Introductory Guide to Light Microscopy - Biomedical Confocal Microscopy

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$E = h\nu$

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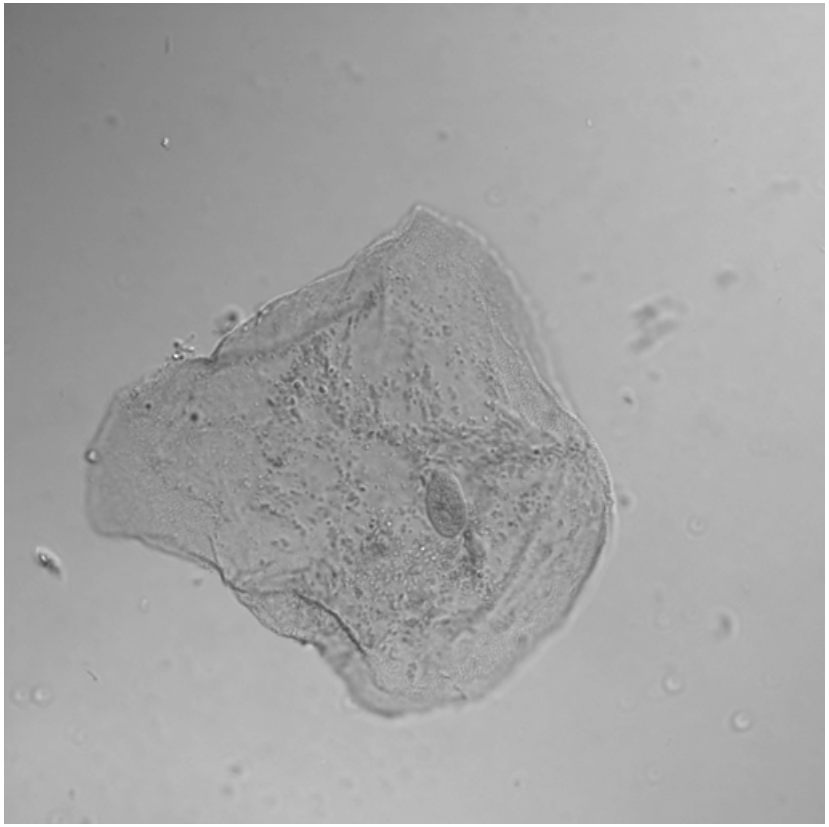
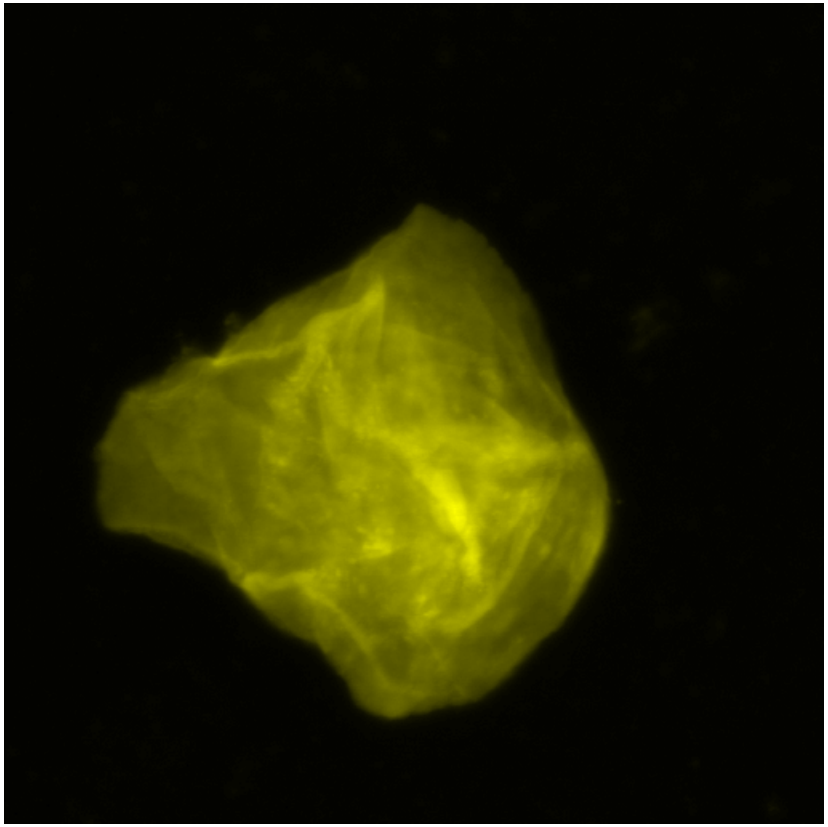
Confocal Microscopy

- Limitation of wide field microscopy
- Origins of Confocal Microscopy
- Confocal Principal
- Confocal Laser Scanning
- Resolution
- X-Z axis scanning
- Visualization

Limitation of wide field microscopy



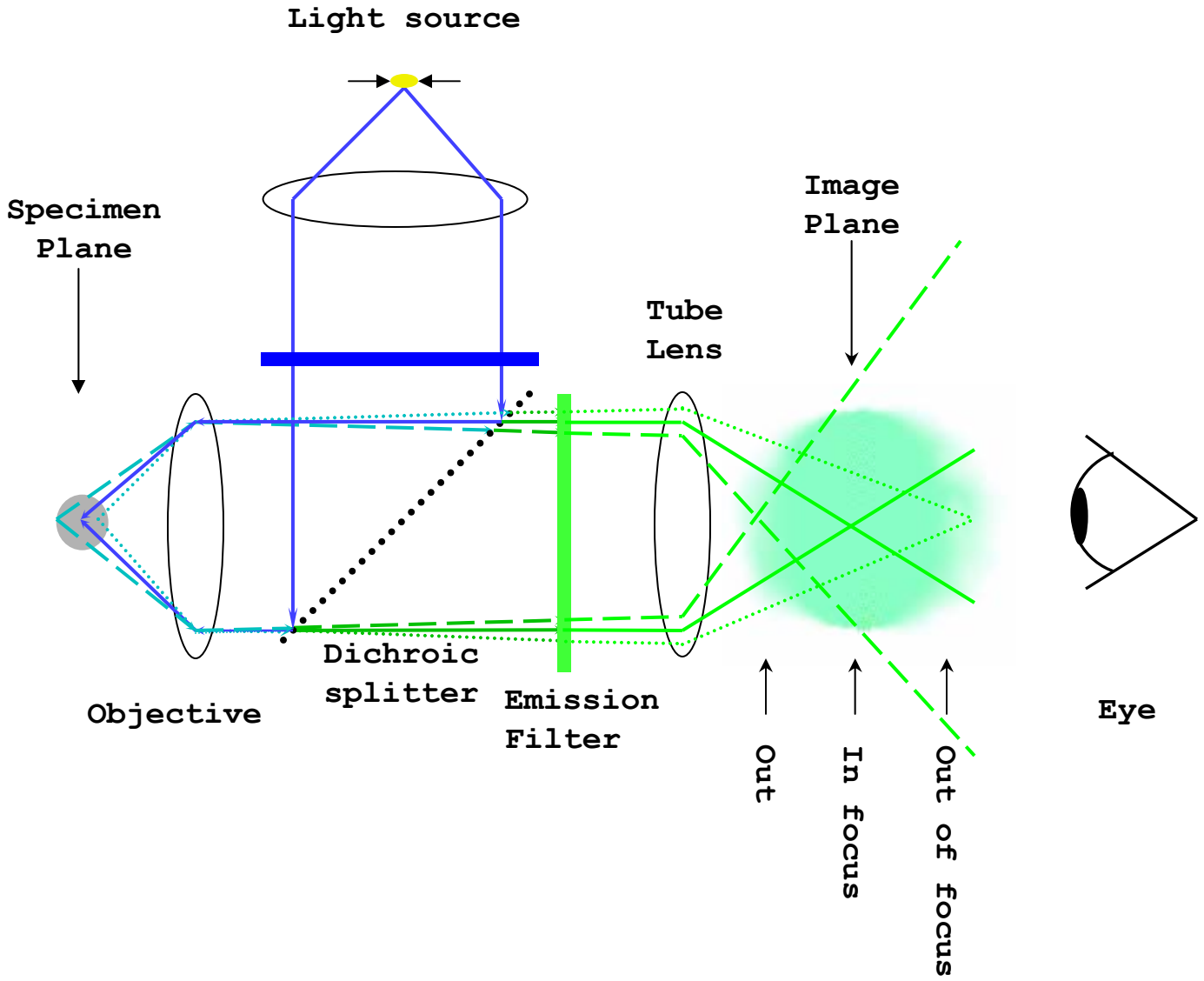


Live Buccal Epithelial cells

(DIC) Transmitted

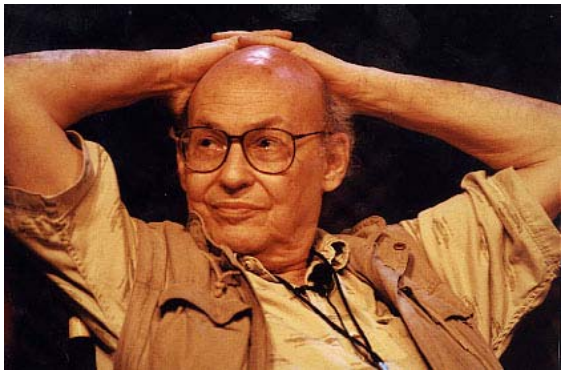
Fluorescence
FM 1-43 membrane dye

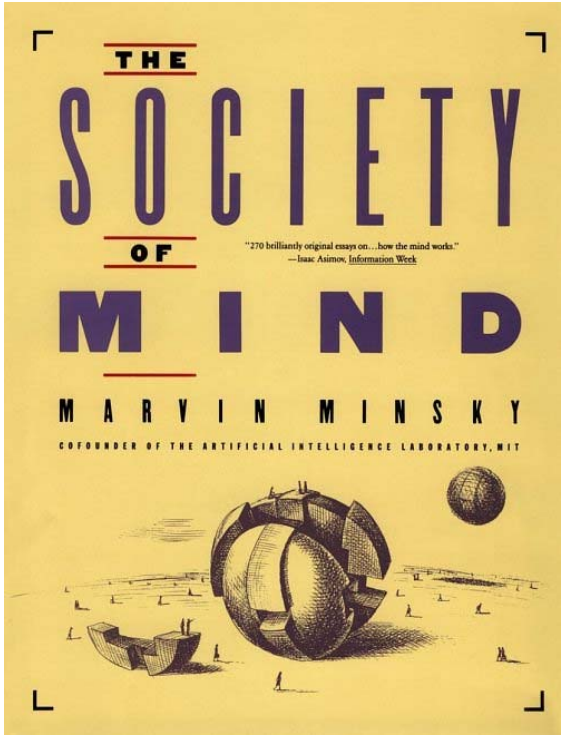
Limitation of wide field microscopy

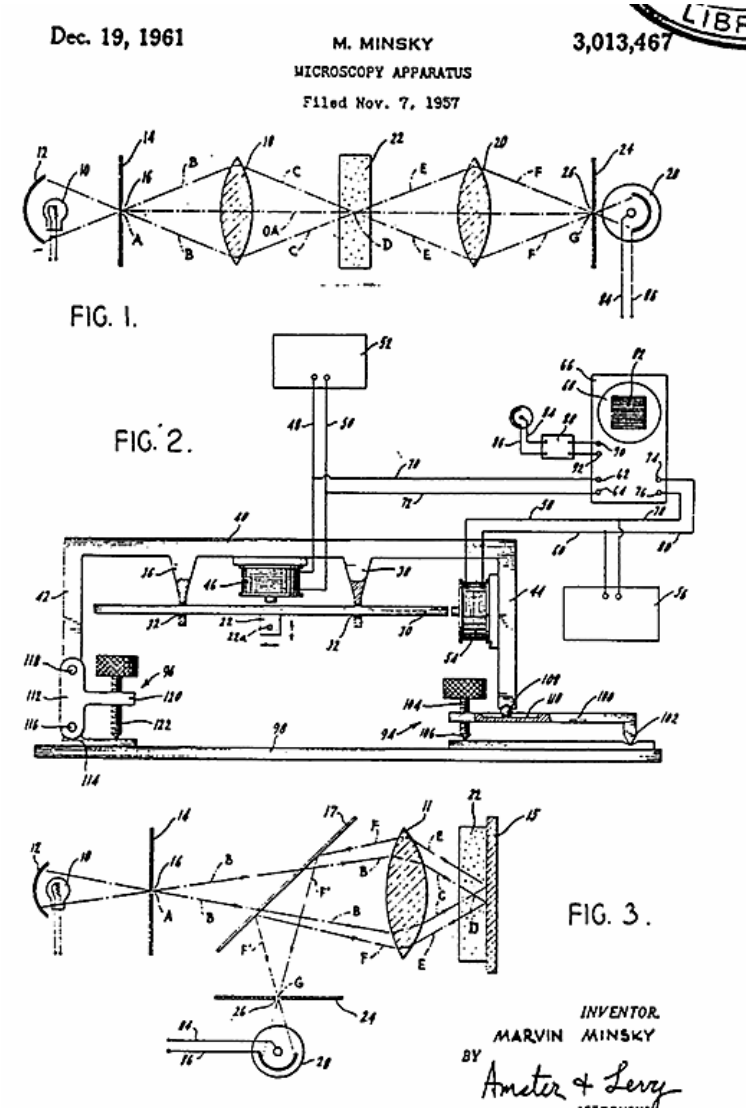


Origins – Marvin Minsky

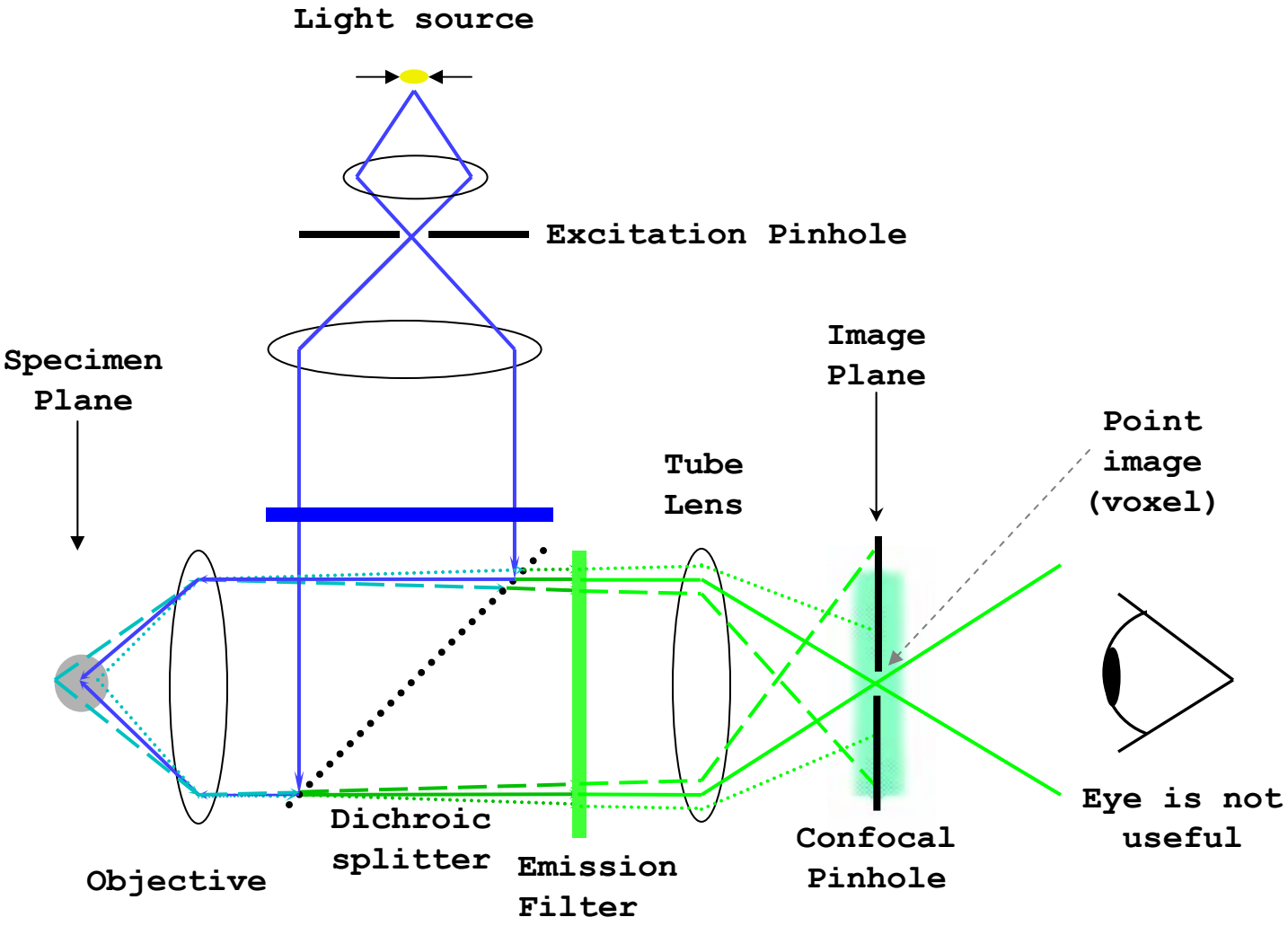
1957 Confocal Patent focal Scanning Microscope: U.S.Patent 3013467



