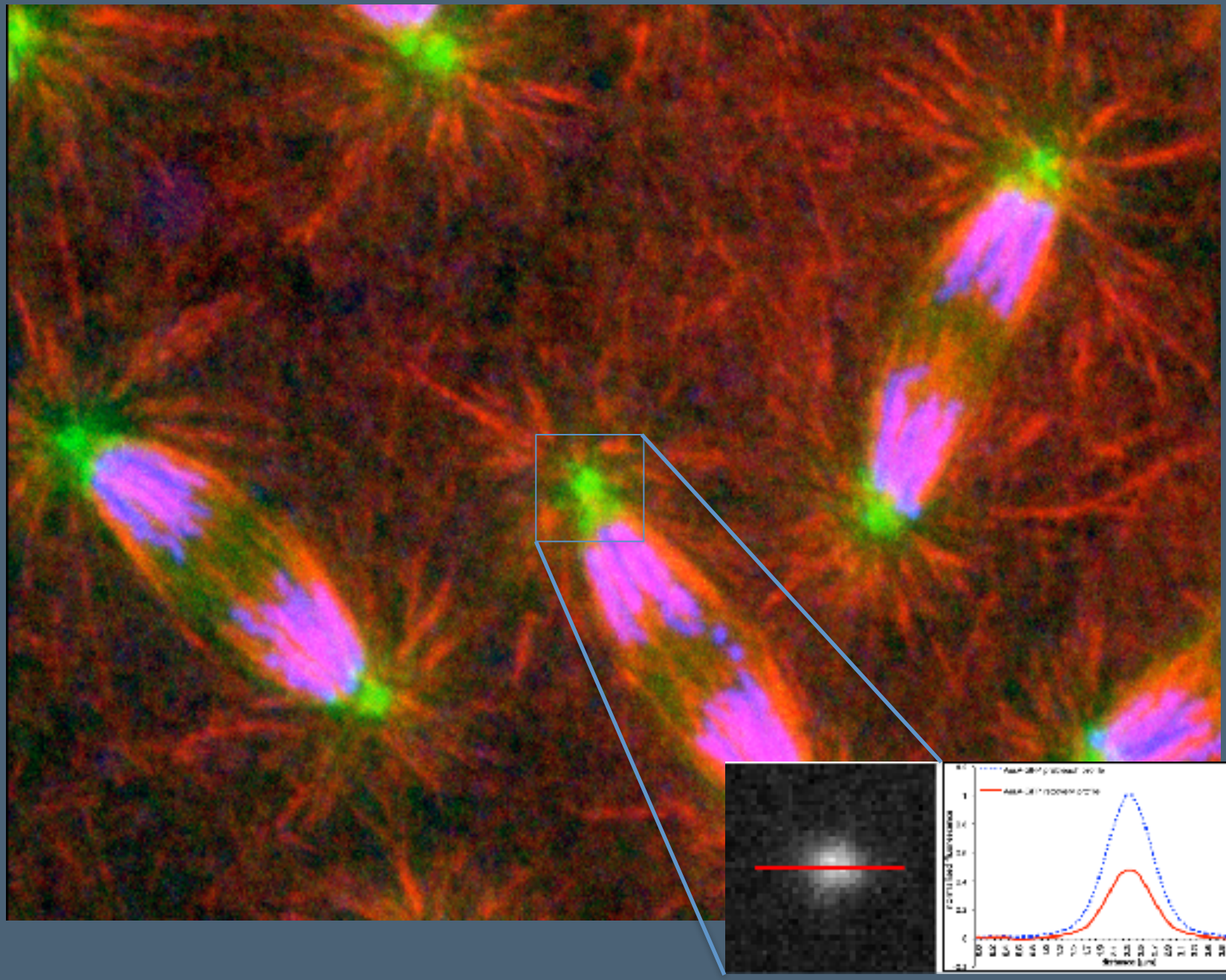
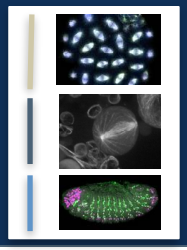
Confocal Microscopy

(Increasing contrast and resolution using optical sectioning)
Lecture 7

November 2017



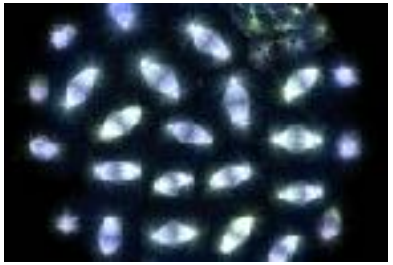


3 Flavours of Microscope

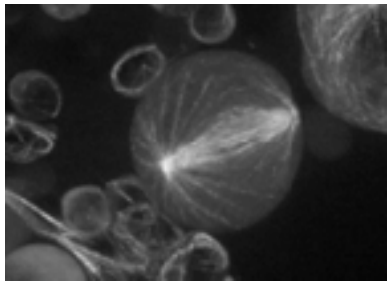
Problem:
Out of Focus
Light

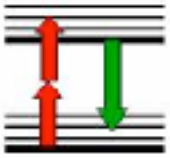
Confocal

Laser
Scanning

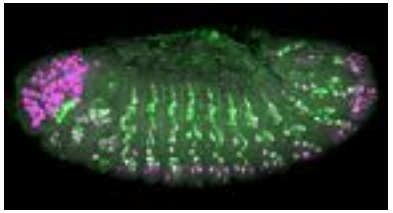


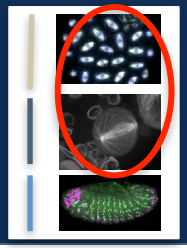
Spinning disc





2-Photon





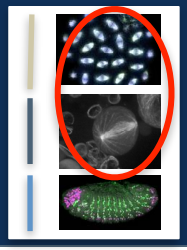
short History of Confocal Microscope

Confocal “concept’ patented by Marvin Minsky in 1957

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

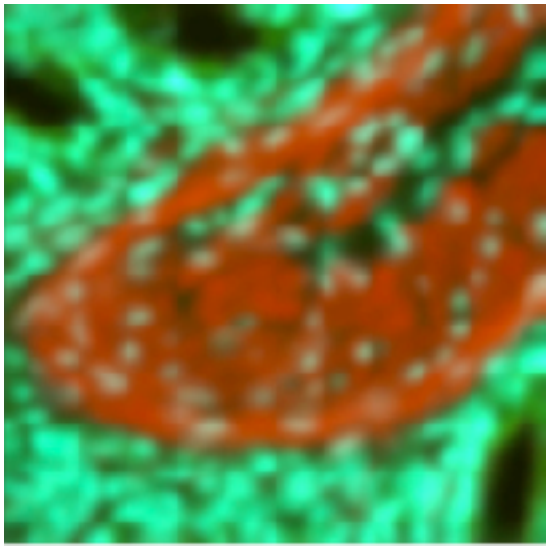
Brakenhoff, Stelzer developed “stage” scanning confocal in late 1970

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



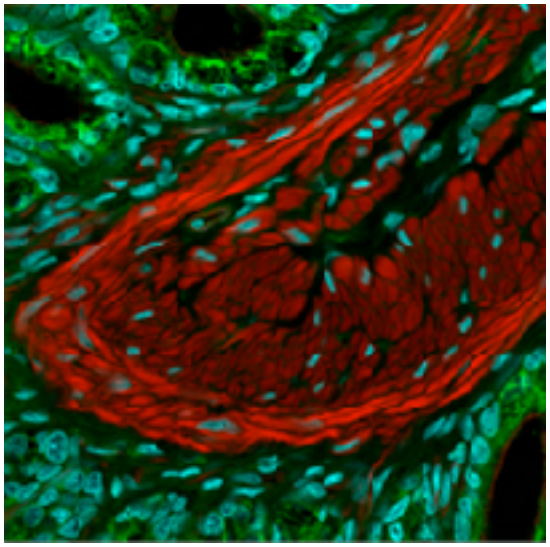
Comparison
Widefield Vs Confocal

Widefield

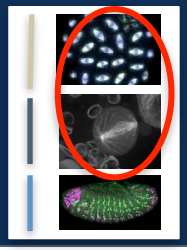


Out of focus light ‘blurs’ image

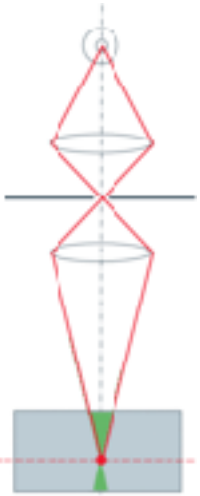
Confocal



Out of focus light is blocked

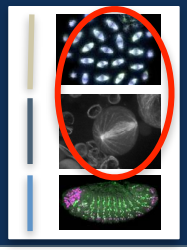


Principle of Confocal Microscopes Pinhole

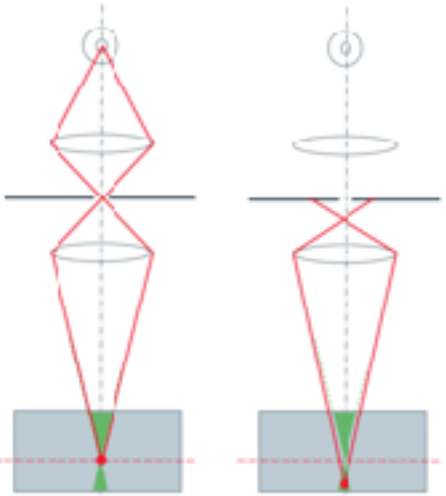


Pinhole diaphragm in the Conjugated focal plane = CONFOCAL

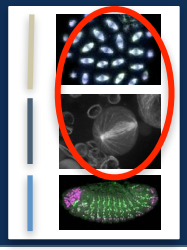
in focus light (from the optical section) passes through the pinhole and into the detector



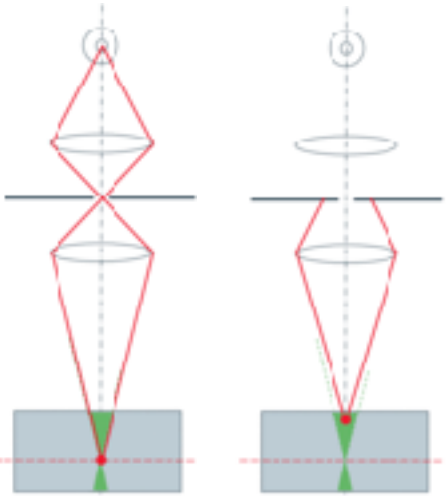
Pinhole – blocks out-of-focus light



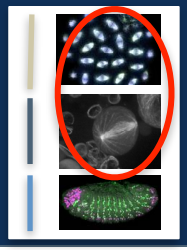
light from below the optical section crosses infront of the pinhole and doesn’t pass through the pinhole aperture



Pinhole – blocks out-of-focus light



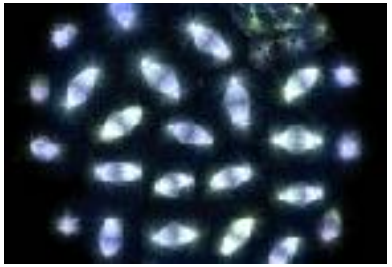
light from above the optical section also doesn't pass through the pinhole aperture



