

Volocity User Guide



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Printing History

Document Edition	Date	About this edition
1.0	January 2001	The first edition of the Volocity for Macintosh User's Guide.
2.0	March 2001	Updated to include information about installing and using Volocity on a PC.
3.0	June 2001	Updated to include more information about library formats, creating QuickTime movies and other minor amendments.
4.0	July 2001	Updated to include information about the Volume Sequencer.
5.0	September 2001	Updated to include information about Measurements.
6.0	December 2001	First printed edition.
7.0	June 2002	Updated to include information about Classifier.
8.0	December 2002	Updated to reflect new structure of software modules: Volocity LE, Volocity Visualization, Volocity Restoration and Volocity Classifier. Major additions to Classifier and new palette, Voxel Spy.
9.0	November 2003	Updated for Volocity 2.5 and to include information about the Improvion License Server for both the client user and server system administrator.
10.0	January 2005	Updated for Volocity 3.0. Transferred information about the Improvion License Server for the server system administrator to a separate guide.
11.0	May 2005	Updated for Volocity 3.5. Added Volocity Acquisition chapter and Colocalization to Volocity Classification chapter.
12.0	August 2005	Updated for Volocity 3.6. Changes to Classifier setup dialog and palette and added FRET and ratio analysis. Changes to Navigation palette and Visualization chapter, to Acquisition chapters and to Restoration chapter.
13.0	January 2007	Updated for Volocity 4.0. New Quantitation chapter. Changes to Visualization chapter. Major reorganization of content to facilitate generation of Help files, accessed within product.
14.0	April 2007	Updated for Volocity 4.1.
15.0	October 2007	Updated for Volocity 4.2.
16.0	February 2008	Updated for Volocity 4.3.

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What is Volocity?

Overview

Volocity is an innovative, high performance 3D imaging software product designed specifically for Life Science research. It allows the scientist to visualize, explore and analyze multi-channel 3D volumes over time, providing information that is difficult to obtain in any other way.

Volocity can be used for 2D imaging as well as 3D and 4D imaging and will accept image sequences from confocal and wide field microscopes. The software consists of a core product and four unique, totally integrated extension products that provide a full suite of tools for 3D image acquisition, volume visualization, restoration, publication, and object classification, tracking and charting.

The Volocity products can be purchased individually or in combination, according to requirements. In addition, a reduced version of the core, Volocity LE, is available to download free of charge from the Improvise website.

Volocity Acquisition

Volocity Acquisition is designed specifically for high performance acquisition of time-resolved, multi-channel 3D sequences. It supports a range of hardware suitable for 3D imaging, including wide field microscopes, scientific grade digital cameras, fast focus drives and wavelength changers.

Volocity Quantitation

Volocity Quantitation is designed to identify, measure and track biological structures in 2D, 3D and 4D. This unique module incorporates innovative classification technology for rapid identification and quantitation of populations of objects in 2D, 3D and 4D.

Volocity Restoration

Volocity Restoration includes restoration algorithms and measured or calculated PSF generation options for confocal and wide field microscopes. The Volocity restoration algorithms are designed for rapid, high-quality restoration of 4D and 3D volumes and for accurate comparison and quantitation of time-resolved changes.

Volocity Visualization

Volocity Visualization provides an extensive range of visualization and publication features. The Volocity 3D View enables the user to interactively explore a 3D rendered object. The user can, in real time, rotate, zoom and animate the 3D object whilst simultaneously varying the contribution and rendering mode for each channel. The user can also position the viewing point either outside or inside the 3D object, and then watch as structures change and develop over time. Bookmarks may

About this Manual

be used to store the position and appearance of the 3D volume and the movie interface may be used to animate transitions between bookmarks.

About this Manual

This manual describes all the features of Volocity. The initial chapters describe the features that are available to all users. Each extension product then has its own chapter, so that the reader can easily locate relevant information. The manual is designed for users of both Mac and PC versions of Volocity. The screenshot examples show the Mac interface. The screenshots often assume that all features are enabled. If you do not have access to a command, it probably belongs to one of the extension products.

Technical Support

If you have any queries about installing and using Volocity, contact Improvion Support:

USA:

E-mail: support@improvision.com

Telephone: +1 (866) 310 0932

Europe and Rest of World:

E-mail: support@improvision.com

Telephone: +44 (0) 24 7669 8121

Web Page

You can also get additional information about Improvion and its products from the **w**eb page:

<http://www.improvision.com/support>

Information to Provide

When contacting Technical Support please be prepared to provide information about your system including the dongle or serial number.

You may be asked to provide information about your computer hardware and from the Volocity system Session Log, see page 33.

Installation

Minimum System Requirements

The computer hardware market changes rapidly and, as a result, information about minimum and recommended system requirements also changes. Please refer to the technical notes published on the Support area of the Improvisation web site, <http://www.improvisation.com>, for the up-to-date specifications.

Volocity is available for Windows and Mac. The exact specification for your computer depends on the type of Volocity configuration that you intend to run. If you are running Volocity extension products you will require a much higher specification than if you are using Volocity LE. Similarly, if you are working with large volumes you will require a fast, powerful processor and the maximum possible capacity of RAM for the machine.

Please note: Volocity 2.5 was the last version of the software to include support for the following operating systems: Mac OS 9, Windows 98, Windows NT4 and Windows ME.

Note: A Volocity dongle will work with both Volocity for Macintosh and Volocity for Windows.

Installing Volocity (Single License)

To install Volocity LE you require an unlock code and a serial number. You obtain these when you download Volocity LE from the website. This is all you need to install the software.

To use any of the chargeable products you must purchase a dongle, which is a security device that will be registered for your required package. It must be plugged into an unused USB port. Software for the full version of Volocity with chargeable products is also available on CD-ROM. Additional functionality can be added later using a serial number provided when you purchase the product. Please ensure that you read the accompanying license before installing the product.

Follow the instructions supplied with the web download or CD-ROM to install Volocity.

Installation for Server Systems

If you are using the Improvisation License Server, you will first need to install and configure the license server application on a machine with a static IP address that all client machines will be able to access. See the "*License Server User Guide*" for complete instructions on how to do this.

Install Volocity on machines that will access the License Server as described in "Installing Volocity (Single License)". These client machines will not need their own dongle, they will obtain Volocity licenses by connecting to the license server.

Starting Volocity for the First Time

- Volocity LE requires a serial number and unlock code which you may obtain by registering on the Improviation website.
- If your license is on a dongle, ensure that the dongle is plugged in and the light inside the dongle is illuminated. This indicates that the dongle drivers are correctly installed.
- If your license will be obtained from the license server, make sure that you know the license server IP address and port number. You will also need a user ID and, possibly, a password for the license server. This is normally set up by the person who installs and manages the license server.

1. Start Volocity.

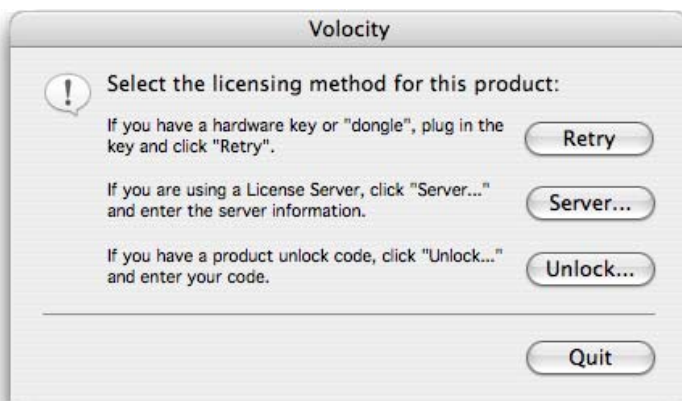
On the Mac: Volocity is installed in the Applications folder. Start by double clicking on the icon.

On Windows: Volocity can be found by navigating through **All Programs** in the start menu.

2. If you have a dongle installed you should see no further messages, Volocity will run. If a licensing message does appear, make sure the dongle is plugged in and click **Retry**.

To use a license server click **Server...** at the dialog shown and enter the details of the Improviation License Server for which you have a user ID and password.

To use Volocity LE click **Unlock...** at the dialog shown and enter the serial number and unlock code.



Changing Your License Server Password

1. When the **Connect To Server** dialog is shown click on the **Change Password...** button.



2. Enter the new password twice to confirm it and click **OK**.

You may also be required to change passwords, when a dialog to that effect is shown. This may be to change a default password or at the request of the Improvisation License Server administrator.

Switching from a License Server to Individual Licenses

Volocity LE: Start up Volocity holding down the 'Apple' key (Mac OS) or the 'R' key (Windows) to bring up the License dialog. Click **Unlock...** to enter the serial number and registration code for your individual license.

Volocity: Plug a Volocity dongle (not an Improvisation License Server dongle) into a free USB port on the machine. Start Volocity. Volocity will recognize the presence of the dongle.

Switching from Individual Licenses to a License Server

Volocity LE: Start up Volocity holding down the 'Apple' key (Mac OS) or the 'R' key (Windows) to bring up the License dialog. Click **Server...** and enter the server IP address and port number.

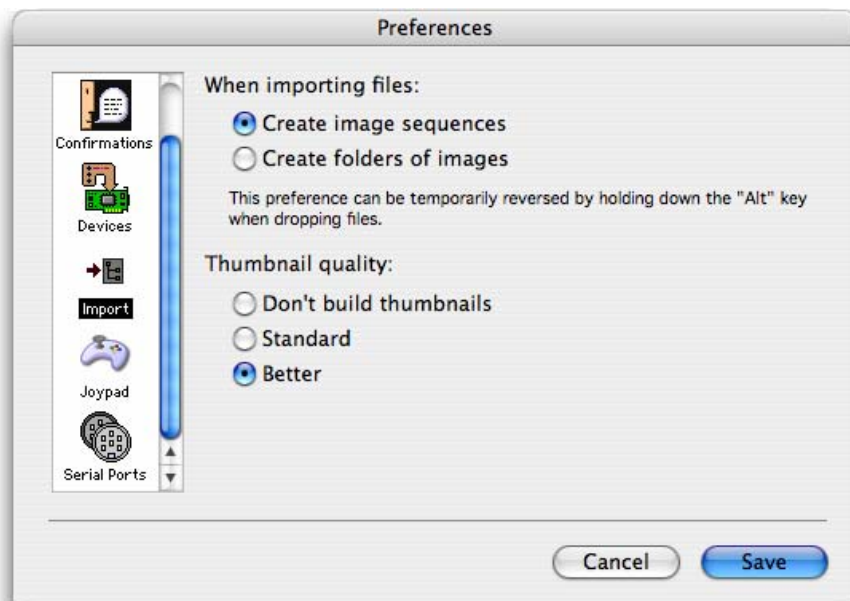
Volocity: Remove the Volocity dongle from the USB port that it is connected to. Start up Volocity. You will be shown the license dialog. Click **Server...** and enter the server IP address and port number.

Preferences

The Preferences dialog allows you to customize various settings.

1. Mac OS X users select **Preferences...** from the Volocity menu. All other users select **Preferences...** from the Edit menu.
2. Select the required preference by clicking on the icon in the left-hand pane.

Preferences



3. Make the required changes and click on **Save** (Mac) or **OK** (PC).

The **Import** preferences allow you to define how images will be imported and to either switch thumbnails off or select the quality (Standard or Better).

The **Confirmations** preferences include settings for displaying hints and checking for software updates. Click on **Reset Hints** if you want the hints to be displayed. Click on **Automatically check for software updates** to ensure that you are informed when new software is available from Improvise.

Other preferences are only applicable if you are running extension products and are described in those chapters.

Session Log

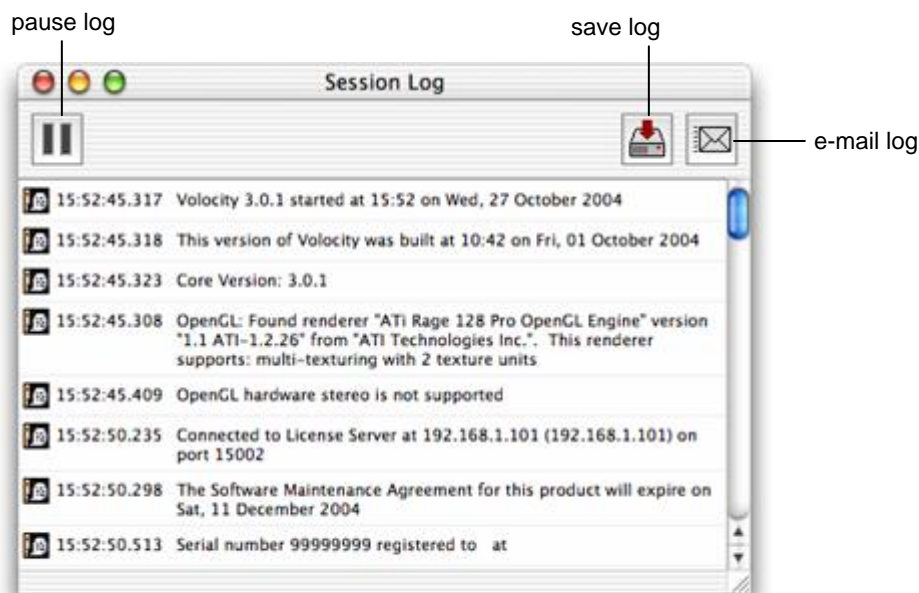
The Session Log records information from when Volocity is started up until it is quit. Session logs always display:

- The version of Volocity that is being run
- The build date of that version which will be on or before the SMA expiry date for the license
- The serial number for that product
- Information about the computer hardware on which Volocity is running
- Any products that are loaded
- Any error messages.

Session logs provide valuable information to Improvion Technical Support engineers when diagnosing hardware or software problems.

Show the Session Log by selecting **Show Session Log** from the Window menu.

Session logs may be saved by clicking on the save button or emailed directly from the Session Log window.



Quitting Volocity

Select **Quit** from the Volocity menu (Mac) or **Exit** from the File menu (PC).

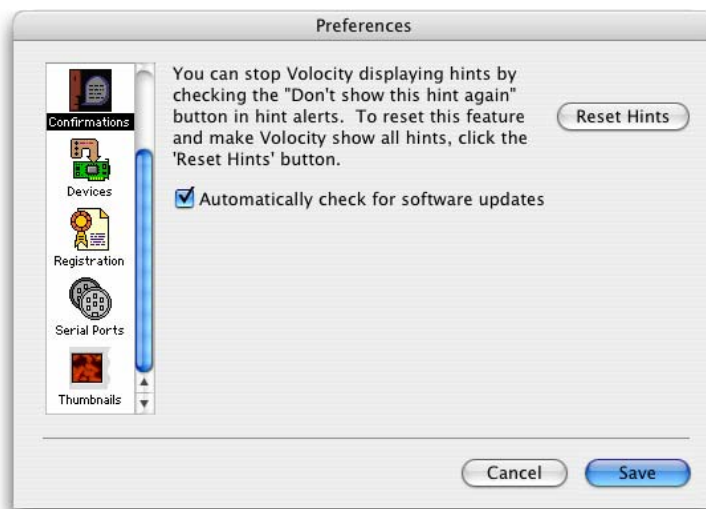
Volocity automatically saves all changes to a library before it quits.

Upgrades from the Website

For Mac OS or Windows simply download the latest installer from the Improviation website and double click to start the installation process. To perform installations you will need an administrator user name and password for the computer. Follow the instructions on screen and restart your computer when requested.

Automatic Software Updates

Alternatively, use the Preferences dialog to enable automatic checking for software updates. Select **Confirmations** and check the box next to **Automatically check for software updates**.



The Improviation server is checked for a newer version on start up. In order to query the Improviation server, the machine running the software must be connected to the Internet. When a later version is detected a message similar to the following is displayed:



Click **Download** and download the installer for the application you wish to upgrade. Run the installer and follow the instructions on screen.

Note: During the upgrade process, the installer will check that you have a valid Software Maintenance Agreement. No information is sent to Improviation during this check, but the upgrade will fail if you do not have one.

Upgrading Applications on Client Machines for a License Server System

Upgrading an application served by the Improviation License Server is simple. All the information relating to users, groups and configurations is stored on the server so there is no special process for installation. See the instructions for Upgrades from the Website on page 34.

Activating Additional Products after Installation (Single License)

Note: This section is not relevant if your organization has a network license. Refer to the separate "Improviation License Server User Guide" for instructions on adding licenses to a server.

If you purchase additional products to add to your original installation, you will receive an unlock code which you must enter in the Unlock Codes dialog. You can do this at any time after installing the software, as long as you have a valid dongle attached to your computer.

Note: If you are upgrading from Volocity LE, you will need to purchase a dongle. When you attach the dongle and run Volocity, the purchased extension modules are automatically enabled.

1. Mac OS X users: Select **Unlock Codes...** from the Volocity menu.
Windows users: Select **Unlock Codes...** from the Help menu.
2. You now need to enter a **Code** for each additional product that you have purchased.

Activating Additional Products after Installation (Single License)



3. Enter the **Code** for the first product and click on **Unlock**. There is no need to specify which product is being added and you can enter codes for products in any order.
4. Repeat step 3 for each product that you have purchased.
5. Quit and restart the software so that the unlock code can take effect.

Getting Started

Overview

This chapter describes:

- important concepts
- what a library is
- how to create libraries
- how to open existing libraries
- how to close libraries and windows
- how to acquire and add images to a library
- how to create an image sequence
- how to create volumes
- how to import items
- supported file formats

Important Concepts

- Data is kept and organized in libraries. A library may contain many experiments and associated information. See page 39.
- Libraries are files which are created using the **New** option in the file menu. Existing libraries are opened using **Open** from the File menu.
- Libraries are databases, changes to databases are saved as they are made, there is no Save command.
- Image sequences organize images that represent channels, time and/or z slices. See page 74.
- Data imported into Volocity have properties. Properties record information about the experiment that acquired the information and affect how items are visualized or processed elsewhere in the software. Properties may be searched for.
- Image data is explored in different ways using views. When an item is opened in the library the views are shown in tabs along the top of the window. See page 61.
- Toolbars update when views change to show the appropriate tools. Tooltips, shown when the cursor is moved over a tool, indicate the purpose of that tool.
- View-specific menus with the same name as the view configure the appearance of the view and access processing specific to that view. For example, when working with the Image View the menu bar will show an Image menu.
- Most menu commands have shortcut keys. If you look at the command in the menu, you will see any shortcut keys to the right of the command name. PC users can use the standard Windows shortcut; right click on the mouse, to display a list of valid menu commands.
- Work with views is enhanced by tools in palettes, which are floating windows containing tools and information that are relevant to different views. Choose **Show Palette** from the Window menu to see available palettes.
- Menu items open dialogs allowing data processing and image enhancement. Dialogs support batch processing of many data sets with one command. See page 96.
- Choose to apply a process to items from a group by selecting them, this highlights them in the interface.

What is a Library?

A library is the term given to the data storage format used by Volocity. A library must be created and images added to it before they can be viewed in Volocity. A library must be opened for Volocity to acquire data.

A Volocity library is a collection of digital images, but the images themselves do not have to be physically stored within the library. The library can act both as:

- an index with links to (and thumbnail views of) the images that are physically stored somewhere else on a hard disk, CD-ROM or other storage medium, and
- a repository for images that have been acquired or edited by Volocity and are physically located within the library (adopted).

The icons on the right hand side of each item in a library indicate where the originals of unadopted data are stored.

This distinction between files that are linked to the library and files that are adopted in the library is important. You will be able to sort and create different views of linked files, but you will not be able to change the source data in any way. Only files that are adopted (physically stored) within a library may be edited.

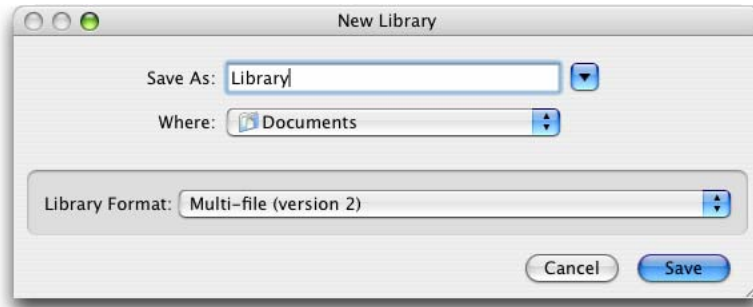
Libraries are based on a database structure and will store changes as they are made.

Creating Libraries

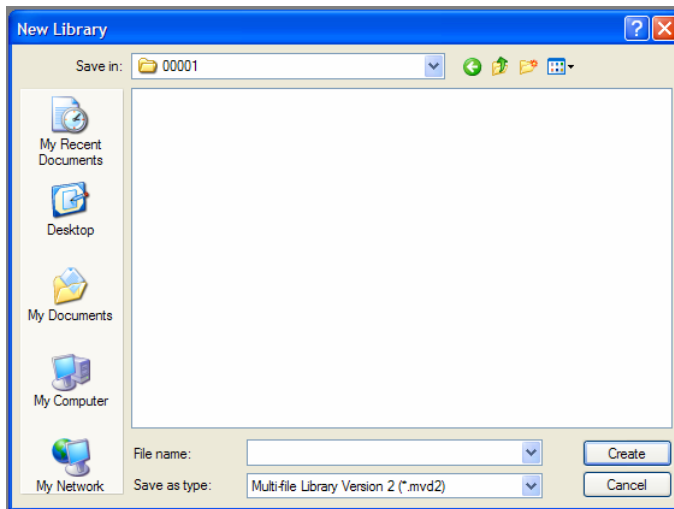
1. You can select **Create a new library** from the prompt shown when Volocity starts (unless you have disabled it).
Or, once you are running the application, select **New Library...** from the File menu. (You can only have one library open at a time. If you already have a library open, you will be prompted to close it first.)
2. Use the dialog to name the library and choose a location to store it.

Creating Libraries

On the Mac, a dialog similar to the following is displayed. Enter a **Name** for the library. The library format is called **Multi-file (version 2)** with the extension **.mvd2**. Click on **Save**.



On the PC, a dialog similar to the following is displayed. Enter a **File name** for the library. Currently, the only library format is **Multi-file Library Version 2 (*.mvd2)**.



3. A library window is displayed. You are now ready to add items to the library.

Library Format

The application creates libraries in a multi-file format, which handles large data sets well and gives fast data access. When you create a multi-file library, it is created within a folder. This folder is labeled with an icon and the name of the library (as entered in the New Library dialog).



a multi-file library folder

Inside the folder are the library file and a data folder, which contains all the data required to view the images and volumes kept in that library. Multi-file library files have a .mvd2 suffix.

It is essential that the library file and data folder are kept together, which is why the application creates them together in a separate folder. To open the library, you must first open this folder and locate the library file. If you want to move a multi-file library to another location you must move the whole folder. If you only move the library file, the application will not be able to display any of its contents.

Opening Libraries

Once you have created a library, you can either double click on the library icon to open it, or select **Open Library...** from the File menu and choose the required library in the dialog. All libraries must have a .mvd2 extension or they will not be visible in the file list.

Only one library may be open at a time. If you already have a library open, the application will prompt you to close it. Once the Library window is displayed, you can add new files to it, as described on page 42.

Closing Windows and Libraries

The application conforms to the standard Mac and PC conventions for closing windows.

On a Mac, close the current window by selecting **Close** from the File menu. If the current window is the Library window, this command closes the library. You can also close any window by clicking on the top left-hand corner of the window.

On a PC, close the library by selecting **Close Library...** from the File menu. Close the current window(s) by selecting **Close** (or **Close All**) from the Window menu. You can also close any window by clicking on the top right-hand corner of the window.

Acquiring Data

Data may be acquired during experiments using hardware for acquisition, that is, cameras and devices for microscope automation. All hardware must be installed and configured before use. This is described in full detail in “Volocity Acquisition Installation and Reference” starting on page 125. This chapter describes how to:

- Install and configure devices
- Use the Video preview to work with devices
- In the Video Preview use light paths to coordinate changes to devices to meet the requirements of imaging modes

Adding Data

Once light paths have been configured they may be used in acquisition protocols. Acquisition protocols allow different combinations of channels, focus movement and XY control to be used in experiments that capture data.

Example Acquisition Protocols for a variety of different experiments are described in the section starting on page 148.

Adding Data

Libraries can contain 2D and 3D images, which may be viewed in a number of different ways. The way that you add items to the library depends upon the type of images and how you want to view and work with them.

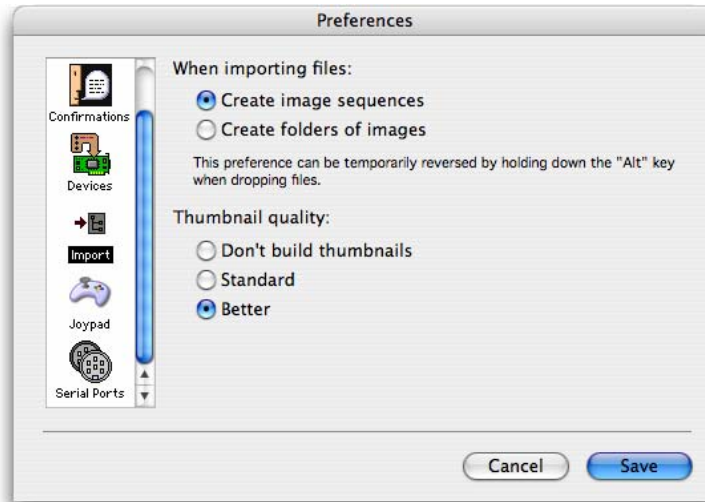
- Acquisition from a camera adds images directly to image sequences. Image sequences are used to work with time and/or channels.
- You can also drag and drop folders of images into an open Library window to add them to the library.
- If you are adding a series of slices from one object at one timepoint on one channel, import the data as a folder of images and then create a volume as described in "Creating volumes." on page 46. For example, you may be creating a volume of data from which to measure a PSF (Volocity Restoration only).

Note: You can also use the **Import** command from the File menu to add single, unrelated items.

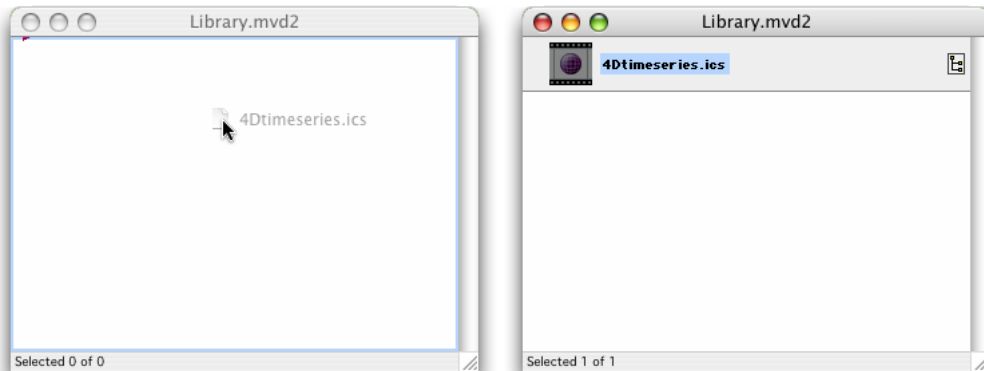
Adding Images Using Drag and Drop

The easiest way of adding items to the library is to drag an item from the desktop and drop it onto the Library window.

For ease of use when image sequences are required set the **Import** preference to create image sequences rather than create folders of images. To do this, select **Preferences...** from the Edit menu (Windows) or the Volocity menu (Mac OS).

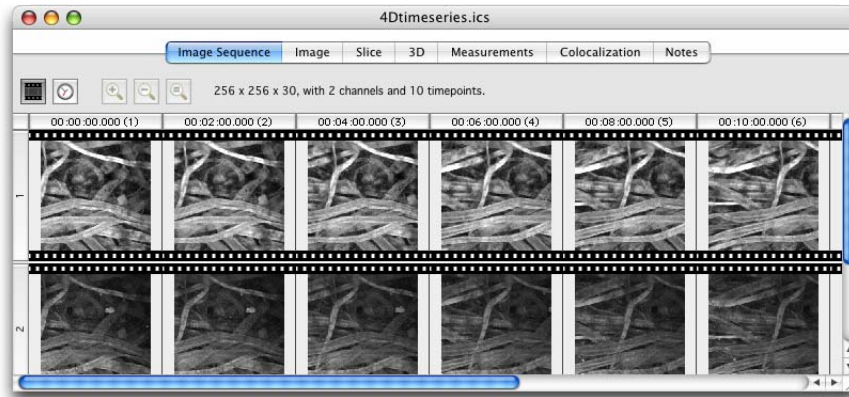


In the following drag and drop example, an image sequence is created.



Double click on the library item to view the image sequence. For a detailed description of the different views, see the “Views on Data” chapter starting on page 61.

Adding Data



If the item dragged is a folder, such as a series of slices for a volume, the application will scan the contents of the folder and add all the files it recognizes to the library. Any hierarchy within the item that you drag and drop onto the Library window is retained.

You can continue to drag as many folders or files as required onto the library, and you can add items to any level of folder structure. Items are inserted into the library at the red drag insertion arrow. The amount of indentation indicates the level at which an item is located within the library hierarchy.

Note: The only limit on library size is the hard disk space. However, it is usually more practical to separate work into more manageable sets and create separate libraries for each one.

Creating an Image Sequence

Image sequences are a collection of images arranged by channel and timepoint. Multi-channel and/or time-resolved data with one or more planes in z is best viewed using an image sequence.

Set the **Import** Preference to **Create image sequences** to make image sequences automatically when importing related data.

The Image Sequencer is a powerful tool for working with data related by time and/or channel. Once you have created an image sequence, you will be able to interact with the data in a number of different ways. See page 76.

There are rules about the type of data that may be made into an image sequence.

- All images in a channel must have the same depth and color look-up table.
- The images must make up volumes that have identical X, Y and Z dimensions.

To Create an Image Sequence Item:

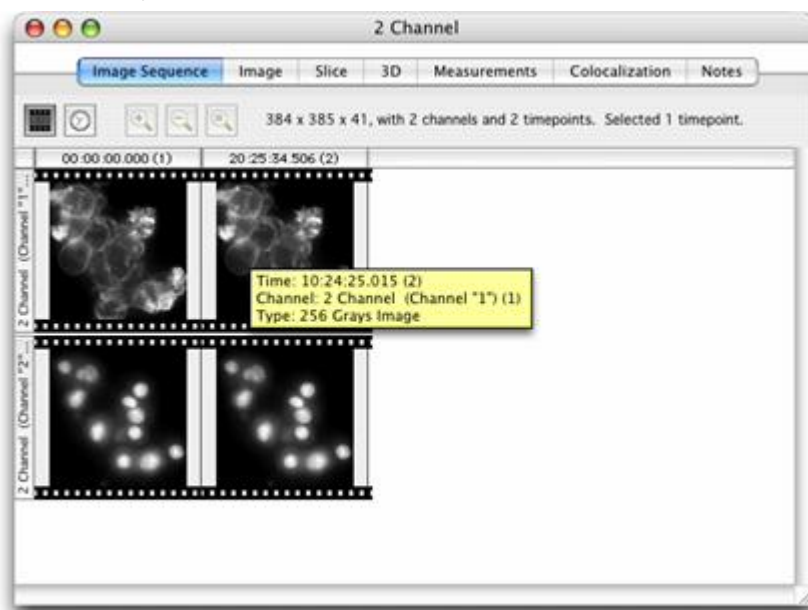
1. Select **Create New** from the Actions menu.
2. Select **Image Sequence** to create a new image sequence item in the library.
3. The Image Sequence window opens automatically.



4. Drag and drop all the data for an experiment into the open Image Sequence view. Tabs in this window allow you to display different views of the data. You use the Image Sequence tab to add items to the sequence; it provides an overview of each channel and timepoint.

Creating Volumes

Hold the cursor over an item in the Image Sequence view to show a tip that gives the timestamp, channel and type



Creating Volumes

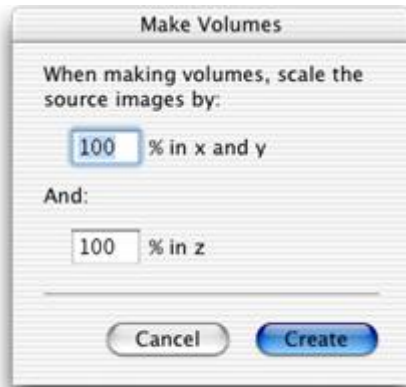
A volume is the name for a 3-dimensional object within Volocity, it is made up of a stack of images.

If you are working with images from a single object at one timepoint or one channel, use the **Make Volumes** command to create a volume. For example, once you have added an image sequence that contains 2D images related in the Z-dimension, such as confocal microscope, wide field microscope or magnetic resonance image stacks, you can use this command to create a volume within Volocity.

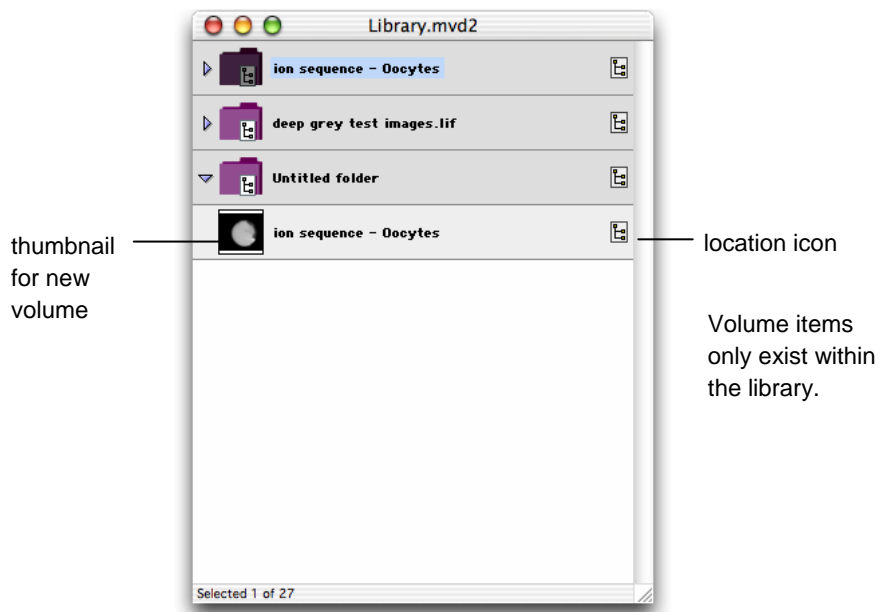
Note: If you are working with multi-channel or time-resolved data, the easiest way of doing this is to use the Image Sequencer.

1. Select the item(s) in the library. From any folder or file containing many images you can select individual images; for example, all the images belonging to a channel.
2. Select **Make Volumes** from the Tools menu.
3. The PC version of the software always gives you the option of scaling down the source images in the volume.

Mac users should hold down the Alt key when selecting **Make Volumes**, if they want to apply scaling.



4. Enter scaling values as a percentage and click on **Create**. Volocity creates the volume and the Library window now contains an image item for the 3D data.



thumbnail
for new
volume

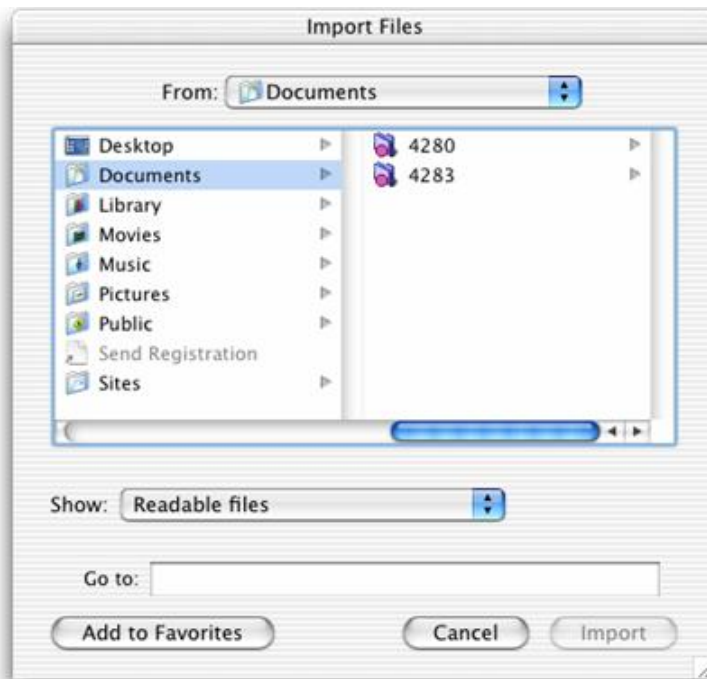
location icon

Volume items
only exist within
the library.

Importing Items (File Menu)

To add single files to a library you can use the Import command.

1. Select **Import...** from the File menu to display a dialog similar to the following.



2. The **Show:** drop-down menu at the bottom of the dialog allows you to specify a file format, so that you can locate the required file more quickly.
3. Locate the required image. If it is in a folder, select the folder and click on **Open** to open it. Once you have located and selected the image, click on **Import** to add it to the library.

Supported File Formats

For reading, Volocity currently supports the following formats:

- BioRad PIC
- Windows BMP
- Volocity Library Clipping
- DeltaVision
- ICS/IDS (Image Cytometry Format)
- JPEG
- Leica LIF (generated by Leica confocals)
- NeuroLucida Tracing XML format
- Openlab LIFF
- PerkinElmer AIC
- Apple PICT
- QuickTime Images (this includes formats such as Photoshop PSD and any other still image format supported by QuickTime)
- TIFF
 - Improvision TIFF extensions (metadata included in the ImageDescription tag)
 - Leica TIFF (generated by Leica confocals)
 - Olympus TIFF (generated by Olympus confocals)
 - PerkinElmer TIFF (generated by PerkinElmer Ultra View systems)
 - GE TIFF (generated by GE Healthcare INCell 1000 systems)
 - OME TIFF (as described by the Open Microscopy Environment <http://openmicroscopy.org>)
- Zeiss LSM (this is a TIFF variant)
- MetaMorph STK (this is a TIFF variant)
- Olympus OIF (this is a TIFF variant)
- TILLVision

Importing Items (File Menu)

For writing, Volocity supports the following formats:

- Windows BMP
- Volocity Library Clipping
- JPEG
- Openlab LIFF
- TIFF (with Improvion extensions)
- OME-TIFF (2007 schema, as described by the Open Microscopy Environment <http://openmicroscopy.org>)
- TIFF for Publication
- QuickTime Movies
- AVI Movies

Volocity supports writing views as QuickTime movies and as AVI movies.

Organizing Data in a Library

Overview

This chapter describes:

- the library format
- the library menu and toolbar
- library thumbnails and icons
- how to tell where data is stored
- how to set item properties
- how to create new folders
- how to copy and move folders and items
- how to find items
- how to compact libraries

Library Format

The application creates libraries in a multi-file format, which handles large data sets well and gives fast data access. When you create a multi-file library, it is created within a folder. This folder is labeled with an icon and the name of the library.

Inside the folder are the library file and a data folder, which contains all the data required to view the images and volumes kept in that library. Multi-file library files have the suffix .mvd2.

It is essential that the library file and data folder are kept together, which is why the application creates them together in a separate folder. To open the library, you must first open this folder and locate the library file. If you want to move a multi-file library to another location you must move the whole folder. If you only move the library file, the application will not be able to display any of its contents.

Library View

The Library view shows you a list of all the items in your library. If there are sub-folders or single files containing multiple images within your library, click on the arrow next to the folder/file thumbnail to expand and contract the folder and view/hide the contents. See also page 62.

Library Menu and Toolbar

Whenever a Library view is active, there is a Library menu and a Library toolbar.

Library Menu

Use the Thumbnail commands on the menu to control the size of the thumbnails in the library. Use the **Show data** and **Sort by** commands, to determine what information is displayed with a library item and how items are sorted in the library.



Library Toolbar



Use the rotate tools to rotate single items 90 degrees in a clockwise or counter-clockwise direction.

The rotate commands are available in the toolbar and from the Actions menu. (You can also flip single images using the Flip commands in the Actions menu, but you must first adopt the item, see page 96.)

Library Thumbnails and Icons

The item's thumbnail tells you what kind of item it is.



This is a folder or multi-layered file.



This is an Openlab LIFF file containing multiple images.



This is a thumbnail of the image (2D) or volume (3D).



This is a PSF (used with Volocity Restoration, see also the glossary).



This is an image sequence.



This is a movie sequence (only available with Volocity Visualization).



This is a measurement item (only available with Volocity Quantitation).



This is a background correction.



This is a registration correction.



This is a spectral signature.

How Do I Tell Where the Data is Stored?

When you create a library, folders and images are catalogued within the library structure. However, the source image remains in its original location - on the hard disk, CD or server - and the application will need access to this information for certain operations, unless you explicitly adopt it into the library structure. Icons indicate whether an item in the Library View is stored within the library hierarchy or is located somewhere else. The following list provides examples of the icons.



The item only exists within the library or has been adopted into the library. All volumes have this icon as they are created within the library. Files copied within or between libraries also have this icon.



The item is stored on the hard disk and catalogued within the library.



The item is stored on a CD and catalogued within the library.



The item is stored on a network server and catalogued within the library.



The item is stored on a Zip disk and catalogued within the library.



The item is stored on a floppy disk and catalogued within the library.

Setting Item Properties (Edit menu)

Every item has a number of properties associated with it.

Some properties are fixed and are assigned by the system, such as the kind, time modified and date modified. Others are user-defined, which provides you with a useful method of recording additional data against an item by which you can also search for items. You can set properties on both 2D and 3D items.

The correct XYZ properties (in micrometers per pixel) are essential for generating correct scale bars, calibrated measurements in Quantitation and the correct application of PSFs in Restoration. These properties effectively set the calibration of the data.

The Volocity file filters read XYZ calibration information from supported file formats when they are imported. Acquisition directly into the application will set the relevant properties automatically.

In the following example, we have set up properties for a single item. To change common properties for several items at the same time, select the required items before selecting **Properties...** from the Edit menu.

1. Select an item and then select **Properties...** from the Edit menu. We have selected an item called "Neuron".
2. Enter properties as appropriate. Use commas to separate multiple entries, for example keywords.

Properties

Change the properties of "Neuron ":

Name	Neuron
Keywords	Fitc]
Created by	
Channel	
Timepoint	
$\mu\text{m}/\text{pixel}$ (X)	1 μm
$\mu\text{m}/\text{pixel}$ (Y)	1 μm
$\mu\text{m}/\text{pixel}$ (Z)	1 μm
Microscope	
Camera/Detector	
Wavelength	500 nm
Black Level	10 levels

Cancel Change

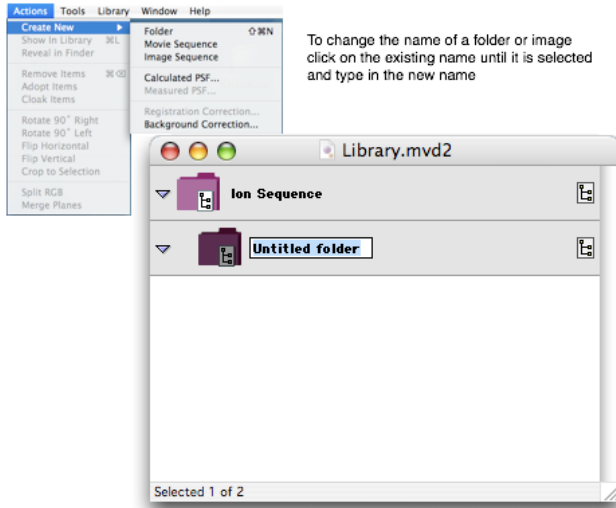
3. Click on **Change**.

Creating New Folders

Create folders to organise data within a library.

1. Select **Create New** from the Actions menu and then select **Folder**. This will create a new, untitled folder underneath whatever item is currently selected in the library. If nothing is selected, the untitled folder will be the last item in the library.

Copying and Moving Folders and Items



2. Drag and drop items into the new folder as required.

Copying and Moving Folders and Items

Copy Items Between Libraries

There are several ways of copying items from one library to another. You can use the Copy and Paste commands from the Edit menu. Select the items that you want to move in the first library. Select **Copy** from the Edit menu, close the library, open the other one and select **Paste** from the Edit menu.

Alternatively, you can create a clipping file. Select the item in the open library, use the **Export...** command and select the Library Clipping format. The Export command allows you to specify a unique name for the clipping file; it will have an .acff suffix. See also page 120.

To copy the clipping file to a new library, open the library and drag and drop the clipping into the library view. You can also use the **Import...** command to import the .acff file into the library. See page 48.

Move Folders into Other Folders

Click on the folder and drag it to the required position in the library hierarchy.

Move Items from One Folder into Another

You can move sub-folders and individual, unrelated image files, such as TIFF files. You cannot move items from multi-layered files, such as Openlab LIFF files or multi-layered TIFF files, because the relationship is set by the source data. Moving the item would change the way the original entry in the library is linked to the source data. If you try to do this, you will be prompted to create a copy of the item.

Copy Items from One Folder into Another

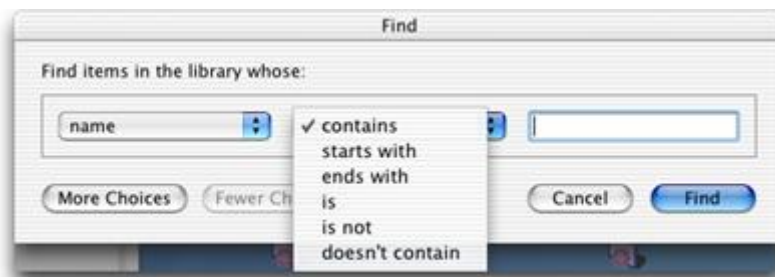
- If you have tried to move an item from a multi-layered file, click **Copy** at the prompt.
- Or select the item and use the **Duplicate** command in the Edit menu. This duplicates the item in the same folder and you can drag and drop it into another folder.
- On a Mac, hold down the Alt key as you drag the item into a different folder. A progress message is displayed telling you that the application is copying the item into the folder.
- On a PC, hold down the Ctrl key as you drag the item into a different folder. A progress message is displayed telling you that the application is copying the item into the folder.

The copied item is automatically adopted into the library so you will be able to make changes to it without changing the source data.

Finding Items (Edit menu)

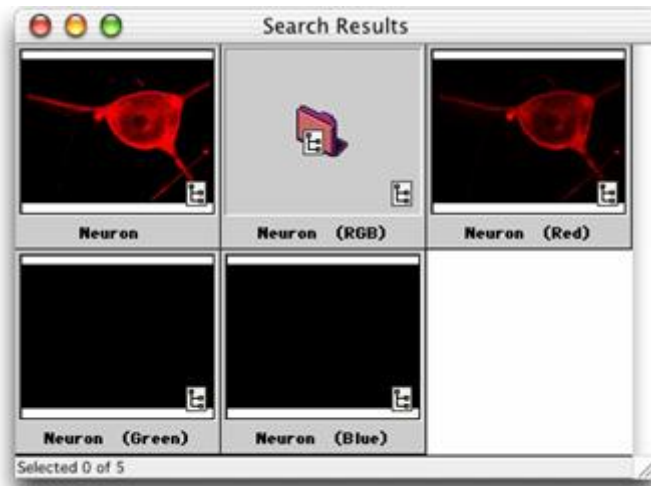
The Find command allows you to quickly locate images within a library.

1. Select **Find** from the Edit menu.
2. Enter the search criteria, as appropriate. You will be able to select from a number of search criteria.



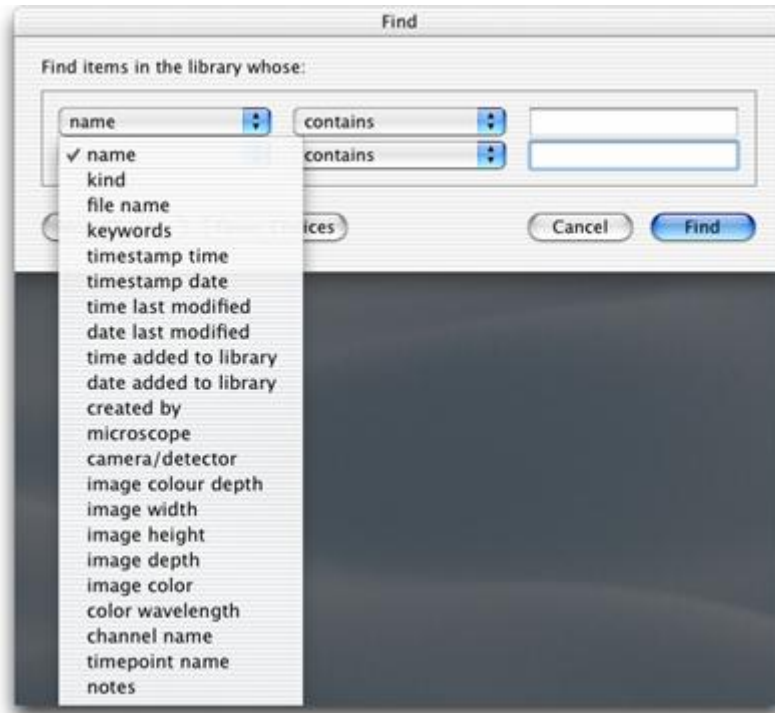
3. Click **Find** to display the Search Results window, which is displayed as a Gallery window. There is also a Results menu which has the same commands as the Gallery menu described on page 87.

Finding Items (Edit menu)



4. Double click on the required item in the Search Results window to open it in a new window.

5. If you want to enter multiple search criteria, select **Find** again and click on the **More Choices** button.



6. Enter as many search criteria as required and click on **Find** to display the search results.

Note: Find remembers the search criteria, so use the **Fewer Choices** button if you want to remove search criteria that you have already set up.

Delete/Remove Items from a Library

Highlight the item(s) to be removed and select **Remove Items** from the Actions menu. If you change your mind, use the **Undo** command from the Edit menu.

Items cannot be removed if they are part of a linked file that is not adopted into the library.

To remove part of an item, for example a selected channel, an ROI, a measurement item row, select the part that is to be removed and use delete (Windows) or backspace (Mac OS).

Locating an Item (Actions menu)

The **Show in Library** (see page 90) and **Reveal in Finder/Explorer** commands in the Actions menu allow you to quickly locate an image link in the library or the source data in the Finder (Mac) or Explorer (PC).

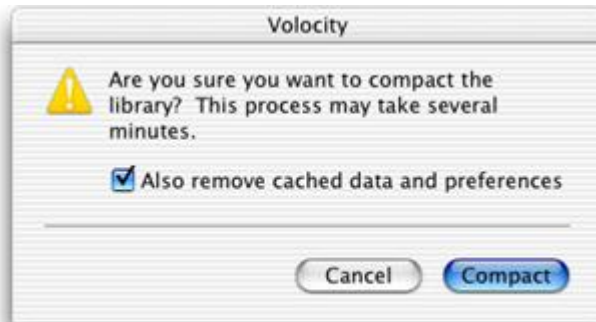
Reveal in Finder/Explorer

This command works from a Library, Single Image or Gallery View and highlights the location of the source data for the selected item in the Macintosh Finder or Windows Explorer. If the source data has been removed from the hard disk, the application just displays the Finder or Explorer. If the source data was on a removable medium, it prompts you to insert the relevant disk or medium.

Compacting Libraries

Even though libraries will increase in size under normal use, Volocity will always make best use of available disk space. However, when moving or backing up libraries you may like to use the Compact Library command. This optimizes the library size. Cached data includes stored rendering settings, therefore, it may take longer to view data once the library has been compacted.

1. Select **Compact Library...** from the File menu.
2. Leave the checkbox selected to **remove cached data and preferences**. Then click on **Compact**.



Views on Data

Overview

Once you have added data, you can view library items and items within an image sequence in a number of different ways. Each type of view has its own window, menu and toolbar. View-specific menus and toolbars contain commands that are particularly relevant to that view. The menu title and/or contents will update according to the active view.

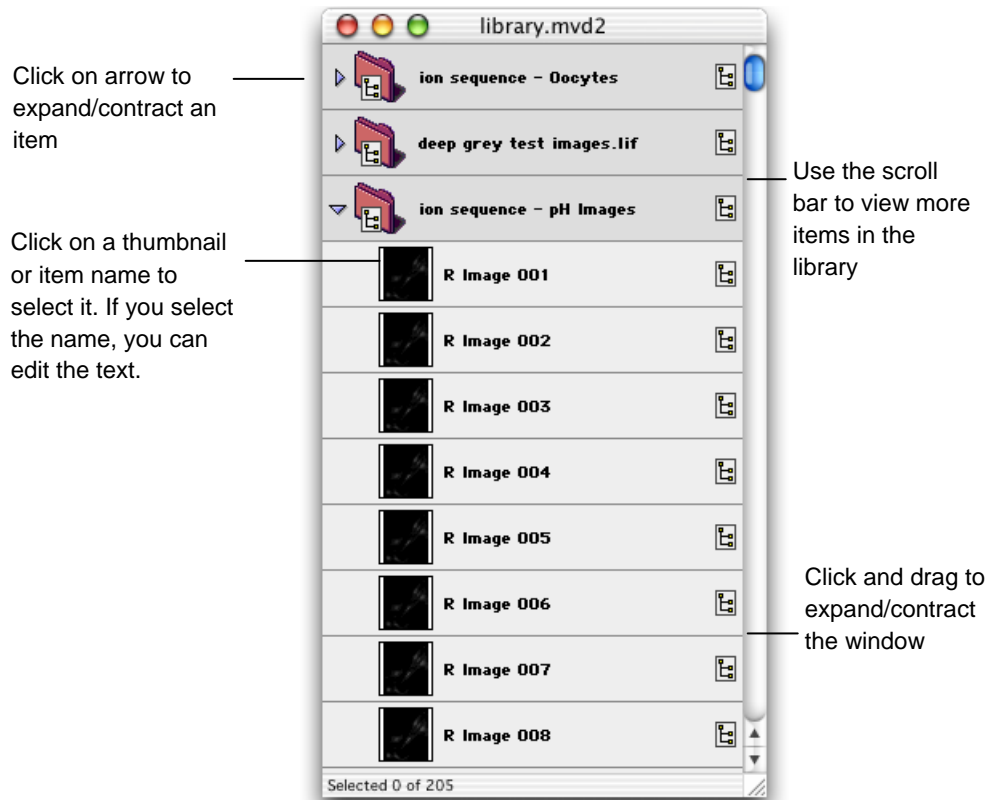
In addition, there are two palettes that may appear in a separate window on the screen: the Navigation palette and the Voxel Spy palette. (If these are not visible, use the **Show Palette** command in the Window menu to display them.)

This chapter describes:

- the Library View
- how to look at and select items in the library
- the Image View
- the Image Sequence view
- the Voxel Spy palette
- the Navigation palette
- the Gallery View, menu and toolbar
- the Movie View, menu and toolbar
- the Notes tab
- how to capture snapshots
- how to show data
- how to sort items

Library View

The Library View shows you a list of all the items in your library. If there are sub-folders or single files containing multiple images within your library, click on the arrow next to the folder/file thumbnail to expand and contract the folder and view/hide the contents.



How Do I Look at Items in the Library?

Double click on an item in the library to open it in a separate window. The window will have a toolbar and a number of tabs that allow you to display different views of the image data. The type of item determines what tabs and tools are available.

- If you select a single image from the library, you will display the item in an Image View. See page 64.
- If you select an image sequence, you will display the item in an Image Sequence view. See page 76. You can click on the Image tab within the sequence to display it in an Image window and then use the Navigation palette to move to other images in the sequence.
- If you select a folder or multi-image file, you will display the contents in the Gallery View. See page 87. If there are sub-folders, you can double click on those to open them. Once you have displayed a Gallery View, you can create a Movie View of the images within the gallery.
- If you select a movie sequence, you will open the item in the Movie Sequence View. This item is only available with Volocity Visualization, see page [249](#).
- If you select a PSF, you will display the item in an Image window together with the PSF data that was used to carry out restoration. This item is only available with Volocity Restoration, see page 339.

Note: It is possible to open the same data multiple times. This is an extremely useful feature as it allows you to compare different views of the data.

Selecting Items in the Library

You select items in the library in the same way that you select items in Macintosh Finder or Windows Explorer.

- Click on an item thumbnail or item name to select the item.
- To select a group of consecutive items, hold down the shift key and click on the first and last item in the group.
- To select multiple items not in sequence, hold down the Apple key on the Mac or the Ctrl key on the PC and click on the required items.

See “Organizing Data in a Library” on page 51 for more information about working with the Library View.

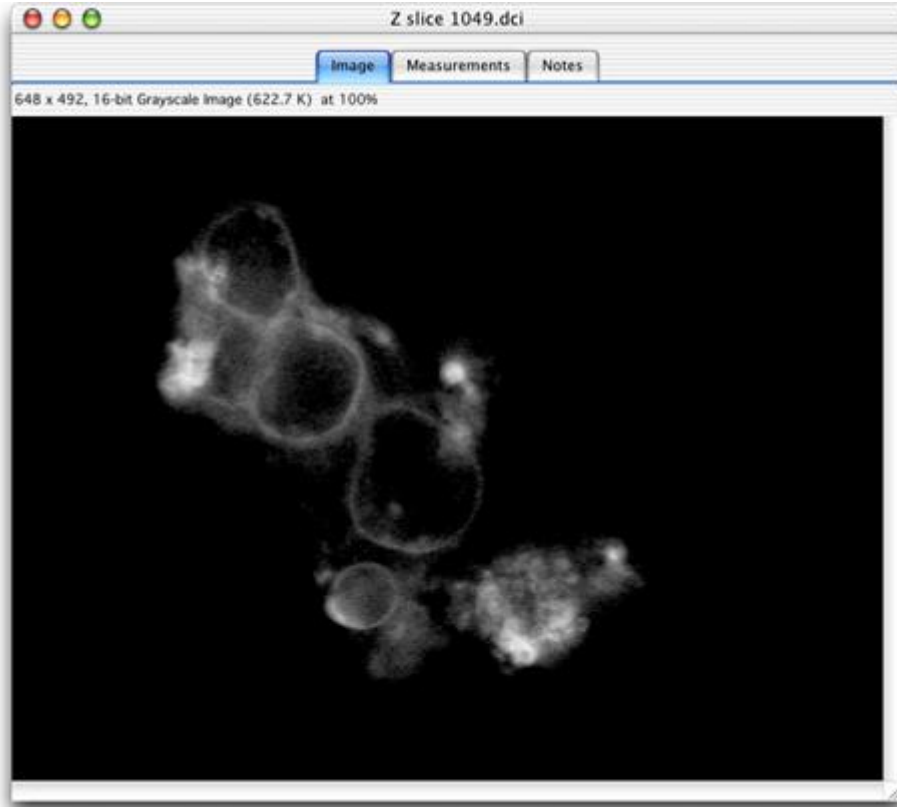
Status Bar on Windows

The status bar is a standard Windows feature that appears in all views on the PC at the bottom right-hand corner of the application window. This is also the first place that progress information is displayed, even before a progress bar is posted to the screen. Error messages may also appear in the status bar.

Image View

When you double click on an image item in the library or click on the Image tab within an image sequence, the data is displayed in an Image View. Whenever an Image View is displayed, you will see an Image menu and toolbar, which contain commands and tools that are appropriate for the image data.

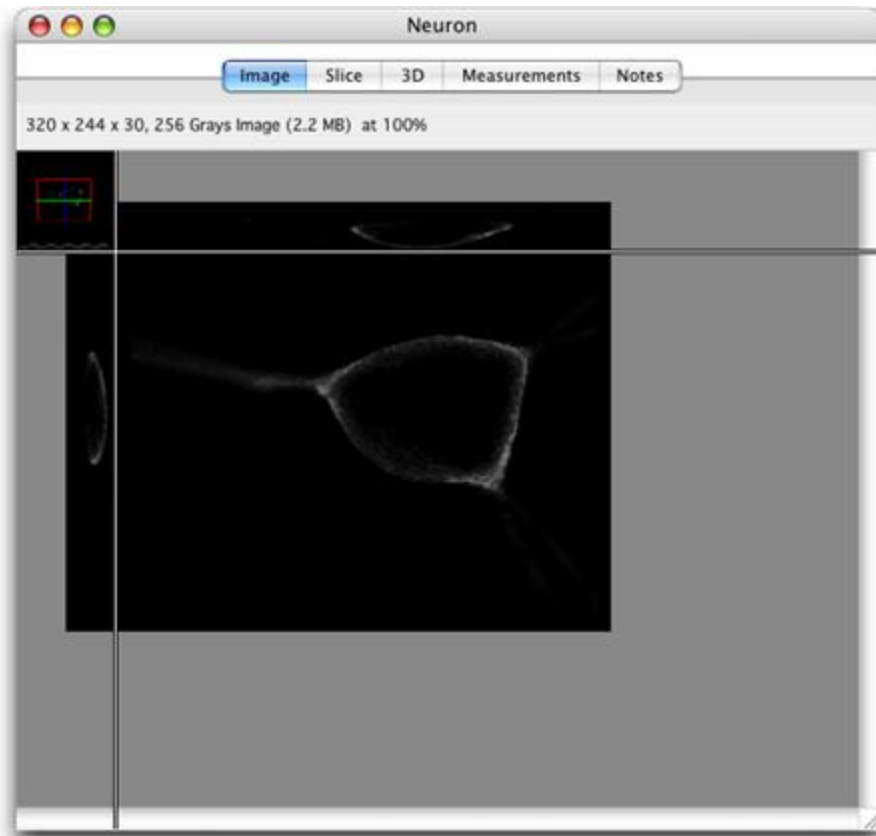
2D Items



If the image is from an image sequence you can use the Navigation palette to move to different timepoints in the sequence, turn channels on and off, and increase or decrease brightness.

3D Items

When you double click on a volume item in the library or click on the Image tab within an image sequence consisting of 3D data, it is also displayed in an Image window. However, the window is now sectioned into four to show the 3-dimensional cross-section of all planes (top left section) and each of the axes/planes.



In an image sequence, the view displays a brightest point merge of all the channels at the current location in the object.

You can use the Navigation palette to play through all the volumes in the sequence, turn channels on and off, and increase or decrease brightness.

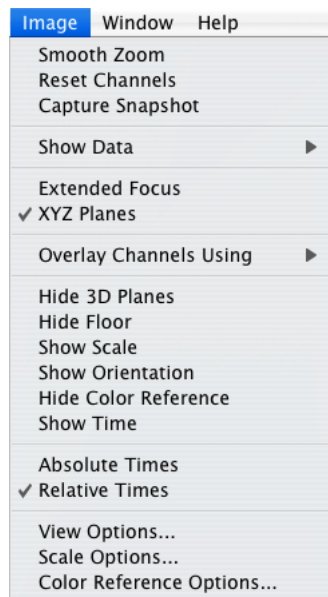
Image Menu

Whenever an Image View is active, there is an Image menu available in the menu bar. The menu commands are appropriate to the type of image data. For example, the **Reset Channels** command only applies to an image that has been selected from an image sequence.

Use the **Smooth Zoom** command on the Image menu to apply interpolation to the view to give better quality when viewing zoomed images and automatically set the best quality for printing.

When viewing the image view of an image sequence consisting of two or more channels, the user can choose the method that is used to merge the two channels for display from the **Overlay channels using** menu.

- **Brightest point:** The brightest intensity in the source images is displayed.
- **Accumulate:** The total intensity of the voxels in the source images is displayed.
- **Average:** The average intensity of the source images is displayed.



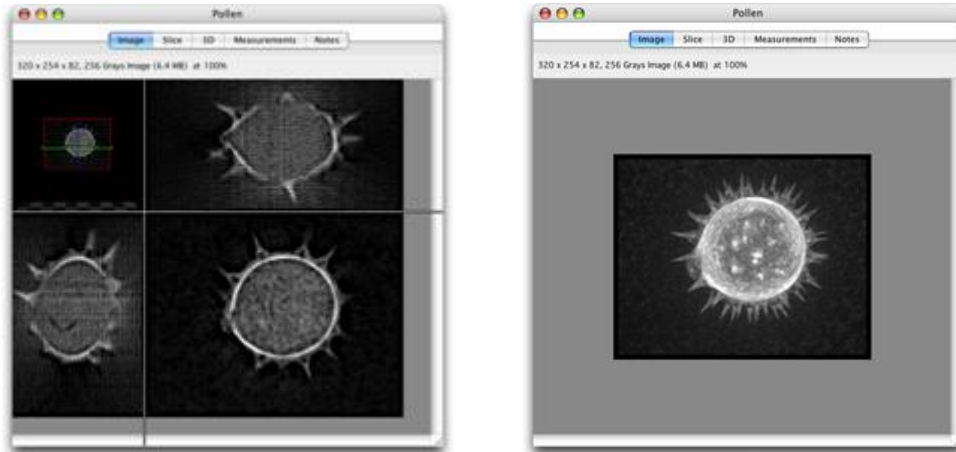
Scale Options... are described on page 69, **View Options...** are described on page 72, **Color Reference Options...** are described on page 67, **Show Data** is described on page 92, **Show Time** is described on page 67, **Capture Snapshot** is described on page 91,

Other commands allow you to show or hide elements of the view. Some of these will be grayed out for 2D data. For example: with a volume item you can select **Hide 3D Planes** to simplify the 3D cross-section part of the view.

Extended Focus (not available in Volocity LE)

This option is available when viewing 3D images in the Image View. It displays a single image by making a brightest-point merge of all the Z-slices of a volume.

Use the Image Menu to toggle between **XYZ Planes** and **Extended Focus**. The XYZ view displays a single plane in each of the three dimensions. The Extended Focus view displays a single image, which is a brightest-point merge of all the Z-slices of a volume.



Selections can be made within the volume, using the tools in the Image View toolbar, and measurements can be made from these selections. Note that the volume and all selections made are three-dimensional and have depth and volume.

Show Time

The timestamp of an image in an image sequence can be shown when viewing the Image View.

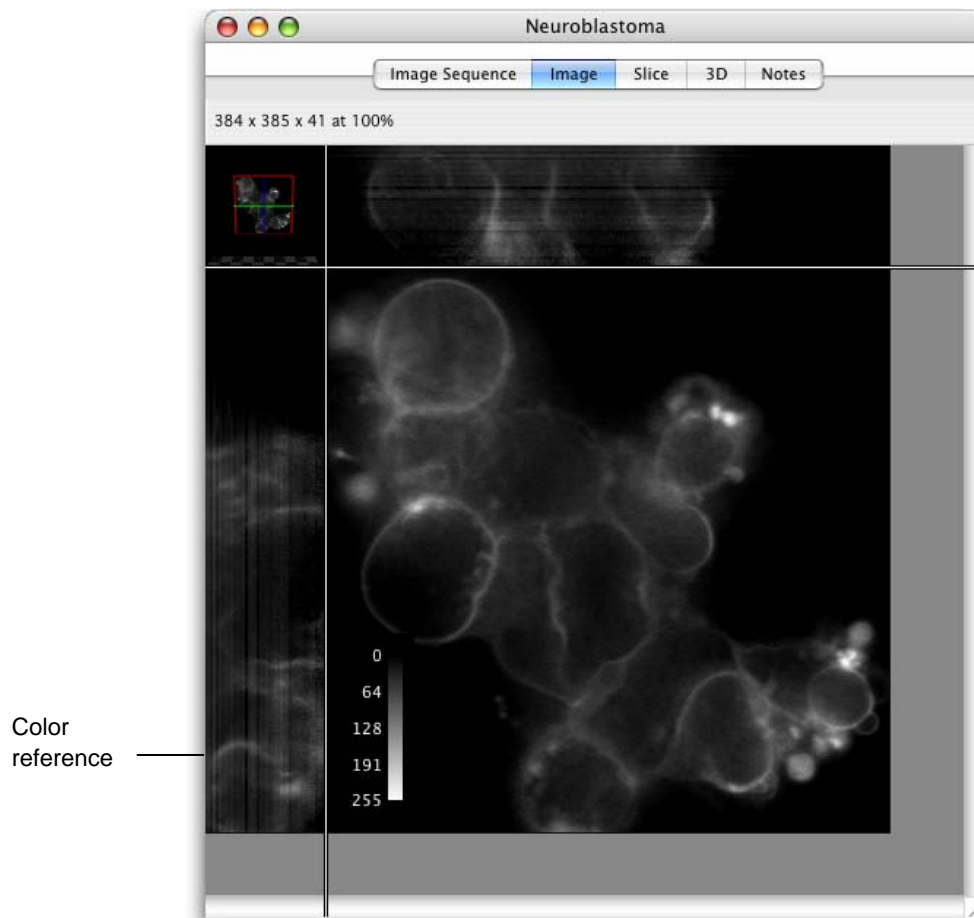
- Select **Show Time** or **Hide Time** in the Image menu to toggle the timestamp on or off.
- Select **Absolute Times** or **Relative Times** to choose the style of timestamp to be displayed.
- Select **View Options...** and change the **Frame time color** to change the color of the timestamp.

Showing Color Reference

On all Image Views, apart from millions of colors images, you can display a legend that shows the colors in the image and the values they represent.

Image View

To display the legend, select **Show Color Reference** from the Image menu.



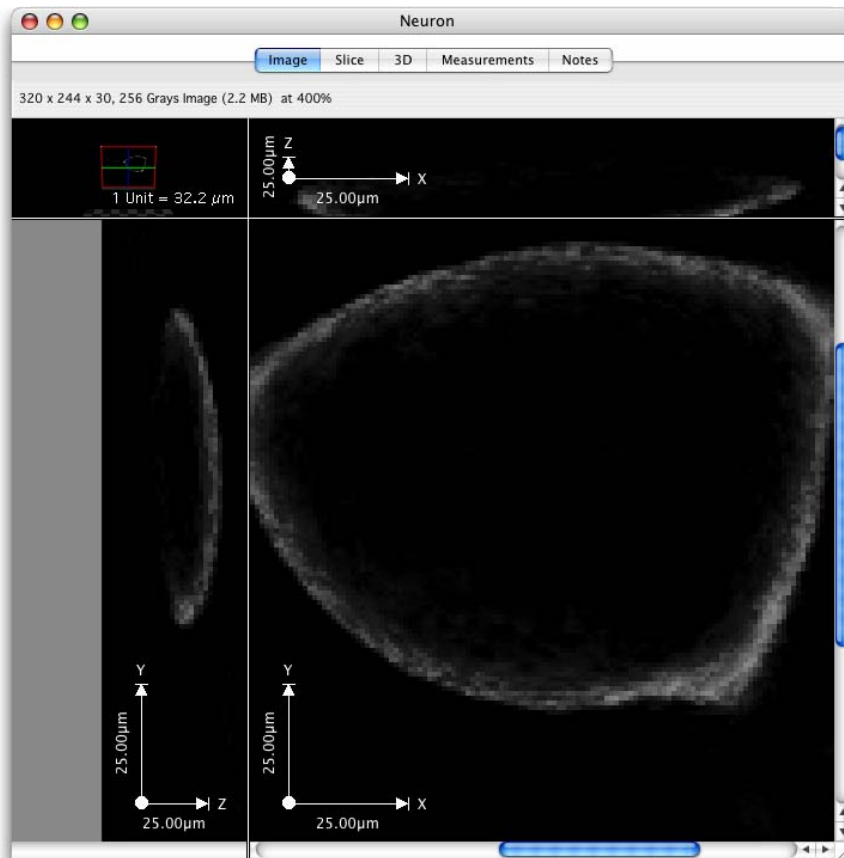
The color reference may be moved to any corner of the XY area of the image view.

Note: There is a separate color reference for each visible channel. Therefore, if multiple channels are selected in the Navigation palette, multiple legends will be displayed. Hide unwanted color references by hiding the channels from which they are derived on the Navigation palette.

Select **Color Reference Options...** from the Image menu to choose how the color reference is displayed.

Showing Scale on Images

Whenever an Image View is active, you will also be able to display scaling information. Select **Show Scale** from the Image menu to activate this feature.



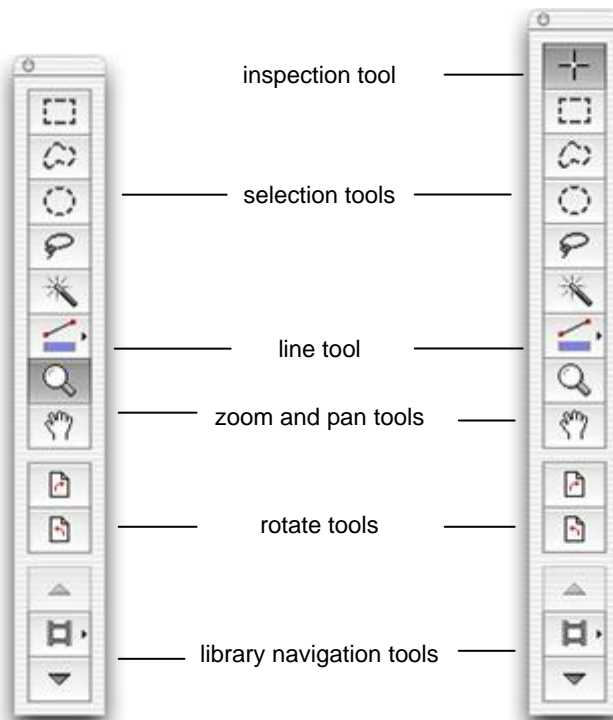
Use **Scale Options...** to show/hide labels

- The scale displayed will depend on the properties set for the size of voxels in the X,Y and Z dimensions. Choose **Show Properties...** from the Edit menu to check these values and ensure the correct scale is displayed.
- Use **Scale Options...** from the Image menu to change how scale is displayed.
- Move the scale to any of the four corners of the image by dragging it.
- Extend or shorten the scale bar by clicking and dragging one end of it.

Image View

Image Toolbar

The image toolbar contains the rotate and library navigation tools, and a number of other tools for zoom and pan, and selecting regions of interest (ROI) on the image. Tooltips, shown when the cursor is held over the tool, indicate its purpose.



Inspection Tool

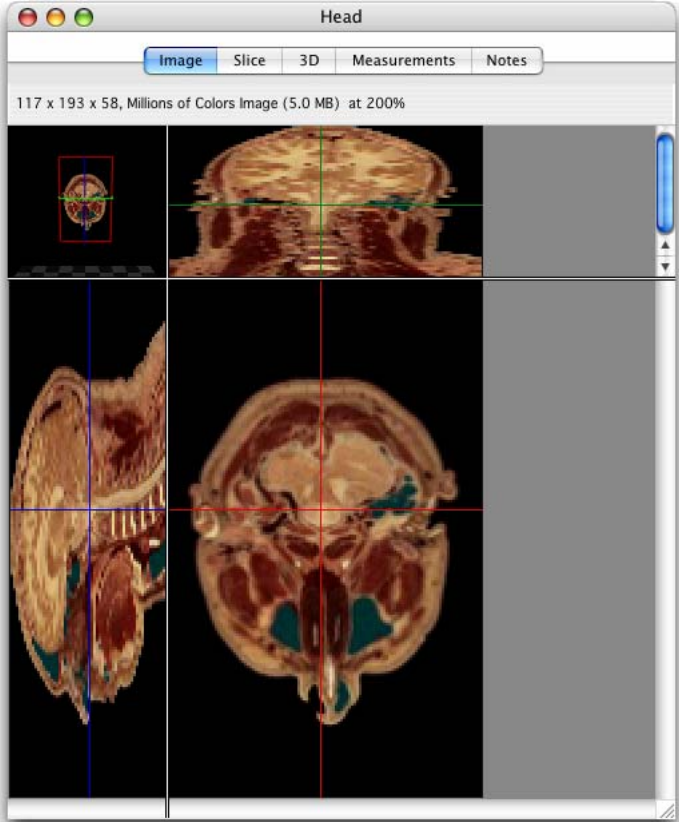


Shortcut key I

This tool is only available with a 3D Image View. Use the cross-hair tool to position the pointer on any of the planes and drag to explore the Image View moving through the different parts of the slices.

Note on the screen how the cross-hair colors are different for each plane and are also shown on the 3D preview in the top left-hand corner (the colors will show on the electronic version of this manual, but not on the printed version).

blue = YZ plane
red = XY plane
green = XZ plane



XZ, green

YZ, blue

XY, red

Zoom and Pan Tools



Shortcut key Z

Use the Magnifying tool to zoom in on a region of interest. Click with the tool to zoom in. On a Mac, Alt-click zooms out again. On a PC, use Ctrl-click. On a 3D Image View, the zoom command allows you to magnify any of the three planes. If you zoom in on one of the planes, then all three planes will be magnified to the same factor and the 3D cross-section has a fixed, relative size.



Shortcut key H

On 2D Image and Movie Views, use the Hand tool to pan through a magnified image. On 3D Image Views, use the Hand tool to pan the XYZ planes. Over the 3D cross-section the cursor will always appear as a hand. Click and hold, then spin and release to make the preview tumble. Use Alt-click (Mac) or Ctrl-click (PC) and drag to zoom the 3D cross-section.

View Options

The View Options dialog is available in the Image menu. This is a tabbed dialog with options for:

- **General:** showing line length with line drawn using the line tool and the color that image timestamps are shown on the Image view.
- **Color Reference:** to change the nature of the color reference overlay, its orientation, color and caption.
- **2D- Scale:** to change the style, color and labeling of the scale bar overlay.
- **ROI:** to change the appearance of ROIs in the image view

Selection Tools

Use the Selection tools to draw regions of interest (ROIs) and lines on the image. Although the tools are available with all versions of Volocity, they are mainly used for measuring with Volocity Quantitation. Refer also to the chapter on "Volocity Quantitation" for worked examples using these tools for measurements.



Shortcut key R

The Rectangular ROI selection tool is available for all image data. It allows you to draw rectangles or squares.



Shortcut key S

The Lasso tool allows you to trace the outline of a selection. The Lasso tool selects regions based upon the color similarities of adjacent pixels. The initial point clicked sets the color to compare and the tool has the effect of shrinking around the pixels that fall outside of the tolerance value.



The Elliptical ROI selection tool allows you to draw ovals or circles.

Shortcut key C



The Freehand tool is available for all image data. It allows you to make a freehand selection of an area on the image.

Shortcut key F



The Magic Wand tool is available for all image data. It allows you to select regions based upon the color similarities of adjacent pixels. The initial point clicked sets the color to compare. This tool has the effect of expanding outwards from the original color.

Shortcut key W



The Line tool is available for all image data, but is of limited use without the Velocity Quantitation functionality. Use it to add lines in the Image View. The tool draws a straight line between points that you specify by holding down the Shift key and clicking with the cursor. You can have any number of points in a single draw action, as long as you hold down the Shift key. When you release the Shift key, Velocity assumes that the last click was the end point.

Shortcut key L

Tips on Using the Selection Tools

Once you have selected an area, you can also use the **Copy** command from the Edit menu. **Cut**, **Paste** and **Clear** commands are only active if the item is adopted (see page 96).

As long as the selection is still active, you can use the pointer to click in the selected area and drag it to another part of the image.

The following key combinations also apply:

Macintosh

- Control-drag on a selection centers the selection on the image.
- Command-drag locks the dimensions, to make a square or circular selection rather than oblong or oval.
- Shift allows you to add to the ROI or draw multiple ROIs.
- Shift + alt (option) allows you to subtract from the ROI or subtract the whole of the existing ROI by drawing around it.
- Alt (option) carries out an AND operation leaving only the intersection of the two ROIs.

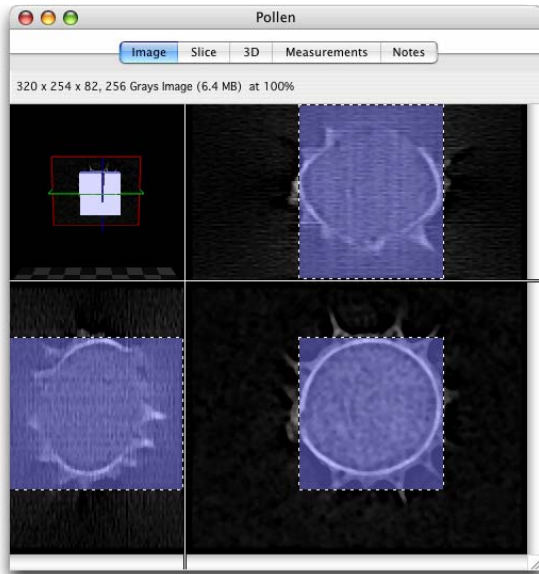
Image View

Windows

- Shift allows you to add to the ROI or draw multiple ROIs.
- Shift + ctrl allows you to subtract from the ROI or subtract the whole of the existing ROI by drawing around it.
- Ctrl carries out an AND operation leaving only the intersection of the two ROIs.

ROI Options

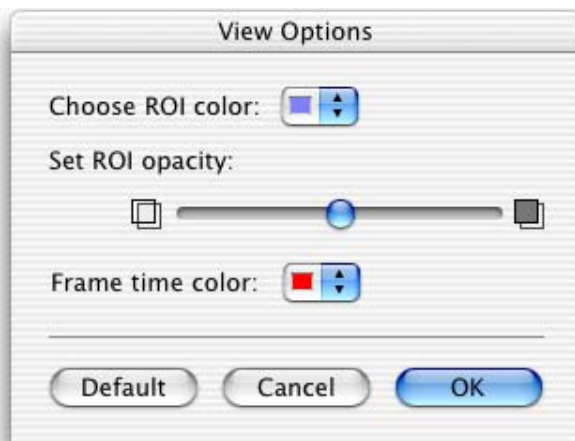
The ROI is shown as marching ants on the Image View with color projected onto the image to highlight the area of interest. You can define the color and transparency used for the ROI.



marching ants
outline the ROI

color block
shows projection
of ROI on each
plane

1. Select **View Options...** from the Image menu.



2. Use the ROI color drop-down menu to select the required color and use the slider control to set the ROI opacity.
3. Click on **OK**.

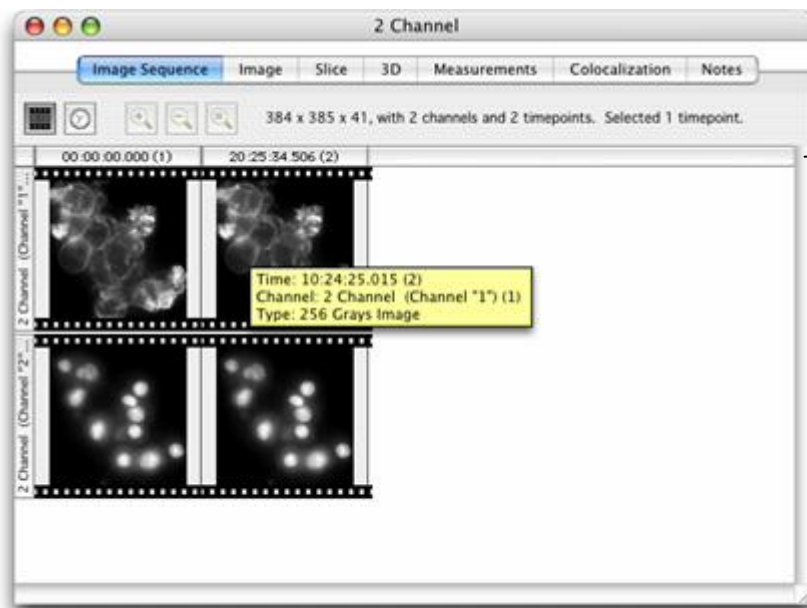
Image Sequence View

Image sequences are collections of images arranged by channel and timepoint. Multi-channel and/or time-resolved data with one or more planes in z is best viewed using an image sequence.

Image sequences are created when the data is added to a library or by dragging all the related images onto a new Image Sequence View.

The Image Sequence View shows the brightest point merge of data for each channel and timepoint as thumbnails. The header information shows the XYZ dimensions of the source image data and the number of channels and timepoints in the sequence.

1. Click on the title bar of any channel or timepoint to select it.
2. Hold the cursor over an item in the Image Sequence View to show a tip that gives the timestamp, channel and type



Note the time stamp at the top of each timepoint.

Channels run horizontally across the view. Channel 1 has been selected.

Timepoints run vertically down the view.

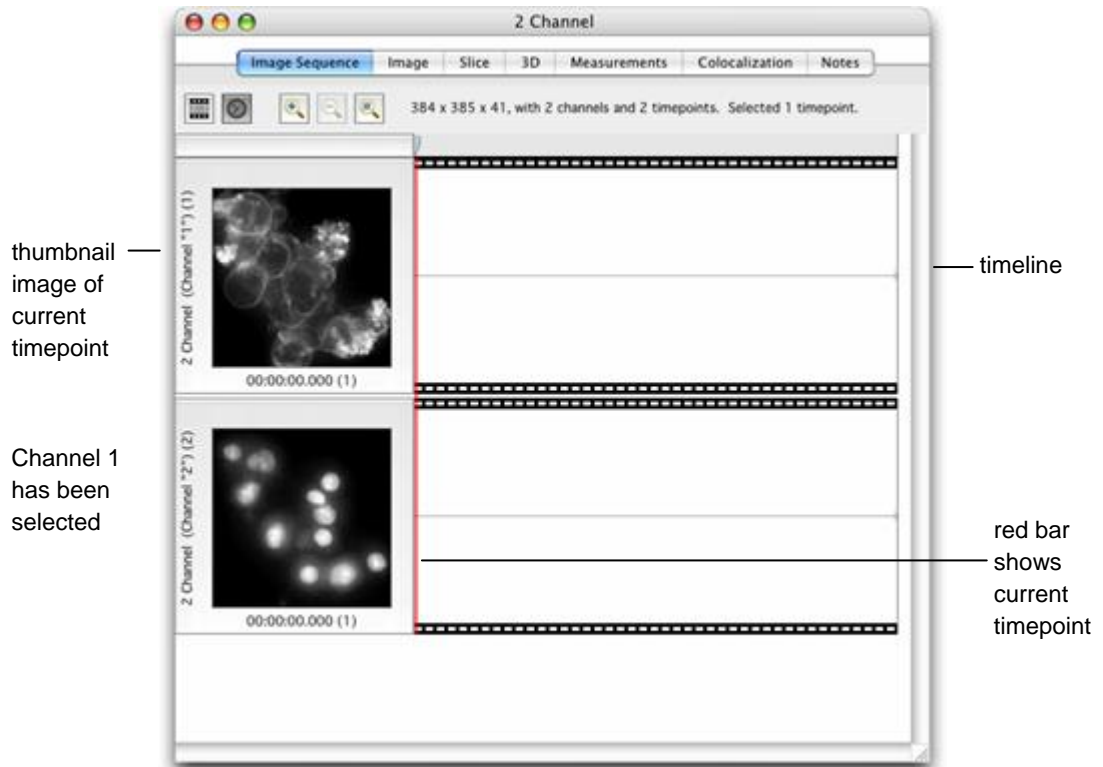
- To remove a channel or timepoint select it and either choose **Clear** from the Edit menu or use the backspace or delete keys.
- Double click on any channel to rename it. Type in a new name and click on **Change**.

Image Sequence Timeline View (not available in Volocity LE)



The Timeline View provides a way of viewing time-resolved image sequences. Click on the **Switch to Timeline** button at the top of the Image Sequence View.

Each row in the Timeline View represents a channel in the image sequence. At the left of the row is a thumbnail image of the current timepoint. To the right of this thumbnail is a “timeline”. On the timeline a vertical red bar signifies the current timepoint.



Use the Navigation palette to ‘play’ the timeline view of the image sequence. The red bar will move across the timeline and the current timepoint thumbnail on the left will update as the timepoint is changed.

Note: The red bar can also be moved by using the mouse to drag it across the timeline.

Image Sequence View

Relative and Absolute Times

Thumbnails in the Image Sequence View are labeled with a timepoint number and timestamp. The display of these timestamps can be altered to show:

- **Absolute Times:** the timestamp given to the volume or the image, or
- **Relative Times:** when timepoint one is designated as time zero and all other times are displayed relative to that.

Use the appropriate option in the Sequence menu to choose which time is shown.

Merge Channels

This command creates a brightest point merge of the selected channels in an Image Sequence.

1. Select two or more channels in an image sequence and select **Merge Channels** from the Sequence menu.
2. A new channel will be created in the sequence. The new channel will have the same dimensions as all other channels in the sequence, and the previously selected channels remain unaltered. The new channel will be named "Merged Channel".

Copy Timepoints to a New Sequence

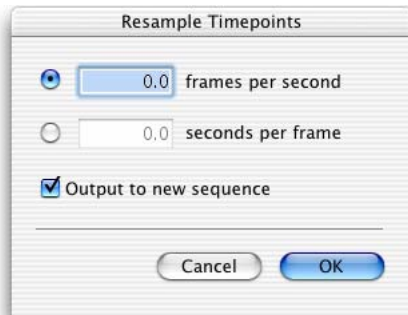
Create a copy of part of an image sequence by dragging the portion required into the library.

1. Select the timepoints that you wish to copy, using the Timeline View, as described above.
2. Click and hold the mouse button on the column header of one of the selected columns.
3. Drag the selected timepoints out of the sequence and into the library.
4. A new image sequence will be created containing the selected timepoints. The new image sequence is named appropriately to indicate which timepoints were selected.

Resample Timepoints

This command allows the image sequence to be resampled according to a desired frequency of timepoints. This will reduce the number of timepoints in the sequence and therefore its size. A reduced number of timepoints may assist analysis of a time-resolved sequence.

1. Select **Resample Timepoints...** from the Sequence menu.



2. Click the appropriate button to resample timepoints according to a desired number of **frames per second** or **seconds per frame**.
3. Check **Output to a new sequence** if the results are to be used to create a new image sequence; it will have "(resampled)" appended to the name.
4. Click **OK**.

Note: If timepoints in the image sequence are already at or below the selected frame rate, **Resample Timepoints...** will make no change.

Image Sequence View Zoom

The Timeline View can be zoomed to view more or less of the sequence in the window. This is done using the three buttons at the top of the Image Sequence View: Zoom In, Zoom Out, and Zoom to Fit.



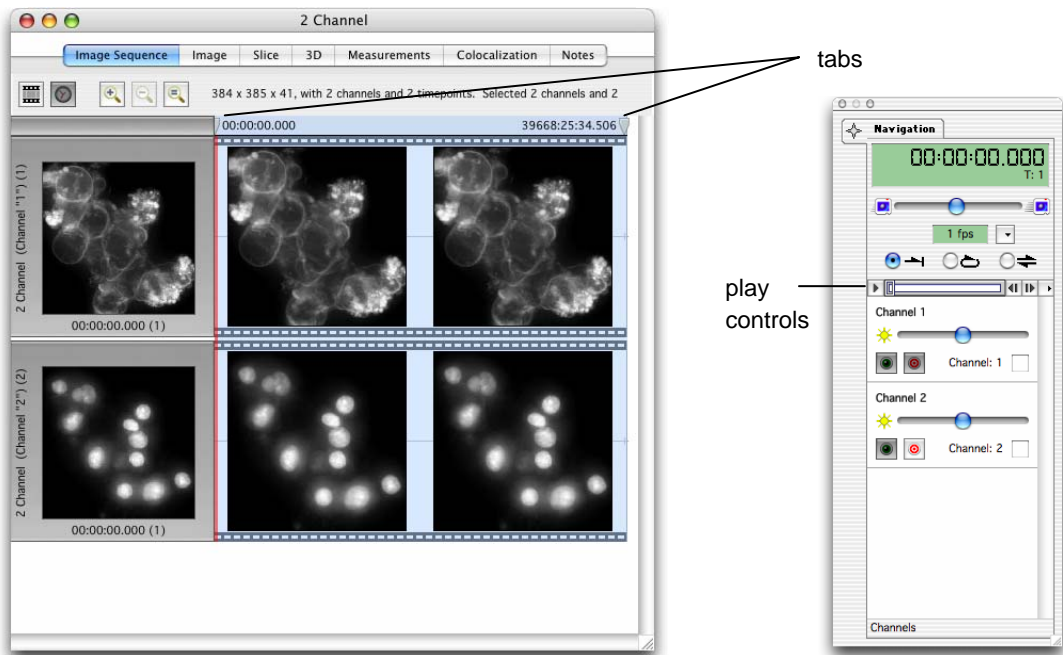
Image Sequence View

Play Back Timepoints

Use the Navigation Palette movie type play controls to play through the thumbnails of each timepoint.

You can also make a selection in the image sequence and use the Navigation palette to play back the selected timepoints.

Click and drag the tabs at the top of the timeline. A shaded selection will appear between these tabs, corresponding to the period of time selected. If space permits, the start and end timestamps of the selection will be shown, and a thumbnail of the first and last images in the selection. A shaded selection will also appear on the slider control in the Navigation palette.

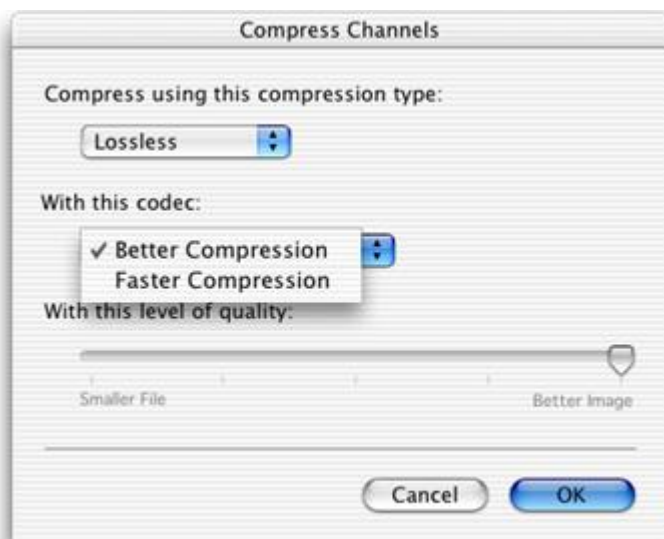


Compress Channels

This option allows you to compress channels within image sequences, which will reduce the file size of libraries containing large image sequences.

Note: Only image sequences created in version 2.5 or higher may be compressed.

1. View an image sequence and select the channels to be compressed.
2. Select **Compress Channels** from the Sequence menu.
3. Select the **compression type**, and the **codec** (the encoding to use) for that compression type from the drop-down menus. When using the compression type, QuickTime, the codec drop-down will offer all installed QuickTime codecs.



Lossless compression means that no data is lost from the image. The data compressed in this way will remain effectively unaltered. The **Better Compression** codec will result in smaller file sizes, while the **Faster Compression** codec will result in less delay when opening image sequences.

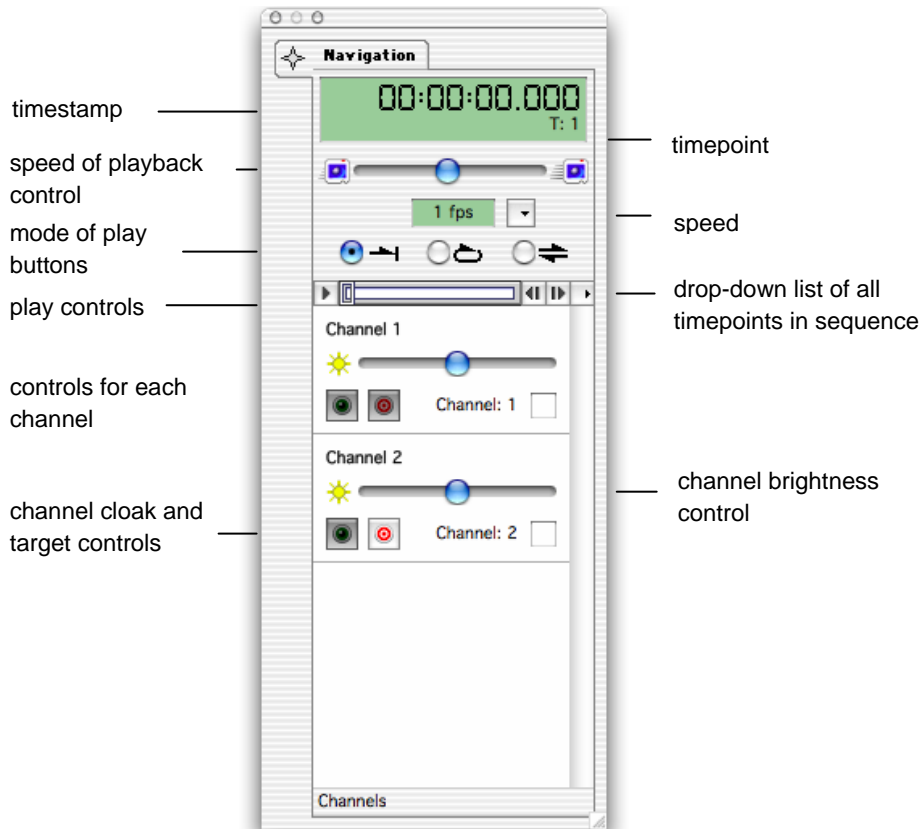
When compressing 8-bit channels, **QuickTime** compression is available. Not all QuickTime codecs are lossless, which means that data may be slightly altered after compression; however, it will usually result in an efficient and fast data compression of large sequences. When using QuickTime compression, use the slider bar on the dialog to choose a **Smaller File** size or a **Better Image** with less data loss. Using lossy compression on a data set will affect the results of measurements, deconvolution, and such like, therefore, it is not recommended for data that is to be processed further.

4. Click **OK**.

The Navigation Palette

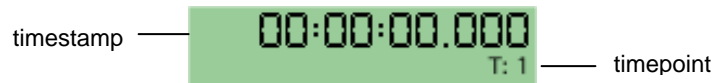
The Navigation palette is available whenever you are working with an image sequence. If it is not visible select **Show Palette** from the Window menu.

Note: The Navigation palette is also available with Movie Views.

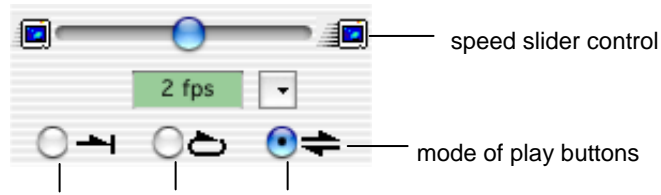


The Navigation palette is useful if you want to play back an image sequence in the Timeline, Image or Rendering views and provides the following controls.

- The digital display gives the timestamp of the image and the timepoint number in the sequence.

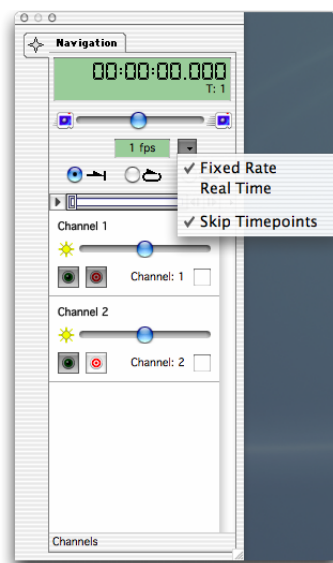


- Select the required mode of play by clicking on the appropriate button, and adjust the speed, if required, by using the slider.



once through loop shuttle

- Click on the selection button to switch between **Fixed Rate** and **Real Time**, and to **Skip Timepoints**, if required.



Use the slider to set the multiple of real time, based on the timestamp of the image. Volocity will play the sequence at the desired multiple, even if the time gap between successive frames is uneven. When playing back large data sets or attempting high rates of playback, there may not be enough capacity to maintain the rate shown in the palette and play back will slow. Select **Skip Timepoints** to maintain the rate required.

The Navigation Palette

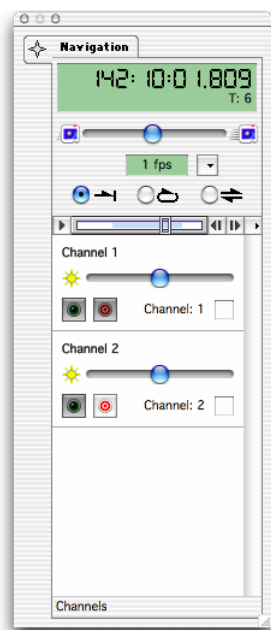
- Use the play controls to play through the volumes in the sequence.



- Use the drop-down list of timepoints for rapid viewing of the required timepoint.



- To play only a selected range selection hold down the Shift key and click on the slider area. Drag to mark the required range. A shaded selection will appear.



If the loop option is selected as the mode of play, the sequence will start again from the beginning of the selected area, once the end of the selected area is reached.

- While you play the sequence, you can use the channel slider control to adjust the brightness on individual channels, as required.



- The two buttons in the channel controls allow you to show or hide the channel, and to target a channel for selections.

show/hide channel —   — target channel for selections

Some tools make different selections depending on which channel is used as the basis for selection. For example, the magic wand tool works on voxel intensity so the size and shape of the selection made by the tool can be very different according to which channel is targeted when the selection is drawn.

The show/hide button cloaks and uncloaks the channel in the Image Sequence View so you can choose to work with a channel or not. If a channel is cloaked in the Image Sequence View, it is also cloaked in the Image View.

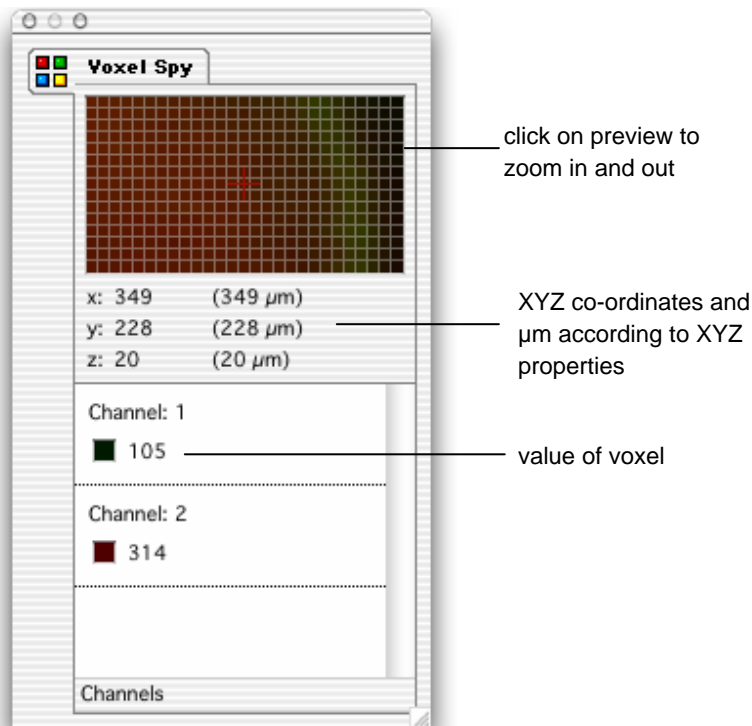
Voxel Spy Palette

The Voxel Spy palette may be used on any Image View. For the voxel under the cursor and for each channel, it shows:

- The X, Y and Z co-ordinates.
- The color according to the active look up table.
- Value(s).