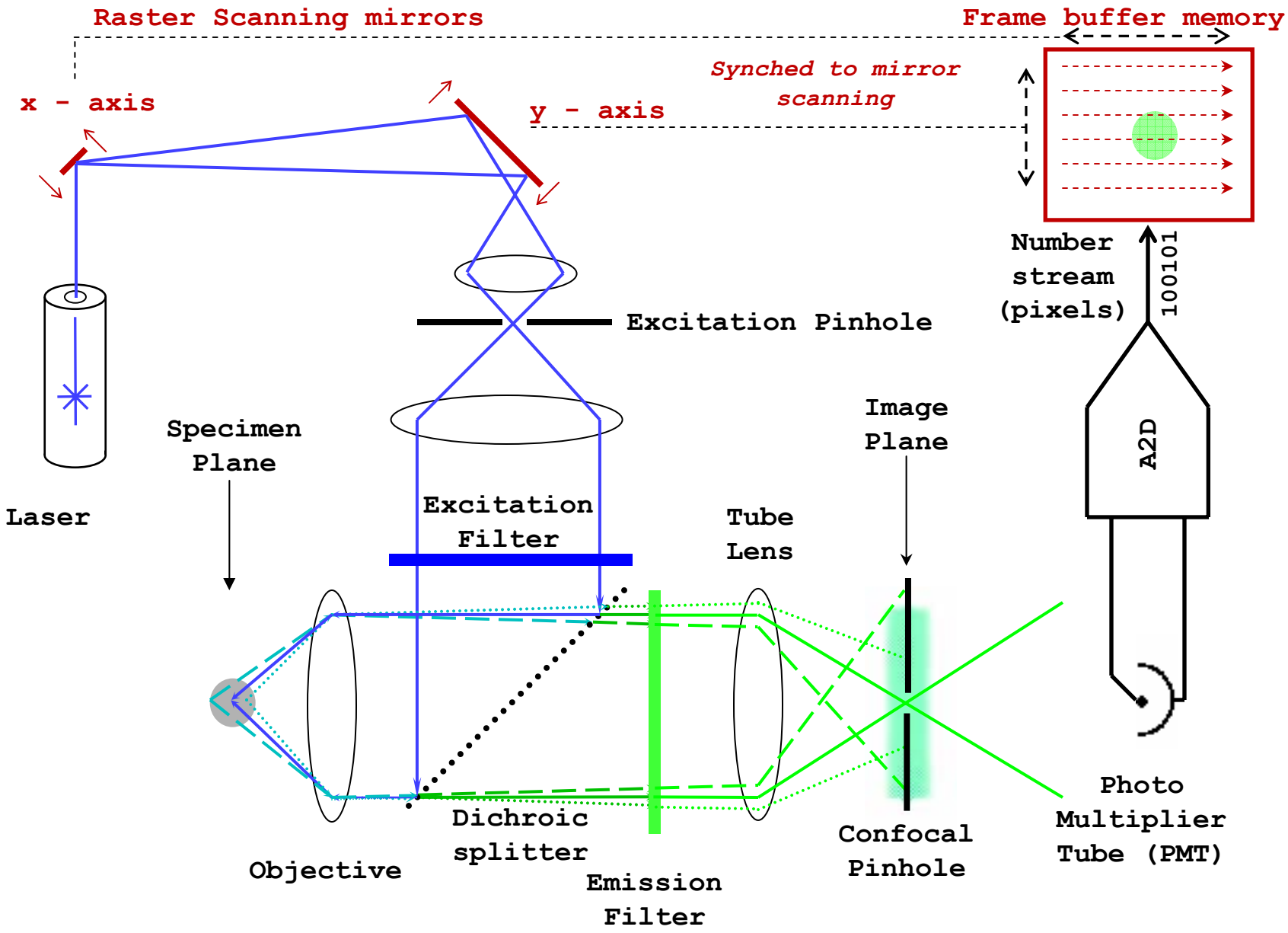




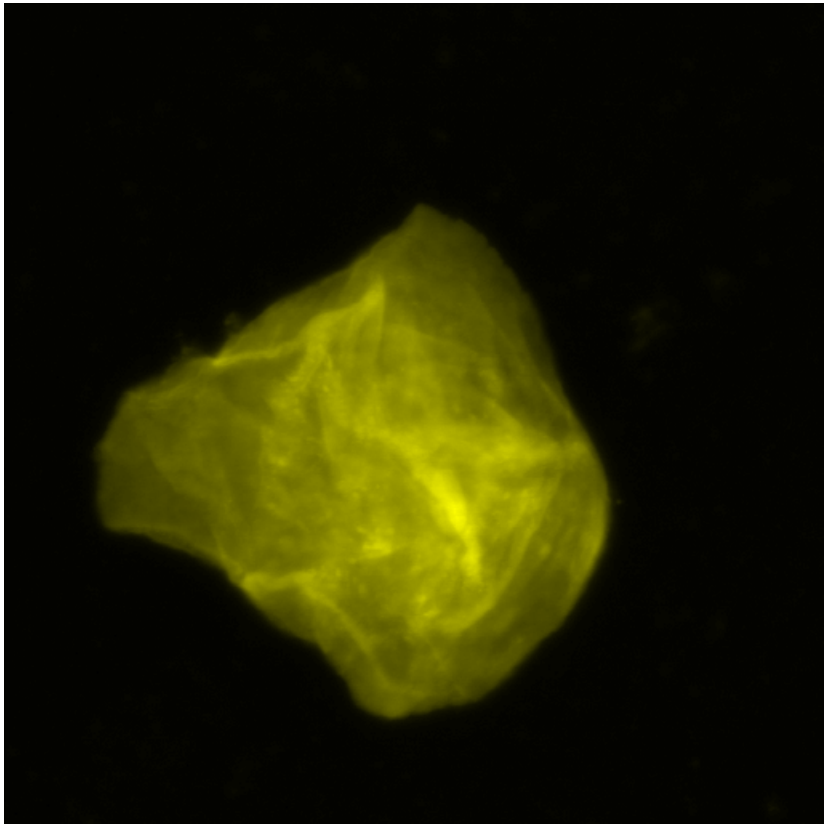
Confocal Principal – the confocal pinhole

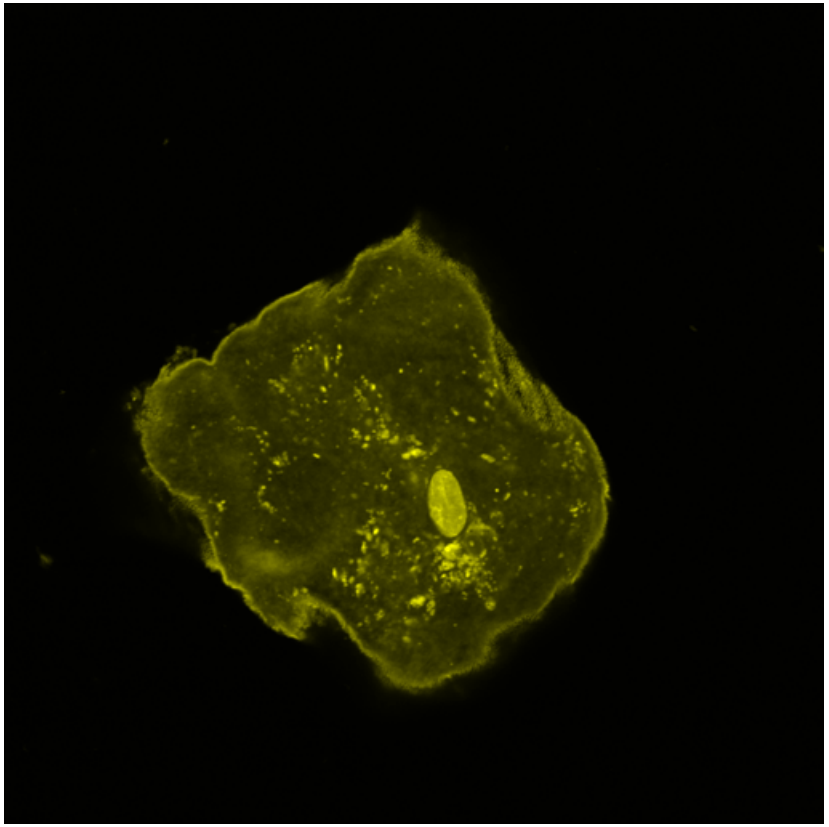


Confocal Laser Scanning Microscope



Confocal Principal – Live Buccal Epithelial cells





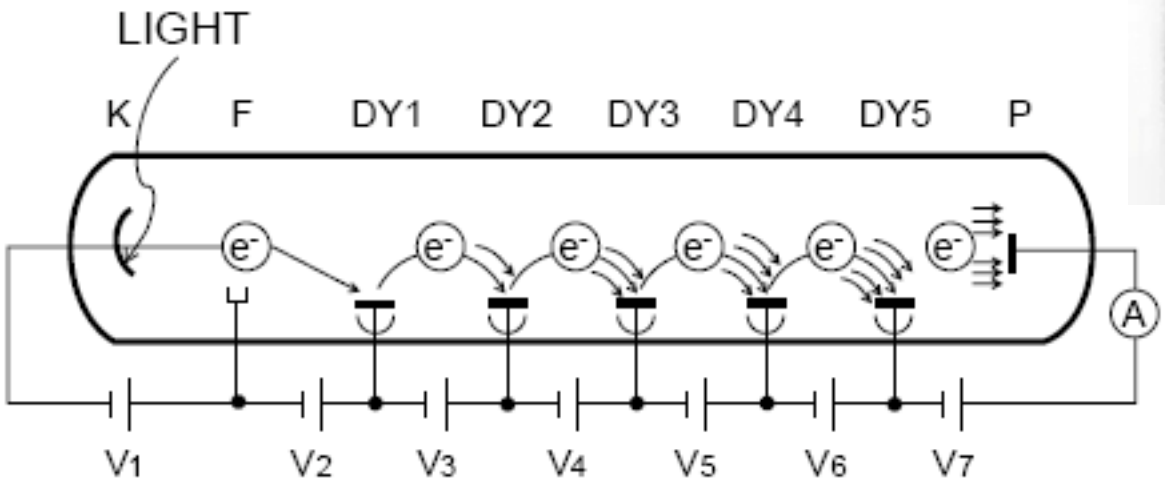
Widefield

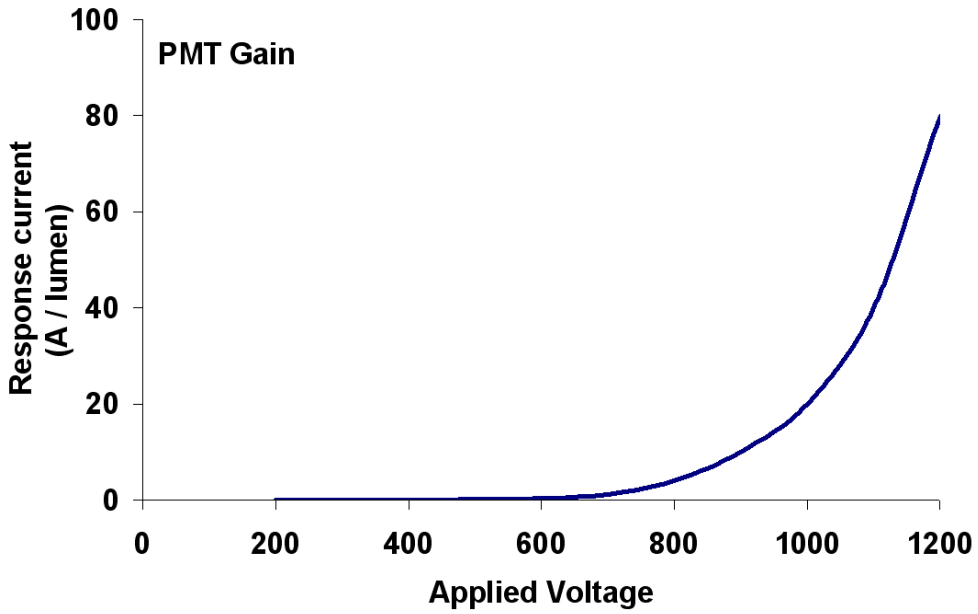
Confocal

FM 1-43 membrane dye

Confocal Laser Scanning Microscope – PMT

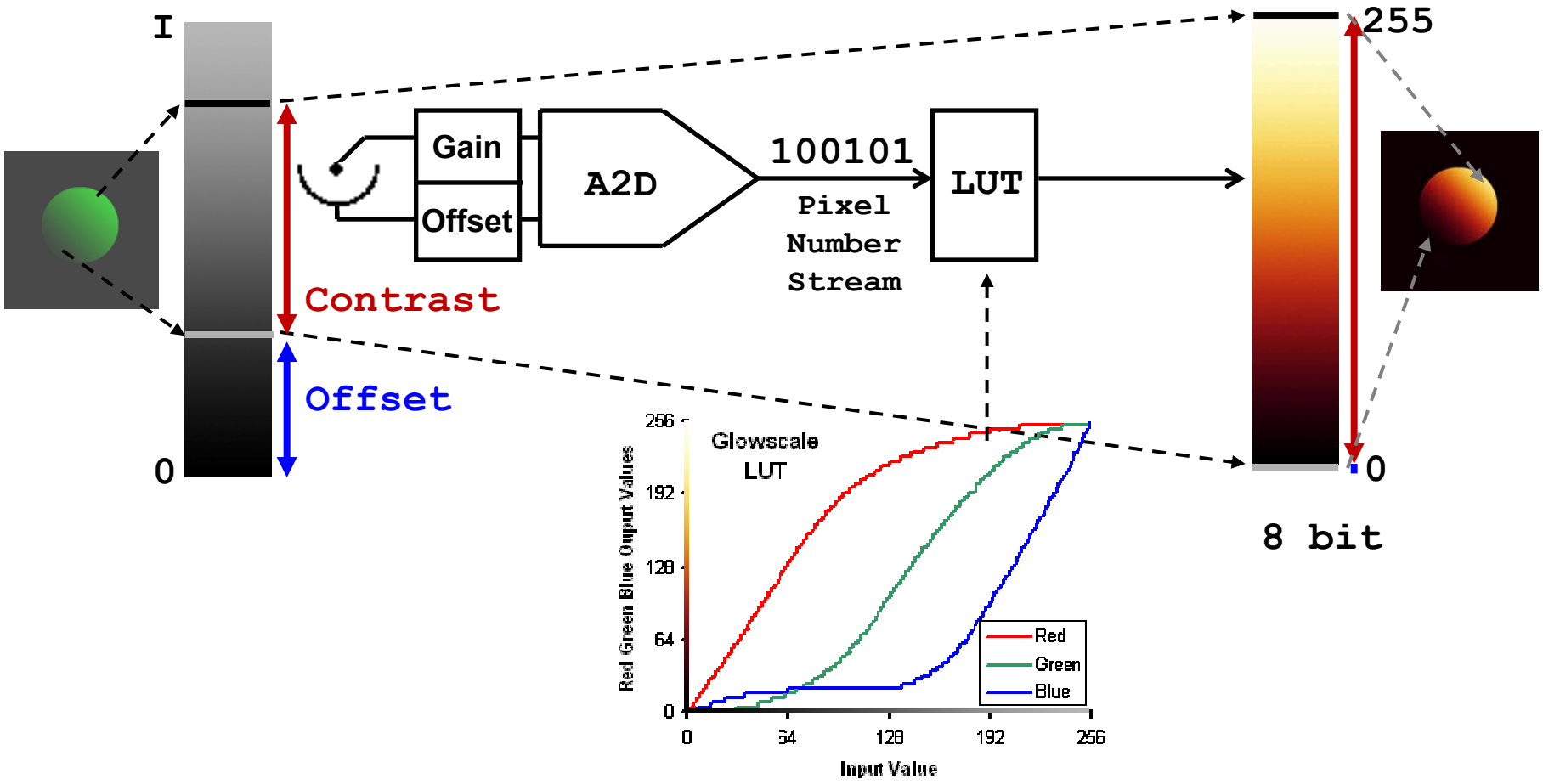
Photo Multiplier Tube (PMT)





