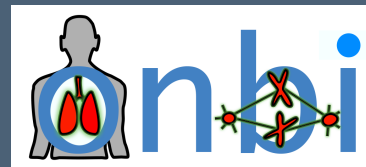
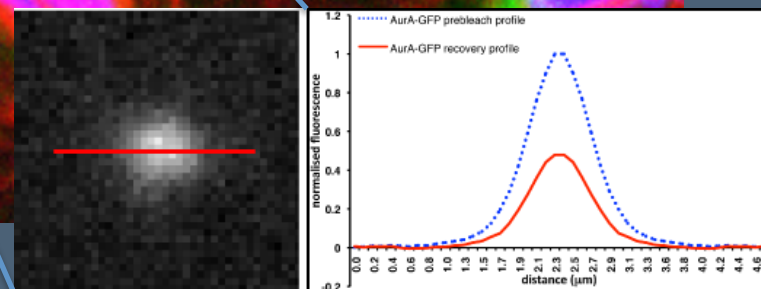
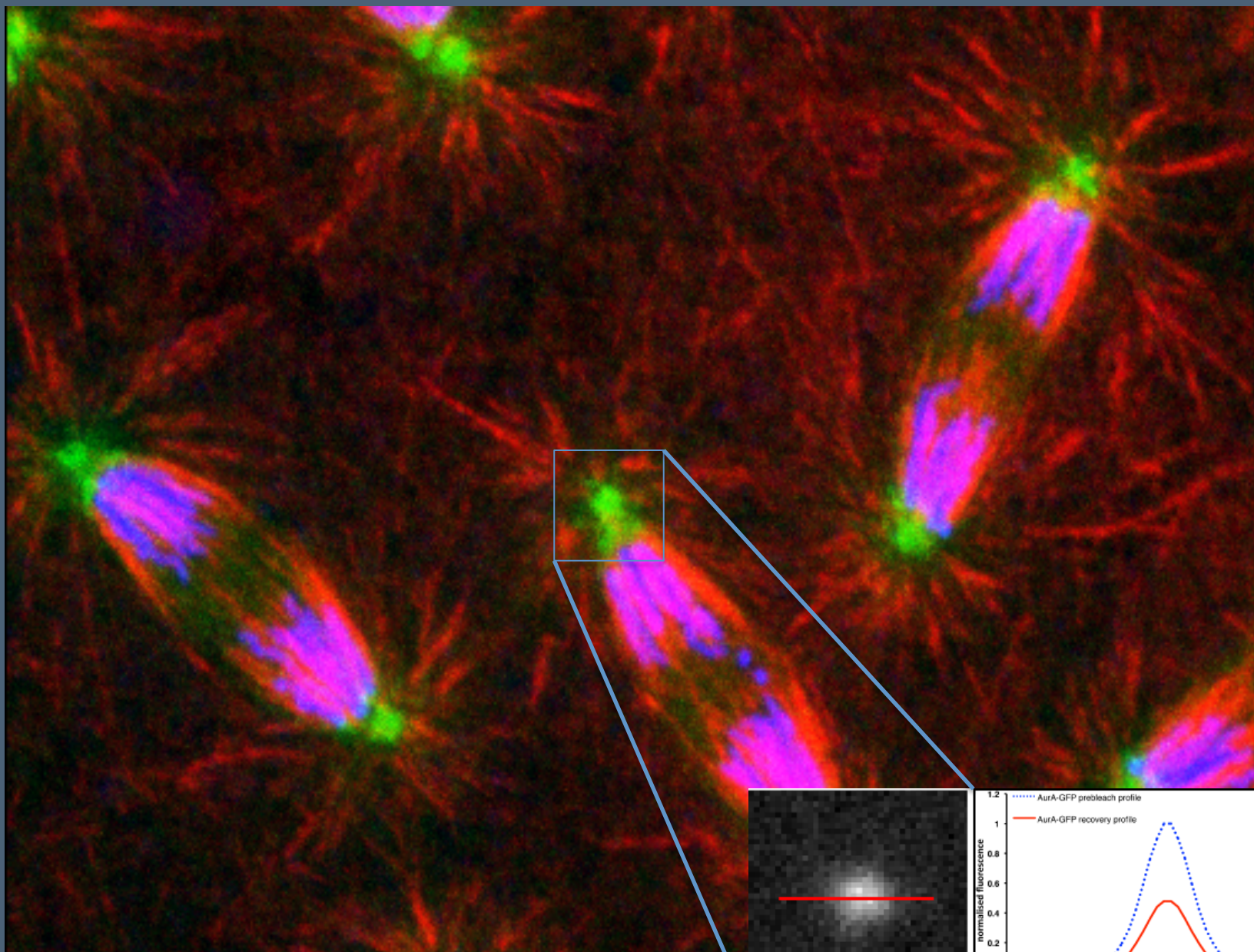


Confocal Microscopy

Lecture 9

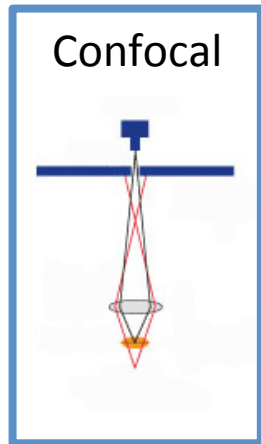
12th November 2014



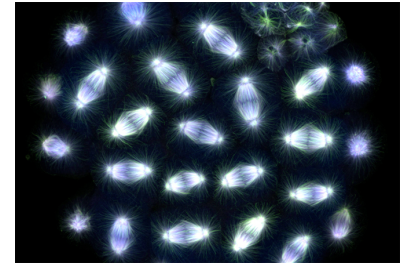


3 Flavours of Microscope

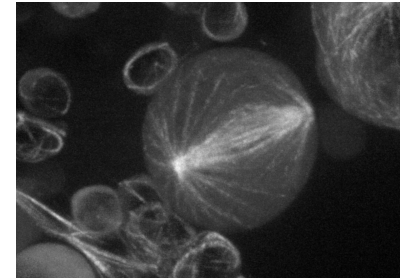
Problem:
Out of Focus
Light



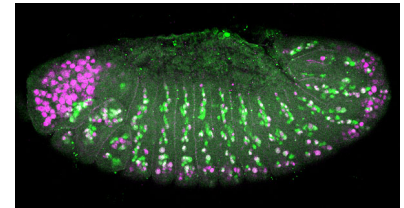
Laser
Scanning



Spinning
disc



2-Photon



A short History of Confocal Microscope



Confocal “concept” patented by Marvin Minsky in 1957



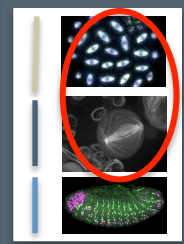
Eggar and Petran developed “spinning disc” confocal in late 1960s

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



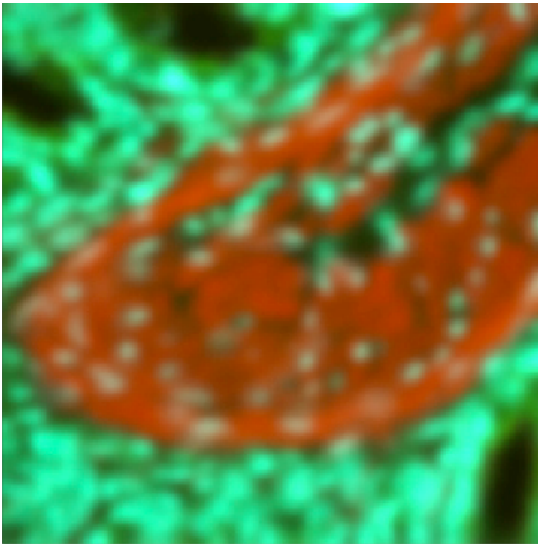
White, Amos and Wilson developed the MRC500 point scanning confocal
-Marketed commercially in 1987





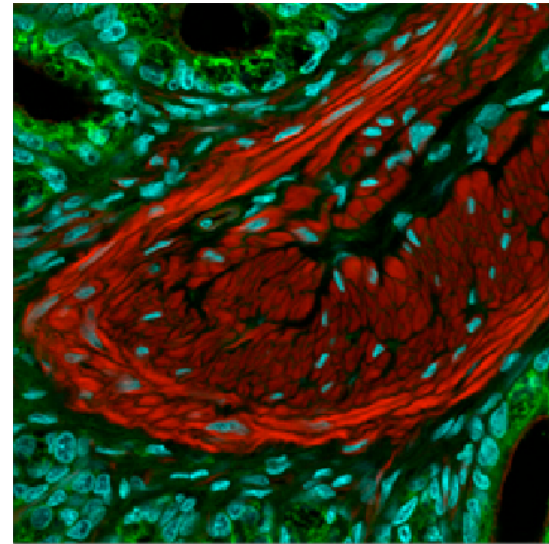
Comparison Widefield Vs Confocal

Widefield



Out of focus light 'blurs' image

Confocal

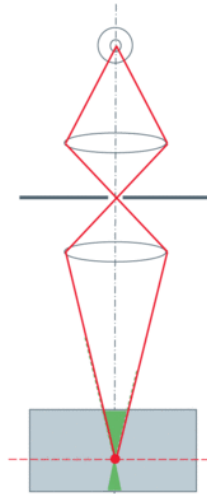


Out of focus light is blocked



Principle of Confocal Microscopes

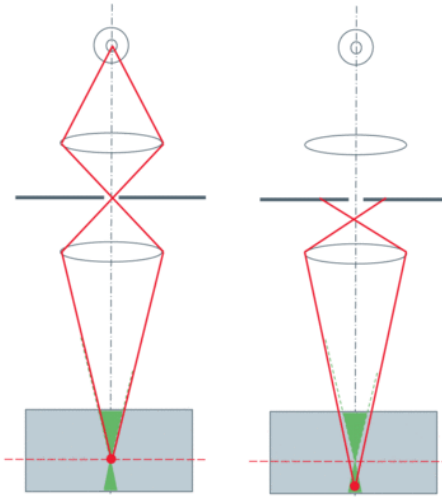
Pinhole



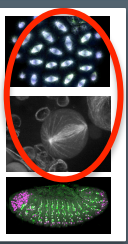
Pinhole diaphragm in the
Conjugated focal plane =
CONFOCAL

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

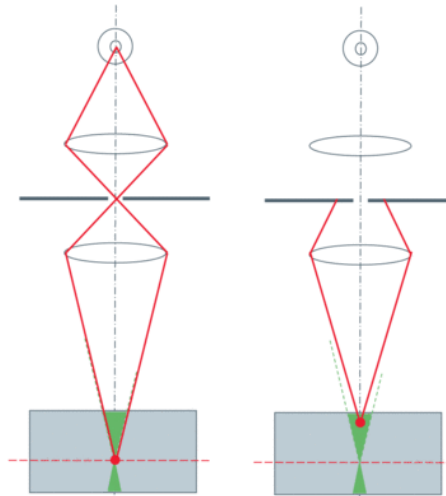
Pinhole – blocks out-of-focus light



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

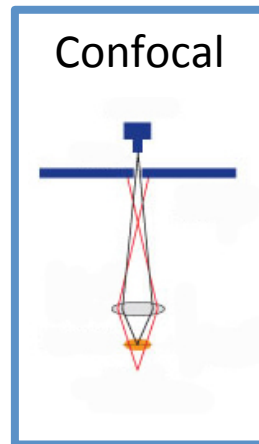


Pinhole – blocks out-of-focus light

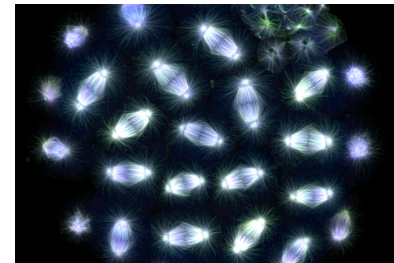


light from above the optical section also doesn't
pass through the pinhole aperture

