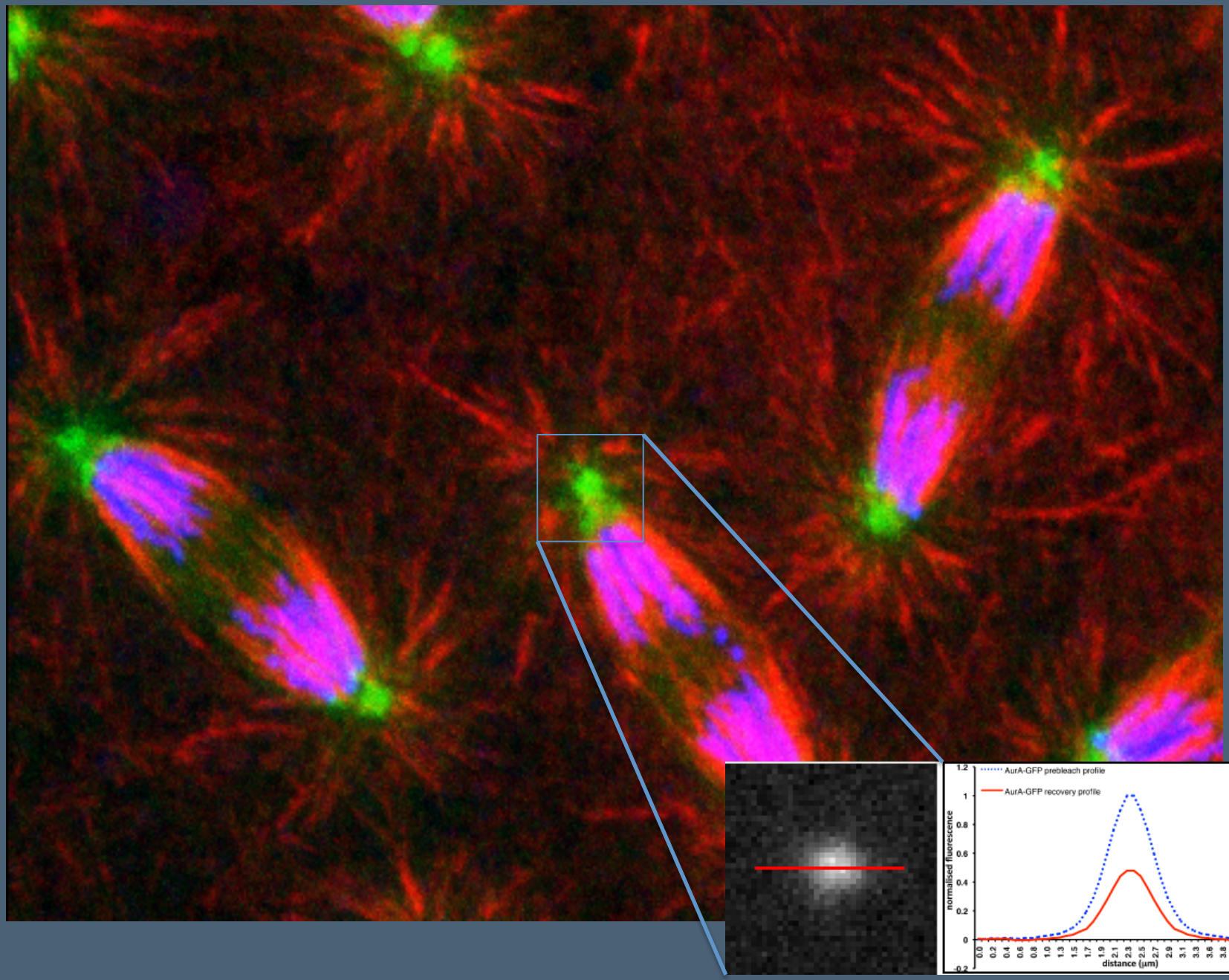
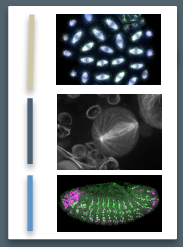
Confocal Microscopy
Lecture 8

March 2015


AurA-GFP prebleach profile
AurA-GFP recovery profile
normalised fluorescence
distance (µm)

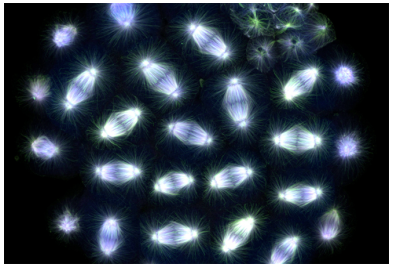


3 Flavours of Microscope

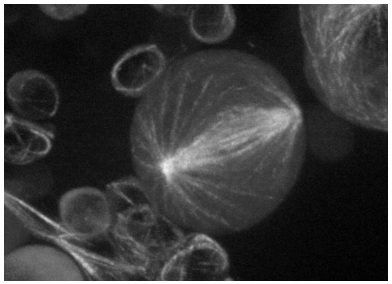
Problem:
Out of Focus
Light

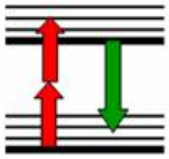
Confocal

Laser
Scanning

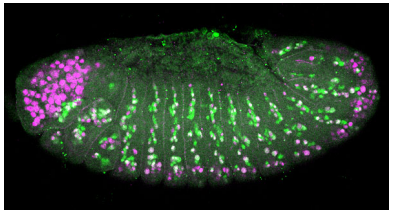


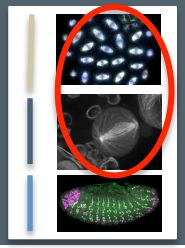
Spinning
disc





2-Photon





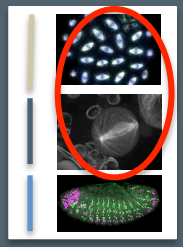
A short History of Confocal Microscope

Confocal “concept’ patented by Marvin Minsky in 1957

Eggar and Petran developed “spinning disc” confocal in late 1960s

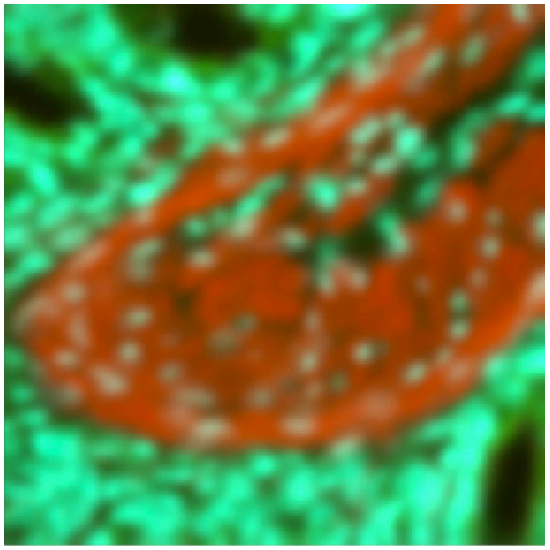
Brakenhoff, Stelzer developed “stage” scanning confocal in late 1970

White, Amos and Wilson developed the MRC500 point scanning confocal
-Marketed commercially in 1987



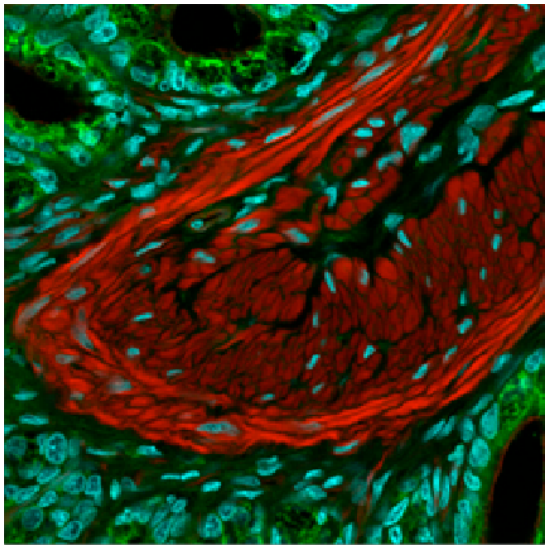
Comparison
Widefield Vs Confocal

Widefield

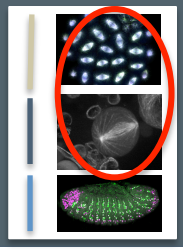


Out of focus light ‘blurs’ image

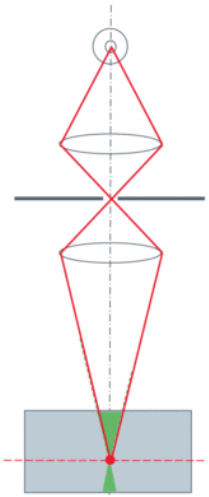
Confocal



Out of focus light is blocked

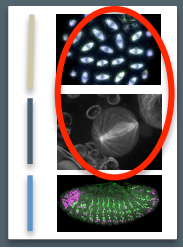


Principle of Confocal Microscopes
Pinhole

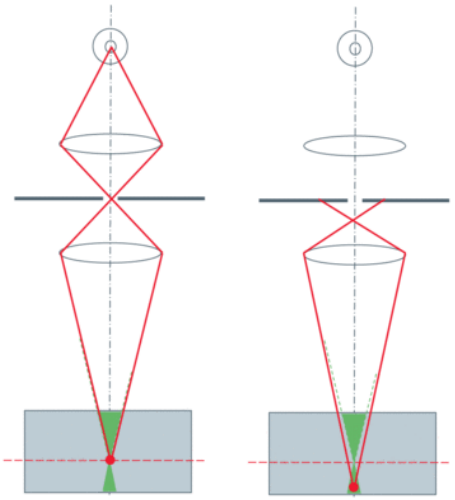


Pinhole diaphragm in the Conjugated focal plane = CONFOCAL

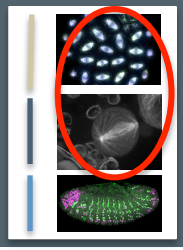
in focus light (from the optical section) passes through the pinhole and into the detector



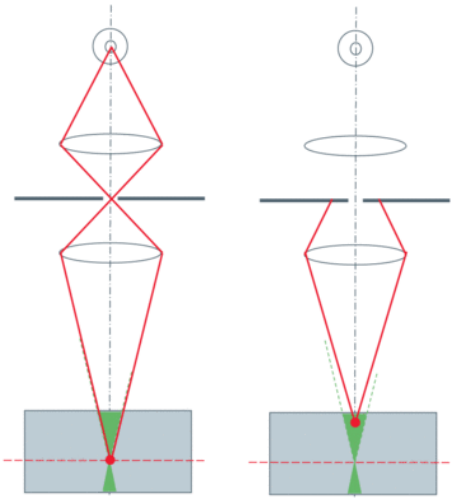
Pinhole – blocks out-of-focus light



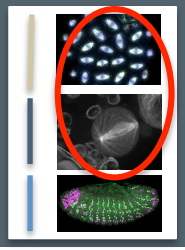
light from below the optical section crosses infront of the pinhole and doesn't pass through the pinhole aperture



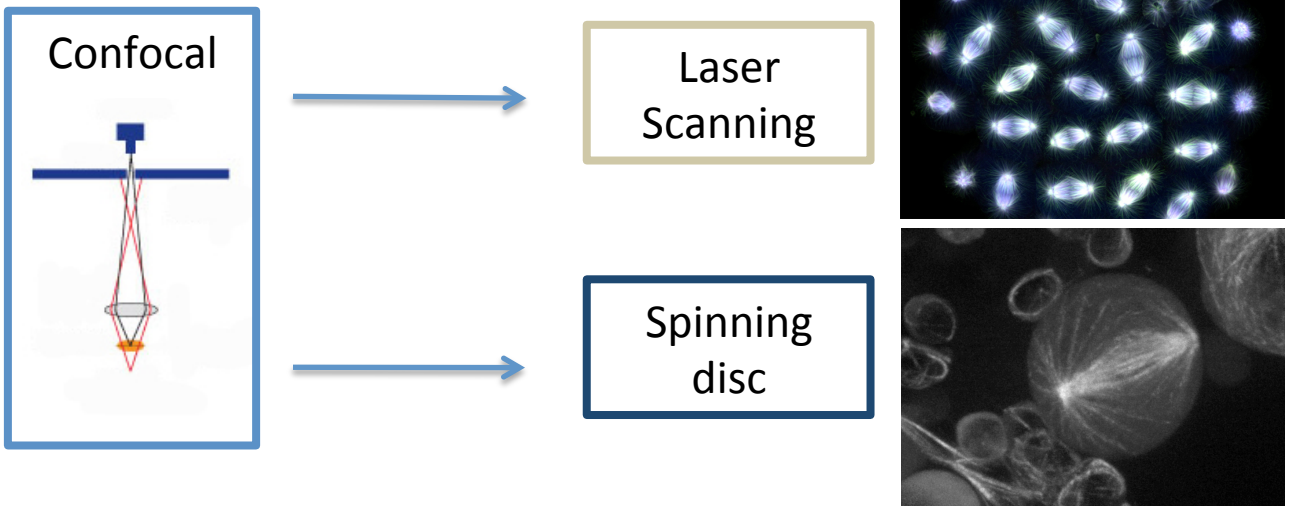
Pinhole – blocks out-of-focus light

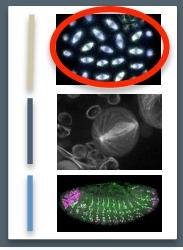


light from above the optical section also doesn't pass through the pinhole aperture