- Quantum yield <= 0.3
- Gain can be very large $>10^7$
- Gain is exponential function of applied voltage
- Noise increases disproportionably at high gain
- Significant dark current

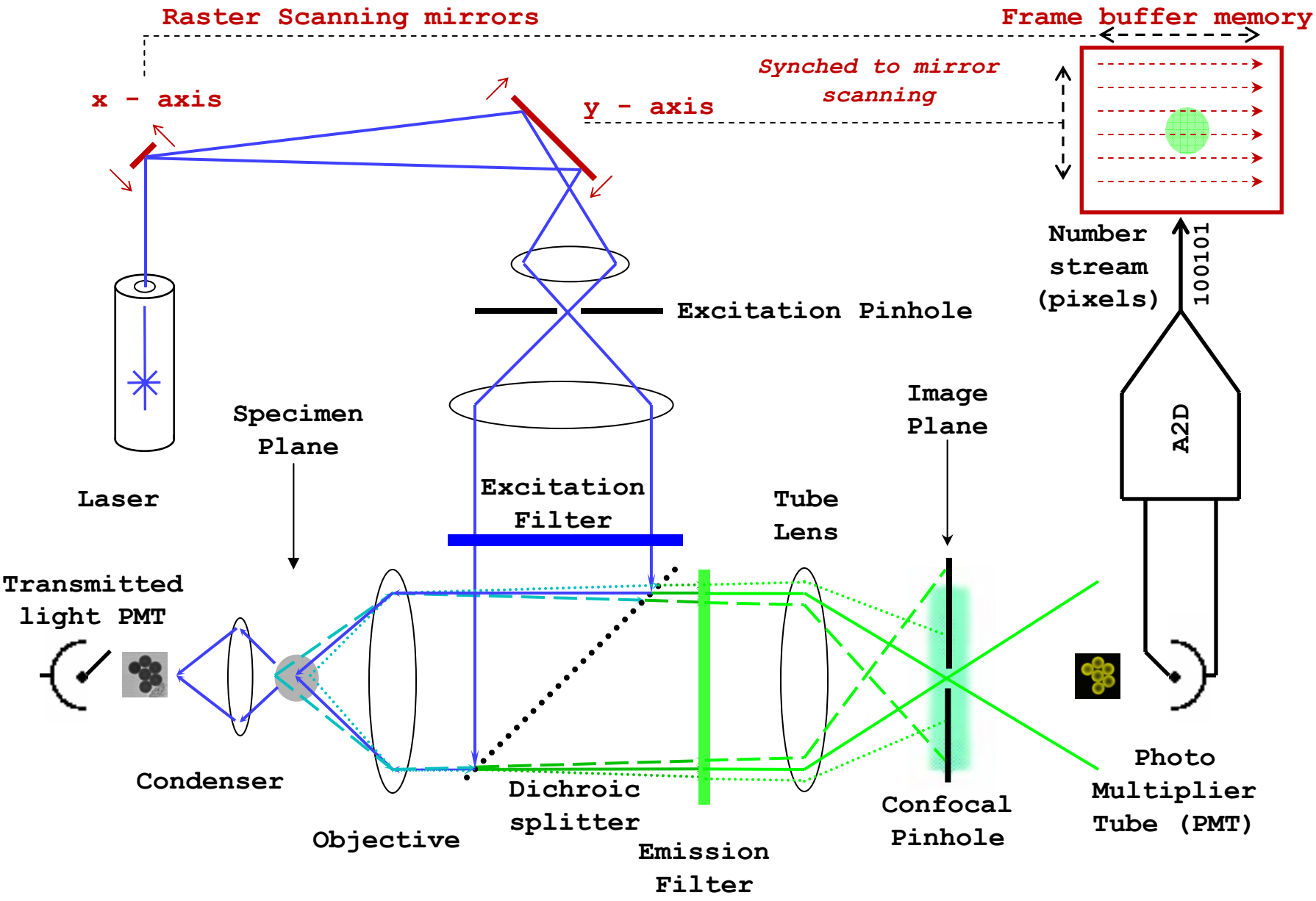
Confocal Laser Scanning Microscope– Analogue to Digital Converter & Frame Buffer Display



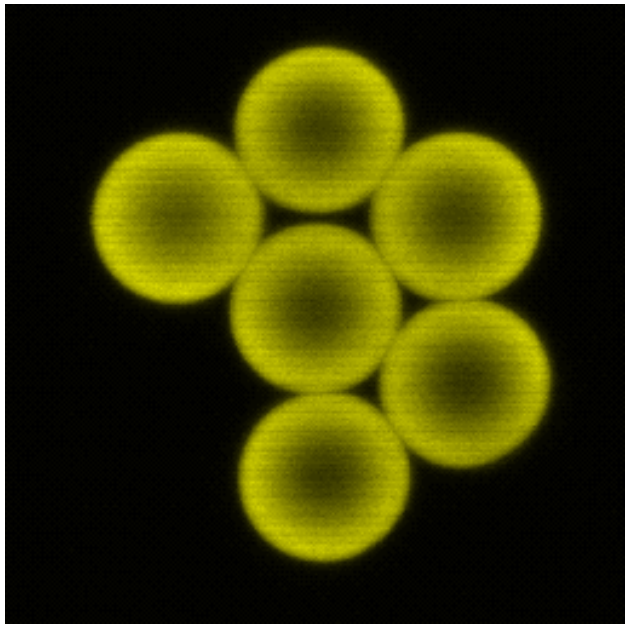
A2D = Analogue to Digital Converter

LUT = Look Up Table

Confocal Laser Scanning Microscope

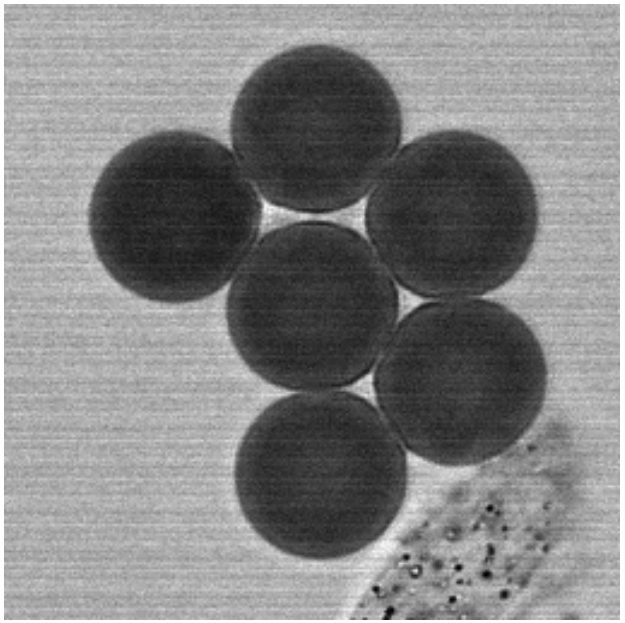


10 um beads – xy view



y
x

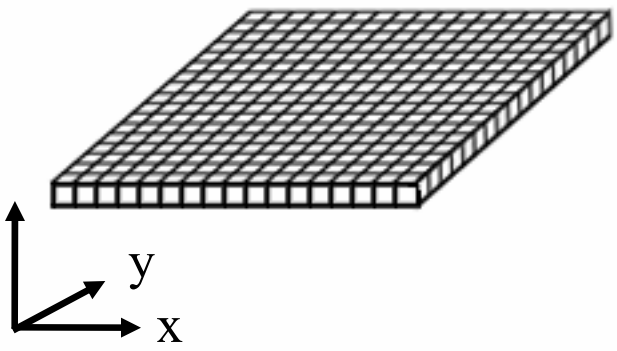
Confocal fluorescence



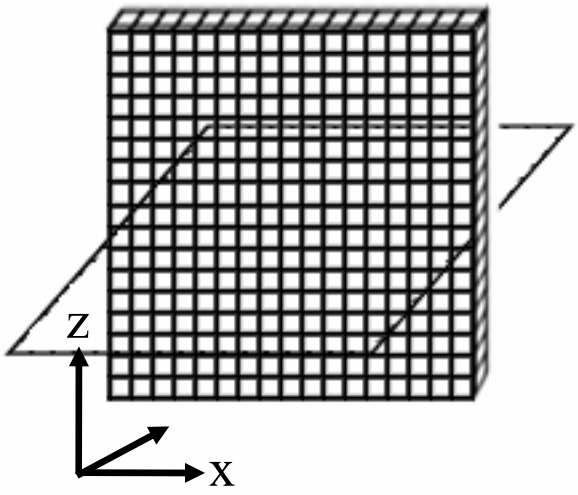
y
x

Wide field transmitted light

Scanning modes

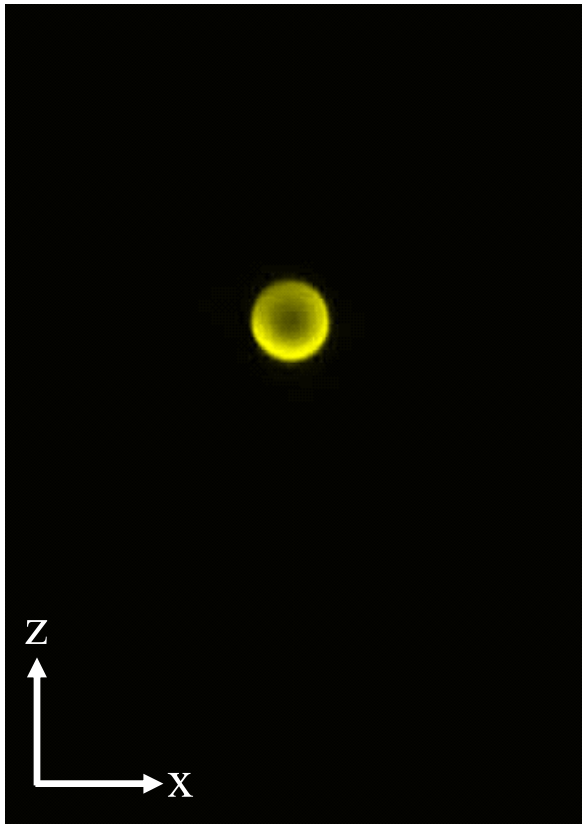


x - y scanning
(conventional slice)

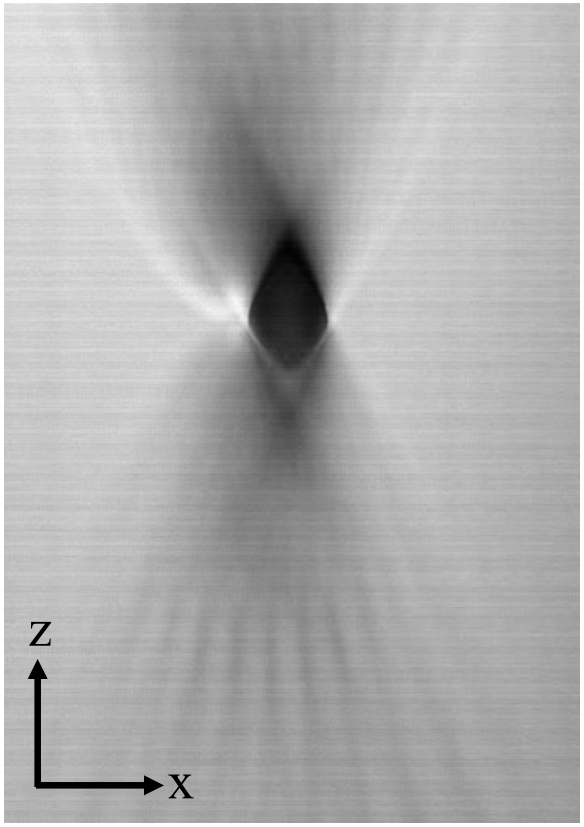


x - z scanning
(vertical slice)