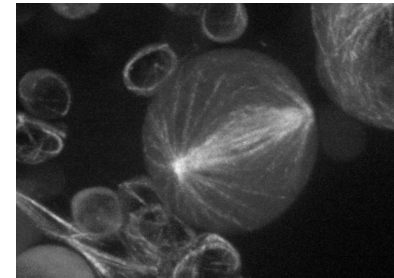
Confocal Microscopes

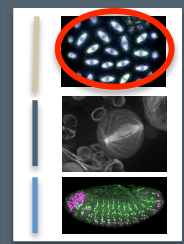


Laser
Scanning



Spinning
disc

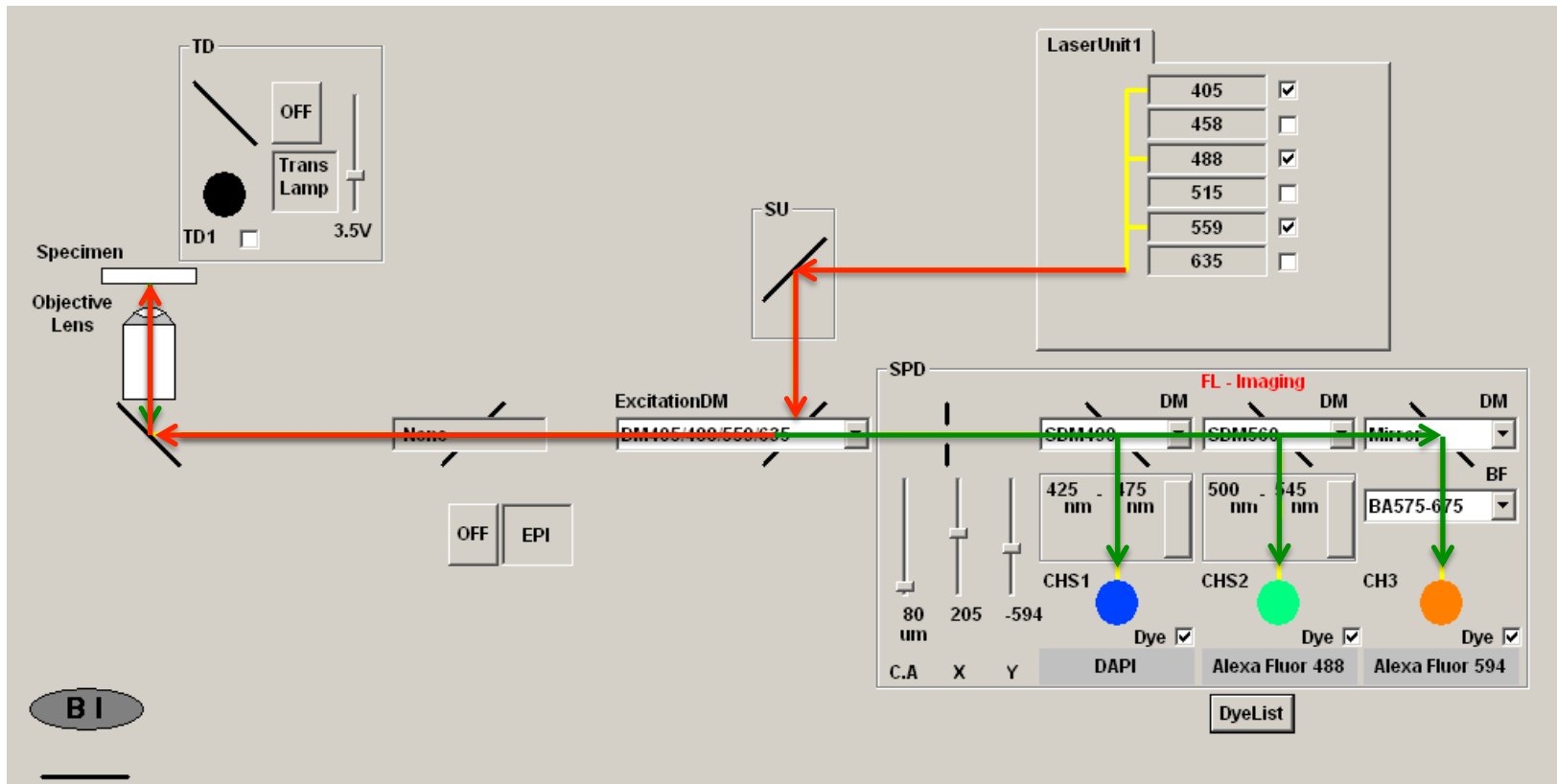




Laser Scanning Confocal Microscope

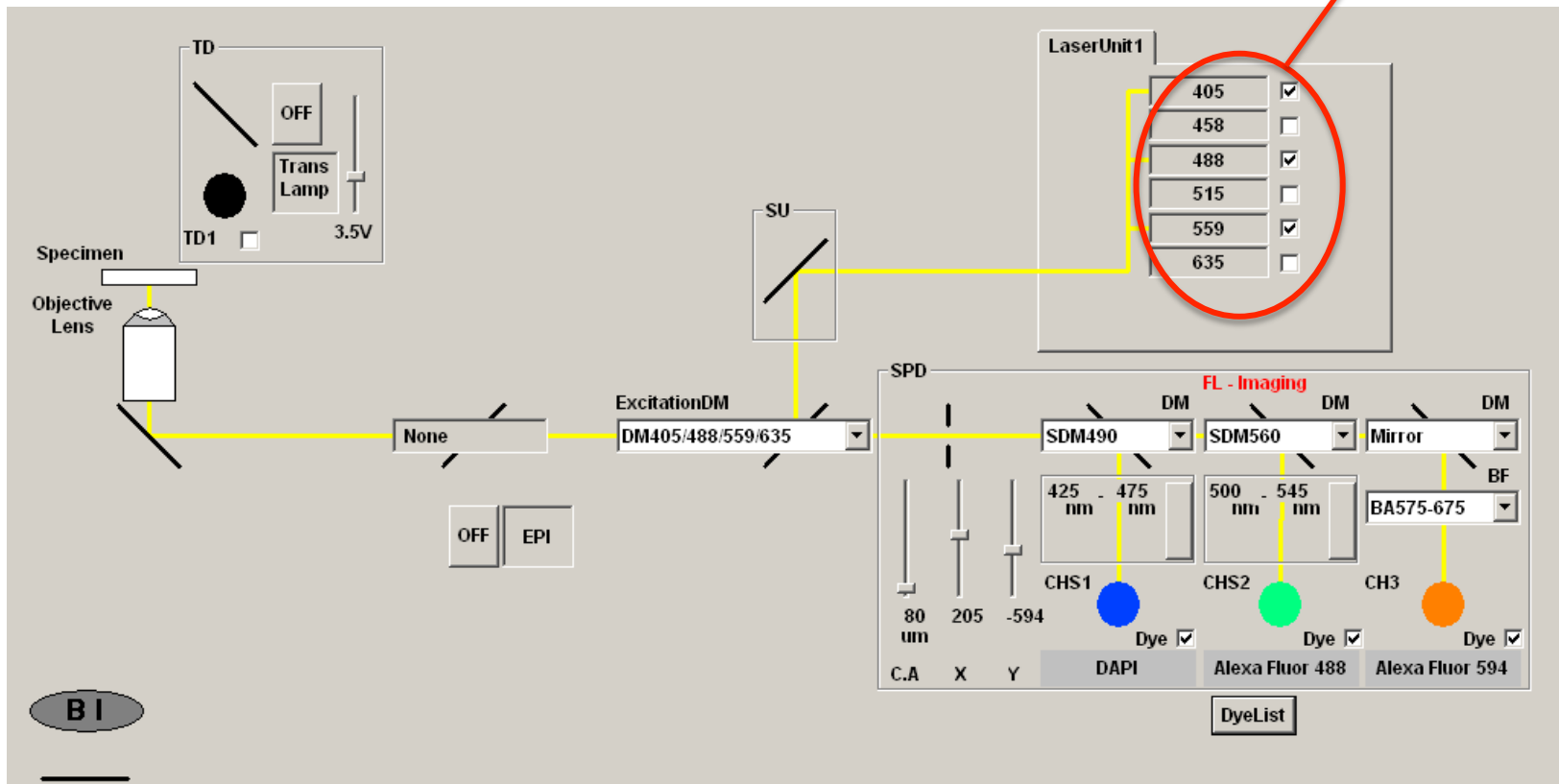


Laser Scanning Confocals are great to get 'pretty' images



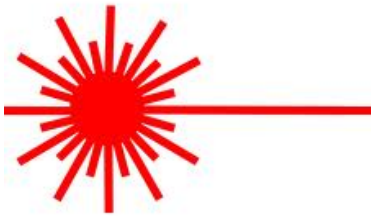
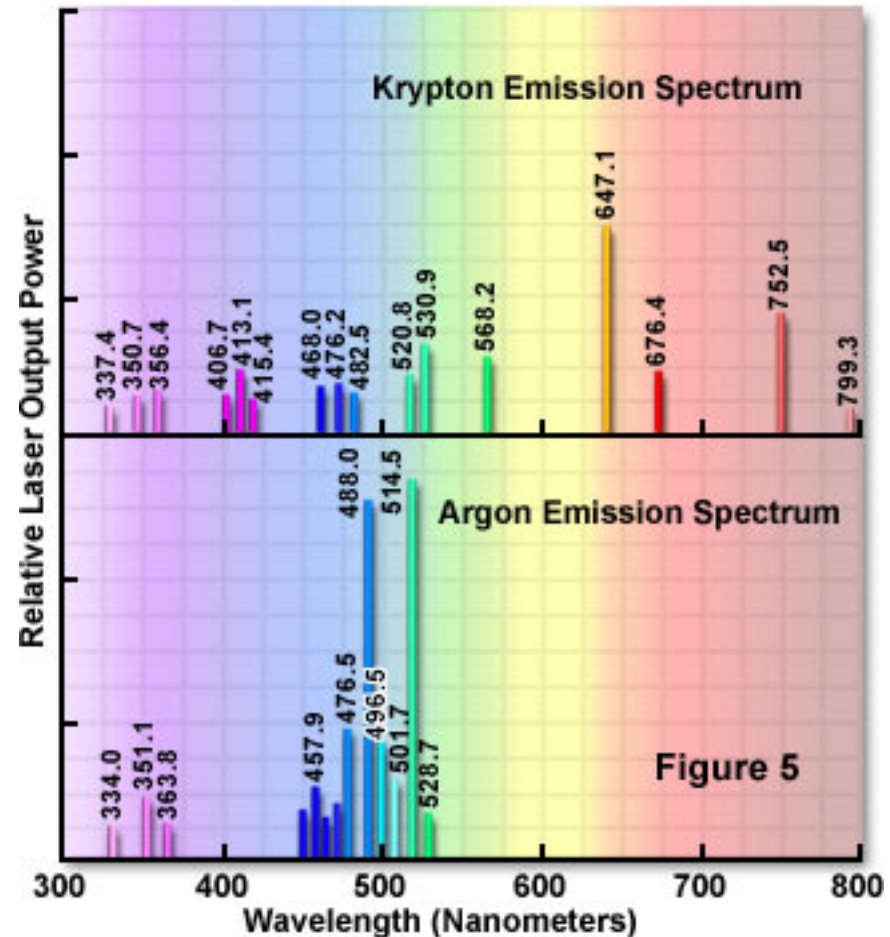
Laser Light Source

laser light source



Laser Light Source

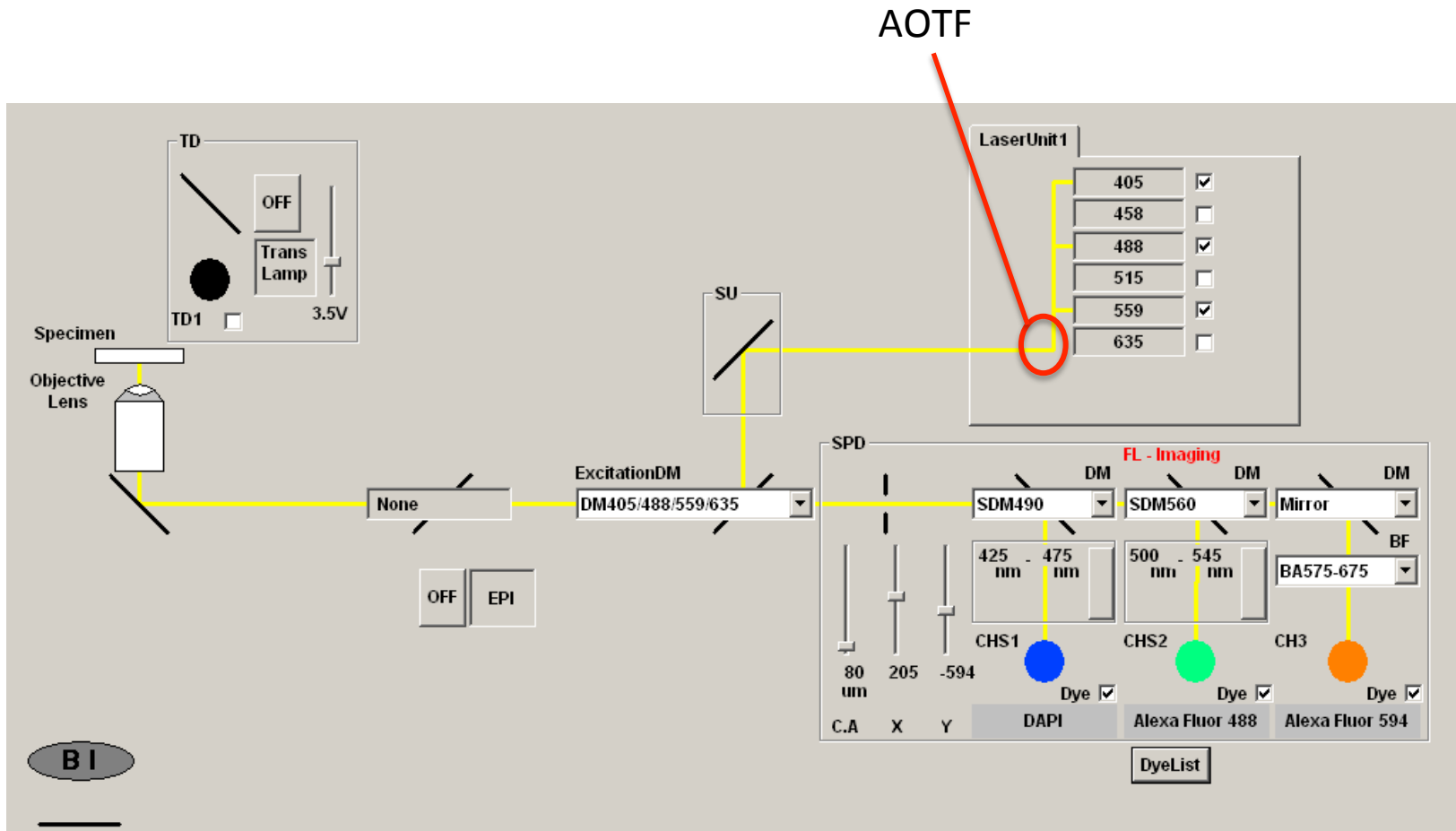
Laser Emission Spectra

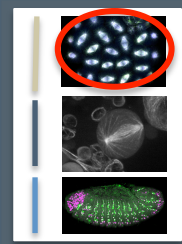


enables tighter control of
fluorophores excited

AOTF

Acousto-Optic Tunable Filter

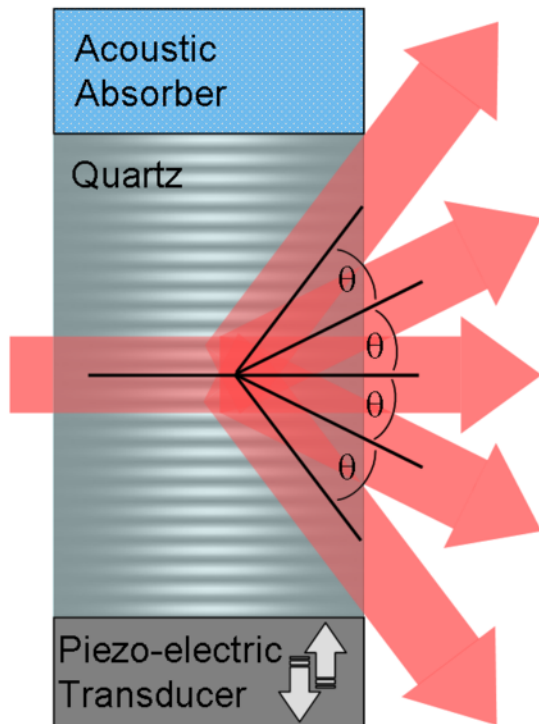




THEORY

AOTF

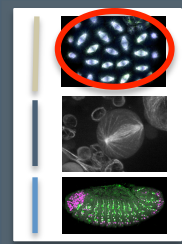
Acousto-Optic Tunable Filter



acousto-optic effect:

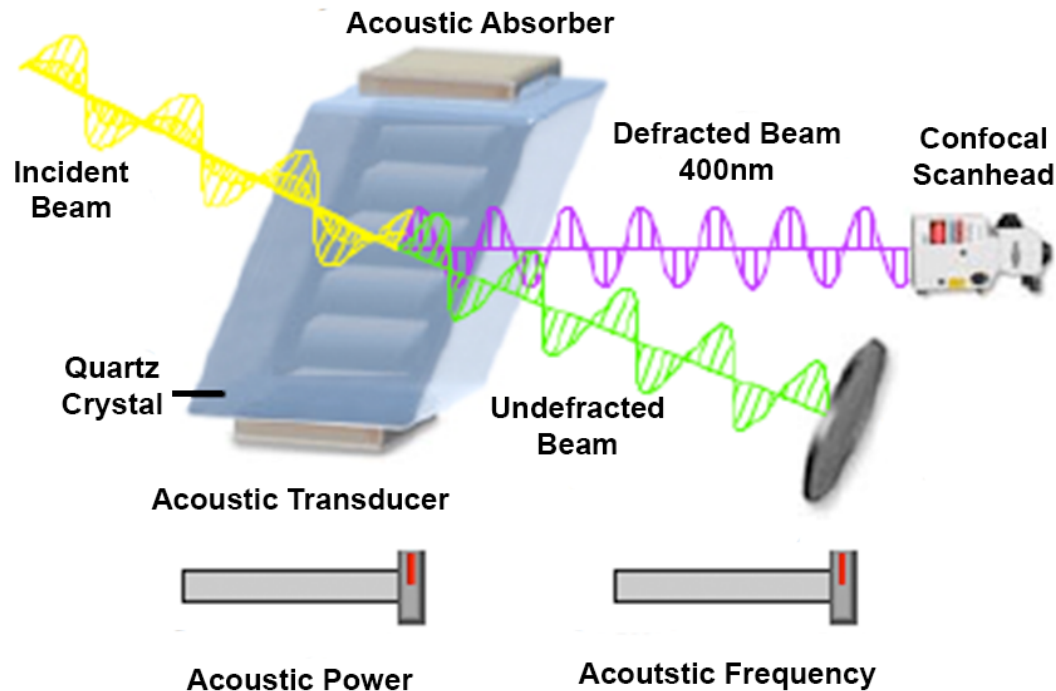
Acoustic wave excited within the quartz gives rise to variations in the refractive index

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.



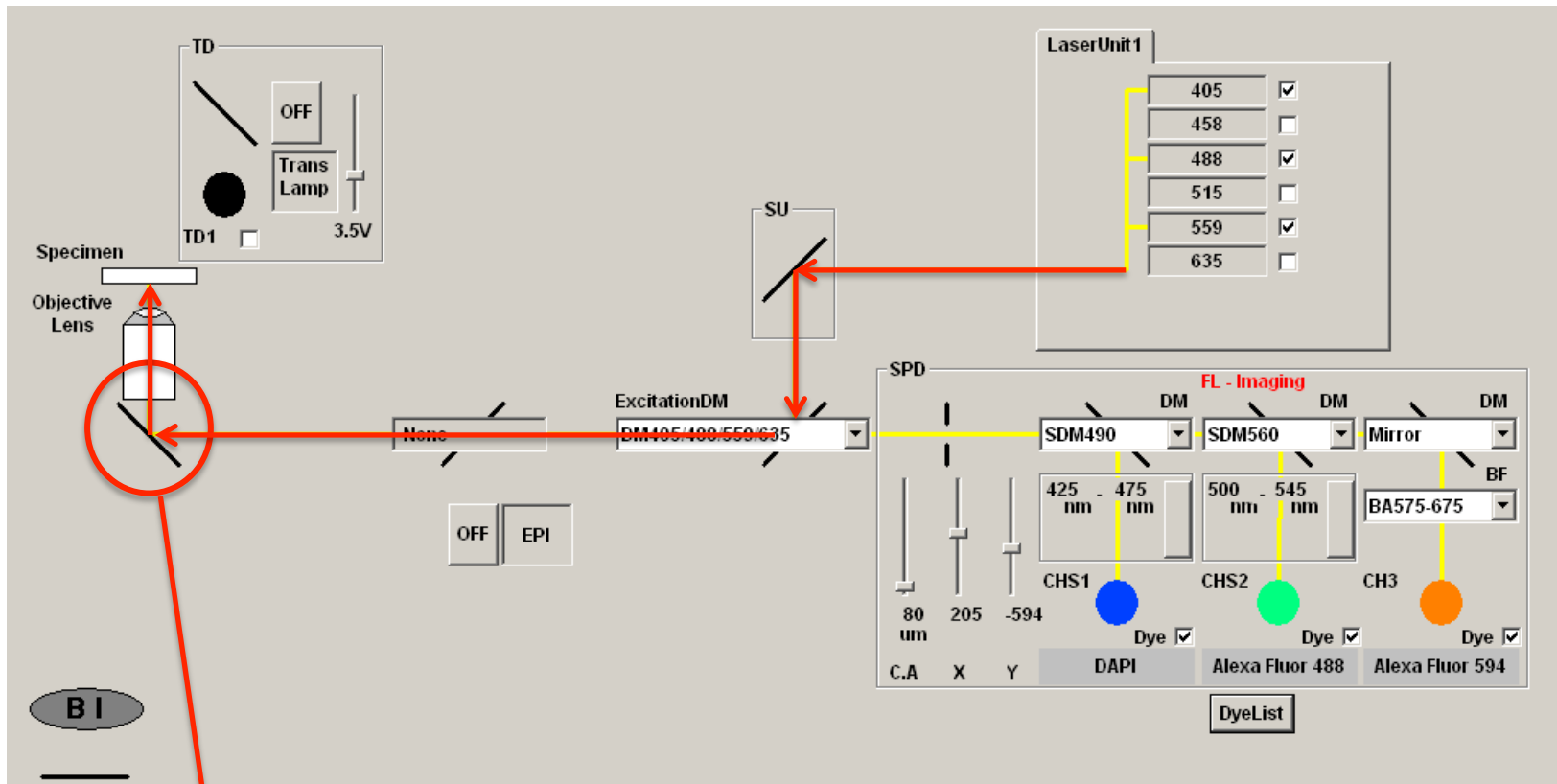
AOTF

Acousto-Optic Tunable Filter



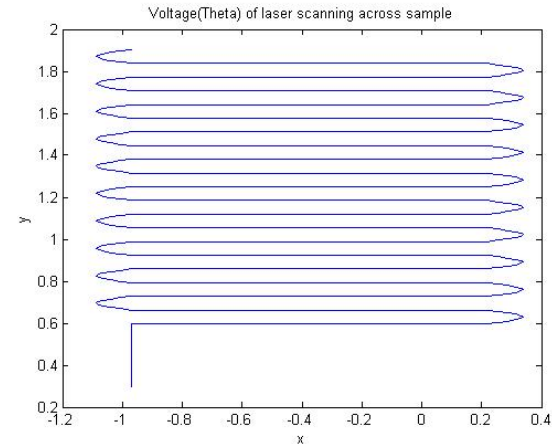
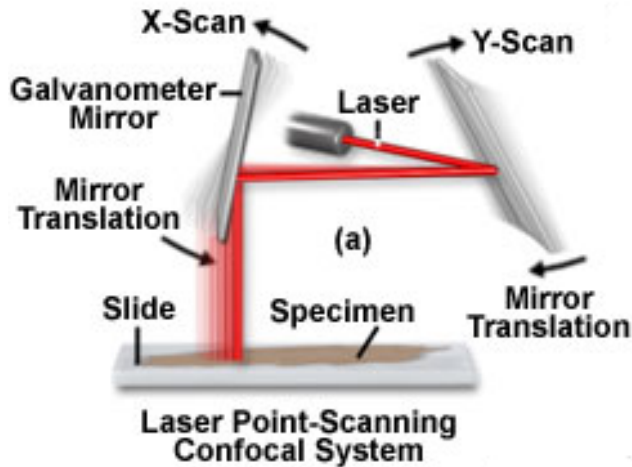
Quick On/Off of lasers
Very fast changes between excitation wavelengths

Galvo Scanning Mirrors



Galvo Scanning Mirrors

Galvo Scanning Mirrors



Sample excited at one point at a time
Relatively slow

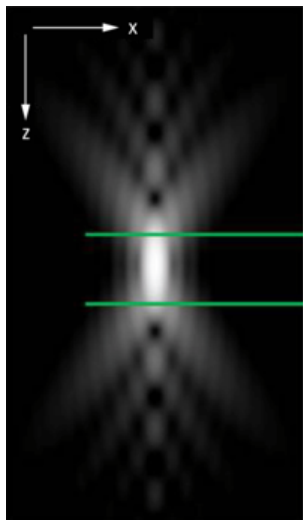


AOTF



THEORY

Pinhole – Optical Sectioning



FWHM=Full Width Half-Maximum



Shorter the wavelength the thinner the optical section

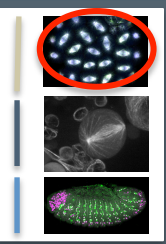
Diameter of the pinhole: Smaller pinhole thinner optical section