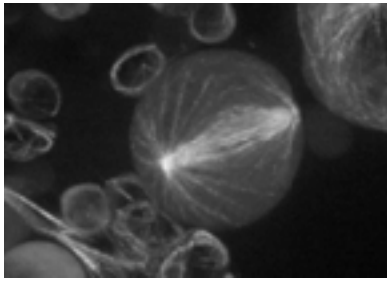
Confocal Microscopes

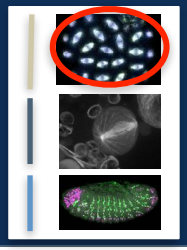
Confocal

Laser Scanning



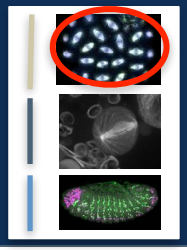
Spinning disc



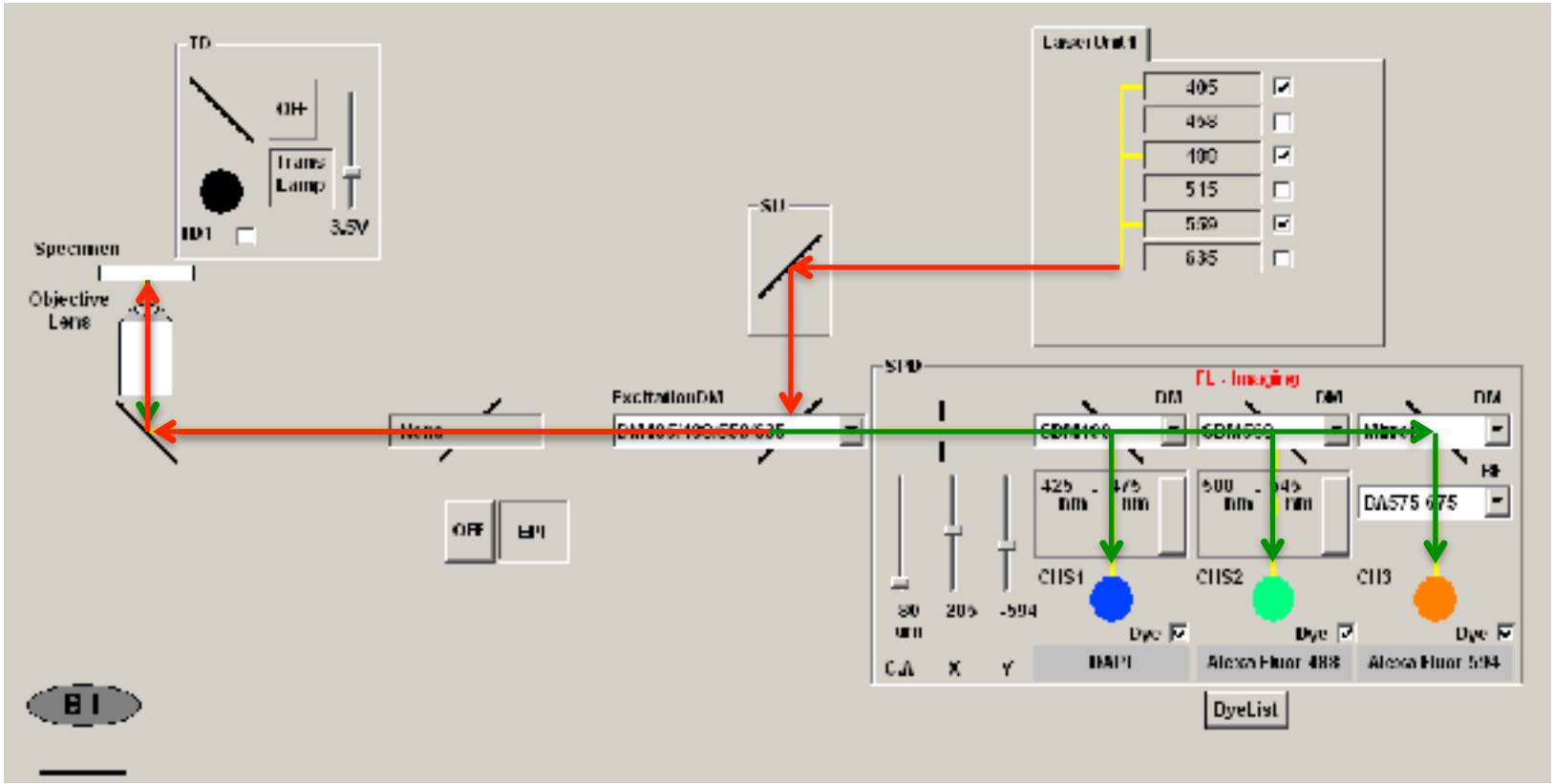


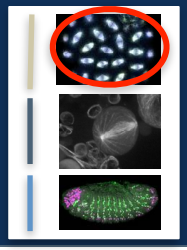
Laser Scanning Confocal

Laser Scanning Confocals are great to get ‘pretty’ images



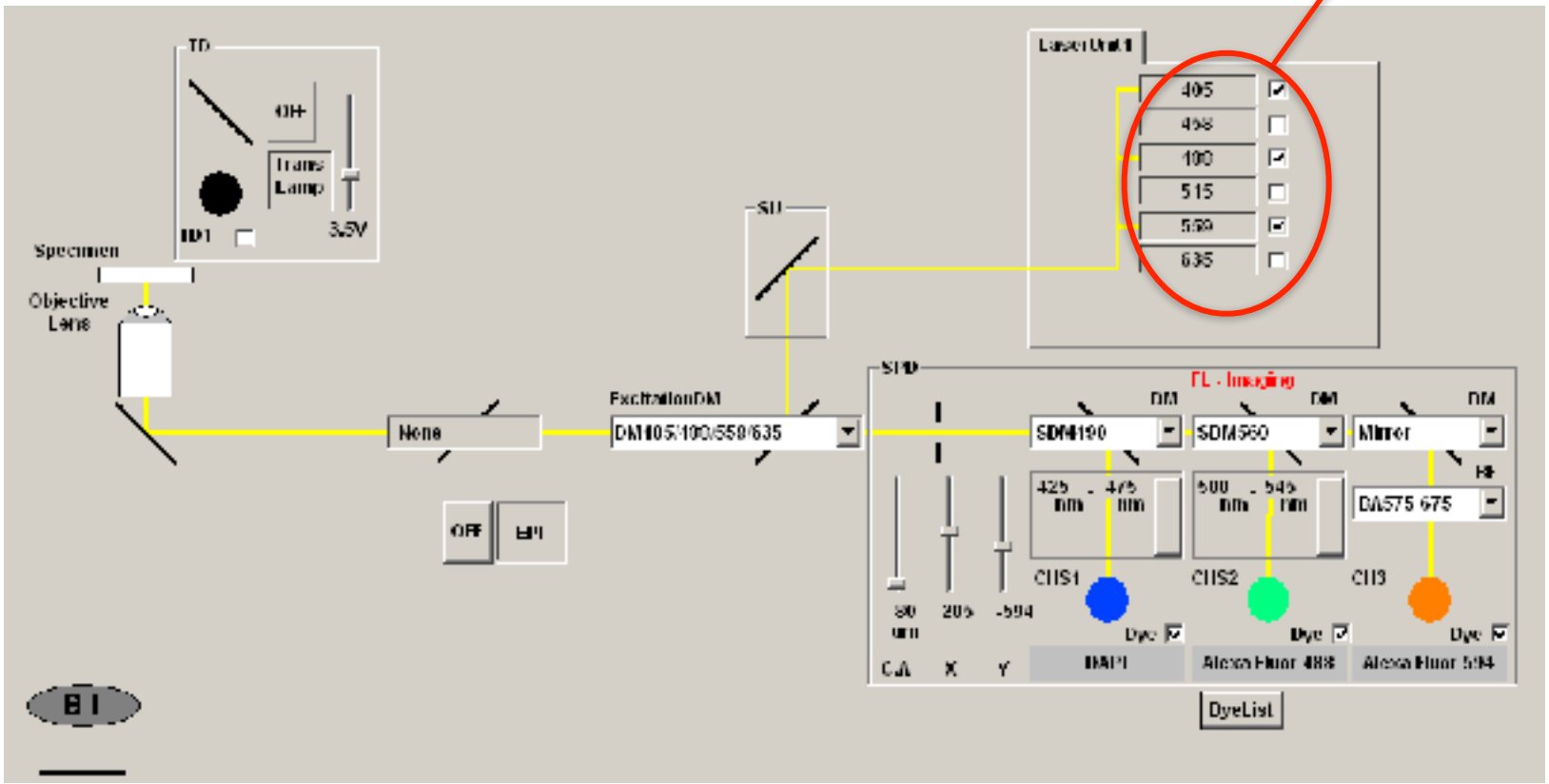
Laser Scanning Confocal

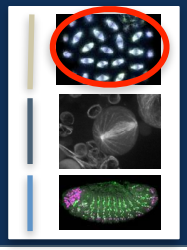




Laser Light Source

laser light source

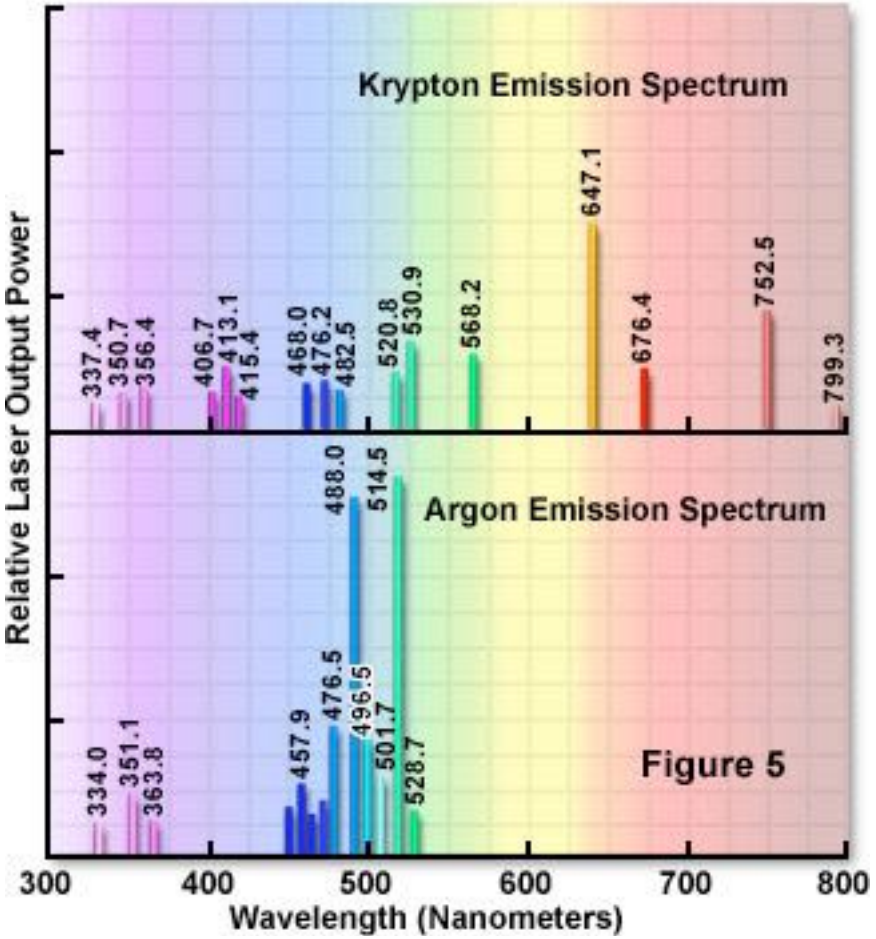


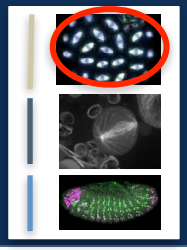


Laser Light Source

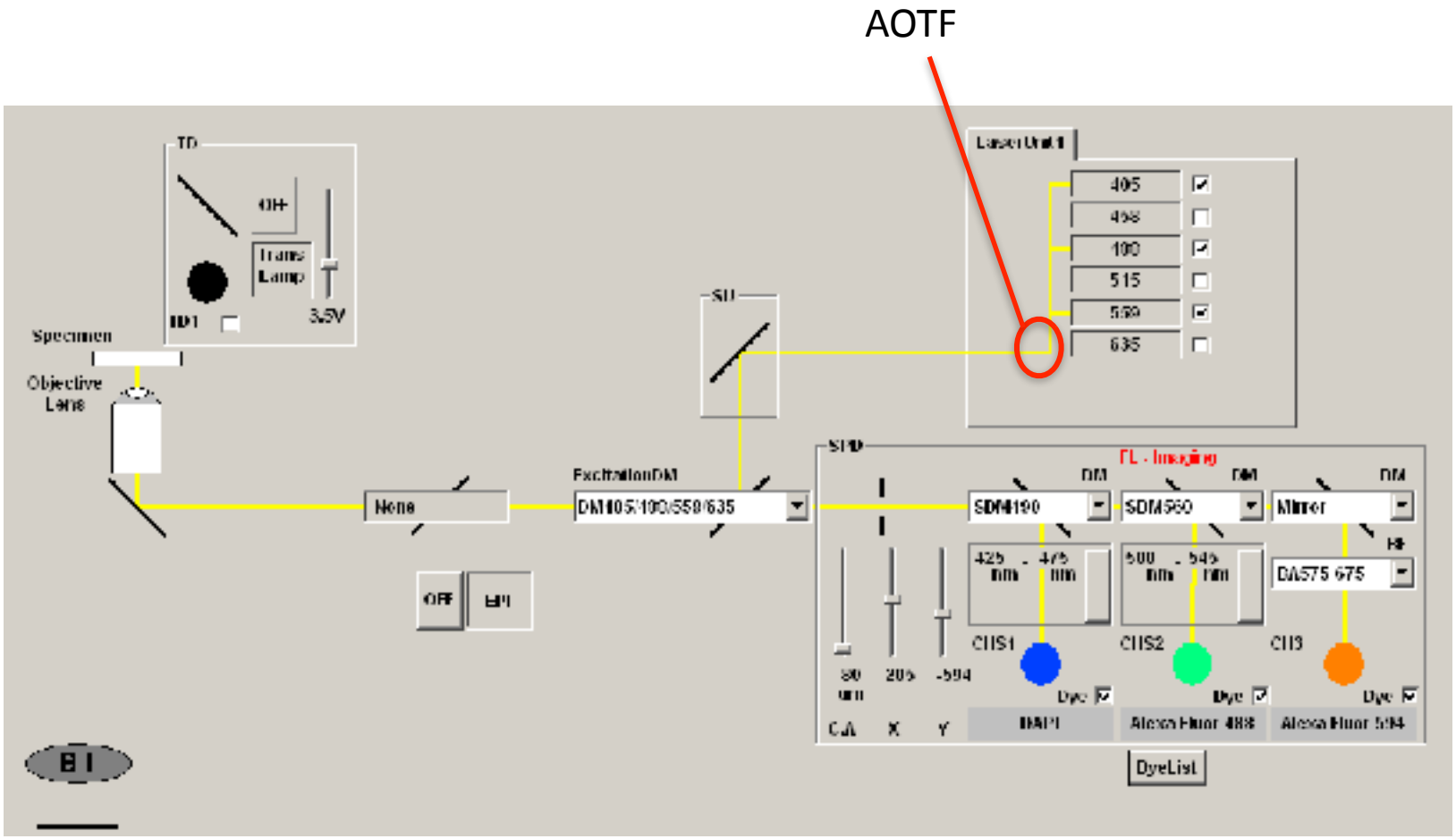
enables tighter control of fluorophores excited

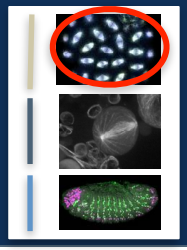
Laser Emission Spectra





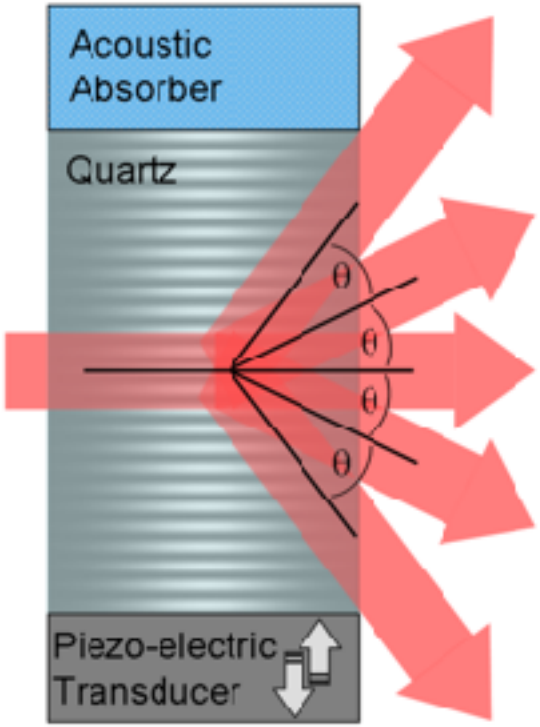
AOTF
Acousto-Optic Tunable Filter





THEORY

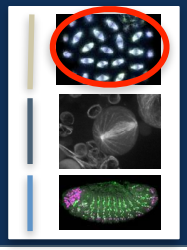
AOTF
Acousto-Optic Tunable Filter



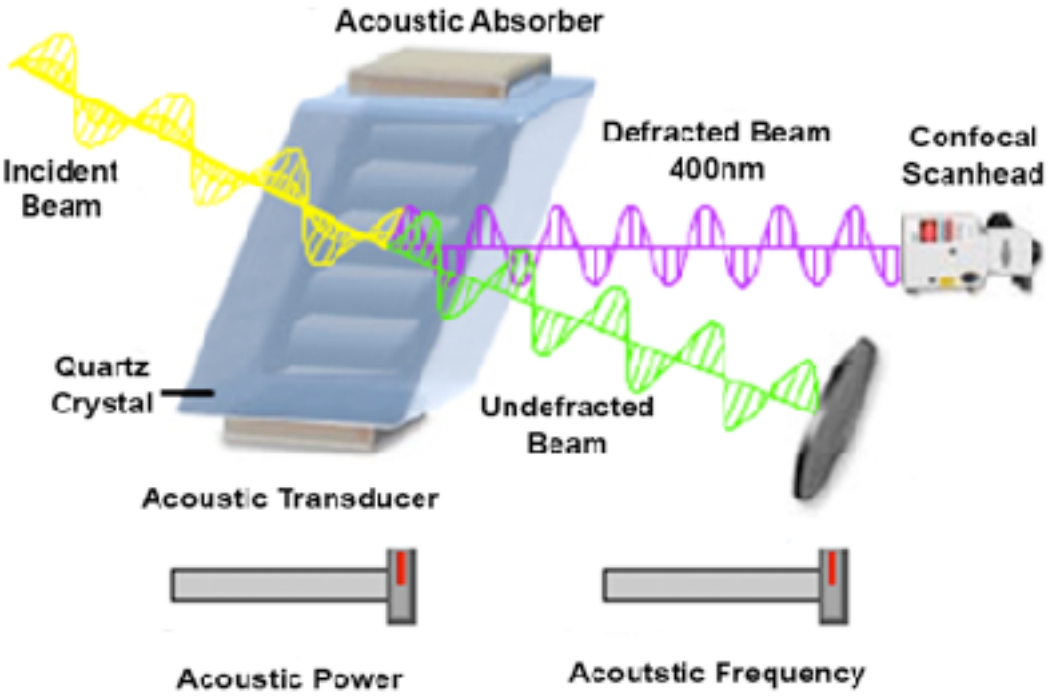
acousto-optic effect:

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.

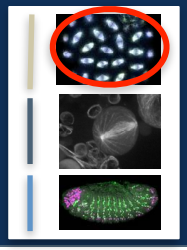


AOTF
Acousto-Optic Tunable Filter

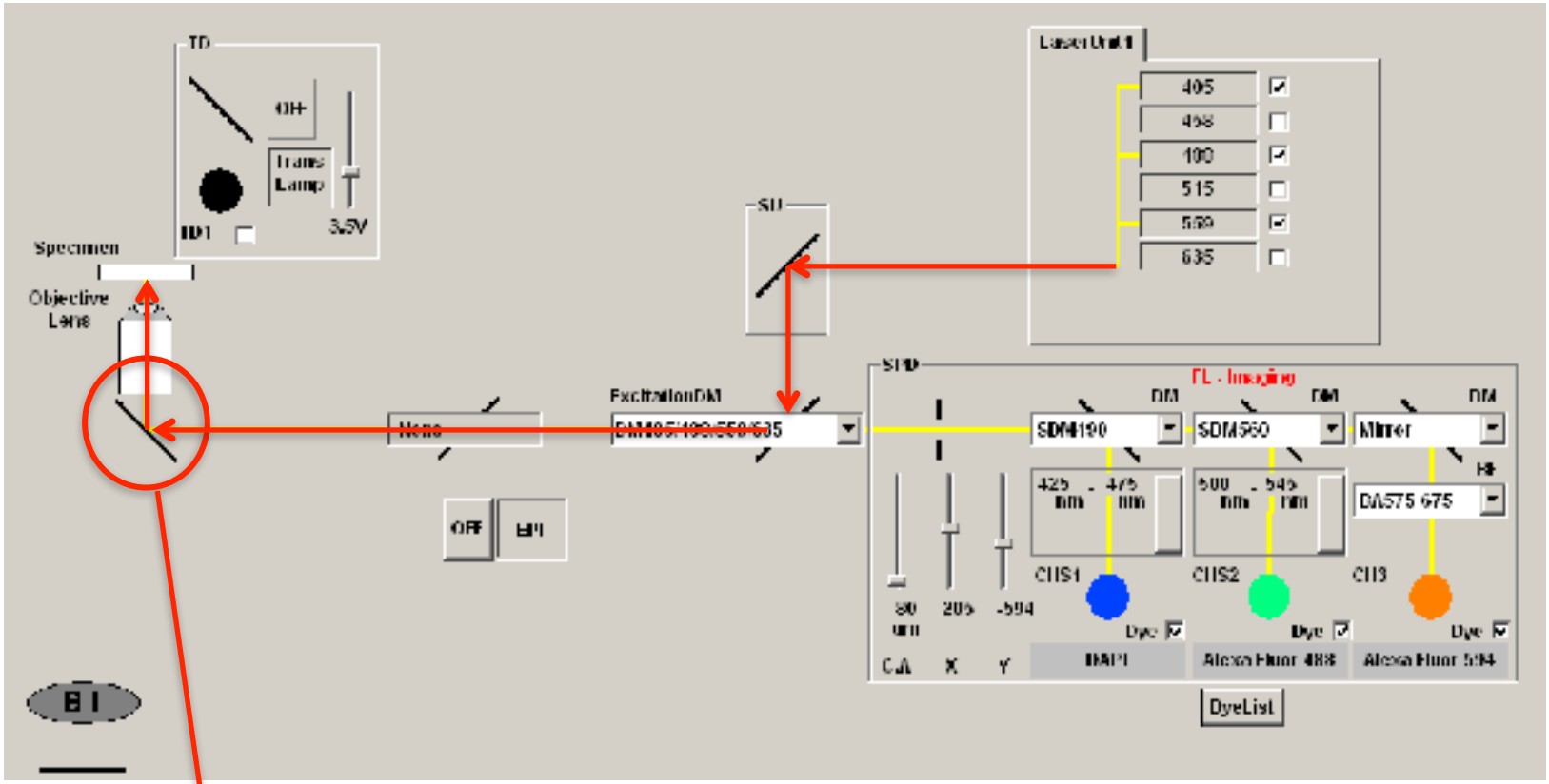


Quick On/Off of lasers

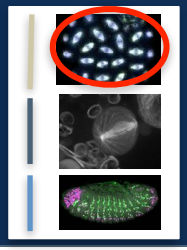
Very fast changes between excitation wavelengths



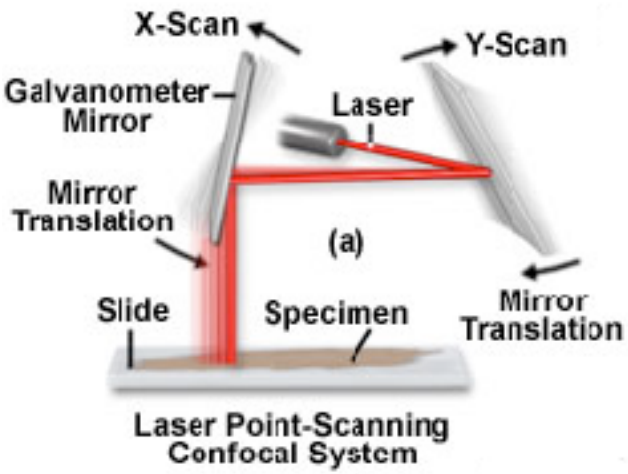
Galvo Scanning Mirrors

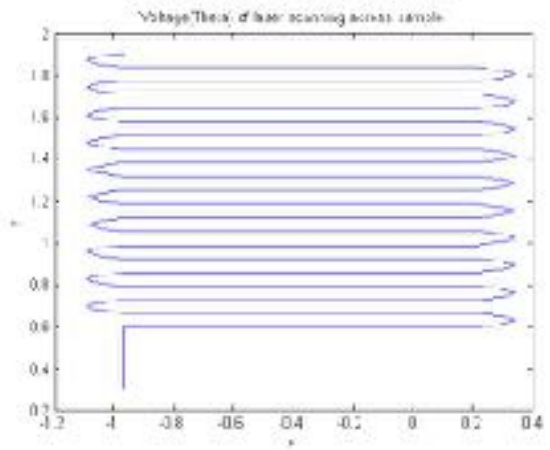


Galvo Scanning Mirrors



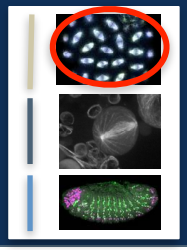
Galvo Scanning Mirrors





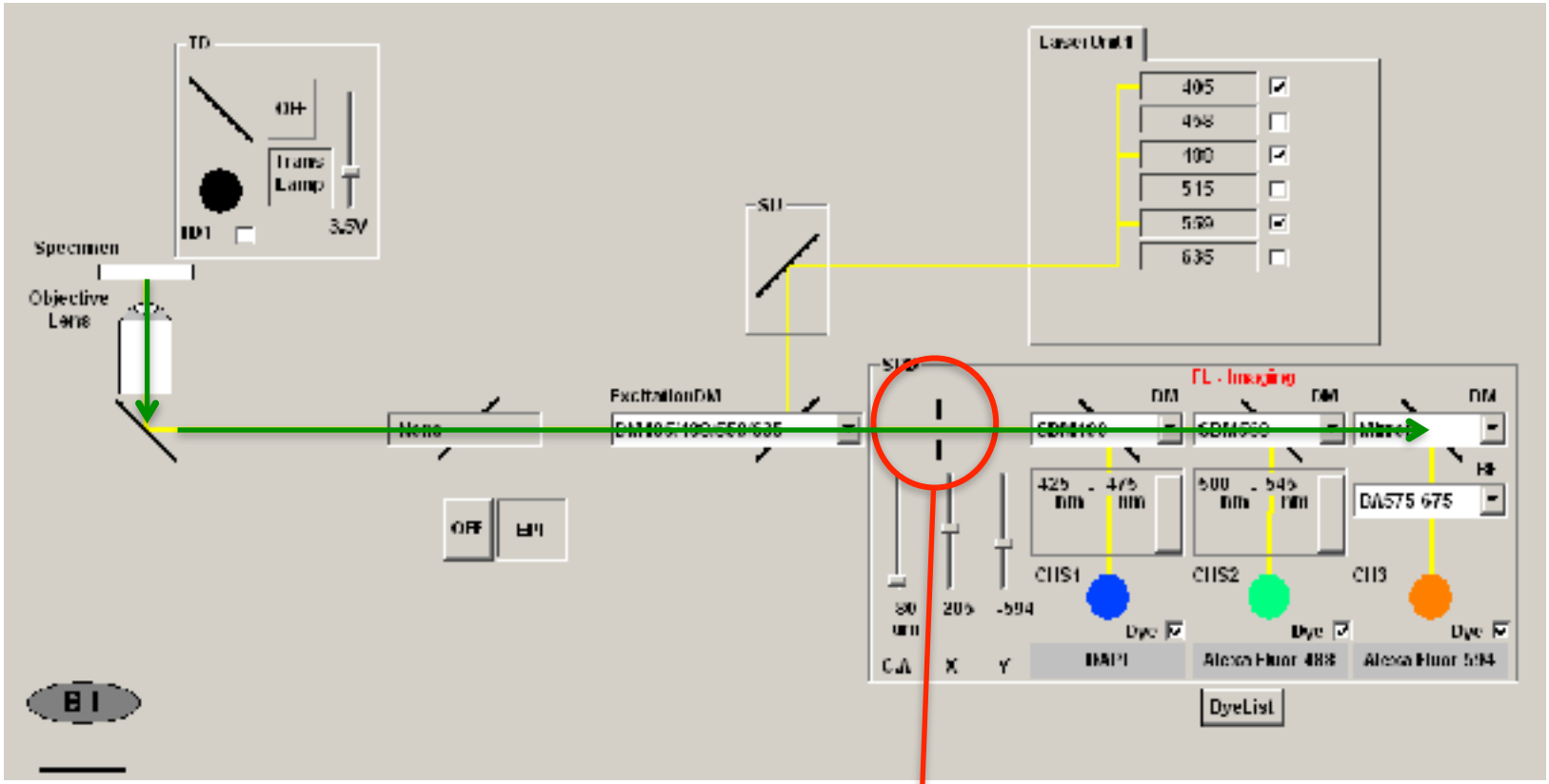
Sample excited at one point at a time

Relatively slow

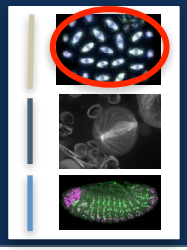


Adjustable Pinhole

AOTF

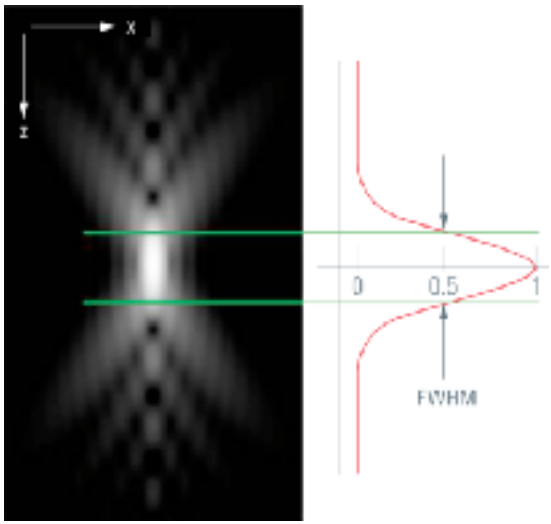


pinhole



THEORY

Pinhole – Optical Sectioning



FWHM=Full Width Half-Maximum

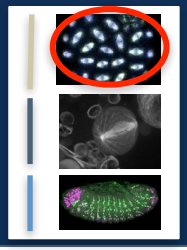
Shorter the wavelength the thinner the optical section

Diameter of the pinhole: Smaller pinhole thinner optical section

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

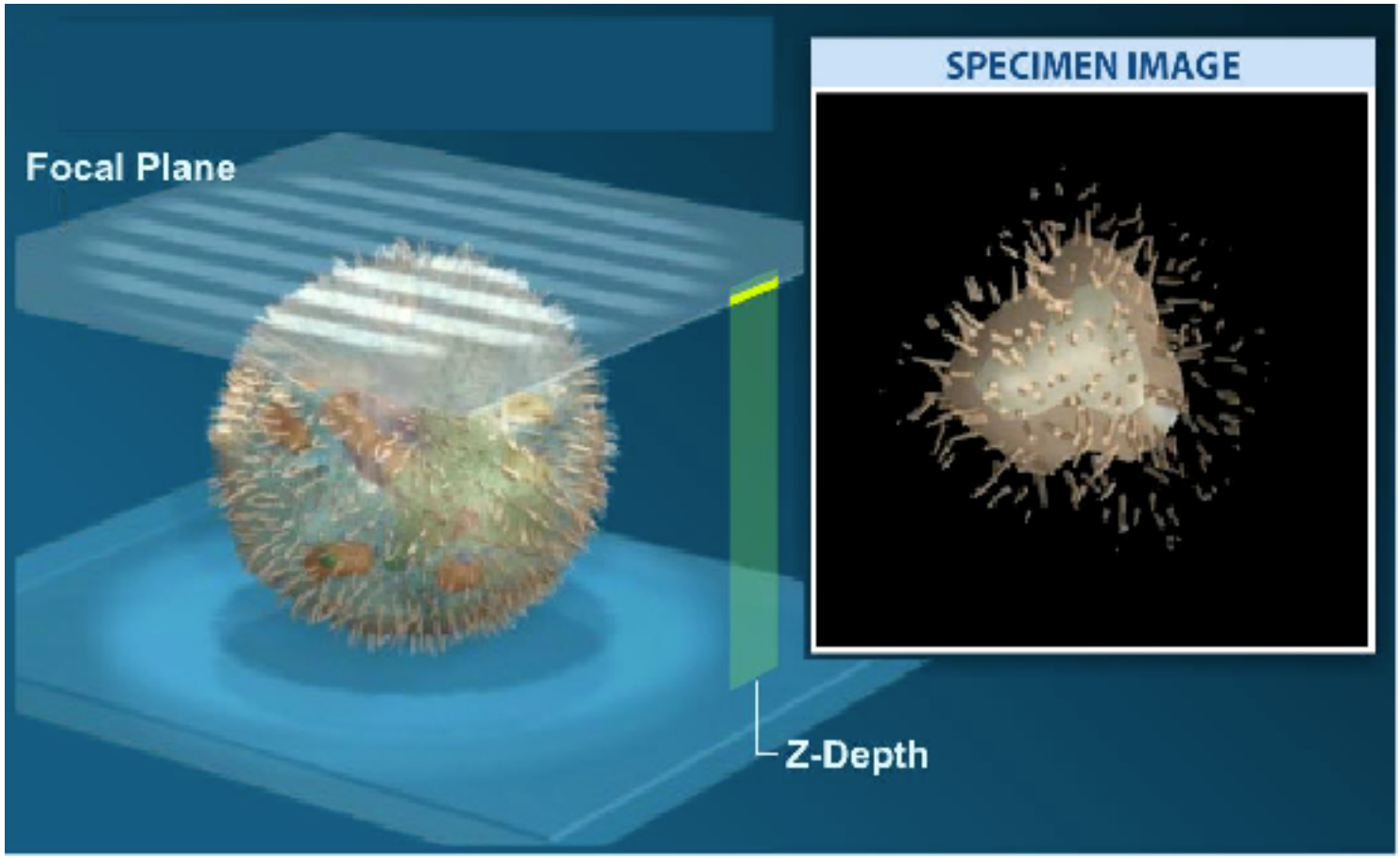
The higher the NA.
the thinner the section