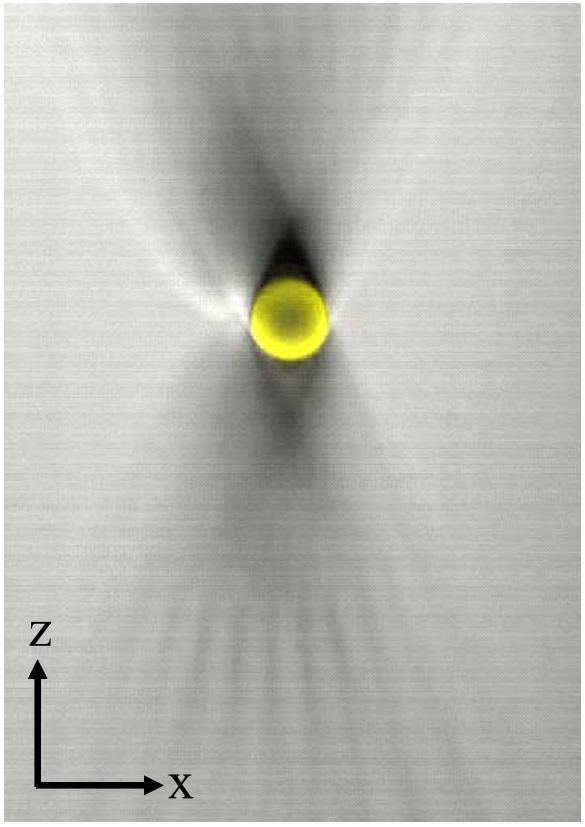
10 um bead – xz side view



Confocal fluorescence

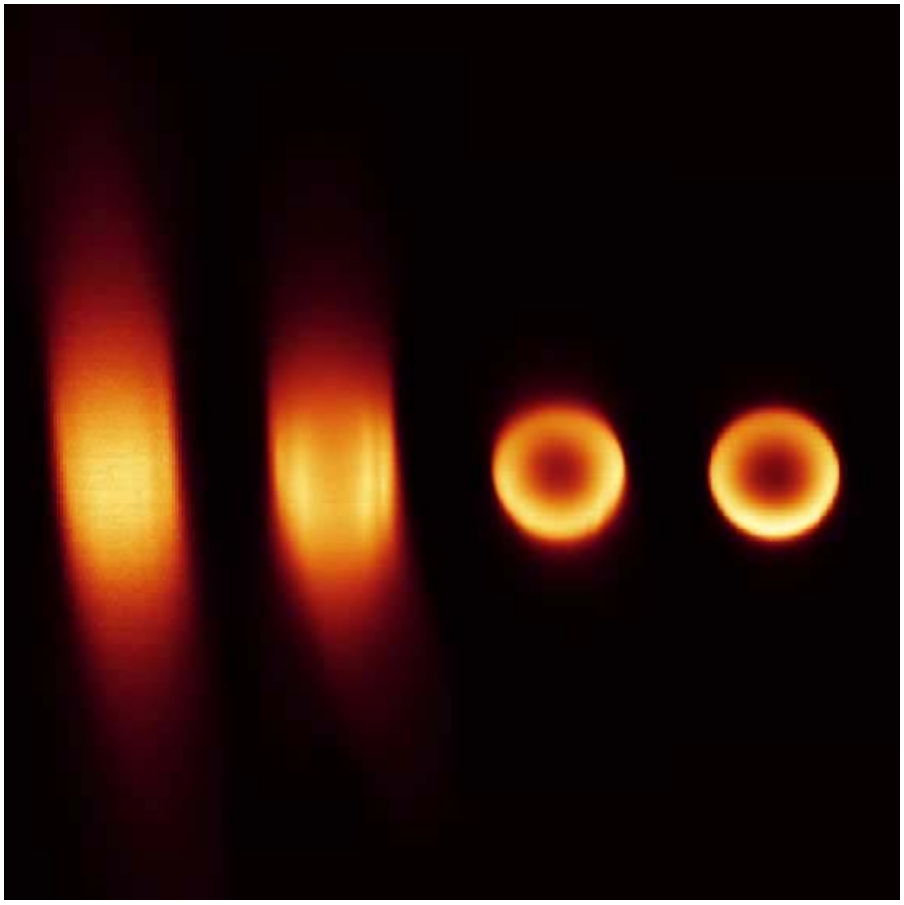


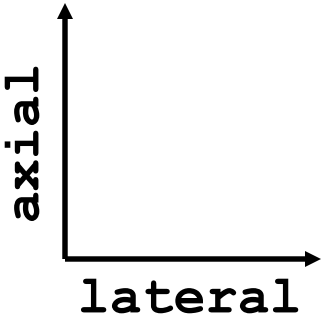
Wide field transmitted light



Overlay

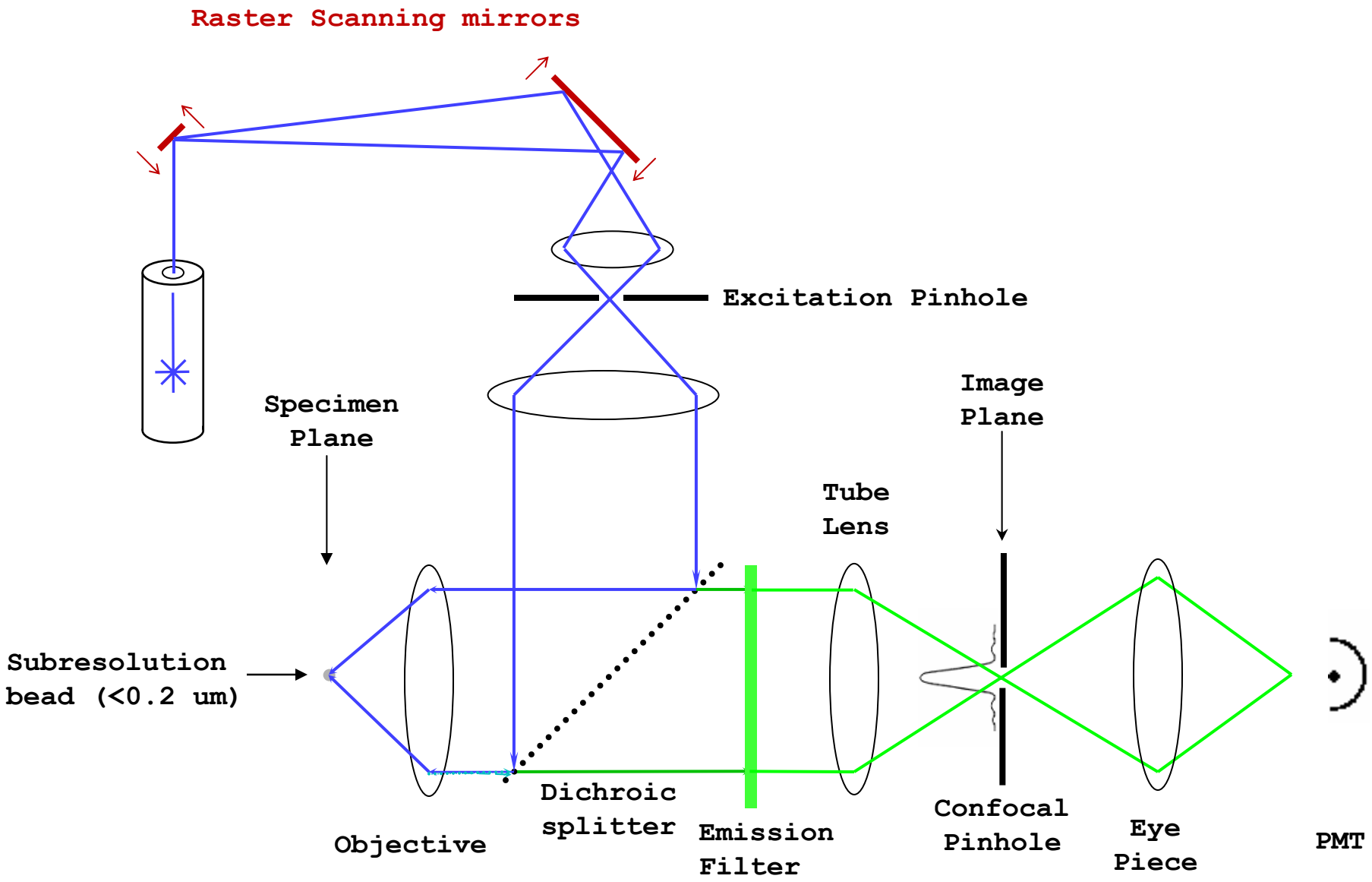
10 um bead – confocal xz view



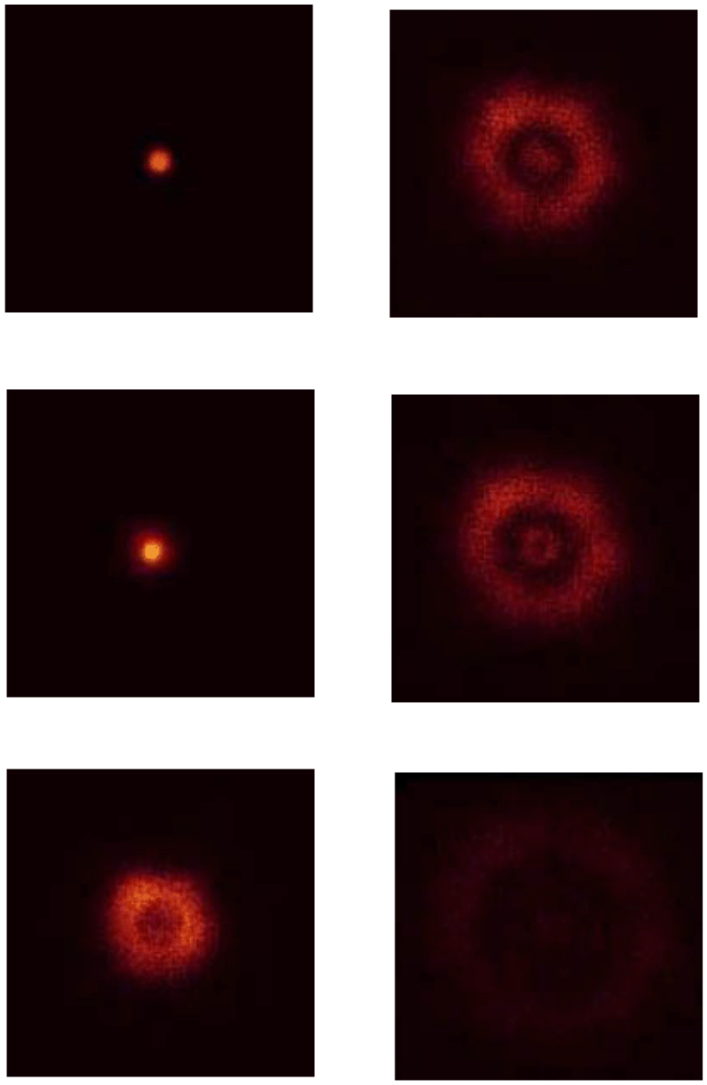


10x	20x	40x	100x	objective
0.3	0.7	1.25	1.4	numerical aperture

Airy Disk

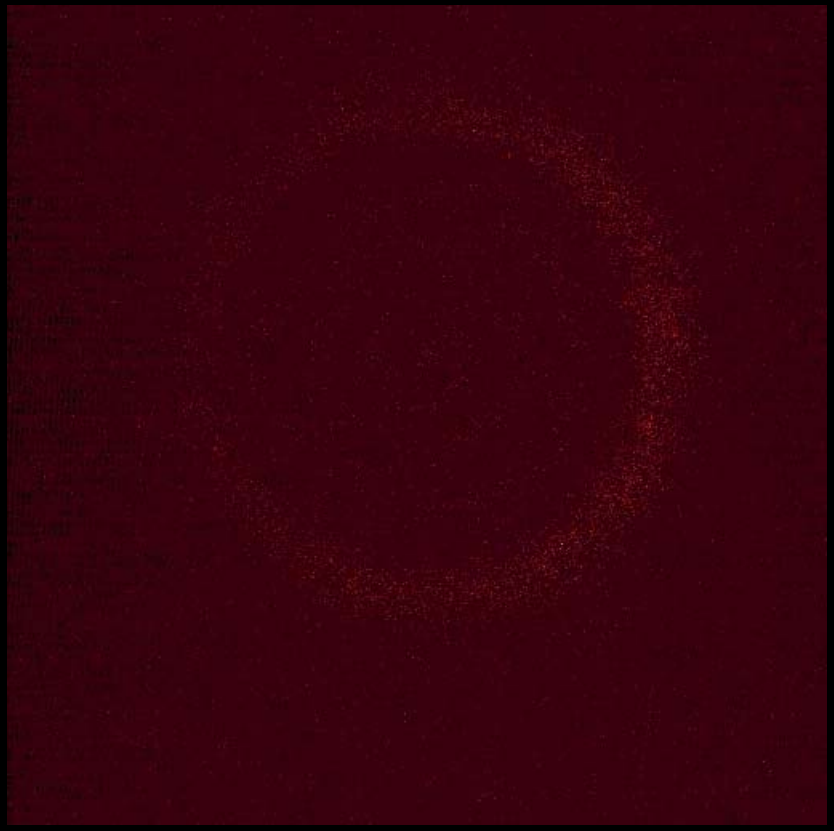

Raster Scanning mirrors
Excitation Pinhole
Specimen Plane
Image Plane
Tube Lens
Subresolution bead (<0.2 um)
Dichroic splitter
Objective
Emission Filter
Confocal Pinhole
Eye Piece
PMT

Airy Disks



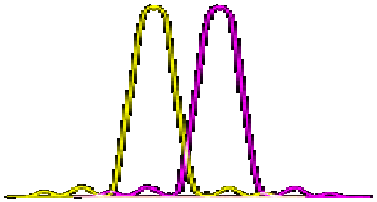
0.5 μm bead Plan Apo 100x 1.4 NA oil

Airy Disk

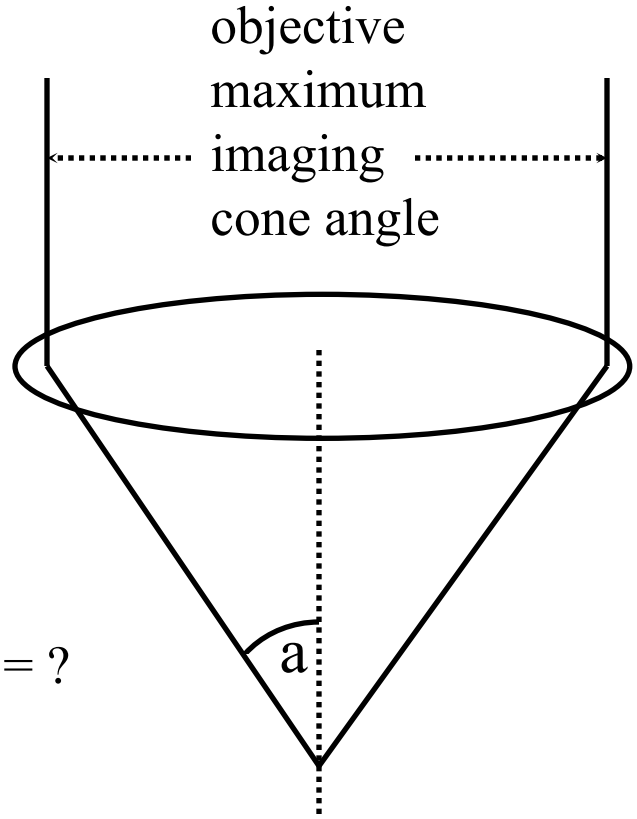


0.5 um bead Plan Apo 20x 0.7 NA oil

Numerical Aperture (NA) - resolution



Rayleigh Criterion: 2 points are said to be resolved when there separation causes the center of the Airy pattern of one point to fall on the 1st minimum of the other Airy disk

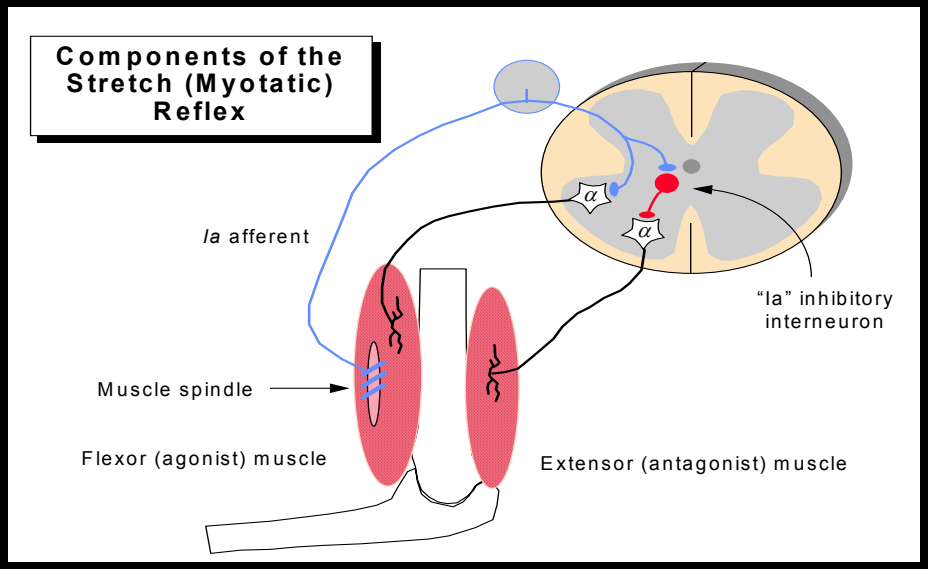


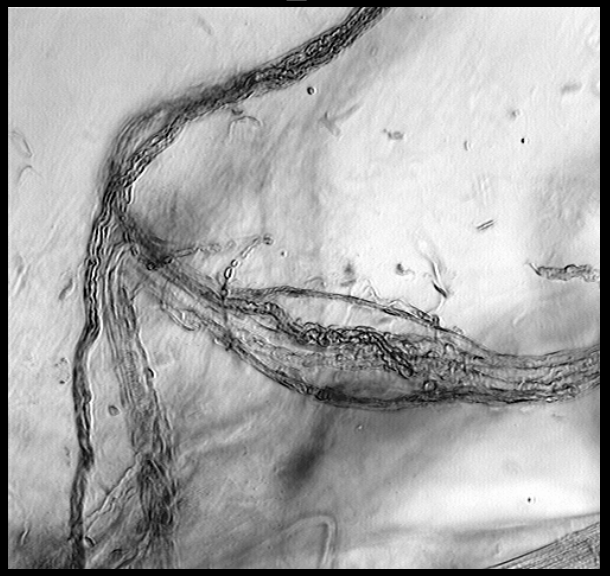
NA = n.sin (a) , a = half cone angle
n = refractive index of medium
λ = wavelength