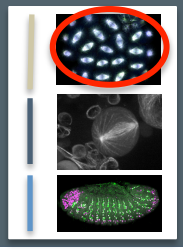
Confocal Microscopes



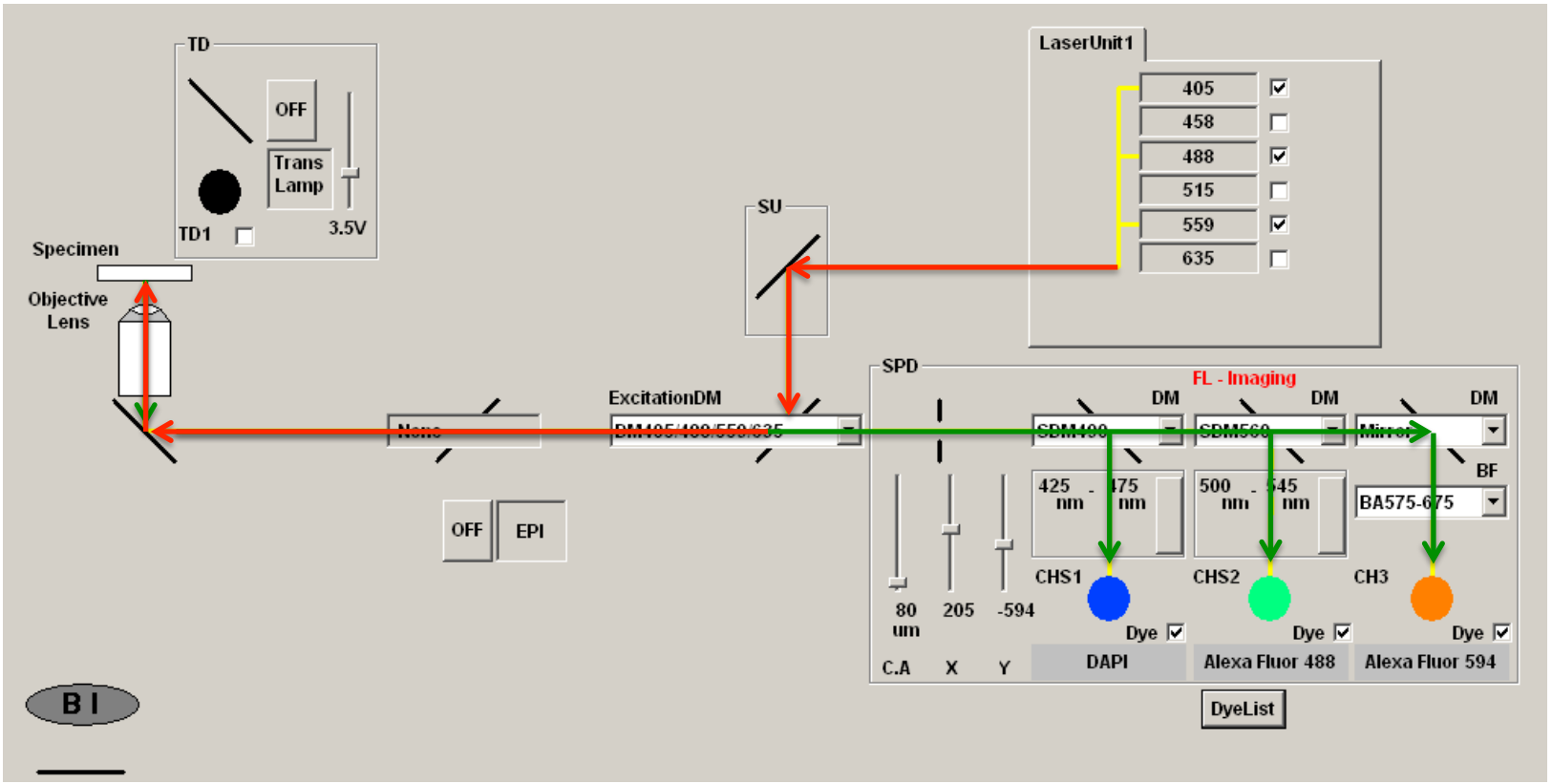


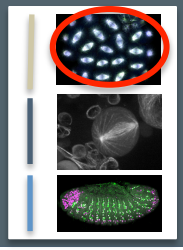
Laser Scanning Confocal Microscope

Laser Scanning Confocals are great to get ‘pretty’ images

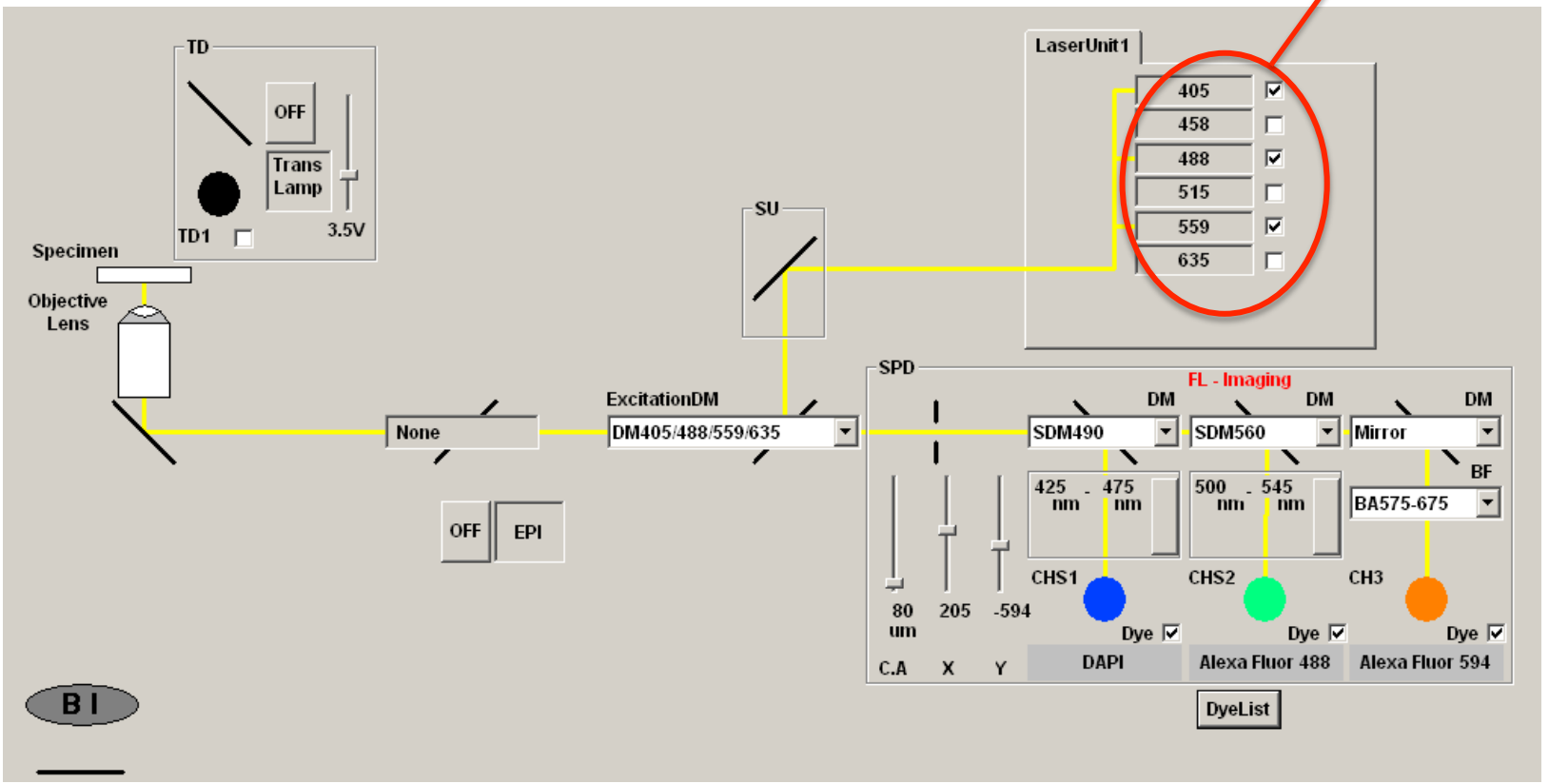


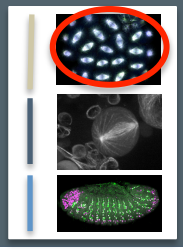
Laser Scanning Confocal - components



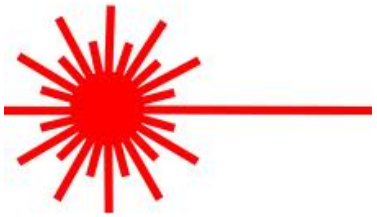


Laser Light Source



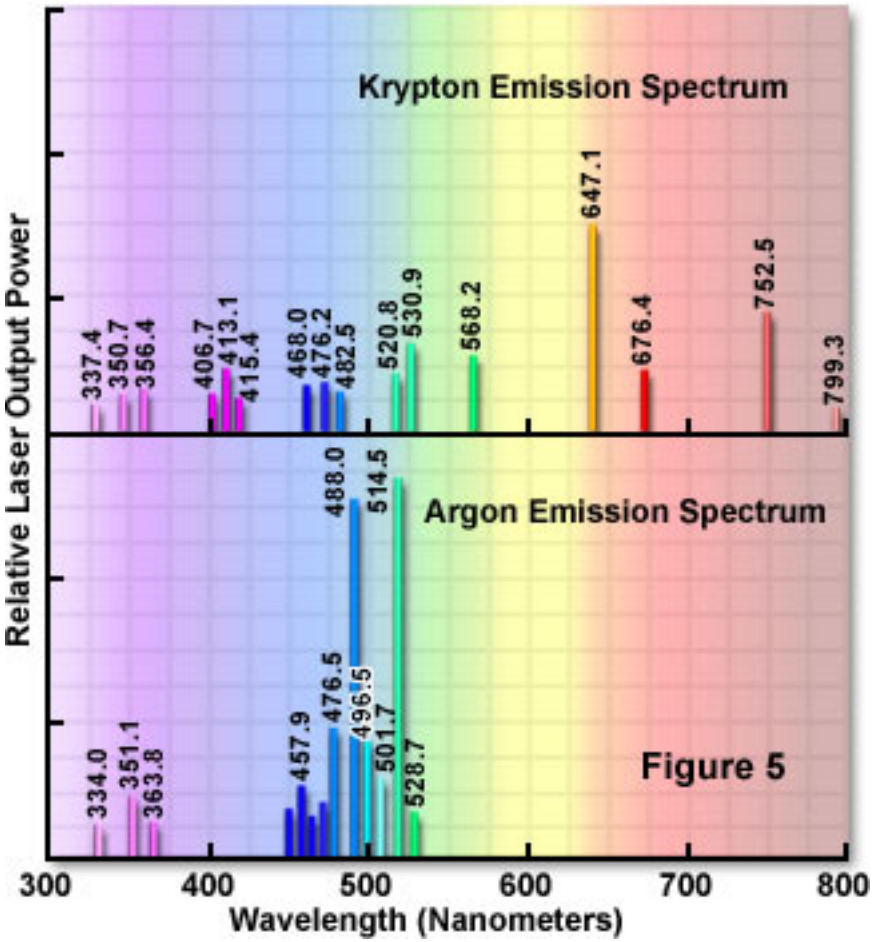


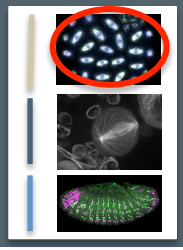
Laser Light Source



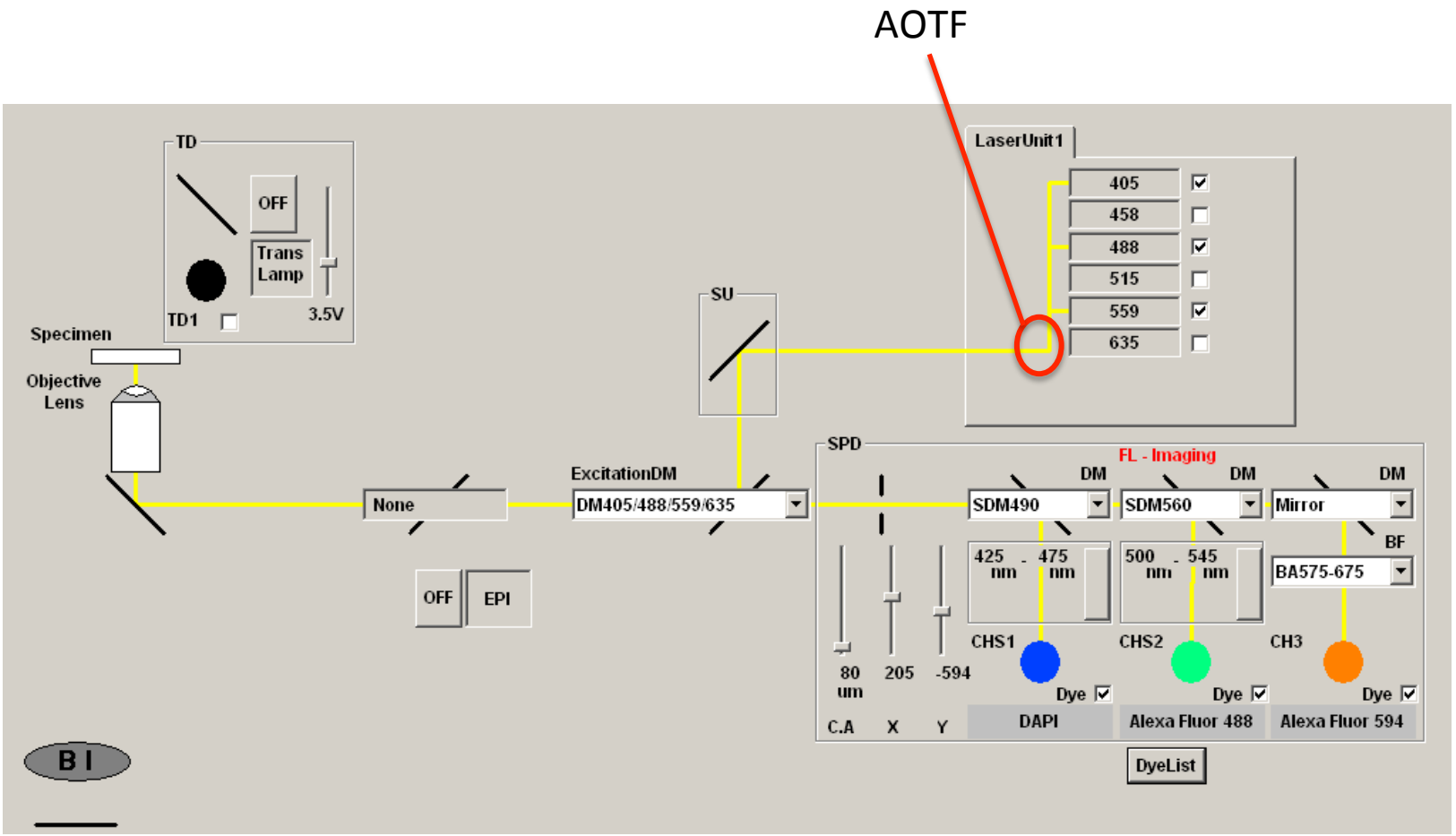
enables tighter control of fluorophores excited

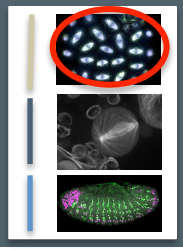
Laser Emission Spectra





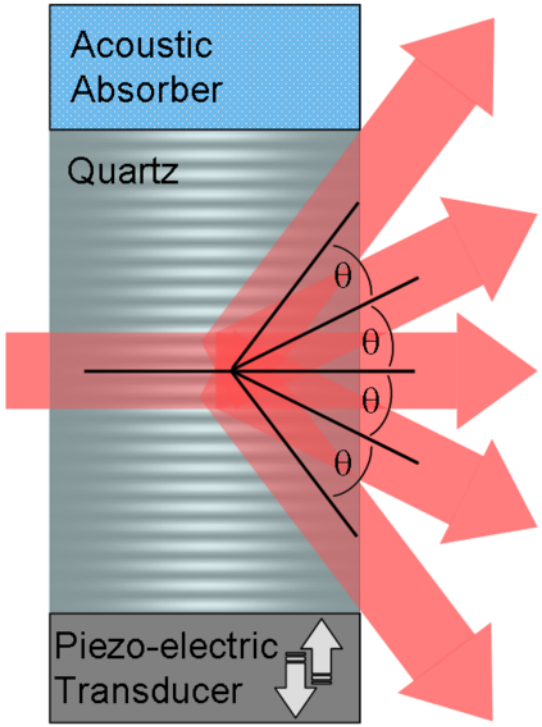
AOTF
Acousto-Optic Tunable Filter





AOTF
Acousto-Optic Tunable Filter

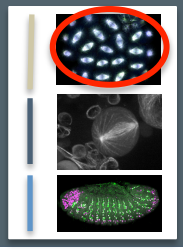
THEORY



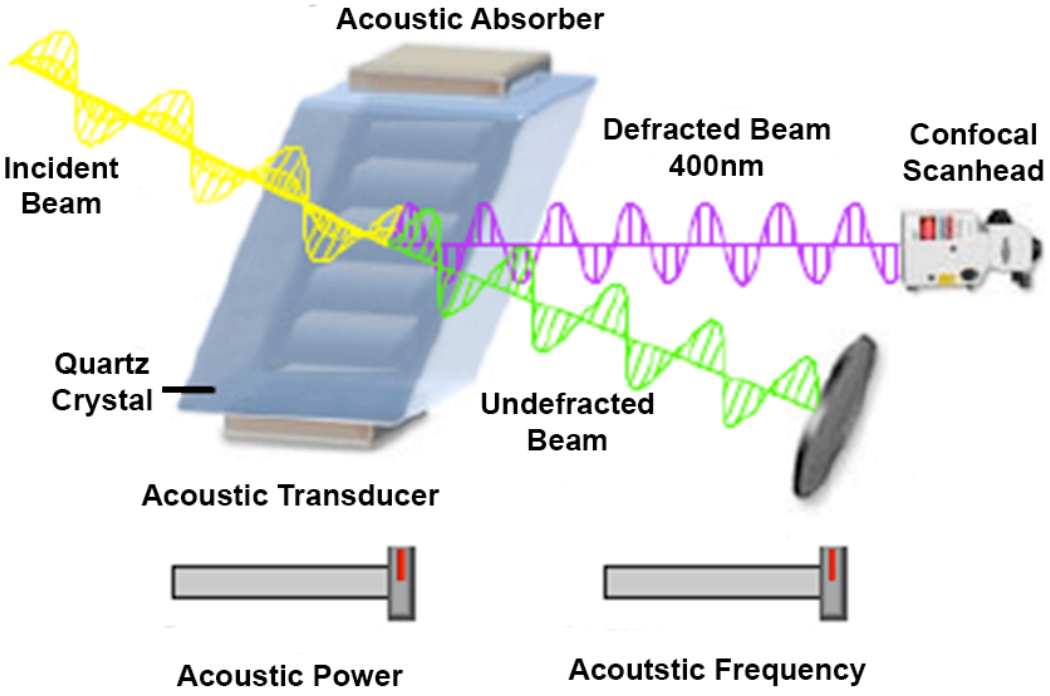
acousto-optic effect:

Acoustic wave excited within the quartz gives rise to variations in the refractive index

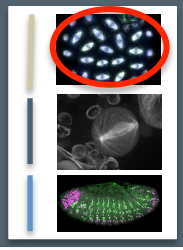
The wavelength of the diffracted light is dependent on the acoustic frequency in the quartz. By tuning the frequency of the acoustic wave, the desired wavelength of the optical wave can be diffracted acousto-optically.



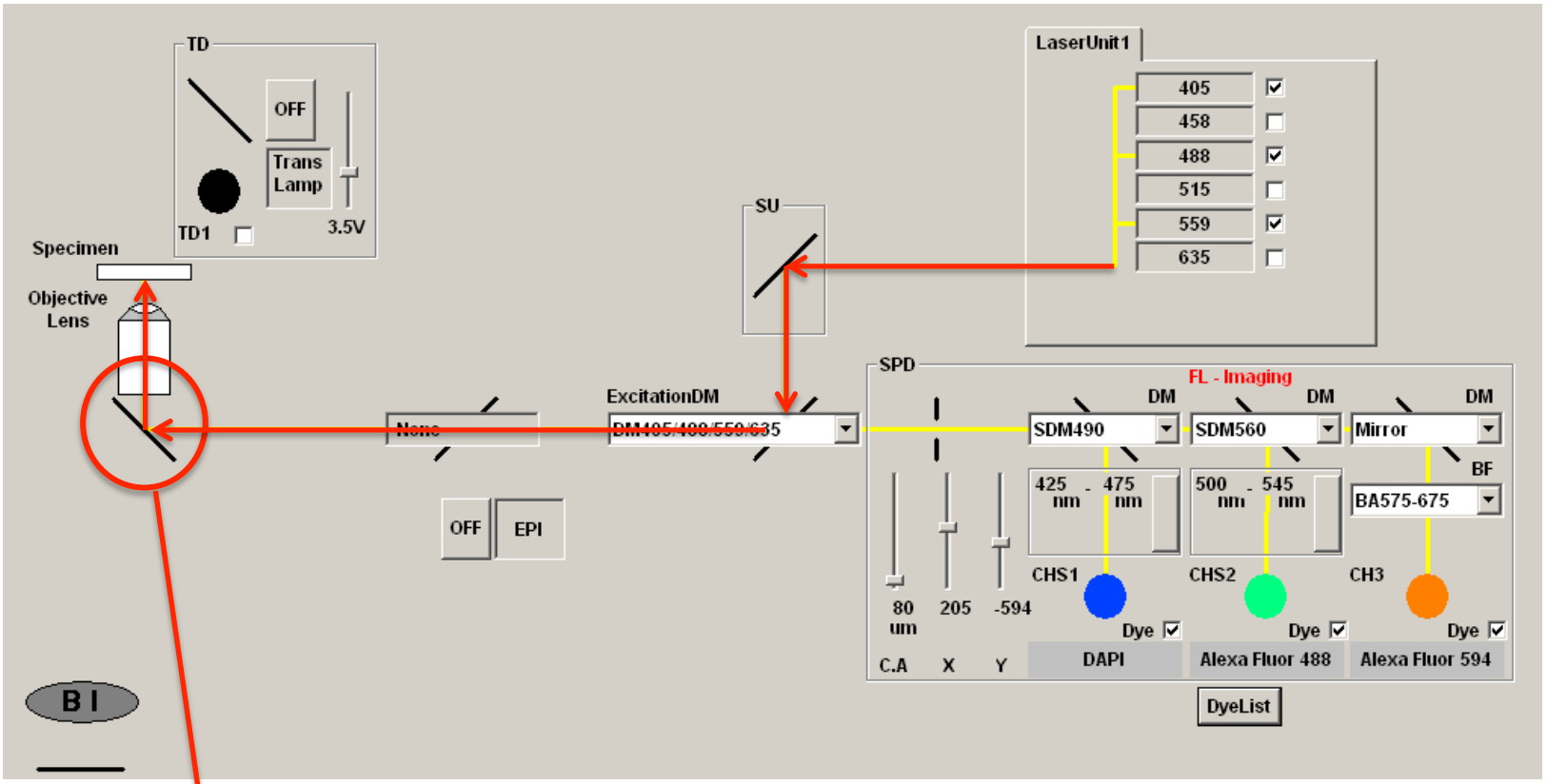
AOTF
Acousto-Optic Tunable Filter



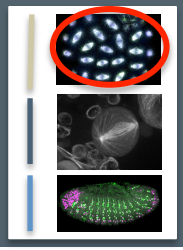
Quick On/Off of lasers
Very fast changes between excitation wavelengths



