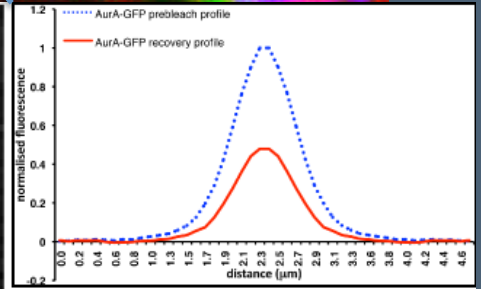
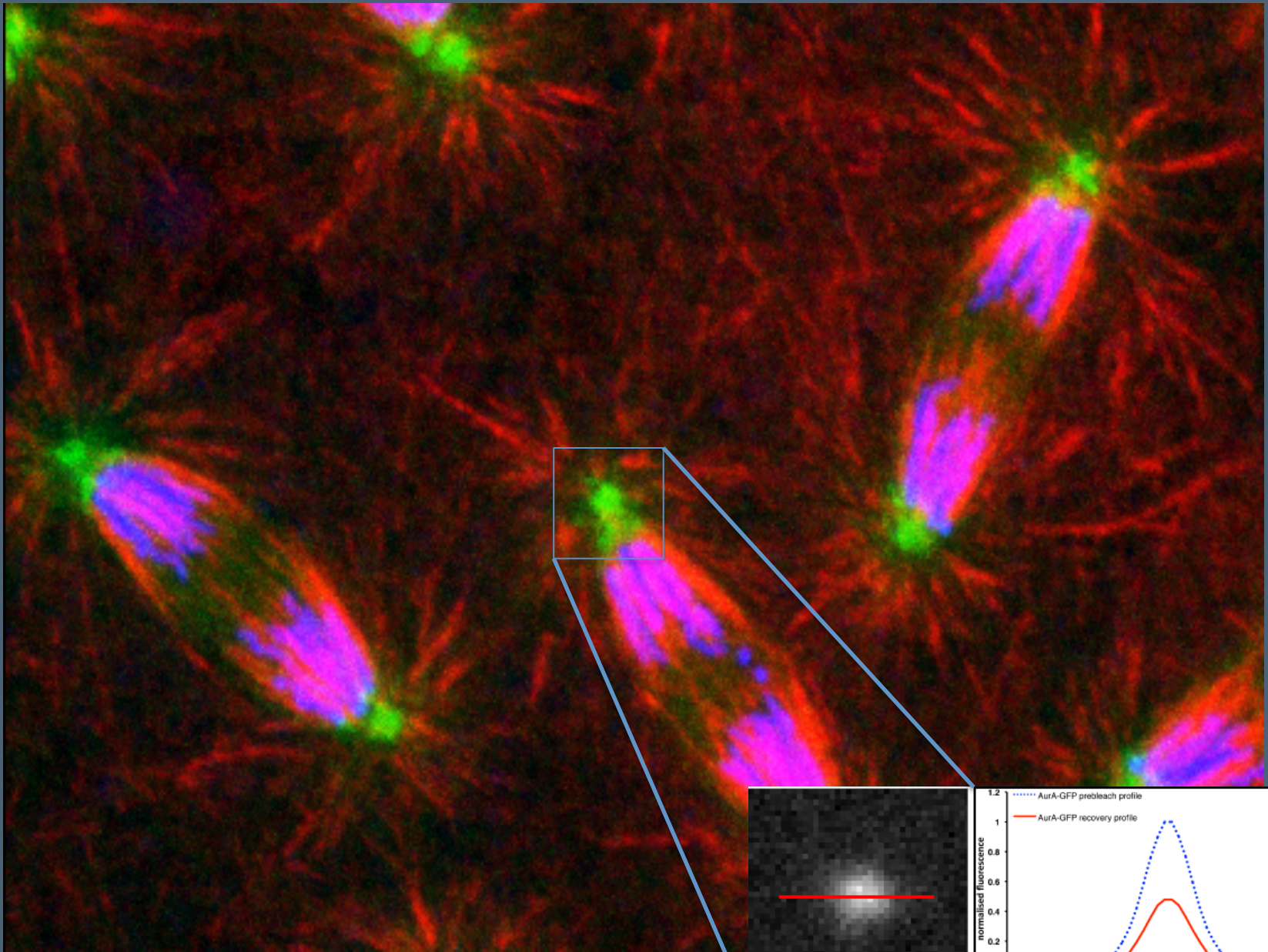


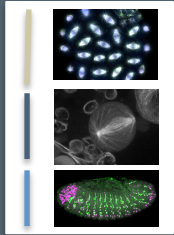
Confocal Microscopy

Lecture 8

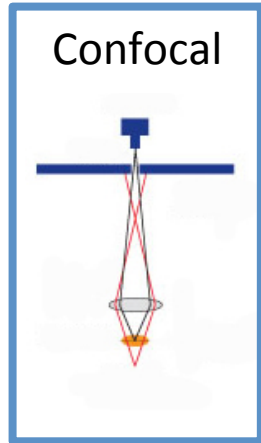
May 2014



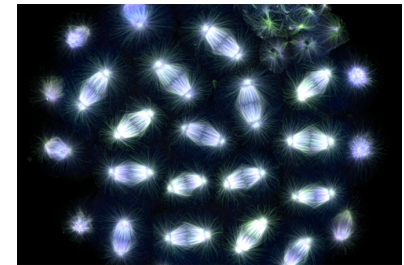
3 Flavours of Microscope



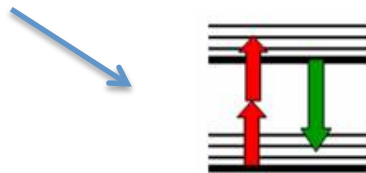
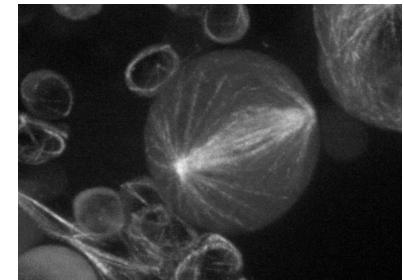
Problem:
Out of Focus
Light



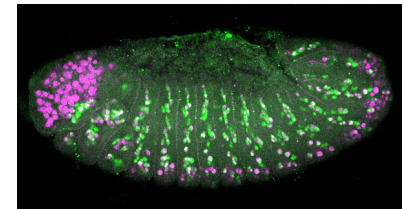
Laser
Scanning



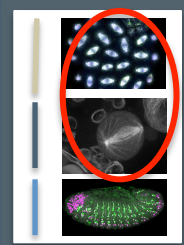
Spinning
disc



2-Photon



A short History of Confocal Microscope



Confocal “concept’ patented by Marvin Minsky in 1957



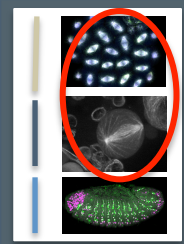
Eggar and Petran developed “spinning disc” confocal in late 1960s

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



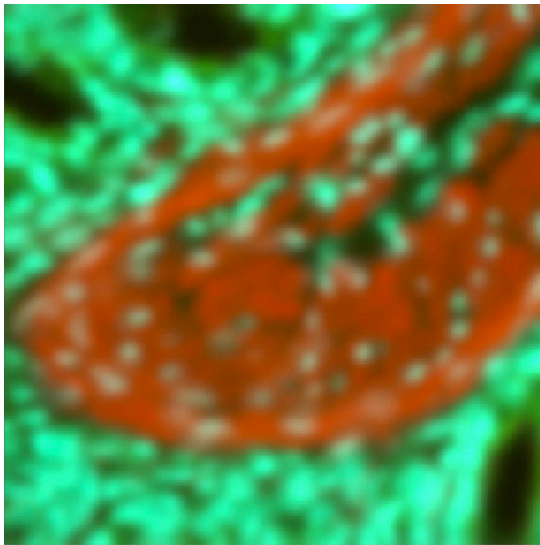
White, Amos and Wilson developed the MRC500 point scanning confocal
-Marketed commercially in 1987





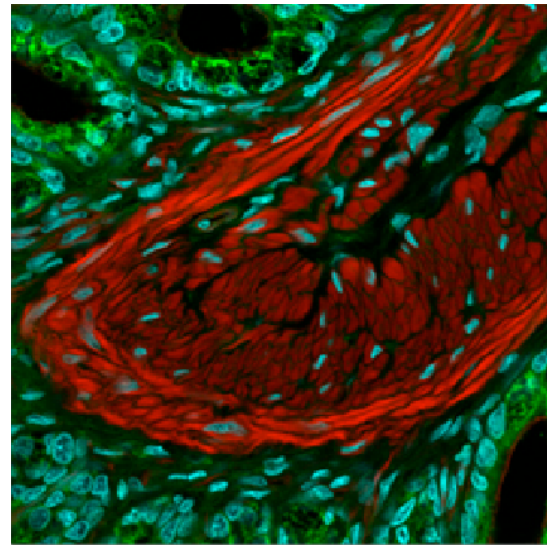
Comparison Widefield Vs Confocal

Widefield

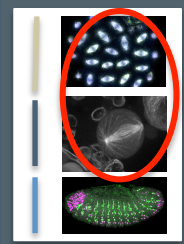


Out of focus light 'blurs' image

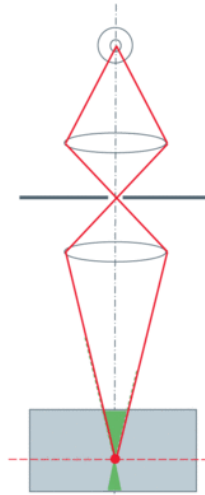
Confocal



Out of focus light is blocked

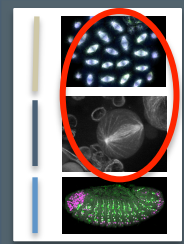


Principle of Confocal Microscopes Pinhole

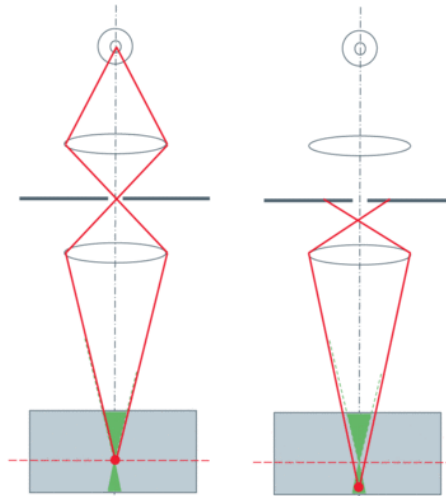


Pinhole diaphragm in the
Conjugated focal plane =
CONFOCAL

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

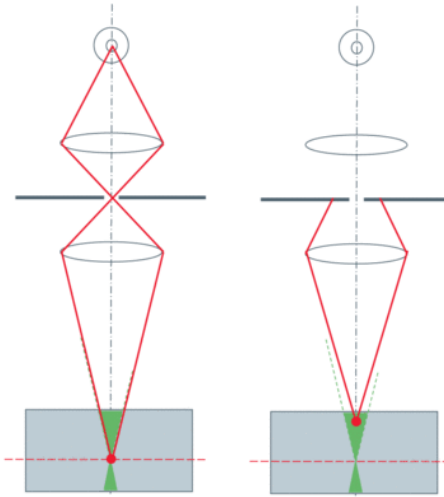


Pinhole – blocks out-of-focus light



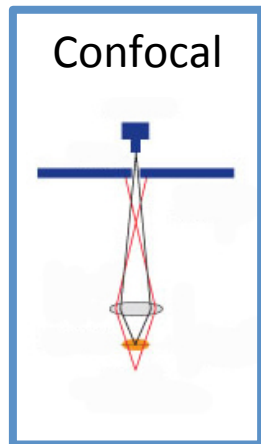
light from below the optical section crosses in front of the pinhole and doesn't pass through the pinhole aperture

Pinhole – blocks out-of-focus light

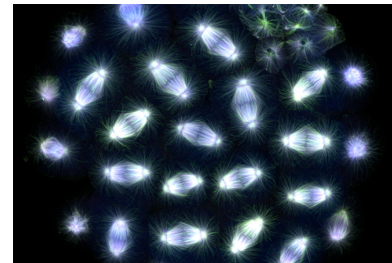


light from above the optical section also doesn't
pass through the pinhole aperture

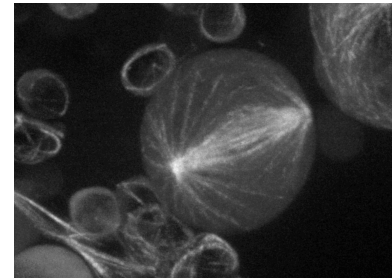
Confocal Microscopes

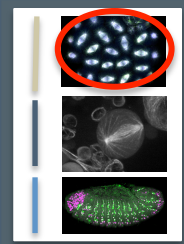


Laser
Scanning



Spinning
disc



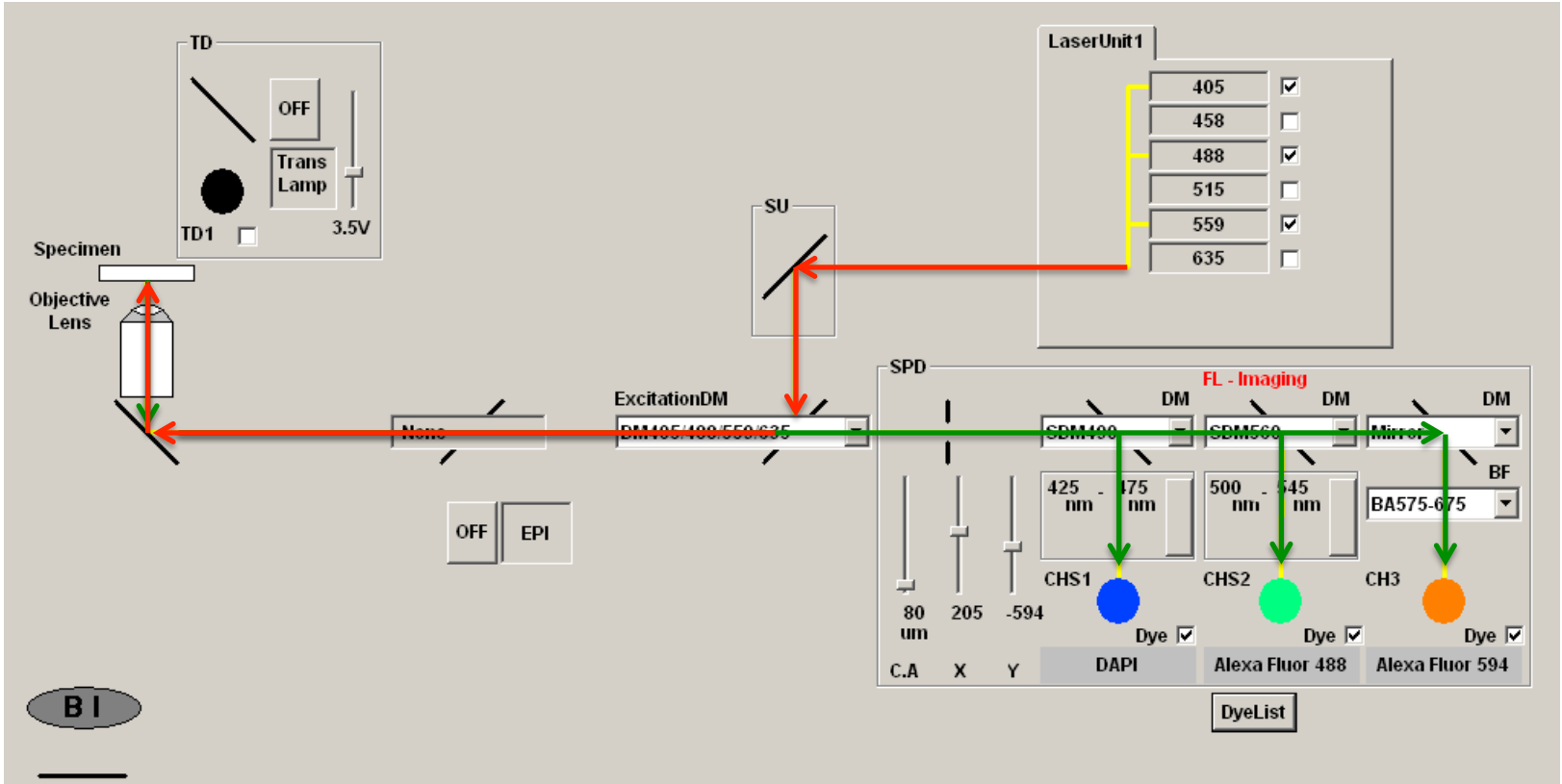
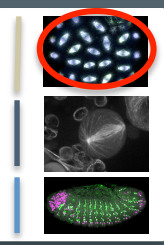


Laser Scanning Confocal Microscope



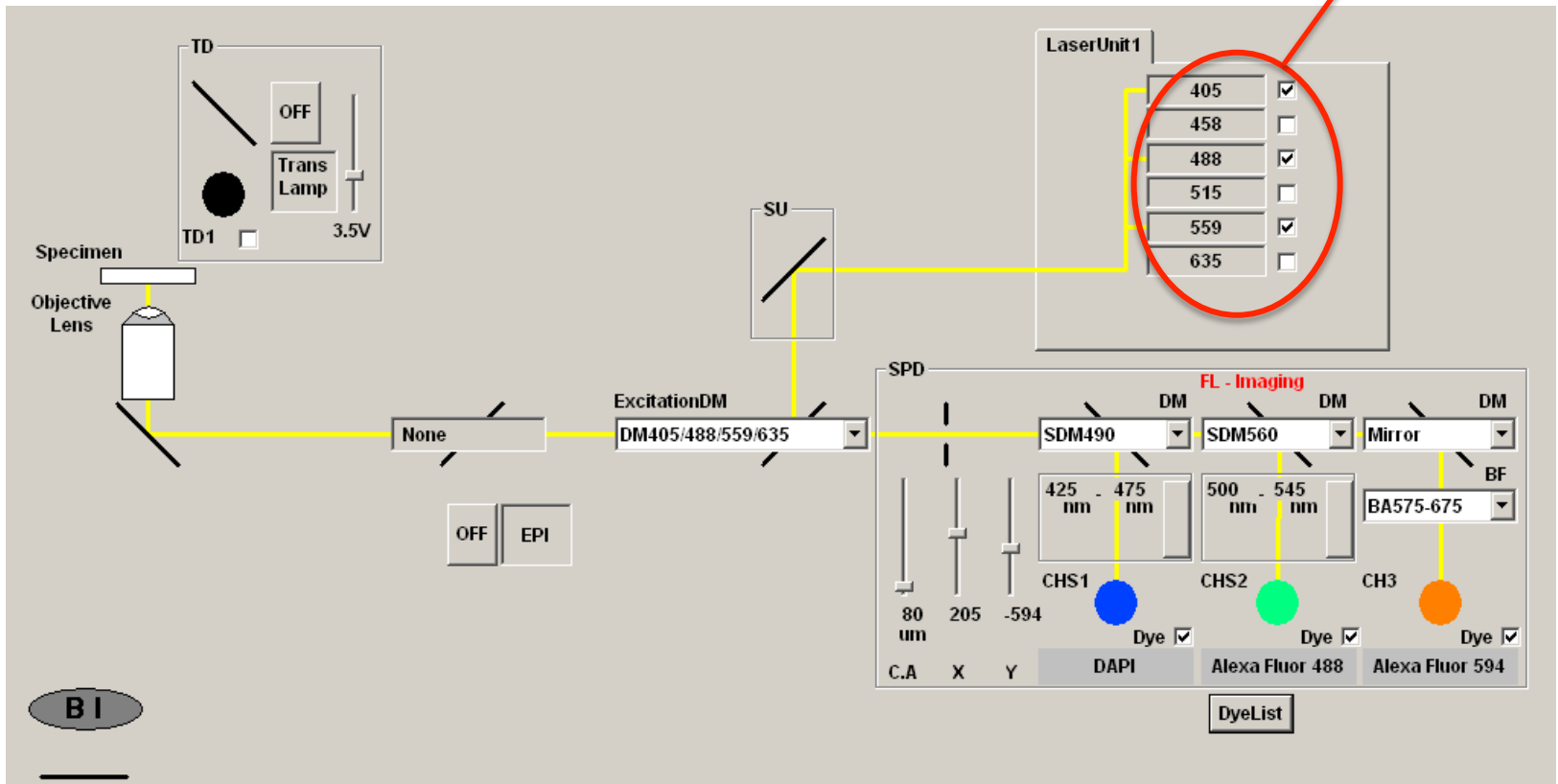
Laser Scanning Confocals are great to
get 'pretty' images