Wide Field $r_{airy} = 0.61\ \lambda / NA$ $r_{airy.axial} = ?$
(fluorescence) $= 0.22\ \mu m$

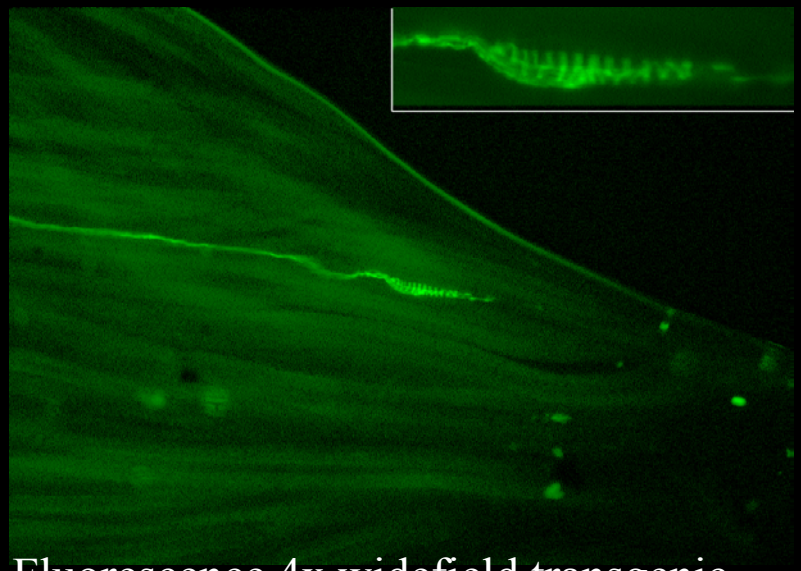
Confocal $r_{airy} = 0.61\ \lambda / NA / \sqrt{2}$ $r_{axial\text{-}fluor} = 1.77\ \lambda / NA^2$
$= 0.15\ \mu m$ $= 0.4\ \mu m$ (for green emission)

Visualization - Example Muscle Spindle





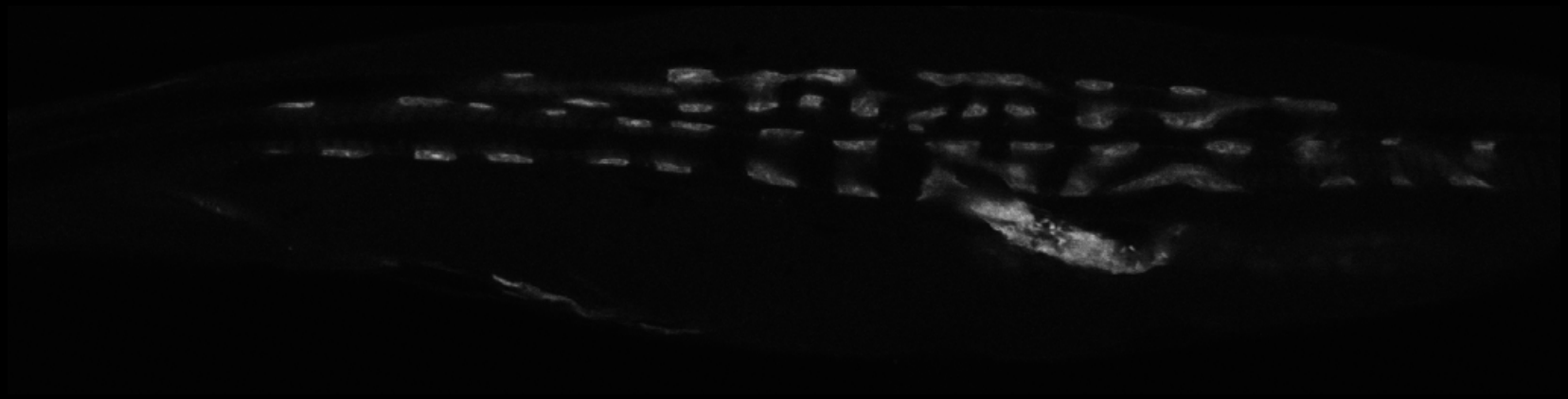
Transmitted light 16x widefield



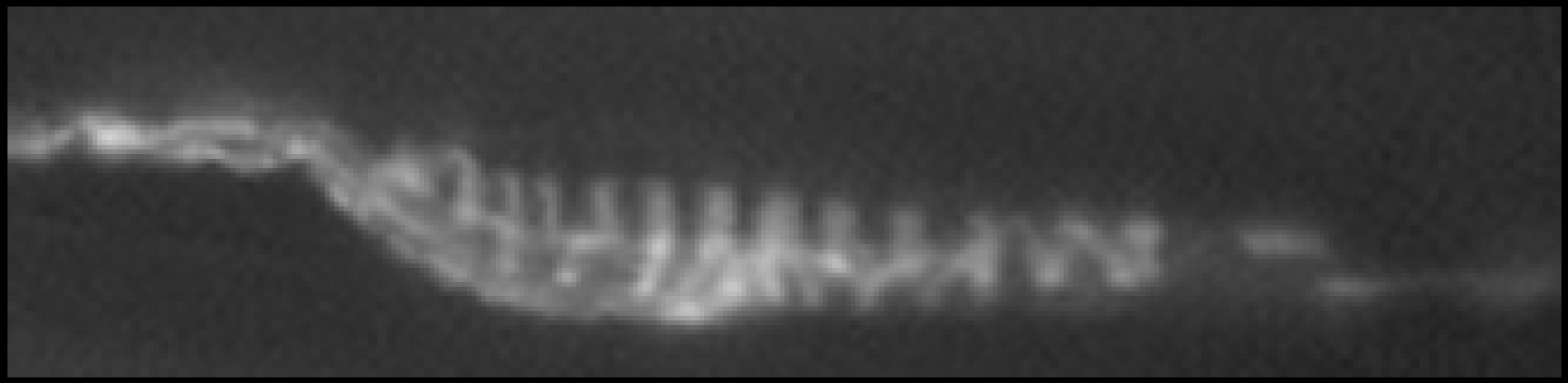
Fluorescence 4x widefield transgenic mouse Thy1-YFG

Wide field – non confocal
Thin slice and z-series
Gallery
Serial slices
Z-axis sampling
Orthogonal view
Average & Maximum projections
Volume render
Multiple channels (dyes / fluorophores)

Visualization - Live Muscle Spindle (Thy1-YFP)

