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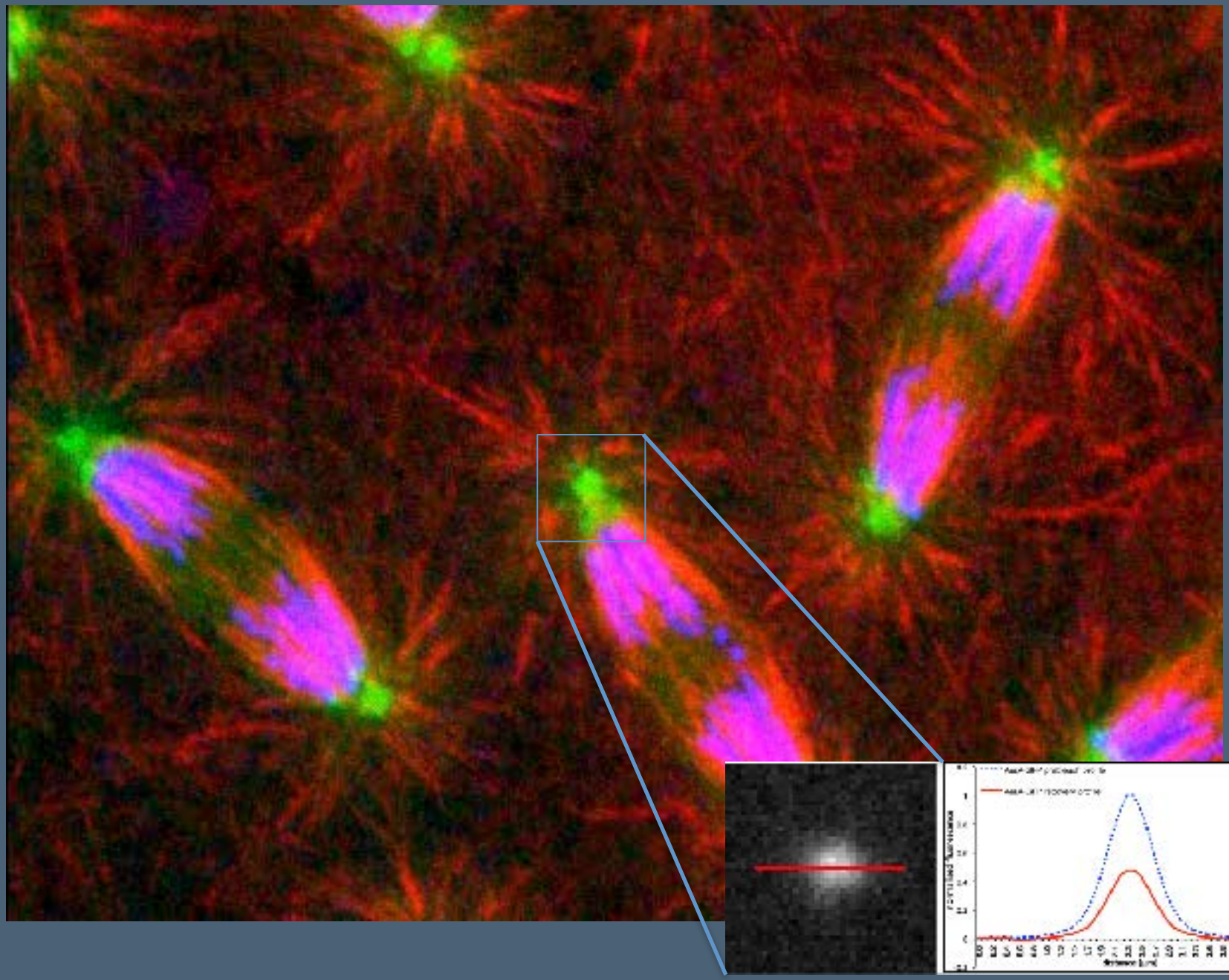
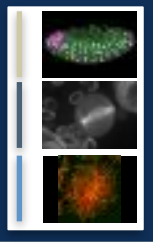
Confocal microscopy and optical sectioning

November 2020





Widefield Microscope

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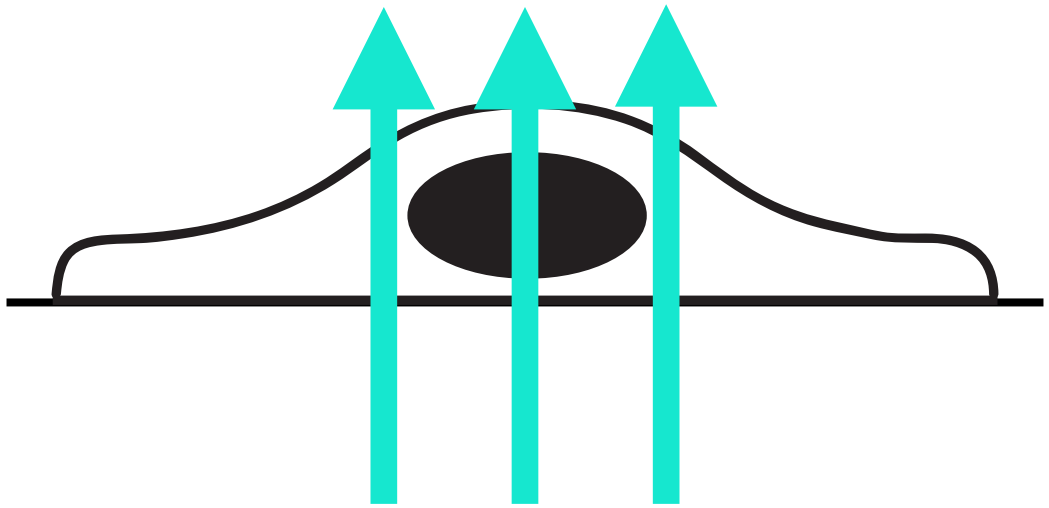
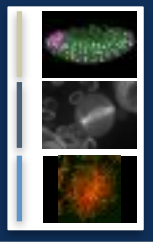


excite entire thickness of your sample



Widefield Microscope

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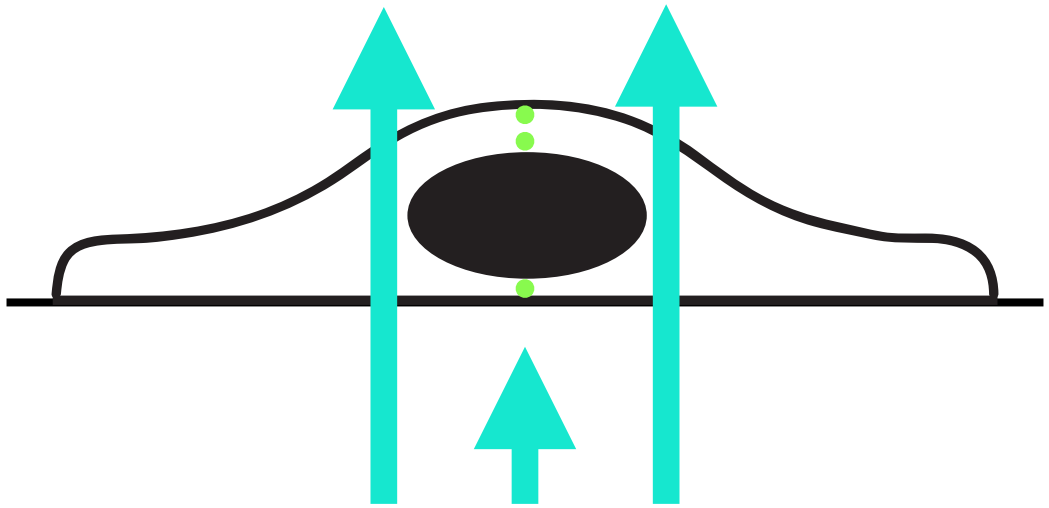
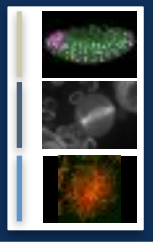
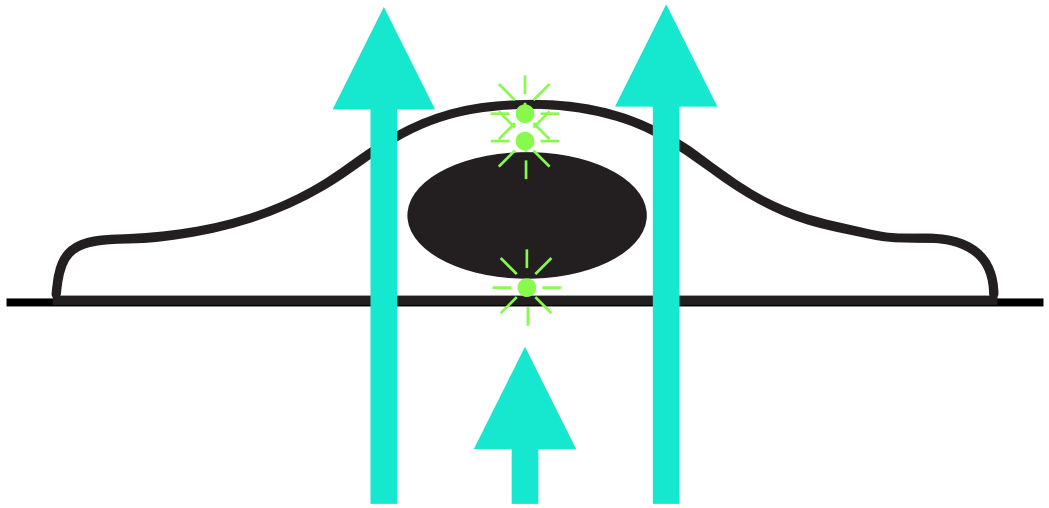