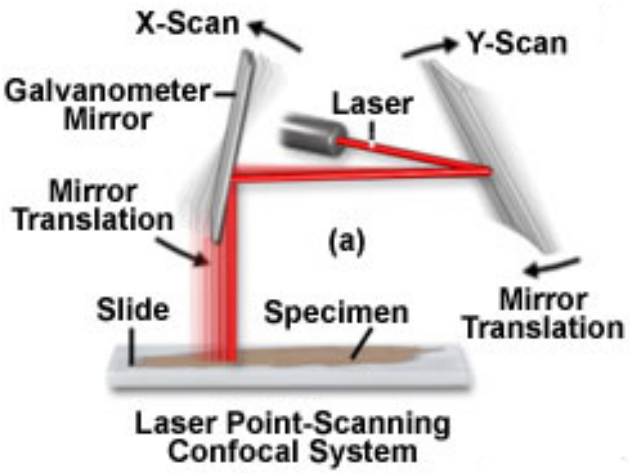
Galvo Scanning Mirrors

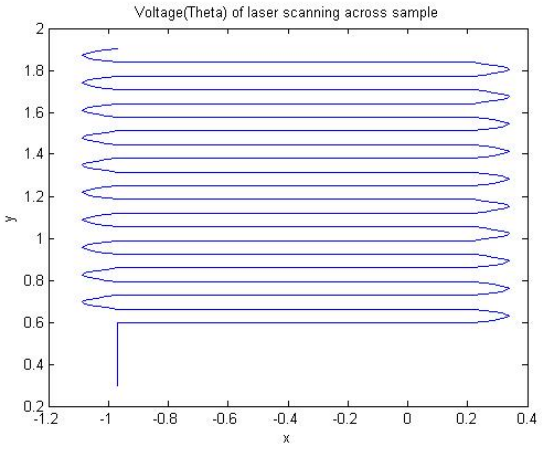


Galvo Scanning Mirrors

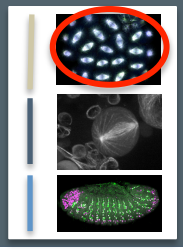


Galvo Scanning Mirrors



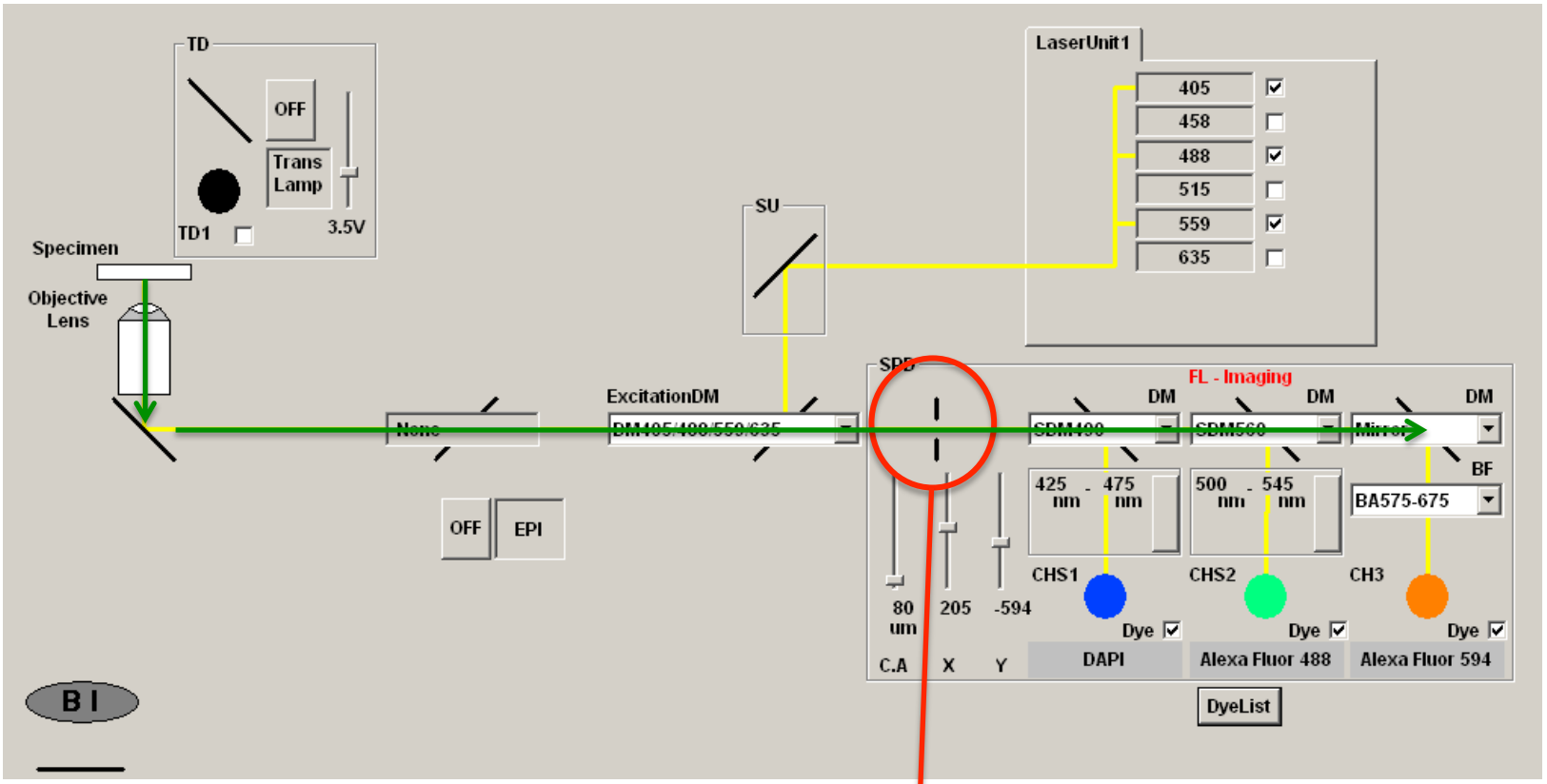


Sample excited at one point at a time
Relatively slow

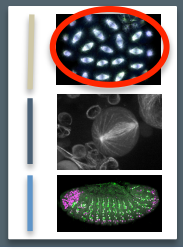


Adjustable Pinhole

AOTF



pinhole

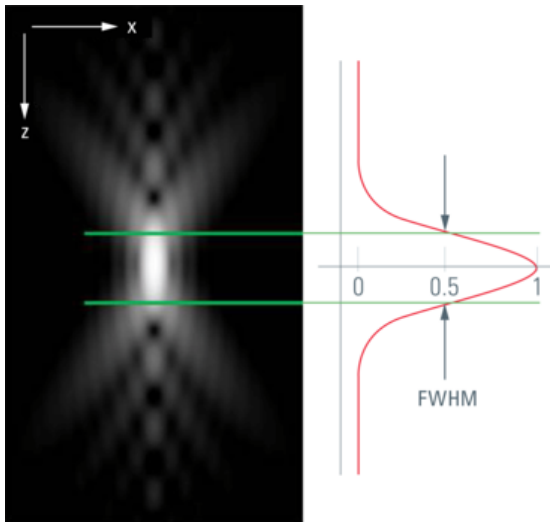


THEORY

Pinhole – Optical Sectioning

Shorter the wavelength the thinner the optical section

Diameter of the pinhole: Smaller pinhole thinner optical section

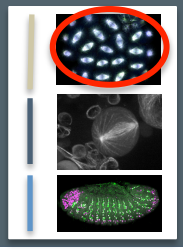


$$FWHM_{axial} = \sqrt{\left(\frac{\lambda_{exc} \cdot n}{NA^2}\right)^2 + \left(\frac{n \cdot \sqrt{2} \cdot PH}{NA}\right)^2}$$

FWHM=Full Width Half-Maximum

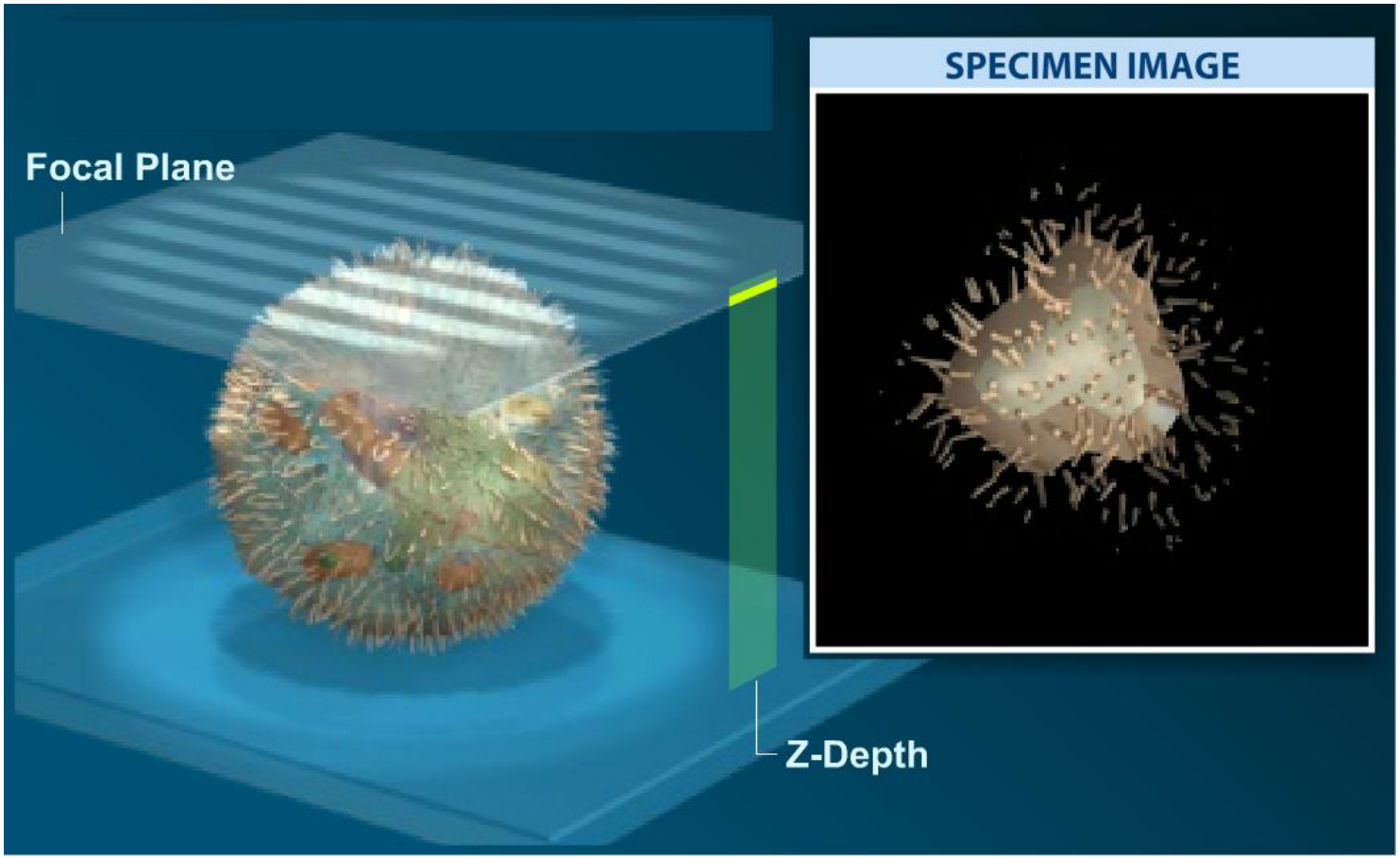
The higher the NA.
the thinner the section

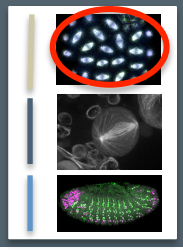
Weak signal > open pinhole > more light but thicker section



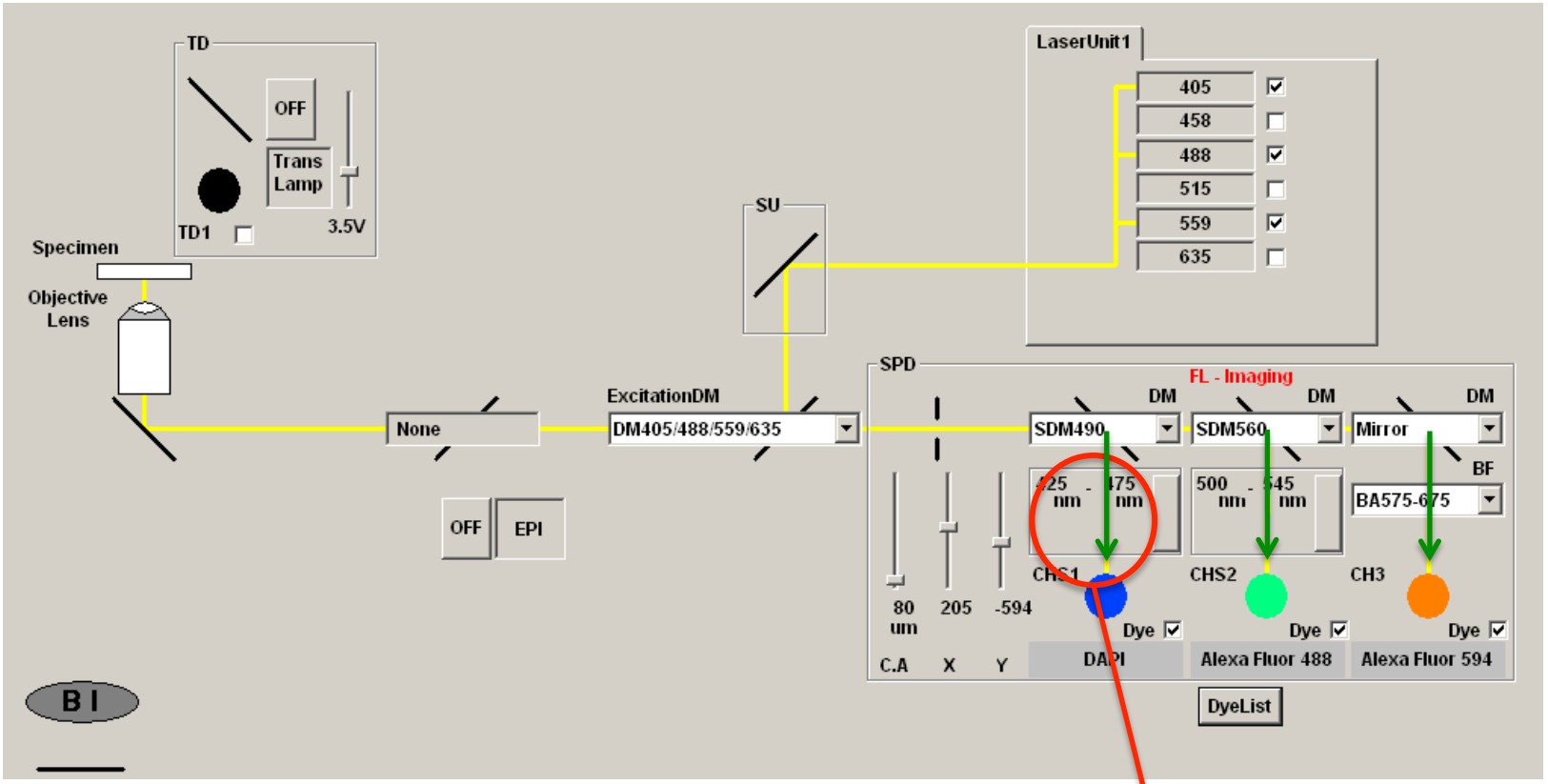
Confocal enables 3D reconstruction

Optical section

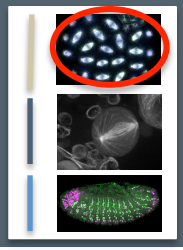




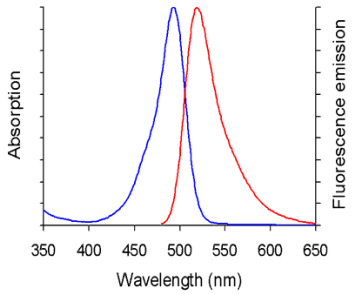
Variable Detector Slit



variable detector slit

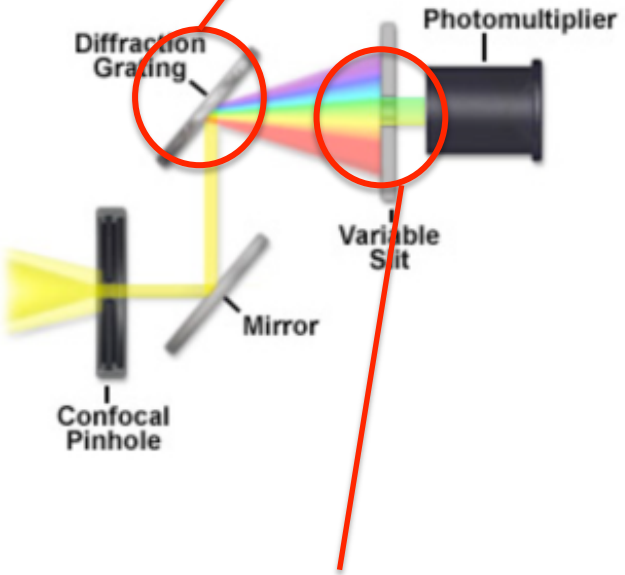


Spectral Unmixing

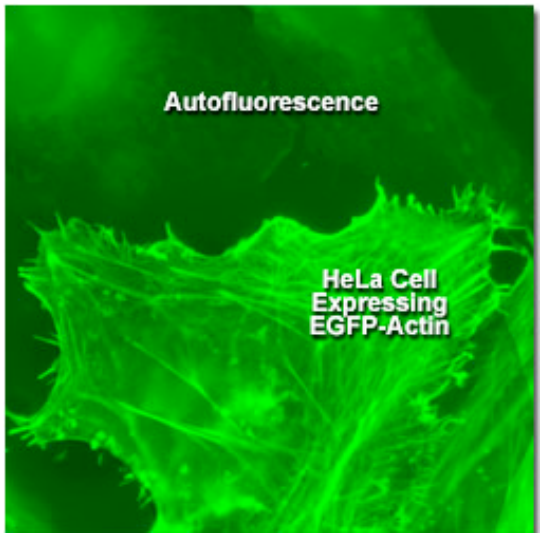


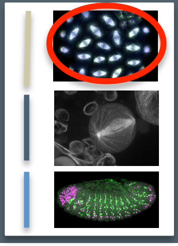
Light emitted from fluorophore as a spectrum

Defraction grating separates wavelengths over physical area

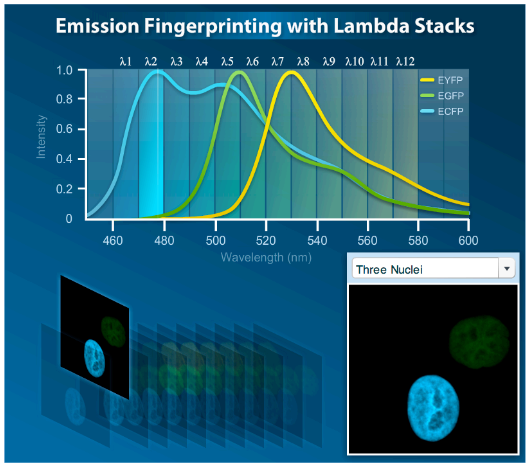


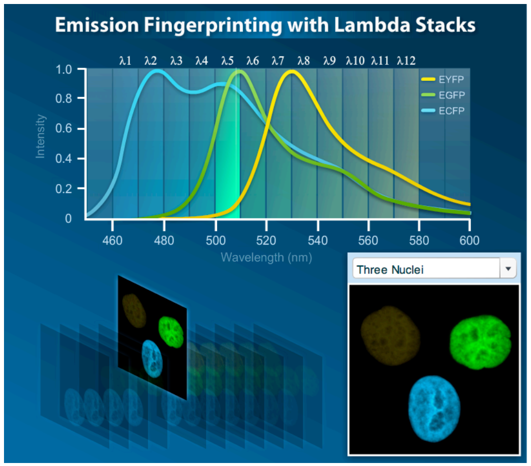
Variable slit lets through only certain wavelengths