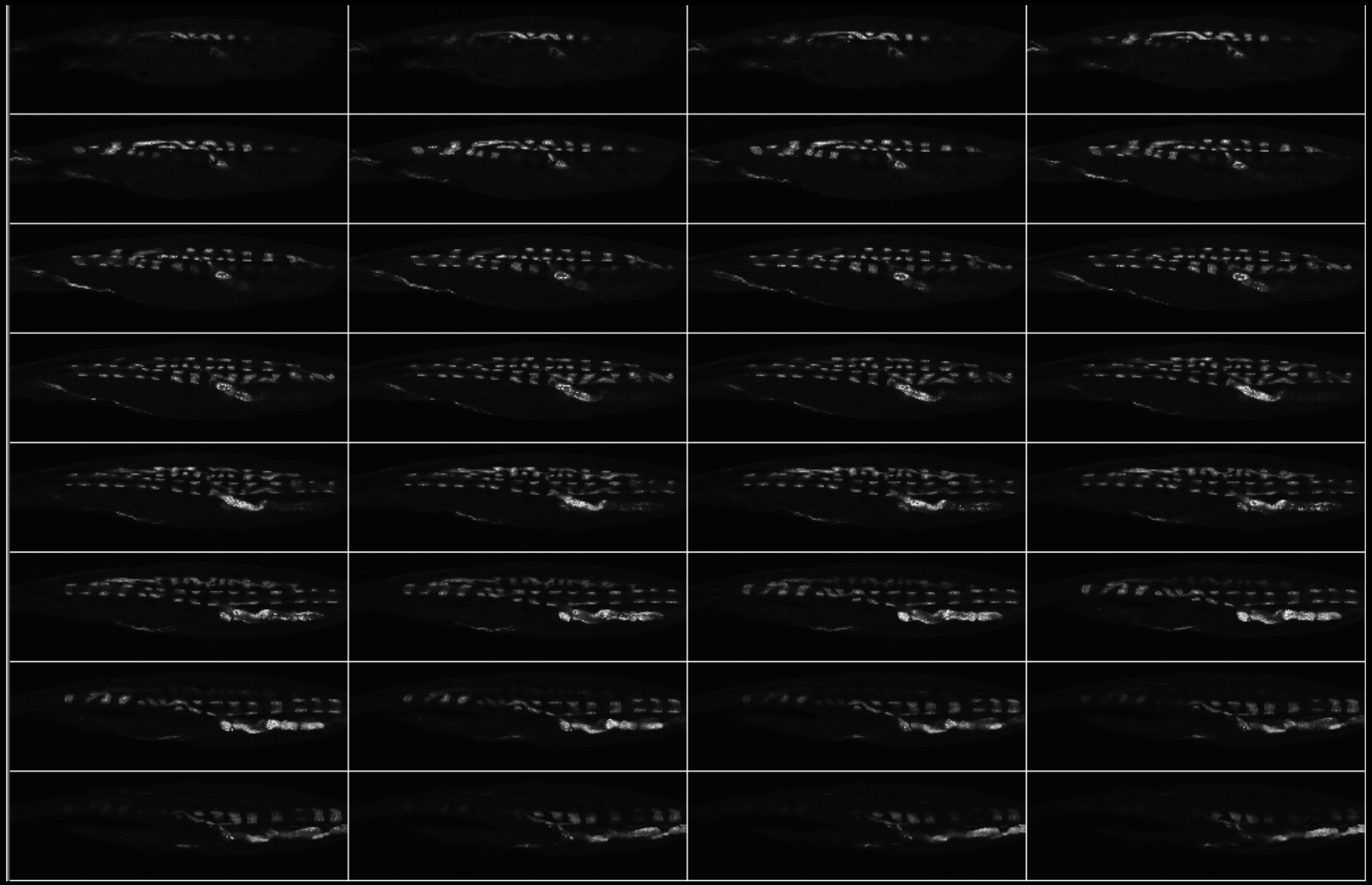


single confocal slice 40x 1.3 NA 230 um

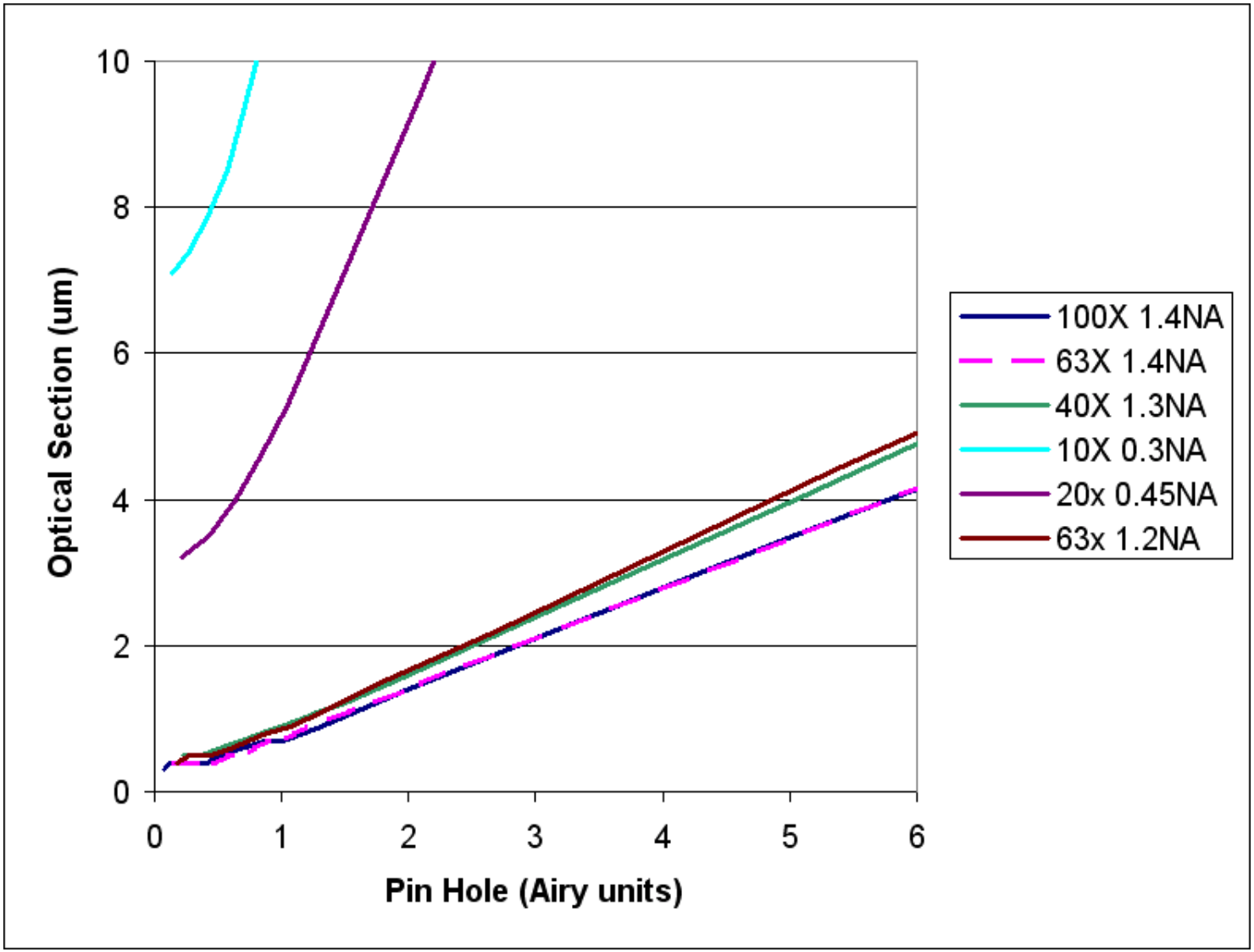


wide field

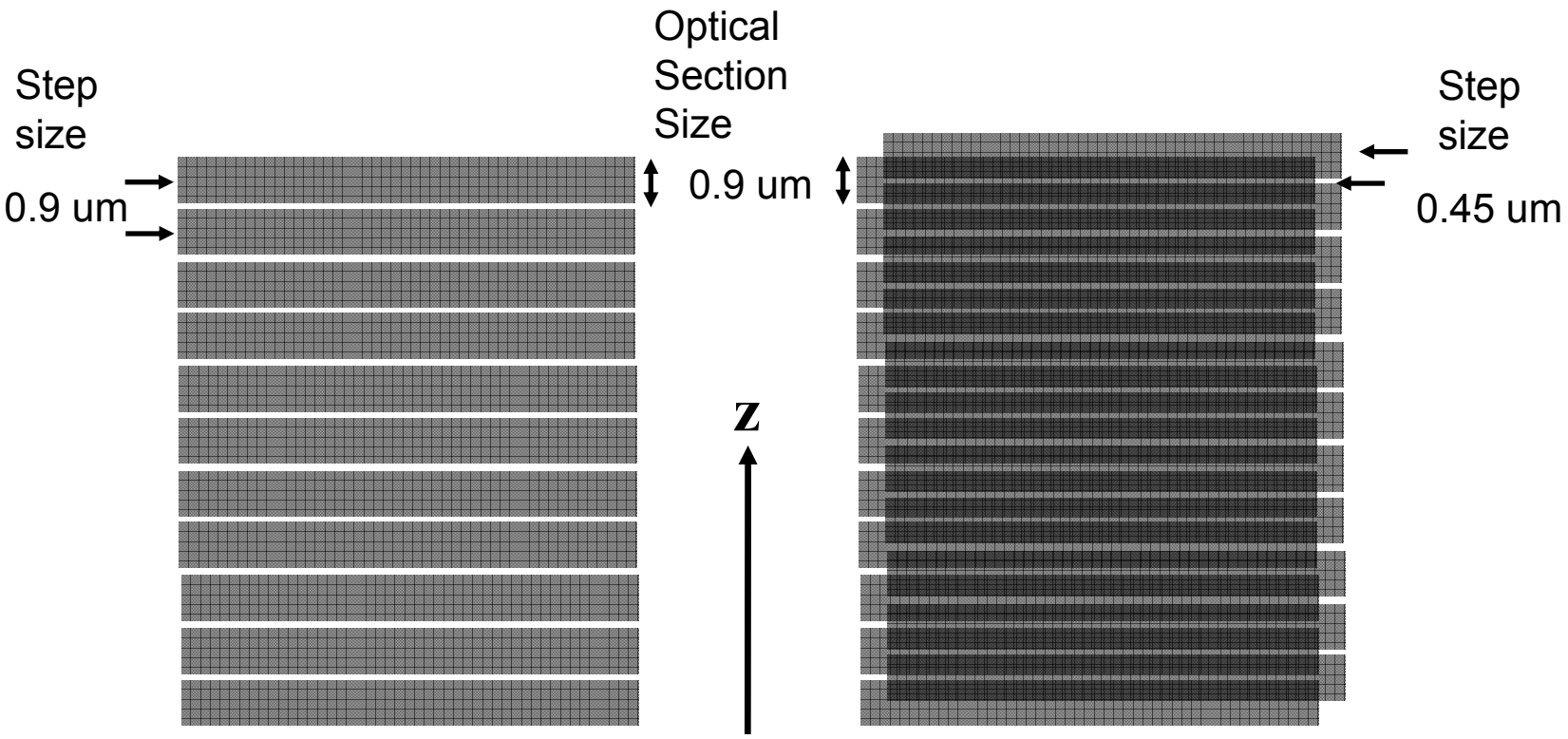
Visualization - Focus to Different Planes
Serial Sections



Pinhole Size versus optical Section



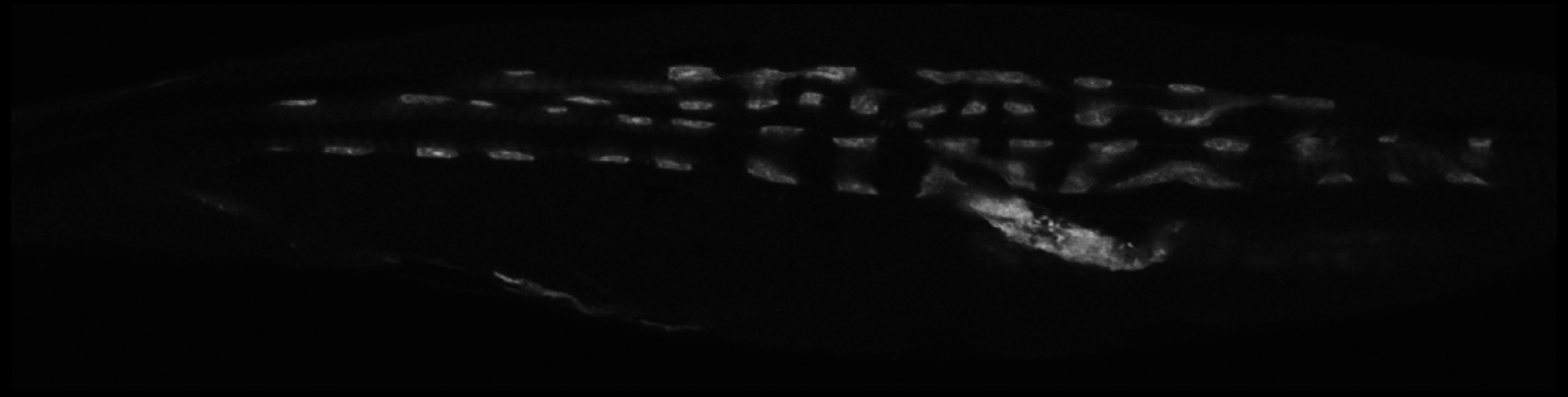
Optical Sectioning



Inadequately Sampled

Just Adequately Sampled
(Nyquist criterion at least twice frequency, ~2.3x)

Visualization - Live Muscle Spindle (Thy1-YFP)

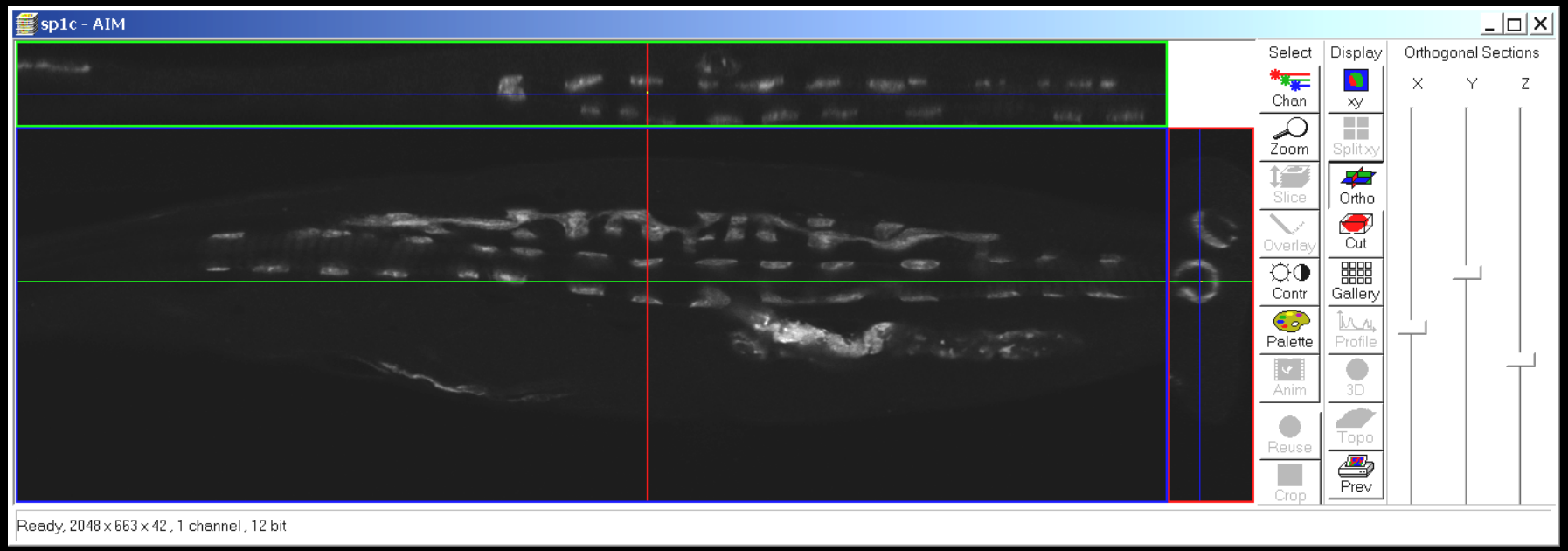


single slice

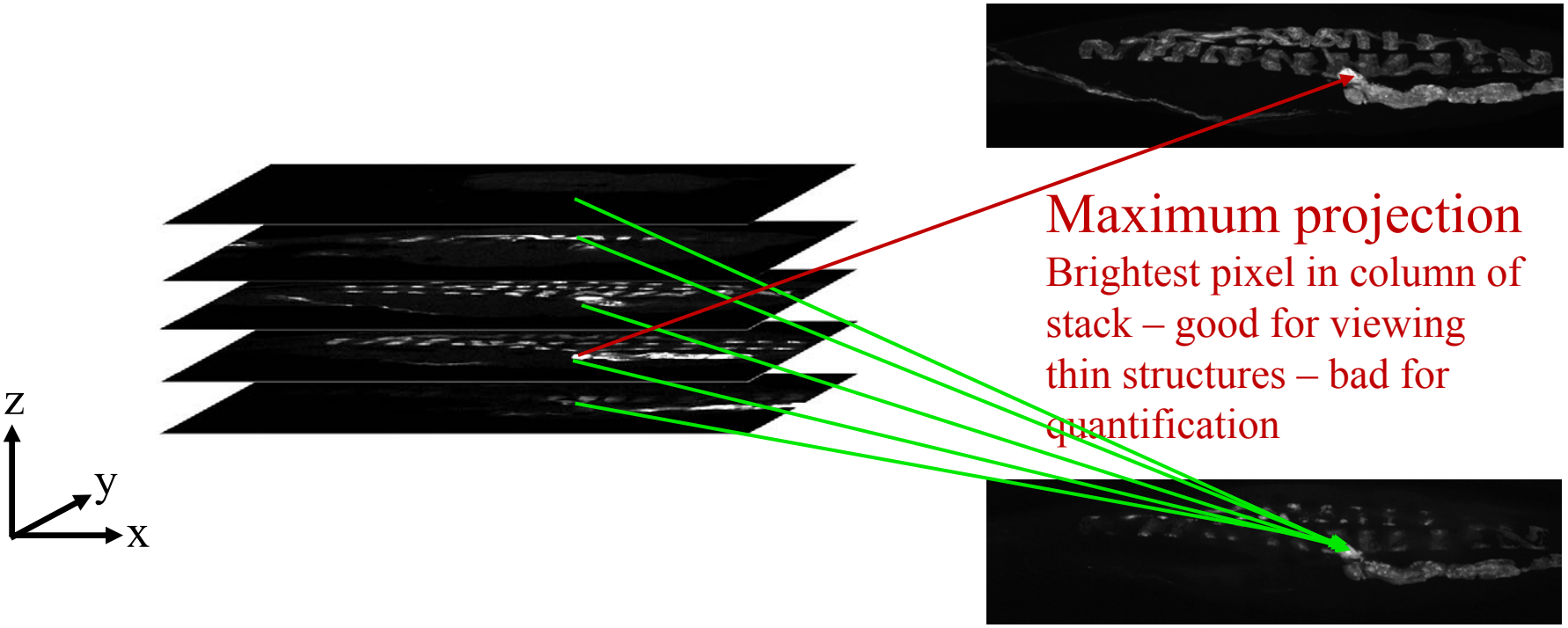
serial slices

40x 1.3 NA 230 um

Visualization - Orthogonal View



Visualization - Extended Focus 40x



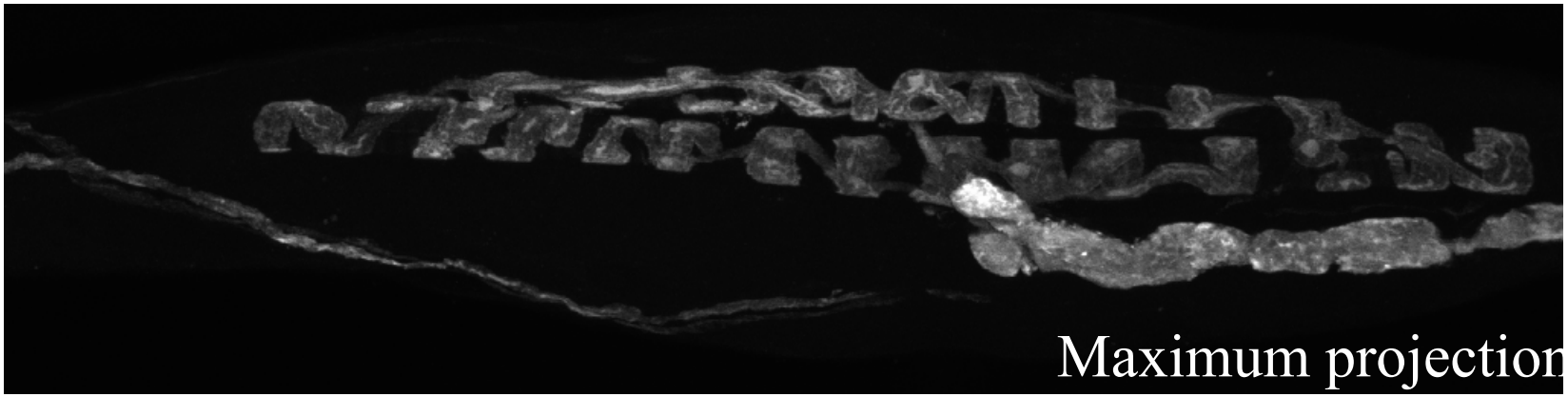
Maximum projection

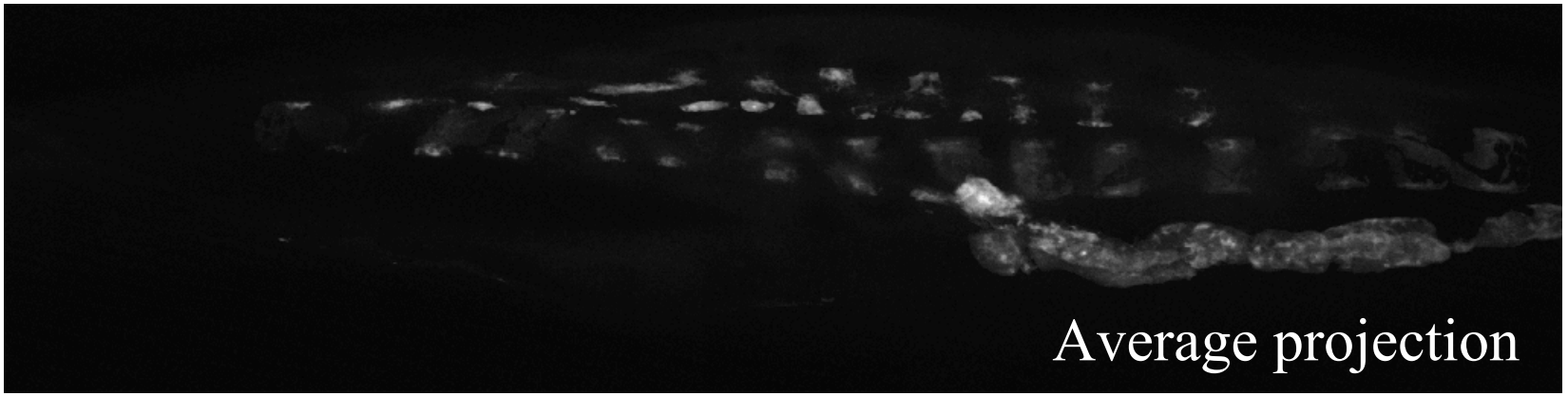
Brightest pixel in column of stack – good for viewing thin structures – bad for quantification

Average projection

Sum corresponding pixels and divide by the number of slices – good for quantification

