Light Microscopy for Biomedical Imaging

27 September 2007

E = hν

Michael Hooker
Microscopy Facility

1. Some Other Confocal Modes
2. Bleed Through
3. Co-localization
4. Live Cell Imaging
5. Digital images (II)

MHMF acknowledges the support of the Roadmap Initiative, School of Medicine, UNC

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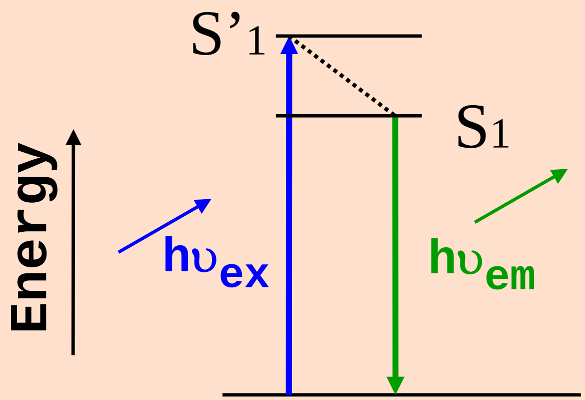
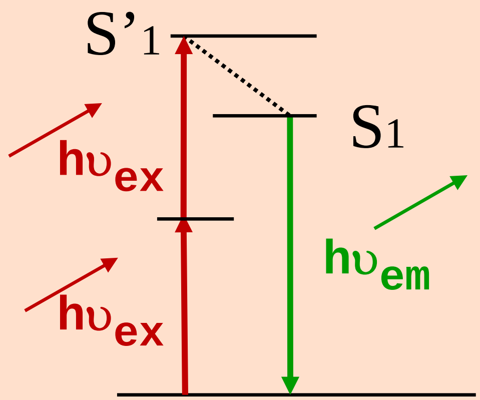
2 Photon Confocal

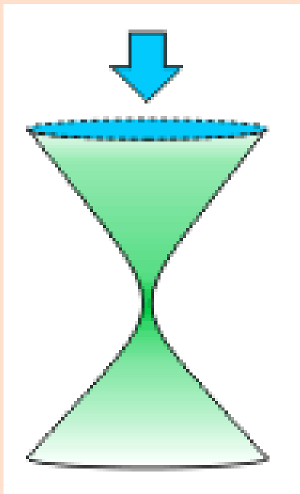


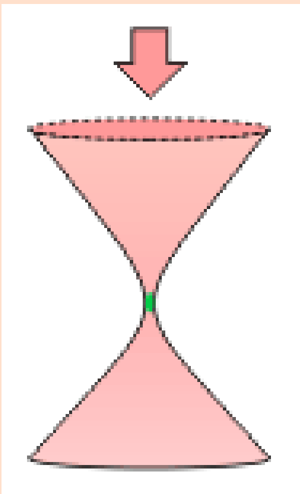
Single Photon

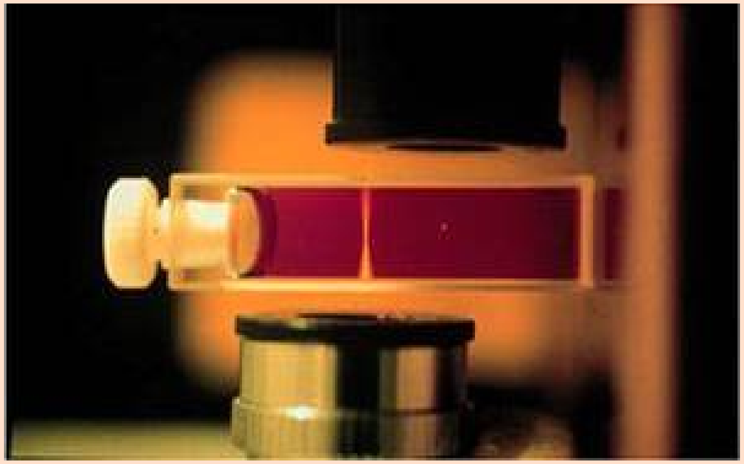


Two Photon

Photons concentrated by;
1. Using 100 fs pulses at a repetition rate of 80 MHz; 2. Focusing the beam with an objective.

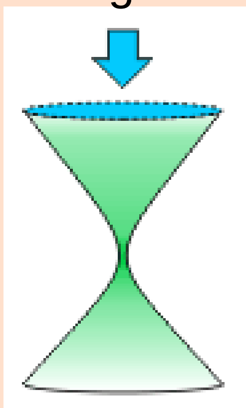


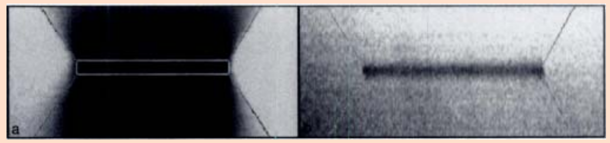




2 Photon Confocal

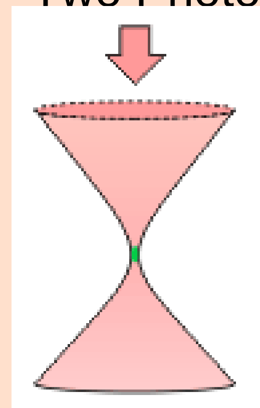
Single Photon





Bleached block of plastic
(x-z scans)

Two Photon



Cheaper
Easier to do
Excite fluorochromes specifically
Ubiquitous

but

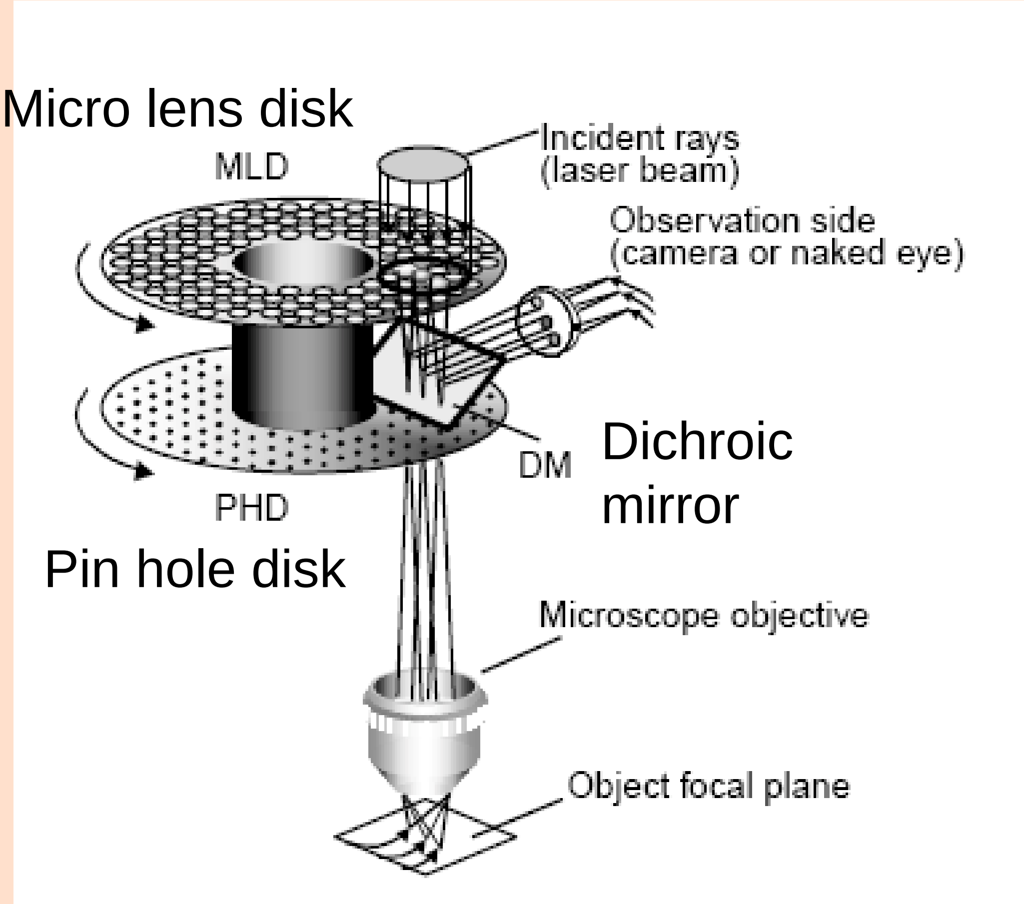
Bleach cone of dye

IR has good depth penetration
Less scattering
Less phototoxicity
Collect all returning photons – no pinhole

but

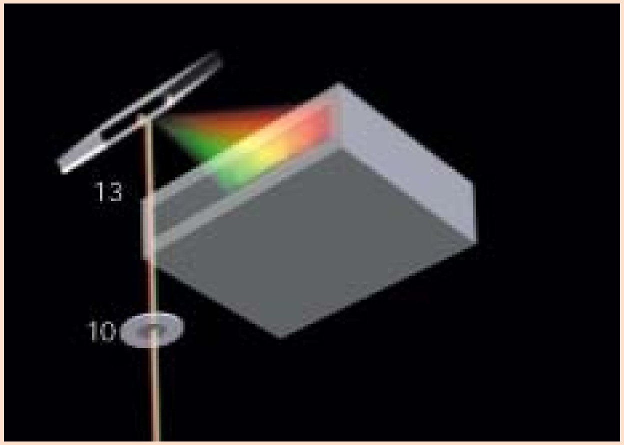
Dye excitation not well characterized
Tend to excite all fluorochromes present
Higher excitation of autofluorochromes
High cost
Need objectives good in IR to blue range
Optics needs good group delay response