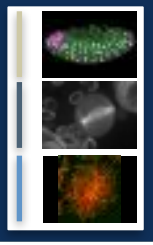
excite entire thickness of your sample



Widefield Microscope



excite entire thickness of your sample



Widefield Microscope

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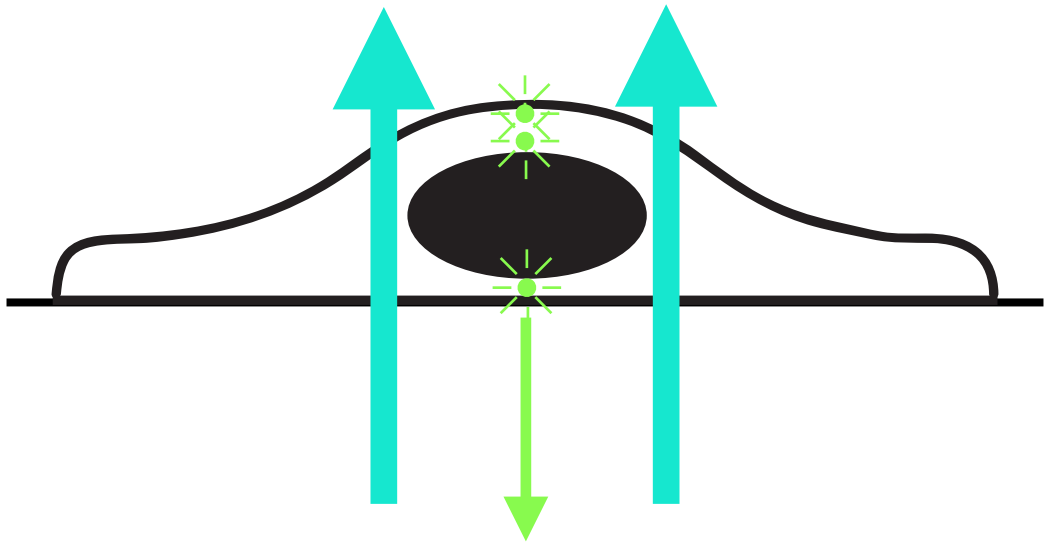
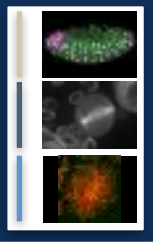


collect all the light emitted from you sample



Widefield Microscope

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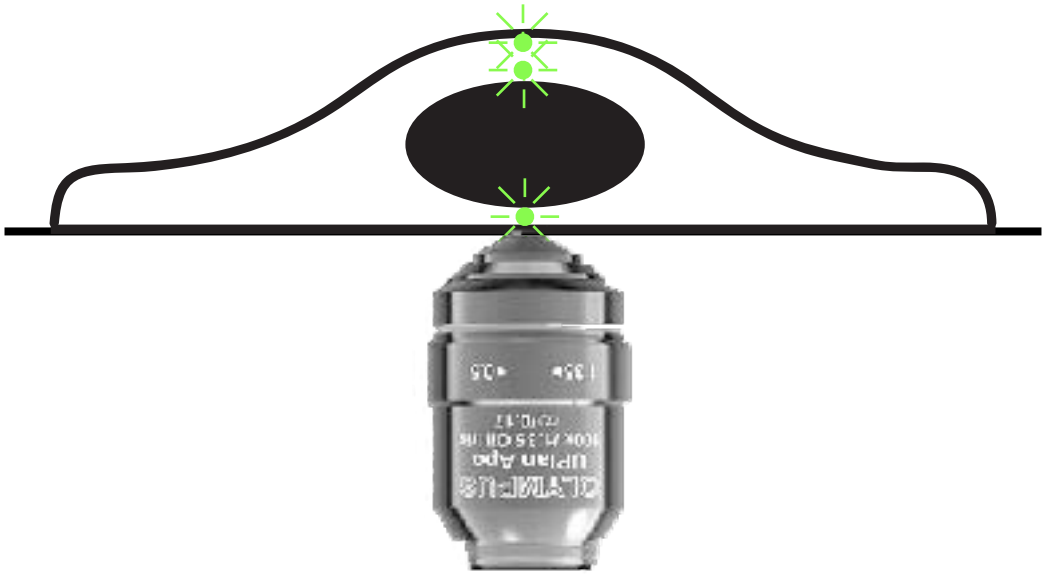
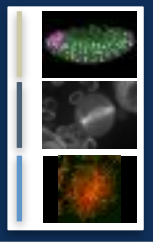


follow light through the optics



Widefield Microscope

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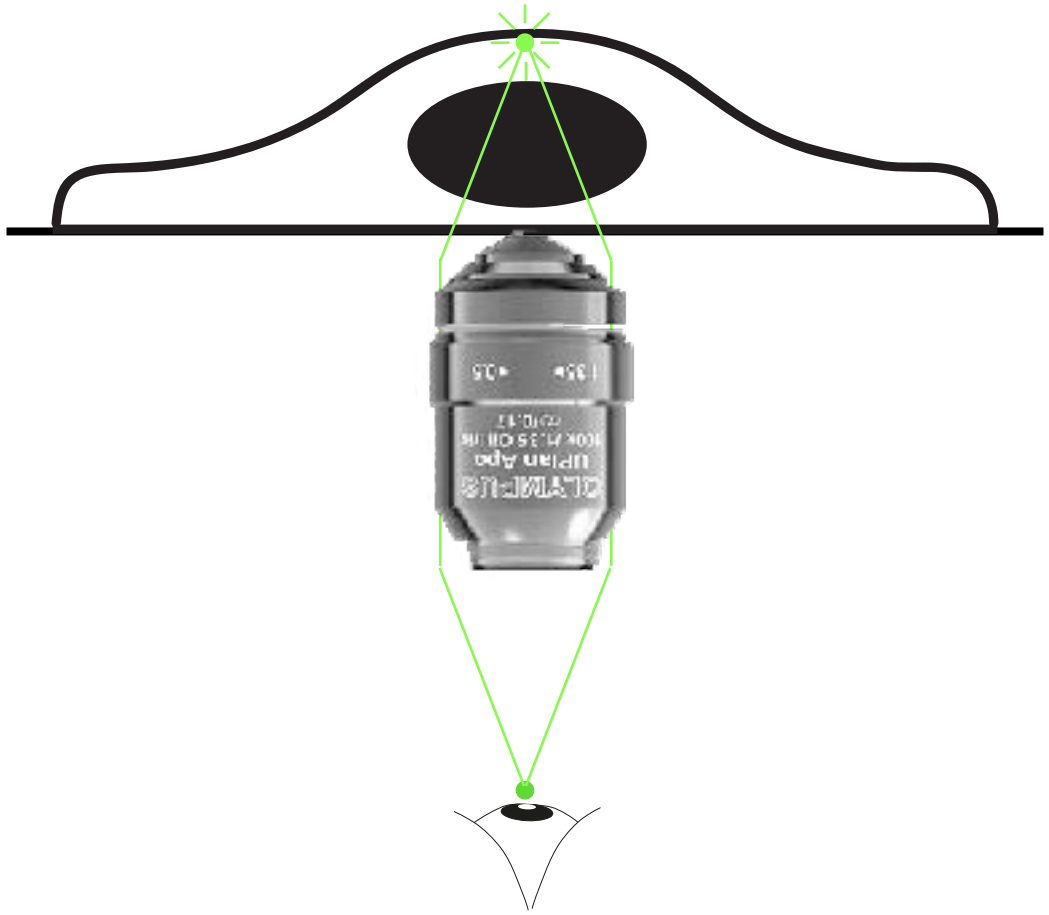

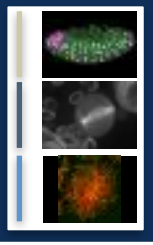

Widefield Microscope

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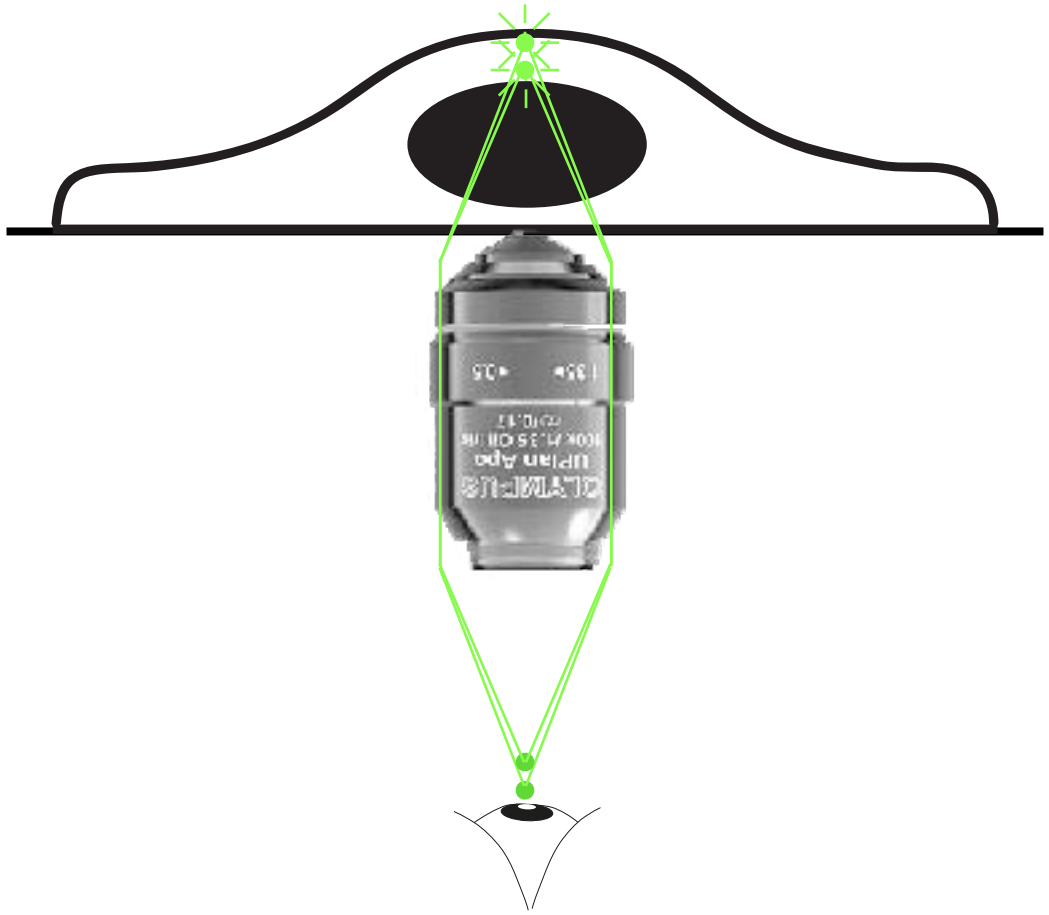