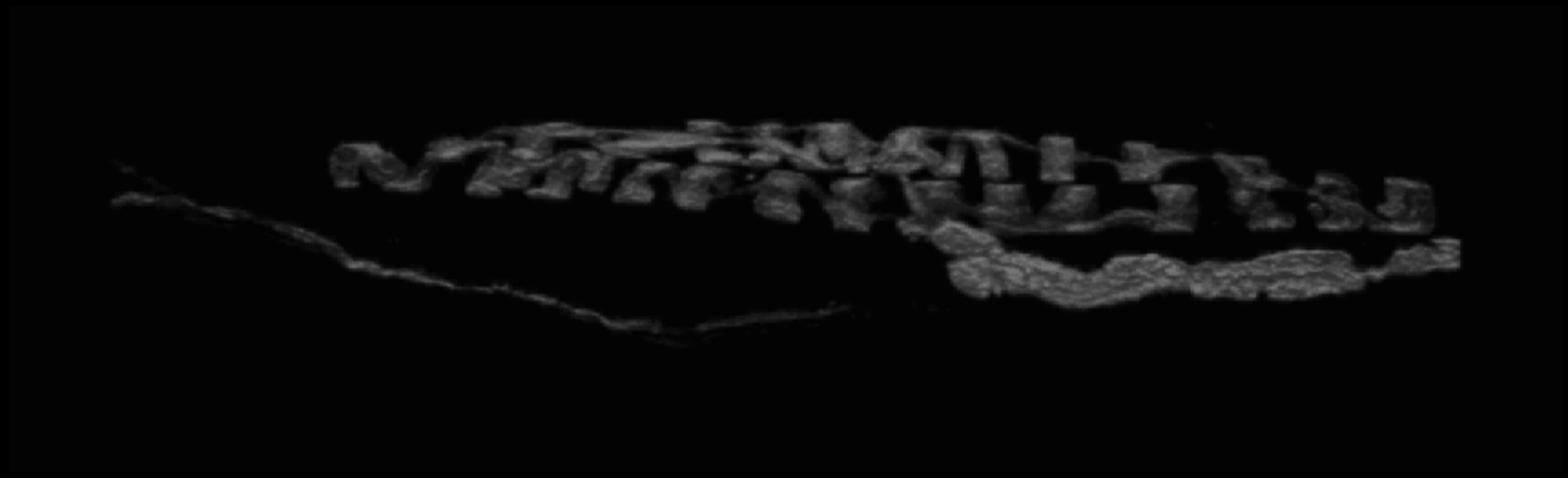
Visualization - Extended Focus 40x



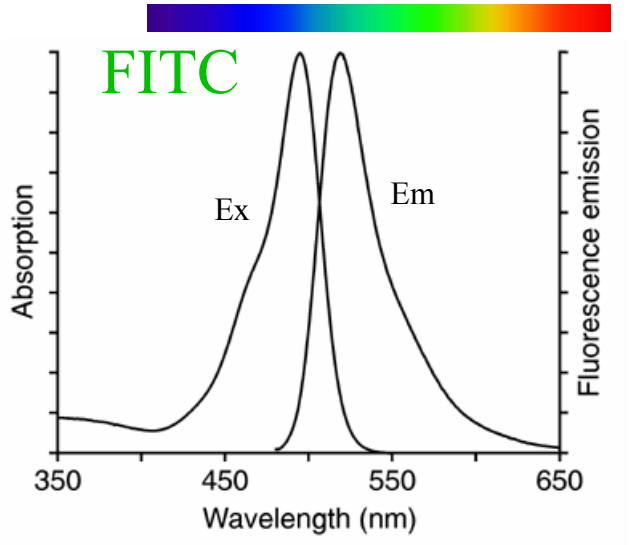


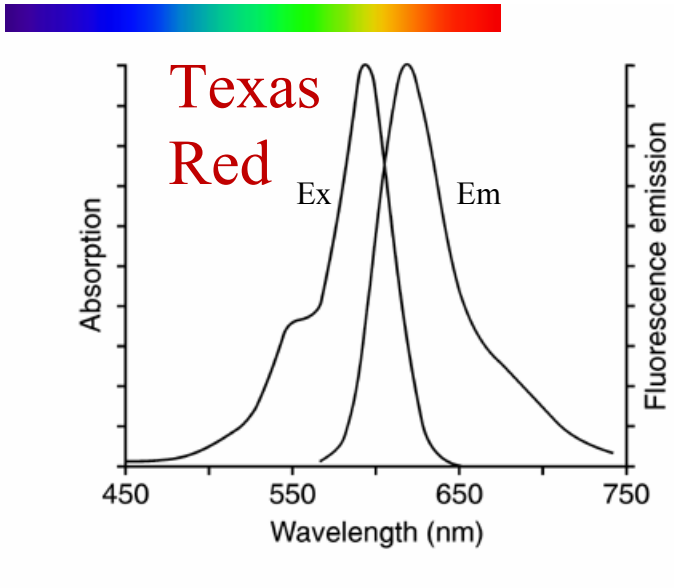
Visualization - Volume Renderimg

Each voxel – intensity * opacity

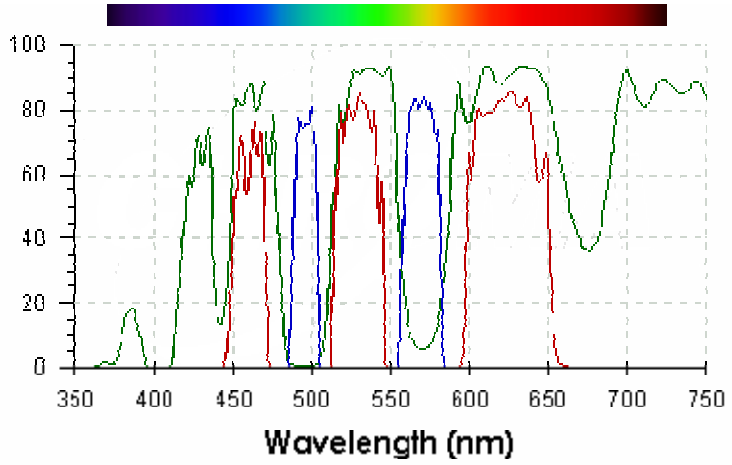


Confocal Multichannel Fluorescence



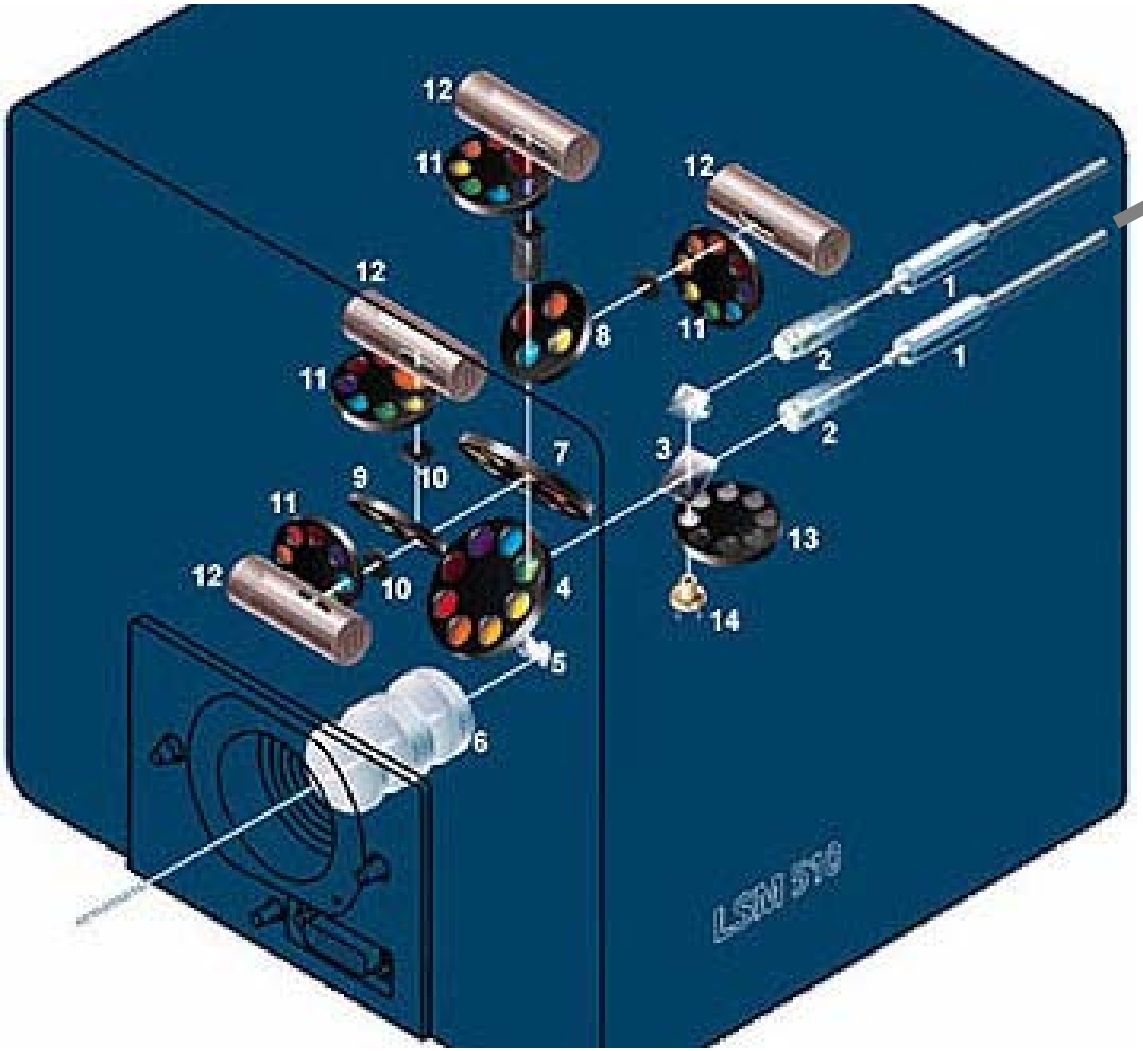


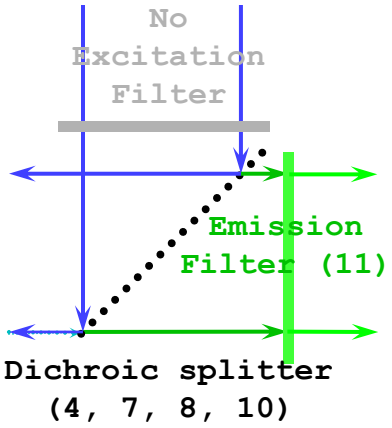
Triple band
Filter (Chroma Corp.)



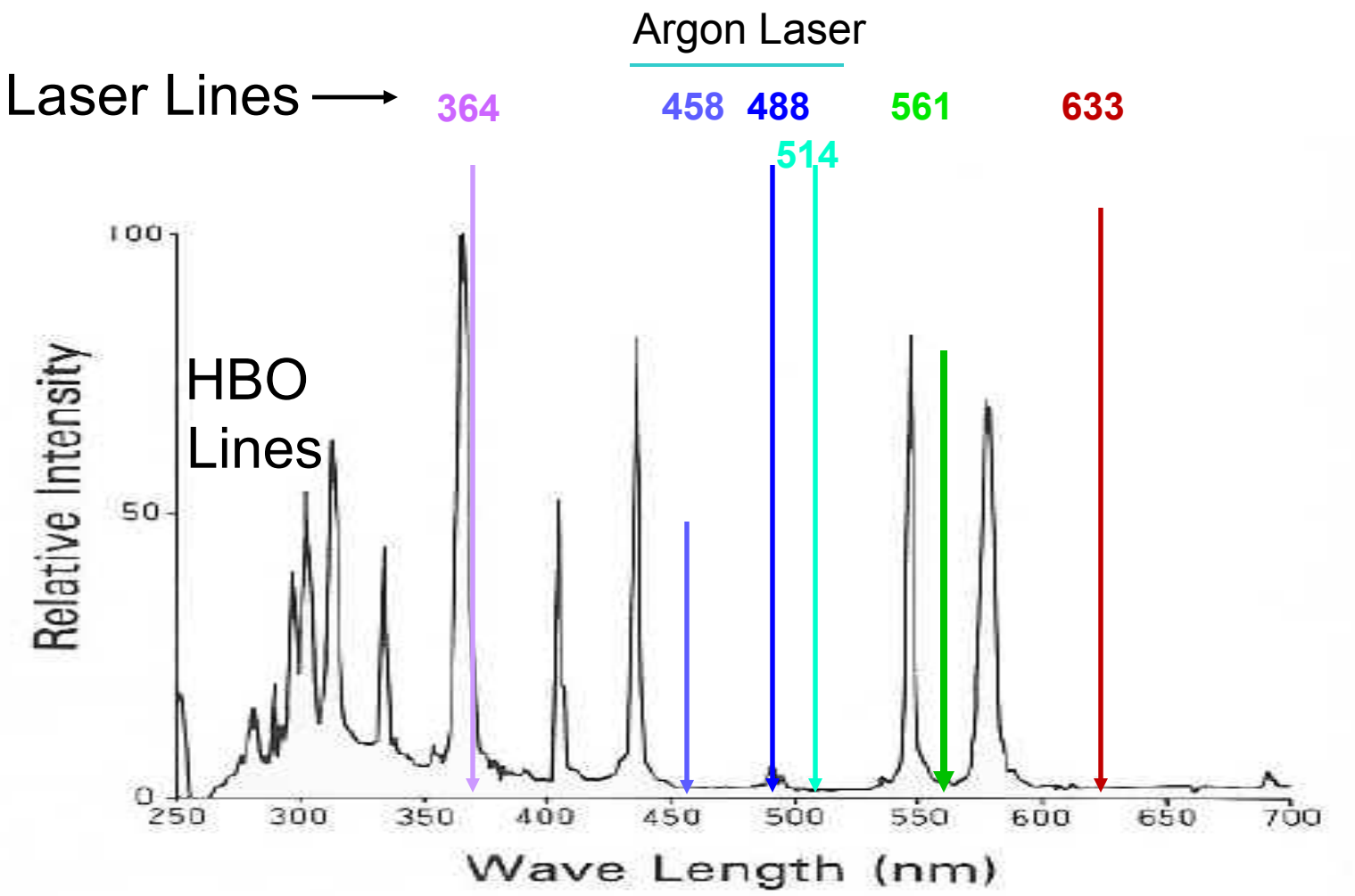
- Exciter
- Dichroic
- Emission

Confocal Multichannel Fluorescence

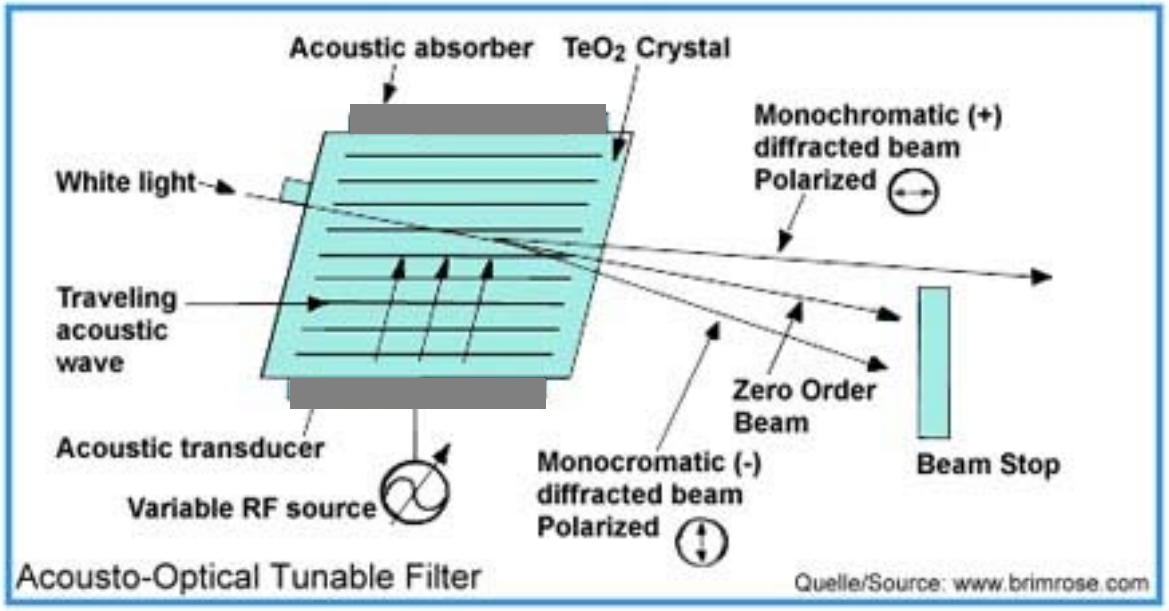




ARC Lamp versus Laser Excitation



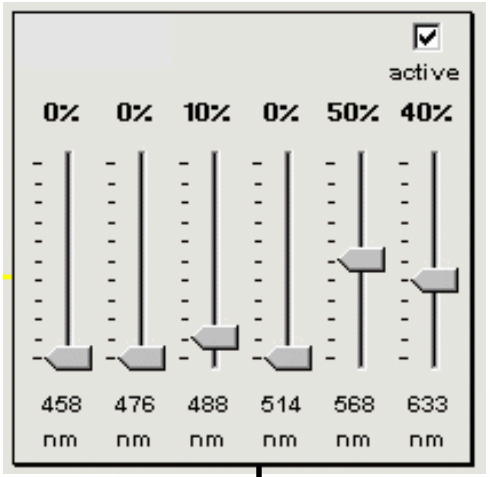
Acousto-Optical Tunable Filter (AOTF)