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

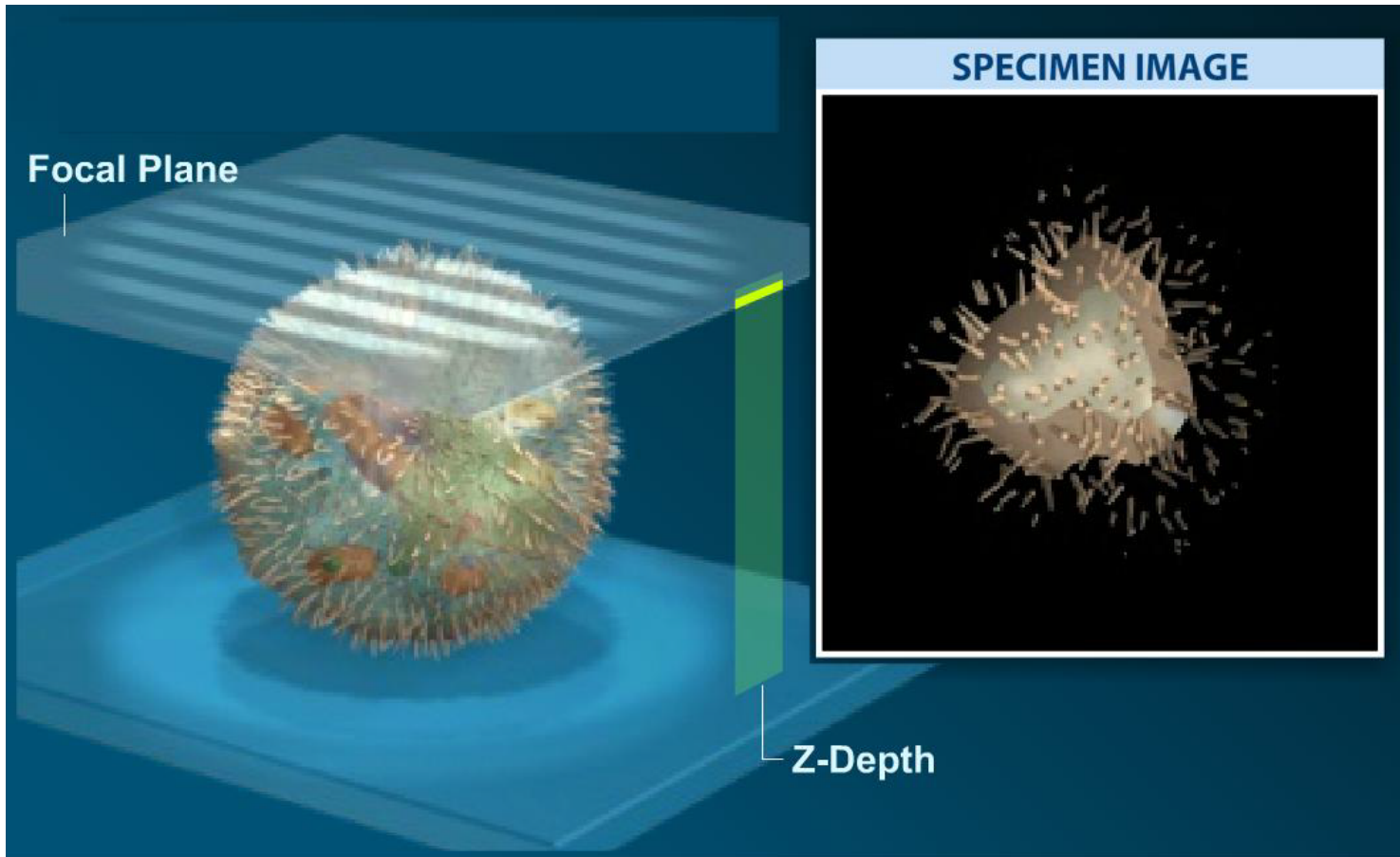
The higher the NA.
the thinner the section

Weak signal > open pinhole > more light but thicker section

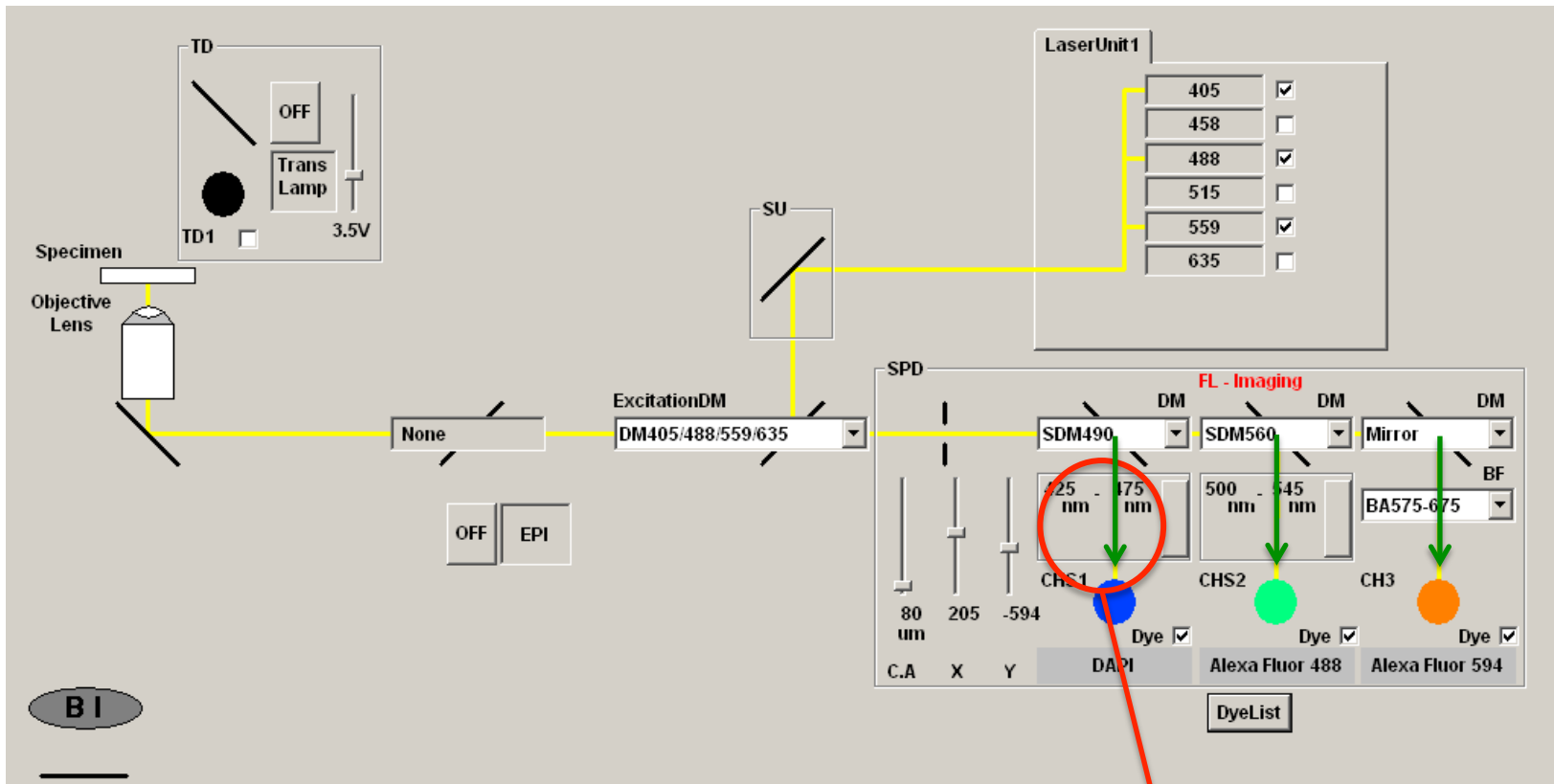
Confocal enables 3D reconstruction



Optical
section {

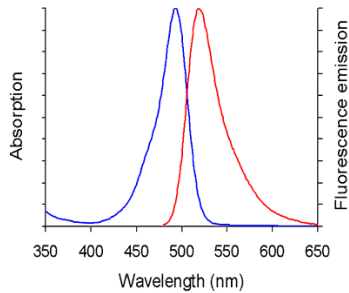


Variable Detector Slit

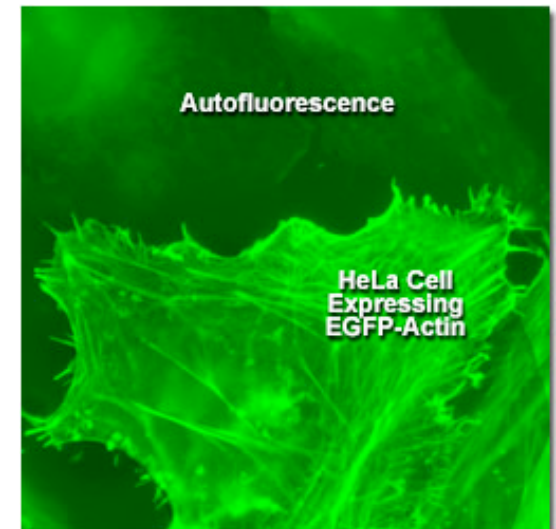
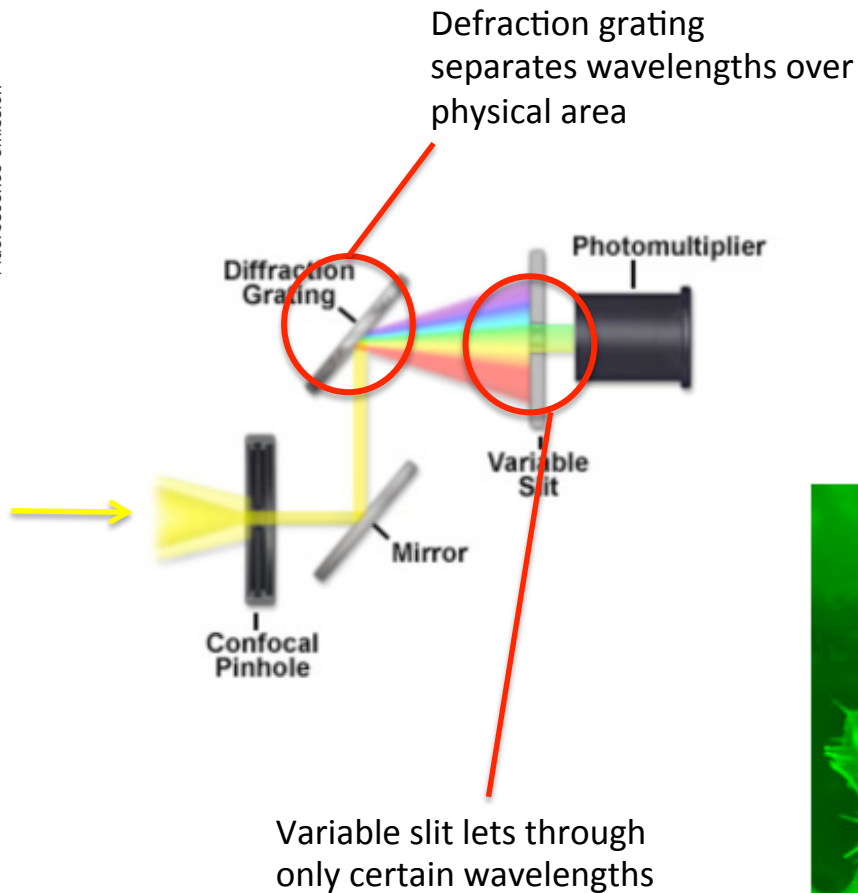


variable
detector slit

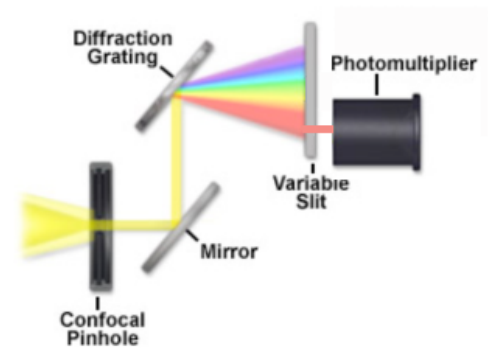
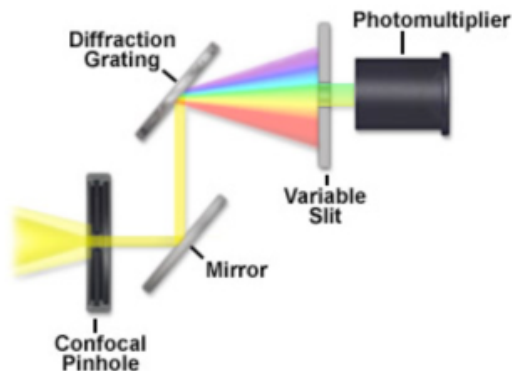
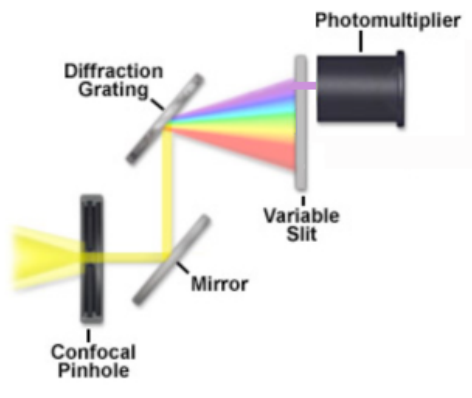
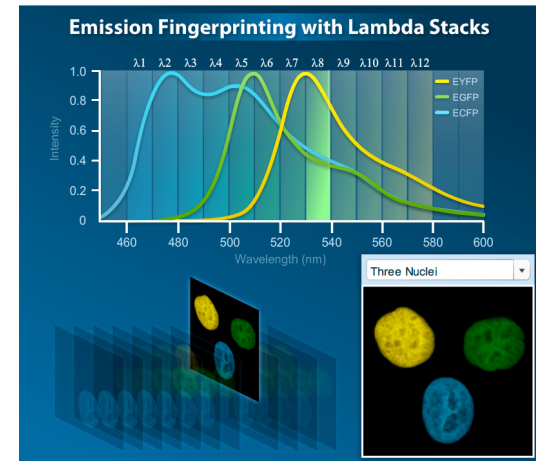
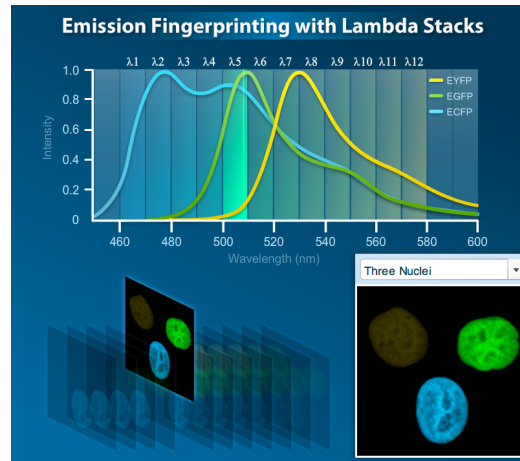
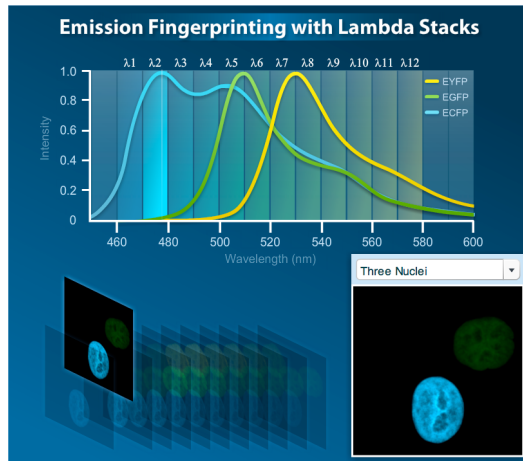
Spectral Unmixing



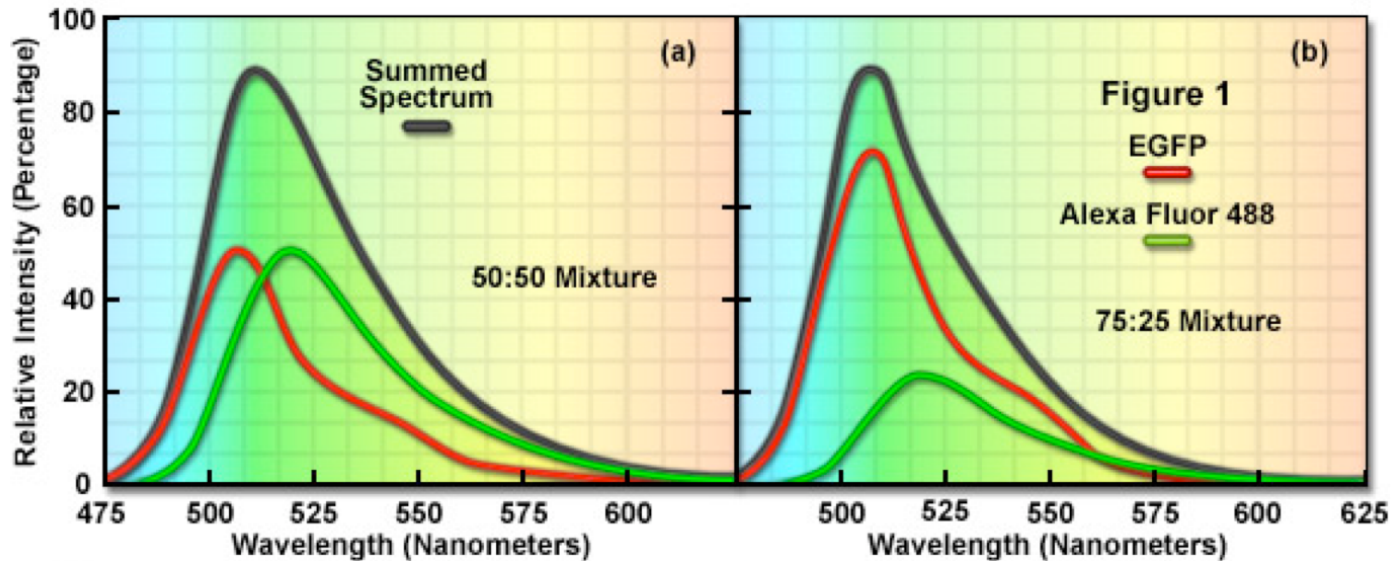
Light emitted from fluorophore as a spectrum



Spectral Unmixing



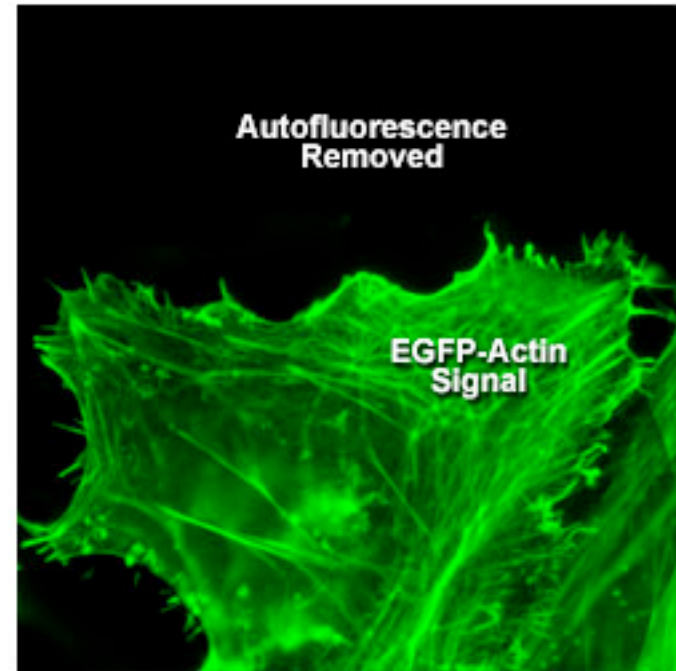
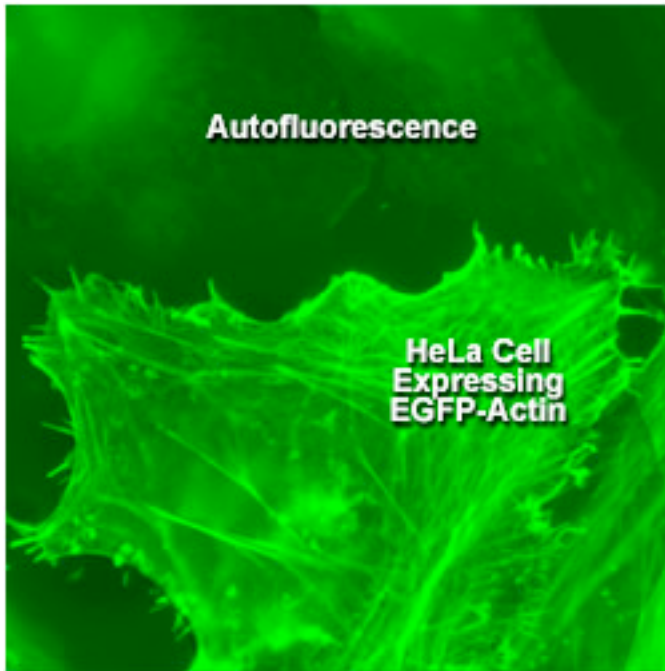
Spectral Unmixing