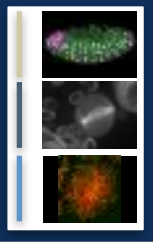

Widefield Microscope

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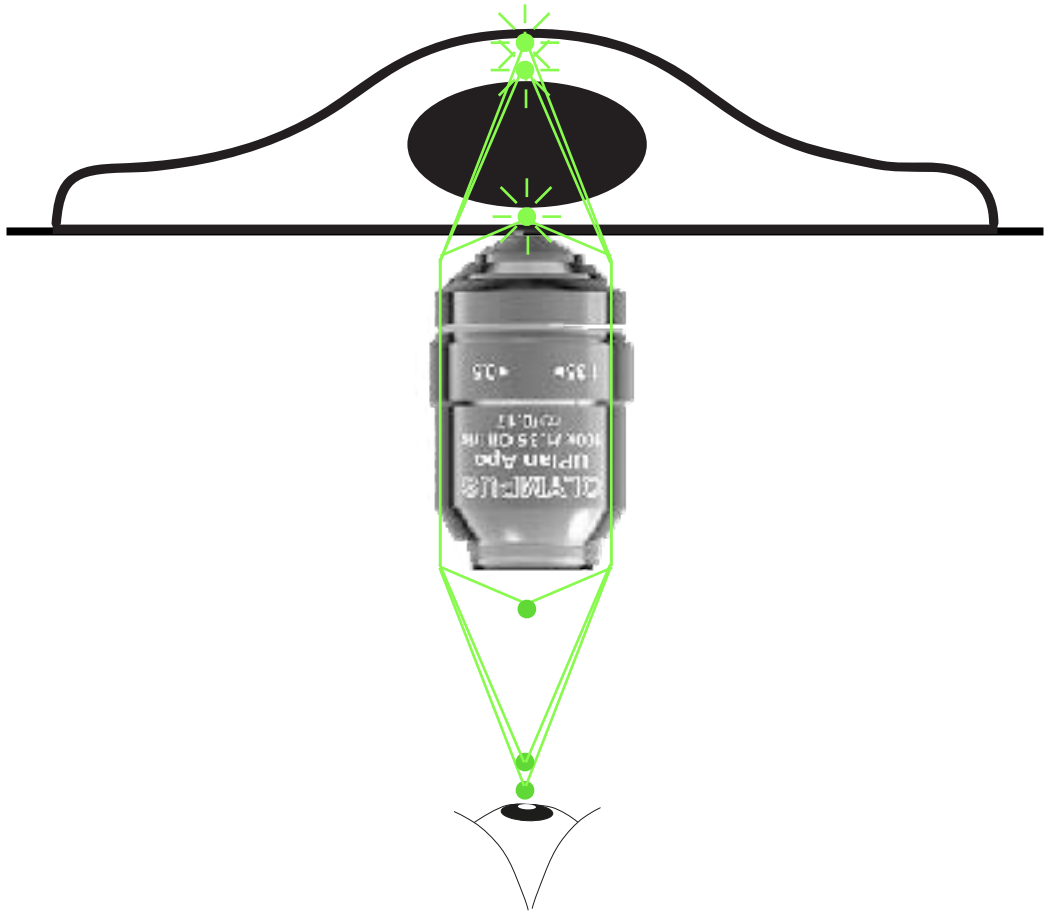

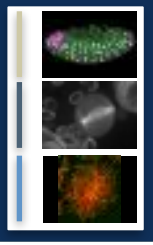

Widefield Microscope

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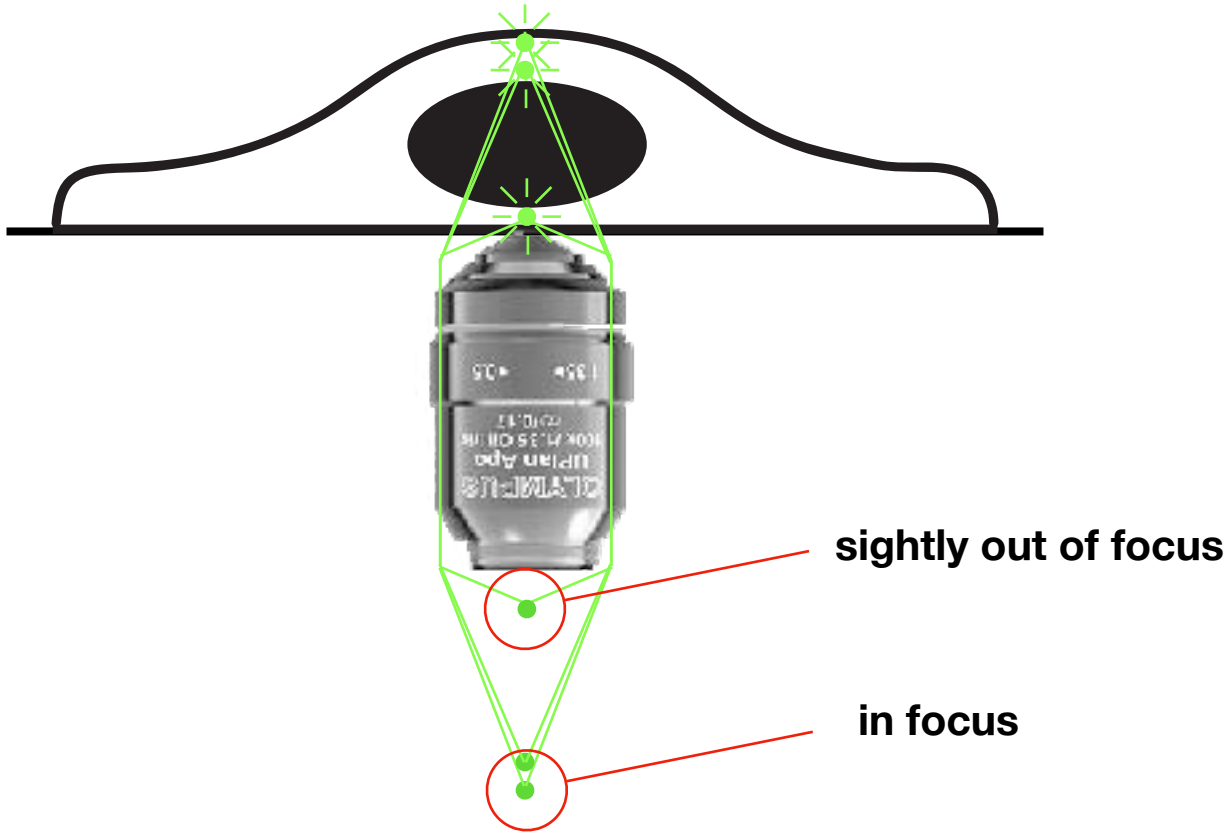
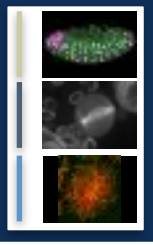
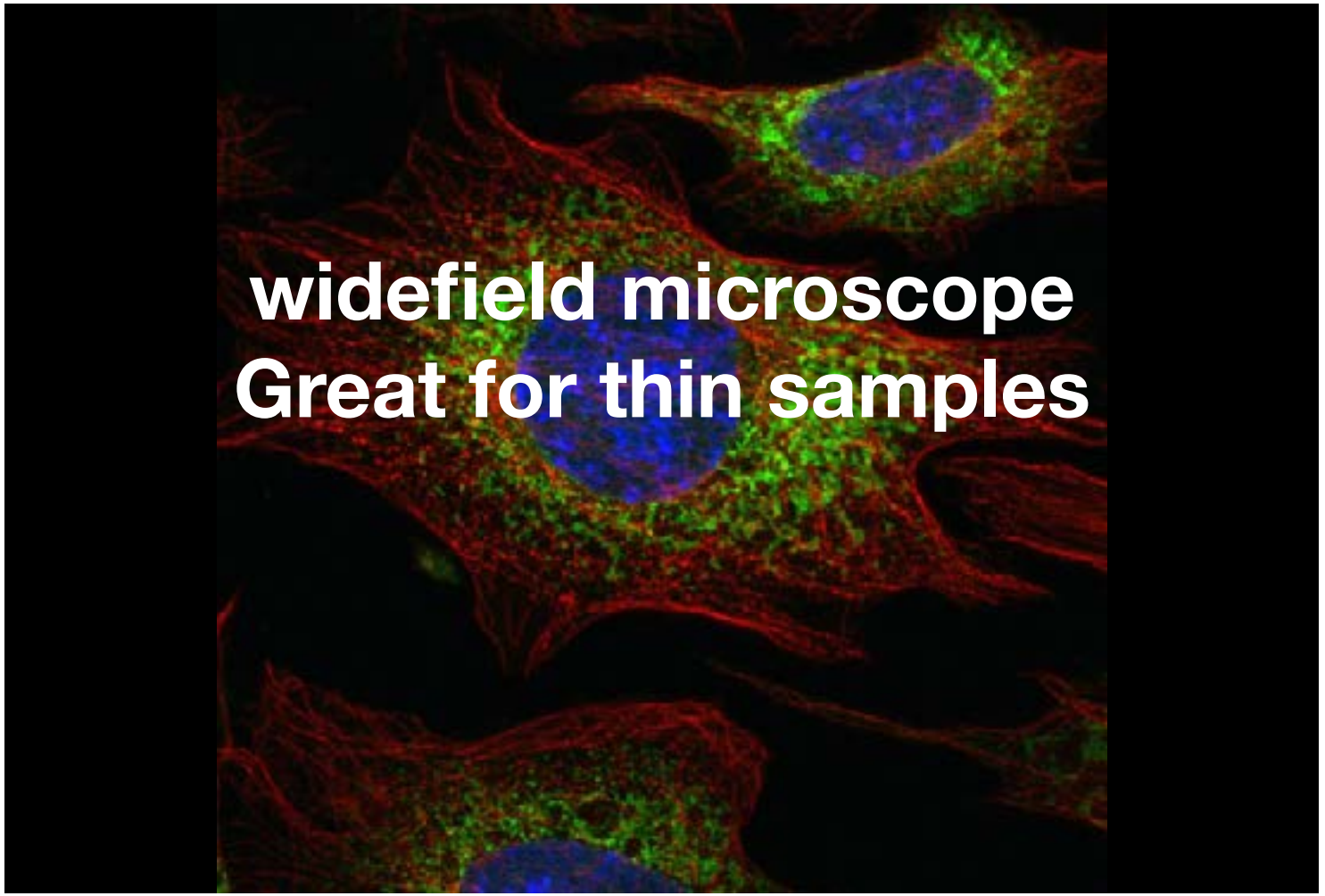
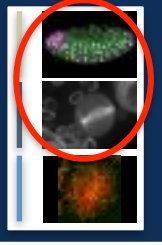