Laser Scanning Confocal - components



Laser Light Source

laser light source



Laser Light Source

Laser Emission Spectra

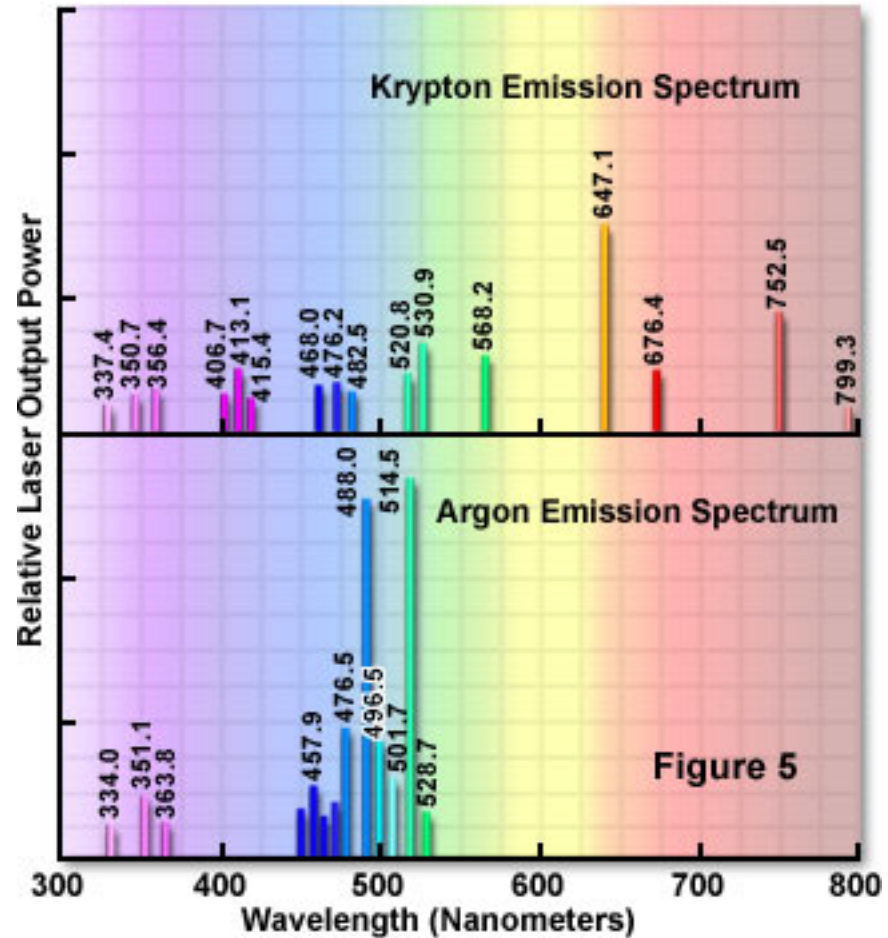
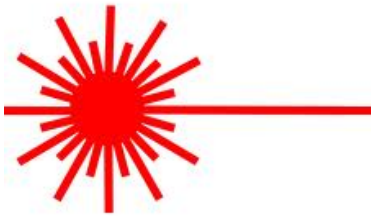
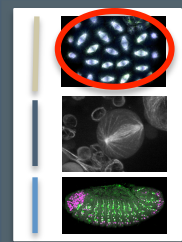


Figure 5

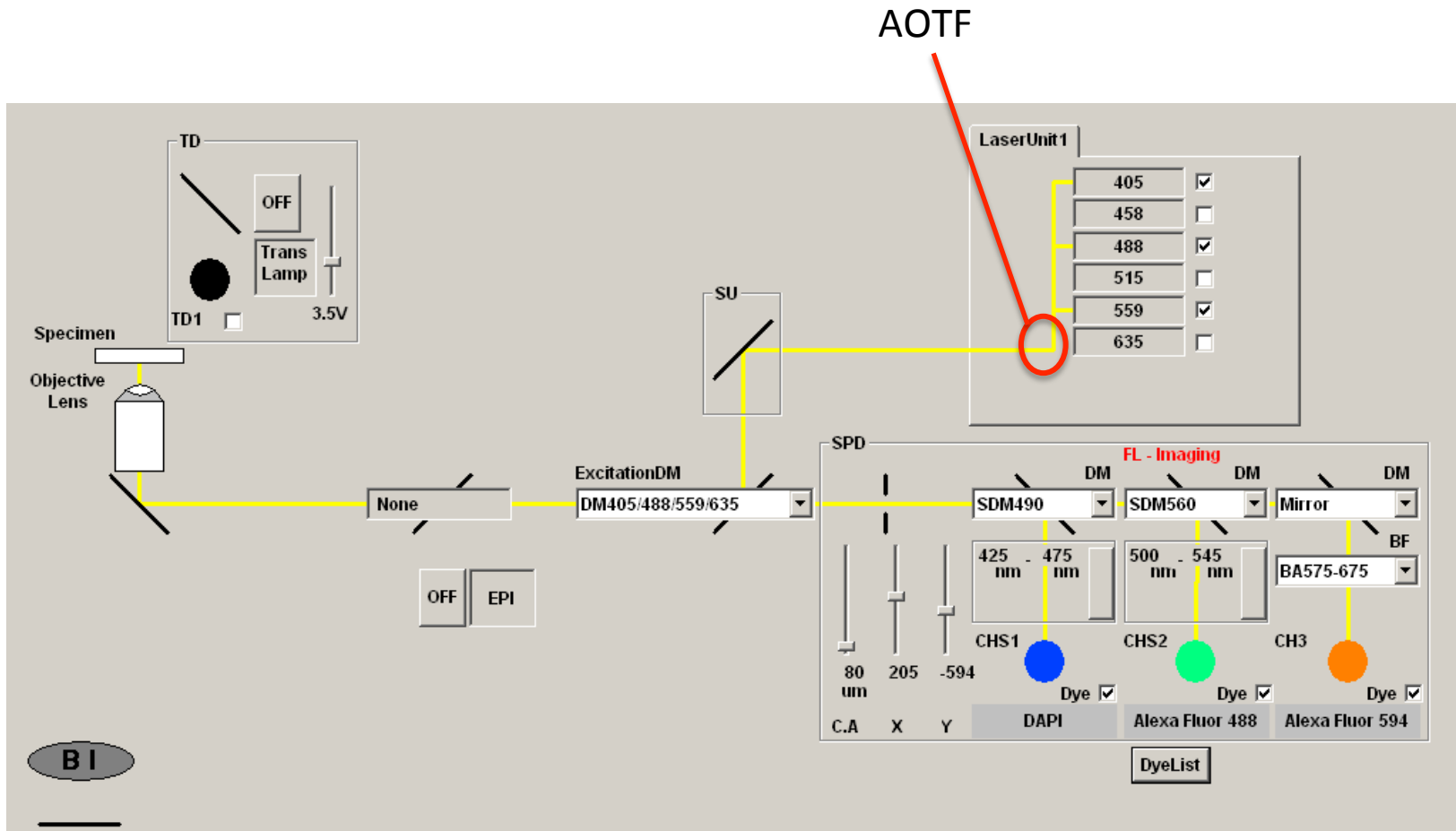


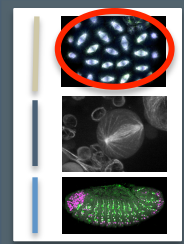
enables tighter control of fluorophores excited



AOTF

Acousto-Optic Tunable Filter

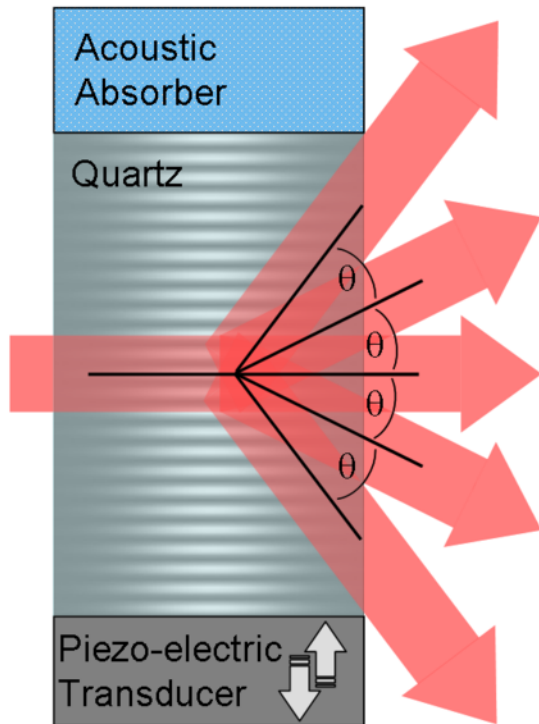




THEORY

AOTF

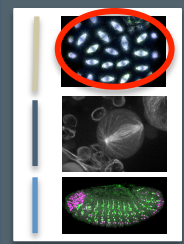
Acousto-Optic Tunable Filter



acousto-optic effect:

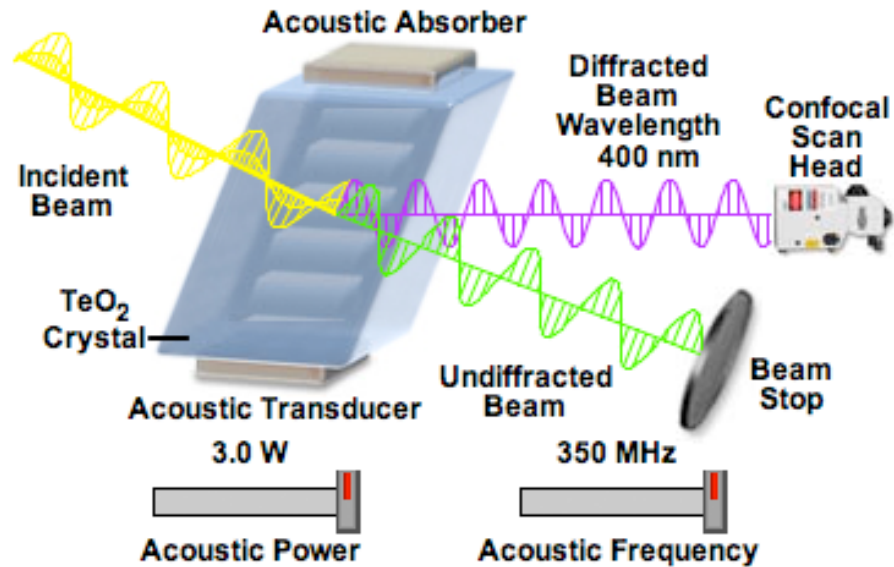
Acoustic wave excited within the quartz gives rise to variations in the refractive index

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.



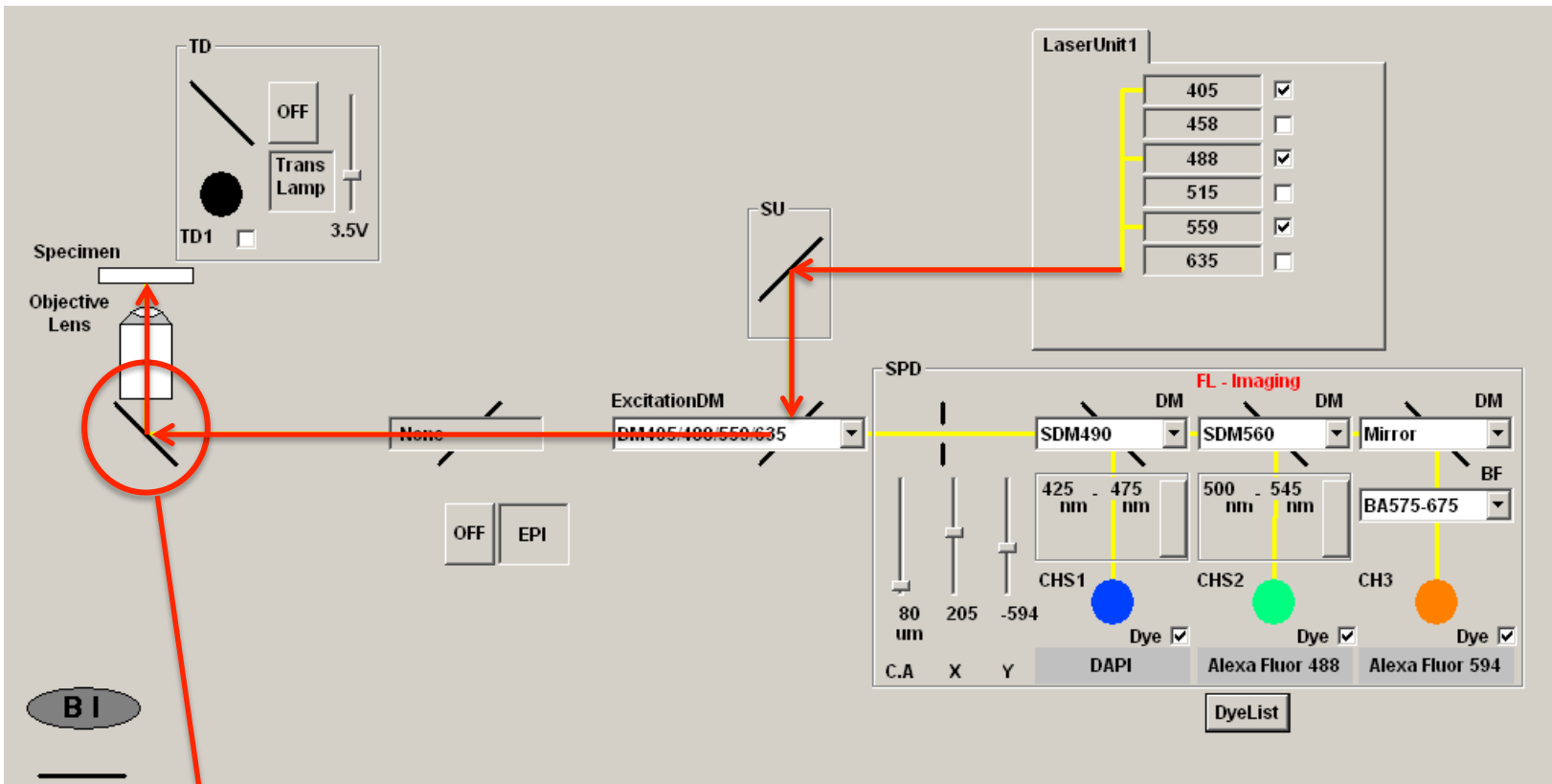
AOTF

Acousto-Optic Tunable Filter

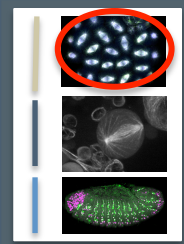


Quick On/Off of lasers
Very fast changes between excitation wavelengths

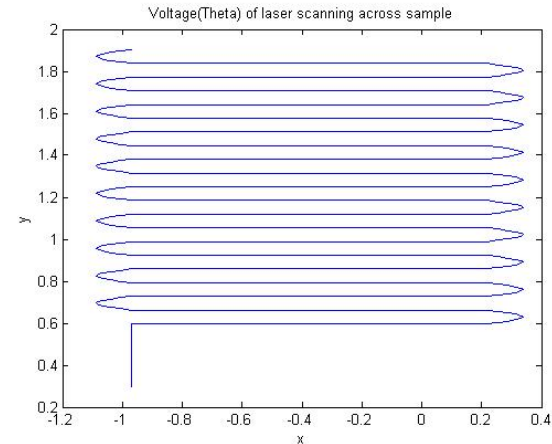
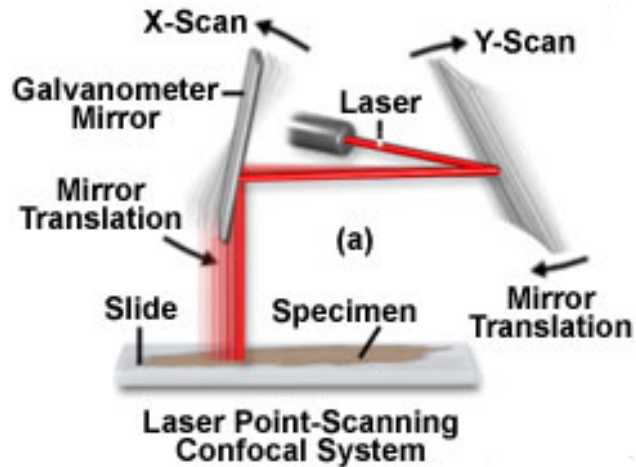
Galvo Scanning Mirrors



Galvo Scanning Mirrors



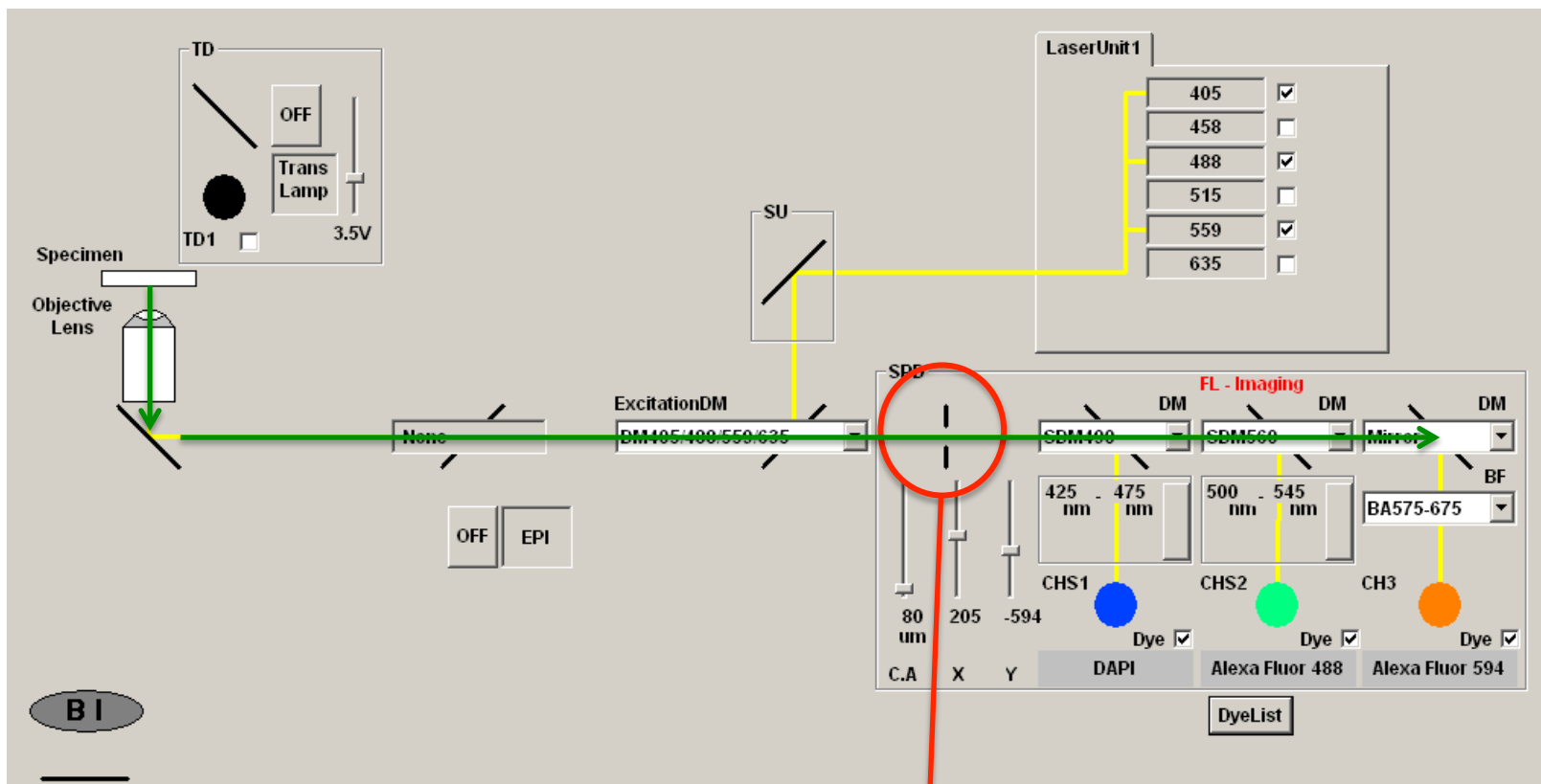
Galvo Scanning Mirrors



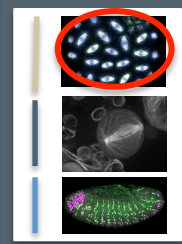
Sample excited at one point at a time
Relatively slow