Choose **Show Palette** from the Window menu, if the Voxel Spy palette is not already visible on the screen, and position the cursor on the Image View at the point of interest.

Click on the preview area in the palette to zoom in and out on the voxel.

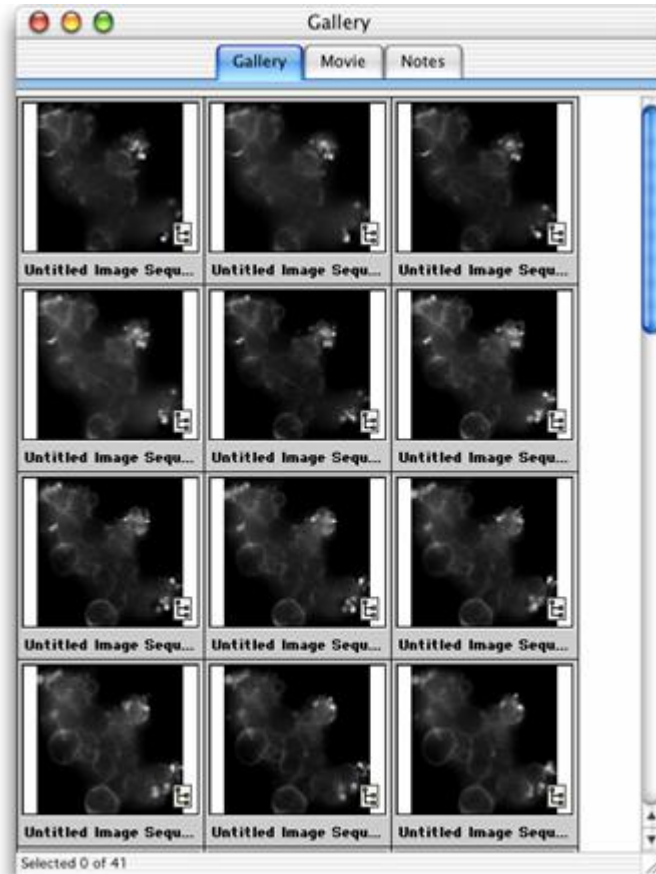


Note: Values shown for voxels are typically intensity values but may represent other properties depending on the source of the channel.

Gallery View

If you double click on a folder in the library, you will see the contents of that folder in a Gallery View.

Use the scroll bars to move through all the contents of the gallery. Once you have displayed a Gallery View of image items, you can use the Movie tab to play these frames as a movie in the Movie View. The Notes tab is used to store text. It is described on page 91 and is common to all items in a library.



Note: Double click on an image item within the Gallery View to display that item.

Gallery Menu and Toolbar

Whenever a Gallery View is active, there is a Gallery menu and a Gallery toolbar, which contain relevant commands and tools.

Gallery View

Gallery Options

When you have a Gallery View active, you will be able to use the Options command under the Gallery menu. This command allows you to set the display options and table style for the gallery. It is particularly useful if you want to print or publish the Gallery View.



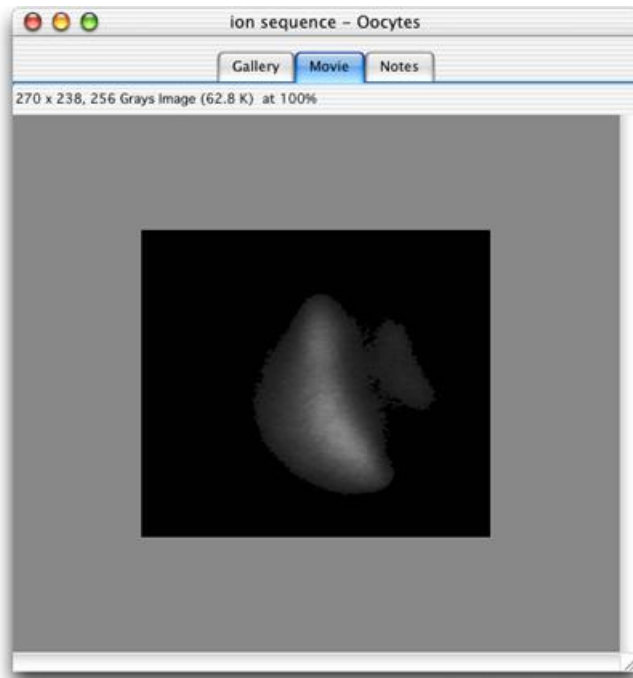
Select a table style of **Normal** or **Minimalist** (which removes the gray border).

Check the appropriate display options and click on **OK**. The "source" icon indicates the location of the source image data and, when selected, appears in the bottom right-hand corner of each image in the Gallery View. If you select a Minimalist table style, you will also be able to show/hide the thumbnail borders.

Movie View

Once you have displayed a multi-layered item in the Gallery View, you can also play the contents of that gallery as a movie.

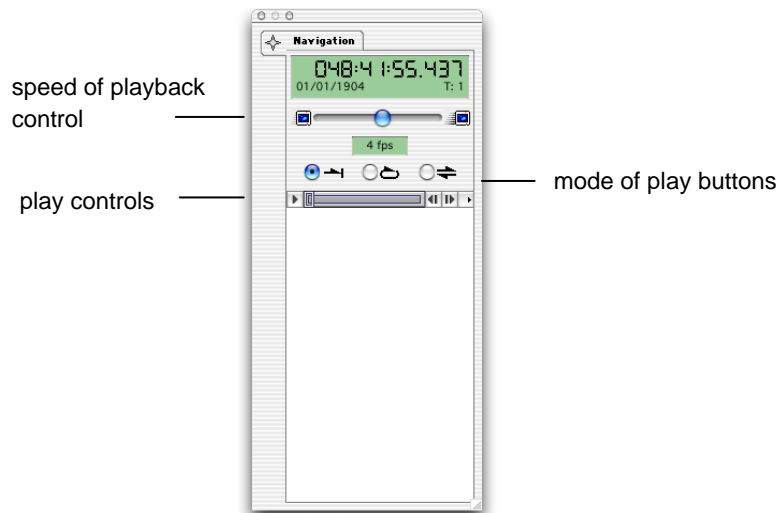
1. Click on the Movie tab.



2. Use the Navigation palette to play the movie.

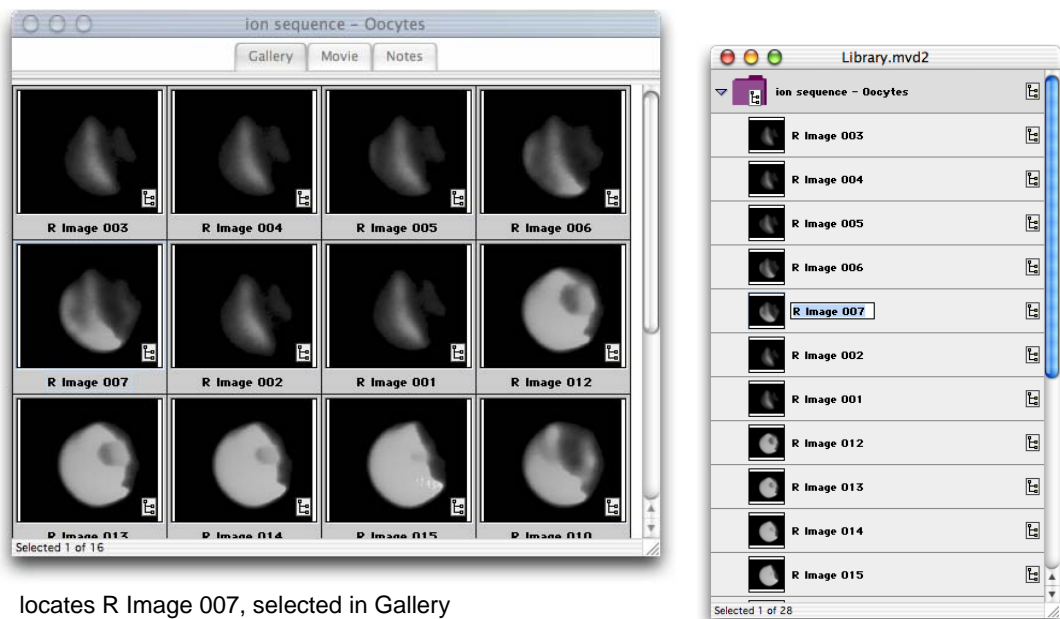
See page 82 for a detailed description of this palette. Note that in Movie mode the palette does not show any controls for individual channels as these are not relevant.

Show in Library



Show in Library

If you have a single image displayed in the active window or a single image selected in a Gallery View, this command highlights the image in the library structure. From a Movie or Gallery View (with no images selected), it highlights the folder or multi-layered file in the library structure.



locates R Image 007, selected in Gallery View, in the library

Notes View

For all items in a library, there is a Notes View. You can add notes to any library item and view or edit the notes at any time. To add or edit notes, just select the Notes View and type in the text. You can use the **Copy** and **Paste** commands from the Edit menu and you can also print the notes, using the **Print** command from the File menu.

Capture Snapshot Command (View-Specific menu)

Although print commands are available from the 3D Views, they are not useful for publishing volumes. However, **Capture Snapshot** in the Image and/or 3D menu allows you to capture 2D snapshots of the 3D image data that you can then print.

When used with the Image View, **Capture Snapshot** captures a 2D snapshot of the 3D image data. It creates a new folder in the library containing four 2D images: the XY, XZ, and YZ planes plus the 3D cross-section.

The **Capture Snapshot** command also captures any scale information, lines or ROIs that are displayed on the active view.

Volocity Visualization users only

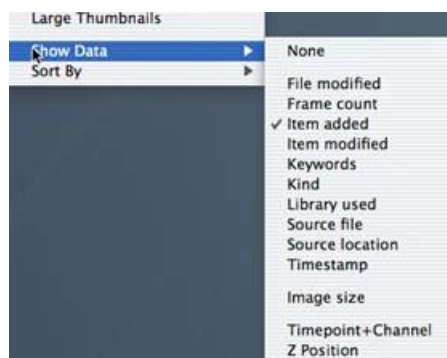
When used with the 3D View, **Capture Snapshot** creates a single 2D image and adds it as a new image to the library. If you want to publish a volume and preserve the 3D effect, use the **Create QTVR Movie...** command from the Tools menu.

Show Data (View-Specific menu)

Show Data (View-Specific menu)

You can select one item of data to display next to a library item. In the Library and Gallery Views the data appears below the thumbnail. For the Image and Movie views on the Mac the data appears just below the tab in the view-specific window; on a PC data appears in the status bar in the bottom right-hand corner of the application window.

1. Select **Show Data** from the view-specific menu.
2. Select a property from the drop-down menu.



3. The additional data is displayed. For Image and Movie views, you may need to resize the window to see all of the data.

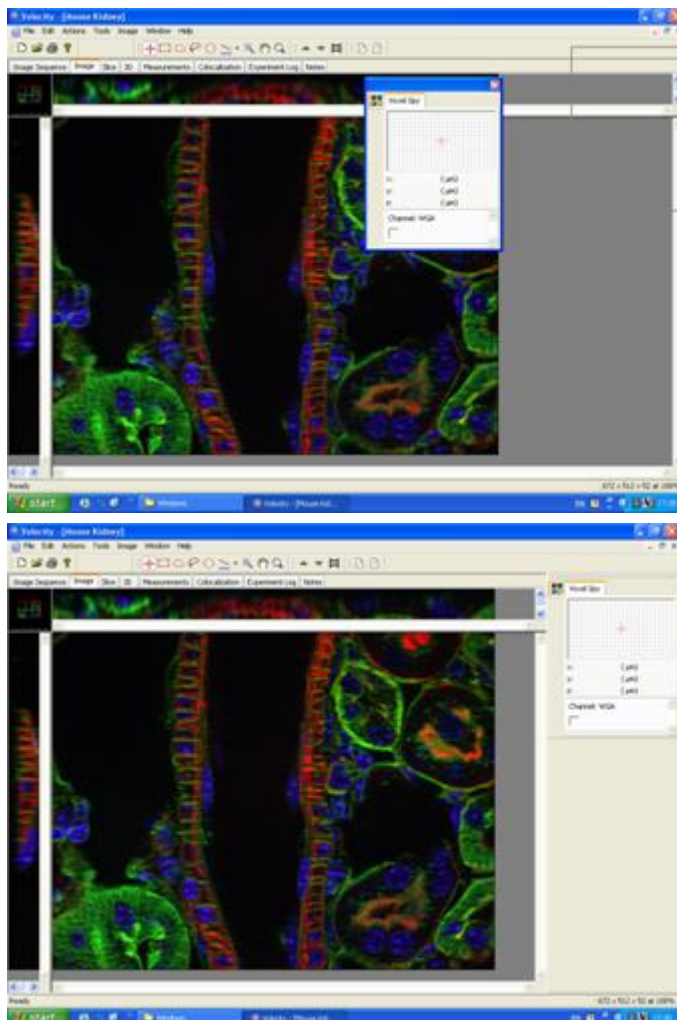
Sort Items (View-specific menu)

You can sort items in a number of different ways.

1. Select **Sort By** from the view-specific menu.
2. Select a property or image brightness or color as the sort criterion.
3. A progress meter is displayed briefly as the application sorts the images and rearranges them.

Docking Palettes (Windows Only)

Palettes may be docked to the left or right-hand edge of the application window. Drag the palette to one side of the application window and release it when the outline of the palette changes. In the example below the Voxel Spy palette is dragged to the right and will dock to the right-hand side of the application window when the mouse is released.



Open views will be sized accordingly. Palettes may be further docked on top of each other into the same window or stacked vertically in their fixed location. Palettes may also be arranged side by side. Drag the palette back onto the main window to release it.

Docking Palettes (Windows Only)

Manipulating Data

Overview

The actions described in this chapter are applicable to both 2D and 3D image data. The commands are ordered alphabetically.

- Undoing Commands
- Batch Processing
- Adopting Items (Actions menu)
- Applying and Uses of Auto Contrast (Tools menu)
- Changing Color Depth (Tools menu)
- Changing Colors (Tools menu)
- Changing Size (Tools menu)
- Cloaking Items (Actions menu)
- Color Balance (Tools menu) (Not available in Volocity LE)
- Contrast Enhancement (Tools menu) (Not available in Volocity LE)
- Creating Background Corrections (Actions Menu)
- Correcting Background (Tools menu)
- Creating Registration Corrections (Actions menu)
- Correcting Registration (Tools menu)
- Cropping Items (Actions menu)
- Merge Planes (Actions Menu)
- Removing Noise (Tools menu)
- Splitting Multi-Colored Items (Actions menu)
- Splitting Volumes (Tools menu)

Undoing Commands

The Edit menu has an Undo command that allows you to undo the most recent actions. It supports multiple undo actions, starting with the most recent and working backwards in sequence.

Batch Processing

Most image processing and enhancement tasks may be applied to batches of image sequences. Typically, the user will select a series of image sequences in the Library view and the dialog for the process concerned will present a list of unique channel names against which the user selects a process. If channels or items are selected, the option to change selected items will be presented.

Adopting Items (Actions menu)

Permanent changes to data may only be made if that data is saved in the library rather than linked to a source file which remains outside the library. Permanent changes include changing data size, cropping data and many image processing tools. Changing a file so that it is part of the library is called adopting. Adopting increases the library file size.

1. Select the required item(s) in the Library or Gallery window.
2. Select **Adopt Items** from the Actions menu. The application searches for the source item(s). If they are not on the hard disk, it will prompt for the removable medium. It will be unable to adopt an item that was once on the hard disk but has since been deleted or moved.
3. The icon indicating the location of the file changes to show that the file is in the library.



Note how the icon on the right shows that the images are now held within the library structure.

If you adopt a single layer within a multi-layered item, all layers within that item are adopted.

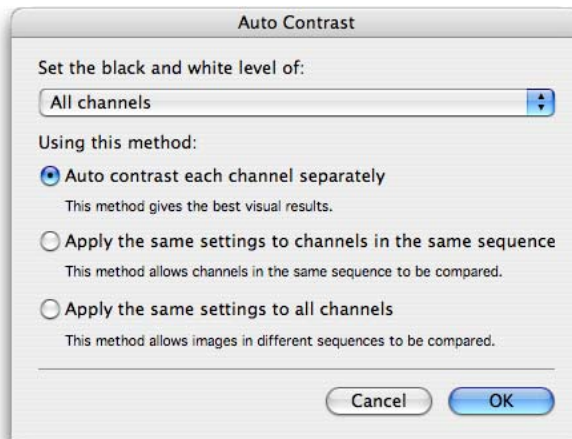
Note: There is no Unadopt command. You can use the Undo command in the Edit menu to undo the most recent action(s), but you cannot select items and expressly apply an Unadopt command.

Applying Auto Contrast (Tools menu)

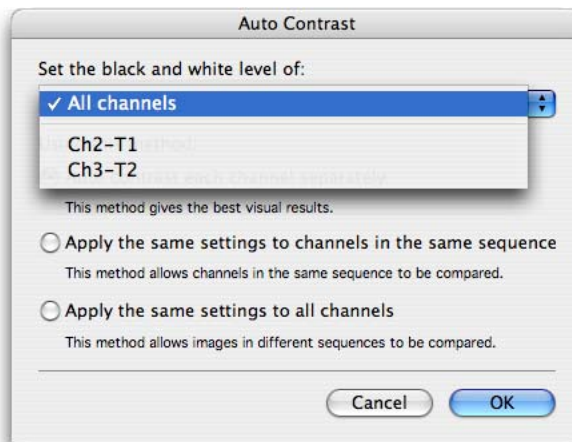
Auto Contrast automatically sets the black and white points of the display image.

Auto Contrast finds the brightest and darkest pixels or voxels in an item and modifies the lookup table so that the brightest point is at the maximum display value, the darkest point is at the minimum display value and all others are adjusted according to a linear best fit. The underlying intensity values within the data are not changed.

1. Select the required item(s). Multiple image sequences can be processed at once.
2. Select **Auto Contrast** from the Tools menu.



3. The Auto Contrast dialog will offer a list of available channels. Selected channels, if applicable, or all channels may be adjusted.



Changing Color Depth (Tools menu)

4. Choose the appropriate option depending on how data will be compared. The dialog explains the options.

To manually adjust:

- Black and white points: use **Contrast Enhancement**, see page 103.
- Gamma: use **Contrast Enhancement**, see page 103.
- Red, green and blue contributions in color images: use **Color Balance**, see page 102.

Uses for Auto Contrast

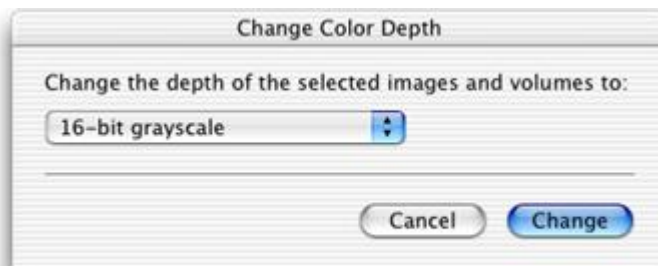
Auto Contrast provides a tool to adjust the LUT on imported data that appears extremely dark or completely black due to a mismatch between LUTs from different software applications.

Auto Contrast can be applied to any item. However, as the contrast achieved depends on the brightest point in each item, the result of applying Auto Contrast to a series of 2D sections (many separate items) will be very different from the result of applying it to a volume (one single item) made from them. In most cases you will want to reapply Auto Contrast after making a volume or image sequence so that the whole data set is treated in the same way.

When working with Auto Contrast, it is important to note that a single voxel that is very much brighter than any other will be enough to set the brightest level. This can lead to Auto Contrast causing no apparent change to data.

Changing Color Depth (Tools menu)

1. Select the required item(s.) Multiple image sequences may be processed at once, simply select them in the library. Select **Change Color Depth...** from the Tools menu.
2. Click on the drop-down menu and select the required color depth.

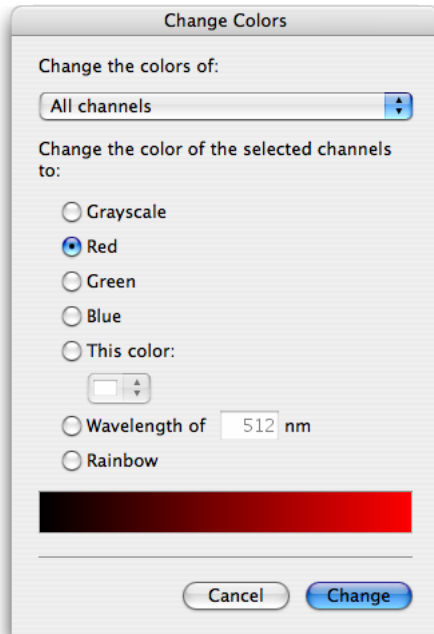


3. Click on **Change**.

Changing Colors (Tools menu)

This command allows you to apply a different color lookup table to images, it does not change any underlying data. Multiple items may be changed in a batch by selecting them all in the library view.

1. Select the required item(s) and select **Change Colors...** from the Tools menu.



2. Select the channel or channels to change
3. Click on the appropriate grayscale or RGB color. You can also click on **This color** and select a color from the drop-down menu, enter a wavelength value or apply a Rainbow LUT.
4. Click on **Change**.

Changing Size (Tools menu)

Use **Change Size** to scale data changing the spatial resolution of the data and preserving the field of view.

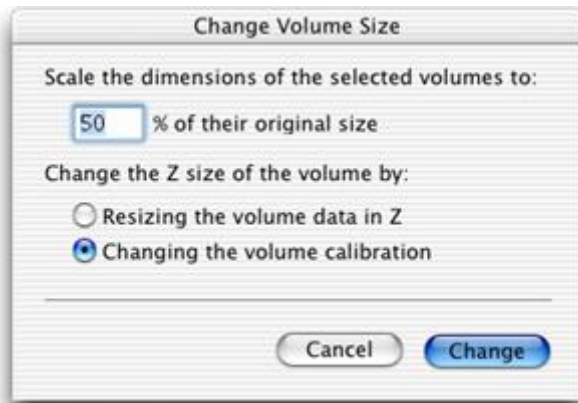
With 3D image data you can change the size of a volume in all axes, which uses less memory and saves time for some processes.

Where resolution of data is important, for example when performing measurements or restoration it is preferable to preserve resolution and crop data instead, using the **Crop to Selection** command in the Actions menu.

To scale data when making single volumes see page 46.

The following example illustrates how to change the size of 3D items. (The process is the same for 2D items, but step 3 is not required.)

1. Select the required item(s) and select **Change Size...** from the Tools menu.

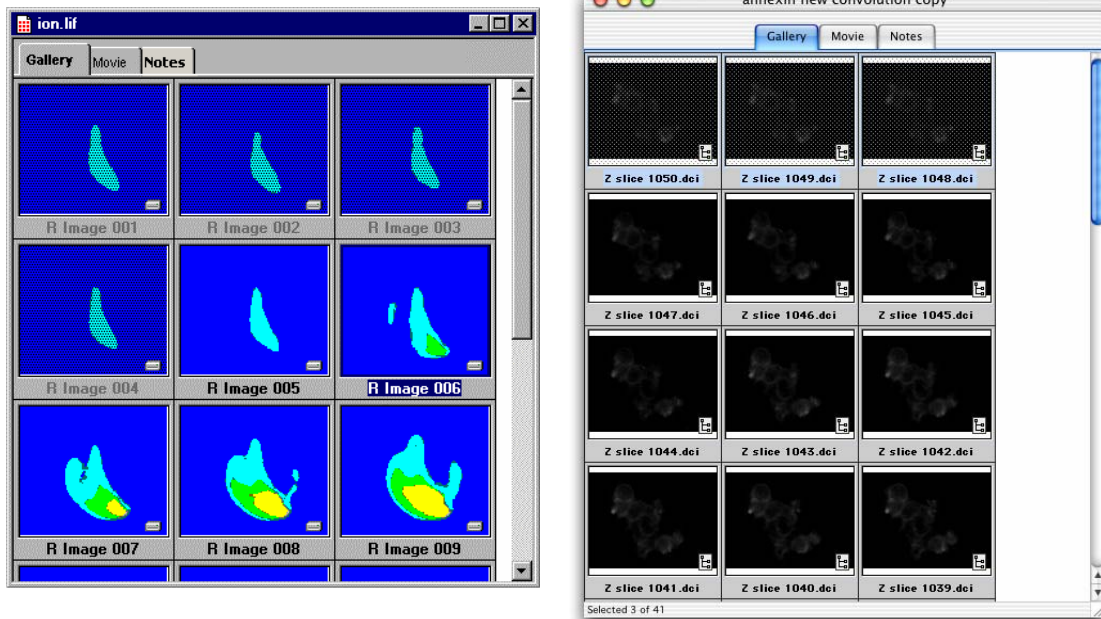


2. Enter a scaling factor.
3. For 3D images only, enter a scaling value as a percentage. The X and Y dimensions are scaled automatically, but you can choose how you want to change the Z size of the volume.
4. Select **Resizing the volume in Z** to change the amount of data in the volume by discarding some of the z slice data. This command changes the number of frames in z.
5. If the volume is short of z data, select **Changing the Z calibration** to retain all of the data but recalibrate the volume in z. This command does not change the data. It maintains calibration, which is taken into account in other processes.
6. Click on **Change**.

Cloaking Items (Actions menu)

Temporarily hide an image to exclude it from movie playback or inclusion in an image sequence by cloaking it. Show or Hide channels in image sequences by cloaking them.

1. Select the item(s) that you wish to hide in the Library or Gallery View. Select the channel in the Image Sequence View.
2. Select **Cloak** from the Actions menu. Note how the icon changes to show that the images are now cloaked. On both Mac and PC, the shading indicates a cloaked item. On a PC, the item's title is also grayed out.

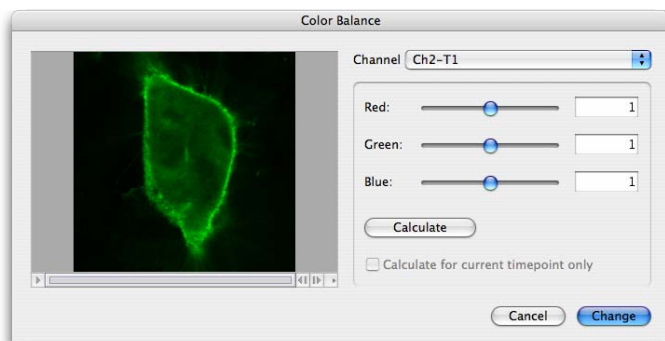


To Uncloak the item select it again and choose **UnCloak** from the Actions menu.

Color Balance (Tools menu)

Color Balance is a dialog that allows the user to adjust the contributions of red, green and blue in a color image.

1. Select the item(s) to be adjusted in the library.
2. Select **Color Balance...** from the Tools menu.

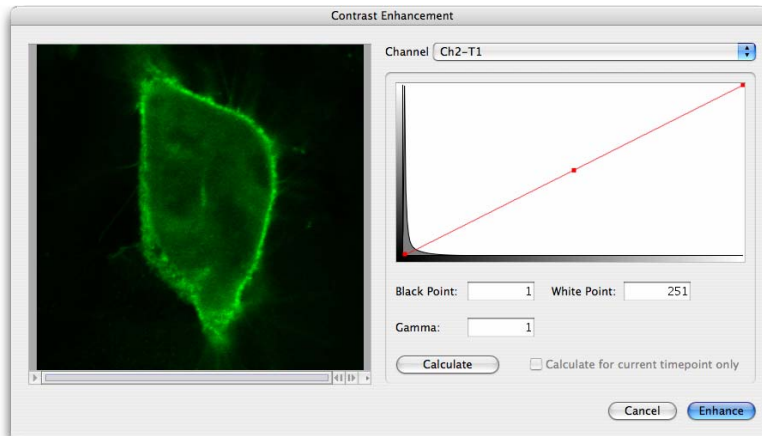


- If multiple 2D images are selected, the preview shows the first in the list; the same contrast enhancement settings are applied to all images.
 - If multiple 3D volumes are selected, the preview shows a brightest point merge of the first in the list; the same contrast enhancement settings are applied to all images.
 - If multiple image sequences are selected, the preview shows the first of the selected sequences. Only one channel at a time is previewed as a brightest point merge. The drop-down at the top of the screen shows a list of the unique channel names detected in the selected sequences. Controls under the preview allow navigation to different timepoints.
1. Use the sliders to increase or decrease the contribution of the colors to the final image.
 2. Adjust one channel at a time; clicking **Apply** will change the selected channel but keep the dialog open allowing more changes to be made. Only one channel at a time may be enhanced but channels with the same name in multiple sequences may be enhanced in a batch.
 3. Click **Change** to apply the last change and close the dialog

Contrast Enhancement (Tools menu) (Not available in Velocity LE)

Contrast Enhancement is a dialog that allows the user to manually set the black and white points of the display lookup table for an image, thus changing the contrast. It also allows gamma adjustment which will set a non-linear distribution of that lookup table between the black and white points.

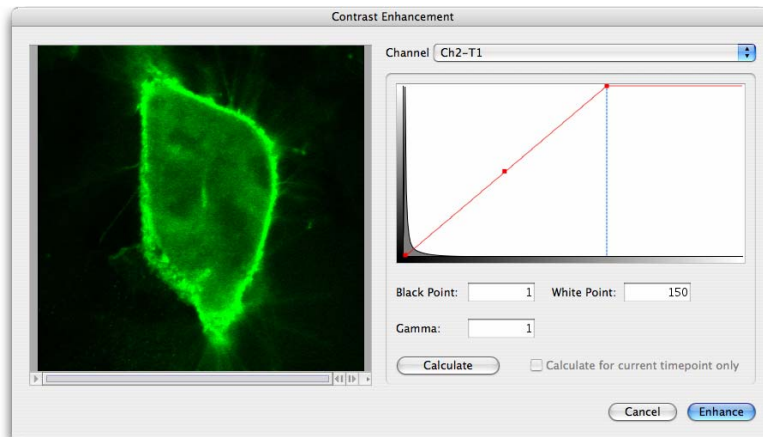
1. Select the required item(s) in the library. If working with an image sequence, select the channel to be enhanced.
2. Select **Contrast Enhancement...** from the Tools menu.



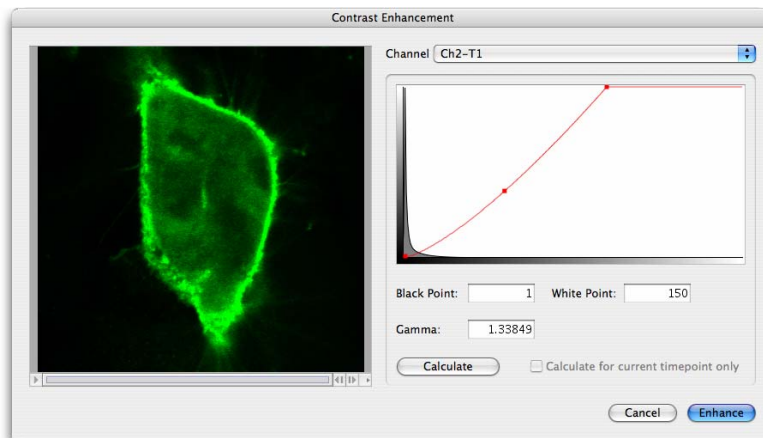
- If multiple 2D images are selected, the preview shows the first in the list; the same contrast enhancement settings are applied to all images.
- If multiple 3D volumes are selected, the preview shows a brightest point merge of the first in the list; the same contrast enhancement settings are applied to all images.
- If multiple image sequences are selected, the preview shows a brightest point merge of the first channel in the first sequence. A drop-down at the top of the screen shows a list of unique channel names detected in the selected sequences. Controls under the preview allow navigation to different timepoints, if applicable.

Contrast Enhancement (Tools menu) (Not available in Volocity LE)

The red line across the histogram shows the current relationship between the input intensity values (X-axis) and the output LUT (Y-axis).



Use the control points (red squares on the red line) to move the black, white and gamma points.



- Change the Y-axis of the histogram to see detail more clearly, cycling through four possible resolutions; linear, log, base 10 and base 100. To do this:

PC: Ctrl-click in the histogram area

Mac: Alt-click in the histogram area

3. Click **Apply** to enhance all the channels with the selected name and leave the dialog open so that other channels may be enhanced.

Calculate black and white points based on the darkest and brightest intensities in the data set by clicking on the **Calculate** button. For time series data choose to set these using just the current timepoint.

Note: The controls on the Contrast Enhancement dialog cannot be used on data sets that have been colored with rainbow colors, see page 99.

4. Once you are satisfied with the adjustments in the preview area, click on **Enhance**.

To adjust contribution of red, green and blue in a color image use Color Balance, see page 102.

Background Corrections

In order to perform accurate analysis of your images or to improve their appearance, you may need to perform **Background correction**. Background corrections include dark current correction, background subtraction, shading correction or offset application. Bright or dark reference images may be required for your correction.

Shading

Shading defects can be introduced into images by poorly aligned light sources, stray light and dirt in the light path.

Shading artefacts are corrected by including division of a bright reference image in your background correction. A suitable bright reference image for fluorescence images can be acquired from an illuminated, uniformly fluorescent sample. A bright reference image for bright field images can be acquired from a blank field (with no specimen present), which has been slightly defocused.

Dark Current, DC Offset.

Ideally, acquiring images from a digital camera in complete darkness would result in images with gray levels close to zero. However, this is not the case. A combination of factors including dark current and DC offset will result in gray levels 10s or 100s of levels from zero. These offset values are present in all images acquired with digital cameras.

This is of no consequence for purely observational experiments when only the appearance, position or size of structures is of interest. However, when the intensity of objects is of interest these factors introduce error to all measurements made. The margin of error is small compared to the intensity of very bright objects, however, it becomes increasingly significant the fainter objects of interest become.

A background correction that subtracts a “dark reference” image from all images corrects this problem, increasing the accuracy of all intensity measurements made. “Dark reference” images are acquired by blocking all light from the camera and acquiring an image. Ideally, the exposure time of

Creating Background Corrections (Actions Menu)

the “dark reference” should be the same as the exposure time of the experimental images to be corrected.

A background correction that subtracts a fixed value from all images could also be used to correct this problem, but this is likely to be less accurate than using a dark reference image.

Background (fluorescence)

Further error can be introduced into intensity measurements by the presence of background fluorescence in a sample. This can be corrected by building a background correction that subtracts a “dark reference” image from all images. In this case the dark reference should be acquired from an illuminated area of your sample in an area in which no cells (or debris) are present. Again, the exposure time of the “dark reference” should match the exposure time of the experimental images to be corrected. A “dark reference” image acquired under these circumstances would correct background fluorescence in addition to dark current and DC offset.

Defects that can be corrected are summarized in the table below:

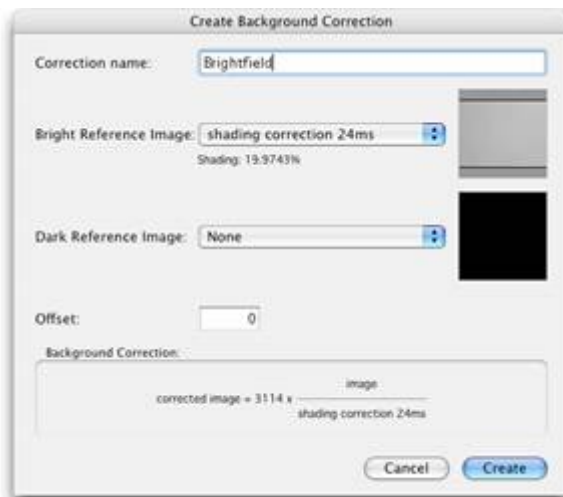
Defect	Correction	Image Required for correction
Shading (fluorescence)	Division with a bright reference	Illuminated uniformly fluorescent slide
Shading (bright field)	Division with a bright reference	Slightly-defocused bright field image (with no specimen present)
Dark current, DC offset	Subtraction of dark reference	Image acquired with light to camera blocked
Dark current, DC offset	Subtraction of a fixed value from every pixel	None, set a negative offset in the correction (this correction is less accurate than 3a.)
Background (fluorescence)	Subtraction of a background image as a dark reference	Illuminated sample with no cells in the field of view

Creating Background Corrections (Actions Menu)

You should acquire or add bright and/or dark reference images to your library before you build background corrections. Bright and dark reference images should be the same dimensions in X and Y, and the same depth as your images to be corrected.

1. Select **Create New** from the **Actions** menu.
2. Select **Background Correction...** to create a new background correction item in the library.

3. The Create Background Correction dialog opens.



You can use the dialog to create a variety of corrections. As you build your correction, it is summarized at the bottom of the dialog. When you first open the dialog the summary reads “corrected image=image” to reflect that you have yet to specify any corrections.

You may apply as many or as few elements of the equation as you wish in your corrections, depending on what you wish to correct for and how accurately you wish to do so.

Volocity uses the following equation when building background corrections:-

$$\text{Corrected Image} = \text{Scaling Factor} \times ((\text{Raw Image} - \text{Dark Reference} + \text{Offset}) / (\text{Bright Reference} - \text{Dark Reference}))$$

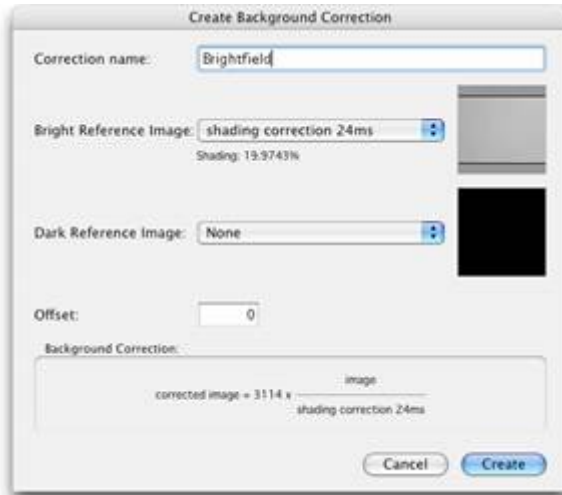
Bright Reference Images

If you wish to divide your raw images by a bright reference image, select a bright reference image from the drop-down menu. All of the images in the library will be displayed in the list. The degree of shading in the bright reference is displayed in the dialog using the formula:

$$\text{Shading} = (\text{Intensity}_{\text{max}} - \text{Intensity}_{\text{min}} / \text{Intensity}_{\text{max}}) \times 100\%$$

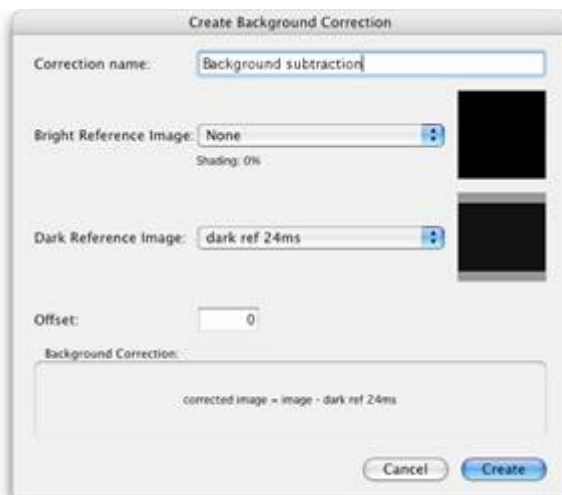
Creating Background Corrections (Actions Menu)

If you select a bright reference image, your corrected image will be multiplied by a scaling factor to bring its gray levels into a convenient range. The scaling factor is the brightest pixel value in the bright reference image (after subtraction of the dark reference image, if selected).



Dark Reference Images

To subtract a dark reference image from your raw images, and bright reference images if you have selected one, select an image from the dark reference drop-down menu. All of the images in the library are listed in the drop-down menu.



Offsets

To apply an offset to your images, enter a positive or negative value into the offset field of the dialog. Positive values will be added to raw images, negative values will be subtracted.

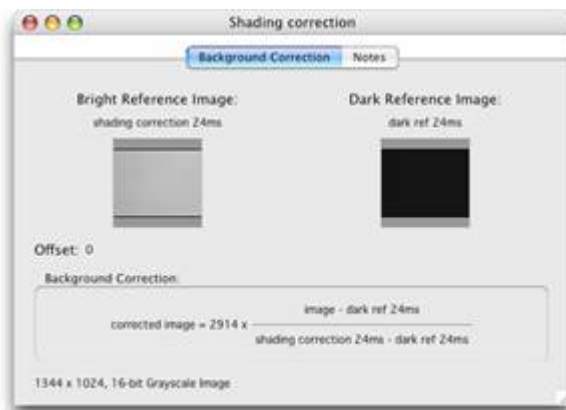
When you have built a satisfactory background correction, select **Create**. A new background correction item is added to the library.



Background correction items can be exported from libraries as library clippings and imported into other libraries.

Inspecting Background Corrections

Double click a background correction item in the library to inspect it. The background correction will open and you can review the names and thumbnails of images that were selected as either bright or dark reference images, as well as a summary of the background correction that will be applied.



Correcting Background (Tools menu)

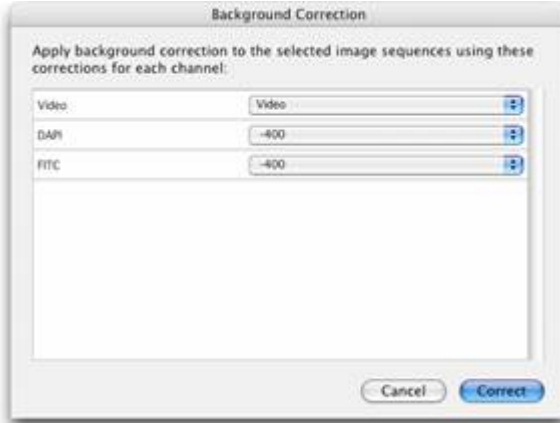
In order to perform accurate analysis of your images or to improve their appearance, you may need to perform **Background correction**. Background corrections include dark current correction, background subtraction, shading correction or offset application. Bright or dark reference images may be required for your correction.

Correcting Background (Tools menu)

For further information on background correction and the images required please see page 105.

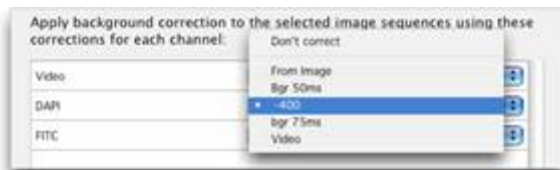
To use the **Correct Background...** command the library must contain appropriate background correction items. See page 105.

1. Select any images or image sequences that you wish to background correct.
2. Select **Correct Background...** from the Tools menu. The **Background Correction** dialog opens.



3. Velocity will attempt to match the names of the background correction items in your library to the names of the channels in your selected images or image sequences. If you have many correction items in your library, you may need to manually select the background correction by clicking on the drop-down menu for each channel and making a more appropriate selection.

Background corrections can be applied to 3D image sequences in the same way that they are applied to 2D datasets. The same background correction will be applied to each plane of your volume.



- For image sequences: Velocity will create new background corrected channels in image sequences in which your background corrections have been applied.
- For images: background corrected images will be placed beneath the original images in the library.

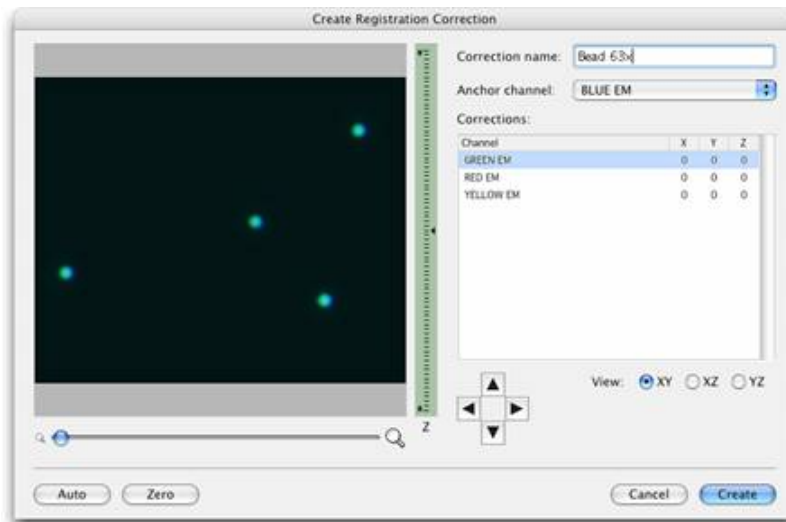
Creating Registration Corrections (Actions menu)

“Registration Corrections” are items that record the degree by which channels need to be moved in X,Y and Z in order to bring them into perfect register. Once Registration Corrections have been generated they can be applied to image sequences indefinitely, provided that the degree of shift between channels remains constant.

We recommend that you first acquire an image sequence of a fluorescent bead set from which registration shifts can be accurately calculated. You should acquire a multi-channel image sequence and ensure that you acquire every channel and every magnification with which you intend to work in your experiments. If you intend to do 3D work, you should acquire 3D image sequences of the beads so that you can detect and correct any registration problems in the Z axis.

1. Select the image sequence on which you wish to base your corrections in the library.
2. Select **Create New** from the **Actions** menu.
3. Select **Registration Correction...**

The Create Registration Correction dialog opens.



4. You should enter a name for your registration correction.
5. Volocity will automatically select an anchor channel, this is the channel relative to which the remaining channels will be shifted. If you wish to use another channel as the anchor, select one from the drop-down menu.

Creating Registration Corrections (Actions menu)

Manual Creation of Registration Corrections

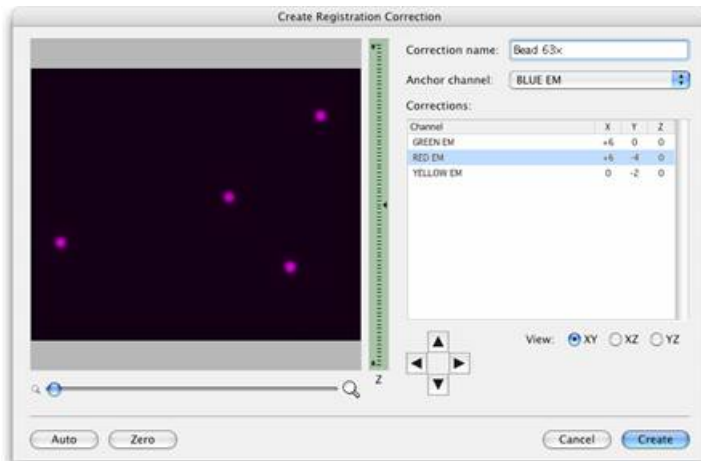
1. Select one of the remaining channels from the list to see its position relative to the anchor channel.
2. Change the **View** displayed in the preview from **XY** to **XZ** or **YZ** by selecting the appropriate radio button, and change the magnification of the preview by dragging the magnification slider.
3. Change the position of the currently displayed plane by either dragging the current plane arrow on the depth control or by clicking anywhere on the depth control.
4. Move the currently selected channel relative to the anchor channel by clicking on the four direction arrows, which will move the selected channel relative to the anchor channel. Move the channel so that it is in perfect register with the anchor channel in the X, Y and Z planes.
5. When you are satisfied that your channel is in perfect register with the anchor, select your next channel and repeat the process.
6. Ensure that all your channels are correctly registered with the anchor. Ensure that you have checked all channels and all views. Registration Corrections are likely to be applied to many image sequences so accuracy at this stage is essential.
7. Click **Create**.

Automated Creation of Registration Corrections

1. To make Volocity attempt to automatically place each channel in register with the anchor channel, click **Auto**.
2. A progress bar is displayed as Volocity calculates the degree of shift between each of the channels.



3. Velocity displays the number of pixels by which each channel has been shifted in X, Y and Z in order to place it in perfect register with the anchor channel.



4. To check the correction of a channel, select the channel from the list. Velocity will display only that channel and the anchor channel in the preview.
5. Manually edit the position of a channel relative to the anchor channel by clicking on the four direction arrows.
6. When you are satisfied that the corrections are accurate, click **Create**.

Registration correction items can be exported from libraries as library clippings and imported to new libraries, so you should not have to repeat the process of generating registration corrections.

Inspecting Registration Corrections

Velocity adds registration corrections to the library. Corrections made at the same time are placed in a folder. The folder and the corrections have the name that was entered in the Create Registration dialog.



Correcting Registration (Tools menu)

You can inspect registration corrections by double clicking on the associated item in the library. You can check the anchor channel and the relative pixel shift associated with each correction.



Correcting Registration (Tools menu)

When working with multi-channel image sequences you may find that channels are not always aligned with each other in perfect register. Channels may be shifted in the X, Y, or Z (or a combination of) planes relative to each other. Often this problem can be corrected by making improvements to your acquisition hardware such as purchasing shift-free fluorescence filter sets. Should this prove impractical you can use Volocity to correct registration problems.

To use the **Correct Registration...** command the library must contain the appropriate registration corrections. See page 111.

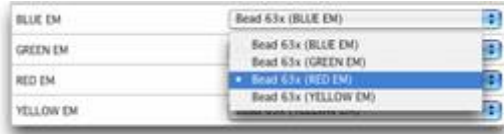
To apply registration corrections to image sequences:

1. Select the relevant image sequences in the library.
2. Select **Correct Registration...** from the Tools menu.
3. The registration correction dialog will open.



4. Volocity will attempt to match the names of the registration correction items in your library to the names of the channels in your selected image sequences. If you have many correction items in your

library you may need to manually select the registration correction by clicking on the drop-down menu for each channel and making a more appropriate selection.



5. We recommend that you avoid mixing corrections that were generated at different times. Volocity will warn you if you choose to do this.
6. When you are satisfied that each of your channels is paired with the appropriate correction item, click **Correct**.
7. Volocity will create new image sequences in which your registration corrections have been applied, each sequence will have the original name with the suffix (registration corrected).

Any areas in registration corrected sequences for which Volocity does not have data from all the channels in the sequence are cropped from the sequence, so registration corrected image sequences always have slightly smaller dimensions than the originals.

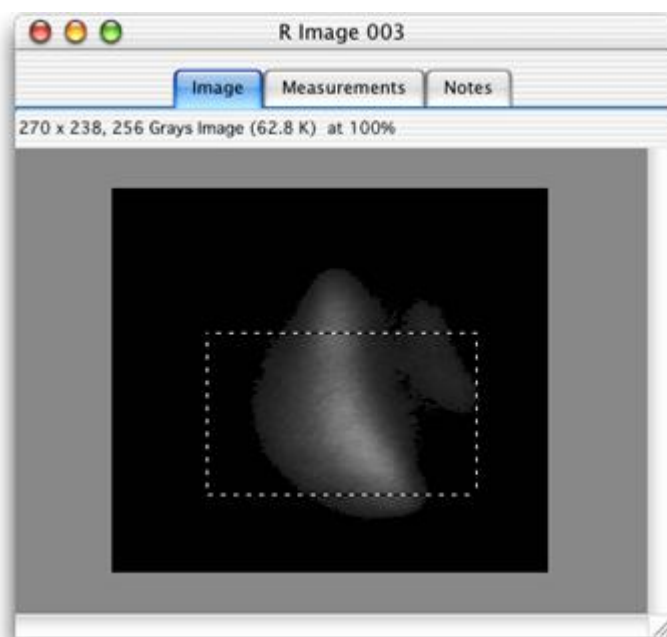
Cropping Items (Actions menu)

Cropping data to a selection reduces the physical size and therefore the file size of an item in the library. Cropping a dataset means information outside the selection is lost but the spatial resolution of the remaining data remains the same.

1. In the Image or Movie window, use one of the selection tools to outline the ROI (region of interest). See page 72 for more information about using Selection tools
2. Select **Crop to Selection** from the Actions menu.

In a 2D Image, this crops to the selected area. In a Movie window, this crops all frames to the selected area. In a 3D Image, this crops all Z slices. In an Image Sequence, this crops the XY dimensions of all Z slices in all channels in all timepoints.

Smaller files use less memory and take less time to process.



Change the size of data sets by scaling, changing resolution but preserving the field of view using the Change size command see page 100.

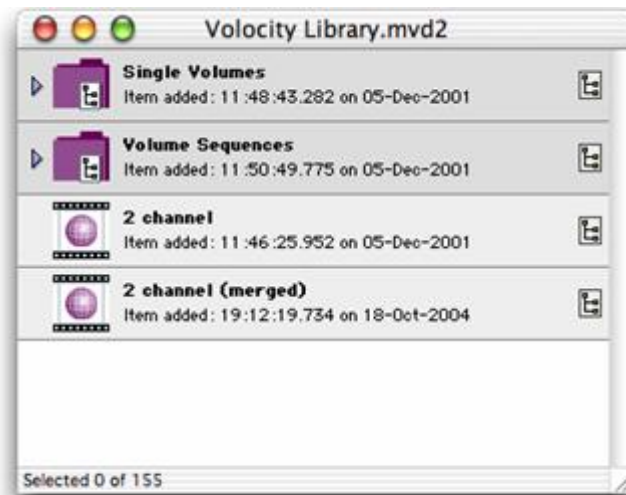
Scale data when making single volumes see page 46.

Merge Planes (Actions Menu)

Merging planes creates a brightest point merge of 3-dimensional data to create a 2-dimensional image. It effects a permanent change to the data set by creating a copy of the original data.

It may be used to reduce data size or simplify movie playback.

1. Select the item(s) which are to be merged from 3-dimensional to 2-dimensional.
2. Select **Merge Planes** from the Actions menu.
3. A new item is created in the library. It has the same name as the old item with “(merged)” appended. The actual number of channels and timepoints in the image is not altered, but the image at each timepoint will be two-dimensional.



Note: Unlike the Extended Focus view described on page 67, which creates a brightest-point merge of all the Z-slices in a volume, **Merge Planes** does actually create a two-dimensional image, and any selections and measurements performed on this image will be two-dimensional.

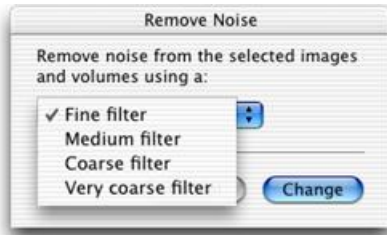
Removing Noise (Tools menu)

The application provides a number of median filters that can be applied to remove noise from an image. These filters range from fine to very coarse offering an increasing size of filter matrix and degree of filtering.

1. Select the required item(s) and select **Remove Noise...** from the Tools menu. The item must be adopted. Multiple image sequences may be selected for processing at once.

Splitting Multi-Colored Items (Actions menu)

2. Select All Channels or the channel name to be adjusted. Click on the drop-down menu and select the required filter.



Fine = 3x3(x3)
Medium = 5x5(x5)
Coarse = 7x7(x7)
Very coarse = 9x9(x9)

3. Click on **Change**. This command may take some time to complete if you have selected a large number of items.

Splitting Multi-Colored Items (Actions menu)

If you have a multi-colored item, you can split it into its three channels. Select the required item(s) and select **Split RGB** from the Actions menu.

In our example we have selected a multi-colored volume item (Neuron). The Split RGB command creates a new folder using the name of the volume (Neuron RGB) and creates a separate volume item for each channel.



item for splitting
selected in the library

new folder item created
containing volume item
for each channel

Splitting Volumes (Tools menu)

You can split a volume from the library or from any of the volume views. This converts the volume into a layered format to produce a stack of two-dimensional images.

1. Select the volume(s) and select **Split Volumes** from the Tools menu.
2. At the prompt choose how you want the volume to be split. You can put all images into a single folder or organize them into a number of folders, in which case you will also need to specify **Order by** criteria from the drop-down menu.



3. Volocity creates new folder item(s) in the library containing stack(s) of 2D images.



Printing and Exporting Data

Overview

This chapter describes how to:

- Print from the application.
- Export items.

Printing from the Application (File menu)

You can print the contents of the active window. This may be the Library View, a Single Image View or a Gallery View, as appropriate. Although print commands are available from the 3D Views, they are not useful for publishing volumes—you can use the **Capture Snapshot** command from the Image menu to create 2D images of the 3D View that you can print.

The dialogs for print and page setup commands vary according to the type of printer that you are using.

1. Click with the cursor in the window that you want to print.
2. Use the view-specific menu options, as appropriate to control how the image appears.
3. Select **Print** from the File menu. (Or from the Print Preview window, if that is displayed.)

Print Preview

If you want to check the result before printing, select **Print Preview** from the File menu. You can usually access the Page Setup and Print commands directly from the preview.

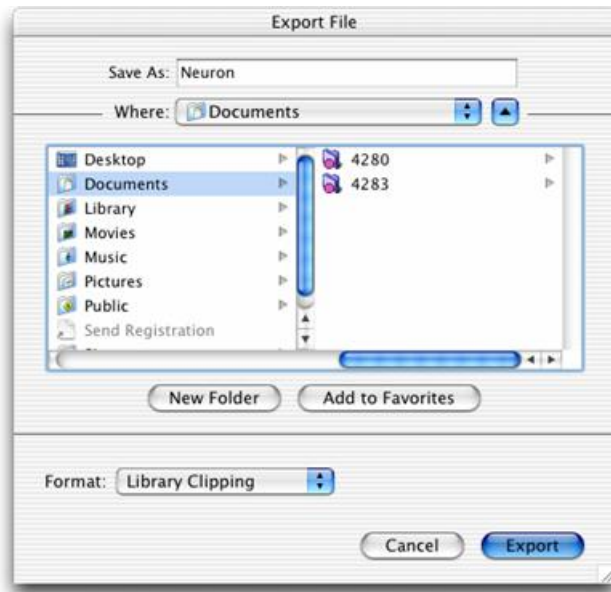
Page/Print Setup

1. Click on **Page Setup...** in the File menu (Mac). Click on **Print Setup...** in the File menu (PC).
2. The Setup dialog varies, depending upon which printer you are using. Amend the settings, as appropriate and click on **OK**.

Exporting Items (File menu)

The Export command allows you to export an item from the library as any of a variety of formats. You can also use it to copy files between libraries.

1. Select the required items in the library or switch to the appropriate view.
2. Select **Export...** from the File menu.



3. Change the file name, if required, and select a folder for the exported file(s).
4. Select the **Format**. Some file formats have an **Options...** button which allows configuration of the setting relevant to that format. If multiple items are selected for export the Export file dialog will show a **Naming...** button allowing you to define how the individual files will be named. Set the options, as appropriate, and click **OK**.



Exporting Items (File menu)

5. Click on **Export**.

The export options depend on the view open and the product being used:

- Export single images to create single images in formats such as TIFF or JPEG. Select multiple single images to export all the images as the same format in one step.
- Exporting a folder of images will make a multi-layered item such as a QuickTime movie or a multilayered TIFF.
- Export volumes and image sequences to export the item as a multi-layered file: Openlab LIFF, Openlab Raw, QuickTime movie or TIFF. The order of layers in the exported file will be z-slices then channels then time.
- Working with an image sequence which contains time resolved data, selecting export from an Image (Extended Focus) or 3D View, offers the ability to export the view as a movie which will make a simple movie of that view stepping through timepoints.
- Quantitation: Export Measurements as tab delimited or comma separated text.
- Exporting Movies: Use the library clipping format to move any library item easily between libraries.

Volocity LE does not allow the creation of QuickTime or AVI movies.

It is also possible to export settings from many parts of the software. Users of Volocity Acquisition are able to export acquisition protocols, users of Volocity Quantitation are able to export measurement protocols. Users of Volocity Visualization are able to export movies. In the dialog where these settings are saved there will be an **Export...** option allowing users to save their settings.

For the latest information on supported file formats, please look at the Release Notes on Improvion's Support web pages at <http://www.improvion.com>.

Export as AVI Movie for Windows Only (Not available in Volocity LE)

Windows users have an additional movie format available for export, the AVI format.

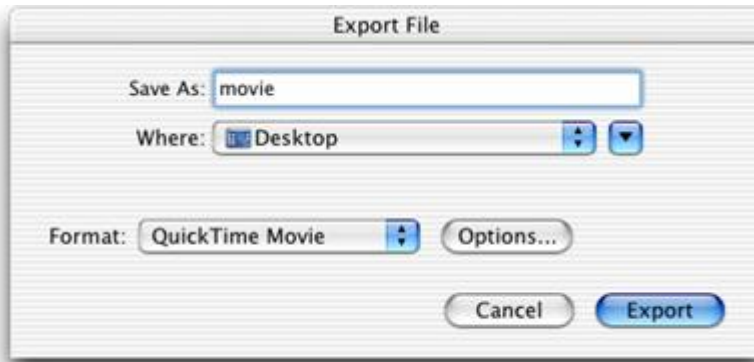
1. Select a folder of images in the Library view.
2. Select **Export...** from the File menu.
3. Enter the file name and select a location for the completed movie.
4. Choose **AVI Movie** from the **Format** drop-down and click **Export**.

Export as QuickTime Movie (Not available in Volocity LE)

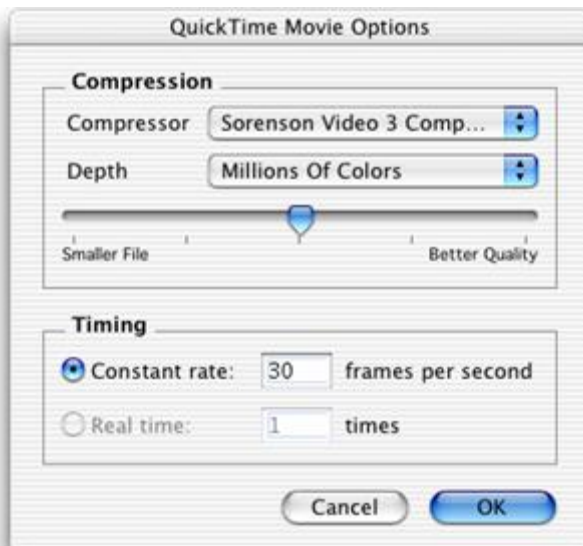
This option allows you to export a QuickTime movie with a frame rate based on the timestamps of the original files.

1. Select a folder of images in the Library view.
2. Select **Export...** from the File menu.
3. Choose a name for the movie file.

4. Select **QuickTime Movie** from the **Format:** drop-down menu and click on **Options...**



5. Select the Compression codec that should be used. The **Compressor** type and the bias towards a **Smaller File** or **Better Image** will affect the size and quality of the file created. This is a subjective decision, however, we suggest that Apple Animation or the Sorenson compression may provide a good balance between quality and file size.



6. Select the timing required for the finished movie and click **OK**.

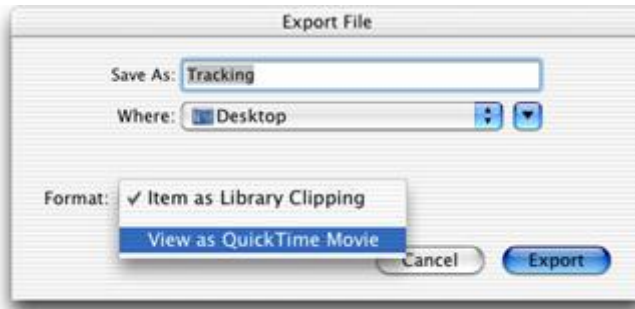
Exporting Items (File menu)

Export View as QuickTime or AVI Movie (Not available in Velocity LE)

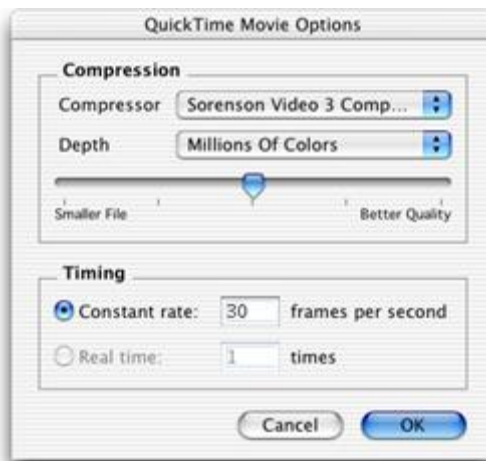
When working with an image sequence which contains two or more timepoints and viewing the Image View, it is possible to make a simple QuickTime or AVI (Windows only) movie from that view.

Note: If each timepoint contains more than one plane in z, use the Extended Focus view.

1. Configure the view, as required with scale bars, selections, and so on.
2. Select **Export** from the File menu, change the file name, if required, and select a folder for the exported movie. Select **View as QuickTime movie**.



The QuickTime Movie format provides additional export options. Select the compression codec and quality, and the timing required for the finished movie, and click **OK**.



3. Click **Export**. The movie will be made by stepping through each of the timepoints.

Volocity Acquisition Installation and Reference

Overview

Volocity Acquisition is designed specifically for high performance acquisition of time-resolved, multi-channel 3D sequences. It supports a range of hardware suitable for 3D imaging, including wide field microscopes, scientific grade digital cameras, fast focus drives and wavelength changers.

It is very important to install and configure acquisition devices correctly before you start capturing images. This chapter describes how to:

- Install and configure devices
- Use device controls
- Calibrate objective lenses
- Measure device latencies
- Configure the Light Path Manager
- Use the Video Preview window
- Carry out fast restoration
- Set up and use the PerkinElmer Ultra *VIEW*
- Set up and use the OptiGrid.
- Set up and use a motorized XY stage for multi-point acquisition.

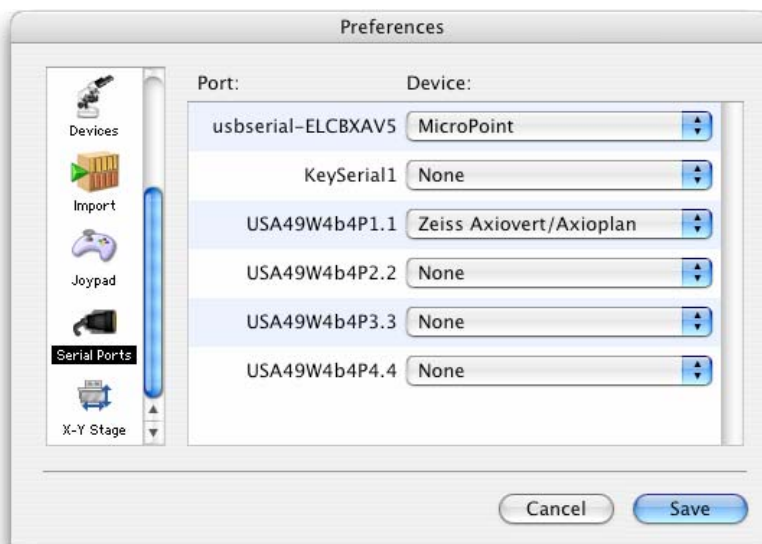
Please refer to “Volocity Acquisition Worked Examples” on page 148 for comprehensive instructions on carrying out single-point and multi-point acquisition and information about setting up specific acquisition protocols.

Installing and Configuring Devices

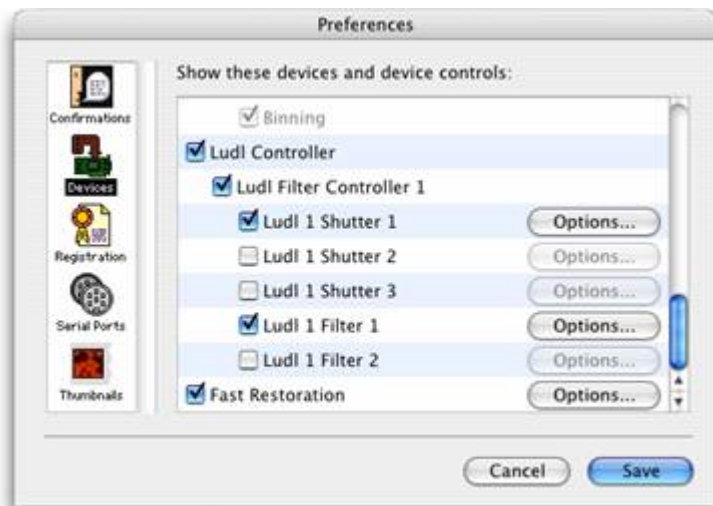
1. Install your camera driver and camera (and frame grabber card, if applicable) according to the manufacturer's instructions.
2. Connect any further hardware (such as microscope, Piezo focus drive, OptiGrid, etc.) to your computer via the appropriate port. Serial devices can be connected directly to serial ports (COM ports) on your computer, if you have them, or via a Keyspan USB serial adaptor.
Note: After installing a Keyspan USB serial adaptor or a major upgrade to the operating system, make sure the drivers for the adaptor are installed before connecting the serial devices.
3. Ensure that all your hardware is switched on.

Setting Preferences

1. Start the application and select **Preferences...** from the Edit menu (Windows) or the Volocity menu (Macintosh).
2. If you have connected serial devices to your computer, you must configure the serial ports. Click on the **Serial Ports** icon in the left-hand panel. If you have no serial devices connected, go to step 4.
3. In the right-hand panel locate each device that is connected and select the appropriate port from the drop-down menu. Devices in the list that are not part of your imaging system may be ignored.



4. Click on the **Devices** icon in the left-hand panel.



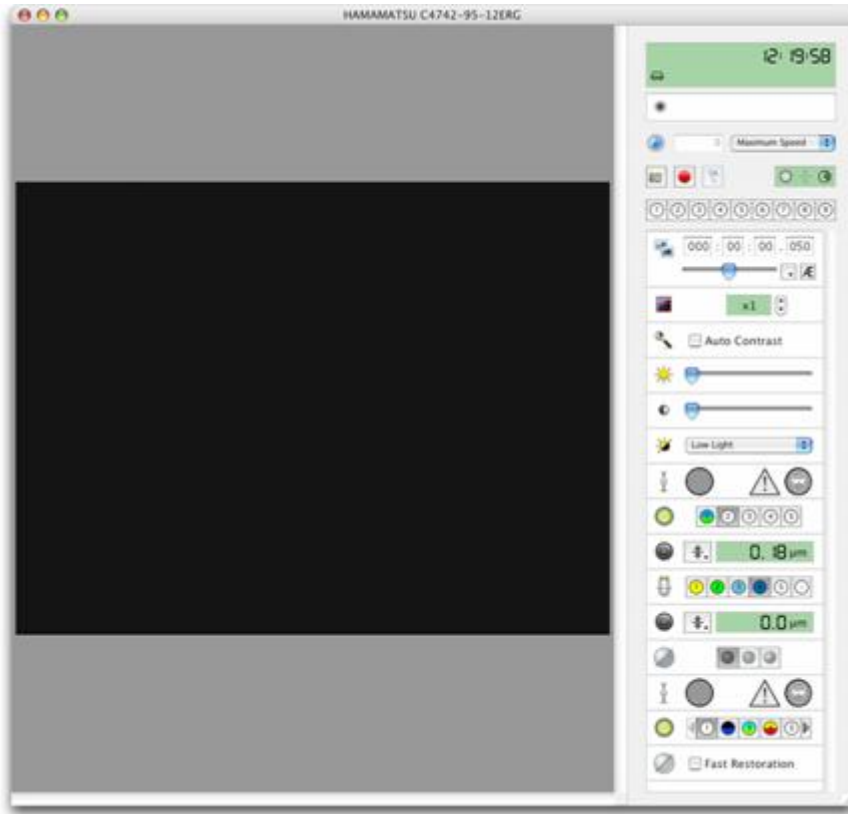
5. A list of devices that have been correctly connected to your computer will appear in the right hand panel. Each device has a subset of device controls that are listed beneath the device.

To appear in the list:

- Cameras and USB devices must be correctly connected to your computer and switched on before starting Volocity.
 - Serial devices must be correctly connected to your computer, switched on, and the appropriate serial port must be selected in the Preferences (see step 3).
6. Carefully review all of the devices listed in the preferences; make sure that you scroll to the bottom of the list so that no devices are overlooked. If a device has an **Options...** button, you must click on the button to review the settings in the device's setup dialog. If necessary, you should edit the device's setup dialog. Failure to review and edit the dialog may result in difficulty using hardware. This may be due to incorrect labeling of devices, or because Volocity has not been given the information it requires to move the hardware accurately. See "Reviewing and Editing Setup Dialogs" below for some examples.

Setting Preferences

7. Select **Show Video Preview** from the Window menu. The devices and device controls that were selected in the Preferences are displayed on the right-hand side of the Video Preview window.



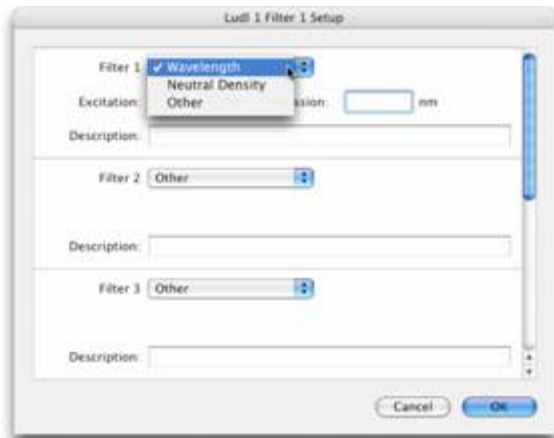
Reviewing and Editing Setup Dialogs

Some devices may require only a single value to be entered into a text field. Some motorized stages, for example, will require that you enter the correct step size for the stage.



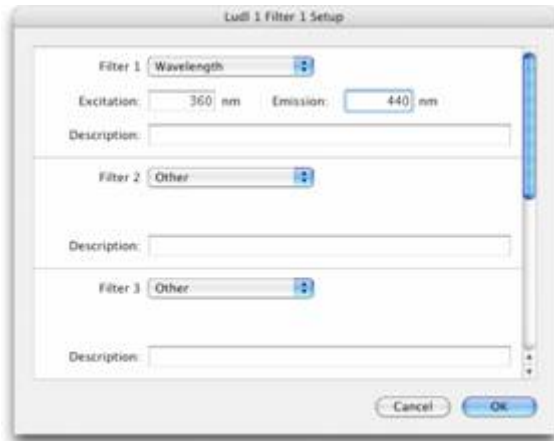
Other devices may require more information to complete the Setup dialog, which should be completed in steps. For example, when completing the dialog for a filter wheel or fluo turret:

1. Select the filter type for each filter position from the drop-down menus.

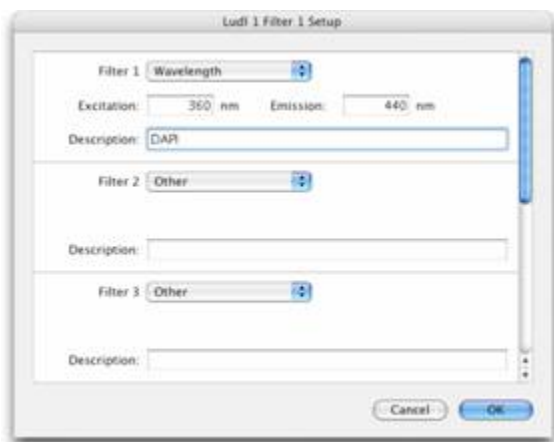


Device Controls

2. Enter details of the filters in the text fields.



3. Name the filter in the description field.



Provided that you carefully review and edit each of your device's setup dialogs, each of your devices can be used easily and accurately with Volocity. Remember to select appropriate options from drop-down menus and to carefully enter accurate information into text fields.

Device Controls

Using Device Controls

Each device that you control with Volocity will have one or more controls with which you can interact. Volocity is compatible with several classes of devices from a number of manufacturers, so the

number and types of control present for each of your devices will vary. The list of controls below is general, different controls may be present on your system.

To determine to which device a control belongs, move the cursor over the icon to the left of the control. The device name will be displayed in the tooltip.



Many devices can be controlled from elsewhere, such as the microscope stand or the control box. In most cases, if you change the state or position of a device from elsewhere, the change will be displayed on the device's control in Volocity.

Double clicking a control's icon is a shortcut to the control's setup dialog.

Button Arrays



Each button in an array represents a different position for the device, for example different filter positions in a filter wheel. The button representing the current position of the device is shaded. Hold the cursor over a button to display a tooltip with a description of its position. Click on the button to move the device to that position. Filter wheels, fluo turrets, objective nosepieces and the light path manager are controlled by button arrays.

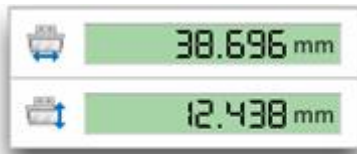
Checkboxes



Checkboxes allow you to control devices that only have two states, on and off. When checkboxes are selected the device is on, when deselected the device is off. Auto Contrast and Fast Restoration are examples of devices controlled with checkboxes.

Device Controls

Digital Displays



Digital displays show the current value of a device as a number, usually representing the device's current position.

Exposure Controls



The exposure control determines the length of time over which your camera will acquire an image. The hours, minutes, seconds and milliseconds components of the time are separated for clarity. To adjust the time click in one of the number fields and edit the number; you must click in another field or press the Return key for your change to take effect. You can also drag the exposure slider to make quick changes, the exposure time will automatically update. Use the drop-down menu to change the range.

If you click the Auto exposure button, **AE**, exposure time will be automatically set.

Focus Controls



Focus controls display the current position of focus drives as a numeric value. As you change the position of the focus drive from the microscope stand the value will be updated.

Select the focus drive menu button to display the following options:



Set Zero	Sets the current position of the focus drive to 0 μ m.
Set Top	Sets the current position of the focus drive as the upper mark in the focus range. This range will be used during acquisition of 3D datasets.
Set Bottom	Sets the current position of the focus drive as the lower mark in the focus range. This range will be used during acquisition of 3D datasets.
Reset Top/Bottom	Resets the focus drive upper and lower marks to the default values of +50 μ m and -50 μ m respectively.
Move to Zero	Moves the focus drive from the current position to 0 μ m.
Move to Top	Moves the focus drive from the current position to the upper mark.
Move to Bottom	Moves the focus drive from the current position to the lower mark.
Move to Hard Center	Moves a piezo focus drive to the centre of its range of travel. This option is not available for non-piezo focus drives.
Move To...	Opens a dialog that allows you to enter the position to which the focus drive will move.
Set Top To...	Opens a dialog that allows you to enter the position to which the upper mark will be set.
Set Bottom To...	Opens a dialog that allows you to enter the position to which the lower mark will be set.
Auto-Focus	Initiates the Auto-Focus function of Volocity. Volocity will move the focus within the Auto-Focus limits to the position in which it detects the most in-focus information.
Set Auto-Focus Limits...	Opens a dialog in which you can set the Auto-Focus limits. Volocity will not move beyond the limits when executing the Auto-Focus function.

Device Controls

Grid Controls



There are three grid controls. From left to right:

- **Grid Off:** Switches the grid off. There is a stationary grid pattern on the Video Preview and from the ocular lenses of the microscope.
- **Grid Confocal:** Activates the grid. There is a confocal image on the Video Preview and a moving grid pattern from the ocular lenses of the microscope.
- **Grid Wide Field:** Activates the grid. There is a wide field image (without grid lines) on the Video Preview and a moving grid pattern from the ocular lenses of the microscope.

Numeric Spin Controls



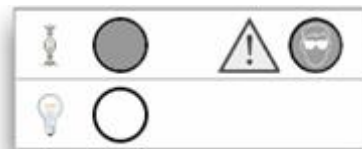
Numeric spin controls allow you to quickly increment or decrement the current value of the control by clicking on the arrows next to the number display. Camera binning is an example of a device controlled with a numeric spin control.

Drop-down Menus



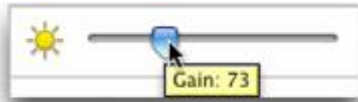
Drop-down menus allow you to select from a number of options for a device. Click on the drop-down menu to see all of the options, and select the option you require. Camera depth and grid quality are examples of devices controlled with drop-down menus.

Shutters



Click directly on the shutter icon to change its state. Solid indicates shutter closed. White indicates shutter open.

Sliders



Sliders allow you to quickly set a device control anywhere between its maximum and minimum values. Hold the cursor directly over the slider to display a tooltip showing the current value of the control. Camera gain and offset are examples of devices controlled by sliders.

Calibrating Objective Lenses

In order to ensure that the data acquired with your Acquisition system is correctly calibrated in X and Y, each lens should be calibrated with a stage graticule.

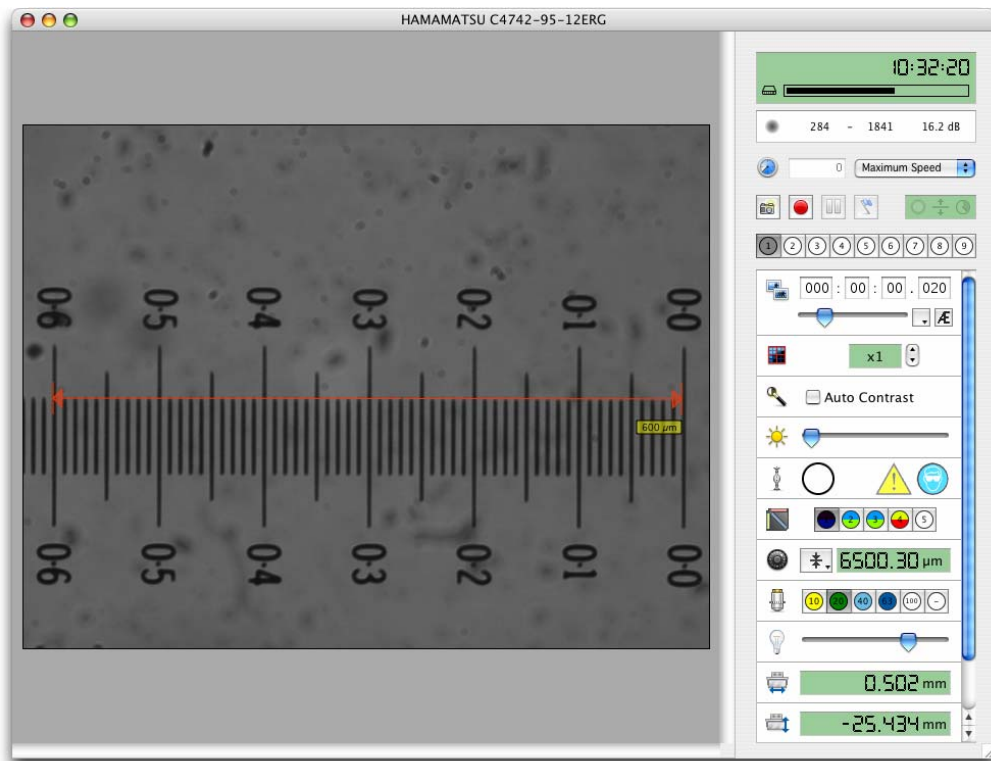
1. Place the graticule slide on the stage and focus on the scale divisions.
2. Select the Calibration tool from the toolbar.



3. Draw a line of known length on your Video Preview. Click and hold your mouse to start the line, drag the mouse to the endpoint of your line and release the mouse button.

Calibrating Objective Lenses

- Remember to draw from the left of your start division to the left of your end division, and make the line as long as possible to minimize the effect of drawing errors.



- Select **Calibrate...** from the **Video** menu or double click on the Calibration tool to open the calibrate window.



6. Enter line length in μm and click **Calibrate.**

To confirm that your objective lens has been correctly calibrated, select **Show Scale** from the Video menu. You can then compare your scale bar to the calibration divisions.

Repeat this process for every objective lens on your microscope.

If you already know how many $\mu\text{m}/\text{pixel}$ your camera has for your current objective lens, double click on the calibrate tool without first drawing a calibration line on the video preview. You will be able to enter the $\mu\text{m}/\text{pixel}$ value directly into the Calibrate window. You should repeat this for each lens.

Measuring Device Latencies

You should use this option when you first install and configure Volocity and also whenever you attach new equipment or use different equipment. Volocity will measure the latencies of all computer-controlled hardware on your system, which will allow Volocity to make intelligent decisions about closing shutters during acquisition.

1. Select **Measure Device Latencies** from the Video menu to display the following prompt.



2. Check that there are no samples and that your microscope is in a safe position (for example, check that your objectives will not collide with your stage if your objective turret is moved). Click **Continue**.

When you have completed these steps your Acquisition system will be correctly configured and ready to use.

Note: The latency measurements are noted in the Session Log. If you wish to know them, view the Session Log at the time of measurement.

Light Paths

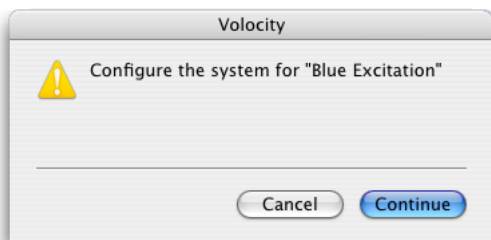
Configuring the Light Path Manager

The Light Path Manager is above the device controls in the Video Preview window and allows you to configure up to nine different light paths and store them for future use.

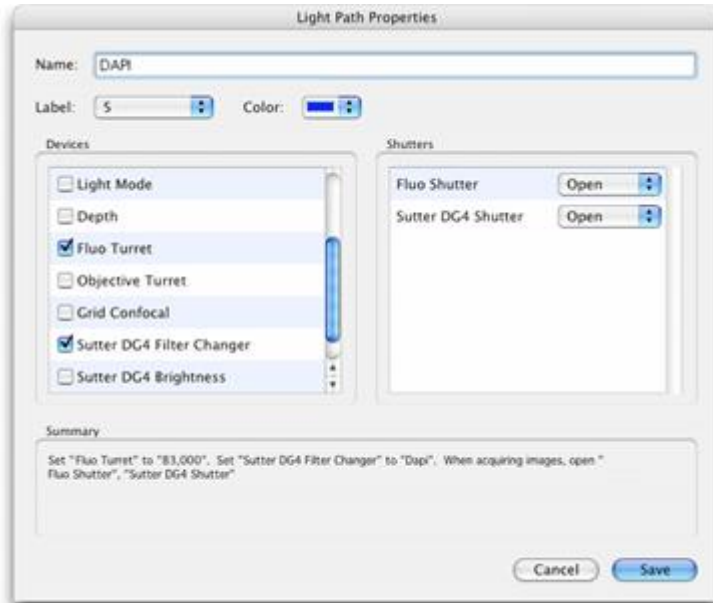


The Light Path Manager allows you to record the correct positions of all your automated hardware for each of your fluorescence filter combinations, as well as transmitted light paths. This greatly simplifies the use of your system and allows you to easily restore the correct light path manually (using the Video Preview window) or during acquisition. You can record the positions or settings of as many or as few devices as you require. For example, you may keep your light path simple and include the positions of only wavelength changing devices and shutters. Under some circumstances you may add other settings to the light path, such as your camera exposure or gain.

1. Configure your hardware for a particular light path. For example, if you are setting a fluorescence light path and you are using an excitation filter wheel and a microscope fluo turret, ensure that they are in the correct positions for your fluorescence filter set of interest.
2. Double click on an unused light path manager button. The Light Path Properties window opens. From the **Devices** list select those devices that you wish to include in the light path. Select the appropriate shutter positions for your light path from the drop-down menus in the **Shutters** list.
3. Provide a **Name** for the light path and select a **Color**. The color will be used for the light path button, and to color channels acquired using the light path. If you are setting a transmitted light path, the color should be white. The **Summary** box provides a useful textual description of the settings that you have included in the light path.
4. If the light path requires changes in the microscope setup that are not controlled by Volocity, for example moving a manual filter changer, check the box for **Requires manual intervention**. A message will be shown each time the light path is called prompting you to make the manual change.



In the following example we have set up the light path for a DAPI channel. We have saved the current positions of the Fluo Turret and Sutter DG4 Filter Changer in the light path. The Fluo Shutter and Sutter DG4 Shutter will be opened whenever the light path is restored.



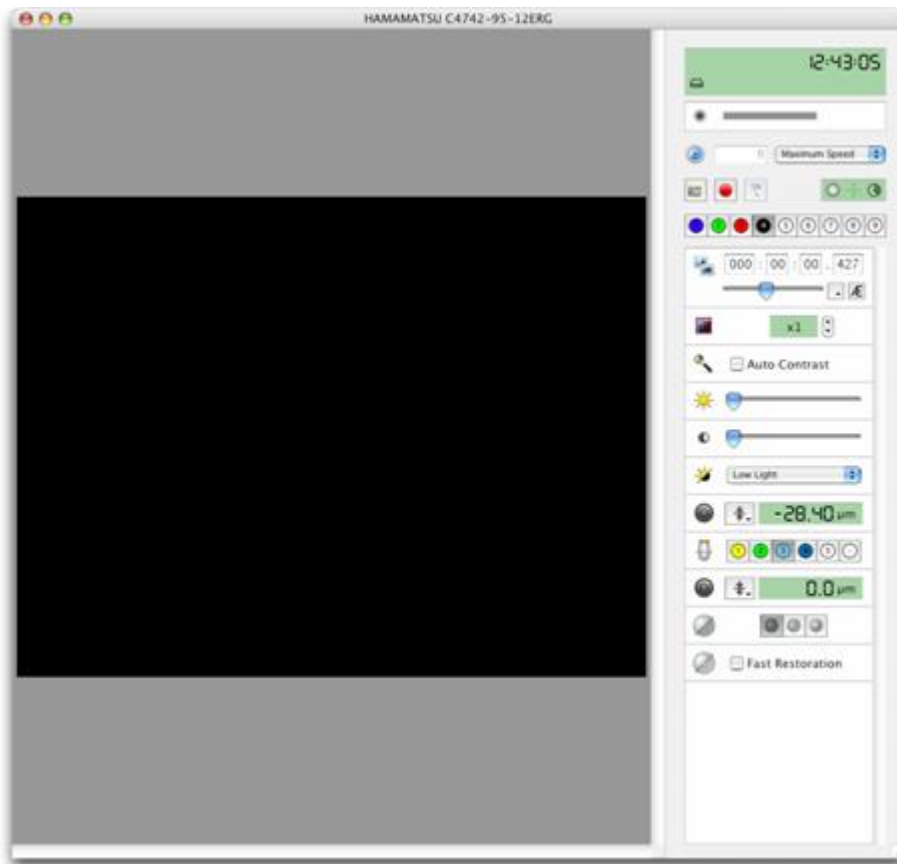
5. Click **Save**.
6. Set up a separate light path for each fluorescence filter and transmitted light path that you will use on your system.

Once you have configured your light paths, we strongly recommend that you hide the individual devices included in the light paths from the Video Preview. You should control these devices only with the Light Path Manager. This will make the system easier to use and make the Video Preview less cluttered.

Light Paths

In our example we have deselected the Fluo Turret, Fluo Shutter, Sutter DG4 Filter changer and the Sutter DG4 Shutter in the **Devices** preferences. The devices are still active, but we will use the Light Path Manager to control them.

Note: We have also configured a light path that closes the shutters.



Editing Light Path Settings

You can easily edit a light path, for example if you want to use the same device settings but with a different camera exposure.

1. Select the light path and change the camera settings on the Video Preview window.

2. Click and hold (Macintosh) or right-click (Windows) on the light path and select:
 - **Overwrite** to change the recorded settings to the current settings.
 - **Edit...** to open the Light Path Properties window. Select the additional devices from the **Devices** list and select **Save**, if you wish to add the positions or settings of additional devices to the light path.




The Video Preview Window

Open an existing library or create a new library and select **Show Video Preview** from the Window menu.

video preview

use the zoom tool to zoom in and out



shortcut key = Z

timing feedback

video feedback

acquisition protocol feedback

light path manager

controls

click and drag to resize window

Acquiring Images

Create light paths to control the devices necessary to image in different channels and an acquisition protocol to manage an experiment.

Contrast Enhancement

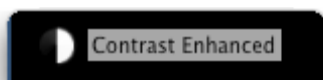
Enhance contrast in the live video to reveal detail captured by the camera but not shown by the default display contrast. Check Auto Contrast on the video controls to apply an automatic contrast setting which will adjust the black and white level of the display to the darkest and brightest captured intensity respectively.

Alternatively manually set black and white levels of the video preview using the live enhancement tool.



Select the live enhancement tool from the toolbar. A histogram of the grey levels in the preview is displayed. To adjust the white level click and drag the mouse to the left (to decrease) or to the right (to increase). To adjust the black level, hold down Ctrl and click and drag the mouse down (to decrease) or up (to increase). Double click the icon in the tool bar to reset the contrast enhancement. Select another tool from the tool bar to hide the display.

When contrast enhancement is being applied an indication will be shown in the top left of the video preview image.



Zooming the Video Preview



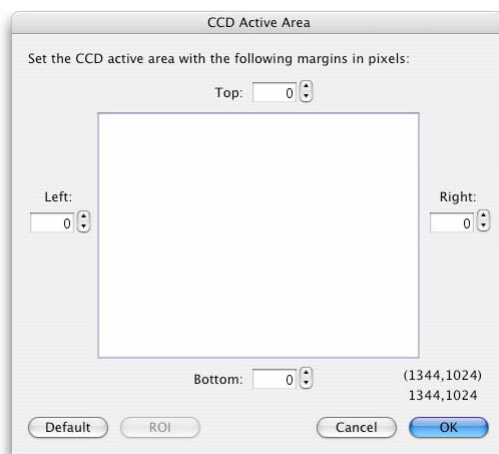
The Video Preview can be zoomed in or out. Select the Zoom tool and click on the Video Preview to zoom in. Click and hold the **Ctrl** key (Windows) or the **Alt** key (Macintosh) to zoom out.

A zoomed window can be scrolled from left to right or up and down using either the scroll bars or the window panning tool.

Setting CCD Active Area

Some combinations of optical components do not generate an image to fill the full CCD of the camera leaving margins around the images. To avoid storing these set the CCD active area to exclude them.

Select **CCD Active Area...** from the Video menu.



Set margins to exclude or set the active area to the dimensions and position of an ROI which can be drawn on the video preview before selecting this dialog.

Cropping the Video Preview



The Video Preview can be cropped, so that smaller images will be acquired by Volocity. With some cameras cropping will provide a speed advantage. This is also known as a sub array acquisition.

- To crop the preview select the Crop tool, click and drag a crop boundary on the Video Preview. Everything that you include within your crop boundary will continue to be updated on the Video Preview, everything outside the boundary is not updated. You can also crop the Video Preview to preset sizes by selecting **Crop Video** from the **Video** menu and selecting one of the options.
- To restore the full resolution Video Preview, select the crop tool and click anywhere outside the crop boundary.

The Video Preview Window

Selecting an ROI on the Video Preview



An ROI can be drawn on the Video Preview. With an ROI on the preview, only the area within the ROI will be used for the video feedback panel.

To draw a region of interest on the Video Preview, select the ROI tool, click and drag your region of interest.

Centering an XY Stage



On a system that includes a motorized XY stage the centering tool will move the XY stage so that the point that the tool clicks on within the Video Preview is centered in the field of view.

The stage and the objective must be calibrated for this tool to work correctly.

Video Feedback Panel

Information on the gray levels in your Video Preview can be found in the Video Feedback panel, in the top right of the Video Preview window. The information can be displayed in four different modes, **numbers**, **histogram**, **bar** and **Max Intensity graph mode**.

To toggle between the four modes, click on the Video Feedback panel.

Numbers mode



The Video Feedback panel displays the minimum and maximum gray level in the Video Preview, and the signal to noise ratio.

Histogram mode



The Video Feedback panel displays the distribution of gray levels in the Video Preview. The minimum gray level is displayed on the left of the histogram, the maximum gray level (which depends upon the bit depth of your camera) is displayed to the right of the histogram.

Bar mode



The Video Feedback panel shows the range of gray levels in the Video Preview. The length of the bar indicates how much of your camera's dynamic range you are currently using. The longer the bar, the better your signal to noise ratio.

Max Intensity graph mode



The Video Feedback panel shows the maximum intensity of gray levels in the Video Preview or ROI over time. An update is made for each frame.

If you have a color camera, you will see feedback for each of the three channels of your camera in addition to a luminance channel.

Using Fast Restoration

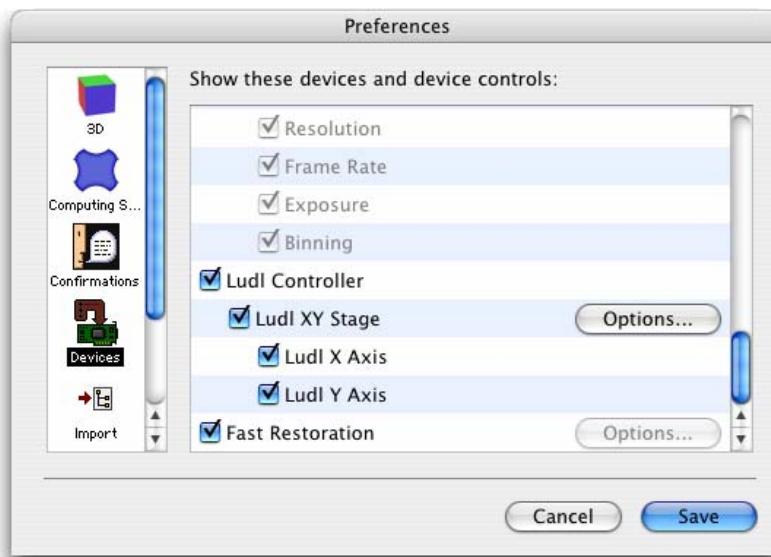
Volocity Acquisition supports Fast Restoration deconvolution. This option is available in the Video Preview window if you have purchased the Volocity Restoration extension product.

Fast Restoration should only be used with single plane acquisition protocols. If you have multiple planes, you should capture them first and deconvolve them later using Volocity Restoration.

Important: You must create or import a PSF for each channel that will be captured before you can set up the acquisition.

Using Fast Restoration

1. Make sure that **Fast Restoration** is selected in the **Devices** Preferences window.



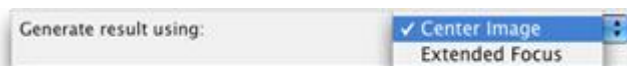
2. You can configure the settings from the **Options...** button or by double clicking on the Fast Restoration icon on the Video Preview window.
3. Ensure that you have the correct PSFs for the channels that you wish to acquire in your Volocity library.
4. Double click on the **Fast Restoration** icon.



The Fast Restoration dialog opens.



5. Select a PSF for each channel that will be captured from the drop-down menus.
6. Decide on the number of neighboring slices that will be included in the restoration algorithm and enter the value in **Capture this many slices above and below** field. The more neighboring slices you include, the longer the process will take.
7. Select the appropriate focus drive from the **Change focus using** drop-down menu.
8. Enter an appropriate value for **Use this Z spacing**.
9. Select the appropriate option for **Generate result using**.



Center Image displays only the deconvolved center image.

Extended focus creates a single image by making a brightest-point merge of all the deconvolved Z-slices of a volume.

10. Click **OK**.
11. Check the **Fast Restoration** box on the Video Preview window. The first time you do this after configuring the settings can take some time as Volocity applies the algorithm and generates the deconvolved image.

PerkinElmer UltraVIEW

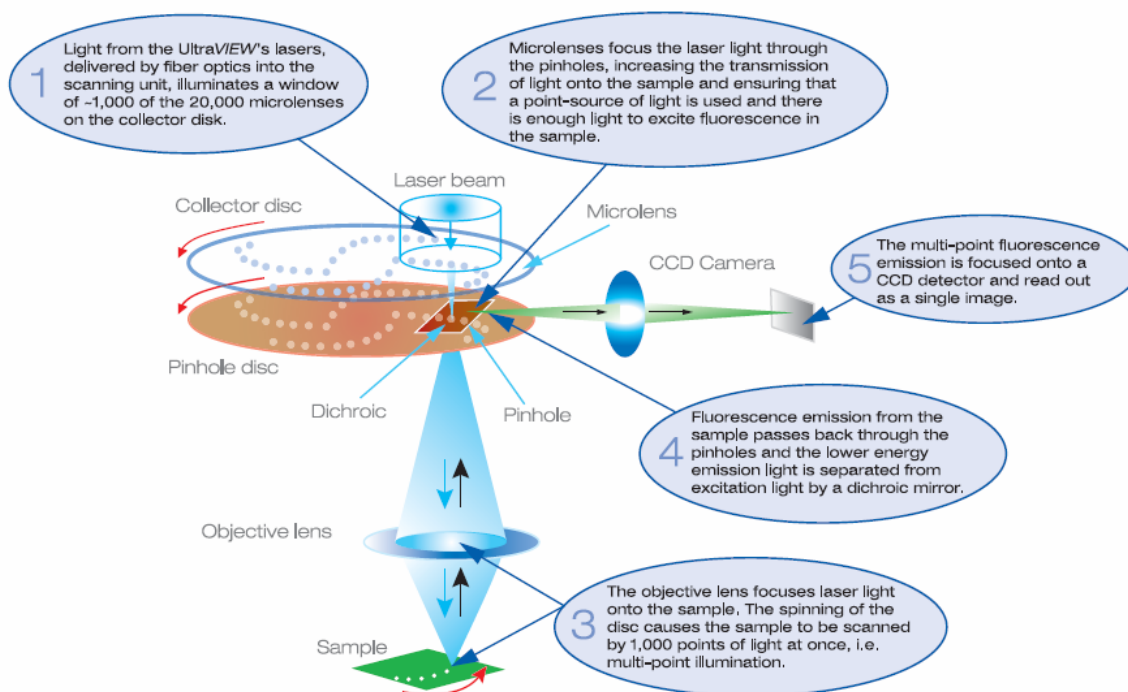
The UltraVIEW VoX is a complete system for live cell imaging using the CSU-X1 spinning disk head from Yokogawa.

The system consists of:

- CSU-X1 spinning disk
- Emission filter wheel
- Modular laser system
- Piezo focus
- CCD camera
- Brightfield shutter
- ProSync control box

Optional accessories include the PhotoKinesis accessory and a motorized XY stage.

How does the spinning disk illumination work?



UltraVIEW models

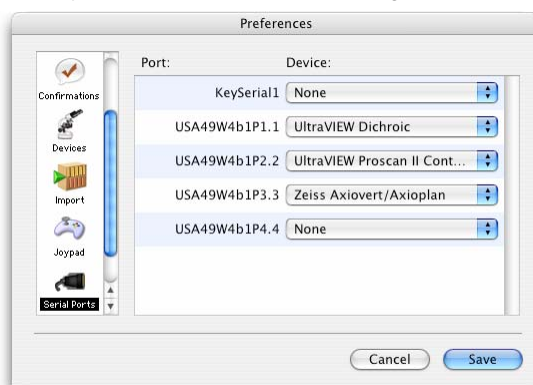
All information refers to the use of Volocity with the UltraVIEW VoX only.

Configuring the UltraVIEW in Preferences

For the hardware of the spinning disk device to be correctly identified and controlled in Volocity it must be configured in the preferences. There are two devices which communicate with Volocity via serial ports: UltraVIEW Dichroic (within the CSU head) and the UltraVIEW Proscan II Controller.

The following information assumes the hardware has been installed and aligned by a qualified PerkinElmer service engineer. These settings will also have been configured at installation but are provided here for reference.