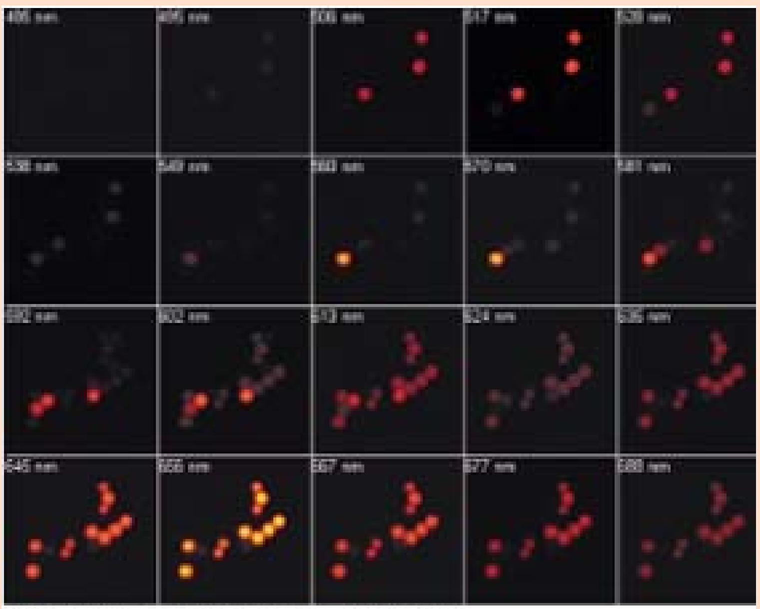
Spinning Disk Confocal



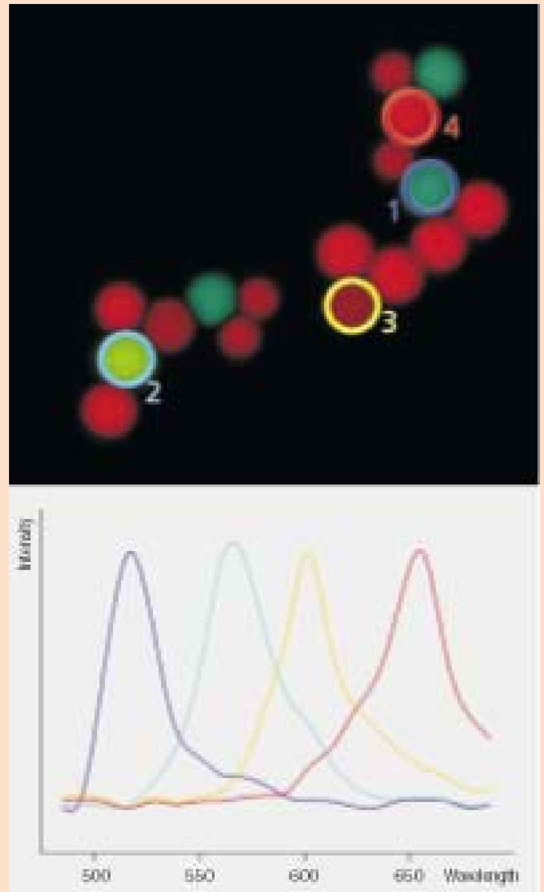
Spinning disk – faster acquisition, less light (photobleaching)

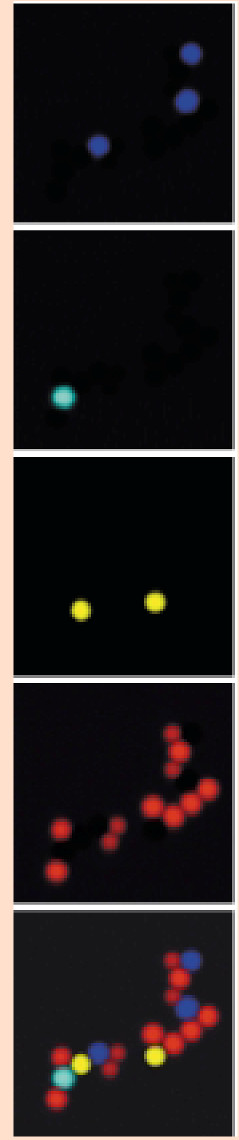
Spectral Detection – Zeiss Meta Confocal





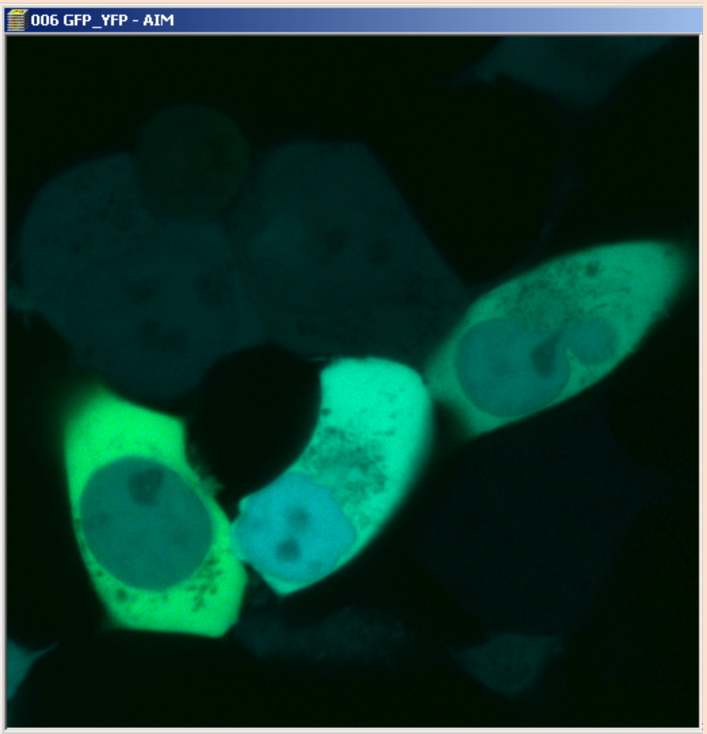
Four-population mix of single-labeled polystyrene beads:
Lambda Stack with spectral distribution of fluorescence emissions



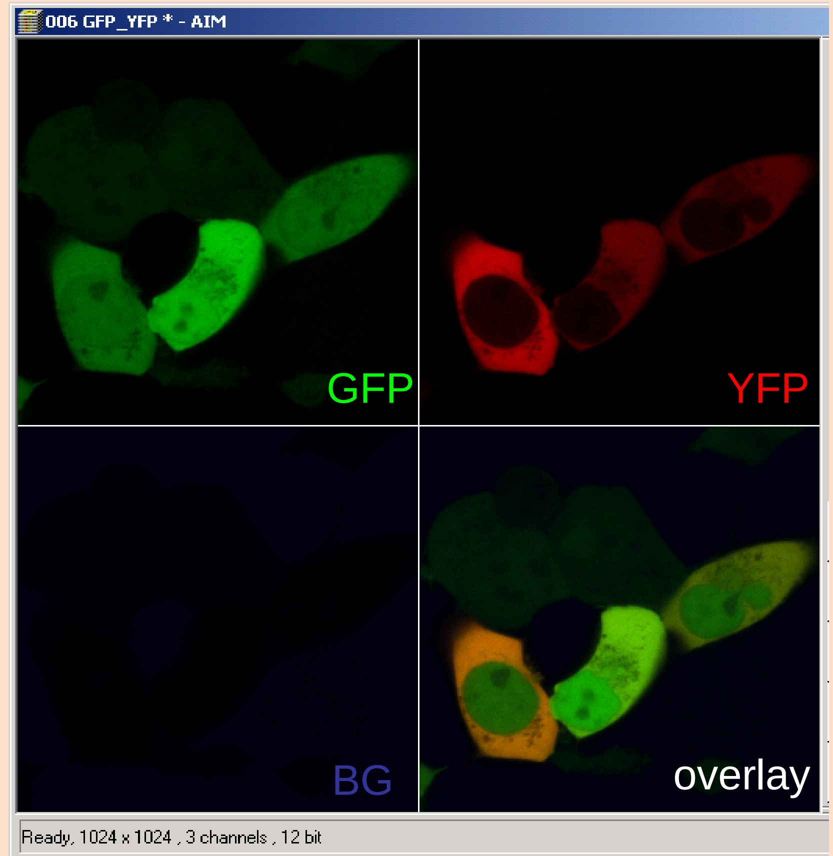


Meta - Emission Fingerprinting

Original Lambda stack



Separation into individual channels
after unmixing



Some Serious Confocal Scanning Oversights

- Not checking for cross talk
- Loosing Resolution - Not ensuring zoom and image size gives a pixel size which matches the optical resolution of the objective – also z-step size too big
- Acquiring too much resolution – photobleaching, wasted time
- Too strong laser excitation – causes photobleaching
- Scanning at a high zoom factor or too long – laser bean dwells too long on a small region
- Not letting forced air cooled lasers cool down at for least 5 minutes after turning off the lasing
- Using Alexa 594 with CY5 or Alexa 633 – cross excitation
- Using a FITC secondary antibody conjugate (use Alexa 488 instead)
- Failing to do no primary antibody controls – Secondary antibodies alone can sometimes have remarkably specific looking distributions
- ……

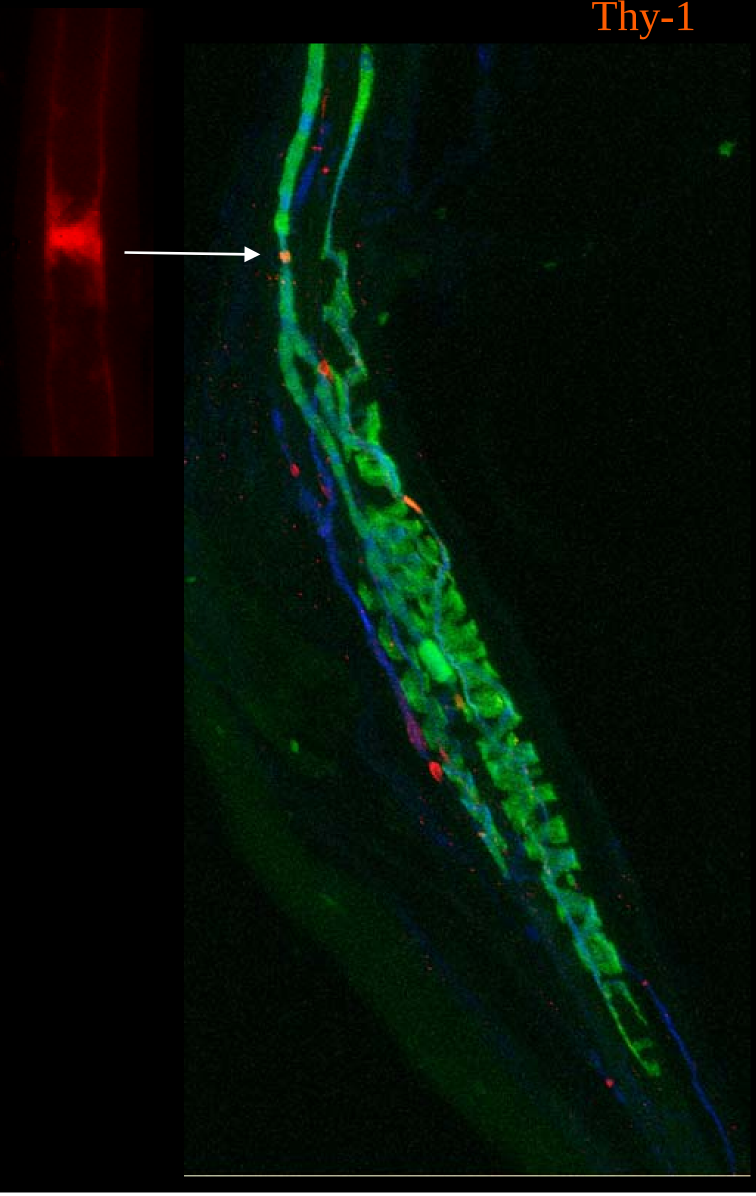
2. Bleed Through

- 1. Some Other Confocal Modes
- 2. Bleed Through
  - Confocal simultaneous scanning
  - Sequential scanning
- 3. Co-localization
- 4. Live Cell Imaging
- 5. Digital Images II