Adjustable Pinhole

AOTF

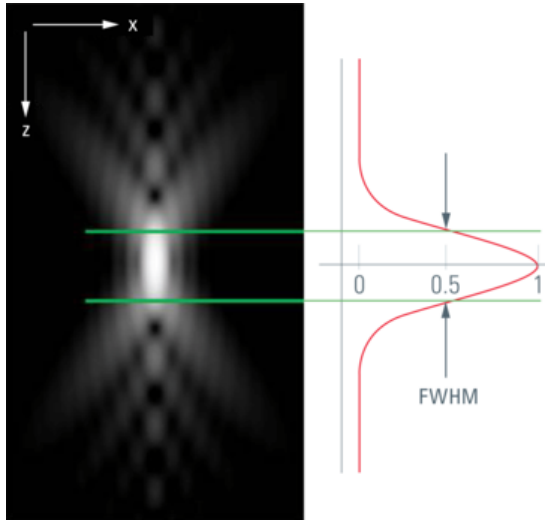


pinhole



THEORY

Pinhole – Optical Sectioning



FWHM=Full Width Half-Maximum

Shorter the wavelength the thinner the optical section

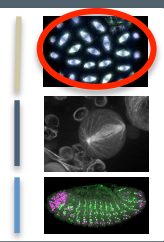
$$FWHM_{tot,axial} = \frac{0.64 \bar{\lambda}}{(n - \sqrt{n^2 - NA^2})}$$

Diameter of the pinhole Larger pinhole thicker section

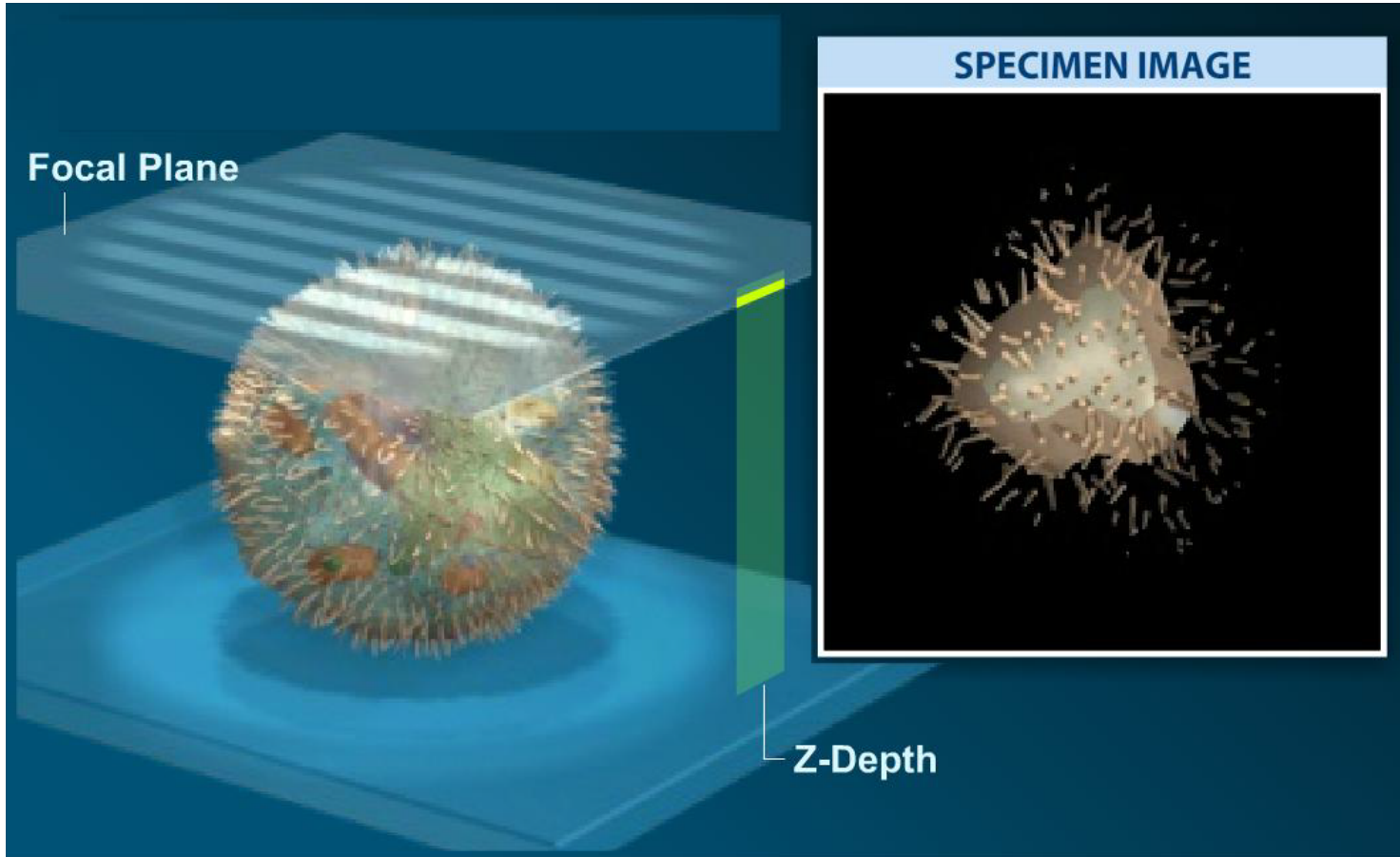
The higher the NA. the thinner the section

Weak signal > open pinhole > more light but thicker section

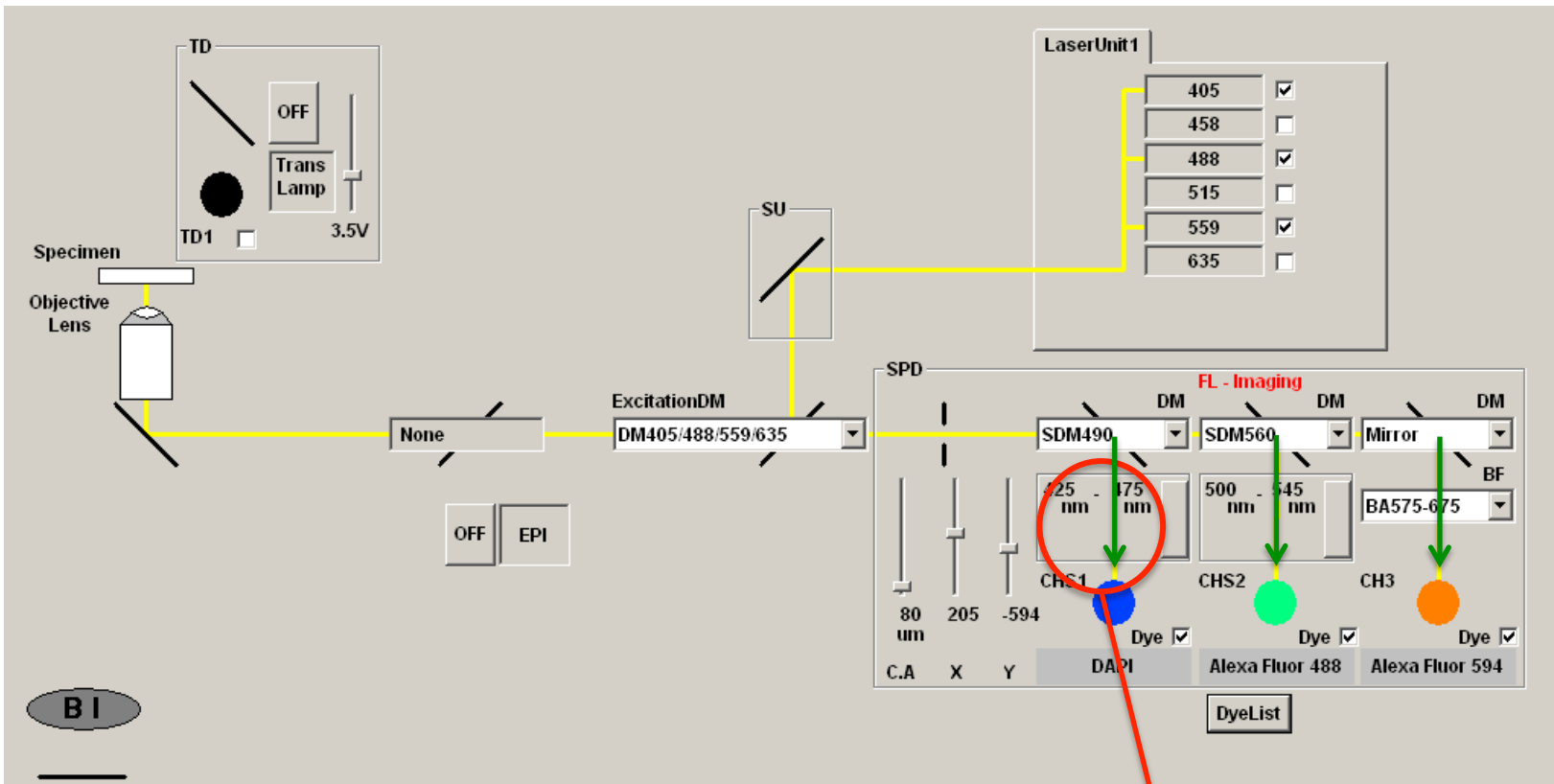
Confocal enables 3D reconstruction



Optical section

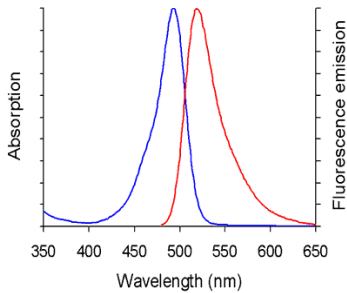


Variable Detector Slit

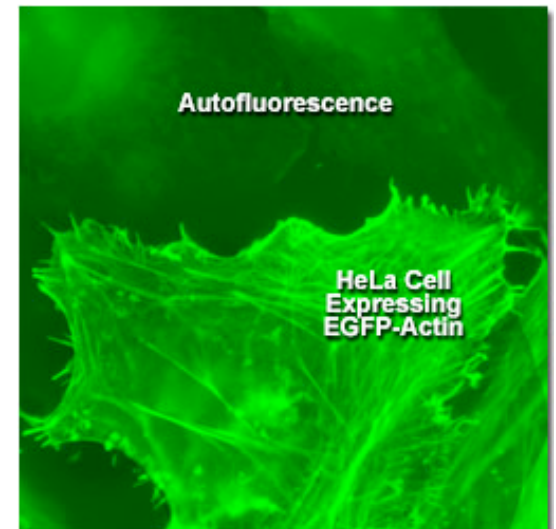
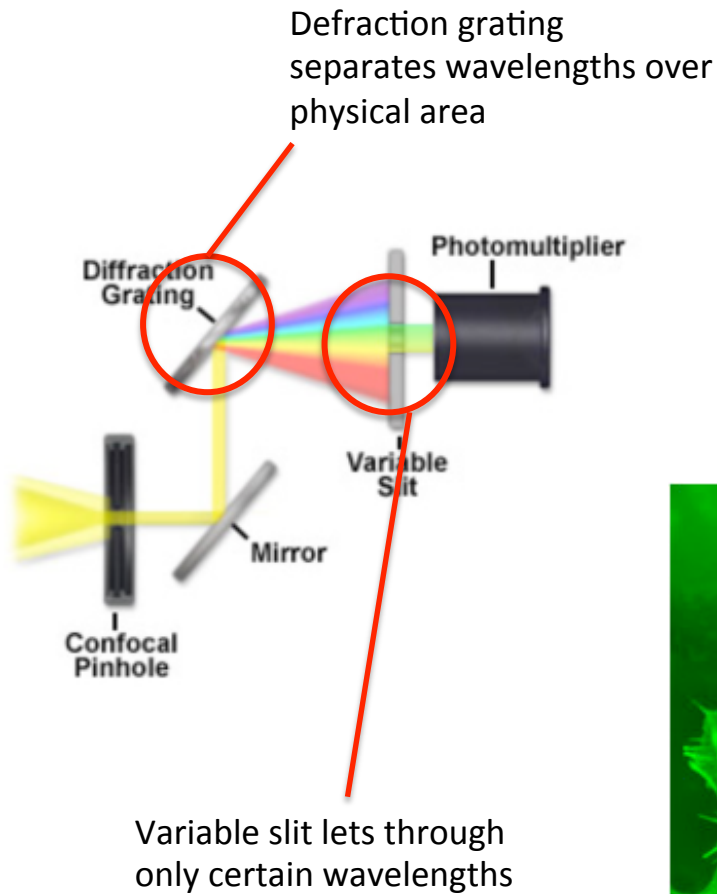


variable
detector slit

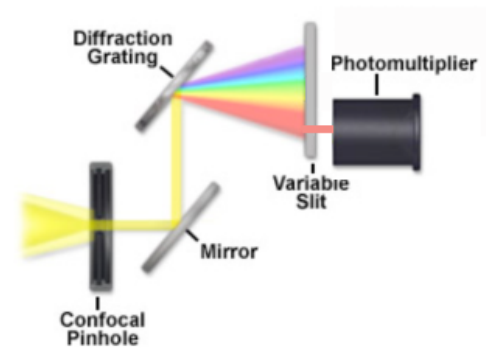
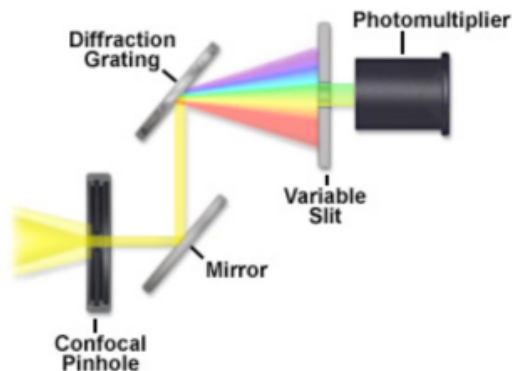
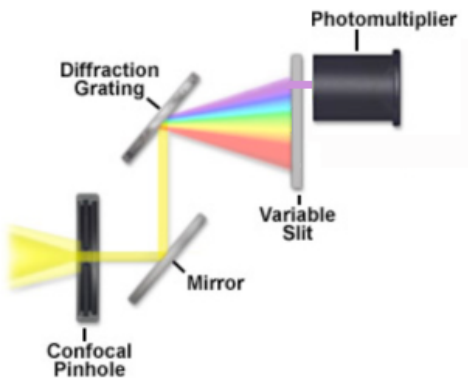
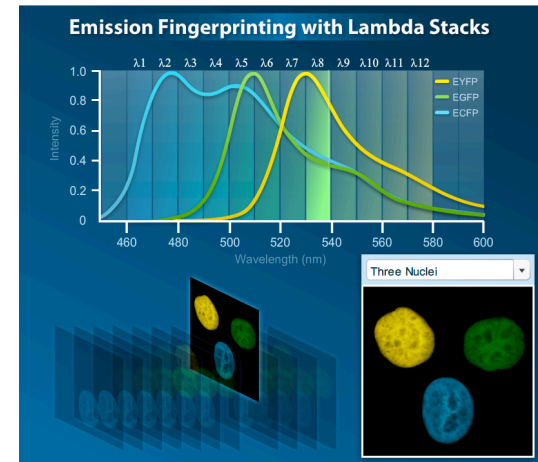
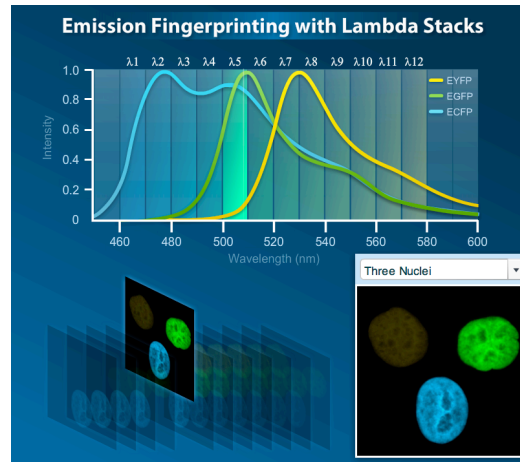
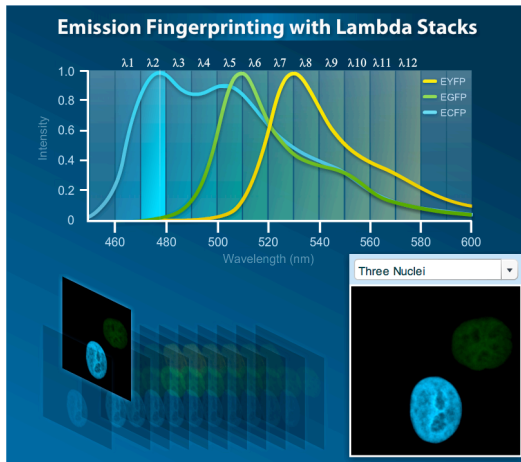
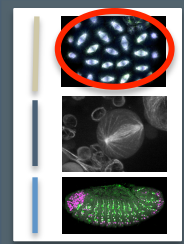
Spectral Unmixing



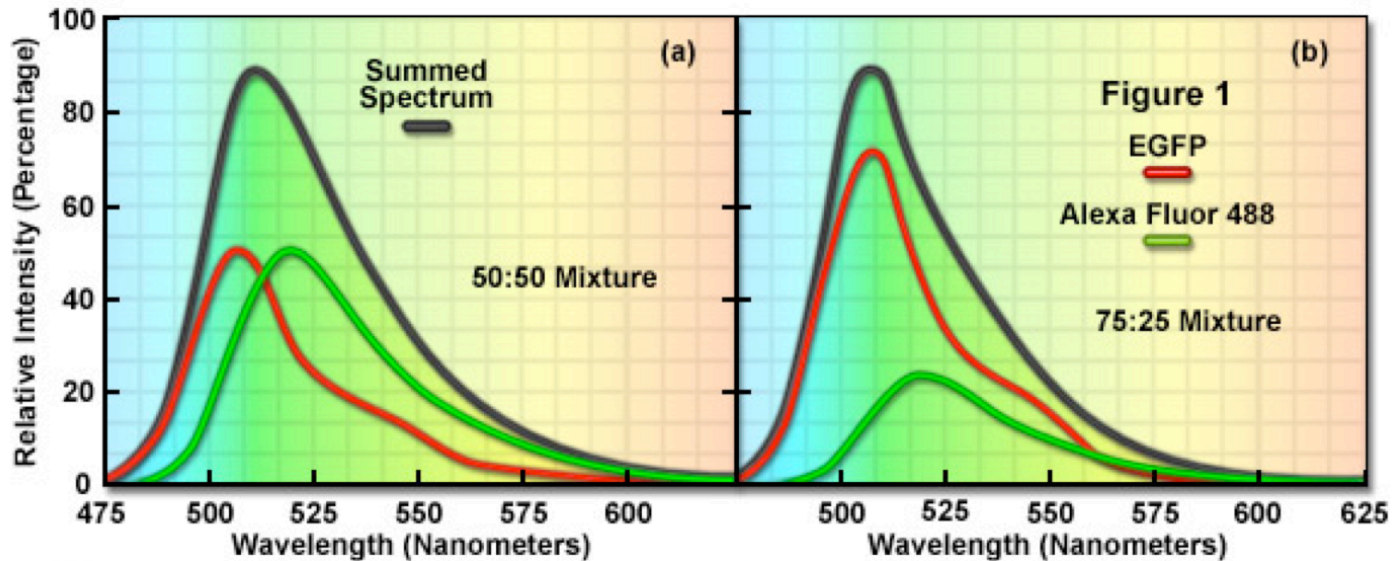
Light emitted from fluorophore as a spectrum