with thin samples this is not a problem




widefield microscope
Great for thin samples



Thin Vs Thick Sample

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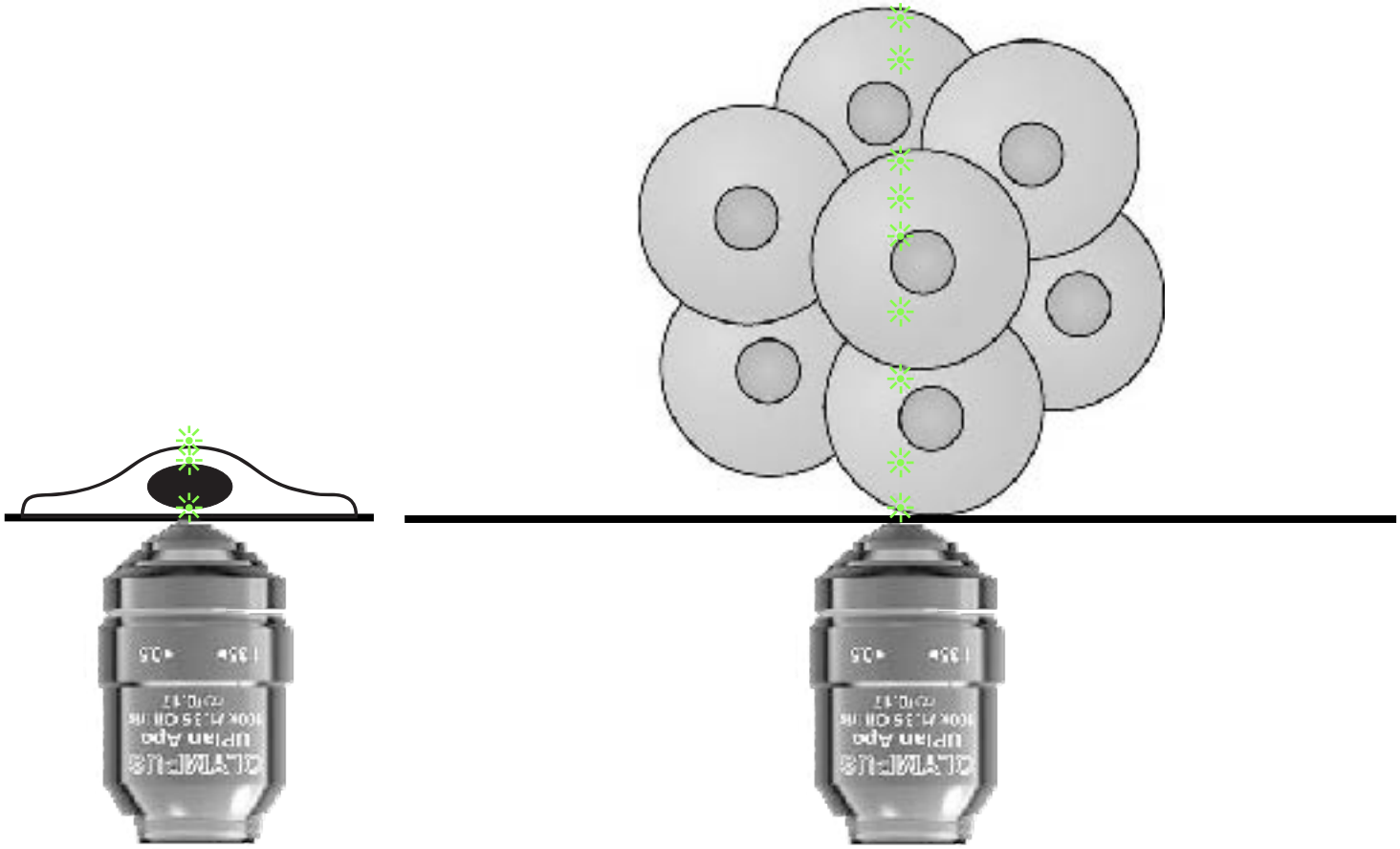
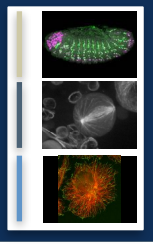


Very little out-of-focus light

Lots of out-of-focus light



3 Flavours of Microscope

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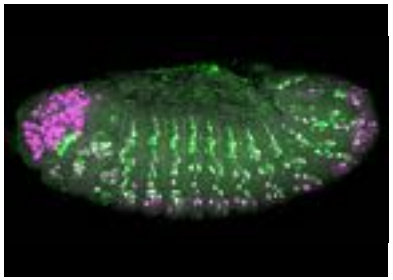
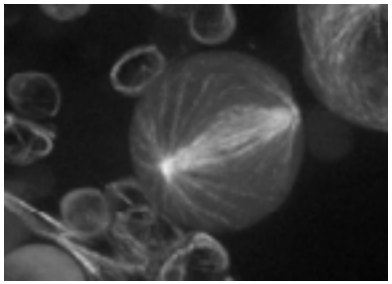
Problem:
Out of Focus
Light

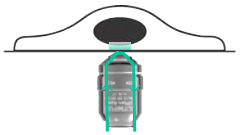
Confocal

Laser
Scanning

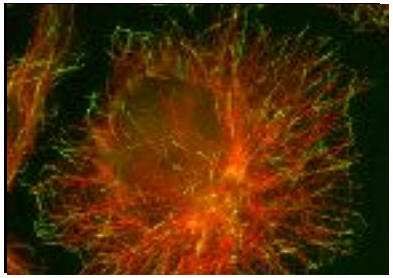


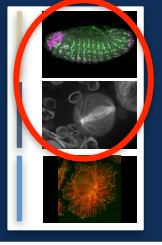
Spinning disc





TIRF





Widefield Vs Confocal

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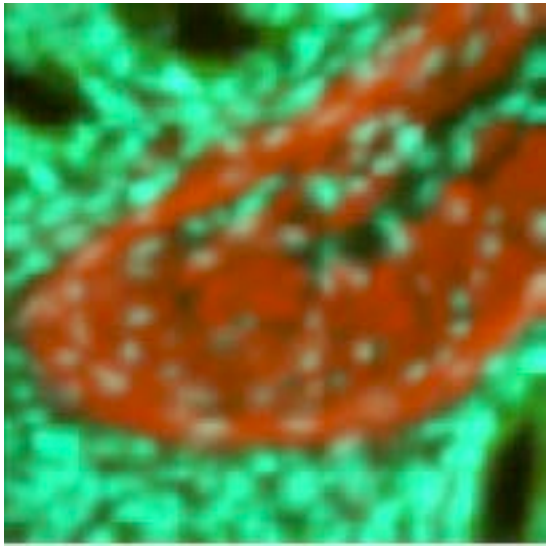
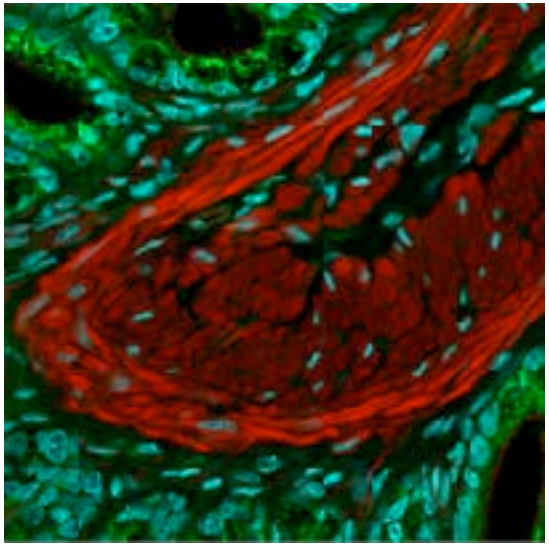
thick samples

Widefield

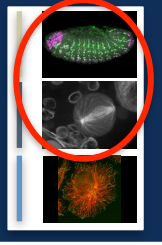


Out of focus light ‘blurs’ image

Confocal



Out of focus light is blocked



Confocal Microscope

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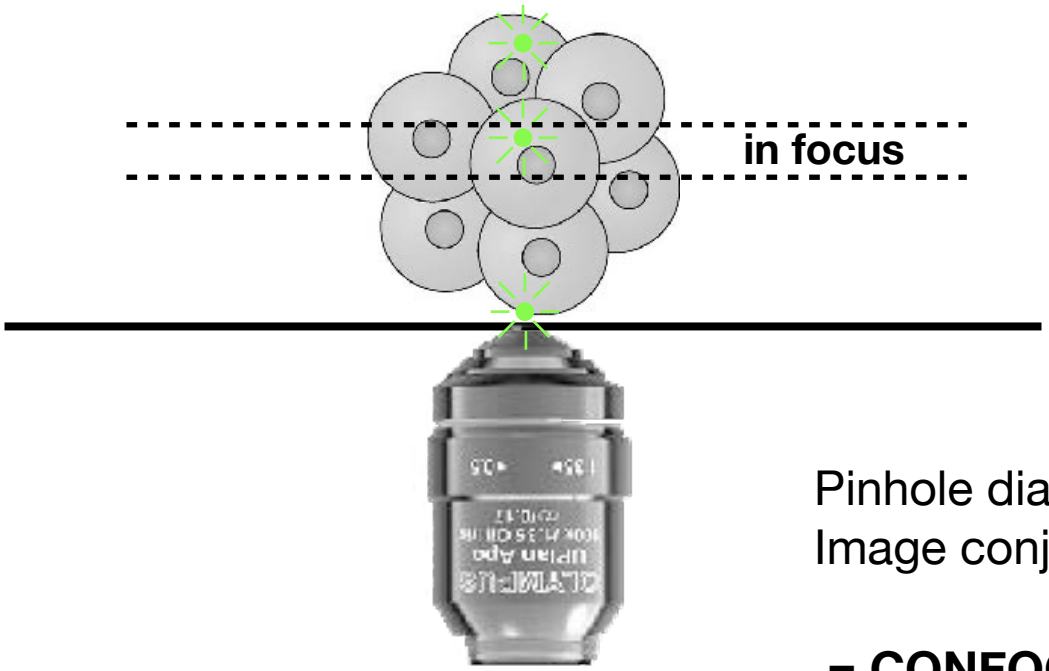
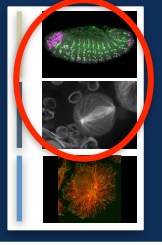