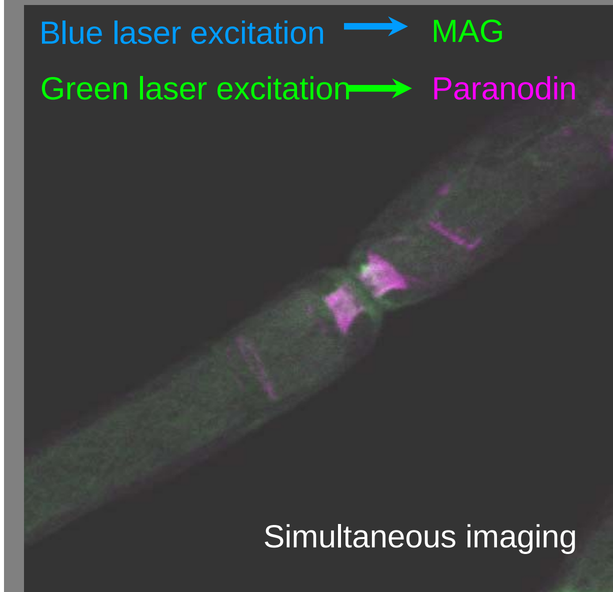
Muscle Spindle

Ab-Neurofilaments 200 (blue) YFP (gray/green) VNaCh (red)

Thy-1 Sodium Channel



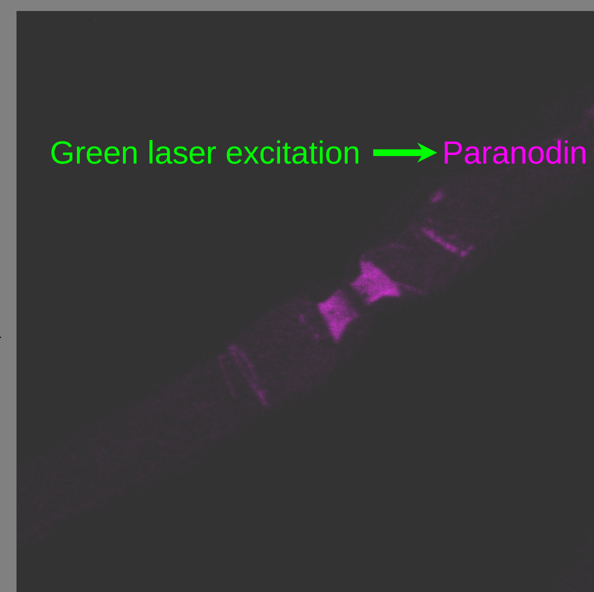
Emission Bleed Through Test



→ Turn off green excitation →

Bad!

→ Turn off blue excitation →

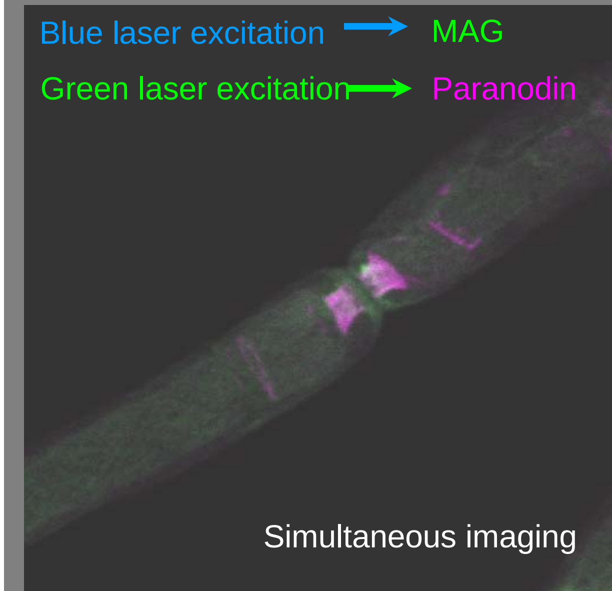


Expected

(MAG = Myelin Associated Glycoprotein)

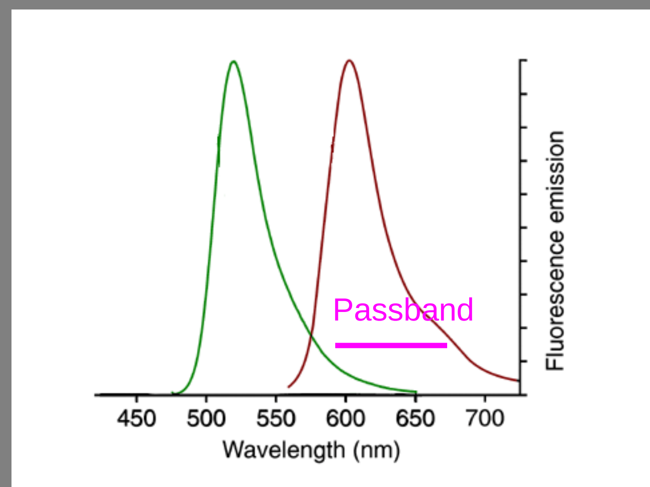
Paranodin = RED

Emission Bleed Through Test

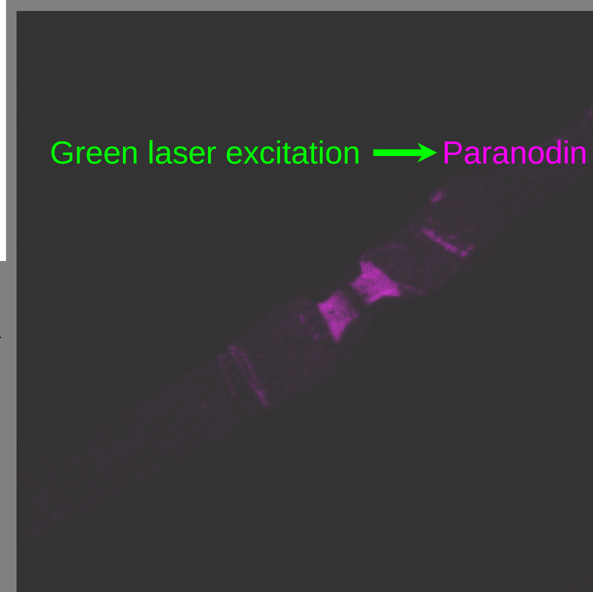


→ Turn off green excitation →

Bad!



Turn off blue excitation →



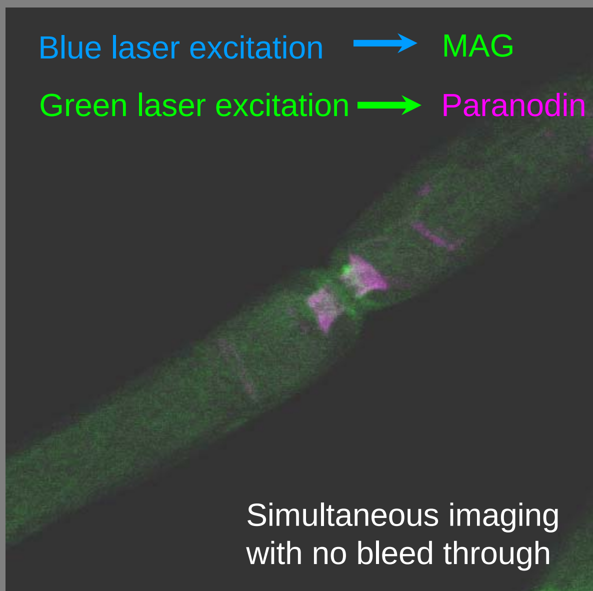
(MAG = Myelin Associated Glycoprotein)

Paranodin = RED

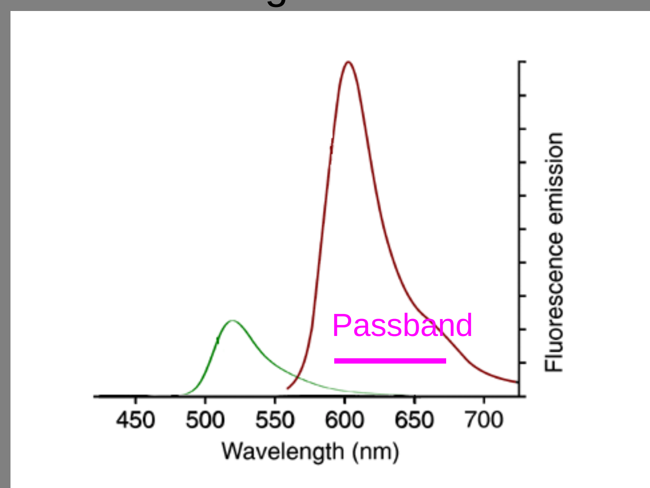
Expected

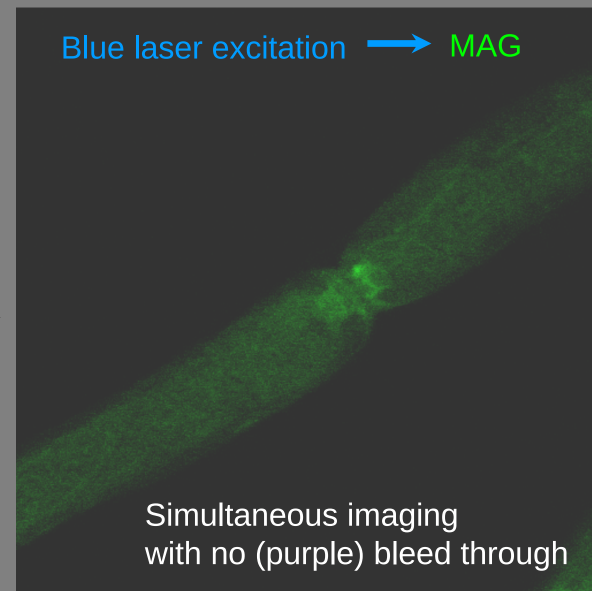
Emission Bleed Through Corrected

Blue excitation reduced to 25%
Green detection sensitivity increased 4 fold



→ Turn off green excitation →





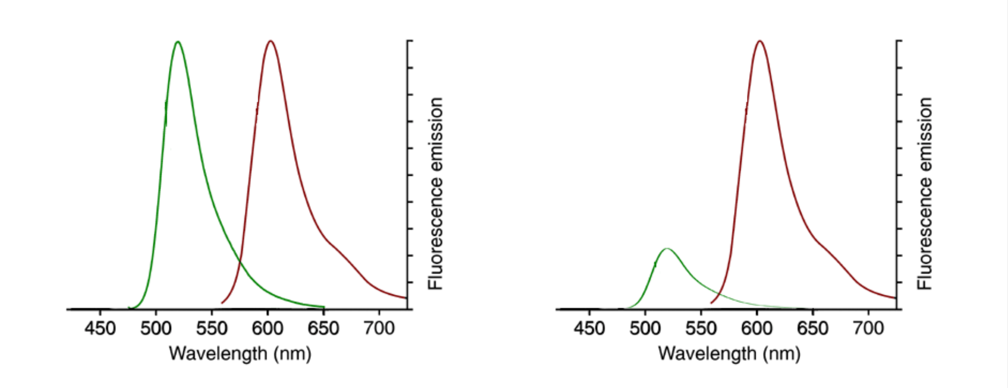
Good!