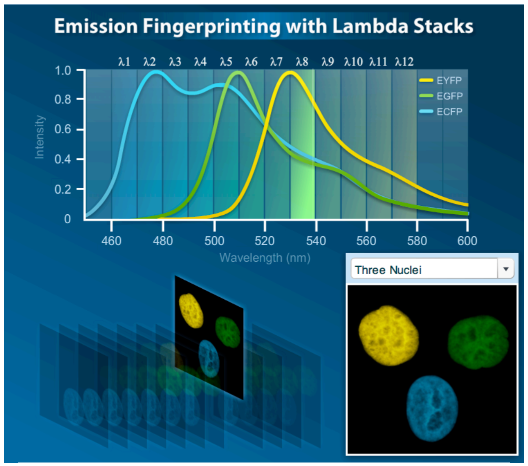


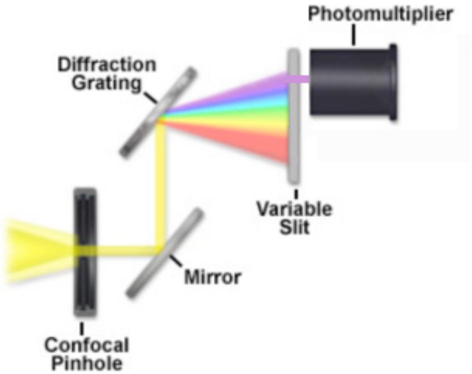


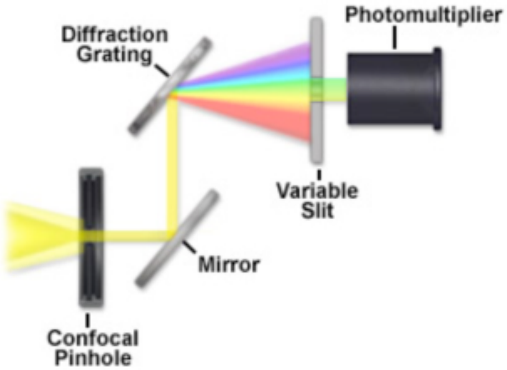
Spectral Unmixing

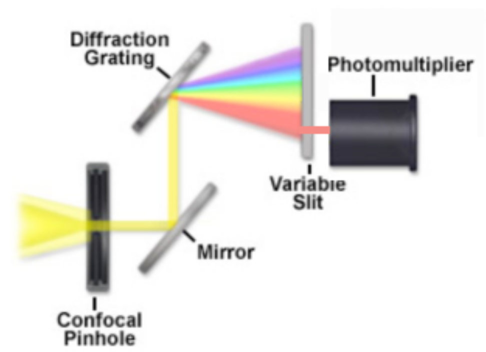


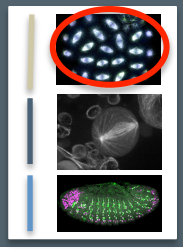




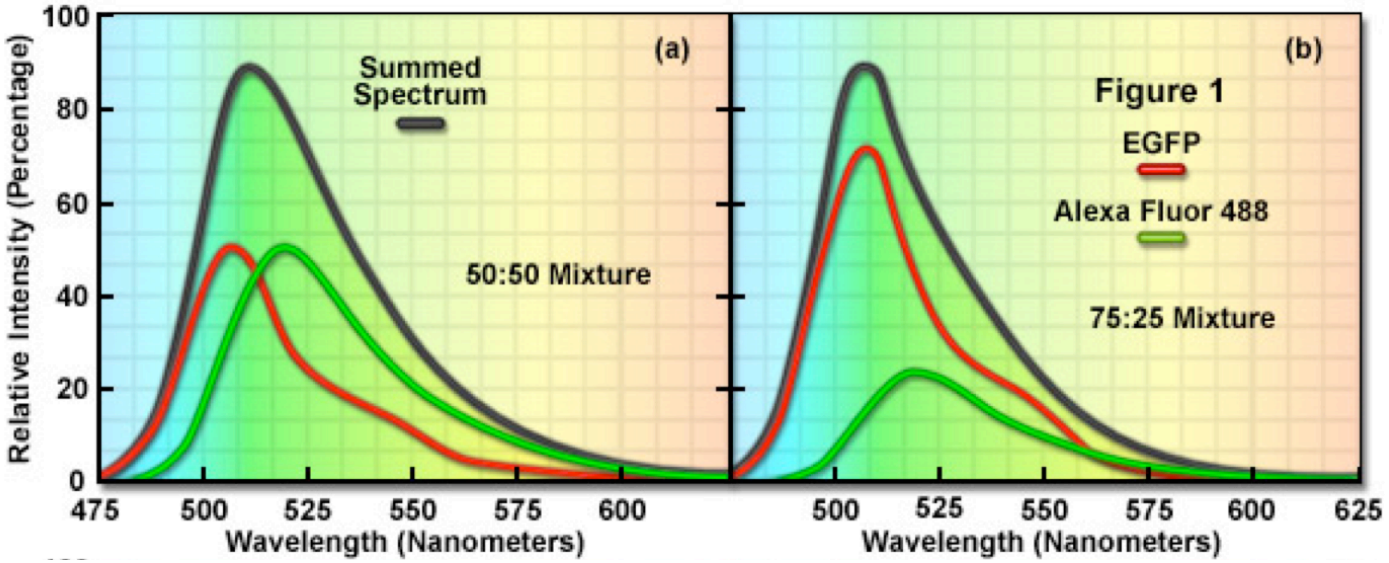






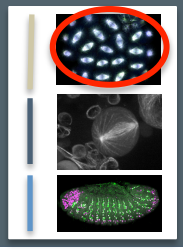


Spectral Unmixing

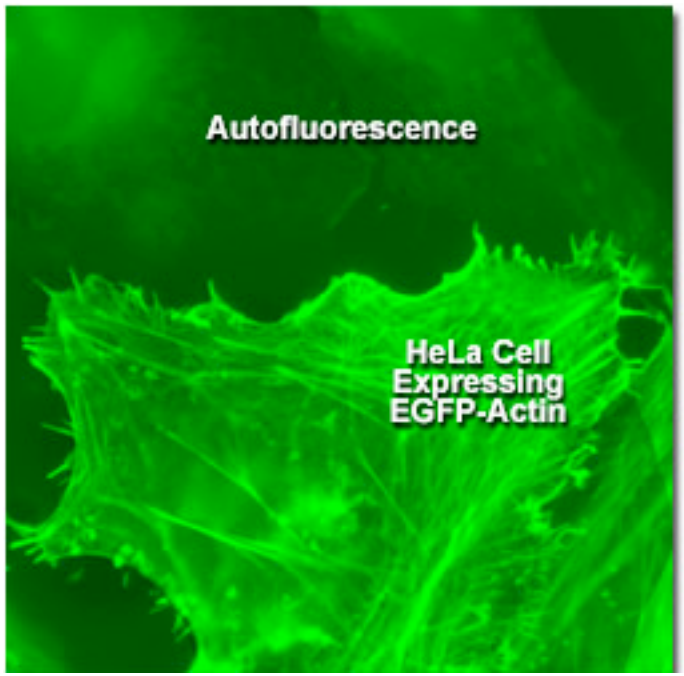


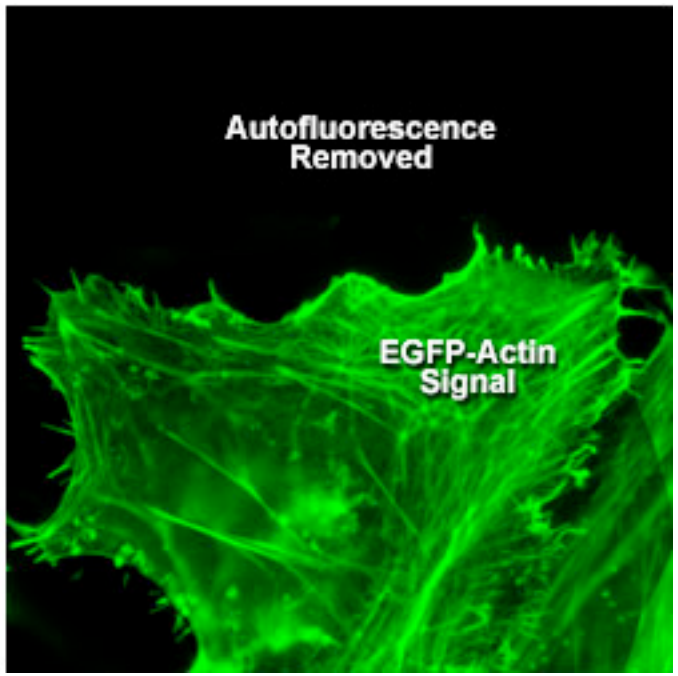
At each pixel:

Match the summed spectrum with all possible summed combinations from a library
At each pixel you therefore know the proportion of each fluorophore present



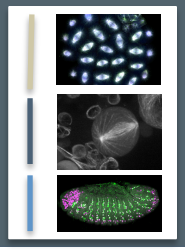
Spectral Unmixing removal of autofluorescence





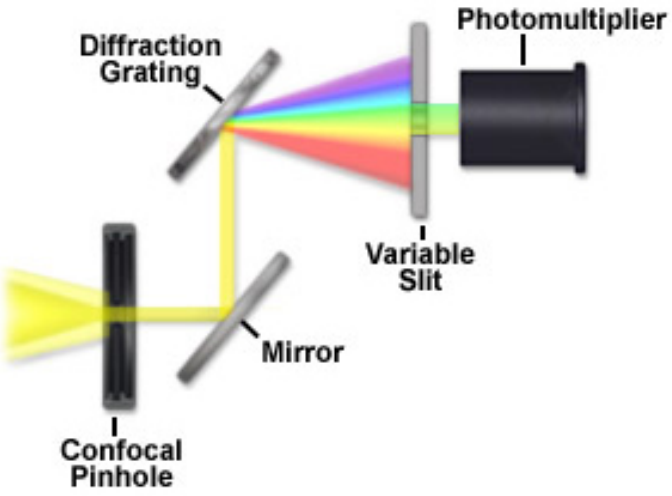
At each pixel:

Calculate the proportion of the pixel is due to autofluorescence.
Subtract the autofluorescence from the ‘true’ GFP value.

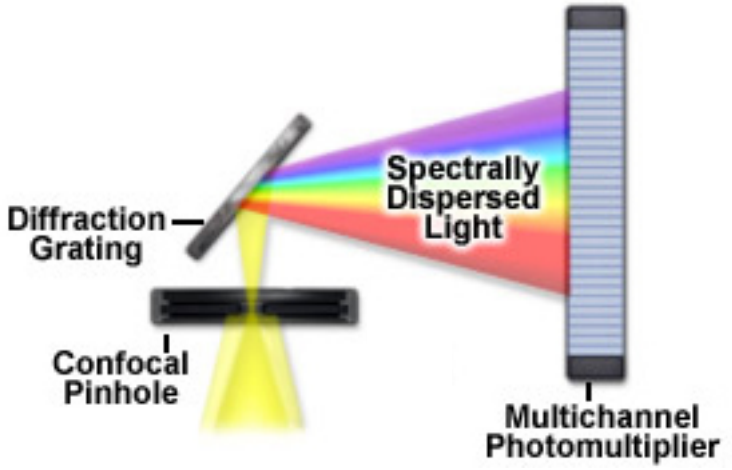


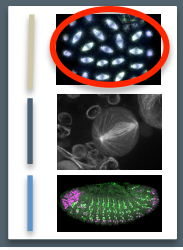
Spectral Unmixing

Olympus FV1000

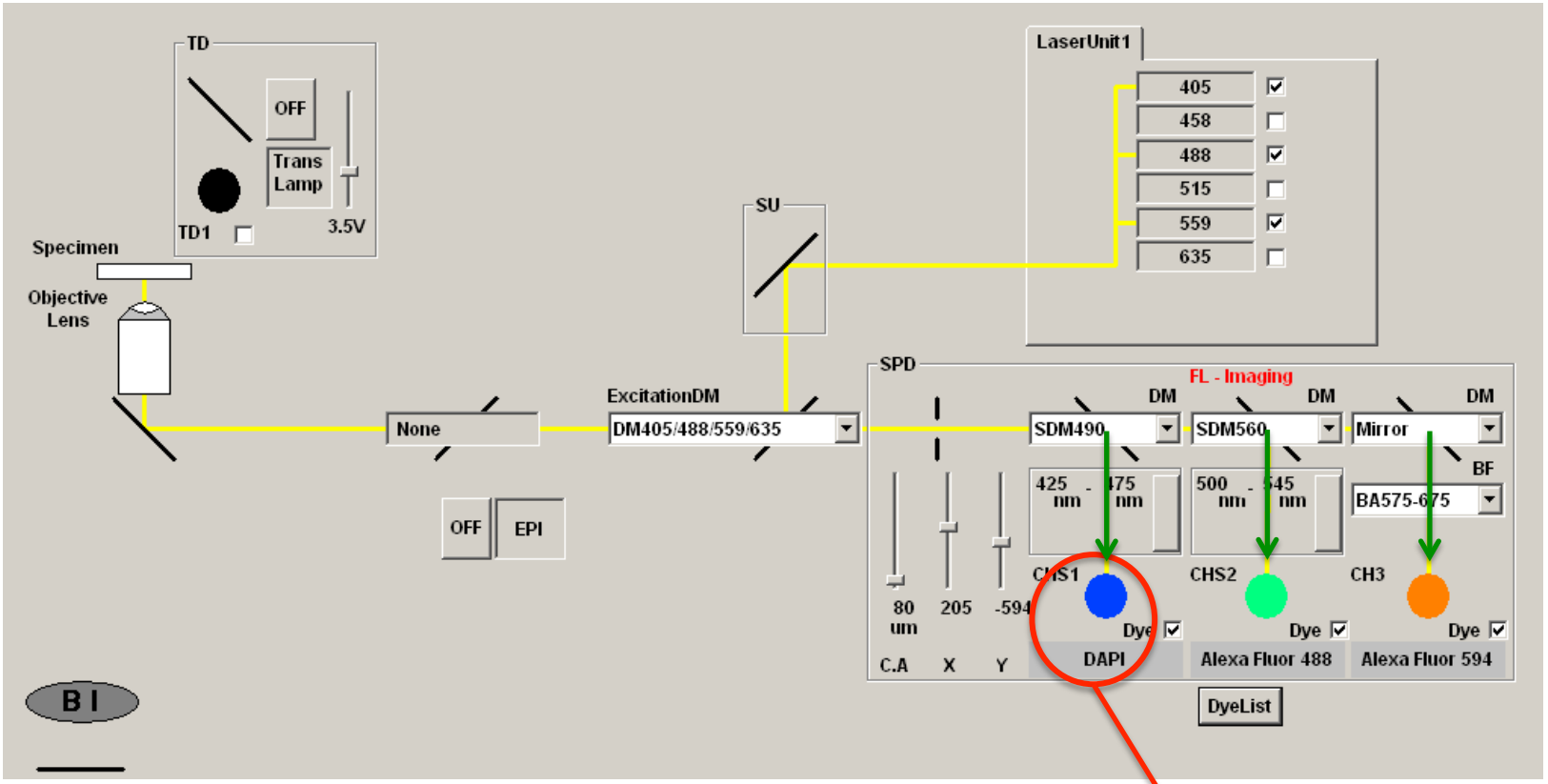


Zeiss 510 META

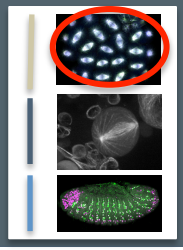




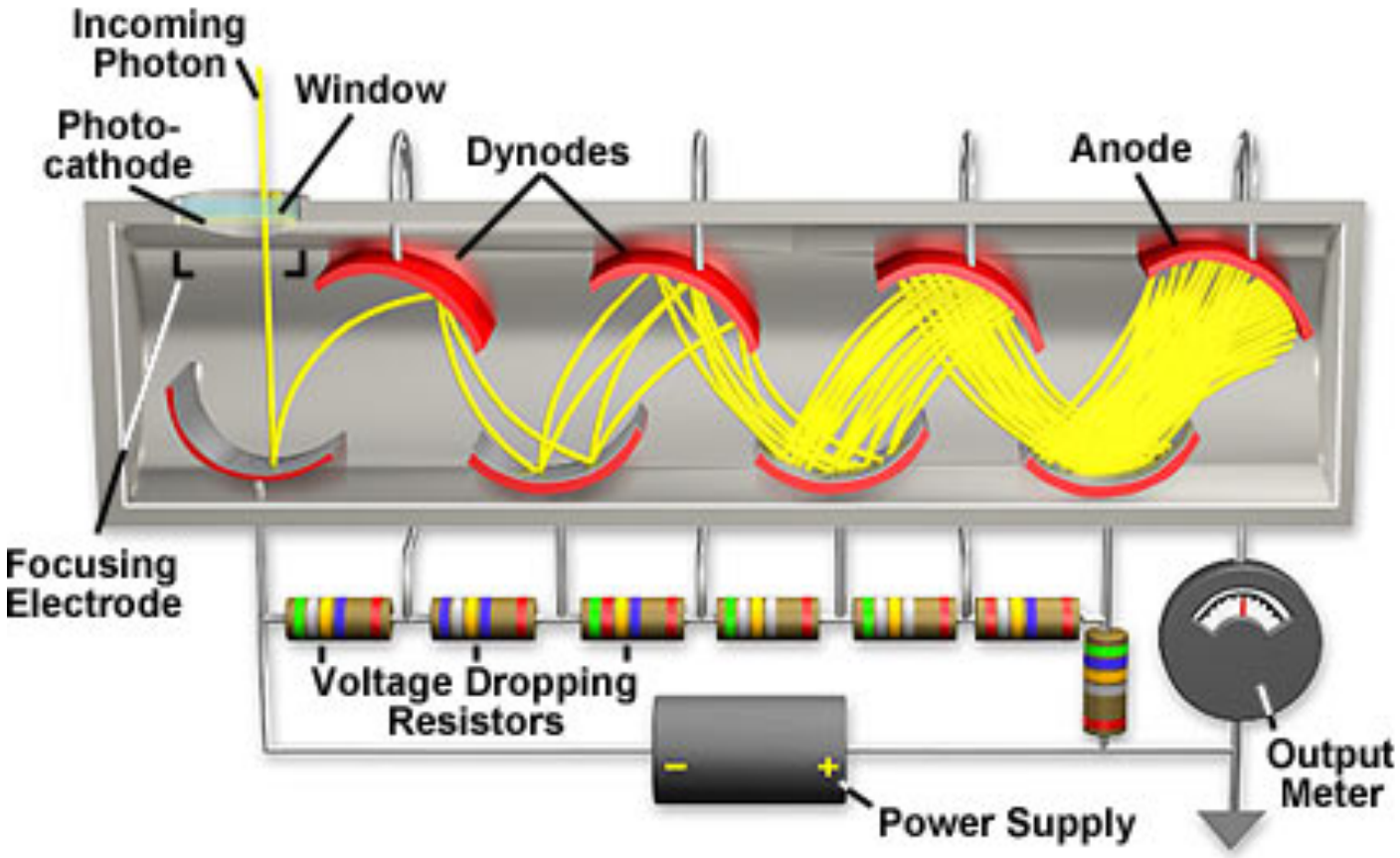
PMT – Photon Multiplier Tube



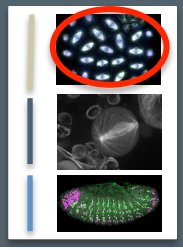
PMT detectors



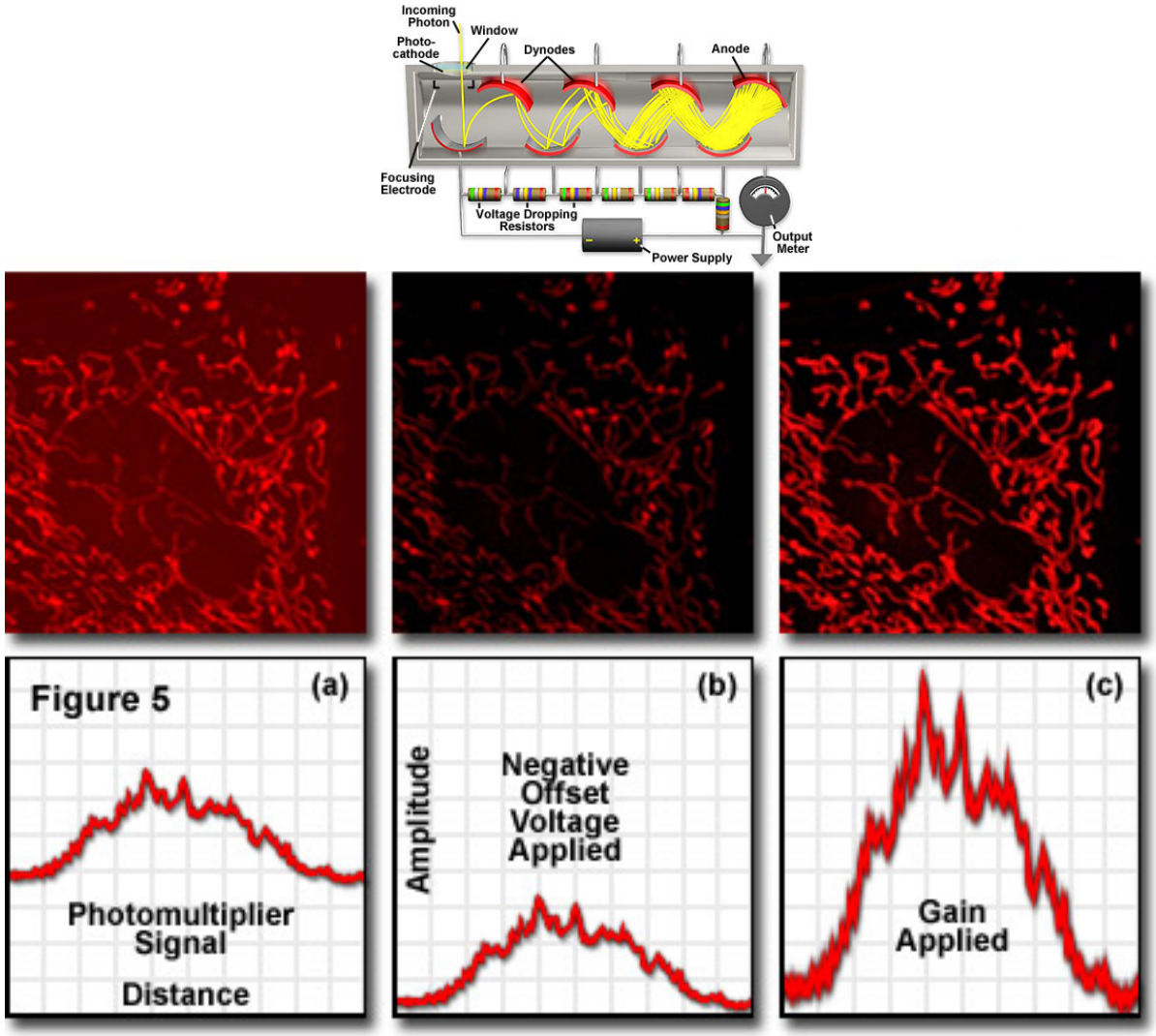
PMT – Photon Multiplier Tube



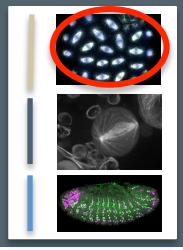
Very Low Noise
Huge Signal Amplification (~1x10)



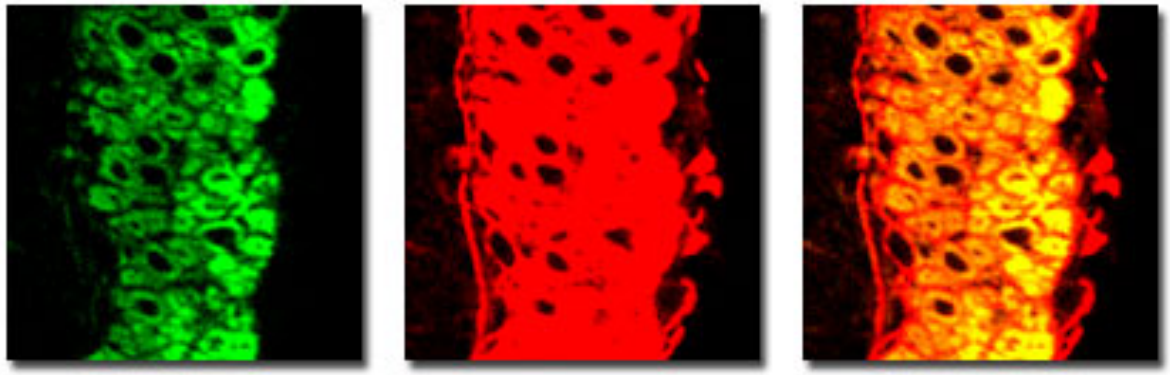
PMT – Photon Multiplier Tube Adjusting Gain and Offset



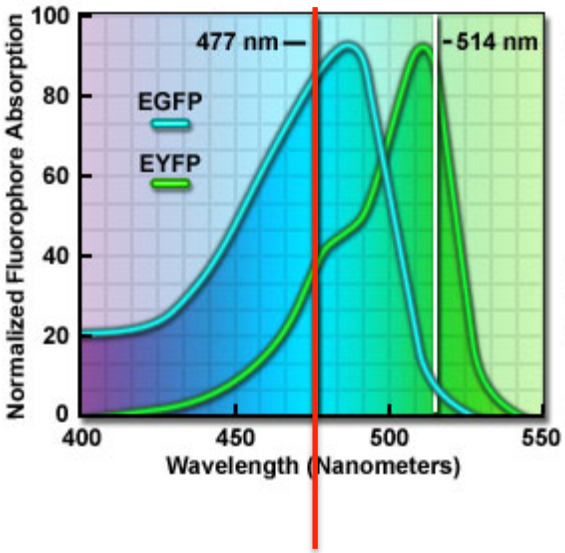
Beware - this is how your image will be saved!