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

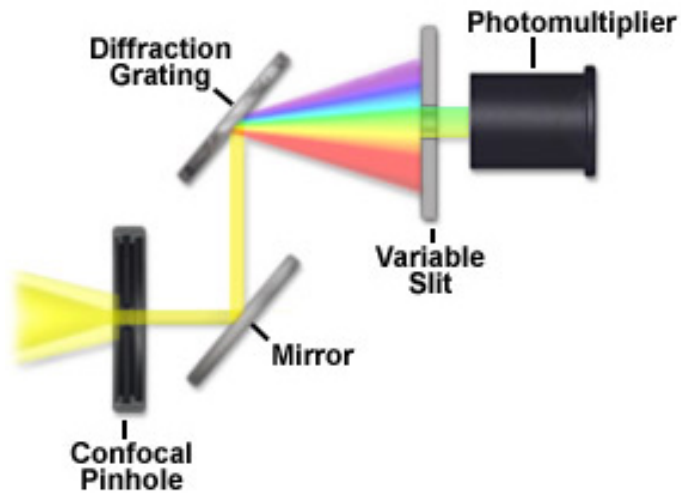


At each pixel:

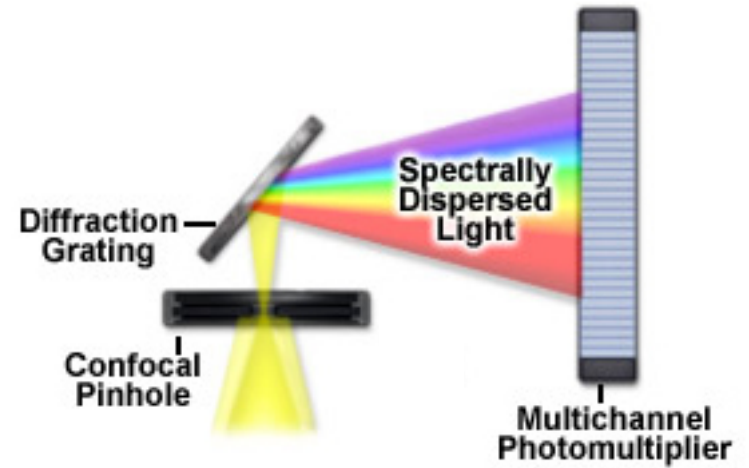
Calculate the proportion of the pixel is due to autofluorescence.
Subtract the autofluorescence from the 'true' GFP value.

Spectral Unmixing

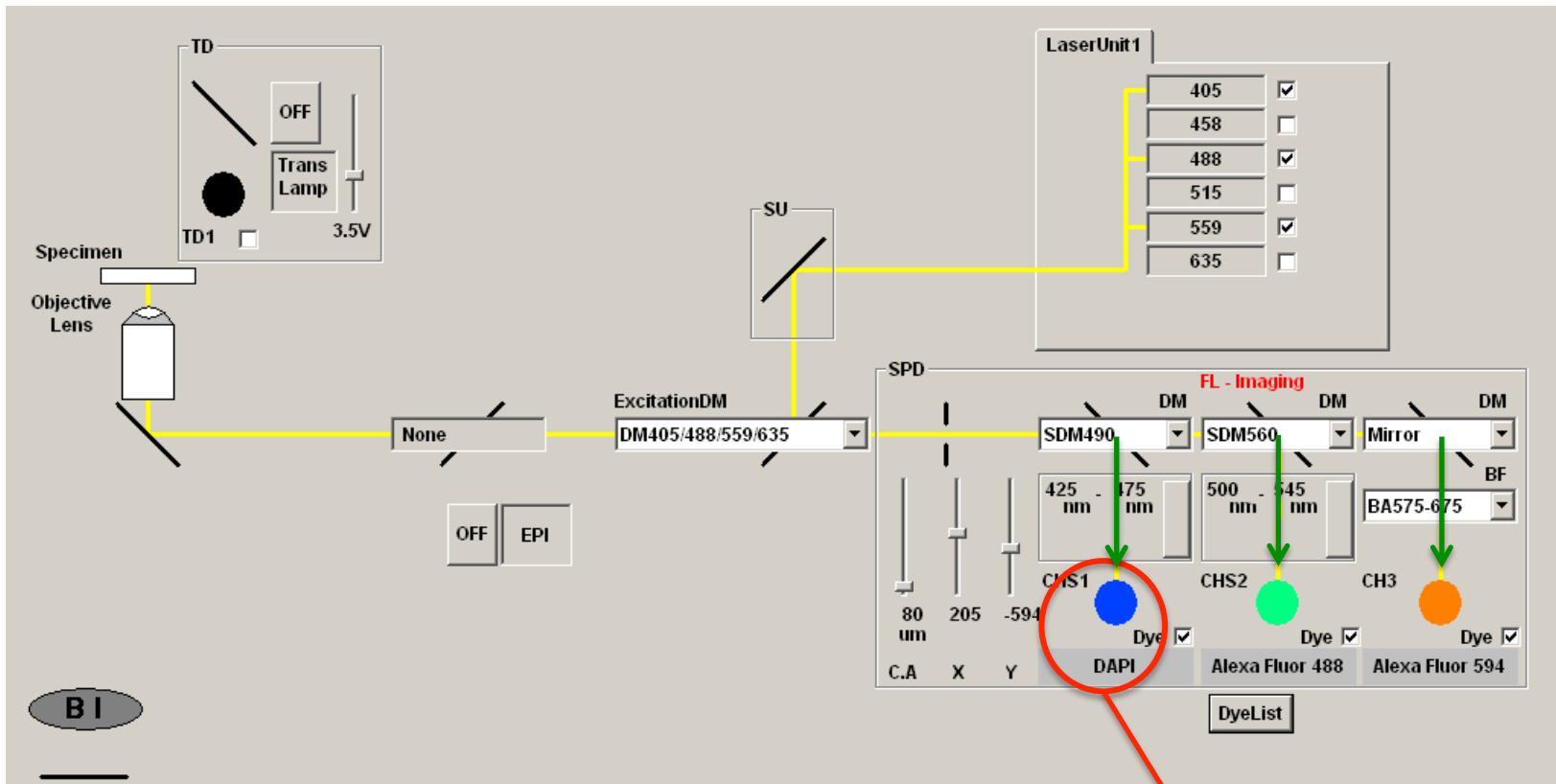
Olympus FV1000



Zeiss 510 META

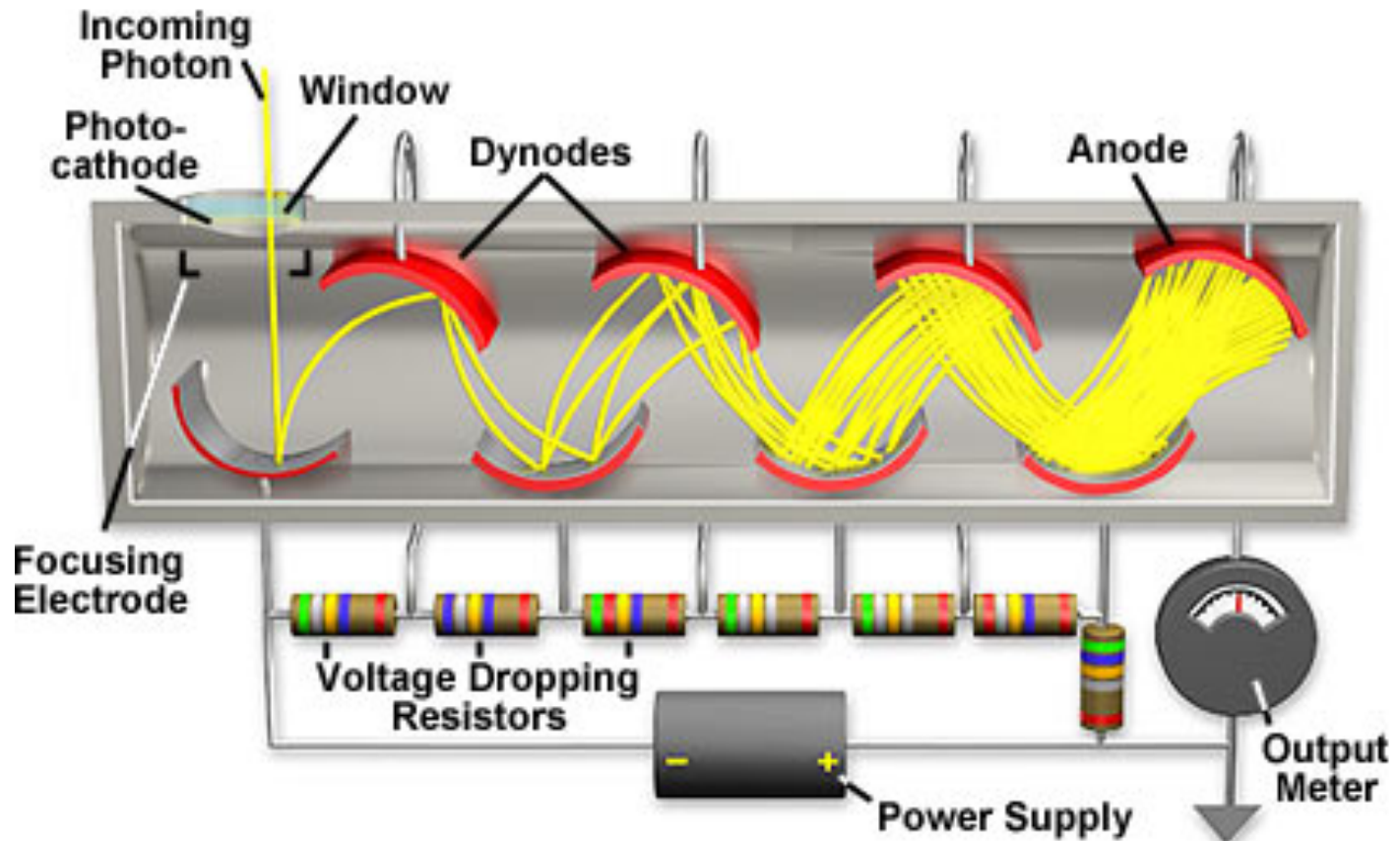


PMT – Photon Multiplier Tube



PMT detectors

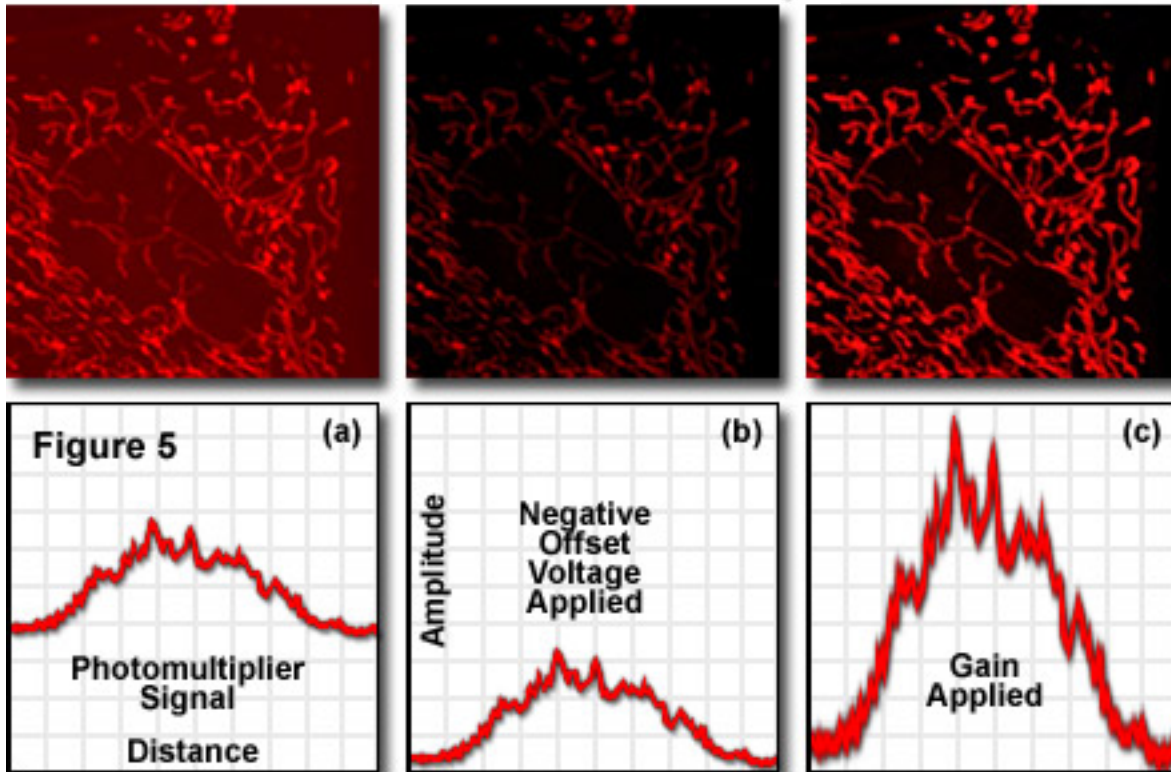
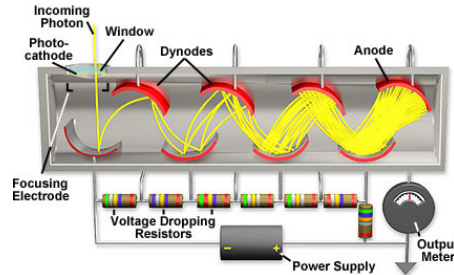
PMT – Photon Multiplier Tube



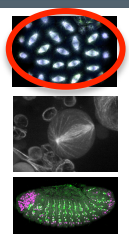
Very Low Noise
Huge Signal Amplification ($\sim 1 \times 10^6$)

PMT – Photon Multiplier Tube

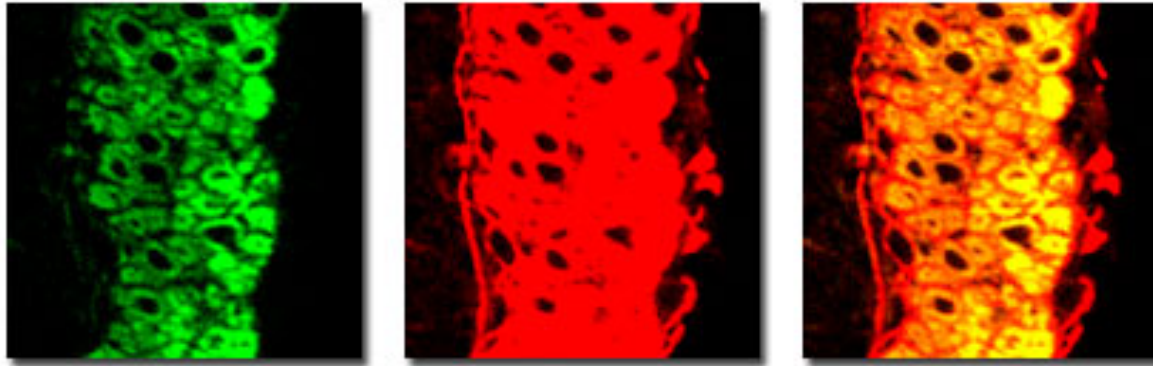
Adjusting Gain and Offset



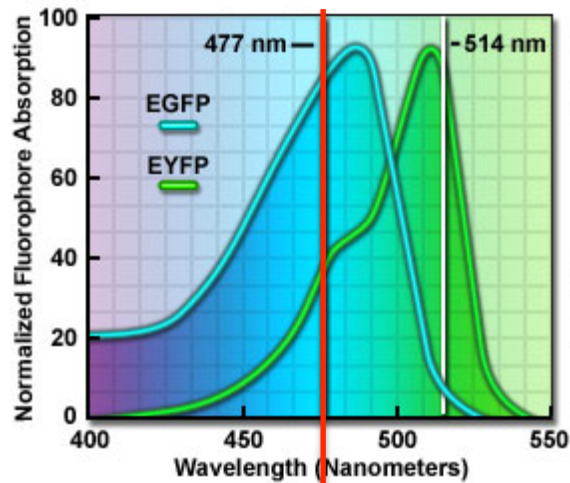
Beware - this is how your image will be saved!