Note: short wavelength fluorophores bleed into longer wavelength detection channels

Simultaneous/Sequential Scanning

Simultaneous – scan multiple dyes at once

- No filter switching, faster
- Can reduce cross talk by lowering excitation power of shorter wavelength excitation



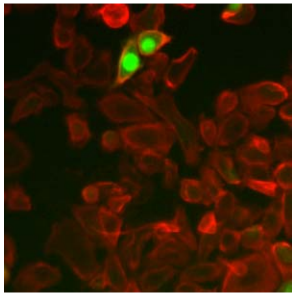
Sequential

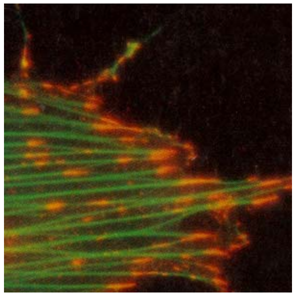
- Excite and detect one dye at a time
- Emission cross talk eliminated
- Be careful of cross excitation, e.g. strong blue laser can excite Rhodamine
- Can speed up filter switching with fully electronic filters e.g. Leica AOBS confocal systems- AOTFs instead of dichroics

3. Co-localization

- 1. Live Cell Imaging
- 2. Bleed Through
- 3. Co-localization
- 4. Deconvolution
- 5. Software

Determining Co-localization with Microscopy





Determining whether fluorescent tags co-localize is not necessarily a trivial issue.

Simple to complex considerations and techniques for addressing this question will be presented.

Such considerations are important to all who view photomicrographs in journals, during seminars, as well as practicing microscopists.

Introduction

• Co-localization at the whole cell level

• Co-localization at the subcellular level

• Resolution, resolution, resolution, i.e. better to use confocal microscopy

• Red / Green pseudo color overlay – the pitfalls

• Fluorograms

• Is there interaction?

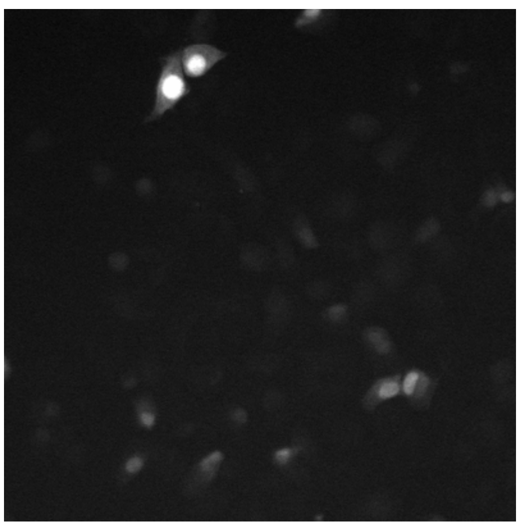
Co-location at the cell level

mixed cells +

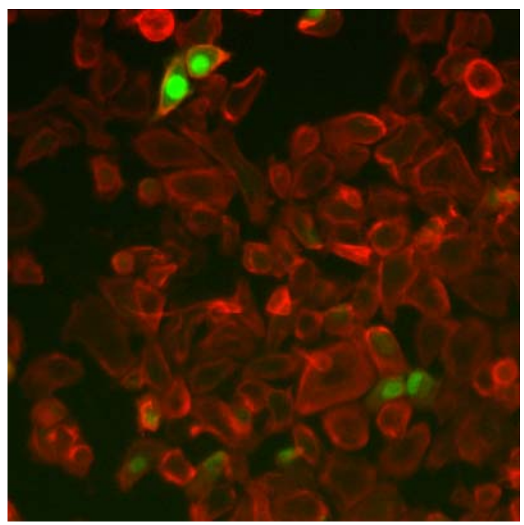
GFP vector +

dsRed vector =

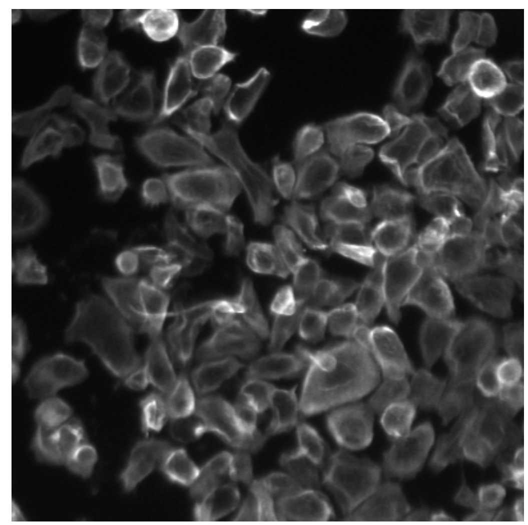
GFP



Overlay

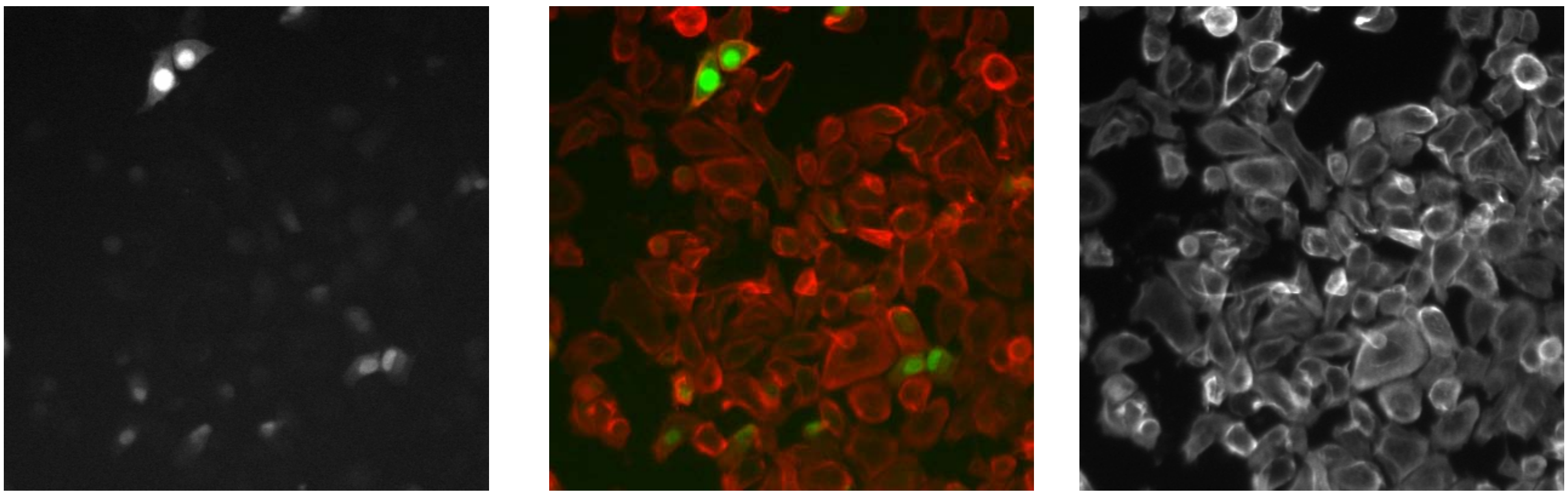


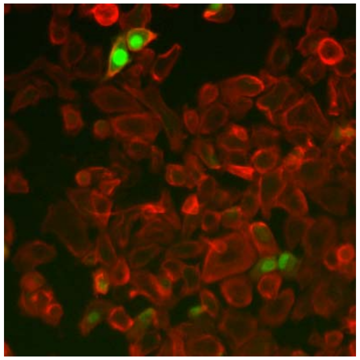
dsRed



Co-location at the cell level

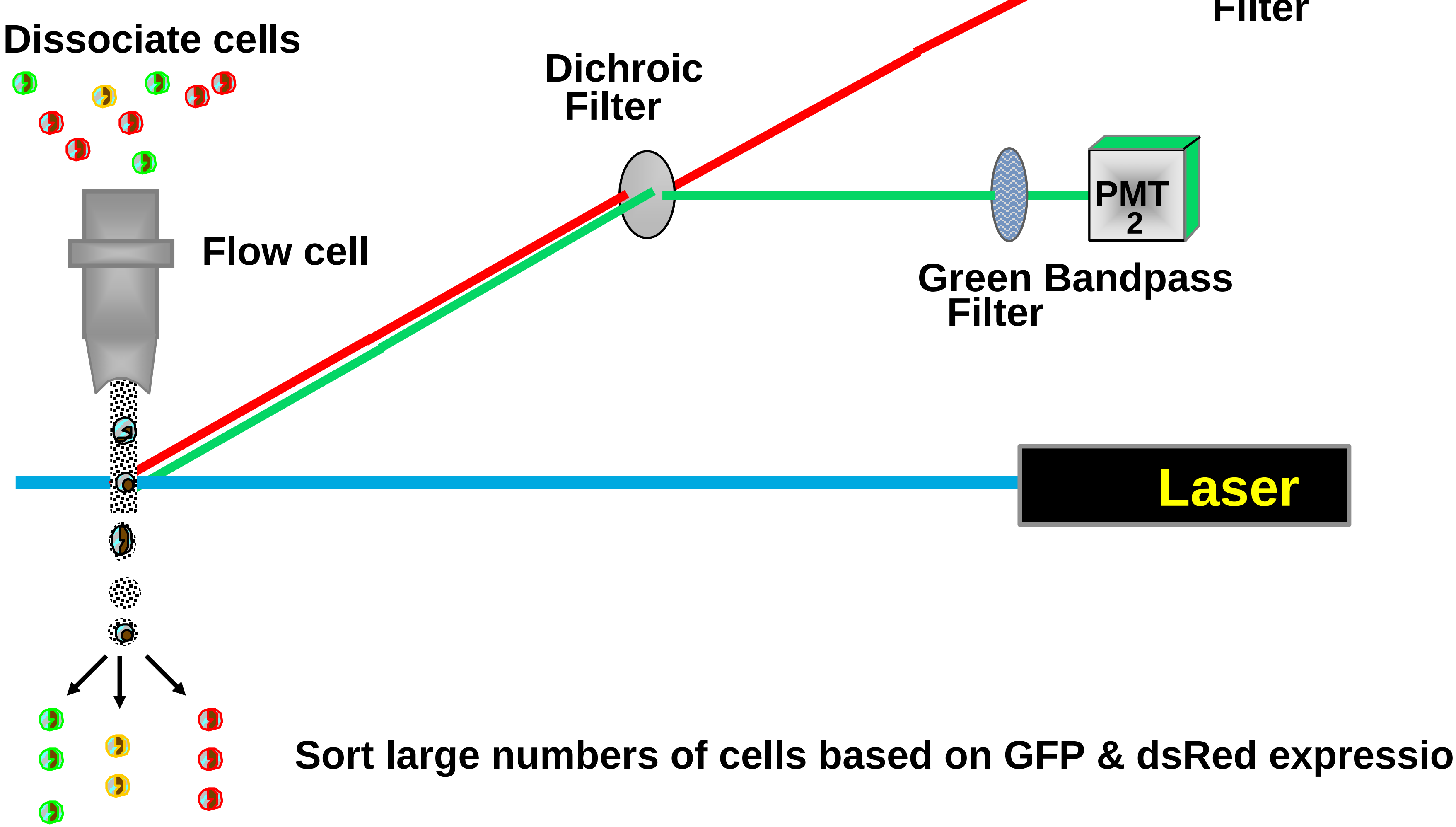
Count cell types based on expression in cell using a microscope