RF = radio frequency

Excitation Light System – AOTF Controlled

Replaces excitation filters



RF modulator

Laser Systems

To scanhead

Fiber optic

Piezo Electric Crystal

Undeflected Light Beam

Acousto-Optic Tunable Filter

Tellurium Oxide

488 nm

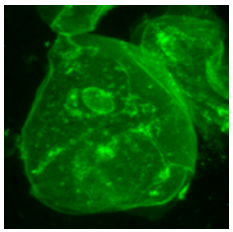
561 nm

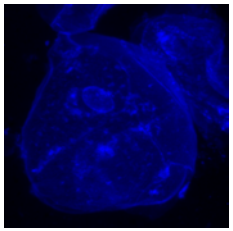
633 nm

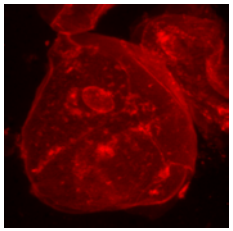
Merging Dichroic Mirrors

RF = radio frequency

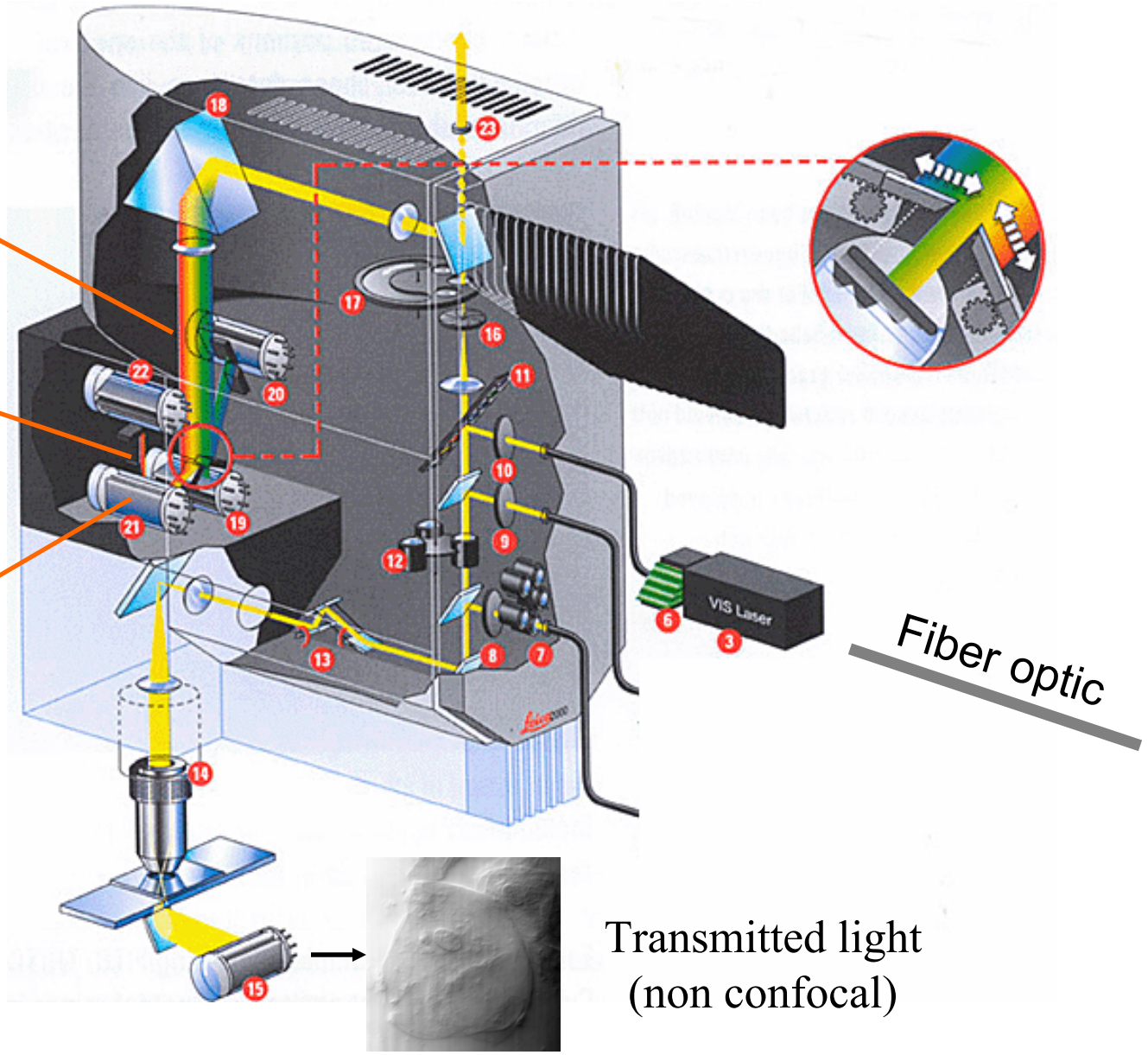
Confocal Multichannel Fluorescence







Fluorescence
(confocal)

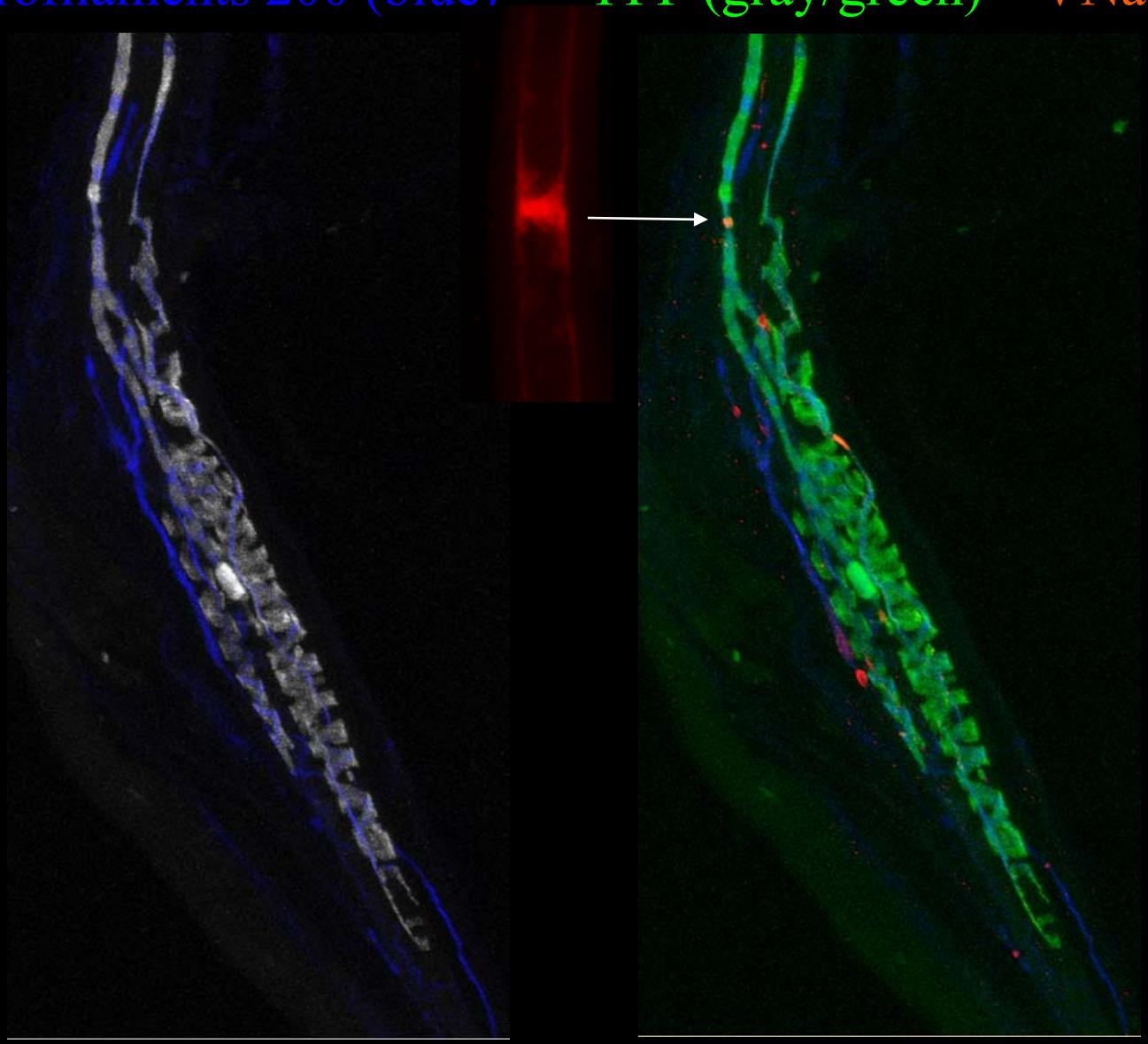


6 = AOTF
11 = dichroic
19 to **21 = PMTs**

Transmitted light
(non confocal)

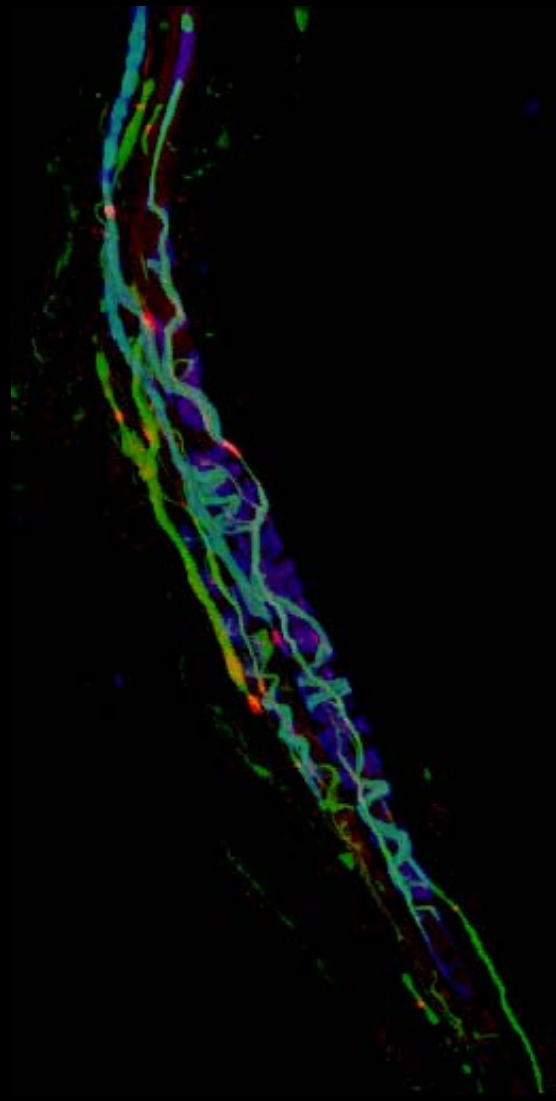
Confocal Multichannel Fluorescence

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



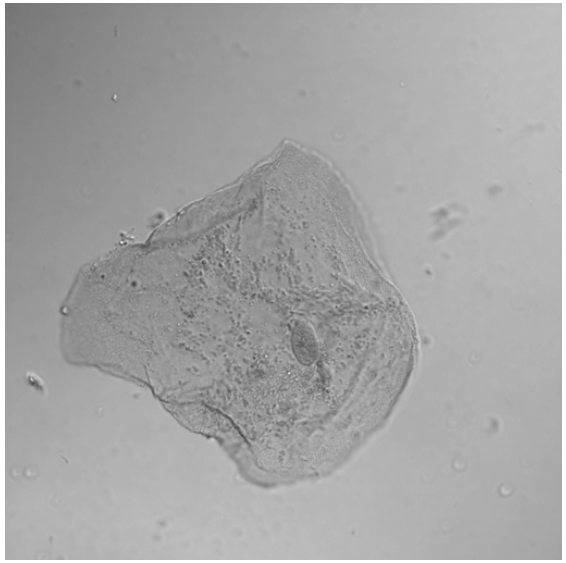
Confocal - Multichannel Fluorescence

Ab-Neurofilaments 200 (green) YFP (Blue) VNaCh (red)

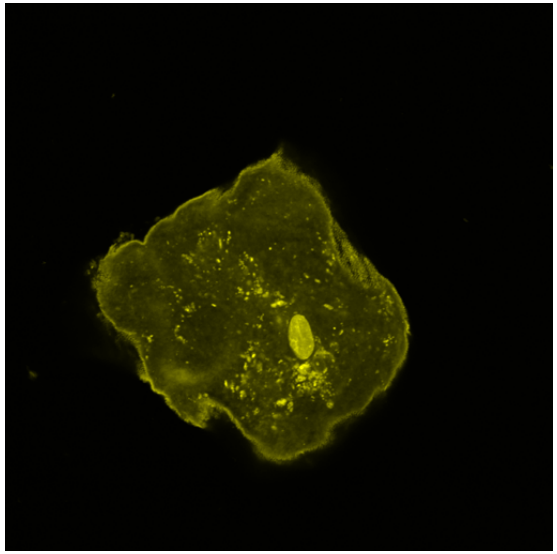


Confocal Microscopy – Summary

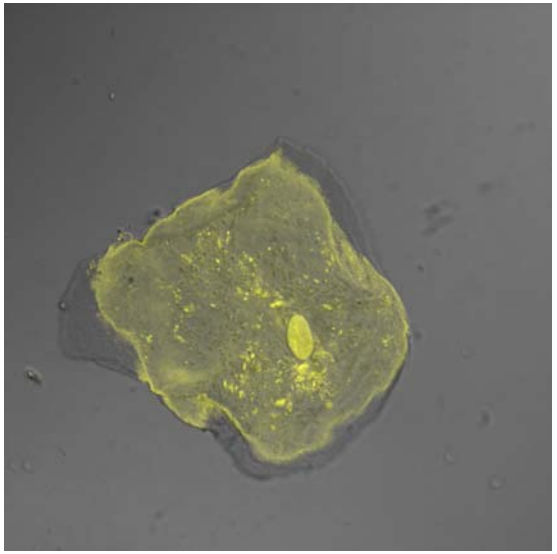
- A microscope system which removes out of focus information optically.
- Better lateral and much improved axial resolution
- Higher contrast
- Reduced glare
- Single voxel scanning permits 3-D reconstructions



(DIC) Transmitted
(widefield)



Fluorescence
FM 1-43 membrane dye
(confocal)



Overlay

References

- Confocal Microscopy Methods and Protocols, in Methods in Molecular Biology, Stephen W. Paddock, 1999

- Handbook of Biological Confocal Microscopy, 2nd ed., James Pawley, 1995

- Handbook of Biological Confocal Microscopy, 3rd ed., James Pawley, 2006

- Confocal Microscopy for Biologists, Alan R. Hibbs, 2004

  (Missing at UNC, Duke & NC State)

Advanced Fluorescence/Confocal
May 14

- Multi-channel bleed through/cross talk
- Co-localization
- Live cell imaging
- FRAP, fluorescence recovery after photobleaching
- Deconvolution

- Your requests?