Pinhole diaphragm in the
Image conjugate plane

= CONFOCAL

pinhole



Confocal Microscope

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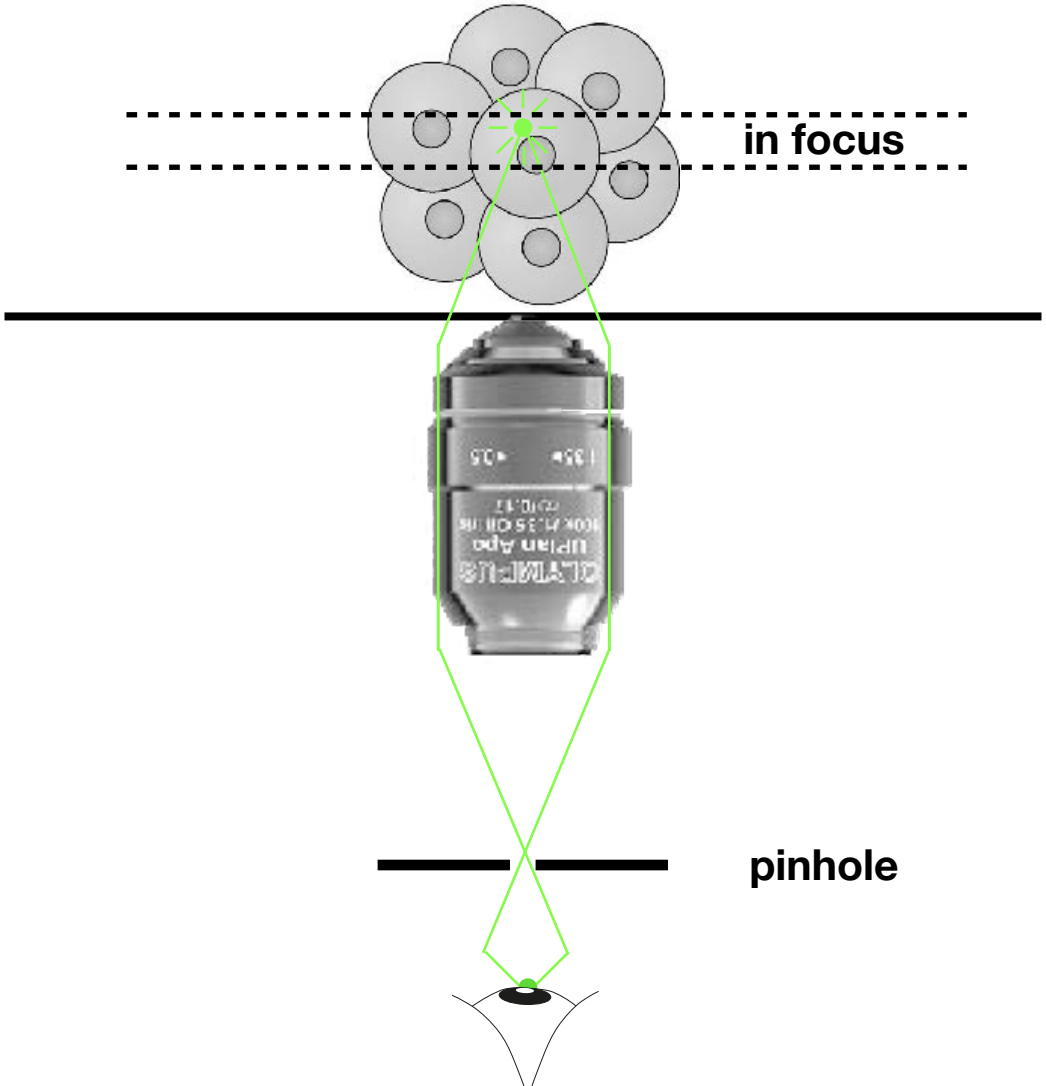
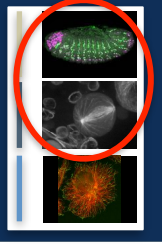
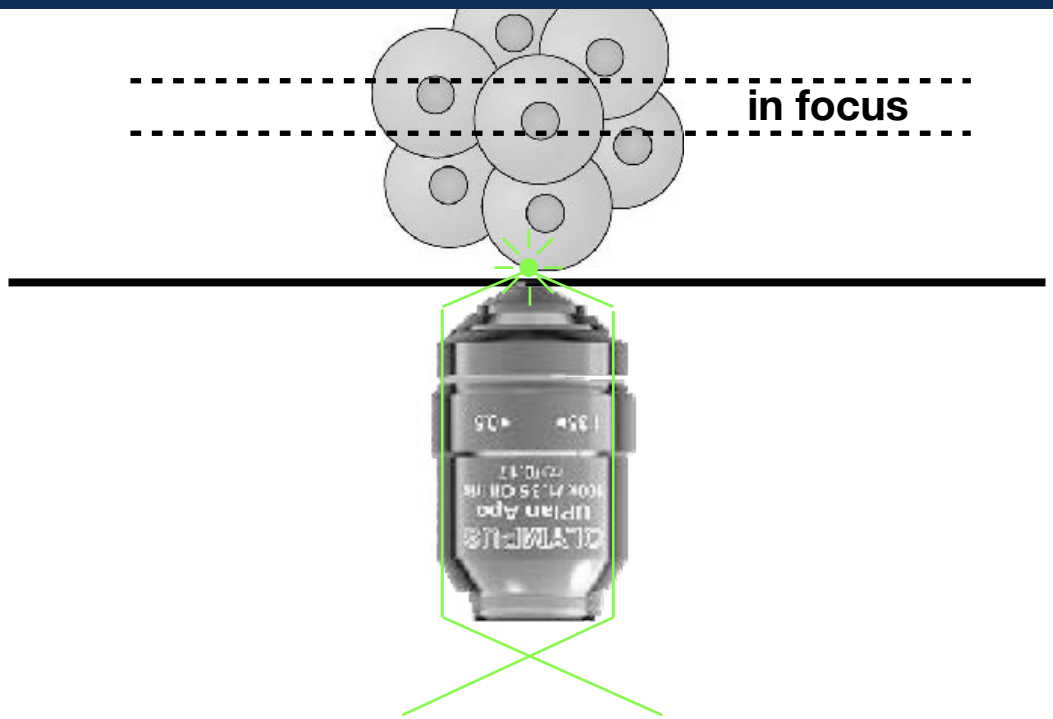
In focus light (from the optical section) passes through the pinhole and into the detector

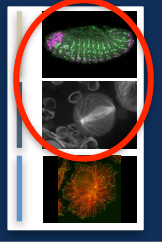




Confocal Microscope

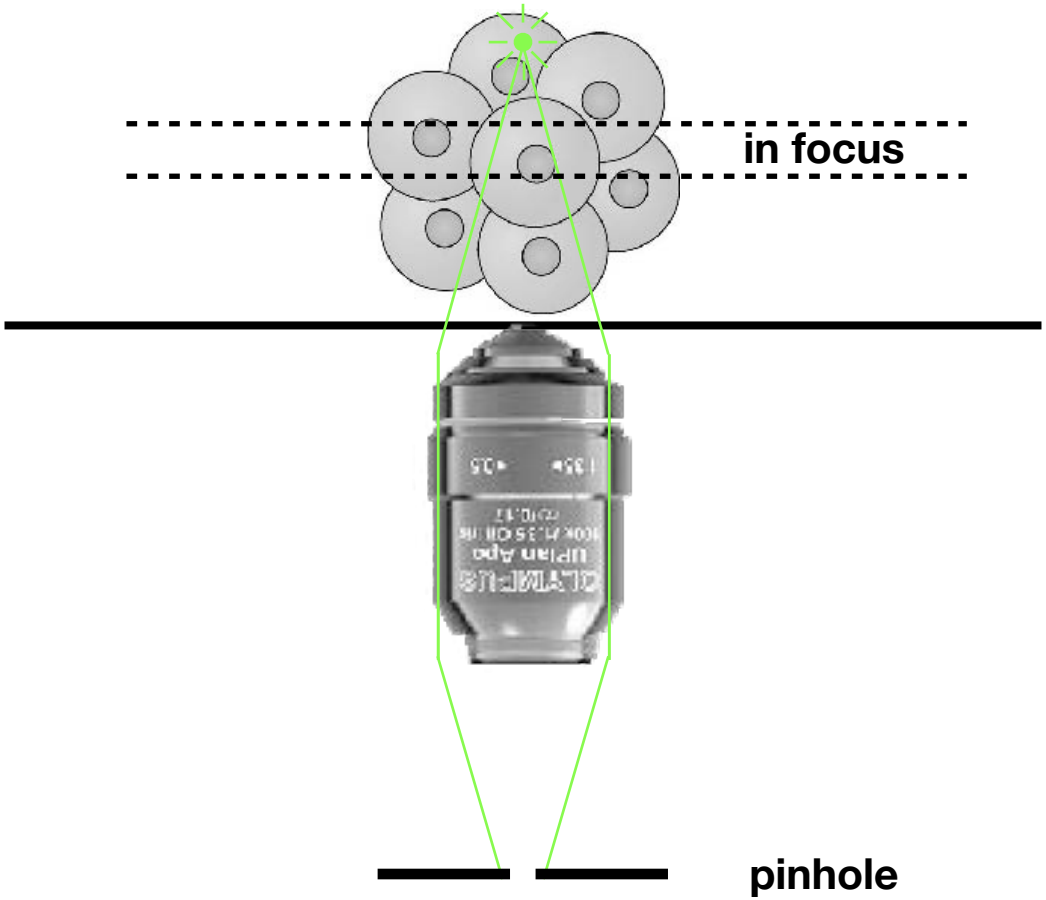
light from below the optical section crosses in front of the pinhole and doesn't pass through the pinhole aperture

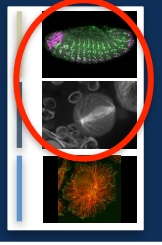




Confocal Microscope

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





Confocal Microscope

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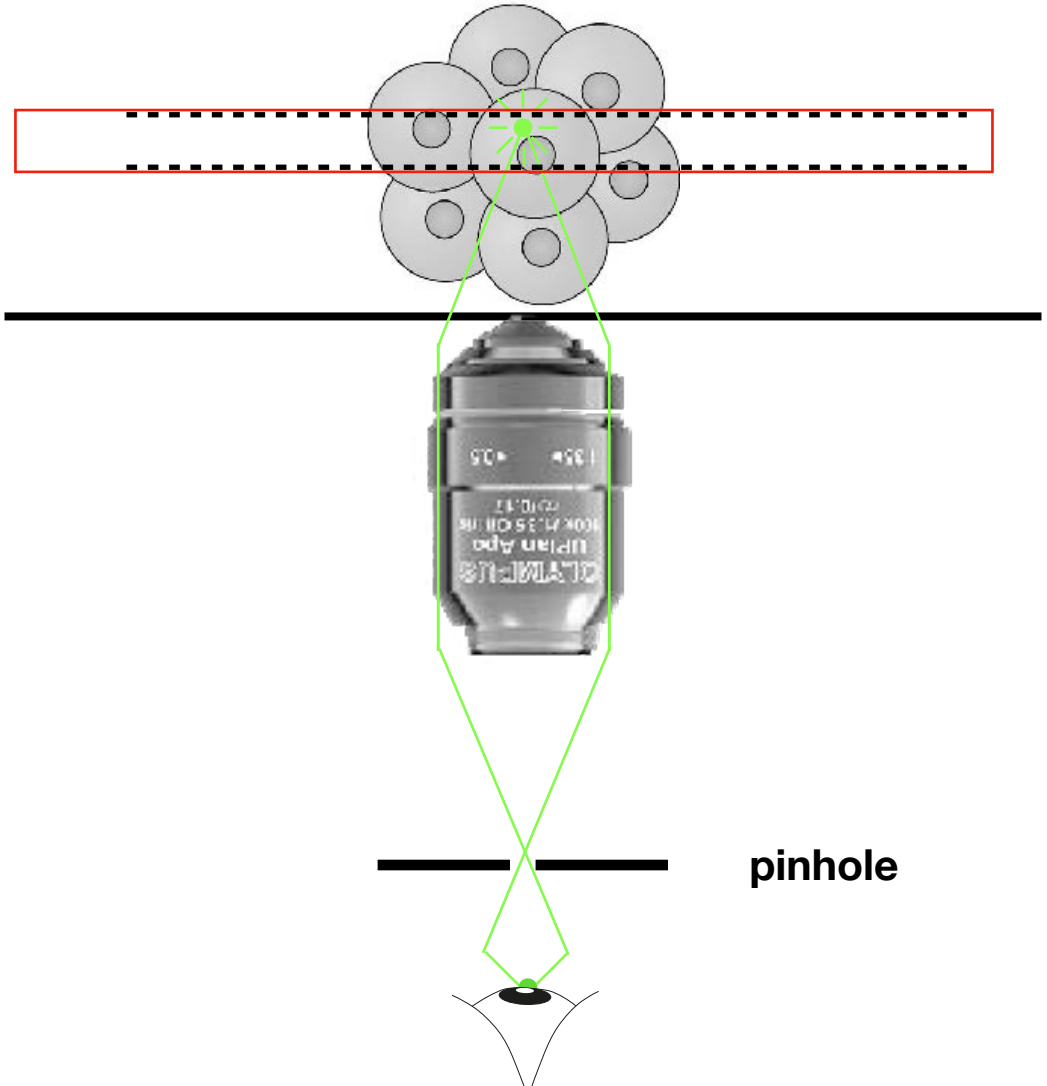
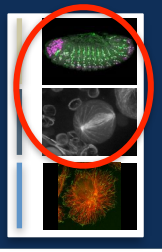
In focus light (from the optical section) passes through the pinhole and into the detector