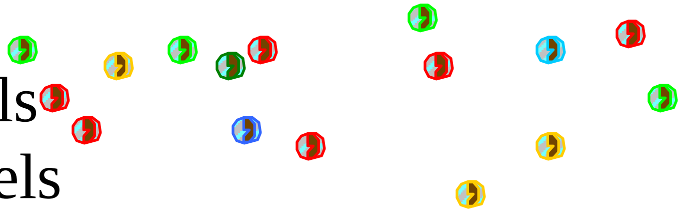
Co-location at the cell level

Flow Cytometer



Sort large numbers of cells based on GFP & dsRed expression

Flow Cytometry Data



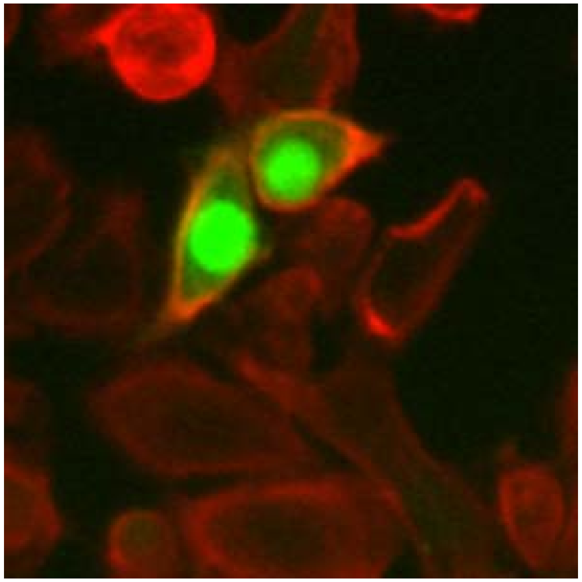
Advantages

- Count large number of cells
- Sorting using multiple labels
- Fast sorting

Minuses

- Cells can be damaged by dissociation
- Mechanical stress to cells through flow plumbing
- Spatial resolution is low.
  - Effectively size of cell.
  - No information of location of label in cell.

Microscopy

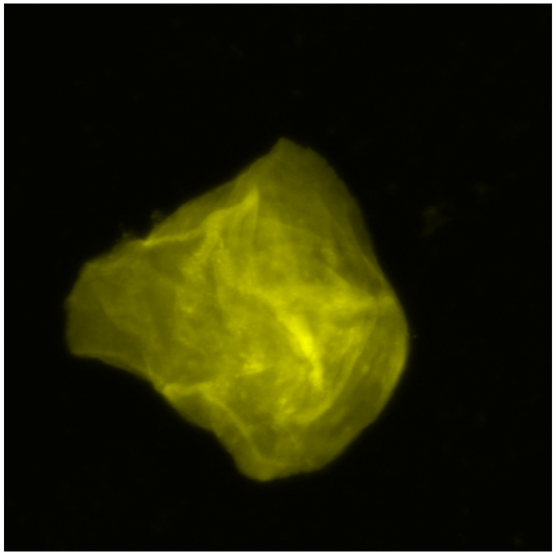


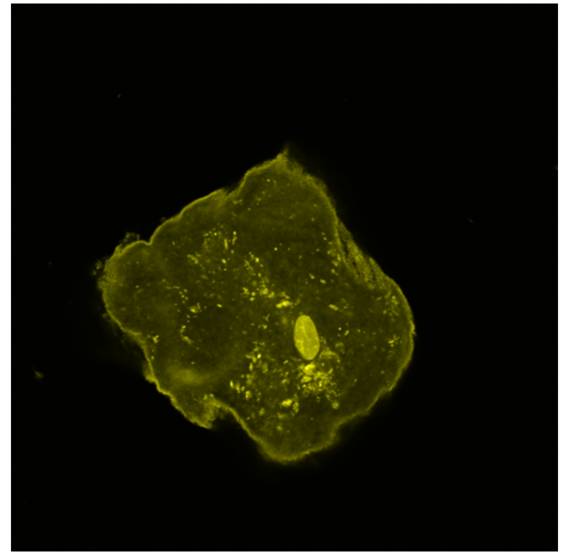
Spatial information – see structures inside the cell

With Fluorescent labels can also see sub resolution structures
- See emitted light without necessarily resolving them

Wide Field versus Confocal

Wide field 63X NA 1.4 FM 1-43 Confocal





r_{airy} = 0.61 λ / NA
= ~0.22 μm
~8 μm thick (measured)

Structures in same x-y position may not be in the same plane

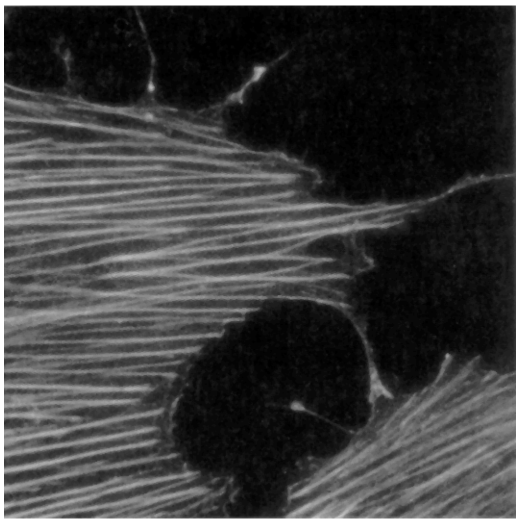
r_{airy} = 0.61 λ / NA / √2
= ~0.15 μm

r_{axial} = 1.77 λ / NA^2
= ~0.45 μm

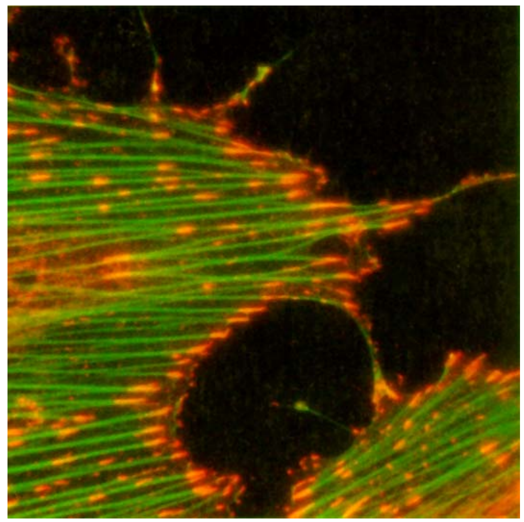
Optical sectioning separates structures

Therefore need to use confocal scanning in order to separate structures in the z axis.

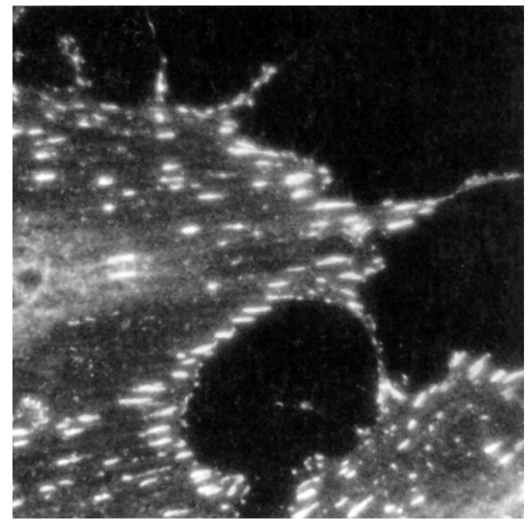
Microscopy – Red Green Overlay



Green
(Actin)



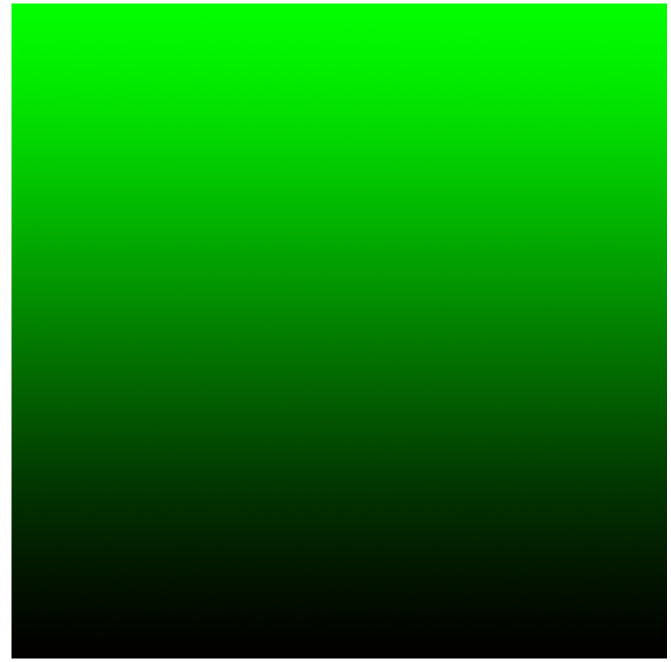
Does anyone
see yellow at the
focal adhesions?