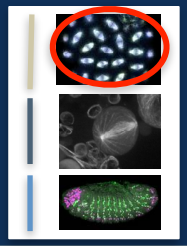
Weak signal > open pinhole > more light but thicker section



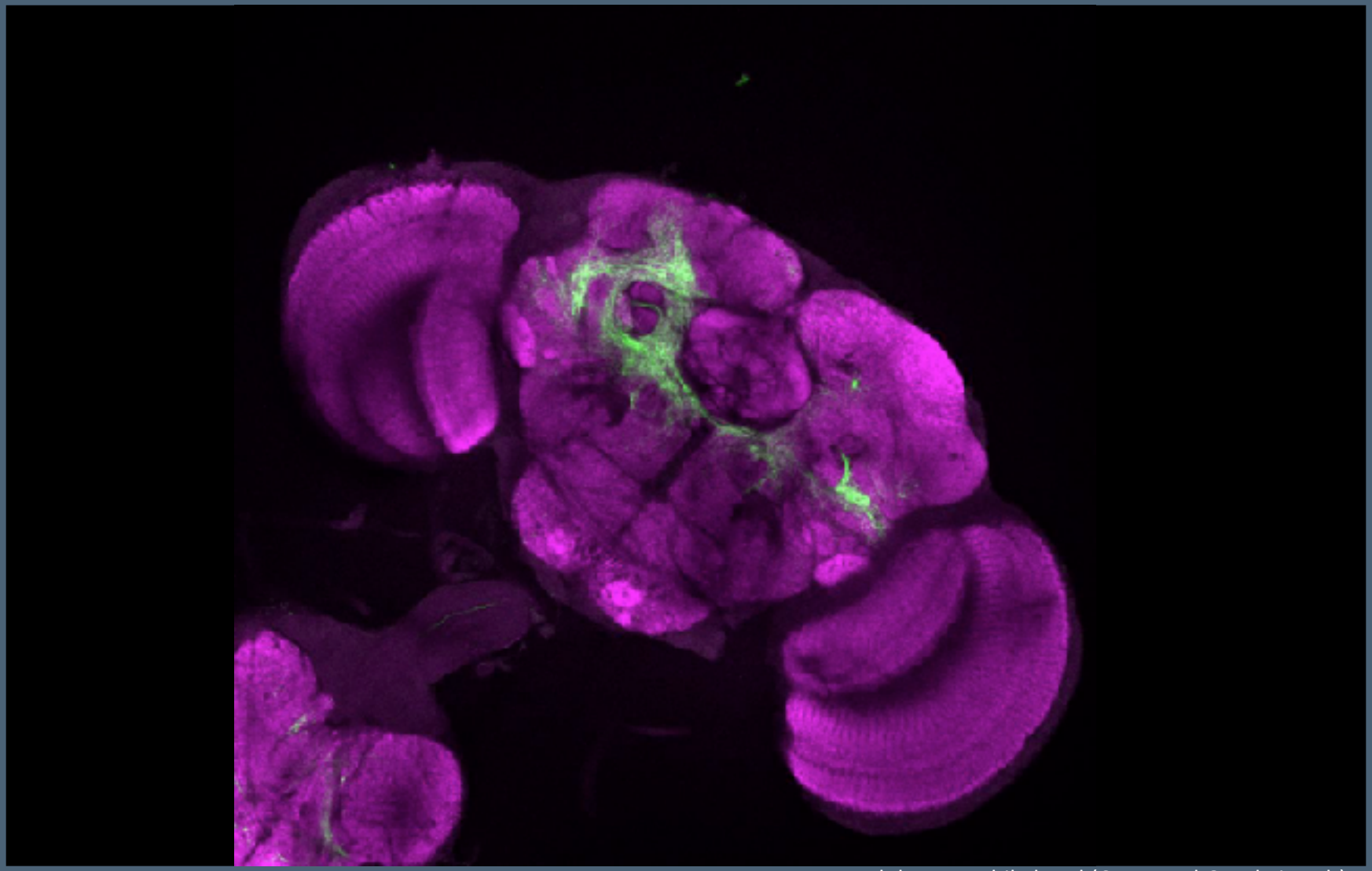
Confocal enables 3D reconstruction

Optical section

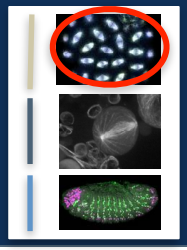




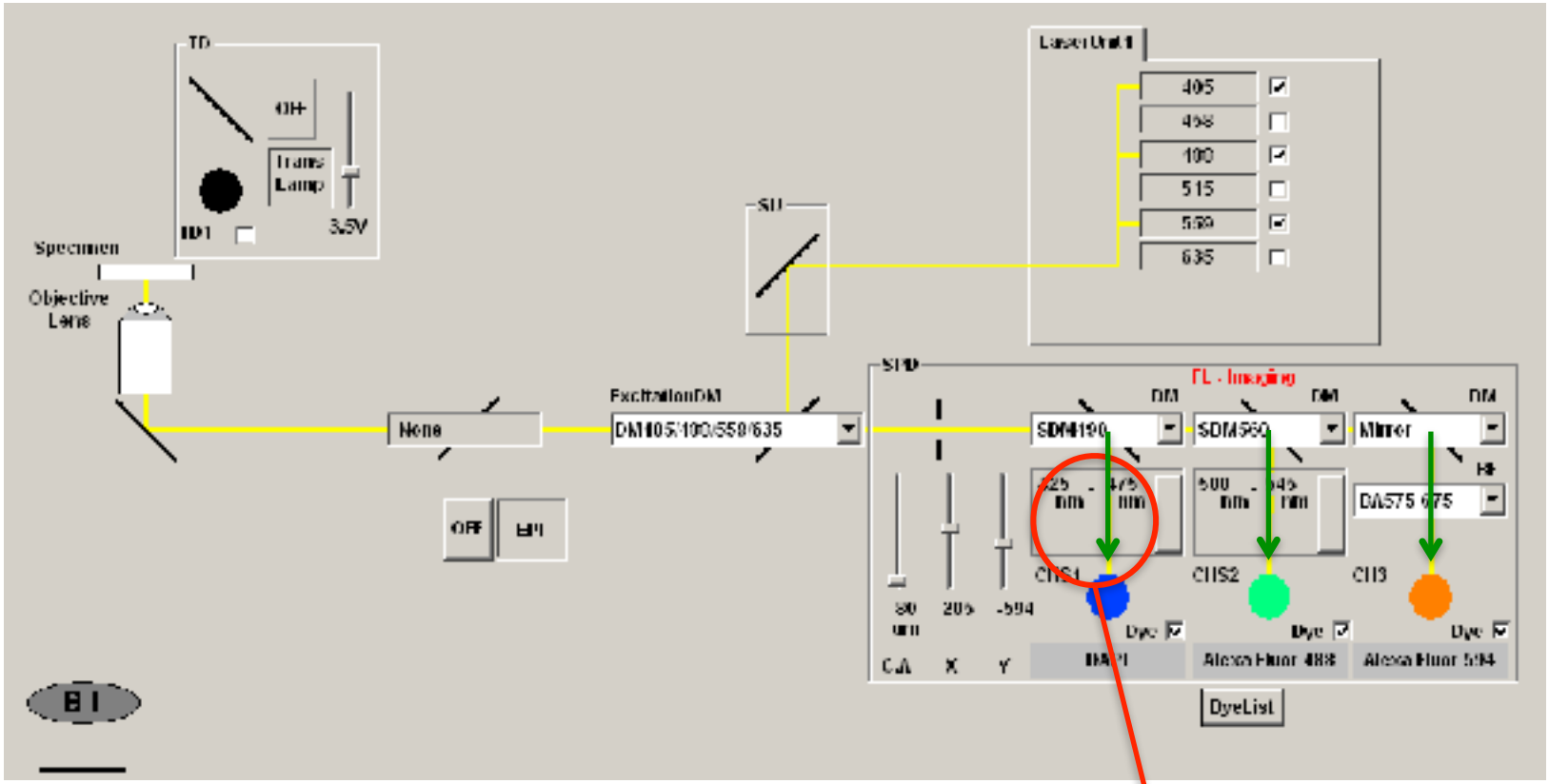
Confocal enables 3D reconstruction

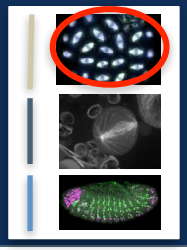


Adult Drosophila head (C. Rezeval Goodwin Lab)

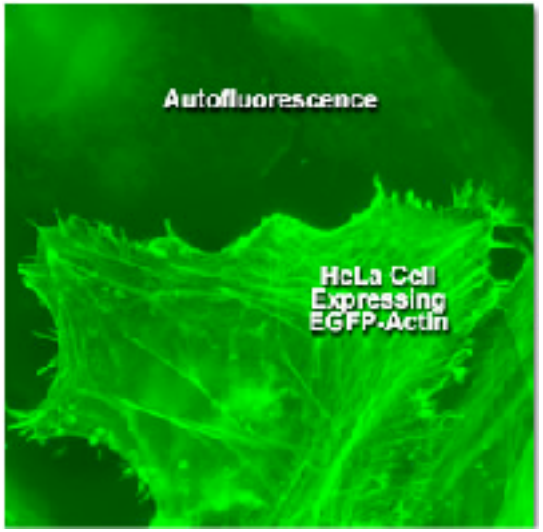


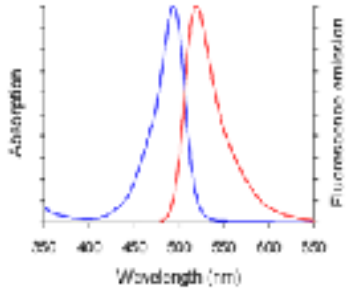
Variable Detector Slit





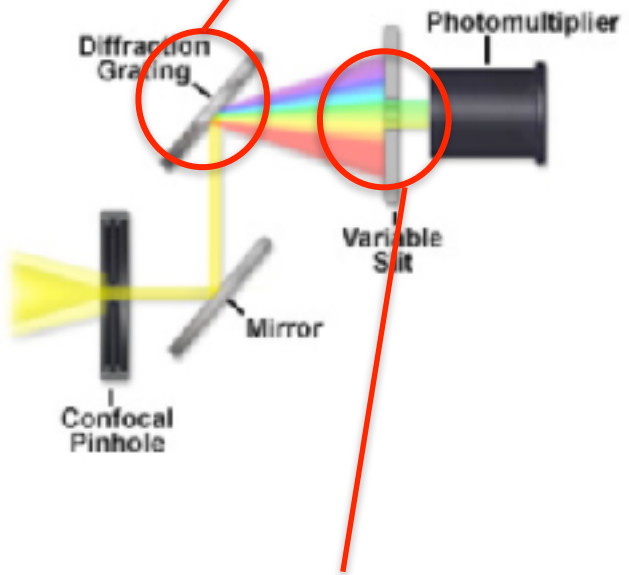
Spectral Unmixing



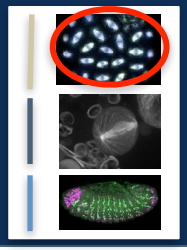


Light emitted from fluorophore as a spectrum

Defraction grating separates wavelengths over physical area

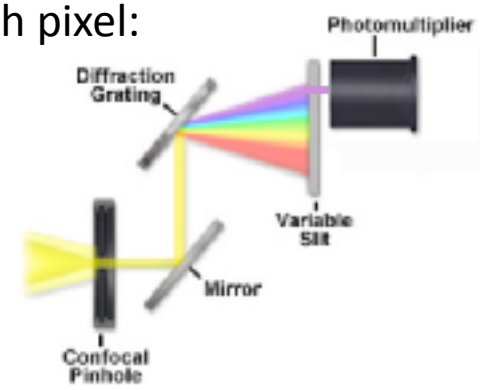


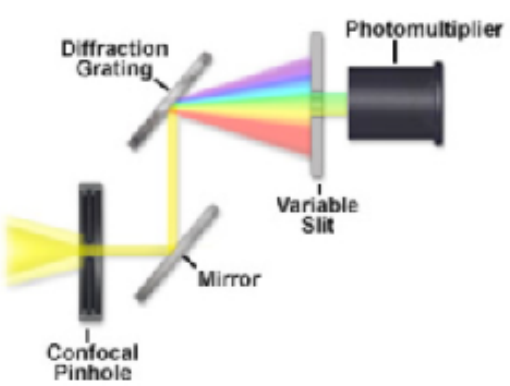
Variable slit lets through only certain wavelengths

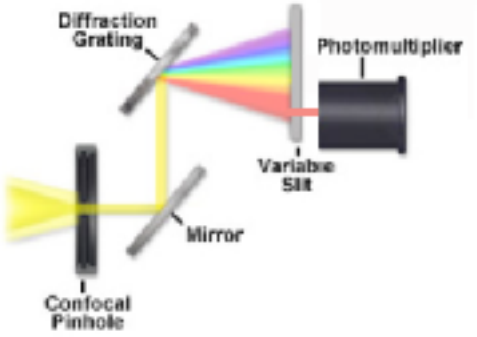


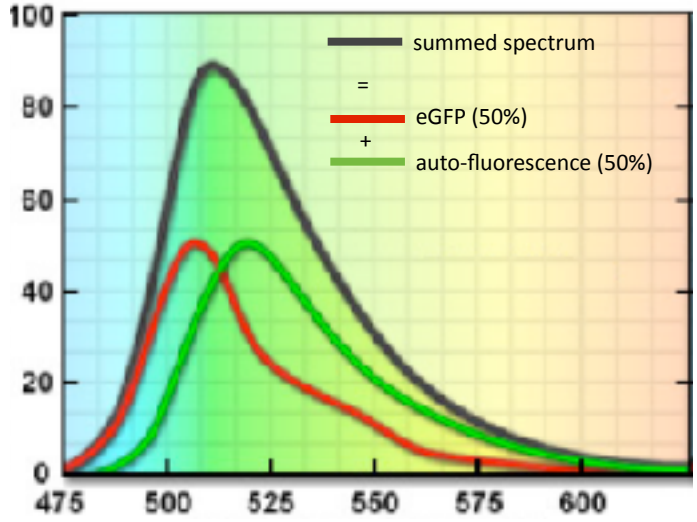
Spectral Unmixing

At each pixel:

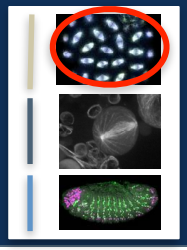




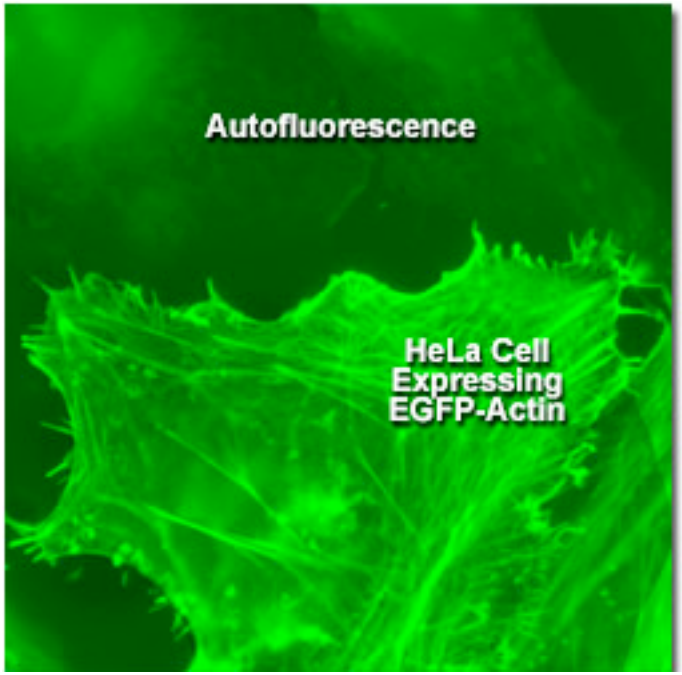


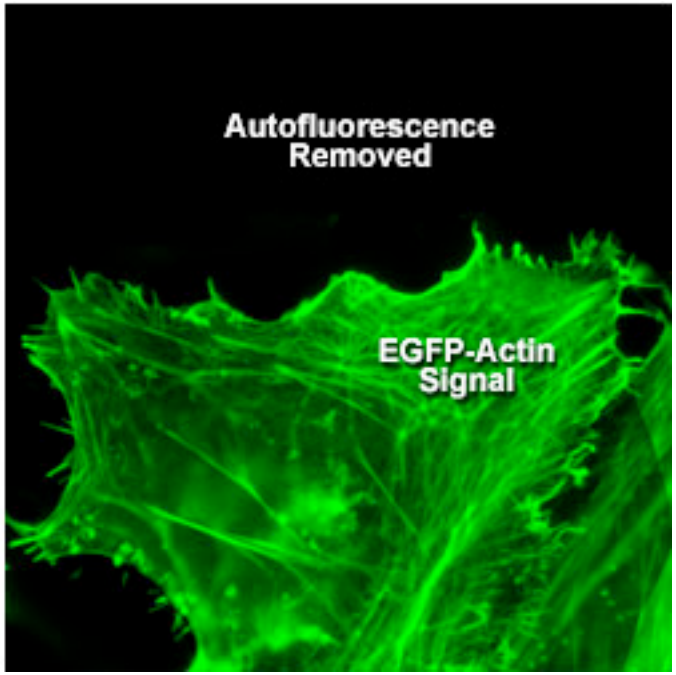


Match the summed spectrum with all possible summed combinations from a library
At each pixel you therefore know the proportion of each fluorophore present



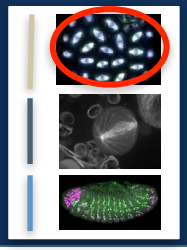
Spectral Unmixing removal of autofluorescence



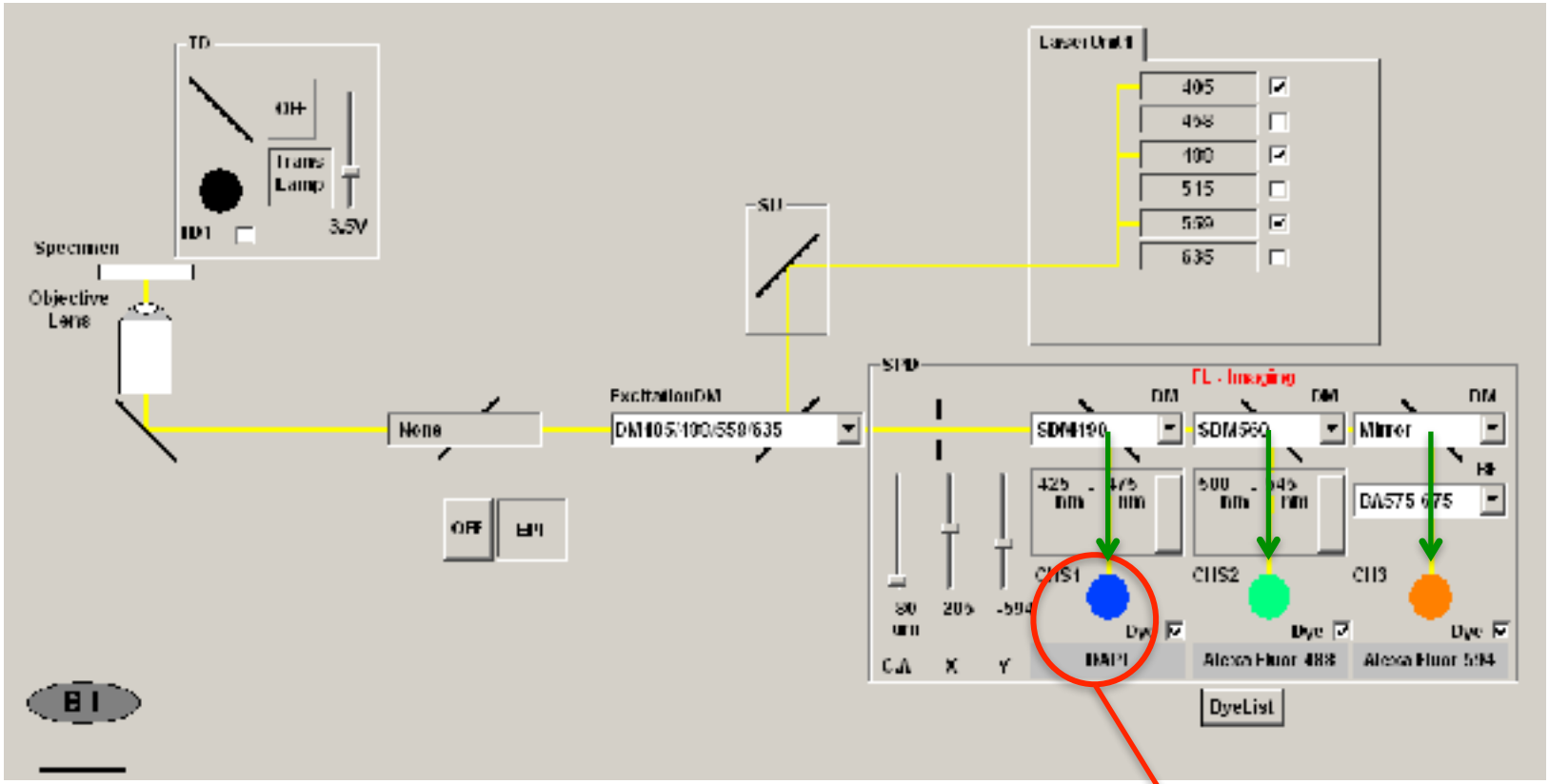


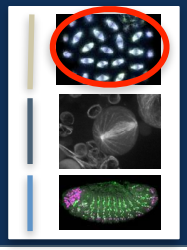
At each pixel:

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

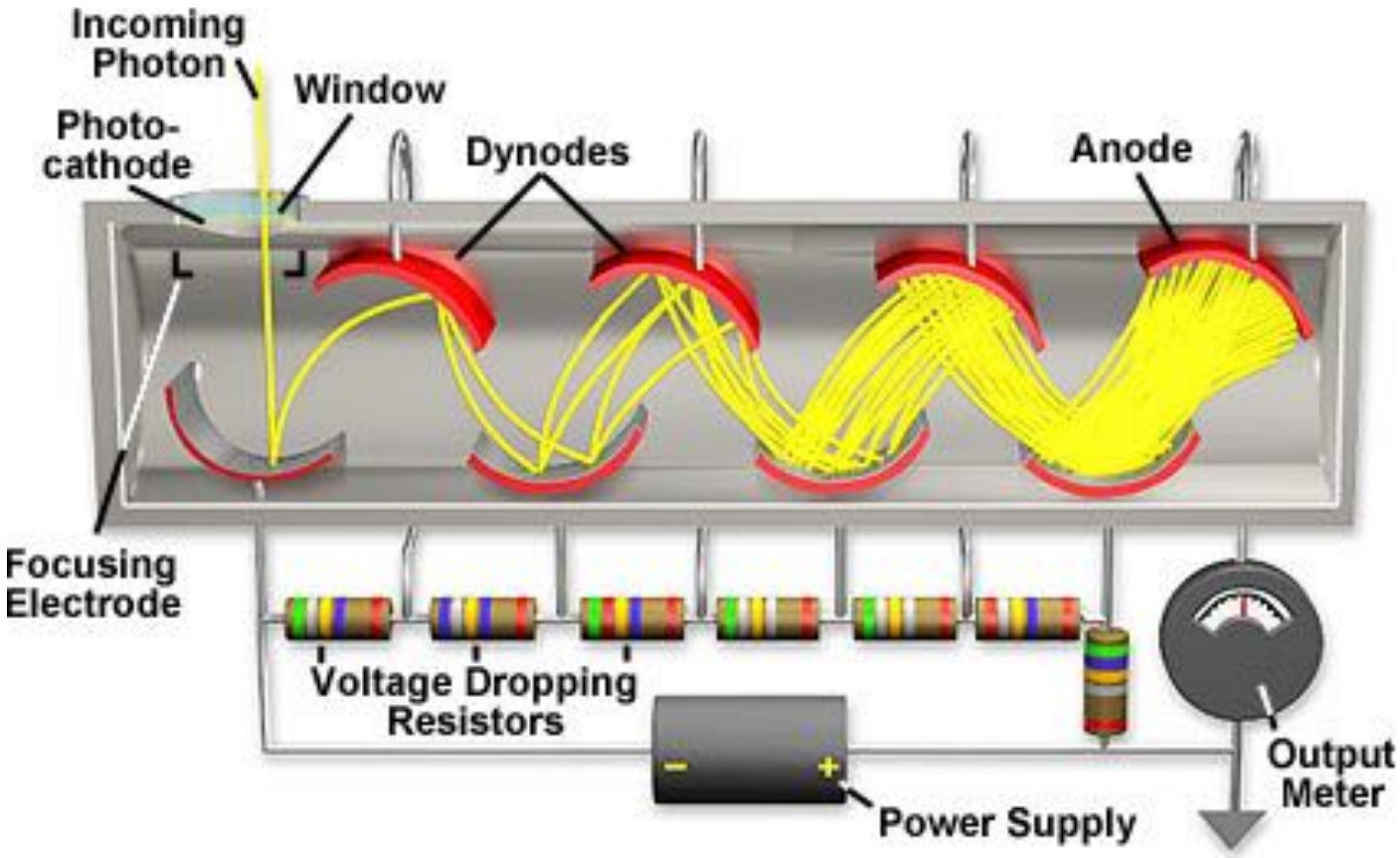


PMT – Photon Multiplier Tube



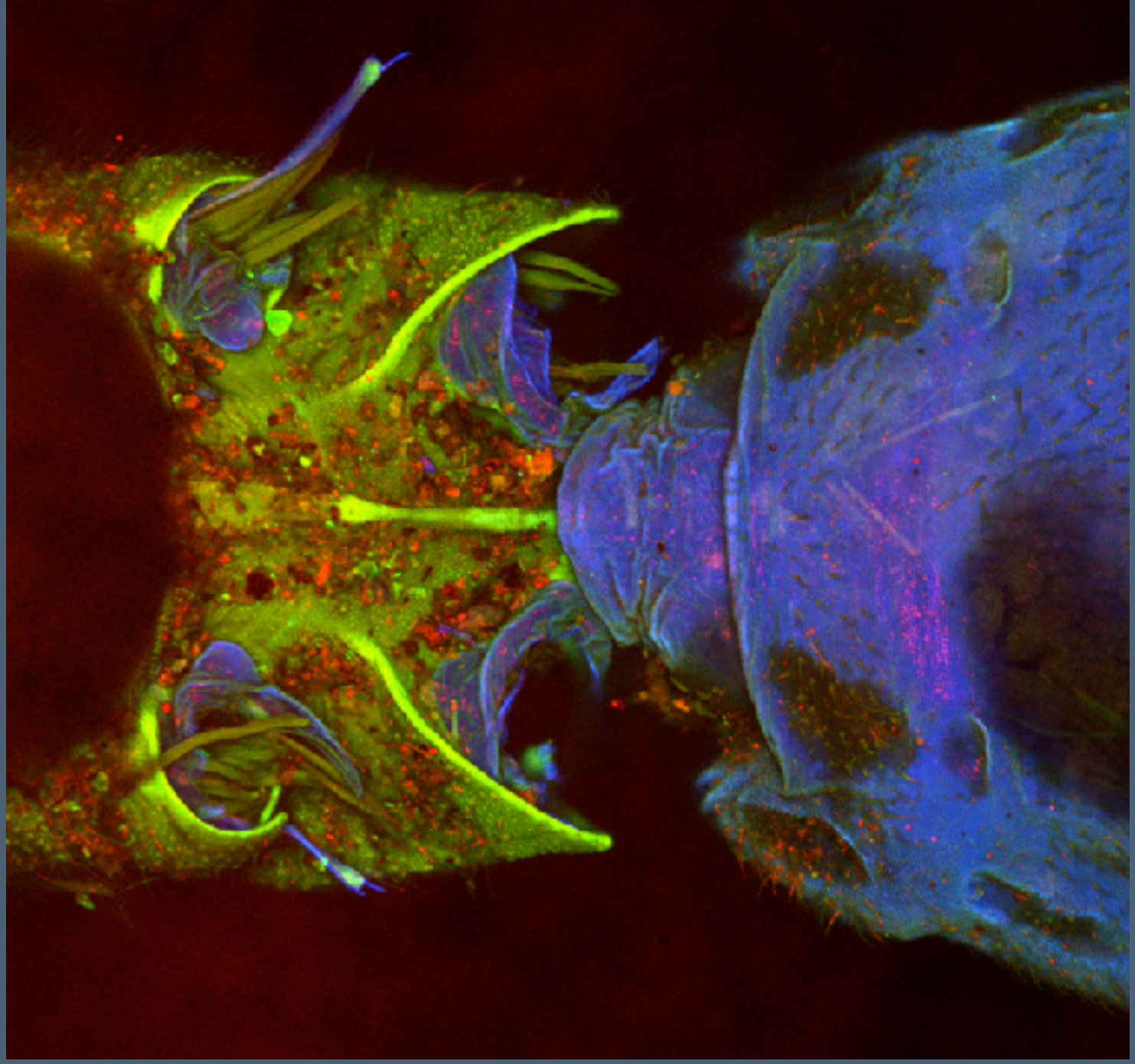


PMT – Photon Multiplier Tube

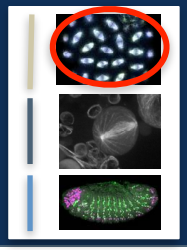


Very Low Noise

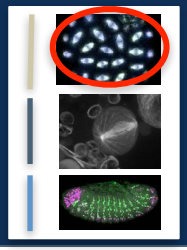
Huge Signal Amplification (~1x10)



insect autofluorescence



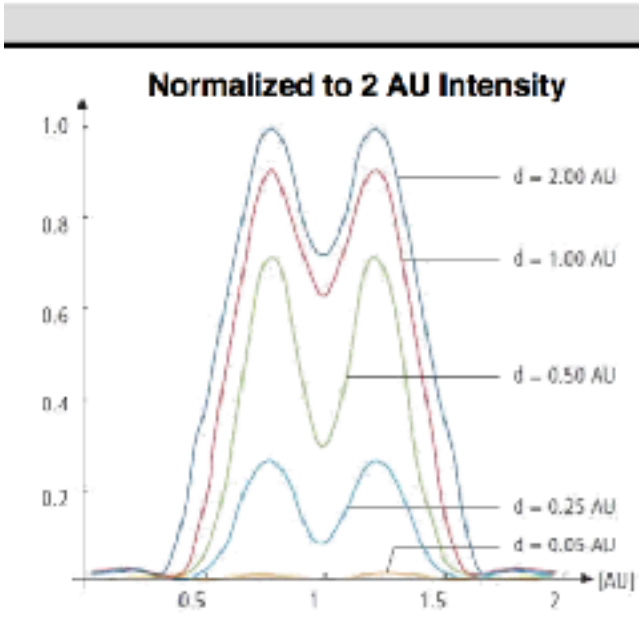
‘Airy-Scan’ technology



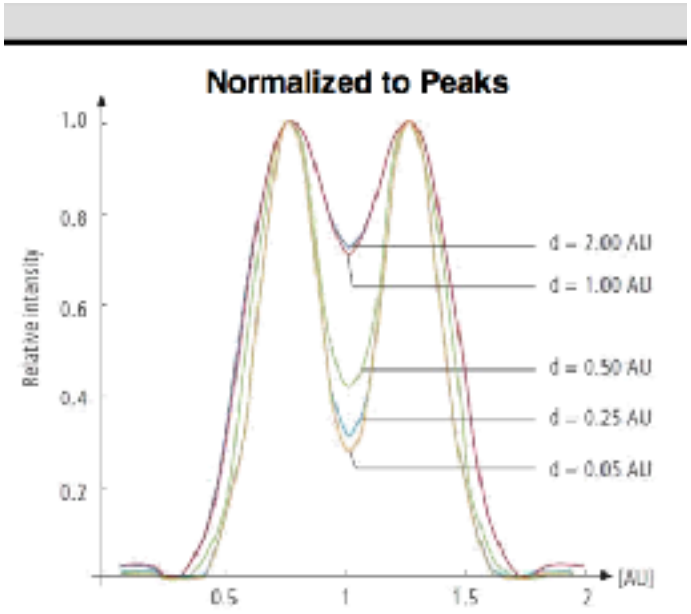
THEORY

‘Airy-Scan’ technology

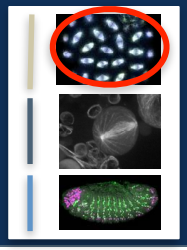
Small Pinhole, signal loss but resolution gain..



However, constricting the pinhole actually yields a drastic reduction in signal below 1 AU



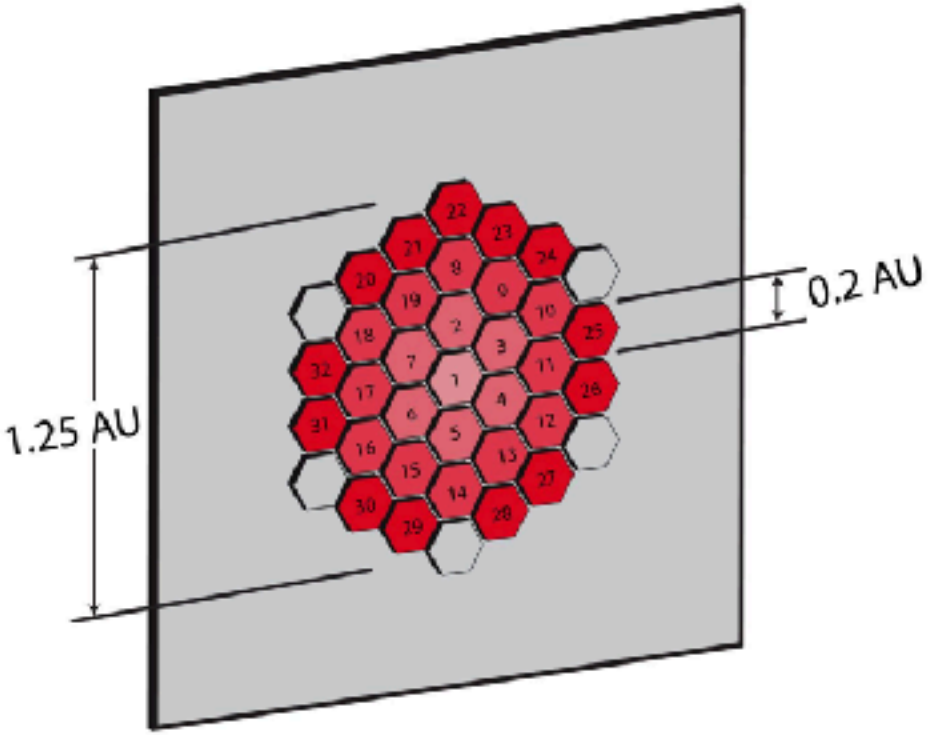
Small pinhole diameters lead to improved resolution steadily until about 0.2 AU, results in deeper dips between two objects



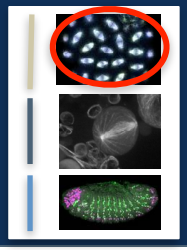
THEORY

‘Airy-Scan’ technology

let through all the emitted light
capture 0.2AU on each detector



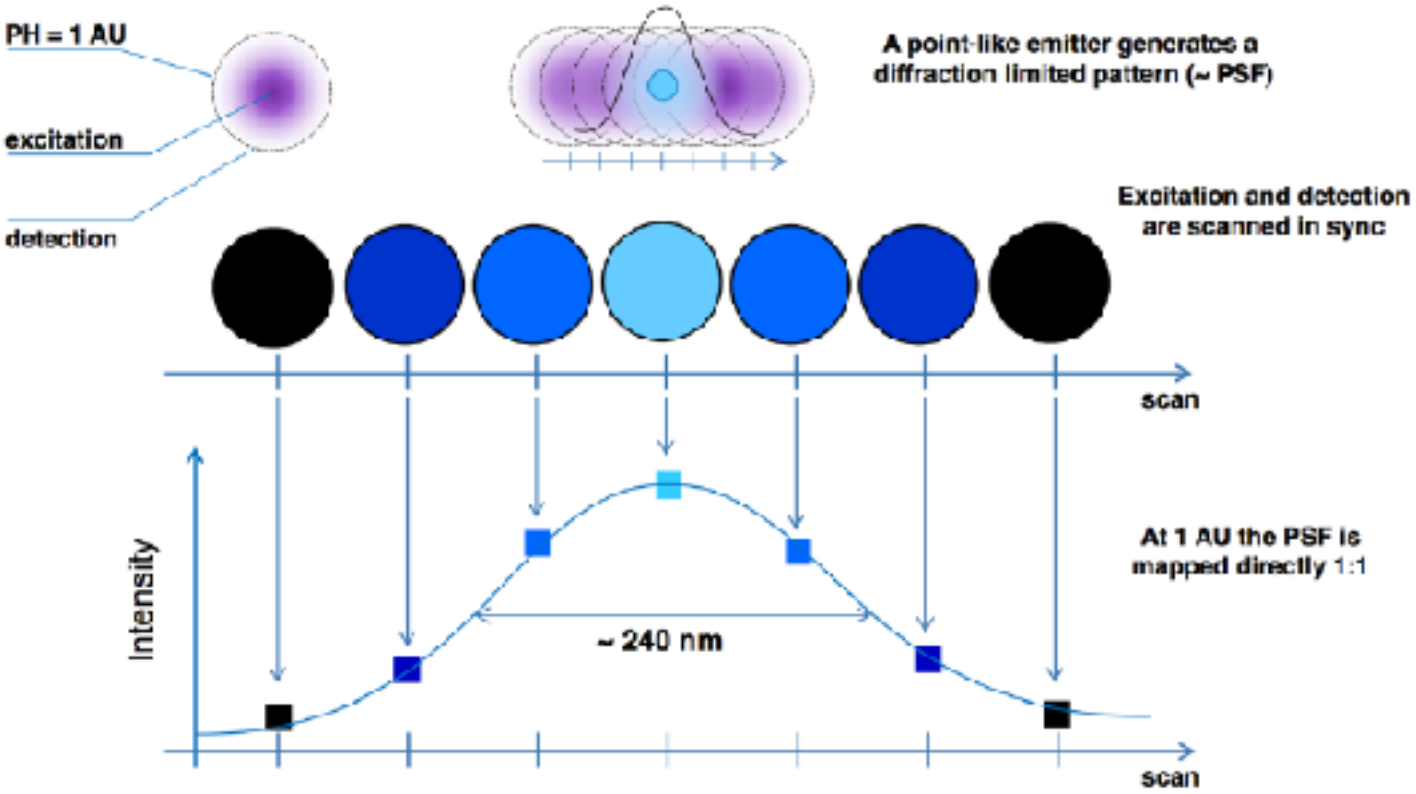
- 32 GaAsP detectors in hexagonal lattice
- Each detector approximately 0.2 AU in diameter
- Total detection area approximately 1.25 AU in diameter
- **Simultaneous improvement in resolution and signal**