Confocal Microscopes

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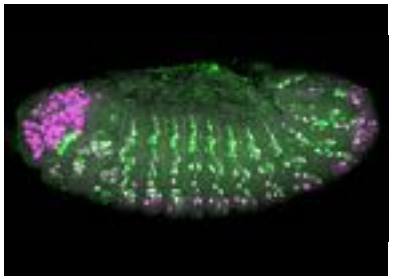
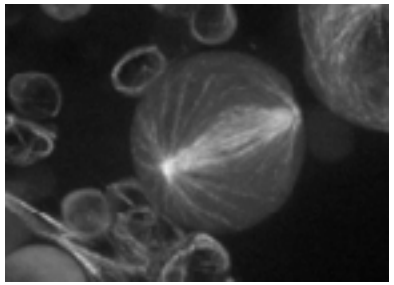
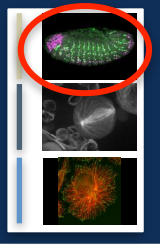
Confocal

Laser Scanning



Spinning disc





Laser Scanning Confocal Microscope

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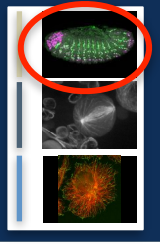
Laser Scanning Confocals are great to get ‘pretty’ images



Laser Scanning Confocal - components

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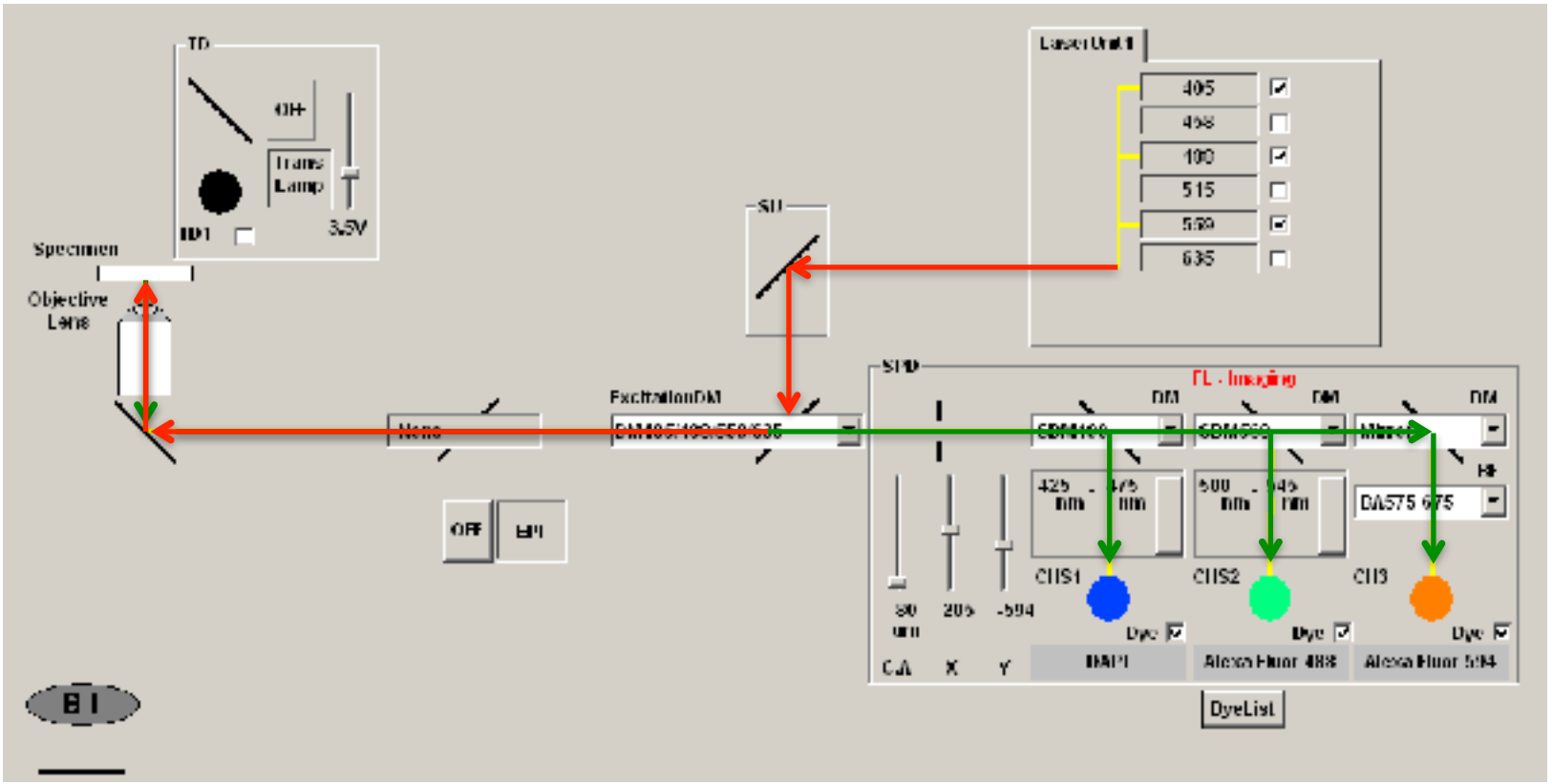

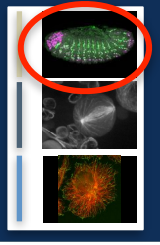

Laser Light Source

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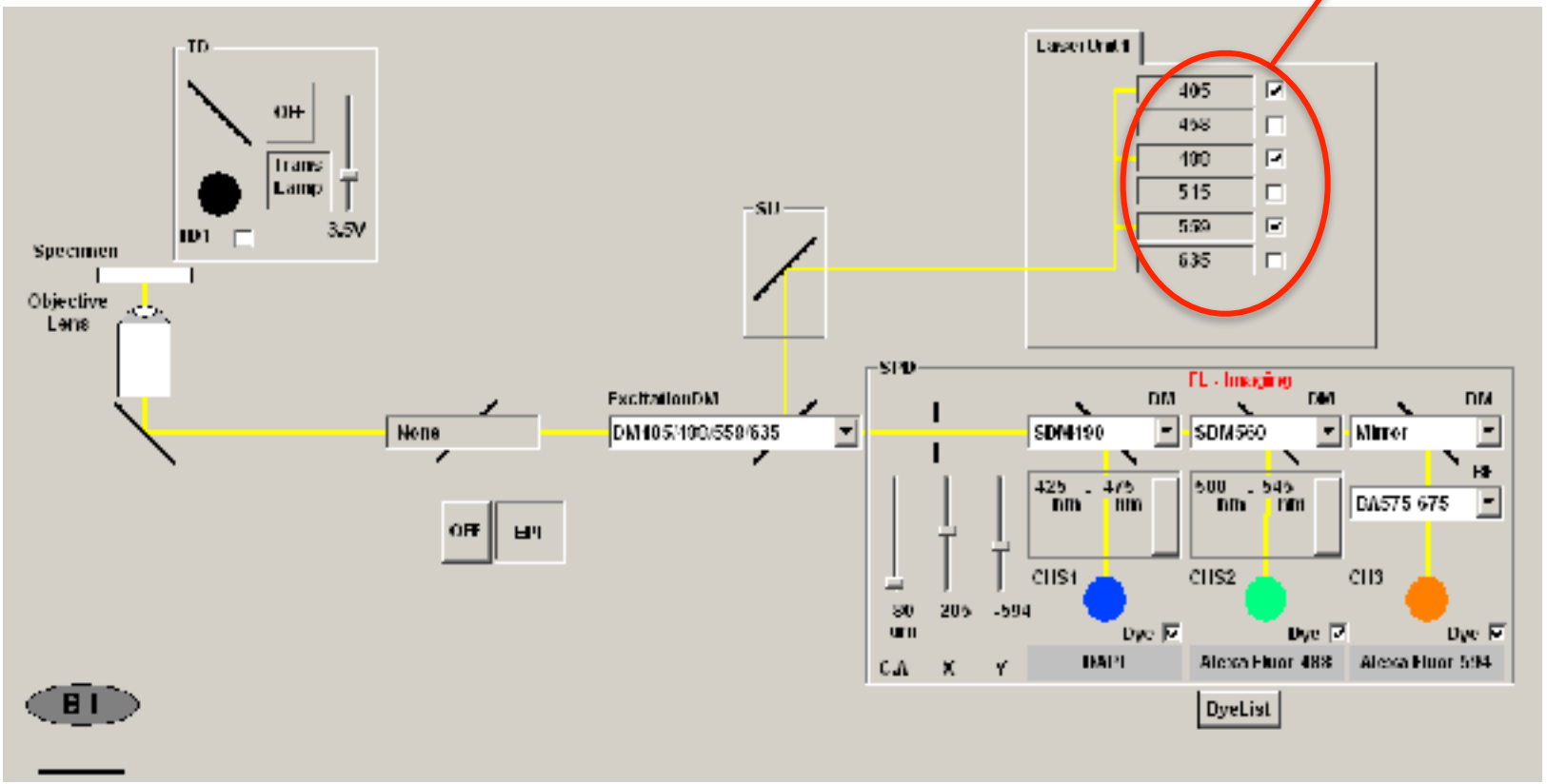
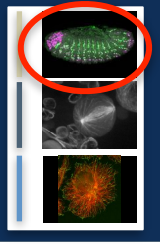
laser light source