Spectral Unmixing



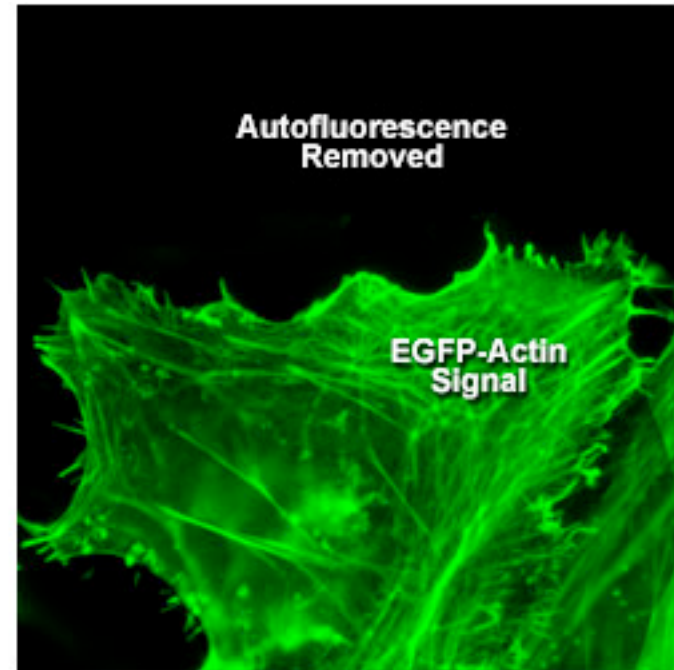
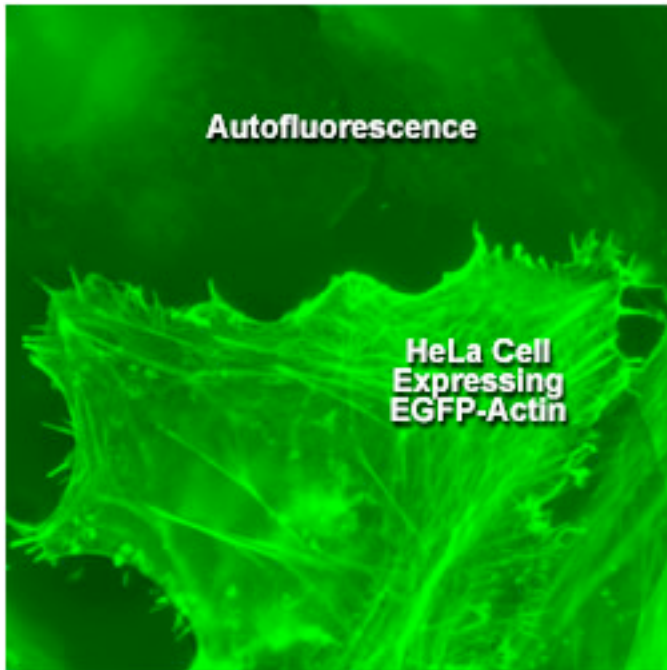
Spectral Unmixing



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

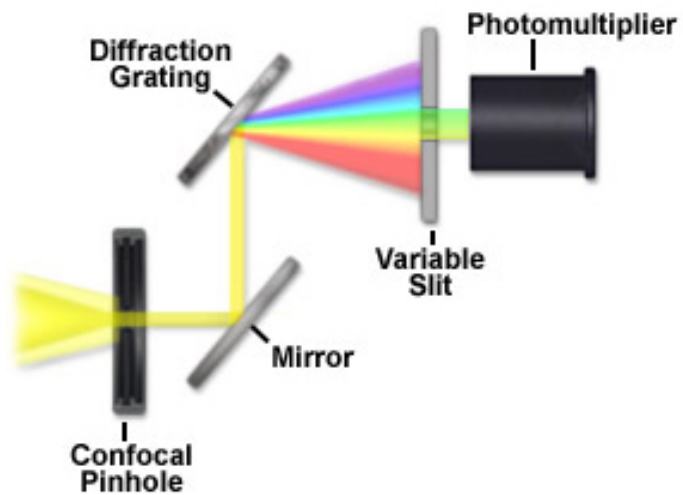


At each pixel:

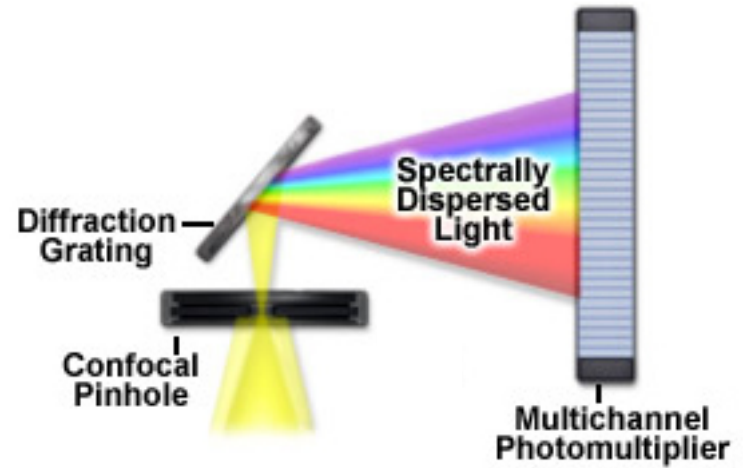
Calculate the proportion of the pixel is due to autofluorescence.
Subtract the autofluorescence from the 'true' GFP value.

Spectral Unmixing

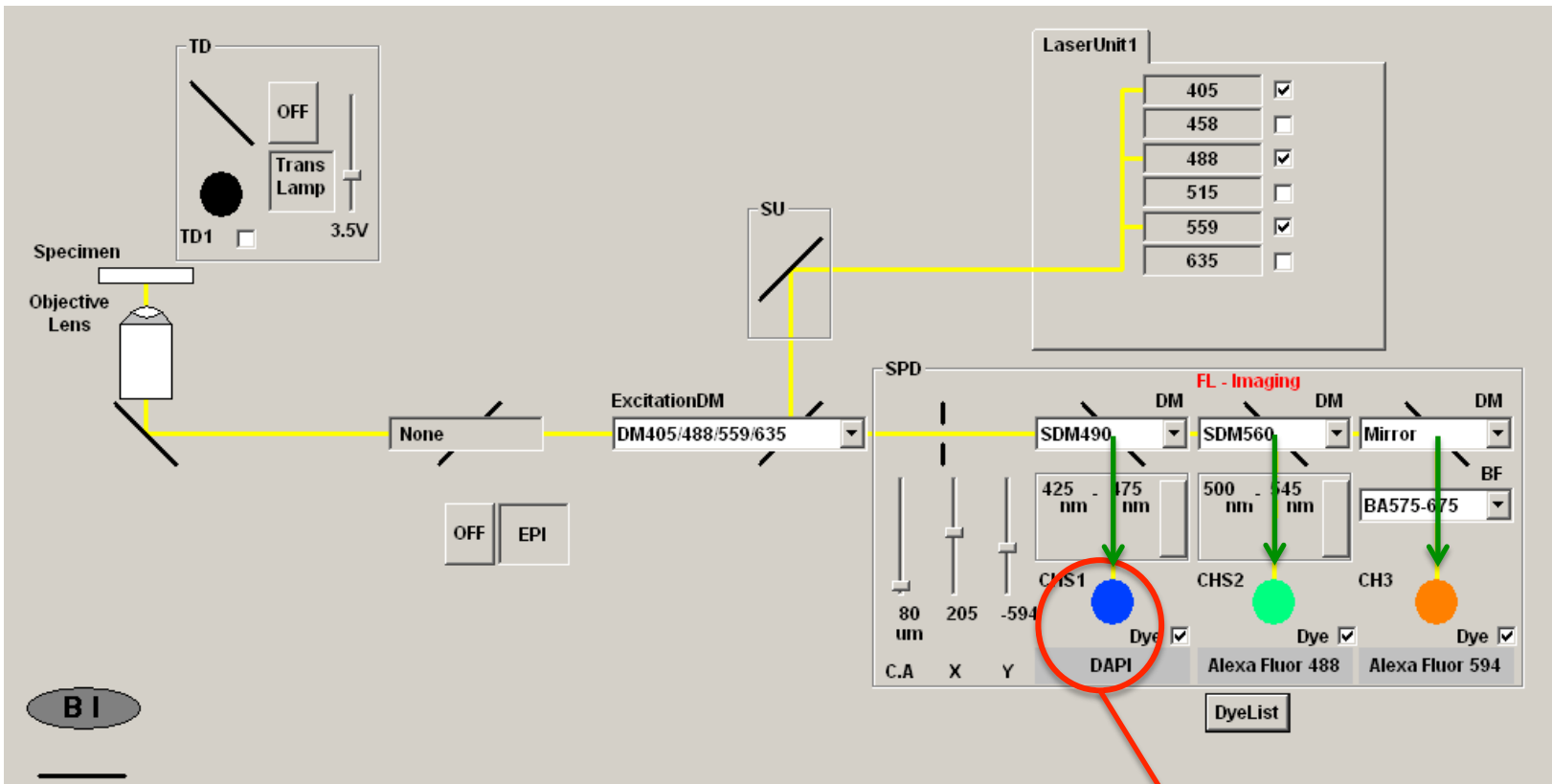
Olympus FV1000



Zeiss 510 META

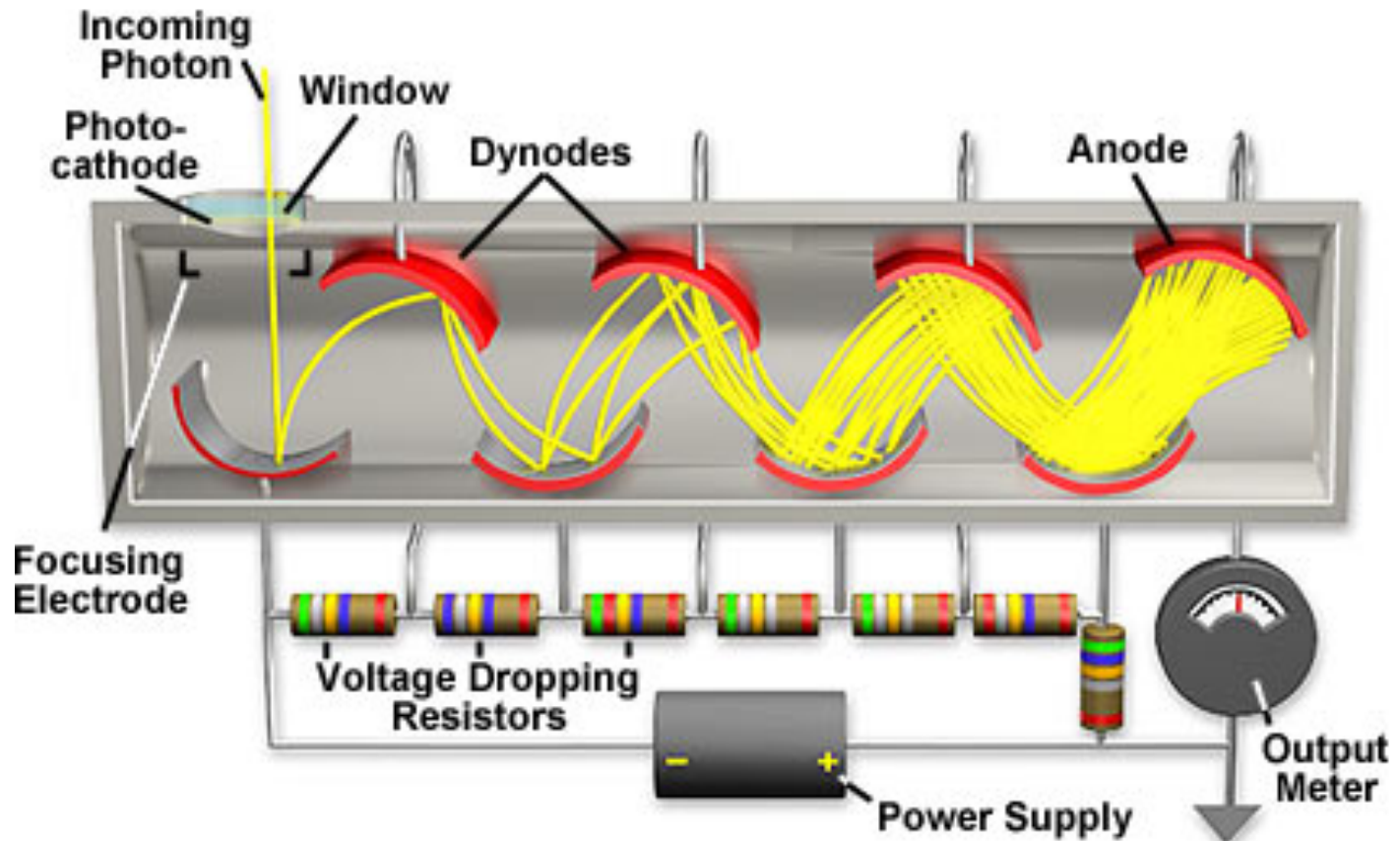


PMT – Photon Multiplier Tube



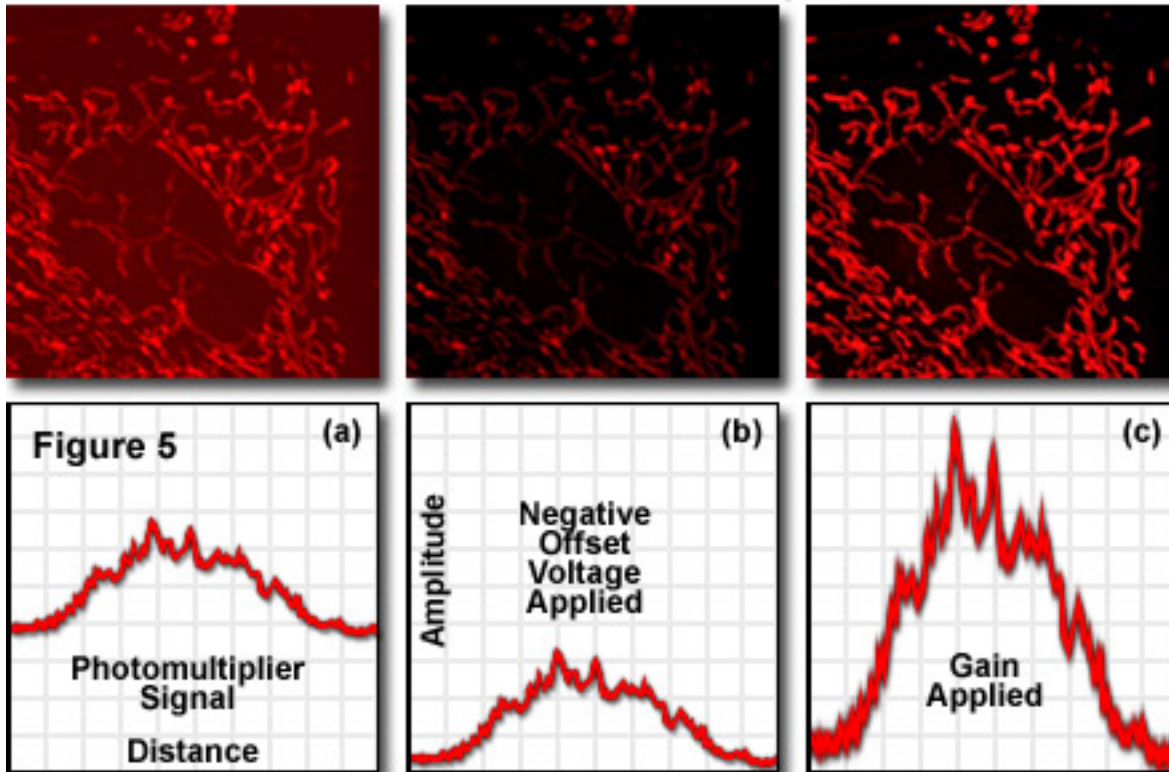
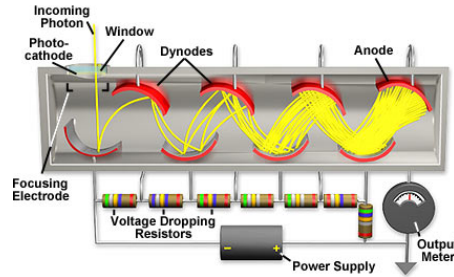
PMT detectors

PMT – Photon Multiplier Tube



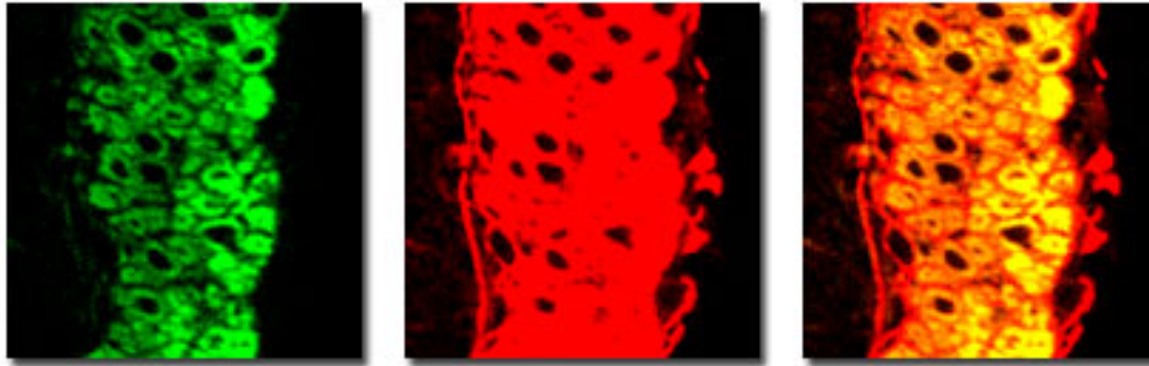
Very Low Noise
Huge Signal Amplification ($\sim 1 \times 10^6$)

PMT – Photon Multiplier Tube Adjusting Gain and Offset

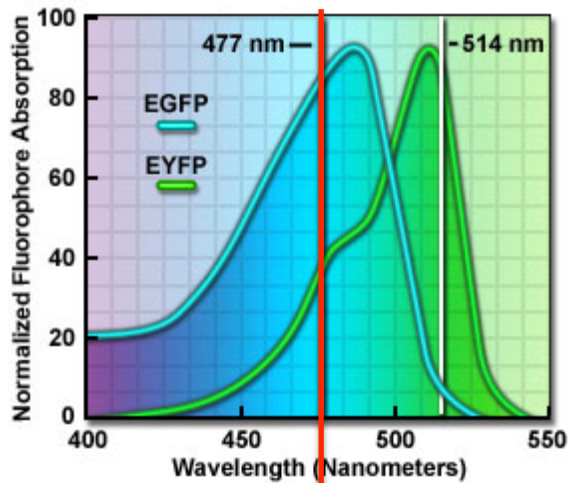


Beware - this is how your image will be saved!