‘bleed-through’

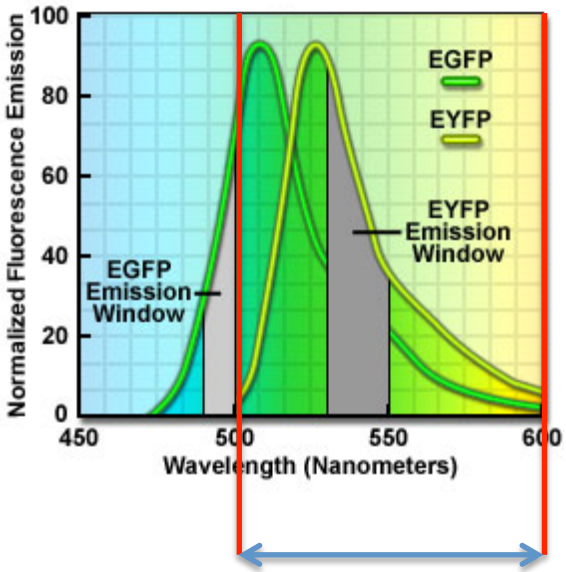


Absorption spectral profiles

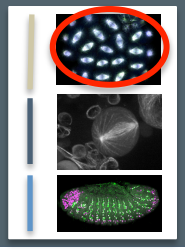


Excite at 477nm

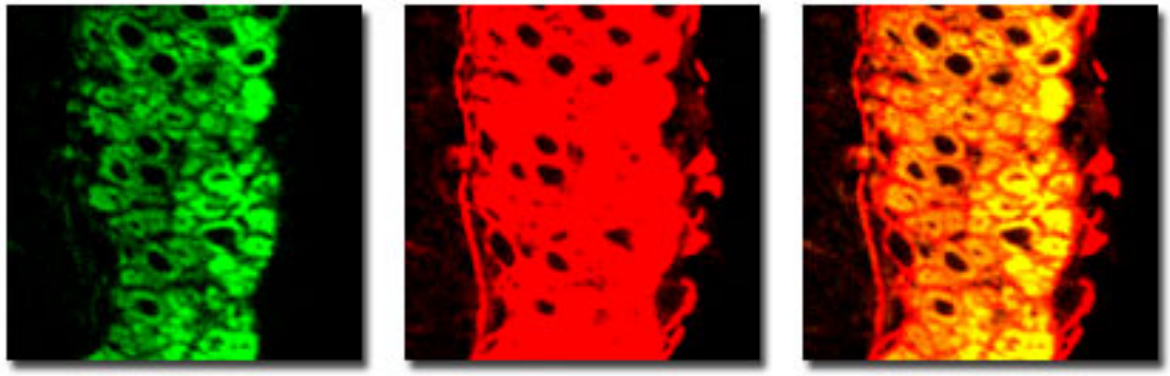
Emission spectral profiles



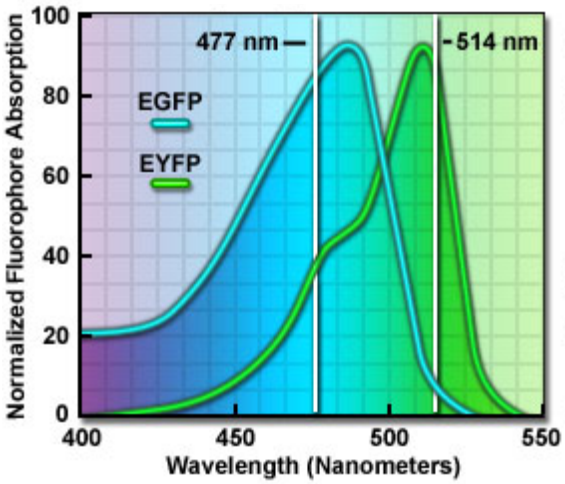
overlapping emission



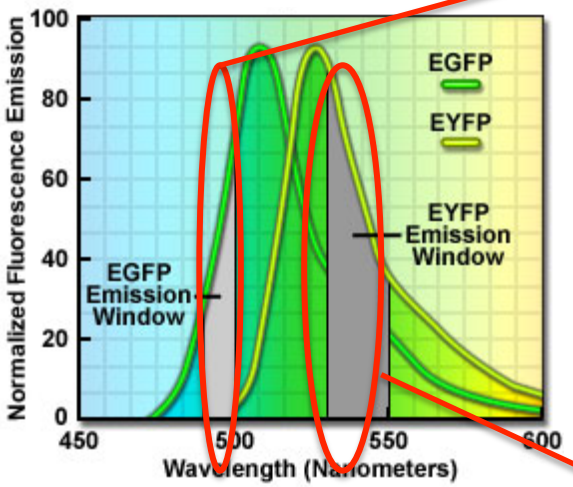
minimising‘bleed-through’ Variable Slits

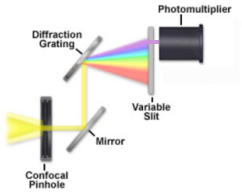


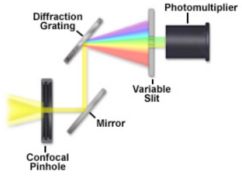
Absorption spectral profiles

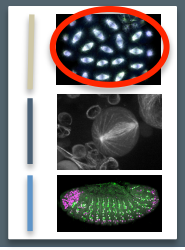


Emission spectral profiles

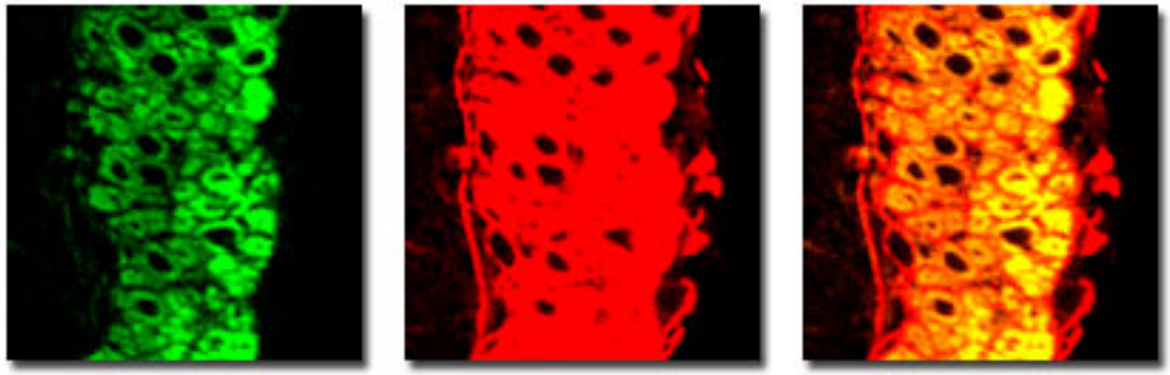




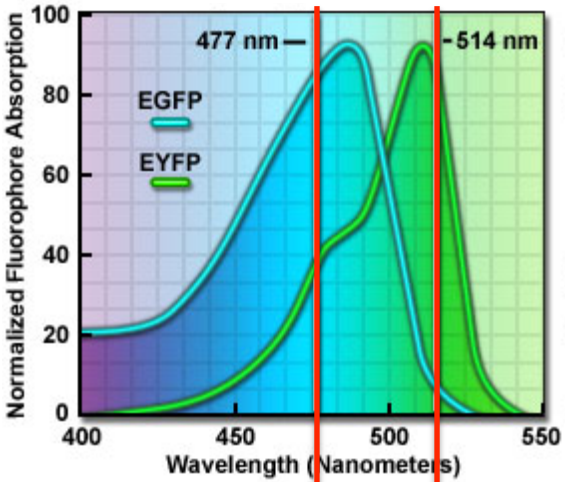




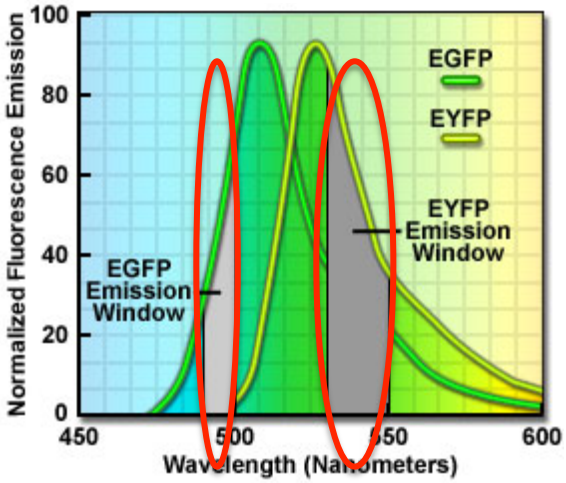
minimising‘bleed-through’ Sequential Scanning



Absorption spectral profiles



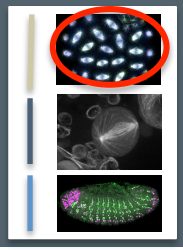
Emission spectral profiles



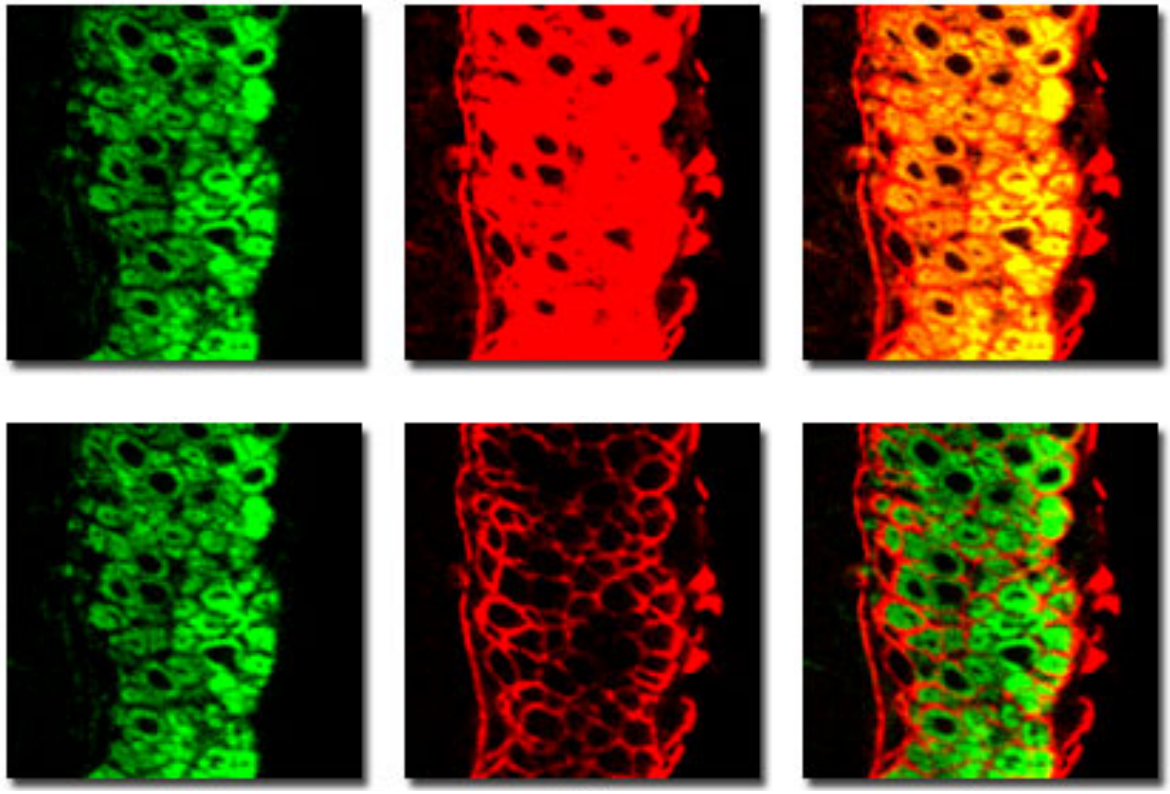
Excite at 477nm

Excite at 514nm

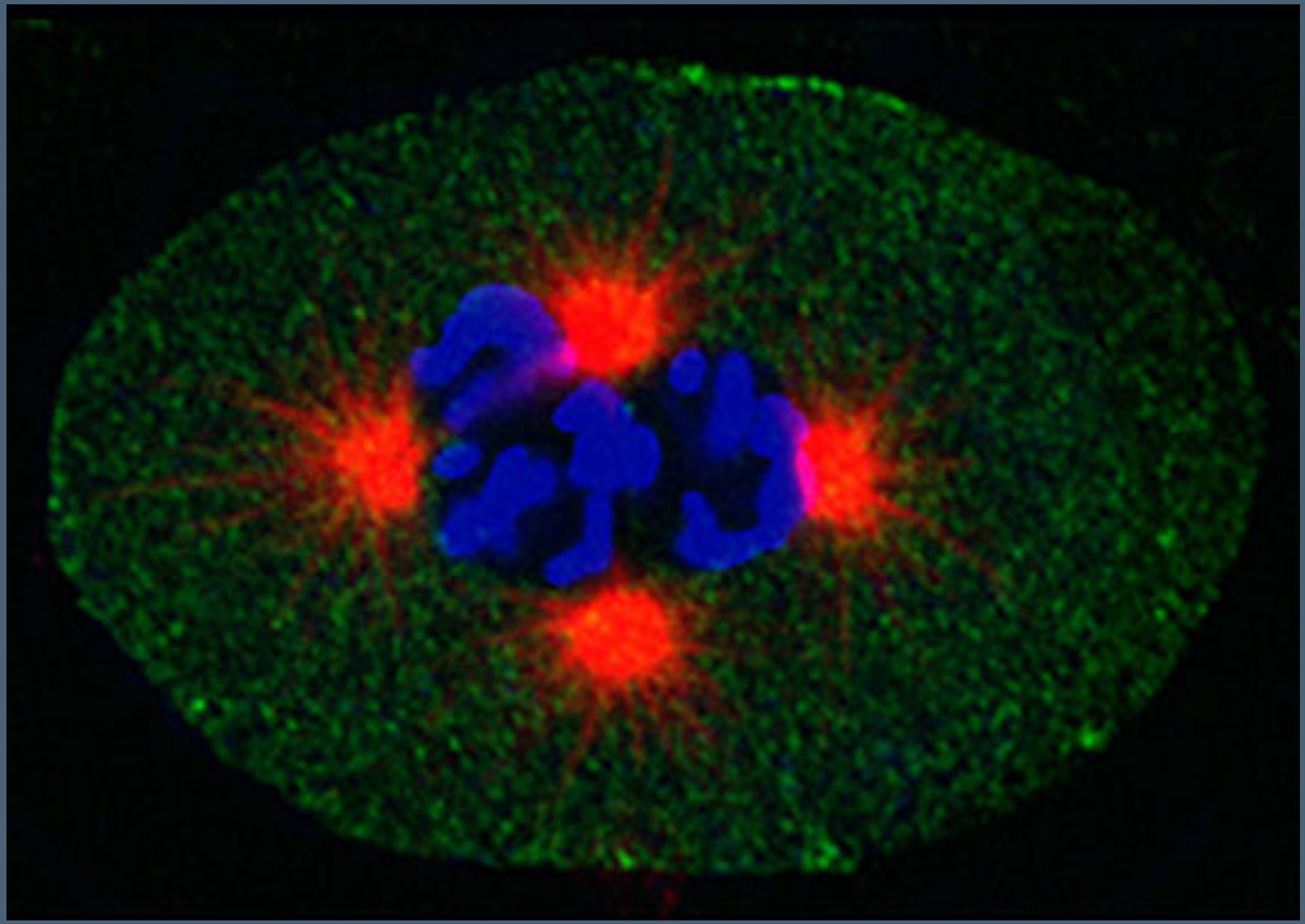
Temporal separation

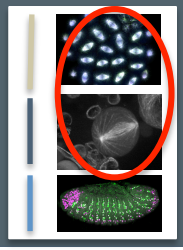


minimising ‘bleed-through’