Red
(Phosphotyrosine)

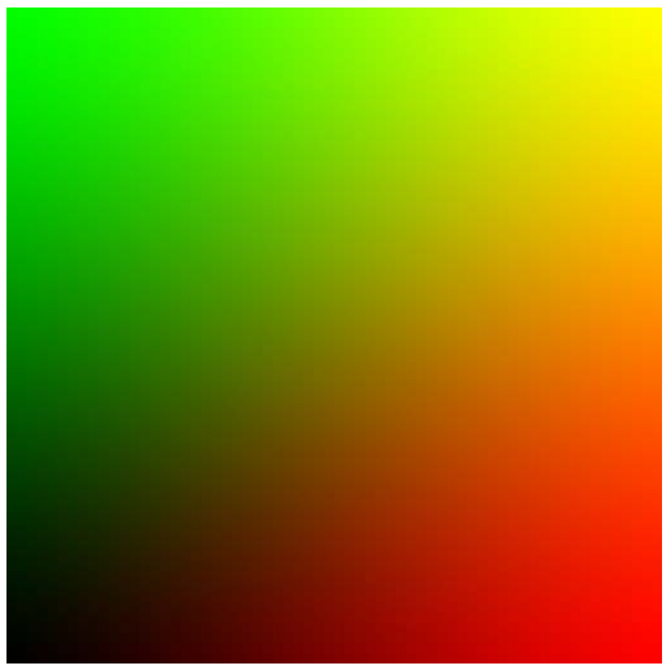
Red + Green

Red 0% to 100%

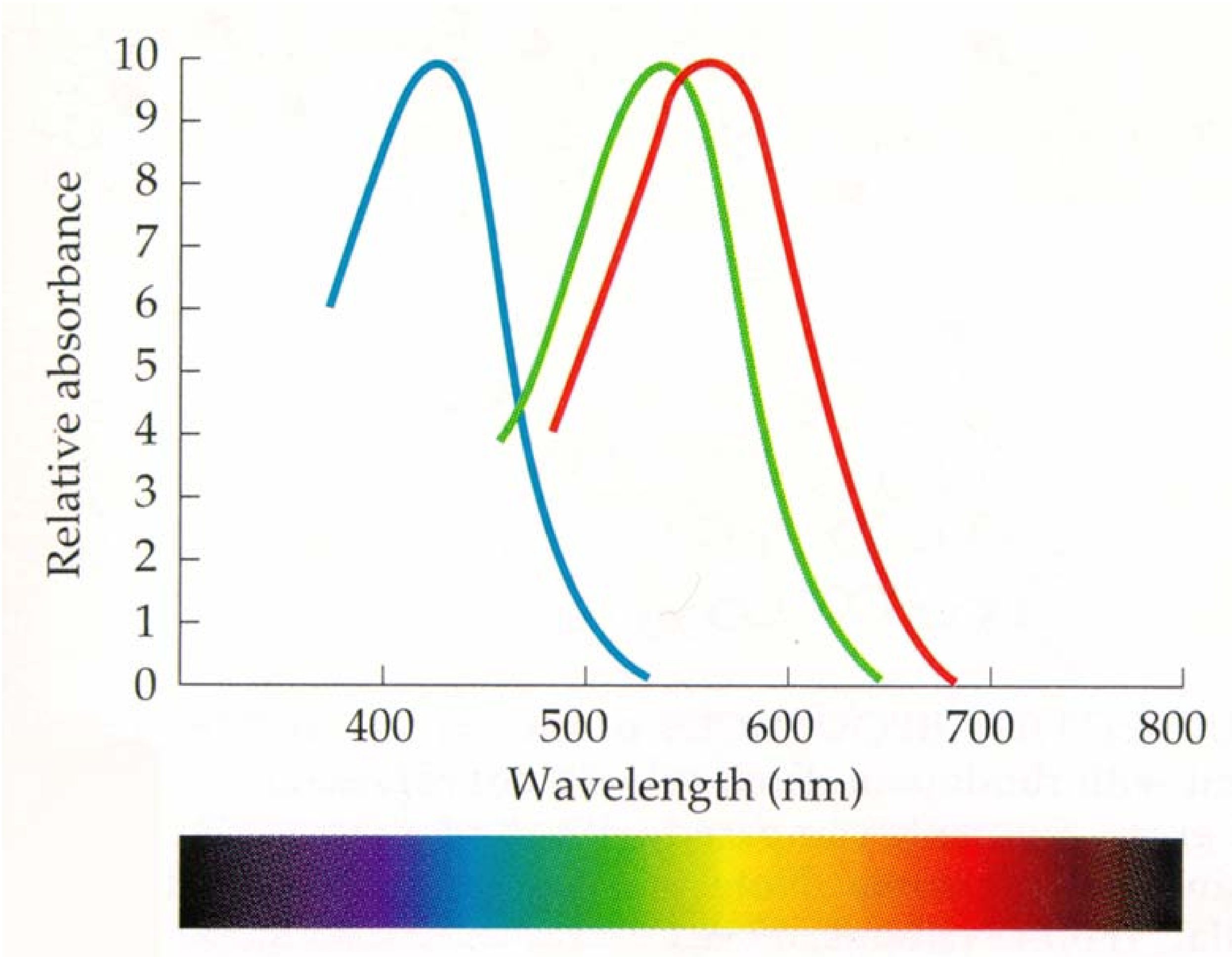


Green 0% to 100%

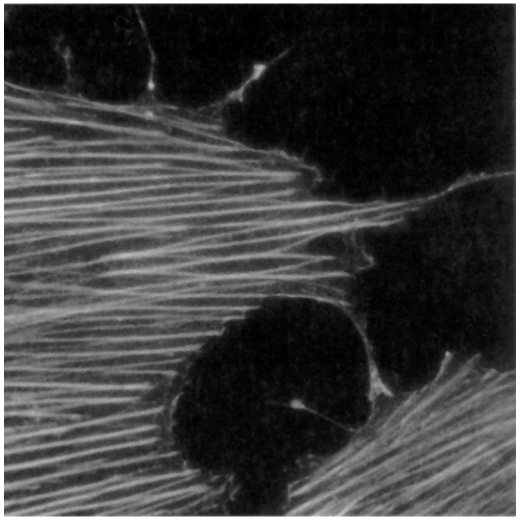
Sum of Red &
Green gradients



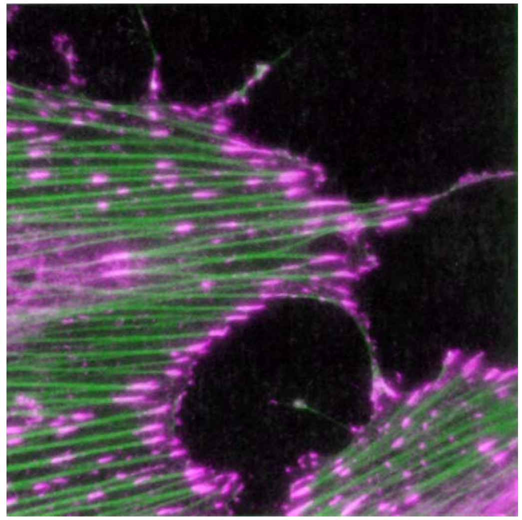
Cones – Trichromat (normal color vision)



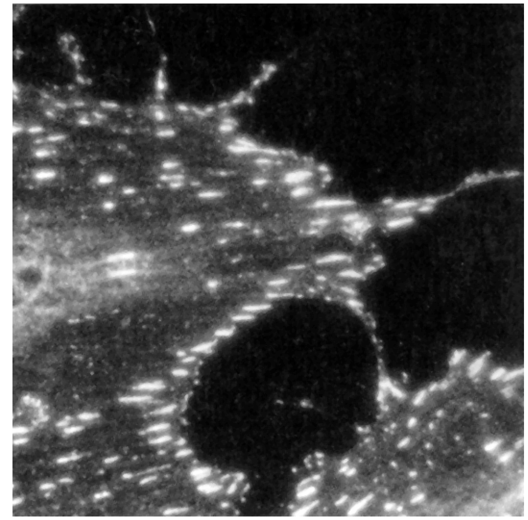
Microscopy –Green Magenta Overlay



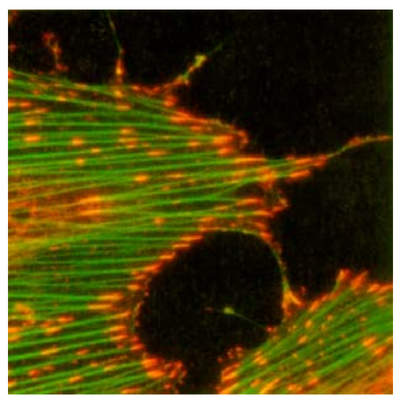
Green
(Actin)



Dichromats
are happy

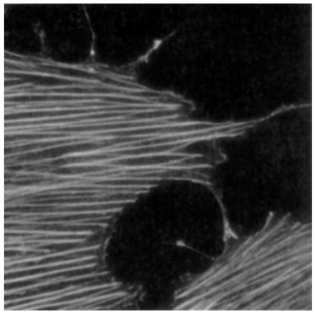


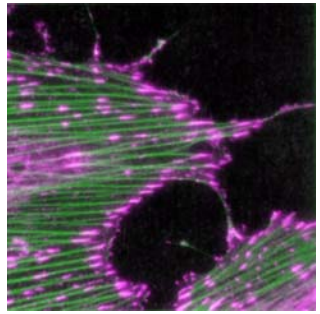
Magenta
(Phosphotyrosine)

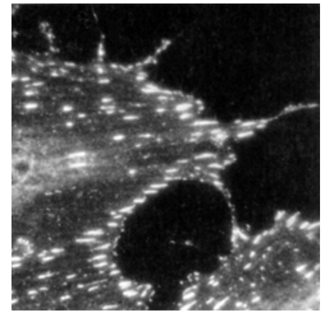


Dichromats
are unhappy

Overlay Summary







Display channels separately in gray scale

Include color overlay for pizzazz factor

Green and magenta good for colorblind viewers

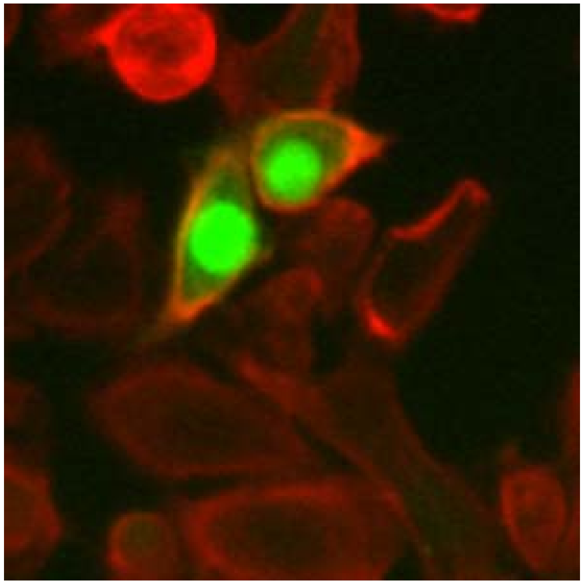
Still can not see different color where green and magenta structures overlay.