'bleed-through'

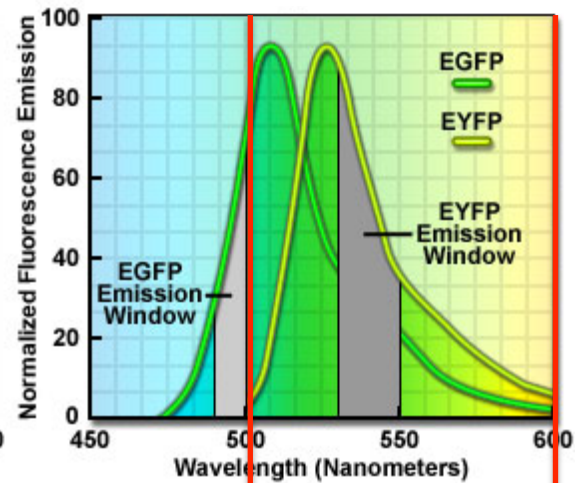


Absorption spectral profiles



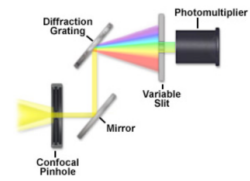
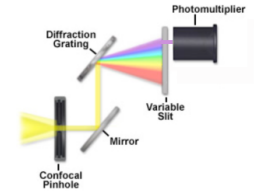
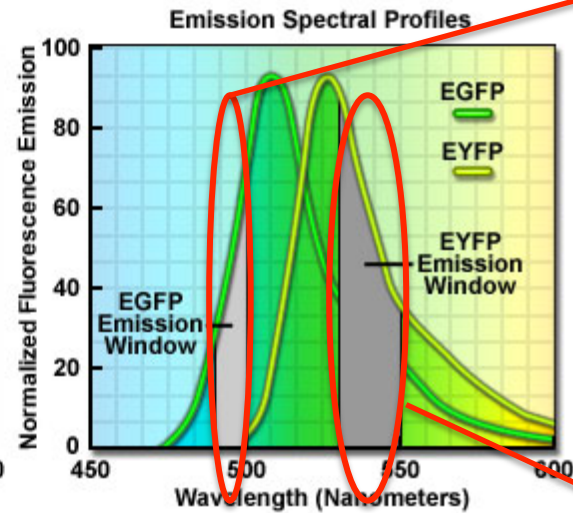
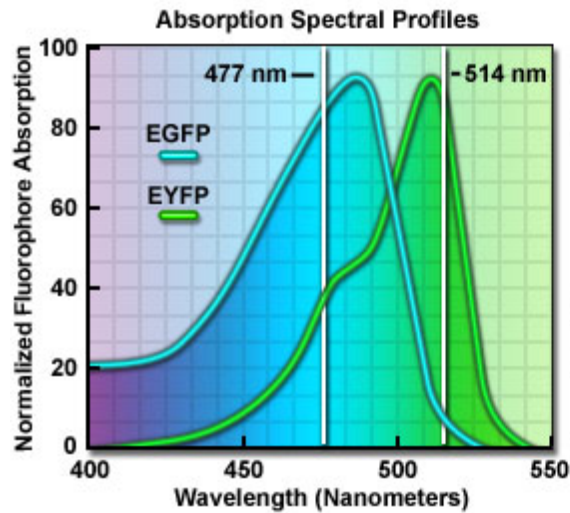
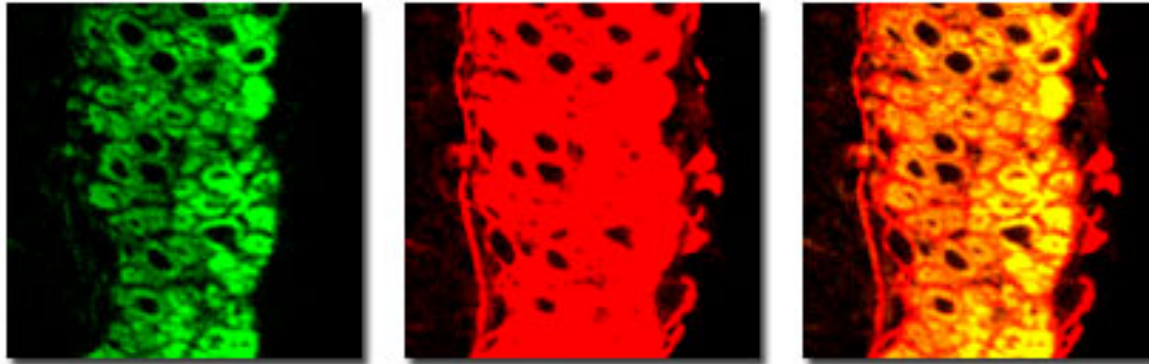
Excite at 477nm

Emission spectral profiles

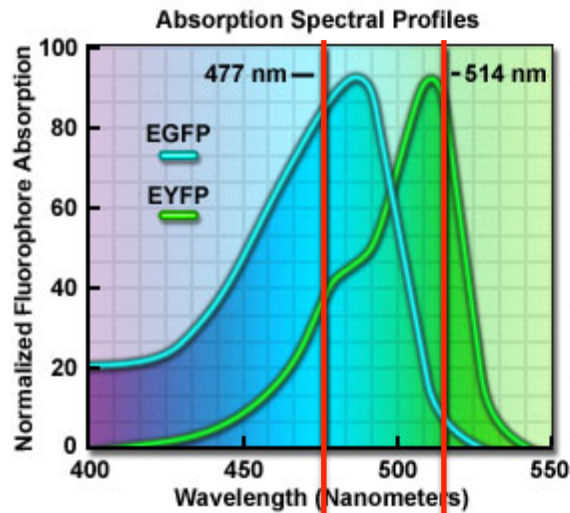
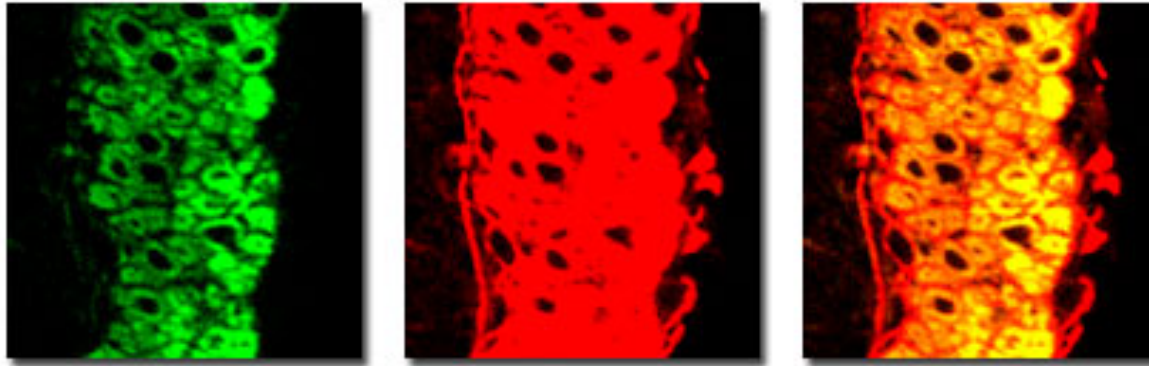


overlapping emission

minimising 'bleed-through' Variable Slits

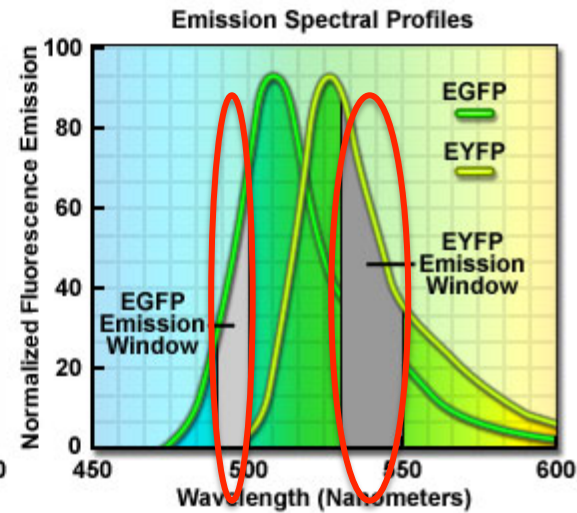


minimising 'bleed-through' Sequential Scanning



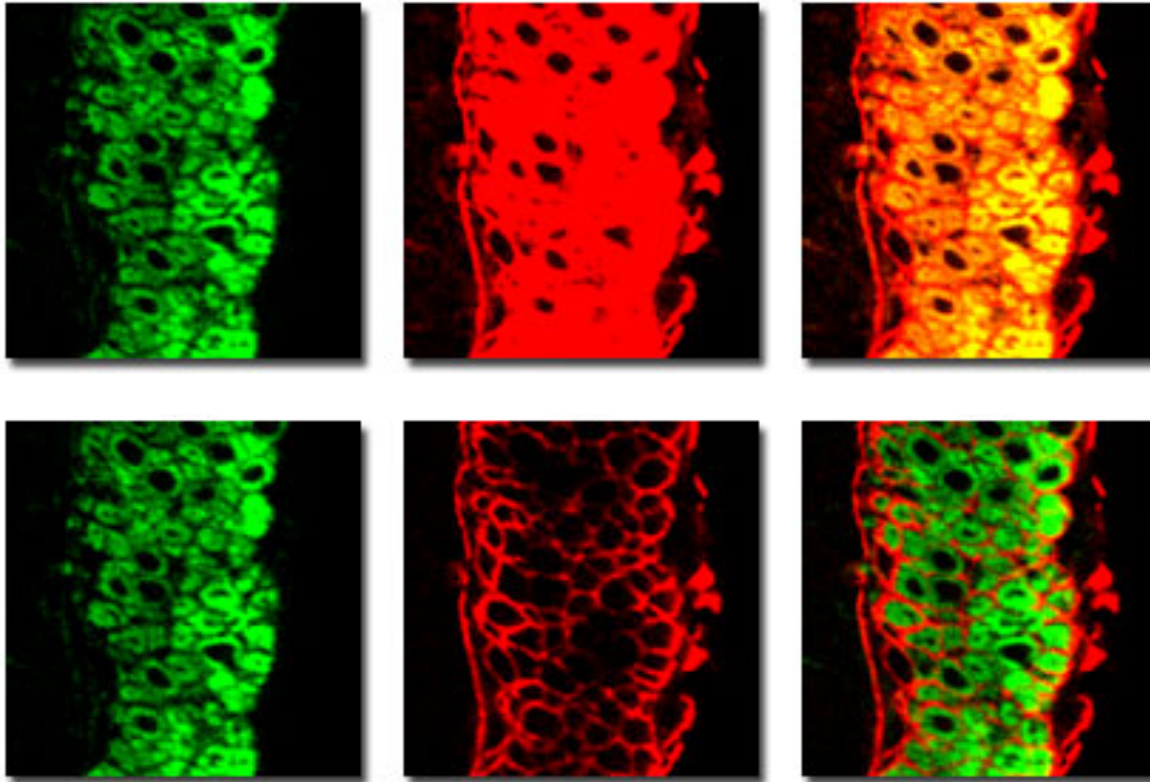
Excite at 477nm

Excite at 514nm

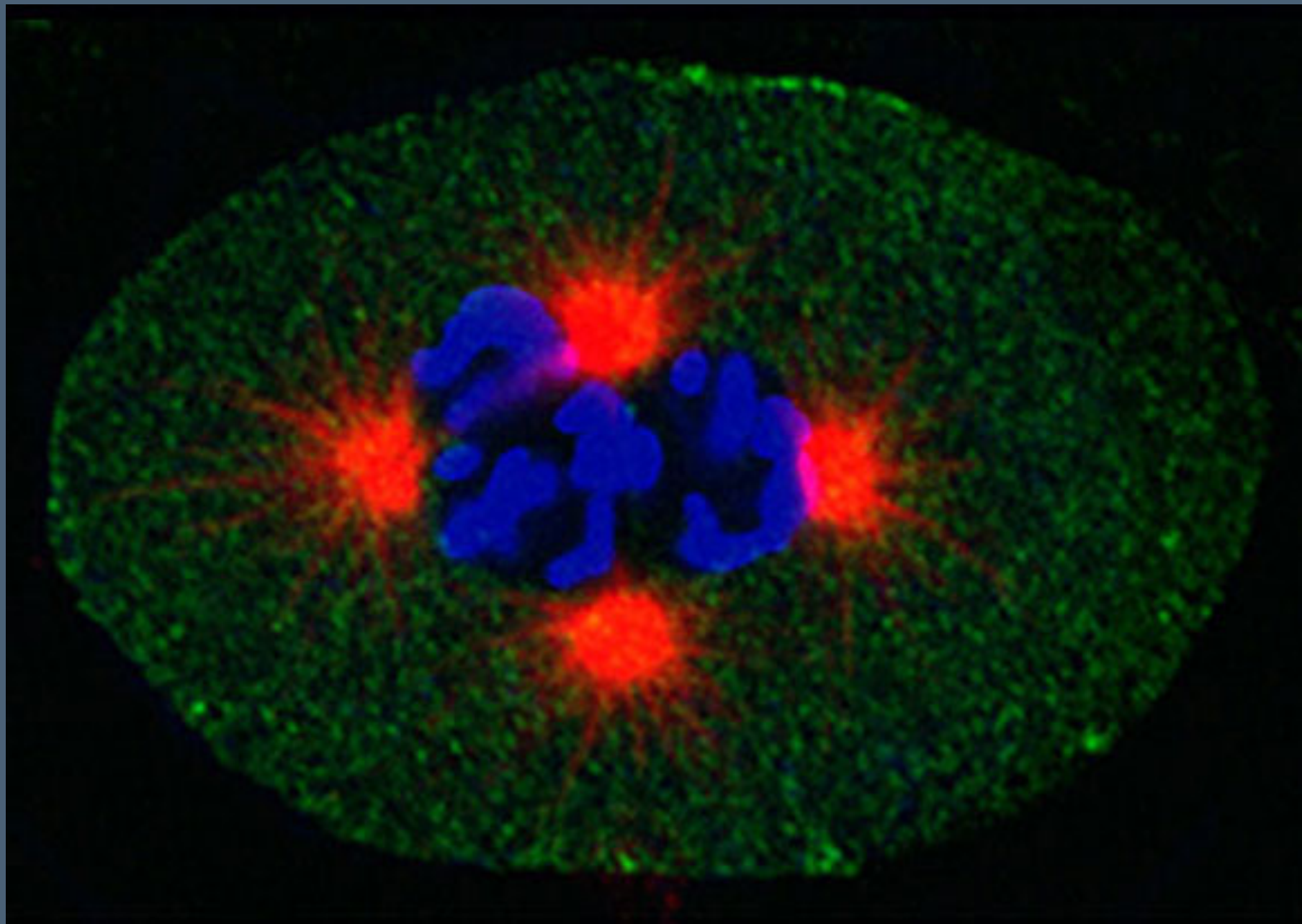


Temporal separation

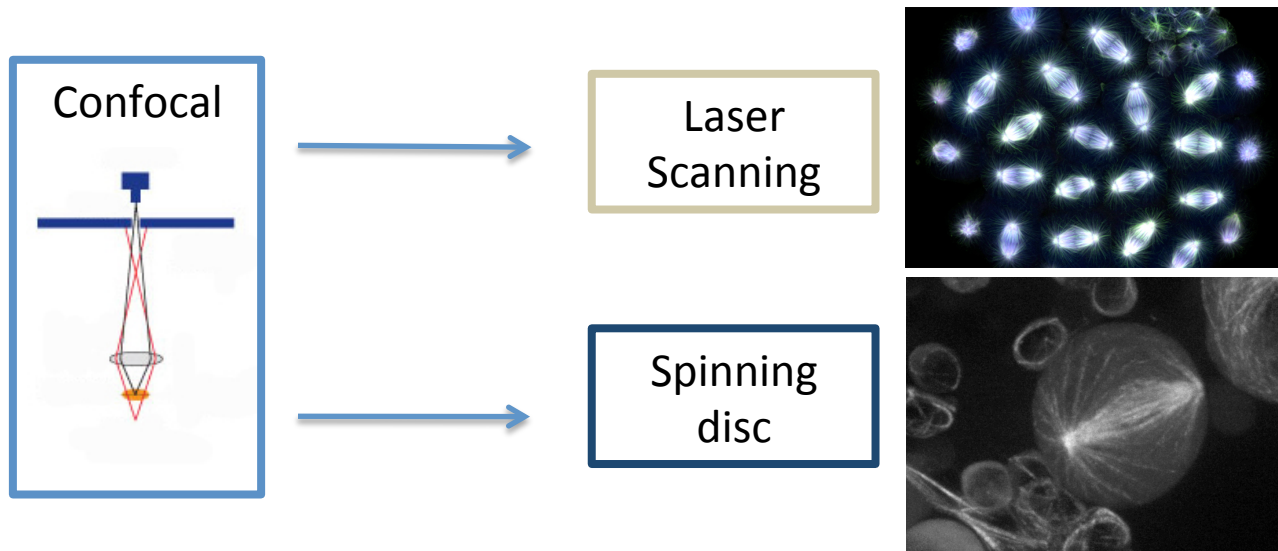
minimising 'bleed-through'



Adjust detector slit widths
Use sequential scanning

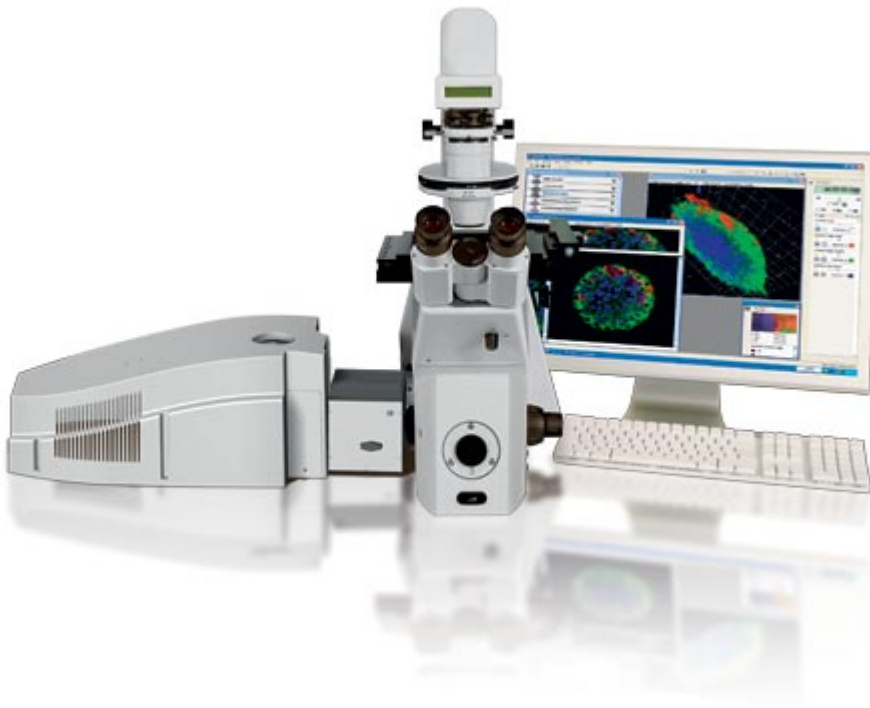


Confocal Microscopes



Both are confocals

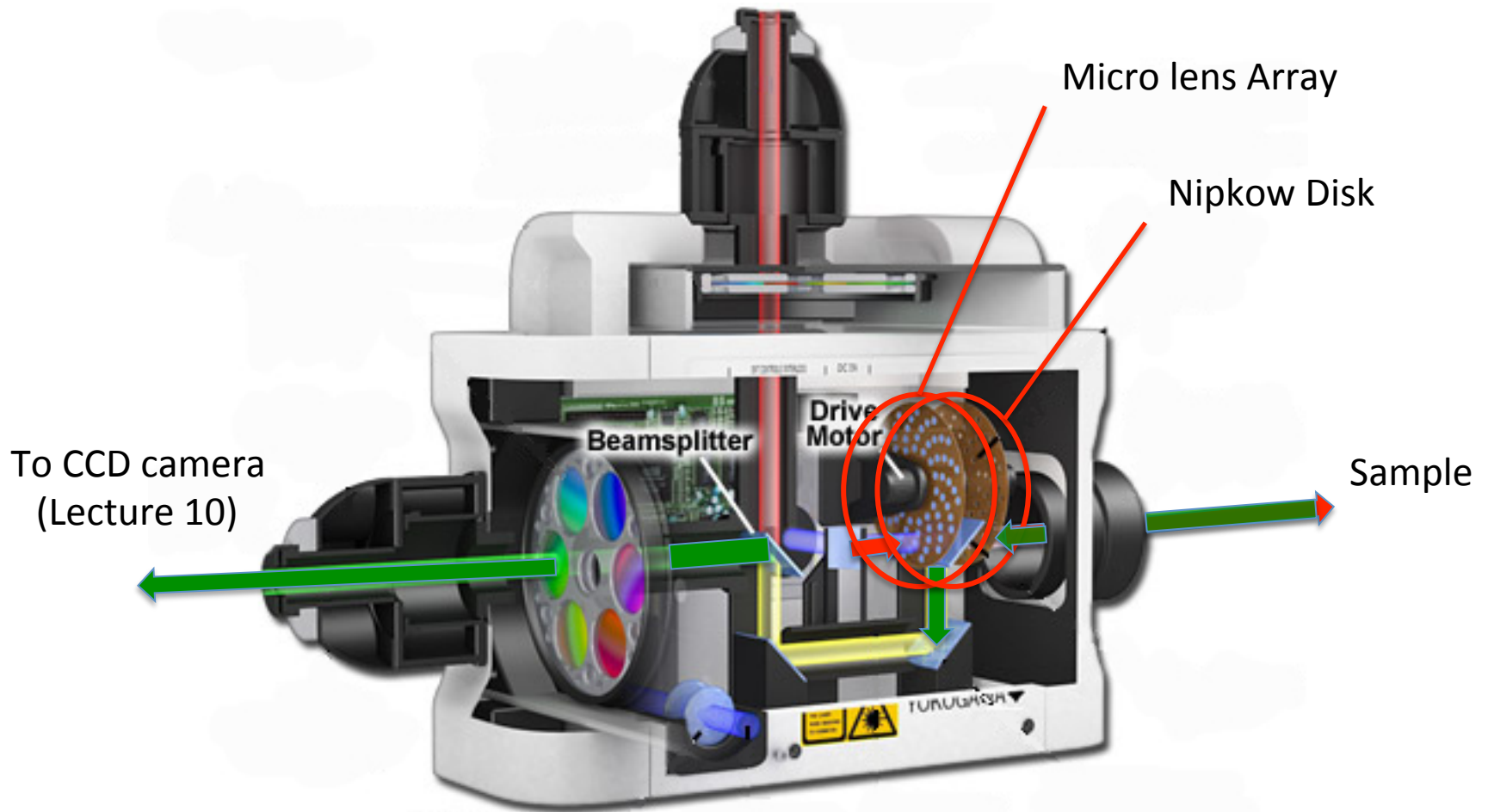
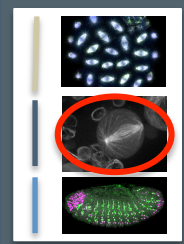
Spinning Disc Confocal