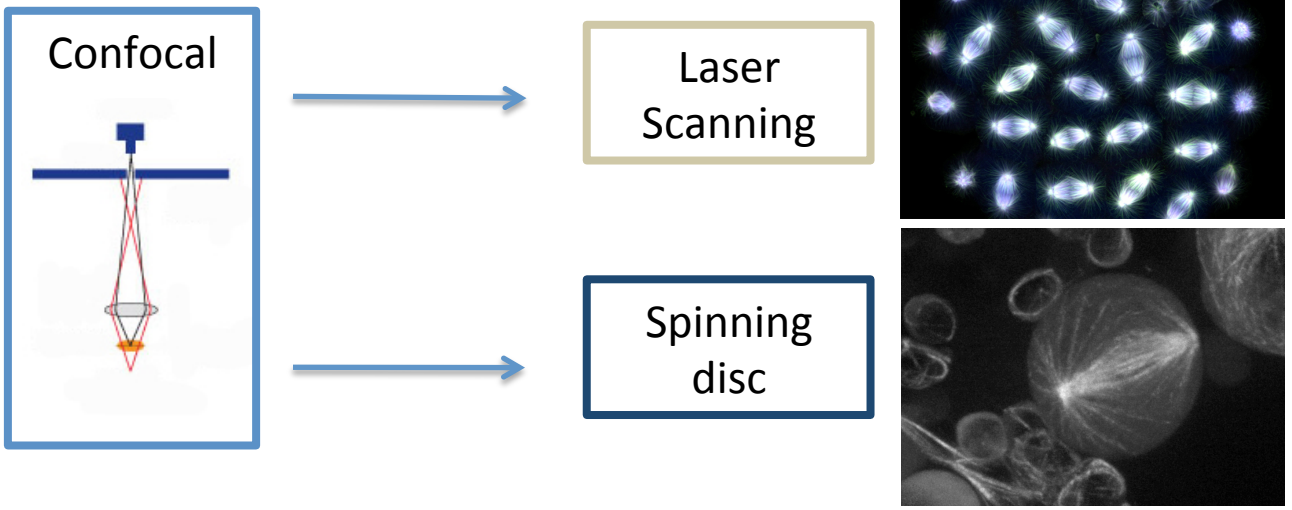


Adjust detector slit widths
Use sequential scanning



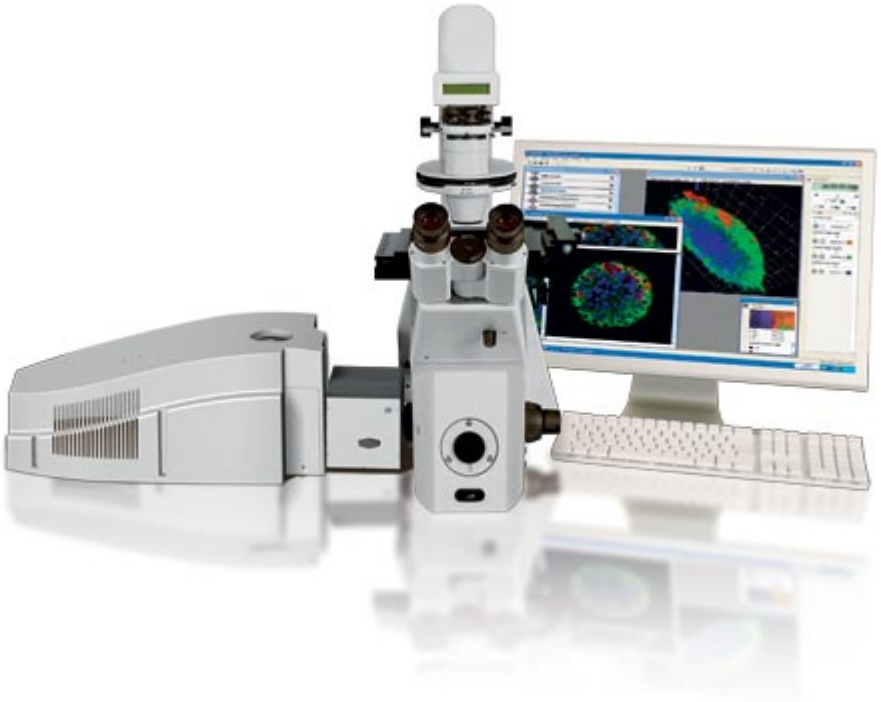


Confocal Microscopes



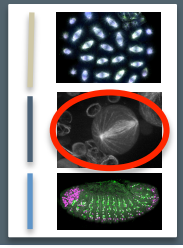
Both are confocals

Spinning Disc Confocal

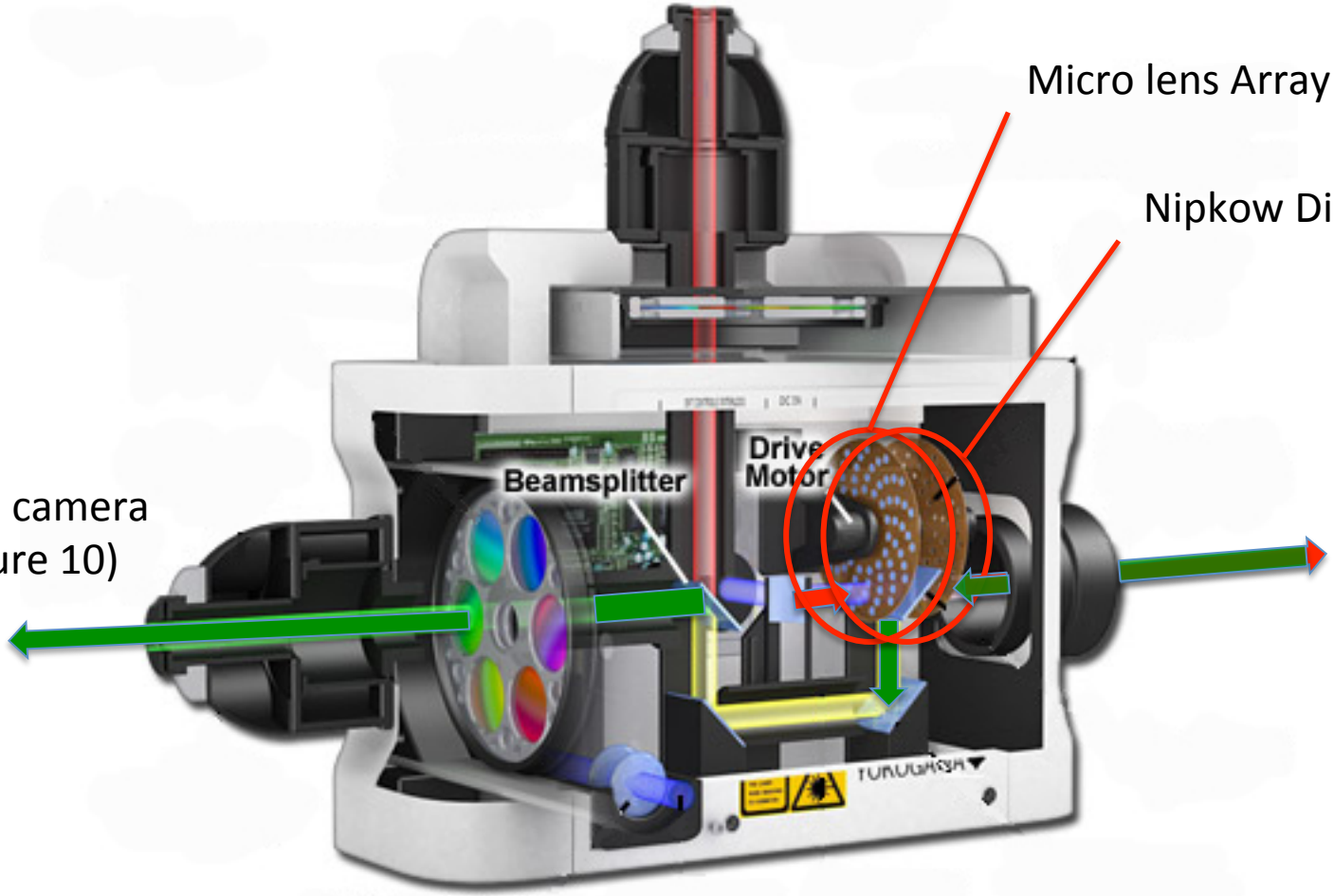


Great for live cell imaging

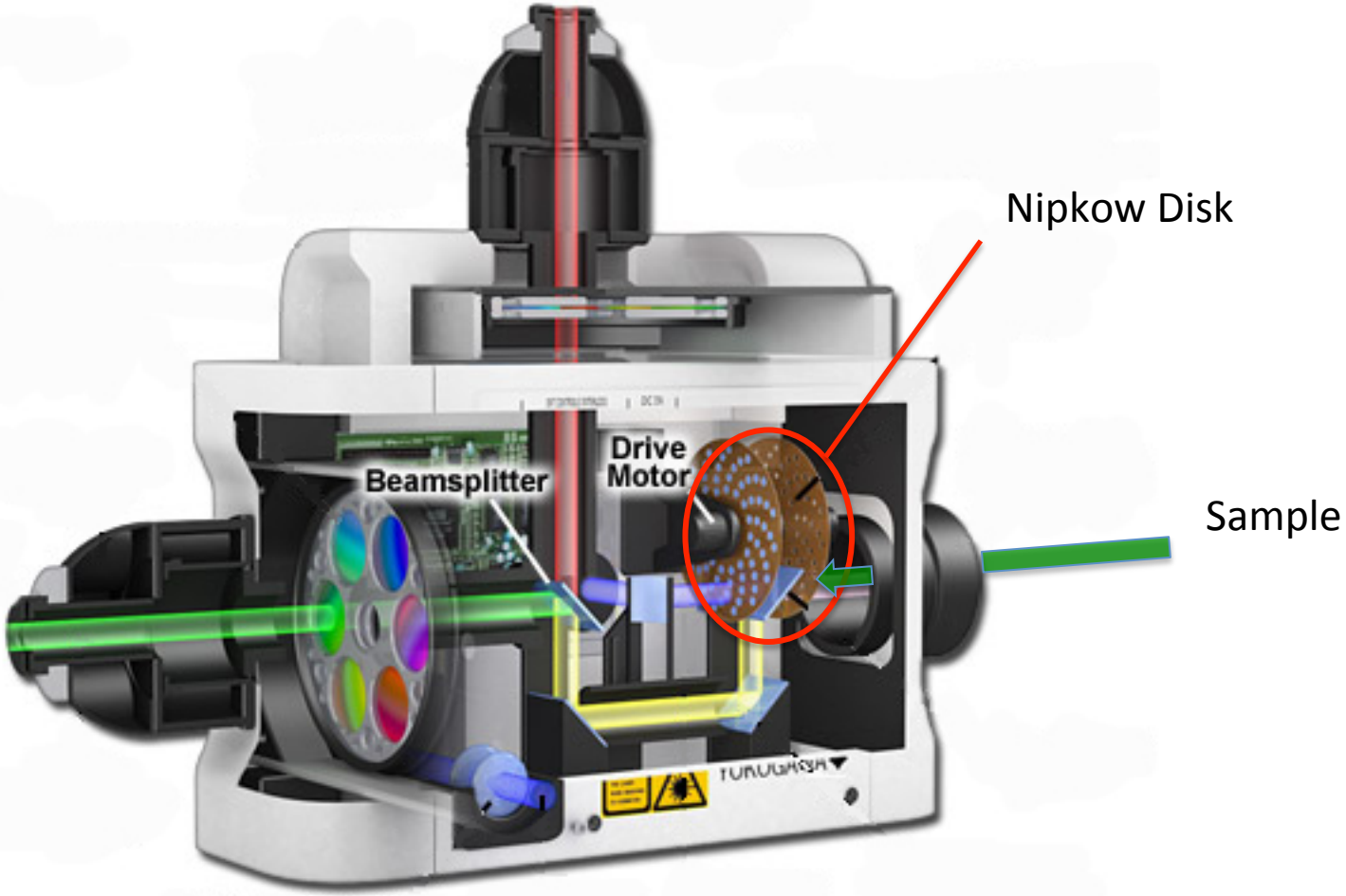
Can collect hundred images per second



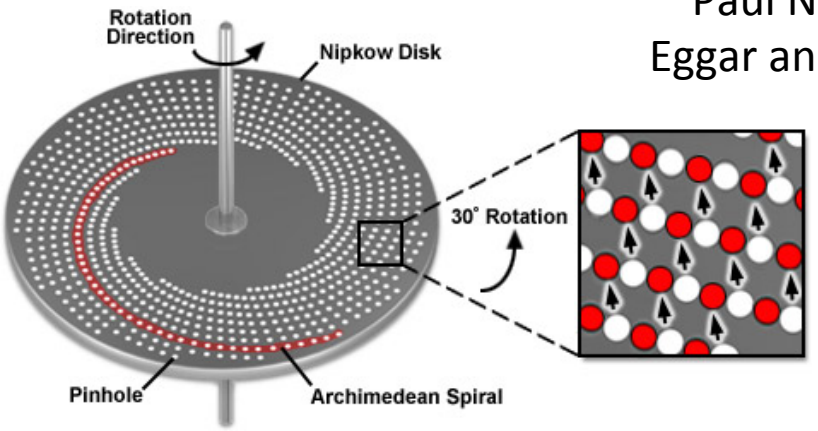
Yokogawa CSU-X1 Spinning Disc



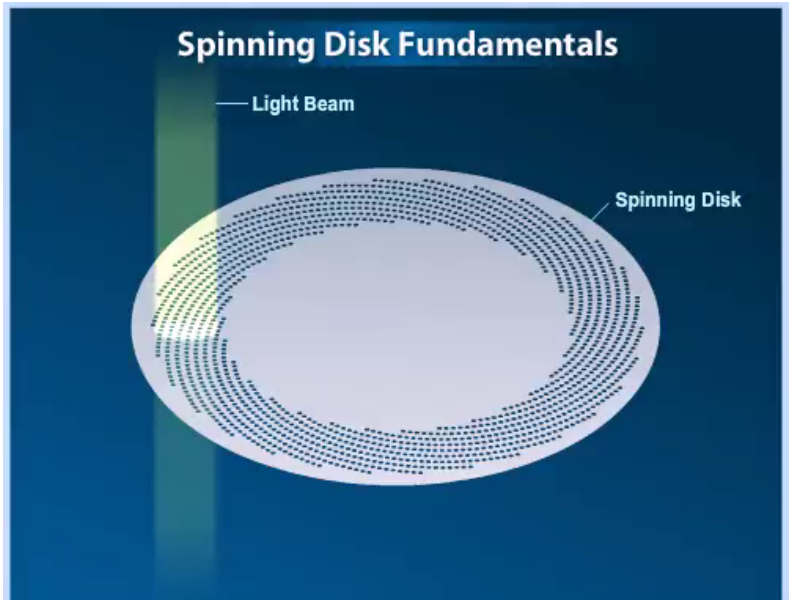
Yokogawa CSU-X1 Spinning Disc



The Nipkow Disk

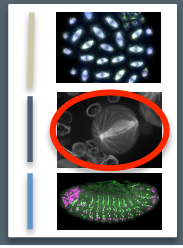


Paul Nipkow, 1884
Eggar and Petran, 1967

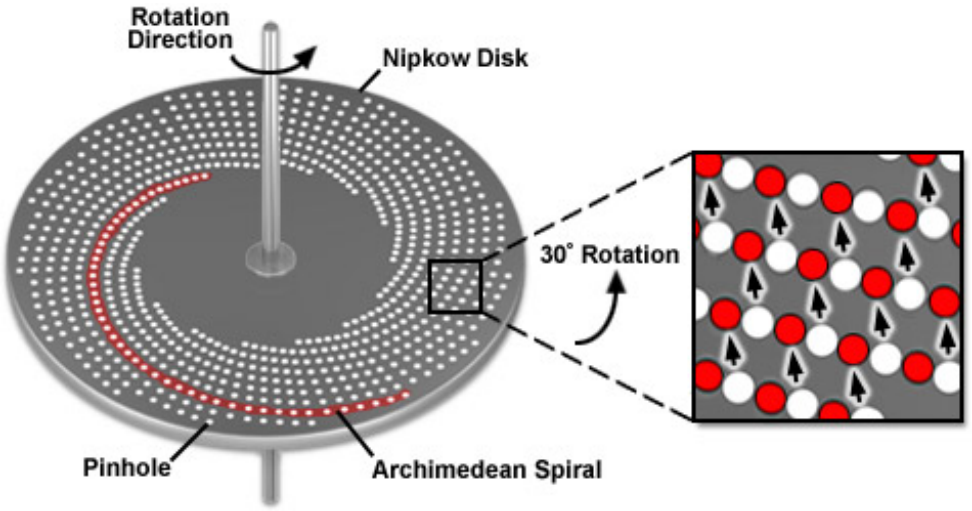


Approx. 1000 pinholes

Single frame created with each 30-degree of rotation of disc (12 frames per rotation)



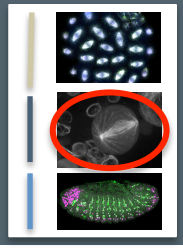
The Nipkow Disk



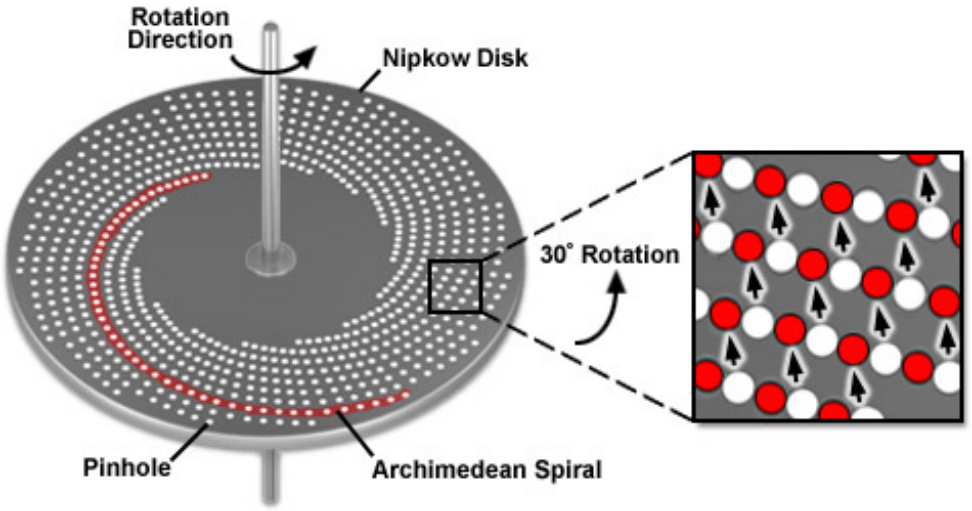
Larger pinholes - brighter image, but less “confocal”

Pinholes fixed size: Typically = 50um

(optimised for biology)

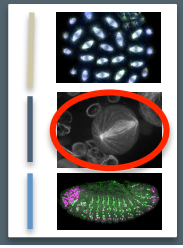


The Nipkow Disk

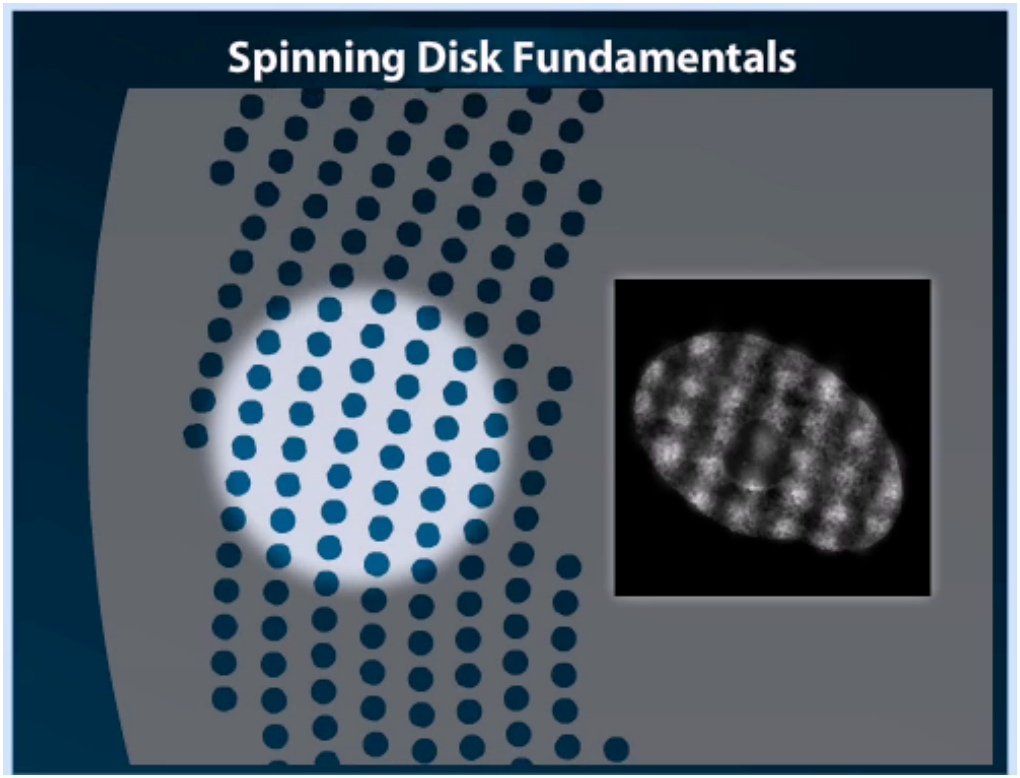


Constant Battle:
Smaller spacing - more light gets through, but “crosstalk”