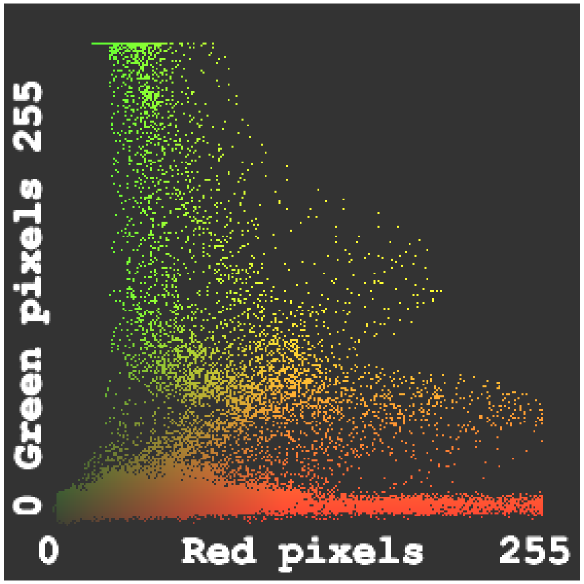
Quantitative solution to measuring overlay - fluorogram

Fluorograms

Use confocal images in order to get good spatial resolution in the x-y & z-axes

correlation 0.147

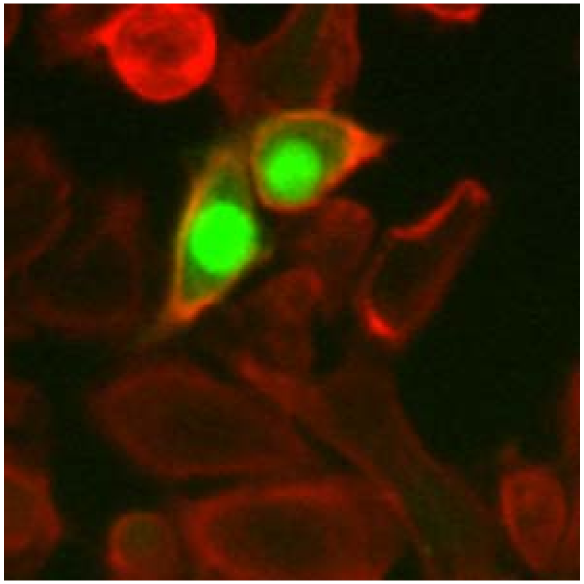


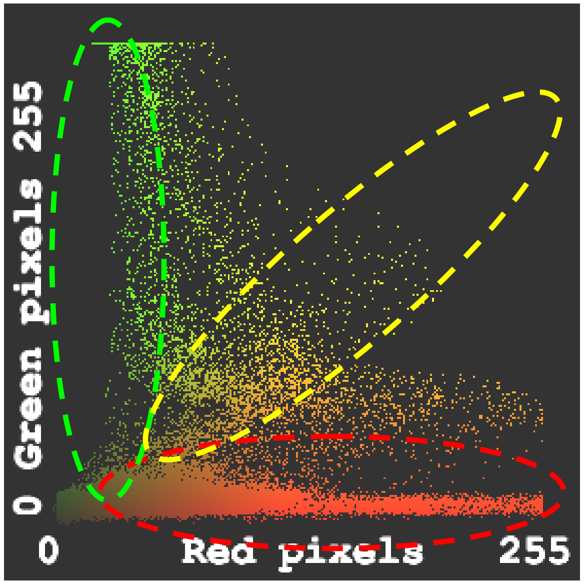


Fluorograms

Use confocal images in order to get good spatial resolution in the x-y & z-axes

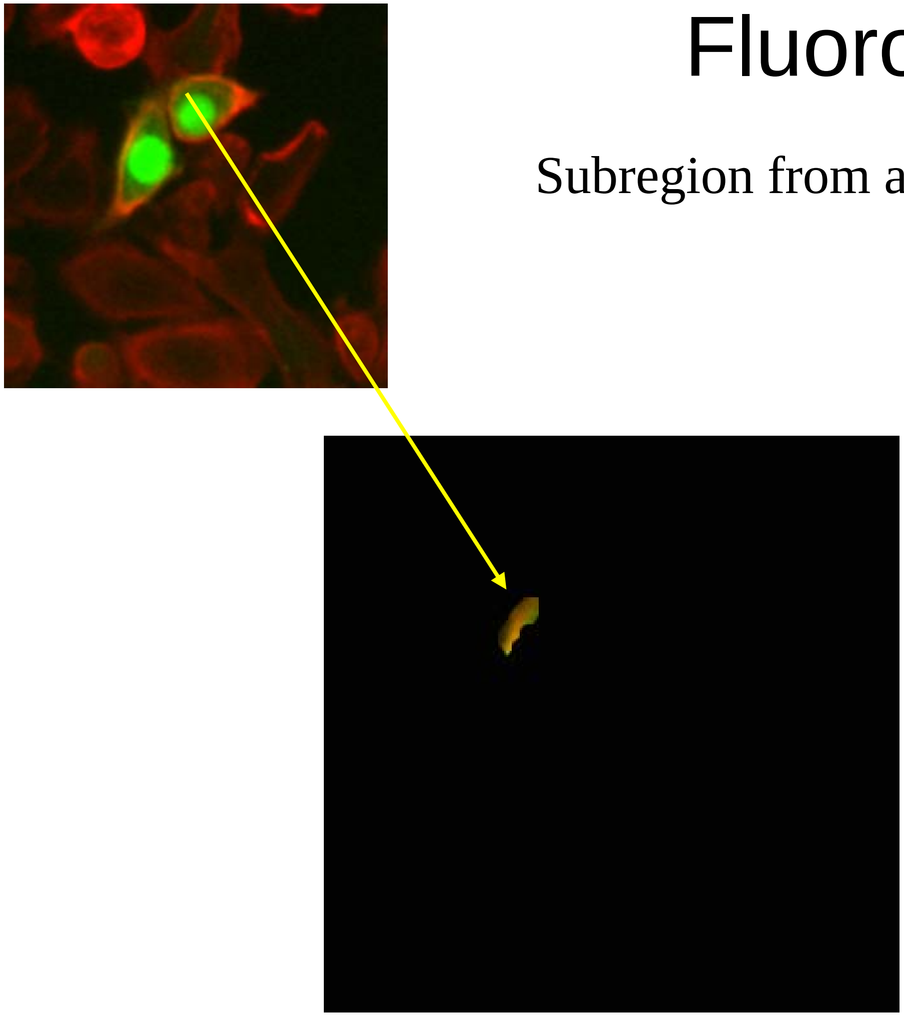
correlation 0.147

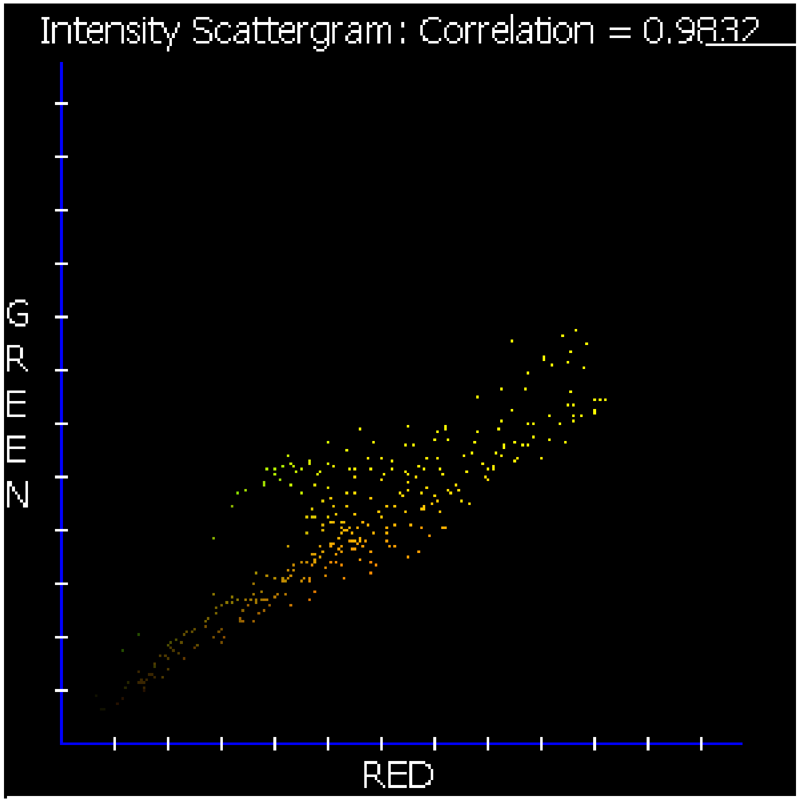




Fluorograms

Subregion from a patch of cell membrane





Fluorogram summary

- Fluorograms are a objective measure of co-localization

- Images must be of thin sections

- Region to analyze must be carefully chosen

- Dyes, filters & excitation must be carefully chosen to avoid cross talk

- Software for generating fluorograms is available
  e.g. C-Imaging, Zeiss and Leica software, ImageJ plugin

- Co-localization does not necessary imply interaction

- Interaction can be measured with FRET, FCS and other techniques

1. Live Cell Imaging

- 1. Live Cell Imaging
  - Morphology (natural structure)
  - Photometric analysis (intensity, e.g. [Ca],)
  - Dynamics (changes in shape &/or intensity with time)

1. Live Cell Imaging

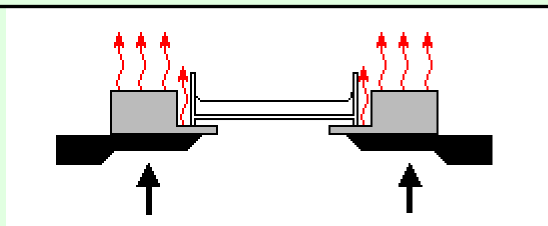
- Conditions
  - Oxygen
  - Temperature
  - CO_2 / pH
  - Physiological ions
  - Osmolarity - humidity – drying - condensation
  - Flow – drug addition, O_2, metabolites
  - Working distance