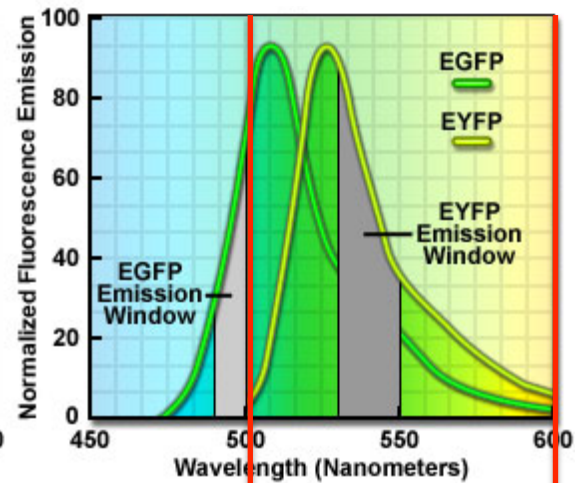
'bleed-through'



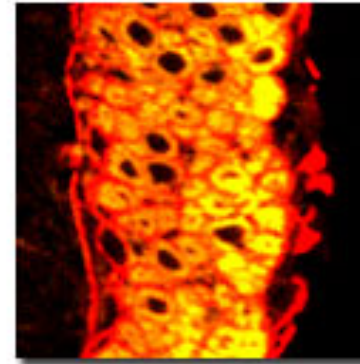
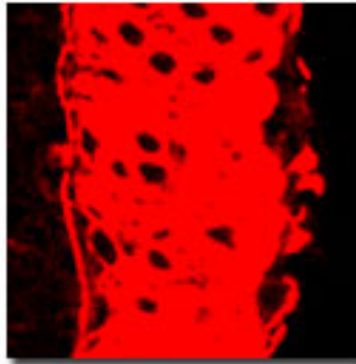
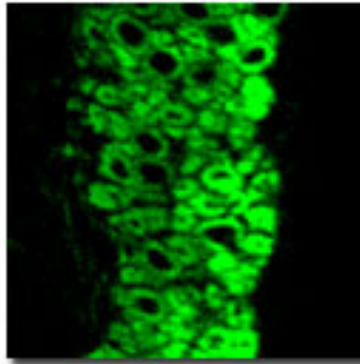
Absorption spectral profiles



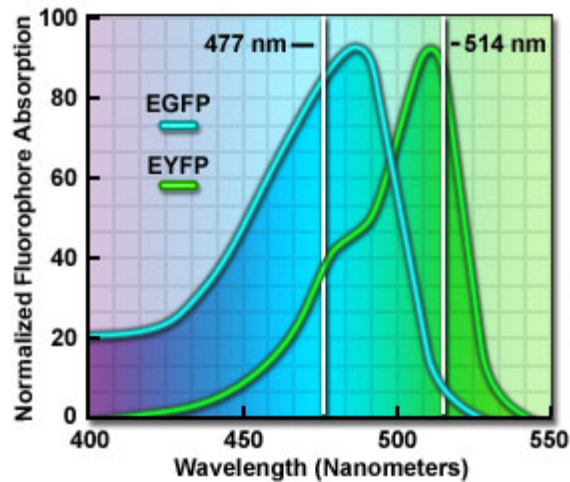
Emission spectral profiles



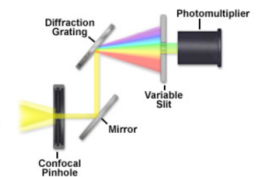
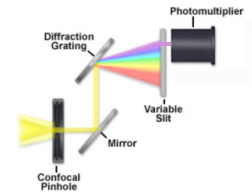
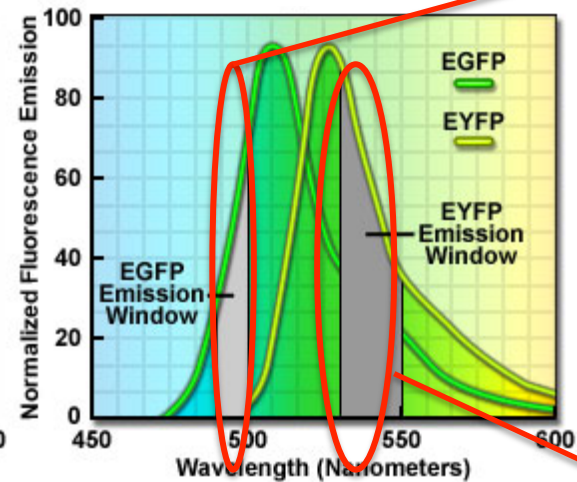
minimising 'bleed-through' Variable Slits



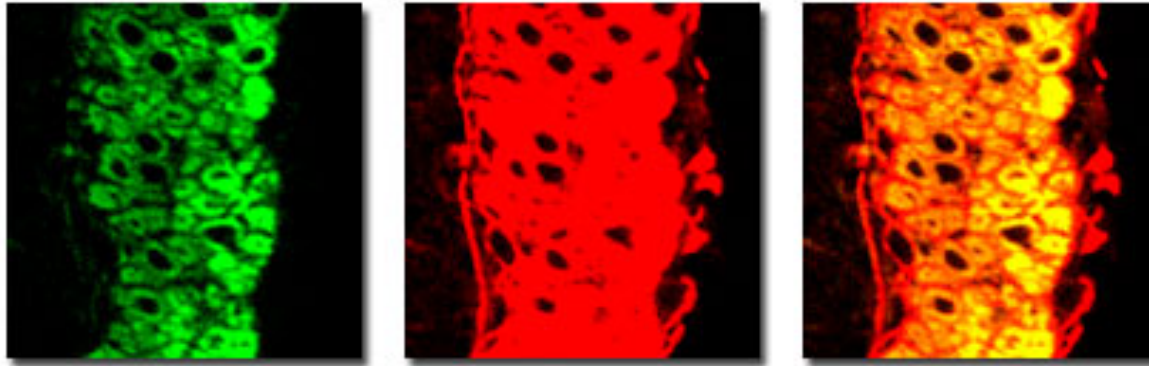
Absorption spectral profiles



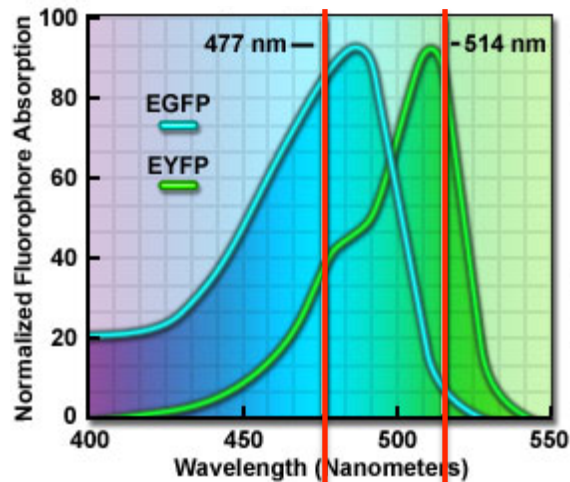
Emission spectral profiles



minimising 'bleed-through' Sequential Scanning



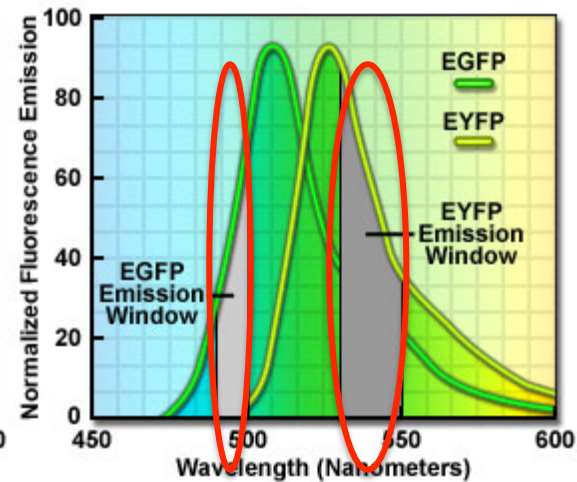
Absorption spectral profiles



Excite at 477nm

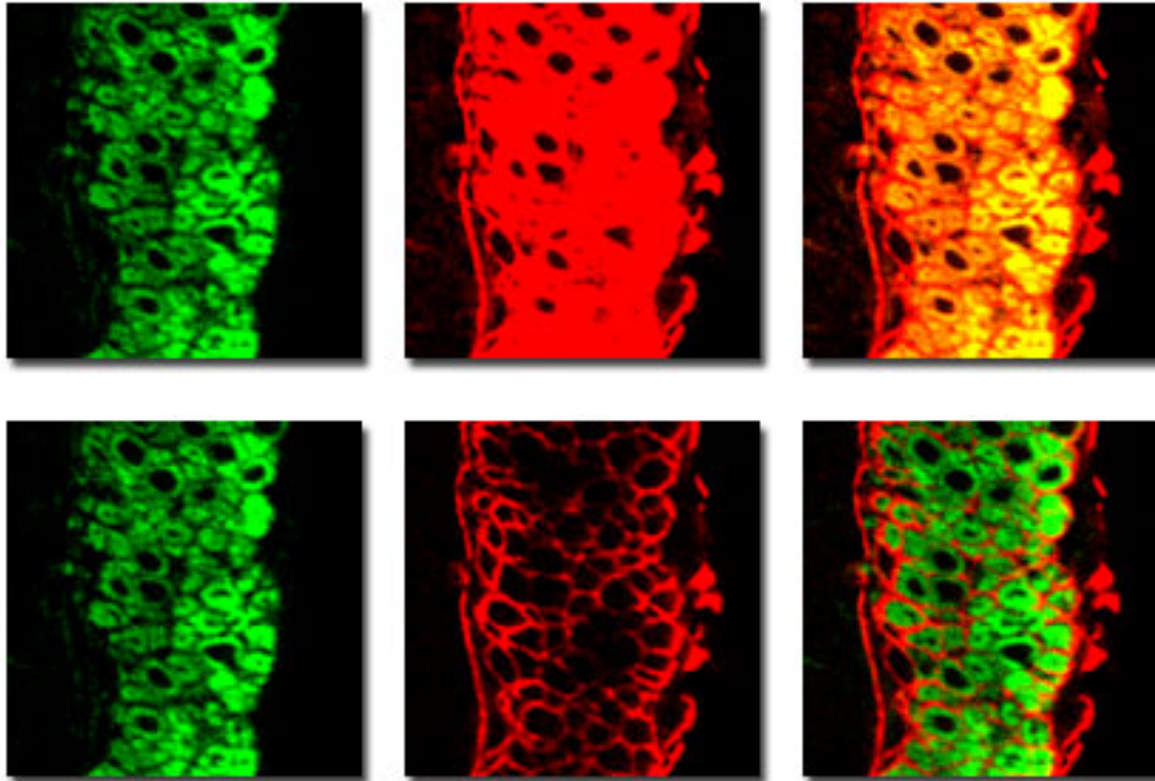
Excite at 514nm

Emission spectral profiles

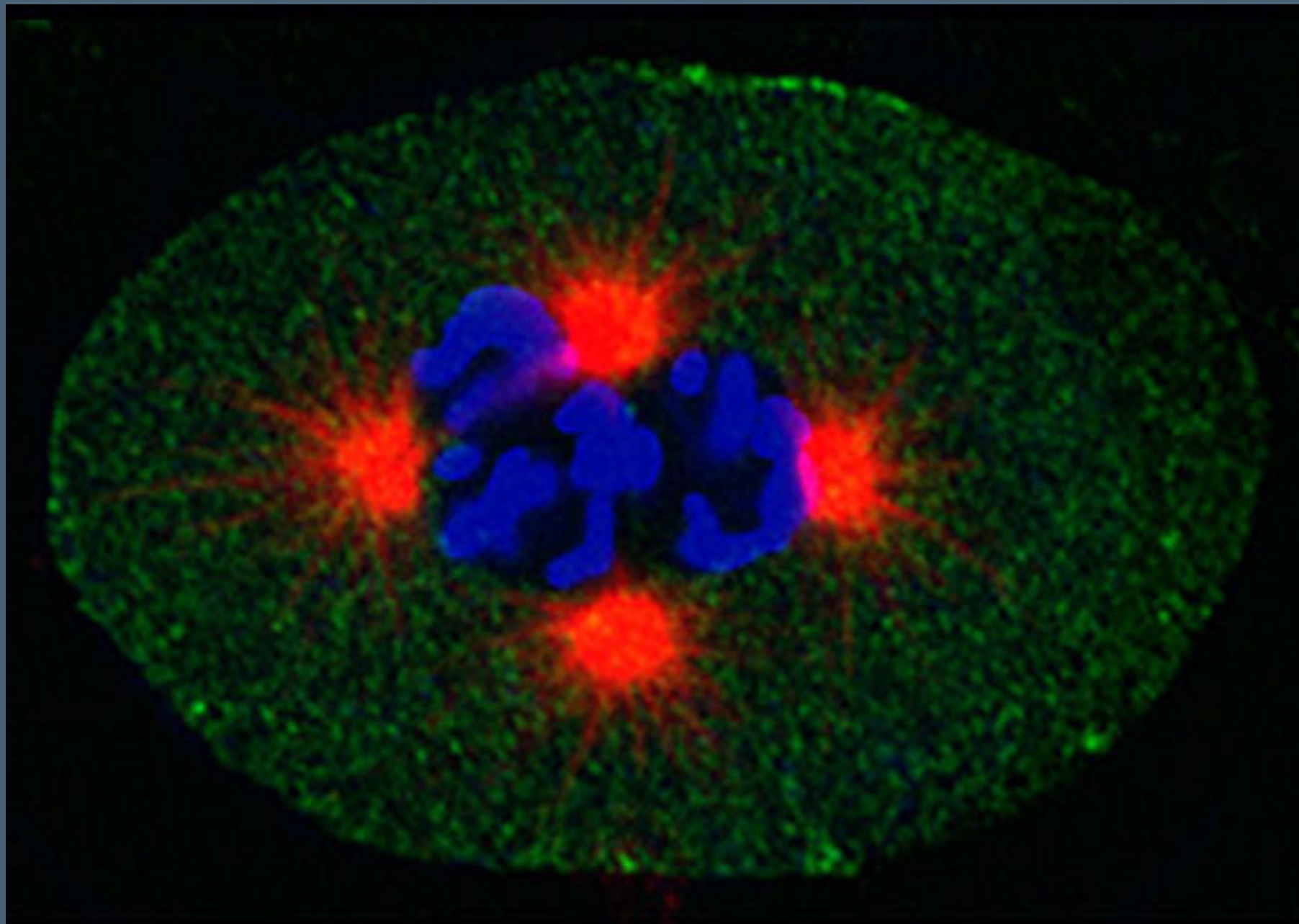


Temporal separation

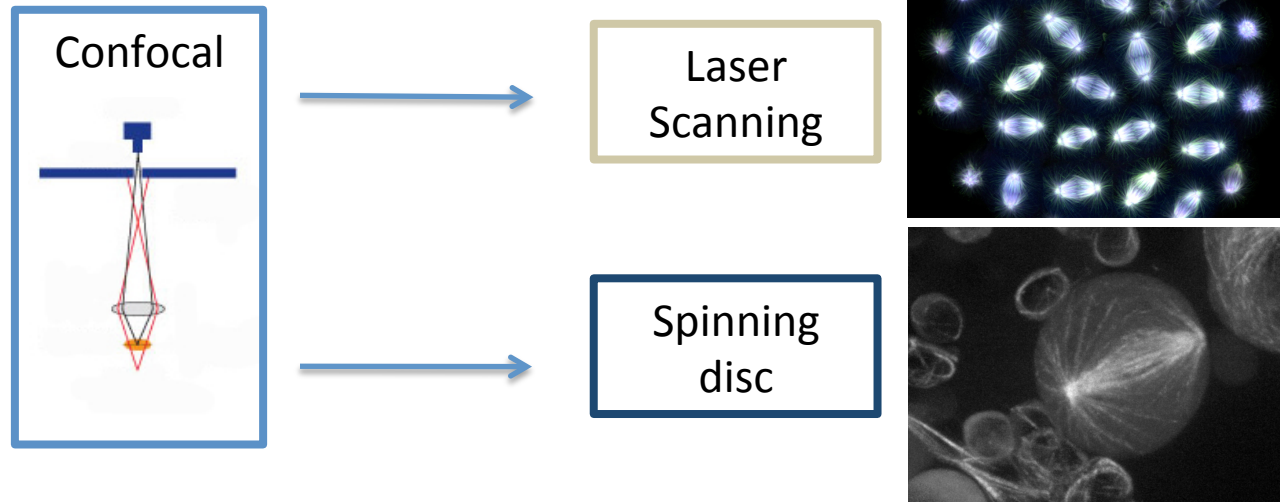
minimising 'bleed-through'