Great for live cell imaging

Can collect 2000 images per second

Yokogawa CSU-X1 Spinning Disc



Micro lens Array

Nipkow Disk

To CCD camera
(Lecture 10)

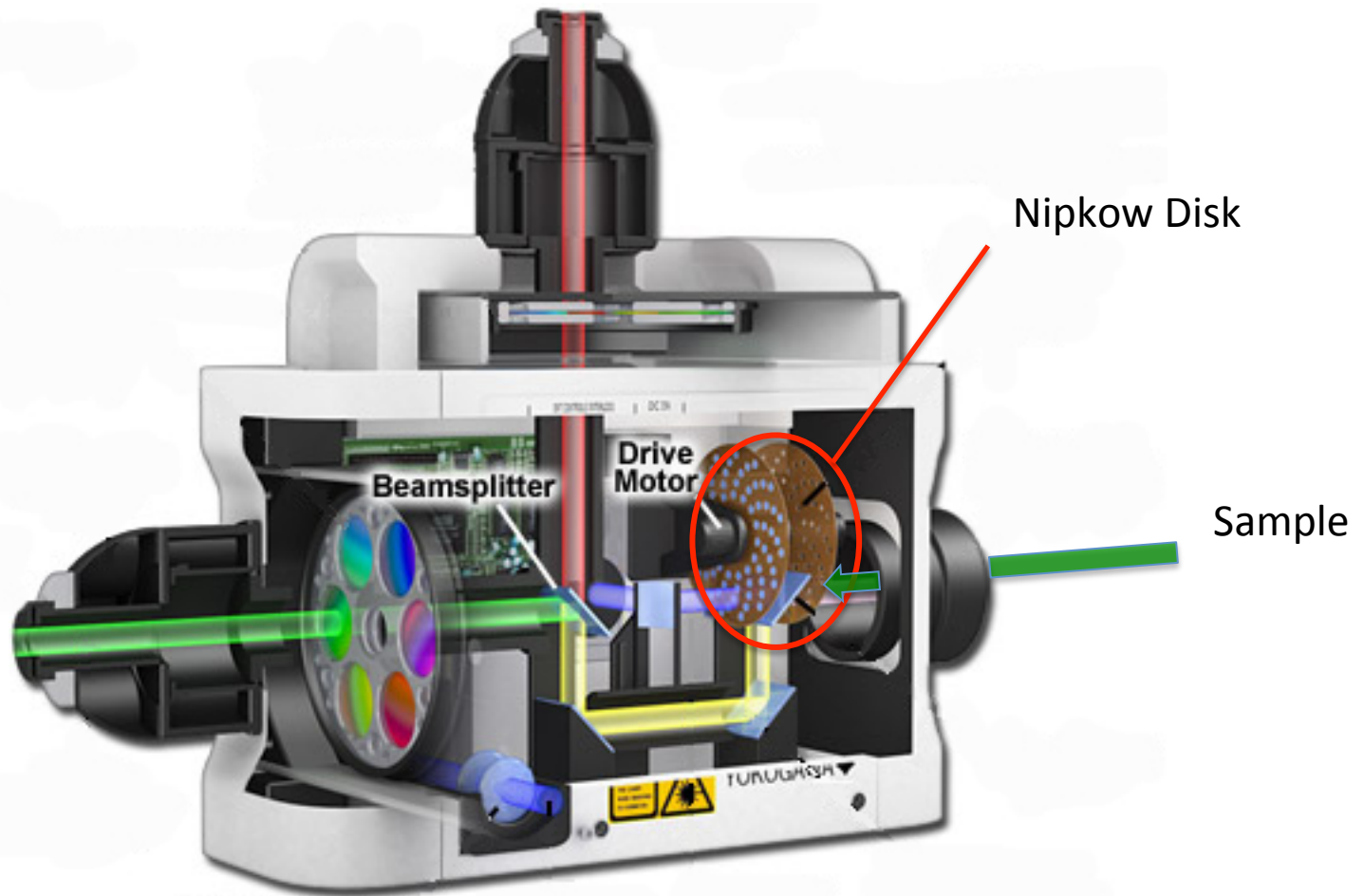
Sample

Beamsplitter

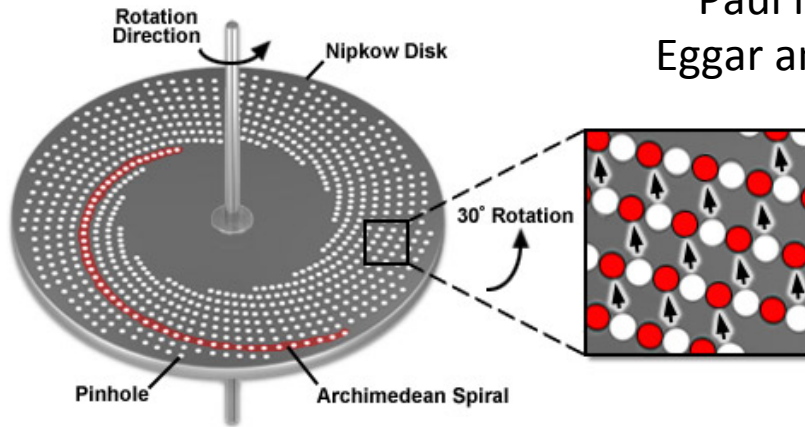
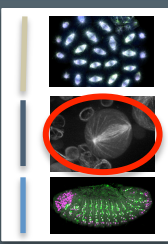
Drive Motor

YOKOGAWA

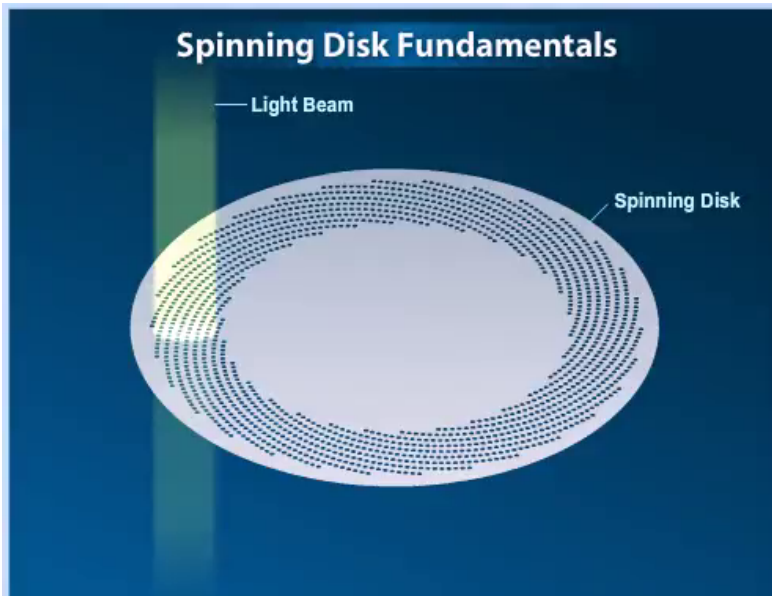
Yokogawa CSU-X1 Spinning Disc



The Nipkow Disk



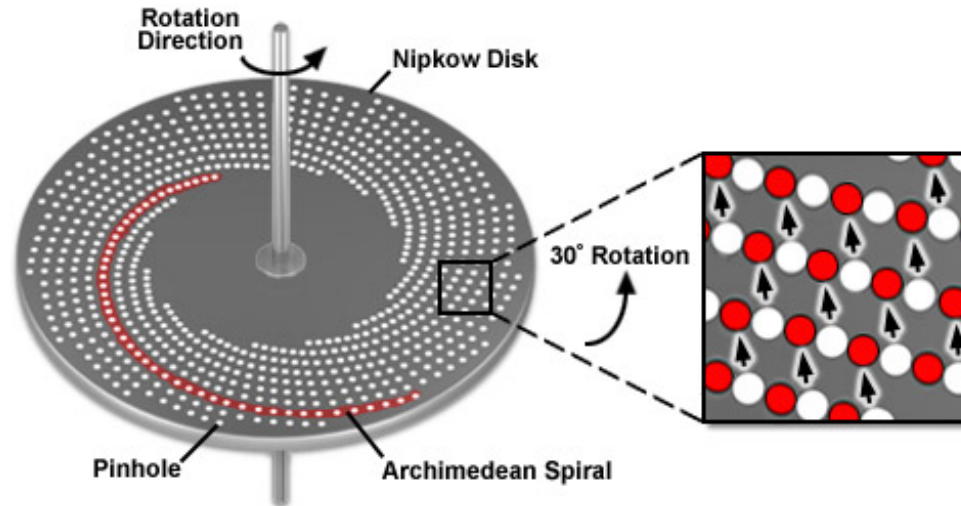
Paul Nipkow, 1884
Eggar and Petran, 1967



Approx. 1000 pinholes

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

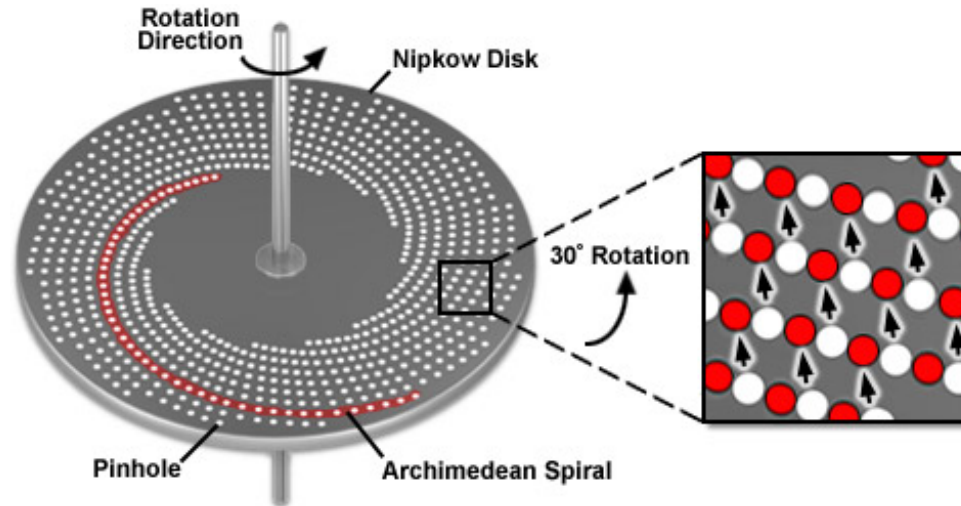
The Nipkow Disk



Larger pinholes - brighter image, but less “confocal”

Pinholes fixed size: Typically = 50 μ m
(optimised for biology)

The Nipkow Disk

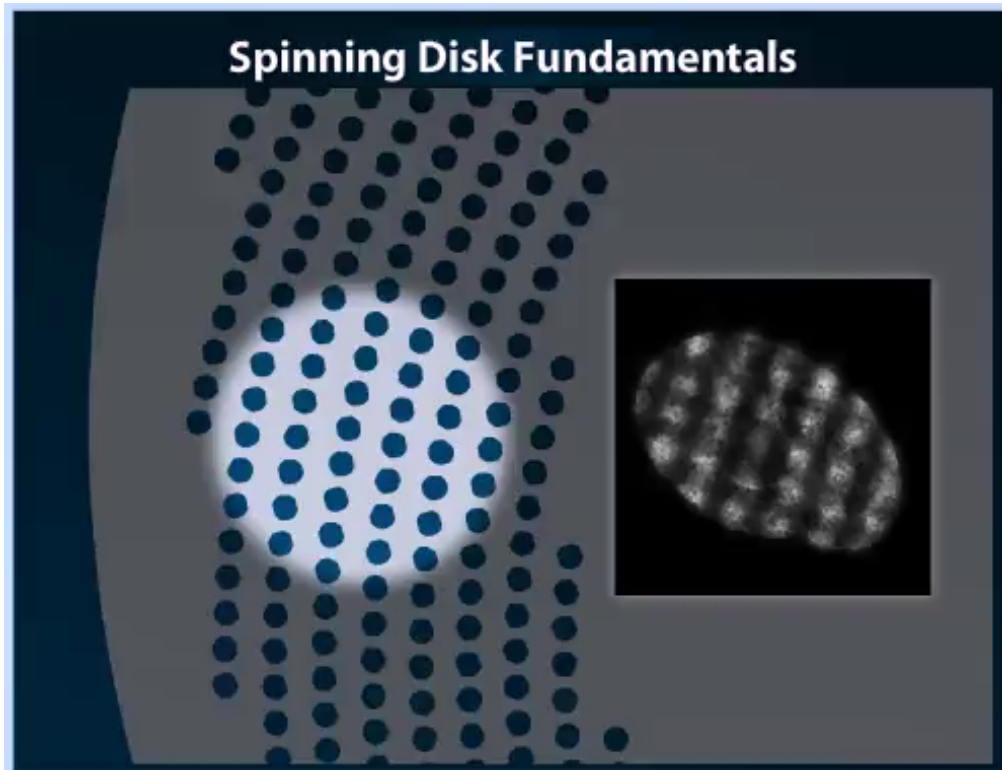


Constant Battle:

Smaller spacing - more light gets through, but "crosstalk"