Laser Light Source

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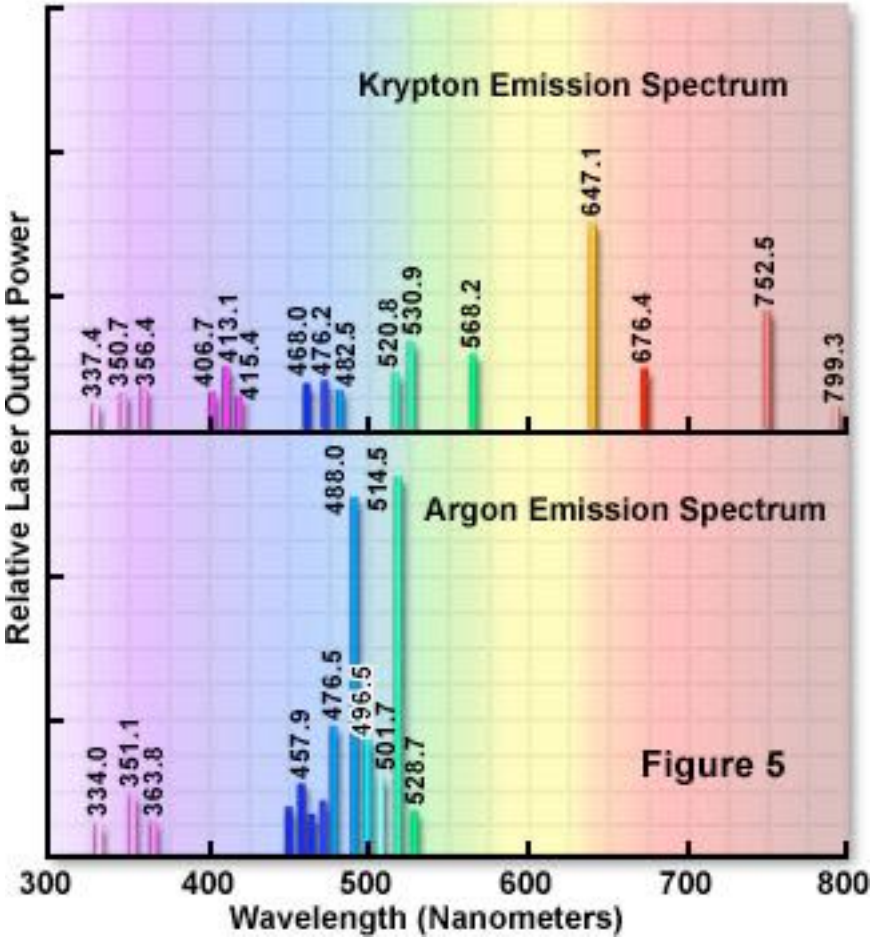
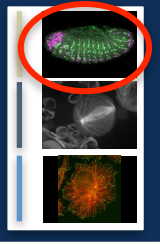
Enables tighter control of fluorophores excited

Laser Emission Spectra





AOTF
Acousto-Optic Tunable Filter

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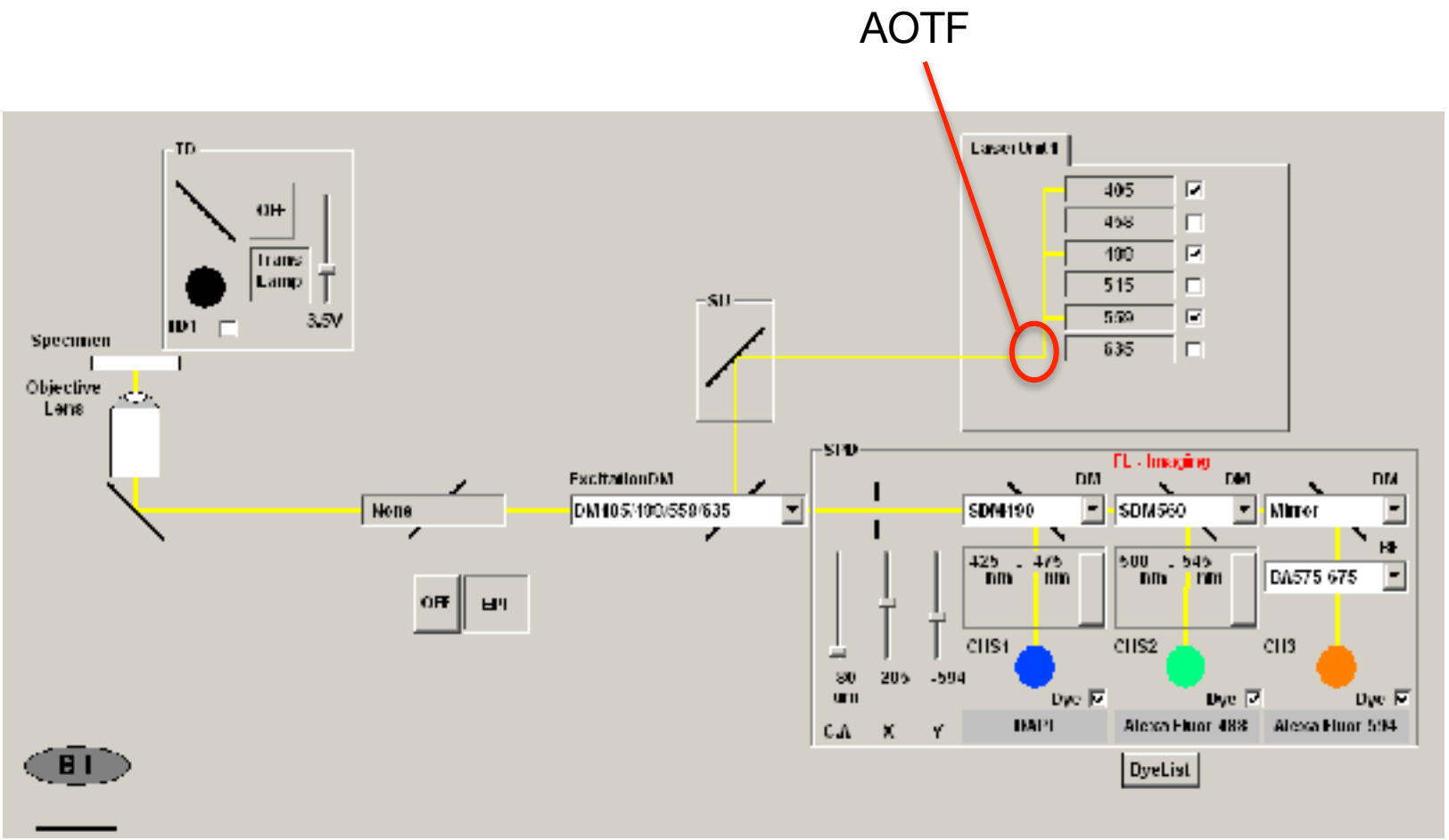

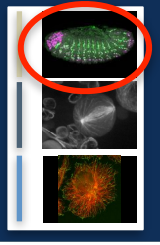

AOTF

THEORY

Acousto-Optic Tunable Filter

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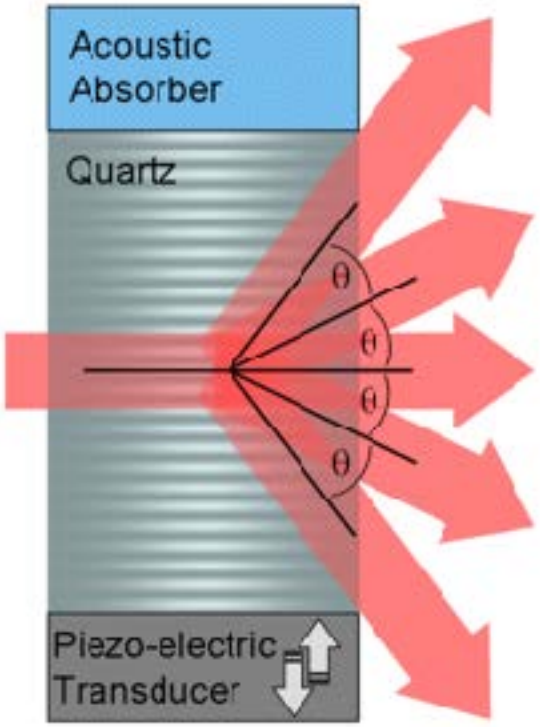
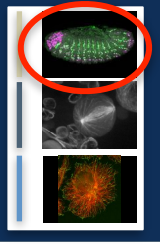


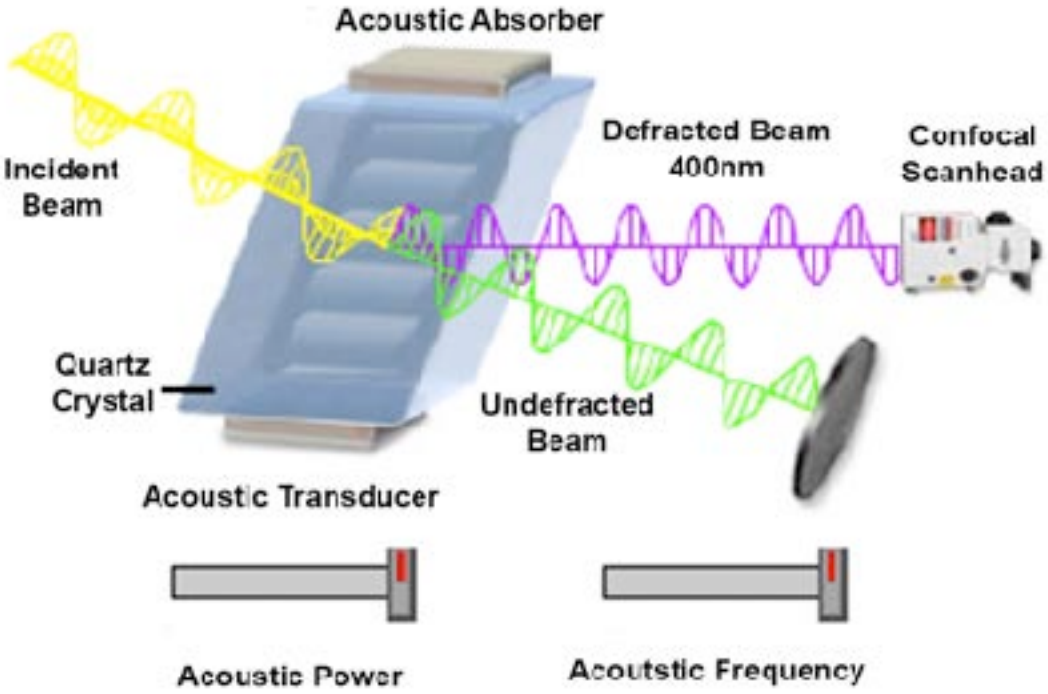
Acousto-optic effect:

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.