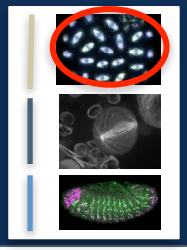


THEORY

‘Airy-Scan’ technology

point of light scanned with 1AU ‘standard’ detector

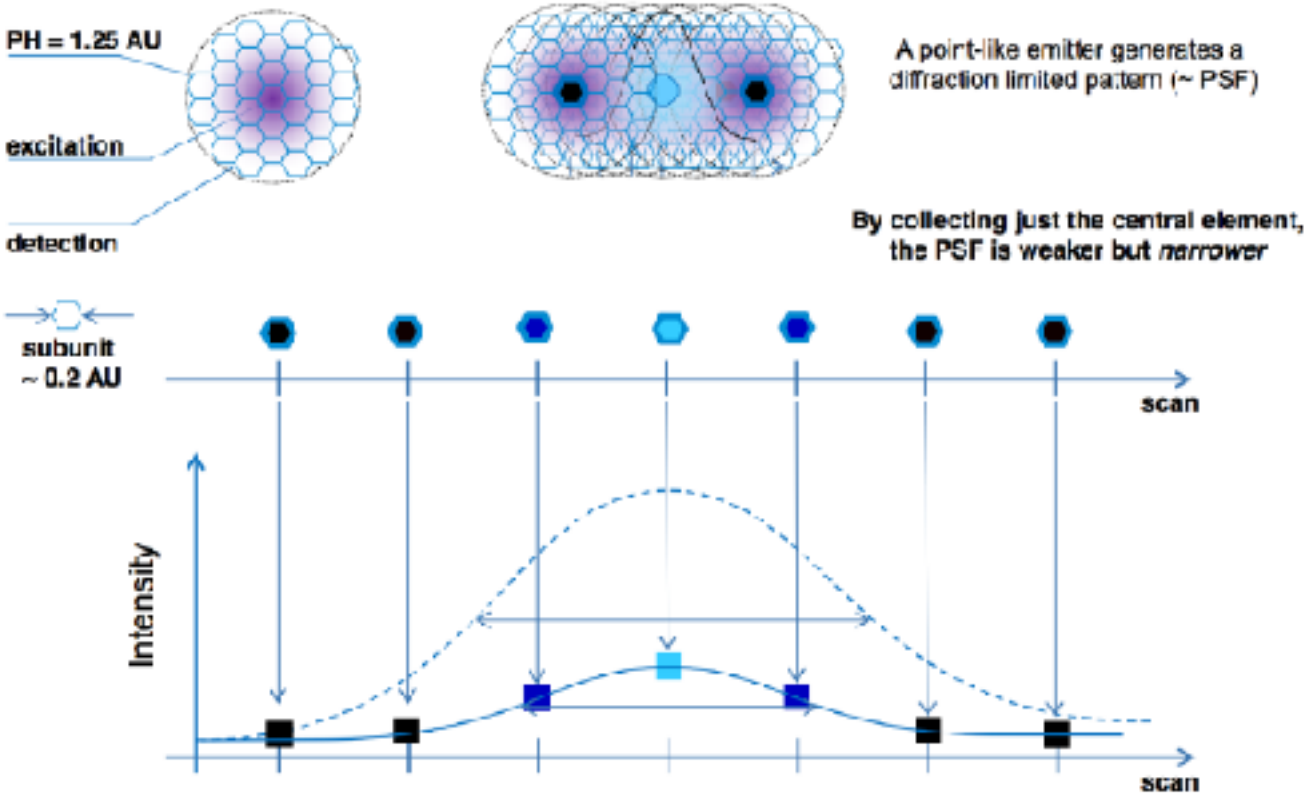


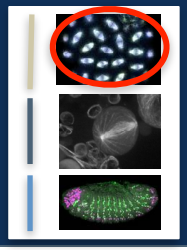


THEORY

‘Airy-Scan’ technology

point of light scanned with 0.2AU ‘Airyscan’ detector
>increased resolution

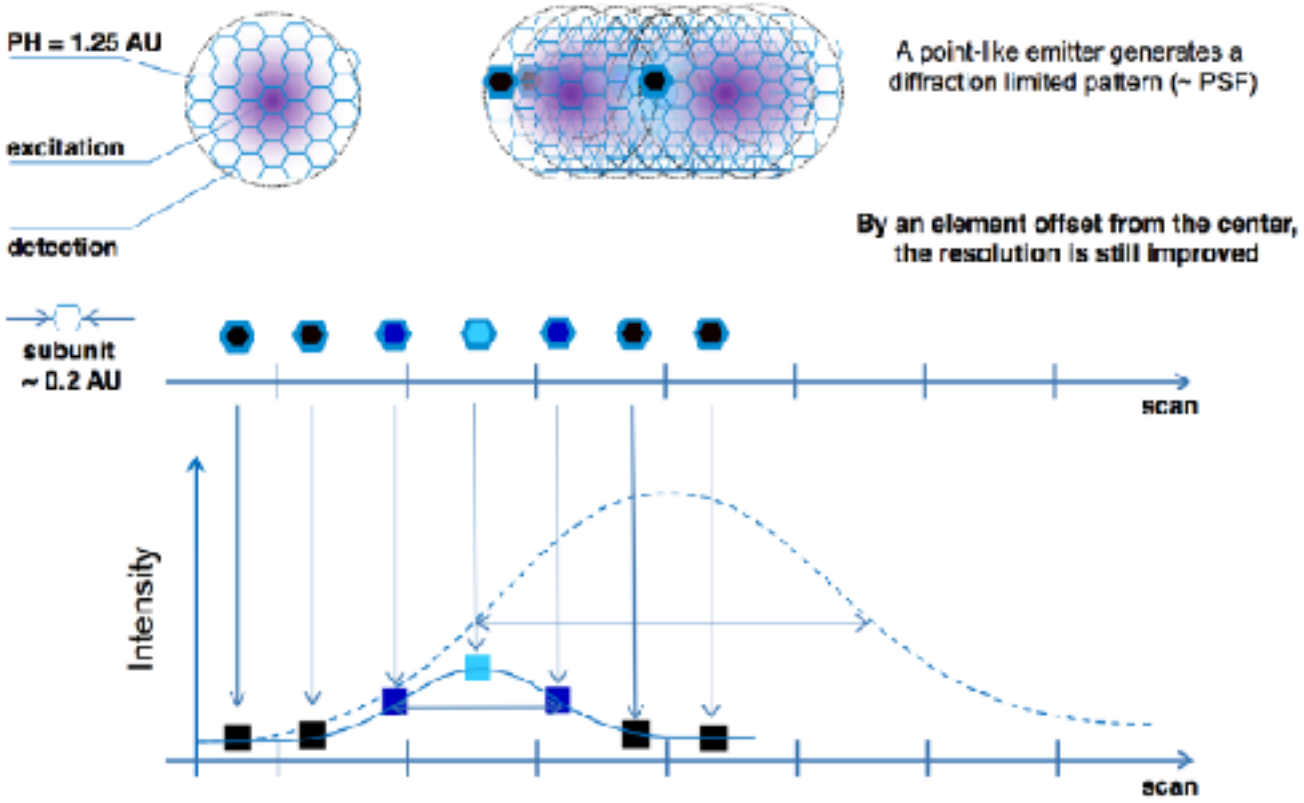


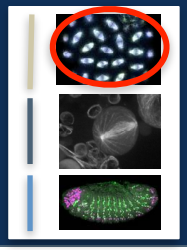


THEORY

‘Airy-Scan’ technology

each 0.2AU ‘Airyscan’ detector provides
>increased resolution

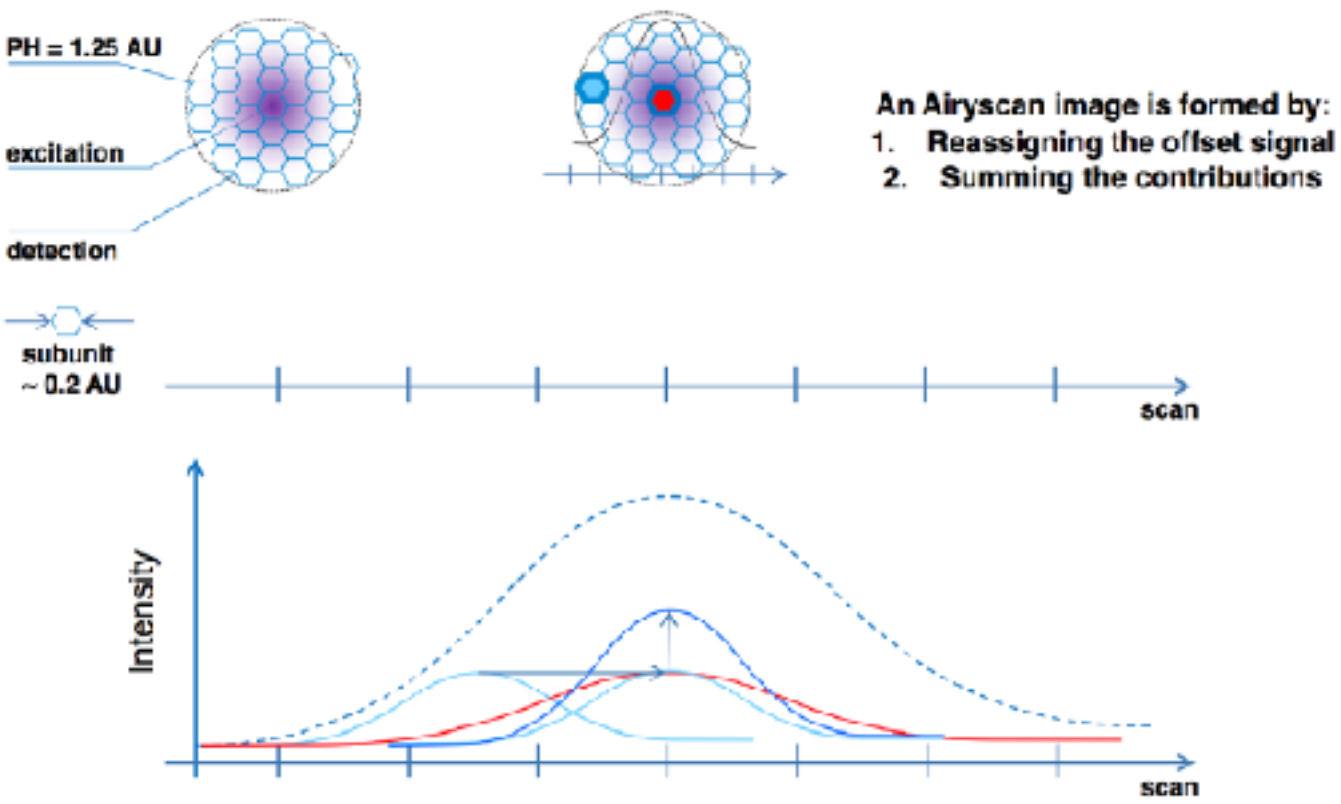


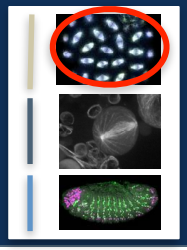


THEORY

‘Airy-Scan’ technology

each 0.2AU ‘Airyscan’ detector info
is reassigned and summed

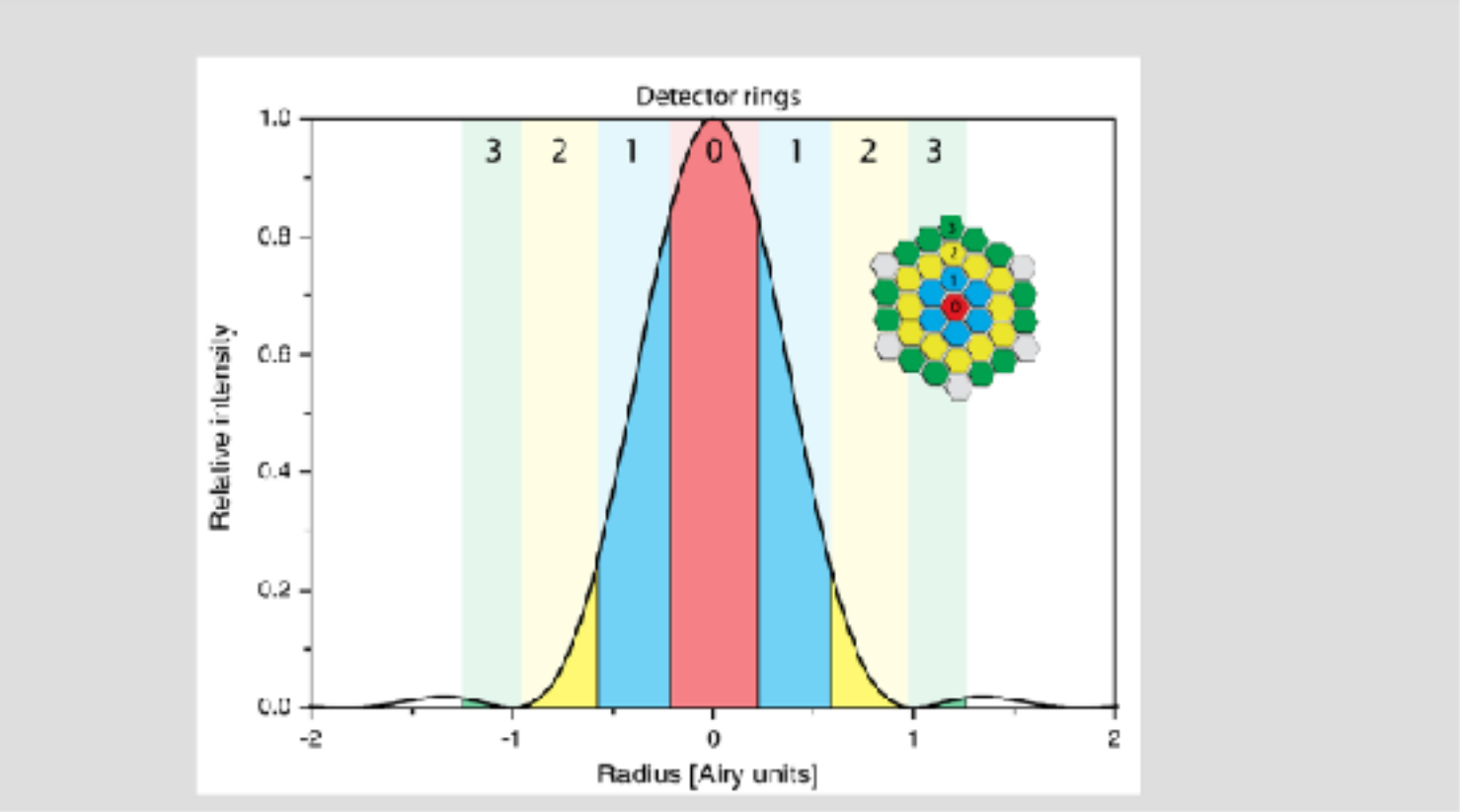


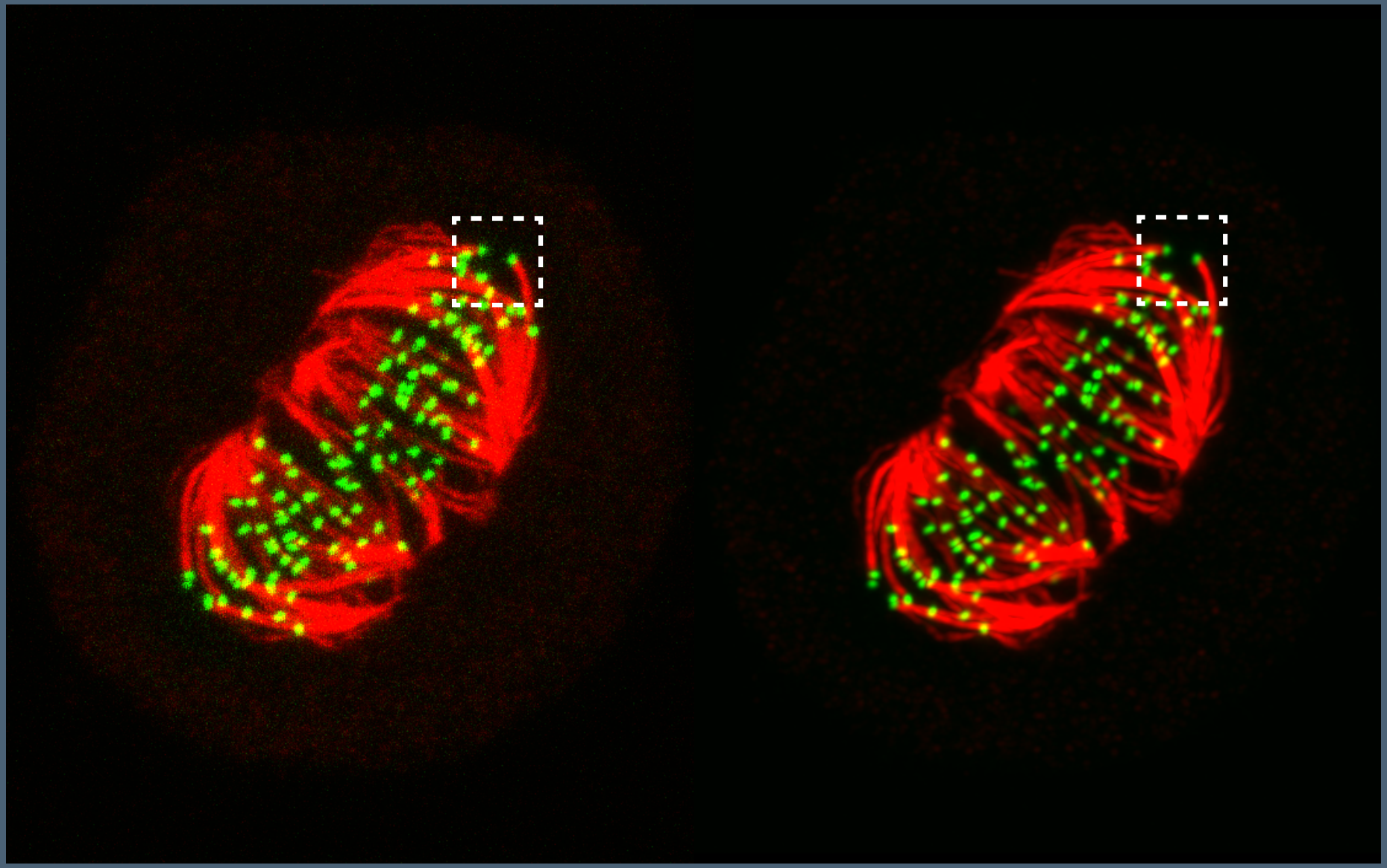


THEORY

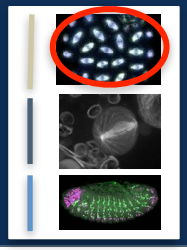
‘Airy-Scan’ technology

effective PSF is now smaller.. > increased resolution (1.4x - 1.7x)

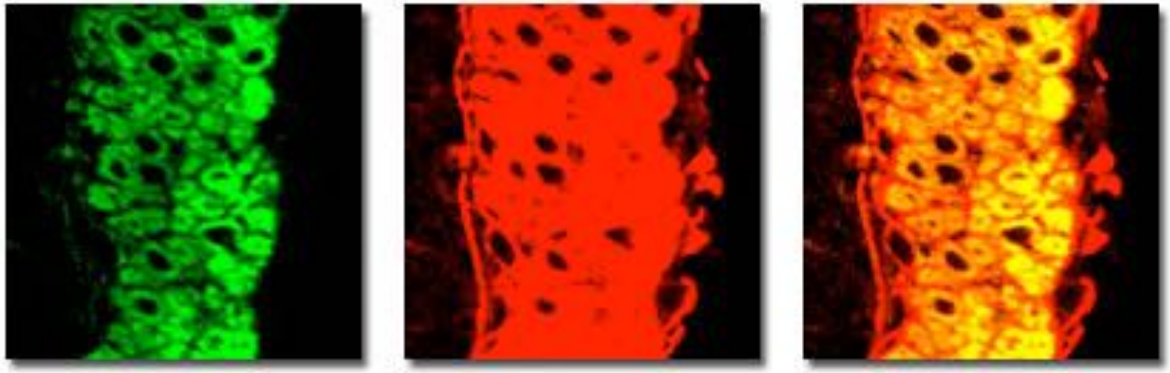




kinetochores (James Banecroft, Gruneberg Lab)

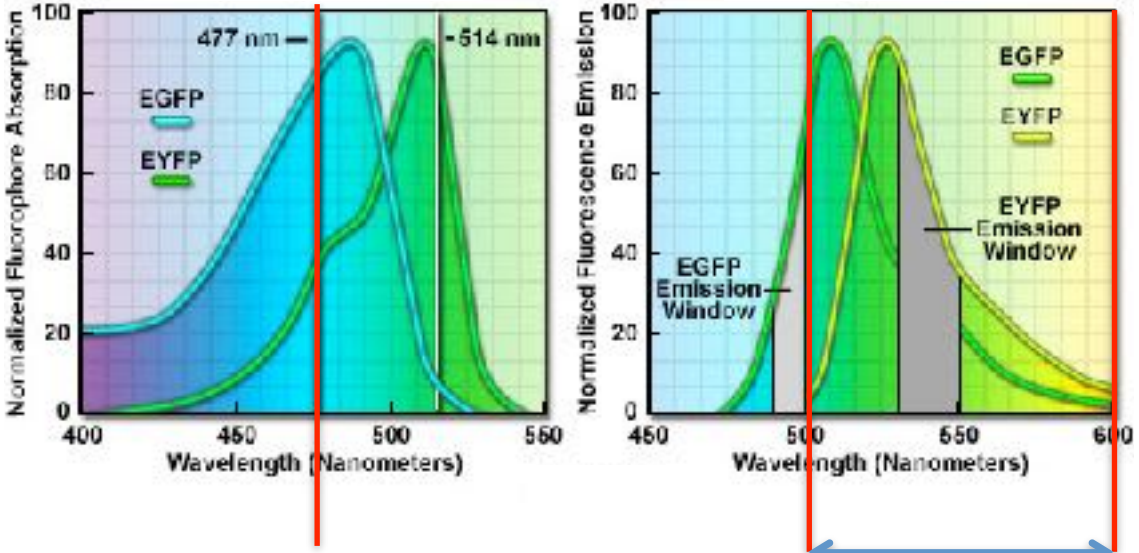


‘bleed-through’



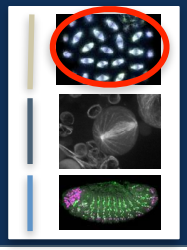
Absorption spectral profiles

Emission spectral profiles

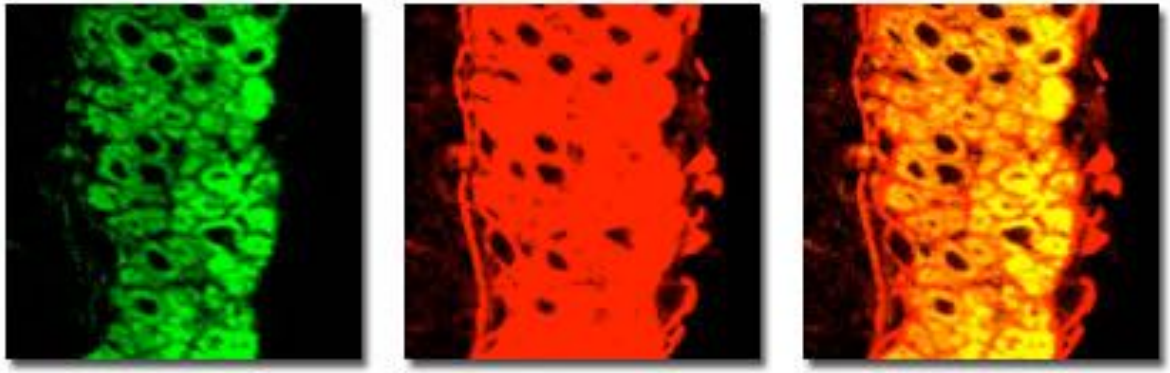


Excite at 477nm

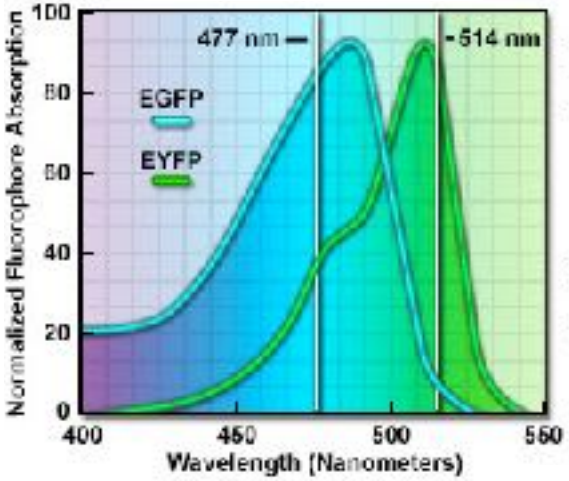
overlapping emission



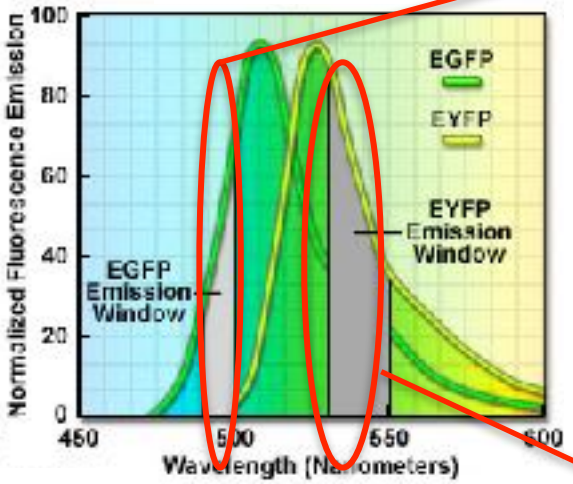
minimising‘bleed-through’ Variable Slits