1. Select **Preferences** from the Edit menu (Windows) or the Volocity menu (Mac OS). Choose **Serial Ports** from the list. The serial ports detected on the system will be listed and to connect a device in Volocity its name must be selected against the port to which it is physically connected.

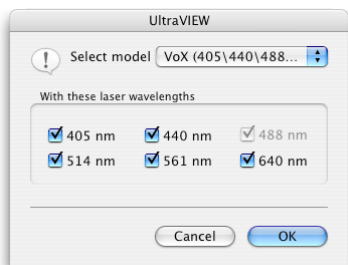


2. Scroll to the Devices pane and select the controls to be displayed in the Video Controls panel. Devices unchecked in this list will not be seen in the Video Controls panel and therefore cannot be controlled from that view.

Configuring the UltraVIEW in Preferences

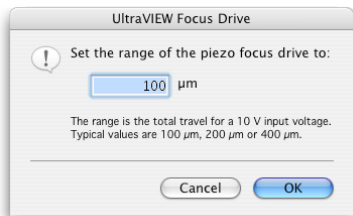


3. Next to the UltraVIEW item click **Options...** to configure the lasers present on the system. Select the UltraVIEW model from the list. Laser lines are not automatically detected.



Check the box next to laser line(s) that are present on your system. The 488 nm laser is standard on all systems; therefore, this option will be checked and cannot be changed.

4. Next to the UltraVIEW Focus Drive item click **Options...** to configure the range of travel for the piezo focus drive.



For the objective piezo drive this distance is 100 micrometers. For the stage insert this distance will be 200 micrometers or 400 micrometers. Check the specification of your particular system.

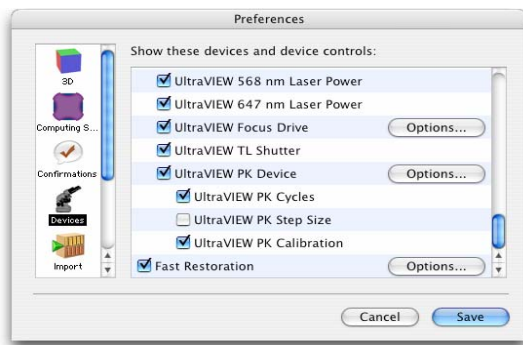
If the microscope focus is also controlled by Volocity it will have its own entry under the heading of the microscope. The piezo focus drive will be the fastest to use in experiments but the microscope focus might have a greater range of travel. Where both are available either may be chosen to acquire

stacks in the Acquisition Setup dialog, which is used to configure the experiment. See “Volocity Acquisition Worked Examples” on page 199 for examples of setting up this dialog.

5. Next to the UltraVIEW Emission Wheel click **Options...** to configure the filters held in the wheel. This should be already configured correctly for a standard system.

PhotoKinesis Accessory

If the optional PhotoKinesis accessory is included in the system there will also be check boxes for the controls of that device.



Click **Options...** to set laser power for bleaching. This dialog is also accessed from video controls while configuring an experiment.



Preparing to Use the UltraVIEW in Volocity

In order to use the UltraVIEW with Volocity all hardware must be configured.

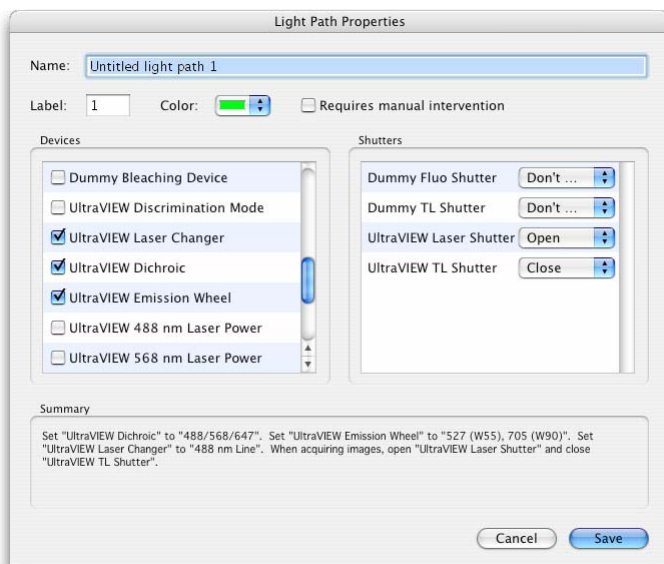
- Components of the UltraVIEW which connect via the ProSync controller and serial ports must be connected, switched on and configured in the Volocity preferences.
- Camera drivers must be installed, the camera connected and switched on. The camera will then be automatically recognized in the Devices list in the Volocity preferences.

If using a motorized microscope that must be connected to a serial port on the computer, use the serial cable supplied by the manufacturer. If there are no spare serial ports, it may be necessary to use a serial adapter.

Choose **Show Video Preview...** from the Window menu to see the image from the camera and the controls for the devices.

To ensure images are correctly calibrated set calibrations for each lens position. Calibration is then applied to each image according to the lens in position when it is acquired.

Light paths may be created for each of the laser lines to enable easy switching between excitation and emission wavelengths for different fluorochromes. It is advisable to create light paths using the Emission Discrimination mode of the UltraVIEW, and for each laser line set and store the laser line, filter wheel position and set the laser shutter to open.

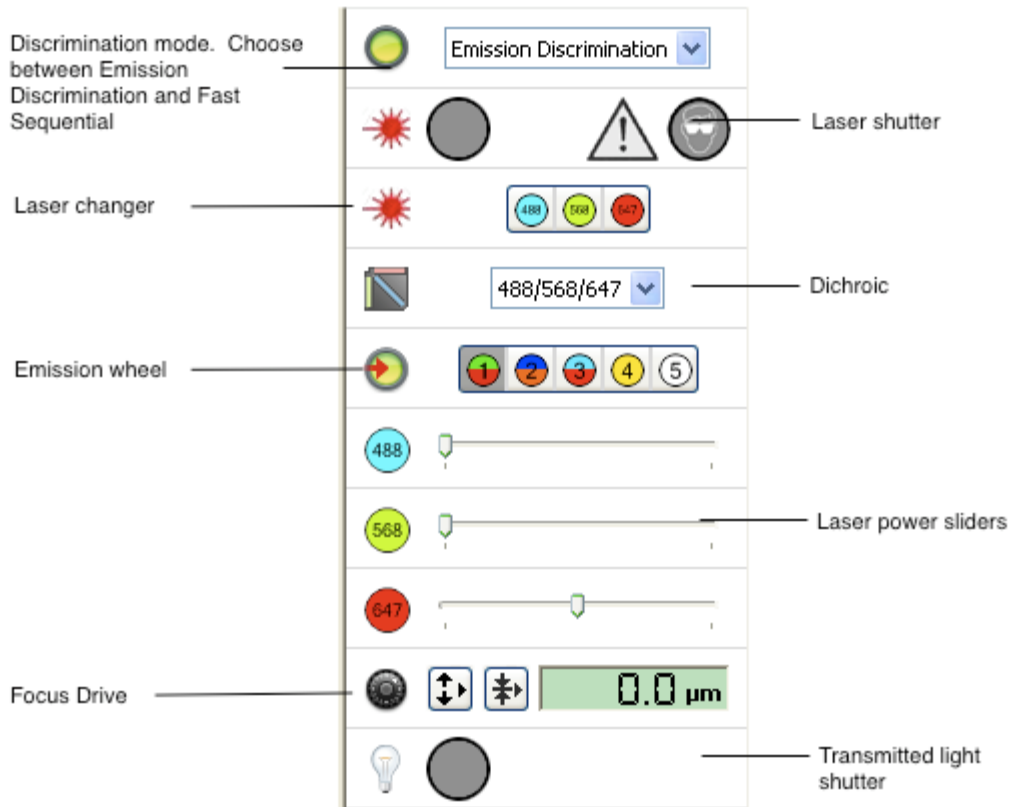


These light paths will then work satisfactorily with both Emission Discrimination and Fast Sequential modes. Storing other settings with these light paths are a matter of personal preference and will vary depending on how the system is being used. See page 138 for information on configuring the light path manager.

If the image generated by the confocal head does not fill the full field of view, adjust the CCD active area using **CCD Active Area...** dialog accessed from the Video menu.

UltraVIEW Controls

When the UltraVIEW is correctly configured in the preferences the following controls will be visible in the Video Controls. Select **Show Video Preview...** from the Window menu to see these controls and the live video.



Discrimination Mode

When experiments are carried out using multiple wavelengths two different methods of capture can be used: emission discrimination or fast sequential.

Emission Discrimination mode will restore the emission filter wheel position associated with the light path. Changing the emission filter provides better discrimination between different dyes in one sample. The disadvantage of Emission Discrimination is a reduction in capture speed, due to the time taken for the emission filter wheel to turn from one position to another.

In Fast Sequential mode the filter wheel is not moved during the experiment. Instead the wheel is automatically turned to the position containing the appropriate broadband barrier filter for the current dichroic to prevent laser breakthrough. Any emission filter wheel positions stored with light paths will be ignored in fast sequential mode. Fast sequential provides the highest capture speed, as switching laser wavelengths is very quick. However, because the same broadband emission filter is used for all images, there is an increased risk of crosstalk between the emissions of different dyes.

The emission filter wheel position must be stored in the light paths that are switched between by the acquisition protocol for emission discrimination mode to work correctly.

Dichroic

Switch between the dichroics installed in the CSU head using this pop-up. Some dichroics are only compatible with specific laser lines. This will be indicated in the interface as the lines that are not compatible with the current dichroic are grayed out.



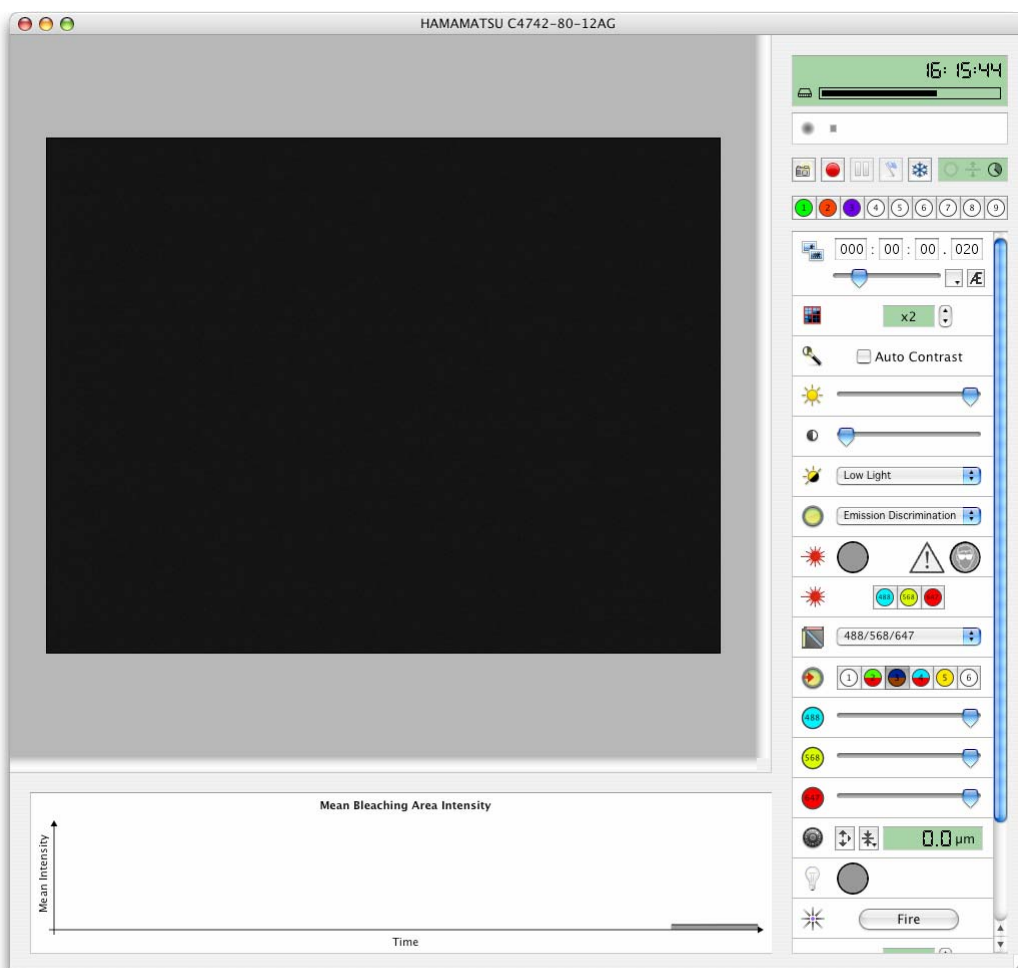
Laser Power

One laser power slider will be shown for each line configured in the preferences. The higher the power used the more excitation light will be sent to the sample. This may increase the emission signal allowing shorter exposures to be used, however it will also lead to faster photobleaching and possible phototoxic effects.

PhotoKinesis Accessory

Calibrating the PhotoKinesis Accessory

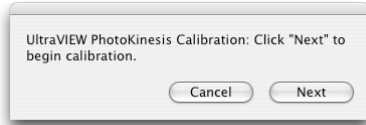
1. Select **FRAP Preview** from the Window menu.



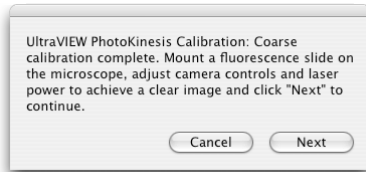
PhotoKinesis Accessory

2. Select **Calibrate UltraVIEW PhotoKinesis Device...** from the FRAP menu.

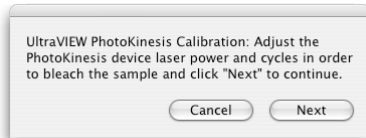
The calibration wizard runs.



3. Follow the instructions and the calibration will run automatically. A spot of laser light will be imaged and moved to all edges of the field to complete a coarse calibration. The next step, fine calibration, requires a sample to image. This works best with a homogenous specimen such as ink from a highlighter pen, allowed to dry. Stick a coverslip over the dried ink to best reproduce the imaging conditions of the sample.



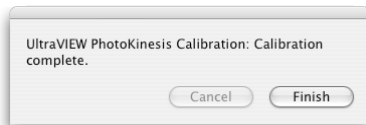
4. When the specimen is in focus and the image is clear on the screen, click **Next**.



5. To determine the laser power and number of cycles required to bleach the calibration sample it may be necessary to test bleach by drawing an ROI and using the **Fire** button to see whether the sample is bleached.



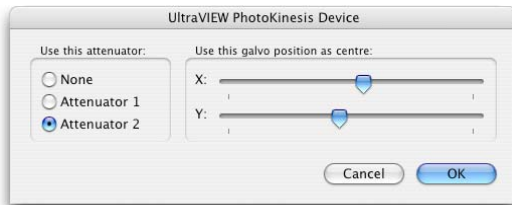
6. Do not be concerned if the ROI and the resultant bleach area are not perfectly aligned, fine adjustment is performed in this step. Click **Next**. Volocity will bleach a specimen area and adjust accordingly.



7. Click **Finish**. The calibration is completed and you may wish to perform a test bleach.

If the calibration is not able to run automatically you will be asked to adjust camera and laser settings to achieve a well-formed spot and to ensure that the spot is approximately centered in the field. The spot is formed by the laser light reflecting off the calibration mirror in the PhotoKinesis unit.

Double click on the bleaching device icon in the interface to access the dialog to insert attenuators and move the position of the spot in the X and Y axes.

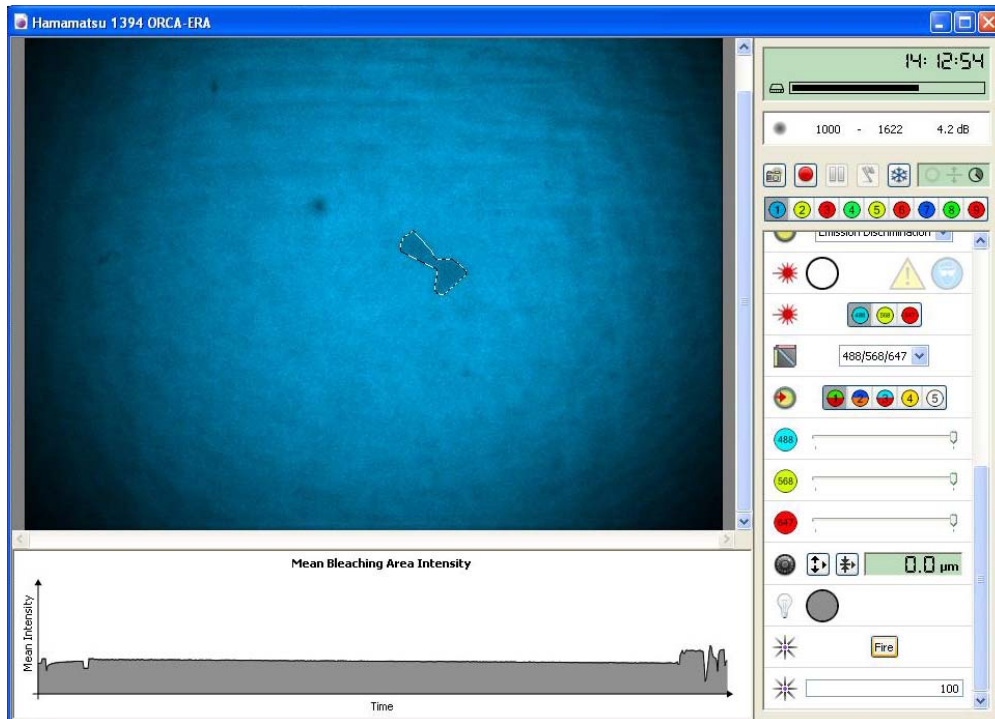


Click **Finish**. The calibration wizard will close and you may wish to test the calibration.

OptiGrid

Testing Calibration

1. Draw an ROI in the FRAP preview, using the ROI drawing tools from the toolbar.
2. Click **Fire** and check the accuracy of the offset. If the region drawn is not matched by the bleached shape you may need to repeat the calibration process using different settings.



OptiGrid

The OptiGrid is an optical sectioning technology that, when combined with image analysis software, confers confocal capabilities to wide field microscopes.

The OptiGrid consists of two pieces of additional hardware:

- The OptiGrid paddle, which is inserted into the microscope's fieldstop location and is connected to
- The OptiGrid controller box

The OptiGrid is based on a technique called "Structured Light" (Neil et al 97).

This technique modifies the illumination system of a microscope to project a grid pattern onto the specimen. The grid is moved into three different positions, and an image is acquired at each position. An algorithm is then used to produce a single, derivative, confocal image from which the grid pattern has been removed.

The algorithm takes advantage of the fact that in-focus information changes as the grid moves, whilst out-of-focus information remains constant. Therefore, if information does not change between images, it is out of focus and can be removed from the final image.

Please see the appendix on page 464 for more information about the algorithm and related publications.

OptiGrid Models

With some models of microscope, chromatic aberration problems have made it difficult or impossible to place the grid pattern in common focus for all excitation wavelengths in use. With earlier OptiGrid models, this restricted the range of excitation wavelengths that could be used together to acquire image sequences, and created a need for manual refocusing of the OptiGrid.

The second-generation OptiGrid, sometimes referred to as the 'Focusing OptiGrid', incorporates a motor that enables it to be moved axially into different focal positions in the light path of the microscope. Once the correct focal position for an excitation wavelength is established it can be consistently and automatically restored. This extends the range of excitation wavelengths that can be sequentially acquired into a single image sequence.

All OptiGrid models are supported in versions 4.1 or higher for Mac OS X and Windows.

You need a free USB port in order to run an OptiGrid.

Note: Manual focus models of the OptiGrid are connected via the Improvion Acquisition Hub.

Connecting the OptiGrid

Motorized Focus Model

This section describes how to connect the OptiGrid model that includes motorized focus control.

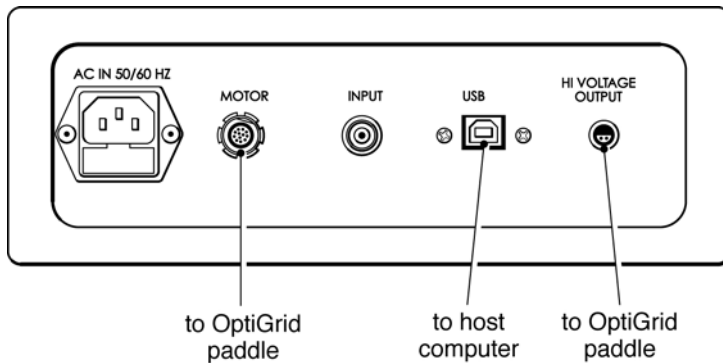
The following components are supplied with your OptiGrid and are required for installation:

- OptiGrid paddle
- OptiGrid controller box
- USB cable
- Focus motor cable
- High voltage cable

Connecting the OptiGrid



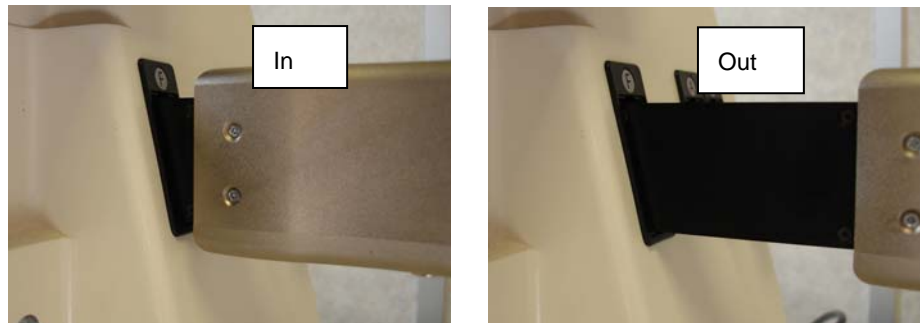
1. The OptiGrid controller box has a high-voltage output. The OptiGrid controller box must be switched off before you connect the OptiGrid paddle using the power switch which is on the front of the controller box.
2. Connect one end of the high voltage cable to the OptiGrid paddle and the other end to the Hi Voltage Output connector on the rear of the OptiGrid controller box.
3. Connect one end of the focus motor cable to the OptiGrid paddle and the other end to the MOTOR connector on the rear of the OptiGrid controller box.
4. Connect one end of the USB cable to the USB connector on the rear of the OptiGrid controller box and the other end to a spare USB port on your computer.



5. If you have a field stop (field diaphragm) installed in you microscope, remove it from the field stop port.



6. Install your OptiGrid in the field stop port. Your OptiGrid “In” position can be found by pushing the OptiGrid into the field stop port until the “clickstop” engages. In this position the OptiGrid should be visible in the ocular lenses or with the camera when fluorescence images are observed. The “Out” position can be found by withdrawing the OptiGrid from the field stop port (disengaging the “clickstop”) until the grid is no longer in the light path, and no elements of the grid housing are in the light path. In this position the OptiGrid can be left in the field stop port.



7. Switch on the OptiGrid controller box and start Volocity. The message “Connected to Volocity” will be displayed on the OptiGrid controller LCD display.
8. You must now set up the OptiGrid for use with Volocity. This requires you to set up the Device Preferences in Volocity and calibrate the OptiGrid. See page 164.

Connecting the OptiGrid

Manual Focus Model

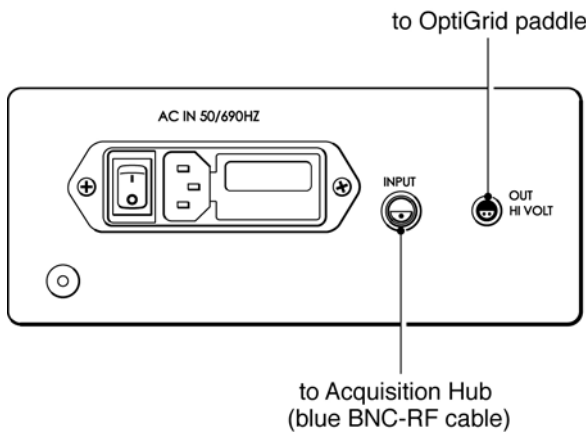
The manual focus model of the OptiGrid is connected via the Improvion Acquisition Hub to the computer system that is running Volocity.

The following components are required for installation:

- OptiGrid paddle
- OptiGrid controller box
- Improvion Acquisition Hub
- Blue BNC-RF cable



1. The OptiGrid controller box has a high-voltage output. The Acquisition Hub and the OptiGrid controller box must both be switched off before you connect the OptiGrid paddle using the power switch is on the front of the Acquisition Hub and the OptiGrid controller box.
2. Connect the OptiGrid paddle to the **OUT HI VOLT** connector on the rear of the OptiGrid controller box.
3. Connect the BNC-RF cable to the **INPUT** connector on the rear of the OptiGrid controller box and connect the other end of the BNC-RF cable to the **OPTIGRID** port on the rear of the Acquisition Hub.



Note: this cable will fit if connected the wrong way around, but the OptiGrid will only work if the cable is connected in the orientation shown below.



4. If you have a field stop (field diaphragm) installed in you microscope, remove it from the field stop port.



5. Install your OptiGrid in the field stop port. Your OptiGrid "In" position can be found by pushing the OptiGrid into the field stop port until the "clickstop" engages. In this position the OptiGrid should be visible in the ocular lenses or with the camera when fluorescence images are observed. The "Out" position can be found by withdrawing the OptiGrid from the field stop port (disengaging the "clickstop") until the grid is no longer in the light path, and no elements of the grid housing are in the light path. In this position the OptiGrid can be left in the field stop port.



7. Switch on the Acquisition Hub and the OptiGrid controller box. The USB, PWR and RDY lights on the Acquisition Hub and the red light on the front of the OptiGrid controller should all be illuminated.

NOTE: If the LEDs on the Acquisition Hub or OptiGrid controller fail to illuminate, check the cabling and make sure that none of the connecting pins are bent. For further information, see page 168.

8. You must now set up the OptiGrid for use with Volocity. This requires you to set up the device preferences in Volocity and calibrate the OptiGrid. See page 11.

Connecting the OptiGrid

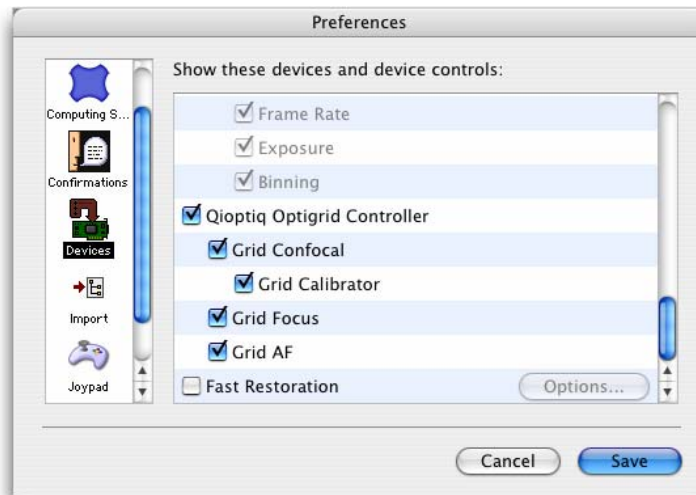
Setting Velocity Preferences

1. Open the Velocity application.
2. Select **Preferences...** from the Edit menu (Windows) or the Velocity menu (Macintosh).
3. Click on the **Devices** icon in the left-hand panel. A list of devices that have been correctly connected to your computer will appear in the right-hand panel. Each device has a subset of device controls that are listed beneath the device.

Motorized Focus OptiGrid Models

Scroll through the list of devices until you find the **Qioptiq Optigrid Controller**.

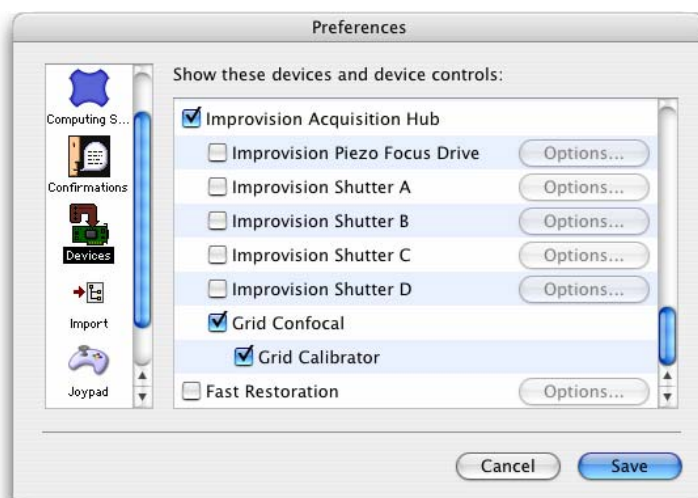
Check the box next to the **Qioptiq Optigrid Controller**, check the boxes for all of its associated devices.



Manual Focus OptiGrid Models

Scroll through the list of devices until you find the Improvisation Acquisition Hub.

Check the box next to the **Improvisation Acquisition Hub**, check the box next to the **Grid Calibrator**.



Calibrating the OptiGrid

It is important to focus the grid manually the first time that you calibrate. The information in this section applies to all OptiGrid models.

In order to calibrate the OptiGrid you must have a uniformly fluorescent sample against which the grid can be imaged. Sealing a small amount of fluorescent ink between a slide and a cover slip can make an ideal calibration slide. Highlighter pens are an excellent source of such ink. Alternatively, you can use the Qioptiq calibration slide.

Calibration process

1. Open Volocity and select **Show Video Preview** from the **Window** menu.
2. Mount the calibration slide on the microscope and select an appropriate fluorescence filter. For example, blue excitation and green emission works well with yellow ink.
3. Expose the calibration slide to the fluorescent light source. Calibration can be done with any lens but you will get better grid contrast with low power lenses.
4. Use the microscope focus drive to focus on the fluorescent material. Air bubbles or the edge of the "inked" area make good reference points.
5. Move to a field of view that is uniformly filled with fluorescent material.
6. Move the OptiGrid to the "in" position in the microscope's fieldstop location.

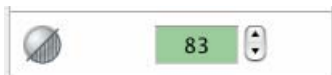
Calibrating the OptiGrid

7. Adjust the aperture diaphragm on the microscope to improve the contrast if necessary.
8. Direct all light to your camera and, if your camera supports it, select **Auto-Exposure** from the Video Preview controls (or the AE button).



Note: If the exposure is low (<20ms), you may see grid lines in your grid confocal images due to lamp flicker. Avoid low exposures by reducing lamp intensity or using neutral density filters.

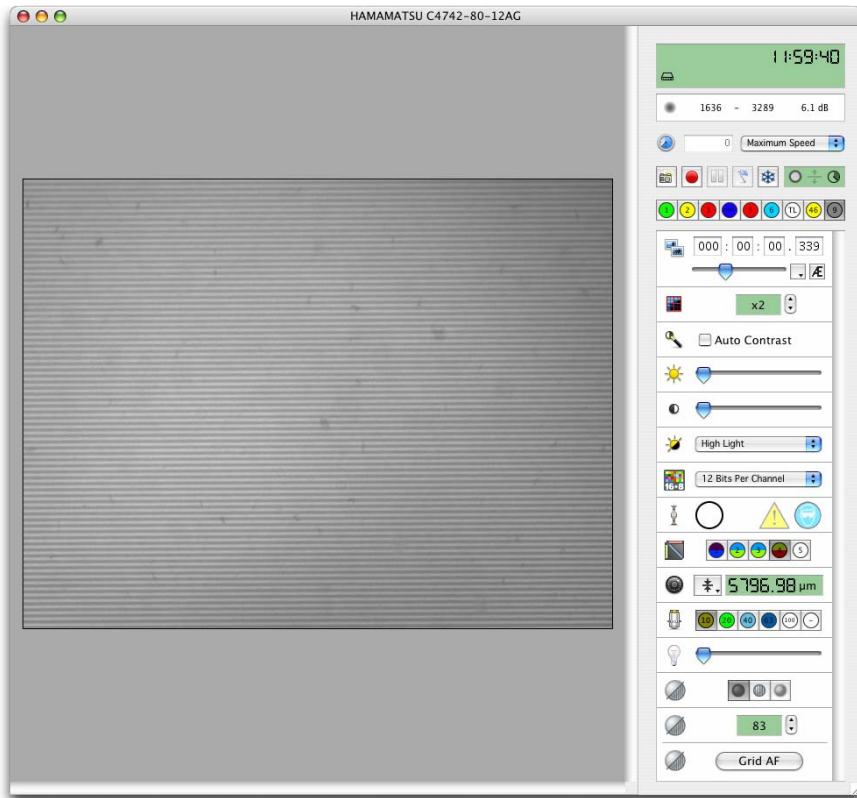
9. Use the grid focus control to adjust the position of the OptiGrid until the image of the grid is maximally focused against the fluorescent material on the Video Preview.



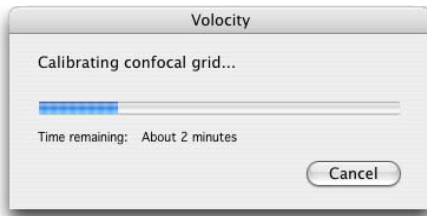
NOTE: With manual focus model of the OptiGrid you must use the “hex wrench” focusing tool to focus the grid. Insert the hex wrench in the middle of the three holes visible on the side of the OptiGrid until it engages. Twisting the hex wrench tool changes the focal position of the OptiGrid. Avoid using excessive force when focusing the OptiGrid as you can damage the grid focus mechanism when the grid has reached the limit of its movement. If significant resistance is met, you are likely to have reached the focus limit and should twist the hex wrench tool in the opposite direction.



10. It is important that the focus is consistent across the grid, see page 181.



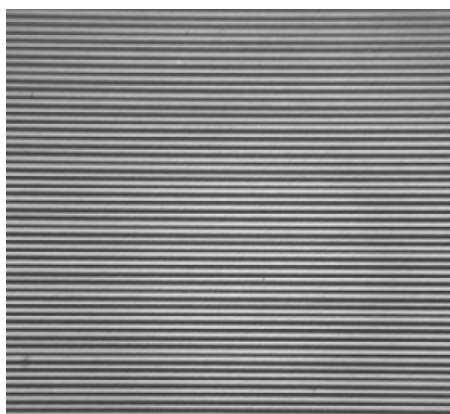
11. Select **Calibrate Confocal Grid...** from the Video menu. A progress bar is displayed as the OptiGrid is calibrated; this may take several minutes.



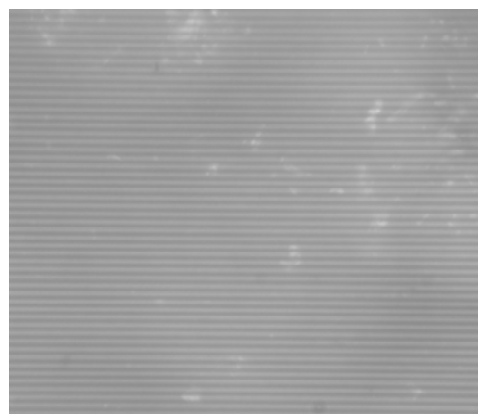
12. The calibration of the grid is now complete and the calibration slide can be removed and replaced with the specimen that you wish to image.

OptiGrid Installation and Calibration Troubleshooting

During calibration, you require an image where the focus is consistent across the grid. Here are examples of good and poor grid images. Note the high grid contrast in the good image:



Good grid image



Poor grid image

If you are unable to focus your grid against your calibration slide, despite moving the grid through its full focus range, check the manufacturer of your fluorescence filter set. Filters manufactured by Semrock do not work with the OptiGrid and should be avoided.

Insufficient contrast warning or error 13901 during calibration (all models)

These errors indicate that the grid is not consistently in focus or that the grid contrast is not high enough for imaging. To rectify this, follow these steps:

1. Ensure that the grid paddle is in the light path.
2. Turn off "Auto Contrast" if your camera supports this function. "Auto Contrast" can lead you to believe that grid contrast is better than it actually is.
3. Use the microscope focus drive to focus on the calibration slide.
4. Adjust the focus of the OptiGrid, using the Grid Focus Control (motorized focus OptiGrid) or the hex wrench tool (manually focusing OptiGrid).
It is important that the focus is consistent across the grid; use the focus adjustment to position the grid such that contrast is as consistent as possible.
5. Repeat steps 2 and 3 to maximize the grid contrast. It can help to use the numerical readout of the minimum and maximum pixel values in the "Video Preview" window to do this. See page 141.
6. Closing the aperture diaphragm will improve grid contrast.
7. Reducing the excitation light intensity and increasing the camera exposure time may improve contrast.

8. Different objectives may give better contrast. Calibration is best performed with a good-quality Plan-Apochromat air objective.

Grid does not move during calibration (all models)

During calibration the grid should move, first slowly in one direction, then more quickly in different directions. If the grid does not move or the calibration fails with error 13902, follow these steps.

Motorized Focus OptiGrid Models



1. Switch off the OptiGrid controller. The OptiGrid controller must be switched off while connecting and disconnecting cables because it has a high-voltage output.
2. Check that the cables are connected correctly. The OptiGrid paddle should be connected to the OptiGrid controller via two cables; one to the Hi Voltage Output connector and the other to the MOTOR connector on the OptiGrid controller. The host computer should be connected to the OptiGrid controller via a USB cable to the USB connector.
3. Check both ends of the high-voltage cable and make sure that no pins are bent on the connectors. If a pin is bent, carefully bend it back into position with a small screwdriver.

Manual Focus OptiGrid Models



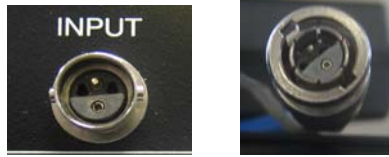
1. Switch off the OptiGrid controller and the Acquisition Hub. The OptiGrid controller must be switched off while connecting and disconnecting cables as it has a high-voltage output.
2. Check cables are connected correctly. The OptiGrid paddle (or microscope connection) should be connected to the “hi volt” output of the black OptiGrid controller. The “input” on the OptiGrid controller should be connected to the “OptiGrid” port on the Acquisition Hub with the blue cable. Note that the blue cable is not a standard BNC-BNC cable; it is a BNC-RF cable. A standard BNC-BNC cable may physically fit, but will not work.
3. Check that no pins are bent on the high-voltage output of the OptiGrid controller. There should be four pins, two in the OptiGrid controller socket and two in the grey paddle cable. If a pin is bent, carefully bend it back into position with a small screwdriver.



There are four pins, two on each side of the connector

Calibrating the OptiGrid

4. Check that no pins are bent on the low-voltage input of the OptiGrid controller. There should be two pins, one in the blue cable and one in the OptiGrid controller socket. If a pin is bent, carefully bend it back into position with a small screwdriver.



There are pins on both sides of the connector – the ones in this picture are OK.

5. Check that the pin is not bent at the Acquisition Hub end of the blue BNC-RF cable.



6. Check that the “OptiGrid” socket on the Acquisition Hub is not damaged – it is possible for the central part of the socket to get distorted if the pin in the blue cable is not central. If the socket is damaged, it is normally possible to repair it using a pin to tease the metal parts of the socket back into place.



This OptiGrid socket has been slightly damaged and the metal parts teased back into place.

Compare with the Piezo Z socket on the left.

7. Switch on both the Acquisition Hub and OptiGrid controller. The USB, PWR and RDY lights on the Acquisition Hub and the red light on the front of the OptiGrid controller should all be illuminated.
8. Retry calibration. If calibration fails again, contact Improvision or the Improvision dealer that supplied your OptiGrid.

Using the OptiGrid

Once you have calibrated the OptiGrid, you are ready to use it for acquisition. If you intend to acquire multi-channel image sequences you may need to ensure that the grid is correctly focused for each of your excitation wavelengths. The worked examples in this chapter illustrate this process.

Grid controls



All versions of the OptiGrid have the grid mode controls. The focus and auto-focus controls are not available with manual focus models of the OptiGrid.

Auto focus

Click **Grid AF** to allow Volocity to auto focus the grid for you. Volocity will test a number of grid focus positions until it finds the position in which grid confocal images with the best contrast are generated. Auto focus will only work after your grid has been calibrated for the first time and provided you have a specimen mounted and in focus.

Focus

Use this control to adjust the focus position of the grid. You can use this control as an alternative to **Grid AF**, or to fine tune the result of **Grid AF**.

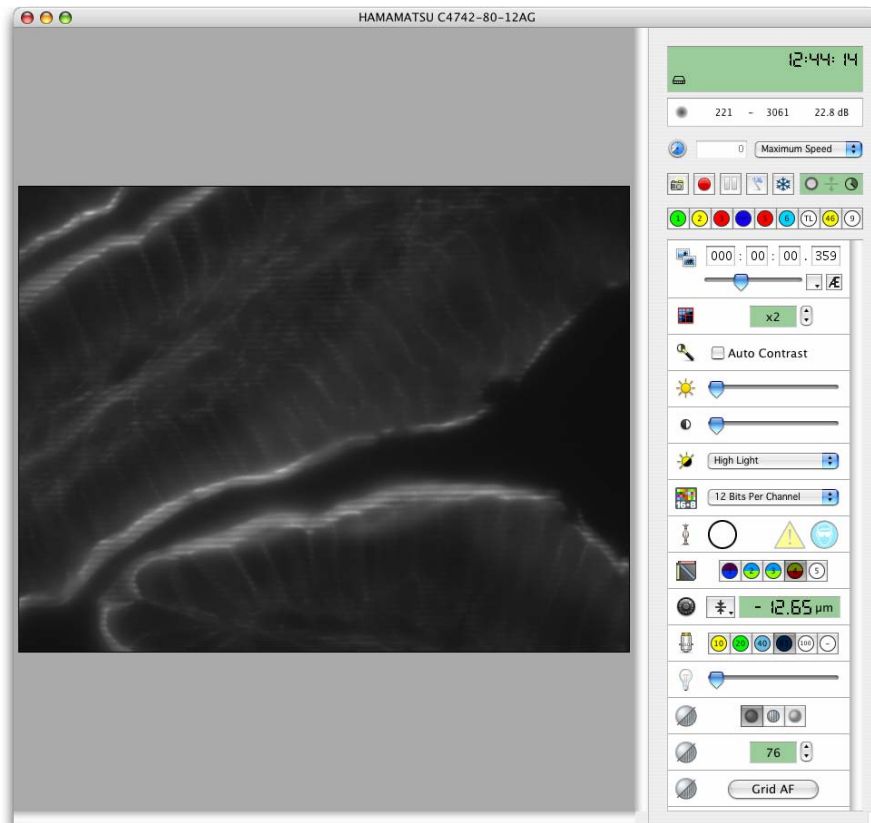
Using the OptiGrid

Grid modes

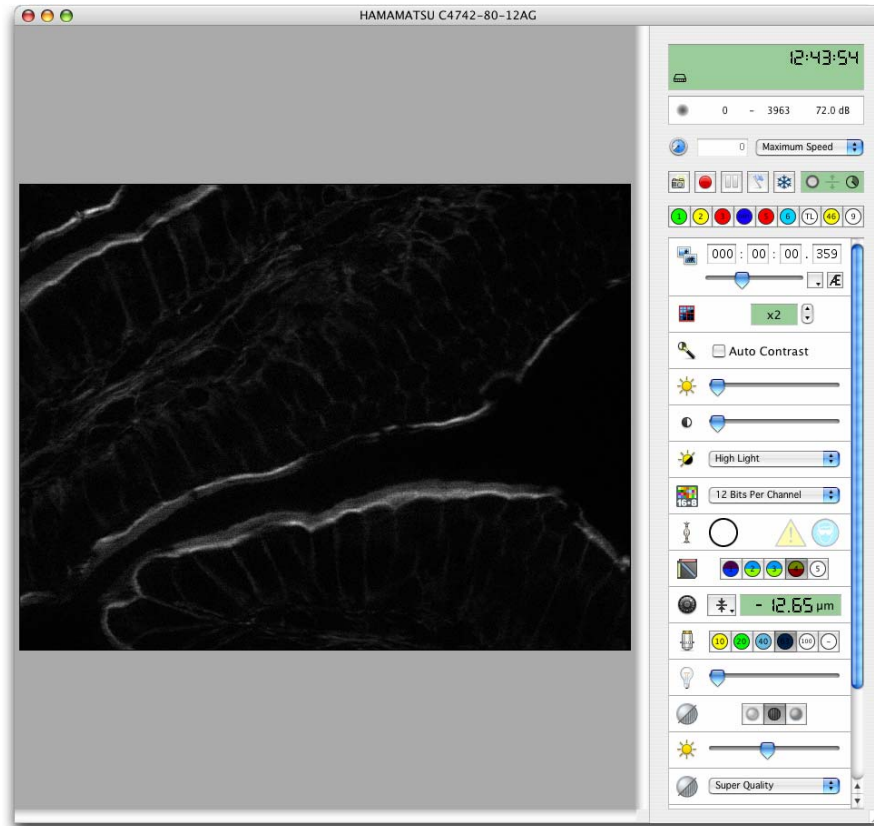
There are three grid mode controls. From left to right:



- **Grid Off:** Switches the grid off. There is a stationary grid pattern on the Video Preview and from the ocular lenses of the microscope.

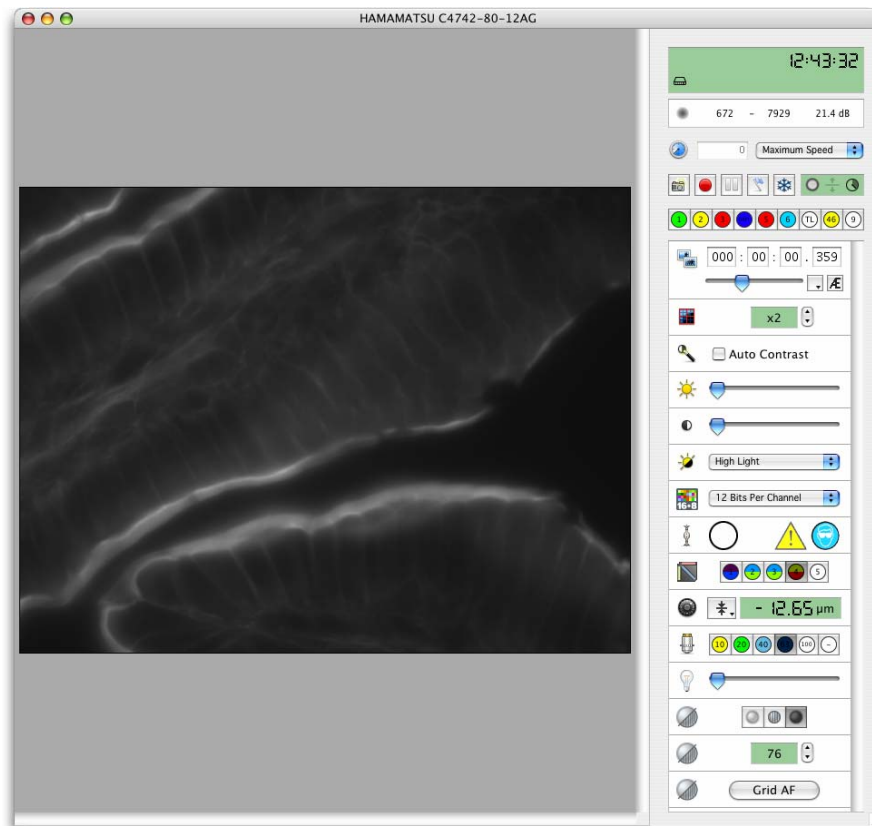


- **Grid Confocal:** Activates the grid. There is a confocal image on the Video Preview and a moving grid pattern from the ocular lenses of the microscope.



Using the OptiGrid

- **Grid Wide Field:** Activates the grid. There is a wide field image (without grid lines) on the Video Preview and a moving grid pattern from the ocular lenses of the microscope.



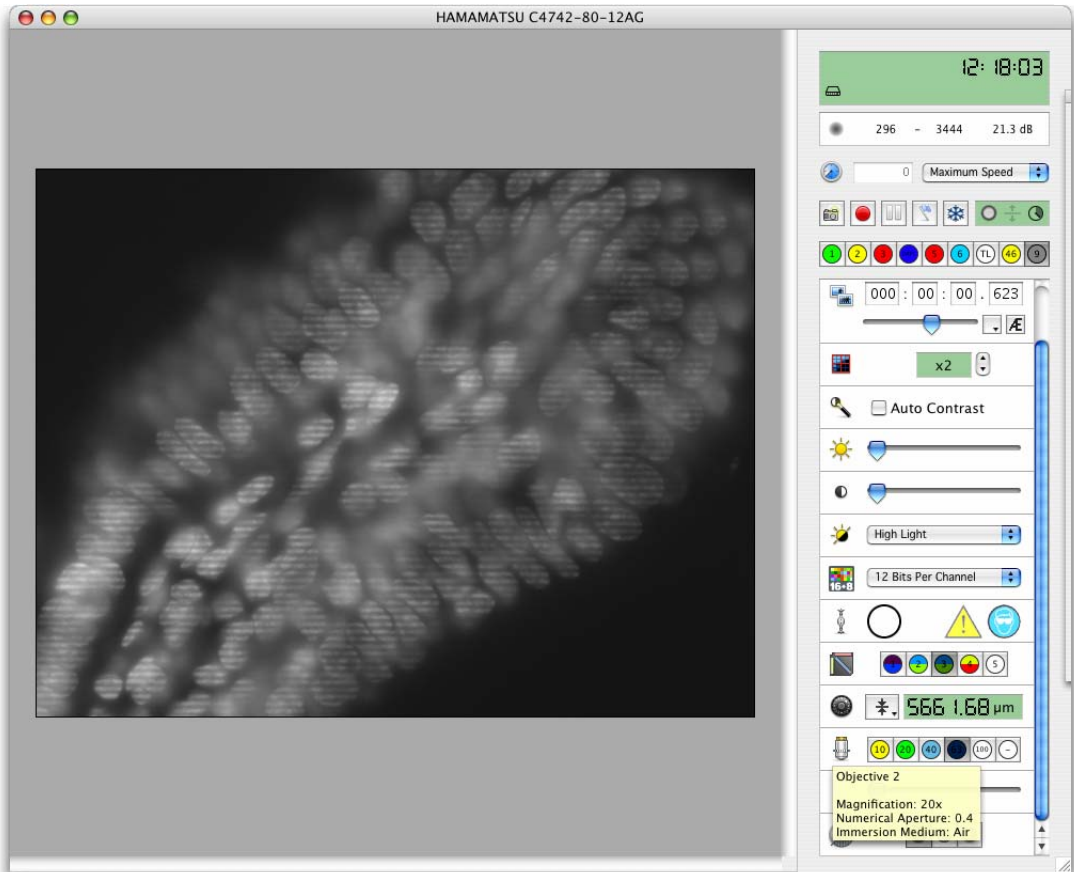
Single-channel grid confocal images

1. Open Volocity and select **Show Video Preview** from the Window menu.
2. Mount your specimen slide on the microscope stage and select an appropriate fluorescence filter set.
3. Expose the specimen slide to the light source and select a suitably powered lens.
4. Move to the field of view that interests you.
5. Ensure that the OptiGrid is in the "in" position in the microscope's fieldstop location.

- Direct all light to your camera and, if your camera supports it, select Auto-Exposure from the Video Preview controls (or the AE button).

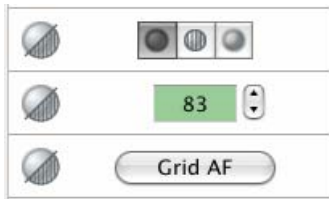


- Set the Grid activation control in the Video Preview to **Off**. You should see clear horizontal lines superimposed on your specimen.



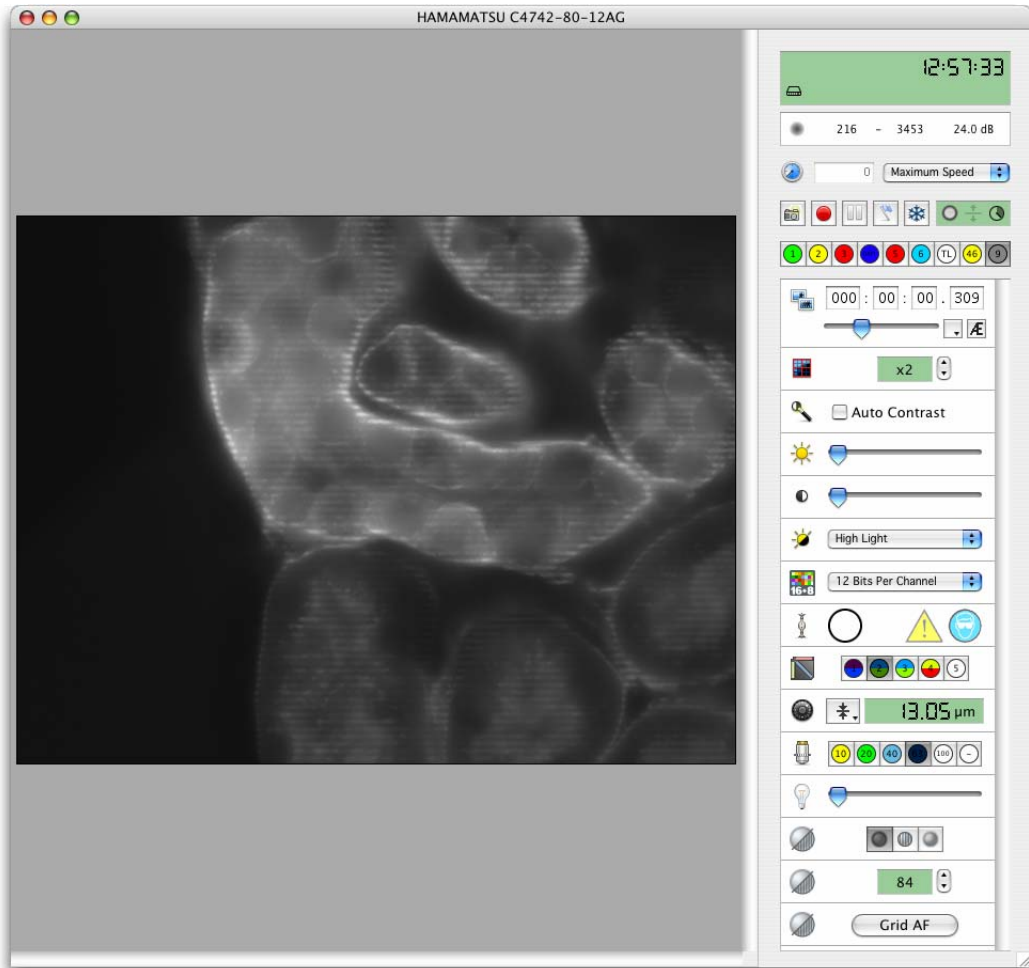
Using the OptiGrid

- Click **Grid AF** to focus the OptiGrid automatically. Alternatively, use the grid **Focus** control to focus the OptiGrid.



NOTE: With the manual focus model of the OptiGrid you must use the “hex wrench” focusing tool to focus the grid. Insert the hex wrench in the middle of the three holes visible on the side of the OptiGrid until it engages. Twisting the hex wrench tool changes the focal position of the OptiGrid. Avoid using excessive force when focusing the OptiGrid as you can damage the grid focus mechanism when the grid has reached the limit of its movement. If significant resistance is met, you are likely to have reached the focus limit and should twist the hex wrench tool in the opposite direction.





Note: You can also adjust the grid position whilst the grid is in “Grid Confocal” mode. As you move the grid, the signal-to-noise ratio of the grid confocal image will improve if the grid’s focus is improved.

9. Set the Grid activation control to “Grid Confocal”.
10. Change the slide or mount the slide in the first place.
11. You are now ready to acquire grid confocal images or image sequences.

After acquisition, remember to move the OptiGrid out of the microscope’s lightpath or ensure that the shutter is closed. If a light source is left on with the grid inserted but not moving, a grid image can be burnt into fixed samples which will cause gridlines.

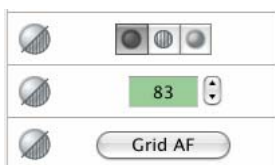
Multi-channel grid confocal images (Motorized Focus OptiGrid)

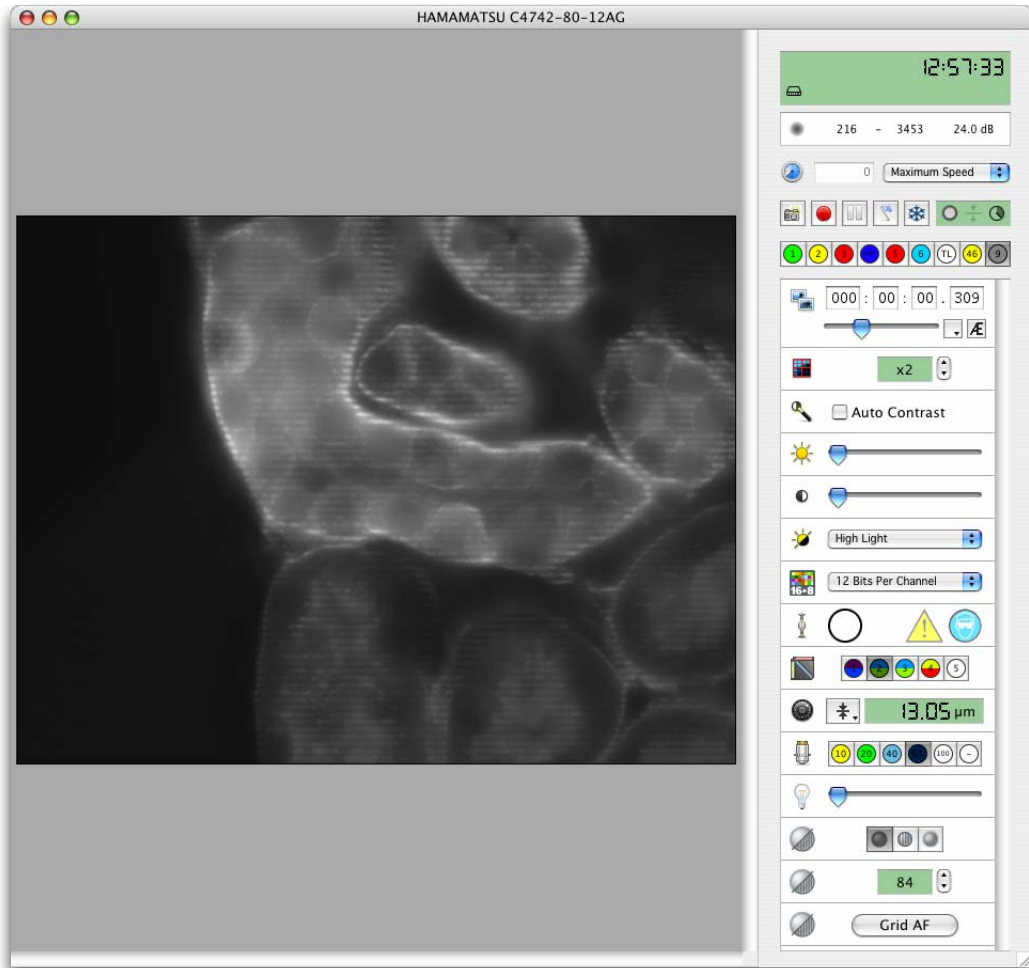
If you wish to acquire multi-channel grid confocal images, you must ensure that the grid is correctly focused for all of your lightpaths before acquisition. See page 138 for more information about lightpaths.

1. Open Volocity and select **Show Video Preview** from the Window menu.
2. Mount the specimen slide on the microscope and select an appropriate light path.
3. Expose the specimen slide to the light source and select a suitably powered lens.
4. Move to the field of view that interests you.
5. Ensure that the OptiGrid is in the “in” position in the microscope’s fieldstop location.
6. Direct all light to your camera and, if your camera supports it, select **Auto-Exposure** from the Video Preview controls (or the AE button).



7. Set the Grid mode control in the Video Preview to Off. You should see clear horizontal lines superimposed on your specimen.
8. Click **Grid AF** to focus the OptiGrid automatically or use the grid focus control to focus the OptiGrid.



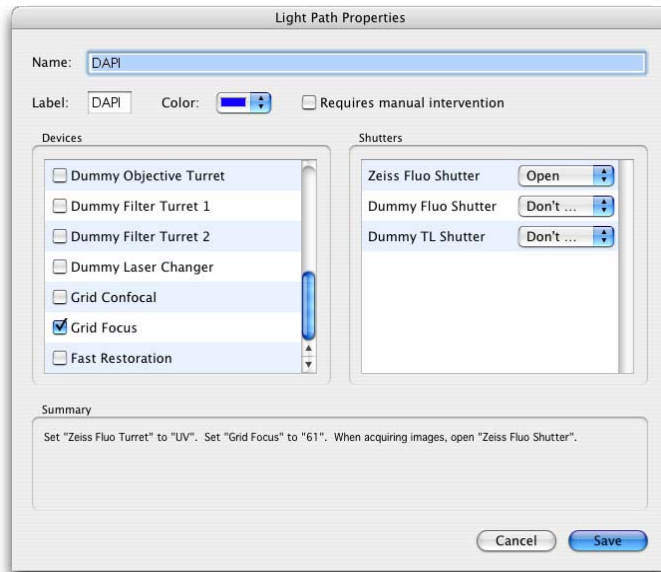


Note: You can also adjust the grid position whilst the grid is in “Grid Confocal” mode. As you move the grid, the signal-to-noise ratio of the grid confocal image will improve if the grid’s focus is improved.

9. Double click on the current light path’s button. The Light Path Properties dialog opens. Scroll through the list of devices until you find **Grid Focus**.

Using the OptiGrid

Click on the **Grid Focus** check box to associate the current grid focus position with the light path.



10. Repeat this process for each of the light paths that you wish to restore for your acquisition.
11. Set the Grid activation control to "Grid Confocal".
12. You are now ready to acquire multi-channel grid confocal image sequences.

After acquisition, remember to move the OptiGrid to the "out" position in the microscope's fieldstop location or ensure that the shutter is closed. If a light source is left on with the grid inserted but not moving, a grid image can be burnt into fixed samples which will cause gridlines.

Multi-channel grid confocal images (Manual Focus OptiGrid)

With the manual focus OptiGrid it is not possible to associate different grid focus positions with each of your light paths. If your OptiGrid cannot be focused perfectly for each of your light paths, it may be necessary to find a compromise grid focus position in which an adequate image of the grid can be seen for all of your excitation wavelengths.

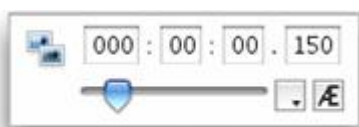
Adjusting the aperture diaphragm

The position of the aperture diaphragm can have an important effect on the quality of your images; you should always carefully consider its position before and during any imaging sessions. Closing the aperture diaphragm tends to improve the contrast of the grid and, therefore, the contrast of the grid confocal images. Closing the aperture diaphragm also increases the exposure times of your images, so you should balance aperture position with acceptable exposure times.

1. Select the grid confocal mode from the Video Preview controls.



2. Adjust the aperture diaphragm on the microscope.
3. Select Auto-Exposure from the Video Preview controls (you will see the grid lines while Volocity adjusts your exposure).



4. Assess whether the quality of the image and the exposure time will meet your experimental needs.

When you are happy with the position of the aperture diaphragm you can proceed with your acquisition of grid confocal images. Acquisition of image sequences can be done as normal, remember to select the grid confocal mode before starting acquisition.

Troubleshooting Grid Imaging

Once you become familiar with the way the grid is working, it becomes clear how artefacts may be generated.

- Vibration may alter the grid movement and prevent it from moving to the correct position for each of the three raw images.
- Inconsistent illumination or photobleaching can produce intensity differences between the raw images. The algorithm will extract artefactual data from these illumination differences and grid lines will appear in the final image.
- A poorly focused (or out of focus) grid will produce little or no difference between the three raw images and a blank confocal image will be generated.

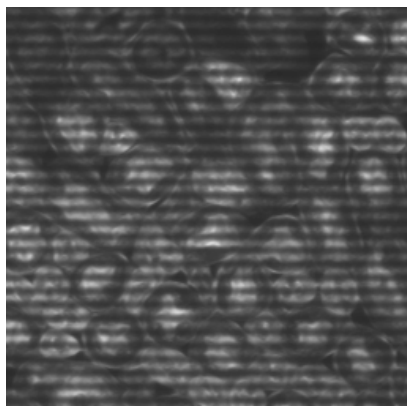
Troubleshooting Grid Imaging

Little or no signal with the grid active

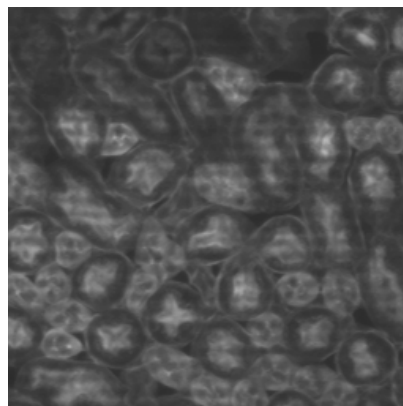
The OptiGrid relies on the grid “marking” in-focus parts of the sample. If the grid contrast in the image is poor, no parts of the sample will be marked and little or no signal will be output.

Recalibrating the grid will not improve the brightness of the grid image – calibration only removes gridlines.

Note: Increasing the camera exposure time will not make the confocal signal brighter – the brightness of the confocal signal is determined purely by the contrast in the grid image.



Good grid contrast



Poor grid contrast

To correct for this:

1. Adjust the grid focus. First focus the sample with the grid off. Select “Grid Confocal” mode, then adjust the grid focus until the confocal signal is as bright as possible.

2. Adjust the imaging system to maximize grid contrast. Closing the aperture diaphragm to reduce stray light will help.
3. It may be necessary to adjust the grid focus for different wavelengths (see page 171) and different objectives.
4. Plan-Apochromat objectives will give better results with multiple wavelengths.

Gridlines in image

Gridlines in the image are caused by the three raw grid images not quite lining up with each other. There are several possible reasons for this:

1. Poor calibration. If gridlines appear, try recalibrating the grid. The grid calibration can change as the grid and microscope heat up in use. Recalibration will correct for this.
2. Vibration can cause grid lines. Isolate the microscope with an anti-vibration table if possible. Equipment containing fans (e.g. camera controllers and light sources) must be isolated from the microscope (e.g. by moving them onto a separate table or onto the floor).
3. Uneven or unstable illumination will cause gridlines. Use a stable light source that offers even illumination. If flicker is a problem, attenuate the light using ND filters and increase exposure times.
4. If the light source is left on with the grid inserted but not moving, a grid image can be burnt into fixed samples which will cause gridlines. Ensure that the fluorescence shutter is closed when possible or that the grid is left running.
5. Fast bleaching samples may show gridlines more than slow bleaching samples. To solve this, reduce the light intensity and increase exposure times.
6. Camera noise will be amplified by the OptiGrid process and will show as gridlines. Avoid using high camera gain with the OptiGrid.

Once these factors are eliminated, recalibrate the grid.

Installing and Configuring Photonics Instruments MicroPoint

Volocity supports the Photonics Instruments MicroPoint Ablation Laser Unit as a bleaching device.

<http://www.photonic-instruments.com/>

Before you use the MicroPoint high-intensity laser unit for the first time with Volocity it must be calibrated. You must calibrate the laser again should the laser unit be rotated relative to the microscope, if the camera is moved relative to the microscope, if the dye cell is changed, or if the microscope's objective lens is changed.

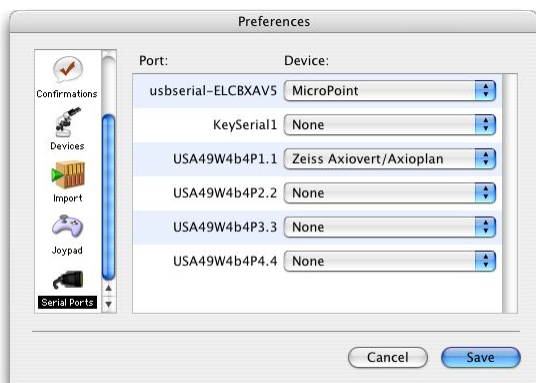
Connect the Ablation Laser Unit to the microscope, as instructed in the Photonics Instruments documentation.

Connect the MicroPoint control box to the computer via a free USB port.

Calibrating Photonics Instruments MicroPoint

Although the MicroPoint control box connects to Volocity via a USB connection it will function as a “Virtual COM Port” (VCP). In order for this to work you must download and install the driver appropriate for your operating system from <http://ftdichip.com/Drivers/VCP.com>

1. Open Volocity and select Serial Ports from the preferences.
2. Set the usbserial-ELCBXAV5 port to the MicroPoint.



3. Select Devices from the preferences.
4. Ensure that all of the MicroPoint devices are selected.



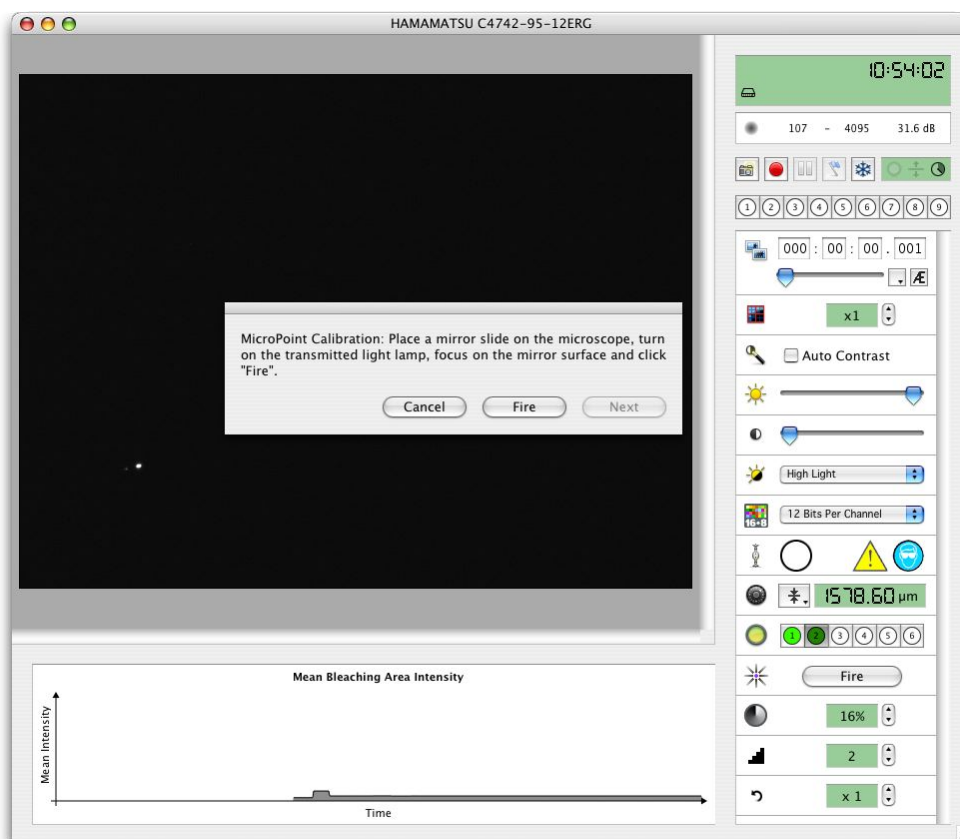
Calibrating Photonics Instruments MicroPoint

Make sure that the laser unit is horizontal (relative to the microscope) before you start, focus and align the laser as described in the Photonics Instruments documentation.

Ensure that your “Laser Safety Filter Block” is always in the light path when using the MicroPoint laser.

1. Mount your mirror slide on the microscope with the reflective surface nearest to the objective lens.

2. Select **Show FRAP Preview** from the Window menu.
3. Switch on your transmitted light lamp.
4. In order to focus on the mirror's surface you should search for a scratch or pit on the surface and focus the microscope on that.
5. Direct all light to the camera and adjust the camera exposure time if necessary.
6. Ensure that any shutters in the laser's light path are open.
7. Select **Calibrate MicroPoint...** from the FRAP menu.
8. The MicroPoint Calibration wizard is opened.
9. Click **Fire**.

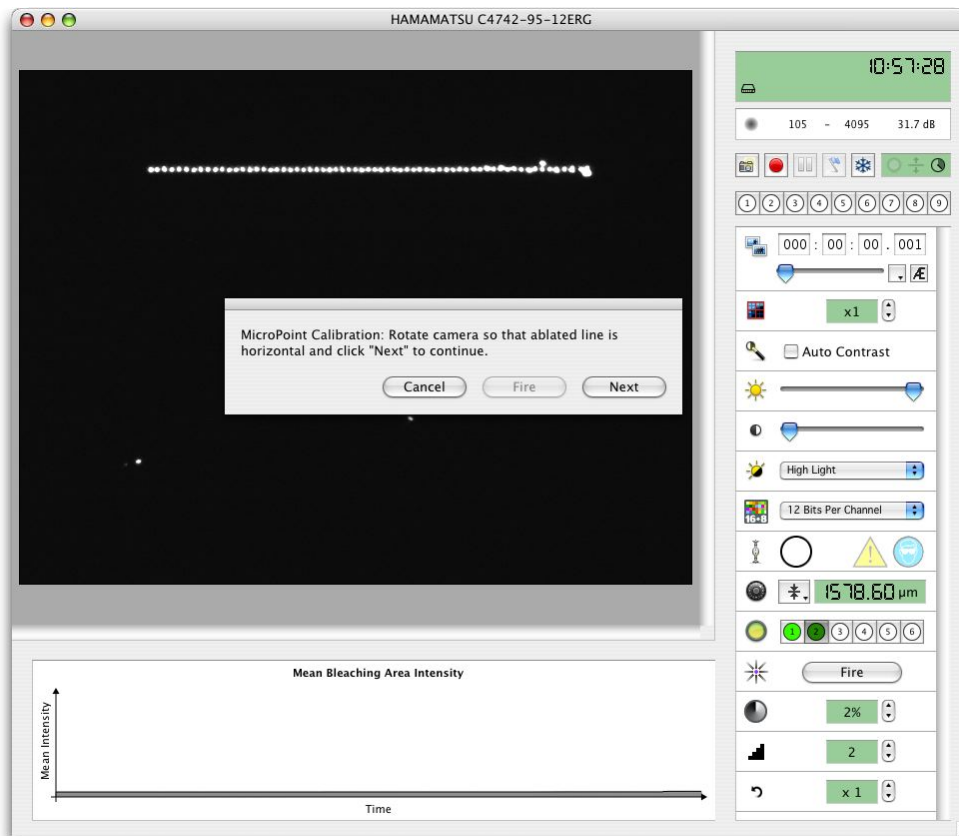


10. The MicroPoint laser is fired. If the laser is correctly focused on the mirror a hole will be cut in the mirror's reflective surface allowing transmission of light and creating a spot. If you are unhappy with the shape and size of the spot, adjust the focus or the intensity of the laser and click **Fire** again.

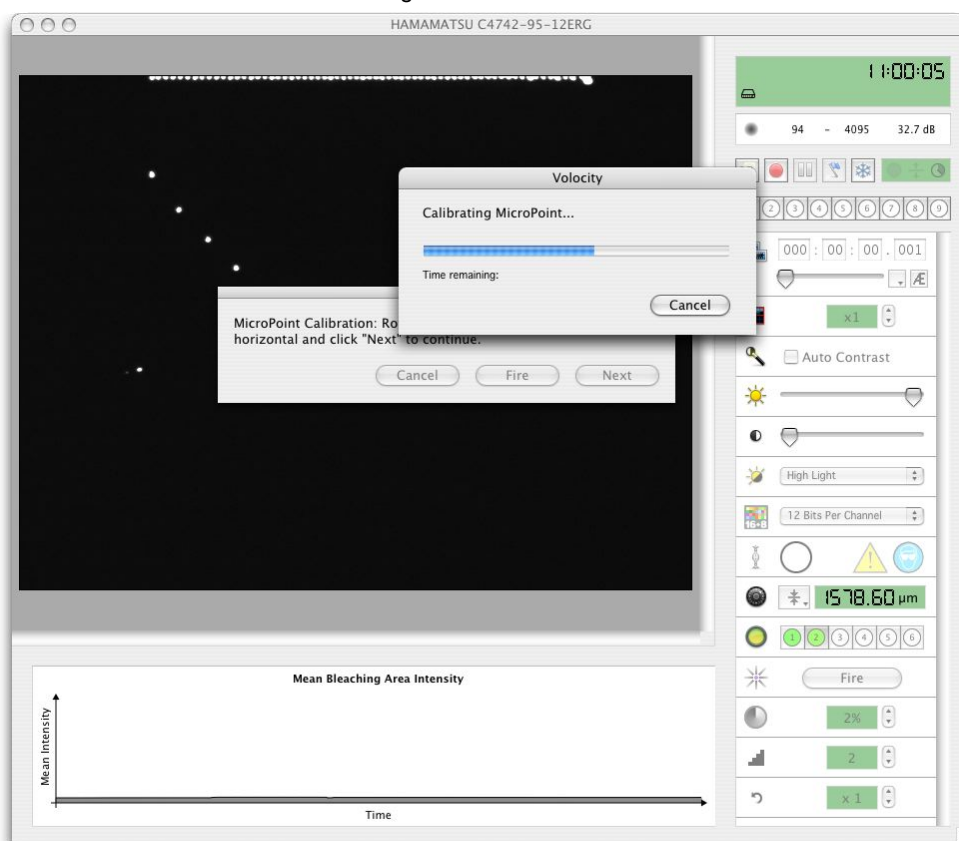
Calibrating Photonics Instruments MicroPoint

11. Once you are happy with the size and shape of the spots, click **Next**. A line of holes is fired onto the mirror. Adjust the rotational position of your camera so that the line is horizontal relative to the FRAP preview.

Tip: You may find it easier to judge whether the line is horizontal, if you move it (using the stage) higher up or lower down the Preview window where you can align it with the top or bottom of the screen.



12. Click **Next** to calibrate the laser. A diagonal line of holes is fired across mirror.



13. Click **Finish**. The calibration wizard will close.
14. You should now check the result: use one of the ROI tools to draw a region of interest on the Preview and click the MicroPoint **Fire** control within the video controls. The cut area should correspond exactly to the ROI. If this is not the case repeat the calibration procedure.

FRAP Acquisition

Velocity provides full support for observing and analyzing the movement of intracellular materials after photobleaching of fluorescence. It supports both Fluorescence Recovery after Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP) analysis.

FRAP Acquisition and Analysis functionality are provided by the FRAP plugin. This is an additional module available for purchase which extends the functionality of Velocity Acquisition to add features

FRAP Acquisition

specifically for FRAP acquisition and extends the functionality of Volocity Quantitation for analysis of data acquired with the FRAP acquisition.

The FRAP plugin requires both Volocity Acquisition and Volocity Quantitation to function.

Preparing a FRAP Experiment

Mount your specimen and expose it to your low intensity light source.

1. With your specimen in focus ensure that the all light is directed to your camera. You should see your specimen in the Video Preview. Adjust the camera controls until you are happy with your image.
2. You may wish to observe your specimen for a while to see whether you have an acceptable level of photobleaching from your low intensity light source, using the live intensity plot should help you make this judgment. If you make adjustments to your light intensity, remember to adjust your camera settings.
3. If possible, adjust the intensity of your bleaching device to the correct level. You should aim for maximum photobleaching without damaging or excessively heating your specimen.
4. When you are happy with the intensity of both your low intensity light source and bleaching device move to a new (unexposed) area of your specimen.



Select the Freeze button. Any shutters associated with your currently selected light path will be closed. The last frame previewed before the shutter was closed will be retained in the FRAP Preview.

5. With the FRAP Preview frozen you can set the bleaching area for your experiment without photobleaching your sample. ROIs are used to mark bleaching areas. Use any ROI tool to draw an ROI or restore an ROI by selecting **ROI** then **Restore...** from the Edit menu. To set an ROI of a particular size we recommend the use of the ROI stamp tool.

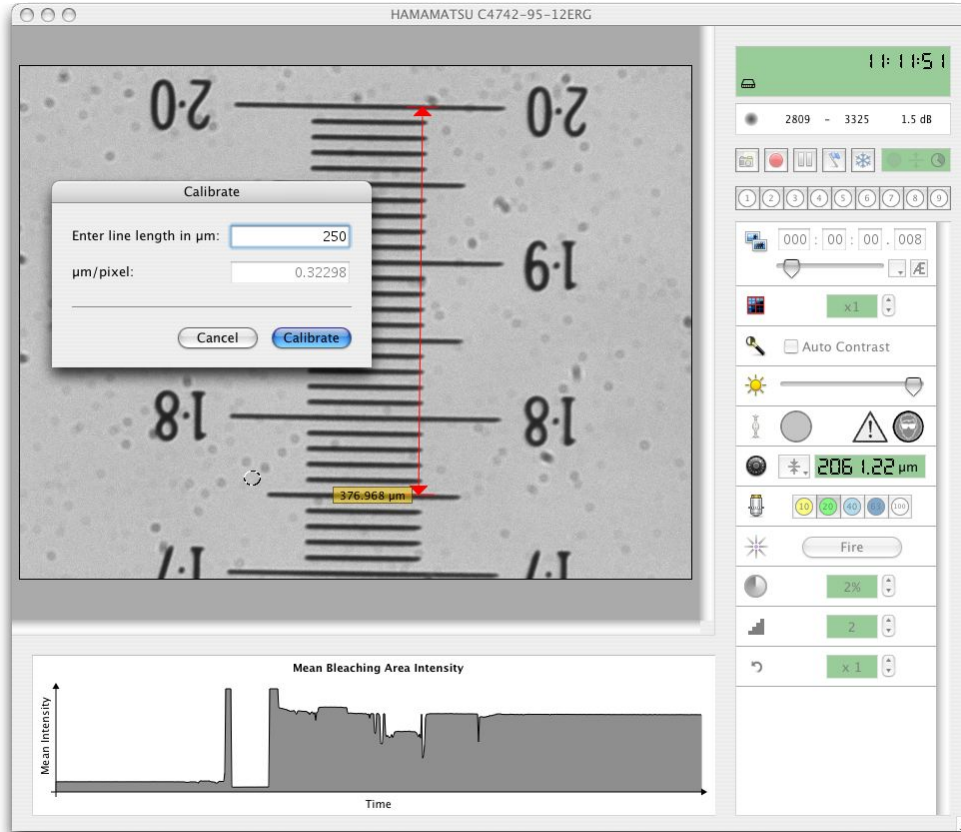
Preparing for FRAP Acquisition

FRAP Acquisition is a plugin to Volocity Acquisition. If you are not yet familiar with Volocity Acquisition we suggest that you consult that part of the guide first.

Before you start your FRAP experiment:

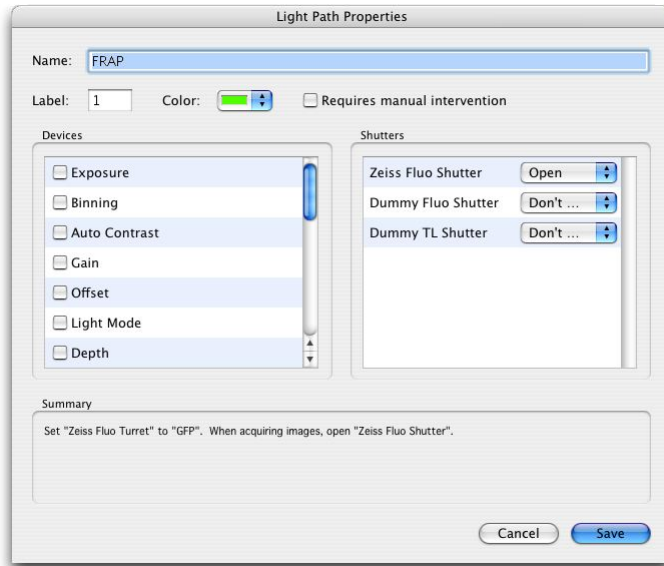
- Install and configure all devices that you wish to use for your FRAP acquisition.
- Select Show **FRAP Preview** from the Window menu.

1. Calibrate each objective lens, if they are not already calibrated.



FRAP Acquisition

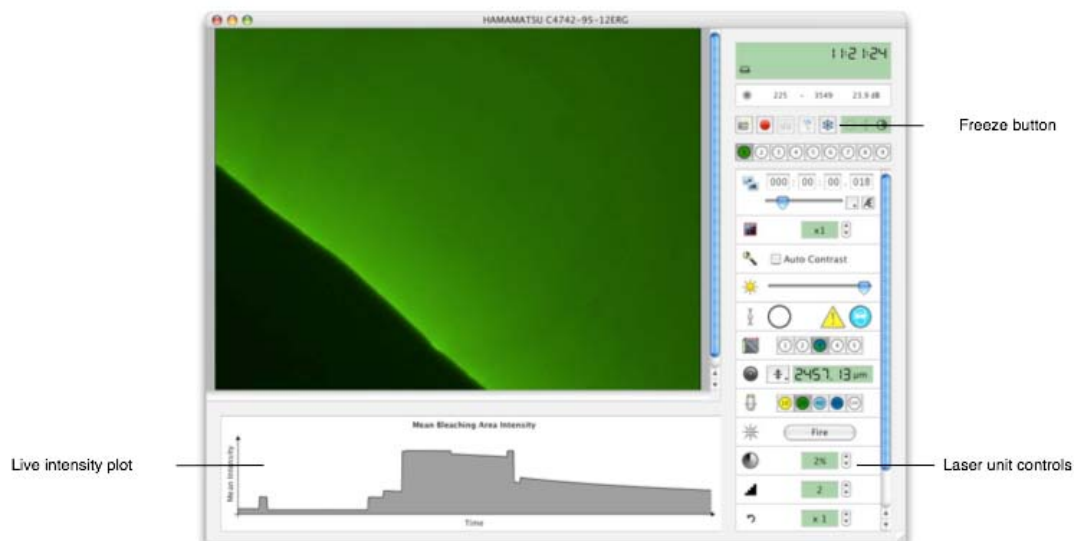
2. Configure any light paths that you require for FRAP acquisition. You will need one light path for each of the channels that you're going to image during the experiment.



The FRAP Preview

This section describes features that are specific to the FRAP Preview window or are of particular relevance to the FRAP Preview window. Details of more controls that are common to the FRAP Preview and the Video Preview are described on page 141.

To display the FRAP preview select **Show FRAP Preview** from the Window menu.

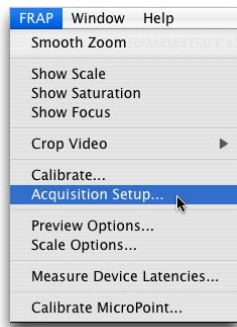


- **Laser unit controls.** These may differ depending upon the type of bleaching device that you are using.
- **Live Intensity plot.** This live plot shows the mean intensity of ROIs (or the mean intensity of the whole image if no ROIs have been selected). Prior to acquisition the mean intensity of all ROIs are shown in a single plot, during acquisition the mean intensity of each ROI is plotted individually. The Intensity plot is useful for assessing the level of photobleaching from the low intensity light source before acquisition, for adjusting the intensity of the high intensity light source, and for monitoring the progress of your experiment during acquisition.
- **The Freeze button.** This allows you to “freeze” the Preview when you find an area of interest. Any shutters associated with the current light path are closed to prevent photobleaching, but the last frame prior to closing the shutter is retained in the Preview. This allows you to select ROIs (bleaching areas) without illuminating your specimen, reducing photobleaching.

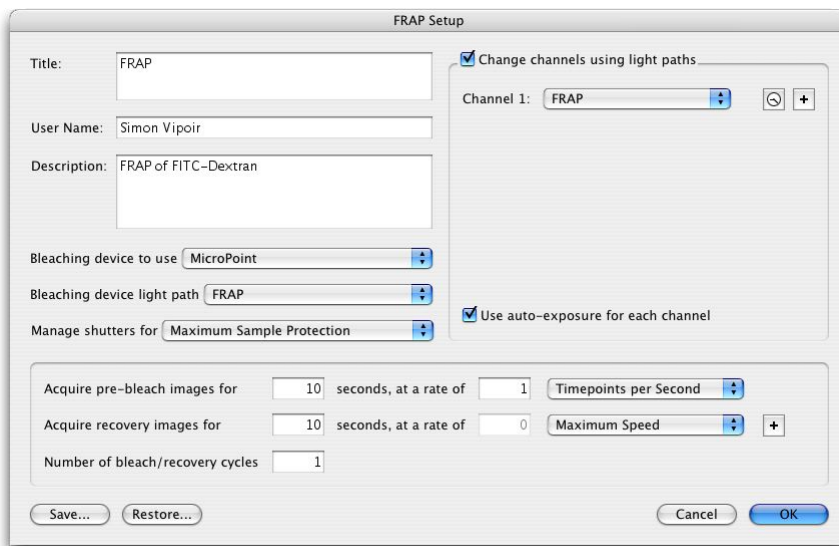
The FRAP Preview also provides additional ROI drawing tools to those available in the Video Preview. In the FRAP preview ROIs are used to mark regions that will be bleached during FRAP experiments. The additional tools provide greater flexibility when setting a bleaching area.

Creating a FRAP Acquisition Protocol

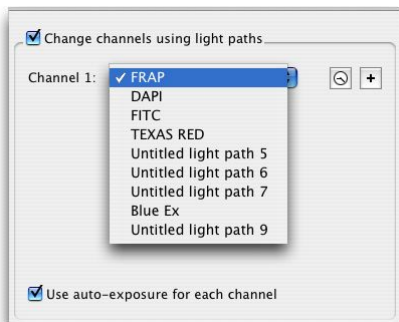
1. Select **Acquisition Setup...** from the FRAP menu.



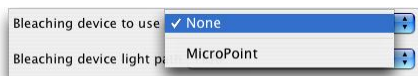
2. The FRAP Setup dialog opens.



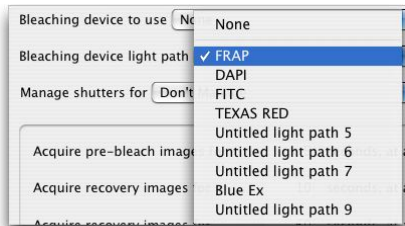
3. If you wish to restore one or more light paths for your pre-bleach and recovery phases, click on the **Change channels using light paths** check box. If you wish to acquire more than one channel for these phases click on the "+" button to add more light paths. Select the appropriate light paths from the drop down menus. The same light paths will be used for both the pre-bleach and recovery phases of your acquisition.



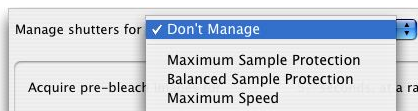
4. If you want Volocity to set the exposure for each of your light paths check **Use auto exposure for each channel**.
5. Select the bleaching device that you wish to use for the experiment from the **Bleaching device to use** drop down menu.



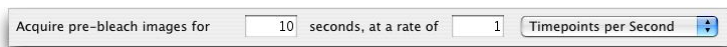
6. Select the light path that you wish to use for the bleaching phase of the experiment from the **Bleaching device light path** drop down menu. You may need to select a light path that is different to the light path you have selected for the pre-bleach/recovery phases of your experiment. Doing so is likely to increase the time taken to acquire your first frame following bleaching so we recommend that you try to avoid changing light paths between bleaching and recovery phases.



7. Select a shutter management option from the **Manage shutters for** drop down menu.



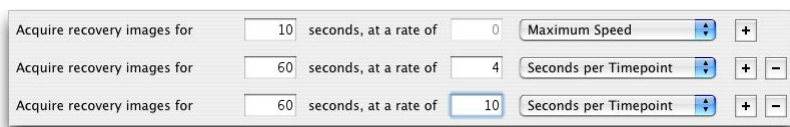
8. In, **Acquire pre-bleach images for**, specify the duration and the frame rate of the pre-bleach period.



FRAP Acquisition

9. In **Acquire recovery images for**, set the duration and frame rate of the recovery phase.

You may wish to set a very high frame rate for initial recovery period, but slow down the acquisition as your recovery approaches plateau in order to reduce photobleaching of your specimen. This can be achieved by setting a number of recovery phases.

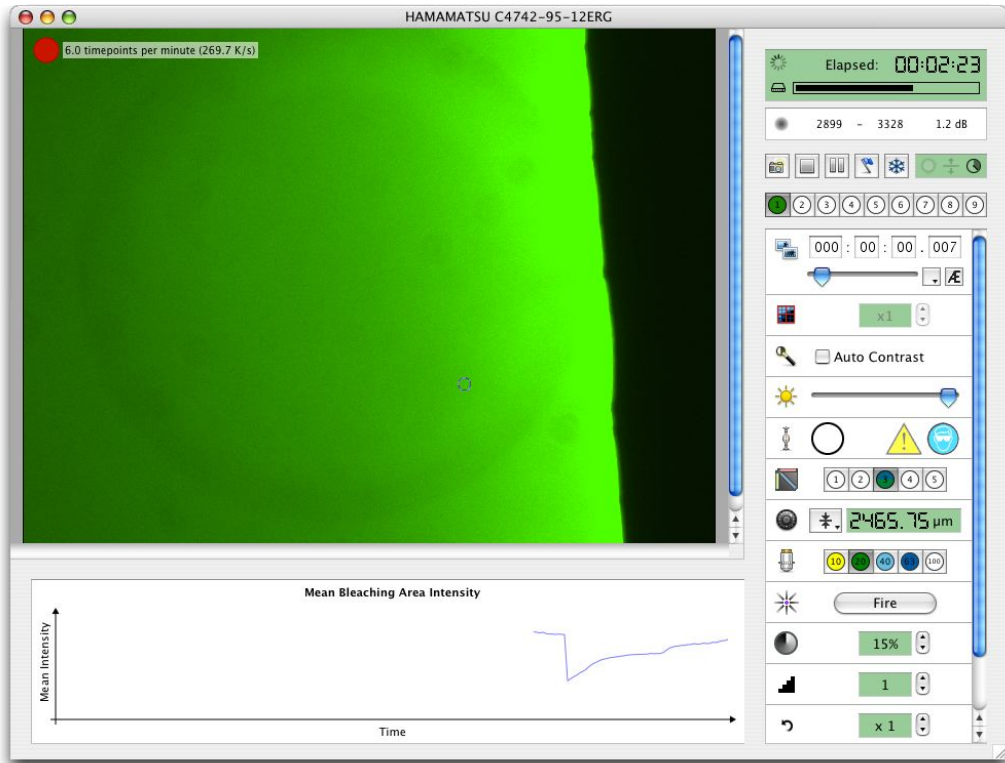


To add an additional recovery phase click the “+” button. Set the duration and acquisition rate of the new phase. You may add up to six different recovery phases as to your protocol. To remove a recovery phase click on the “-“ button.

In the example shown above an initial recovery period of ten seconds in which images are acquired at maximum speed is followed by a sixty second phase in which images are acquired every four seconds, which is followed by a final recovery period of sixty seconds in which images are acquired every 10 seconds.

10. Set the Number of bleach/recovery cycles. This will usually be set to 1 for FRAP experiments. With the number of cycles set to 1 the protocol will finish after completing the recovery phases. You may wish to repeatedly bleach your specimen, for FLIP experiments for example. With the number of cycles set to greater than 1 the bleaching and all of the recovery phases will be repeated. Note: the pre-bleach phase is never repeated.
11. When you are happy with your protocol click **OK**.
12. Click on the “Capture” video control to apply the acquisition protocol that you have just set up. (A Volocity library must be open to store the image sequence that you acquire; you will be prompted to open one, if necessary.)

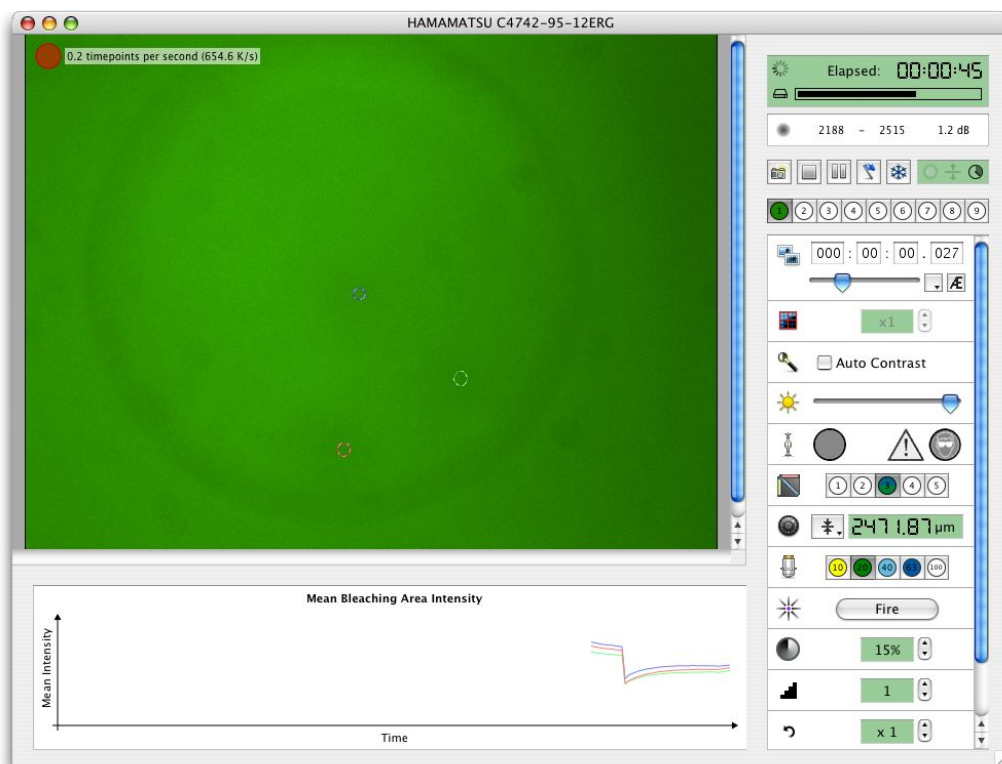
Volocity will carry out the pre-bleach phase, fire the bleaching device according to its configuration and continue with acquisition of the recovery images.



When acquisition begins ROIs change to colored “Bleach areas”, the intensity within a “Bleach area” is plotted in a matching color on the live intensity plot.

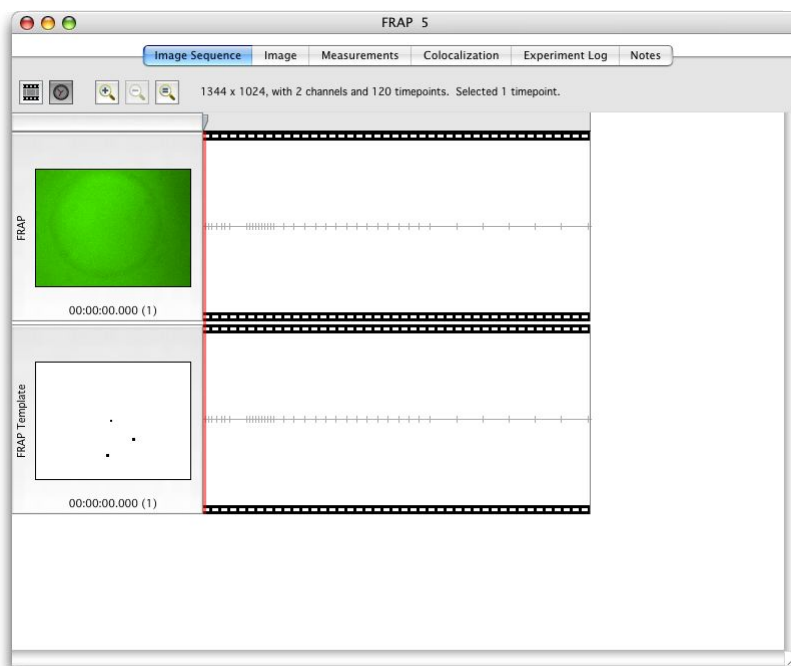
If you have set multiple ROIs on the Preview, which become multiple “Bleaching areas”, each is given its own color and plot when acquisition begins.

FRAP Acquisition



You can study the intensity of the bleached areas in the live intensity plot over the course of the recovery. When the protocol comes to an end the preview will be automatically frozen so that the final image of the recovery and the intensity plot can be reviewed. The plot and the FRAP preview will be overwritten when the preview is “unfrozen”.

Your bleaching/recovery data is stored as an image sequence in your Volocity library, a channel is recorded for each of the light paths that you selected for the pre-bleach/recovery phases. Volocity also adds a template channel which records the positions of your bleach areas for analysis. Areas within the bleach area have a value of zero in the template channels, areas outside the bleach area have a value of 255. Volocity also records the time of the last frame before bleaching, and the first frame following bleaching as meta-data for analysis purposes. The experiment time is set to zero at the end of the bleaching phase, any frames acquired before this will have negative time stamps.



Data acquired with Velocity FRAP acquisition may be analyzed using the Velocity FRAP analysis.

FLIP Acquisition

FLIP is a technique that is also possible with the FRAP plugin. An image sequence making up a FLIP experiment consists of three elements:

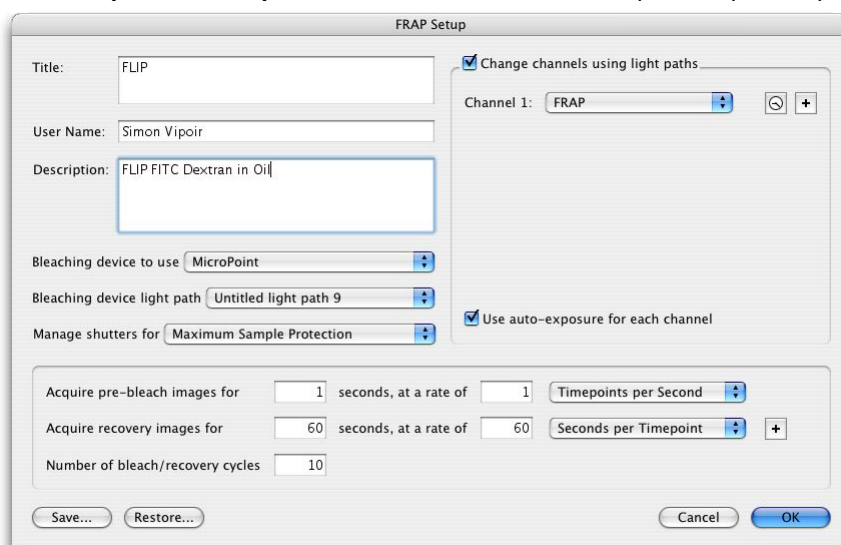
- a bleached area
- a bleached area that contains labeled molecules of interest that may be free to enter and leave the bleached area. The experiment is to determine whether the molecules are moving between the two bleached areas and at what rate.
- A control cell or region of interest from a compartment that is not connected to the bleached areas in any way

1. Select **Show FRAP Preview** from the Window menu.
2. Mount your specimen and expose it to your low intensity light source.
3. With your specimen in focus ensure that the all light is directed to your camera. You should see your specimen in the Video Preview. Adjust the camera controls until you are happy with your image. You may wish to observe your specimen for a while to see whether you have an acceptable level of

FLIP Acquisition

photobleaching from your low intensity light source, using the live intensity plot should help you make this judgment. If you make adjustments to your light intensity, also adjust your camera settings.