Adjust detector slit widths
Use sequential scanning

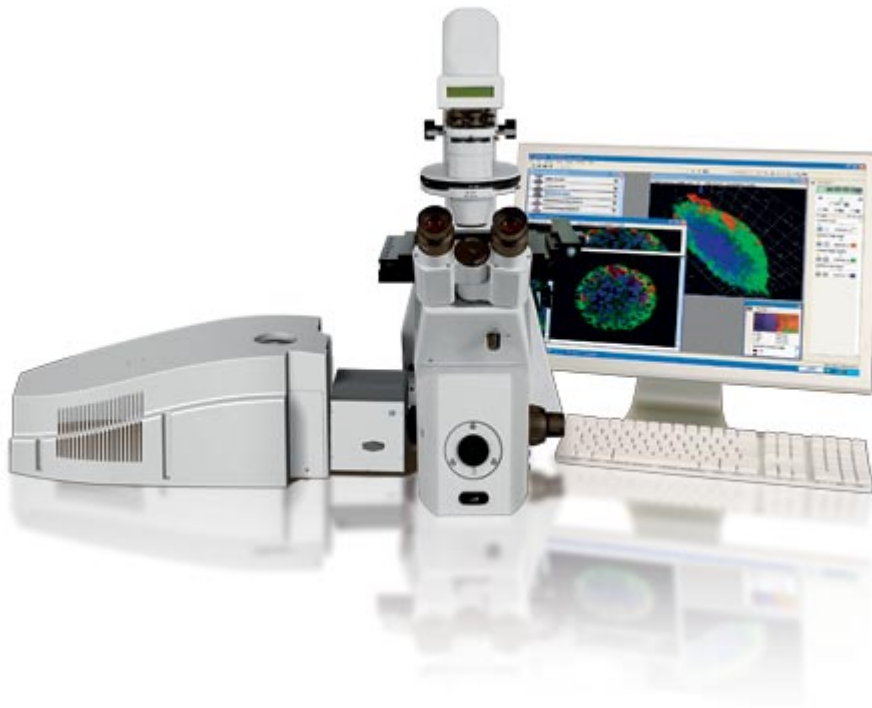


Confocal Microscopes



Both are confocals

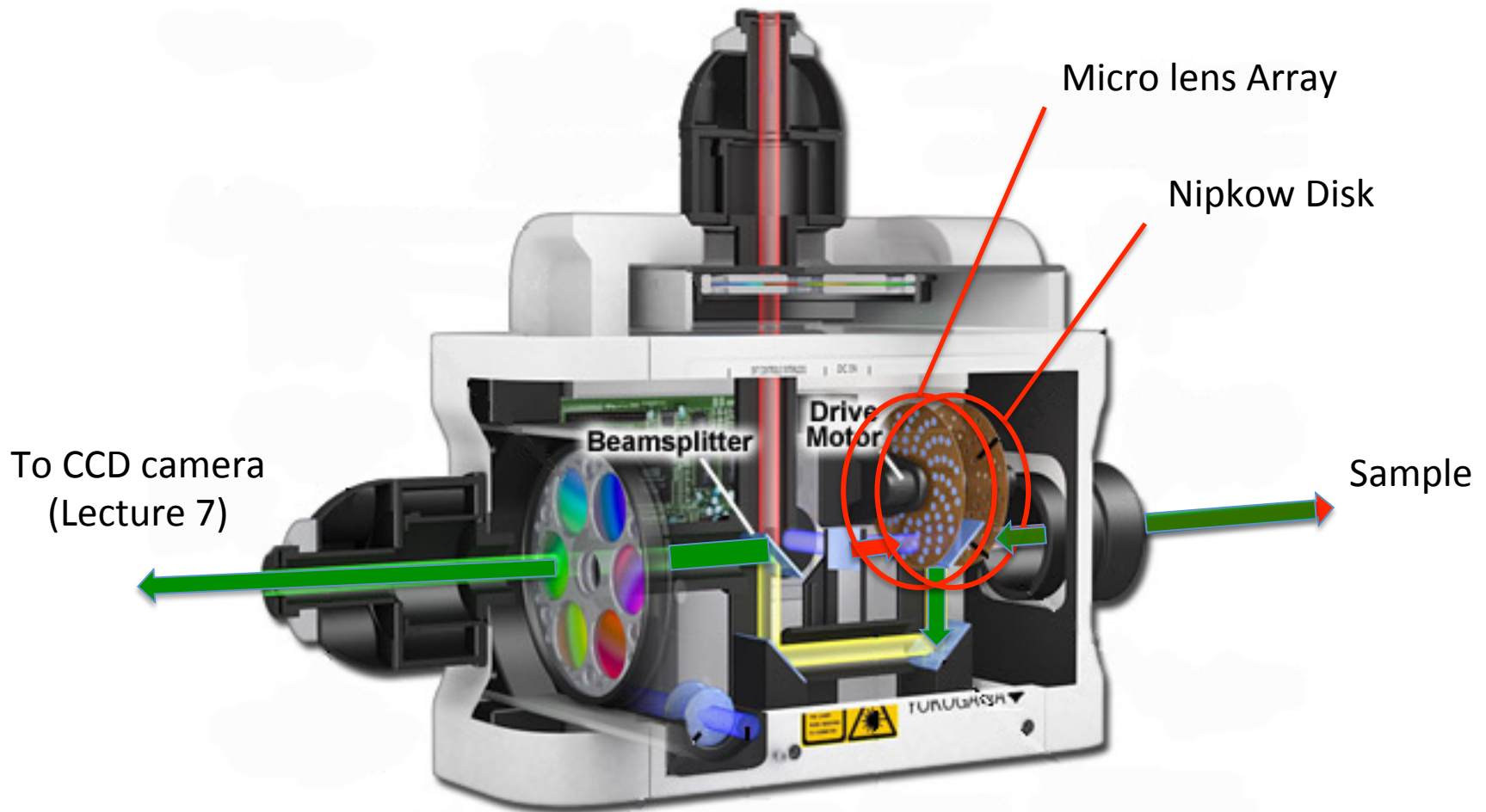
Spinning Disc Confocal



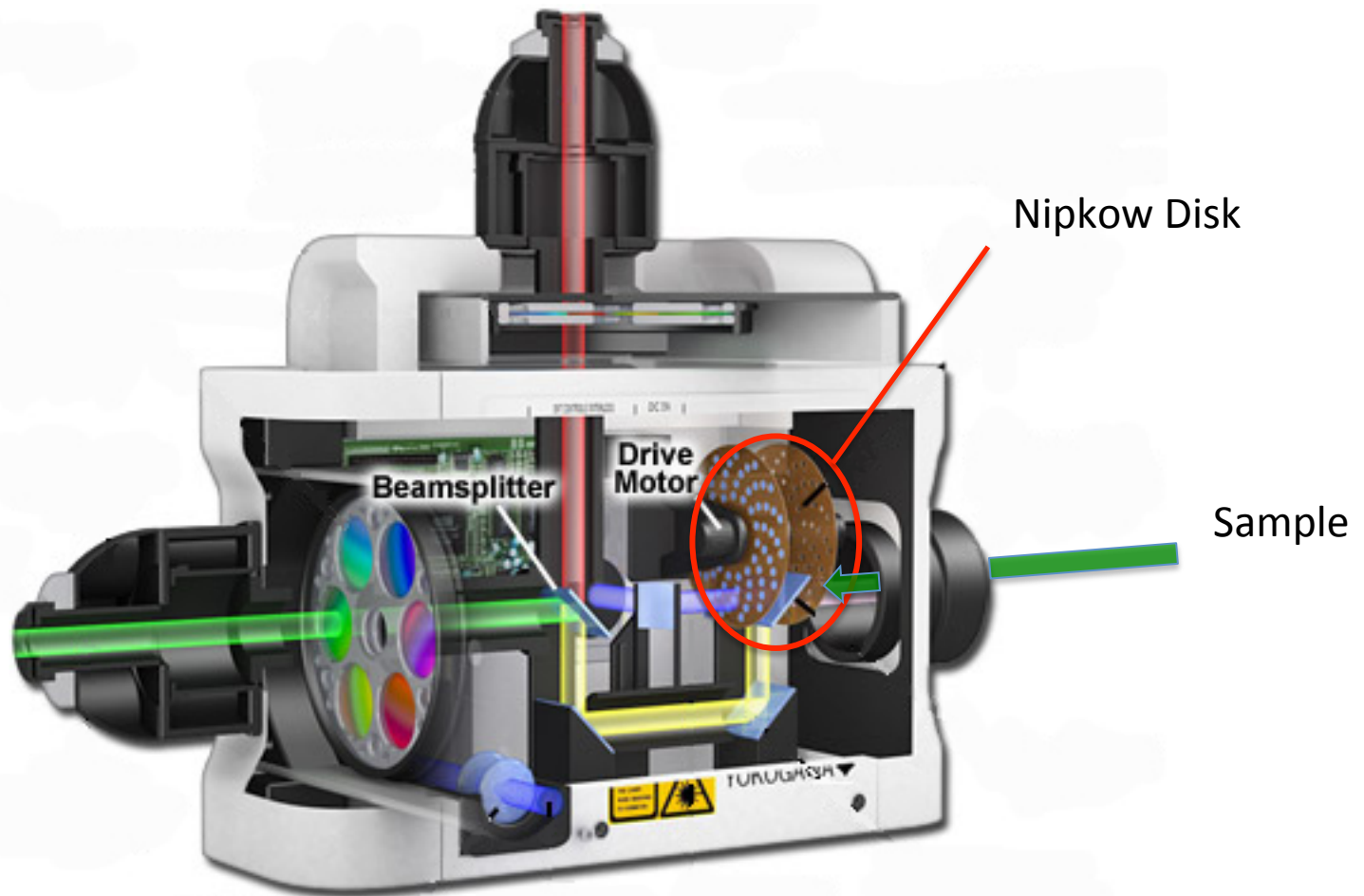
Great for live cell imaging

Can collect 'many' images per second

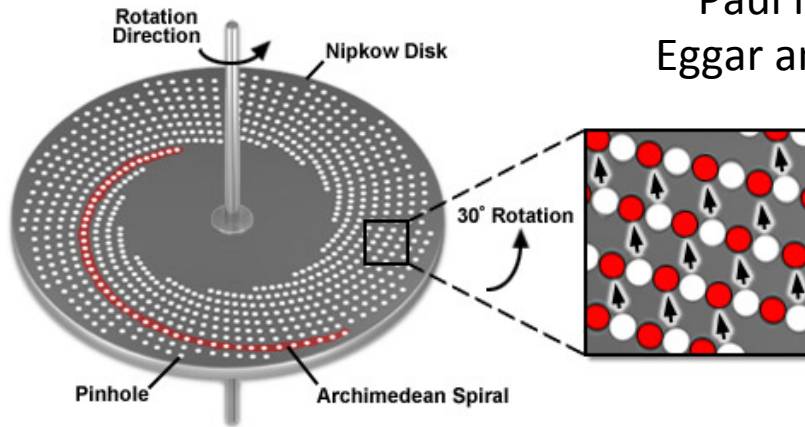
Yokogawa CSU-X1 Spinning Disc



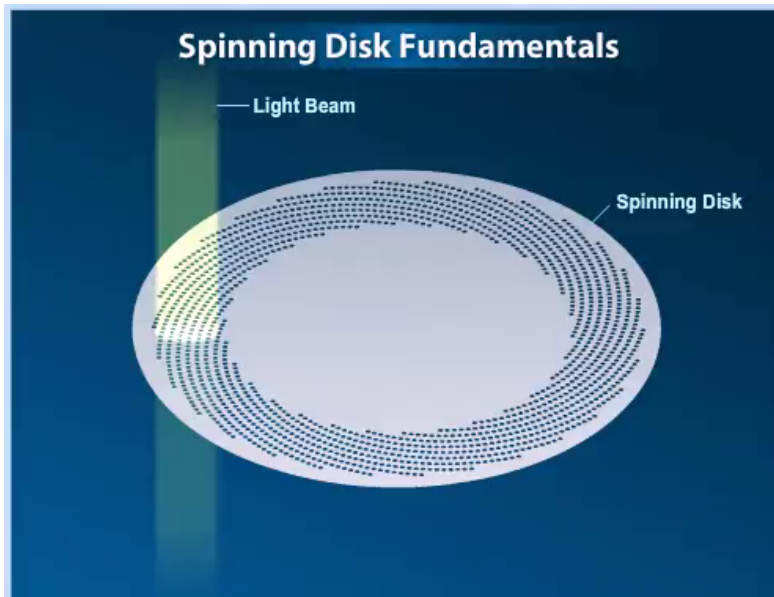
Yokogawa CSU-X1 Spinning Disc



The Nipkow Disk



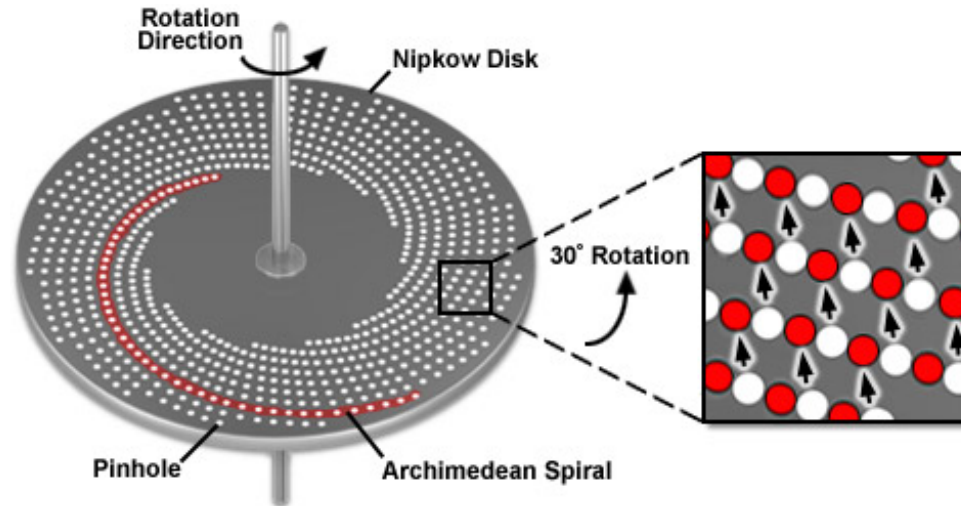
Paul Nipkow, 1884
Eggar and Petran, 1967



Approx. 1000 pinholes

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

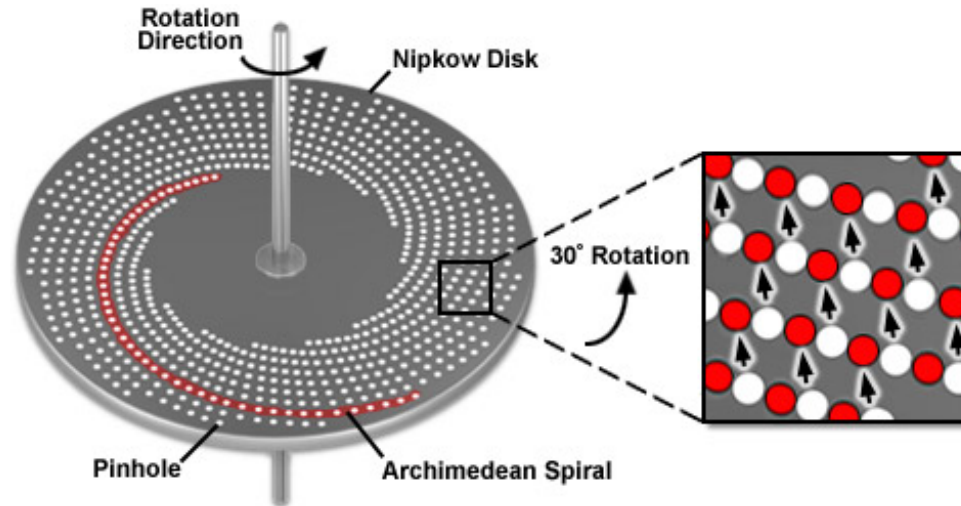
The Nipkow Disk



Larger pinholes - brighter image, but less “confocal”

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

The Nipkow Disk

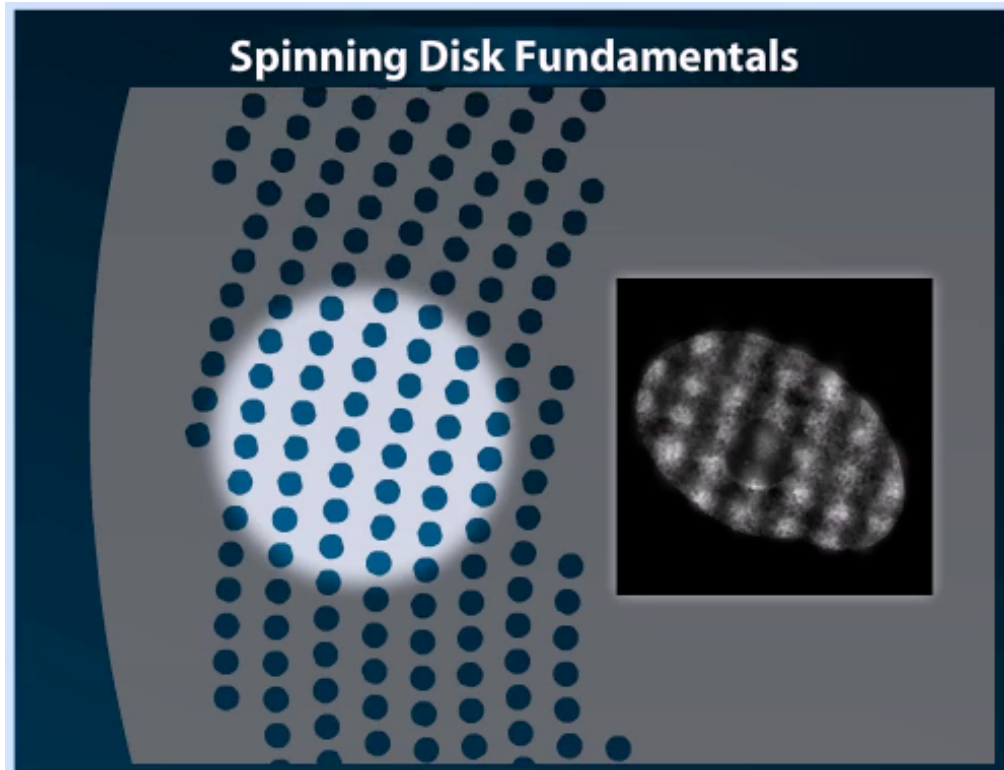


Constant Battle:

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

Pinhole Spacing Typically = 2.5um apart

Spinning Disc Confocal

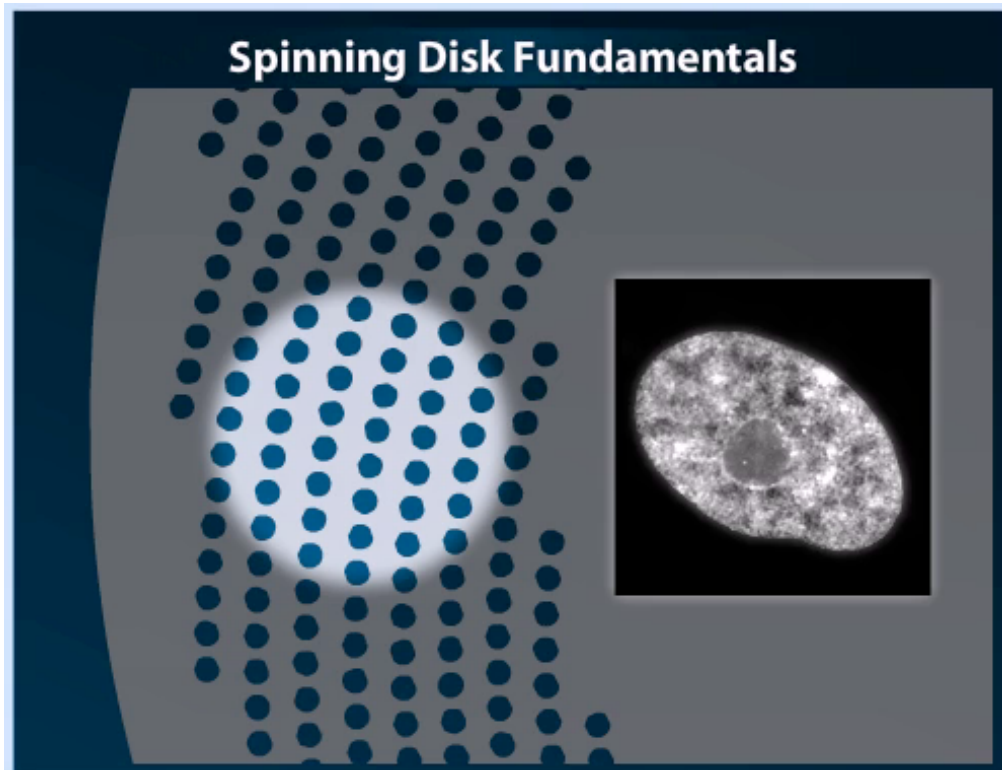


Disc spins to illuminate sample

spin speed – disc spinning
too slowly

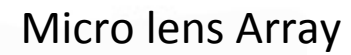
you see the pinholes on the
sample

Spinning Disc Confocal

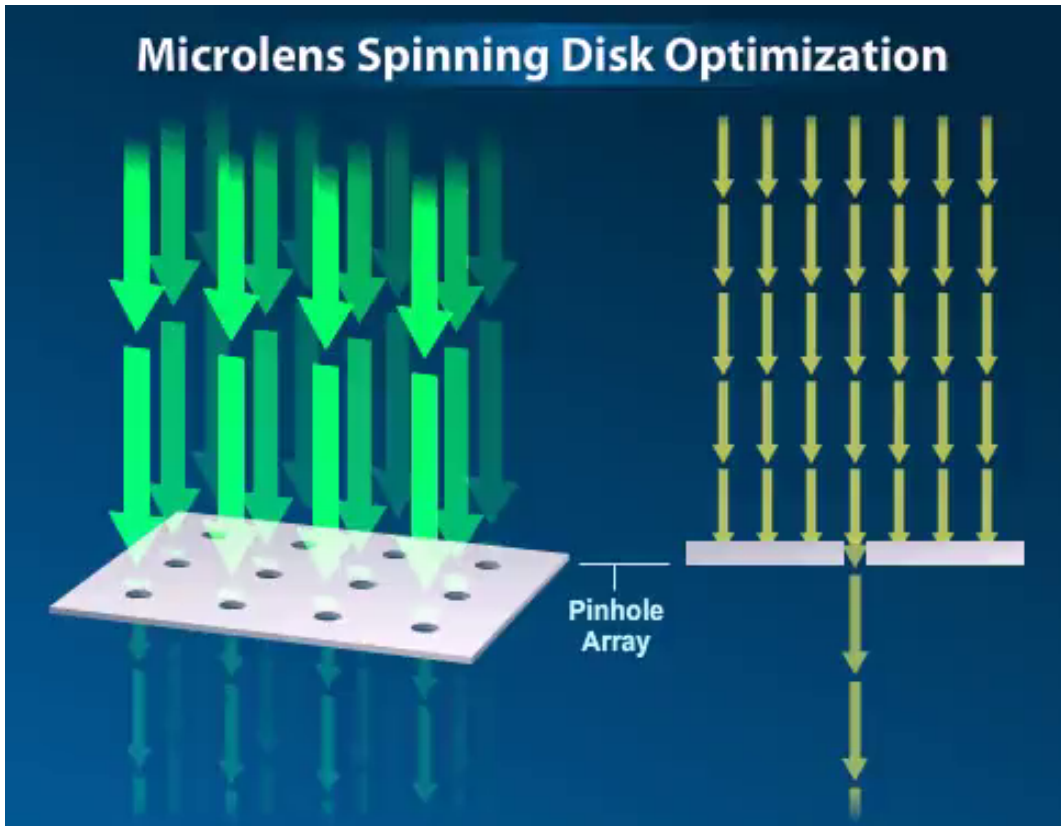


Mismatches between disk rotation speed and camera integration periods are generally not a problem for long exposure times (greater than 100 milliseconds)