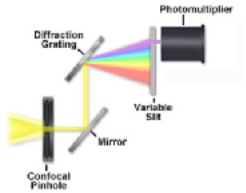


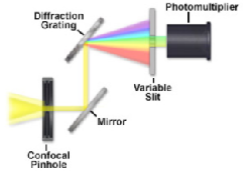
Absorption spectral profiles

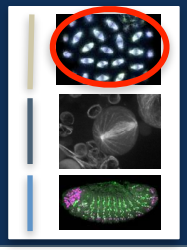


Emission spectral profiles

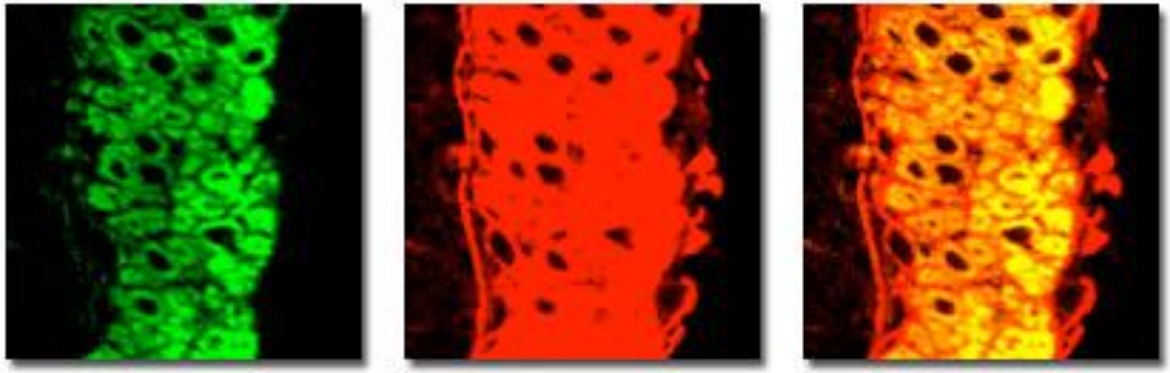




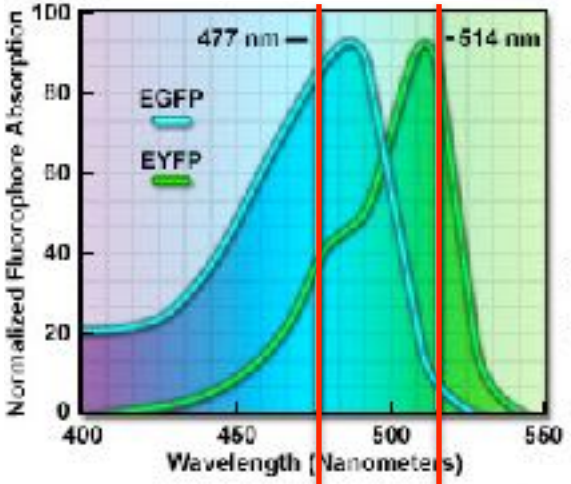




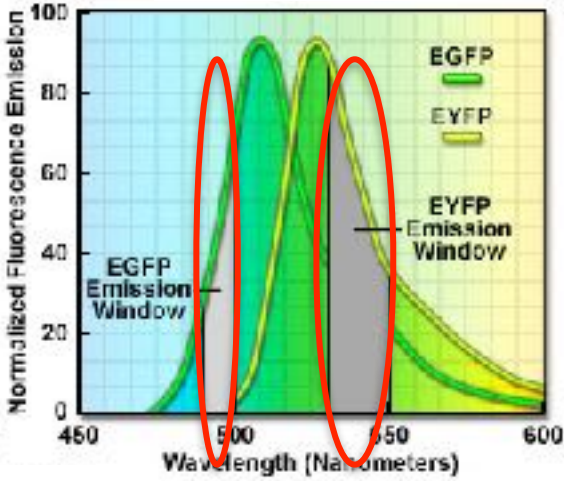
minimising‘bleed-through’ Sequential Scanning



Absorption spectral profiles



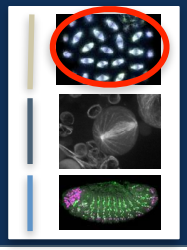
Emission spectral profiles



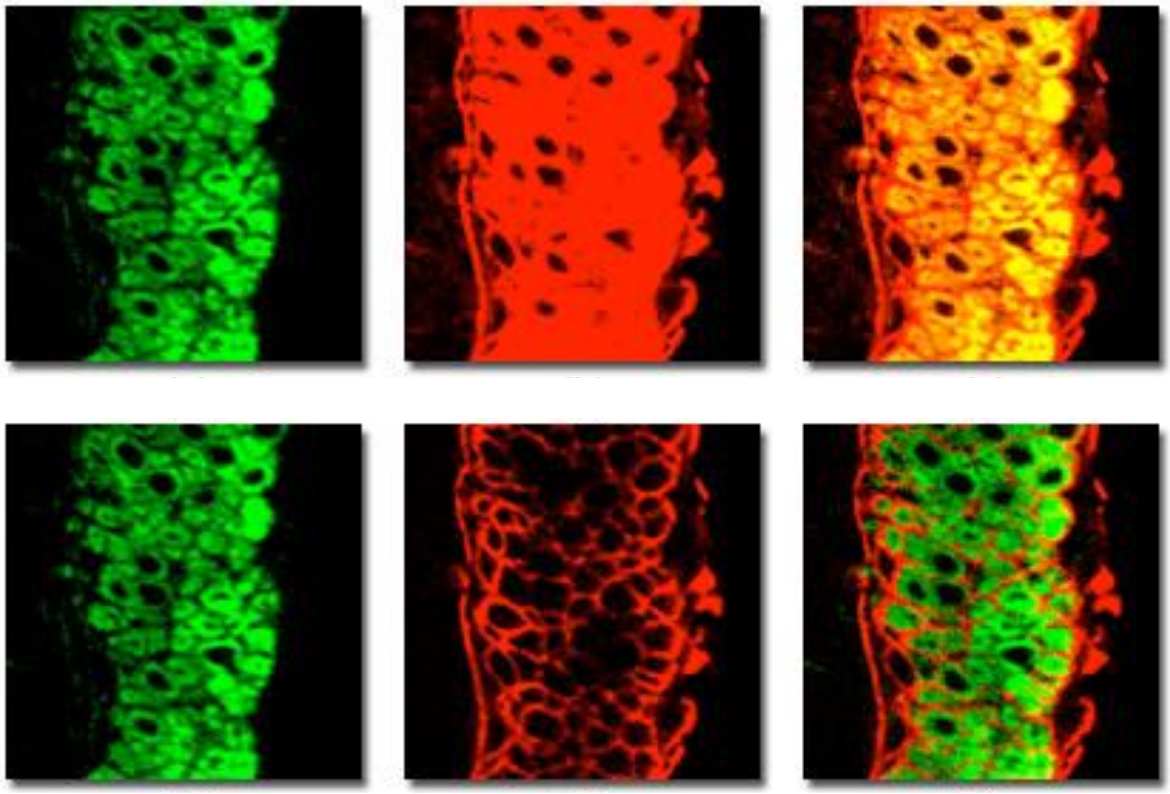
Excite at 477nm

Excite at 514nm

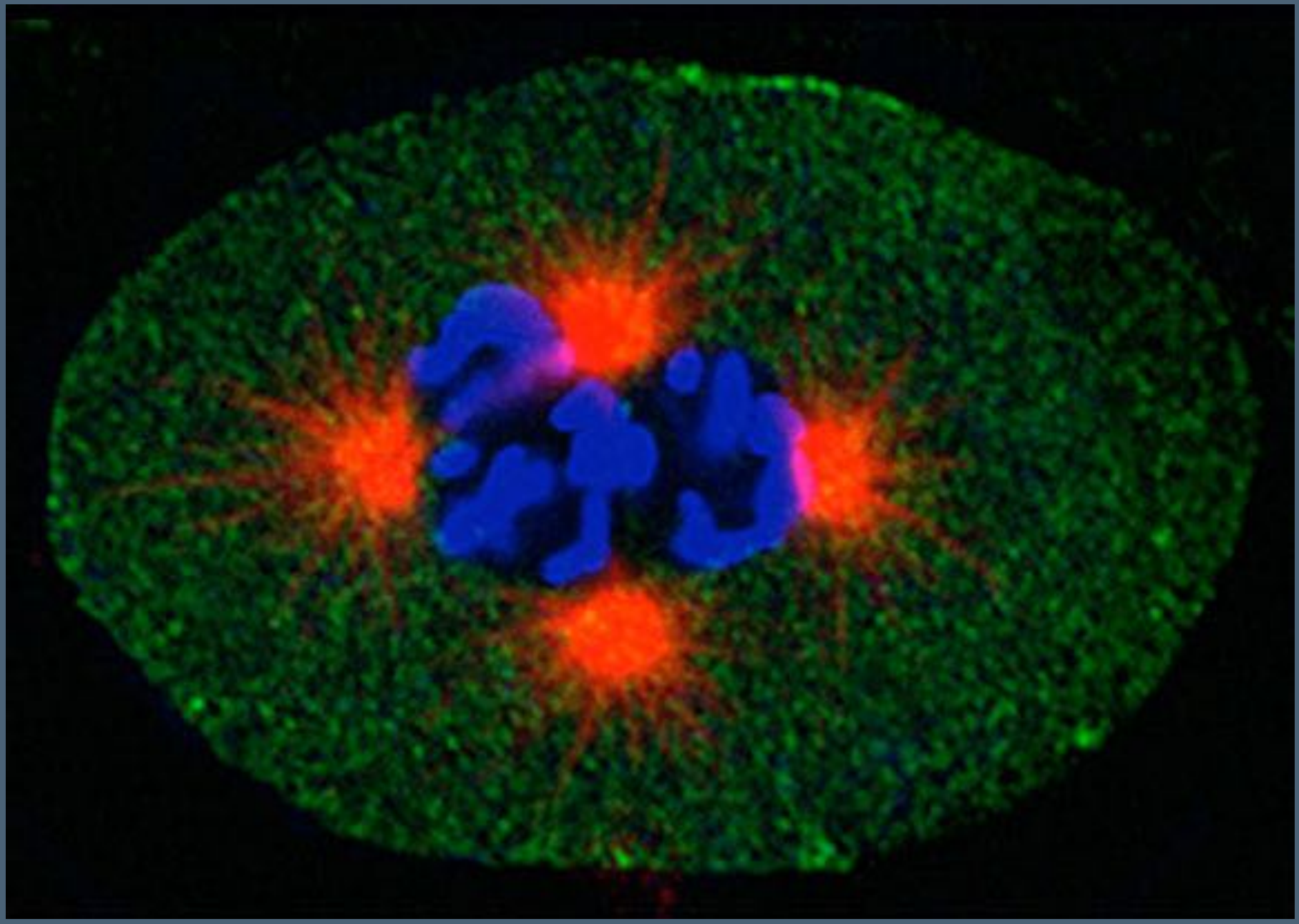
Temporal separation

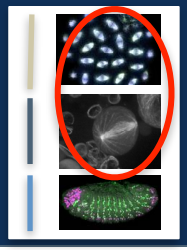


minimising ‘bleed-through’



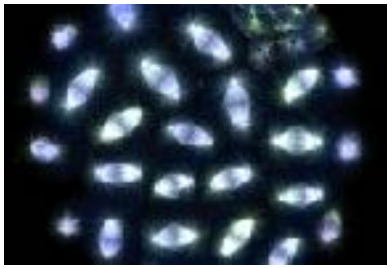
Adjust detector slit widths
Use sequential scanning