Pinhole Spacing Typically = 2.5um apart

Spinning Disk Confocal



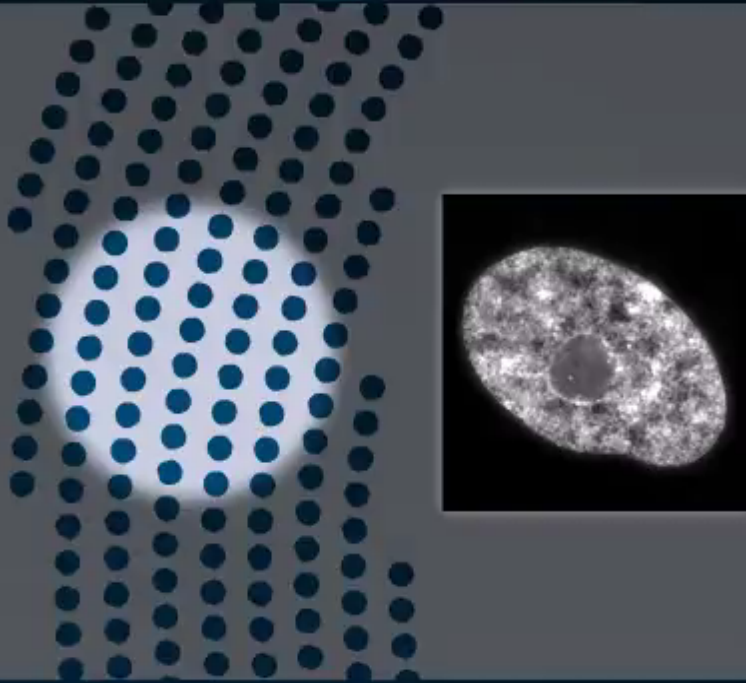
Disc spins to illuminate sample

spin speed – disc spinning
too slowly

you see the pinholes on the
sample

Spinning Disc Confocal

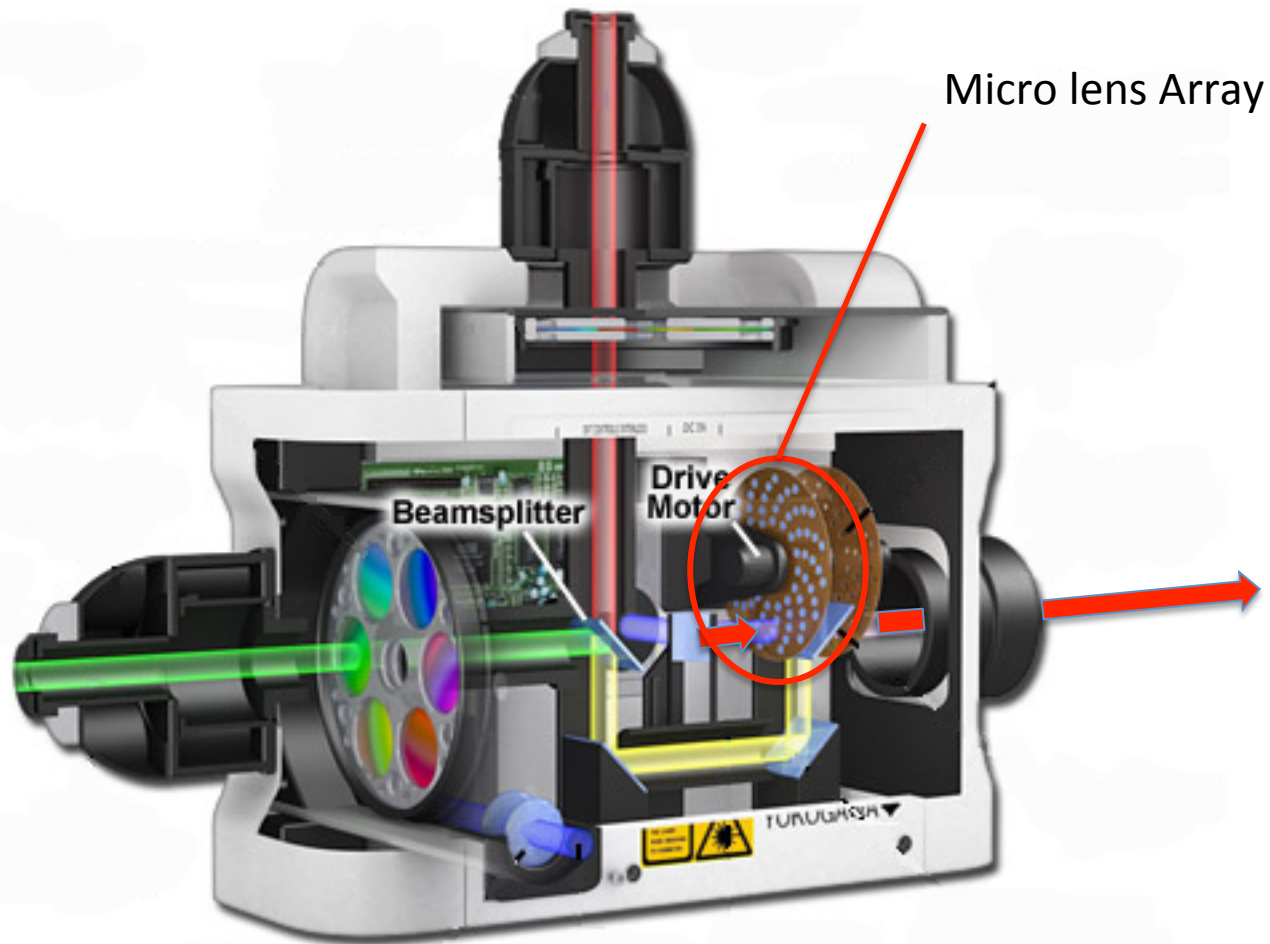
Spinning Disc Fundamentals



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

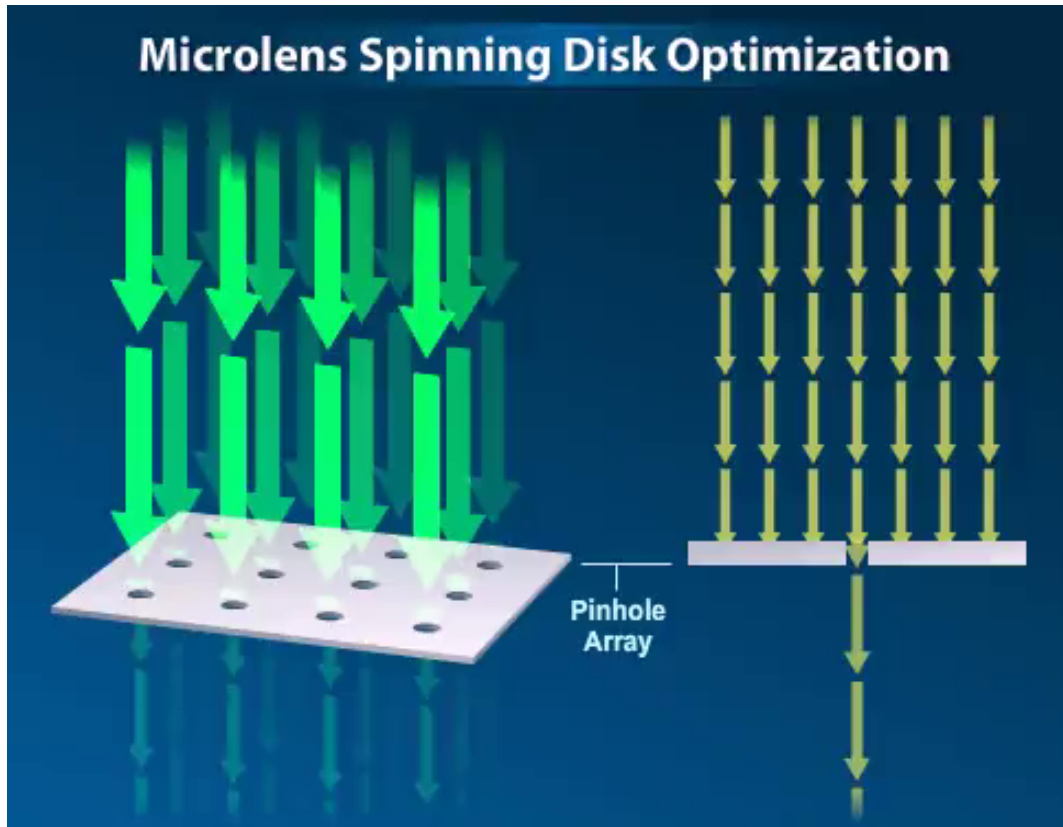
Disc speed must carefully match camera frame rate

Yokogawa CSU-X1 Spinning Disc



Yokogawa Spinning Disc Confocal Microlenses

Microlens Spinning Disc Optimization

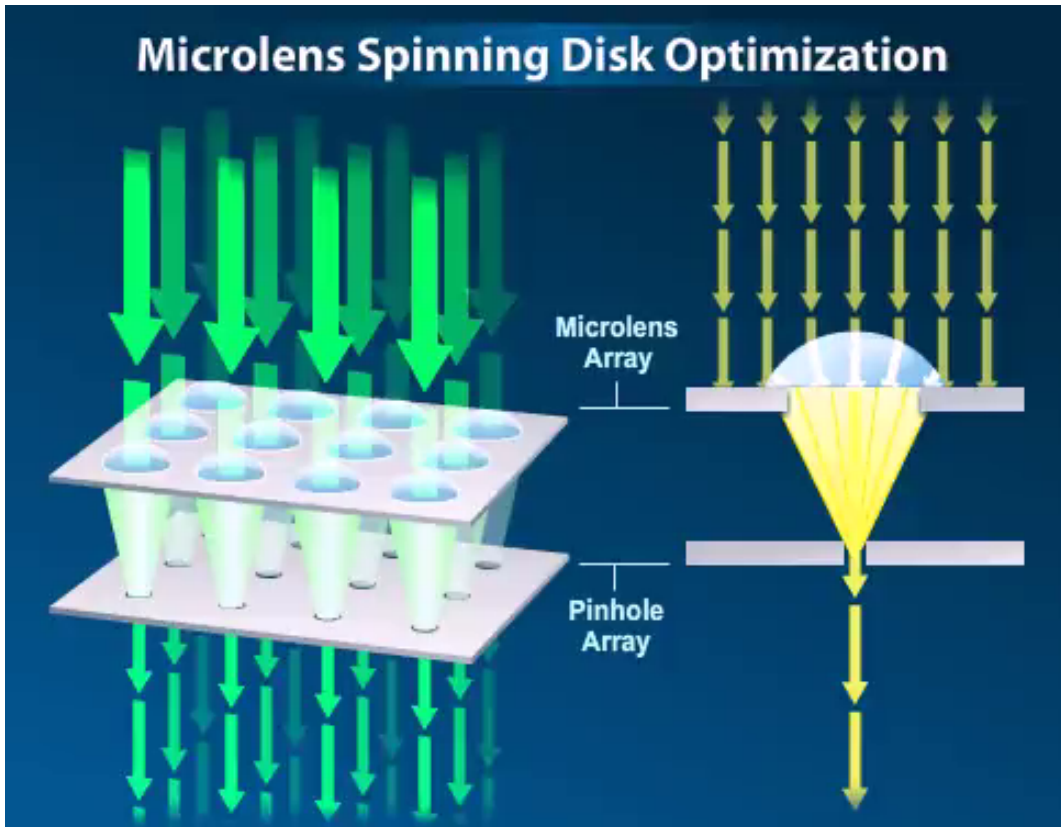


just a pinhole array –
Optimised for ‘cofocality’
and ‘crosstalk’

too much light is blocked
from reaching the specimen

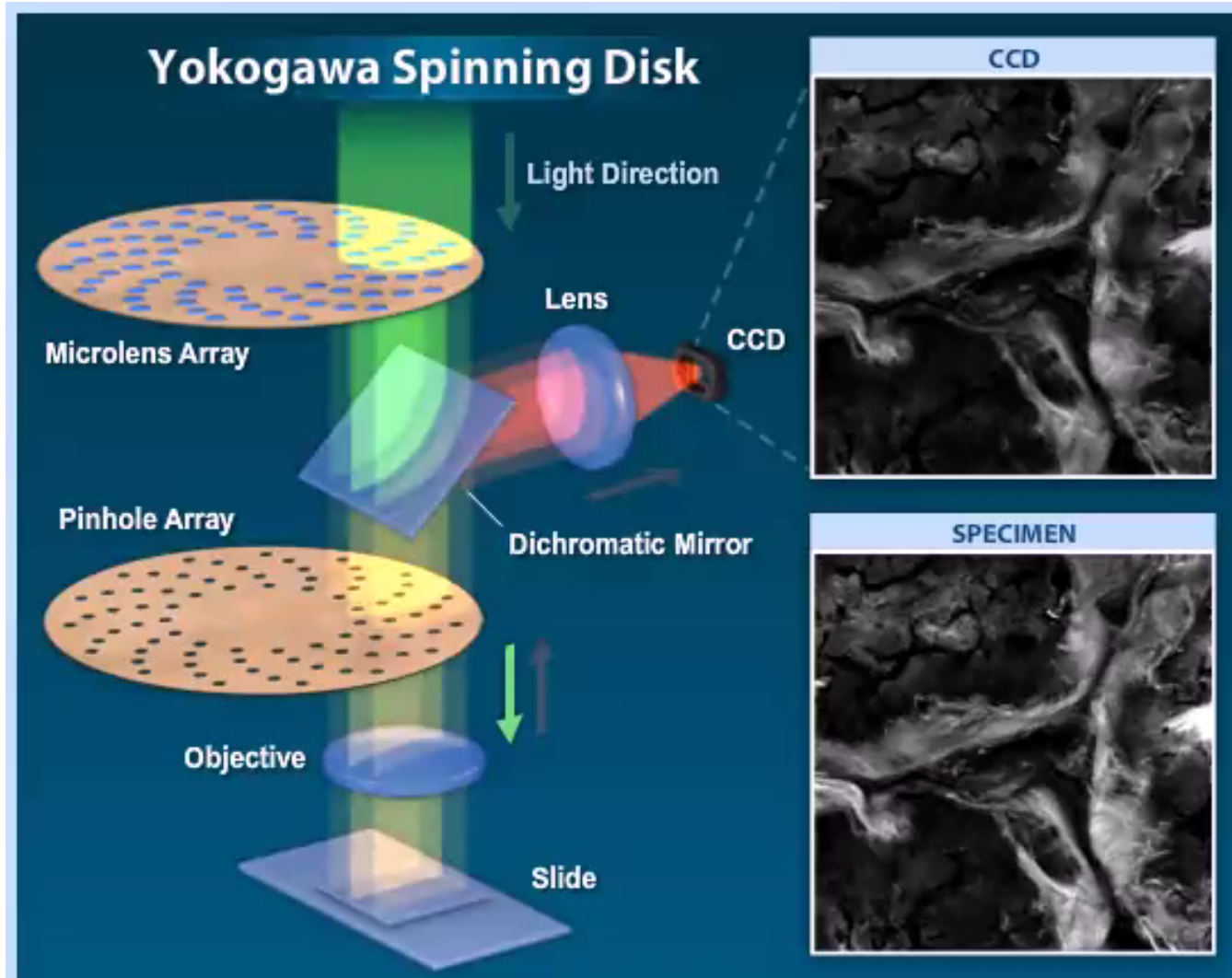
Only 4% light passes through disc

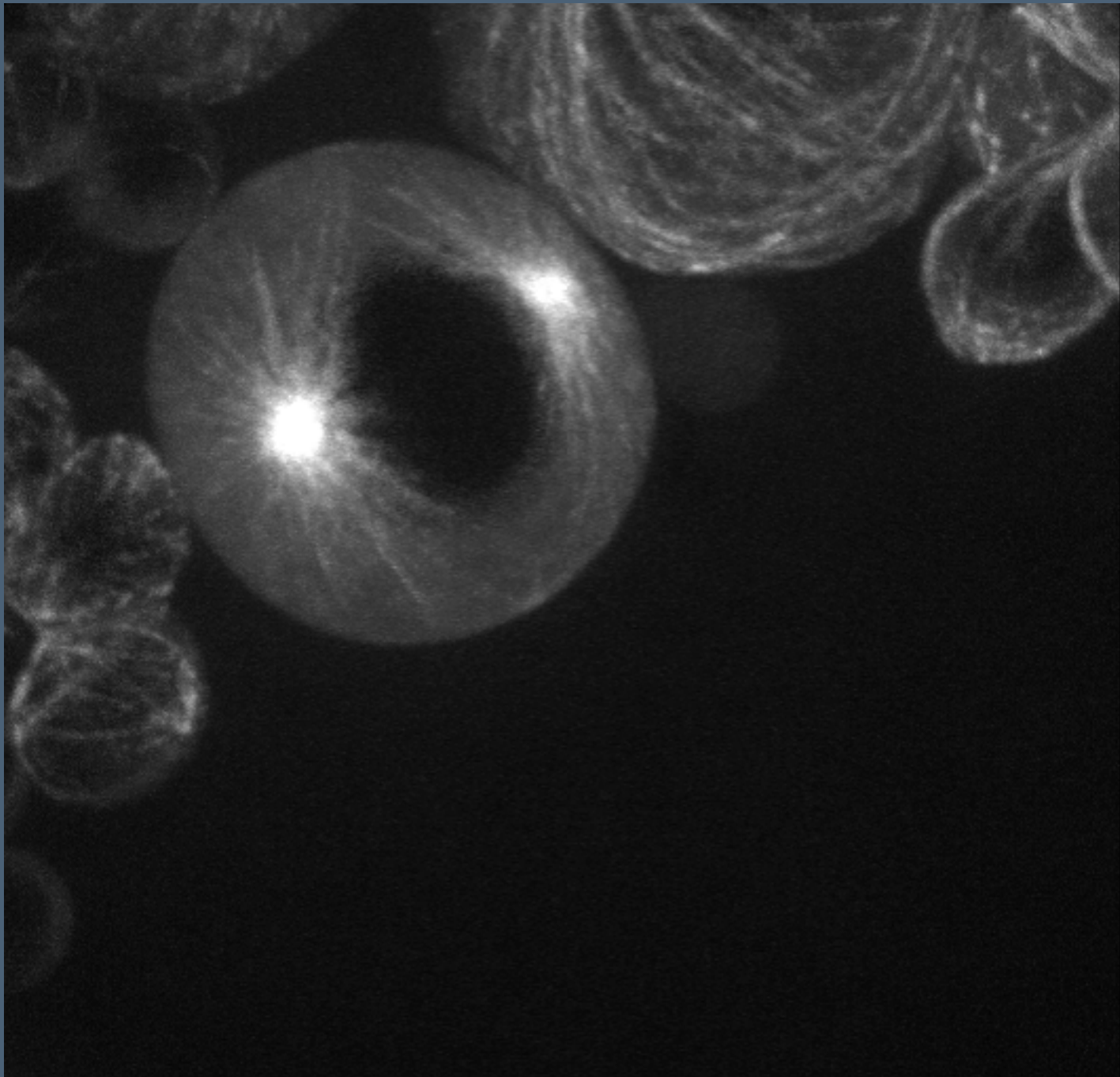
Yokogawa Spinning Disc Confocal Microlenses



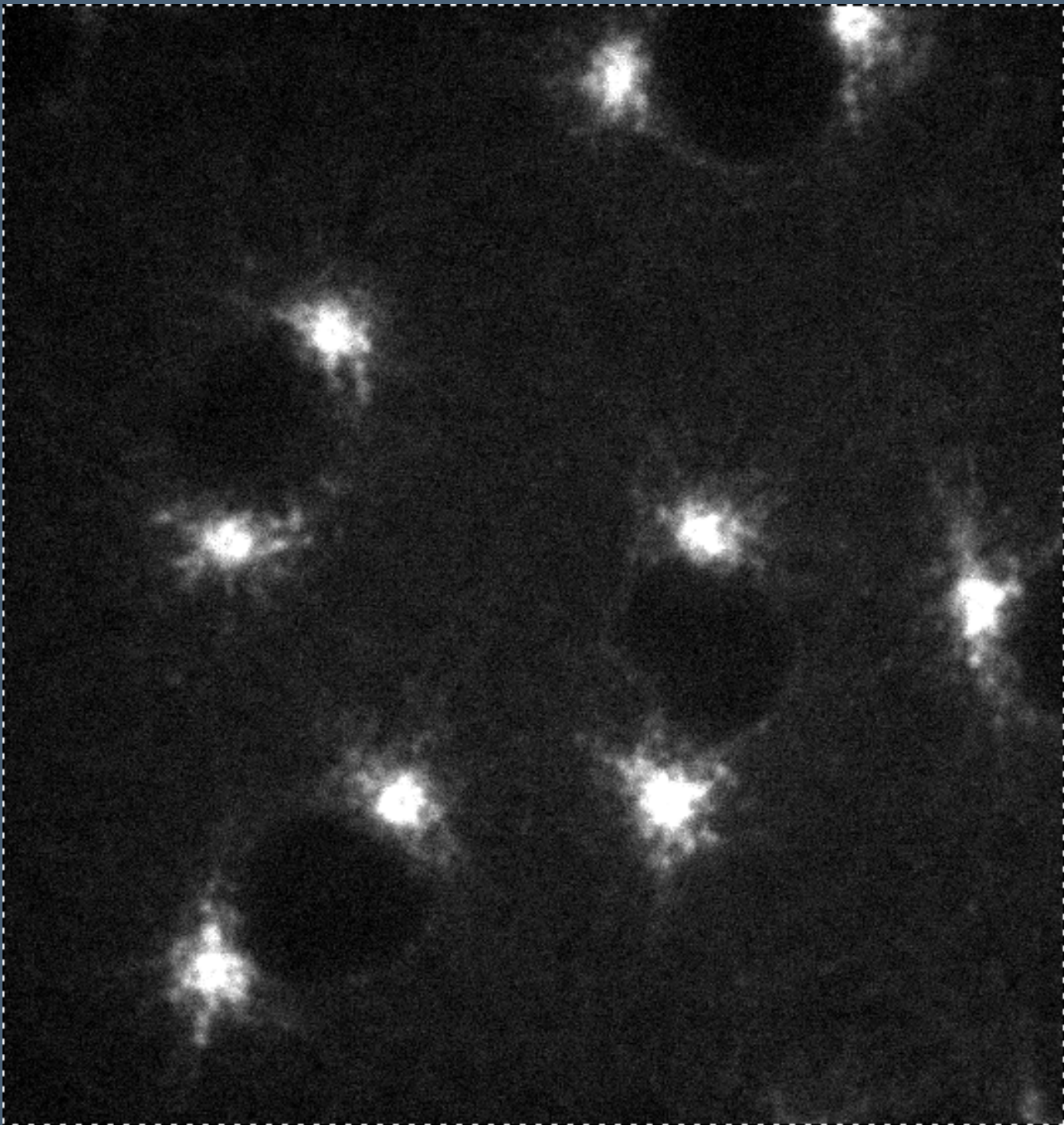
Typically 56% light passes through disc

Yokogawa Spinning Disc Confocal

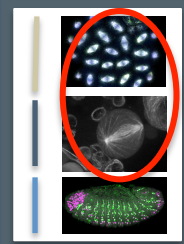




Cell division in brain stem cells (neuroblasts), Raff Lab

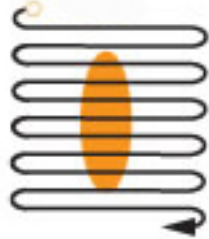


MT binding protein in *Drosophila* embryo, Raff Lab



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!

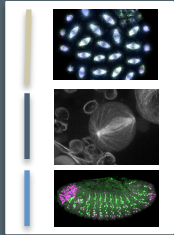
Pretty damn good!

Pretty Movies

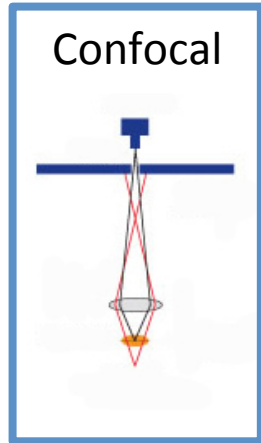
Good – if process slow

Unbeatable!