Disc speed must carefully match camera frame rate



Yokogawa Spinning Disc Confocal Microscopes

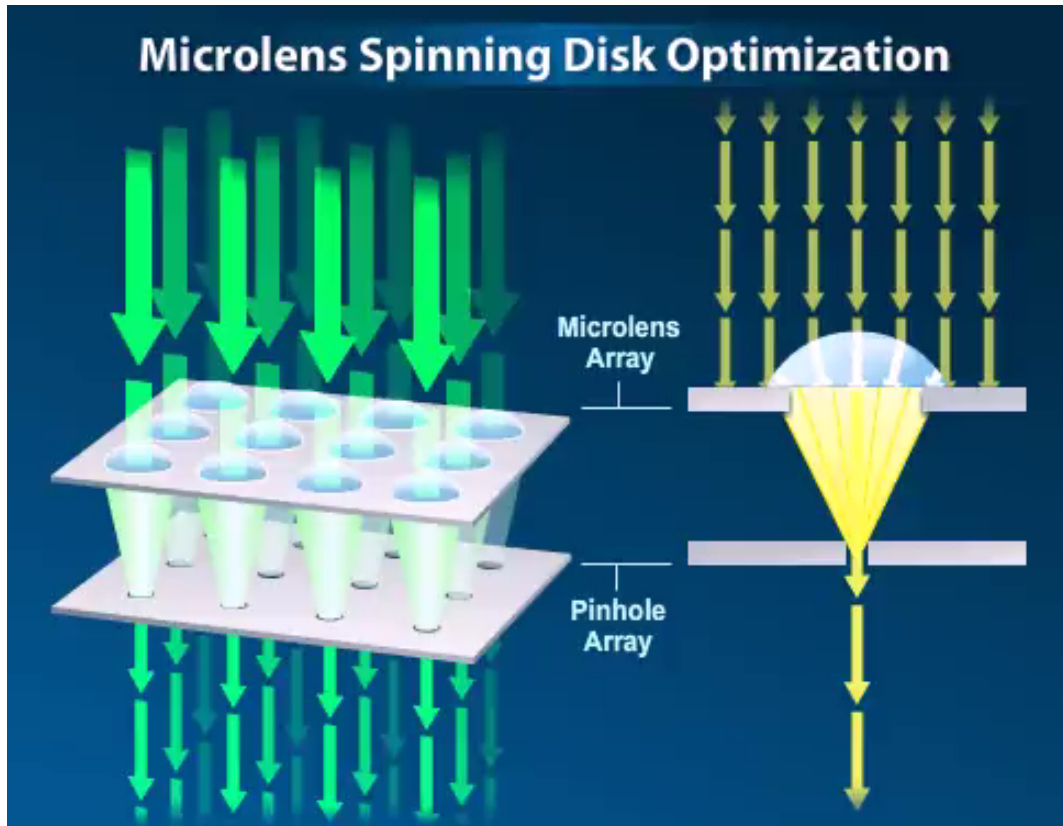


just a pinhole array –
Optimised for 'cofocality'
and 'crosstalk'

too much light is blocked
from reaching the specimen

Only 4% light passes through disc

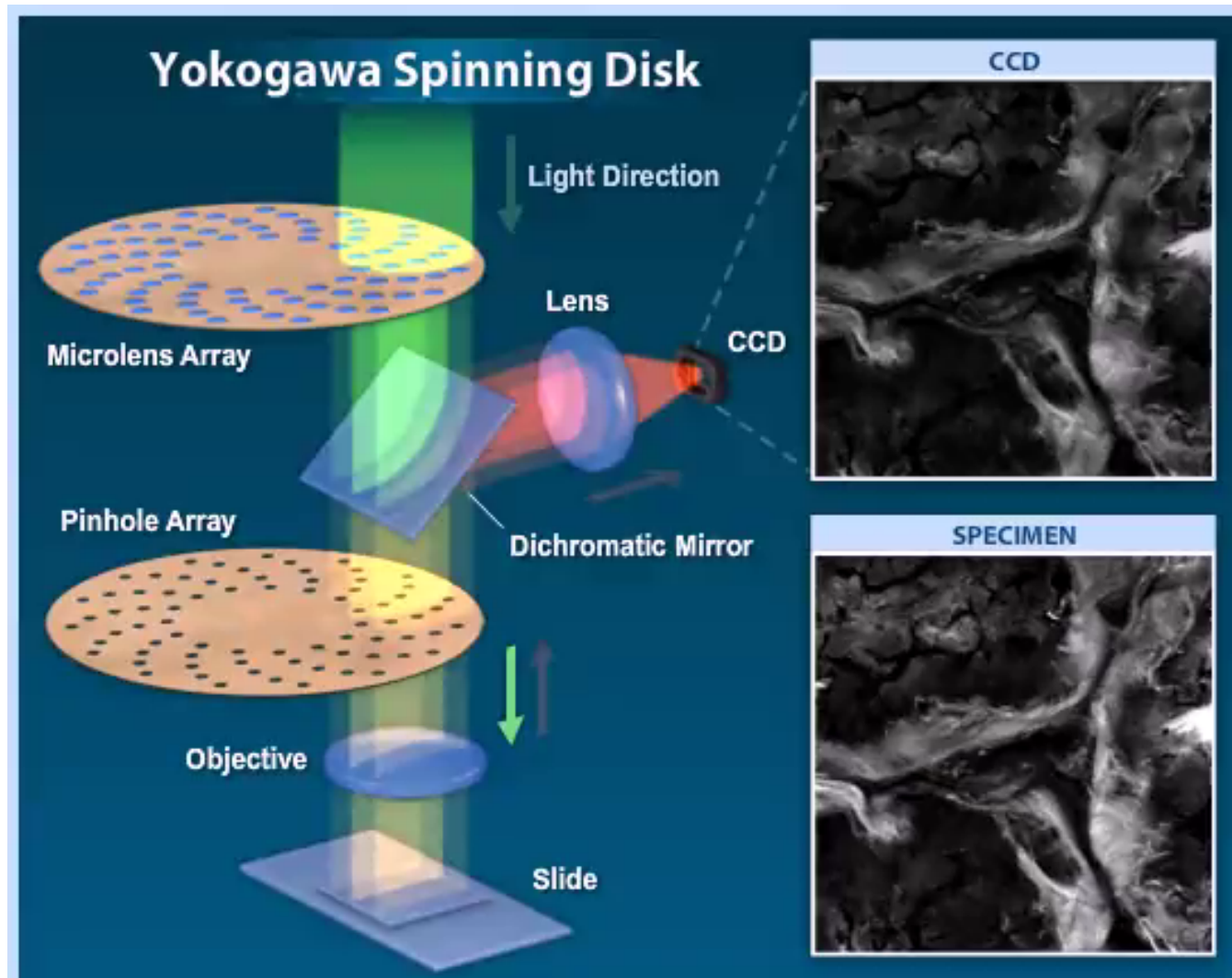
Yokogawa Spinning Disc Confocal Microscopes

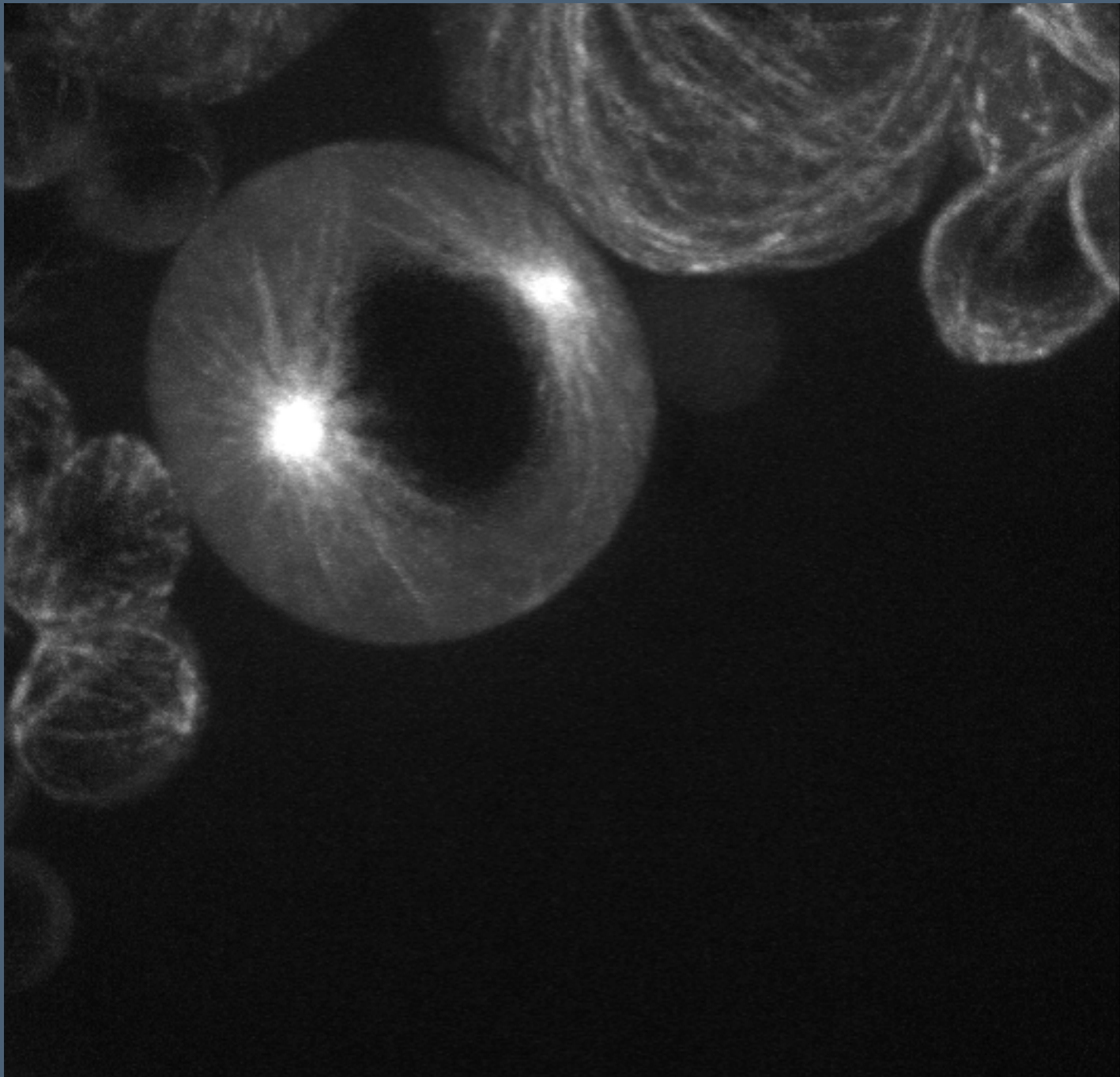


micro-lens array
increase the light
reaching the specimen

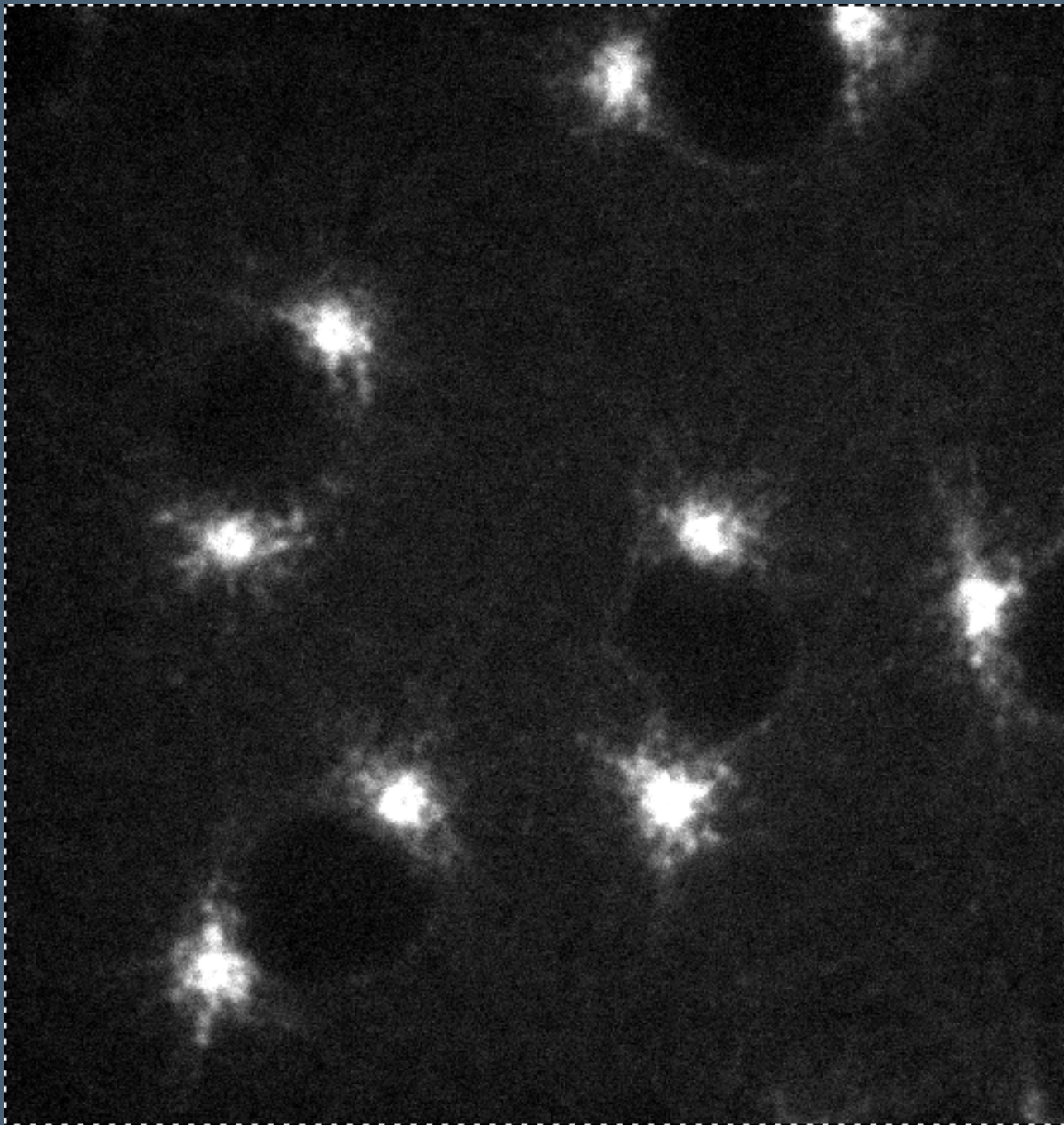
Typically 56% light passes through disc

Yokogawa Spinning Disc Confocal

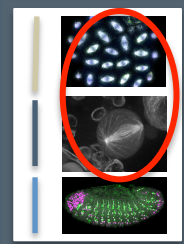




Cell division in brain stem cells (neuroblasts), Raff Lab

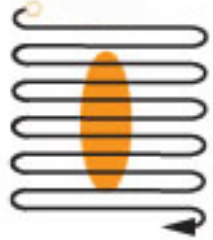


MT binding protein in *Drosophila* embryo, Raff Lab



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!

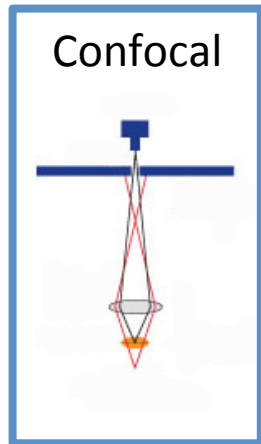
Pretty Movies

Good – if process slow

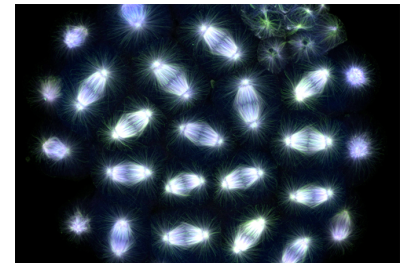
Unbeatable!

3 Flavours of Microscope

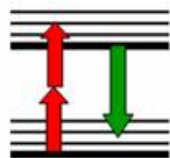
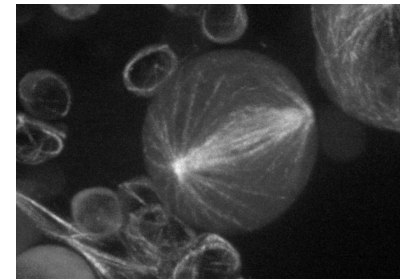
Problem:
Out of Focus
Light



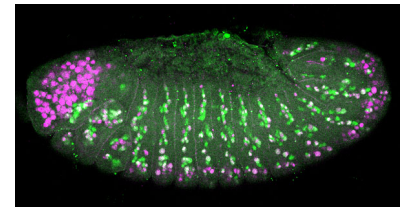
Laser
Scanning



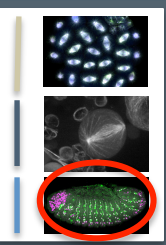
Spinning
disc



2-Photon



2-photon Microscope



Not a 'confocal'