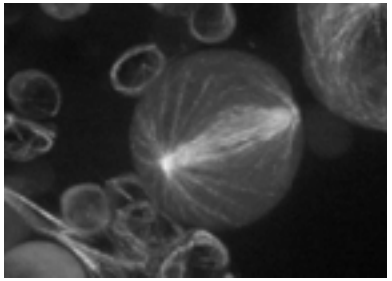


Confocal Microscopes

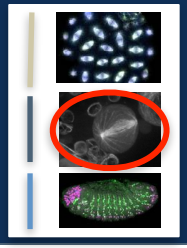
Laser Scanning



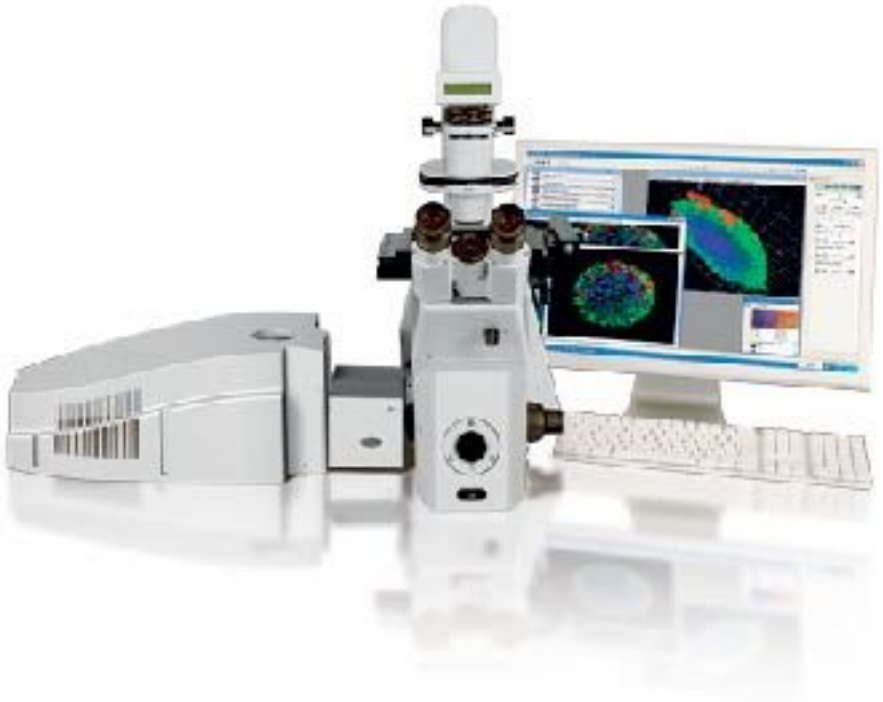
Spinning disc



Both are confocals

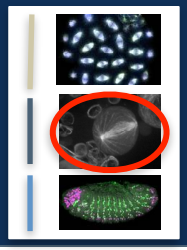


Spinning Disc Confocal

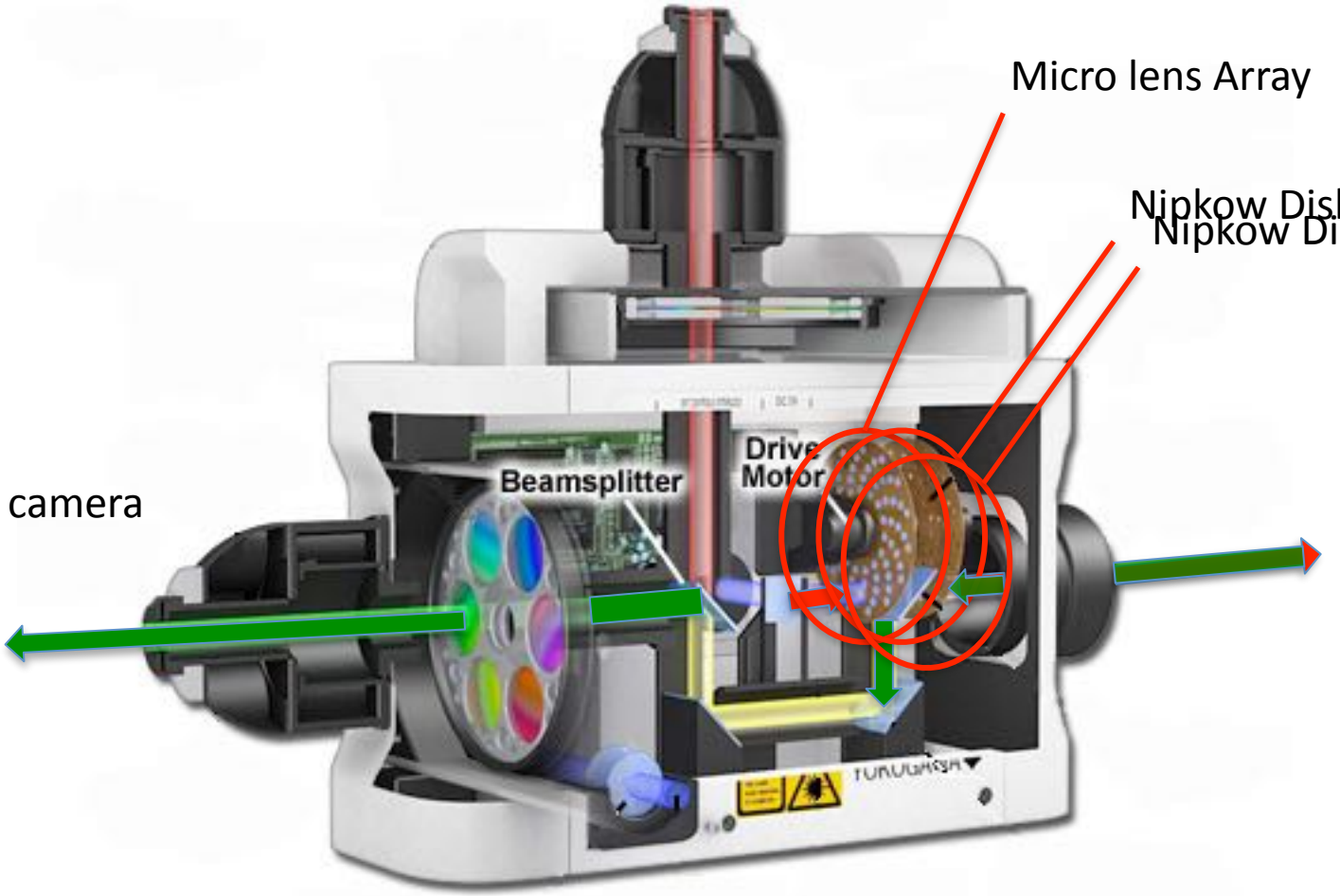


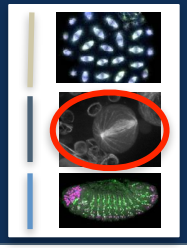
Great for live cell imaging

Can collect many images per second

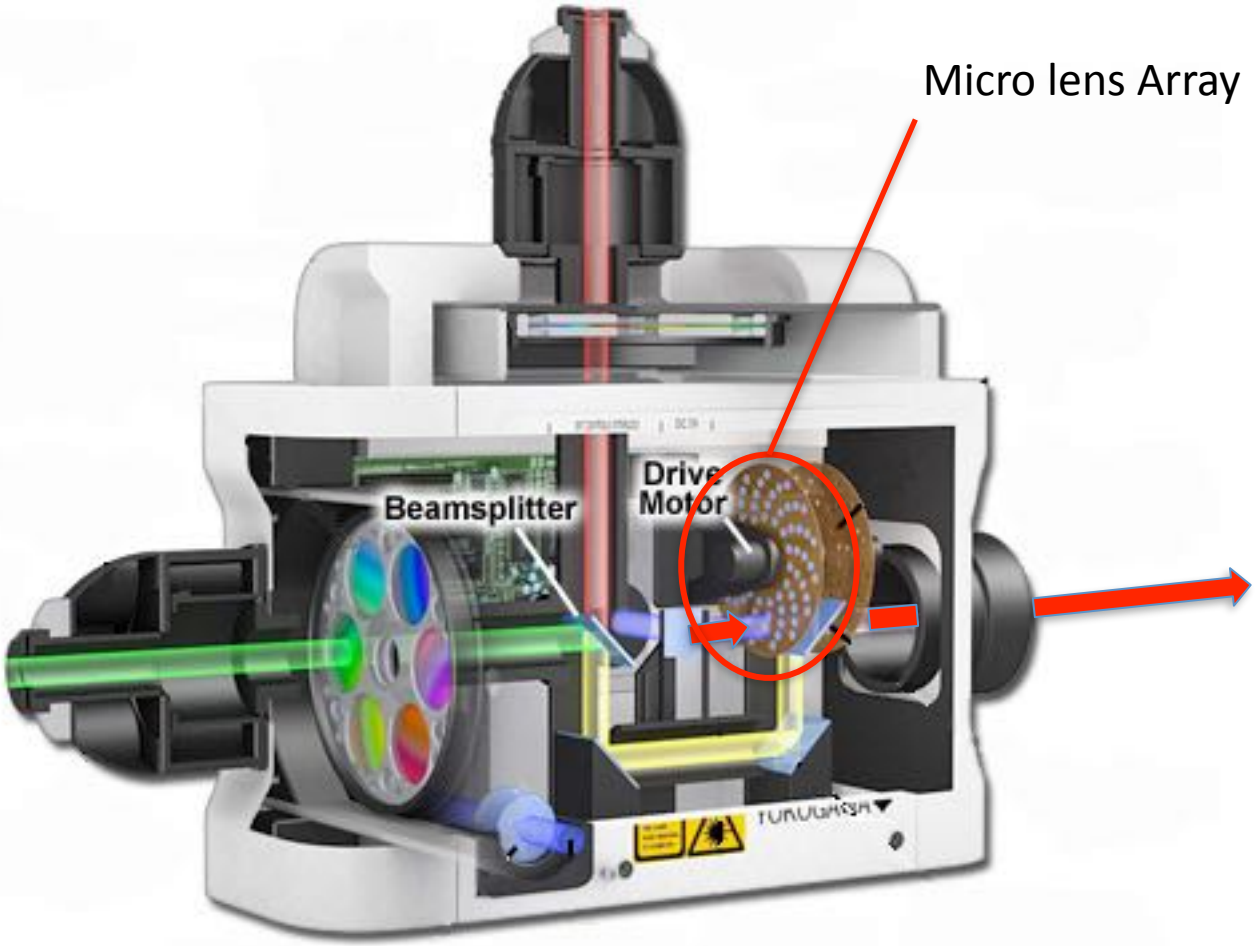


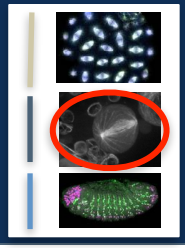
Yokogawa CSU-X1



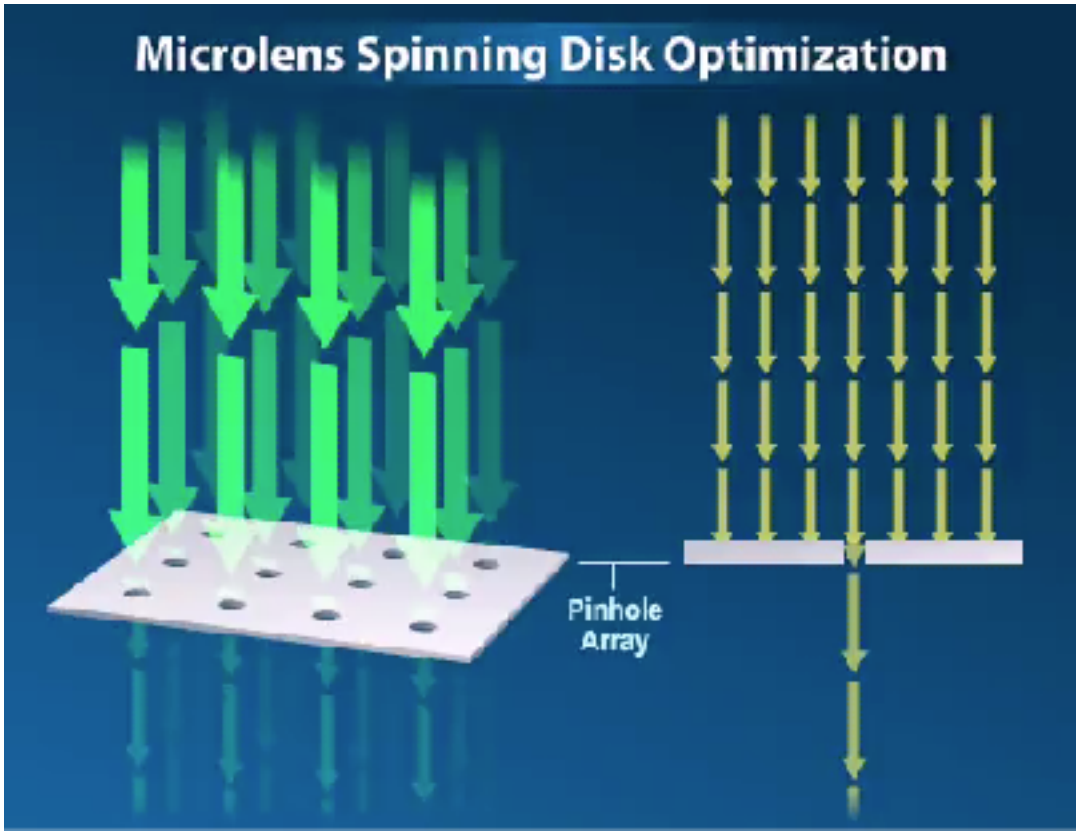


Yokogawa CSU-X1





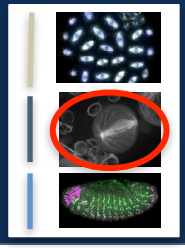
Yokogawa Spinning Disc Confocal



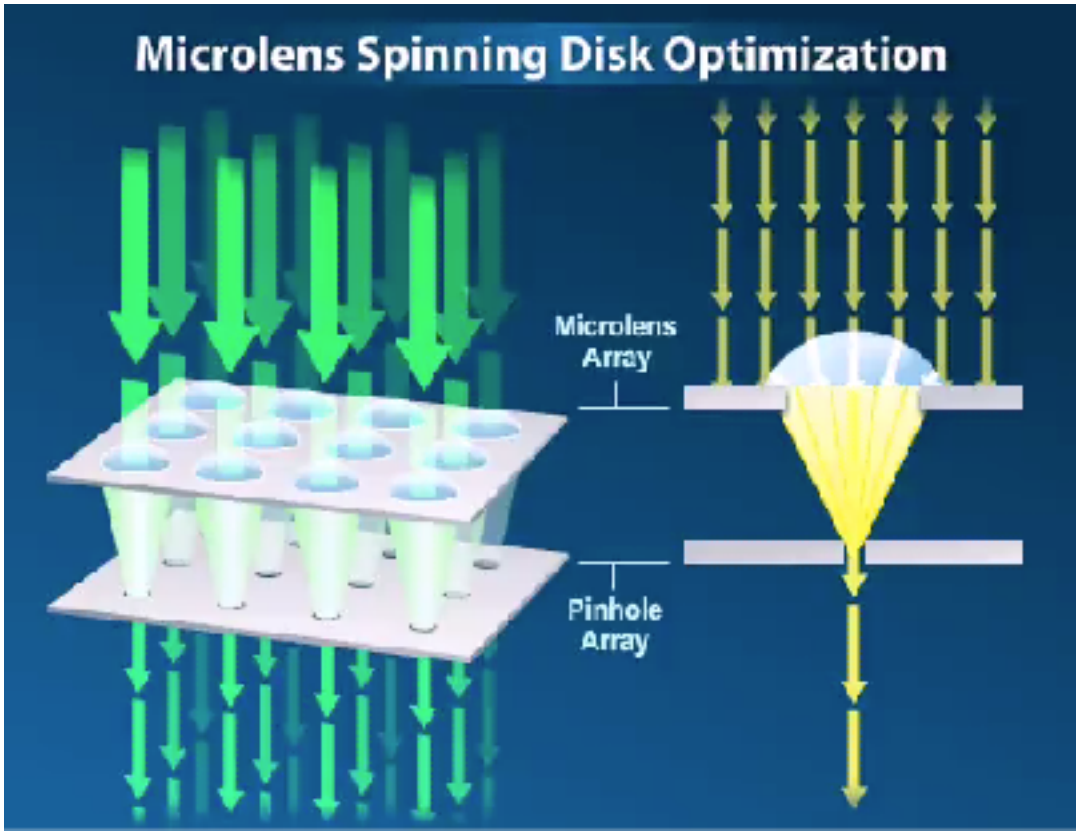
just a pinhole array –
Optimised for ‘cofocality’
and ‘crosstalk’

too much light is blocked
from reaching the specimen

Only 4% light passes through disc

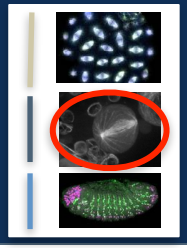


Yokogawa Spinning Disc Confocal

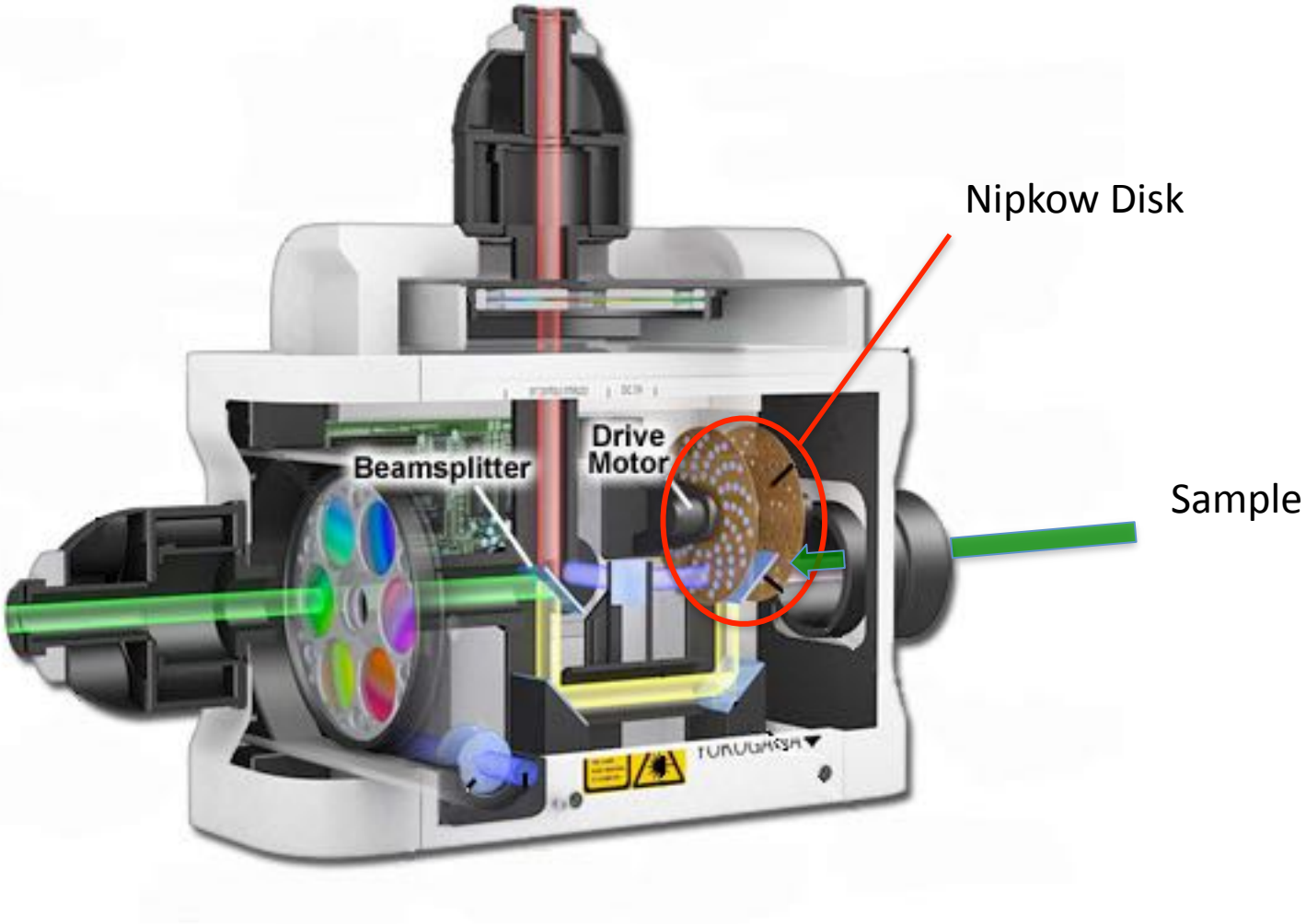


micro-lens array increase the light reaching the specimen

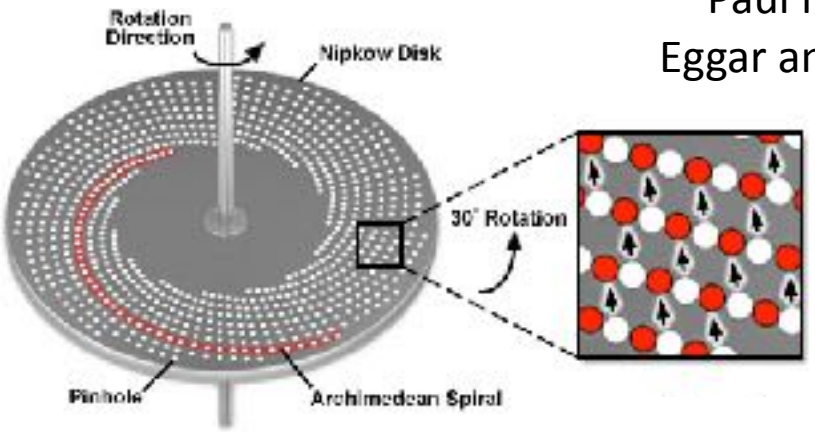
Typically 56% light passes through disc



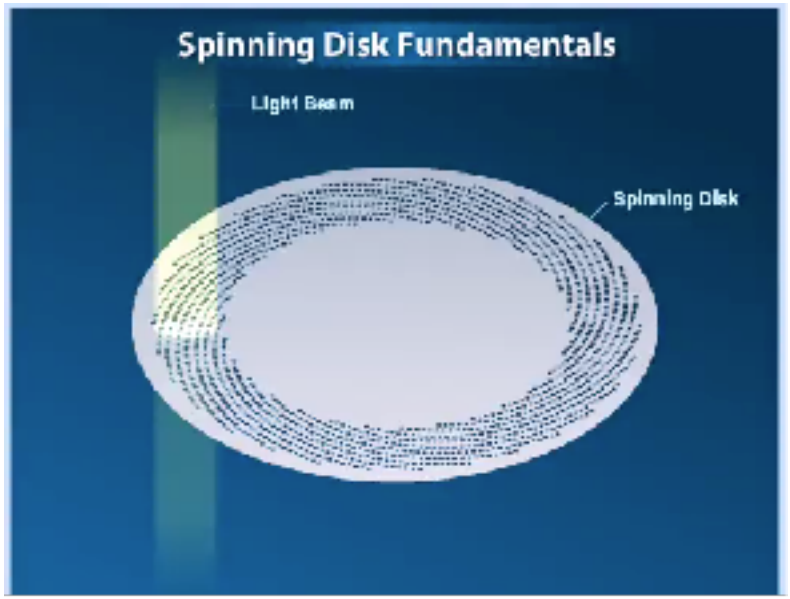
Yokogawa CSU-X1



The Nipkow Disk

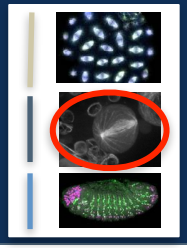


Paul Nipkow, 1884
Eggar and Petran, 1967

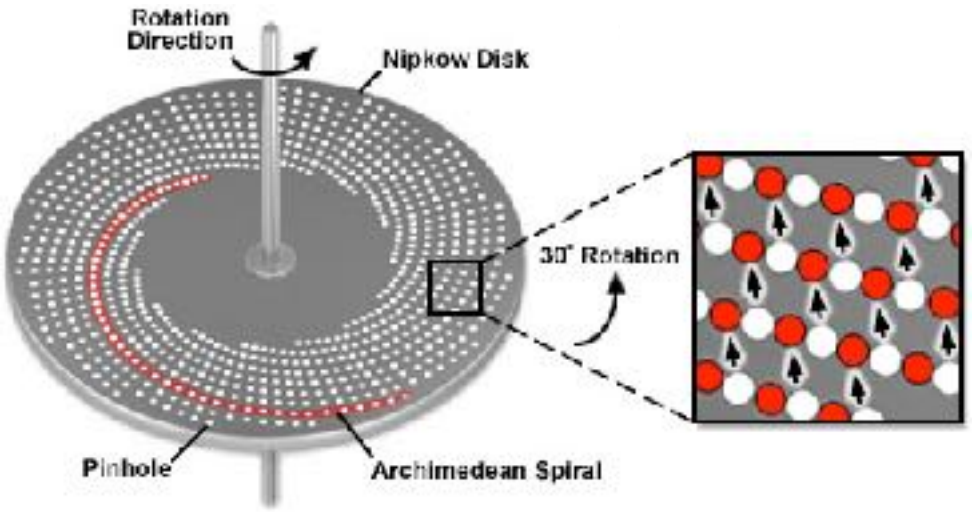


Approx. 1000 pinholes

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



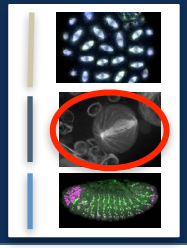
The Nipkow Disk



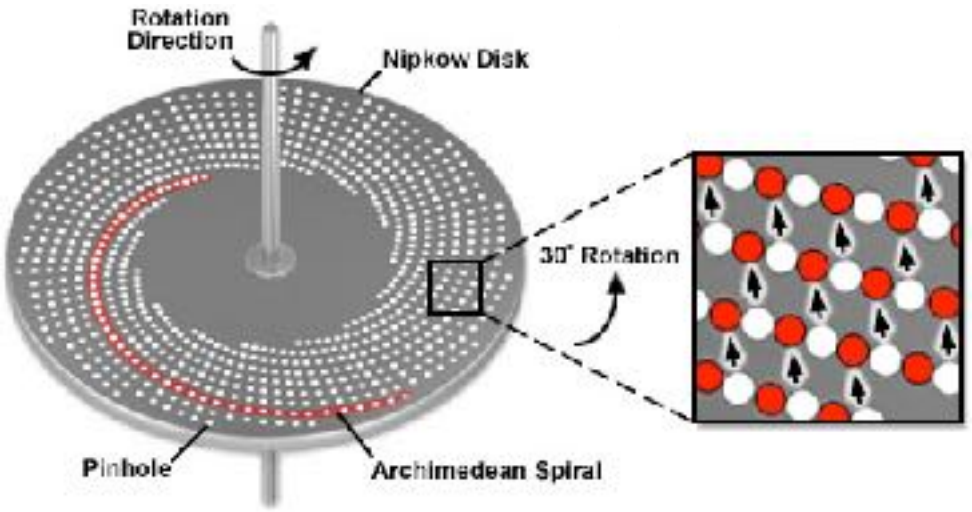
Larger pinholes - brighter image, but less “confocal”

Pinholes fixed size: Typically = 50um

(optimised for biology)



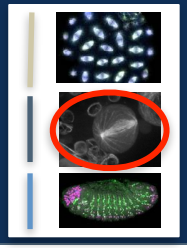
The Nipkow Disk



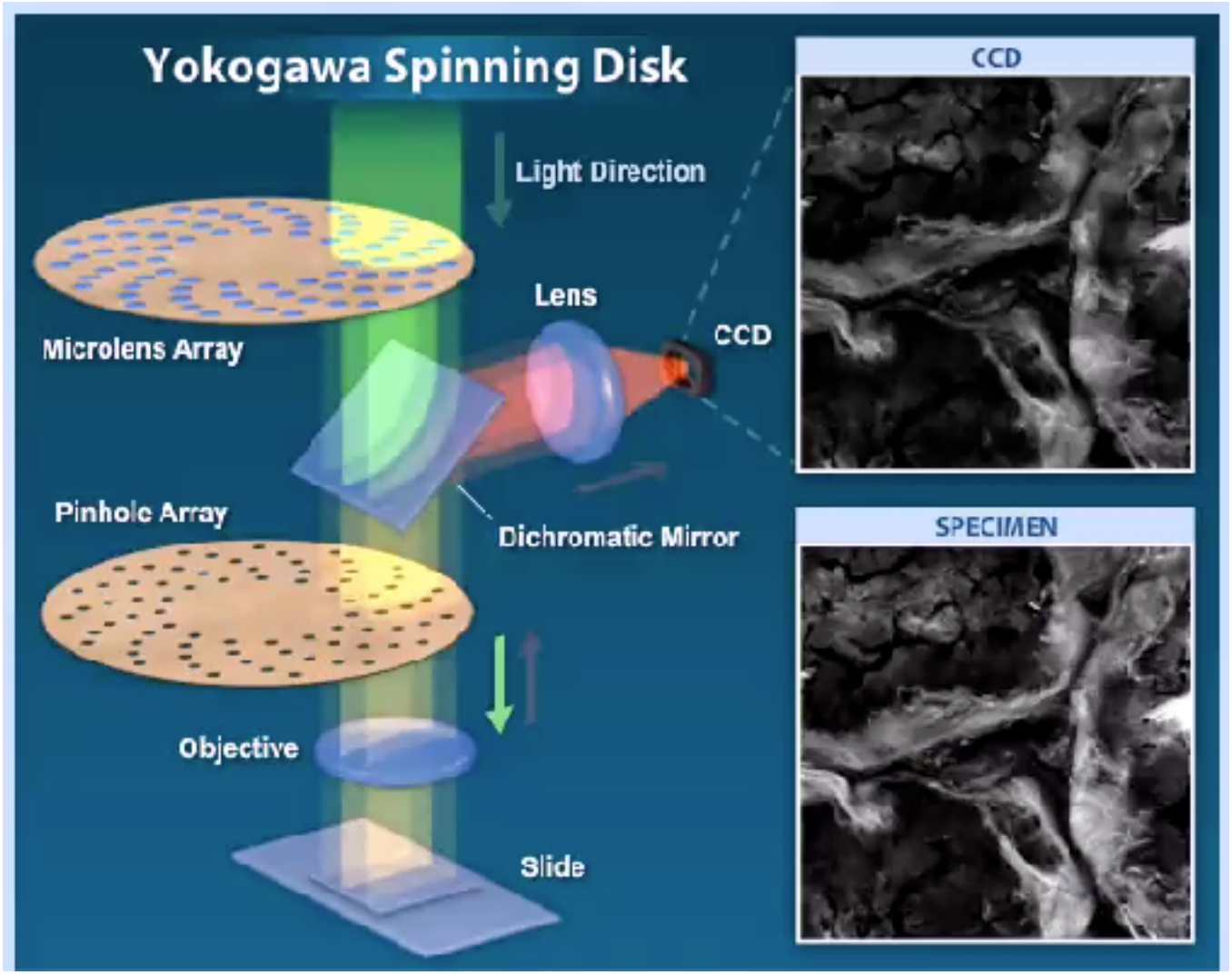
Constant Battle:

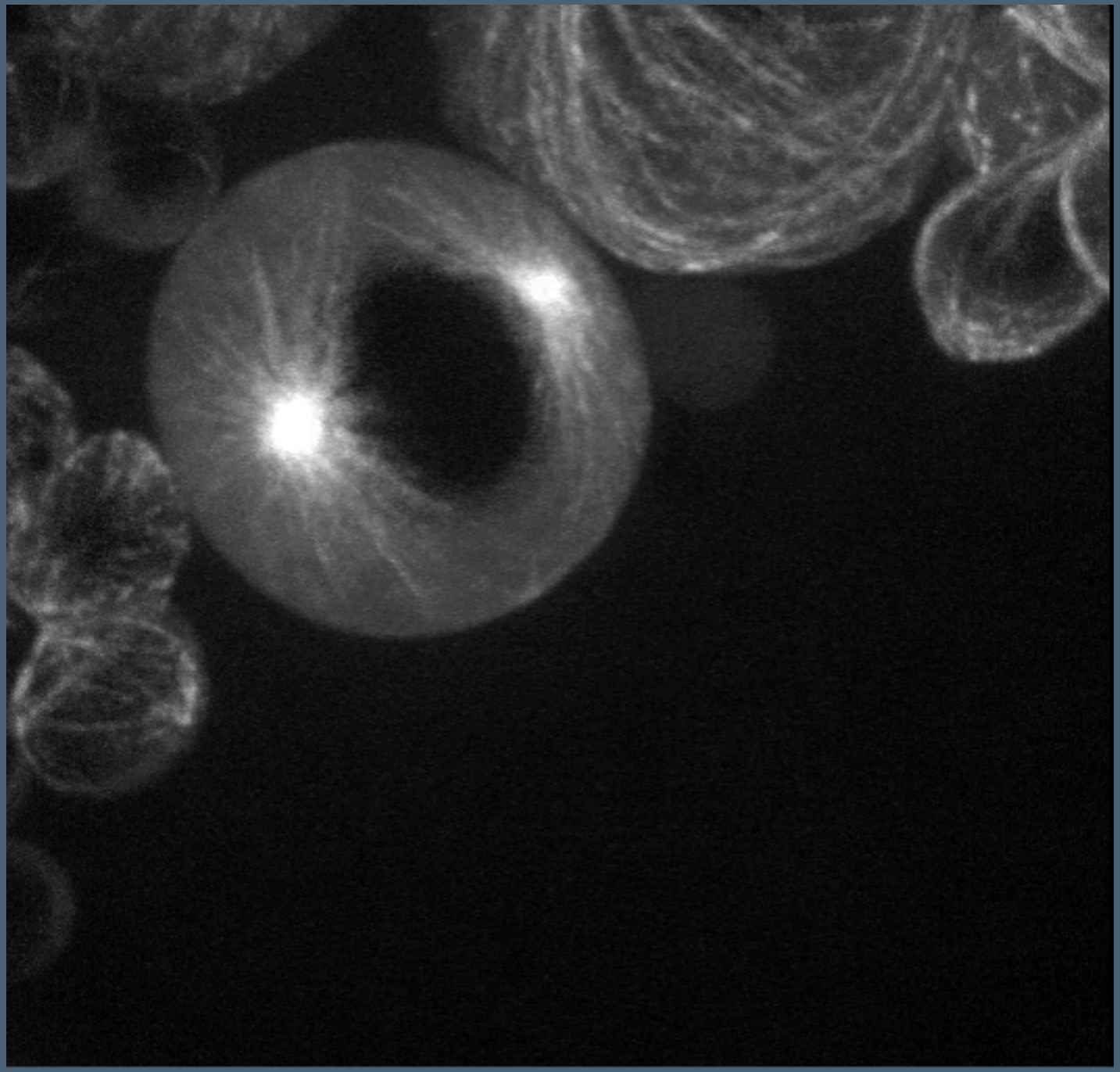
Smaller spacing - more light gets through, but “crosstalk”