4. If possible, adjust the intensity of your bleaching device to the correct level. You should aim for maximum photobleaching without damaging or excessively heating your specimen.
5. When you are happy with the intensity of both your low intensity light source and bleaching device move to a new (unexposed) area of your specimen.
6. Select the **Freeze** button. Any shutters associated with your currently selected light path will be closed. The last frame previewed before the shutter was closed will be retained in the FRAP preview.
7. With the FRAP preview frozen you can set your bleaching area for your experiment without photobleaching your sample. ROIs are used to mark bleaching areas. Use any ROI tool to draw an ROI or restore an ROI by selecting ROI then **Restore...** from the Edit menu. To set an ROI of a particular size we recommend the use of the ROI stamp tool.
8. Select **Acquisition Setup...** from the FRAP menu and set up the acquisition protocol.



In this example, the bleach and recovery phase is repeated 10 times. Two images are acquired during the recovery phase, one immediately following the bleach and a second a minute after the bleach. Click **OK**.

9. The acquisition protocol is now stored and can be run by clicking the capture button on the FRAP preview.
10. The pre-bleach images are acquired only once, but the bleach and recovery will be repeated, in this case 10 times. These images may then be measured using the standard measurement tools in Volocity. An example of this is described in "FLIP Analysis" on page 423.

Volocity Acquisition Worked Examples

Overview

Volocity Acquisition allows you to acquire single or multi-channel, 2D, 3D or 4D image sequences at a single position or at multiple XY points. Before you start acquisition of your image sequences you must configure the Video Preview window and the Acquisition Setup window. Once these steps are complete you can repeatedly execute your acquisition protocol.

This chapter describes how to set up Volocity to carry out single-point and multi-point acquisition and summarizes required settings for different acquisition protocols.

It assumes that you have installed and configured your acquisition device(s) correctly, and set up any additional options, such as Fast Restoration, that you require for your experiment.

For comprehensive installation, configuration and reference information about Volocity Acquisition see the separate chapter starting on page 120. In particular, you may like to review the two sections that describe the Video Preview window, page 141, and how to control devices, page 130.

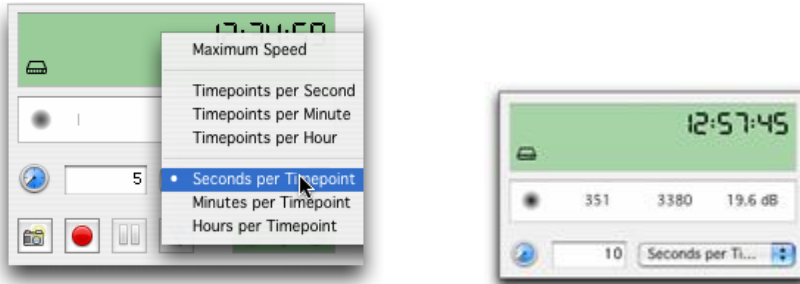
Single-Point Acquisition Process

The following steps describe all options available during single-point acquisitions.

You only need to set up those steps that are appropriate for your acquisition protocol.

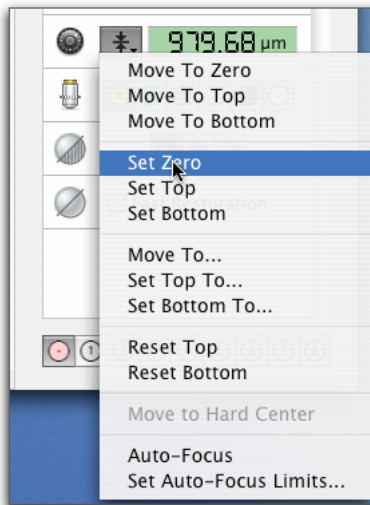
1. On the Video Preview window, select the appropriate timelapse option. You can change the timelapse option at any time during an acquisition. If you wish to acquire only a single timepoint, you can ignore the timelapse options.

For example, to acquire one timepoint every 10 seconds, select **Seconds per Timepoint** from the drop-down menu, and enter **10** into the text field.



If you wish to acquire images at the fastest possible rate, select **Maximum Speed**, Volocity will then acquire the images at the fastest rate supported by your hardware.

2. If you wish to capture a single plane only, ignore the focus drive options and go to step 3. If you wish to capture a number of Z sections within your specimen, you will need to set the range through which the focus drive will move in the focus interface in the Video Preview.



First, move to the midpoint of your specimen using the focus control of your microscope.

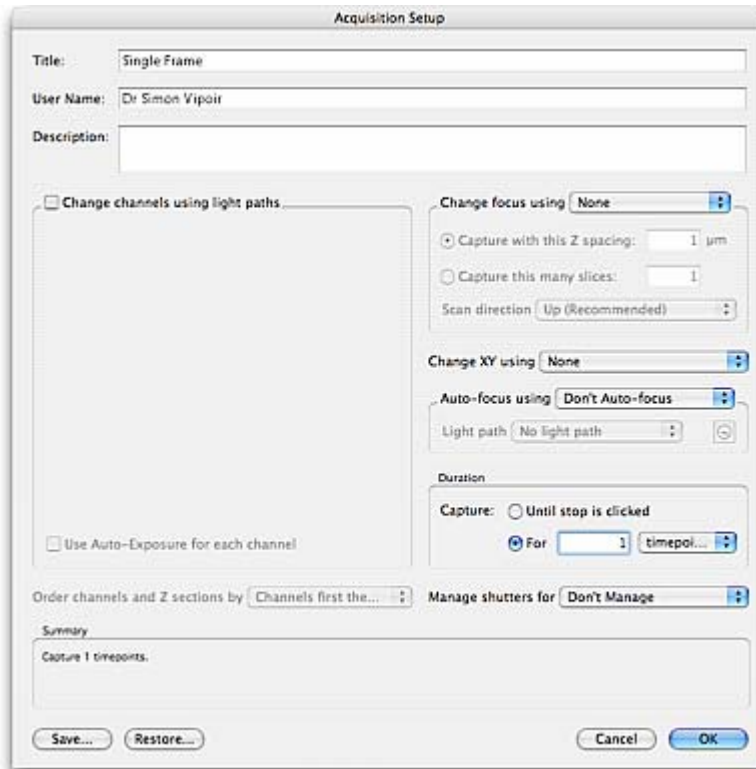
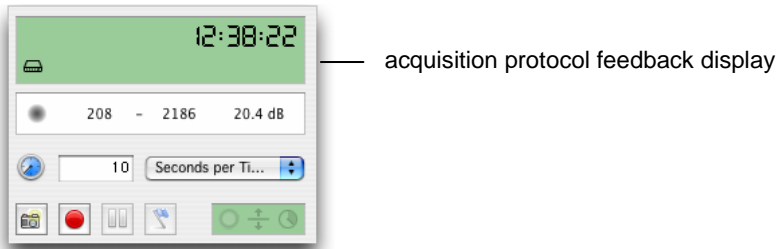
Select **Set Zero** from the focus drive drop-down menu on the Video Preview window.

Move to the lower limit of your specimen using your microscope focus drive and select **Set Bottom** from the focus drive drop-down menu.

Move to the upper limit of your specimen using your microscope focus drive and select **Set Top** from the focus drive drop-down menu.

3. Select **Acquisition Setup...** from the Video menu.

Or double-click on the acquisition protocol feedback display on the Video Preview window.

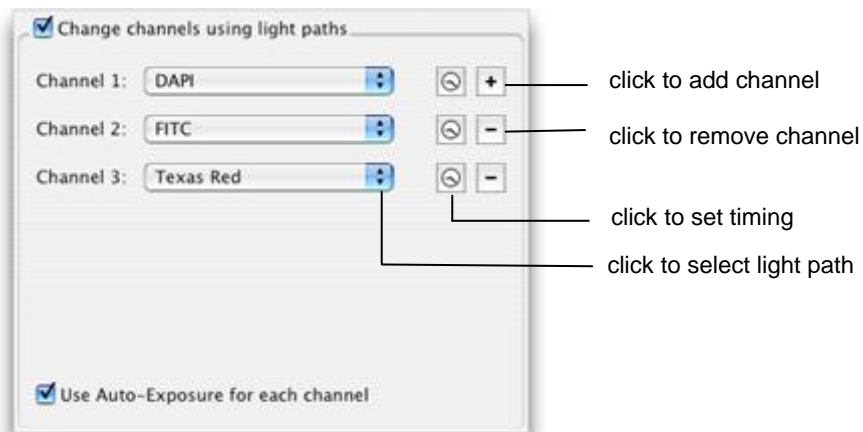


4. Enter a **Title**, **User Name** and **Description**. The Title will be used to name the images that you acquire; both the User Name and Description will be stored with the properties of the image. Complete the dialog by selecting the appropriate settings for your acquisition protocol.

Single-Point Acquisition Process

5. Change channels using light paths

If you wish to acquire a multi-channel sequence check the **Change channels using light paths** box.



Add or remove channels using the “+” and “-” buttons and select the correct light path for each channel by clicking on the drop-down menus.

If you wish to acquire a channel at a rate different to other channels (for example, you may wish to acquire a fluorescence or brightfield reference image periodically), click on the timing icon. The **Channel Timing** dialog opens. Select the **Acquire a new frame for this channel every:** option, and enter the new frame rate.



A warning symbol is displayed on the Acquisition Setup dialog next to the channels that will not be captured at the normal rate.

During timepoints in which a channel is not acquired the last timepoint that was acquired will be duplicated.

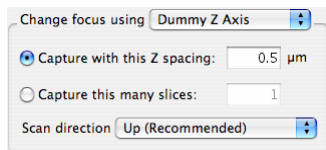
Auto-Exposure

If you wish Volocity to perform an Auto-Exposure for each channel, check the **Use Auto-Exposure for each channel** box. With this option selected, Volocity will perform an Auto-Exposure at the midpoint of your specimen for each of your channels before starting the acquisition.

Changing the exposure between channels reduces the speed of the acquisition protocol and should be avoided for speed-critical acquisition. Often a compromise exposure that is adequate for all channels can be found.

6. Change focus using

If you are acquiring multiple Z sections, select the appropriate device from the **Change focus using** drop-down menu and enter either the **Z spacing** that you require or the number of **slices** that you require.



Using the Z spacing option Volocity will calculate the number of slices required to move through the range between the top and bottom positions as configured in the Video Preview. If the Z spacing is not a factor of the range, an additional slice will be captured.

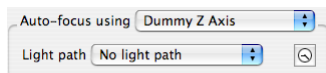
Using the number of slices option Volocity will calculate the spacing between slices required to capture that number of slices within the range between top and bottom.

The scan direction option allows you to select between:

- **Up:** This is the recommended option; it moves the focus device against gravity for the most accurate positioning.
- **Down:** This option moves the focus device with gravity.
- **Bi-Directional:** This option alternates the up and down direction of focus movement. This may minimize disturbance of the system by avoiding the relatively large movement otherwise required to return the focus to its starting position.

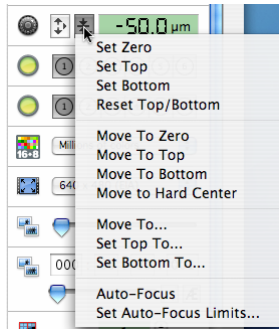
7. Auto-focus using

Select a focus device to use for auto-focus during the experiment.



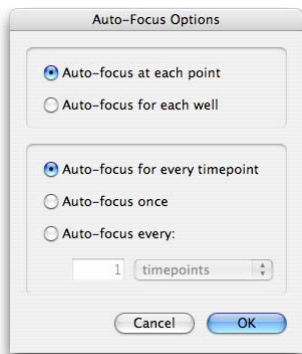
Single-Point Acquisition Process

Auto-focus operates by seeking the highest contrast image when sampling within a range in the z-axis. This range is set in the focus drive settings on the Video Preview window.

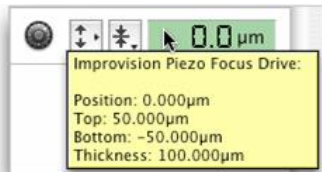


Using auto-focus will reduce the speed of acquisition by the time taken to determine the best plane to image.

Choose the frequency to apply auto-focus by configuring the timing options.



If you are using auto-focus and planning to acquire a Z stack around the position determined by the auto-focus, the setting of top and bottom positions in the focus drive settings become vitally important. Each of your acquisition points will be treated as zero, regardless of its actual Z value. So the distance between zero and your top and bottom position will be the same as the distances between each of your points and their top and bottom positions (see the tables below for examples). You can quickly check what values are set for top and bottom by inspecting the focus drive tool tip.



Top and Bottom marks with top set to 5 and bottom set to -5

Position (Z) of acquisition point (μm)	Bottom mark for acquisition (μm)	Top mark for acquisition (μm)
0	-5	5
10	5	15
20	15	25
30	25	35



Top and Bottom marks with top set to 10 and bottom set to -10.

Position (Z) of acquisition point (μm)	Bottom mark for acquisition (μm)	Top mark for acquisition (μm)
0	-10	10
10	0	20
20	10	30
30	20	40



If you wish to change the top and bottom positions, use the commands in the focus drive drop-down menu, as described in step 2 of this section.

Note: You should avoid re-setting the zero value of your focus drive after setting your acquisition points. If you do so, you will invalidate the Z coordinates stored with each point.

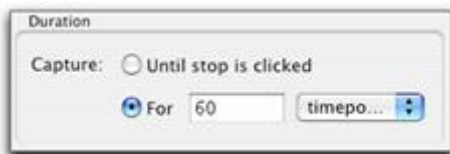
8. Change XY using

Set to **None** for single-point acquisition.



Single-Point Acquisition Process

9. Duration



If you wish to acquire multiple timepoints, select either **Until stop is clicked** (Volocity will acquire data until it is manually stopped) or select **For**, enter a number into the text field and select either “timepoints”, “ms”, “seconds”, “minutes” or “hours” from the drop-down menu.

If you wish to acquire a single timepoint, select **For**, enter “1” into the text field, and select “timepoints” from the drop-down menu.

10. Order channels and Z sections by

If you are acquiring both multiple channels and multiple Z sections you must decide in which order Volocity will acquire your images.

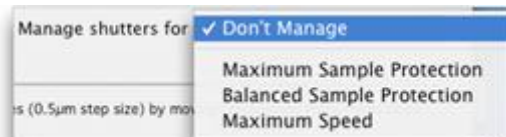


There are two options in the **Order channels and Z sections by** drop-down menu.

The **Channels first then Z** option acquires all channels at each Z section before the Z section is changed. This option has the advantage of acquiring all channels at precisely the same Z section, but may be slow with a slow wavelength changing device.

The **Z first then Channels** option acquires all Z sections for each channel before the channel is changed. This option has the disadvantage of introducing potential inaccuracies in Z positions (depending upon the reproducibility of your focus drive), but it does allow acquisition protocols to be conducted at high speed, even with relatively slow wavelength changing devices.

11. Manage shutters for



These options determine how Volocity will manage the shutters between channels and timepoints. Volocity uses the latency threshold measurements for each device in the light path to determine how it will control the shutter. You must have measured your device latencies for this option to work effectively, see page 137.

12. When you have set up the necessary acquisition parameters, click **OK**.

13. With your specimen in focus ensure that the light path is directed to your camera. You should see your specimen in the Video Preview. Adjust the camera controls until you are happy with your image. A good starting point is to select the Auto-Exposure (AE) button (if this feature is supported by your camera); Volocity will automatically adjust the exposure of your camera. Fine adjustments of both the exposure and other camera controls can then be made.



14. Click the appropriate Capture button on the Video Preview window.

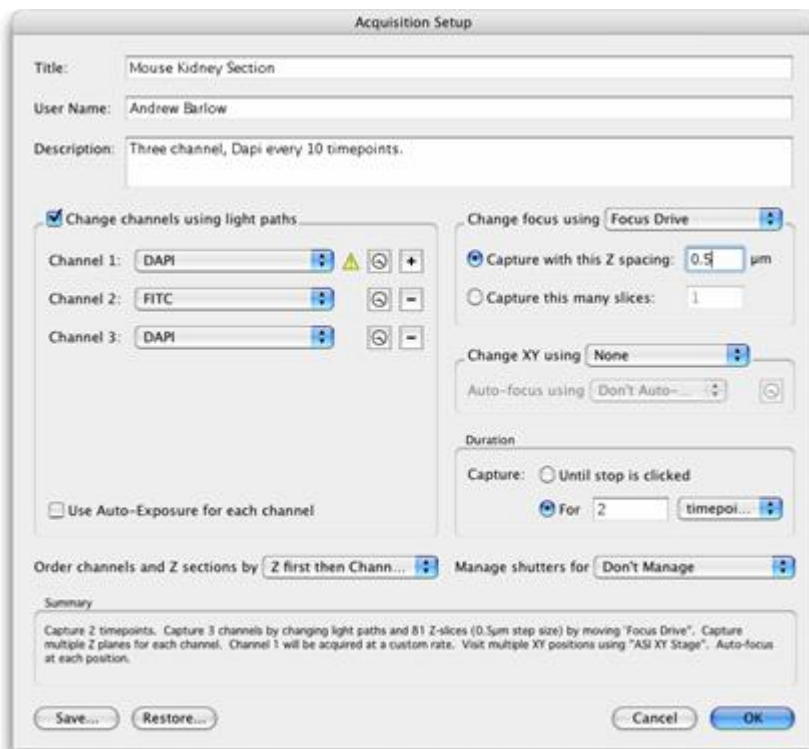


The image is given the name that you entered in the acquisition Setup window followed by a number. All images and image sequences acquired by Volocity are automatically saved to your hard disk.

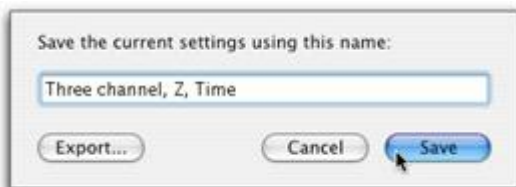
Single-Point Acquisition Process

Saving Acquisition Protocol Settings

1. Click **Save...** on the Acquisition Setup dialog



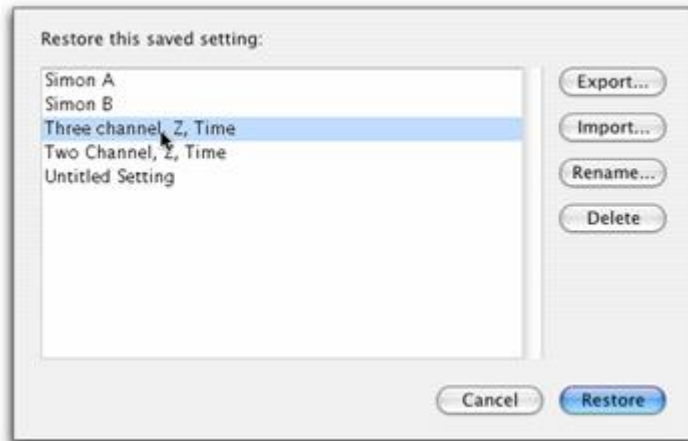
2. Enter a descriptive name for your acquisition protocol and click **Save**.



Note: You can also use this dialog to export settings, for example, if you wish to share those settings with a colleague.

Restoring Acquisition Protocol Settings

1. Click on **Restore...** on the Acquisition Setup window to display a list of saved acquisition protocols.



2. Select the protocol that you wish to restore and click **Restore**.

XY Stage, Multi-Point Acquisition Process

This section describes how to use Volocity to carry out multi-point acquisition using a motorized XY stage.

There are several steps to this process:

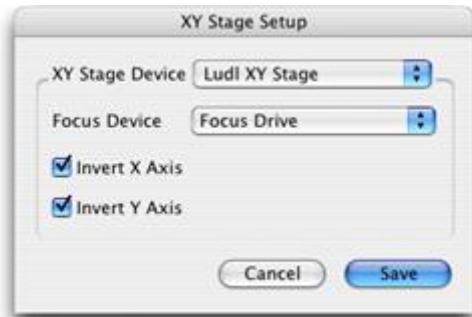
- Set up and calibrate the XY stage.
- Create overlays and scans.
- Find specimens of interest and record their positions as “Acquisition Points”.
- Set up the acquisition protocol and capture the image sequences.

Set Up and Calibrate the XY Stage

The XY stage must first be configured as a device in the preferences. See page 126.

Note: Each time you start Volocity or turn the stage controller on, and are planning to use the two together, calibrate the stage.

1. Select **Stage Setup...** from the Stage menu.



2. Select the appropriate **XY Stage Device** and correct **Focus Device** from the drop-down menus. The focus drive that you select here will be used to store the positions of your acquisition points; you may use a different focus drive for your acquisitions.
3. Use the checkboxes to set the X and Y axes correctly and click **Save**.
4. Clear the stage area and select **Calibrate Stage...** from the Stage menu.

5. At the warning prompt, check the stage area is clear. When you are satisfied that neither your specimens nor your microscope (in particular your lenses) will be damaged by large stage movements, click **Continue**. Volocity moves the stage to the bottom right and top left positions to establish the full range of travel and current position of the stage relative to the range of travel.



XY Stage View

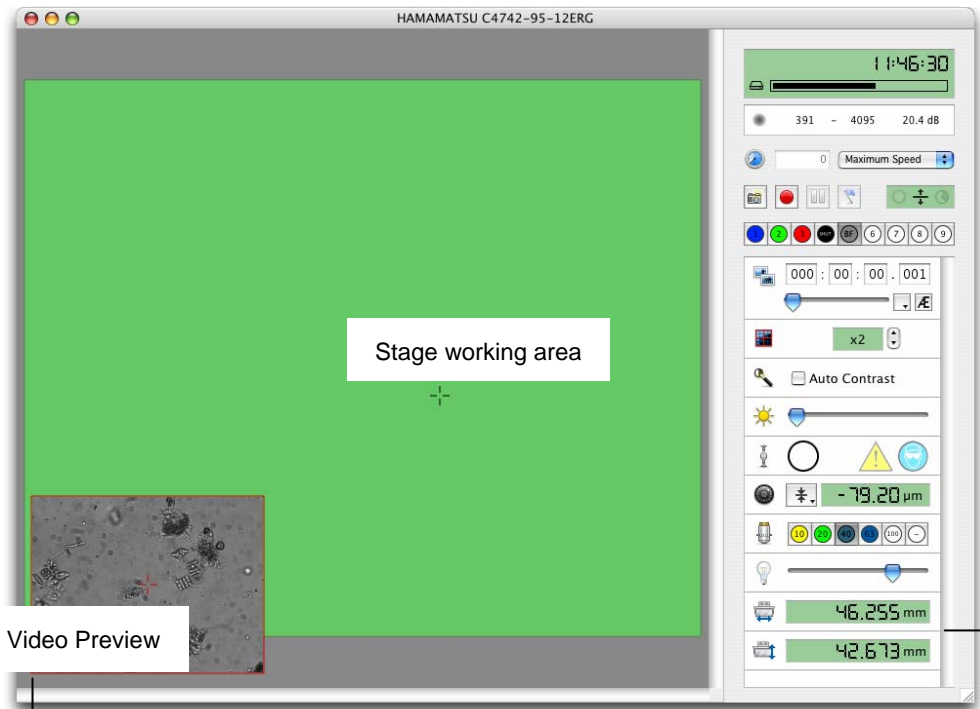
Once the stage is calibrated, Volocity provides a choice of views: the XY Stage view and the live Video Preview (described on page 141). In the XY Stage view the green area represents the full working area of the stage. The gray crosshairs represent your current position on the stage.

If you move the stage manually you will see the position of the crosshair change to reflect the movement; the X and Y coordinates of the stage will also be updated. You can quickly move the stage by selecting the ROI tool (Shortcut key, R) and double clicking on the point on the XY Stage work area to which you want the stage to move.

Note: The shortcut key **R** selects the ROI tool.

A small Video Preview is displayed in the bottom left hand corner of the XY Stage view. This allows you to navigate around the stage whilst seeing the current Video Preview. The Video Preview can be moved to any corner of the XY Stage view. To move the Video Preview to another corner of the XY Stage view, click and drag the video preview to its new location.

XY Stage, Multi-Point Acquisition Process



You may click and drag the preview to another corner

stage X and Y coordinates

You can toggle between the views by selecting the required view from the Video menu or by using the shortcut keys.

- On a Mac:
Shift+Apple+L for live video
Shift+ Apple+X for XY stage
- On a PC:
Ctrl+Shift+L for live video
Ctrl+Shift+X for XY stage

Adding Overlays

You can add overlays to your XY Stage view that will allow you to easily and quickly navigate around your culture vessels or slides.

1. Select **Make Well Overlay...** from the Stage menu.

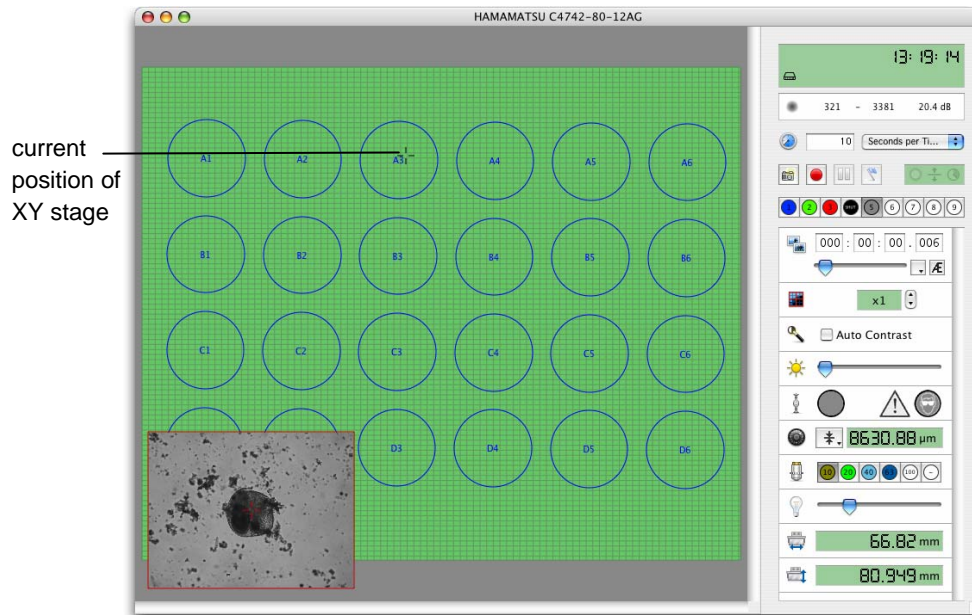


2. Select the type of slide or culture vessel from the drop-down menu and click **Create...** Volocity now provides a wizard which guides you through the steps for setting the position of the XY stage. In our example, we are making an overlay for a multi-well plate.
3. At each step of the wizard, move the stage to the requested position and click **Next**.



XY Stage, Multi-Point Acquisition Process

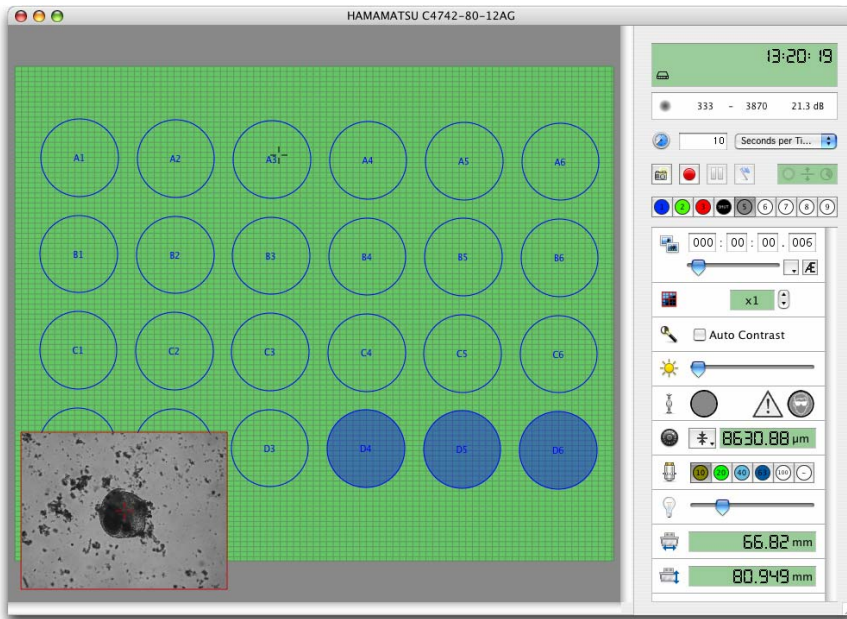
After the last requested setting has been entered, the wizard closes and an overlay is shown on the XY Stage view. Again, the cursor shows the current position of the stage.



Once you have set up the overlay you can:

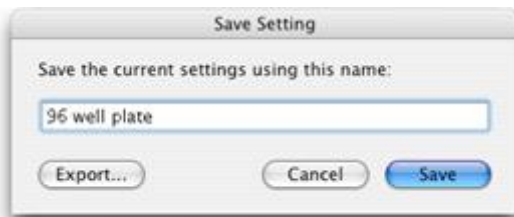
- Move to a specific well. Select the ROI tool and double click within a well to move quickly to that well.
- Select individual wells or groups of wells to work with. Use the ROI tool to select the required well(s) and select **Disable Selected Wells/Enable Selected Wells** from the Stage menu, as appropriate.

The following example shows three wells disabled.



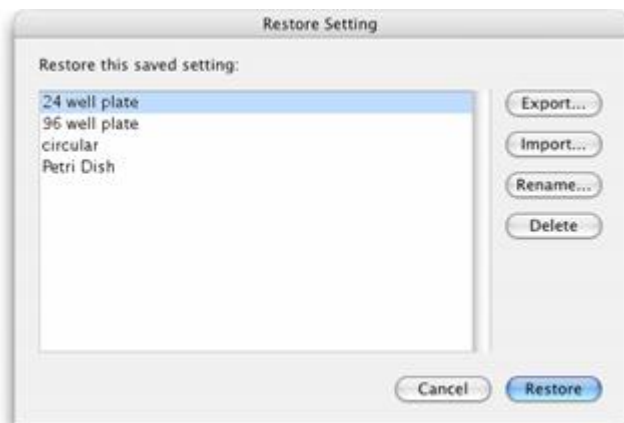
Saving and Restoring Overlays

To save overlays select **Save Overlay...** from the Stage menu. Enter a descriptive name for the overlay in the dialog that opens and select **Save**.



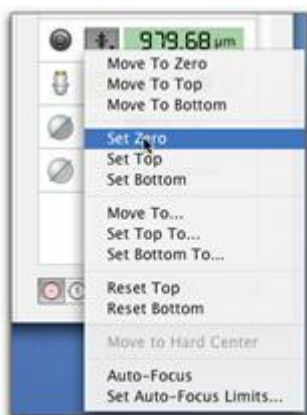
XY Stage, Multi-Point Acquisition Process

To restore a previously saved overlay select **Restore Overlay...** from the Stage menu. Select the overlay that you wish to restore from the list and select **Restore**. The overlay will be displayed in the XY Stage view.



Adding Points

Before adding acquisition points, we recommend that you focus on your specimen and select **Set Zero** from your focus drive drop-down menu (if you have more than one focus drive in your imaging system ensure that you reset the focus drive that you will use for your acquisition protocol).

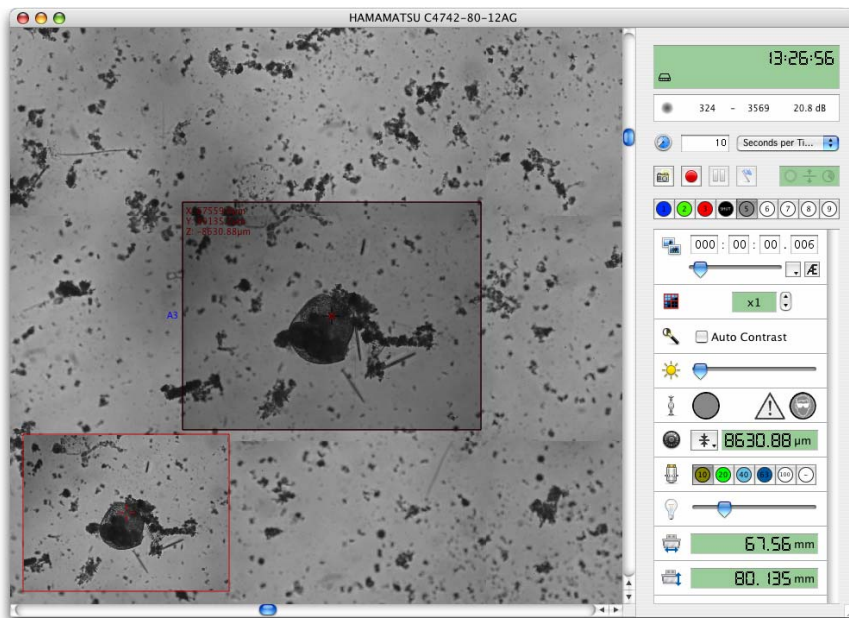


You can add points manually or ask Volocity to create random points for you.

Manually Adding Points

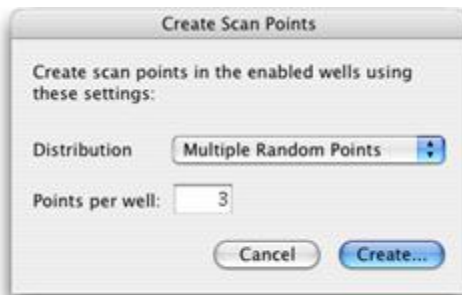
1. Move the stage to the area of interest and focus on your specimen. You can do this by screening your specimen using your microscope's ocular lenses, or by using the Video Preview in Volocity to scan your specimen.

2. Select **Add point** from the stage menu or use the shortcut key:
On Mac: Shift+Apple+A
On PC: Ctrl+Shift+A
3. Move the stage and continue to add points manually, as required.
4. The point is shown as a cross on the XY Stage view. The X, Y and Z co-ordinates are recorded with the point and displayed next to the point (you may have to zoom in to see the coordinates).



Creating Random Points

1. Select **Create Points from Well Overlay** from the Stage menu.

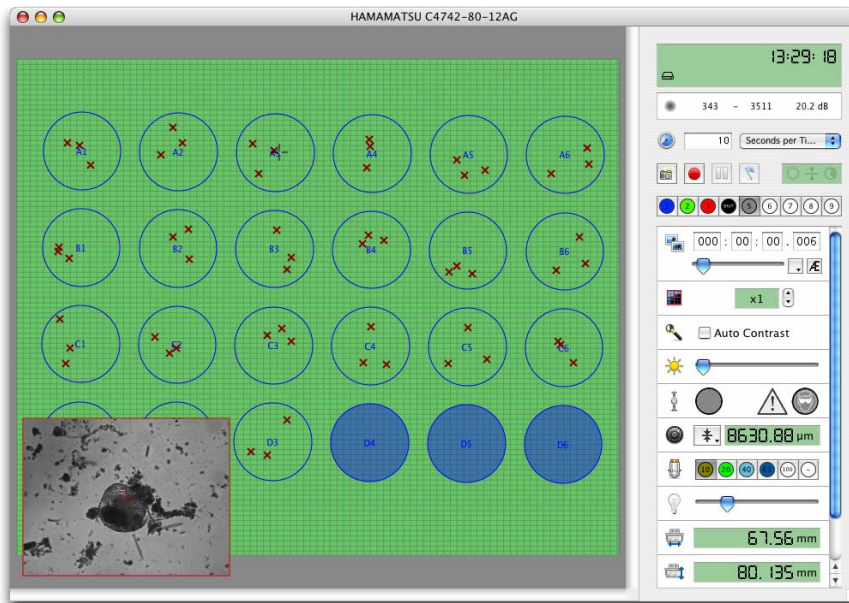


2. Select **Multiple Random Points** from the drop-down menu to add single acquisition points to random positions within each enabled well. Select **Multiple Tiled Points** from the drop-down menu

XY Stage, Multi-Point Acquisition Process

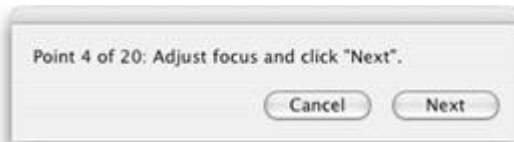
to add a matrix of multiple points at the centre of each well. Specify the required number of **Points per well**.

3. Click **Create...**



Inspecting and Editing Points

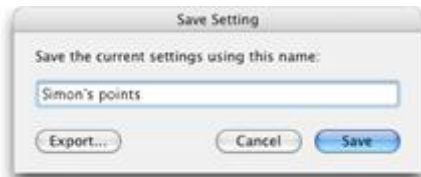
1. Use the ROI tool to double click on a point. This moves the stage and focus drive to the X, Y, and Z position of the point. You can use the **Go to Next Point** or **Go to Previous Point** commands or their shortcuts from the Stage menu to cycle through your points.
2. If you change the focal position of a point, select **Update Point** from the Stage menu.
3. If you have created a lot of points, use the **Review Points...** command on the Stage menu. The Review Points Wizard opens and takes you to each stored point.



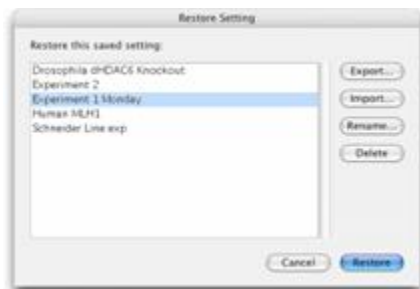
4. At each point you can review and edit the focal position. The current focal position when you select **Next** in the wizard will be stored with each point.

Saving and Restoring Points

To save a set of acquisition points, select **Save Points...** from the Stage menu. Enter a descriptive name for your points in the dialog that opens and select **Save**.



To restore previously saved acquisition points select **Restore Points...** from the Stage menu. Select your points from the list and select **Restore**.



Scanning

Note: Always make sure that the lenses are calibrated accurately and that the camera head is positioned squarely on the microscope before scanning. We also recommend that you enable binning on your camera prior to scanning; this will allow Volocity to scan fields of view more quickly.

You can set up scanning areas in three ways. You can scan regions of interest, whole wells from overlays, or you can scan acquisition points.

Scanning Regions of Interest

Set a region of interest by selecting the ROI tool, clicking and holding the mouse, and dragging out a region of interest on the XY Stage view.

You can also set an ROI in several steps by moving the stage to opposite corners of your ROI.

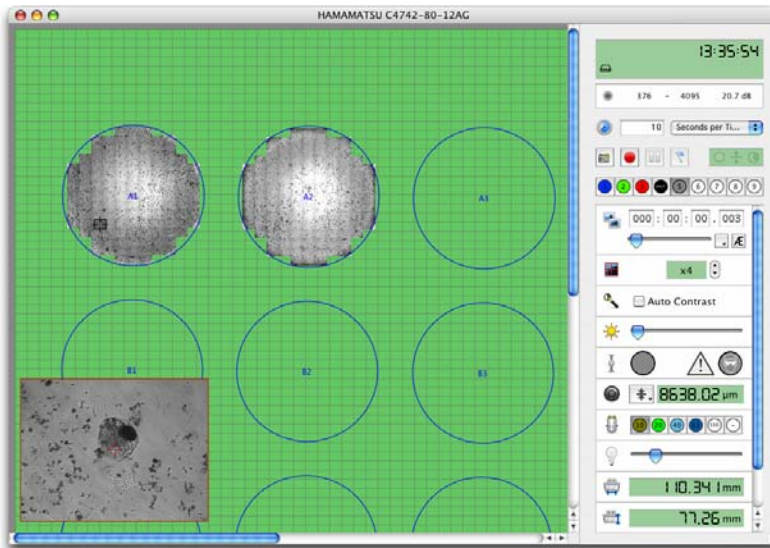
- Move the stage to the top left hand corner of you region of interest and select **Set ROI Top-Left** from the Stage menu.
- Move the stage to the bottom right-hand corner and select **Set ROI Bottom-Right** from the Stage menu.

Once you have set a region of interest, select **Scan Selected Area** from the Stage menu.

XY Stage, Multi-Point Acquisition Process

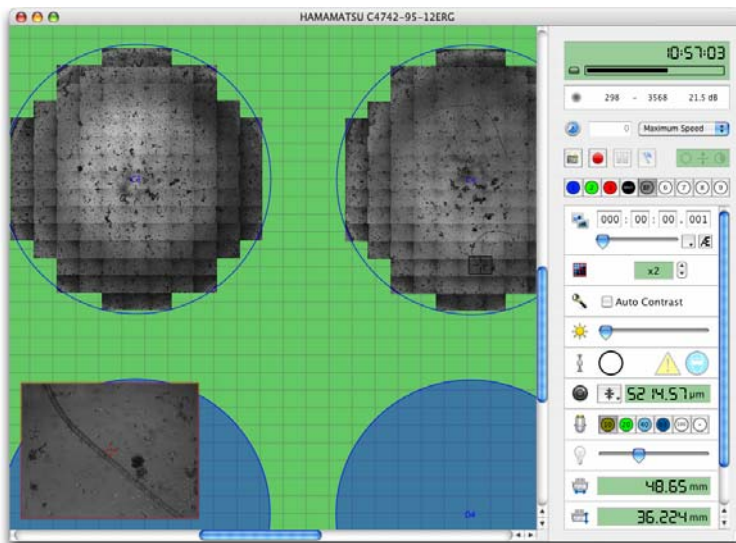
Scanning Enabled Wells

After making an overlay and ensuring that only the relevant wells are enabled, select **Scan Enabled Wells** from the Stage menu. Volocity will scan all fields of view within the enabled wells.



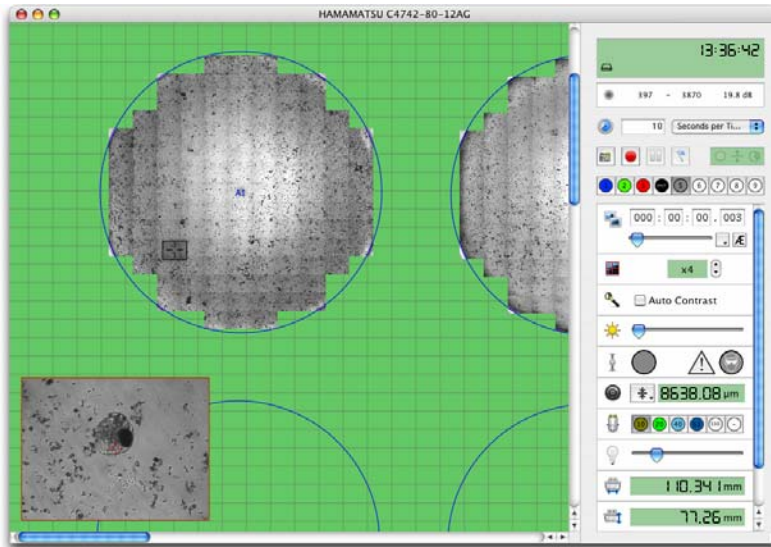
Use the zoom tool to zoom on scanned areas. This will help you to find areas of interest in your specimen.

Note: The shortcut key **Z** selects the zoom tool.



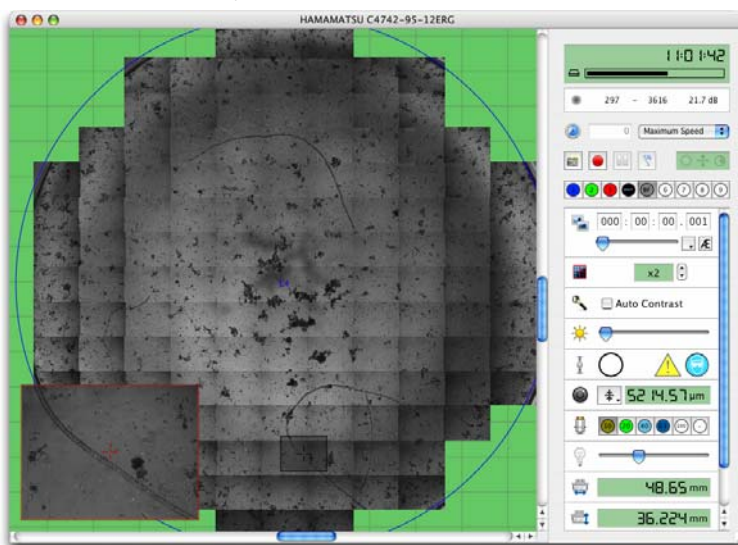
Scanning Acquisition Points

After you have added your acquisition points (see page 216) select **Scan Points** from the Stage menu. Velocity will scan your specimen and acquire a field of view for each of your acquisition points.



Capture snapshots of scanned areas by selecting **Capture Snapshot** from the Stage menu. Each well that you have scanned will be captured as a separate snapshot.

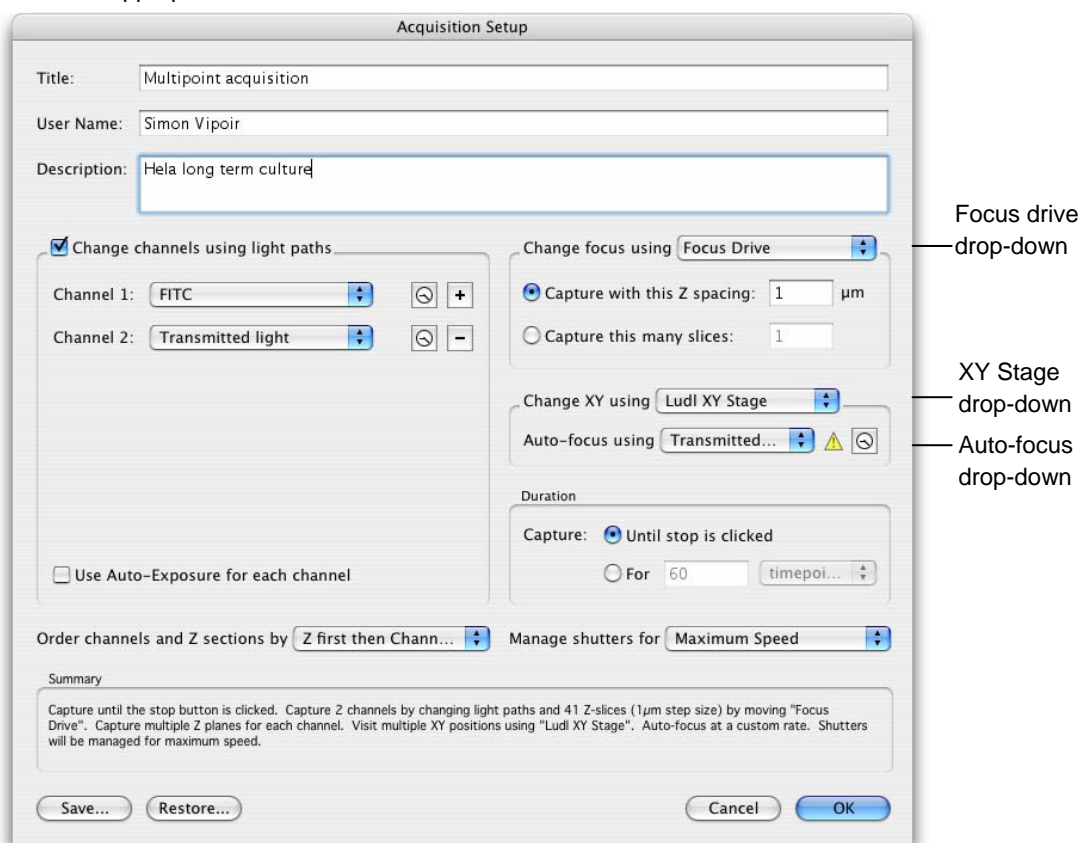
Note: The shortcut key **F5** captures a snapshot.



Set Up and Run the Acquisition Protocol

After storing points in the stage area an acquisition protocol is used to image the points as required.

1. Select **Acquisition Setup...** from the Video menu or double-click on the acquisition protocol feedback display on the Video Preview window.
 - You must select the appropriate focus drive and XY stage from the drop-down menus.
 - The focus drive that you select for acquisition may be different from the focus drive that you selected to store the positions of acquisition points (see page 216). This will allow you to move between acquisition points with a microscope focus drive, and to capture Z series with a piezo drive.
 - Make other choices for change channels, duration, image order and shutter management as appropriate.



2. Auto-Focus

If you want Velocity to auto-focus on your points during the acquisition, select the light path that you wish to use for auto-focusing from the drop-down menu.



If you choose to auto-focus during the acquisition, you can also set timing options by selecting the “timing” icon.

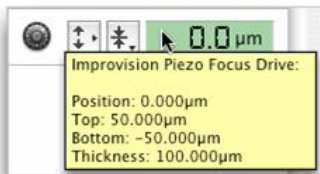
You may choose to auto-focus at each point, or at each well. If you choose to auto-focus at each point, each point will have its own focus point. If you choose to auto-focus for each well, all the points within a well will share a common focus point.



You may also choose to auto-focus for every timepoint, to only auto-focus for the first timepoint, or to auto-focus periodically.

3. If you have chosen to acquire multiple Z planes during your experiment, you now need to ensure that the top and bottom focus limits are set correctly.

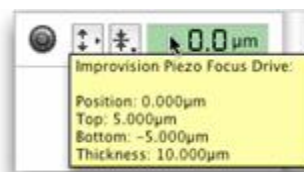
Each of your acquisition points will be treated as zero, regardless of its actual Z value. So the distance between zero and your top and bottom position will be the same as the distances between each of your points and their top and bottom positions (see the tables below for examples). You can quickly check what values are set for top and bottom by inspecting the focus drive tool tip.



XY Stage, Multi-Point Acquisition Process

Top and Bottom marks with top set to 5 and bottom set to -5

Position (Z) of acquisition point (μm)	Bottom mark for acquisition (μm)	Top mark for acquisition (μm)
0	-5	5
10	5	15
20	15	25
30	25	35



Top and Bottom marks with top set to 10 and bottom set to -10.

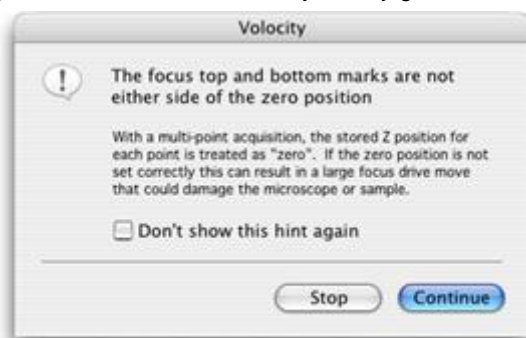
Position (Z) of acquisition point (μm)	Bottom mark for acquisition (μm)	Top mark for acquisition (μm)
0	-10	10
10	0	20
20	10	30
30	20	40



If you wish to change the top and bottom positions, use the commands in the focus drive drop-down menu.

Note: You should avoid re-setting the zero value of your focus drive after setting your acquisition points. If you do so, you will invalidate the Z coordinates stored with each point.

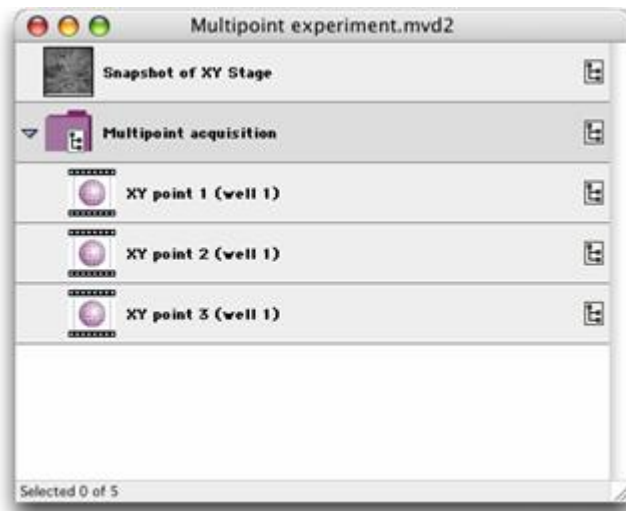
4. Make sure a Velocity library is open and run the acquisition. If you have not set the top and bottom positions either side of zero, you may get the following warning.



We recommend that when this warning is generated you stop the acquisition and check your top and bottom positions. In situations where the top and bottom are a long way from zero there is a danger that the focus drive may move long distances during the acquisition. This may result in damage to your microscope or specimen.

Velocity visits each acquisition point and conducts your acquisition protocol. The number of times Velocity visits each point is determined by the duration option that you set with the protocol, the frequency is determined by the time lapse option that was set on the Video Preview window.

Each acquisition point has its own image sequence. All the image sequences from a single multipoint acquisition are organized within a folder in your library.



Capturing Image Sequences

Click the capture button to acquire an image sequence using the current acquisition protocol.

Once capture has started a red dot is shown on the Video Preview and the capture button changes to a stop button.



Click the capture button to acquire an image sequence using the current acquisition protocol.



Once capture has started a red dot is shown on the Video Preview and the capture button changes to a stop button.

Note: The shortcut key **F6** starts/stops the acquisition.

Capturing Image Sequences

If you have configured your acquisition protocol to acquire a fixed number of timepoints, the protocol will stop when the correct number of timepoints has been acquired. If you wish to stop the protocol before the end is reached, or if you have chosen the **Capture until stop is pressed** option, you must click the Stop icon on the Video Preview window.

You may re-run your acquisition protocol by selecting the Capture icon as many times as you wish.

Pause Acquisition while Capturing



You may pause acquisition by selecting the pause button during acquisition of an image sequence.

Any timepoints that have been partially acquired when you select pause will be completed before the acquisition is paused. Once partially acquired timepoints are completed no further acquisition will take place until the acquisition is resumed.

While acquisition is paused the pause button is highlighted and the red record icon in the top left corner of the Video Preview window changes to a gray pause icon.

- To resume acquisition click on the highlighted pause button.
- To stop acquisition whilst paused click on the capture/stop button.

If a timepoint is overdue when you resume acquisition, it will be acquired immediately and at the time intervals that you have specified in the time-lapse options thereafter.

Freeze the Video Preview



Freeze the image being shown in the Video Preview. This has two effects:

- When viewing a live image, an acquisition protocol is not running. Freezing the preview will leave the last captured frame visible on the screen but will close any shutters associated with the current light path. This allows an image to be viewed and evaluated without exposing the sample to further illumination light.
- When an acquisition protocol is running, freezing the preview will not affect any of the settings controlled by the protocol but the preview will not update with each captured frame. This maximizes the resources available to the acquisition and can improve performance.

While the Video Preview is frozen, the snowflake icon and the text "Preview frozen" will be shown in the top left-hand corner.

Acquisition Feedback

The acquisition protocol feedback panel provides a graphical summary of the current acquisition protocol. Double clicking on the acquisition protocol feedback panel opens the acquisition setup dialog.



Multiple timepoints

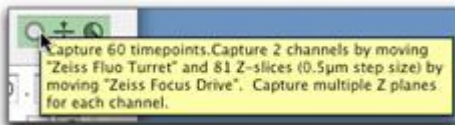


Multiple timepoints and multiple Z sections



Multiple timepoints, multiple Z sections, and multiple channels

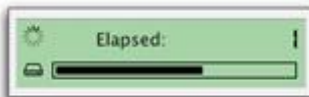
A more detailed summary is displayed in the acquisition feedback panel tooltip (move the cursor over the LCD display to see the tooltip).



Click on the number in the timing feedback panel to toggle between the feedback modes. The display changes to show:



the elapsed time

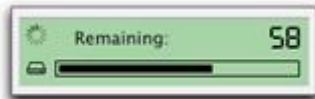


the number of elapsed timepoints



the remaining time

Events and Logs



the number of remaining timepoints



the time until the next timepoint

Note: If **Capture until stop is clicked** was selected in the Acquisition Setup window, the **Remaining** option will display the approximate time or timepoints remaining until your hard disk is full.

Events and Logs

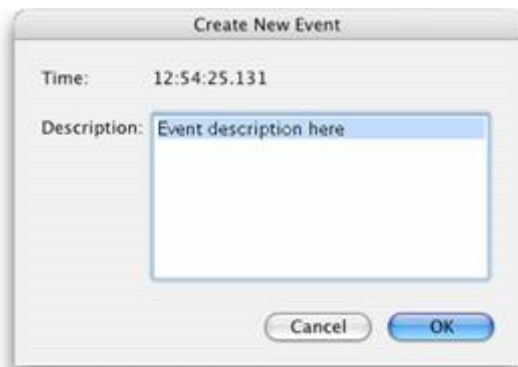
If something happens while observing acquisition that you wish to record, click on the Event flag.

click to record event during acquisition



Note: The shortcut key **F7** creates an event.

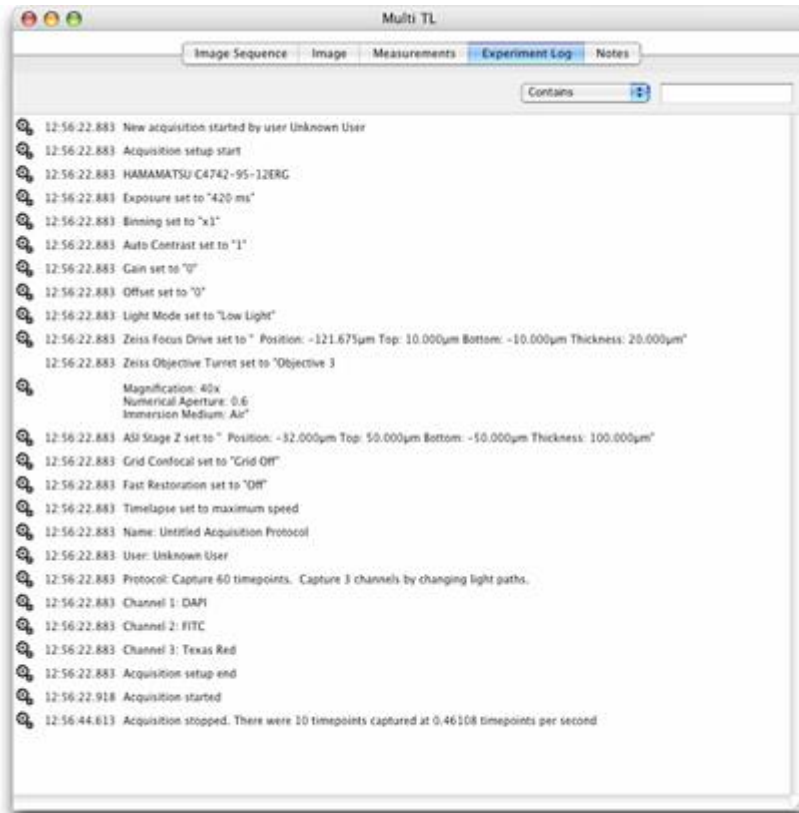
Enter the appropriate text to identify the event and click **Create**.



The text will be stored in the Experiment Log of the image sequence.

All of the image sequences that you acquire with Volocity Acquisition will have an Experiment Log View, which can be displayed by selecting the Experiment Log tab.

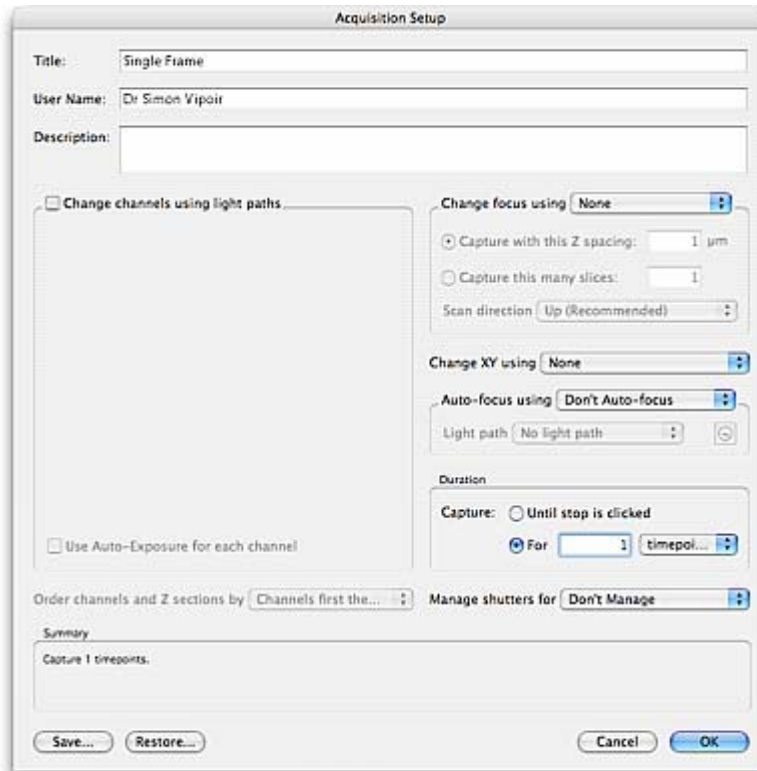
The settings that were used for acquisition and any events that you recorded during acquisition are recorded in the Experiment Log.



Example Acquisition Protocols

Acquiring a Single Frame

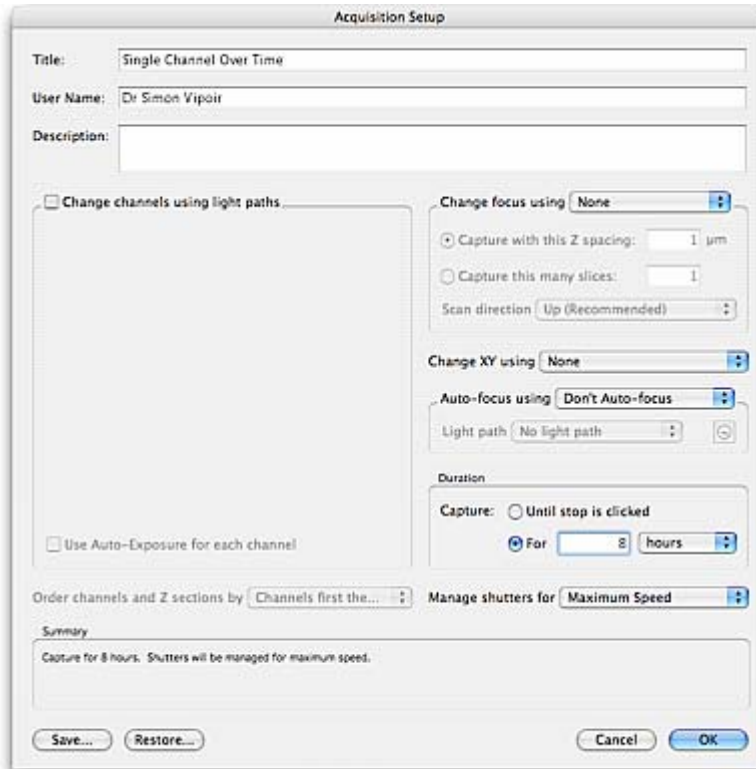
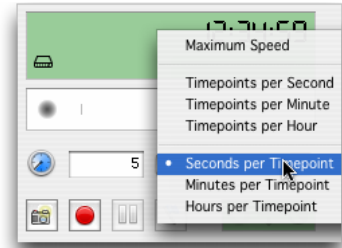
1. On the Video Preview controls no timelapse options are required.
2. Adjust the camera settings until you are happy with the image.
3. On the Acquisition Setup window enter information fields only and click **OK**.



4. Click the capture single image icon on the Video Preview window.

Acquiring a Single Channel over Time

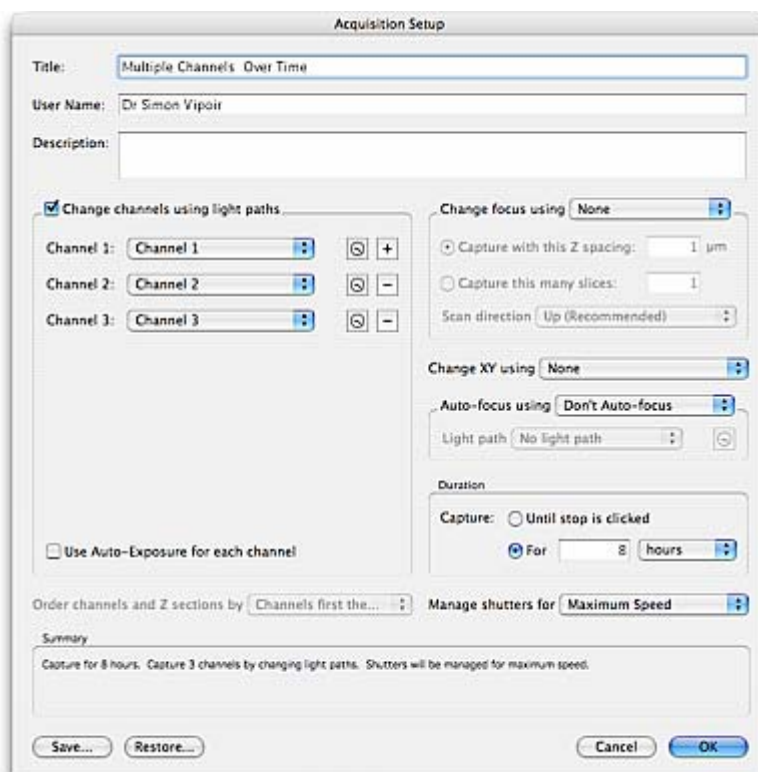
1. On the Video Preview controls set up timelapse options.
2. Adjust the camera settings until you are happy with the image.
3. On the Acquisition Setup window, enter information fields and:
 - Ensure that **Change channels using light paths** is not selected.
 - Set **Change focus using** to None.
 - Choose the **Duration** for the experiment.
 - Set the **Shutters**.



4. Click the capture image sequence icon on the Video Preview window.

Acquiring Multiple Channels over Time

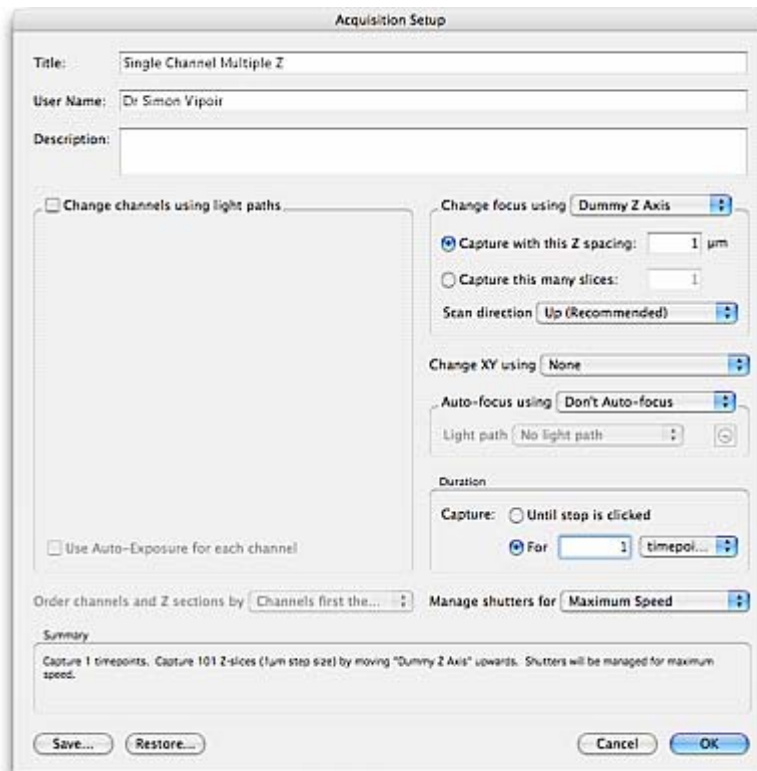
1. On the Video Preview controls set up the timelapse options.
2. If you wish to use the same exposure for each channel, ensure that the **Auto Contrast** option has been selected in the Video Preview window.
3. If you wish to manually set exposures for each channel ensure that they are included in your light paths.
4. On the Acquisition Setup window, enter information fields and:
 - Ensure that **Change channels using light paths** is selected. Add the correct number of channels.
 - Set **Change focus using** to None.
 - Enter a number in the **Duration** section and set the units.
 - Set the **Shutters**.



5. Click the capture image sequence icon on the Video Preview window.

Acquiring a Single Channel at Multiple Z-Planes and Single Timepoint

1. On the Video Preview controls no time lapse options are required.
2. At the midpoint of your specimen adjust the camera settings until you are happy with the image.
3. Set up the **Top** and **Bottom** values for the focus drive.
4. On the Acquisition Setup window enter information fields and:
 - Ensure that **Change channels using light paths** is not selected
 - Select the appropriate device from the **Change focus using** drop-down menu and set an appropriate **Z spacing**
 - Set the **Duration** = 1 timepoint
 - Set the management of **Shutters**.



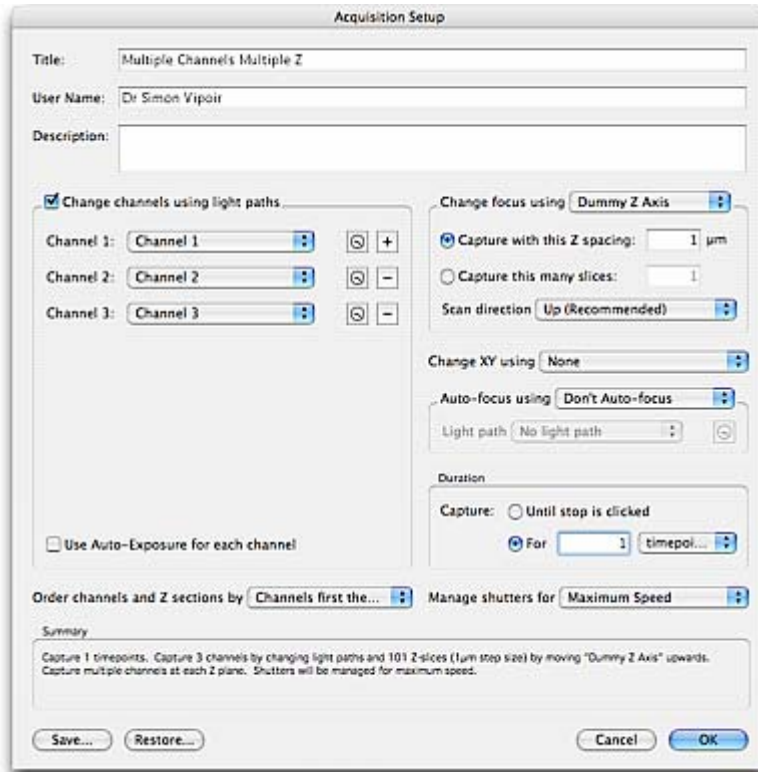
5. Click the capture image sequence icon on the Video Preview window.

Acquiring Multiple Channels at Multiple Z-Planes and Single Timepoint

1. On the Video Preview controls no timelapse options are required.
2. If you wish to use the same exposure for each channel, ensure that the **Auto Contrast** option has been selected in the Video Preview window. This will ensure the optimum contrast settings to assist setting one exposure for all channels.



3. If you wish to manually set exposures for each channel ensure that they are included in your light paths.
4. Set up the **Top** and **Bottom** values for the focus drive.
5. On the Acquisition Setup window enter information fields and:
 - Ensure that **Change channels using light paths** is selected. Add the correct number channels.
 - Select the appropriate device from the **Change focus using** drop-down menu and set an appropriate **Z spacing**.
 - Set the **Duration** = 1 timepoint.
 - Select the appropriate option from the **Use this ordering for channels and Z sections** drop-down menu.
 - Set the **Shutters**.



- Click the capture image sequence icon on the Video Preview window.

Example Acquisition Protocols

Acquiring a Single Channel at Multiple Z-Planes Over Time

1. On the Video Preview controls set up the timelapse options.
2. At the midpoint of your specimen adjust the camera settings until you are happy with the image.
3. Set the **Top** and **Bottom** values for the focus drive.
4. On the Acquisition Setup window enter information fields and:
 - ensure that **Change channels using light paths** is not selected
 - select the appropriate device from the **Change focus using** drop-down menu and set an appropriate **Z spacing**
 - enter a number in the **Duration** section and set the units
 - set the **Shutters**.

Acquisition Setup

Title: Single Channel Multiple Z Over Time

User Name: Dr Simon Vipoir

Description:

Change channels using light paths

Change focus using: Dummy Z Axis

Capture with this Z spacing: 1 µm

Capture this many slices: 1

Scan direction: Up (Recommended)

Change XY using: None

Auto-focus using: Don't Auto-focus

Light path: No light path

Duration

Capture: Until stop is clicked

For: 8 hours

Order channels and Z sections by: Channels first the...

Manage shutters for: Maximum Speed

Summary

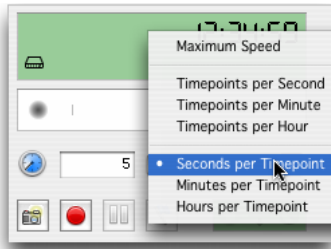
Capture for 8 hours. Capture 101 Z-slices (1µm step size) by moving "Dummy Z Axis" upwards. Shutters will be managed for maximum speed.

Save... Restore... Cancel OK

5. Click the capture image sequence icon on the Video Preview window.

Acquiring Multiple Channels at Multiple Z-Planes Over Time

1. On the Video Preview controls set up the timelapse options.



2. If you wish to use the same exposure for each channel, ensure that the **Auto Contrast** option has been selected in the Video Preview window.



3. If you wish to manually set exposures for each channel ensure that they are included in your light paths.
4. Set the **Top** and **Bottom** values for the microscope.
5. On the Acquisition Setup window enter information fields and:
 - Ensure that **Change channels using light paths** is selected. Add the correct number of channels.
 - Select the appropriate device from the **Change focus using** drop-down menu and set an appropriate **Z spacing**.
 - Enter a number in the **Duration** section and set the units
 - Select the appropriate option from the **Use this ordering for channels and Z sections** drop-down menu.
 - Set the **Shutters**.

Example Acquisition Protocols

Acquisition Setup

Title:

User Name:

Description:

Change channels using light paths

Use Auto-Exposure for each channel

Change focus using

Capture with this Z spacing: µm

Capture this many slices:

Scan direction

Change XY using

Auto-focus using

Light path

Duration

Capture: Until stop is clicked

For hours

Order channels and Z sections by

Manage shutters for

Summary

Capture for 8 hours. Capture 101 Z-slices (1µm step size) by moving "Dummy Z Axis" upwards. Shutters will be managed for maximum speed.

6. Click the capture image sequence icon on the Video Preview window.

Spectral Separation

Overview

Spectral separation is a technology that allows the separation of images containing data from more than one fluorophore into channels that contain data from only a single fluorophore. In order to achieve this separation or “un-mixing”, sample images must be acquired at a minimum of two different wavelengths. Color cameras lend themselves naturally to this multiple wavelength sampling because they simultaneously sample at three different wavelengths (red, green and blue). However, multiple wavelengths can just as effectively be acquired using monochrome cameras and wavelength changing devices such as filter wheels.

Why Separate Color Images?

Acquiring a single color channel instead of separate blue, green and red channels is an effective means of reducing acquisition time. You may still wish to analyse each of the fluorophores in your experiment in a single monochrome channel. Spectral separation will allow you to acquire the data for up to three fluorophores into a single color channel and separate each fluorophore into its own monochrome channel post-acquisition.

Why Separate Monochrome Images?

Spectral separation can be used to un-mix the contribution of spectrally overlapping fluorophores from a series of grayscale images sampled at different wavelengths (lambda stacks). You can therefore improve the separation of dyes that you have found difficult to separate adequately by more conventional means using filter sets.

Lambda stacks can be composed of a series of images in which the excitation wavelength is constant and the emission wavelength is changed, reflecting the shape of the emission wavelengths of fluorophores. Alternatively, the emission wavelength can be constant and the excitation wavelength changed, reflecting the shape of the excitation wavelengths of fluorophores. It may also be possible to successfully un-mix fluorophores from Lambda stacks in which both excitation and emission wavelengths are varied.

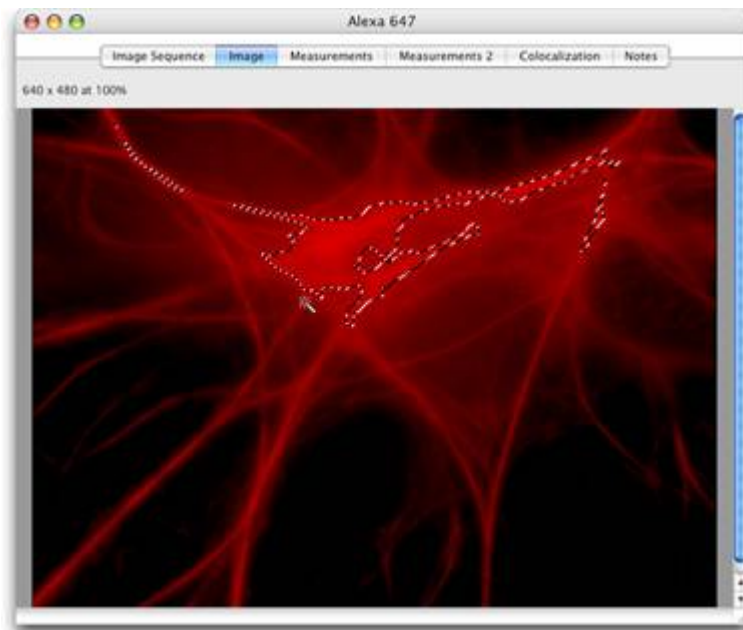
There should be a minimum of two channels in a Lambda stack. The maximum number of fluorophores that can be separated from a lambda stack is equal to the number of images in the lambda stack.

Creating Spectral Signatures from Color Images

In order to accurately separate your fluorophores into individual channels you must first create accurate spectral signatures. A spectral signature allows Volocity to calculate how a sample of only a single fluorophore contributes to the red, green and blue elements of color images.

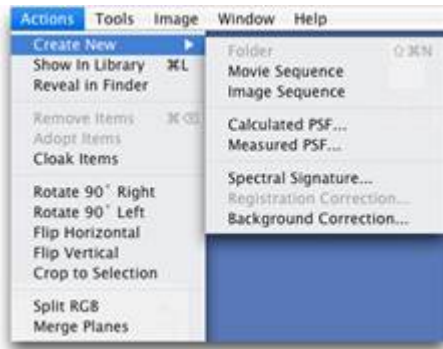
To generate a spectral signature:

- First prepare a sample that contains only a single fluorophore.
- Acquire an image or image sequence of the sample.
- Open your image or image sequence in the Image View and select a region brightly stained with your fluorophore with a region of interest tool, such as the magic wand tool. You should ensure that no pixels in this region are saturated.



1. Select **Create New** from the Actions menu.

2. Select **Spectral Signature...**



3. The Create Spectral Signature dialog opens.



4. Velocity will assign a sample color and perceived peak wavelength to your new spectral signature. Give the spectral signature a meaningful name and click **OK** to create the signature.

5. Spectral signatures are added to your library.



6. You may inspect a spectral signature by double clicking on its library item.



You should make a spectral signature for each of the fluorophores that you wish to include in your experiments. You can un-mix up to three fluorophores in each experiment.

Creating Spectral Signatures from Monochrome Images

Successful spectral separation is dependent on the creation of accurate spectral signatures. A spectral signature allows Volocity to calculate how a single fluorophore contributes to each point of a lambda stack.

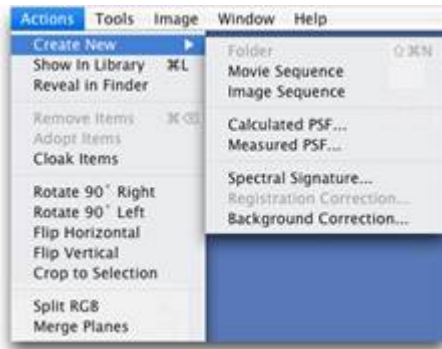
To generate a spectral signature:

- First prepare a sample that contains only a single fluorophore.
- Acquire a lambda stack of the specimen into an image sequence.
- Open your image or image sequence in the Image View and select a region brightly stained with your fluorophore with a region of interest tool, such as the magic wand tool. You should ensure that no pixels in this region are saturated.

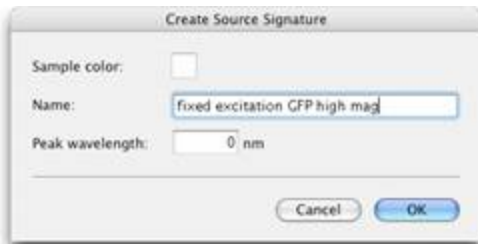


1. Select **Create New** from the Actions menu.

2. Select **Spectral Signature...**



3. The Create Spectral Signature dialog opens.



4. Enter a name for your spectral signature and click **OK**.

5. Spectral signatures are added to your library.



6. You may inspect a spectral signature by double clicking on the library item.

You should make a spectral signature for each of the fluorophores that you wish to include in your experiments.

Separating Color Images

In order to accurately separate each of the channels from your color image, Volocity will need to calculate the contribution made by background. This can be done automatically or you can select a region of interest that contains only background. Volocity can calculate the background directly from this region of interest or you can create a background spectral signature, which you may re-use if your background is consistent between multiple image sequences. To make a background spectral signature:

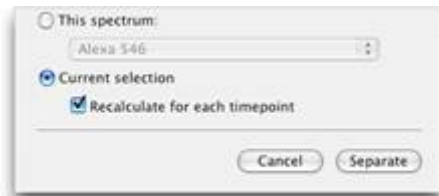
- With your region of interest selected, select **Create New** then **Spectral Signature...** from the **Actions** menu.
 - Give the background spectral signature a name and click **OK**.
1. Select an image or image sequence of a multiply (two or three fluorophores) labeled color specimen.
 2. Select **Separate Spectra...** from the **Tools** menu.



3. In the Separate Spectra dialog select the spectral signatures of the fluorophores in your color image from the drop-down menus. Each of the spectral signatures in your library will be listed in the drop-down menus.



4. Select one of the **Calculate background from** radio buttons.
 - If you want Volocity to automatically calculate the background, select the **Default background** radio button.
 - If you have made a background spectral signature, select the **This spectrum** radio button and select your spectral signature from the drop-down menu.
 - If you have specified a region of interest containing only background, choose the **Current Selection** radio button. If your image sequence is time resolved, the background will be calculated from the first timepoint only unless you select the **Recalculate for each timepoint** checkbox.

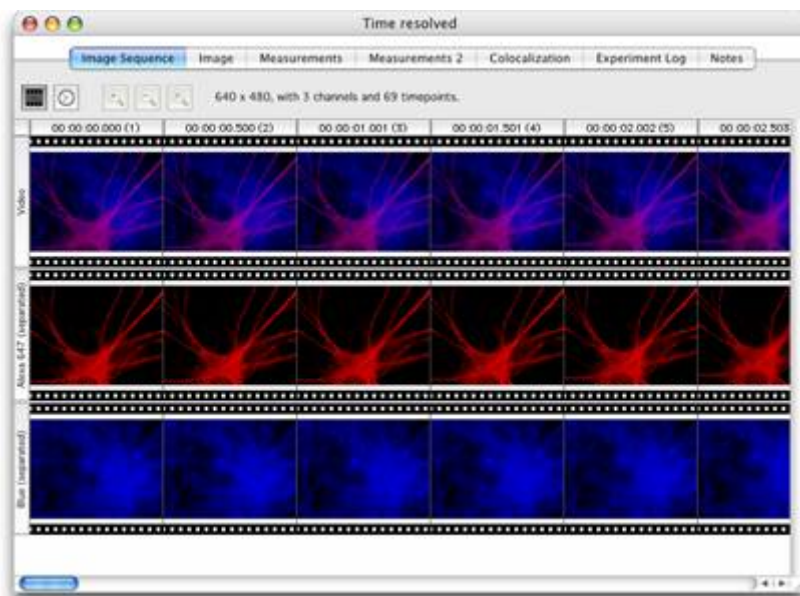


When you select **Recalculate for each timepoint** the background is recalculated for every timepoint from the same region of interest. You must be certain that your region of interest always contains only background (and that no objects of interest stray into your region during the course of the time series).

5. Click **Separate**.

New channels based on your selected spectral signatures will be added to your image sequence. The new channels are monochrome and are colored based on the color of the spectral signature from which they were derived.

Separating Monochrome Images (Lambda Stacks)



If you un-mix a color image, new monochrome images will be added to a folder of images beneath your original image.

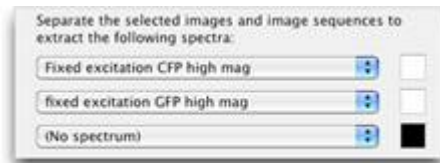
Separating Monochrome Images (Lambda Stacks)

Acquire lambda stacks of specimens labelled with the combination of fluorophores that you wish to separate. You must acquire your experimental specimens at exactly the same wavelengths and relative exposures as your spectral signatures.

In order to accurately separate each fluorophore into its own channel from your lambda stacks, Volocity will need to calculate the contribution made by background. This can be done automatically or you can select a region of interest that contains only background. Volocity can calculate the background directly from this region of interest or you can create a background spectral signature, which you may re-use if your background is consistent between image sequences. To make a background spectral signature:

- With your region of interest selected, select **Create New** then **Spectral Signature...** from the **Actions** menu.
 - Give the background spectral signature a name and select **OK**.
1. Open an image sequence in the image view.
 2. Select **Separate Spectra...** from the **Tools** menu.
 3. In the Separate Spectra dialog select the spectral signatures from the drop-down menus of the fluorophores that you wish to un-mix from your lambda stacks.

- The number of drop-down menus will be equal to the number of images in your lambda stacks.
- Each menu contains a list of all the spectral signatures in your library that were derived from lambda stacks containing the same number of images as the image sequence that you wish to un-mix.
- If you have over-sampled (i.e. the number of images in each lambda stack is greater than the number of fluorophores in your experiment) then you may leave some of the drop-down menus as the default (**No spectrum**).

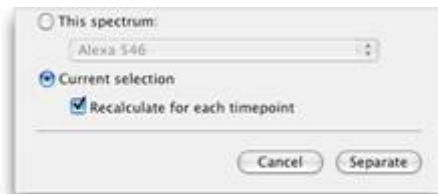


4. Select one of the **Calculate background from** radio buttons.

- If you want Volocity to automatically calculate the background, select the **Default background** radio button.
- If you have made a background spectral signature select the **This spectrum** radio button and select your spectral signature from the drop-down menu.



- If you have a region of interest containing only background selected (in the image view) choose the **Current Selection** radio button. If your image sequence is time resolved the background will be calculated from the first timepoint only unless you select the **Recalculate for each timepoint** checkbox.



- When you select the **Recalculate for each timepoint**, the background will be recalculated for every timepoint from the same region of interest. You must be certain that your region of interest always contains only background (and that no objects of interest stray into your region during the course of the time series).

Separating Monochrome Images (Lambda Stacks)

5. Click **Separate**.

New channels, based on your selected spectral signatures, will be added to your image sequence. The new channels will contain only information from a single fluorophore.



Volocity Visualization

Overview

Volocity Visualization includes a renderer that provides a versatile range of rendering modes for different sample types, allowing interactive rendering of 3D and 4D data, and a Slice View that allows reslicing of image data in any orientation. Once you have activated this set of features you will have an additional view of image data, the 3D View. These views, like all other Volocity views, have their own specific menu and tools.

Note: Volocity Visualization is an additional product that must be purchased separately. See page 35 for more information about activating additional products purchased to expand your system.

Specific recommendations are made for the graphics cards. These are detailed in the System Requirements technical note 263. This technical note will be updated as new graphics cards become available and are tested.

If the installed graphics card does not meet this specification, or the required modules (called HR Rendering and IP Rendering, which performs HR Intensity Rendering) are absent from the Volocity modules folder, the Classic 3D Renderer will load.

When you select a 3D View, Volocity calculates a projection—a screen image of how the volume appears from a specific viewpoint—using direct rendering from the 2-dimensional data. The speed of the rendering engine allows you to interact with the volume on screen to view it from different angles and/or use different rendering methods. The intense computation required is carried out as you interact with the rendered view and the Renderer Controls in the Navigation palette.

Types of Renderer

There are different renderers within Volocity, their availability depends on the license purchased for the system and the hardware configuration. There is a section on each Renderer in this chapter. The Renderers differ in the features supported and the style of rendering that is used.

- Purchasers of Volocity Visualization Lite will have the HR Intensity Renderer.
- Purchasers of the full Volocity Visualization license will have both the HR Opacity and HR Intensity Renderers.

HR Rendering is a perspective rendering, whereas 3D Rendering is orthographic. In HR Rendering zooming into a volume allows movement into that volume rather than continuing to magnify the voxels. The HR Renderer can also display feedback from rows selected in a Measurements session (you will need both the Volocity Visualization and the Volocity Quantitation modules).

Some systems support a choice of renderers and have an additional choice of **Ray Tracer**, which is a software renderer.

3D View

Movie Sequencer

Volocity Visualization also includes the Movie Sequencer, which allows you to create and store a volume animation template that can be applied to any Rendering View to create a series of pictures for export as an AVI or QuickTime movie file.

Bookmarks and Movies

Bookmark and Movie panes of the 3D View allow you to store volume positions and then animate transitions between bookmarks.

Slice View

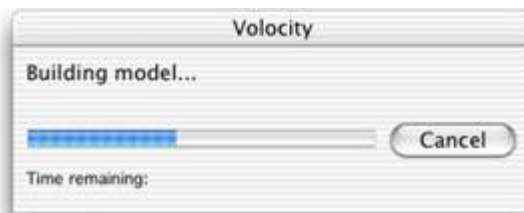
The Slice View allows 3D or 4D data to be viewed at a different orientation of slicing and, if required, that data may be re-sliced to create another version of the data at the new orientation.

The Slice View is available with the full Volocity Visualization licence only.

3D View

Select the item that you wish to view in 3D, or render and double click to open it.

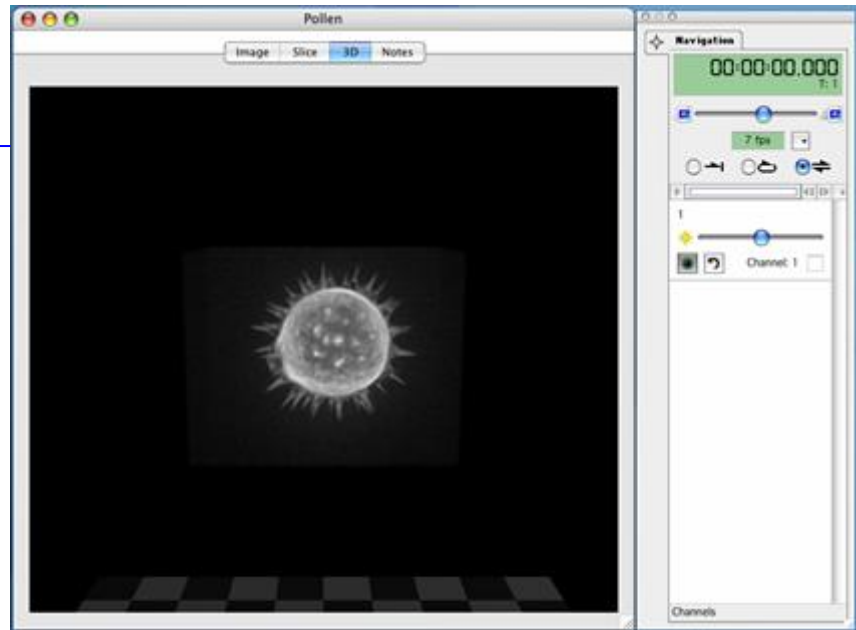
1. Click on the 3D tab. If the item is part of an image sequence, Volocity will render all the channels for the timepoint at once.
2. Creating the rendered view for the first time may be a lengthy process, depending upon the volume and complexity of the data. A progress bar is displayed as Volocity renders the volume.



3. A 3D View will be opened. There is more detail on the individual Renderers used to produce the image shown on the screen in the relevant sections.

The following example shows a view generated by the HR Intensity Renderer.

a progress bar will be displayed here as you work with rendered data



Once you have created the rendered 3D View you can use the tools in the toolbar to work with the image and the Navigation palette to adjust Renderer settings and play through the channels. When using HR Renderers Volocity displays a progress bar in the top left-hand corner of the view as it applies any changes that you make. Select **Reset View** from the Renderer menu to reset any values applied to the Renderer Controls from the Navigation palette.

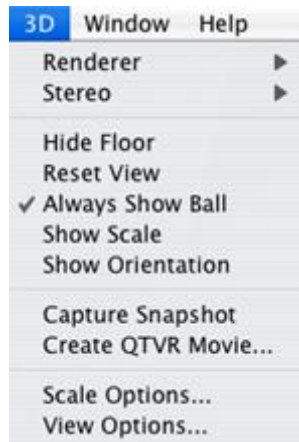
Note: When Volocity renders a volume, it stores sufficient information in its cache memory so that it can display the rendered volume quickly when you switch between different types of view. The cache is saved with the library. If you are moving the data or sending it to another user, you may like to use the Compact Library command, with the option to remove cached data enabled, to delete this information and reduce file size. See page 60.

Using Contrast Enhancement with 3D Views

Changes made using the Contrast Enhancement dialog (Tools menu) are carried through into the 3D View. Remember that it is possible to alter the black point, white point and gamma of a dataset using the Contrast Enhancement and this will affect the appearance in the rendered view.

3D Menu

Whenever a 3D View is active, the view specific menu will be called 3D and it contains controls that allow you to show/hide elements of the view and apply special effects.



Renderer

If available, this option allows selection of the rendering style. This choice is only available if using Volocity Visualization.

Stereo

This option allows selection of a stereo mode of viewing which will generate the image so that it is viewed with specialized glasses to emphasize the 3D appearance.

Show/Hide Floor

A floor is shown below the object in the 3D View to emphasize the orientation of the object. **Hide Floor** becomes **Show Floor** when the floor is not visible. Use the View Options to change the position and appearance of the floor.

Reset View

This option resets the orientation of the object to the default position applied when the data is first seen in the 3D View.

Always Show Ball

This option always displays the graphical ball which is used to move the object around in the view.

Show/Hide Scale

Scale is shown using a grid behind the object. The grid moves to stay behind the object as it is rotated. The distance represented by each square in the grid, the calibration information, is shown in the bottom right corner of the screen.

Use the 3D Scale tab in the **View Options...** dialog to change the color of the scale grid and switch calibration information on or off.

Show/Hide Orientation

Orientation is a marker to identify x, y, and z axes. This multicolored pointer is shown in the bottom left hand corner of the view

Capture Snapshot

This option creates a 2D image of the view as it currently appears. This image may then be exported.

Create QTVR Movie...

This option creates a specialized movie which can be viewed using a QuickTime player. Rather than playing back frames through time, different orientations of the view are captured and viewers of a QTVR movie may manipulate the object themselves within the limits of range of movement set when making the movie. QuickTime must be installed on the system for this option to be available.

Show/Hide Time

Select **Show Time** from the 3D menu to display the volume timestamp. Change the color of the timestamp or frame time in the View Options.

View Options

Use the **View Options...** command to change the setup of the 3D View. The dialog is tabbed for different settings.

In the **General** tab change the background and/or floor color. Using different colors for these can improve the visibility of details in the data being rendered. Floor position and also pattern can be changed. The **3D Scale** tab changes how the scale grid is drawn.

Show/Hide Bookmarks

This option shows or hides the interface area for storing bookmarks. Other menu items are for creation, editing and navigation between bookmarks.

Show/Hide Movie

This option shows or hides the interface area for designing movies. Other menu items are for editing and saving movies.

3D Toolbar



Use the three library navigation tools at the bottom of the toolbar to navigate through items in the library without making the Library view active.



Shortcut key R

Use this tool to rotate the object. A ball outlines the object and the pointer changes to a hand when you position it on the object. When you let go, the object spins around the pivotal point (see next page for example). Use Alt-click (Mac) or Ctrl-click (PC) and drag to zoom.



Shortcut key F

Click once on this tool to fly around the object.



Shortcut key Z

Click with this tool and drag to zoom in and out.

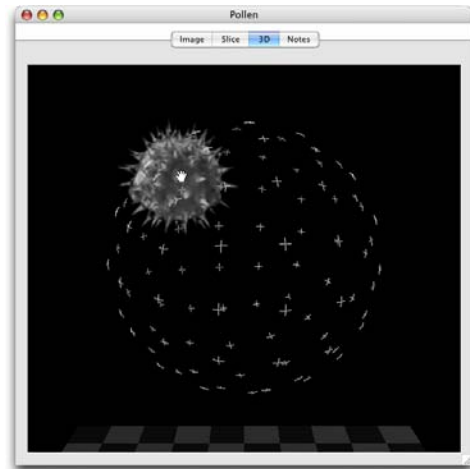
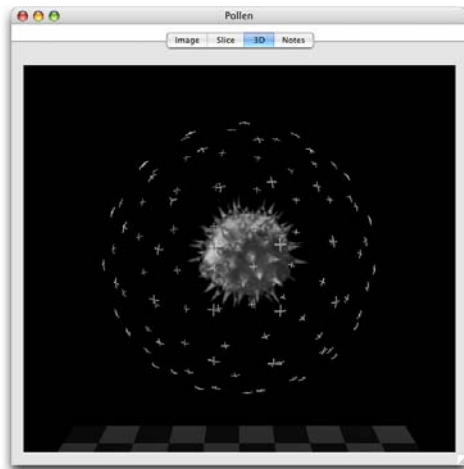


Shortcut key H

Use this tool to move the rendered object within the sphere. For example, this allows you to set the pivotal point for rotation, as described in the next section.

Setting the Pivotal Point of Rotation

1. Click and drag on the sphere with the hand tool to center the object on the first axis.





2. Change to the rotate tool to check the effect.
3. Then continue to adjust for each axis using the hand tool until you have set the pivotal point of rotation, as required.

Note: Select **Reset View** from the 3D menu to return the object to its original position.

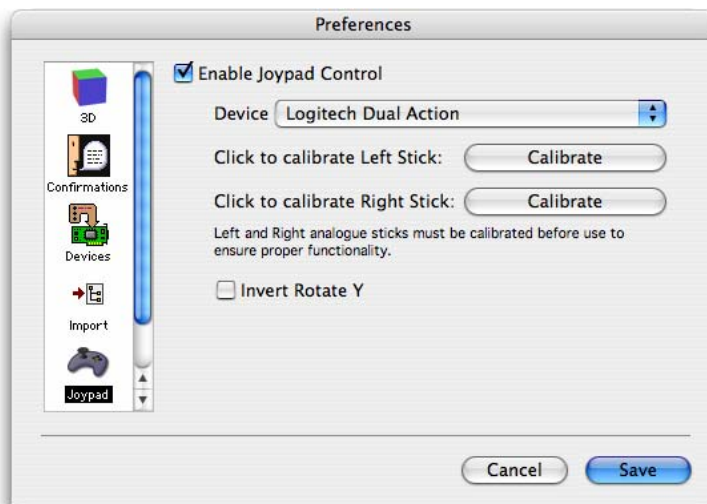
Free Flight Camera Control

It is possible to control the viewing point or camera position for free flight around and through the volume. This is achieved using a USB joystick/gamepad with two analog joysticks and front face buttons.

Joypad Preferences

It is important to enable the joypad and calibrate the sticks before using the joypad for the first time.

1. Select **Preferences...** from the Edit menu (Windows) or the Volocity menu (Mac).
2. Click on the **Joypad** icon.
3. Check to **Enable Joypad Control**, select the **Device** and **Calibrate** both sticks.



Free Flight Camera Control

Controlling the Joypad

The gamepad controls the camera or viewer position allowing completely free movement around and through the volume. The gamepad is, therefore, not moving the object at all.



Left Analog Stick

Move Left, Right, Forwards and Backwards

Top Left Shoulder Button

Modifies action of left analog stick

Move Up and Down

Numeric Buttons

1 Point at center of volume

2 Add bookmark

Right Analog Stick

Turn Left, Right, Up and Down

Top Right Shoulder Button

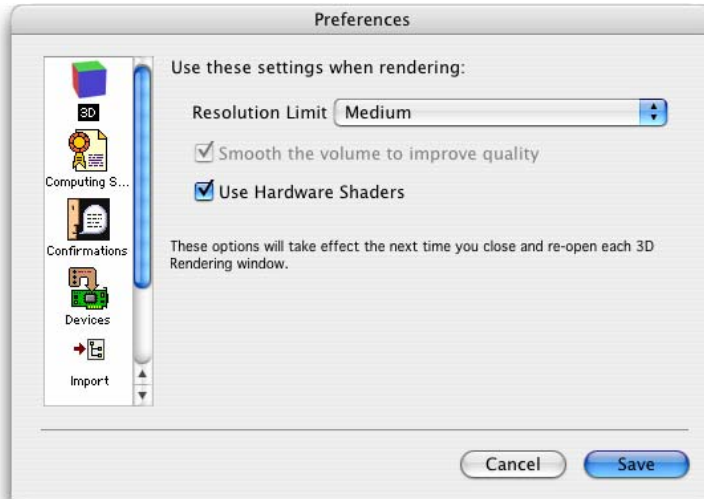
Modifies action of right analog stick

Roll Left and Right

3D Preferences

The 3D Preferences allow you to make adjustments in order to optimize rendering performance for the hardware system.

1. Select **Preferences...** from the Volocity menu (Mac OS X) or the Edit menu (Windows).
2. Click on the **3D** icon to display the settings for 3D Views.



3. The **Resolution Limit** sets the maximum number of voxels that will be rendered. Any data set exceeding the limit for a particular setting will be filtered allowing the user to achieve a balance between quality and interactivity. Reducing the resolution will increase interactivity and increase the size of data set that a particular system is able to render.
4. The HR Renderers support fragment/pixel/hardware shaders, if these are supported by the graphics card. The terms used to describe hardware shaders vary depending on the graphics card chipset manufacturer.

Hardware shaders offer better independent rendering of multiple channels reducing artefacts that can be introduced when channels interfere with each other. Hardware shaders are enabled in the renderer by checking **Use Hardware Shaders**.

Use of hardware shaders requires a powerful graphics card that supports the OpenGL extension 'GL_ARB_fragment_program'. Recommended cards are described in the system requirements technical note.

5. Settings that are grayed out indicate that they are not supported by the system. Make the required changes and click on **Save** (Mac) or **OK** (Windows). The settings will be applied the next time you open a 3D window. Open views will have to be closed and reopened for changes to take effect.

Which Renderer?

Which Renderer?