3 Flavours of Microscope

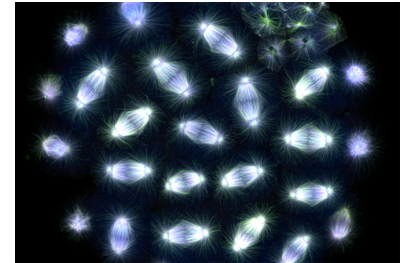


Problem:
Out of Focus
Light

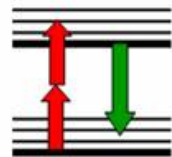
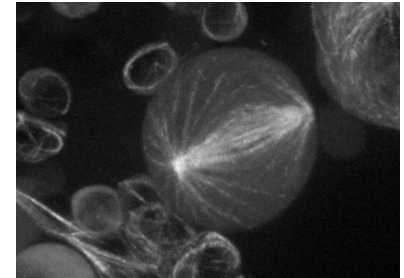


Confocal

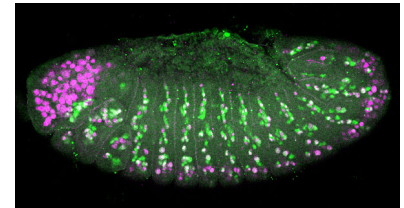
Laser
Scanning



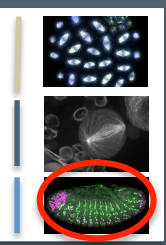
Spinning
disc



2-Photon



2-photon Microscope



Not a 'confocal'

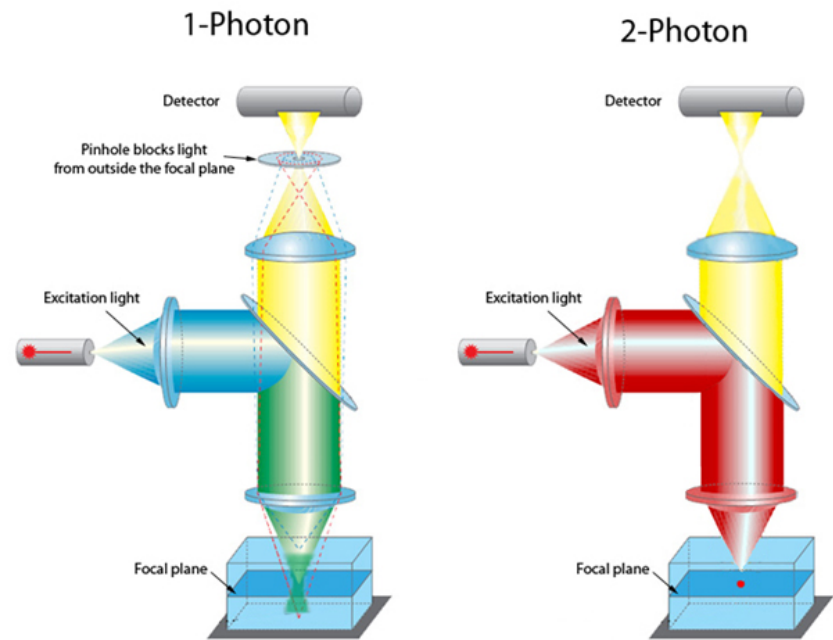
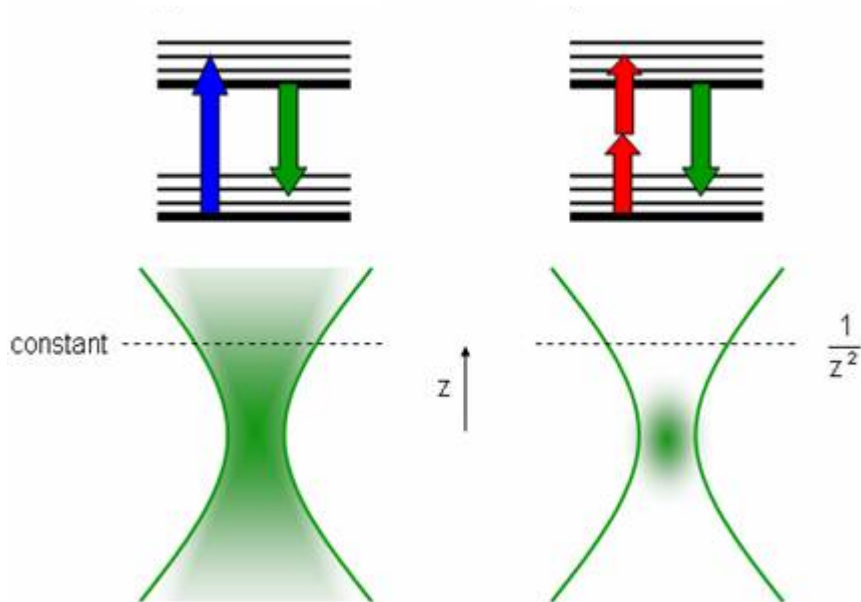
for imaging deeper into thick specimens

less damaging to biological samples

Confocal Vs 2-photon

1 Photon Excitation

2 Photon Excitation

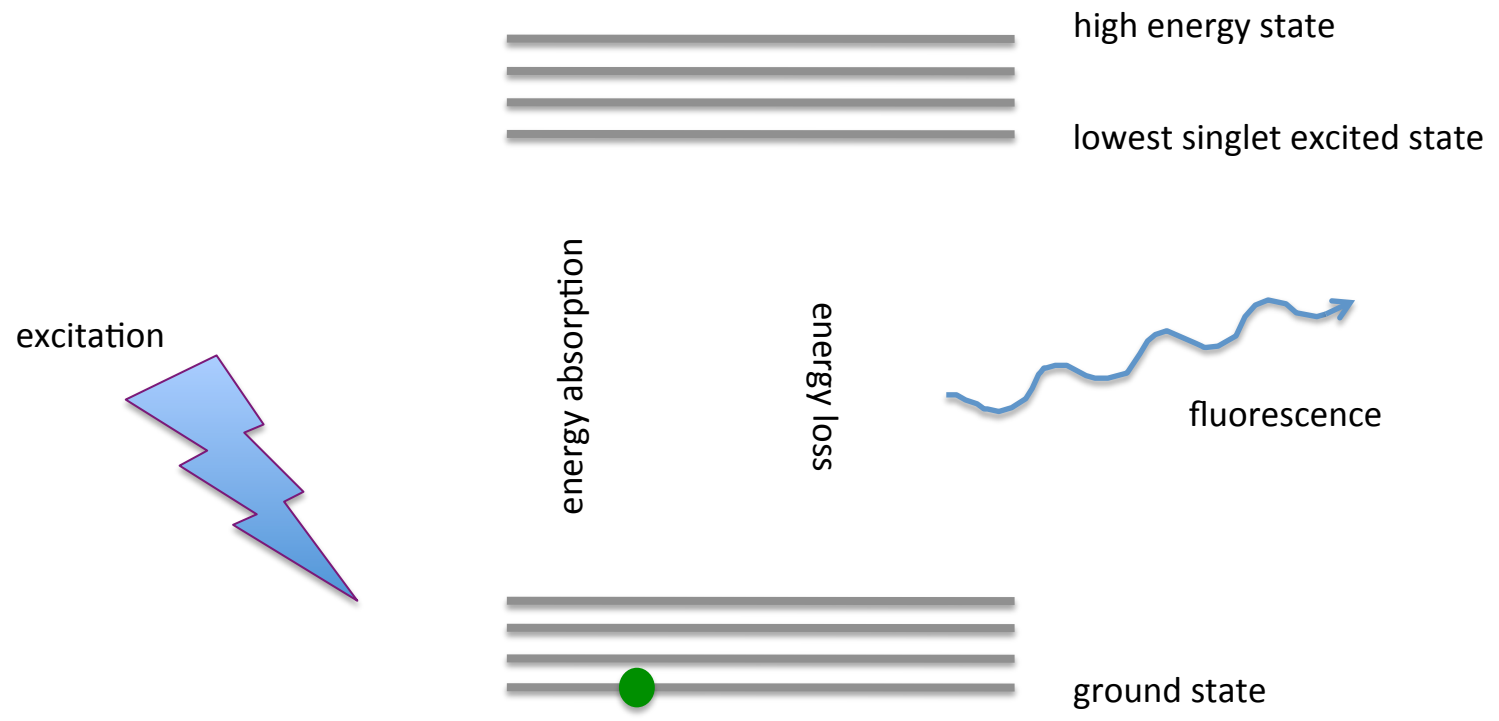


There is no out of focus light



THEORY

1 Photon Excitation





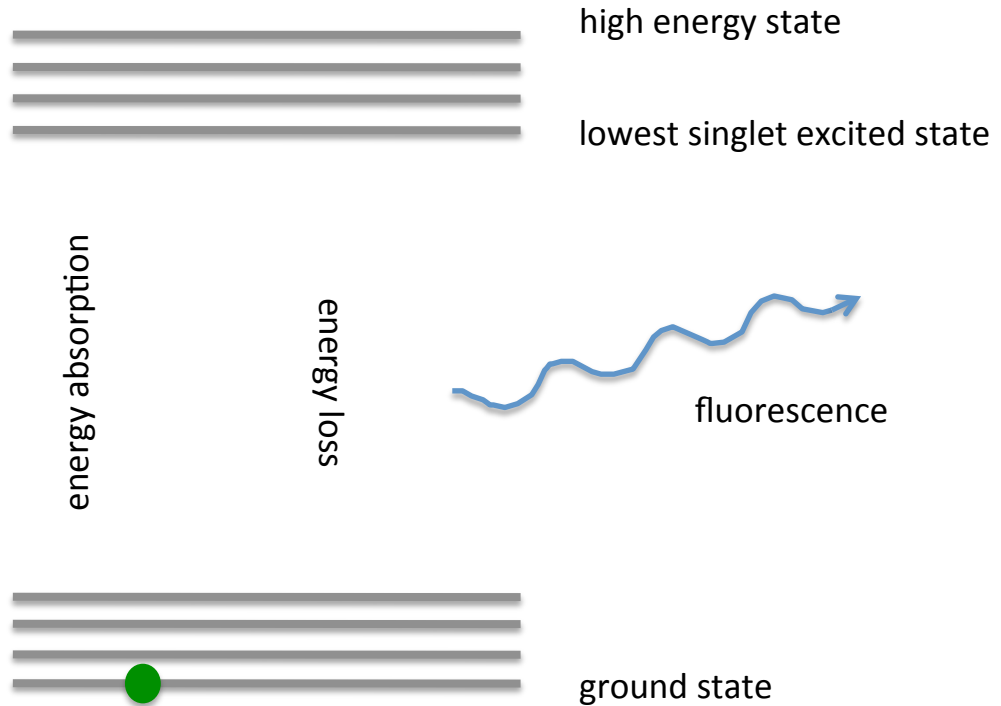
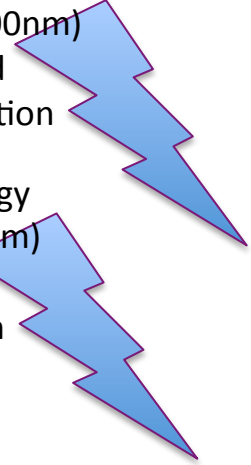
THEORY

2 Photon Excitation

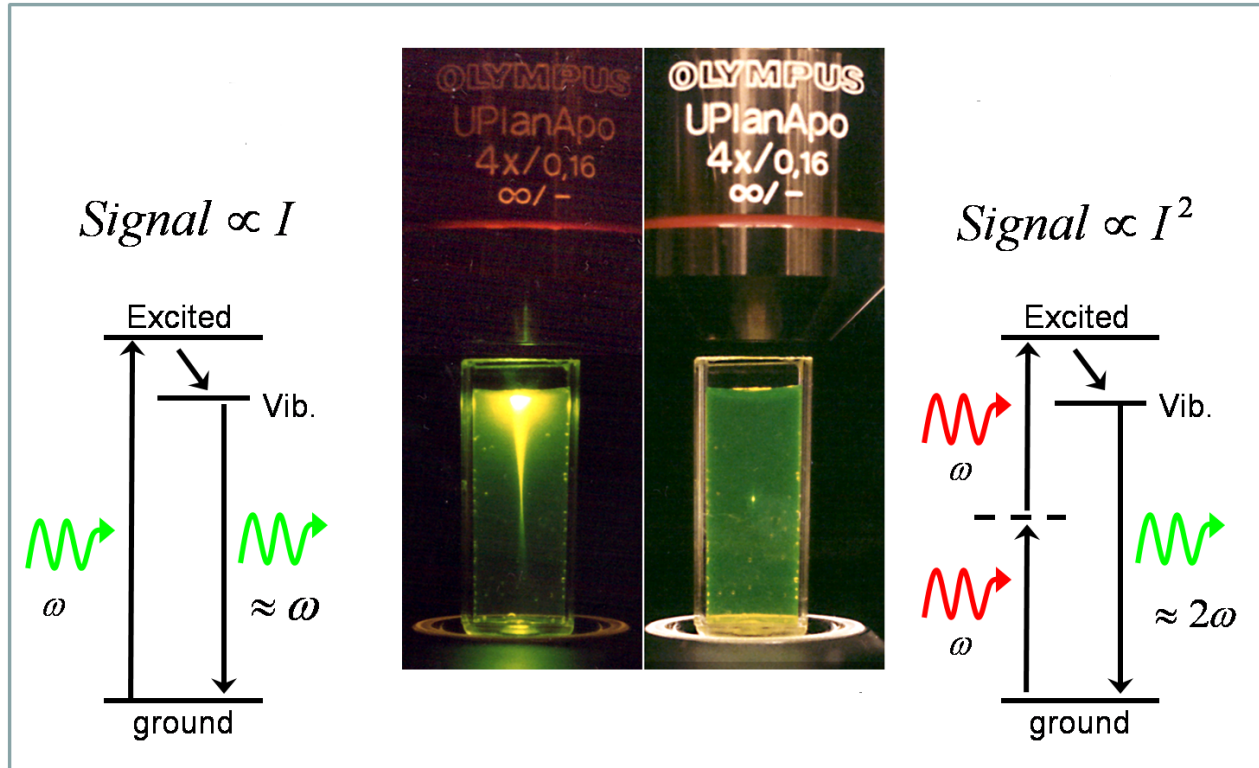
Almost simultaneous

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

Low energy
(IR ~700nm)
Pulsed
excitation

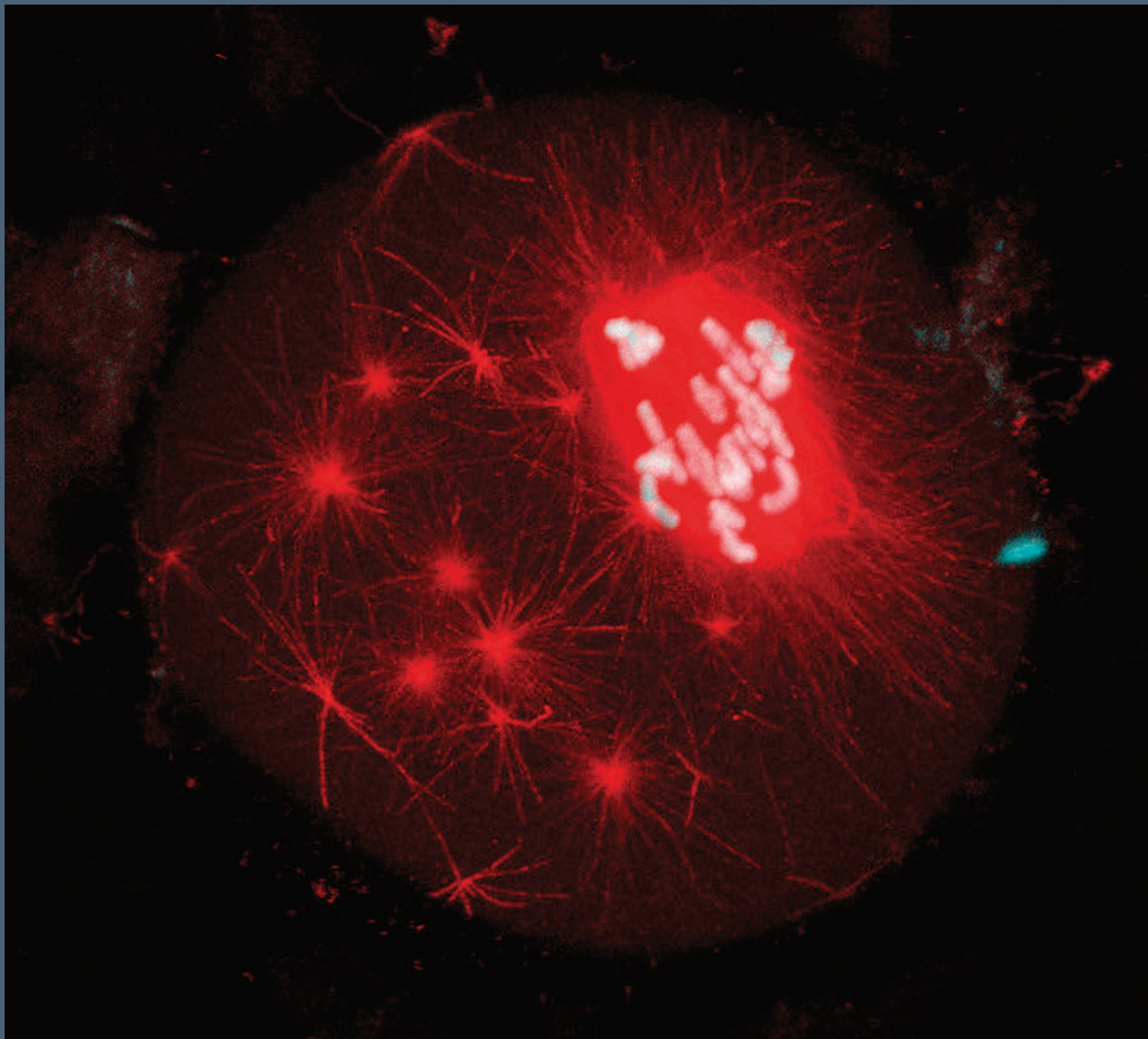


Principle of 2-photon Microscope



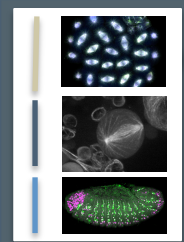
Near simultaneous, two photon event highly unlikely, only really possible a focal point

Tightly focused excitation

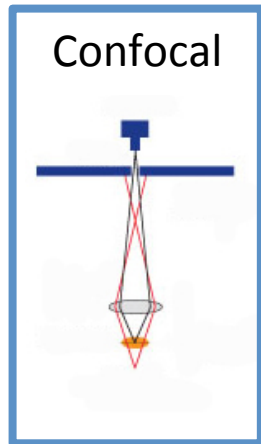


Spindle formation in mouse oocyte, labelled with Hoechst, Alexa 680. M Schuh. EMBL, Heidelberg, Germany

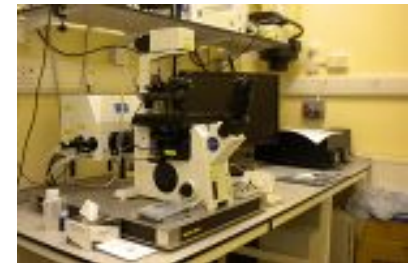
3 Flavours of Microscope



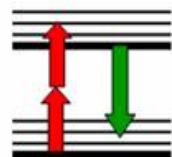
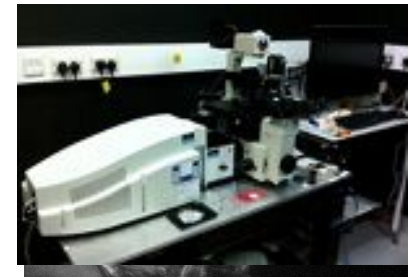
Problem:
Out of Focus
Light



Laser
Scanning



Spinning
disc



2-Photon





<http://www.micron.ox.ac.uk>

Micron
OXFORD