Pinhole Spacing Typically = 2.5um apart

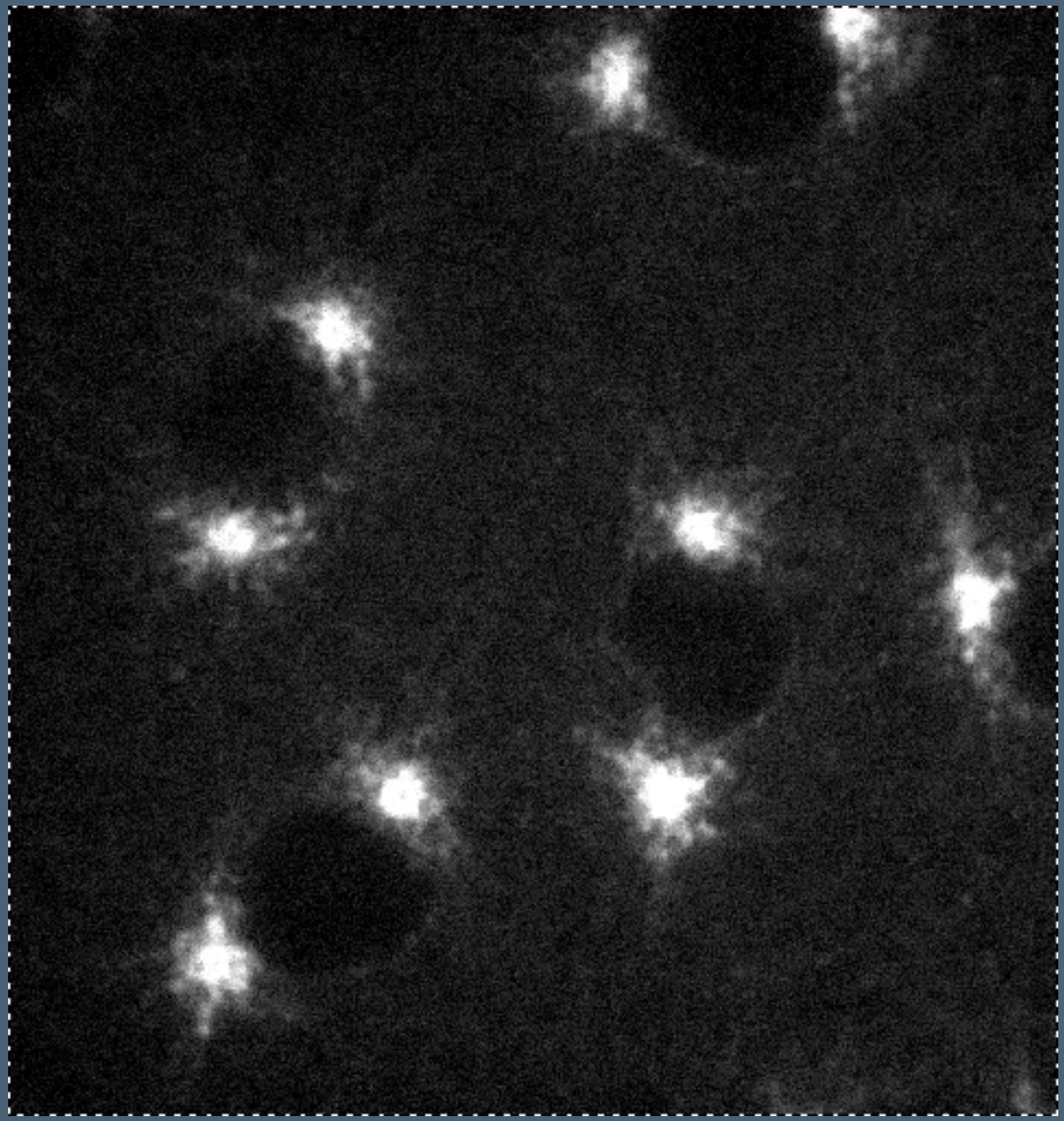


Yokogawa

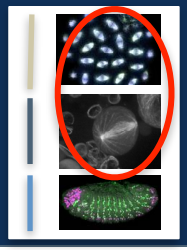

Yokogawa Spinning Disk
Light Direction
Lens
CCD
Microlens Array
Pinhole Array
Dichromatic Mirror
Objective
Slide
CCD
SPECIMEN



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

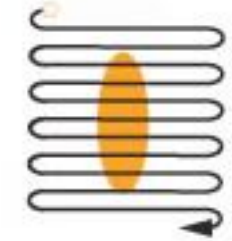


MT binding protein in *Drosophila* embryo, Raff Lab



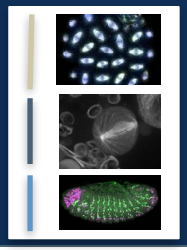
Point Scanning Vs Spinning Disc

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!

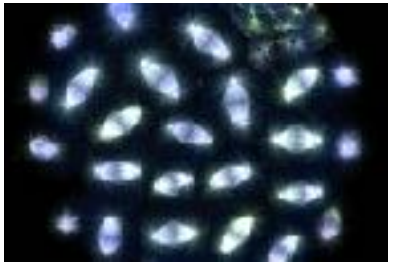


3 Flavours of Microscope

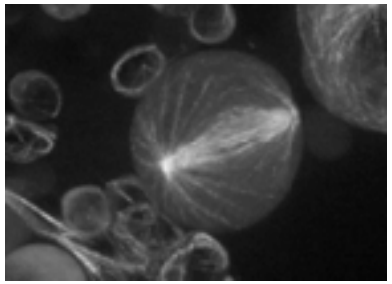
Problem:
Out of Focus
Light

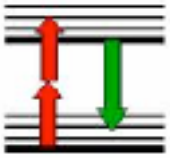
Confocal

Laser
Scanning

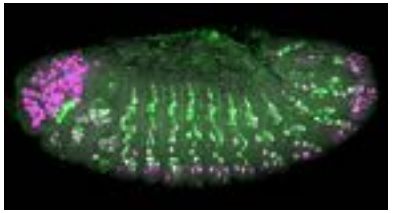


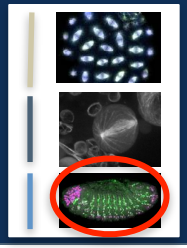
Spinning disc





2-Photon



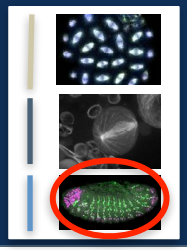


2-photon Microscope

Not a ‘confocal’

for imaging deeper into thick specimens

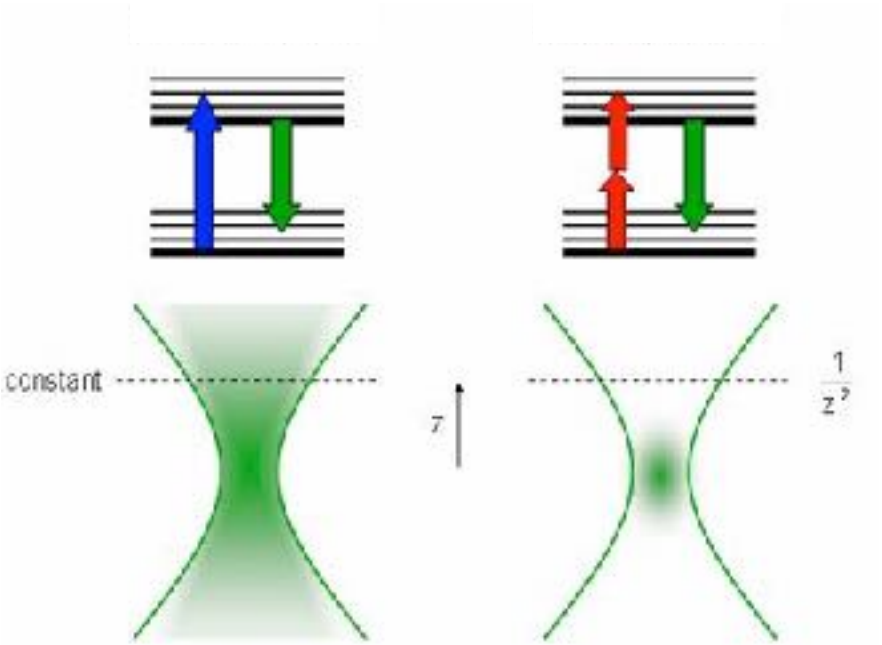
less damaging to biological samples

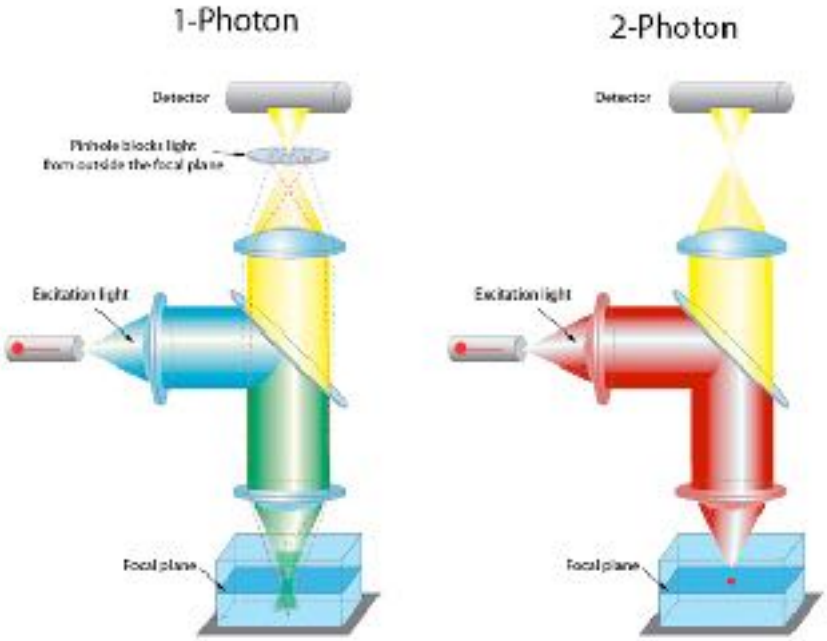


Confocal Vs 2-photon

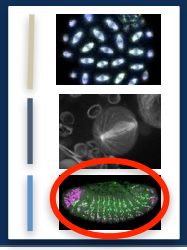
1 Photon Excitation

2 Photon Excitation



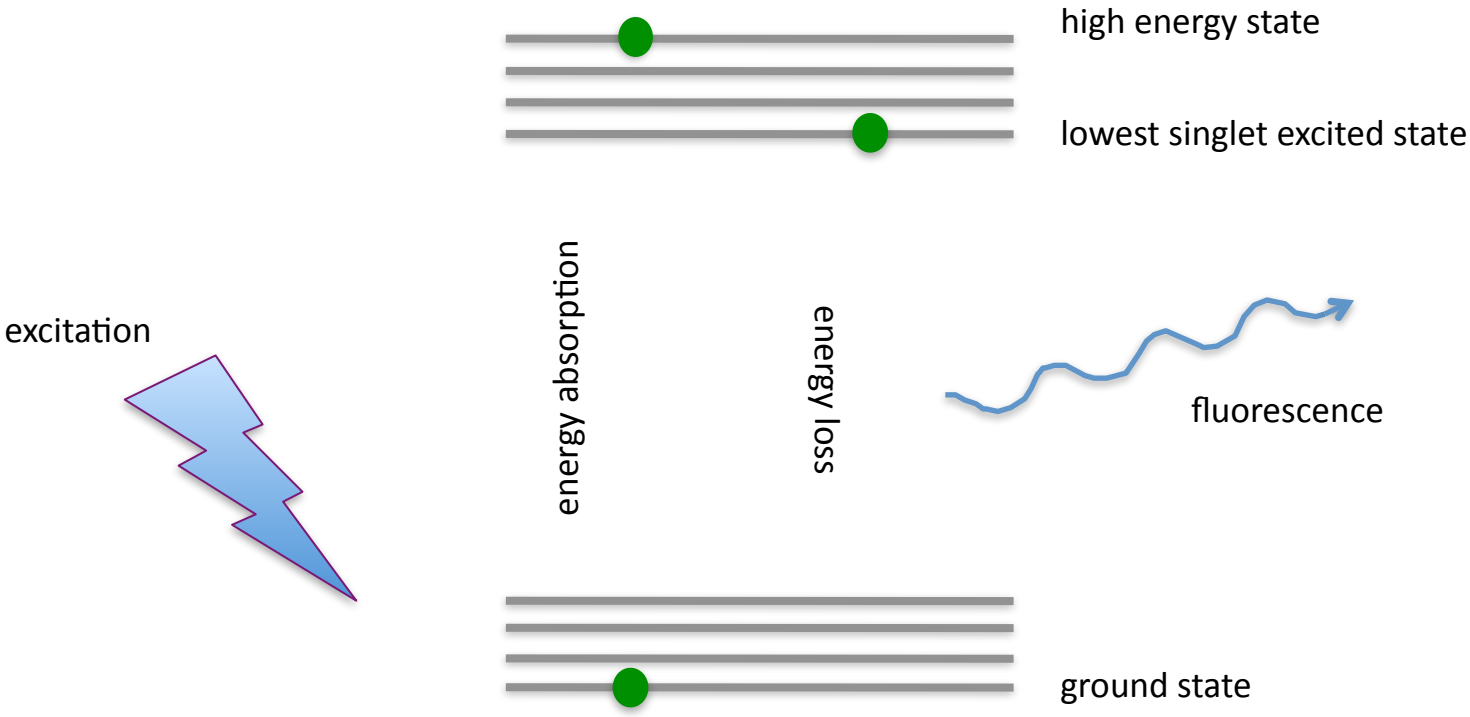


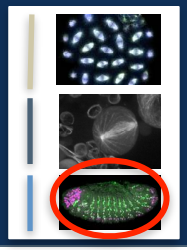
There is no out of focus light



THEORY

1 Photon Excitation





THEORY

2 Photon Excitation

Almost simultaneous

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

Low energy (IR ~700nm) Pulsed excitation

high energy state

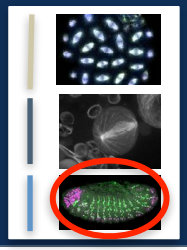
lowest singlet excited state

energy absorption

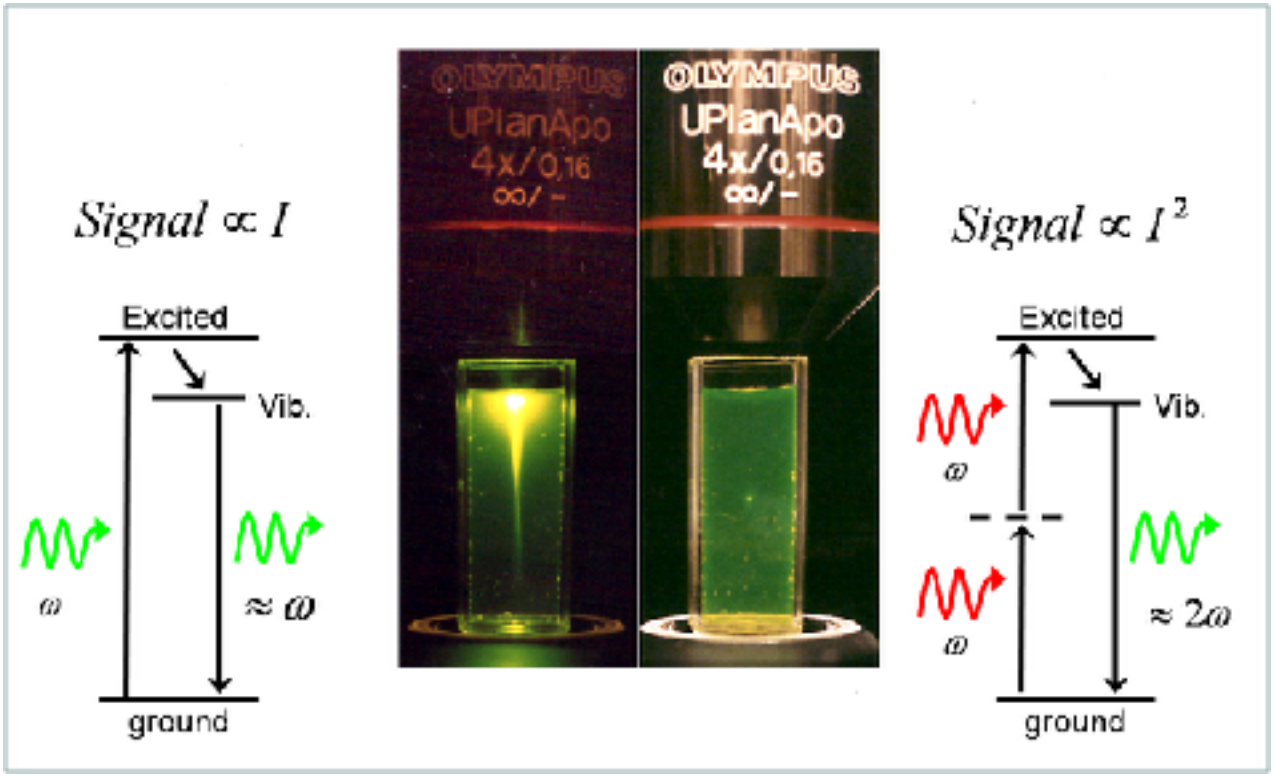
energy loss

fluorescence

ground state

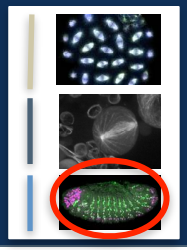


Principle of 2-photon Microscope

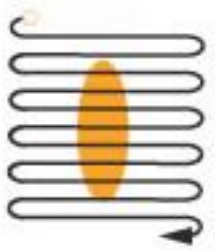


Near simultaneous, two photon event highly unlikely, only really possible a focal point

Tightly focused excitation



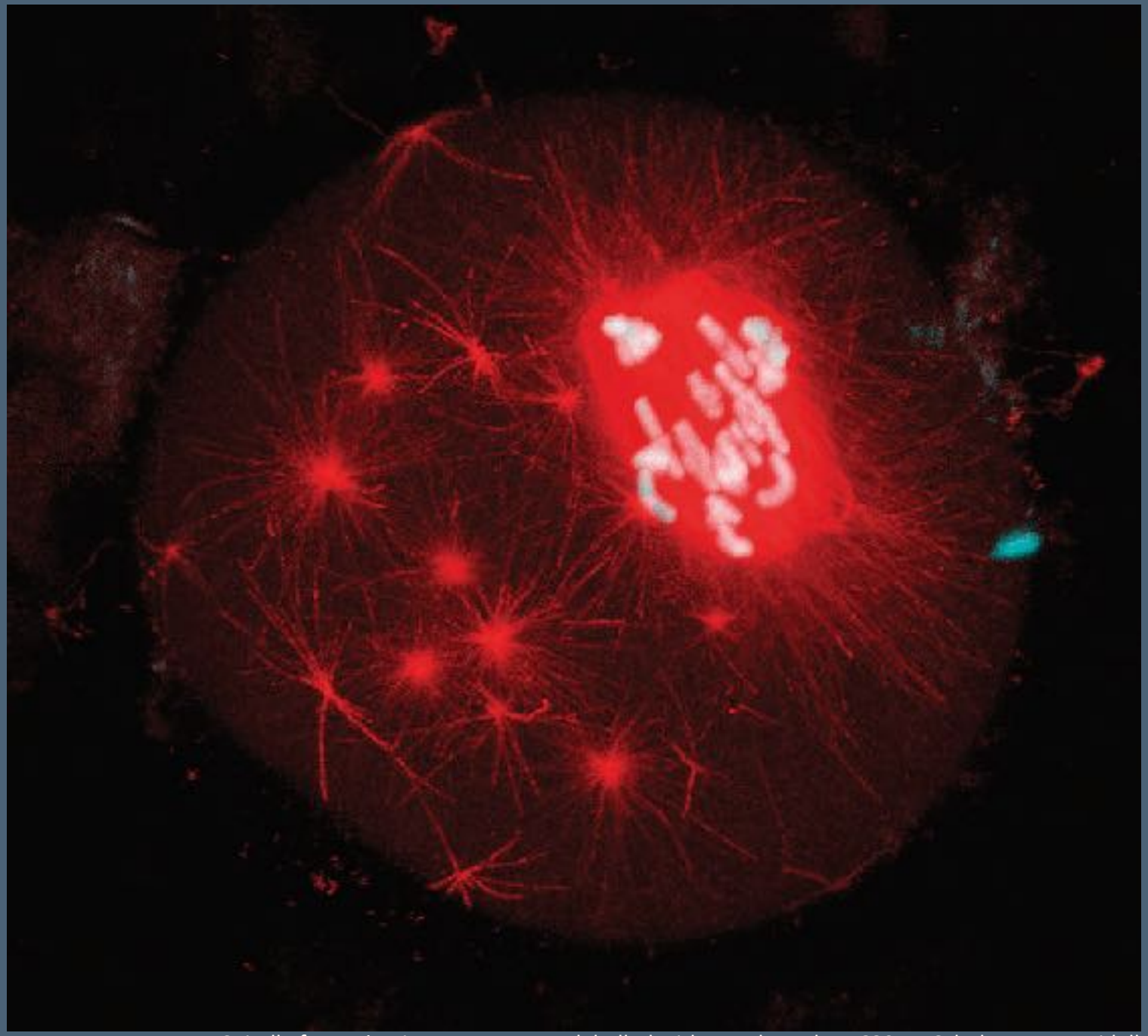
2-photon Microscope



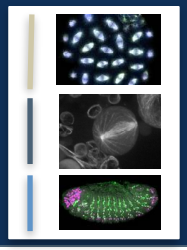
Pulsed excitation laser is then scanned across the sample.

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



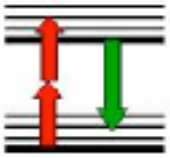
3 Flavours of Microscope

Problem:
Out of Focus
Light

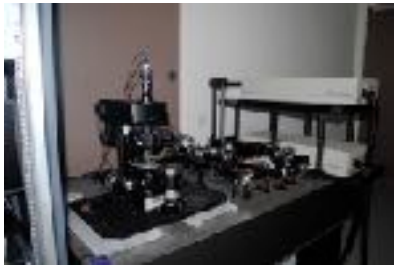
Confocal

Laser
Scanning

Spinning disc



2-Photon

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