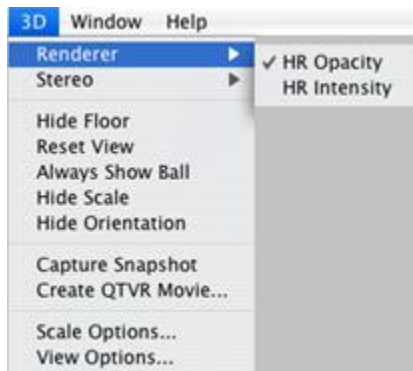
You will have a choice of renderers, if you have the full version of Volocity Visualization and your hardware supports HR Renderers.

- Users of Volocity Visualization Lite will only be able to use the HR Intensity Renderer.
- Users with hardware that does not support the HR Renderers will only be able to use the Classic 3D Renderer.

Selecting the Renderer (Volocity Visualization only)

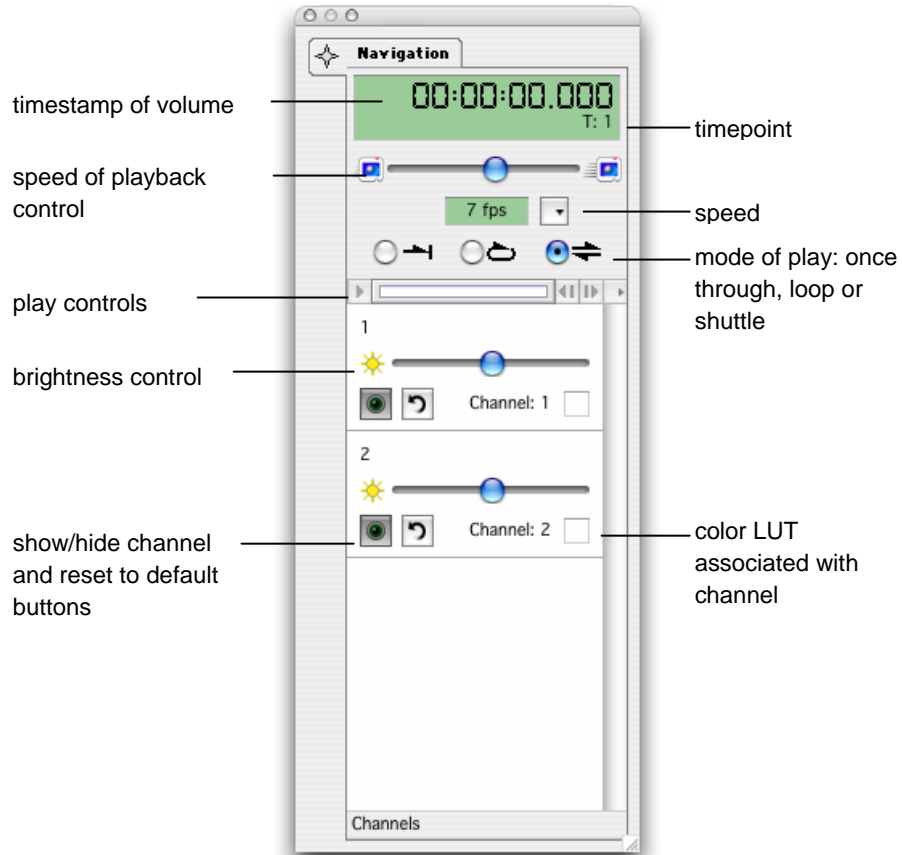
Volocity Visualization users with hardware which supports the HR Renderers will be able to select between the HR Opacity and HR Intensity Renderers.

From the 3D menu select **Renderer** and choose the required type of renderer.

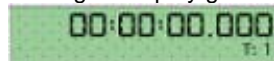


HR Intensity Renderer (Volocity Visualization and Visualization Lite)

Choose **Show Palette** from the Window menu, if the Navigation palette is not already visible on the screen.



- The digital display gives the timestamp of the image and the timepoint number in the sequence.



- Select the required mode of play by clicking on the appropriate button, and adjust the speed, if required, by using the slider.



HR Intensity Renderer (Volocity Visualization and Visualization Lite)

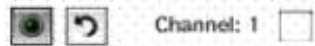
- Use the play controls to play through the volumes in the sequence.



- Use the **Brightness** slider control to control the brightness of the rendered voxels. The HR Intensity Renderer uses the maximum intensity voxel from the path through the volume as the screen image and the brightness slider allows a multiplication factor to be applied to the voxel intensities. Moving the slider to the left reduces the brightness, moving it to the right increases brightness.



- The show/hide button cloaks and uncloaks the channel in the 3D View so you can choose to work with a channel or not. The arrow button resets the slider for that channel to default (the values it had when first opened). The colored square reflects the color lookup table associated with the channel.



- When you change controls or zoom in on the 3D View, while you play the sequence, you can use the channel Renderer Controls to adjust the rendering mode and settings and tailor the data set for optimum visualization, as required. As you do this, you may see a blue progress bar in the top left-hand corner as the Renderer rebuilds part of the model.

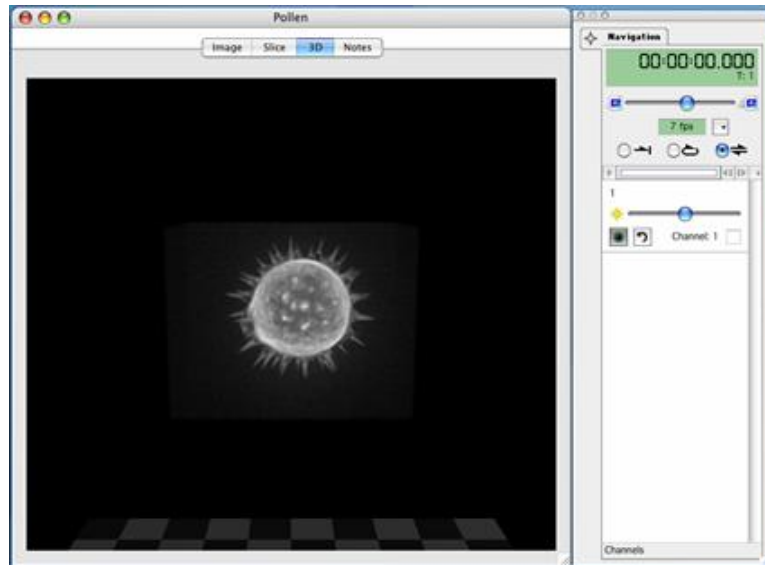
Note: If you set a light background color in the View options (see page 253), this will be interpreted as maximum intensity and you might not get the result you expect.

HR Intensity Renderer Examples

The following sequence shows the effect of applying the brightness control to an image.

HR intensity default

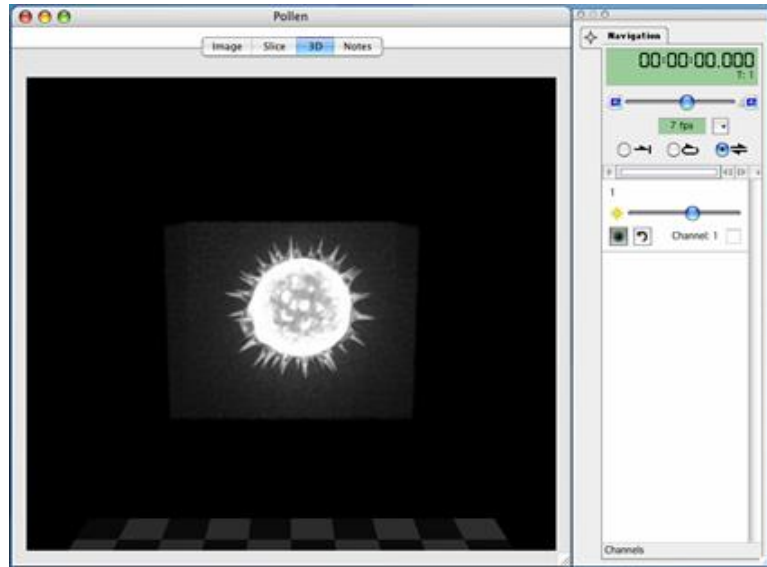
the brightness
slider is in its
default position



HR Intensity Renderer Examples

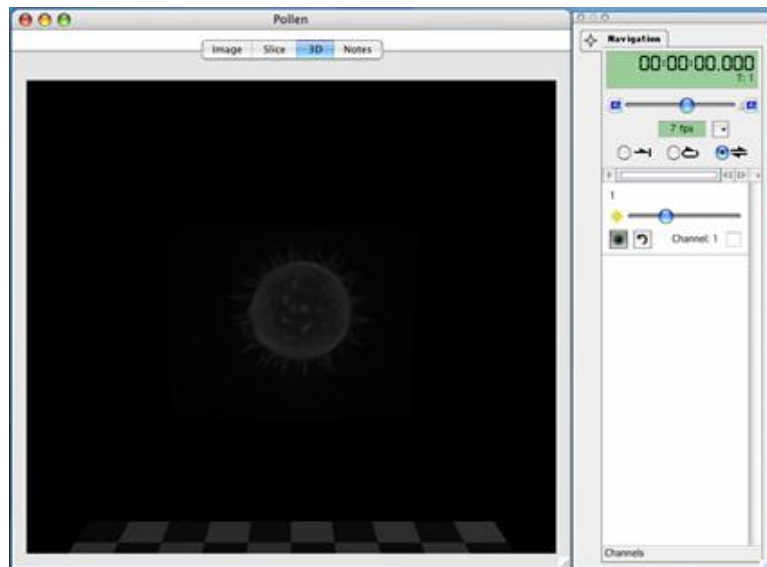
HR intensity brighter

the brightness slider has been moved to the right



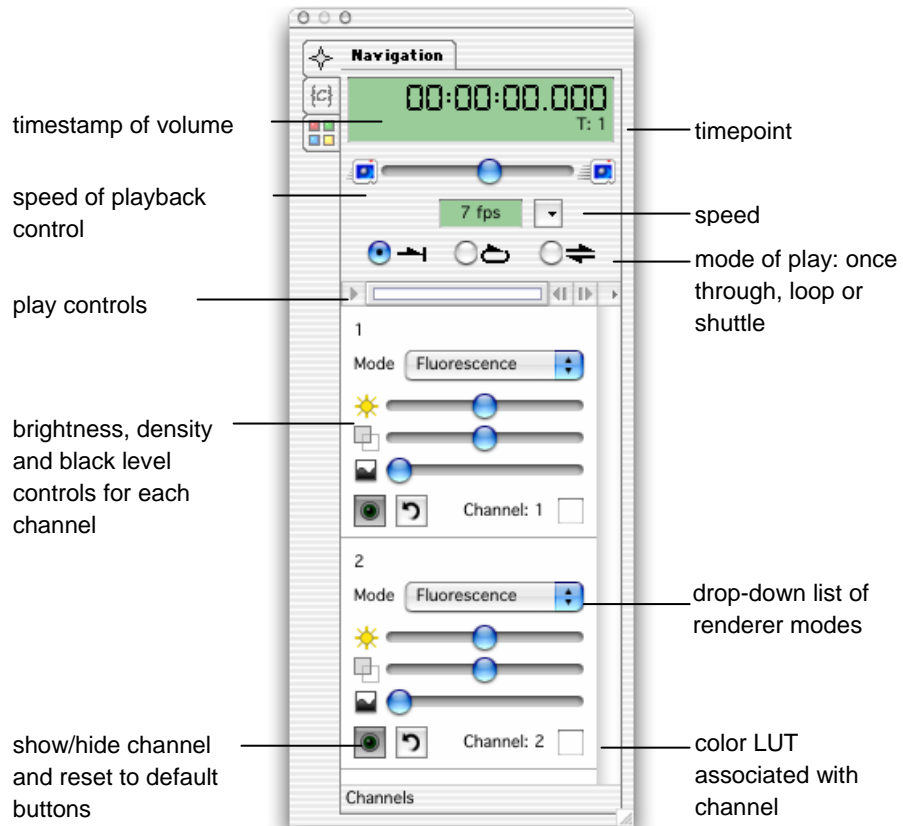
HR intensity dimmer

the brightness slider has been moved to the left



HR Opacity Renderer (Volocity Visualization only)

Choose **Show Palette** from the Window menu, if the Navigation palette is not already visible on the screen.



- The digital display gives the timestamp of the image and the timepoint number in the sequence.



- Select the required mode of play by clicking on the appropriate button, and adjust the speed, if required, by using the slider.



HR Opacity Renderer (Volocity Visualization only)

- Use the play controls to play through the volumes in the sequence.



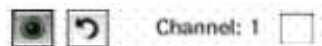
- Select the required rendering mode from the **Mode** drop-down menu.
 - **Fluorescence** applies direct opacity rendering, which is designed for use on data from fluorescence microscopy.
 - **Brightfield** applies direct opacity rendering, which is designed for use on data generated by transmitted light microscopy.
 - **Isosurface** applies indirect surface rendering, which identifies a surface around objects where all voxel intensity values are the same. This intensity is determined by the black level setting. The surface is made up of complex polygons. This generates apparently solid objects which cannot be seen through. No signal from objects behind can pass through an isosurface.
- Use the slider controls to control the brightness, density and black level of the rendered image.



- **Brightness** alters the brightness of the rendered voxels. A multiplication factor is applied to the intensity values of the voxels. Moving the slider to the left reduces the brightness, moving it to the right increases brightness.
- **Density** alters the density of the rendered voxels. This affects how much can be seen through the structure. Denser voxels do not allow signal from the objects behind to pass through to the screen image. Moving the slider to the right increases voxel density, moving it to the left decreases density.
- **Black Level** sets a value at which voxels of that intensity or lower will not be rendered. The remaining data is rescaled across the full display range of the screen. When choosing an appropriate black level, it may help to change the background color of the rendering view. Choose **View Options** from the 3D menu and choose a different color for the background (a lighter color usually helps the process). When the background color has been changed a dark area may be visible around the rendered object. Adjust the black level until this darkness is removed.

Leaving the cursor over any of the Renderer Controls momentarily brings up a tip showing their current values.

- The show/hide button cloaks and uncloaks the channel in the 3D View so you can choose to work with a channel or not. The arrow button resets the slider button for that channel to default (the values it had when first opened). The colored square reflects the color lookup table associated with the channel.



- When you change controls or zoom in on the 3D view while you play the sequence, you can use the channel Renderer Controls to adjust the rendering mode and settings and tailor the data set for optimum visualization, as required. As you do this, you may see a blue progress bar in the top left-hand corner as the Renderer rebuilds part of the model.
- QTVR movies or movie sequences can be made from the HR Opacity view.

HR Opacity Renderer Examples

Example: effect of black level, density and brightness on a single channel

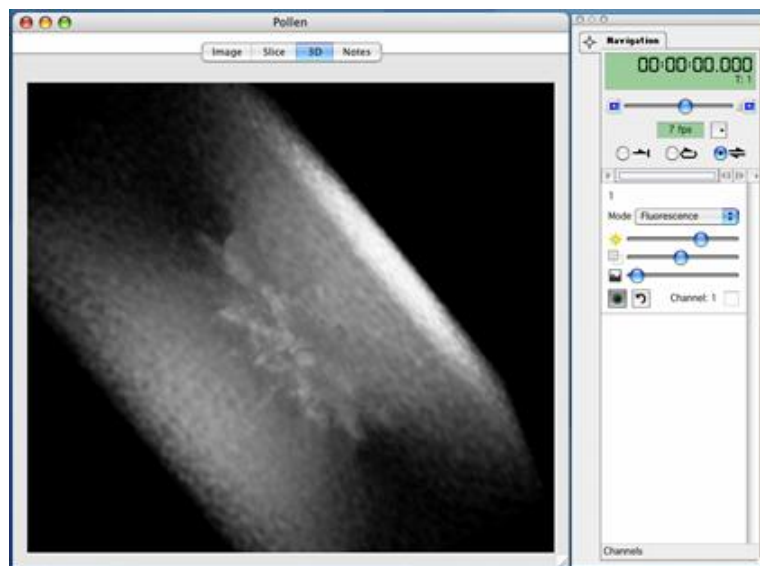
In the first three examples, we have adjusted the black level to stop low intensity voxels causing noise when rendered and obscuring the object of interest.

We can then use the brightness and density sliders to adjust the transparency of the object.

The following sequence shows the effect of applying the brightness control to an image.

Black level is 0%

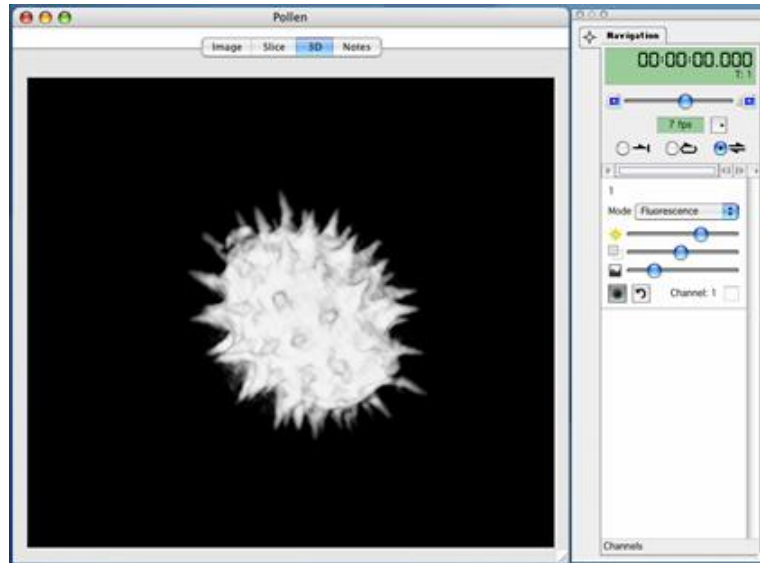
When rendered, the low intensity voxels cause noise and obscure the object of interest.



HR Opacity Renderer Examples

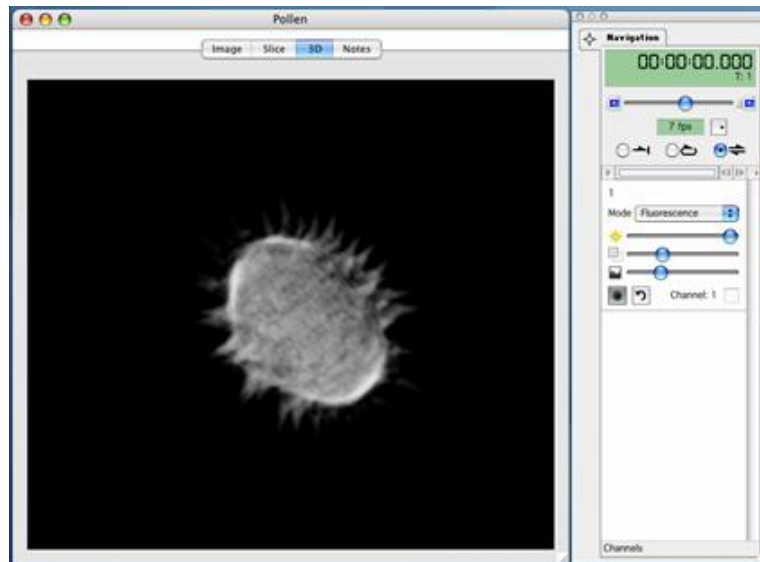
Black level is increased

Unwanted, low intensity voxels are not rendered.



Keeping the same black level

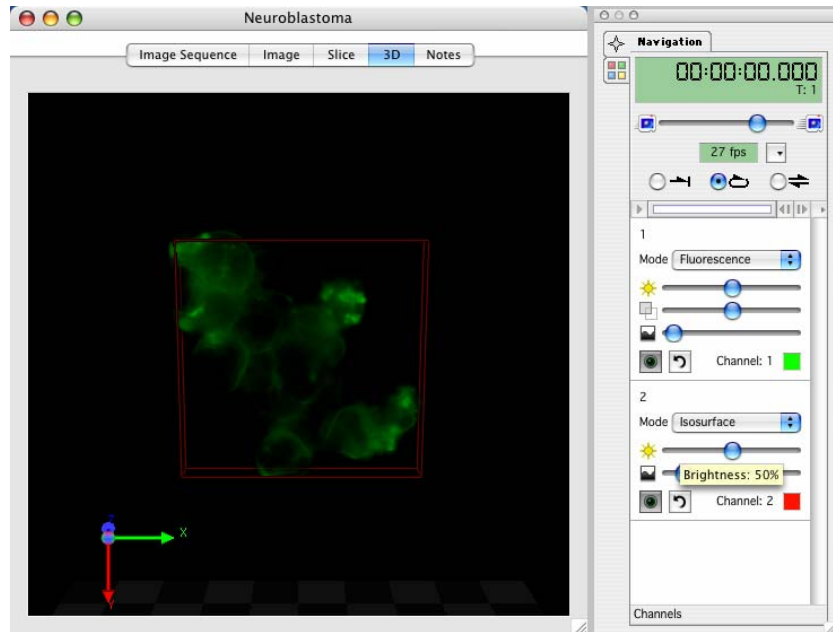
By raising the brightness and lowering the density we can produce a semi-transparent object.



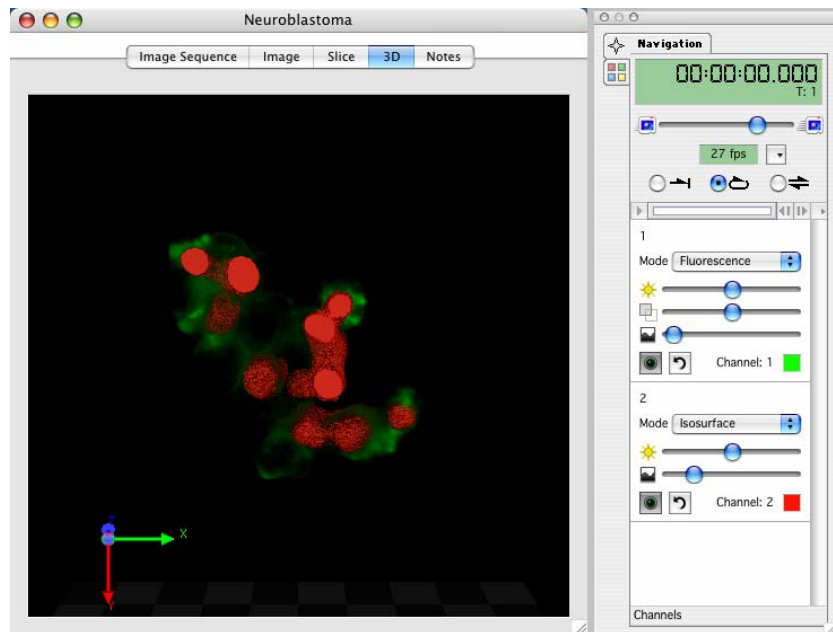
Example: using different rendering modes on a 2-channel data set

In the following example (on the next page) on a two channel data set, one channel is set to Isosurface rendering and the other is set to Fluorescence mode.

While the isosurface is being generated a simple wire box appears to show its approximate location. This is replaced by the isosurface when it is ready.



The black level of the Isosurface channel is adjusted to change the intensities included in the isosurface. The controls for the channel in fluorescence mode can also be adjusted.

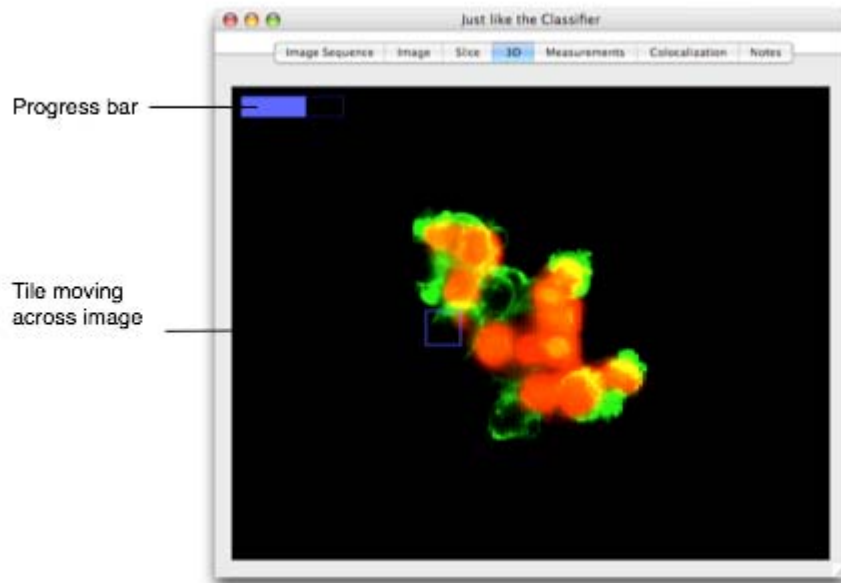


Ray Tracer

If your system supports a choice of renderers, choose **Ray Tracer** from the Renderer sub-menu in the 3D menu.

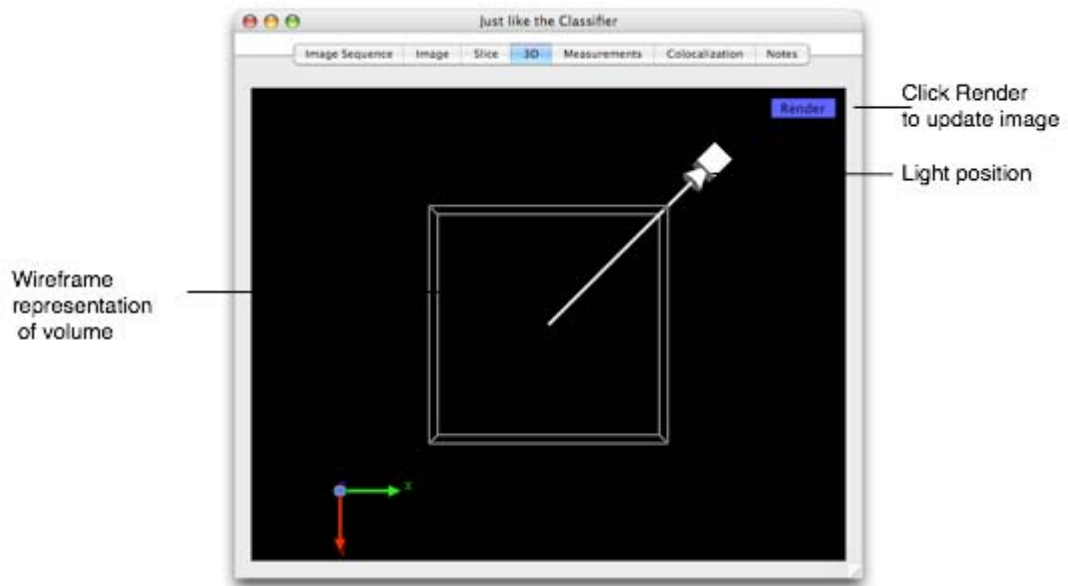
Unlike HR renderers which are real time and update the view quickly, the Ray Tracer is not an interactive renderer. The screen image is built up in tiles over multiple passes. A progress bar in the top left-hand corner of the image shows progress to completion of the screen image.

The Ray Tracer is a software renderer, using the computer CPU to generate the results rather than graphics hardware.



Using the tools in the toolbar to change the position or the orientation of the view will not update the view immediately; the view will switch to a wireframe representation of the volume that can be moved as required.

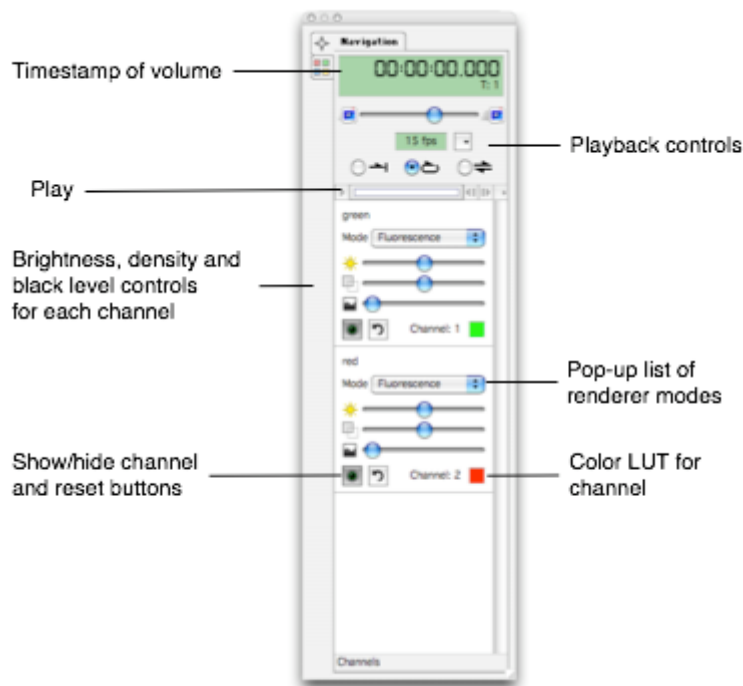
When the cursor is within the view a **Render** button is shown in the top right-hand corner. Click on this button to prompt the building of the screen image in tiles over multiple passes.



There is no need to wait for the image to build if you wish to make further changes; it is possible to interact with the menus, toolbar and palettes while the image is being built. Click on the **Render** button to update the image with your changes.

Ray Tracer

Choose **Show Palette** from the Window menu to show the Navigation palette containing the controls for the appearance of the channels.



Changes to the settings in the Navigation palette do not update the view immediately; the wireframe representation is shown and you must click the **Render** button to update.

- The digital display gives the timestamp of the image and the time point number in the sequence.
- Select the required mode of play by clicking on the appropriate button, and adjust the speed, if required, by using the slider.
- Use the play controls to play through the volumes in the sequence.
- Select the required rendering mode from the drop-down menu.
 - **Fluorescence:** applies direct opacity rendering, which is designed for use on data from fluorescence microscopy.
 - **Isosurface:** applies indirect surface rendering, which identifies a surface around objects where all voxel intensity values are the same. This intensity is determined by the black level setting. The surface is made up of complex polygons. This generates apparently solid objects which cannot be seen through. No signal from objects behind can pass through an isosurface.

Fluorescence and Isosurface rendering modes in the Ray Tracer ensure compatibility for data which is also viewed using other renderers. The Ray Tracer offers further isosurface modes:

- **Matte:** for a non-reflective surface.
- **Metal:** gives the surface properties similar to that of metal, surfaces are shiny and may reflect properties of the surrounding scene, for example the color of the floor.
- **Glass:** generates a surface which mimics the properties of glass. Objects can be given a high degree of transparency and there may be some reflection. Surfaces will be shiny.
- Use the slider controls to control the brightness, density and black level of the rendered image.
 - **Brightness** alters the brightness of the rendered voxels. A multiplication factor is applied to the intensity values of the voxels. Moving the slider to the left reduces the brightness, moving it to the right increases brightness.
 - **Density** alters the density of the rendered voxels. This affects how much can be seen through the structure. Denser voxels do not allow signal from the objects behind to pass through to the screen image. Moving the slider to the right increases voxel density, moving it to the left decreases density.
 - **Black Level** sets a value at which voxels of that intensity or lower will not be rendered. The remaining data is rescaled across the full display range of the screen. When choosing an appropriate black level, it may help to change the background color of the Rendering view. Choose **View Options** from the 3D menu and choose a different color for the background (a lighter color usually helps the process). When the background color has been changed a dark area may be visible around the rendered object. Adjust the black level until this darkness is removed.
- Leaving the cursor over any of the Renderer Controls momentarily brings up a tip showing their current values.
- An additional tool is available to you, the light source position. Select this tool and click in the view, move the light position control. Ray tracing has the ability to cast shadows within the data and on the floor of the scene. It may be necessary to change the floor color to best show shadows on the floor. Do this using the View options in the 3D menu.

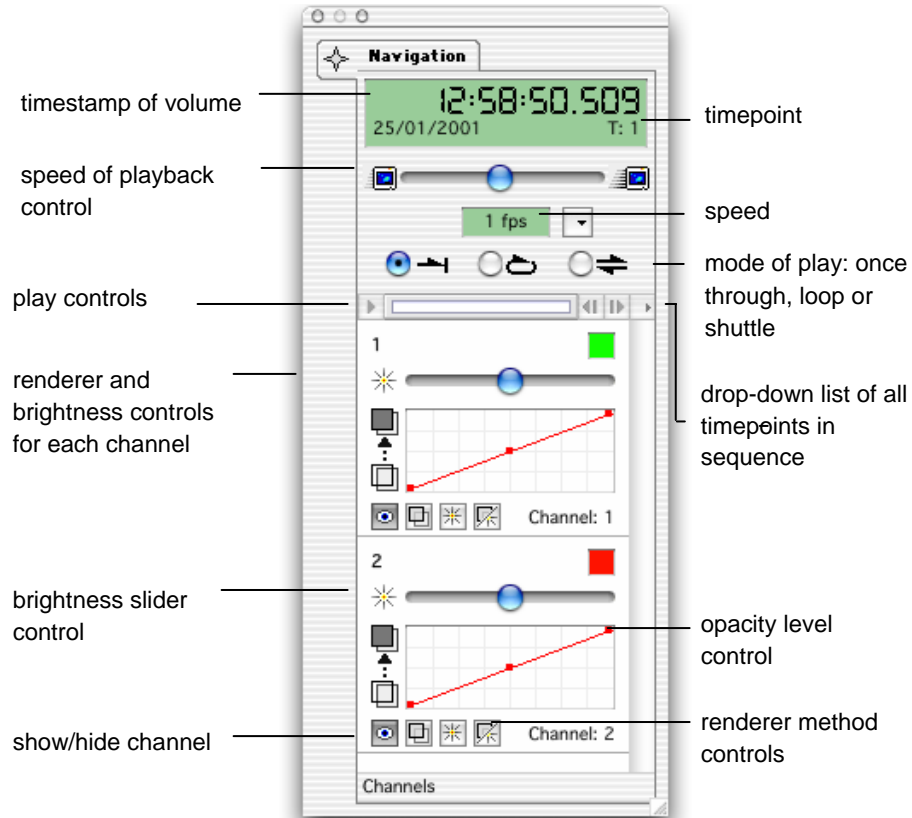
Switching views during tracing will cause the image to be lost and it will have to be traced again.

Note: The Ray Tracer does not currently support measurements feedback display or drawing of the scale grid in the view.

Classic 3D Renderer

This renderer will only load if the HR Opacity and HR Intensity Renderers are not available in the full Velocity Visualization license.

Choose **Show Palette** from the Window menu if the Navigation palette is not already visible on the screen.



- The digital display gives the timestamp of the image and the timepoint number in the sequence.
- Select the required mode of play by clicking on the appropriate button, and adjust the speed, if required, by using the slider.



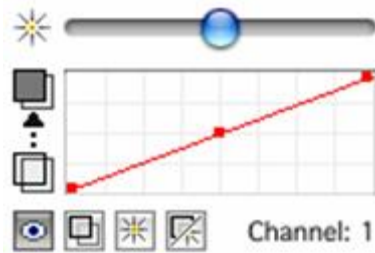
- Use the slider in the play controls to play through the volumes in the sequence.



- The show/hide button cloaks and uncloaks the channel in the Image View so you can choose to work with a channel or not.



- While you play the sequence, you can use the channel Renderer controls to adjust the rendering method and tailor the data set for optimum visualization, as required. Volocity's Classic 3D Renderer offers three rendering methods: Maximum Intensity Projection (MIP), Opacity Rendering and a combination of MIP and Opacity. If you are using the Image Sequencer, each channel will have its own Renderer Controls on the Navigation palette. Leaving the cursor over any of the Renderer Controls momentarily brings up a tip showing their current values.



The simplest way to choose between the three available rendering methods is to click the respective button on the palette.



Note: Select **Reset View** from the View menu to reset any values applied to the Renderer Controls from the Navigation palette

- **MIP:** This method uses the intensity value of the brightest voxel along the ray path in the screen representation.

The slider bar controls a multiplication factor that is applied to the intensity values of the voxels before the screen image is calculated. This slider ranges from 0 to 200. The center point is normal. The far left reduces the brightness level to 0. The far right increases it to 200%.



- **Opacity Rendering:** Using an opacity lookup table (LUT) each voxel in the volume is given an opacity value related to its intensity. The opacity value determines whether a voxel is solid or transparent. Opacity values are then computed along the ray path to create the screen representation.

Classic 3D Renderer Example

The opacity LUT controls determine how intensities are mapped to opacities and, therefore, the appearance of the opacity rendering. Objects that are 100% opaque are solid; those that are 0% opaque are completely transparent.



click and drag on top and bottom squares to adjust opacity level, click and drag middle square to shift line left or right

- **Combination MIP/Opacity Rendering:** The unique facility to combine the two methods produces different results from those using either method in isolation. It offers more realism by allowing bright objects to be seen through others or opaque objects in one channel to obscure objects behind them.
- Movie sequences and QTVR movies can be made from the 3D Rendering view.

Classic 3D Renderer Example

Example: uses the MIP slider only

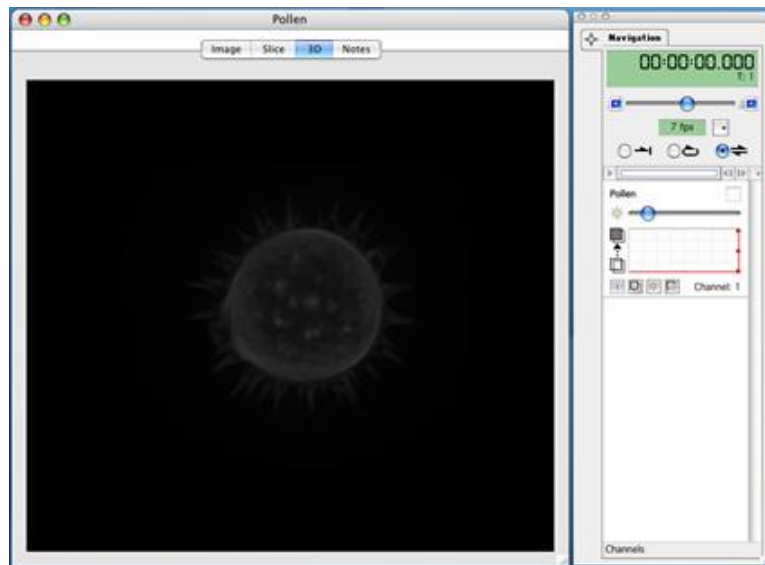
In the first two examples, we have applied brightness rendering and have used the slider control to change the value of the brightest point in the object.

The opacity is off in both examples. Look at the graph in the Renderer Controls; all intensity values are mapped to 0, completely transparent.

The following sequence shows the effect of applying the brightness control to an image.

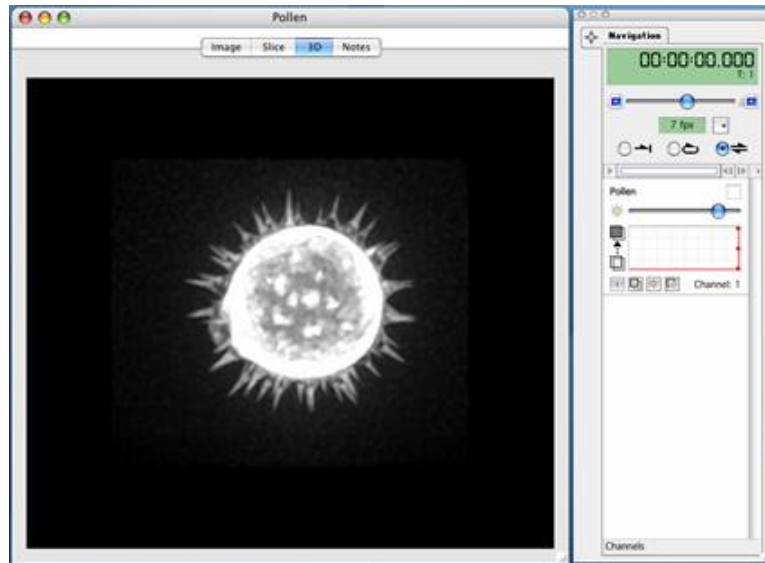
MIP contribution set to 25%

Note how dark the image is.



**MIP
contribution
set to 94%**

The image is brighter and you can see any structures behind it.



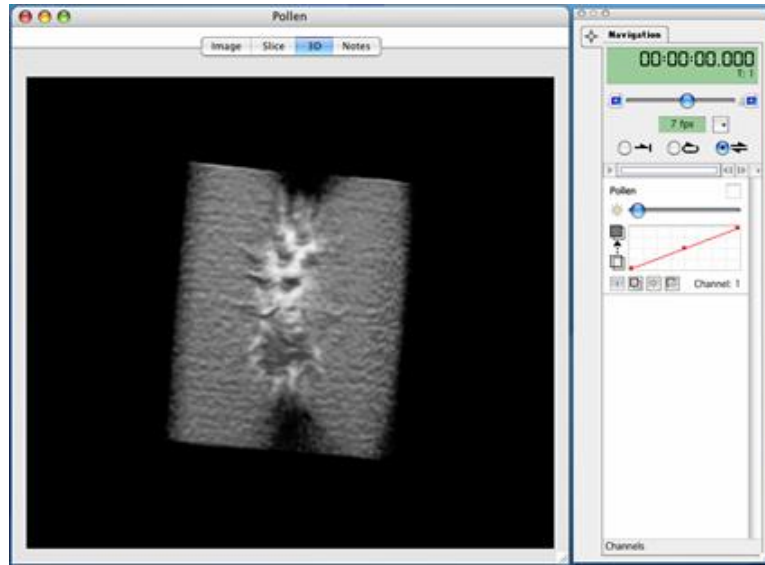
Example: uses the opacity controls only

In the next three examples, we have applied opacity rendering and have used the opacity controls to vary the solidity of each voxel. This can have the effect of peeling away layers to reveal structures within structures.

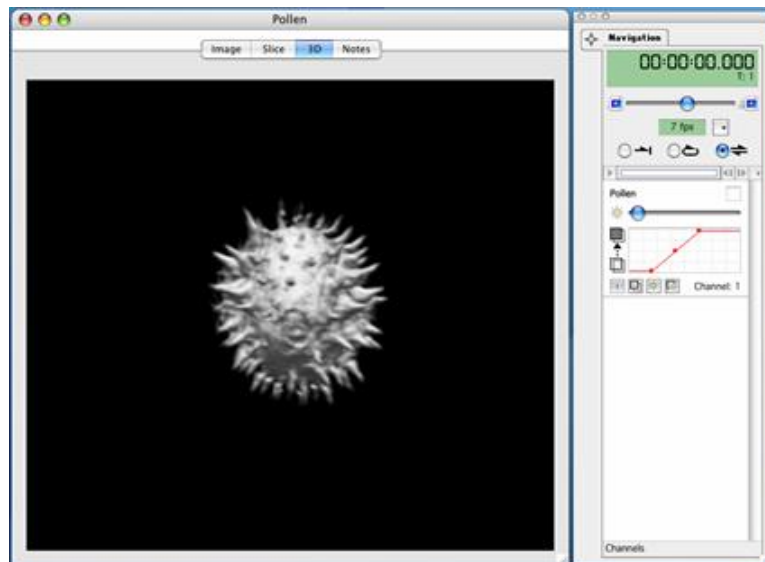
The MIP/brightness control is off in all three examples. Look at the slider in the Renderer Controls; it is set at 0.

Classic 3D Renderer Example

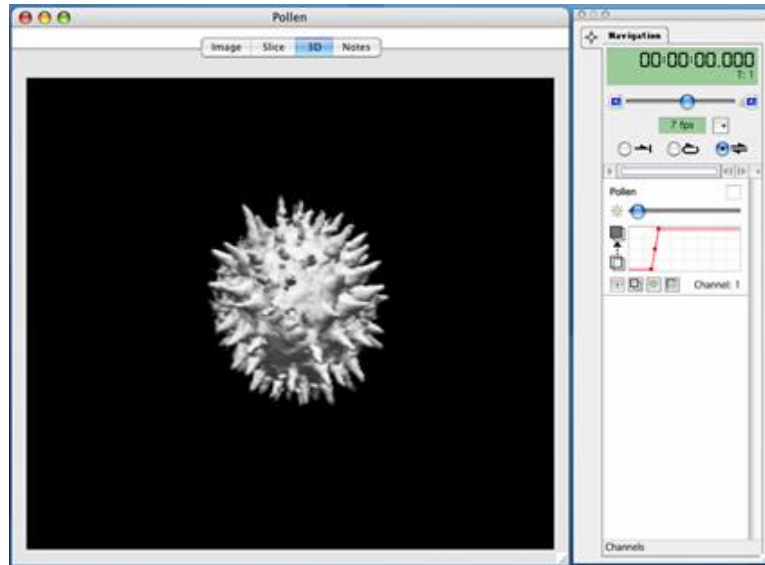
Opacity values set to display data at all gray levels. Underlying structures are only partially visible.



Displays data at gray levels between 50 and 152. This allows us to make parts of the structure transparent and produces the effect of peeling away layers to reveal underlying structures.



Displays data at gray levels between 50 and 80. The image is solid. Underlying structures are not visible.



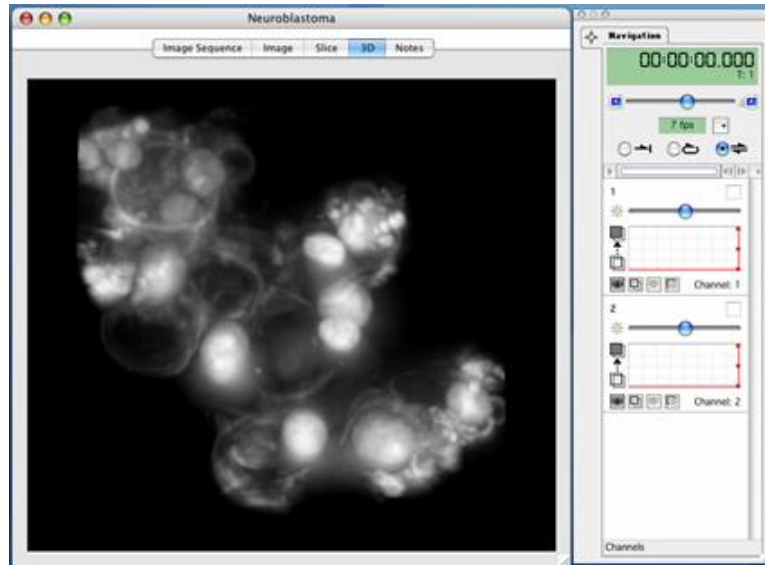
Example: combines MIP and opacity controls

In the final two examples we illustrate the power of the combination rendering method. You will notice that there are two sets of Renderer Controls. This is because the examples are from a multi-channel data set; each channel has its own Renderer Controls in the Navigation palette.

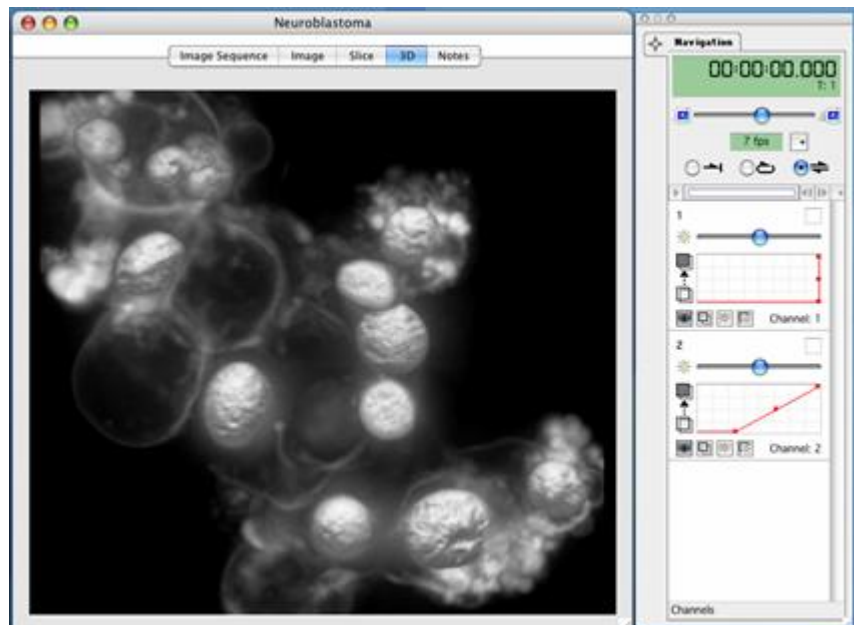
The following examples show how two or more separate channels can be rendered in different ways to better show structures and relationships between those structures.

Classic 3D Renderer Example

MIP only, used identically for both red (nuclei) and green (membrane) channels. Nuclei visible but partially obscured by the membrane.



Opacity rendering used for the red channel, mapping 0 to 100% opacity across intensities from 52 to 255. Nuclei are now solid and the membrane is transparent.



3D View Bookmarks

Bookmarks allow you to store information about the position of the volume and the rendering settings being applied to it.

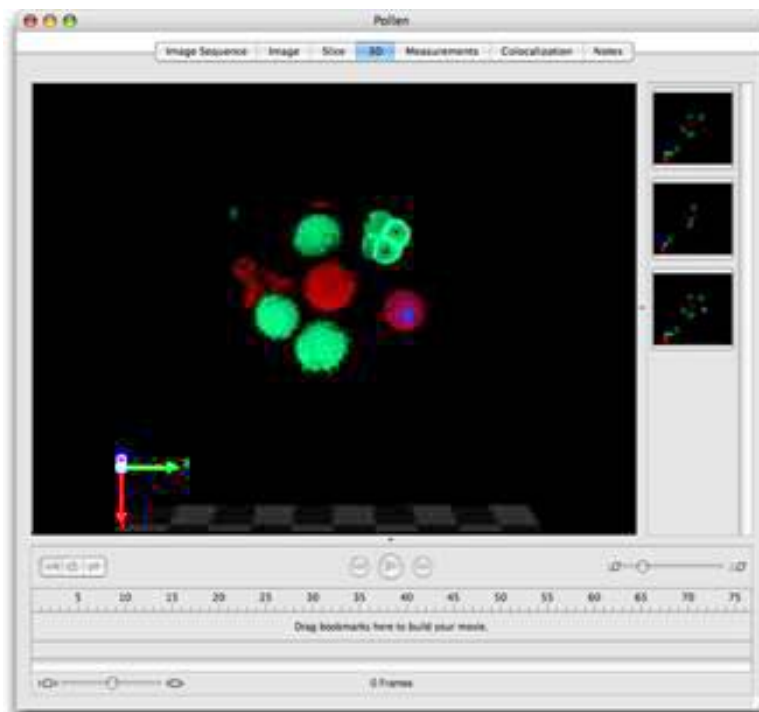
Bookmarks can be used to remember these settings so that they may be retrieved later. For example, to return to a previous position and appearance after making changes or to use the position and appearance of one data set to view another data set with exactly the same settings applied.

To Create a Bookmark

Bookmarks are kept in a Bookmark pane to the right of the 3D View. If this pane is not visible, select **Show Bookmarks** from the 3D menu.

- Position the volume as required and set the Renderer Controls in the Navigation palette to produce the desired appearance.
- Select **Add Bookmark** from the 3D menu.

A thumbnail of the volume will be added to the bookmarks pane.



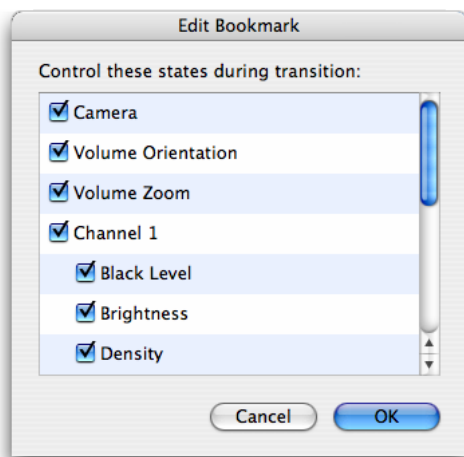
To Use a Bookmark

Double click on the thumbnail of a bookmark in the bookmark pane to use the settings of that bookmark to change the position and rendering settings of the current 3D View to those stored by the bookmark.

Alternatively use **Next Bookmark** or **Previous Bookmark** in the 3D menu to step through bookmarks in the order in which they were created.

To Edit a Bookmark

1. Select the bookmark in the bookmark pane, the borders of the bookmark will become highlighted.
2. Select **Edit Bookmark...** from the 3D menu.
3. A dialog is opened showing the states of the view that are being stored.



4. Uncheck a state and that part of the bookmark setting will not be applied to the 3D View when the bookmark is used.
Check the state again and the setting(s) stored when the bookmark was created will be applied again when the bookmark is used.

To Move a Bookmark.

To use the settings of one 3D View with another data set so that both data sets may be compared move the bookmark from the first 3D View to the second.

With both data sets open drag the bookmark from one bookmark pane to the other.

The bookmark thumbnail will update the first time it is used with the new dataset.

To Delete a Bookmark

Select the bookmark in the bookmark pane, the borders of the bookmark will become highlighted.

Use the Delete (Windows) or Backspace (Mac OS) keys to delete the bookmark.

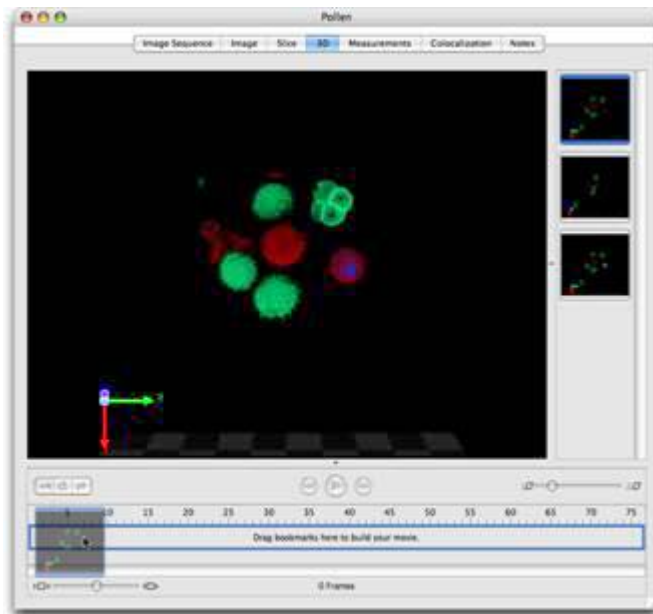
Overlays turned on or off by items in the 3D menu, i.e. the ball control, scale, orientation and time, are not stored by bookmarks.

3D View Movie Interface

You can animate a 3D View by scripting a series of movements and changes to the appearance of the volume using the Movie Sequencer. See page 288.

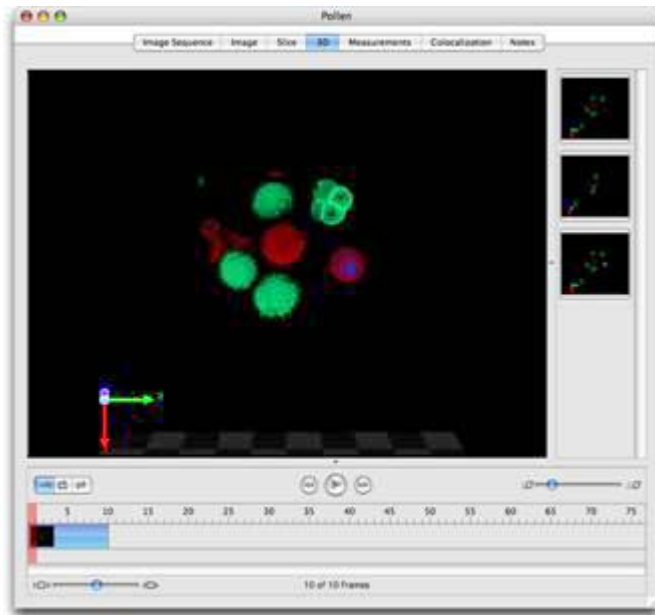
Alternatively, to create a more freely-flowing movie, use bookmarks and the movie interface in the 3D View. Bookmarks are the starting point for making movies in this way. The movie will be an animation of the transition between the appearance of the volume in one bookmark and its appearance in the next bookmark. Each transition takes place over a number of frames determined by you. Select **Show Bookmarks** from the 3D menu to view the bookmarks pane.

1. Choose **Show Movie** from the 3D menu.

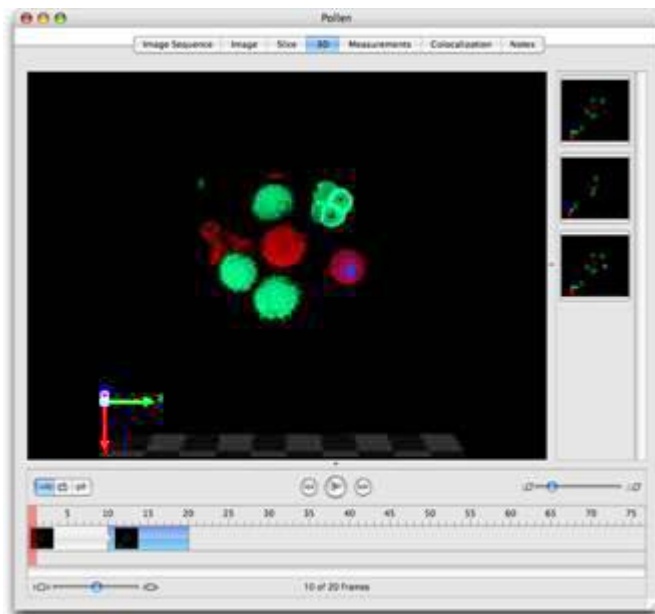


2. Drag a bookmark thumbnail from the bookmark pane to the movie pane to create a keyframe in the movie. By default this keyframe will be 10 frames long. Keyframe length may be changed by clicking and dragging the end of the keyframe.

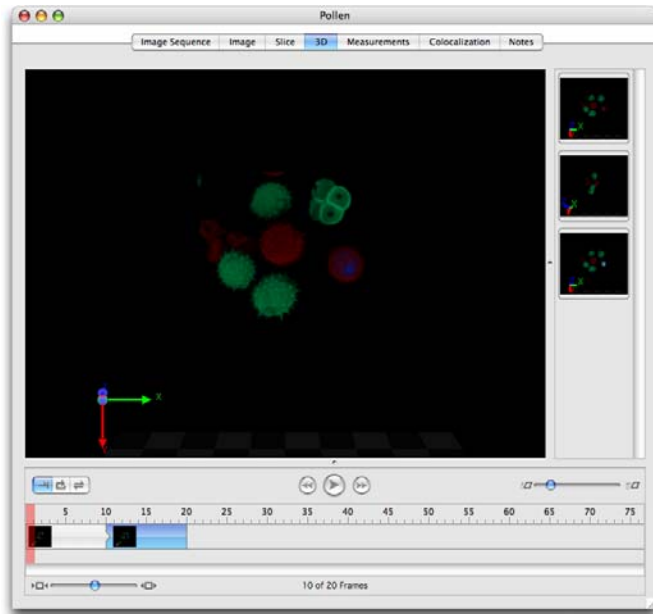
3D View Movie Interface



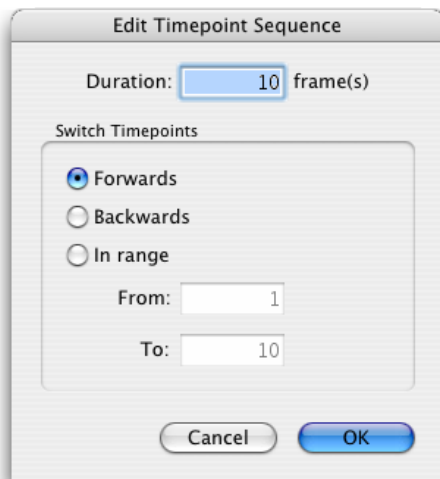
3. Drag a second bookmark into the movie pane. When the movie plays, the volume will be gradually changed in appearance from the first bookmark to the second, this transition taking as long as the number of frames over which the first keyframe extends.



- Continue dragging bookmarks in to make a series of keyframes/transitions.



- To include switching between timepoints in a movie made for a time resolved sequence select **Add Timepoint Sequence...** from the 3D menu. This will add a time bar to the movie which can be configured to last any number of frames, switching between timepoints backwards or forwards and even switching between a subset of timepoints.

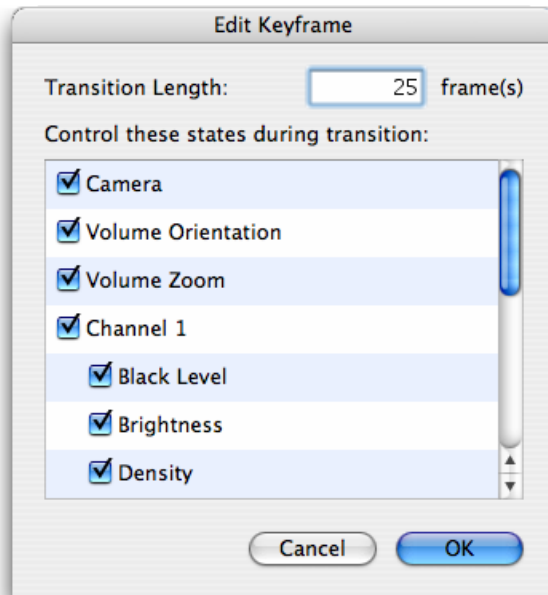


3D View Movie Interface

6. Delete a keyframe from the movie by clicking on it once to select it then using the Delete key (Windows) or the Backspace key (Macintosh)
7. Preview the movie on the current 3D View using the controls at the top of the movie pane.

Edit Keyframe

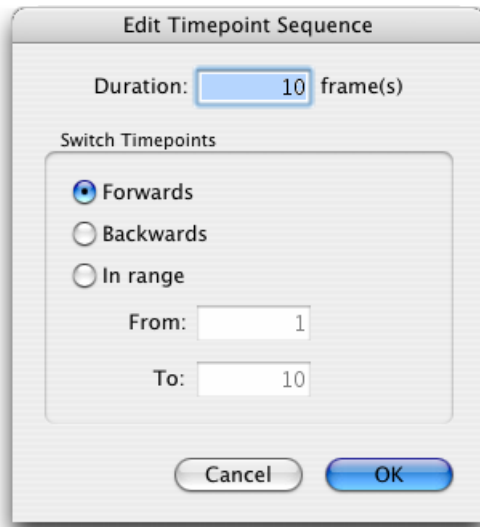
Double click on a keyframe or select **Edit Keyframe...** from the 3D menu to access the Edit Keyframe dialog.



This dialog allows configuration of the length of the keyframe or transition and also selection of the bookmark properties which are used in that transition. It is, therefore, possible to use only the positional information and not the rendering settings stored with a keyframe.

Edit Timepoint Sequence

Double click on a timepoint sequence or select **Edit Timepoint Sequence...** from the 3D menu to access the Edit Timepoint Sequence dialog.



Adjust the number of frames over which the timepoints will be played through, switch between timepoints backwards or forwards.

You can also use this dialog to configure a timepoint sequence to switch between a subset (or range) of timepoints. Additional timepoint sequences may be added to the movie to switch through remaining timepoints.

Resize Movie

Select **Resize Movie...** from the 3D menu to easily change the length of the movie. Movie length may be changed to a percentage of its existing frame number, to a fixed number of frames or to last a known length of time when played at a known frame rate.



Clear Movie

Select **Clear Movie** from the 3D menu to delete all the keyframes and timepoint sequences of a movie.

Once a movie is created you may wish to generate the frames of the movie for the current 3D View to then be able to export them. Also, it may be relevant to save this movie for future use and for use with different data sets.

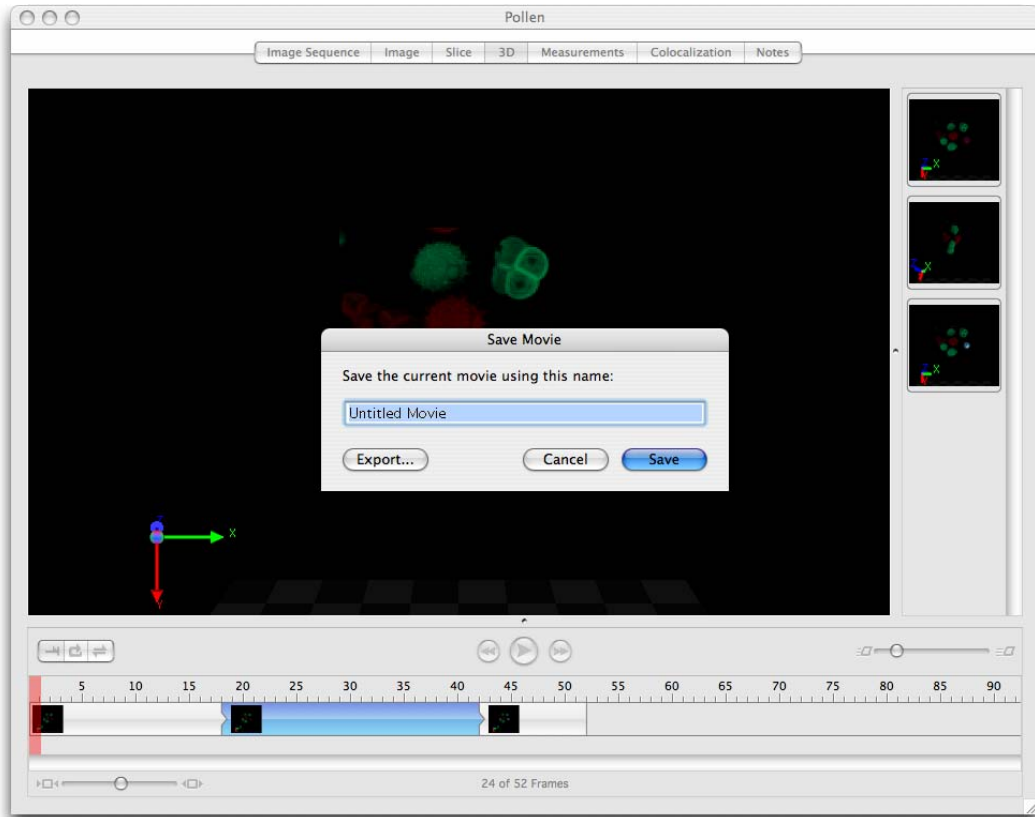
Make Movie

Select **Make Movie** from the 3D menu to create the movie frames and add them to the library. A new folder will be added to the library. The name is the same as the source data with **Movie** appended. Double click on this folder to view the contents.

This folder may be exported as a movie by selecting it and choosing **Export...** from the File menu.

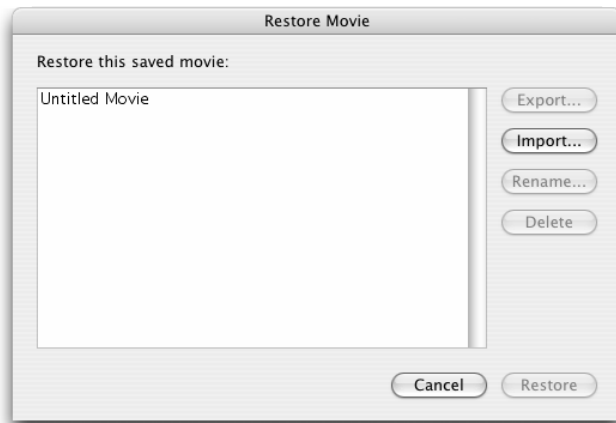
Save and Restore Movie

Save the movie keyframes by selecting **Save Movie...** from the 3D menu. Give the movie a name and click **Save**. This will store the movie as a preference within the system.



Creating Movie Sequences

Use existing movies with a data set by viewing the 3D View and selecting **Restore Movie...** from the 3D menu.



Creating Movie Sequences

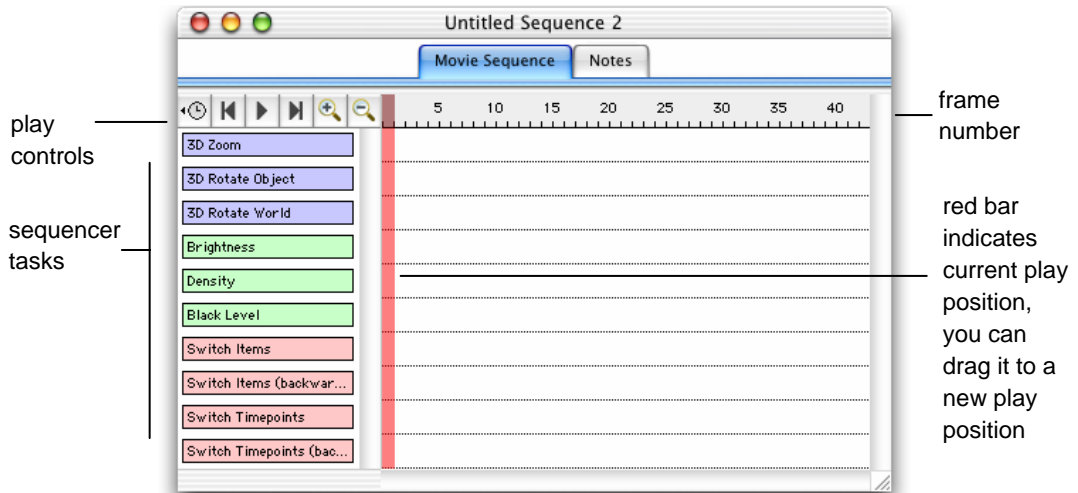
A Movie Sequence is a volume animation template that can be applied to any 3D View.

An alternative method for animating a 3D View is to use bookmarks to build keyframes in the movie pane of the 3D View itself. See page 281.

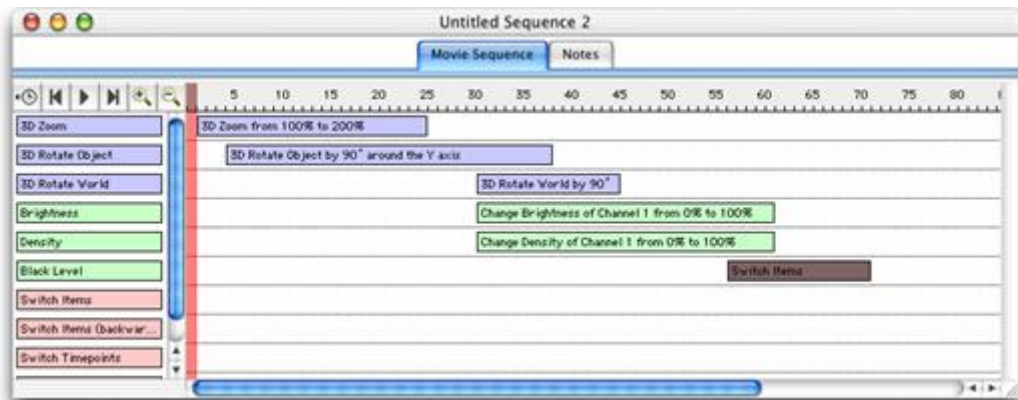
A simple way of animating a rendered view over time is to use the **Export view as movie** command in the Export dialog, see page 124.

Creating a Movie Sequence Item

1. While displaying the 3D View of the item that you wish to animate, select **Create New** from the Actions menu and select **Movie Sequence** to create a new, untitled movie sequence item in the library.
2. The Movie Sequence window opens automatically.

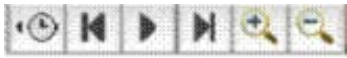


3. Use the Movie Sequence window to set up any number of actions for the movie.
4. Drag the required task(s) across to the main window.
5. Then stretch the task box to define the frames during which the task will be carried out. Tasks may be sequential or concurrent, as shown in the following example.



Creating Movie Sequences

6. Double click on a task and enter setup parameters in the Task dialog, as appropriate. (Not all tasks require setup parameters.)
7. Once you have set up the sequence, use the play controls to preview the animation of the open view. The red bar shows you the current frame in the movie sequence.



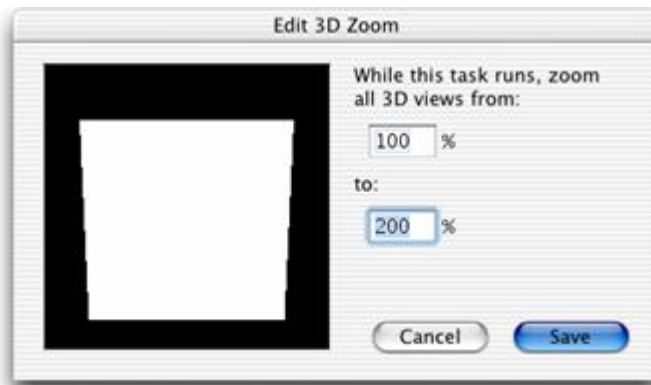
8. Once created, the movie sequence can be used with any open Rendering view.

You may wish to create a folder containing the individual frames of this movie as a separate item in the library. A folder of frames can be opened within Volocity as a Gallery View and played from the Movie tab. The whole folder can also be exported as a QuickTime or AVI file. Select **Make Movie** from the Sequence menu.

3D Zoom Task

Double click on this icon to set the zoom factor for 3D Views during the movie.

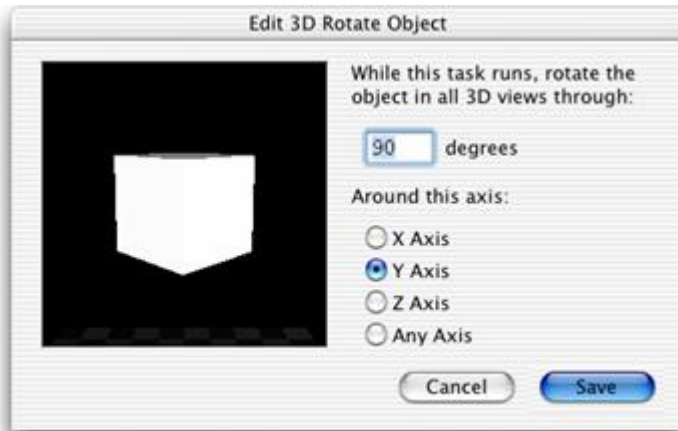
Enter the required percentage and click on **Save**.



3D Rotate Object Task

Double click on this icon to set the rotation for 3D Views during the movie.

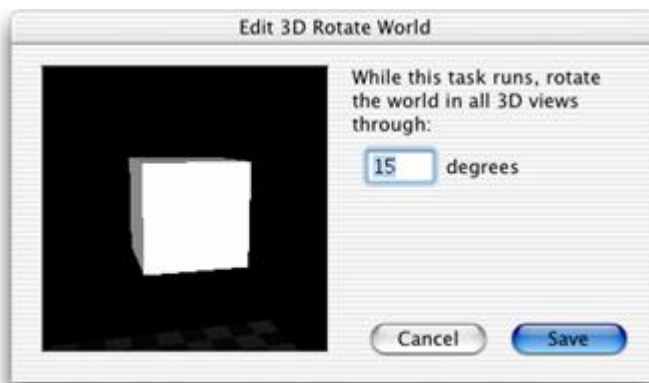
Enter the required rotation angle, specify the axes for rotation and click on **Save**. Note how the position of the red pole changes to reflect your settings.



3D Rotate World Task

Double click on this task to set the rotation for the world (stage floor) during the movie.

Enter the required rotation angle and click on **Save**.



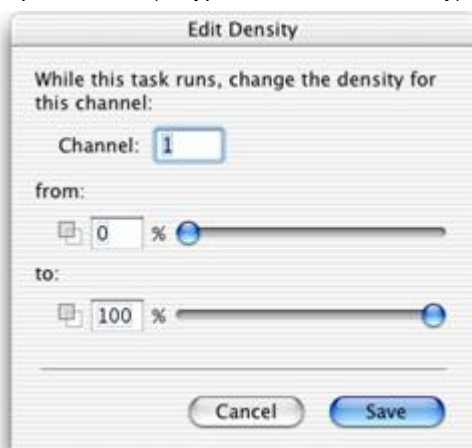
Brightness Task

Double click on the task to set the brightness level for a channel. Enter the number for the channel, use the slider controls to set the initial value and the required value (or type in the values directly) and click **Save**.



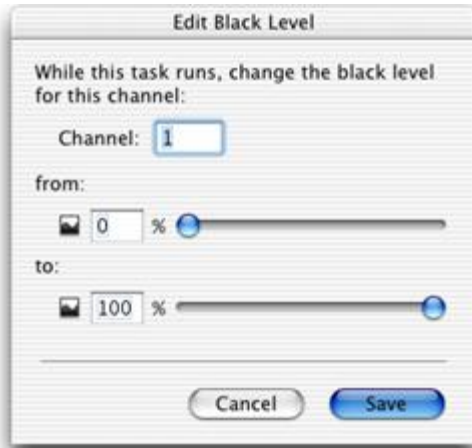
Density Task

This task is only applicable to HR Opacity rendering. Double click on the task to set the density for a channel. Enter the number for the channel, use the slider controls to set the initial value and the required value (or type in the values directly). Click **Save**.



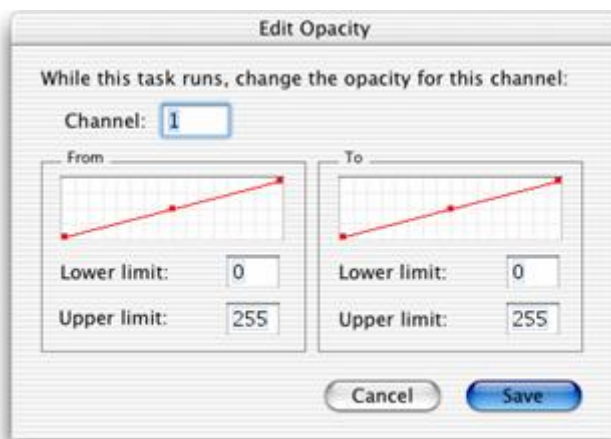
Black Level Task

This task is only applicable to HR opacity rendering. Double click on the task to set the black level for a channel. Enter the number for the channel, use the slider controls to set the initial value and the required value (or type in the values directly). Click **Save**.



Opacity Task

This task is only applicable to Classic 3D Rendering. Double click on the task to set the opacity for a channel. Enter the number for the channel, use the graph window to set the original opacity limits and the required opacity limits (or type in the values directly) and click **Save**.



Creating QuickTime VR Movies

Switch Items Task

Use this task to move through all the contents of a folder in order over the length of the task. There is no setup dialog.

Switch Items (Backwards) Task

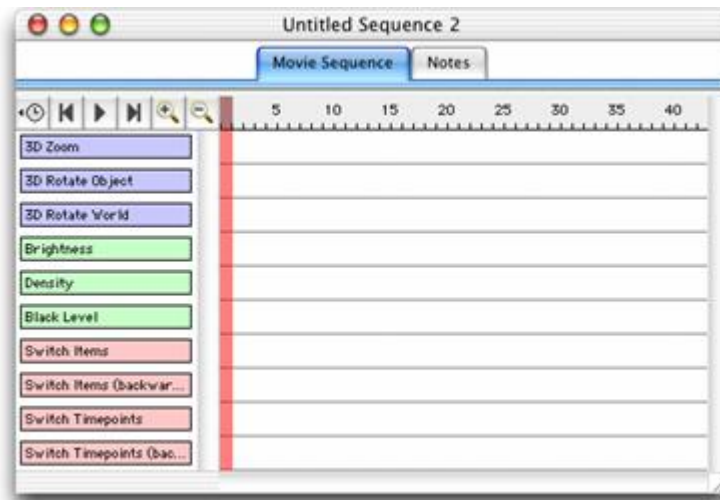
Use this task to move through the contents of a folder in reverse order. There is no setup dialog.

Switch Timepoints Task

Use this task to move through the timepoints in an image sequence over the length of the task. There is no setup dialog.

Switch Timepoints (Backwards) Task

Use this task to move through the timepoints in an image sequence in reverse order. There is no setup dialog.

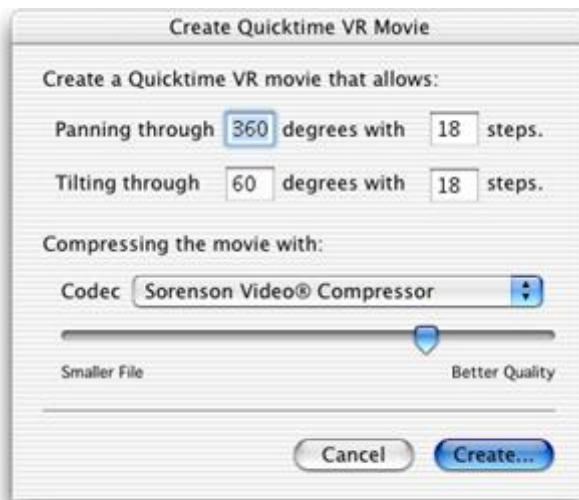


Creating QuickTime VR Movies

A QTVR file is an interactive movie that can be viewed in QuickTime player. If you have QuickTime installed on your machine, you can render a volume and then create a QTVR movie of the rendered view.

Note: A QuickTime VR movie is not a real movie, as such, rather an animation that is created by displaying the object according to a map of user-defined co-ordinates.

1. Select **Create QTVR Movie...** from the 3D menu



2. Amend the settings as appropriate.
3. The panning and tilting values define the rotation that can be applied to the QuickTime object. These values are used to build a map of all the co-ordinates that will be used to display the object on the screen. In our example, you will be able to rotate the object 360 degrees in 36 steps and tilt it through 60 degrees in 18 steps (this means the object will rotate 10 degrees for each step).
4. Select a compression format from the drop-down list and use the slider control to set the compression factor.
5. The available compression options vary according to the version of QuickTime that is installed
6. Click on **Create...**
7. Select a folder and enter a name for the movie.
8. Click on **Save**.

Aligning Volumes

The Velocity alignment tool is designed to compensate for rotational and translational shifts only between the 2-dimensional planes that make up a volume.

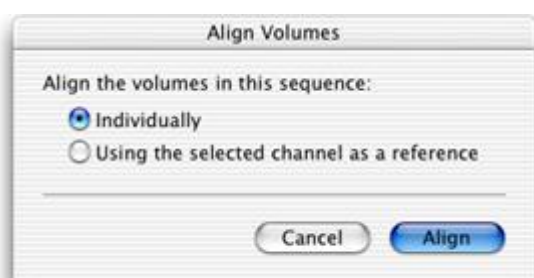
When performing rotational correction the Alignment tool will search for correlation +/- 30 degrees from the origin. The Alignment tool searches for correlation between slices in the data to match features and establish where each slice should be in relation to the next. For alignment to be successful features must be represented in neighboring slices. This means that the slices must be similar.

The Alignment tool will not be able to perform on structures that are, or approximate to, spheres since they show rotational symmetry which makes feature matching impossible.

During rotation of images anti-aliasing is applied which has a smoothing effect on the data. The original data is not overwritten, a new volume or volume sequence is created with the suffix (aligned). The XY dimensions of an aligned volume will be the same as the original volume, areas of image that have been shifted outside the original boundary to allow alignment will be cropped from the result.

There is no benefit to be gained by repeatedly aligning the same data.

1. Select **Align Volumes...** from the Tools menu.
2. If you are aligning a single image there are no further prompts. If you are aligning images in an image sequence, a prompt will be shown offering options to align volumes individually or using a selected channel in the sequence as a reference..

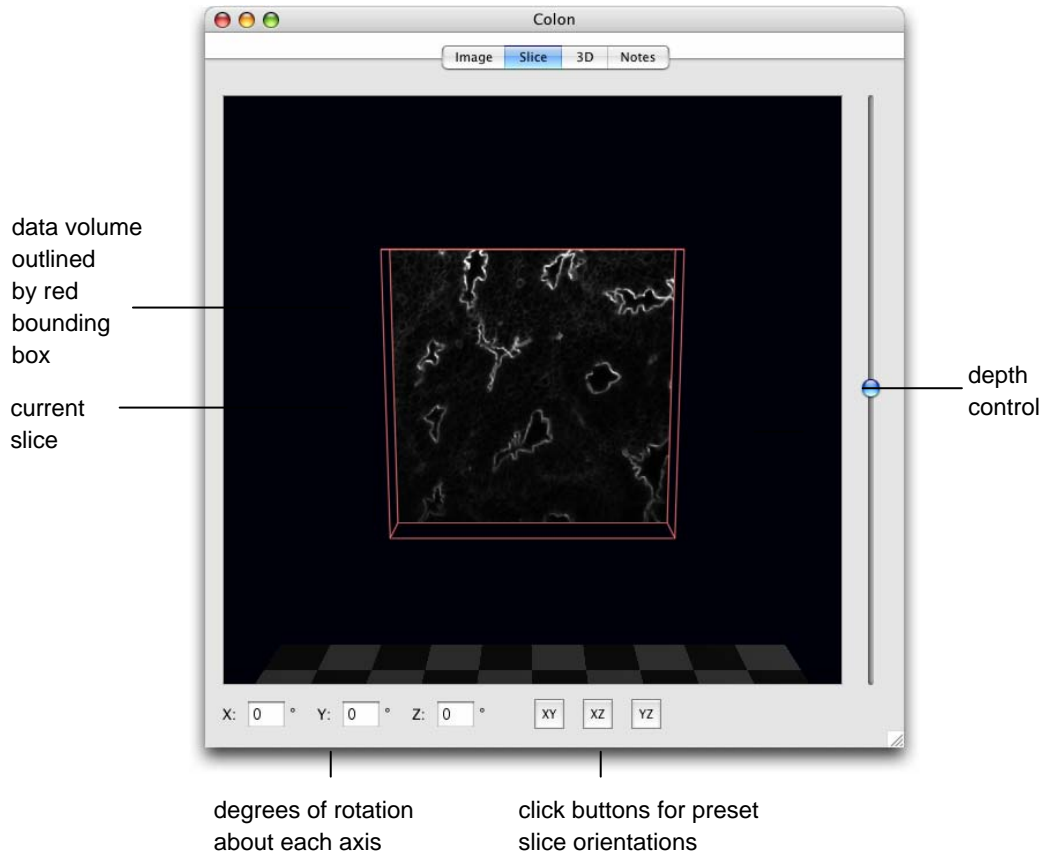


3. If no channel has been selected or you have selected more than one channel, only **Individually** is available. If a channel has been selected, you can click on either button. Click **Align**.

Slice View (Volocity Visualization only)

The XY plane in which the images from a sample have been acquired may not always provide the best presentation of the structures being examined. It may be desirable to re-slice the image data in a different orientation in order to get the required view. The Slice View and associated tools allow the reslicing of data sets in any chosen rotation of X, Y and Z.

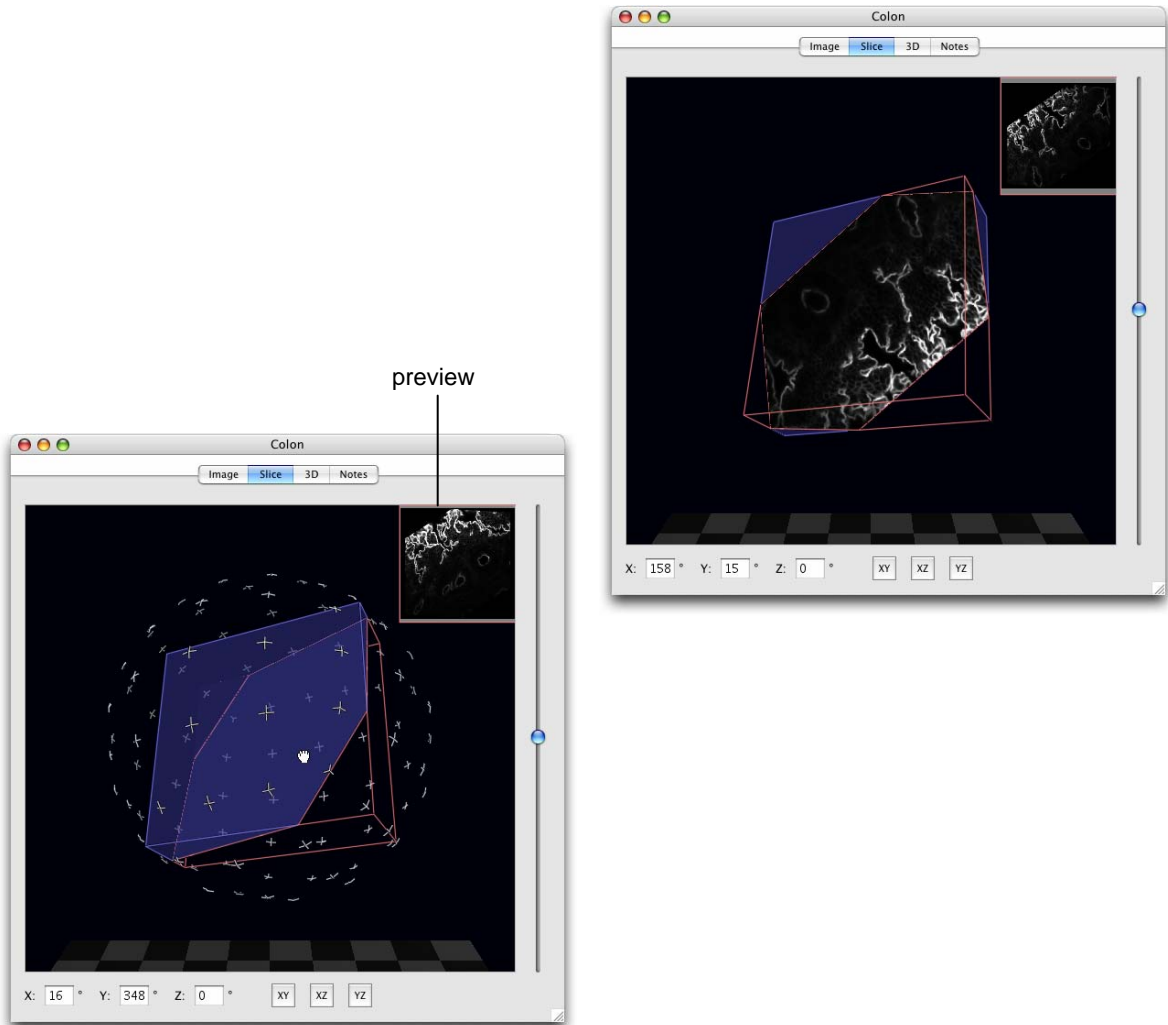
Before reslicing data ensure that the X, Y, and Z calibrations are correct in the item properties, see page 54.



Enter Values to Set Slicing Orientation

You may either enter values for the X, Y, and Z values or use the buttons to step up and down. In order to achieve all possible orientations of the slice, the rotation should be considered to be first around the X axis, then around the new Y axis and finally around the new Z axis.

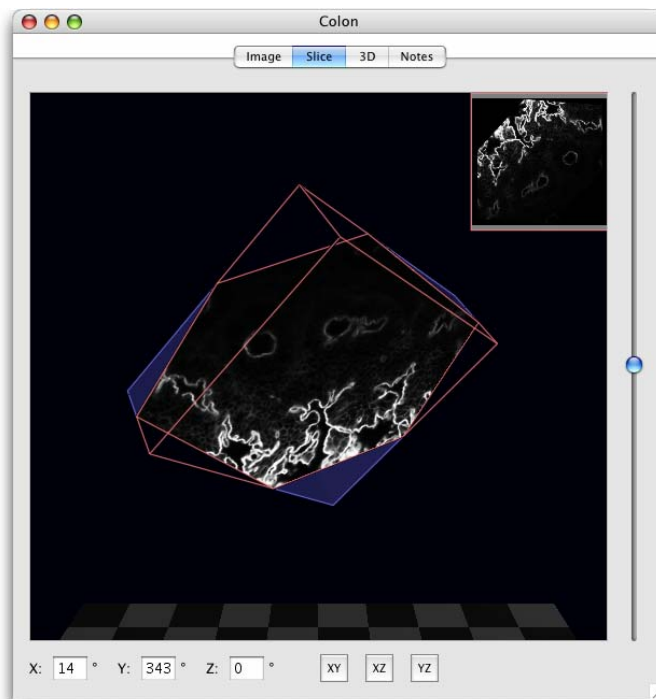
The slice may be rotated through 360 degrees about all axes. A preview of the slice may be displayed in the top right-hand corner select **Show Preview** from the Slice menu. The slice may be rotated through 360 degrees about all axes. A preview of the slice may be displayed in the top right-hand corner, as shown in the second image below (see also page 302).



Slice View (Volocity Visualization only)

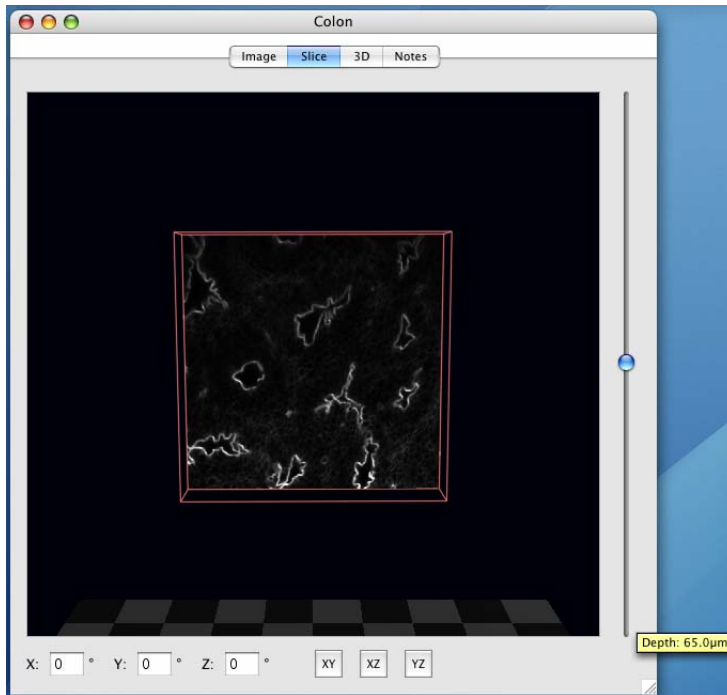
Rotate Volume, shortcut key, **V**.

Use this tool to rotate about the current slice (it is the volume that is rotated).



The slice remains stationary and the volume is moved. The data shown on the slice will update as the volume is moved through it. This tool can be used to allow constant viewing of the slice face while still changing the slice orientation.

When a slice orientation has been chosen, use the depth slider to the right of the view to see the slice moved through the volume. A tooltip for the slider will show the depth through the volume in microns. This value requires accurate calibration of the volume in the Properties dialog, see page 54.



Navigation Palette

The Navigation palette can be used with the Slice View in a very similar way to its use with the Image View. If the Navigation palette is not already visible on the screen, select **Show Palette** from the **Window** menu.

Slice View (Volocity Visualization only)

Show Preview

Choose **Show Preview** to preview new slices that would be created, if the volume were to be sliced according to current settings. The slice preview is updated with the current slice when the depth slider is moved. Choose **Hide Preview** to remove the preview pane from the top right-hand corner of the Slice View.

To Create Data with New Slicing Orientation

Slice Image or **Slice Sequence** from the Slice menu creates a new volume or image sequence with each slice at the new orientation. The volume will typically be larger than the original. Any offset slices will be drawn around to avoid cutting off data.

- Resliced volumes are interpolated using tri-cosine interpolation so that each axis has the same calibration as the highest resolution axis in the original data. Calibration will be changed accordingly. The overall dimensions of the data set will not change and calibrated measurements, such as the volume in square microns, will be the same. Non-calibrated measurements, such as voxel counts, will not be the same as the original data.
- Calibrate data before slicing; corrections on sliced data will not be easy to calculate.
- Rendered views of sliced and unsliced data will look identical.
- The mean intensity of objects will not be changed. Individual voxels may have altered intensity values because of interpolation.

Capture Snapshot

This option adds a new 2D image to the library showing the view as it appears when the snapshot is captured.

Show/Hide Floor, Scale, Orientation

These options change the display of the Slice View. It is possible to show a scale grid and orientation tool as in the Rendering View, see page 253.

Scale and Slice View Options

These options change the color of the scale grid and Slice View background to clearly display data.

Volocity Quantitation Worked Examples

Overview

Volocity Quantitation is designed to identify biological structures automatically. These can then be measured and tracked in 2D, 3D and 4D, analyzed and charted. Volocity Quantitation is an individual product and must be purchased separately. See page 35 for more information about activating extension products.

Volocity Quantitation provides a powerful, easy-to-use interface that allows the user to build up protocols of measurement tasks and then view the feedback as an overlay on the original image and view a table of measurements – all from the same Measurements View.

The Measurements View provides all the tools required to identify objects and make a variety of measurements from those objects. Measurements made in the Measurements View can also be stored as a new item in the library by using **Make Measurement Item** in the Measurements menu.

To assist users wishing to start working immediately, this chapter provides five worked examples describing common applications using Volocity Quantitation:

- Measure the mean volume of structures in a 2-channel data set at a single timepoint.
- Measure multiple timepoints.
- Measure multiple image sequences.
- Measure and track multiple timepoints.
- Colocalization.

For more detailed information please see the separate reference chapter, starting on page 339.

Measuring a Single Timepoint

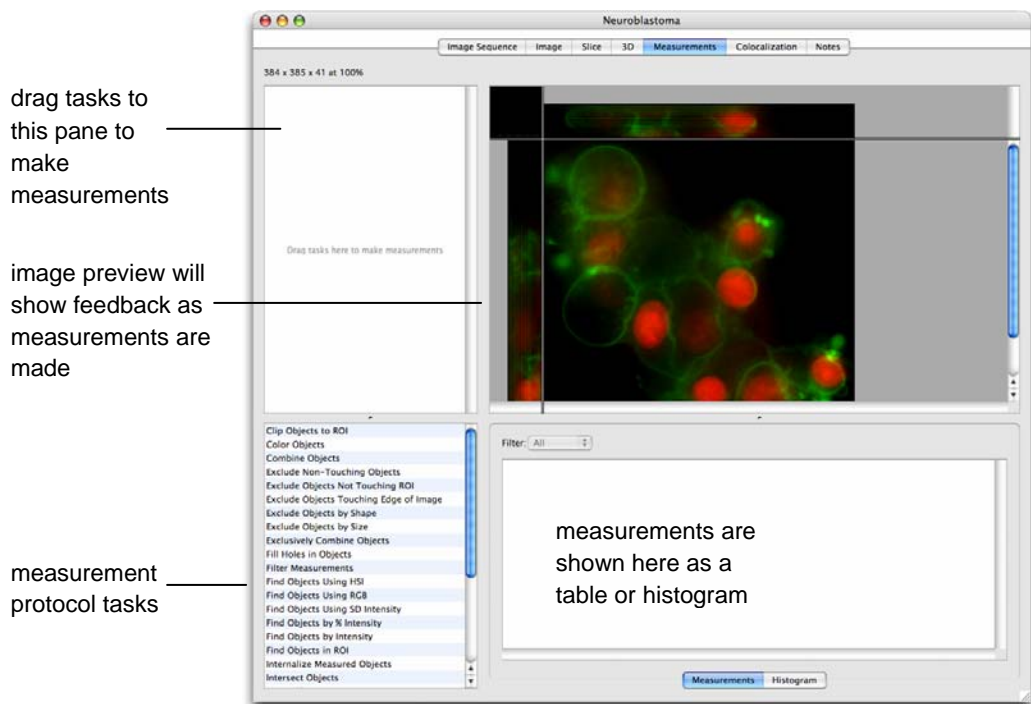
The process has three stages:

- Create a protocol to locate objects of interest in your data set. A protocol is used to threshold data to select areas of interest and, from that selection, include or exclude objects based on a range of criteria.
- Create a Measurement Item to store the measurements.
- Review the results.

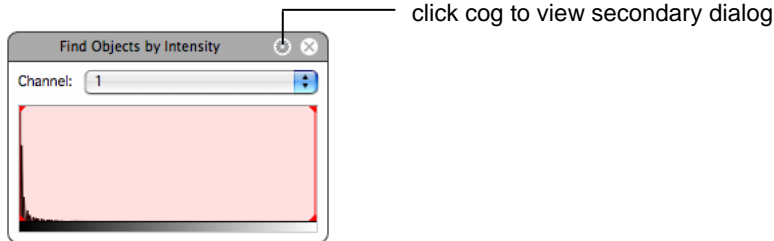
To obtain correct values for calibrated spatial measurements, it is important to ensure that the data is calibrated correctly by setting the X, Y and Z pixel dimensions in the Properties dialog, see page 54.

Creating the Measurement Protocol

1. Open the required data set and view the Measurements tab. The Measurements View contains all the tools and information needed for selecting objects.



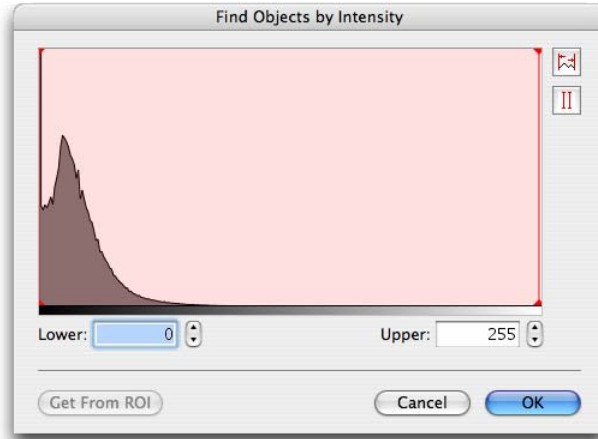
2. Start the protocol by using a “Find Objects” task such as **Find Objects by Intensity**. There are other “Find Objects” tasks which may be appropriate for different types of data. See page 363 for a description of these. If the dataset contains more than one channel, as in this example, make sure that the correct channel is selected in the task.



3. The task shows a histogram of the intensity values present within the data set and selected values are shown as a shaded red area on the histogram. Drag the vertical, red lines to adjust the intensity range selected.
4. The selected objects will be shown as a colored overlay in the image preview and standard morphological and intensity measurements will be displayed in the measurements table. To change the color of objects found use the **Color Objects** task, see page 359 and to change the measurements made use the **Measure Objects** task, see page 373.
5. If the selected objects are not automatically shown, select **Update Feedback** from the Measurements menu. To update feedback automatically after any change to the protocol turn on **Automatically Update Feedback** in the Measurements menu.

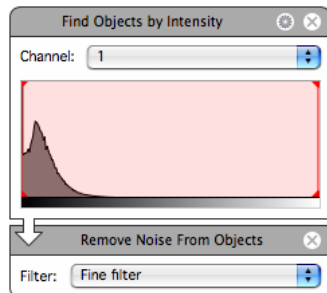
Measuring a Single Timepoint

6. If too many or too few objects have been selected you may need to make adjustments to the protocol.
 - **Find Objects.** The protocol will only select voxels if they are between the minimum and maximum intensity set in the task. Drag the red bars to include more or fewer voxels. Click on the cog icon to access the secondary dialog for the task.