Pinhole Spacing Typically = 2.5um apart



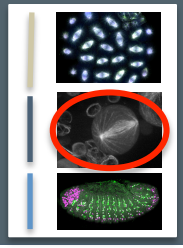
Spinning Disc Confocal



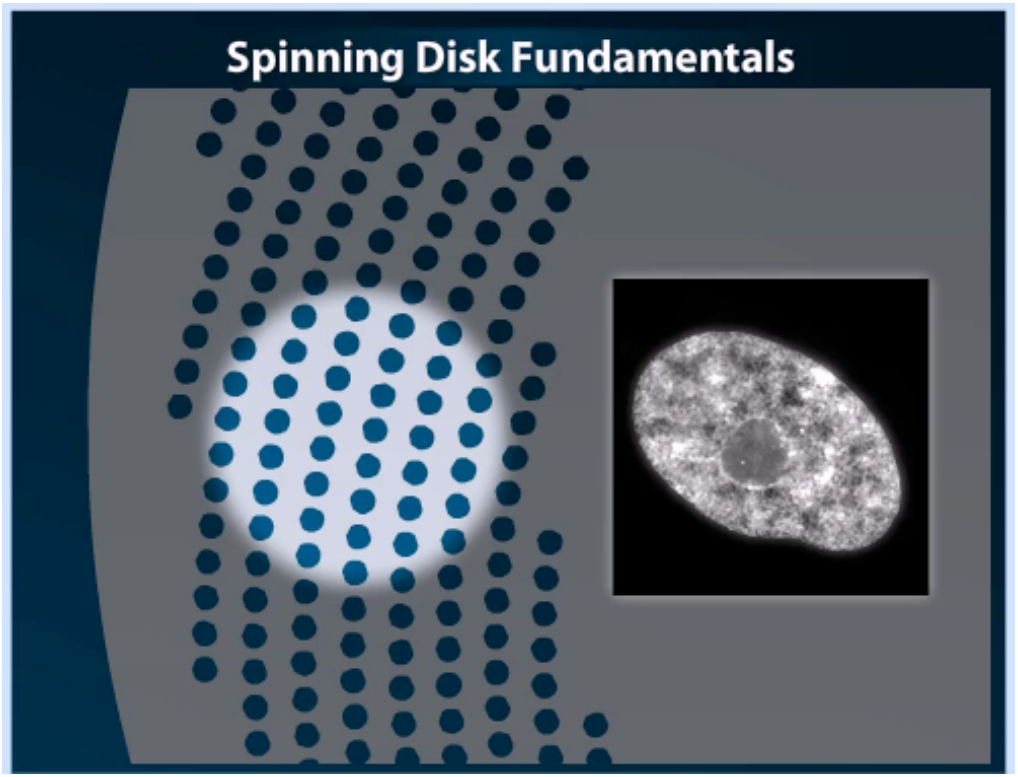
Disc spins to illuminate sample

spin speed – disc spinning too slowly

you see the pinholes on the sample

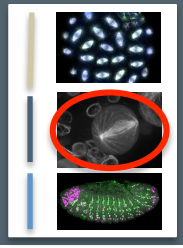


Spinning Disc Confocal

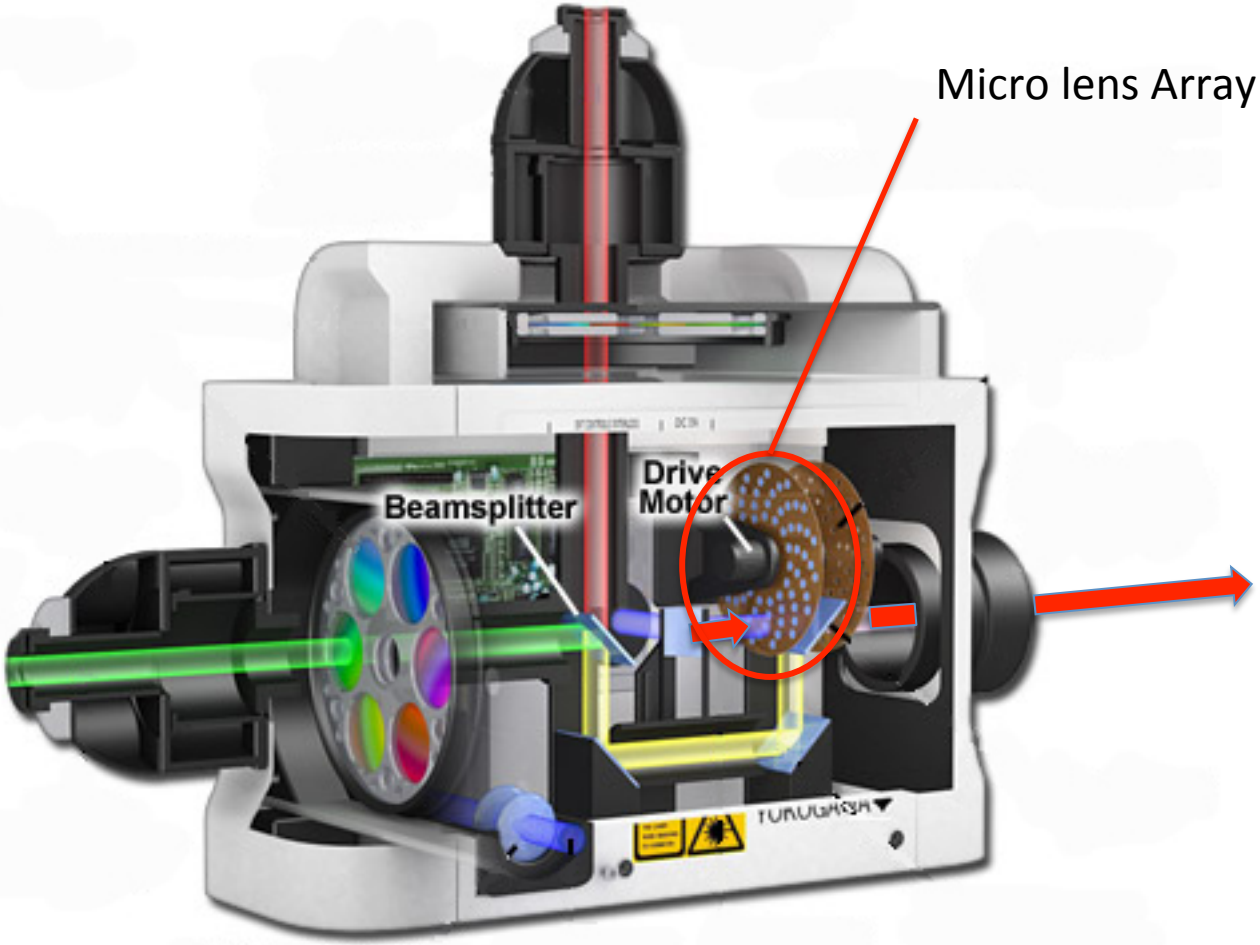


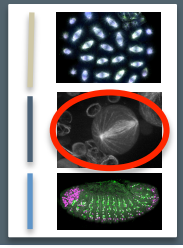
Mismatches between disk rotation speed and camera integration periods are generally not a problem for long exposure times (greater than 100 milliseconds)

Disc speed must carefully match camera frame rate

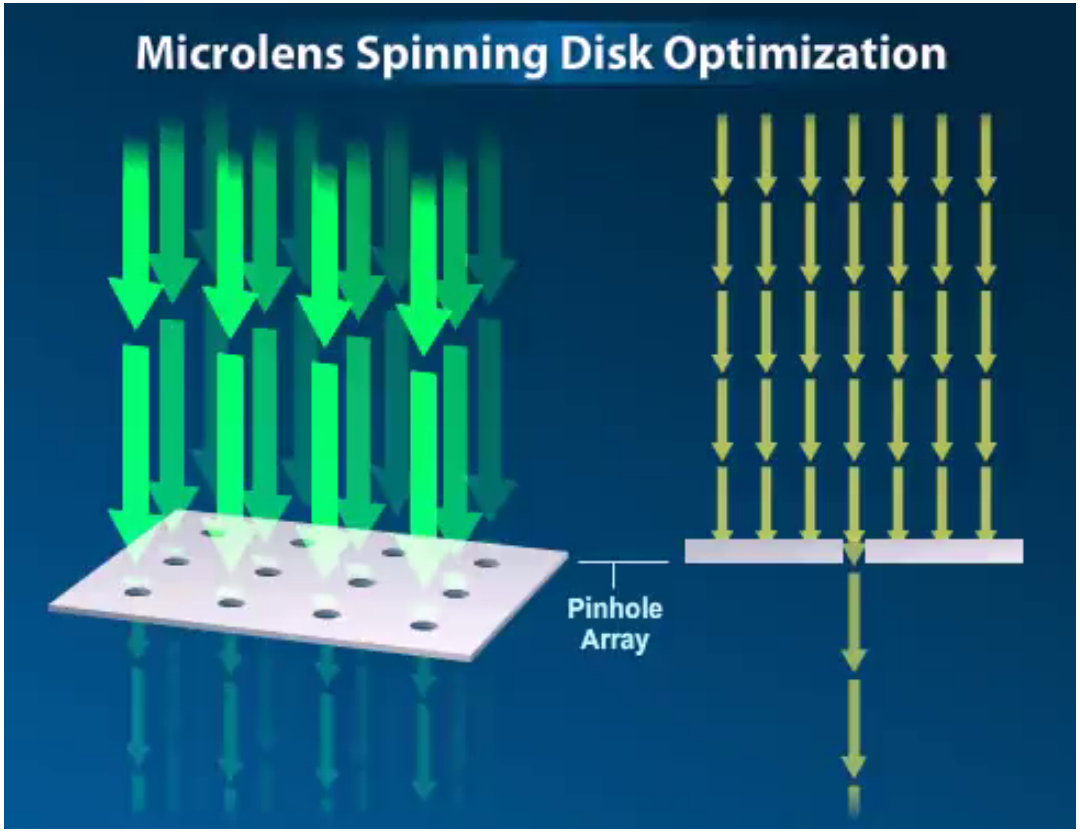


Yokogawa CSU-X1 Spinning Disc





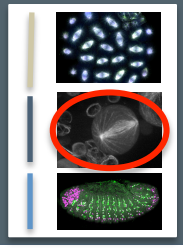
Yokogawa Spinning Disc Confocal Microlenses



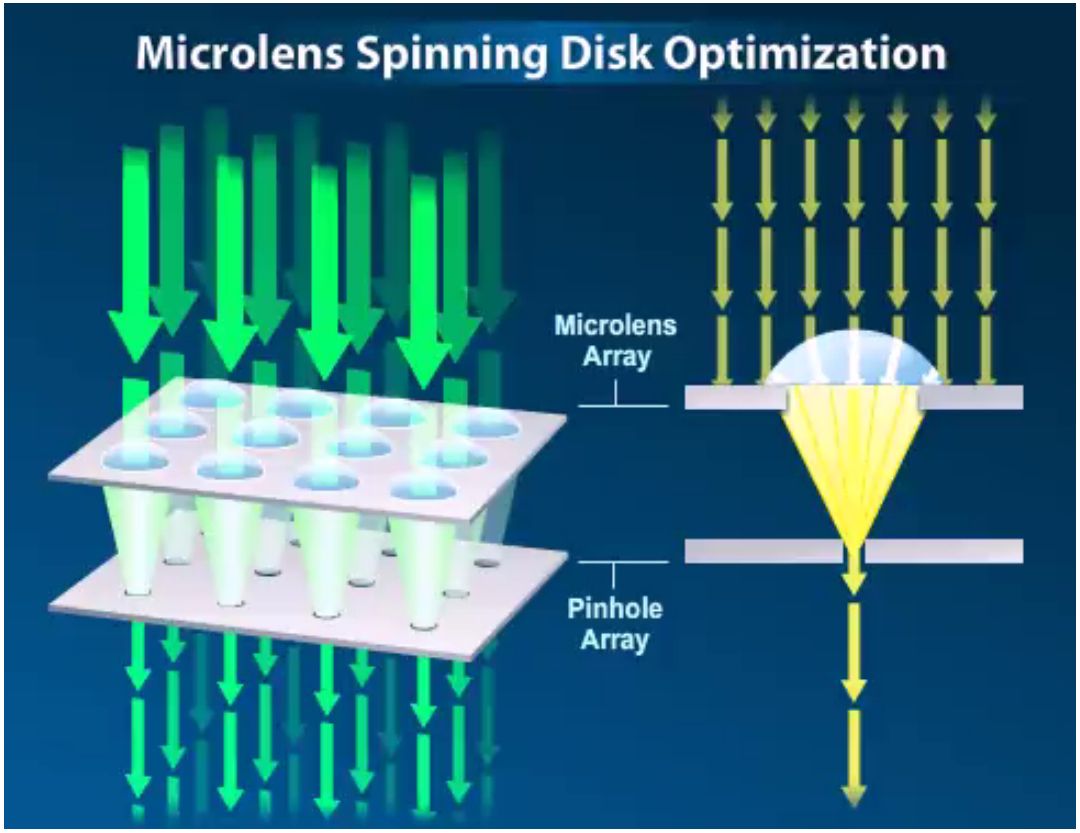
just a pinhole array –
Optimised for ‘cofocality’
and ‘crosstalk’

too much light is blocked
from reaching the specimen

Only 4% light passes through disc

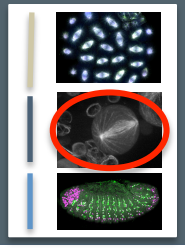


Yokogawa Spinning Disc Confocal Microlenses

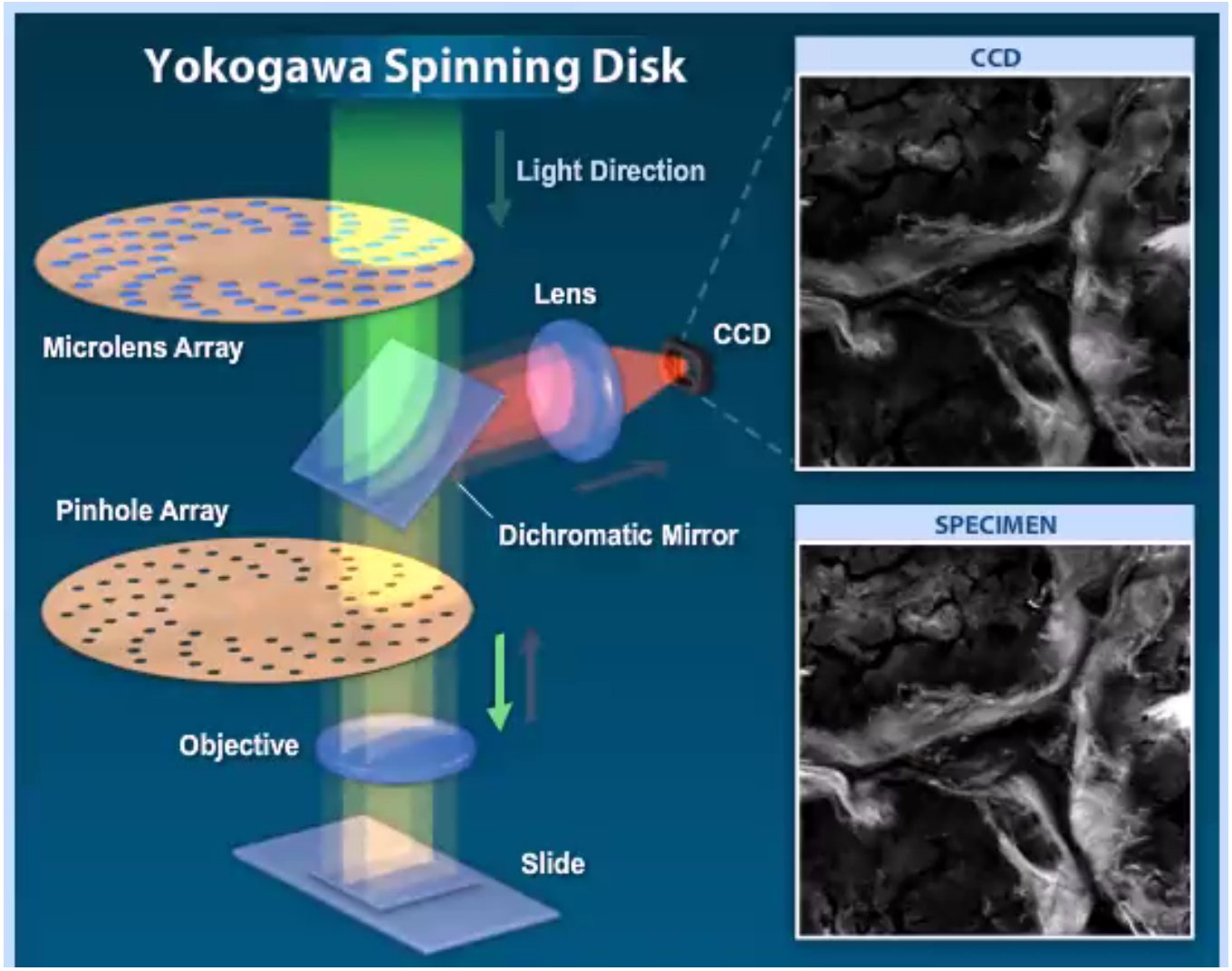


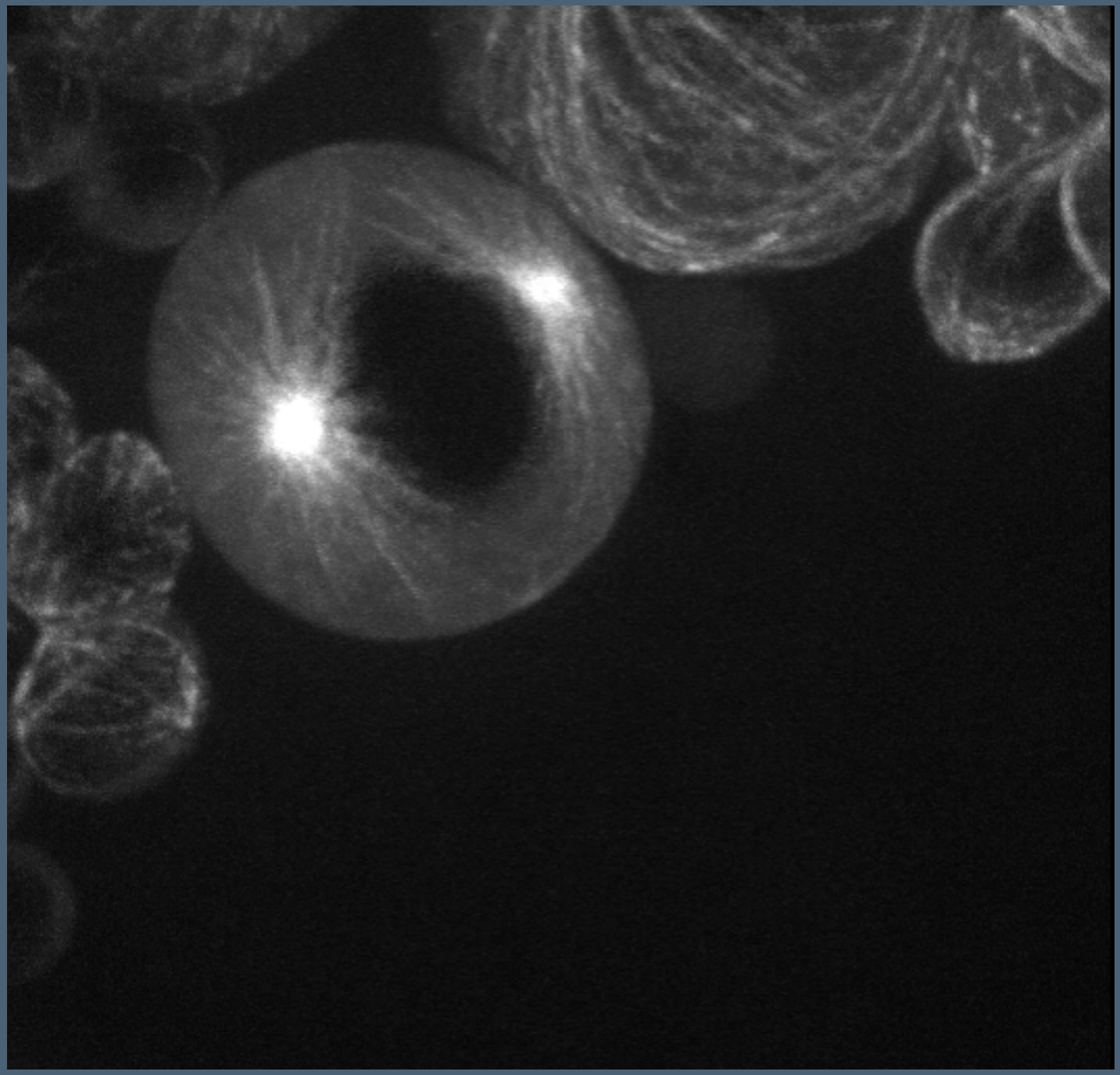
micro-lens array increase the light reaching the specimen

Typically 56% light passes through disc

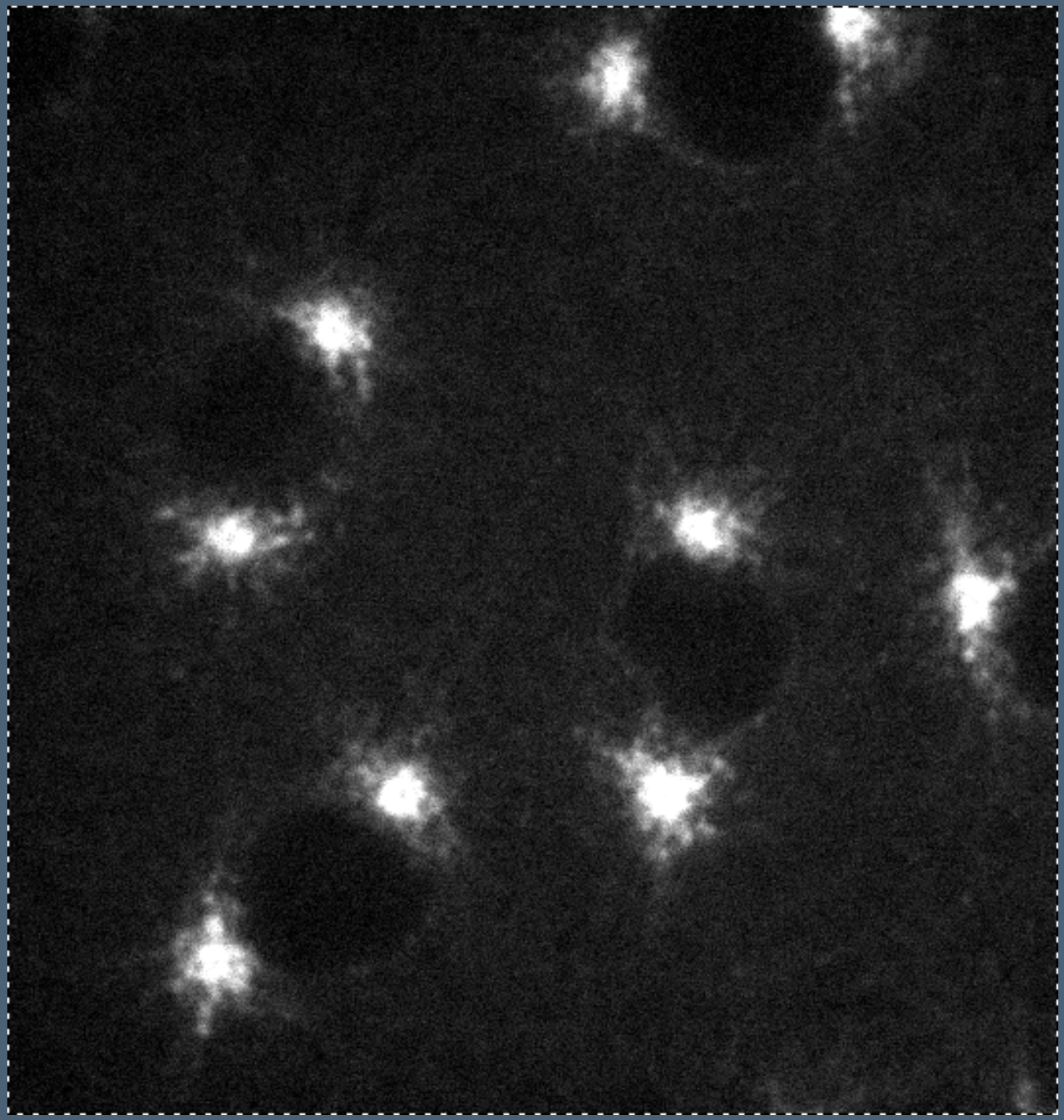


Yokogawa
Spinning Disc Confocal

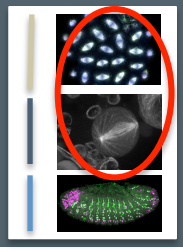




Cell division in brain stem cells (neuroblasts), Raff Lab

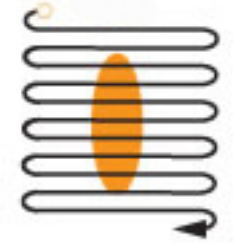


MT binding protein in *Drosophila* embryo, Raff Lab



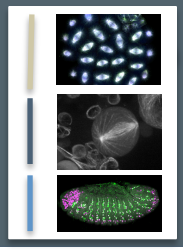
Point Scanning Vs Spinning Disc

Point Scanning



Spinning Disc

Speed	Slow (secs)	Fast (msecs)
Sensitivity	OK	OK
Flexibility	Good	Poor
Bleaching	Poor	Good
Pretty Pictures	Unbeatable!	Pretty damn good!
Pretty Movies	Good – if process slow	Unbeatable!