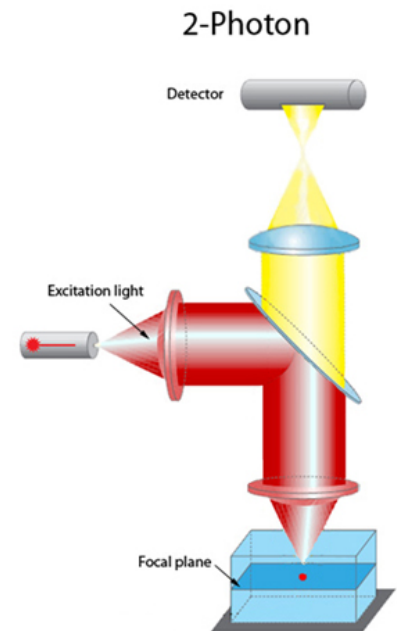
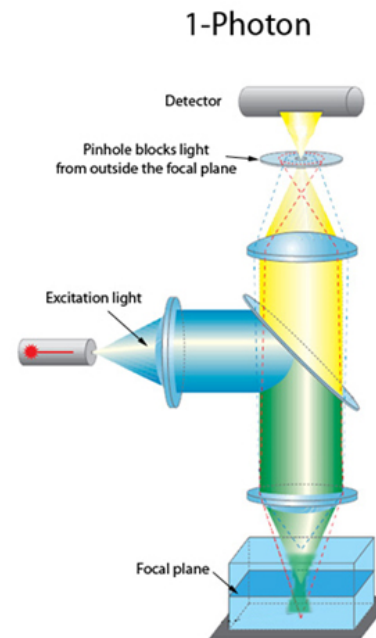
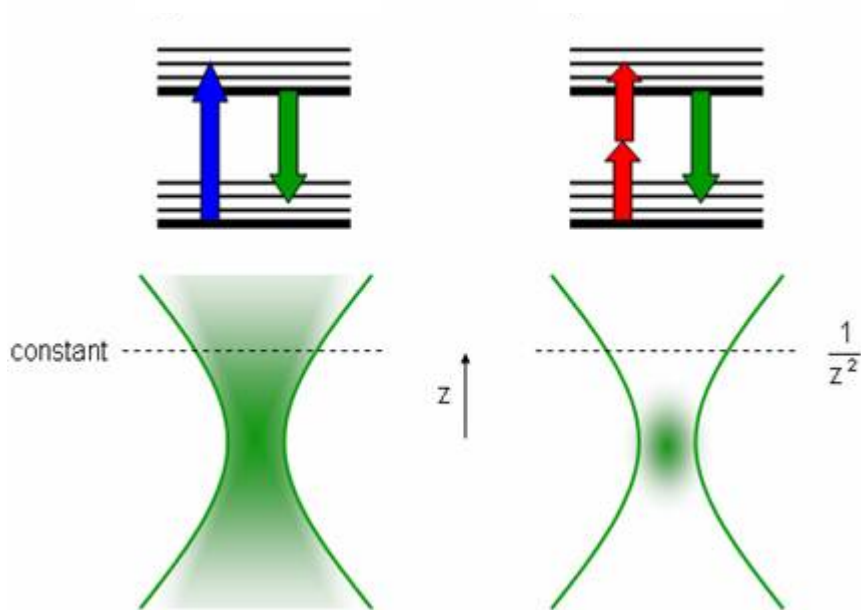
for imaging deeper into thick specimens

less damaging to biological samples

Confocal Vs 2-photon

1 Photon Excitation

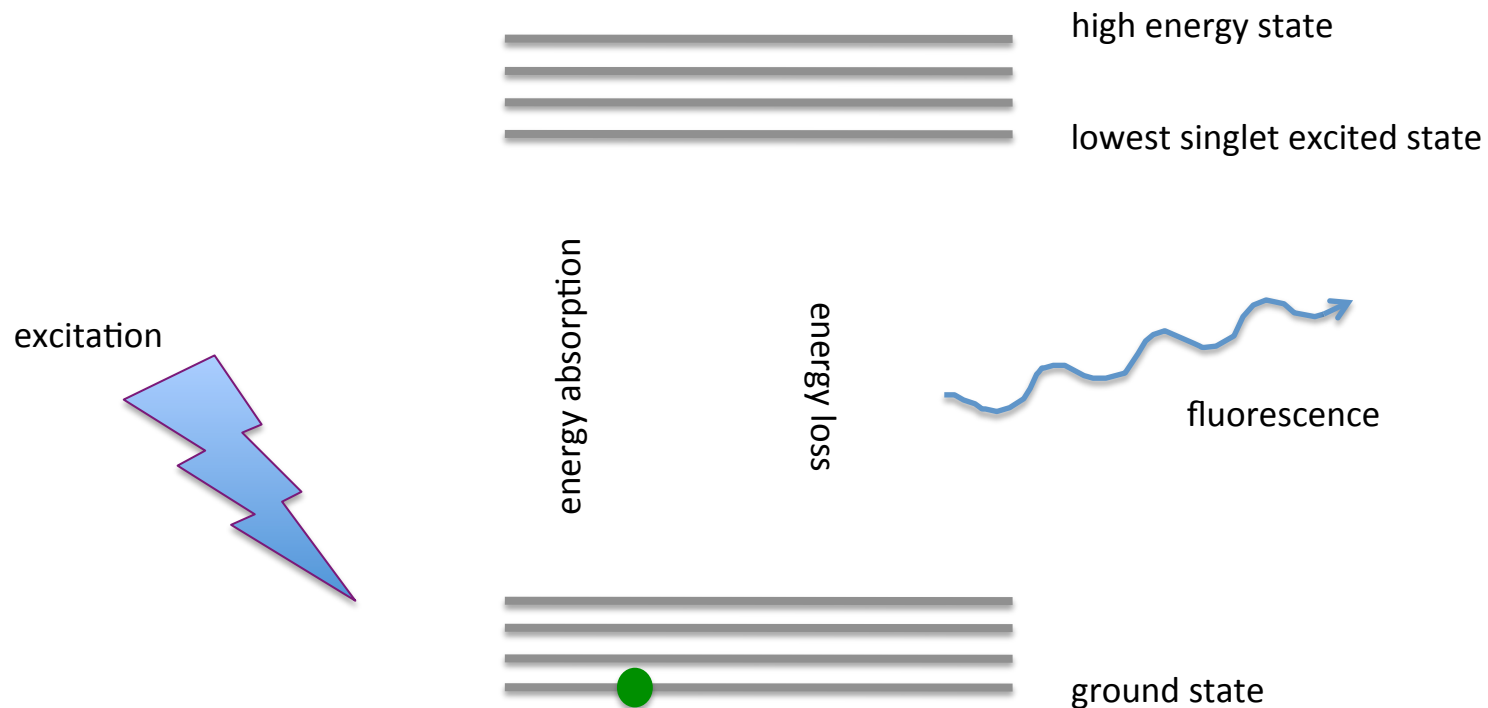
2 Photon Excitation



There is no out of focus light

THEORY

1 Photon Excitation



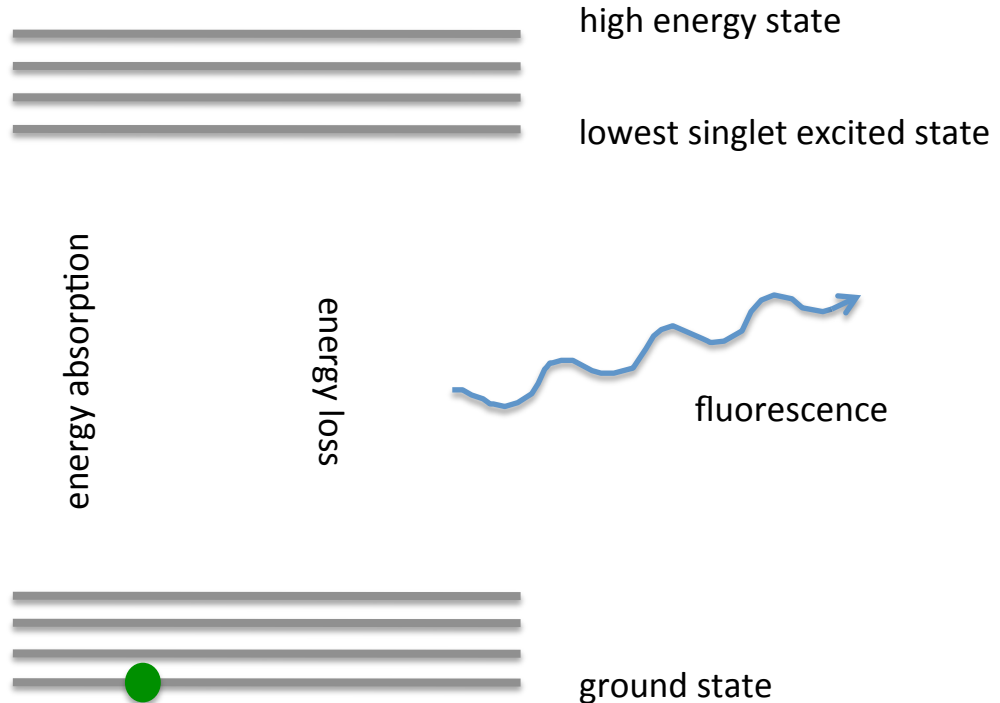
THEORY

2 Photon Excitation

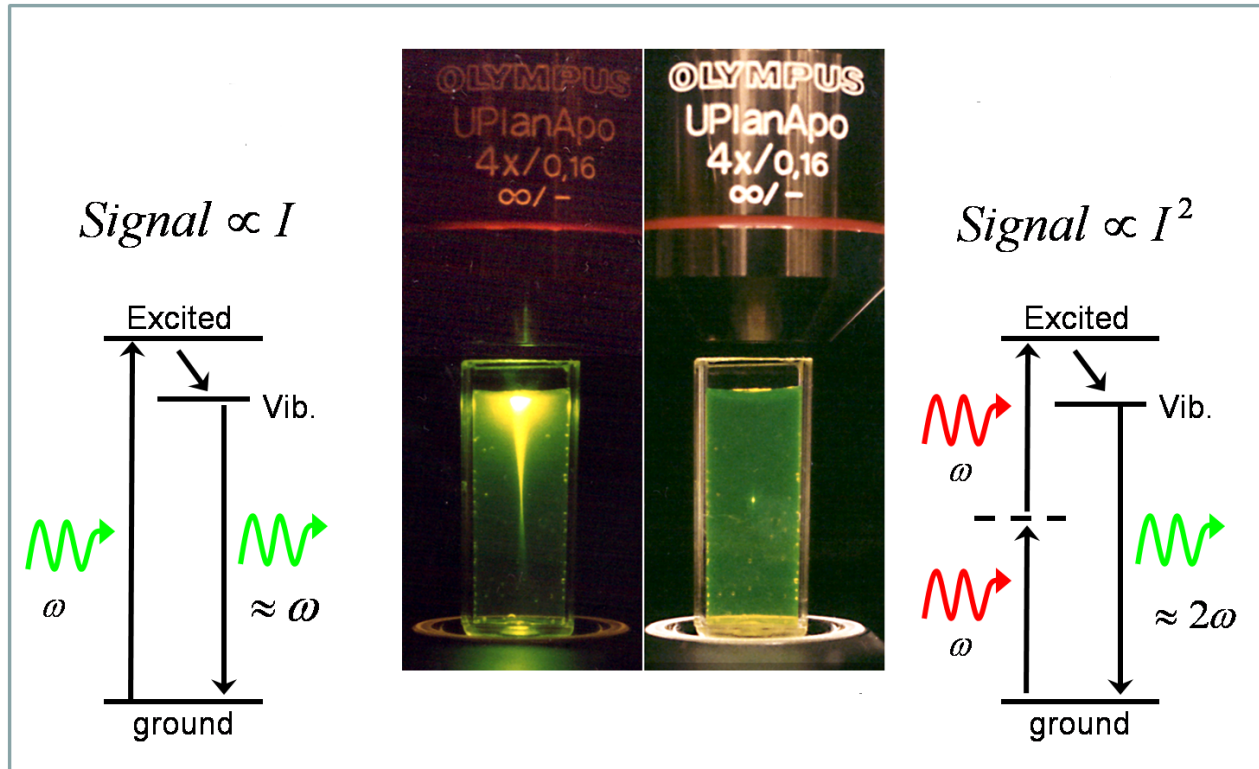
Almost simultaneous

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

Low energy
(IR ~700nm)
Pulsed
excitation



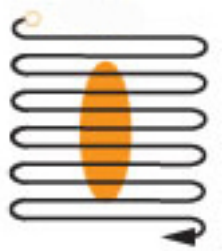
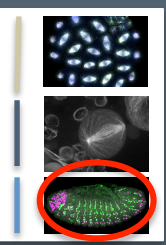
Principle of 2-photon Microscope



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

Tightly focused excitation

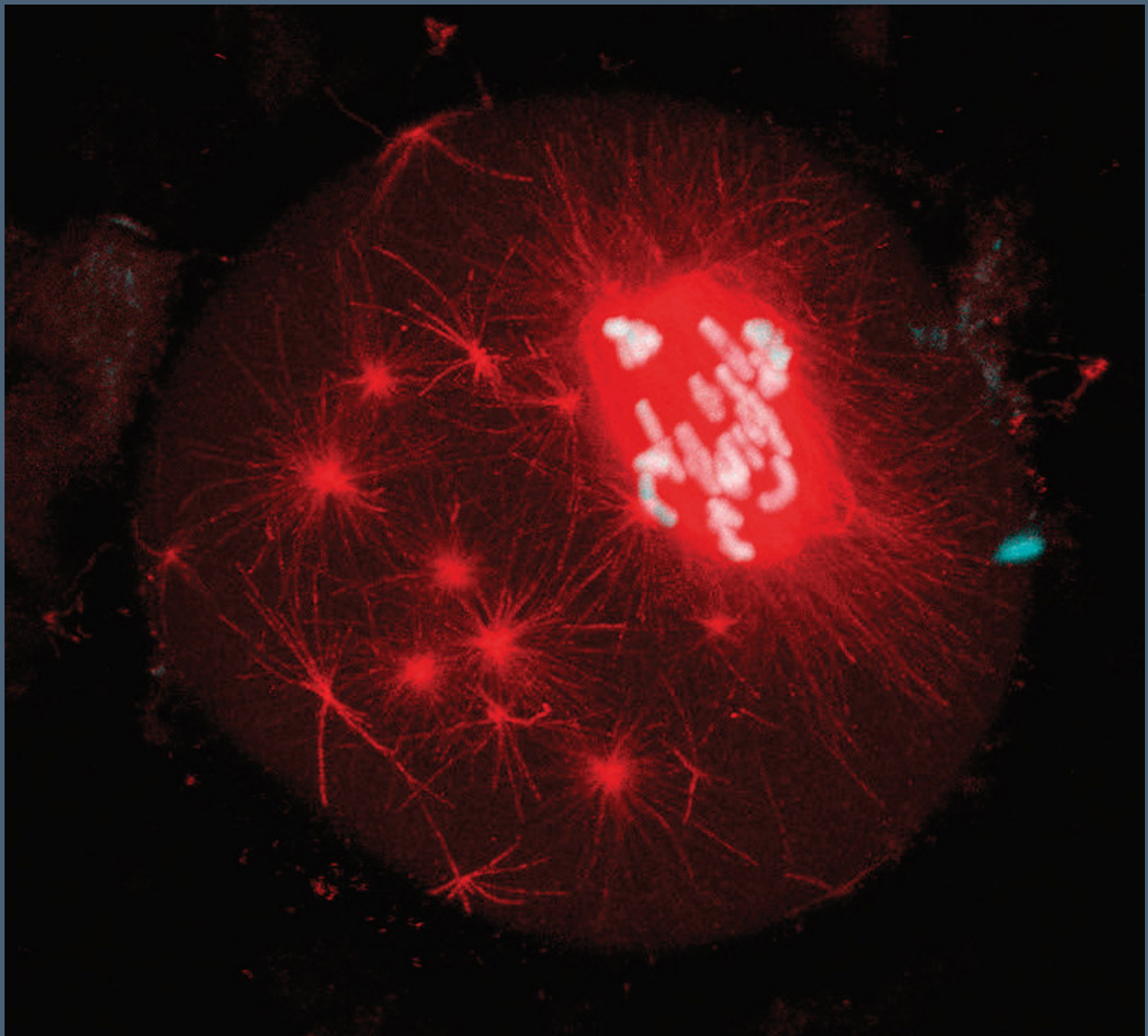
2-photon Microscope



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.

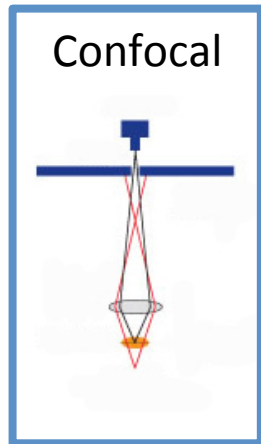
In addition, these lower-energy photons are less likely to cause damage outside the focal volume.



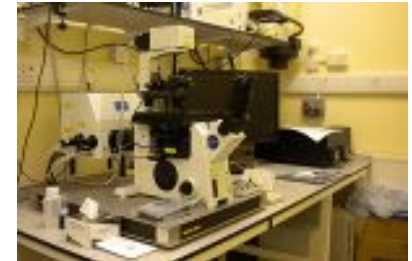
Spindle formation in mouse oocyte, labelled with Hoechst, Alexa 680. M Schuh. EMBL, Heidelberg, Germany

3 Flavours of Microscope

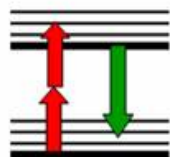
Problem:
Out of Focus
Light



Laser
Scanning

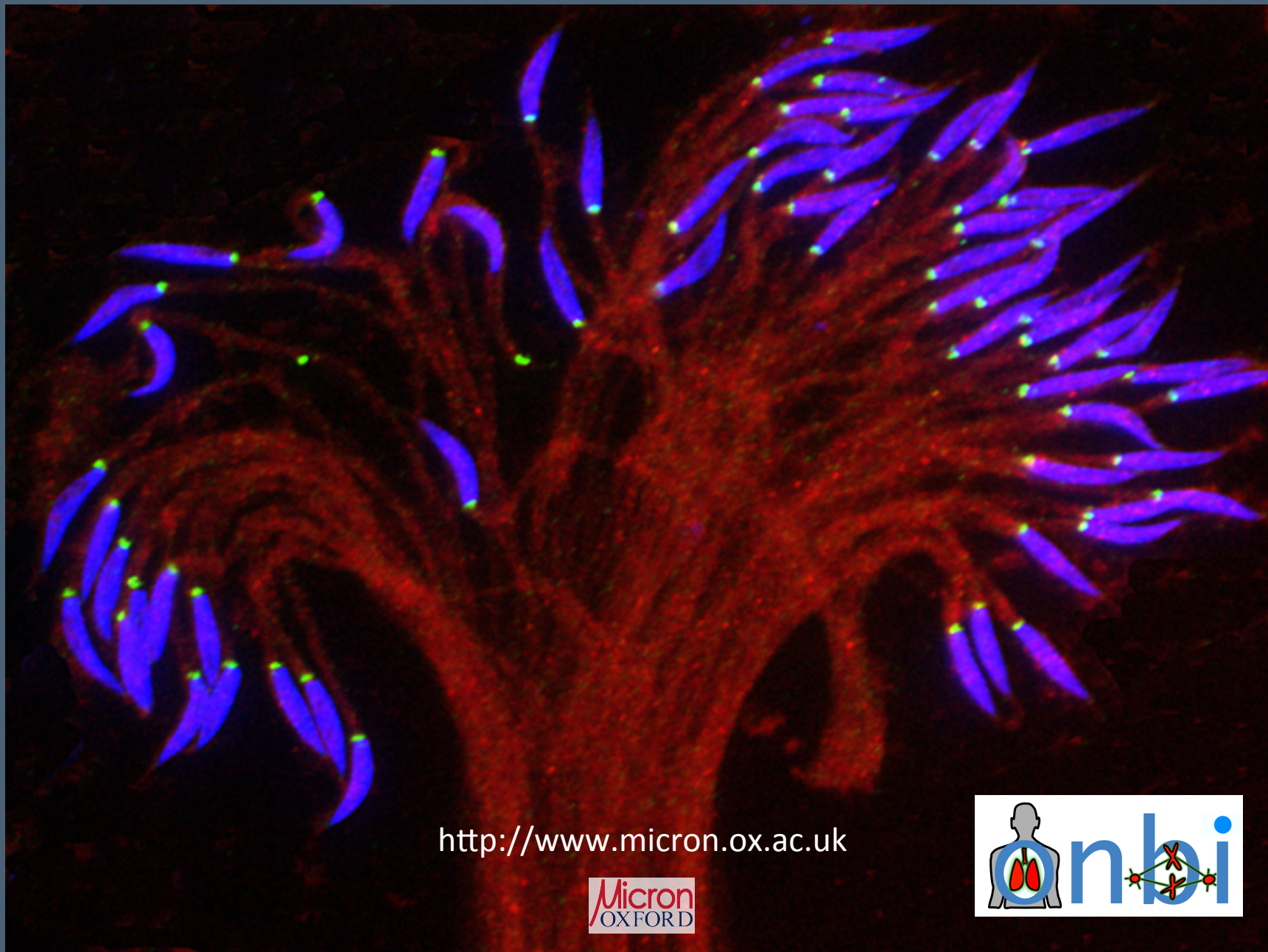


Spinning
disc



2-Photon





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

Micron
OXFORD