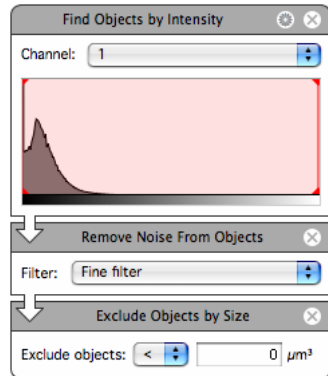


This dialog shows a larger histogram and value entry fields for more precise setting of threshold values. It is also possible to set the thresholds to the maximum and minimum intensity values found within a region of interest drawn on the image. The ROI must be drawn before this dialog is opened. Choose **Get From ROI**.

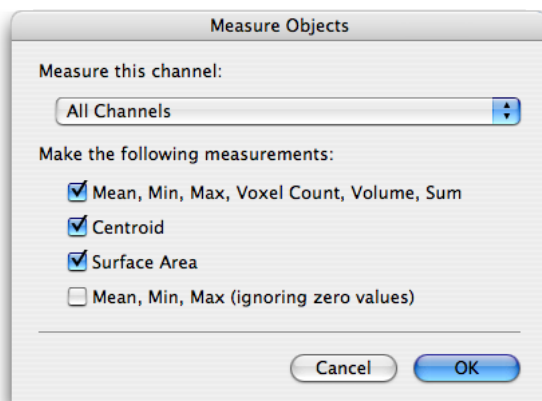
- **Modify the Selection.** It may be necessary to remove noise from the selection to avoid selecting voxels that are not relevant to the objects required. Add a **Remove Noise From Objects** task to the protocol and select the size of filter to be applied.



- Exclude Objects.** It is also possible to exclude objects based on certain characteristics such as their size, shape or position relative to the edge of the image or an ROI. To exclude by size use the table of measurements to view the sizes of the objects found by the current protocol. Click on a column heading to sort the table by the contents of that column. Add an **Exclude Objects by Size** task to the protocol to exclude objects larger or smaller than the size entered.



- Adjust the protocol, as required, until the preview shows the required selection.
- If the table does not show the measurements required, or shows too many, add Measure task(s) to the protocol. Click on the cog icon in a Measure task to set up the measurements required. For example the **Measure Objects** task dialog will look like this:

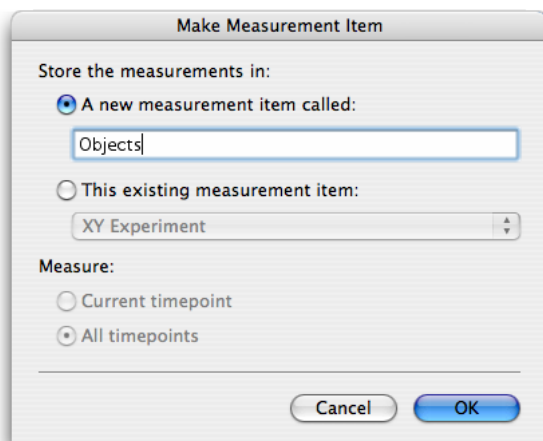


- If you wish to save the protocol for future use, select **Save Protocol...** from the Measurements menu.

Measuring a Single Timepoint

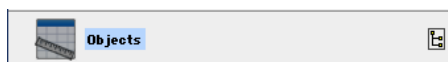
Storing Measurements in a Measurement Item

1. The measurements visible in the Measurements View are present because a protocol is active in the Measurements View. As soon as the protocol is changed the measurements will change. To store measurements and work with them further, you must create a Measurement item.
2. Select **Make Measurement Item...** from the Measurements menu.



3. Select to make a new measurement item and enter a name for it, or add these measurements to an existing table, which may be made from the same data or from different data. Measurement items may combine measurements from different sources.
4. Click on **OK**.

A progress bar may be shown on screen while the measurements are being made. A new item will be added to the library.



Viewing the results

1. Double click the Measurement item to open the Raw View showing the table of numbers.

This table may be sorted, re-organized and filtered. See page 383 for more information on working with the Raw View.

If required, the measurements may be exported as tab or comma-delimited text. To do this, display the Raw View and select **Export...** from the File menu.

Measuring Multiple Timepoints

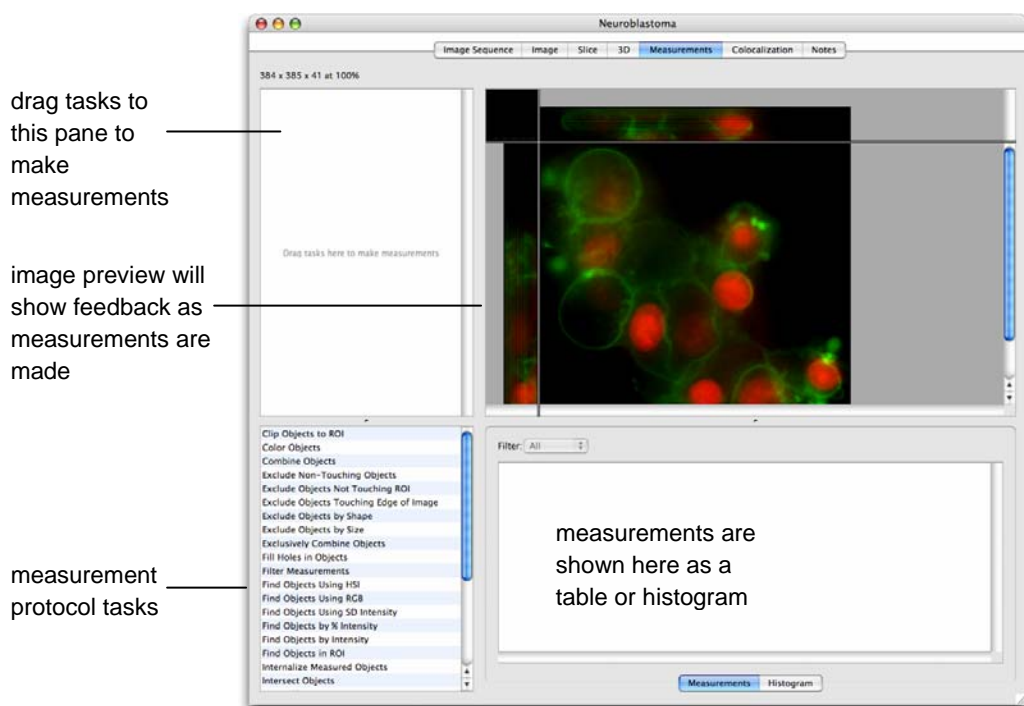
The process has three stages:

- Create a protocol to locate objects of interest in your data set. A protocol is used to threshold data to select areas of interest and, from that selection, include or exclude objects based on a range of criteria.
- Create a Measurement Item to store the measurements.
- Review the results.

To obtain correct values for calibrated spatial measurements, it is important to ensure that the data is calibrated correctly by setting the X, Y and Z pixel dimensions in the Properties dialog, see page 54.

Creating the Measurement Protocol

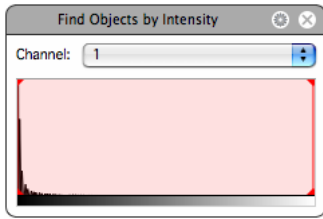
1. Open the required data set and view the Measurements tab. The Measurements View contains all the tools and information needed for selecting objects.



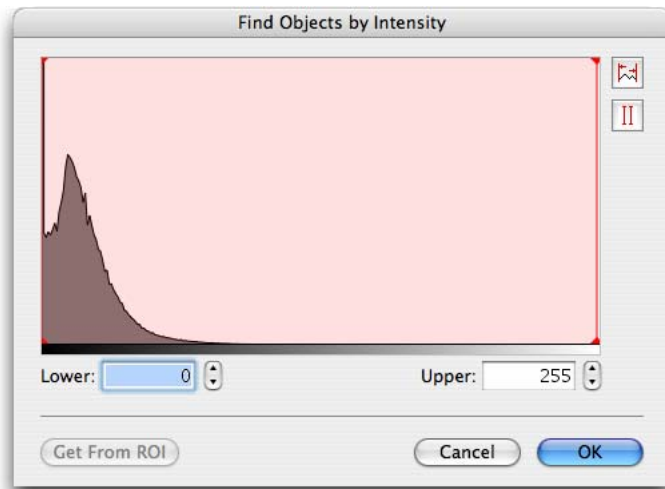
2. Start the protocol by using a “Find Objects” task such as **Find Objects by Intensity**. There are other “Find Objects” tasks which may be more appropriate for time resolved data (for example, **Find Objects by % Intensity** and **Find Objects by SD Intensity**). These tasks do not use absolute intensity values and are, therefore, better suited to data where the range of intensity values does not

Measuring Multiple Timepoints

remain constant. See page 363 for a description of these tasks. If the dataset contains more than one channel, make sure the channel containing the objects required is selected in the task.

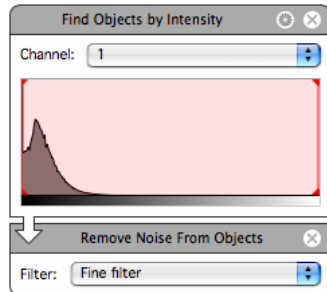


3. The task shows a histogram of the intensity values present within the data set; selected values are shown as a shaded red area on the histogram. Drag the vertical, red lines to adjust the intensity range selected.
4. The selected objects will be shown as a colored overlay in the image preview and standard morphological and intensity measurements will be displayed in the Measurements table. To change the color of objects found use the **Color Objects** task, see page 359, and to change the Measurements made use the **Measure Objects** task, see page 373.
5. If the selected objects are not automatically shown, select **Update Feedback** from the Measurements menu. To update feedback automatically after any change to the protocol, turn on **Automatically Update Feedback** in the Measurements menu.
6. If too many or too few objects have been selected, you may need to make adjustments to the protocol.
 - **Find Objects.** The protocol will only select voxels if they are between the minimum and maximum intensity set in the task. Drag the red bars to include more or fewer voxels. Click on the cog icon to access the secondary dialog for the task.

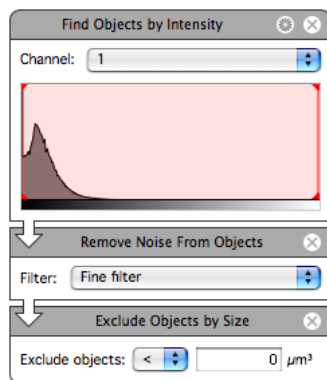


This dialog shows a larger histogram and value entry fields for more precise setting of threshold values. It is also possible to set the thresholds to the maximum and minimum intensity values found within a region of interest drawn on the image. The ROI must be drawn before this dialog is opened. Choose **Get From ROI**.

- **Modify the Selection.** It may be necessary to remove noise from the selection to avoid selecting voxels that are not relevant to the objects required. Add a **Remove Noise From Objects** task to the protocol and select the size of filter to be applied.



- **Exclude Objects.** It is also possible to exclude objects based on certain characteristics such as their size, shape or position relative to the edge of the image or an ROI. To exclude by size use the table of measurements to view the sizes of the objects found by the current protocol. Click on a column heading to sort the table by the contents of that column. Use **Exclude Objects by Size** in the protocol to exclude objects larger or smaller than the size entered.



- Adjust the protocol, as required, until the preview shows the required selection.
7. You should check the protocol on a number of different timepoints before storing the measurements. This saves time and avoids making unnecessary measurements.

To do this:

- Display the Measurements View and the Navigation palette on the screen at the same time.

Measuring Multiple Timepoints

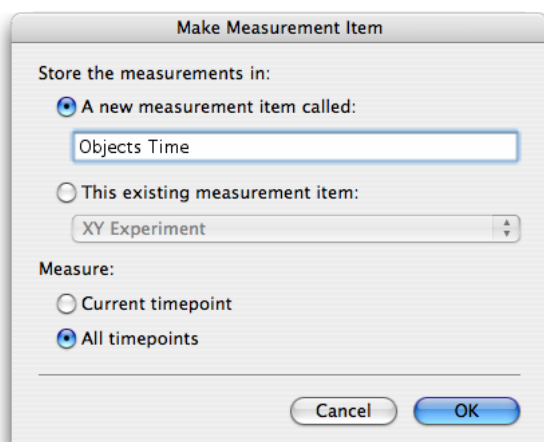
- Set up the protocol, then use the Navigation palette (as described on page 82) to move between timepoints. Stop at intervals and **Automatically Update Feedback** will apply the protocol to the new timepoint. If the protocol is not applied when the timepoint changes, select **Update Feedback** from the Measurements menu.

You can then see the effect on the Image View and adjust the protocol for a number of timepoints to obtain the best fit.

8. Once you are happy that the protocol is optimized you may wish to save it. Select **Save Protocol...** from the Measurements menu.

Storing Measurements in a Measurement Item

1. The measurements visible in the Measurements View are present because a protocol is active in the Measurements View. As soon as the protocol is changed, the measurements will change. To store measurements and work with them further, you must create a Measurement item.
2. Select **Make Measurement Item...** from the Measurements menu.



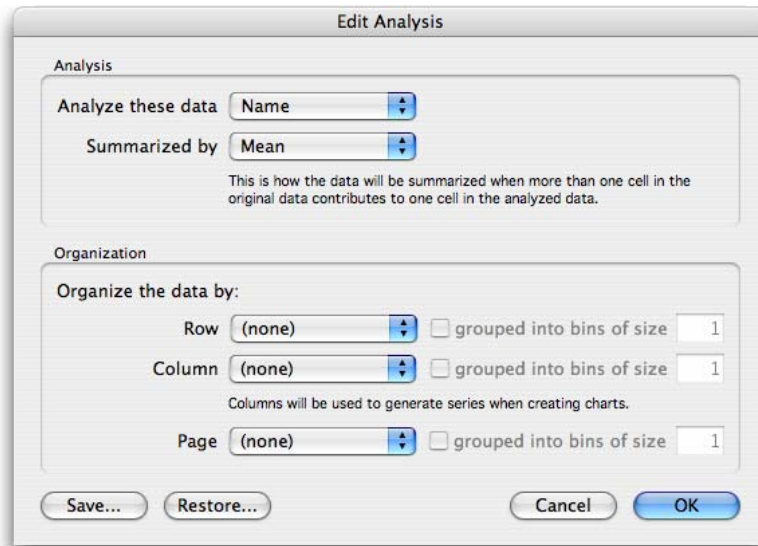
3. Select to make a new Measurement item and enter a name for it, or add these measurements to an existing table, which may be made from the same data or from different data. Measurement items may combine measurements from different sources.
4. Choose to measure **All timepoints** and Click on **OK**.

A progress bar may be shown on screen while the measurements are being made. A new item will be added to the library.



Viewing the Results

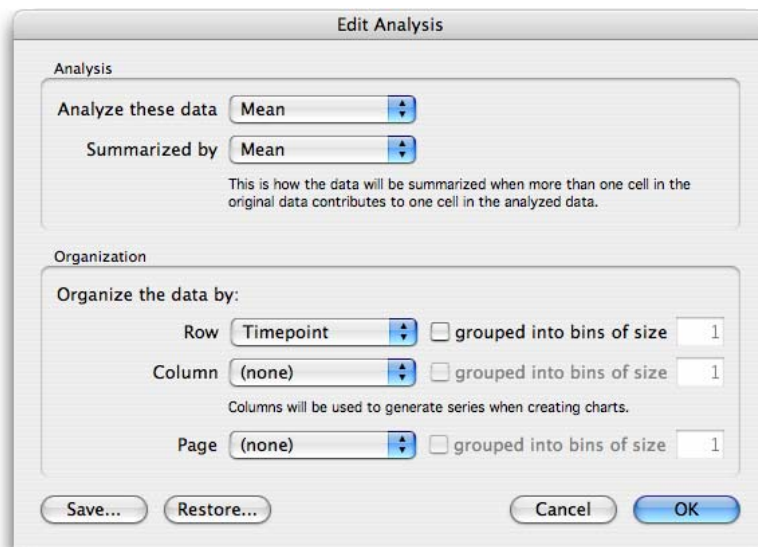
1. Double click the Measurement item to open the Raw View showing the table of numbers.
 This table may be sorted, re-organized and filtered. See page 383 for more information on working with the Raw View.
 If required, the measurements may be exported as tab or comma-delimited text. To do this, display the Raw View and select **Export...** from the File menu.
2. The Analysis View may be used to show relationships of data over time. For example the mean intensity of all the measured objects for each timepoint. Click on the Analysis tab and select **Analyze...** from the Analysis menu.



The data of interest is the mean intensity for each object.

Measuring Multiple Timepoints

There are several objects per timepoint, so these can all be summarized by finding the mean, then organizing the data by timepoint to show the relationship between mean intensity and timepoint.



The top area of the Analysis View now shows a table with the mean of the mean intensities arranged in rows according to timepoint.

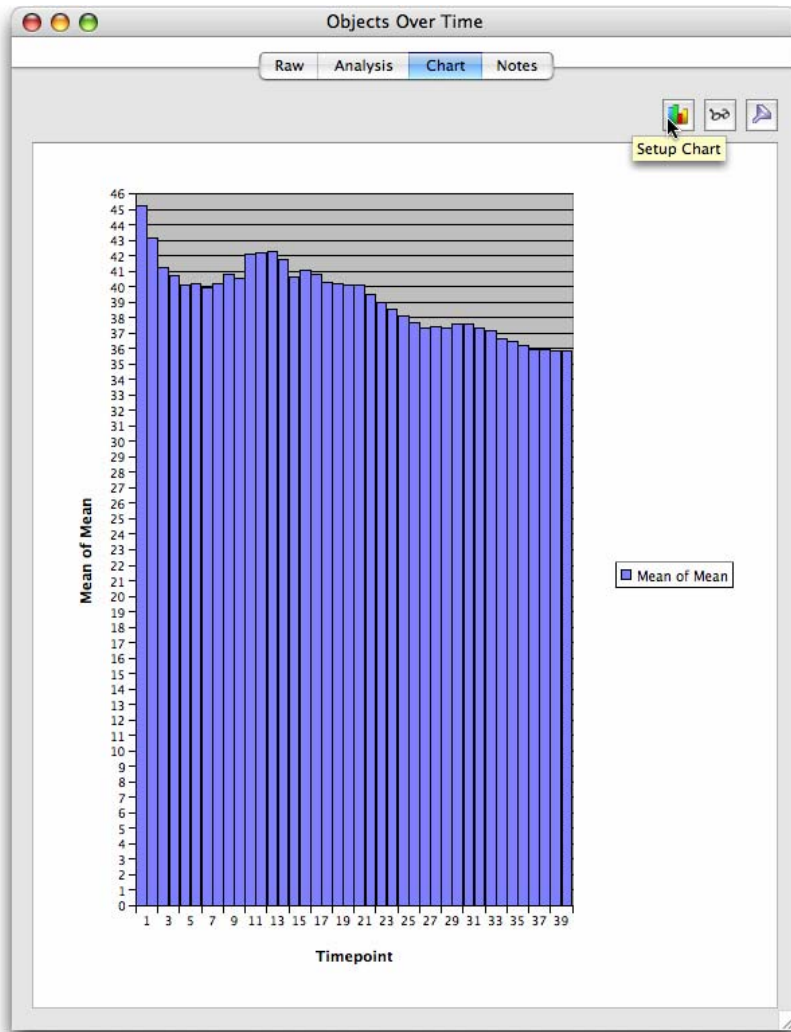
Objects Over Time

Raw Analysis Chart Notes

Mean of data from Mean, row values from Timepoint.

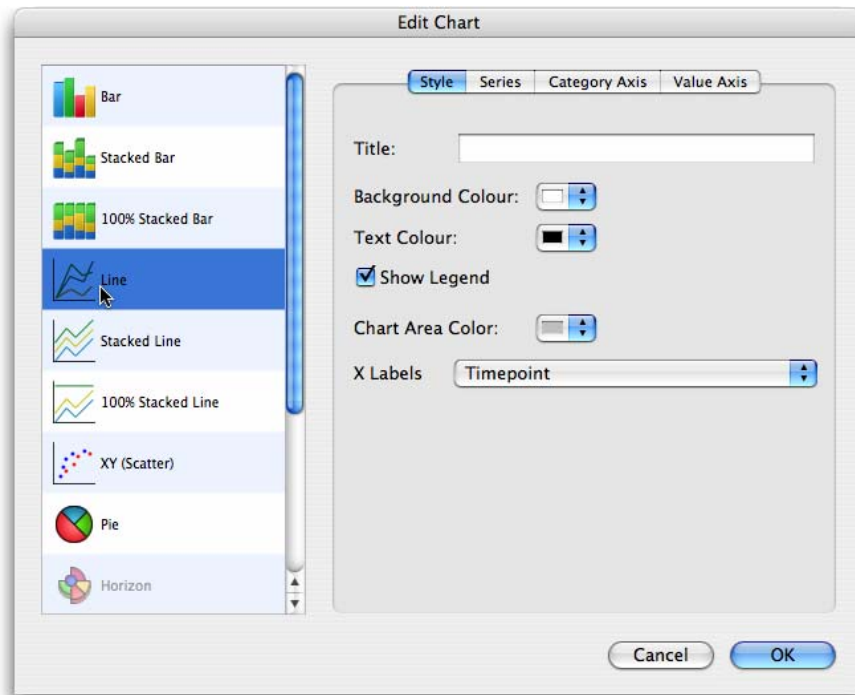
Timepoint	Mean of Mean
1	45.2106
2	43.153
3	41.2418
4	40.7353
5	40.1004
6	40.2019
7	39.9116
8	40.1747
9	40.7632
10	40.5481
11	42.1173
12	42.1551

This can easily be plotted as a chart by clicking on the Chart tab.



Measuring Multiple Timepoints

There are many different chart types and options to configure the appearance of the chart, accessed by clicking on the Chart Setup button or selecting **Edit Chart...** from the Chart menu.



Measuring Multiple Image Sequences

Volocity supports batch processing of image sequences. One Measurements protocol is applied to multiple selected sequences and the results are collected in a single Measurements item.

The process has four stages:

- Create a protocol to locate objects of interest in your data set. A protocol is used to threshold data to select areas of interest and, from that selection, include or exclude objects based on a range of criteria.
- Save the protocol.
- Apply the saved protocol to selected image sequences in the library.
- Review the results.

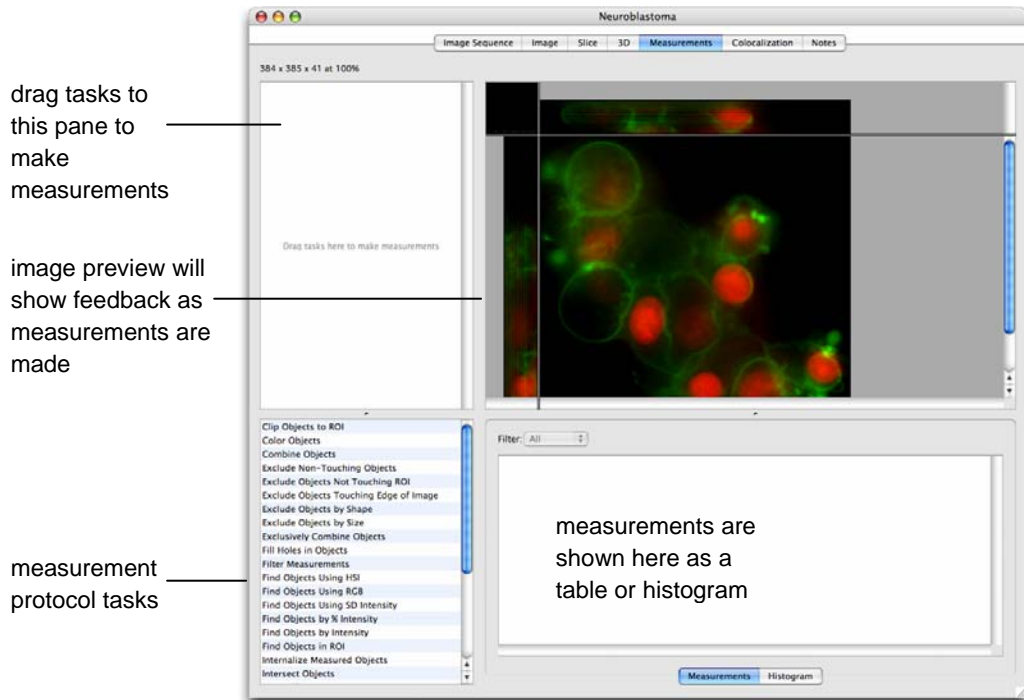
If you have a saved measurements protocol that you would like to apply to multiple image sequences, start at “Apply the Protocol to Selected Sequences” on page 321. Otherwise, you must first create a protocol by opening one of the image sequences that you will measure and viewing the Measurements tab.

To obtain correct values for calibrated spatial measurements, it is important to ensure that the data is calibrated correctly by setting the X, Y and Z pixel dimensions in the Properties dialog, see page 54.

Measuring Multiple Image Sequences

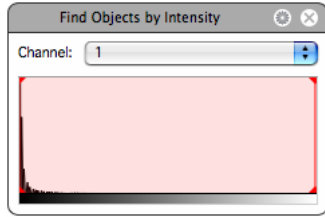
Creating the Measurement Protocol

1. Open the required data set and view the Measurements tab. The Measurements View contains all the tools and information needed for selecting objects.

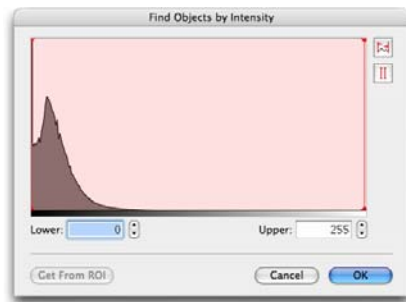


2. Start the protocol by using a “Find Objects” task such as **Find Objects by Intensity**. There are other “Find Objects” tasks that may be more appropriate when working with multiple data sets containing images that have widely-varying intensity values (for example, **Find Objects by % Intensity** and **Find Objects by SD Intensity**). These tasks do not use absolute intensity values and are, therefore, better suited to data where the range of intensity values does not remain constant. See page 363 for a description of these tasks.

If the dataset contains more than one channel, make sure the channel containing the objects required is selected in the task.



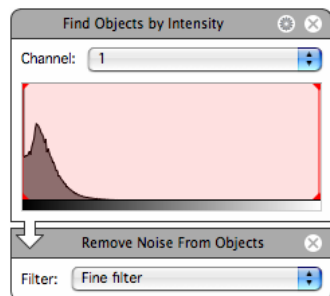
3. The task shows a histogram of the intensity values present within the data set and selected values are shown as a shaded red area on the histogram. Drag the vertical, red lines to adjust the intensity range selected.
4. The selected objects are shown as a colored overlay in the image preview and standard morphological and intensity measurements are displayed in the Measurements table. To change the color of objects found use the **Color Objects** task, see page 359, and to change the measurements made use the **Measure Objects** task, see page 373.
5. If the selected objects are not automatically shown, select **Update Feedback** from the Measurements menu. To update feedback automatically after any change to the protocol, turn on **Automatically Update Feedback** in the Measurements menu.
6. If too many or too few objects have been selected you may need to make adjustments to the protocol.
 - **Find Objects.** The protocol will only select voxels if they are between the minimum and maximum intensity set in the task. Drag the red bars to include more or fewer voxels. Click on the cog icon to access the secondary dialog for the task.



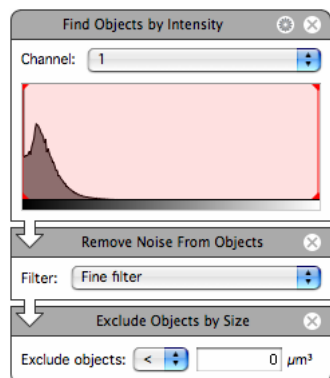
This dialog shows a larger histogram and value entry fields for more precise setting of threshold values. It is also possible to set the thresholds to the maximum and minimum intensity values found within a region of interest drawn on the image. The ROI must be drawn before this dialog is opened. Choose **Get From ROI**.

Measuring Multiple Image Sequences

- **Modify the Selection.** It may be necessary to remove noise from the selection to avoid selecting voxels that are not relevant to the objects required. Add a **Remove Noise From Objects** task to the protocol and select the size of filter to be applied.



- **Exclude Objects.** It is also possible to exclude objects based on certain characteristics such as their size, shape or position relative to the edge of the image or an ROI. To exclude by size use the table of measurements to view the sizes of the objects found by the current protocol. Click on a column heading to sort the table by the contents of that column. Add an **Exclude Objects by Size** task to the protocol to exclude objects larger or smaller than the size entered.



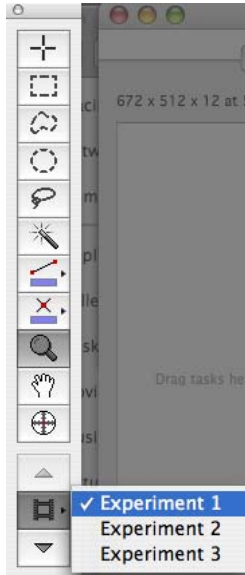
Adjust the protocol, as required, until the preview shows the required selection.

7. It is possible to review the protocol applied to other image sequences to check that it is appropriate. This saves time and avoids making unnecessary measurements.

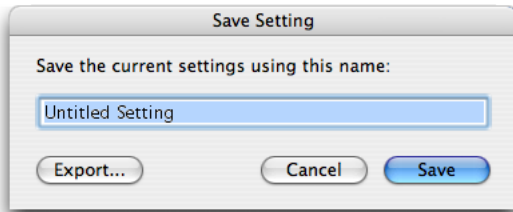
To do this:

- Set up the protocol in the Measurements View of one of the items.
- Use the library navigation tools in the toolbar to move up or down through items in the library or jump straight to one item.

You can then see the effect and adjust the protocol if necessary without having to leave the Measurements View.



8. Select **Save Protocol...** from the Measurements menu.

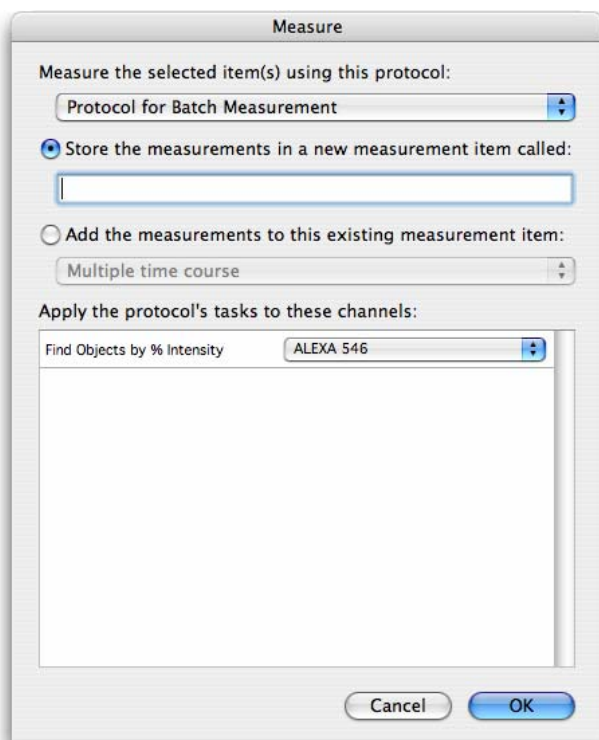


Apply the Protocol to Selected Sequences

1. In the Library View select the items to be batch processed by measuring with the protocol.

Measuring Multiple Image Sequences

2. Select **Measure...** from the Tools menu.



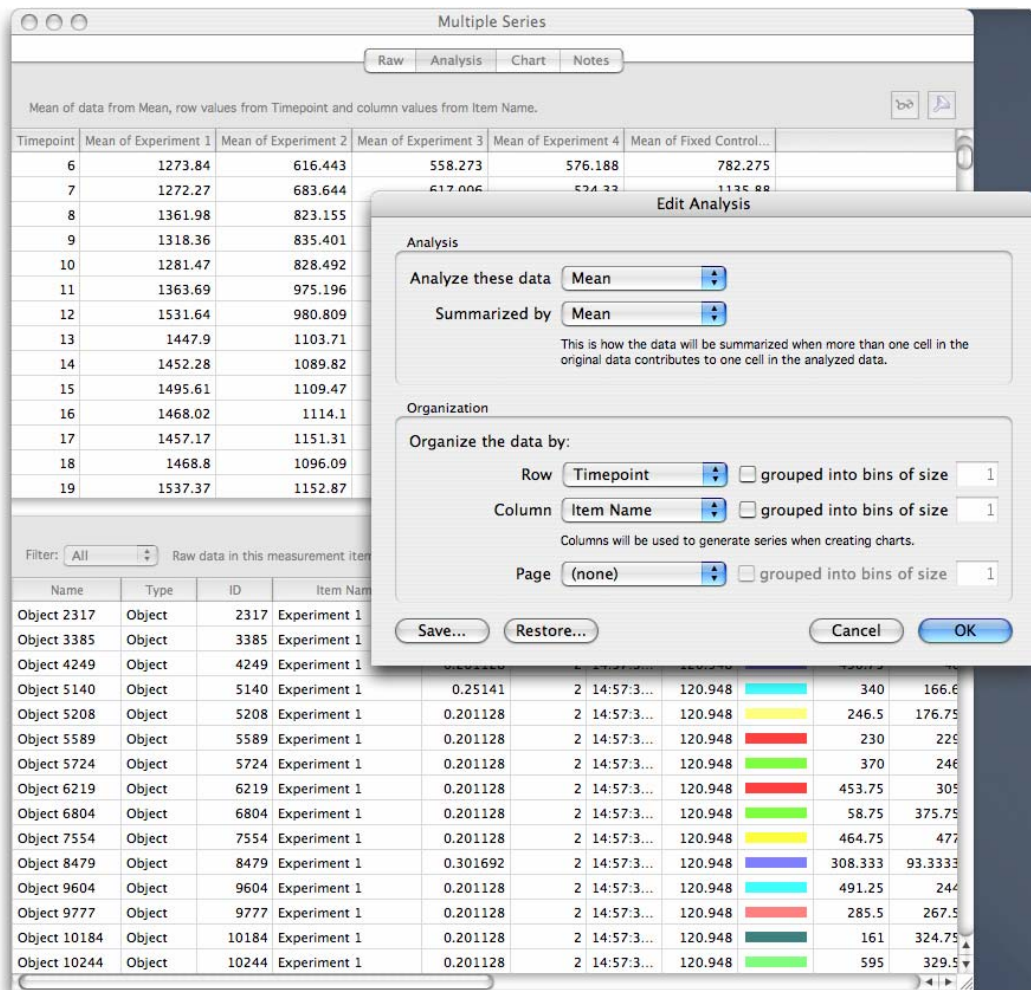
Select the protocol to be applied to the selected items from the drop-down menu, which lists all saved protocols by name.

Either, select **Store the measurements in a new measurement item called:** and enter a name for the Measurement item that will contain all the measurements made by the selected protocol on the selected items. Or, select **Add the measurements to this existing measurement item:** and choose from the measurement items that already exist in the library. In this case, all the measurements from all the selected items will be added to the measurements already in the table.

For each task in the protocol that operates on a specific channel (i.e. the Protocol dialog contains a drop-down for channel selection when the task is configured), there will be an entry under **Apply the protocol's tasks to these channels:** The drop-down lists the unique channel names found within the selected items. Channels with the same name will all be processed.

3. Select the correct channel for each task in the list and click **OK**.
4. A progress bar is shown as each of the selected items is measured with the protocol and the Measurement item is created.
5. Double-click the Measurement item to view the Raw View of all the measured objects.

6. To perform further analysis of this large amount of data, go to the Analysis tab and select **Edit Analysis...** from the Analysis menu.
7. To show the changing mean intensity over time, the Analysis dialog could be configured as shown below:

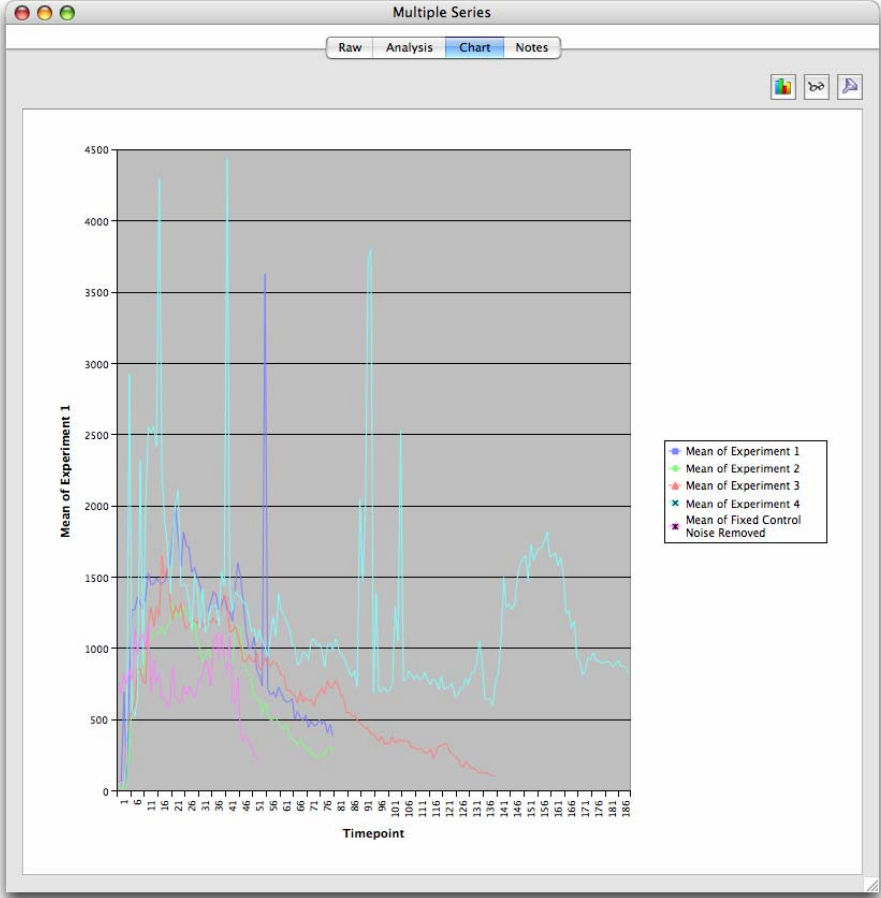


Time may be represented as absolute or relative.

Item Name will organize the data according to the name of the image sequence. Since Item Name is selected for **Column** in the table, these will become individual series in a chart allowing us to visually compare the data sets.

Measuring Multiple Image Sequences

Click on the **Chart** tab to view the chart. The default chart type is a bar chart. Select **Edit Chart...** from the Chart menu to change the chart type. The following example shows a line chart.



Measuring and Tracking Multiple Timepoints

Velocity's unique time-based measurement architecture allows you to measure and track biological objects as they move in time in both 2D and 3D.

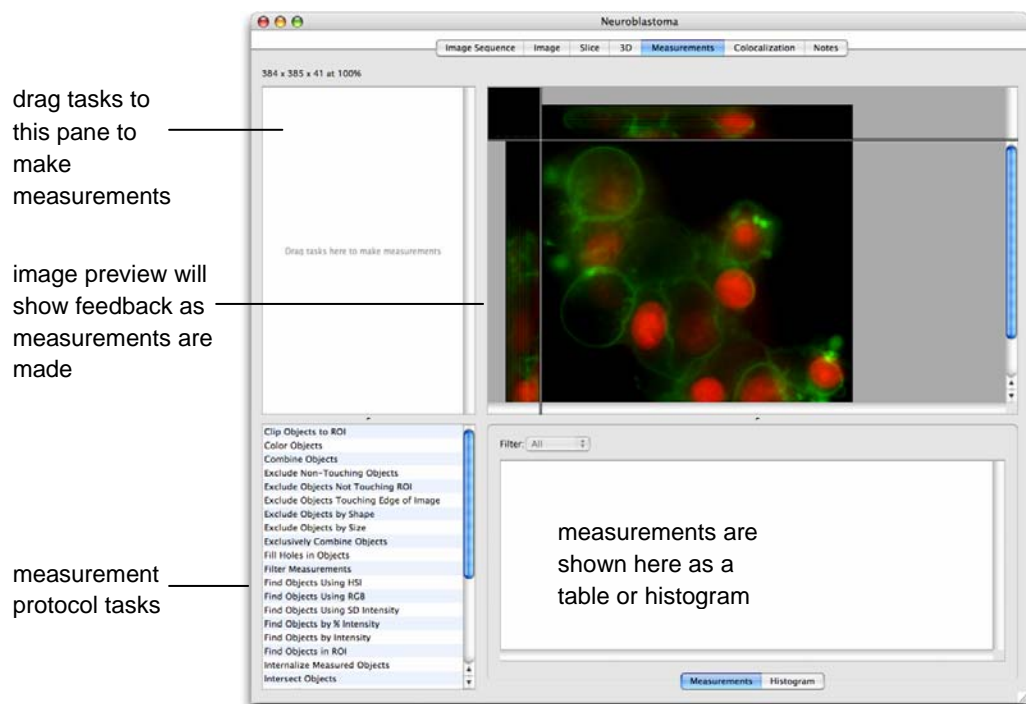
Tracking uses the centroid measurement for each object to identify whether objects have moved between timepoints. It does not use any of the other measurements.

The process has three stages:

- Create a protocol to locate objects of interest in your data set. This protocol must locate objects as accurately as possible in each timepoint.
- Measure the objects found by the protocol and generate tracks based on the centroid position of each object.
- Review the results.

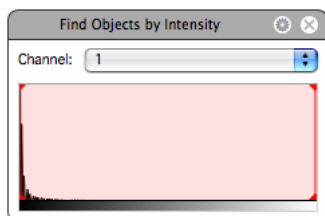
Creating the Measurement Protocol

1. Open the required data set and view the Measurements tab. The Measurements View contains all the tools and information needed for selecting objects.



Measuring and Tracking Multiple Timepoints

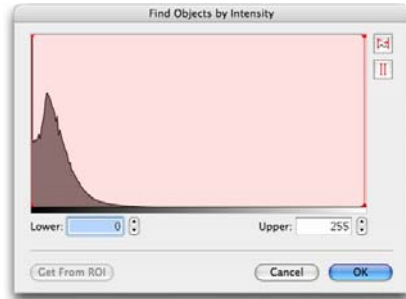
2. Start the protocol by using a “Find Objects” task such as **Find Objects by Intensity**. There are other “Find Objects” tasks that may be more appropriate for time resolved data, for example **Find Objects by % Intensity** and **Find Objects by SD Intensity**. These tasks do not use absolute intensity values and are, therefore, better suited to data where the range of intensity values does not remain constant. See page 363 and page 369 for description of these. If the dataset contains more than one channel make sure the channel containing the objects required is selected in the task.



3. The task shows a histogram of the intensity values present within the data set and selected values are shown as a shaded red area on the histogram. Drag the vertical, red lines to adjust the intensity range selected.
4. The selected objects are shown as a colored overlay in the image preview and standard morphological and intensity measurements are displayed in the Measurements table. To change the color of objects found use the **Color Objects** task, see page 359, and to change the measurements made use the **Measure Objects** task, see page 373.
5. If the selected objects are not automatically shown, select **Update Feedback** from the Measurements menu. To update feedback automatically after any change to the protocol, turn on **Automatically Update Feedback** in the Measurements menu.

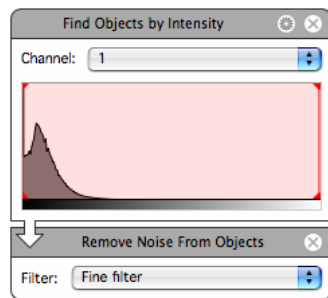
6. If too many or too few objects have been selected, you may need to make adjustments to the protocol.

- **Find Objects.** The protocol will only select voxels if they are between the minimum and maximum intensity set in the task. Drag the red bars to include more or fewer voxels. Click on the cog icon to access the secondary dialog for the task.



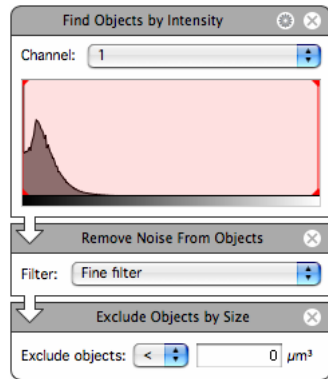
This dialog shows a larger histogram and value entry fields for more precise setting of threshold values. It is also possible to set the thresholds to the maximum and minimum intensity values found within a region of interest drawn on the image. The ROI must be drawn before this dialog is opened. Choose **Get From ROI**.

- **Modify the Selection.** It may be necessary to remove noise from the selection to avoid selecting voxels that are not relevant to the objects required. Add a **Remove Noise From Objects** task to the protocol and select the size of filter to be applied.



- **Exclude Objects.** It is also possible to exclude objects based on certain characteristics such as their size, shape or position relative to the edge of the image or an ROI. To exclude by size use the table of measurements to view the sizes of the objects found by the current protocol. Click on a column heading to sort the table by the contents of that column. Add an **Exclude Objects by Size** task to the protocol to exclude objects larger or smaller than the size entered.

Measuring and Tracking Multiple Timepoints



- Adjust the protocol, as required, until the preview shows the required selection.
7. You should check the protocol on a number of different timepoints before storing the measurements. This saves time and avoids making unnecessary measurements.

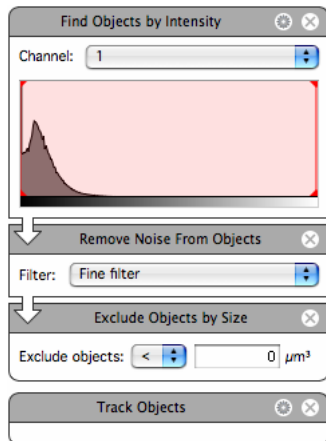
To do this:

- Display the Measurements View and the Navigation palette on the screen at the same time.
- Set up the protocol, then use the Navigation palette (as described on page 82) to move between timepoints. Stop at intervals and **Automatically Update Feedback** will apply the protocol to the new timepoint. If the protocol is not applied when the timepoint changes, select **Update Feedback** from the Measurements menu.

You can then see the effect on the Image View and adjust the protocol for a number of timepoints to obtain the best fit.

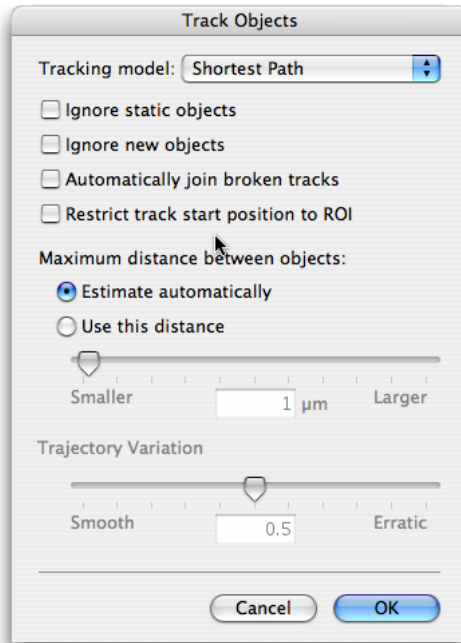
Adding a Tracking Task to the Protocol

1. To make the protocol track centroid position when objects are made in more than one timepoint, add the **Track Objects** task to the protocol.



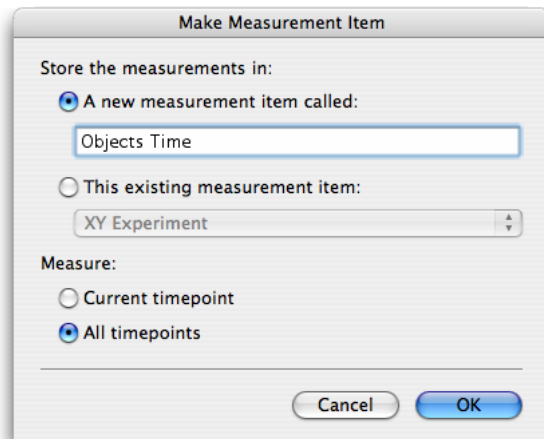
The **Track Objects** task will always go to the bottom of the list of tasks in the protocol. It is not possible to add it between other tasks or to add other tasks after it.

2. Click on the cog icon to access the secondary dialog for the **Track Objects** task.



3. Select the appropriate options. These are described fully on page 377. For this example, we will select the **Shortest Path** and allow Volocity to calculate the **Maximum distance between objects** automatically.
4. Click **OK** to close the dialog.
5. Tracking will only be carried out if all timepoints are being measured. Either turn on **Measure All Timepoints** in the Measurements menu to see tracking in the Measurements View, or select **Make a Measurements Item** in the Measurements menu and ensure **Measure: All timepoints** is selected; this makes a Measurements item that contains tracks.

Measuring and Tracking Multiple Timepoints



Note: Turning on **Measure: All Timepoints** in the Measurements View increases the amount of time taken to apply the protocol each time a change is made.

6. When **Measure: All Timepoints** is turned on Velocity will apply the protocol to all the timepoints and track the centroid positions of the objects found. The Measurements table will show all the objects found in each timepoint and the tracks made from them. Select individual tracks to check and adjust the **Track Objects** task, if necessary.

Viewing the Results

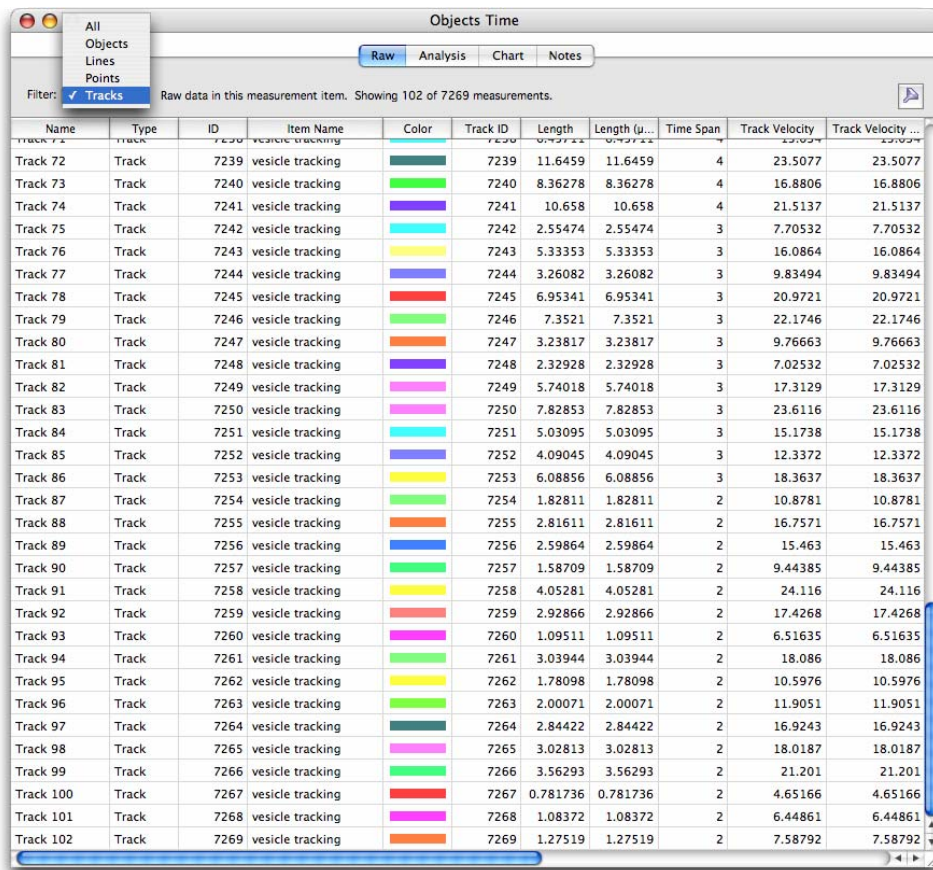
1. Open the Measurement item created above.

The Raw table shows objects found in each timepoint and the tracks made from them with measurements relevant to each.

Name	Type	ID	Item Name	Volume (µm³)	Timepoint	Abs. Time	Rel. Time...	Color	Centroid X	Centroid Y	Centroid Z
Object 7145	Object	7145	vesicle tracking	39	40	17:22:1...	8.04958	[Color]	68.6923	197.897	68.69
Object 7146	Object	7146	vesicle tracking	40	40	17:22:1...	8.04958	[Color]	38.125	200.975	38.1
Object 7147	Object	7147	vesicle tracking	81	40	17:22:1...	8.04958	[Color]	44.9136	202.704	44.91
Object 7148	Object	7148	vesicle tracking	105	40	17:22:1...	8.04958	[Color]	47.4	71.0381	4
Object 7149	Object	7149	vesicle tracking	20	40	17:22:1...	8.04958	[Color]	37.7	73.05	3
Object 7150	Object	7150	vesicle tracking	19	40	17:22:1...	8.04958	[Color]	34.7895	75.9474	34.78
Object 7151	Object	7151	vesicle tracking	13	40	17:22:1...	8.04958	[Color]	113.769	94	113.7
Object 7152	Object	7152	vesicle tracking	23	40	17:22:1...	8.04958	[Color]	126.565	118.87	126.5
Object 7153	Object	7153	vesicle tracking	19	40	17:22:1...	8.04958	[Color]	100.947	191.053	100.9
Object 7154	Object	7154	vesicle tracking	74	40	17:22:1...	8.04958	[Color]	74.6081	202.324	74.60
Object 7155	Object	7155	vesicle tracking	26	40	17:22:1...	8.04958	[Color]	70.3077	201.731	70.30
Object 7156	Object	7156	vesicle tracking	74	40	17:22:1...	8.04958	[Color]	60.4054	70.7838	60.40
Object 7157	Object	7157	vesicle tracking	16	40	17:22:1...	8.04958	[Color]	75.6875	71.1875	75.68
Object 7158	Object	7158	vesicle tracking	15	40	17:22:1...	8.04958	[Color]	117.333	94.2	117.3
Object 7159	Object	7159	vesicle tracking	23	40	17:22:1...	8.04958	[Color]	91.913	198.913	91.9
Object 7160	Object	7160	vesicle tracking	36	40	17:22:1...	8.04958	[Color]	80.7778	204.222	80.77
Object 7161	Object	7161	vesicle tracking	4	40	17:22:1...	8.04958	[Color]	4.25	174.75	4
Object 7162	Object	7162	vesicle tracking	7	40	17:22:1...	8.04958	[Color]	111.143	184.429	111.1
Object 7163	Object	7163	vesicle tracking	13	40	17:22:1...	8.04958	[Color]	96	198.769	96
Object 7164	Object	7164	vesicle tracking	5	40	17:22:1...	8.04958	[Color]	32.8	200	3
Object 7165	Object	7165	vesicle tracking	4	40	17:22:1...	8.04958	[Color]	76	67.5	76
Object 7166	Object	7166	vesicle tracking	4	40	17:22:1...	8.04958	[Color]	34.5	69.5	3
Object 7167	Object	7167	vesicle tracking	2	40	17:22:1...	8.04958	[Color]	133	141.5	1
Track 1	Track	7168	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A
Track 2	Track	7169	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A
Track 3	Track	7170	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A
Track 4	Track	7171	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A
Track 5	Track	7172	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A
Track 6	Track	7173	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A
Track 7	Track	7174	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A
Track 8	Track	7175	vesicle tracking	N/A	N/A	N/A	N/A	[Color]	N/A	N/A	N/A

Measuring and Tracking Multiple Timepoints

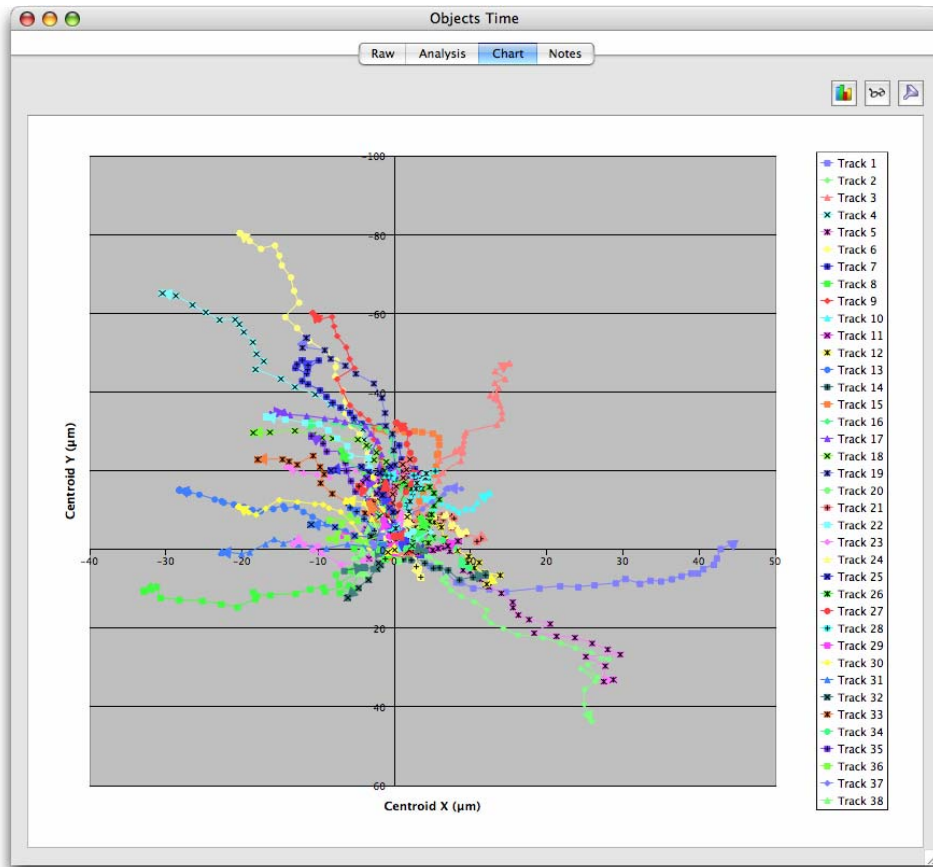
2. Choose **Tracks** from the **Filter:** drop-down to quickly view just the tracks.



The screenshot shows the 'Objects Time' application window. A filter menu is open, showing options: All, Objects, Lines, Points, and Tracks (which is selected). Below the menu, the text reads 'Raw data in this measurement item. Showing 102 of 7269 measurements.' The main table displays the following data:

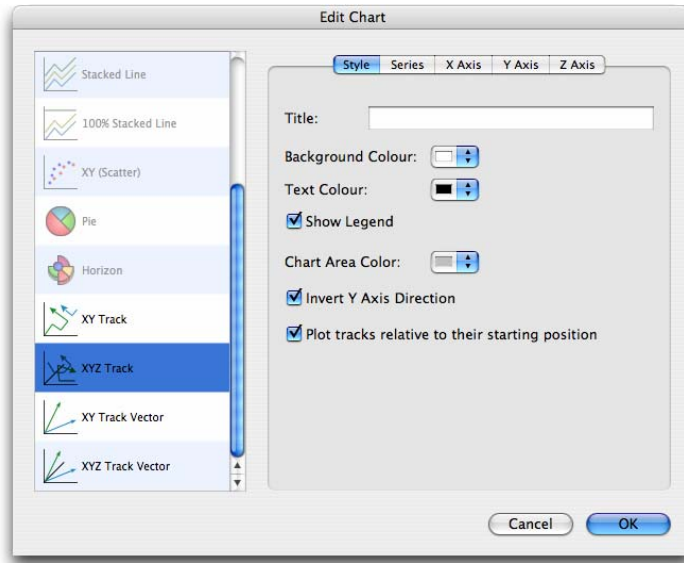
Name	Type	ID	Item Name	Color	Track ID	Length	Length (μ...)	Time Span	Track Velocity	Track Velocity ...
Track 72	Track	7239	vesicle tracking	Dark Green	7239	11.6459	11.6459	4	23.5077	23.5077
Track 73	Track	7240	vesicle tracking	Light Green	7240	8.36278	8.36278	4	16.8806	16.8806
Track 74	Track	7241	vesicle tracking	Purple	7241	10.658	10.658	4	21.5137	21.5137
Track 75	Track	7242	vesicle tracking	Cyan	7242	2.55474	2.55474	3	7.70532	7.70532
Track 76	Track	7243	vesicle tracking	Yellow	7243	5.33353	5.33353	3	16.0864	16.0864
Track 77	Track	7244	vesicle tracking	Blue	7244	3.26082	3.26082	3	9.83494	9.83494
Track 78	Track	7245	vesicle tracking	Red	7245	6.95341	6.95341	3	20.9721	20.9721
Track 79	Track	7246	vesicle tracking	Light Green	7246	7.3521	7.3521	3	22.1746	22.1746
Track 80	Track	7247	vesicle tracking	Orange	7247	3.23817	3.23817	3	9.76663	9.76663
Track 81	Track	7248	vesicle tracking	Purple	7248	2.32928	2.32928	3	7.02532	7.02532
Track 82	Track	7249	vesicle tracking	Pink	7249	5.74018	5.74018	3	17.3129	17.3129
Track 83	Track	7250	vesicle tracking	Magenta	7250	7.82853	7.82853	3	23.6116	23.6116
Track 84	Track	7251	vesicle tracking	Cyan	7251	5.03095	5.03095	3	15.1738	15.1738
Track 85	Track	7252	vesicle tracking	Blue	7252	4.09045	4.09045	3	12.3372	12.3372
Track 86	Track	7253	vesicle tracking	Yellow	7253	6.08856	6.08856	3	18.3637	18.3637
Track 87	Track	7254	vesicle tracking	Light Green	7254	1.82811	1.82811	2	10.8781	10.8781
Track 88	Track	7255	vesicle tracking	Orange	7255	2.81611	2.81611	2	16.7571	16.7571
Track 89	Track	7256	vesicle tracking	Blue	7256	2.59864	2.59864	2	15.463	15.463
Track 90	Track	7257	vesicle tracking	Light Green	7257	1.58709	1.58709	2	9.44385	9.44385
Track 91	Track	7258	vesicle tracking	Yellow	7258	4.05281	4.05281	2	24.116	24.116
Track 92	Track	7259	vesicle tracking	Red	7259	2.92866	2.92866	2	17.4268	17.4268
Track 93	Track	7260	vesicle tracking	Magenta	7260	1.09511	1.09511	2	6.51635	6.51635
Track 94	Track	7261	vesicle tracking	Light Green	7261	3.03944	3.03944	2	18.086	18.086
Track 95	Track	7262	vesicle tracking	Yellow	7262	1.78098	1.78098	2	10.5976	10.5976
Track 96	Track	7263	vesicle tracking	Light Green	7263	2.00071	2.00071	2	11.9051	11.9051
Track 97	Track	7264	vesicle tracking	Dark Green	7264	2.84422	2.84422	2	16.9243	16.9243
Track 98	Track	7265	vesicle tracking	Pink	7265	3.02813	3.02813	2	18.0187	18.0187
Track 99	Track	7266	vesicle tracking	Light Green	7266	3.56293	3.56293	2	21.201	21.201
Track 100	Track	7267	vesicle tracking	Red	7267	0.781736	0.781736	2	4.65166	4.65166
Track 101	Track	7268	vesicle tracking	Magenta	7268	1.08372	1.08372	2	6.44861	6.44861
Track 102	Track	7269	vesicle tracking	Orange	7269	1.27519	1.27519	2	7.58792	7.58792

3. Click directly on the Chart tab to show the tracks as plots of centroid position.



Measuring and Tracking Multiple Timepoints

Select **Edit Chart...** from the Chart menu to change the type of chart and customize many features of its appearance.

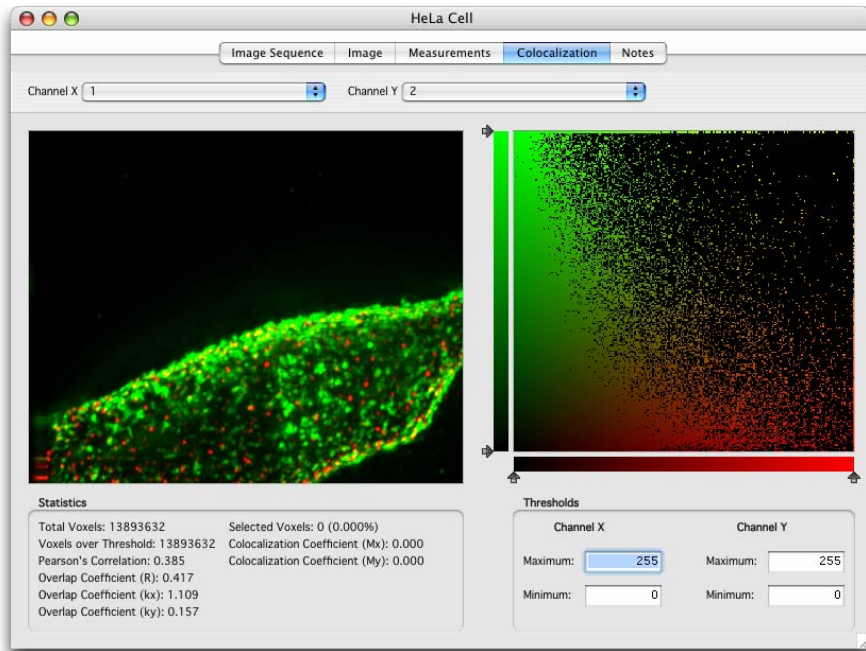


Colocalization

Colocalization is part of the Volocity Quantitation product designed specifically for Colocalization analysis as described by Manders et al (1993). See also page 412.

Using the Colocalization View

1. Open the image sequence containing the two channels to be analyzed. The two channels must be the same color depth. It is much easier to visualize colocalization if each channel has a different color. Colocalization does not operate on single volumes.
2. Select the Colocalization tab. Set Channel X and Channel Y using the drop-downs.



The preview image shows an extended focus merge of the two channels. Volocity calculates statistics, displayed under the preview, on the intensity information in the whole image. While Volocity is generating the scatter plot and calculating statistics a progress indicator is shown in the top right-hand corner of the view.

Colocalization

3. Working with the scatter plot on the right, set thresholds for each axis so that the lower threshold is above background. It may not be necessary to change the upper threshold.

There are three ways of setting thresholds.

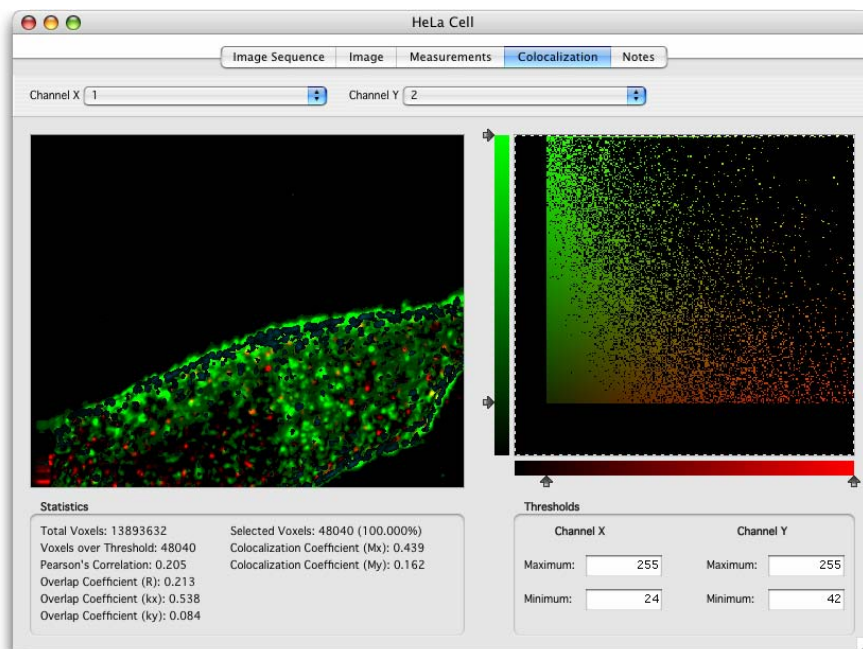
- Use the ROI tool to draw a region of interest on the Preview. Select **Set Thresholds from ROI** from the Colocalization menu.
- Enter the required values manually into the Threshold **Maximum** and **Minimum** boxes for each channel or drag the slider controls on the scatter plot.
- Select **Automatic Thresholding** from the Colocalization menu to generate thresholds automatically.

Any voxels of intensity outside threshold will be removed from the preview image for that channel. Any point on the scatter plot that is outside threshold for either channel will be removed from the scatter plot leaving only points that are positive for both x and y, that is, colocalized.

If thresholds are not set, voxels where both images show background intensity levels may be interpreted as colocalized.

4. Select voxels on the scatter plot to be analyzed.

Select All and **Select None** in the Edit menu operate on the scatter plot. Use the shortcuts Apple-A on Mac OS and Ctrl-A on Windows to select all.

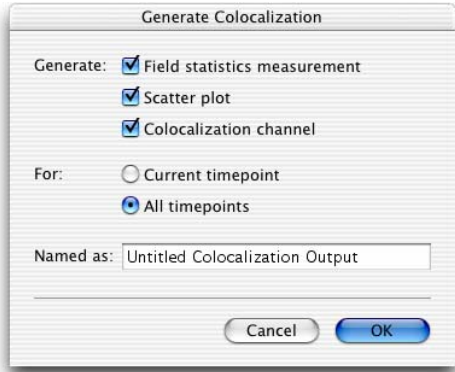


5. Colocalized voxels are shown in an overlay on the preview.

If required, select sub-regions of the scatter plot for analysis. Use the ROI tools to draw a region of interest on the scatter plot. The preview will update so the overlay shows the location of just the selected, colocalized voxels. The statistics will also update to reflect the selected voxels.

6. Create the colocalization output.

Select **Generate Colocalization** from the Colocalization menu and choose to make the following output for the current timepoint or all timepoints in the image sequence.



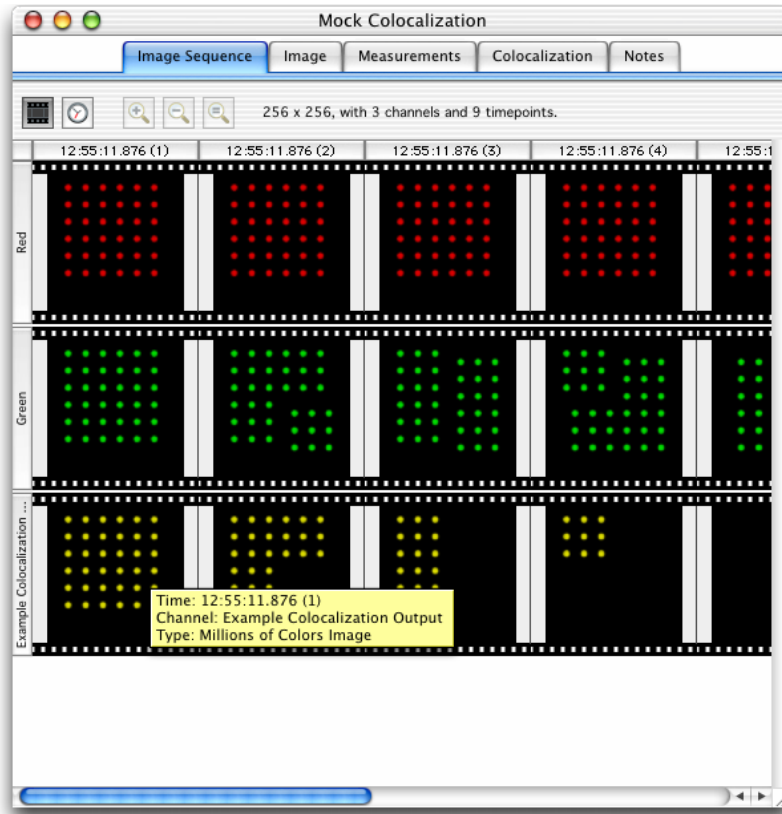
- Field statistics are measurements representing the whole volume rather than objects within it. One timepoint in an image sequence will give rise to one measurement row in the Measurements table. Colocalization statistics (Mx and My) are generated for regions of interest selected on the scatter plot as described at step 3.

Timepoint	Pearsons Correlation	Overlap Coefficient R	Overlap Coefficient Kx	Overlap Coefficient Ky	Colocalization Coefficient Mx	Colocalization Coefficient My	X Min Threshold	X Ma
18/2005 1	1	1	1	1	1	1	1	258
18/2005 2	0.726063	0.75	0.75	0.75	0.75	0.75	0.5	258
18/2005 3	0.452126	0.5	0.5	0.5	0.5	0.5	0.5	258
18/2005 4	0.178189	0.25	0.25	0.25	0.25	0.25	0.25	258
18/2005 5	-0.0957481	0	0	0	0	0	0	258
18/2005 6	0.230651	0.286675	0.25	0.333333	0.25	0.333333	0.5	258
18/2005 7	0.312313	0.353553	0.25	0.5	0.25	0.5	1	258
18/2005 8	0.48296	0.5	0.25	1	0.25	1	1	258
18/2005 9	0.232356	0.25	0.0833333	0.75	0.0833333	0.75	0.75	258

- The scatter plots are saved to a folder in the current library. Each scatter plot is stored as a 2D image and may be exported.

Colocalization

- A Colocalization channel is made from the selected colocalized voxels. These voxels are marked in the Colocalization View preview with the colored overlay. The channel is produced from a brightest point merge honoring the color and intensity of those voxels in the original channels.



Volocity Quantitation Reference

Overview

This is the reference chapter for Volocity Quantitation. For worked, step-by-step examples on measuring and analyzing data at a single timepoint and at multiple timepoints, please see page 148.

This chapter describes:

- The Measurements View
- How to draw points, lines and ROIs to create objects manually
- How to create measurement protocols and use them to select objects
- Raw Data, Analysis and Chart Views
- How to visualize and quantify colocalization between pairs of images
- FRAP and FLIP analysis
- Ratioing
- FRET analysis

Measurements View

Measurements View

To measure objects, they must first be identified. The Measurements View provides all the tools required to identify objects and make a variety of measurements from those objects. Use the Region of Interest drawing tools to manually draw around objects (see page 341), or create a measurement protocol using the Measurement Protocol tasks (see page 357).

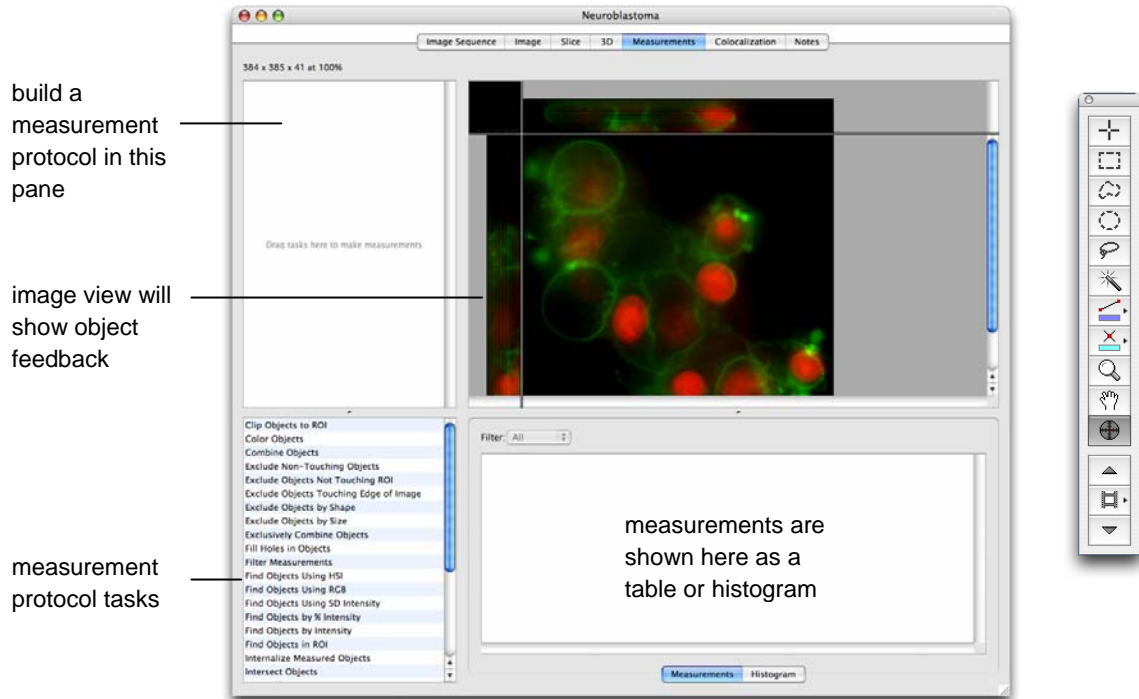


Image View

This is an image view within the Measurements View. Options, such as Extended Focus, XYZ planes, Show/Hide time and Show/Hide color reference, in the Measurements menu allow you to configure this view in the same way as you would configure the Image View, see page 66.

Measurements and Histogram

The Measurements table provides numerical information about the objects found in the image directly above it. This numerical information is only shown while objects are selected either by a user-drawn ROI or a measurements protocol.

Click on the **Histogram** tab to display the numerical information as a simple histogram, one column from the table at a time. Use the histogram to find patterns in a selected item property.

Choose the data to be shown from the drop-down list of those available.

Measurements Protocol

The most powerful tool for selecting and measuring objects is the Measurements protocol. You build a protocol by selecting measurements tasks in the lower half of the Measurements View and dragging them into the top left-hand pane. Descriptions of individual measurement tasks start on page 358.

As tasks are added to the protocol, feedback updates to show the objects found as an overlay in the image and as rows of measurements in the table below.

Drawing Objects to be Measured in the Measurements View

Select objects to be measured in the Measurements View using tools for drawing regions, lines and points.

Alternatively find objects by creating a Measurement protocol, see page 357.

Using the Drawing Tools

Drawing Lines with the Line Tool



Use the Line tool to add lines in the Image View. Connect points by holding down the Shift key and clicking with the cursor. Release the Shift key to end a line.

shortcut key = L

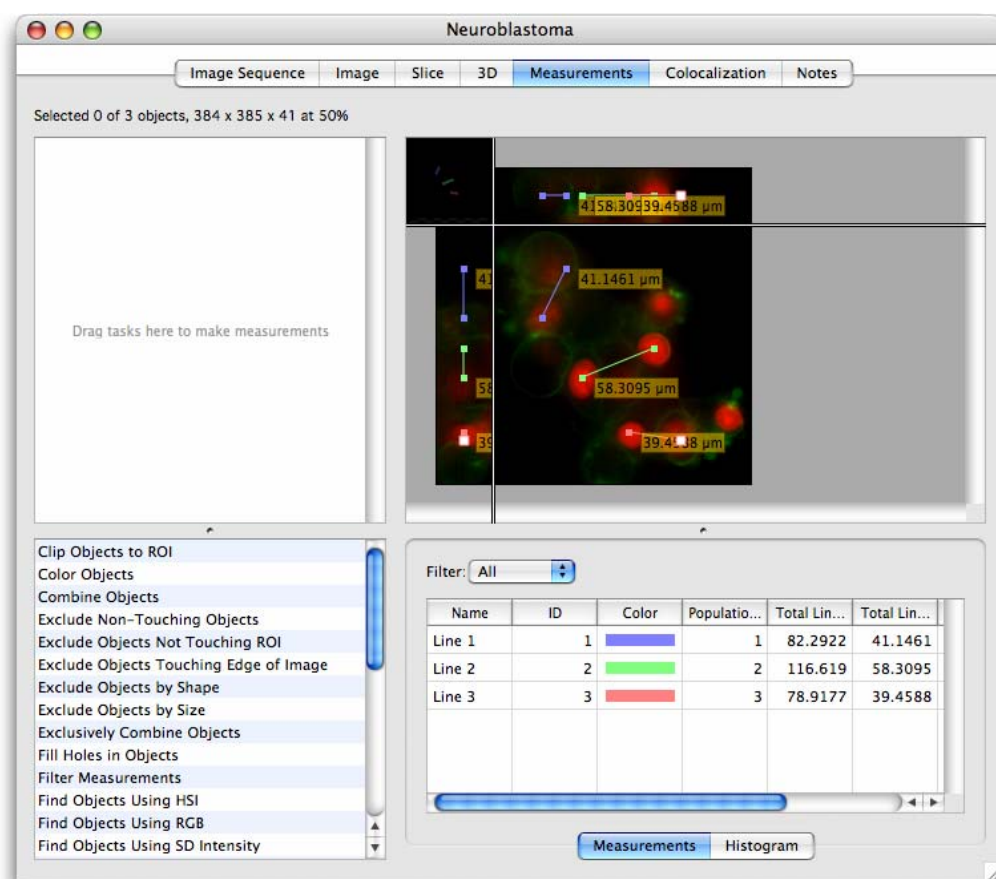
You can also move between planes when specifying points in the line. This makes it easier to accurately identify the points between which a measurement is required. For example, the line will still be connected even if one end starts in the XY part of the view and the other end is marked in the YZ part.

The total length of the line is shown as the line is drawn. Turn off this display in the **View Options...** dialog found in the Measurements menu.

Line measurements are also shown in the table below the Image View. Export this table as a text file by selecting **Export...** from the File menu. Save this table by selecting **Make Measurement Item...** from the Measurements menu.

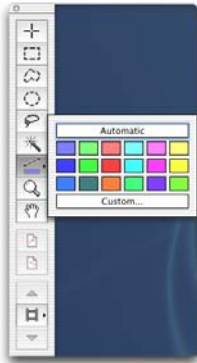
Once drawn, you can move control points of lines by passing the cursor over a line. To do this the line tool must be selected. The cursor changes shape over a control point indicating that you can grab and move it. Again, this can be done in the most convenient part of the view including the 3D cross-section part.

Using the Drawing Tools



As lines are drawn automatic feedback updates the table below the image to show the measured lengths. For each line a row is shown in the table below the image preview. The length of the line is shown uncalibrated and as calibrated units. Export this table as a text file by selecting **Export...** from the File menu. Save this table as an item in the library by selecting **Make Measurement Item...** from the Measurements menu.

Setting Up the Line Tool

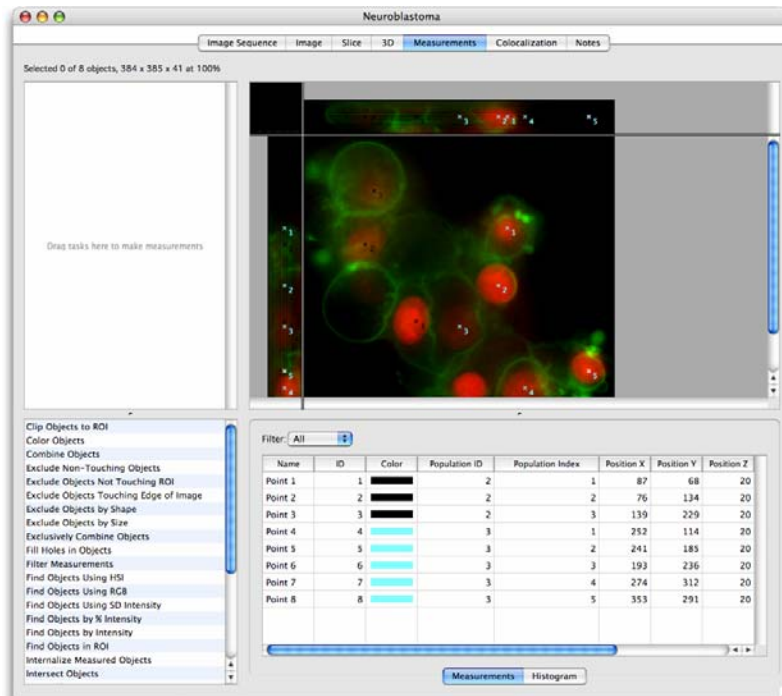


Velocity will assign a different color for each successive line automatically or you can specify a color. Click and hold on the Line tool to display the color picker dialog. Click on **Automatic** or click on either one of the displayed colors or use the **Custom...** dialog to specify a particular color. Note how the line tool reflects the color selected.

To delete part of a line double click on a control point to highlight that section of the line and use the Backspace (Mac) or Delete (PC) key. To delete all the lines active on the view, use the **Select None** command in the Edit menu.

Point Counting with the Point Tool

Use the Point tool to count objects in the image. Select the Point tool and, for each click on the image area, a point count increments by one.



Using the Drawing Tools



Change the color of the point tool by clicking on the menu next to the tool. A new color will start a new count at one.

To delete a point, click on it to highlight it and use the Backspace (Mac) or Delete (Windows) keys. Other points of the same color will be renumbered.

For each point, a row is shown in the table below the image preview. The position of the point as X, Y and Z coordinates is stored. All points of the same color are assigned the same population ID.

- Export this table as a text file by selecting **Export...** from the File menu.
- Save this table as an item in the library by selecting **Make Measurement Item...** from the Measurements menu.

Drawing Regions Using ROI Tools

Use these tools to draw a region of interest. The selected area will be outlined with "marching ants", a moving outline. Each object (i.e. contiguous block of voxels) within the ROI will be assigned a different color. Regions of interest are shown in the Image View using a colored overlay. Select **View Options...** from the Image menu to change this.

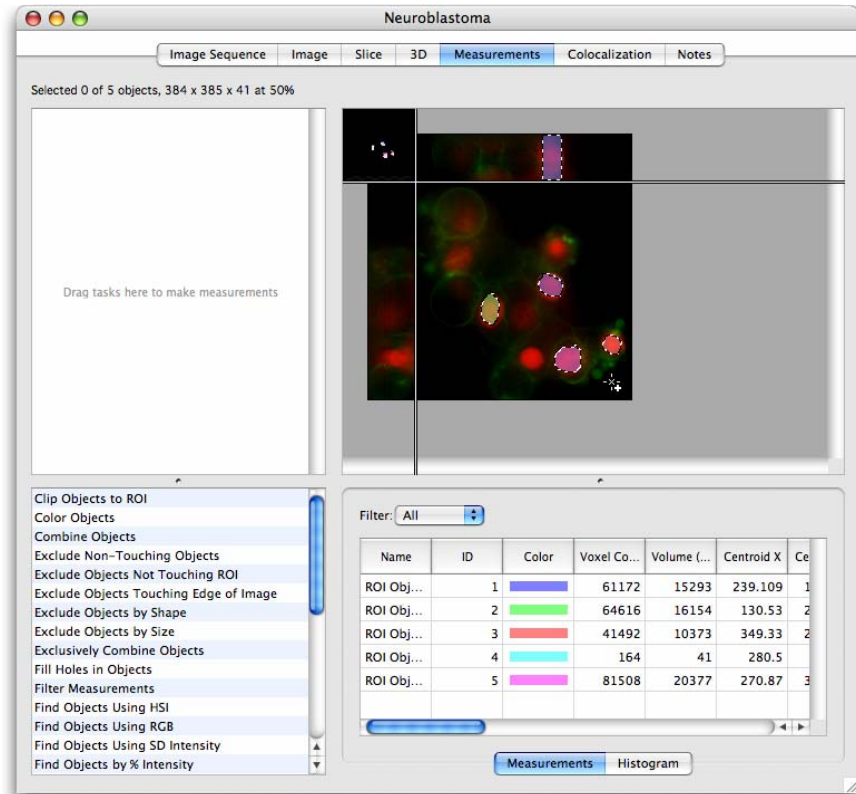
For each object a row is shown in the table below the image preview. Some default morphological and intensity information for each object is stored.

- Export this table as a text file by selecting **Export...** from the File menu.
- Save this table as an item in the library by selecting **Make Measurement Item...** from the Measurements menu.



shortcut key = **R**

The Rectangular ROI selection tool allows you to draw rectangles or squares, as illustrated in the following example.



shortcut key = **F**

The Freehand tool allows you to make a freehand selection of an area on the image.



shortcut key = **S**

The Lasso tool allows you to trace the outline of a selection. It selects regions based upon the color similarities of adjacent pixels. The initial point clicked sets the color to compare and the tool has the effect of shrinking around the pixels that fall outside of the tolerance value.



shortcut key = **W**

The Magic Wand tool has the effect of expanding the selection outwards from the original pixel clicked on until the threshold set by the Setup Magic Wand is reached.



The ROI stamp tool makes a selection by placing an ROI of a predetermined shape and size on the image, centered on the cursor position. This is useful for making ROIs of a known size and/or repeating a selection of a known size.

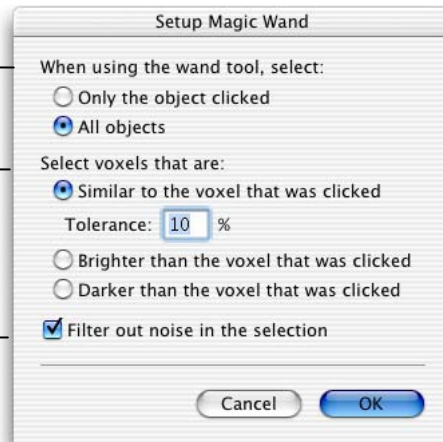
Setting Up the Magic Wand

Double click on the Magic Wand tool to display the Setup dialog.

click to select only contiguous voxels,
or all groups that meet criteria

click to select one of the threshold
criteria

if required, check to enable filter

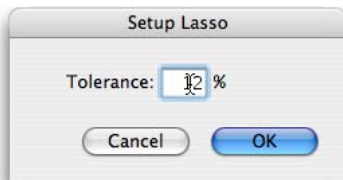


Note: If you are trying to isolate regions of interest from background data, it may be easier to use the Wand tool to select the background, use the **All objects** criterion and then apply the **Reverse Selection** command in the Actions menu.

Filter out noise in the selection will apply a 3x3x3 median filter. This will remove very small objects that are often described as noise and can add significantly to the time taken to make the measurement.

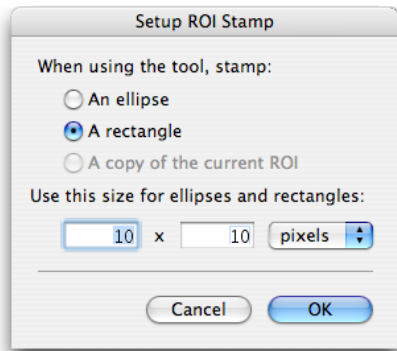
Setting Up the Lasso Tool

Double-click on the Lasso Tool in the tool bar to display a dialog in which you may adjust the **Tolerance** of the selection.



Setting up the ROI Stamp Tool

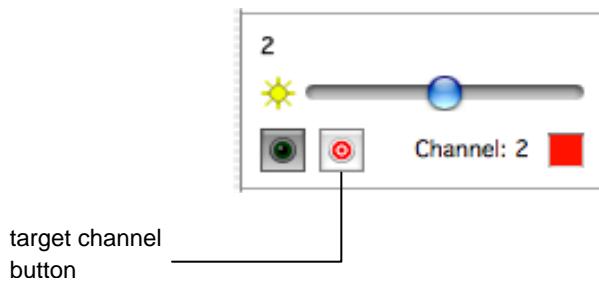
Double click on the ROI Stamp Tool to display the Setup dialog.



The size of the ROI may be set in pixels or micrometers. An ROI size set by micrometers will vary in the number of pixels depending on the calibration of the item.

Target Channel

The Magic Wand and Lasso tools work on voxel intensity. So, within multi-channel data sets in an image sequence, the size and shape of the selection made by the tool can be very different according to which channel is targeted when the selection is drawn. Use the target channel button in the Navigation palette to choose the channel on which the selections will be made. See page 82 for more information about working with the Navigation palette.



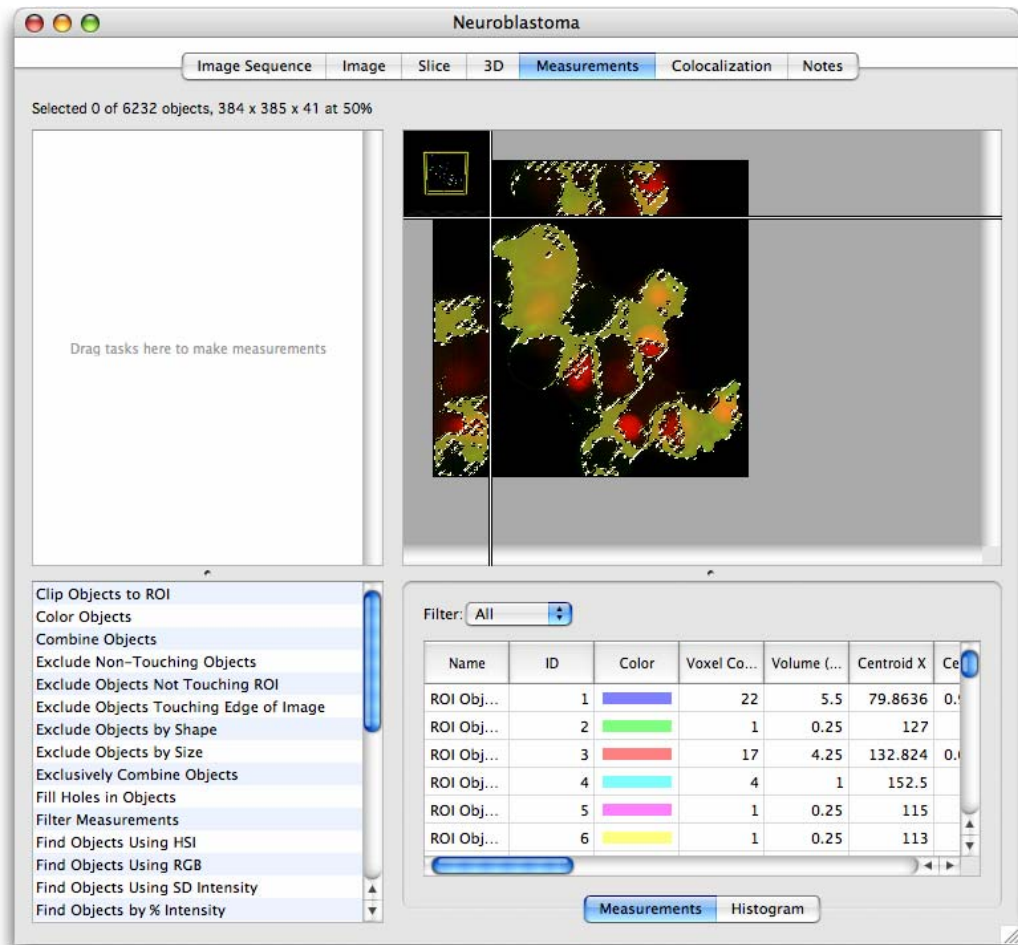
Using the Drawing Tools

The following example shows the selection made if the magic wand is clicked when the red channel is targeted.

The screenshot shows the Neuroblastoma software interface. The main window displays a 3D image of a neuroblastoma with several colored regions (red, green, yellow, cyan) overlaid on a dark background. The interface includes a menu bar with options: Image Sequence, Image, Slice, 3D, Measurements, Colocalization, and Notes. Below the menu bar, there is a status bar indicating "Selected 0 of 306 objects, 384 x 385 x 41 at 50%". The main image area is divided into a top-left corner showing a small thumbnail of the full image and a larger central area showing the current slice. Below the image area, there is a list of tasks to make measurements, including "Clip Objects to ROI", "Color Objects", "Combine Objects", "Exclude Non-Touching Objects", "Exclude Objects Not Touching ROI", "Exclude Objects Touching Edge of Image", "Exclude Objects by Shape", "Exclude Objects by Size", "Exclusively Combine Objects", "Fill Holes in Objects", "Filter Measurements", "Find Objects Using HSI", "Find Objects Using RGB", "Find Objects Using SD Intensity", and "Find Objects by % Intensity". To the right of the task list, there is a table with columns: Name, ID, Color, Voxel Co..., Volume (...), Centroid X, and Centroid Y. The table contains six rows of data, each representing a selected region. Below the table, there are buttons for "Measurements" and "Histogram".

Name	ID	Color	Voxel Co...	Volume (...)	Centroid X	Centroid Y
ROI Obj...	1	Blue	7029	1757.25	40.1386	40.1386
ROI Obj...	2	Green	1	0.25	27	27
ROI Obj...	3	Red	1	0.25	104	104
ROI Obj...	4	Cyan	1	0.25	108	108
ROI Obj...	5	Magenta	1	0.25	229	229
ROI Obj...	6	Yellow	1	0.25	234	234

In the following example the magic wand is clicked at the same location, but the selection is different because the green channel is now targeted.



Working with Regions

Once you have selected an ROI, you can also use the **Copy**, **Cut**, **Paste** and **Clear** commands from the Edit menu. The **Crop to Selection** command in the Actions menu will reduce the data set to what is contained within the ROI. When cropping, the selection is always simplified to the smallest straight-sided shape that will contain the selection made, even if you have used the Freehand or Magic Wand tool.

ROI menu

The sub-menu **ROI** under the **Edit** menu contains commands for working with ROIs once they have been drawn.

- **Undo** to undo the last change made to an ROI
- **Redo** to redo the last change made to an ROI
- **Save** to store the currently active ROI as a settings file in the preferences
- **Restore** to recall a previous stored ROI and apply it to the current view
- **Grow** to expand the ROI by one voxel in all directions
- **Shrink** to reduce the ROI by one voxel in all directions

Key combinations

The following key combinations also apply:

Mac OS key combinations

- Control-drag on a selection centers the selection on the image.
- Command-drag locks the dimensions, to make a square or circular selection rather than rectangular or oval.
- Shift allows you to add to the ROI or draw multiple ROIs.
- Shift + alt (option) allows you to subtract from the ROI or subtract the whole of the existing ROI by drawing around it.
- Alt (option) carries out an AND operation leaving only the intersection of the two ROIs. This allows you to select in one part of the XYZ view and refine the selection based on what can be seen in another part.

Windows key combinations

- Shift allows you to add to the ROI or draw multiple ROIs.
- Shift + ctrl allows you to subtract from the ROI or subtract the whole of the existing ROI by drawing around it.
- Ctrl carries out an AND operation leaving only the intersection of the two ROIs. This allows you to select in one part of the XYZ view and refine the selection based on what can be seen in another part.

Object Feedback

As objects are selected in the image, feedback is shown both in the image and in the table. You can relate measurement rows to objects shown in the image.

- To show which object in the image is represented by a row in the table, select the row. Visual feedback for all other objects will be hidden, leaving just the selected object shown. Deselect the row in the table to return to showing all objects again.

The screenshot shows the Neuroblastoma software interface. At the top, the title bar reads "Neuroblastoma". Below it are tabs for "Image Sequence", "Image", "Slice", "3D", "Measurements", "Colocalization", and "Notes". The "Measurements" tab is active. The main window displays a microscopy image with several segmented objects in red, green, and purple. A text box on the left says "Selected 1 of 1202 objects, 384 x 385 x 41 at 100%". Below the image is a list of tasks: "Drag tasks here to make measurements". At the bottom, there is a table with columns: Name, ID, Color, Voxel, Volume, Min, Max, Mean, Sum, and M. The table is filtered to show all objects. The row for ID 1025 is selected, and its corresponding object in the image is highlighted in purple.

Name	ID	Color	Voxel ...	Volume (...)	Min (1)	Max (1)	Mean (1)	Sum (1)	M (1)
ROI Obj...	1068	Yellow	202090	202090	0	255	48.5667	9.8148...	
ROI Obj...	1088	Green	167442	167442	0	255	48.3661	8.0985...	
ROI Obj...	1076	Dark Green	90439	90439	0	113	27.2246	2.4621...	
ROI Obj...	1023	Orange	82180	82180	0	149	54.0554	4.4422...	
ROI Obj...	1025	Purple	60589	60589	11	205	63.3301	3.8371...	
ROI Obj...	866	Light Green	38175	38175	0	255	67.6896	2.5840...	
ROI Obj...	597	Red	21	21	40	69	60.381	1268	
ROI Obj...	457	Blue	16	16	22	54	32.625	522	
ROI Obj...	515	Magenta	14	14	13	72	36.8571	516	
ROI Obj...	688	Cyan	13	13	90	113	97.6154	1269	

Object Feedback

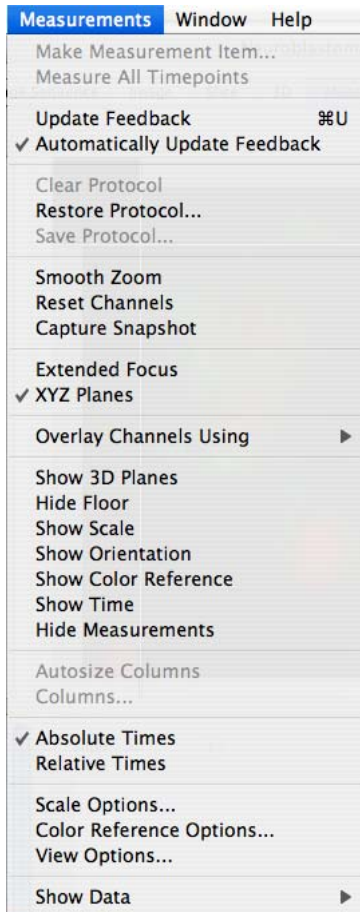
- To find measurements for objects use the Select object tool from the tool bar. The cursor changes to a pointing hand when it is over voxels which have been measured as part of an object, click to select the object, scroll to the relevant row in the measurements table and select the measurement row.

The screenshot shows the Neuroblastoma software interface. The main window displays a 3D visualization of brain cells with red and green regions. A mouse cursor is positioned over one of the red regions. Below the visualization is a table of measurements for various objects. The table has columns for Name, ID, Color, Voxel count, Volume, Min, Max, Mean, and Sum. The table is filtered to show all objects.

Name	ID	Color	Voxel ...	Volume ...	Min (1)	Max (1)	Mean (1)	Sum (1)	M (2)
ROI Obj...	1068	Yellow	202090	202090	0	255	48.5667	9.8148...	
ROI Obj...	1088	Green	167442	167442	0	255	48.3661	8.0985...	
ROI Obj...	1076	Dark Green	90439	90439	0	113	27.2246	2.4621...	
ROI Obj...	1023	Orange	82180	82180	0	149	54.0554	4.4422...	
ROI Obj...	1025	Purple	60589	60589	11	205	63.3301	3.8371...	
ROI Obj...	866	Light Green	38175	38175	0	255	67.6896	2.5840...	
ROI Obj...	597	Red	21	21	40	69	60.381	1268	
ROI Obj...	457	Blue	16	16	22	54	32.625	522	
ROI Obj...	515	Magenta	14	14	13	72	36.8571	516	
ROI Obj...	688	Cyan	13	13	90	113	97.6154	1269	

Measurements Menu

Many of the options in the Measurements menu are identical to those in the Image View. These allow you to configure the image to assist in object selection. You can change how the image is displayed and, for 3D data, switch between XYZ and Extended Focus.



The rest of this section describes those menu options that are specific to measurement.