1. Live Cell Imaging

- Control - simple to complex

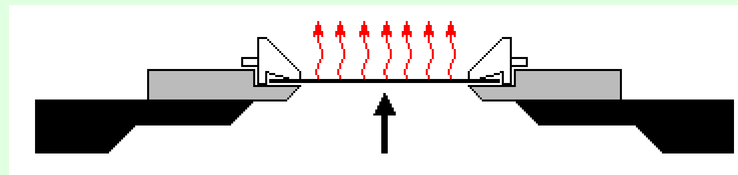
#1.5 cover slip glued to hole in petri dish

•Ambient temperature
•Ambient O_2 good!
•No CO_2 (poorly controlled $[pH]_i$)
•Drying – OK short term
•Simple
•Commercially available



Electrically heated metal block

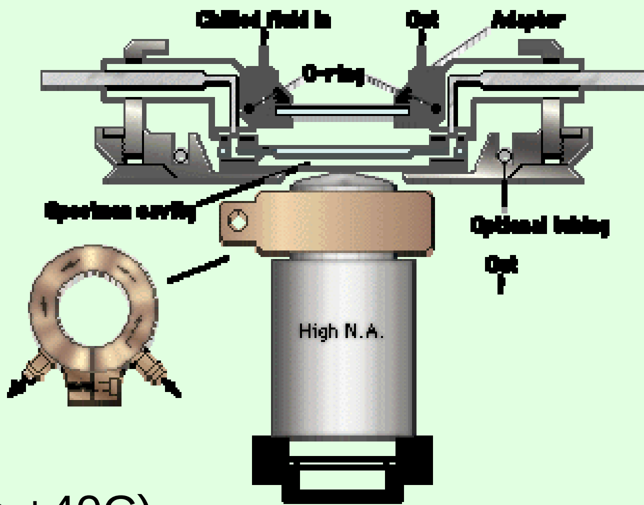
Better temperature control

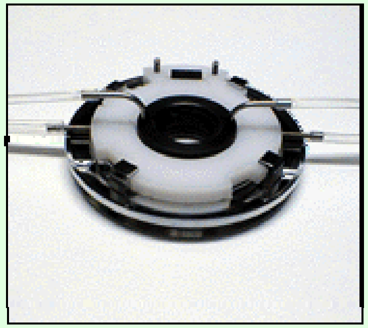


Electrically heated Indium Tin Oxide transparent coating on petri dish

1. Live Cell Imaging

- Control – complex!





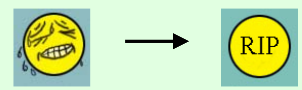
- Well controlled temperature (to +40C)
- Controlled flow though solution with:
  - CO_2 (intracellular pH controlled)
  - Drugs
  - O_2
  - No drying
- Good long term, e.g. 24 hr
- Heat loss through oil immersion to objective negated by heated collar

1. Live Cell Imaging

- CO_2 / pH
- 15 mM HCO_3 + 5% CO_2 → intra & extra cellular pH control → long term viability
- Hepes, etc, does not cross membrane → extra cellular pH control only
- Cell types have different resiliency to non ideal conditions

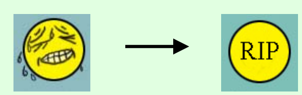
External pH indicated by Phenol Red

pH = 6.5 5% CO_2
Hepes (10 – 25 mM)

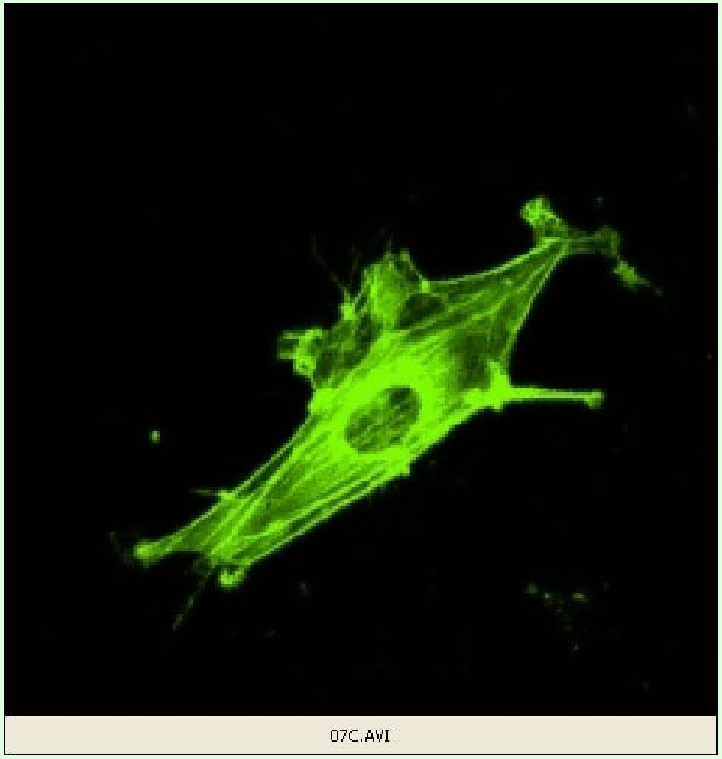


pH = 7.4 5% CO_2 + HCO_3
Hepes (10 – 25 mM)

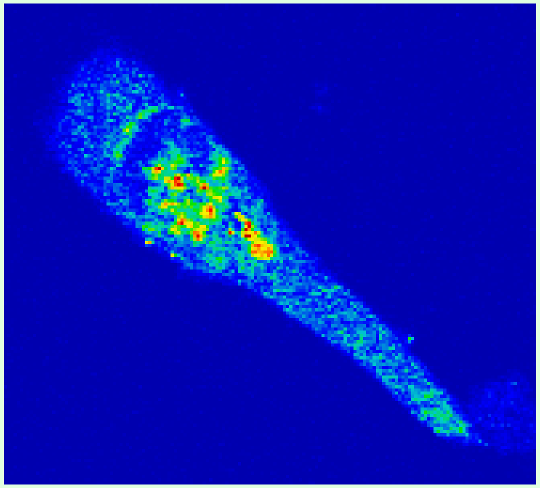
pH = 8.0 air only + HCO_3
Hepes (10 – 25 mM)



1. Live Cell Imaging



Cultured neuron transfected with GFP - migration



Cardiac myocyte labeled with Fluo-3 – $[Ca]_I$ showing spontaneous activity

Digital Images (II)

- **Summary:** majority of images are 2-D arrays of 8 bit monochrome, 24 bit RBG color, Indexed color, 8 bit monochrome

- Image processing not easy or meaningful unless image is a linear gray scale or RGB image. (photometrically correct, i.e. intensity corresponds to pixel value)

File Formats

- There are a large number of ways images can be represented and stored in a computer file. All formats have variations, sub-formats. Common formats are TIFF, GIF, JPG. Some software will even use a proprietary format, and force the use of proprietary software or conversion programs.

- Details of formats can be found readily via Google.

Image File Representation - Header

- Header (typical)
  – Code for format type *4949*=TIF or *GIF87a*=GIF or *FFD8FF*=JPG, etc.
  – Size of image file on disk
  – X dimension (horizontal pixels)
  – Y dimension (vertical pixels)
  – Bits per pixel (e.g. 8, 12, 16)
  – Channels per pixel (e.g. 1=monocrhome, 3=RGB, 2=indexed)
  – Calibration (e.g. pixels per inch)
  – Other information both useful and redundant

- Number stream saved as a 2-D array – f(x,y)
  – Pixel intensity values

Typical Digital Image

- Grayscale – 8 bit usually. Most commonly 12, 14, 16, 32bit! No color information.

- Color – RGB 8 bit red – 8 bit green – 8 bit blue for each pixel. Referred to as 24 bit (handled in most imaging software). Can have more bits per pixel, but rarely so.

- Index Color (can also have index which is gray scale) f(x,y) array– 8 bit photometric correct data passed through a red, green blue look up table (LUT). Can only have 256 different colors per image. But colors can be chosen from 16 million (24 bit RGB). Intensity of translated data may not be related to original intensity of detected pixel. Color is pseudo color.

File Formats - Typical

- TIFF – tagged image file format – blocks of various data are proceeded by a tag (information about what the nature of the block is) – tags describe whether block is text, 8, 12, 16 bit numbers, start of block, length of block, location of next tag. This format is very flexible but can have software which does not know how to handle a specialized TIF file image structure. Compression is generally **lossless**. However lossy compression can be specified.

- GIF – 8 bit indexed (can not handle RGB color). OK for single channel data. Can colorize using the look up table. Data is compressed. Compression is **lossless**.

- JPG – RGB or 8 bit. Compression is ***lossy*** **compression**. Not good for data to be analyzed or measured. Compression is reapplied on every save.

File Formats – Some Video

- AVI – audio visual interleaved tagged – video for Windows – Uncompressed, compressed with several compression codecs (compression-decompression algorithms) possible. 8, 16 or 24 (RGB) bit numbers. Compression is lossy.

- MOV – Movie for Apple systems.   8 bit indexed (can not handle RGB color). OK for single channel data. Can colorize using the look up table. Data is compressed. Compression is lossy.

- Choose codecs carefully – None, or Cinepac if movie is large

- Can convert with Quick Time Pro ($29.95 for an unlock code) – do open movie then export to convert to AVI or File Save as for MOV

- Always check that movies plays in the program the program(s) one is using to display with on the computer to be used to view with, especially for seminars. E.g. PowerPoint will internally use Media Player, and test on computer used in seminar room!

References

Easy Reading:

- Microscopy from the Very Beginning, 2nd ed., Carl Zeiss Microscopy (pdf at http://microscopy.unc.edu/iglm)
- Optical Imaging Techniques in Cell Biology, Guy Cox, 2007

Good for basic details on practical confocal, easy reading:

- Confocal Microscopy for Biologists, Alan R. Hibbs, 2004
  (Missing at Duke & NC State library)

Detailed & Technical:

- Handbook of Biological Confocal Microscopy, 2nd ed., James Pawley, 1995 or
- 3rd ed., James Pawley, 2006

Detailed Image Processing (not specific to microscopy):

- The Image Processing Handbook, 5th, John C. Russ, 2006

Photoshop basics for science (up to version 7 only):

- Quick Photoshop for Research: A Guide to Digital Imaging for Photoshop 4x, 5x, 6x, 7x, Gerald Sedgewick, 2002

Colocalization:

- Demandox. D/ & Davoust, J., Journal of Microscopy, (1997) vol 185, pp 21-26. Multicolor analysis and local image correlation in confocal microscopy.
- Manders, E.M.M., Verbeek, F.J., & Aten, J.A., (1993) Journal of Microscopy, vol. 169, pp 375-382. Measurement of co-localization of objects in dual-color confocal images.