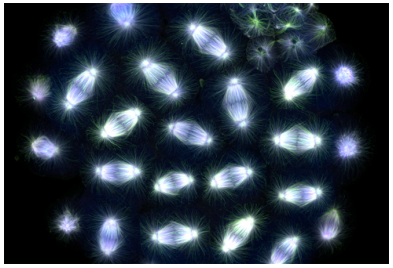


3 Flavours of Microscope

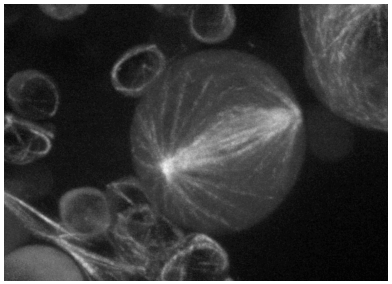
Problem:
Out of Focus
Light

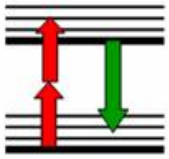
Confocal

Laser
Scanning

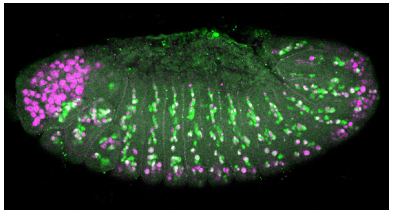


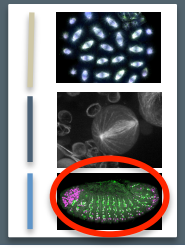
Spinning
disc





2-Photon





2-photon Microscope

Not a ‘confocal’

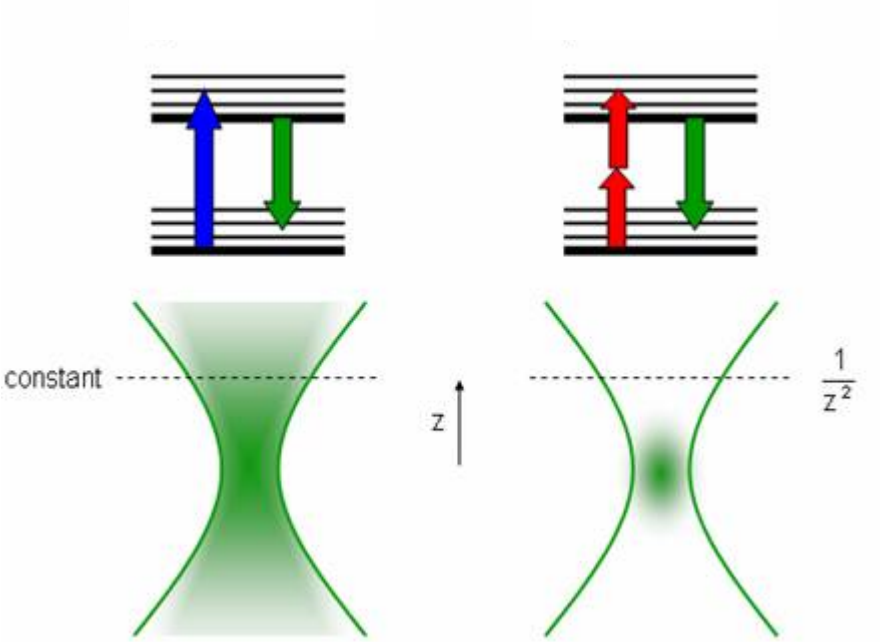
for imaging deeper into thick specimens

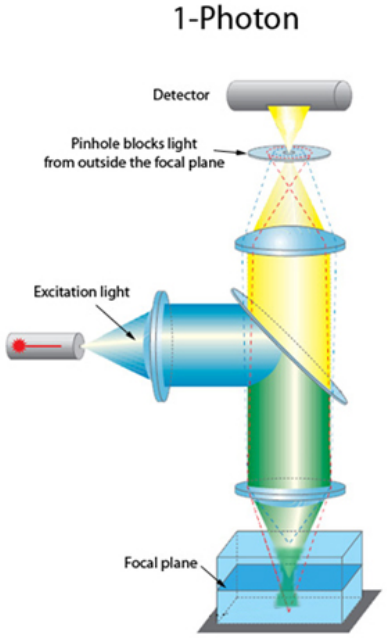
less damaging to biological samples

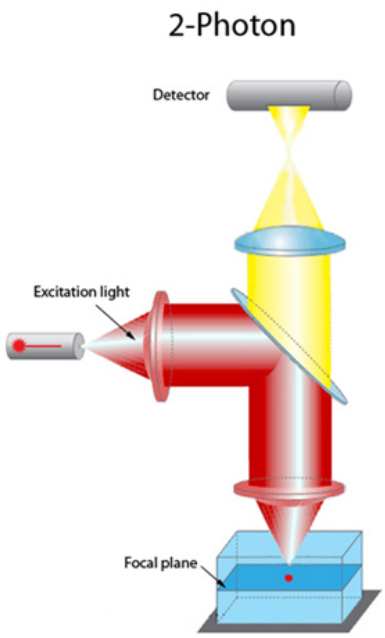
Confocal Vs 2-photon

1 Photon Excitation

2 Photon Excitation



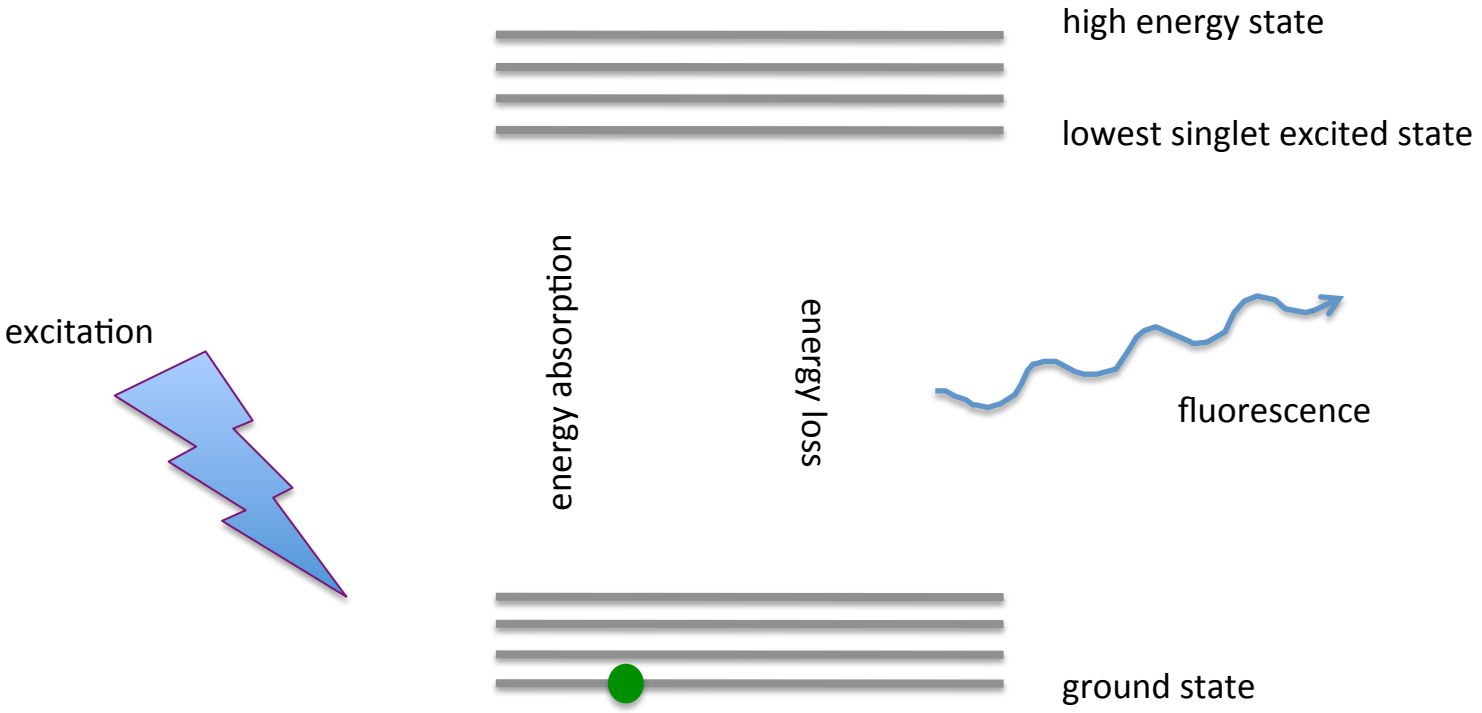




There is no out of focus light

THEORY

1 Photon Excitation



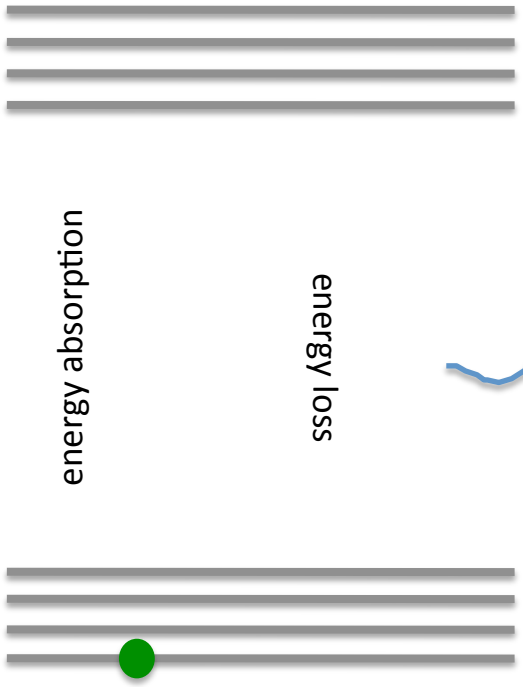
THEORY

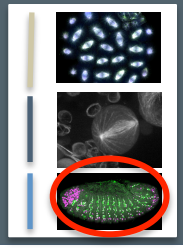
2 Photon Excitation

Almost simultaneous

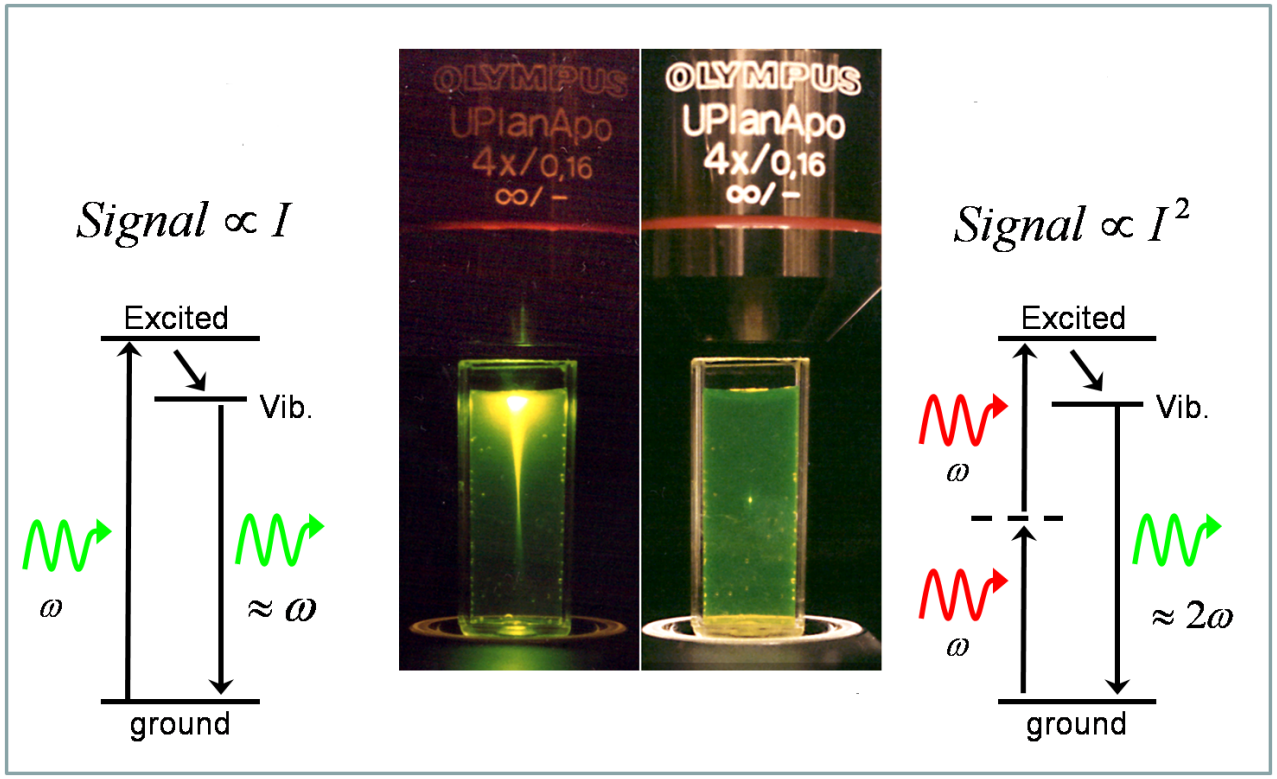
2nd low energy (IR~700nm) pulsed excitation

Low energy (IR ~700nm) Pulsed excitation



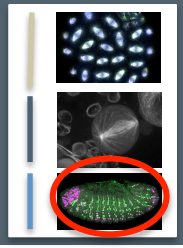


Principle of 2-photon Microscope

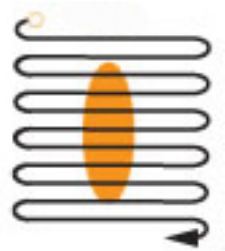


Near simultaneous, two photon event highly unlikely, only really possible a focal point

Tightly focused excitation



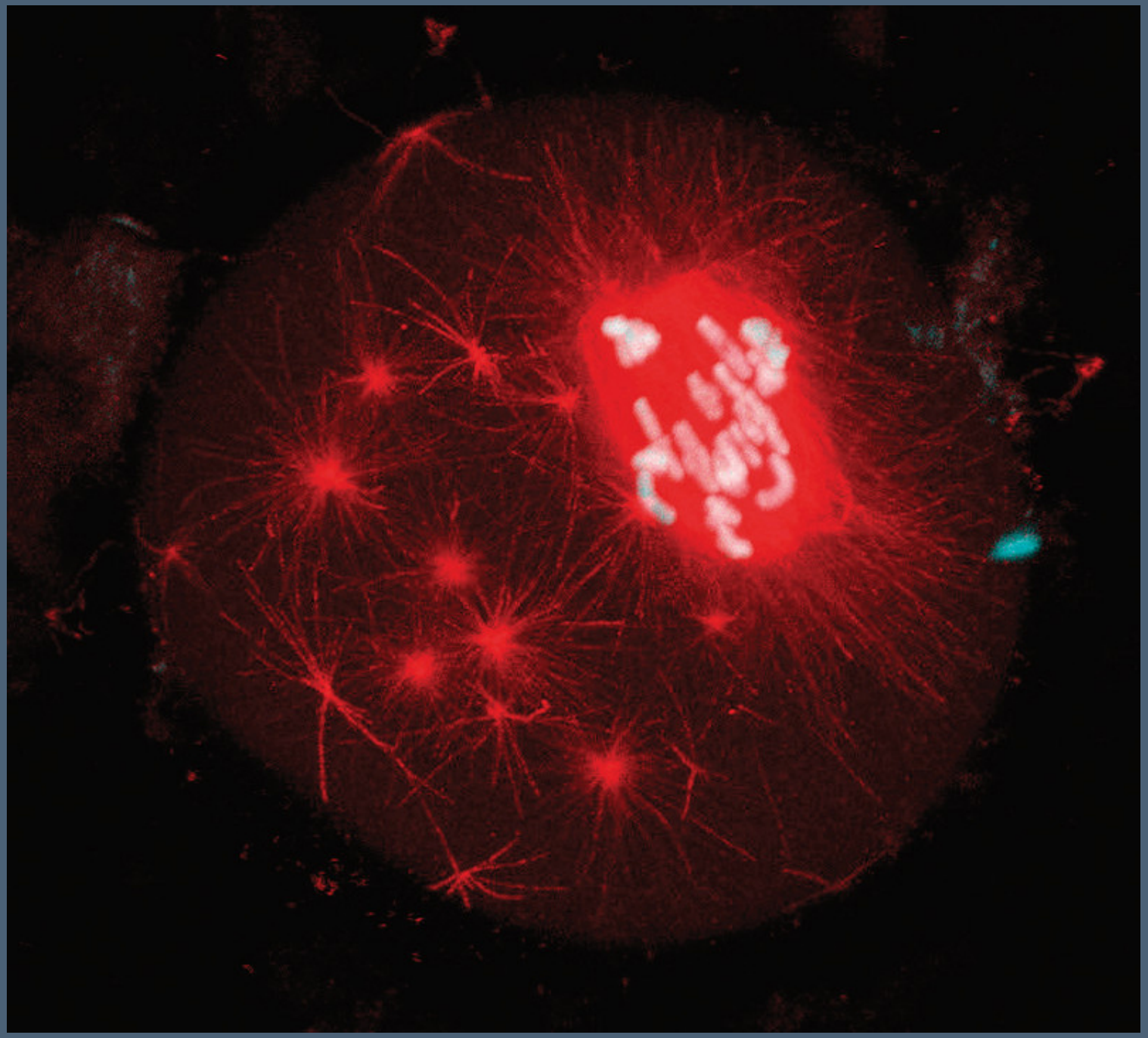
2-photon Microscope



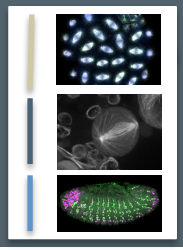
Pulsed excitation laser is then scanned across the sample.

Longer wavelengths are scattered to a lesser degree than shorter ones, and penetrate deeper into the sample.

In addition, these lower-energy photons are less likely to cause damage outside the focal volume.



Spindle formation in mouse ooctye, labelled with Hoechst, Alexa 680. M Schuh. EMBL, Heidelberg, Germany



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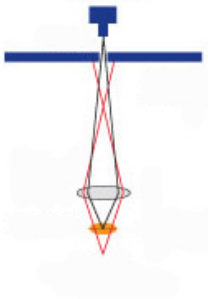
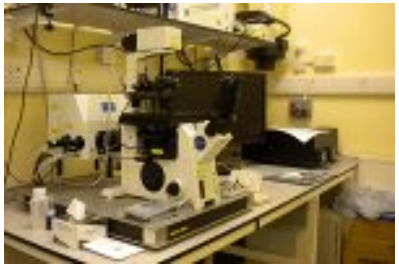
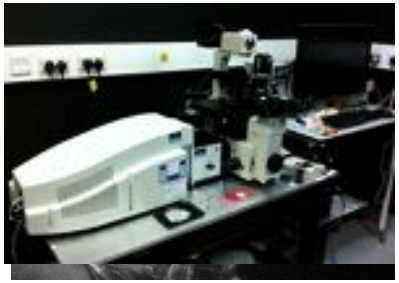
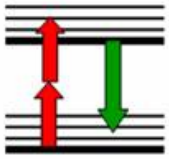


Laser
Scanning



Spinning
disc





2-Photon


http://www.micron.ox.ac.uk
Micron
OXFORD