Measurements Feedback Options

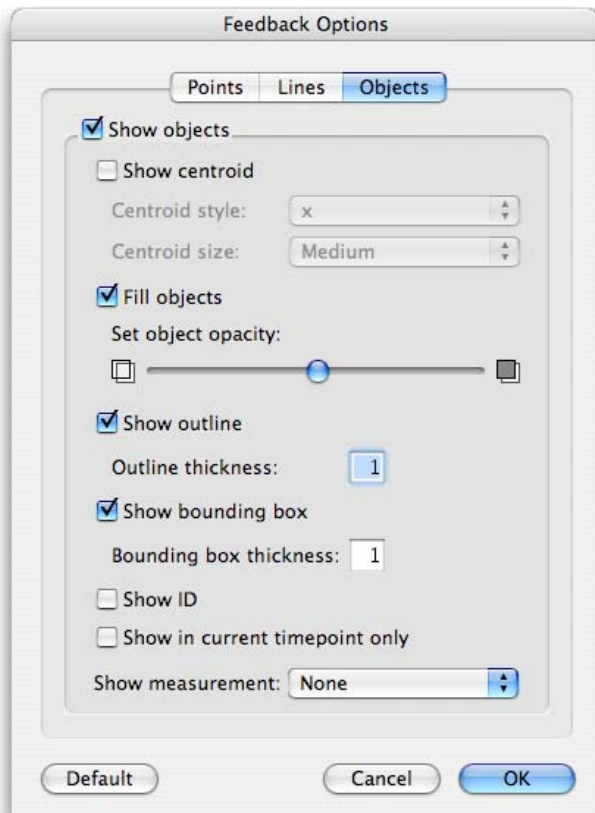
In the Measurements View the location of objects selected in the image is shown using feedback. The updating of feedback is set by the **Automatically Update Feedback** option in the Measurements menu. The visual appearance of the feedback is controlled by the **Feedback Options...** in the same Measurements menu.

Object Feedback

Change the feedback options to show feedback and images more clearly together and to illustrate specific points.

The Feedback Options dialog is divided into tabs, one for each type of measured item that could be shown and the changes that can be made to their display.

You may choose to show each type of feedback or not, and there are sub options within each tab.



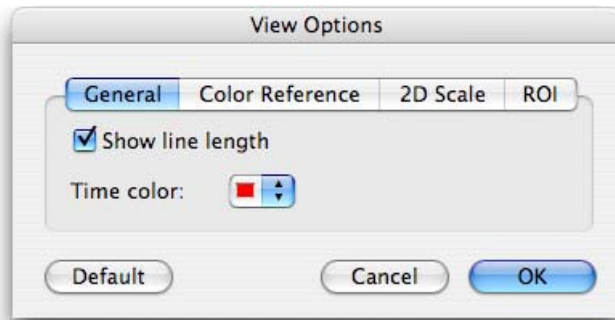
- **Show in current timepoint only** is an option for viewing feedback within time resolved data. When this option is checked only feedback that relates to measurements made from the current timepoint will be shown. Changing the timepoint using the Navigation palette will change the feedback so that only objects in the new timepoint are shown.
- **Show measurement** will allow any of the measured parameters to be shown alongside the colored shape which represents the measurement.

To change the appearance of an ROI drawn on a view use the ROI tab in the View Options dialog for that view.

Measurement View Options

Change aspects of the view display using the **View Options** dialog.

The dialog is divided into tabs for the different features on the view that are to be displayed.

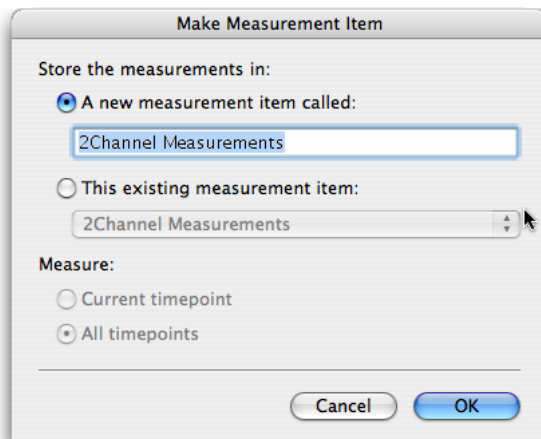


Line length and ROI options refer to objects selected in the Measurements View.

To change how objects selected by a measurements protocol are displayed in the view, use the **Feedback Options** dialog also available from the **Measurements** menu.

Make Measurement Item

Use **Make Measurement Item...** to create a new table item in the library containing the measurements as previewed in the Measurements View.



A new measurement item may be created and named or the measurements may be added to an existing measurement item to build up a table of data.

For time-resolved data you may select measure all timepoints in the Make Measurement Item dialog, even if the measurements have only been previewed on one timepoint.

Measurements and Histogram

Measure All Timepoints

When this option is checked ROI or selections active in the view will be applied to all timepoints in the dataset and the measurements previewed in the table will be from all timepoints. This can take time to apply; therefore, this option may be checked or unchecked. Measurement operations that rely on the time component of the data will not be previewed in the Measurements View unless this option is checked.

Hide Measurements

Allow the image to be displayed in a larger window by hiding the Measurements table, if not required. **Hide measurements** changes to **Show measurements** to allow the table to be revealed again.

Measurements and Histogram

The Measurements table provides numerical information about the objects found in the image above. This numerical information can also be displayed as a simple histogram by clicking on the Histogram tab.

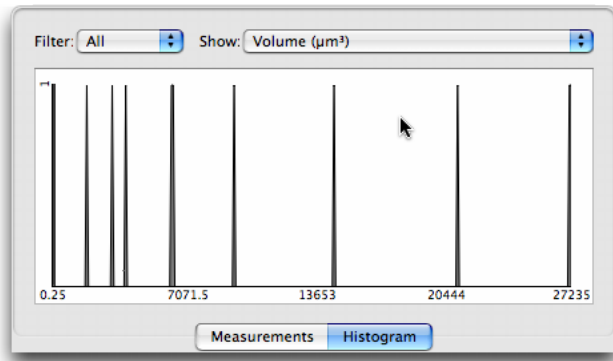
This numerical information is only shown while objects are selected either by a user drawn ROI or a Measurements protocol.

- Store measurement information by creating a measurement item; select **Make Measurement Item...** from the Measurements menu.
- Choose to **Hide** or **Show Measurements** from the Measurements menu to hide this table.
- Show only the measurements of a particular type, objects, lines or points by selecting from the drop-down at the top of the table.
- Sort the table by the contents of one column by clicking on the column heading, click again to reverse the sort. An arrow in the column header indicates that this column is sorting the data.
- Modify the columns shown by checking or unchecking column headings in the Columns dialog.
- Rearrange columns by clicking on the column heading and dragging that heading to another position, a red insertion line shows where the column will be placed.
- Fit the column widths to the data enclosed by selecting **Autosize Columns** from the Measurements menu.

Histogram Tab

Click on the histogram tab to switch to a histogram that displays one column from the table at a time. Use the histogram to find patterns in a selected item property.

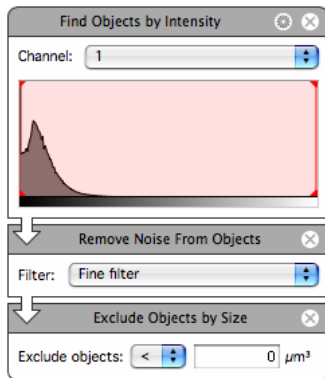
Choose the data to be shown from the drop-down list of those available.



Creating Measurement Protocols

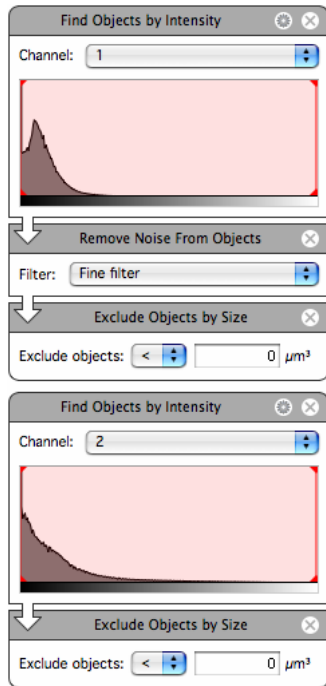
Measurements protocols are the most powerful tools for selecting and measuring objects. Protocols are made up of tasks and are built by dragging tasks from the list into the protocol area. Feedback into the view shows where objects have been found and lists numerical information about those objects. Measurements protocols start with 'Find Objects' tasks, after which the selection is refined by the addition of further tasks.

The order of tasks affects the result. Tasks lower down in the list work only on the results of tasks higher up in the protocol. The task flow is indicated by an arrow.



Measurement Protocol Tasks

A set of tasks starting with a “Find Objects” task is known as a series. It is possible to have more than one series in a single protocol.

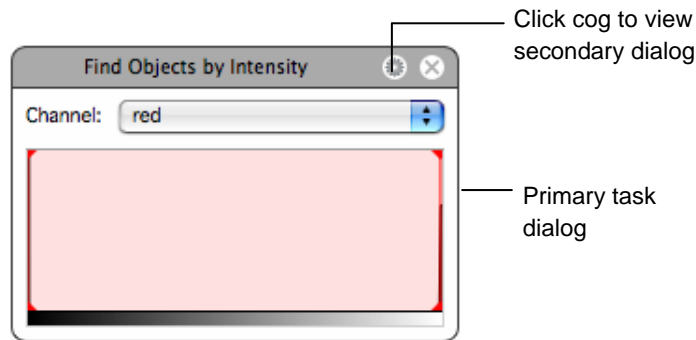


Measurement Protocol Tasks

Protocol tasks are listed alphabetically in the task list.

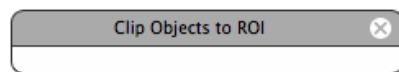
- Drag and drop the task name from the list to add to the protocol. The insertion point shows where the task will go.
- Double click on a task name in the list to add it straight to the bottom of the list.
- Move tasks around by dragging and dropping, the insertion point is shown.
- Remove a task from the list by clicking the cross.

Some information is in the primary task area and always displayed. There may be additional information; this is accessed by clicking on the cog icon to show a secondary dialog.



Clip Objects to ROI

Objects must have been found earlier in the protocol.

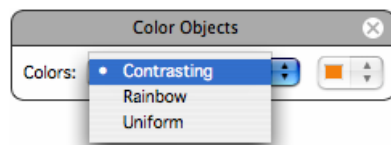


Drawing a region of interest in the image and using this task will clip the result to the bounds of the ROI. This may mean some objects are cropped.

Use with **Exclude Objects not Touching ROI** to ensure that any objects that intersect with the ROI are included.

Color Objects

When objects are found they are shown in the image area of the view using a colored overlay. By default each separate object identified is given a different color. It may be useful when working with objects to color them in a different way.



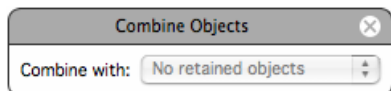
Use **Color Objects** in a series to apply a specific way of coloring a set of objects. Use anywhere below a find object task but above any measure task.

Combine Objects

This task merges the objects found by one series of measurements task with the objects found by another series.

Measurement Protocol Tasks

The first series of objects must include a **Retain Objects** task that names the first set of objects so that they can be specified in this task.



Exclude Non-Touching Objects

This task keeps only those objects in a second series that touch objects in a first series.

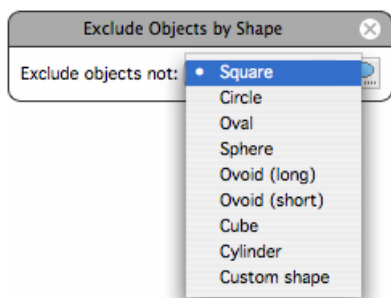
The first series of objects must include a **Retain Objects** task that names the first set of objects so that they can be specified in this task.



Exclude Objects by Shape

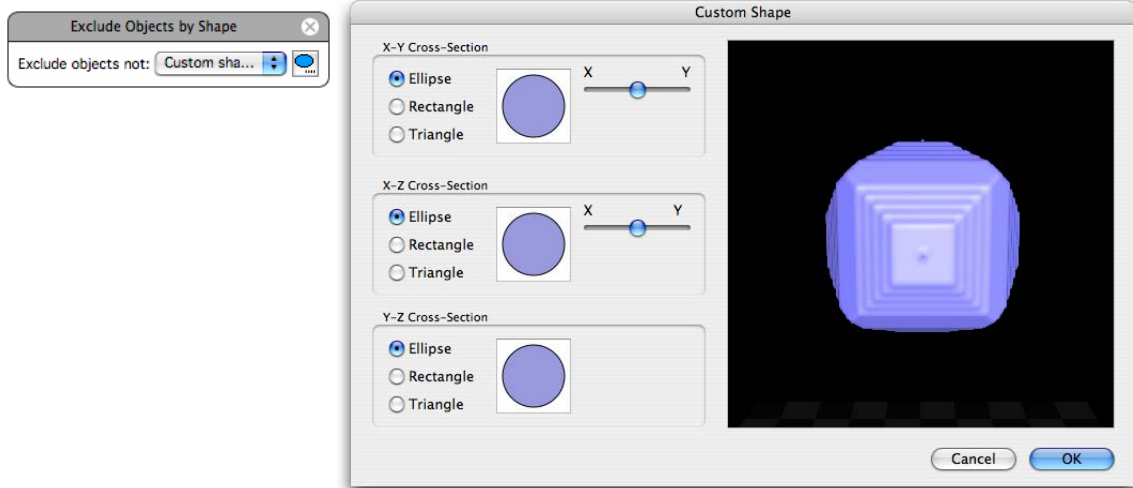
Objects found earlier in the protocol can be removed if they do not meet criteria for shape. This task would be used later than a **Find objects** task in a measurements protocol.

Select the required shape from the drop-down list: either one of the predefined shapes or the Custom Shape.



Custom shape: Custom shape allows you to create bespoke shapes using the XY, XZ and YZ cross sections through an ellipse, rectangle or triangle. The custom shape is saved when the protocol is saved.

To create a custom shape, select **Custom shape** from the drop-down menu and click on the button to launch the Shape Preview window. Select the axes and use the slider controls to adjust values manually.



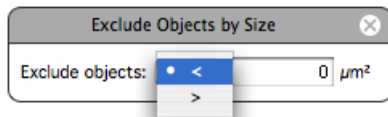
Ellipse and Rectangle: When the XY slider is set to the central point the ratio between the X and Y axis (or X and Z) is 50-50. As the slider is moved towards X, the length of the X axis will reduce and increase in Y and vice-versa. The same rules apply for the XZ sliders. The YZ ratio is taken from the XY and XZ controls.

Triangle: It is possible to skew the angles of the triangle to achieve all possible dimensions from an equilateral triangle (the slider is set at the central point) to a right-angled isosceles triangle (the slider is set to the extreme right or extreme left).

Exclude Objects by Size

Objects found earlier in the protocol can be removed if they do not meet criteria for size. This task would be used later than a **Find objects** task in a measurements protocol.

The task excludes objects greater than or smaller than a defined size.



Exclude Objects not Touching ROI

Objects must have been found earlier in the protocol.

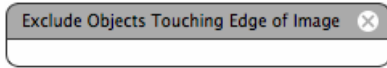
Drawing a region of interest in the image and using this task will show only objects that intersect with the ROI.



Exclude Objects Touching Edge of Image

Objects must already have been found by the protocol. This task will remove any of those objects touching the edge of the sample in the X,Y or Z planes. On datasets with small Z dimensions, this may remove too much from the selection.

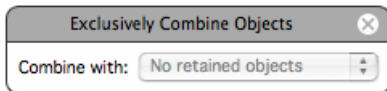
When working with 2D data the Z dimension is not considered as the edge of the image because this would mean every object would be excluded.



Exclusively Combine Objects

The first series of objects must include a **Retain Objects** task that names the first set of objects so that they can be specified in this task.

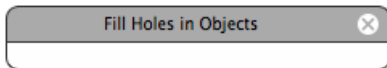
The result shows just those areas where the objects from one series are found, not both.



Fill Holes in Objects

Objects must have been found using any of the **Find Objects** tasks earlier in the protocol.

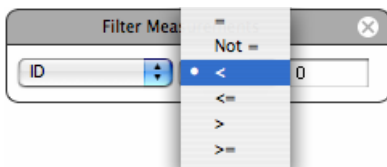
If any of the objects identified have holes in them, this task will fill those holes provided that they are surrounded in all dimensions by a contiguous wall of voxels. For example, a hardboiled egg has a white that completely surrounds the yolk. So, the yolk is completely surrounded on all sides. Therefore, if you select the white and fill holes, the whole volume of the boiled egg will be selected including the yolk.



Filter Measurements

Objects that have been found by the protocol are listed in the table below the image. If this table is not visible choose **Show Measurements** from the Measurements menu.

The list of objects found may be further refined by filtering on any of the measured parameters.

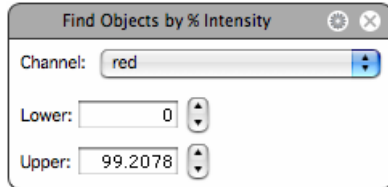


Find Objects by % Intensity

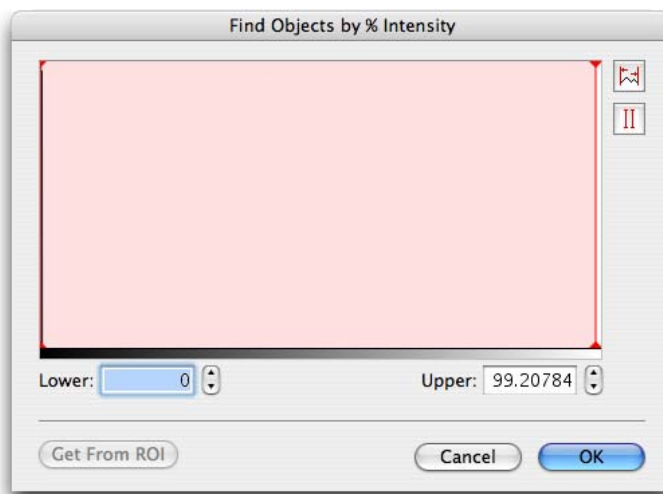
Find Objects tasks select voxels according to intensity or color information. This task selects voxels according to % intensity.

Intensities within the current volume are represented as a percentage considering the highest intensity value in the volume as 100%. This removes the need to select by absolute intensity values and may work better for time resolved data sets.

The primary task shows text entry boxes to set the lower and upper limit of the selection.



Click on the cog icon to show the secondary dialog. This shows a histogram of the current volume. Number of voxels is along the Y-axis; color or intensity values are along the X-axis.



Click on the vertical red lines in the histograms and drag them to change the selected range of values.

Change the Y-axis of the histogram to see detail more clearly, cycling through four possible resolutions; linear, log, base 10 and base 100. To do this:

PC: Ctrl-click in the histogram area

Mac: Alt-click in the histogram area

Measurement Protocol Tasks



Select all Values. Select this tool to display the maximum range of values for the threshold mode that you have selected.

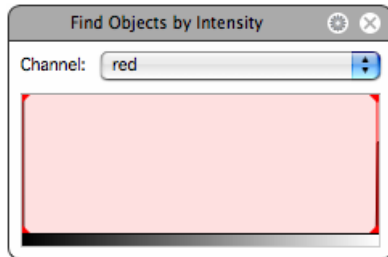


Invert Region of Selection. Select this tool to reverse the area of selection on the histogram. **Get From ROI** will set the minimum and maximum thresholds to the minimum and maximum intensity value found within an ROI active on the Image

Find Objects by Intensity

This task selects voxels according to specified intensity threshold values.

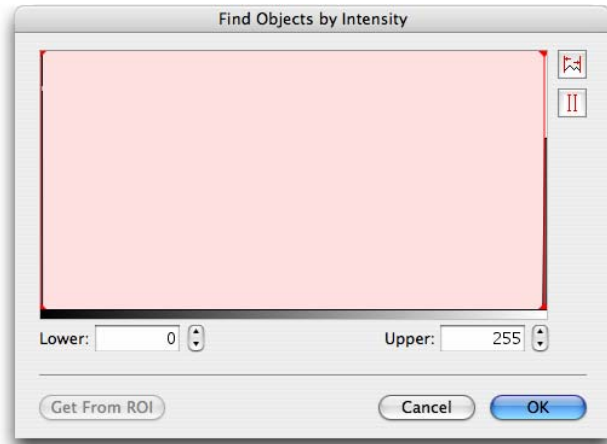
The primary task shows a histogram of the current volume. Number of voxels is along the Y-axis; intensity values are along the X-axis.



Click on the vertical red lines in the histogram and drag them to change the selected range of values. Tooltips show the intensity values.



The secondary dialog shows a larger histogram of the current volume. Text boxes allow direct entry of intensity values for the thresholds.



Click on the vertical red lines in the histograms and drag them to change the selected range of values.

Change the Y-axis of the histogram to see detail more clearly, cycling through four possible resolutions; linear, log, base 10 and base 100. To do this:

PC: Ctrl-click in the histogram area

Mac: Alt-click in the histogram area



Select all Values. Select this tool to display the maximum range of values for the threshold mode that you have selected.

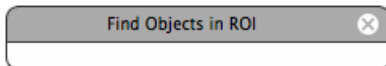


Invert Region of Selection. Select this tool to reverse the area of selection on the histogram.

Get From ROI will set the minimum and maximum thresholds to the minimum and maximum intensity value found within an ROI active on the Image

Find Objects in ROI

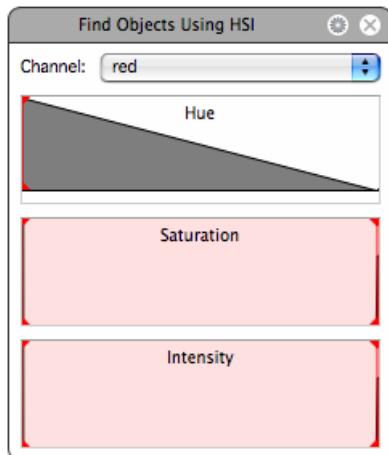
This task will incorporate the objects within an ROI into the protocol. This allows those objects to be included in later processing.



Find Objects Using HSI

This task selects voxels according to HSI color information.

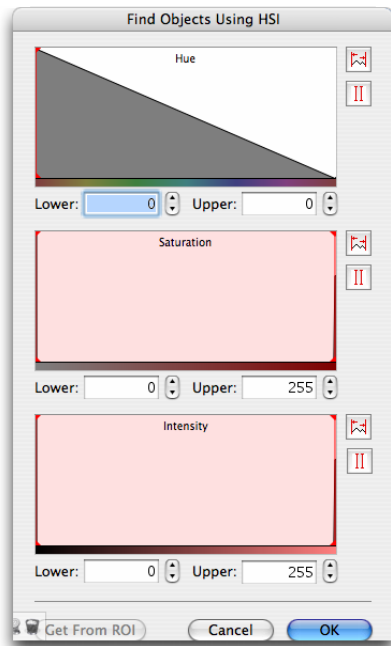
The primary task shows three histograms of the current volume. Number of voxels is represented along the Y-axis; hue, saturation and intensity values are along the X-axis in each histogram.



Click on the red vertical lines in the histograms and drag them to change the selected range of values. Tooltips show values.

The secondary dialog shows a larger histogram of the current volume.

Text boxes allow entry of values as an alternative means of setting thresholds.



Click on the red vertical lines in the histograms and drag them to change the selected range of values.



Select all Values. Select this tool to display the maximum range of values for the threshold mode that you have selected.



Invert Region of Selection. Select this tool to reverse the area of selection on the histogram.

Get From ROI will set the minimum and maximum thresholds to the minimum and maximum values found within an ROI active on the image.

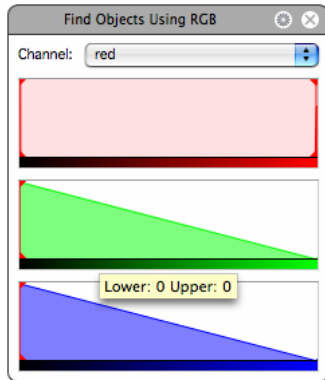
Measurement Protocol Tasks

Find Objects Using RGB

This task selects voxels according to RGB color information.

The primary task shows three histograms of the current volume. Number of voxels is represented along the Y-axis; red, green and blue intensity values are along the X-axis in each histogram

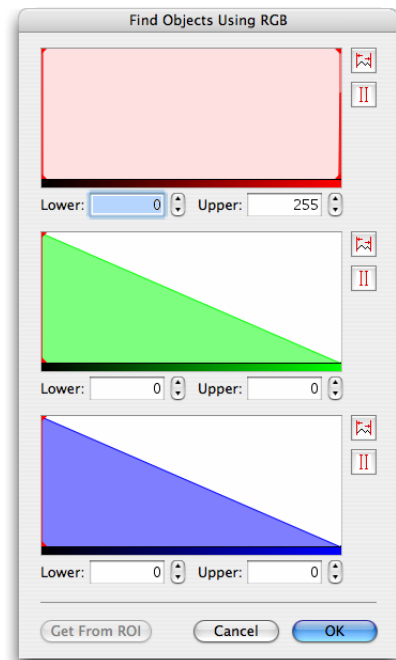
Click on the red vertical lines in the histograms and drag them to change the selected range of values. Tooltips show values.



A secondary dialog shows larger histograms, which because of the increased scale may be easier to use for fine adjustments.

Number of voxels is shown along the Y-axis; color or intensity values along the X-axis.

Text boxes allow entry of values as an alternative means of setting thresholds.



Click on the vertical red lines in the histograms and drag them to change the selected range of values.



Select all Values. Select this tool to display the maximum range of values for the threshold mode that you have selected.



Invert Region of Selection. Select this tool to reverse the area of selection on the histogram.

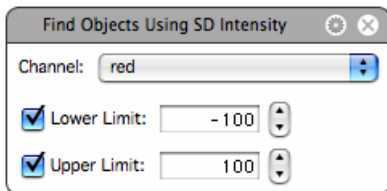
Get From ROI will set the minimum and maximum thresholds to the minimum and maximum values found within an ROI active on the image.

Find Objects Using SD Intensity

This task selects voxels according to intensity information.

Intensities within the current volume are represented as standard deviations above or below the mean intensity. This removes the need to select by absolute intensity values and may work better for time resolved data sets.

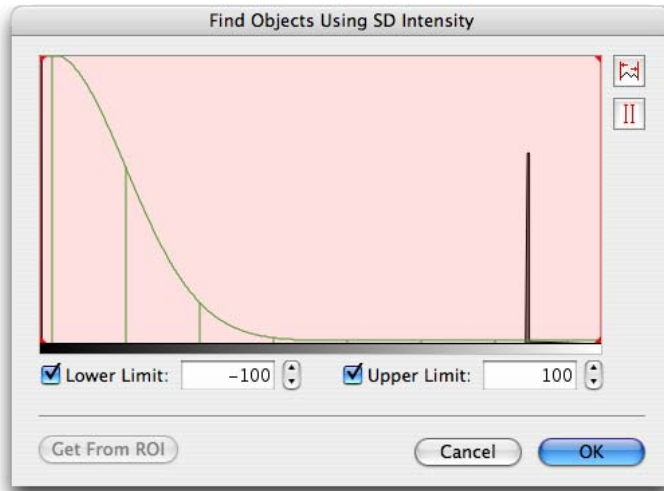
The primary task shows text entry boxes to set the lower and upper limit of the selection in numbers of standard deviations. Positive values are above the mean, negative values are below the mean. Uncheck the upper or lower limit boxes to allow everything above or below a set point to be selected.



Click on the cog icon to open the secondary dialog for this task. The secondary dialog shows a histogram of the current volume; number of voxels is along the Y-axis, color or intensity values are along the X-axis. Superimposed over the histogram is a curve (in green) showing the normalized intensity. Vertical green lines are shown at the mean intensity and at each standard deviation above and below the mean. Data may, therefore, be thresholded based on a set number of standard deviations around the mean.

Measurement Protocol Tasks

Set lower and upper limits by entering the value of standard deviation at which the limit is to be set. The mean intensity is at 0 standard deviations and standard deviations below the mean intensity have negative values.



Click on the red vertical lines in the histograms and drag them to change the selected range of values .



Select all Values. Select this tool to display the maximum range of values for the threshold mode that you have selected.

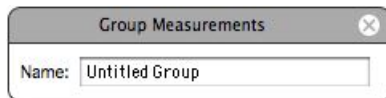


Invert Region of Selection. Select this tool to reverse the area of selection on the histogram.

Get From ROI will set the minimum and maximum thresholds to the minimum and maximum values found within an ROI active on the image.

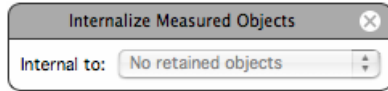
Group Measurements

This task identifies a set of measured objects, points or lines with text in an additional column in measurement tables. This column can be used to identify the measurements as a group, even after tables have been exported as text.



Internalize Measured Objects

Objects found by one series will be related to the objects found by another series according to one set of objects being inside or outside the other.

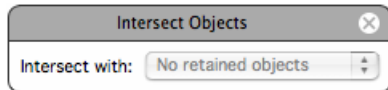


Objects from the second series that are within the same object from the first series will be given the same parent ID.

The first series of objects must include a **Retain Objects** task which will name the first set of objects to enable them to be specified in this task.

Intersect Objects

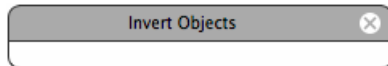
Objects are found by one series, a second series finds a different population. **Intersect objects** will find just the areas where both sets of objects overlap.



A **Retain Objects** task must be used in the first series to name the first set of objects to enable them to be specified in this task.

Invert Objects

This task will invert the selection made by the series so far. This may be useful in situations when it is easier to select background or unwanted objects and reversing the selection serves to select the required objects.

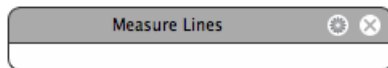


Measure Lines

This task ensures that line measurements are recorded.

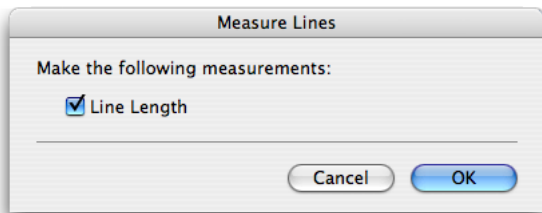
Draw lines using the line tool available from the tool bar. As lines are drawn the line length is displayed in the Measurements table below the image. This is the default display.

The default display may change as other objects are found and the line measurements will no longer be displayed. If they are no longer displayed in the table below the image, they will not be recorded in any measurement item created unless you include a **Measure Lines** task in your protocol.



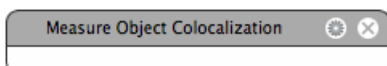
Measurement Protocol Tasks

The secondary dialog of the Measure Lines task shows checkboxes to select the measurements to be made. Currently, line length is the only measurement available for lines.

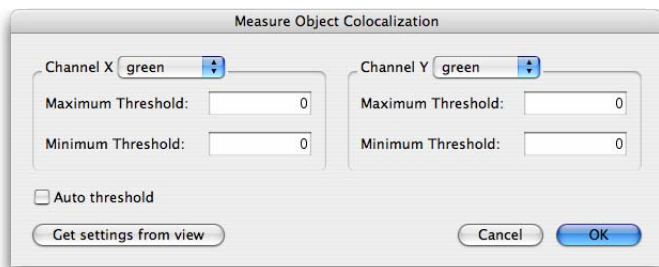


Measure Object Colocalization

Colocalization in the context of digital fluorescence imaging can be described as the detection of signal at the same voxel location in each of two channels. The two channels are made up of images of two different fluorochromes taken from the same sample area.



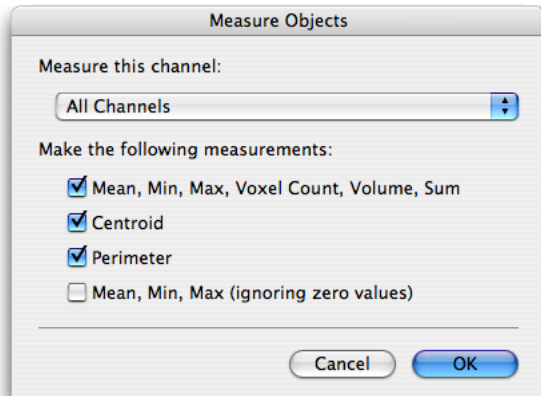
Measure Object Colocalization will calculate the colocalization coefficients M_x and M_y for each object found by the series. Click on the cog icon to access the secondary dialog and configure the task.



In the secondary dialog select the channels to be colocalized.

Thresholds are essential. Enter the intensity values for each channel. Voxels with intensity values below the minimum threshold and voxels with intensity values above the maximum threshold will be excluded from the calculations. Use minimum thresholds to remove intensities in each channel that are not significant in a determination of colocalization.

To measure morphological and intensity information from objects include a **Measure Objects** task as well as the **Measure Object Colocalization** task. Configure the secondary dialog of the **Measure Objects** task to record the required measurements.



Alternatively, configure the colocalization view for the data set before working with the Measurements View and click **Get settings from view** to use these settings in the task.

See also the section on Colocalization starting on page 412.

Measure Objects

Objects are selected either by using the ROI tools available from the tool bar or by using **Find Objects** task in a measurement protocol.

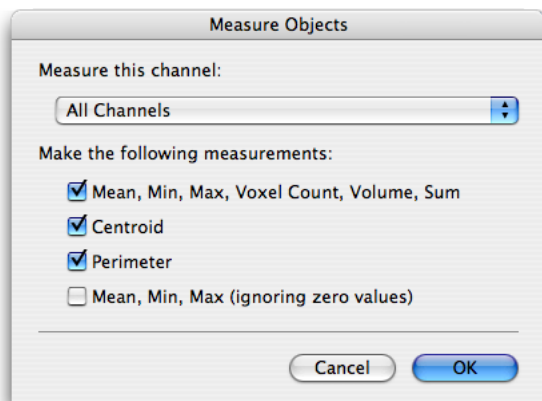
As objects are selected, measurements are displayed in the Measurements table below the image. This is the default display. For each object found the table will display:

- Voxel/Pixel count
- Volume (μm^3)/Area (μm^2)
- Min, Max, Mean and Sum intensity values. Each channel in the data set will have a set of intensity columns.
- Centroid position in pixels and micrometers
- Surface Area (μm^3)/Perimeter (μm^2)

The default display may change as other objects are found and the object measurements will no longer be displayed. If they are no longer displayed in the table below the image, they will not be recorded in any measurement item created.

Measurement Protocol Tasks

Ensure the measurements required are recorded or change the measurements to record by using a **Measure Objects** task.



Intensity measurements ignoring zero are useful when working with data where background values are always zero. This occurs after certain processing steps, for example ratio and FRET calculations.

A **Measure Objects** task may also be used partway through a protocol to record measurements at that point and again later to measure a new set of measurements.

Once the **Measure Objects** task is included, default measurements will not be generated.

The centroid of an object is the unweighted centre. It is calculated by taking the average position of the voxels making up the object. Thus, the average x coordinate of the voxels determines the x coordinate of the centroid and the average y coordinate of the voxels determines the y coordinate of the centroid.

Surface area of objects is measured by fitting a “skin” to the surface of the mask layer identifying the object at the voxel level. Triangles are then fitted to each cluster of voxels on the surface of the object. The area of each triangle is measured; the sum of triangle areas for each object gives the surface area. There is no surface simplification for measurement. It is important, when using any measurement, to ensure that results are statistically significant compared to any measurement error or noise. This is especially important with surface area measurements where noise and surface features can have a considerable impact on the measurement.

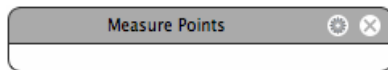
Measure Points

Mark points using the point tool available from the tool bar.

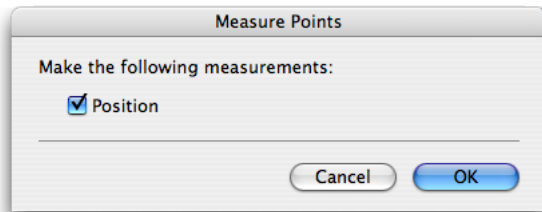
As points are marked, the point location is displayed as a coordinate in the Measurements table below the image. This is the default display.

The default display may change as other objects are found and the point positions will no longer be displayed. If they are no longer displayed in the table below the image, they will not be recorded in any measurement item created.

Ensure the point locations are recorded by using a **Measure Points** task in the protocol.

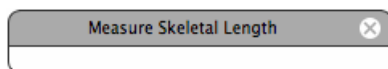


The secondary dialog of the **Measure Points** task shows checkboxes to select the measurements to be made. Currently, **Position** is the only measurement available for points.



Measure Skeletal Length

This is an additional measurement that may be made from objects.



Skeletal length is calculated by first thinning or skeletonising the object from all sides until a single voxel line is left. This measurement is best suited to elongated shapes. The direction of the line is elongated to meet the edges of the object and the length of this line is the skeletal length. For objects where the skeleton branches, the skeletal length is measured as the longest traverse across the object without crossing a point more than once.

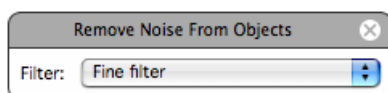
Skeletal diameter is the diameter of a cylinder if it had a length equal to the skeletal length of the object determined as above and a volume equal to the object's measured volume.

It is derived using the equation for the volume of a cylinder.

$$V = \text{Pi } r^2 * l$$

Remove Noise from Objects

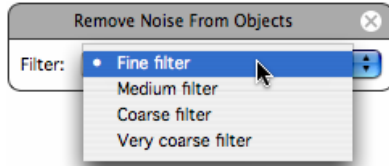
Use this task to apply a median filter to the selection already made. The selection is made by a **Find Objects** task earlier in the series.



The size of the filter applied is selected in the drop-down on the task.

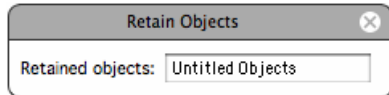
Measurement Protocol Tasks

From the smallest fine filter to the largest very coarse, the median filter matrix is increased in size. The time taken to apply the filter and the degree of filtering also increase.



Retain Objects

This task retains the objects found by that point in the protocol in a temporary way solely during the running of the protocol.



Filling in the name field allows the set of objects to be used later, by for example **Intersect** and **Combine Objects** tasks that process the output of one series of measurements tasks with the output of a previous series that has been retained.

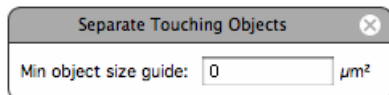
Restore Objects

This task updates the selection to show any objects that have been retained as a set previously.



Separate Touching Objects

This function can assist in the separation of two or more objects that a **Find Objects** task has identified as one. The distance of each voxel from the surface of the selection is examined and, if the distribution of the voxels indicates that two or more objects are present, a one-pixel thick row of voxels is removed between them to create separate objects.



The minimum size guide may be optionally set to the typical size of a single object. This refines the task by preventing separation if the distance between two new objects would be too small.

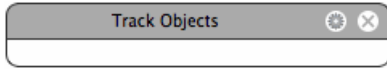
Subtract Objects

Subtract the area masked by the retained objects from one series from the objects found in the current series.



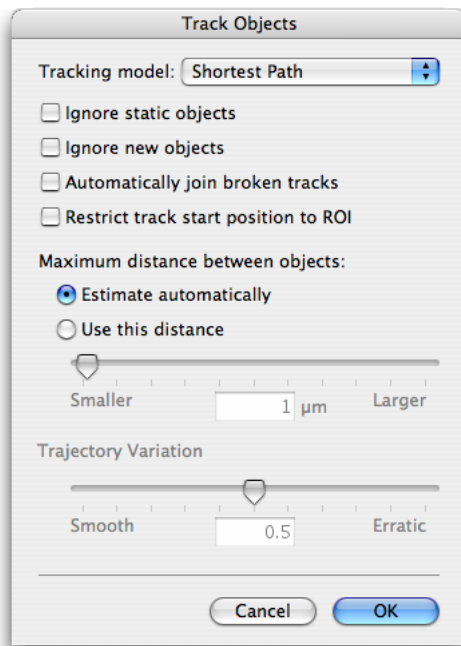
Track Objects

This task is particular to time resolved data. The **Track Objects** task will place itself at the bottom of any protocol since objects must be found before they can be tracked.



The Measurements View shows only the protocol as applied to the current timepoint by default. It may be useful to turn on **Measure All Timepoints** in the Measurements menu to preview all the measurements over time and the tracks.

The Velocity tracking algorithms operate on centroid positions. A track is generated by connecting centroids to trace the path of a moving object. Different models are used to identify the most likely position for each object being followed in each timepoint.



The **Shortest Path** model attempts to create tracks based on the criterion that the chosen matches between the populations of object centroids in any pair of timepoints have the minimum physical distance between them.

The **Trajectory Variation** model uses the Shortest Path model to first establish a likely track for an object between centroids in timepoint 1 and timepoint 2. The next centroid position of that object is determined by that which is consistent in both direction and speed. This model should be effective for objects that cross paths.

Measurement Protocol Tasks

When the **Trajectory Variation** model is selected, the Trajectory Variation slider is used to alter the sensitivity of the algorithm to variation. Lower trajectory variation restricts the calculated tracks to smoother trajectories. If this value is set too low, tracking may fail to find any valid tracks.

Other Options

- If required, enable the **Ignore Static Objects** and **Ignore New Objects** checkboxes.
- **Restrict track start position to ROI** requires an ROI to be drawn on the image. When this option is checked, tracks will only be measured if they start with the drawn ROI.
- **Automatically Join Broken Tracks** is a feature that allows tracks to be continued, even if the object was not detected in periods of up to two consecutive timepoints along the trajectory.
 - With **Automatically Join Broken Tracks** off, the default setting, a track will terminate if a timepoint is reached where no centroid can be found that is likely to be part of the track based on the restrictions determined by the parameters entered in the **Track Objects** dialog.
 - When **Automatically Join Broken Tracks** is on the tracking algorithm will join a partial track to another partial track beyond the missing section as long as the whole track has a consistent trajectory.

It may be appropriate to allow Volocity to **Automatically Join Broken Tracks** where objects have not been measured in all timepoints along their path length. An object may fail to be detected in a timepoint because of factors such as noise or inconsistent illumination.

Users may also join tracks manually after the creation of a measurement item using the **Join Objects** option in the Raw menu. See page 385.

- **Maximum distance between objects** must be set for both tracking models.
 - **Estimate automatically:** Volocity will calculate a maximum, effective matching distance to use as an upper bound for possible motion tracking between timepoints in a sequence based on the size of the dataset ($\frac{1}{4}$ of the diagonal of the volume).

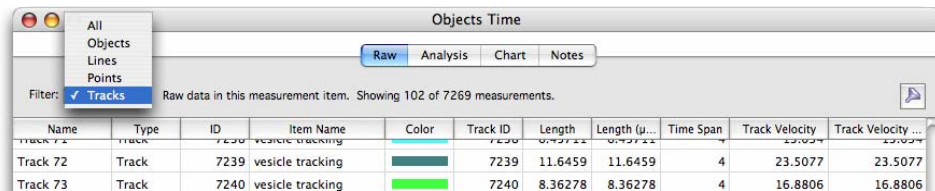
Volocity will then attempt to make a shortest path matching between the first two timepoints using this calculated upper bound. The maximum distance between objects calculated for this dataset is the average distance between all the matched object centroids in the first two timepoints plus one standard deviation. This value is then used for the maximum distance throughout the data set. The text box for **Use this distance** mode displays the last used value for the maximum distance between objects, including when this value was estimated automatically. This feedback may be referred to when refining tracking parameters.
 - **Use this distance:** Volocity will use the user-specified distance as the maximum limit for matching object centroids between timepoints.

Tip: Establish a maximum distance to set by scanning the time series and choosing two timepoints between which the greatest movement is apparent. Obtain a value to enter in the **Track Objects** dialog by measuring this movement using the line tool. Adjustment to this distance can be made for subsequent measurement, if required.

The tracking algorithm assigns a tracking ID to objects. If it determines that an object in a timepoint is the same as an object in a previous location, it gives that object the same ID. The track (or line connecting all the objects with that ID) also has the same ID.

Viewing Tracks

Tracks measured are added to the same Measurements item as the objects. Use the quick filter drop-down to view only tracks.



Select a track row in the Measurements Item to show feedback of the track within the image data that it came from. If that data is open to a view that supports feedback, the objects associated with that track, objects with the same tracking ID, will also be shown as Measurements feedback.

Use the feedback options to show or hide the objects or to show the objects only in the current timepoint. This is useful if you want to make a movie showing the objects moving along their tracks.

Manually Track Objects (Tools menu)

When it is not possible to use Velocity's automatic tracking algorithms to track moving objects within a time-resolved data set, manual tracking will allow you to create track objects in a Measurement item by manually marking the object location in each timepoint.

Open the image sequence containing the data you wish to track.

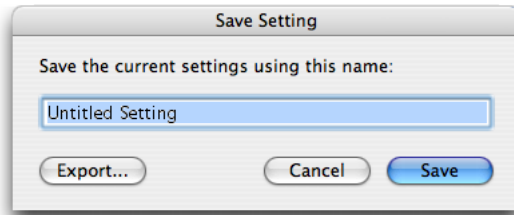
1. View the Image or Measurements View. These views allow use of the point and ROI tools which are needed to mark object locations.
2. Select **Manually Track Objects** from the Tools menu.
3. Follow the instructions given by the Manual Tracking wizard.

Clear, Save, Restore and Export Protocol

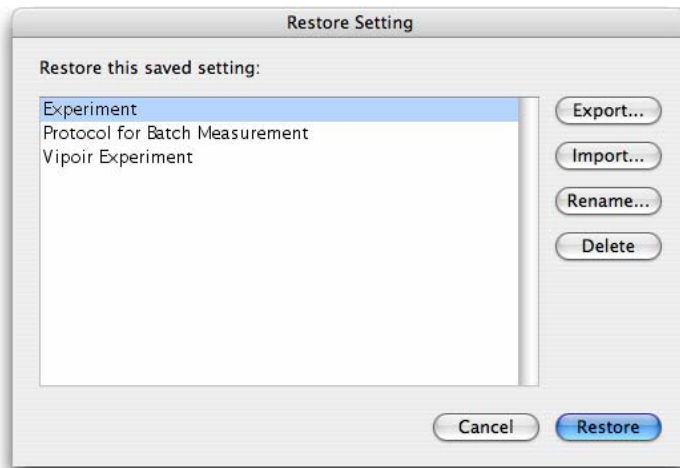
- Select **Clear Protocol** from the Measurements menu to remove a protocol from the Measurements View without saving it.

Clear, Save, Restore and Export Protocol

- Select **Save Protocol...** from the Measurements menu to save a protocol and use it again in the future. Protocols are stored as preferences at user level.

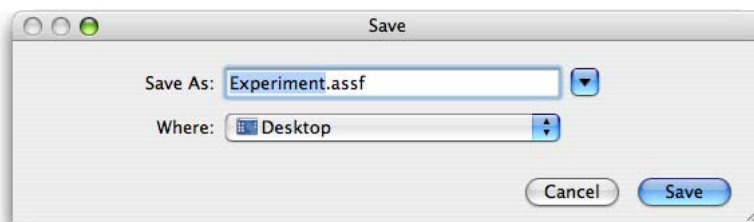


- Select **Restore Protocol...** from the Measurements menu to restore an existing protocol and use it with the active Measurements View.



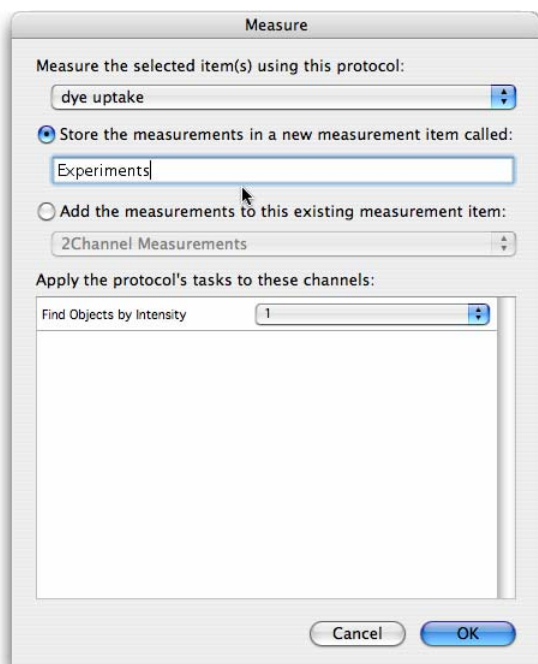
You can also use this dialog to manage protocols. It allows renaming and deletion of existing protocols. Use **Import...** to add a protocol exported as a settings file (.assf extension) to the protocol on the system.

- To share protocols with other users, click **Export...** on either the Save Protocol dialog or the Restore Protocol dialog. This will create a settings file, with the extension .assf. Settings files are not recognized as protocols unless they are imported.



Applying Saved Protocol Batch Processing Measurements

1. Select **Measure...** from the Tools menu.

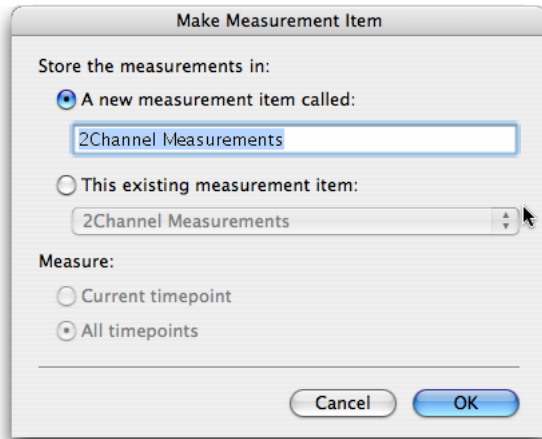


2. Select the protocol to be applied to the selected items from the drop-down menu, which lists all saved protocols by name.
3. Either, select **Store the measurements in a new measurement item called:** and enter a name for the Measurement item that will contain all the measurements made by the selected protocol on the selected items. Or, select **Add the measurements to this existing measurement item:** and choose from the measurement items that already exist in the library. In this case, all the measurements from all the selected items will be added to the measurements already in the table.
4. For each task in the protocol that operates on a specific channel (i.e. the Protocol dialog contains a drop-down for channel selection when the task is configured), there will be an entry under **Apply the protocol's tasks to these channels:** The drop-down lists the unique channel names found within the selected items. Channels with the same name will all be processed.
5. The Measurement item created will contain all the measurements made on all the selected items.

Measurement Items

Make Measurement Item

Once you are happy with the measurements from points, lines and regions marked on the Measurements View, use **Make Measurement Item...** from the Measurements menu to record measurements.



Volocity records measurements to the specified Measurement item. You can then view the measurements as a table. This table has a number of views.

A Measurements item is also created when a saved protocol is applied to selected items in the library (using the Measure dialog from the Tools menu).

Note: The **FRAP Analysis...** command in the Tools menu generates a measurement item. Designed specifically for FRAP analysis this measurement item will appear different from one created by other measurement actions in Volocity and you will interact with it differently. See “FRAP Measurement Item” on page 429.

There are now three stages that you can work through to extract patterns or trends from image data.

- Organize and filter the raw data so that it is ready for analysis.
- Analyze the raw data.
- Chart the results.

Raw Data View

The initial table view shows the **Raw** measurement data.

The screenshot shows a window titled '2Channel Measurements' with tabs for 'Raw', 'Analysis', 'Chart', and 'Notes'. The 'Raw' tab is active, displaying a table with 17 rows of data. The table has columns for Name, Type, ID, Item Name, Volume (µm³), Timepoint, Abs. Time, Rel. Time..., Color, Centroid X, and Centroid Y. The data is as follows:

Name	Type	ID	Item Name	Volume (µm³)	Timepoint	Abs. Time	Rel. Time...	Color	Centroid X	Centroid Y
Object 1	Object	1	Neuroblastoma	1	1 12:58:5...		0		161	
Object 2	Object	2	Neuroblastoma	47172	1 12:58:5...		0		349.662	2
Object 3	Object	3	Neuroblastoma	2	1 12:58:5...		0		172.5	
Object 4	Object	4	Neuroblastoma	14	1 12:58:5...		0		328.5	2
Object 5	Object	5	Neuroblastoma	1	1 12:58:5...		0		80	
Object 6	Object	6	Neuroblastoma	2	1 12:58:5...		0		84	
Object 7	Object	7	Neuroblastoma	2	1 12:58:5...		0		241.5	
Object 8	Object	8	Neuroblastoma	46	1 12:58:5...		0		75.9565	
Object 9	Object	9	Neuroblastoma	1	1 12:58:5...		0		305	
Object 10	Object	10	Neuroblastoma	7	1 12:58:5...		0		304.429	
Object 11	Object	11	Neuroblastoma	110710	1 12:58:5...		0		134.898	2
Object 12	Object	12	Neuroblastoma	189721	1 12:58:5...		0		81.0743	9
Object 13	Object	13	Neuroblastoma	261063	1 12:58:5...		0		232.704	1
Object 14	Object	14	Neuroblastoma	3	1 12:58:5...		0		192.667	1
Object 15	Object	15	Neuroblastoma	1	1 12:58:5...		0		182	
Object 16	Object	16	Neuroblastoma	1	1 12:58:5...		0		182	
Object 17	Object	17	Neuroblastoma	204309	1 12:58:5...		0		246.054	3

There are a number of ways of organizing and filtering the raw data so that you can review it before analysis.

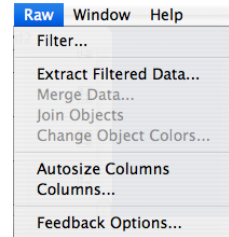
Working with Columns

Column headings show which measurements were made. N/A in a column means the data is not available. For example, if a session contains both line and region measurements, **Total Length** is not a relevant measurement for a region so that column will have N/A against all region rows.

- To reorganize columns click the column heading, drag it to its new position and release.
- To sort columns click on the column heading—an arrow shows that the column is sorted and the direction of the arrow indicates whether the sort order is ascending or descending. Click again to reverse the sort. For example: sort on the **Name** column to group together the same types of measurement. Sort on the **Voxel Count** column to find the largest and smallest objects.
- To change the width of a column click on the cell border and drag.

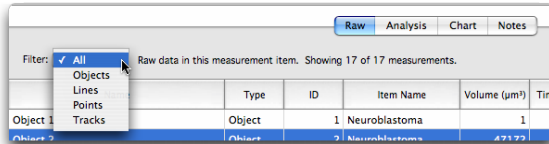
Raw Data View

- To resize all columns to fit contents, select **Autosize Columns** from the Raw menu.
- To hide/show columns select **Columns...** in the Raw menu.
All columns that can currently be shown will be displayed in the dialog.
To show a column, check the box corresponding to the column name and click **OK**. (Click **Hide All** or **Show All** to uncheck/check all boxes respectively.)

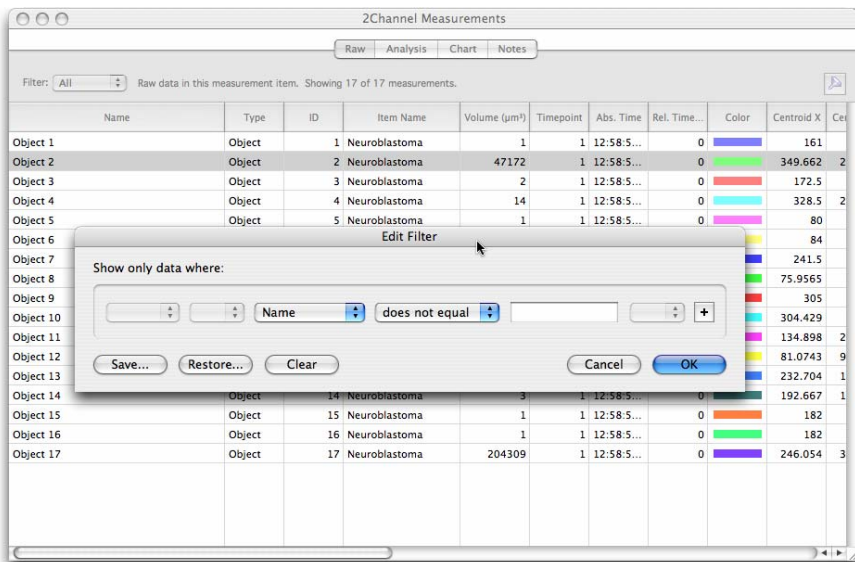


Filter

Set up filters to hide unwanted data. Quick filtering on the basis of type of measurement can be achieved with the **Filter** drop-down. Choose to show just object measurements or just line measurements.



For more advanced filtering select **Filter...** from the Raw menu or click on the filter button at the top of the window.



Filter out rows by selecting the column to filter on and entering values to select by. And/Or expressions are permitted, as is the use of brackets.

Click on the **Save...** button to save a filter as a preference to easily access the same criteria again.

Once a filter is applied the number of filtered measurements is displayed. To cancel a filter open the Filter dialog and click **Clear**.

Extract or Merge Data

You can create new Measurements items from existing items by combining their contents.

Where applying a filter has created a new table that you would like to process further, use **Extract Filtered Data...** from the Raw menu to create a new Measurement session containing just that data. Name the new Measurement Item to be created.

To add the contents of the current table to a Measurement Item that already exists in the library use **Merge Data...**This tool allows you create tables that are combinations of other data.

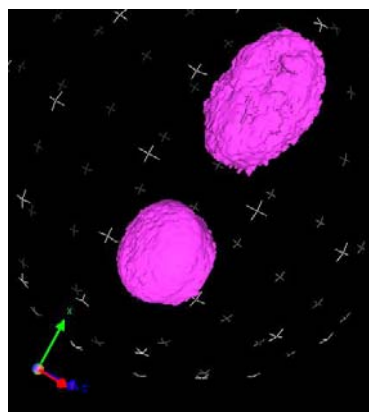
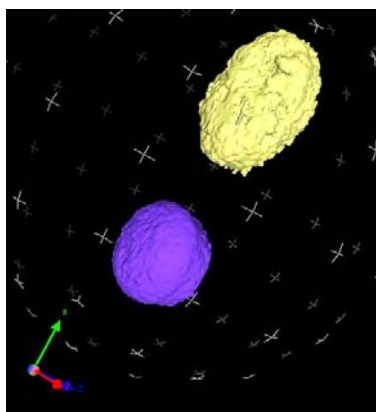
- **Remove duplicate rows** will ensure that the same measurement is not repeated.
- **Merge similar rows** will ensure that different measurement criteria, indicated by the column heading, for the same object are kept in one row, not spread over two or more.

Join Objects

This feature can be used to join two or more objects, polylines or tracks. Any number of objects can be joined.

Select the objects to be joined in the table and click **Join Objects** in the Raw menu. A new object will be created in the table, consisting of the union of all selected objects.

Note that this object may be non-contiguous, that is, it may contain two distinct areas which do not touch. However, for the purposes of measurement and feedback it is considered to be a single object. This new object will be re-measured, with all the appropriate measurements. Note that the previously selected objects are not deleted or altered in any way.

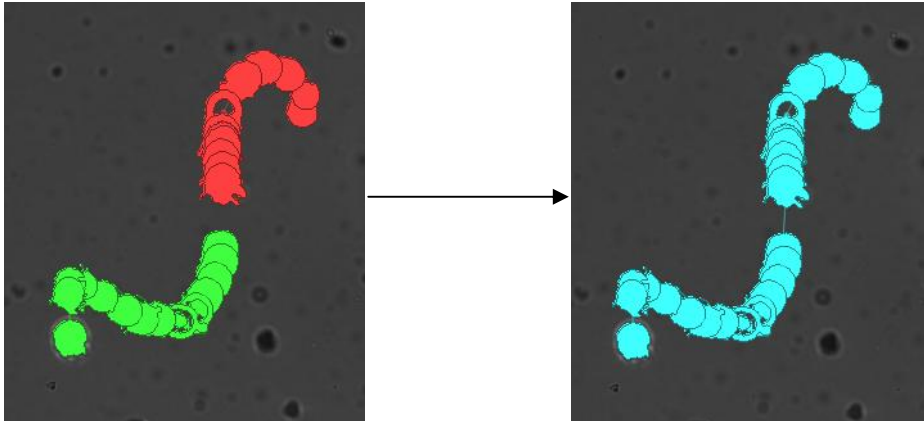


Measured lines can also be joined using this command when two or more lines are selected in the table. The ends of each line which are closest to each other are joined together, creating a single line. This new line is added to the table and re-measured. Again, the previously selected lines are not deleted or altered.

Raw Data View

Finally tracks can also be joined, but only under certain circumstances. Two tracks which track an object through the same timepoint cannot be joined. For example: if Track A tracks an object from timepoint 1 to timepoint 5, and Track B tracks an object from timepoint 5 to timepoint 9, the tracks **cannot** be joined, as they both track an object through timepoint 5.

If Track C tracks an object from timepoint 1 to timepoint 4, then it can be joined with Track B. The tracks do not share a timepoint. If the tracks selected cannot be joined, the command will be disabled in the Measurements menu. Any number of tracks can be joined, providing that no two selected tracks share a timepoint.

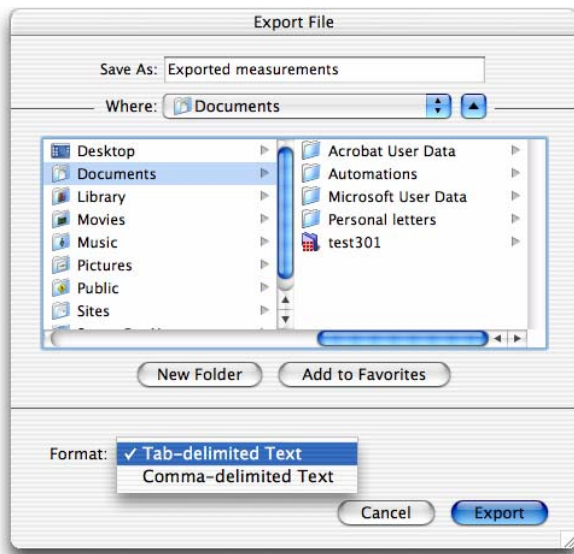


When two or more tracks are joined, a new track is created in the Tracking table. This track is assigned a new Tracking ID, and is re-measured for length, velocity, etc. All objects which were previously associated with the selected tracks are now only associated with the new track, and their tracking IDs will all be reassigned to the new track.

Note that this does not delete or alter the tracks previously selected; however the objects previously associated with them will now be associated with the new track instead.

Exporting Measurements

Once you have created a Measurements table, you can also export the measurements. Select **Export** from the File menu and select the required file format.



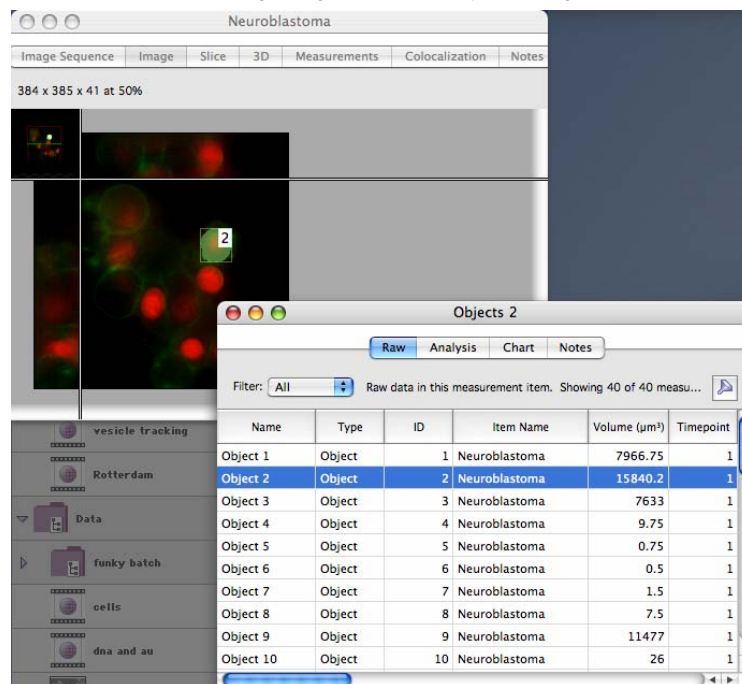
Feedback from the Raw View

If a row in the Raw View of a Measurement Item corresponds to a single measured object, its location in the original image data can be shown using feedback. When the row is selected the object will be shown using colored feedback in any open Image, Measurements or 3D View (if available) of the data from which that measurement was made.

Select measurements in a view by clicking on a row, shift and click to select multiple rows.

Raw Data View

Open the corresponding image data, usually an image sequence, and the feedback will be shown.



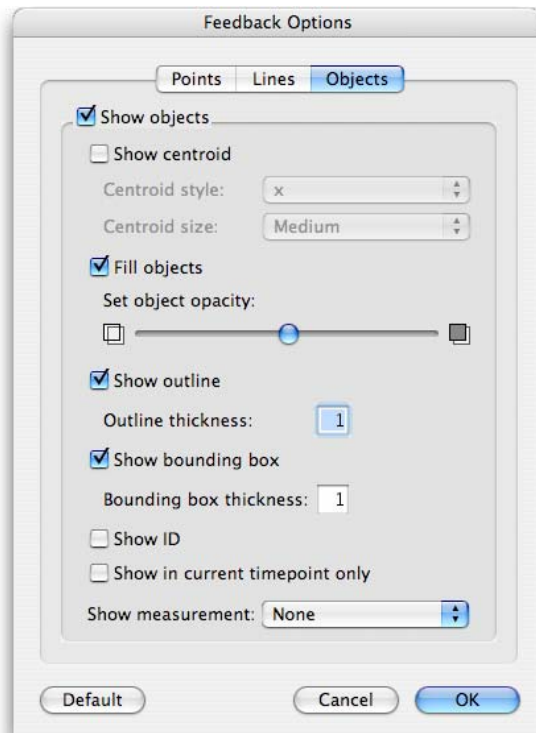
To control how feedback is displayed:

- Use the **Feedback Options...** command in the Measurements menu to define the feedback display.
- Use the **Change Object Colors...** command in the Raw menu to define the color of object feedback.

Feedback Options Dialog

The Feedback Options dialog is tabbed, with one tab for each type of measurement object that may be present in the table. The types available vary with different software products in the Velocity family.

Each option changes how the feedback will be shown in views of the original data that support feedback display.



Use these options to change how the feedback appears:

- **Show in current timepoint only** is an option for viewing feedback within time resolved data. When this option is checked only feedback that relates to measurements made from the current timepoint will be shown. Changing the timepoint using the Navigation palette will change the feedback so that only objects in the new timepoint are shown.
- **Show measurement** will allow any of the measured parameters to be shown alongside the colored shape that represents the measurement.

Note: Some options do not take effect for feedback shown in 3D views. All feedback in 3D views is shown as solid isosurfaces.

Change Object Colors

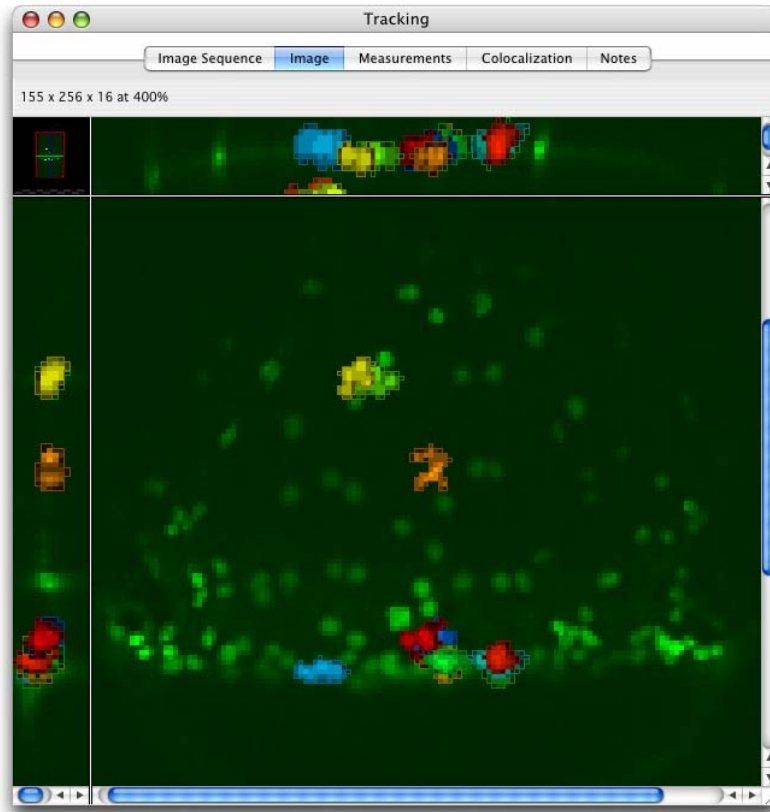
This dialog changes the color used to represent the selected objects with this dialog.

When **Rainbow colors** is applied, all selected objects or tracks will be colored according to their position in the table. Objects at the top of the table will be colored red; objects further down the table will step down through the visible spectrum, with objects at the bottom colored violet.

This feature can be used to color objects according to any attribute measured by Volocity. The following example colors objects according to their measured volume.

1. Click on the header at the top of the **Volume** column to order all objects in the table by their measured volume.
2. Select all the objects in the table that you wish to recolor and select **Change Object Colors...** in the Measurements menu.
3. Select **Use Rainbow Colors** and click **OK**.

The color coding on the measurements feedback in the other views now reflects the objects' measured volume.



If Feedback is not Displayed

If feedback is not displayed as expected, check the item name column for the selected measurements. The correct image data must be open.

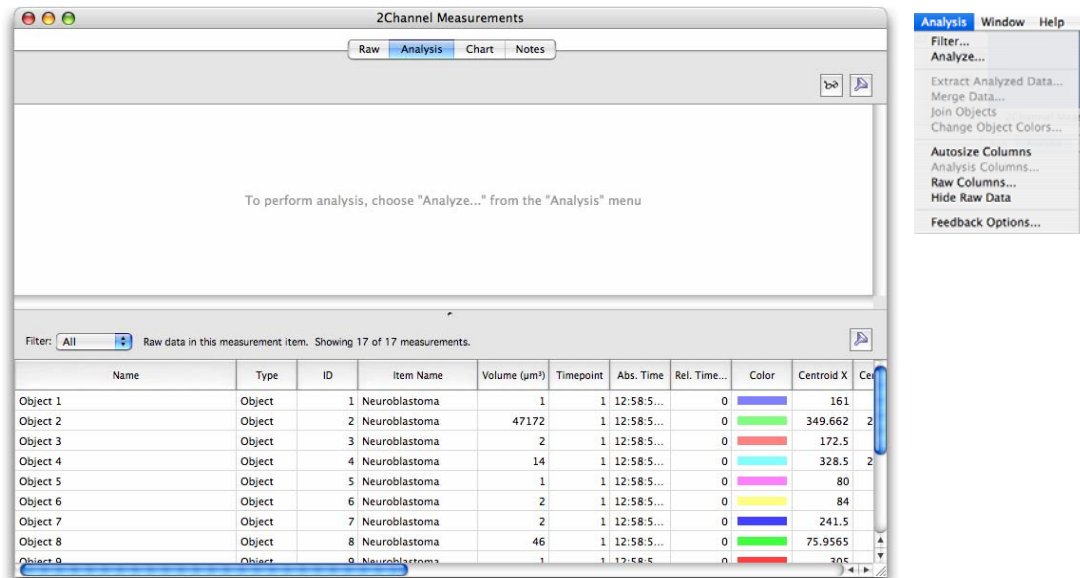
Check the **Feedback Options** dialog to check that the feedback is prominent enough against the image data.

Check the status of **Show objects in current timepoint only**. Use the Navigation palette to move to the correct timepoint or uncheck this option to show all objects regardless of the timepoint in which the object was measured.

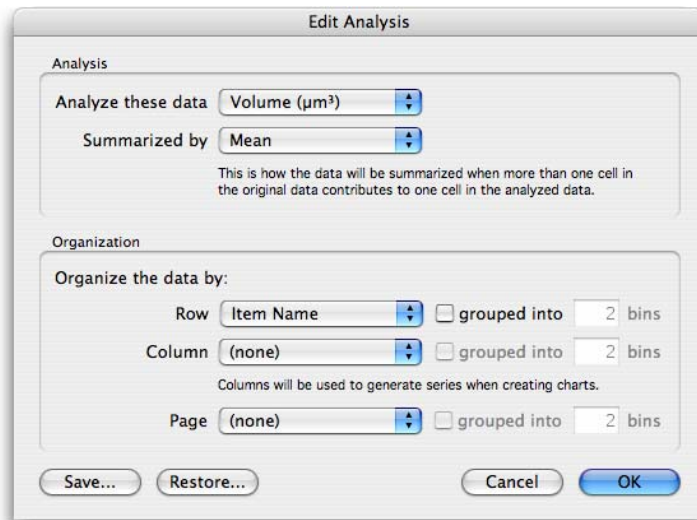
Analysis View

Click on the Analysis tab of a measurement item to work with the data and organize it in different ways. The Analysis View is the second step in the process of extracting patterns or trends from image data.

The top part of the view will show the reorganized data, the lower part shows the raw table. Select **Hide Raw Data** from the Analysis menu to show only the reorganized data.



Select **Analyze...** from the Analysis menu to open the dialog that will allow you to perform this powerful organization.



Analyze these data: The drop-down shows all the measured criteria available for the objects in the Raw table, select one as the subject of analysis. If the analysis is to look at intensity over time, select a column containing intensity values.

Summarized by: Choose from Value, Mean, Sum, Product, Count, Min/Max, Variance/SD.

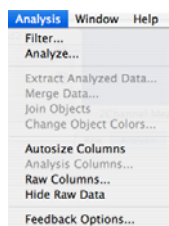
Organize the data by: Select other measured criteria from the raw table to show the relationship between those criteria and the subject of the analysis selected in **Analyze these data:** If the analysis is to look at intensity over time, select the time or timepoint column here.

It is possible to organize the data by up to three criteria, rows, columns and pages.

Grouped into bins will allow you to divide the rows, columns and/or pages into groups to best present the data.

Data filtered out of the Raw View will not be included in analysis but the filter may be edited at any time by selecting **Filter...** from the Analysis menu or clicking on the filter button at the top of the screen.

The Analysis menu contains options for working with the analysis table created.



Extract Analyzed Data... creates a new Measurement item containing just the analyzed or reorganized data, which can be analyzed further.

Merge Data... adds the analysis table to an existing measurement item.

Remove duplicate rows ensures that the same measurement is not repeated.

Merge similar rows ensures that different measurement criteria, indicated by the column heading, for the same object are kept in one row, not spread over two or more.

Analysis View

This could be used to combine two or more different analysis methods in one table. The result of one way of organization is extracted to a new measurement item then the original item reorganized and the result merged with the item created in the first step.

Change the appearance of the tables shown in this view by using the options for columns in the Analysis menu.

- To re-organize columns click the column heading, drag it to its new position and release.
- To sort columns click on the column heading—an arrow shows that the column is sorted and the direction of the arrow indicates whether the sort order is ascending or descending. Click again to reverse the sort. For example: sort on the Name column to group together the same types of measurement.
- To change the width of a column click on the cell border and drag.
- To resize all columns to fit contents, select **Autosize Columns**
- To hide/show columns select **Analysis** or **Raw Columns...** in the Analysis menu.

Analysis Examples

Find the Total Number of Objects Measured

A measurement item Raw View contains one row of information per object. In the following example a protocol was applied to five image sequences and the table contains over 32000 measurements.

Name	Type	ID	Item Name	Volume (µm ³)	Timepoint	Abs. Time	Rel. Time...
Object 8479	Object	8479	Experiment 1	0.301692	2	14:57:3...	120.948
Object 11701	Object	11701	Experiment 1	0.452538	2	14:57:3...	120.948
Object 13547	Object	13547	Experiment 1	0.50282	2	14:57:3...	120.948
Object 33478	Object	33478	Experiment 1	0.402256	2	14:57:3...	120.948
Object 37416	Object	37416	Experiment 1	0.301692	2	14:57:3...	120.948
Object 41711	Object	41711	Experiment 1	0.402256	2	14:57:3...	120.948
Object 44960	Object	44960	Experiment 1	0.351974	2	14:57:3...	120.948
Object 49385	Object	49385	Experiment 1	0.351974	4	15:01:3...	361.01
Object 65239	Object	65239	Experiment 1	0.301692	4	15:01:3...	361.01
Object 66082	Object	66082	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69193	Object	69193	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69314	Object	69314	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69573	Object	69573	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69837	Object	69837	Experiment 1	0.301692	4	15:01:3...	361.01
Object 79561	Object	79561	Experiment 1	0.301692	4	15:01:3...	361.01
Object 88861	Object	88861	Experiment 1	0.553102	4	15:01:3...	361.01
Object 95418	Object	95418	Experiment 1	0.452538	4	15:01:3...	361.01
Object 96716	Object	96716	Experiment 1	0.301692	4	15:01:3...	361.01
Object 96799	Object	96799	Experiment 1	0.301692	4	15:01:3...	361.01

To find the total number of objects measured in each of the image sequences the Analysis dialog in the Analysis View is configured as follows:

- Analyze this data: **ID**. Anything that has been measured for each item could be used here.
- Summarized by: **Count**. The total number of IDs and therefore the total number of objects.
- Row: **Item Name**. Show the name of the library item to organize the data by image sequence.
- Column: Not required.
- Page: Not required.

Analysis Examples

Edit Analysis

Analysis

Analyze these data ID

Summarized by Count

This is how the data will be summarized when more than one cell in the original data contributes to one cell in the analyzed data.

Organization

Organize the data by:

Row Item Name grouped into bins of size 1

Column (none) grouped into bins of size 1

Columns will be used to generate series when creating charts.

Page (none) grouped into bins of size 1

Save... Restore... Cancel OK

The Analysis View looks like this:

Multiple Series Data

Raw Analysis Chart Notes

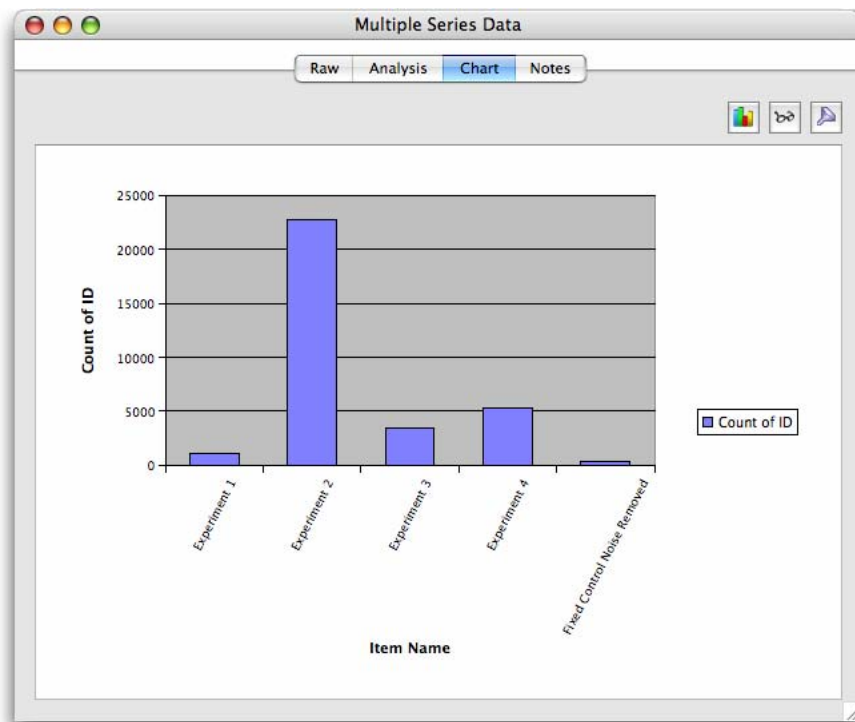
Count of data from ID, row values from Item Name.

Item Name	Count of ID
Experiment 1	1132
Experiment 2	22724
Experiment 3	3395
Experiment 4	5250
Fixed Control Noise Removed	336

Filter: All Raw data in this measurement item. Showing 32837 of 32837 measurements.

Name	Type	ID	Item Name	Volume (μm^3)	Timepoint	Abs. Time	Rel. Time...
Object 11701	Object	11701	Experiment 1	0.452538	2	14:57:3...	120.948
Object 13547	Object	13547	Experiment 1	0.50282	2	14:57:3...	120.948
Object 33478	Object	33478	Experiment 1	0.402256	2	14:57:3...	120.948
Object 37416	Object	37416	Experiment 1	0.301692	2	14:57:3...	120.948
Object 41711	Object	41711	Experiment 1	0.402256	2	14:57:3...	120.948
Object 44960	Object	44960	Experiment 1	0.351974	2	14:57:3...	120.948
Object 49385	Object	49385	Experiment 1	0.351974	4	15:01:3...	361.01
Object 65239	Object	65239	Experiment 1	0.301692	4	15:01:3...	361.01

The analysis table arranges the data for a chart. This table could be displayed as a bar chart:



Find the Total Number of Objects Measured per Timepoint

Using the same table of data as the previous example, to find the total number of objects measured per timepoint. the Analysis dialog in the Analysis View is configured as follows:

- Analyze this data: **ID**. Anything that has been measured for each item could be used here.
- Summarized by: **Count**. Generate a count of the total number of unique IDs and therefore the total number of objects.
- Row: **Timepoint**.
- Column: Not required.
- Page: Not required.

Analysis Examples

Edit Analysis

Analysis

Analyze these data: ID

Summarized by: Count

This is how the data will be summarized when more than one cell in the original data contributes to one cell in the analyzed data.

Organization

Organize the data by:

Row: Timepoint grouped into bins of size 1

Column: (none) grouped into bins of size 1

Columns will be used to generate series when creating charts.

Page: (none) grouped into bins of size 1

Save... Restore... Cancel OK

The Analysis View looks like this:

Multiple Series Data

Raw Analysis Chart Notes

Count of data from ID, row values from Timepoint.

Timepoint	Count of ID
1	120
2	2545
3	9556
4	11934
5	73
6	99
7	195

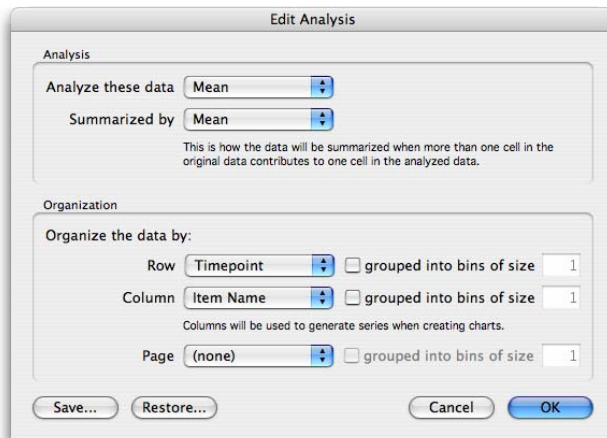
Filter: All Raw data in this measurement item. Showing 32837 of 32837 measurements.

Name	Type	ID	Item Name	Volume (µm³)	Timepoint	Abs. Time	Rel. Time...
Object 44960	Object	44960	Experiment 1	0.351974	2	14:57:3...	120.948
Object 49385	Object	49385	Experiment 1	0.351974	4	15:01:3...	361.01
Object 65239	Object	65239	Experiment 1	0.301692	4	15:01:3...	361.01
Object 66082	Object	66082	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69193	Object	69193	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69314	Object	69314	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69573	Object	69573	Experiment 1	0.301692	4	15:01:3...	361.01
Object 69837	Object	69837	Experiment 1	0.301692	4	15:01:3...	361.01

Find the Mean Intensity of Objects per Timepoint per Image Sequence

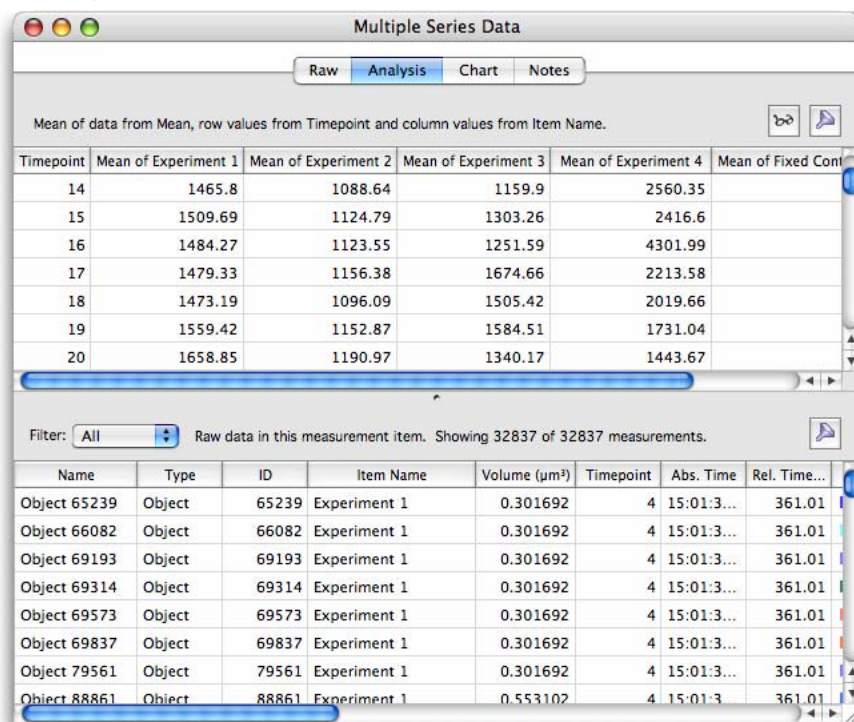
Using the same table of data as the previous example, to find out whether there is a pattern to intensities of objects dependent on timepoint and experiment, the Analysis dialog in the Analysis View is configured as follows:

- Analyze this data: **Mean Intensity**. Where more than one channel has been measured in the data set choose the mean intensity column for the channel of interest.
- Summarized by: **Mean**. If multiple objects have been measured in every timepoint this will present the mean of the mean intensities.
- Row: **Timepoint**.
- Column: **Item Name**. The Item Name is the name of the image sequence so there will be one column for each image sequence measured.
- Page: Not required.



Analysis Examples

The Analysis View looks like this:



Mean Number of Objects per Cell

Objects have been measured to identify those that are within measured cells. Objects have been given a Parent ID according to the objects they are found within, using **Internalize Measured Objects** in a measurement protocol.

Max	Mean	Sum	Voxel Co...	Centroid Z	Centroid ...	Surface A...	Surface A...	Parent ID
689	157.428	423167	2688	4.37426	2.18713	3104.97	439.47	N/A
129	117.5	470	4	0.75	0.375	12.6087	1.76684	N/A
123	115.6	578	5	0	0	14.5605	1.8589	2
113	113	226	2	0	0	10	1.35361	2
348	184.74	19213	104	3.98077	1.99038	146.235	21.9458	4
348	158.976	113191	712	5.18539	2.5927	829.29	120.715	2
121	121	121	1	0	0	6	0.835365	N/A
140	122	1220	10	0.6	0.3	27.6814	3.98682	2
199	142.824	2428	17	0.411765	0.205882	44.4364	6.02348	2
132	126.25	505	4	0	0	16	2.07299	1
112	111.5	223	2	0.5	0.25	10	1.4696	2
129	126	252	2	0	0	10	1.35361	2
121	121	121	1	0	0	6	0.835365	2
110	110	110	1	0	0	6	0.835365	2
144	130.4	652	5	0.4	0.2	13.5123	1.95419	2
116	116	116	1	0	0	6	0.835365	1
152	123.833	2972	24	1.45833	0.729167	52.0475	7.51267	N/A
109	109	109	1	0	0	6	0.835365	2
191	147.938	2367	16	0.75	0.375	34.853	4.96071	N/A

To find out how many objects are within each of the larger objects, the analysis dialog is configured as follows:

- Analyze this data: **ID**. Anything that has been measured for each item could be used here.
- Summarized by: **Count**. The total number of IDs and therefore the total number of objects.
- Row: **Parent ID**. Organize the objects by the ID of the object they were found within.
- Column: Not required.
- Page: Not required.

Analysis Examples

Edit Analysis

Analysis

Analyze these data: ID

Summarized by: Count

This is how the data will be summarized when more than one cell in the original data contributes to one cell in the analyzed data.

Organization

Organize the data by:

Row: Parent ID grouped into bins of size 1

Column: (none) grouped into bins of size 1

Columns will be used to generate series when creating charts.

Page: (none) grouped into bins of size 1

Save... Restore... Cancel OK

The Analysis View looks like this:

Parent IDs

Raw Analysis Chart Notes

Count of data from ID, row values from Parent ID.

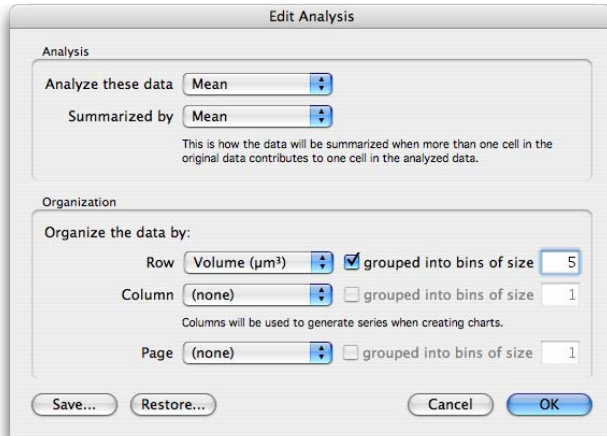
Parent ID	Count of ID
1	45
2	99
3	54
4	40
5	24
6	91
7	108

Filter: All Raw data in this measurement item. Showing 1282 of 1282 measurements.

Name	Type	ID	Item Name	Volume (μm^3)	Timepoint	Abs. Time	Rel. Time...
Object 3	Object	3	Experiment 2	7466.88	35	17:32:1...	9392.26
Object 4	Object	4	Experiment 2	7481.97	35	17:32:1...	9392.26
Object 5	Object	5	Experiment 2	2394.83	35	17:32:1...	9392.26
Object 6	Object	6	Experiment 2	8064.23	35	17:32:1...	9392.26
Object 7	Object	7	Experiment 2	9851.46	35	17:32:1...	9392.26
Object 8	Object	8	Experiment 2	20575.4	35	17:32:1...	9392.26
Object 9	Object	9	Experiment 2	0.150846	35	17:32:1...	9392.26
Object 10	Object	10	Experiment 2	1.15649	35	17:32:1...	9392.26

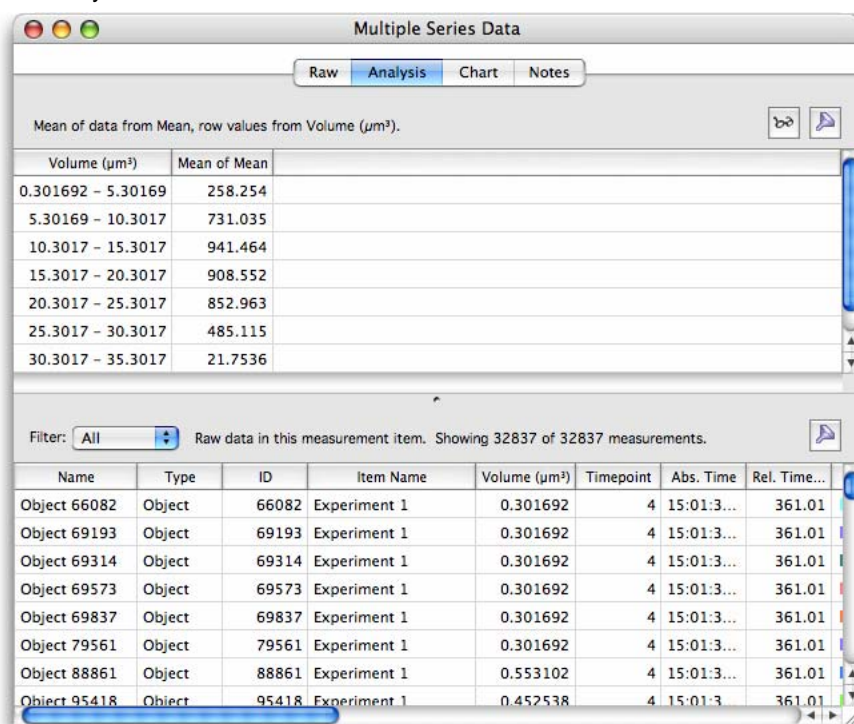
Show Changes in Intensity with Object Volume

- Analyze this data: **Mean**. For a single channel data set this represents the mean intensity of each object.
- Summarized by: **Mean**
- Row: **Volume**. Since there are many volumes represented in the table, showing each one individually will be excessive data to process. Rows, Columns and Pages may all be shown grouped into bins to prepare data in the style of a histogram.
- Column: Not required.
- Page: Not required.



Analysis Examples

The Analysis View looks like this:



Show Changes in Object Velocity over Time

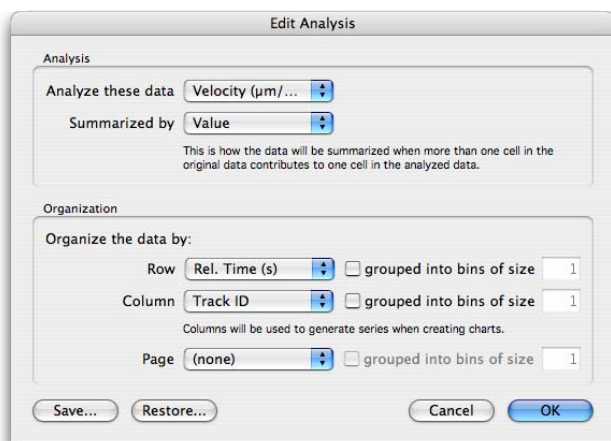
Object tracking measures not only an average Velocity for the whole track, shown in the Raw View in the Track Velocity column but also each object velocity from one timepoint to another. These velocities may be analyzed to illustrate the nature of the object movement over the time course of the experiment.

Name	Type	ID	Item Name	Volume (µm ³)	Timepoint	Abs. Time	Rel. Time...
Object 23	Object	23	vesicle tracking	83	1	17:22:0...	0
Object 24	Object	24	vesicle tracking	87	1	17:22:0...	0
Object 25	Object	25	vesicle tracking	96	1	17:22:0...	0
Object 26	Object	26	vesicle tracking	78	1	17:22:0...	0
Object 27	Object	27	vesicle tracking	83	1	17:22:0...	0
Object 28	Object	28	vesicle tracking	142	1	17:22:0...	0
Object 29	Object	29	vesicle tracking	99	1	17:22:0...	0
Object 30	Object	30	vesicle tracking	53	1	17:22:0...	0
Object 31	Object	31	vesicle tracking	177	1	17:22:0...	0
Object 32	Object	32	vesicle tracking	81	1	17:22:0...	0
Object 33	Object	33	vesicle tracking	63	1	17:22:0...	0
Object 34	Object	34	vesicle tracking	195	1	17:22:0...	0
Object 35	Object	35	vesicle tracking	140	1	17:22:0...	0
Object 36	Object	36	vesicle tracking	227	1	17:22:0...	0
Object 37	Object	37	vesicle tracking	79	1	17:22:0...	0
Object 38	Object	38	vesicle tracking	1	1	17:22:0...	0
Object 39	Object	39	vesicle tracking	35	1	17:22:0...	0
Object 40	Object	40	vesicle tracking	60	1	17:22:0...	0
Object 41	Object	41	vesicle tracking	30	1	17:22:0...	0

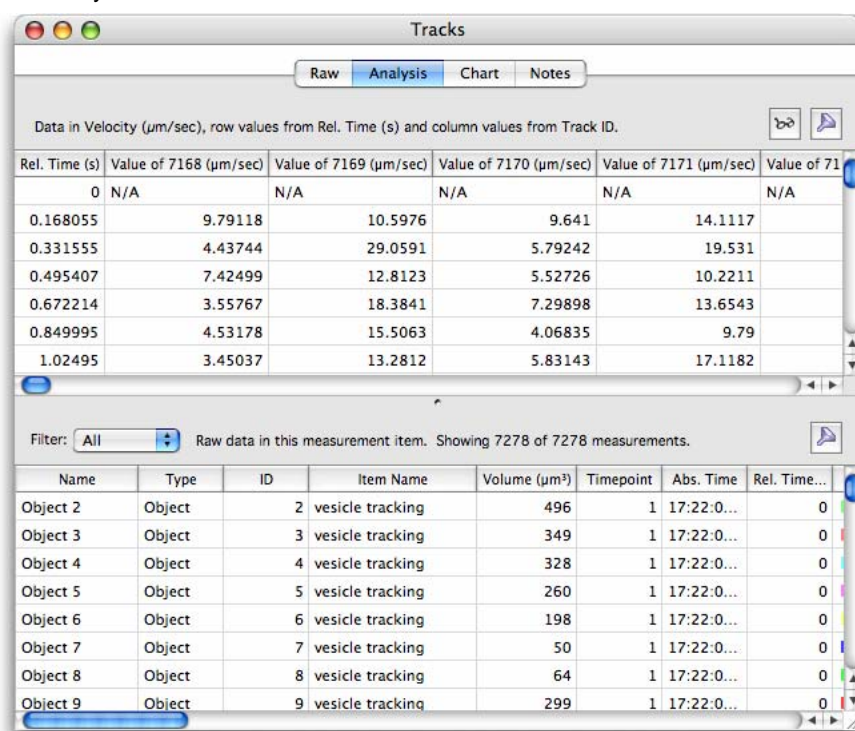
The Analysis dialog is configured as follows:

- Analyze this data: **Velocity**.
- Summarized by: **Value**.
- Row: **Relative Time**. Time may be absolute, relative or by timepoint.
- Column: **Track ID**. All the objects that make up a track are related to each other by the Track ID.
- Page: Not required.

Analysis Examples



The Analysis View looks like this:



This could be illustrated in a line chart. The following chart indicates that the objects accelerate and decelerate throughout the experiment.

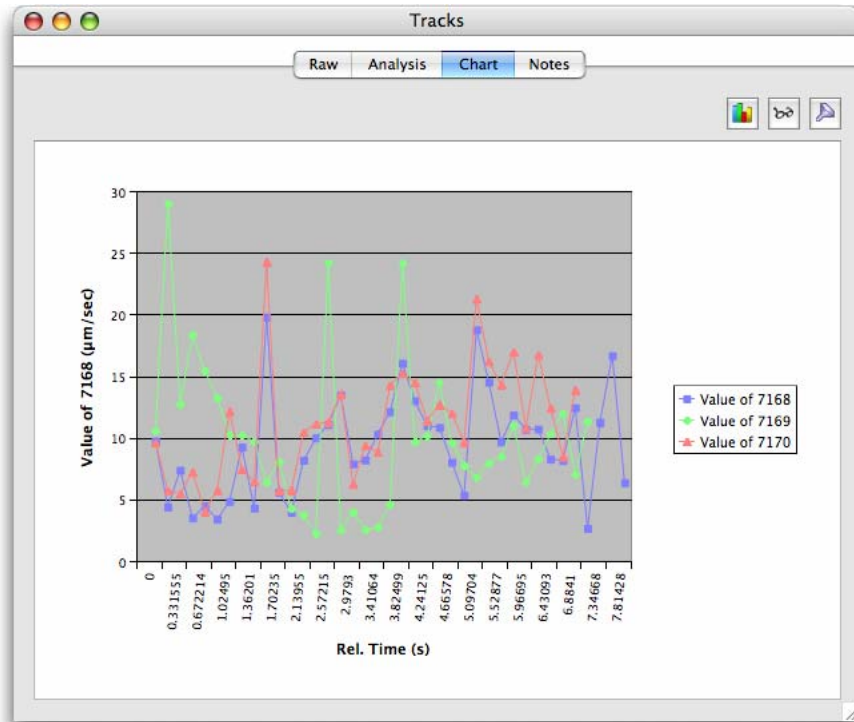
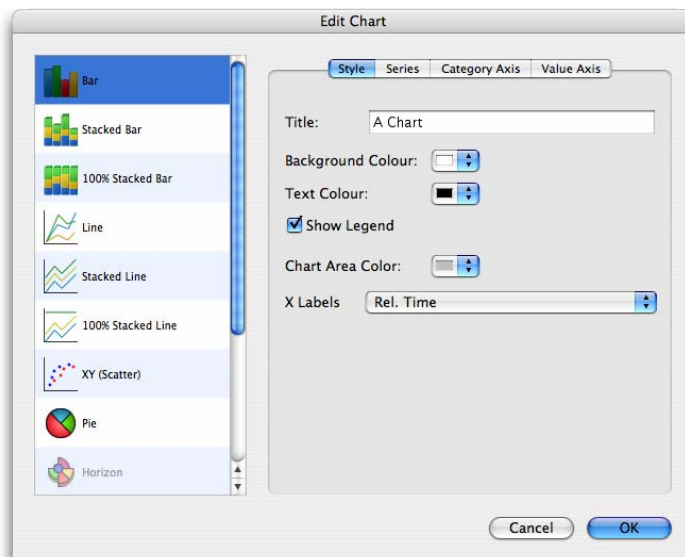


Chart View

The Chart View shows a graphical representation of the data in the analysis table. To generate a chart:

- First, set up an analysis table in the Analysis View.
- Then click on the Chart tab.

To configure chart style and appearance select **Chart...** from the Chart menu or click on the chart button at the top right of the window.



The chart will show only data that is not filtered out. Edit the filter from the Chart View by selecting **Filter...** from the Chart menu or clicking on the filter button at the top right of the window.

The Analysis table which is being represented in the chart can also be changed without returning to the Analysis tab. Choose **Analyze...** from the Chart menu or click on the analyze button at the top right of the window.

Exceptions to this description are the Track Charts. These are examples of special case charts where the information required to generate them is extracted directly from the raw data in the Raw View. When working with tracks click directly on the Chart tab to view XY or XYZ Tracking Charts

Available chart types are:

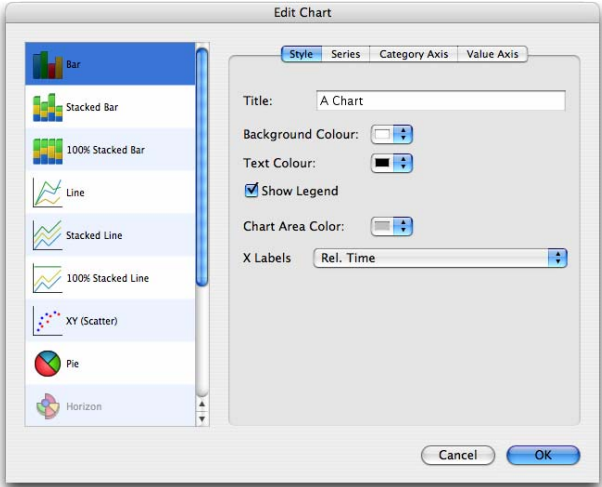
- Bar - compare values across categories
- Stacked Bar - compare contribution of a value to the total across categories
- 100% Stacked Bar - compare percentage contribution across categories
- Line - display trend across categories
- Stacked Line - display trend of contributions to the total across categories
- 100% Stacked Line - display trend of percentage contributions across categories.
- XY (Scatter) - compare pairs of values
- Pie - display contribution of each value to total
- Horizon - compare values
- XY Track - display XY positions over time
- XYZ Track - display XYZ positions over time

To keep an image of the chart select **Capture Snapshot** from the Chart menu. A 2D image of the chart will be added to the library. This may be exported in any of the formats for 2D images.

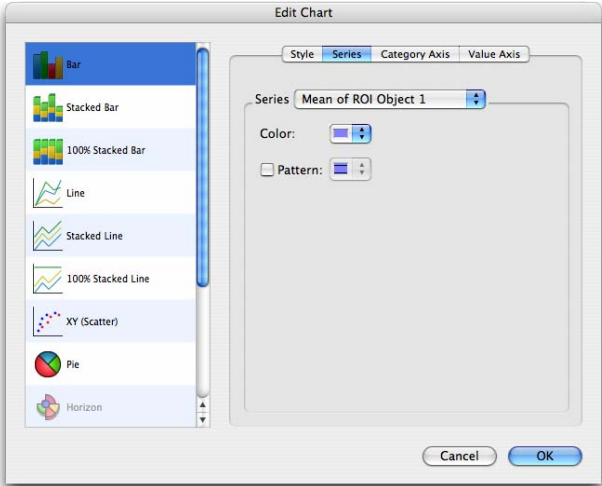
Chart Examples

For each chart type configuration options are offered for style, series, category axis and value axis.

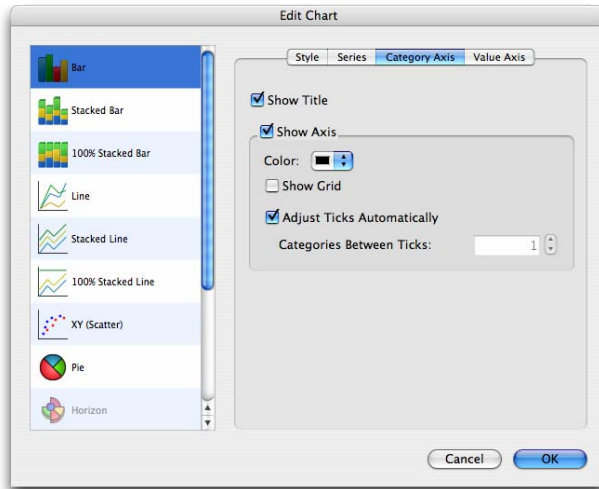
Style: A wide range of styles is available and each style has a number of user configurable options. The following examples illustrates options for a bar chart.



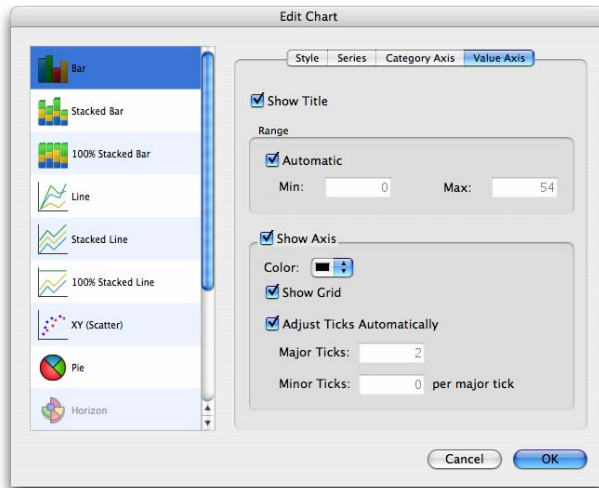
Series: To change the series style, select **Series** from the Edit Chart dialog. Color and pattern may be changed, as required.



Category Axis: To change the categories, select **Category Axis** from the Edit Chart dialog. Axis scale limits and divisions may be changed, as required.



Value axis: To change the categories, select **Category Axis** from the Edit Chart dialog. Axis scale limits and divisions may be changed, as required.



Colocalization

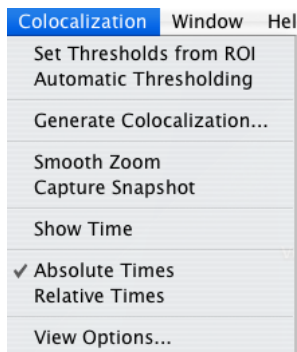
Colocalization is part of the Volocity Quantitation product designed specifically for Colocalization analysis as described by Manders et al (1993).

Colocalization in the context of digital fluorescence imaging can be described as the detection of signal at the same voxel location in each of two channels. The two channels are made up of images of two different fluorochromes taken from the same sample area.

Colocalization calculates measurement statistics based on the voxels and their intensity. It provides the user with a tool for evaluating how similar the images from two channels are. It provides a separate Colocalization view that shows:

- An extended focus merge of the two channels as a preview
- A scatter plot that represents the intensity values found within the images
- Statistics, which consist of Pearson's Correlation and Overlap Coefficients for the whole image and Colocalization Coefficients for regions of interest drawn on the scatter plot
- Any thresholds that have been applied to the data

The Colocalization View has a tool bar and a view specific menu. These are both used when working with the view.

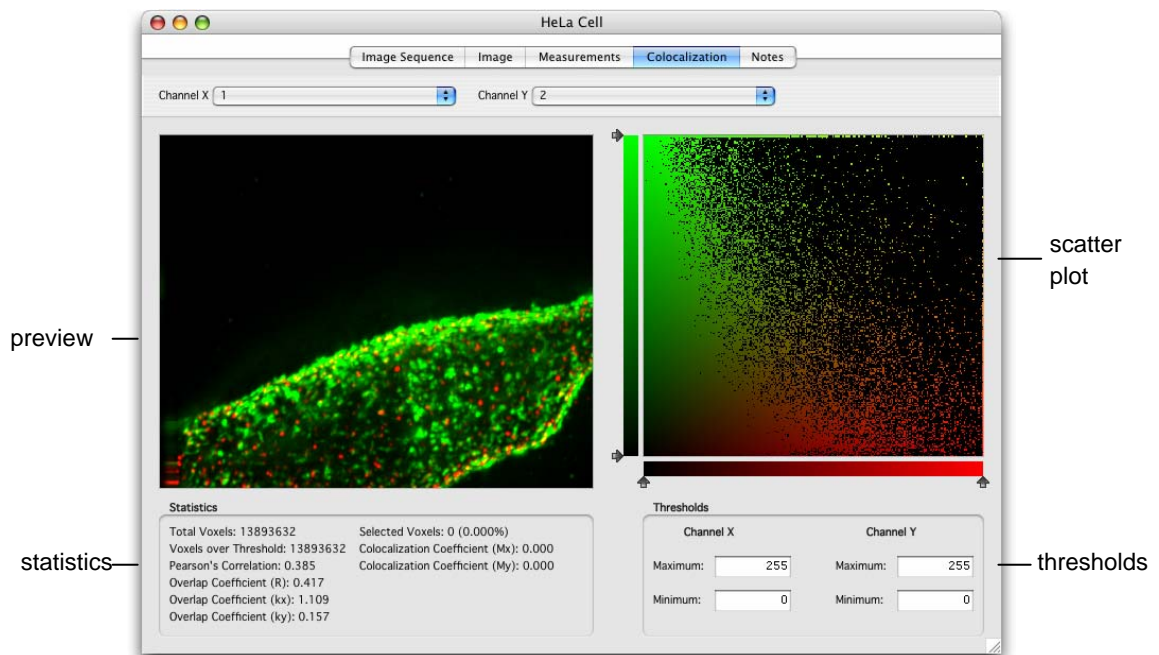


Colocalization menu



Colocalization tool bar

The Colocalization View



Preview

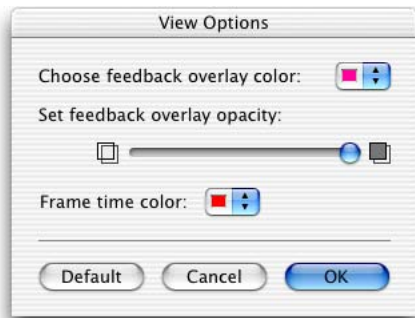
This is an extended focus merge of the two channels selected in the Channel X and Channel Y drop-downs. The current timepoint is shown and controlled by the Navigation palette.

Show the absolute or relative timestamp of the current timepoint in the preview by selecting **Show Time** from the Colocalization menu.

The following tools from the tool bar may be used on the preview image: rectangular, freehand, circular, magic wand and lasso ROI tools, zoom and pan.

Colocalization

Colocalized voxels selected in the scatter plot are shown using colored feedback in the preview. Use **View Options...** in the Colocalization menu to change the color and opacity of this feedback.

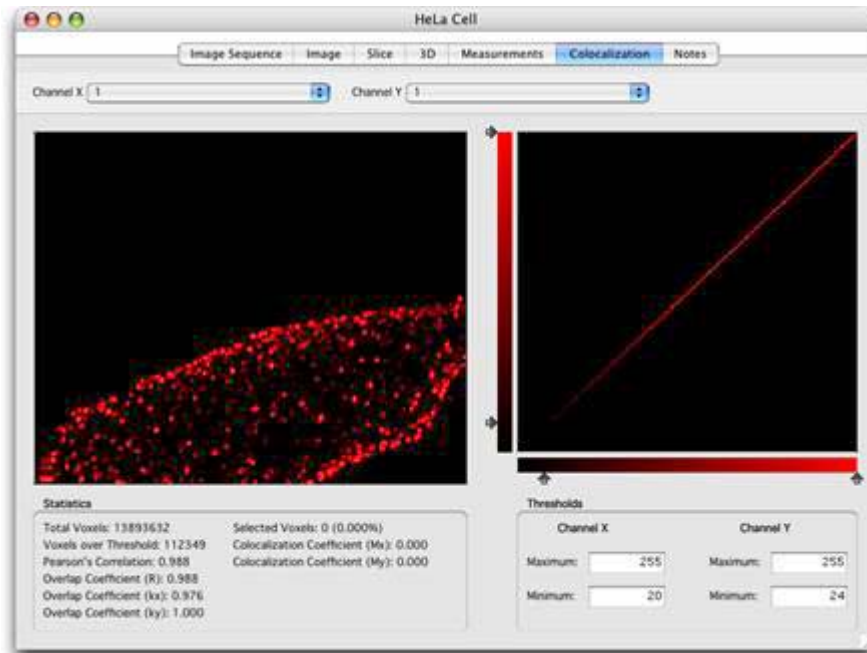


Scatter Plot

Each axis represents the ascending gray levels present within the two analyzed volumes. The axis is assigned according to the drop-downs at the top of the view.

For example: if a voxel in image x has an intensity value of 100 and the corresponding voxel in image y has an intensity value of 30, a point will be plotted at co-ordinates 100,30. It does not matter how many voxels in the two channels have intensity values of 100,30; only one point will be plotted on the scatter plot.

Two perfectly colocalized images, i.e. identical, will generate a scatter plot where the points fall in a line at 45 degrees to either axis.



Select All and **Select None** in the Edit menu operate on the scatter plot. You can also use the shortcuts Apple-A on Mac OS and Ctrl-A on Windows to select all.

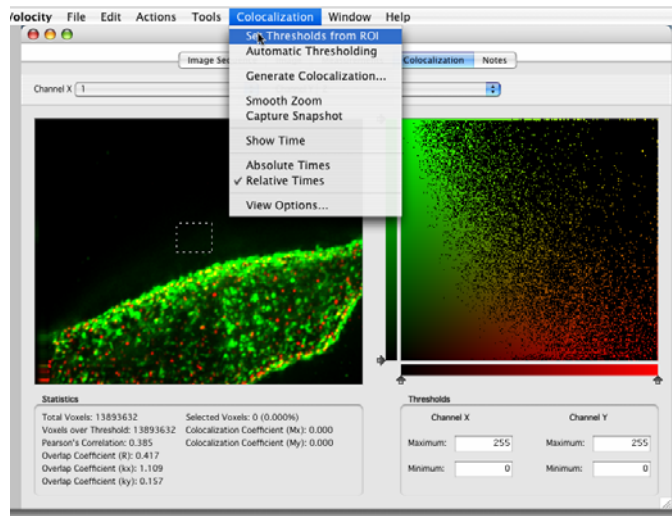
If required, select sub-regions of the scatter plot for analysis. Use the ROI tools to draw a region of interest on the scatter plot. The preview will update so the overlay shows the location of just the selected, colocalized voxels. The statistics will also update to reflect the selected voxels.

Thresholds

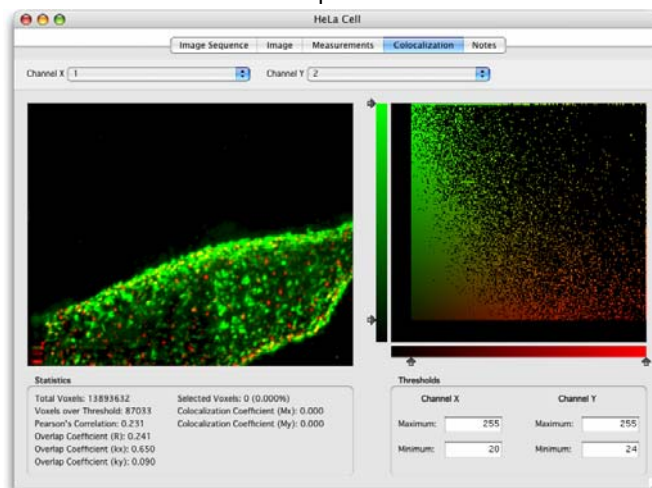
There are three ways of setting thresholds:

Colocalization

- Use the ROI tool to draw a region of interest on the Preview. Select **Set Thresholds from ROI** from the Colocalization menu. Volocity calculates the mean intensity of the ROI for each channel and sets it as the minimum threshold value for that channel.

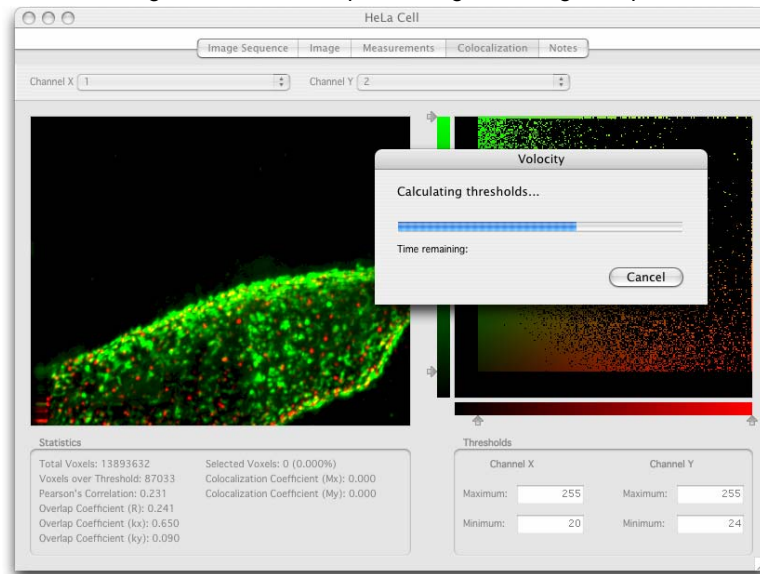


- Enter the required values manually into the Threshold **Maximum** and **Minimum** boxes for each channel or drag the slider controls on the scatter plot. You will be able to see where voxels have been excluded on the scatter plot.



- Select **Automatic Thresholding** from the Colocalization menu to generate thresholds automatically. Volocity uses statistical tests to set thresholds objectively. Automatically calculating thresholds will take time, and this time will increase with increasing dimensions of the data set.

Turning automatic thresholding on disables manual setting of thresholds and **Set thresholds from ROI**. For multi-timepoint data sets, Velocity will recalculate thresholds for each timepoint viewed. Change the current timepoint using the Navigation palette.



Any voxels of intensity outside threshold will be removed from the preview image for that channel. Any point on the scatter plot that is outside threshold for either channel will be removed from the scatter plot leaving only points that are positive for both x and y, i.e. colocalized.

If thresholds are not set, voxels where both images show background intensity levels may be interpreted as colocalized.

Automatic Thresholding

The aim of the Automatic Thresholding is to determine thresholds that exclude voxels for which Pearson's Correlation between the two channels is less than, or equal to, 0. It is a two-step process. First, a best-fit regression line of the Channel X and Channel Y intensities over all the voxels is found. Second, the thresholds are moved along the regression line until the point is reached where Pearson's Correlation for voxels below threshold is less than, or equal to, 0. This technique is based on the method proposed in:

S. V. Costes, D. Daelemans, E. H. Cho, Z. Dobbin, G. Pavlakis & S. Lockett

Automatic and Quantitative Measurement of Protein-Protein Colocalization in Live Cells

Biophysical Journal 2004 Vol. 86, pp. 3339-4003

Automatic Thresholding is turned on or off by selecting **Automatic Thresholding** from the Colocalization menu. It is possible that Automatic Thresholding may not be able to determine thresholds for a data set, in which case a message is shown. Automatic Thresholding may determine

Colocalization

different thresholds depending on the data set; therefore, it should not be used if the same thresholds are to be applied across a series of data sets or timepoints within the same data set.

Any voxels of intensity outside threshold will be removed from the preview image for that channel. Any point on the scatter plot that is outside threshold for either channel will be removed from the scatter plot leaving only points that are positive for both x and y, that is colocalized.

If thresholds are not set, voxels where both images show background intensity levels may be interpreted as colocalized.

Statistics

While Volocity is generating the scatter plot and calculating statistics a progress indicator is shown in the top right-hand corner of the view.

The statistics presented in the Colocalization window are calculated as follows:

X_i = intensity of voxel i in image x unless X_i is outside threshold in which case $X_i = 0$

Y_i = intensity of voxel i in image y unless Y_i is outside threshold in which case $Y_i = 0$

$$\text{Pearson's Correlation} = \frac{\sum_i (X_i - x_{\text{aver}}) \cdot (Y_i - y_{\text{aver}})}{\sqrt{\sum_i (X_i - x_{\text{aver}})^2 \cdot \sum_i (Y_i - y_{\text{aver}})^2}}$$

Pearson's Correlation is a statistical test for a linear relationship between two variables. Pearson's Correlation can vary between -1 and 1. A correlation of 0 means there is no linear relationship between the variables. -1 and 1 indicate perfect negative and perfect positive linear relationships respectively. Interpreting the degree of colocalization from negative values is difficult.

$$\text{Overlap Coefficient (R)} = \frac{\sum_i x_i \cdot y_i}{\sqrt{\sum_i (x_i)^2 \sum_i (y_i)^2}}$$

$$\text{Overlap Coefficient (kx)} = \frac{\sum_i x_i \cdot y_i}{\sum_i (x_i)^2}$$

$$\text{Overlap Coefficient (ky)} = \frac{\sum_i x_i \cdot y_i}{\sum_i (y_i)^2}$$

$$\text{Colocalization Coefficient (Mx)} = \frac{\sum_i x_{i,\text{coloc}}}{\sum_i x_i}$$

$$\text{Colocalization Coefficient (My)} = \frac{\sum_i y_{i,\text{coloc}}}{\sum_i y_i}$$

Where $x_{i,\text{coloc}} = x_i$ if y_i is within the intensity range defined by the ROI, $x_{i,\text{coloc}} = 0$ if y_i is outside the intensity range and $y_{i,\text{coloc}} = y_i$ if x_i is within the intensity range defined by the ROI, $y_{i,\text{coloc}} = 0$, if x_i is outside the intensity range.

Colocalization Coefficients may be values between 0 and 1. 0 indicates that none of the signal within thresholds in that channel exists as colocalized with the other channel. A value of 1 indicates that all of the signal within thresholds in that channel exists as colocalized with the other channel.

For further explanation of these statistics see the following references:

M.M. Manders, P. J. Verbeek & J. A. Aten

Measurement of co-localization of objects in dual colour confocal images

Journal of Microscopy 1993 Vol. 169, pp. 375-382

Generating Output

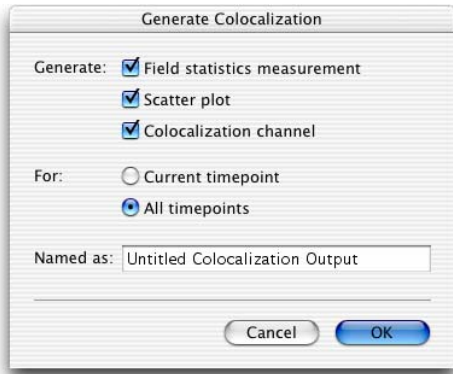
There are two methods for generating output using the principles of the Colocalization View:

- **Generate Colocalization**, which can be set up to generate a field statistics measurement, a scatter plot and a colocalization channel for the current or all timepoints.
- **Measure Object Colocalization** task used in a measurement protocol, which measures the colocalization coefficients Mx and My for each object and displays them in the table area of the Measurements View.

Colocalization

Generate Colocalization

Select **Generate Colocalization** from the Colocalization menu and choose the required output for the current timepoint or all timepoints in the image sequence.



- Field statistics are measurements representing the whole volume rather than objects within it. One timepoint in an image sequence will give rise to one measurement row in the Measurements table. In a row, the colocalization statistics (M_x and M_y) are generated from the region of interest selected on the scatter plot.
- The scatter plots are saved to a folder in the current library. Each scatter plot is stored as a 2D image and may be exported.
- A Colocalization channel is made from the selected colocalized voxels. These voxels are marked in the Colocalization View preview with the colored overlay. The channel is produced from a brightest point merge honoring the color and intensity of those voxels in the original channels.

Measure Object Colocalization

Use this task within a measurement protocol in the Measurement View. Find objects using tasks added to the protocol and then add Measure Object Colocalization to find M_x and M_y for each. As discussed above, it is essential to set thresholds for each channel and the Measure Object Colocalization task allows thresholds to be set. Alternatively, after configuring the Colocalization View the settings in the view may be read into the task.

Important Considerations

It is important to have adequate separation of the two fluorescence signals, with no overlap between emission of one and excitation of the other. This affects choice of filters as well as fluorochromes themselves.

Colocalization can only analyze the voxel values presented in the selected images. It is the user's responsibility to ensure the images are valid for the study they are carrying out.

Users may wish to deconvolve images before processing. Iterative Restoration is appropriate for this application since it preserves voxel intensity values, see page 451.

Colocalization assumes that there has been no voxel shift between the capture of the two channels and that they are perfectly aligned. Adequate controls are also essential to avoid the possibility of false colocalization, for example generated by non-specific labeling.

FRAP Analysis

Carrying Out FRAP Analysis

Only data that has been acquired with Volocity FRAP Acquisition may be analyzed with Volocity FRAP Analysis.

Prior to carrying out FRAP analysis you should consider applying the following corrections to your FRAP recovery data:

- Background correction
- Photobleaching correction

Background Correction

Volocity provides a flexible background correction tool that allows you to correct images based on either division of images with a bright reference image, subtraction of a dark reference image or subtraction of fixed offset values from images (for more details please Correcting Background).

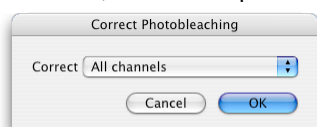
If you wish to apply background correction to your FRAP image sequences, acquire any correction images that you require and create your background correction item in your library. Select the images that you wish to correct and apply background correction from the Tools menu.

Photobleaching Correction

The Volocity photobleaching correction tool corrects for photobleaching by applying correction factors to images that restore their intensity to the intensity of the first image in an image sequence. Without photobleaching correction it is likely that you will get an underestimation of the mobile fraction from your FRAP analysis.

There are two methods of applying photobleaching correction. Apply whichever is appropriate for your experiment.

- **Based on a region of interest.** To apply a correction based on a region of interest you must have your image sequence open in the Image View. Select an ROI and then select **Correct Photobleaching...** from the Tools menu. Make sure that you select the appropriate channel for correction; in our example we are correcting the "FRAP (bg corrected) channel".



FRAP Analysis

When photobleaching correction based on an ROI is applied Volocity multiplies the whole image by whatever correction factor is required for each time point to keep the ROI at 100% intensity throughout the image sequence. Because it requires an ROI to be selected it cannot be used to batch correct multiple channels.

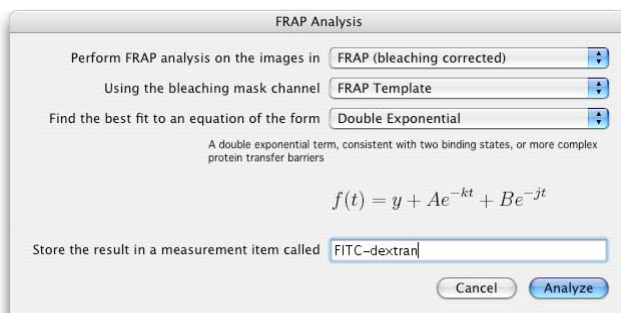
- **Based on the whole image.** Select **Correct Photobleaching...** from the Tools menu. Make sure that you select the appropriate channel for correction. Volocity multiplies the whole image by whatever correction factor is required for each time point to keep the whole image at 100% intensity throughout the image sequence. Photobleaching correction based on the whole image does support batch processing. To batch process several images sequences, select them in your Volocity library before selecting **Correct Photobleaching...** from the Tools menu.

Normalization

You may include as many data sets as you wish in a single FRAP analysis. So that multiple data sets can be pooled, Volocity normalizes FRAP data by setting the intensity of bleach areas in the last frame before bleaching to 1 and by setting the intensity in the first frame after bleaching to 0, all intensity measurements are then made according to this scale.

Select the image sequences that you wish to include in your analysis and select **FRAP Analysis...** from the Tools menu.

FRAP Analysis



1. Select the channel on which FRAP analysis is to be performed.
2. Select the appropriate bleaching mask channel; this is the FRAP template channel created when we acquired the image sequence (the correct mask channel should be selected by default provided that the channel names have not been changed).
3. Select the appropriate equation.
 - The Single Exponential equation is appropriate for a single binding state (see Sprague and McNally (2005) for further information on single binding states).
 - The Single Constrained Exponential equation is appropriate for a single binding state where you wish to constrain the curve fit to have a value of 0 at time 0, i.e. pass through the origin.

- The Double Exponential equation is appropriate for two binding states or more complex transfer barriers. However, be aware that this equation will fit almost all recovery curves and does not necessarily indicate two binding states for your molecule of interest (see Sprague and McNally (2005) for further information on two independent binding states and the caution required to use this equation).
- The Soumpasis analytical solution (Soumpasis1983) provides a solution for pure diffusion.
- Specify a name for the measurement item in which to store the result and click **Analyze**. When analysis is complete your measurement item will automatically open in the Chart view.

References

Axelrod D, Koppel DE, Schlessinger J, Elson E and Webb WW (1976)

Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055-1069

Soumpasis D.M. (1983)

Theoretical analysis of fluorescence photobleaching recovery experiments. *Biophys. J.* 41:95-97

Sprague BL and McNally JG (2005)

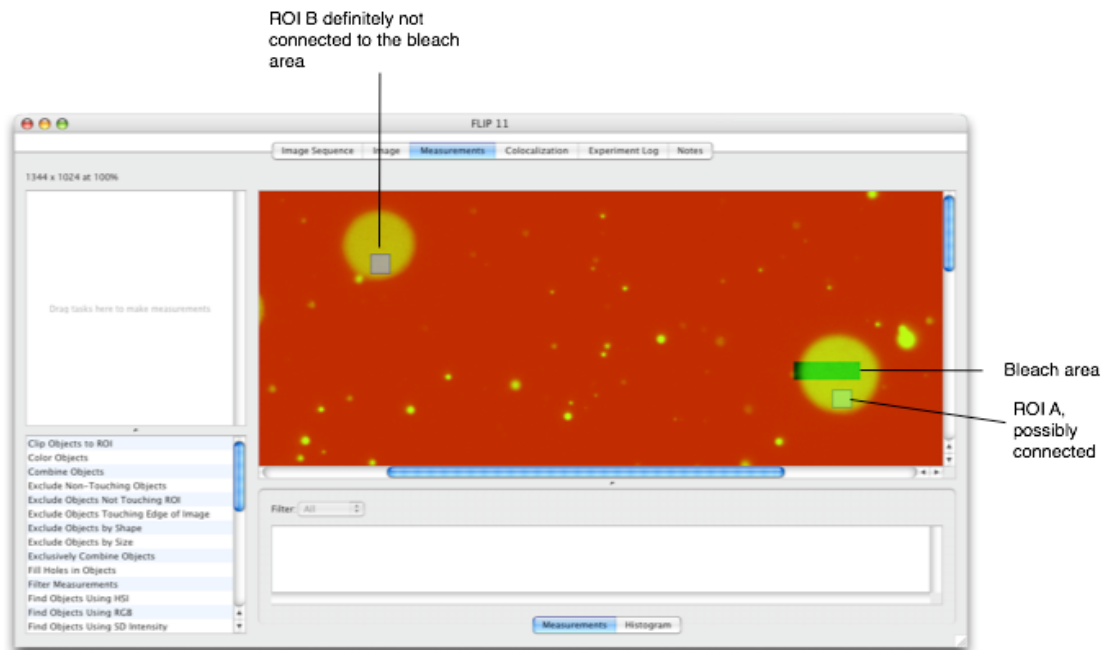
FRAP analysis of binding: proper and fitting. *Trends in Cell Biology.* 15:84-91

FLIP Analysis

As with FRAP analysis, you may wish to background correct and photobleaching correct your image sequence before analysis.

1. Open your image sequence in the measurements view.
2. Select ROIs for analysis. In this case ROI A may be connected to the bleach area (mobile molecules would be free to diffuse between the bleach area and ROI A), but ROI B can not possibly connected to the bleach area. A dramatically higher bleach rate for ROI A than ROI B would demonstrate that molecules are freely diffusing between ROI A and the bleach area.

FRAP Analysis



3. Select **Measure all timepoints** from the Measurements menu.
4. Select **Create measurements item** from the Measurements menu.
5. Name your measurements item and click **OK**.
6. Open the measurements item and select the Analysis tab. Select **Analyze** from the Analysis menu. The Edit Analysis dialog opens.



Set the Analysis dialog as follows:

- Analyze these data = **Mean**
 - Summarize by = **Value**
 - Row = **Timepoint**
 - Column = **Name**
 - Page = **None**
7. Check the **Include normalized data to the control in row** box, and select timepoint 1 from the drop down menu. This results in intensity values being calculated as a percentage of their initial values. This makes changes in intensity clearer, and makes comparison of the intensity changes in the two areas possible.
8. Click **OK**.

FRAP Analysis

FLIP 3

Raw Analysis Chart Notes

Data in Mean (FRAP (bleaching corrected)), row values from Timepoint and column values from Name.

Timepoint	Value of ROI Object 1 (FRAP (bleaching corrected))	Value of ROI Object 1 Normalised to control (%) (FRAP (bleaching corrected))	Value of ROI Object 2 (FRAP (bleaching corrected))	Value of ROI Object 2 Normalised to control (%) (FRAP (bleaching corrected))
1	2732.69	100	3173.34	100
2	2730.28	99.9119	3169.72	99.8859
3	2736.33	100.133	2867.76	90.3704
4	2727.09	99.795	2510.85	79.1231
5	2739.28	100.241	2317.74	73.0378
6	2731.77	99.9665	2056.7	64.8118
7	2722.89	99.6414	1894.57	59.7028
8	2727.01	99.7923	1722.05	54.2661
9	2725.7	99.7442	1605.91	50.6062
10	2714.67	99.3405	1469.79	46.3169
11	2711.69	99.2316	1380.9	43.5155

Filter: All Raw data in this measurement item. Showing 28 of 28 measurements.

Rel. Time (s)	Name	Type	ID	Item Name	Timepoint	Abs. Time	Color	Pixel Count	Area (µm ²)	Min (FRAP)	Max (FRAP)	Mean (FRAP)	Sum (FRAP)	Min (FRAP Template)
0.59576	ROI Object 1	Object	3	FLIP 11	2	10:26:34.95...	Blue	576	5.76	2212	2954	2728.45	1.57159e+06	255
0.59576	ROI Object 2	Object	4	FLIP 11	2	10:26:34.95...	Green	528	5.28	2361	3538	3167.73	1.67256e+06	255
6.5819	ROI Object 1	Object	5	FLIP 11	3	10:26:40.94...	Blue	576	5.76	2188	2868	2626.78	1.51302e+06	255
6.5819	ROI Object 2	Object	6	FLIP 11	3	10:26:40.94...	Green	528	5.28	2220	3018	2752.91	1.45353e+06	255
66.6454	ROI Object 1	Object	7	FLIP 11	4	10:27:41.00...	Blue	576	5.76	2103	2953	2630.72	1.5153e+06	255
66.6454	ROI Object 2	Object	8	FLIP 11	4	10:27:41.00...	Green	528	5.28	1970	2747	2422.16	1.2789e+06	255
72.7464	ROI Object 1	Object	9	FLIP 11	5	10:27:47.10...	Blue	576	5.76	2017	2829	2542.74	1.46462e+06	255
72.7464	ROI Object 2	Object	10	FLIP 11	5	10:27:47.10...	Green	528	5.28	1841	2351	2151.54	1.13602e+06	255
132.806	ROI Object 1	Object	11	FLIP 11	6	10:28:47.16...	Blue	576	5.76	2023	2796	2547.29	1.46724e+06	255
132.806	ROI Object 2	Object	12	FLIP 11	6	10:28:47.16...	Green	528	5.28	1566	2134	1917.91	1.01266e+06	255
138.787	ROI Object 1	Object	13	FLIP 11	7	10:28:53.14...	Blue	576	5.76	1953	2731	2456.78	1.4151e+06	255
138.787	ROI Object 2	Object	14	FLIP 11	7	10:28:53.14...	Green	528	5.28	1461	1928	1709.57	902652	255

- Your Analysis now contains four columns. Two with raw intensity values over time and two with normalized intensity values over time. So that you plot only the normalized values hide the columns containing raw values. Select **Analysis Columns...** from the Analysis menu and uncheck the boxes corresponding to the raw values (leaving the boxes that correspond to normalized values checked) and click **OK**.

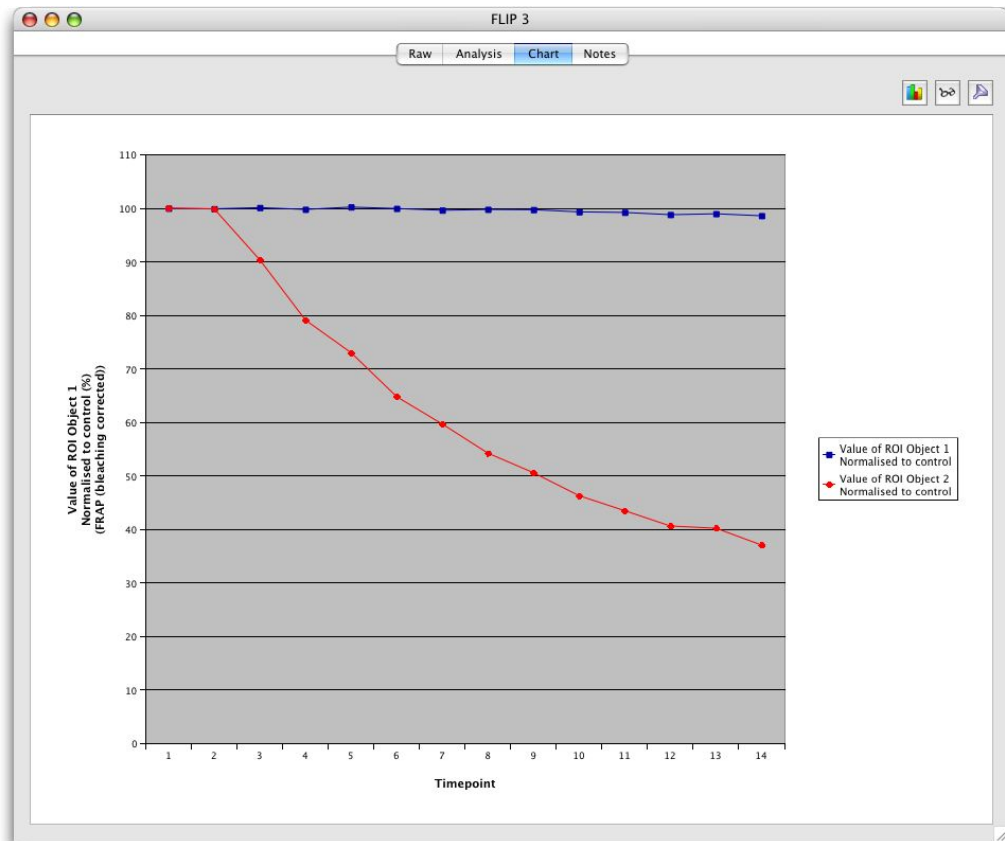


10. Select the Chart Tab.

11. Select the **Setup Chart** button

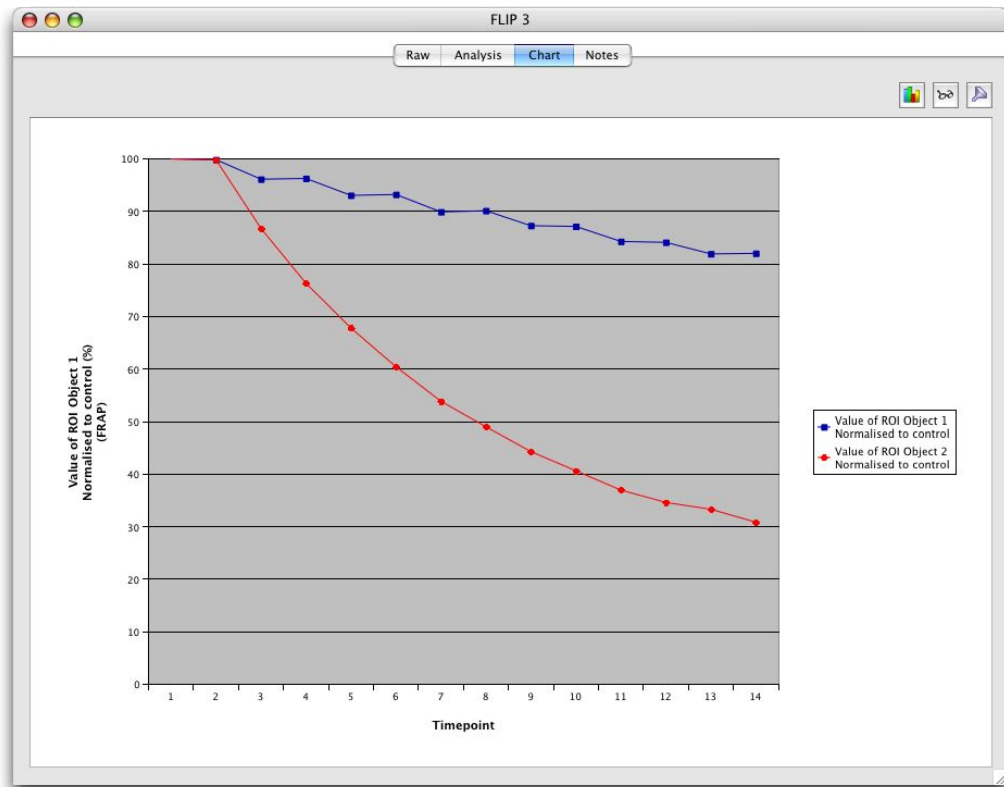
12. In the Edit chart dialog set the chart to "Line" and click **OK**.

FRAP Analysis



In our example we can see that ROI A (plotted in Red) loses fluorescence in comparison to ROI B (plotted in blue). This demonstrates that the fluorescence material in ROI A is able to move freely into and out of the bleaching area, unlike the material in ROI B which can never enter the bleaching area. In this case photobleaching was corrected based on a third ROI, which was unconnected to ROI A or B.

The same analysis without photobleaching correction is shown below. Even without photobleaching correction fluorescence is clearly lost at a much higher rate in ROI A (red) than ROI B (blue).

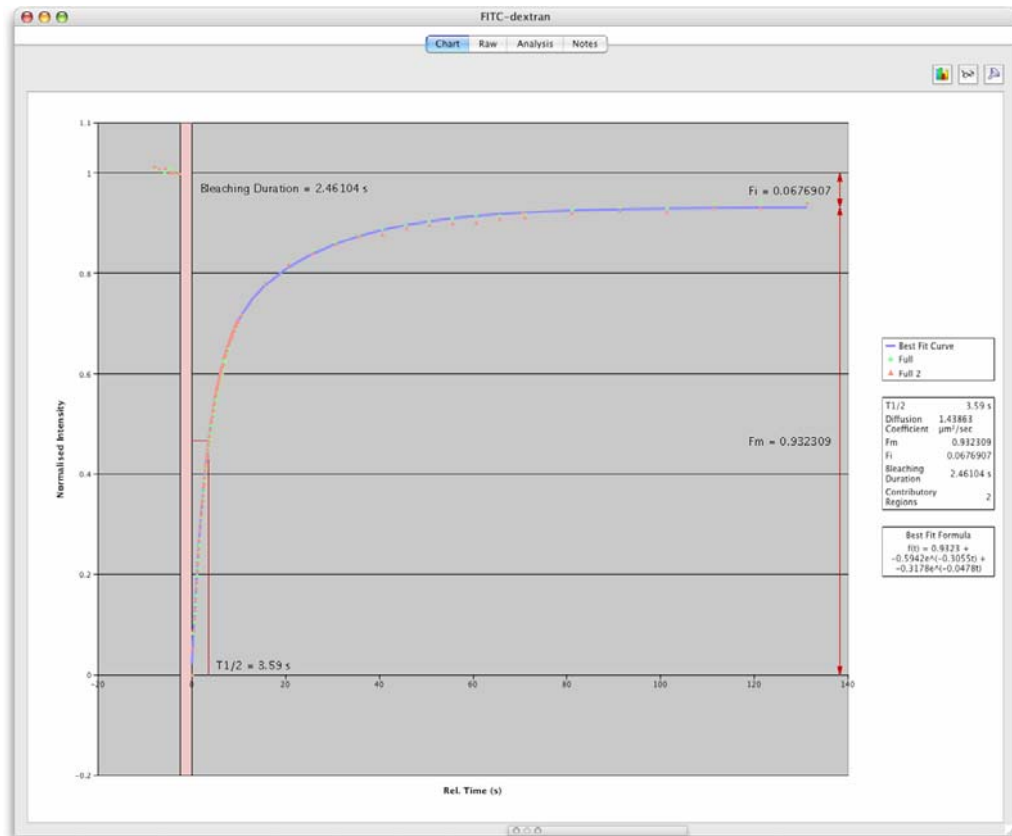


FRAP Measurement Item

The **FRAP Analysis...** command in the Tools menu generates a measurement item. Designed specifically for FRAP analysis the information in this measurement item will be laid out in a different way compared to one created by other measurement actions in Volocity and you will interact with it differently.

The Chart View provides the most accessible view of the results. The FRAP measurement item will open to the Chart View by default.

FRAP Measurement Item



FRAP charts show the normalized recovery for each of your bleached areas (each recovery is plotted in its own color and style), as well as the best fit curve (shown with a line joining each point by default).

Parameters that are displayed either graphically or in the legend are shown in the table below.

Parameter	Method of Calculation	Chart or Legend	Reference
$T^{1/2}$	Time taken for the intensity of the bleached area to recover to half of the plateau intensity	Both	
Mobile Fraction (F_m)	Normalized intensity at plateau	Both	
Immobile Fraction (F_i)	Normalized pre-bleach intensity-normalized intensity at plateau	Both	
Diffusion Coefficient (D)		Legend	Axelrod et al 1976
Fitted Equation	Equations are fitted to the raw data using the least squares method	Legend	
T_D (Characteristic diffusion time, Soumpassis equation only)	Derived by fitting the Soumpassis equation to the raw data	Legend	Soumpassis 1983

For a more detailed view of the normalized intensity values from your recoveries as well as the curve fitted recovery values select the Raw tab. Note the Rel Time column; this represents the time relative to the first frame to be acquired after bleaching, which is set to 0.

Ratioing

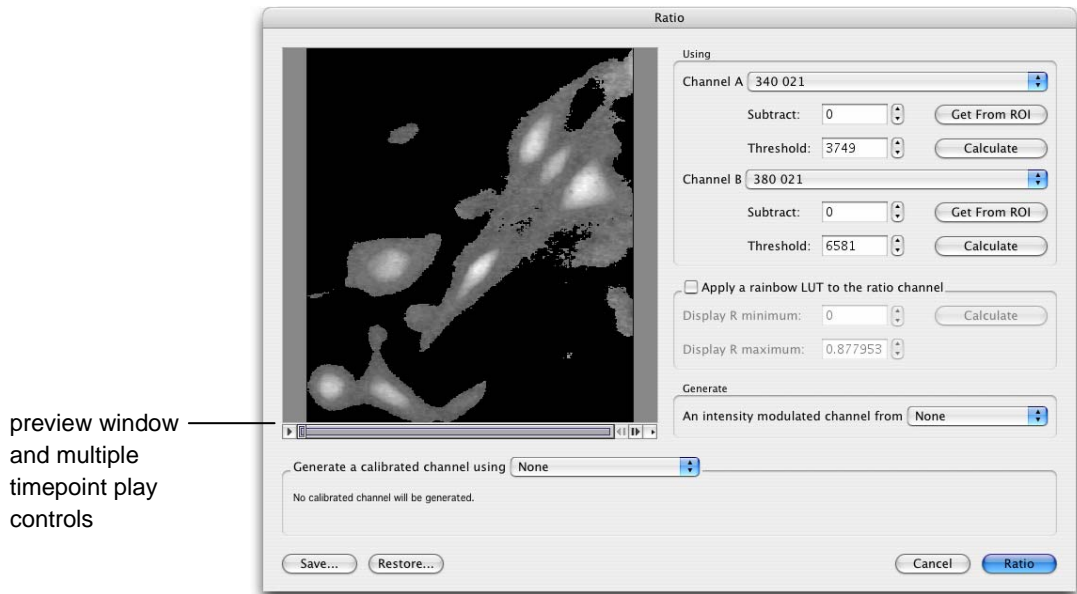
The screenshot shows a software window titled "FITC-dextran" with a "Raw" tab selected. Below the tabs, there is a filter dropdown set to "All" and a status bar indicating "Raw data in this measurement item. Showing 229 of 229 measurements." The main area contains a data table with the following columns: T1/2 (s), Diffusion Coefficient (µm²/sec), Fm, Fa, Best Fit Formula, Bleaching Duration (s), Contributory Regions, Rel. Time (s), Best Fit Curve, Full (FRAP (bleaching corrected)), and Full 2 (FRAP (bleaching corrected)). The table contains 229 rows of data, with most cells containing "N/A" and some containing numerical values.

Ratioing

Velocity ratioing allows you to carry out offline analysis of ratioed pairs of images. It requires an image sequence containing at least two channels. The channels must be the same depth but may be 8-bit, 16-bit or floating point. The ratio is always calculated as Channel A / Channel B, therefore, it is important to specify the channels correctly in the Ratio dialog.

Applying Ratio Calculation

1. Open an image sequence containing 2 or more channels.
2. Choose **Ratio...** from the Tools menu. The ratio option is available when any of the tabs in the image sequence are active, but working from the Image View allows you to use an ROI drawn in that view to set background values for subtraction.



3. Select which channels in the image sequence should be used for **Channel A** and **Channel B**. A constant background value may be subtracted from every voxel in every image of each channel, if required. If source images are already background subtracted, go to step 4.

Note: Subtraction of a background constant is relevant only if the source images are evenly illuminated.

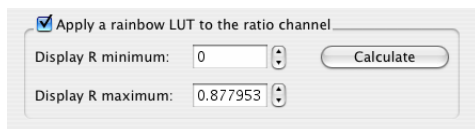
Enter a value in the **Subtract** field or draw an ROI on the Image View before opening the Ratio dialog and click **Get from ROI** next to the Subtract field. Volocity will use the mean intensity from that ROI for each channel as the value to subtract. This value will be subtracted from the intensity value of every voxel before processing the ratio calculation.
4. Set threshold values for each channel by typing a value in the **Threshold** field. Thresholds allow removal of signals that would interfere with interpretation of results.

For example, there will be areas in the images where low intensities in each of the A and B channels produce similar ratio values to higher intensities in other areas. Thresholds will mean a result of 0 (zero) is set when one of the source voxels has an intensity at or below the threshold.

Use the Voxel Spy to pick out an exact threshold value or ask Volocity to **Calculate** the value for you. The calculate option determines the modal intensity value for the channel and uses it as the threshold.
5. You may wish to apply rainbow colors to the ratioed channel. Check the **Apply a rainbow LUT to the ratio channel**. To optimize the use of the rainbow across the range of ratio values present within the results enter **Display R minimum** and **Display R maximum** values. This allows the finite range of colors available in the lookup table to be used to illustrate most effectively any changes in the ratio

Ratioing

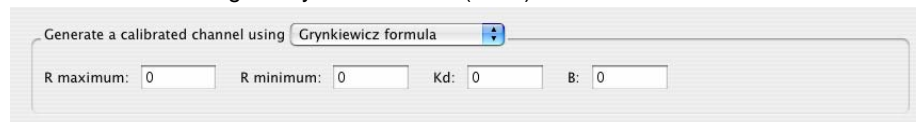
values. Click **Calculate** and the maximum and minimum ratio values for the current timepoint will be used. To easily compare ratio images with rainbow colors applied, they should all have the same **Display R minimum** and **Display R maximum** set. In the rainbow LUT the minimum value is shown as blue, the maximum as white. By setting **Display R minimum** and **Display R maximum** you are setting the black and white point of the images. This is what is done automatically by the Auto Contrast command in the Tools menu. Auto Contrast should, therefore, be used with caution if ratio images are to be used comparatively.



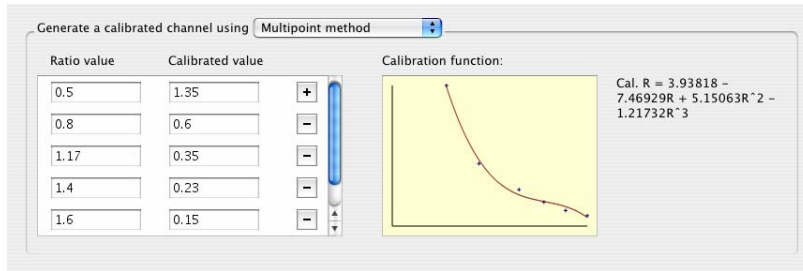
6. If required, generate an intensity modulated channel that will appear as a new channel in the image sequence. Select the modulator image from the **Generate** drop-down list. This generates a millions of colors display channel and may show morphology more clearly at the same time as indicating ratio values.



7. If a calibrated channel is required, select one of the two methods in the **Generate a calibrated channel using** drop-down list and enter the appropriate values in the table.
 - **Grynkiewicz:** Edit the four constants, as appropriate. These values will be applied to the Grynkiewicz equation (see page 436). The relevant constants for the current experiment must be determined according to Grynkiewicz et al (1985).



- **Multi-point:** Use the values previously obtained from a calibration experiment for known correction of ion. Use the + button to create up to 11 (eleven) points in the table as required and enter the **Ratio value** and corresponding **Calibrated value**. These points are fitted to a third order polynomial or S-shaped curve. The preview graph provides a visual check that the calibrated values entered are sensible.



The preview shows a brightest point merge of the resultant ratioed image for the current timepoint. If the sequence contains multiple timepoints, use the play controls to view the effect of the parameters through the time series.

- Click **Ratio** to apply the ratio calculation and create a ratio channel in the image sequence. If specified, Volocity also creates a calibrated ratio and an intensity modulated channel. Channels may be hidden using the controls on the Navigation palette.

Note: If appropriate, use the **Save..** and **Restore...** buttons to save the settings for use in other experiments.

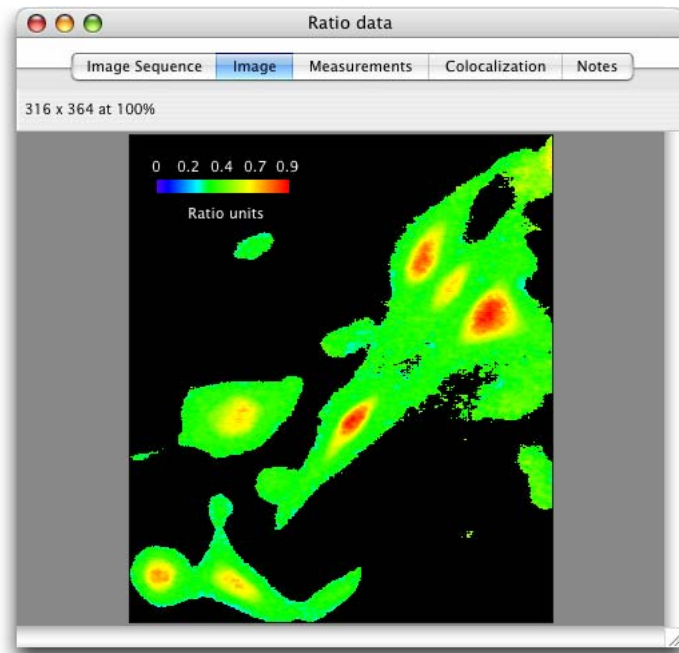
Measuring Ratio Channels

Target the appropriate channel when carrying out measurements, so that you obtain the required measurement units: ratio units or calibrated units. If drawing regions of interest to follow changes over time it may be beneficial to use the intensity measurements that ignore 0.

Using Color Reference with Ratio Images

To display ratio or calibrated images it may be beneficial to show the color reference on the Image View. This will show the values represented by the colors. Select **Show Color Reference** from the Image menu.

FRET Analysis



Grynkiewicz equation

$$[Ca^{2+}]_i = \frac{K_D * B * (R - R_{min})}{R_{max} - R}$$

Ca^{2+} = intracellular Ca²⁺ concentration

K_D = dissociation constant of fura-2

B = fluorescence signal at 340 nm at $0 \mu M \cdot l^{-1} Ca^{2+}$ / fluorescence signal at 380 nm at $39.8 \mu M \cdot l^{-1} Ca^{2+}$.

Grynkiewicz, G. Poenie, M. and Tsien, R. Y.

A new generation of Ca^{2+} indicators with greatly improved fluorescence properties.

Journal of Biological Chemistry 1985 Vol. 260, pp. 3440-3450

FRET Analysis

FRET analysis allows accurate FRET measurements and generation of net FRET (or "corrected" FRET) and normalized FRET images for publication.

The analysis currently offered by Volocity is the 3-image FRET technique, as described by Gordon et al. (1998) and Xia and Liu (2001).

References

The following references should be used to find further information on the technique and guidance on interpretation.

Gerald W. Gordon, Gail Berry, Xiao Huan Liang, Beth Levine, and Brian Herman

Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy. *Biophysical Journal* 1998 Vol. 74, pp. 2702-2713

Zongping Xia and Yeucheng Liu.

Reliable and Global Measurement of Fluorescence Energy Transfer Using Fluorescence Microscopes. *Biophysical Journal* 2001 Vol. 81, pp. 2395-2402

Youvan DC, Coleman WJ, Silva CM, Peterson J, Bylina EJ and Yang MM.

Fluorescence imaging micro-spectrophotometer. *Technology et alia* 1997 Vol. 1, pp. 1-16

FRET

FRET (Fluorescence Resonance Energy Transfer) is the non-radiative transfer of energy from an excited fluorophore (donor) to another fluorophore (acceptor). FRET only occurs when the molecules concerned are in very close proximity (1-10nm). This makes it a valuable technique for studies where distances and interactions between molecules are of interest.

Data Acquisition

Three images are required for each data point: a Donor image, an Acceptor image, and a FRET image. For example, one typical donor/acceptor pair is CFP and YFP.

- Donor image = Excite with CFP filter, CFP dichroic, emit with CFP filter
- Acceptor image = Excite with YFP filter, YFP dichroic, emit with YFP filter
- FRET image = Excite with CFP filter, CFP dichroic, emit with YFP filter

Volocity combines the three input images on a voxel-by-voxel basis according to the chosen FRET method. The output is a floating-point value at each voxel.

- All images should be in proper register before FRET analysis.
- Images must be grayscale 8, 16 or floating bit depth.
- All images must be the same depth.

Calculating Bleed-through Constants

Volocity requires two values that represent the bleed-through or cross-talk of each dye (Donor and Acceptor) into the FRET filter set. These values are calculated at the start of the experiment and should be recalculated if any change is made to the system that could affect transmission characteristics or sensitivity to wavelengths of light.

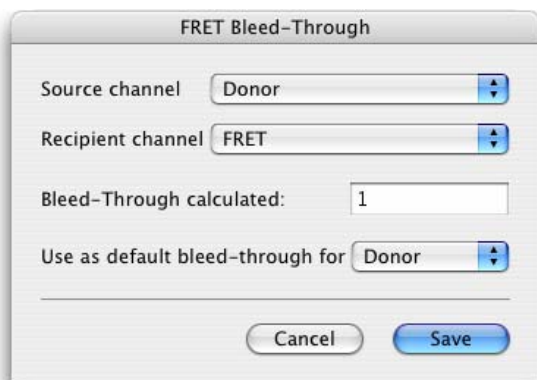
Note: The FRET bleed-through dialog does not offer subtraction of any background values. If you wish to subtract background from the images used to calculate bleed-through, this should be done

FRET Analysis

before using the images in FRET analysis. Alternatively, calculate your own bleed-through using values obtained by measuring appropriate ROIs in the images, as follows:

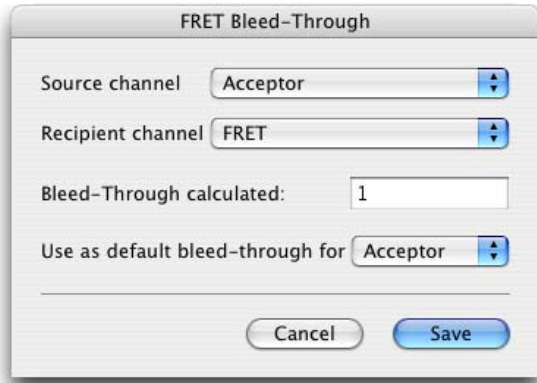
(intensity within cell(s) recipient-background intensity recipient) / (intensity within cell(s) source-background intensity source)

1. First set up the constant for the Donor channel. An image sequence containing donor and FRET channels from a sample containing only the donor fluorochrome is required.
2. Define an ROI or multiple ROIs on the Image View.
3. Select **FRET Bleed-through...** from the Tools menu.



4. Nominate a **Source channel** (where the bleed-through is coming from, in this case the donor channel) and a **Recipient** channel (where the bleed-through is detected, in this case the FRET channel). In a sample containing only donor fluorochrome any signal detected in the FRET channel must be bleed-through.
5. Select the channel to which the bleed-through constant should be applied in the **Use as default bleed-through constant for** drop-down list, in this case **Donor**.
6. Click on **Save**. The constant will be applied when you carry out the FRET calculation, as described in the next section. The bleed-through value is a normalised percentage between 0.0 and 1.0.
 - The value is calculated as the mean intensity of the recipient divided by the mean intensity of the source.
 - If you have defined multiple ROIs, Volocity calculates a mean of the mean values.

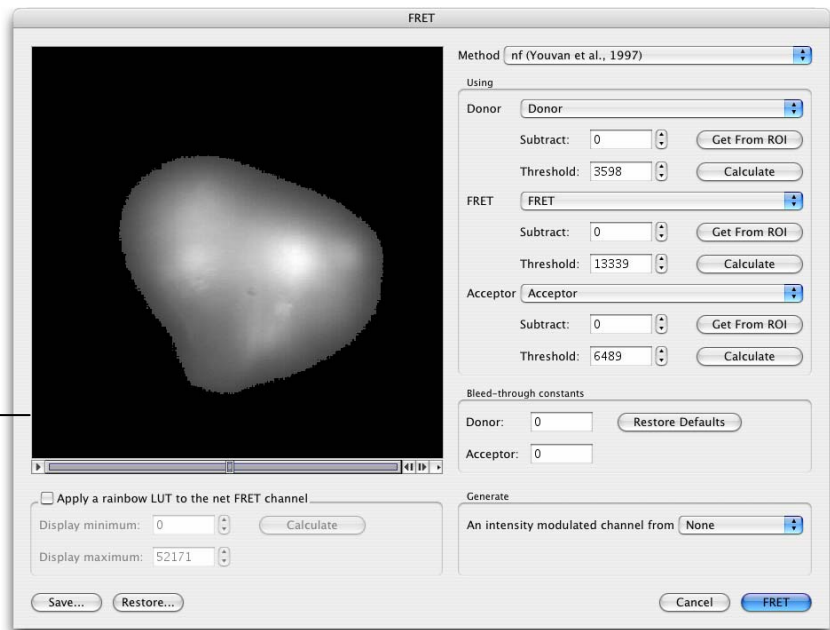
- Now create the constant for the Acceptor channel. An image sequence containing acceptor and FRET channels from a sample containing only the acceptor fluorochrome is required. Repeat steps 2 to 6.



Using the FRET dialog

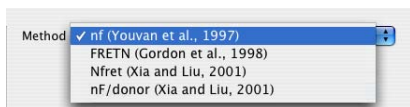
- Open an image sequence containing 3 or more channels, which represent a Donor channel, an Acceptor channel and a FRET channel.
- Choose **FRET...** from the Tools menu.

preview window
and multiple
timepoint play
controls



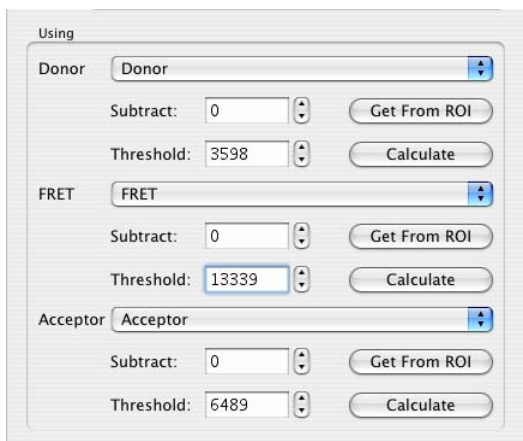
FRET Analysis

3. Select the **Method** of FRET calculation to be used from the drop-down list.



4. Select which channels should be used for **Donor**, **FRET** and **Acceptor**. A constant background value may be subtracted from every voxel in every image of each channel, if required. If source images are already background subtracted, go to step 5.

Note: Subtraction of a background constant is relevant only if the source images are evenly illuminated.



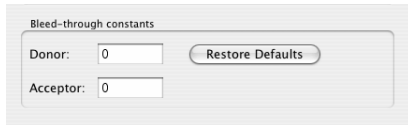
Enter a value in the **Subtract** field or draw an ROI on the Image View before opening the FRET dialog and click **Get from ROI** next to the Subtract field. Volocity will use the mean intensity from that ROI for each channel as the value to subtract. This value will be subtracted from the intensity value of every voxel before processing the FRET calculation.

5. Set threshold values for each channel by typing a value in the **Threshold** field. Thresholds allow removal of signals that would interfere with interpretation of results.

For every voxel position for which one of the source voxels has an intensity value at or below threshold, the value of that voxel position in the result image is set to 0 (zero).

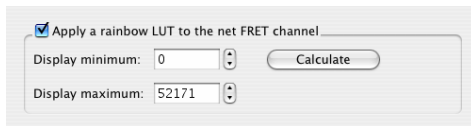
Use the Voxel Spy to pick out an exact threshold value or ask Volocity to **Calculate** the value for you. The calculate option determines the modal intensity value for the channel and uses it as the threshold.

6. Enter bleed-through constants to be used. Click **Restore Defaults** to apply the Bleed-through constants stored from the last bleed-through calculation.

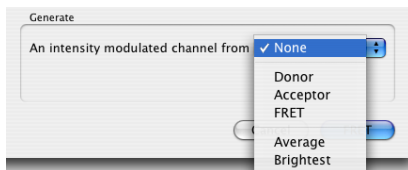


7. Preview the corrected FRET image. The preview shows a brightest point merge of the resultant FRET image. If the sequence contains multiple timepoints, use the play controls to view the effect of the parameters through the time series.
8. You may wish to apply rainbow colors to the output channel. Check the **Apply a rainbow LUT to the net FRET channel**. To optimize the use of the rainbow across the range of values present within the results enter **Display minimum** and **Display maximum** values.

This allows the finite range of colors available in the lookup table to be used to illustrate most effectively any changes in the FRET values. Click **Calculate** and the maximum and minimum FRET values for the current timepoint will be used. To easily compare FRET images with rainbow colors applied, they should all have the same **Display minimum** and **Display maximum** set. In the rainbow LUT the minimum value is shown as blue, the maximum as white. By setting **Display minimum** and **Display maximum** you are setting the black and white point of the images. This is what is done automatically by the Auto Contrast command in the Tools menu. Auto Contrast should, therefore, be used with caution if FRET images are to be used comparatively.



9. If required, generate an intensity modulated channel that will appear as a new channel in the image sequence. Select the modulator range from the **Generate** drop-down list. This generates a millions of colors display channel and may show morphology more clearly at the same time as indicating FRET values.



10. Click **FRET** to apply FRET calculation and create a corrected FRET channel in the image sequence. If specified, Volocity also creates an intensity modulated channel. All channels are also visible in the Navigation palettes and Voxel Spy palette. Channels may be hidden using the controls on the Navigation palette.

Measuring Corrected FRET Channels

FRET channels in image sequences may be measured in the same way as any other channel. When measurements are made on a FRET channel values for min, max, mean will be in the appropriate FRET units. If drawing regions of interest to follow changes over time, it may be beneficial to use the

Intensity Modulated Images

intensity measurements that ignore 0. This will ensure that if background areas are included when the ROIs are drawn, they will not influence the results.

See other sections of this chapter for details on how to use measurement tools and the classifier.

Using Color Reference with FRET images

To display corrected FRET images it may be beneficial to show the color reference on the Image View. This will show the values represented by the colors. Select **Show Color Reference** from the Image menu.

Intensity Modulated Images

The IM image uses pseudocolor and intensity to display a FRET output image. Color is used to represent FRET values, intensity represents the intensity of one of the input images. This technique helps reflect spatial information since the intensity of the original images is reflected in the resultant image.

To produce the IM image the intensity of each voxel of the FRET output image is scaled based on the range of intensities in the input images. The source image used for this is called the modulator image. The modulator image can be one of the source images, the brightest of the source images or the average of the source images.

The range of colors in the IM image is determined by the look up table applied to the FRET output image.

The range of intensities in the IM image is determined by the range of intensities in the modulator image.

The IM image is a millions of colors image. It is, therefore, a visualization tool not suitable for quantitation.

Volocity Restoration

Overview

Volocity Restoration is an additional product that must be purchased separately. See page 35 for more information about activating extension modules.

Volocity Restoration offers two powerful techniques that allow you to apply deconvolution to an image sequence or volume. These techniques are:

- **Iterative Restoration**, which is a restorative technique that reassigns out-of-haze focus (it does not subtract it from the data). It is designed to minimize out-of-haze focus and noise throughout the volume. Iterative restoration not only gives improvement in resolution in X and Y, but most significantly also in Z. The results are applicable for 3D visualization and all 3D measurements provided by Volocity Quantitation, including intensity. The processing required to achieve this is both time and memory intensive.
- **Fast Restoration**, which is a rapid, single pass process that acts on the volume as a whole. It gives resolution improvement in X and Y.

Note: For both techniques, the first time a data set is deconvolved, it may take slightly longer than the second and subsequent times a data set of the same dimensions is processed. This is because one optimization present within Volocity Restoration is the storage of the method by which the Fast Fourier Transform (FFT) of a data set will be calculated. The next time a data set of those dimensions is encountered the method for calculating the FFT is already available, thus saving a processing step.

Data Acquisition

Restoration is performed on z series of images that are managed within Volocity as either individual volumes or within image sequences. The acquisition process requires careful consideration to obtain optimum results from Volocity Restoration. In particular, the following acquisition issues may affect the quality of the deconvolved image data:

- undersampling, particularly in the z axis
- saturation of the signal within images
- uneven illumination due to an unstable light source
- premature cropping of the signal by capturing too little in X, Y and Z around the sample

Please refer to Appendix A on page 459 for a more detailed discussion about the requirements for sampling wide field microscopy data for deconvolution.

PSF items

In order to apply either technique, you must first create a PSF item. This is used to model the behavior of light in the optical system and must be appropriate for the data that is to be deconvolved. You can create a PSF in two ways:

- **Calculated PSF**, which is based on values entered into the **Calculate PSF...** dialog box. These values should be the same as the parameters under which the data for deconvolution was acquired. The application uses the values to calculate an appropriate PSF and create a new PSF item.
- **Measured PSF**, which is based on a volume (typically made from a z series of images of a sub-resolution, fluorescent bead) acquired under the same conditions as the experimental data. The application measures the PSF in the bead volume and creates a new PSF item.

Note: See Appendix B for a discussion of the reasons why beads might appear as ellipses instead of spheres in optical microscopy.

Creating PSF volumes can be time consuming. Improvition, therefore, recommends that you create a library of PSF volumes for different objectives and camera settings and drag them into the active library as they are needed. If you double click on a PSF icon in the library, it will open an Image View with an extra column on the right of the window containing the PSF data.

Note: Please see the Glossary for a definition of PSF parameters.

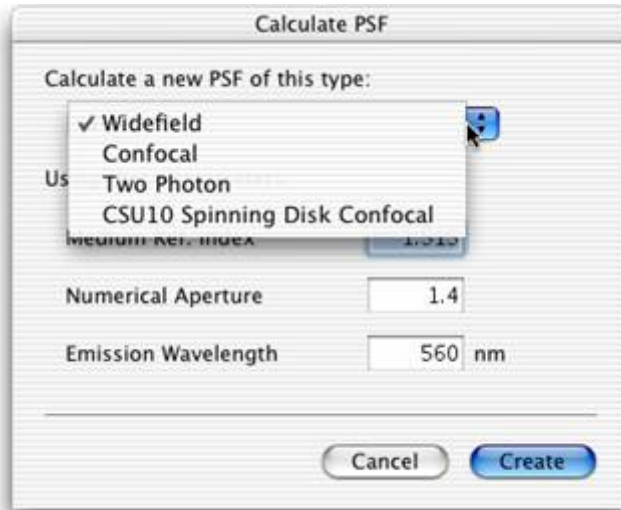
Data Calibration

The method of calculating and using point spread functions within Volocity 2.5 and later requires that all data used within Volocity Restoration be correctly calibrated before use. This applies to raw data for deconvolution as well as volumes from which a PSF is to be measured. Check the data calibration before commencing by selecting **Properties** from the Edit menu. Correct the X, Y or Z micron/pixel values, if necessary. See also page 54.

Calculate PSF

This dialog is used to calculate all types of PSF available in Volocity, including Widefield, Confocal, Two Photon and Spinning Disk Confocal PSFs.

1. From the Actions menu, select **Create New** and **Calculated PSF...**
2. In the dialog select the required type of PSF from the drop-down menu box.



3. Enter the values relevant for the data to which the PSF will be applied. Please see over the page for examples of the various PSF types.
4. Click on **Create** to create the PSF item.

The PSF calculated from the dialog is supersampled. When you enter the values for deconvolution, Volocity will read the calibration from the volume and automatically subsample this supersampled PSF. This ensures an accurate PSF with the correct settings for deconvolution.

Note that when deconvolving the volume, Volocity requires the calibration values to be correctly set, or the PSF used will not be accurate. If you attempt to deconvolve a volume for which the calibration values appear not to have been set, you will be reminded to set these values before proceeding.

The spinning disk is used in such systems as the PerkinElmer UltraView. This PSF is unique to a spinning disk with the spiral pinhole configuration, pinhole size, and pinhole spacing of the CSU10/CSU22 spinning disk and should not be used to calculate the PSF for any other type of spinning disk.

Measure PSF

Measure PSF

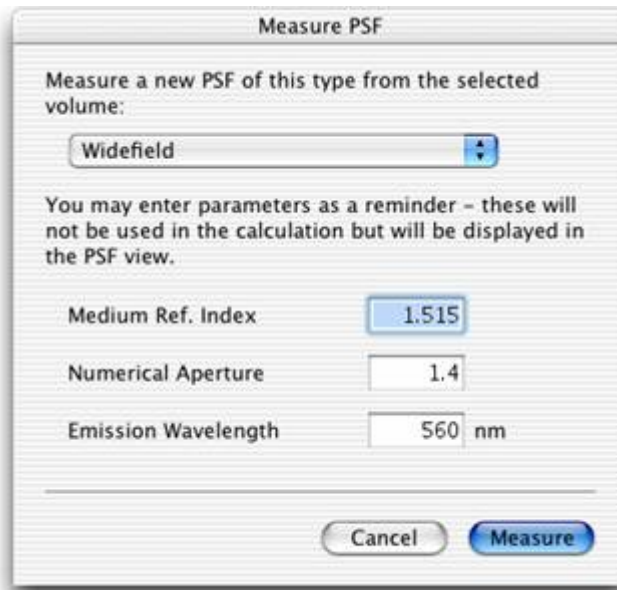
This dialog is used to measure all types of PSF available in Volocity.

1. Make a volume from PSF measurement data, such as z sections taken of a sub resolution, fluorescent labeled bead.
2. Select the PSF bead volume in the library and, from the Actions menu, select **Create New** and **Measured PSF...**



3. In the dialog select the required type of PSF from the drop-down menu box. All other values are optional. You may like to record the other acquisition parameters for the PSF bead as a reminder, but they are not used to create the PSF item.

The following example shows the dialog for a widefield PSF.



4. Click on **Measure**.

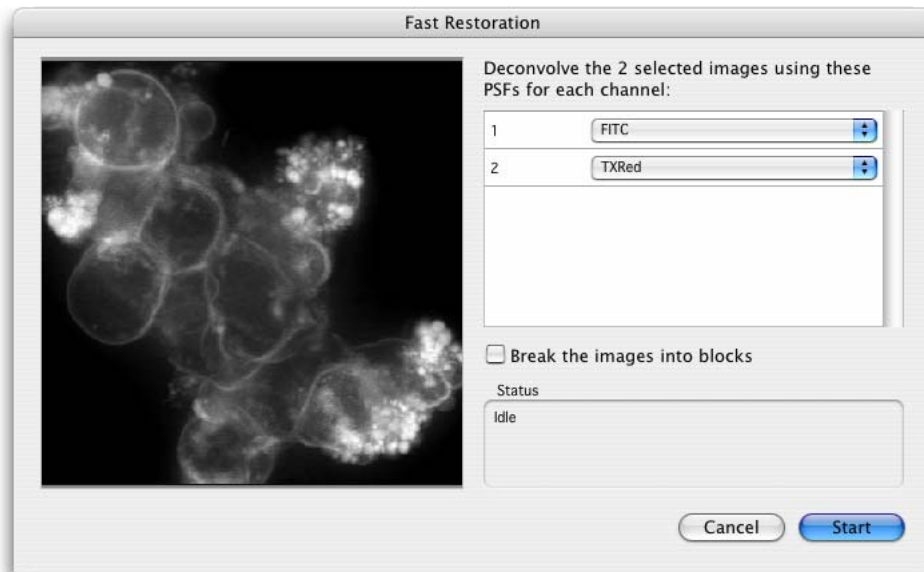
Note that when deconvolving the volume, Volocity requires the calibration values to be correctly set, or the PSF used will not be accurate. If you attempt to deconvolve a volume for which the calibration values appear not to have been set, you will be reminded to set these values before proceeding.

Applying Fast Restoration

You can apply Fast Restoration to a single volume or an image sequence, or multiple volumes or image sequences selected in the library.

Make sure the library contains the required PSF volumes. You will need a PSF for each wavelength and lens type.

1. Select items to be restored in the library. All items must be correctly calibrated, by setting X, Y and Z properties to correctly use PSFs.
2. **Fast Restoration...** from the Tools menu. The dialog will show a list of all the unique channel names identified in the selected data.
3. Select the appropriate PSF for each channel from the drop-down menu. This means channels with the same name will be restored using the same PSF.



4. For large data sets, you may like to check the **Break images into blocks** checkbox. Deconvolution is a very memory-intensive operation and the software needs to hold multiple copies of the volume in memory at the same time so the space occupied in memory during restoration is much greater than the physical space occupied by the original data.

Restoration will proceed fastest if all data required for the processing can be held in physical memory. When the data set size exceeds the amount of physical memory Volocity can break the images down into blocks which will fit, using the following calculation.

Memory per processor = physical RAM/number of processors.

Block size will vary therefore, not only with data set size but also with hardware configuration.

Based on this calculation the volume is divided into blocks allowing an overlap between blocks. This overlap is used in the processing of each block but not copied to the result to minimize the deterioration of quality caused by breaking the images down.

Restoration will proceed more quickly since data will not have to be paged in and out of virtual memory. On systems with multiple processors blocks may be processed simultaneously, further increasing speed. Breaking images into blocks may lead to a slight deterioration in quality; depending on the nature of the data it may be possible to detect the edges of the blocks the data was broken down into.

Note: If it is calculated that the data fits into memory as a single block, it will be restored as a single block, even if the checkbox is checked.

5. Click on **Start** to apply Fast Restoration.

The preview window shows each volume as restoration is applied. While the deconvolution is being carried out the **Start** button will change to **Pause**. You can pause the process at any time, for example if you want to interact with the preview. Click on the button (now labeled **Start**) a second time to restart the processing. If you click **Done** while the deconvolution is paused, the deconvolved data will not be saved.

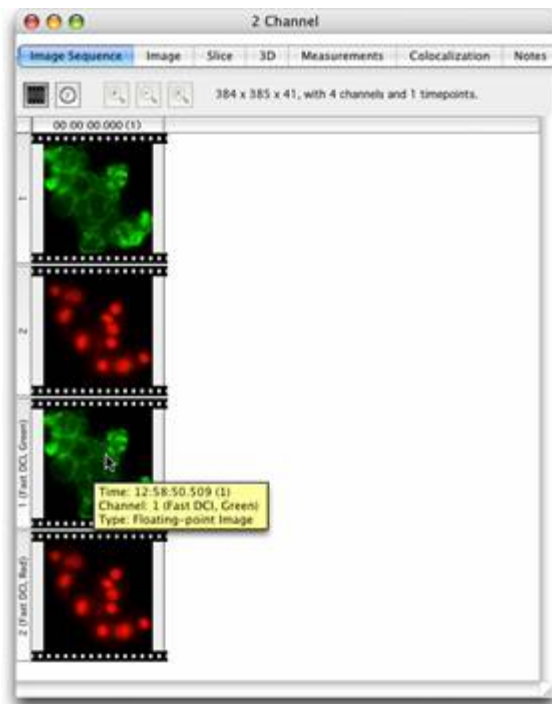
The Status window shows progress details.

6. Once all the selected channels have been deconvolved, click **Done**. When deconvolution is completed, each channel will have a deconvolved equivalent with a name indicating that Fast Restoration was applied; the raw data is not replaced with deconvolved data.

For single volume(s) the name of the new volume includes the text 'Fast DCI' and the name of the PSF used.

Applying Fast Restoration

For image sequences the results are added as new channels, so this information is in the name of the new channels and the tooltip shown when the mouse is hovered over the channel. If the data was broken down into blocks the number of blocks is recorded.

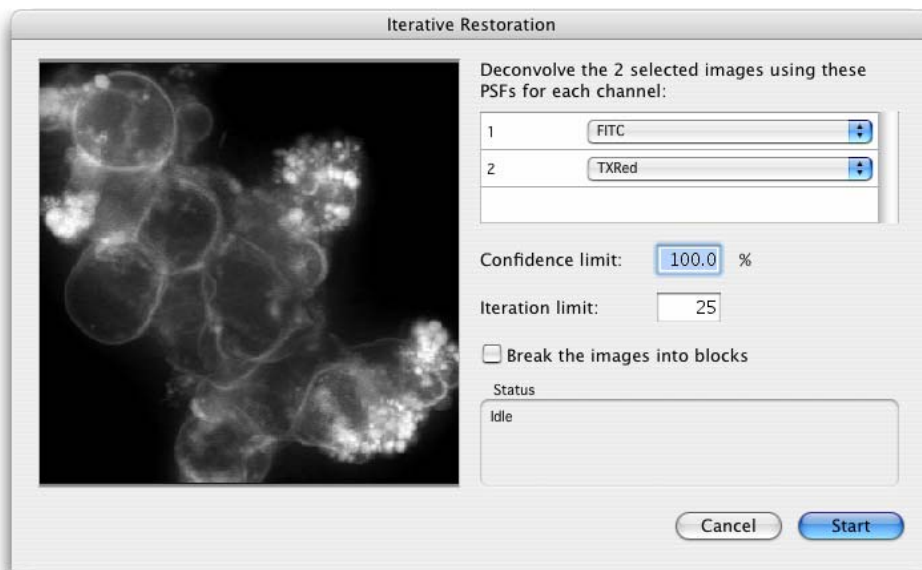


Applying Iterative Restoration

You can apply Iterative Restoration to a single volume, or an image sequence, or multiple volumes or image sequences selected in the library. When you set up the restoration parameters, as illustrated in the following example, you also specify an iteration limit and a confidence limit, as a percentage. After each iteration, Volocity calculates a confidence in the current image. This is expressed as a percentage. The deconvolution process will continue until either the required number of iterations is reached, or the confidence limit of the current iteration exceeds the value entered.

Make sure the library contains the required PSF volumes. You will need a PSF for each wavelength and lens type.

1. Select items to be restored in the library.
2. Select **Iterative Restoration...** from the Tools menu.



3. Select the appropriate PSF for each channel from the drop-down menu. This means channels with the same name will be restored using the same PSF.
4. Set parameters to determine when the deconvolution will end. Either set a confidence limit or an iteration limit. An iteration is one repetition of the restoration algorithm towards the result. The confidence limit is a measure of image convergence. 100% confidence indicates that restoration can offer no further improvement to the image. Whichever is reached first will terminate the deconvolution. Confidence is calculated after each iteration and is the result of testing the results of deconvolution against the original data. 100% confidence may take many iterations to reach. Many datasets can be deconvolved extremely successfully with the confidence limit set to 98% or 99%. It may take a

Applying Iterative Restoration

different number of iterations to reach the same confidence limits for different channels. The iteration limit will stop deconvolution after the required number of iterations. We recommend an iteration limit of 25. You should not need to change this for different data sets.

5. For large data sets, you may like to check the **Break images into blocks** checkbox. Deconvolution is a very memory-intensive operation and Volocity needs to hold multiple copies of the volume in memory at the same time so the space occupied in memory during restoration is much greater than the physical space occupied by the original data.

Restoration will proceed fastest if all data required for the processing can be held in physical memory. When the data set size exceeds the amount of physical memory Volocity can break the images down into blocks which will fit, using the following calculation.

Memory per processor = physical RAM/number of processors

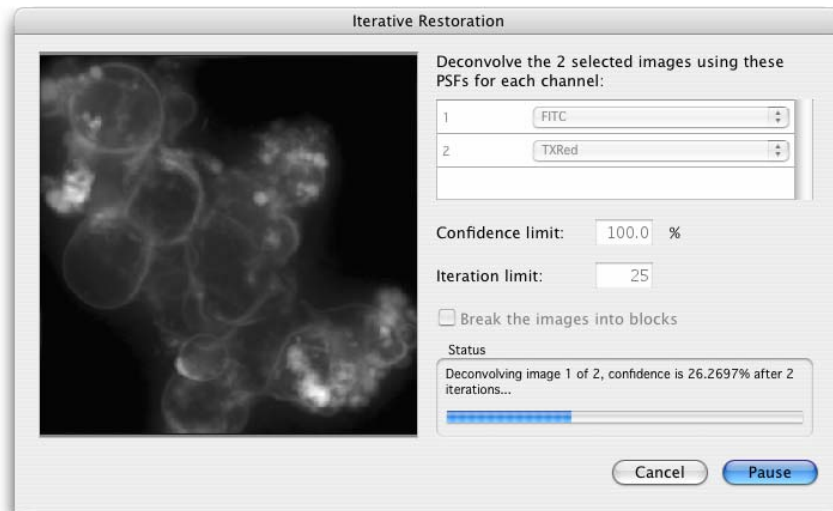
Block size will vary, therefore, not only with data set size but also with hardware configuration.

Based on this calculation the volume is divided into blocks allowing an overlap between blocks. This overlap is used in the processing of each block but not copied to the result to minimize the deterioration of quality caused by breaking the images down.

Restoration will proceed more quickly since data will not have to be paged in and out of virtual memory. On systems with multiple processors blocks may be processed simultaneously, further increasing speed. Breaking images into blocks may lead to a slight deterioration in quality, depending on the nature of the data it may be possible to detect the edges of the blocks the data was broken down into.

Note: If it is calculated that the data fits into memory as a single block, it will be restored as a single block, even if the checkbox is checked..

6. Click on **Start** to apply Iterative Restoration.



Feedback on progress is different depending on the use of **Break images into blocks**. When **Break images into blocks** is not enabled the preview window shows a brightest point merge, updated with each iteration as restoration progresses. It is normal for the results of the first iterations to look worse than the original data since the first estimate of the true nature of the data in the image is based on the image itself.

The confidence factor will rise significantly with each iteration, however, the rate of increase will slow with successive iterations.

Note: While the deconvolution is being carried out the **Start** button will change to **Pause**. You can pause the process at any time, for example if you want to interact with the preview. Click on the button (now labeled **Start**) a second time to restart the processing. If you click **Done** while the deconvolution is paused, the deconvolved data will not be saved.

If **Break images into blocks** is enabled feedback on progress is provided less frequently. The preview image, a brightest point merge of the volume is updated as each block is completed. The status box will show the number of volumes of the total completed and the number of blocks of the current volume completed. Progress will not be updated with each iteration.

7. Once all the selected channels have been deconvolved, click **Done**. When deconvolution is completed, each channel will have a deconvolved equivalent; the raw data is not replaced with deconvolved data.

After Iterative Restoration the result is meaningfully labeled, according to the processing it underwent.

For single volume(s) the name of the new volume includes the number of iterations, the confidence limit reached, the number of blocks, if applicable, and the name of the PSF used.

Illumination Correction

For image sequences the results are added as new channels, so this information is in the name of the new channels and the tooltip shown when the mouse is hovered over the channel. If the data was broken down into blocks the number of blocks is also recorded.

Illumination Correction

Minor anomalies in excitation light intensity over time, such as lamp flicker, can lead to poor deconvolution results. This will manifest itself as one or more inconsistently bright or dark layers in the deconvolved volume. The flicker may not be apparent during inspection of the raw data.

Better results can be obtained by applying illumination correction, then deconvolving the data again.

Select the required volume(s) and select **Correct Illumination** from the Tools menu.

Correct Illumination operates within each individual volume and bases any intensity correction solely on the trends shown within that volume. It does not normalize time resolved data to account for bleaching and cannot correct severe problems with lighting during the experiment. Ideally, lamp flicker should be avoided by using a stable light source.

Glossary

The definitions below explain the terminology used in this guide.

3D Renderer

Volocity calculates a projection—a screen image of how the volume appears from a specific viewpoint—using direct rendering from the 3-dimensional data. 3D Rendering is orthographic.

Adopted item

If you want to edit an image and change it in any permanent way, you need to adopt it first. An item that is adopted into the library has the image data stored within the library. This means that any changes made to the item are only applied to the adopted item not the original image data.

Black Level

The Black Level allows you to control opacity rendering. It sets the lower level for opacity, thus allowing you to create an offset from 0. The 3D Renderer will treat anything below the value specified as completely transparent.

Cloak

There may be times when you want to exclude images from a particular view of the data. For example, you may want to run a movie from a Gallery View, but do not want to include all the images in a gallery. The cloak command allows you to hide the images without removing them from the library.

Detector Pinhole Size

This is a parameter in the calculation of a confocal PSF. It is the diameter of the pinhole used while acquiring the optical sections. Note: this is measured in airy units not micrometers.

Displacement

This is the straight line distance between the first centroid in a track and the last centroid.

Displacement rate

This is the displacement/(time of the last timepoint in the track – time of the first timepoint in the track). Displacement rate may also be defined as the average displacement per second.

Duplicate

The duplicate command allows you to duplicate items in the library, so that you can include items in a number of different folders.

Emission/Light Wavelength

This is a parameter in the calculation of a PSF. It is the emission maximum of the fluorochrome imaged in the data set.

Excitation Wavelength

This is a parameter in the calculation of a confocal PSF. It is the excitation maximum of the fluorochrome imaged in the data set.

Fast Restoration

This is a rapid, single-pass process that acts on the volume as a whole. It gives resolution improvement in X and Y.

Folder

The library may be organised into a number of different folders that best suit your archiving/sorting requirements. Each folder may hold sub-folders or individual items. In this way you can organize your images into a number of different categories, each with a number of different levels.

Gallery

If you have sub-folders in the library of files whose format supports multiple images, for example Openlab LIFF files. The gallery view displays all the images in a single view.

HR Renderer

Volocity calculates a projection—a screen image of how the volume appears from a specific viewpoint—using direct rendering from the 3-dimensional data. HR Rendering is a perspective rendering. Two types of HR Rendering are available: HR Opacity and HR Intensity.

Image

This relates to the bottom-level view of the library - the individual image, which may be 2D or 3D. Double click on an image or volume thumbnail to display the image in a new window where you can view and manipulate it, if required. For 3D items, the view shows the 3-dimensional cross-section of all planes (top left section) and each of the axes/planes.

Image Sequencer

The Image Sequencer allows interaction with multiple channel, time resolved data by providing an easy-to-use interface for this data.

Iterative Restoration

This is a restorative technique that reassigns out-of-focus haze (it does not subtract it from the data). It is designed to minimize out-of-focus haze and noise throughout the volume. Iterative restoration not only gives improvement in resolution in X and Y, but most significantly also in Z. The results are applicable for 3D visualization and all 3D measurements provided by Volocity Classification, including intensity.

Keywords

A keyword is an item property that you enter on the Properties dialog to provide additional data about that item that can be used in the Find command.

Library

This is the structure within which the digital images are collected and organised.

Linked item

When items are added to a library, a link is created to the original source data wherever it is held, for example on the hard disk, on a CD or on a network server. No image data is stored with the item in the library. When you view the linked image, the image data is retrieved from the original image file. You cannot make changes to linked items. You must adopt them into the library if you want to apply any of the commands that change image data.

Note: If the library has been moved from one machine to another or the original data has been deleted, the item thumbnail will still appear in the library and gallery views, but you may not be able to display it as a single image or movie.

Meandering index

This is the displacement rate / velocity, which provides a measure of a track's deviation from a straight line. The meandering index is always a value less than or equal to 1. A meandering index of 1 indicates that the track is a perfect straight line; the smaller the value of the meandering index, the greater the meandering of the track.

Medium refractive index

This is a parameter in the calculation of a PSF. It is the refractive index of the medium between the objective lens and the cover slip. Typical values are 1.0 for air, 1.33 for water and 1.515 for oil.

Movie

From the gallery view, you can select the Movie tab to display the contents of the gallery as a movie.

Movie Sequencer

The Sequencer allows you to define a number of tasks that manipulate the 3D Views and combine them together to produce a movie view of the volume.

Numerical aperture

This is a parameter in the calculation of a PSF. It is the numerical aperture of the objective lens. It can normally be found engraved on the objective lens.

Properties

These describe an item in the library. There are fixed properties, such as image size, source location and date details, which cannot be changed. Other properties, however, allow the user to add or edit information, such as adding keywords or changing the name.

PSF (Point Spread Function)

Images seen through a microscope contain information from the focal plane of interest plus out-of-focus information from other focal planes on the image. Volocity Restoration uses a technique called deconvolution to remove the out-of-focus information. This is a mathematical technique that calculates the out-of-focus information from other images using the "point spread function", or PSF, and subtracts this from the image plane of interest. It processes the Z stack of images as a complete data set, converting pixels to voxels (volume elements) to produce high-resolution, deconvolved images.

Skeletal length

This is the maximum length in the selected object and is shown in the Measurements. The selection is eroded evenly from its border inwards until it consists of a one-voxel thick, skeletal representation along its entire length. This "skeleton" is then measured.

Smooth zoom

The Smooth Zoom command on the View menu applies interpolation to the view to give better quality when viewing zoomed images and automatically sets the best quality for printing.

Thumbnail

The thumbnail is a representation of an item for quick display. This may be a folder or image. The application automatically creates a thumbnail for each image or each layer in a multi-layer image.

Time span

This is the number of timepoints spanned by a track.

Volume

A volume is the name for a 3-dimensional object within Volocity. It can exist as a single item within the library, created from a series of 2D images related in the Z-dimension or within an image sequence, which is a powerful tool for creating a collection of volumes arranged by channel and timepoint.

Voxel

This is the 3-dimensional equivalent of a pixel, the smallest discrete spatial component of a digital volume. Volumes within Volocity consist of voxels, each with an associated color and /or intensity value.

Appendices

Sampling Widefield Data for Deconvolution

Overview

This technical note contains a discussion of some of the requirements of data sampling to achieve optimal deconvolution results when using Improvion deconvolution software. Deconvolution will generate the best results when presented with the highest quality data.

Depth of Sample to Scan

Point spread of light sources from within a sample can extend above and beyond the limits of the physical top and bottom of that sample. Deconvolution uses out of focus information to calculate true sample data therefore it is important to capture that which extends beyond the sample boundary.

This means capturing a large enough image size in X and Y and taking sample slices above below the object. As a rule of thumb, capture from darkness below the object to darkness above or half the thickness of the object of interest above and below, therefore total scanned distance is twice thickness of sample object.

Measured PSFs

The acquisition of data for a measured PSF must be subjected to the same strict controls as the experimental data. Effort must be taken to minimise artefacts or aberrations. If these cannot be avoided then the data for the measured PSF should be collected with the same artefacts and aberrations as the experimental data. The bead used for measuring PSFs must be a speck bead of a size below the resolution of the microscope for the results to be valid. PSFs must be measured from a point source of light.

Optics and Illumination

The illumination pathway must be clean, free from dust and correctly aligned. Refer to the documentation supplied with the microscope to confirm how to align the epi-fluorescence light path.

Use high numerical aperture lenses. Try to match refractive indices through sample, cover slip and immersion medium.

If the illumination source power output varies during the collection of a data stack this will result in there being differences in intensity between z planes. These may be more apparent after deconvolution. Volocity Restoration offers a Correct Illumination function which can alter the data to remove these variations. It is always preferable to avoid introducing artefacts into images rather than correct for them later. Use an illumination source with a stable power supply. Lamp flicker is worse towards the end of the life of an arc lamp, replace bulbs if they show signs of flickering and always at the end of their recommended life.

Practical Considerations

The above information is for an ideal situation and it may be necessary to compromise on some of the recommendations to stay within the performance limits of the hardware and to ensure survival of the specimen for the desired duration of the experiment.

Although operating the camera at its highest resolution will satisfy the Nyquist theorem for sampling frequency, it may be necessary to use binning to minimise sample exposure to UV light. In many cases the XY sampling will still be approximately half the size of the smallest resolvable object.

Imaging many stacks over time or imaging a sample which shows photo bleaching may require compromise on the number and spacing of z slices. It may be worth considering a volume deconvolution technique rather than an iterative restoration if the number of slices has to be reduced.

Sampling Frequency

A biological structure labeled with a fluorescent probe consists of continuous distribution of fluorescence. Imaging a biological sample means taking samples of this continuous fluorescence at discrete points in XYZ space (i.e. images). The frequency at which images are taken therefore affects the quality of the final result.

Nyquist Theorem

The Nyquist theorem states that in order to fully describe objects they must be sampled at a rate of twice the smallest resolvable object in the data.

For iterative restoration the Nyquist sampling rate is the minimum necessary and higher sampling rates will lead to superior deconvolution results.

For volume deconvolution lower sampling rates in z may be acceptable. The lower the number of neighbors being considered in the deconvolution the larger the z spacing that may be acceptable.

Sampling frequency in X and Y is determined by pixel size of the camera and objective magnification. Sampling frequency in z is determined by the distance between slices.

Sampling in x and y

Sampling frequency in x and y is determined by the spacing of the pixels on the camera.

$$\text{sampling frequency} = \text{camera pixel size} / \text{total magnification}$$

$$\text{XY or lateral resolution: } d = 0.61\lambda / \text{NA}$$

Where λ = wavelength and NA = numerical aperture of objective lens

Minimum sampling frequency in X and Y therefore = $d/2$.

If sampling frequency is less than or equal to d then sampling theory is obeyed.

Sampling in z

Find the depth of focus of the system and sample at half that distance.

A method of calculating DOF is:

$$\lambda/2NA^2$$

Where λ = wavelength and NA = numerical aperture of objective lens.

Example

A sample labeled with GFP (emission wavelength 510nm) is imaged with a 63x oil immersion objective with NA of 1.4. The immersion medium has a refractive index of 1.518. The camera has a pixel size of $6.45\mu\text{m}^2$.

XY or lateral resolution = 222 nm or 0.222 μm

Z or axial resolution = 130 nm or 0.13 μm

To follow the Nyquist theorem the sampling interval should be half of these:

XY sampling = 0.111 μm

Based on the description above the camera will sample at 0.102 μm in X and Y.

Z sampling = 0.065 μm

It is as important to sample accurately as it is to sample at the correct frequency. Always use a motorised stage, manual adjustment is not only tedious and slow, it is not accurate enough.

Calculation of the optimum sampling rate will give results of very small values. If the equipment available cannot reproducibly produce such fine movement, compromise is necessary to maintain accuracy. Sample at the smallest reproducible z step. Biological samples may not tolerate exposure to the excitation wavelength for the time necessary for such intensive sampling, and sampling ideals will again need to be compromised, as discussed below.

Avoid backlash in microscope focus mechanisms by moving against the load, or against gravity. Move the focus in one direction only during the acquisition. Once started never reverse the direction. To avoid backlash at the beginning of a stack focus to the first imaging point, move to below that start point then, back up to the start to capture the first frame.

Signal to Noise

Maximise signal to noise ratio in images, use longest exposure possible which the sample will tolerate without bleaching.

Avoid pixel saturation; deconvolution will accentuate this and lead to blooming.

Perform background subtraction on images before deconvolution.

Spherical Aberration

Spherical aberration is asymmetry in the spread of light from a point source. It can be detected by viewing fluorescently labeled beads with the system. Focus through the beads and look for symmetrical spread of light on either side.

Sampling Widefield Data for Deconvolution

Use the correct cover slip. Lenses are designed for optimum performance used with certain cover slips. A lens will not perform to its specification if used with a different thickness of cover slip.

Match refractive indices between sample, sample medium, immersion medium and lens. Mismatched refractive indices can cause spherical aberration and can lead to optical z section size varying from physical z step size, because the path of the emission light is changed at each boundary between different RI.

Try to ensure that all the materials that the light passes through are closely matched for RI.

It may be preferential to use a lower NA, water immersion lens which more closely matches the RI of a watery sample than an oil immersion lens. Also consider dipping objectives which are lowered into the medium surrounding the sample.

Vibration

Vibration of the acquisition system will cause lateral and axial shifts in the image acquired which will degrade the quality of the stack and therefore deconvolution. There is no way to correct for this; therefore, systems must be protected from vibration.

What Causes Axial Elongation of Structures in Optical Microscopy?

Overview

Fluorescently-labeled beads are used for a variety of purposes in optical microscopy, from measuring a point spread function to assessing the performance and calibration of the system. The beads supplied by manufacturers such as Molecular Probes are manufactured under strict controls and can be considered as spheres. Why then do 3D visualisations of these beads, reconstructed from 2D sections, appear, not as spheres, but as ovoid?

The reasons why beads might appear as ellipses instead of spheres in optical microscopy are different depending on the size of the objects being observed.

Working with Small Objects

The z (axial) resolution of a widefield or confocal microscope is normally three to four times less than its xy (lateral) resolution. In other words, the smallest object that can be resolved is approximately 200 nm in xy and 700 nm in z.

When working with small objects, such as 200 nm-point source beads for measuring a PSF, this effect is significant. The beads will appear to be 3-4x elongated in z.

The elongation effect caused by the difference in resolution in z is only significant on small structures around the resolution limit of the microscope. It is a fact of optical microscopy and should be taken into consideration when interpreting data.

Working with Larger Structures

When working with larger structures (>10 μm) you can sometimes also see elongation. The cause of this is normally different. It is due to a mismatching of refractive indices, either between the immersion medium of the objective and the sample medium or between the sample medium and the sample itself.

If the refractive indices are mismatched then the physical distance moved by the focus drive is not the same as the optical distance. This gets worse as you move deeper into the sample, away from the cover slip. Optical distance is less than focus drive distance and the result is stretching in z.

To minimize this effect we recommend that refractive indices are matched as closely as possible. For example if your specimen consists mainly of water and is mounted in an aqueous medium, it would be preferable to use a water immersion lens of a slightly lower NA than an oil immersion lens of the same magnification and higher NA.

Another factor which can also effect elongation is spherical aberration from high NA lenses. Most microscope manufacturers have special flat-field objectives that will reduce this effect. Consider evaluating lenses for spherical aberration before accepting the final example.

These factors apply to structures within biological samples equally as to beads therefore they should be taken into account when interpreting 3-dimensional data.

OptiGrid Structured Light Algorithm

Structured light illumination systems such as the OptiGrid project a sinusoidal pattern onto specimens by placing a one-dimensional grating in the microscope's illumination path.

The grid is moved in the Y plane into three different positions and an image is acquired at each position. The following algorithm is used to produce a single, derivative, confocal image from which the grid pattern has been removed.

$$\text{Confocal Image} = \sqrt{((I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2)}$$

I_1 = Image with the grid in position 1

I_2 = Image with grid in position 2

I_3 = Image with grid in position 3

	I_1	I_2	I_3	$(I_1 - I_2)^2$	$(I_1 - I_3)^2$	$(I_2 - I_3)^2$	Grid Image
Strongly Focused	1	1	0	0	1	1	1.41
Weakly Focused	0.8	0.8	0.2	0	0.36	0.36	0.84
Out of focus	0.5	0.5	0.5	0	0	0	0

Other sources of information

Improvisation

TN424: A study of fluorescence standards confirms that OptiGrid confocal images are suitable for quantitative microscopy

Barlow and Guerin (2007)

Quantization of Widefield Fluorescence Images Using Structured Illumination and Image Analysis Software. *Microscopy Research and Technique* 70:76-84

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