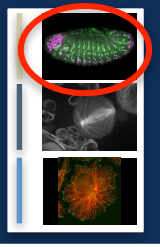


AOTF
Acousto-Optic Tunable Filter



Quick On/Off of lasers

Very fast changes between excitation wavelengths



Galvo Scanning Mirrors

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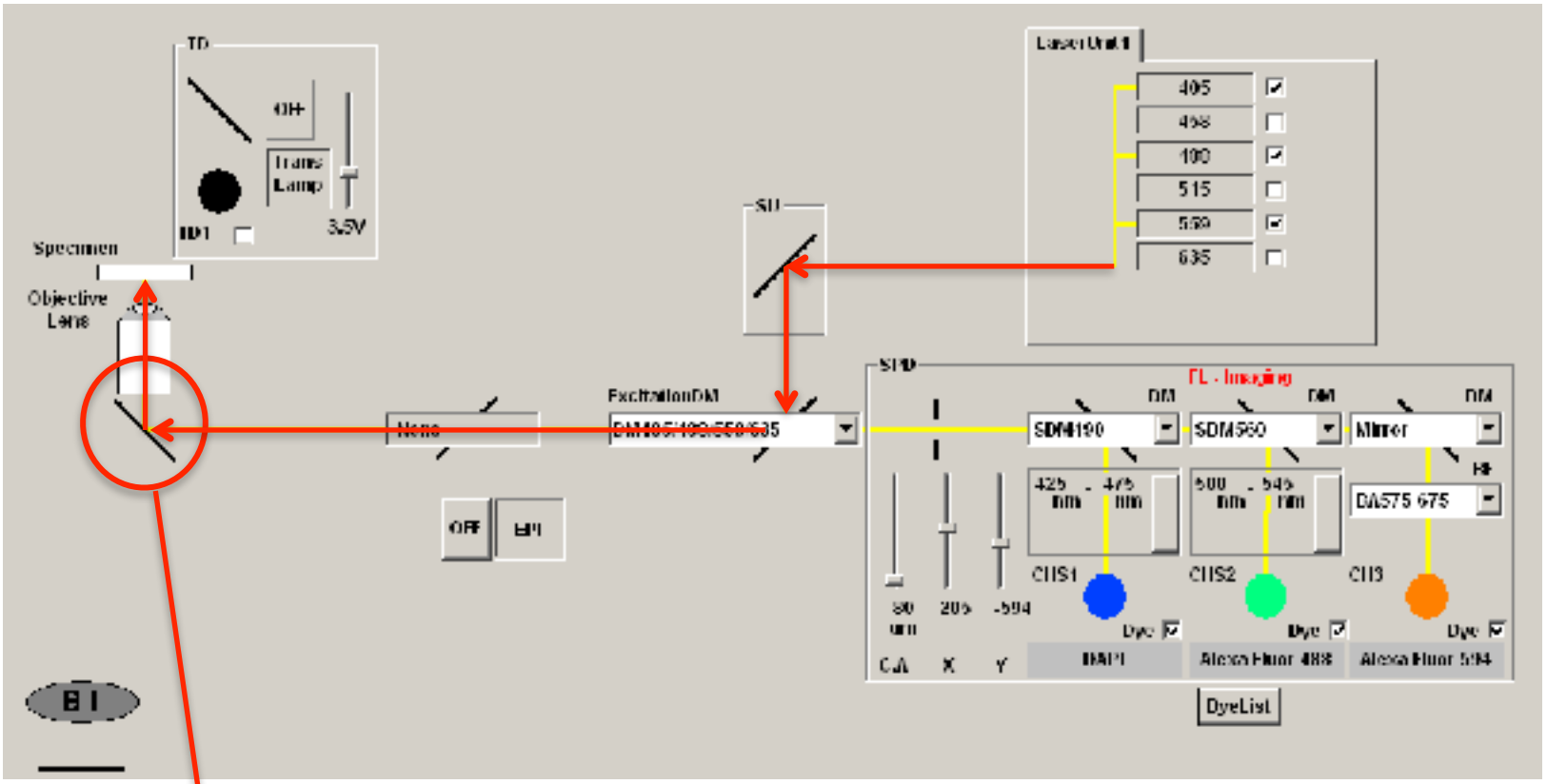
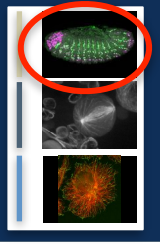


Galvo Scanning Mirrors



Galvo Scanning Mirrors

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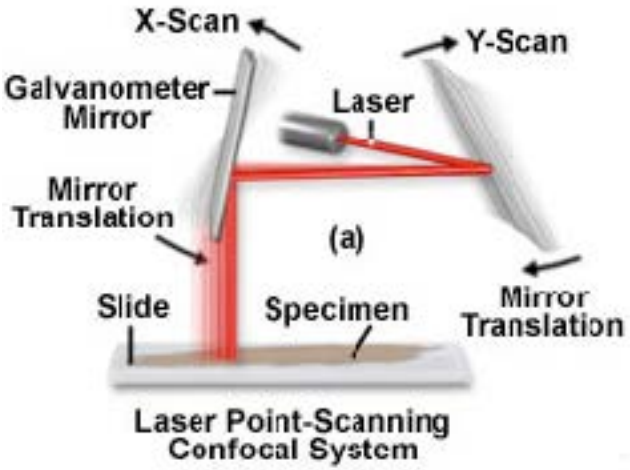
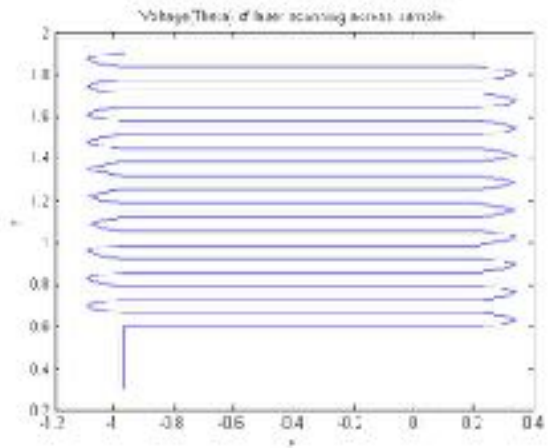
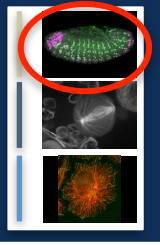


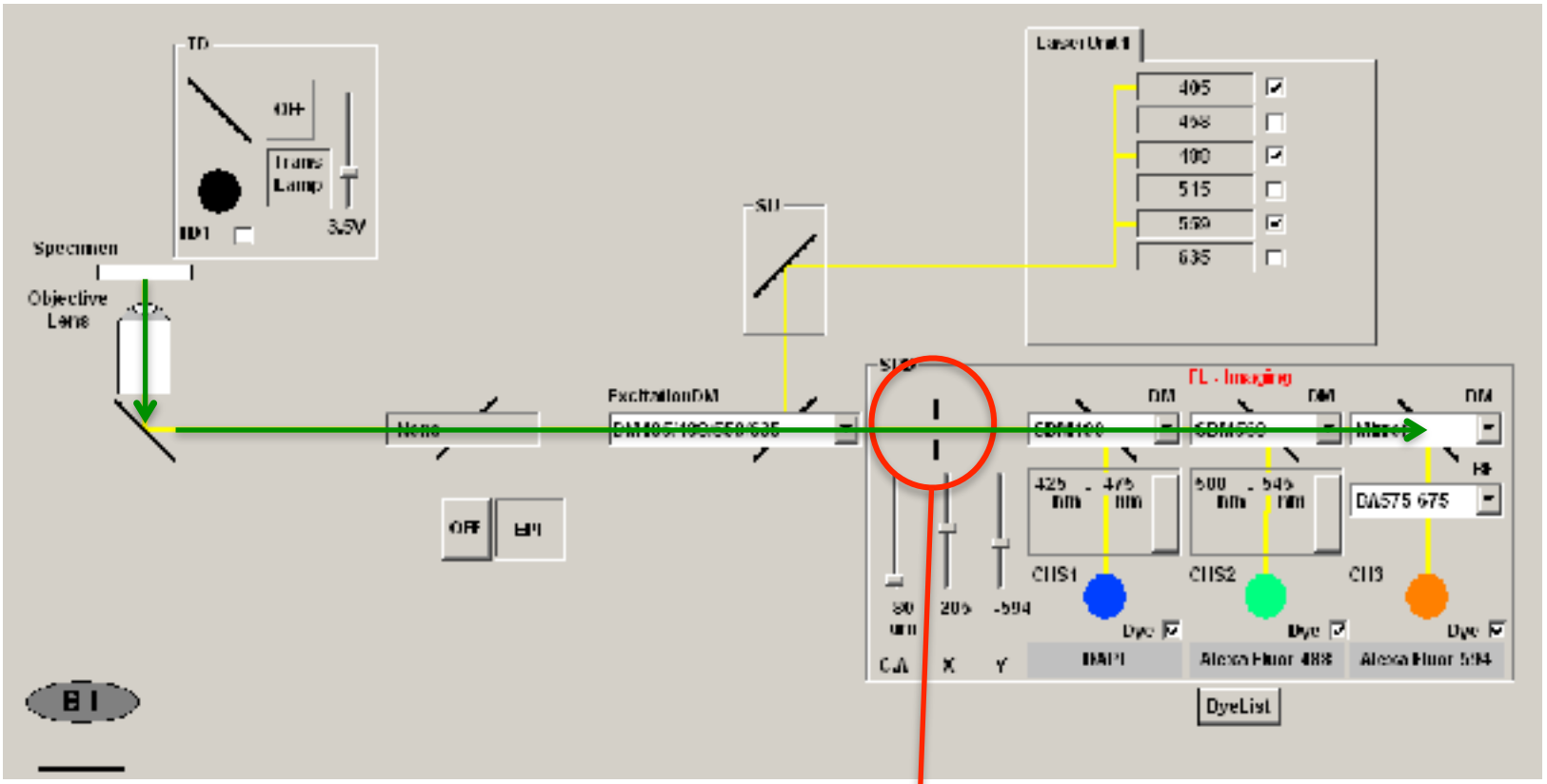


Sample excited at one point at a time
Relatively slow

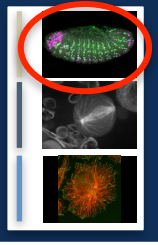


Adjustable Pinhole

AOTF



pinhole



THEORY

Pinhole – Optical Sectioning

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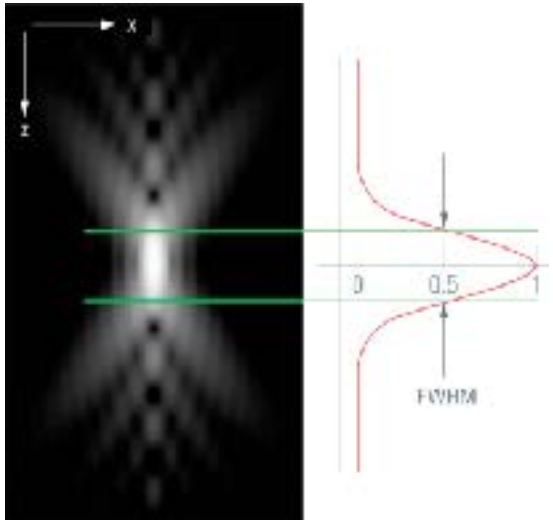
Shorter the wavelength the thinner the optical section

Diameter of the pinhole: Smaller pinhole thinner optical section

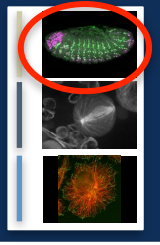


$$FWHM_{axial} = \sqrt{\left(\frac{\lambda_{exc} \cdot n}{NA^2}\right)^2 + \left(\frac{n \cdot \sqrt{2} \cdot PH}{NA}\right)^2}$$

FWHM=Full Width Half-Maximum

The higher the NA.
the thinner the section

Weak signal > open pinhole > more light but thicker



Confocal enables 3D reconstruction

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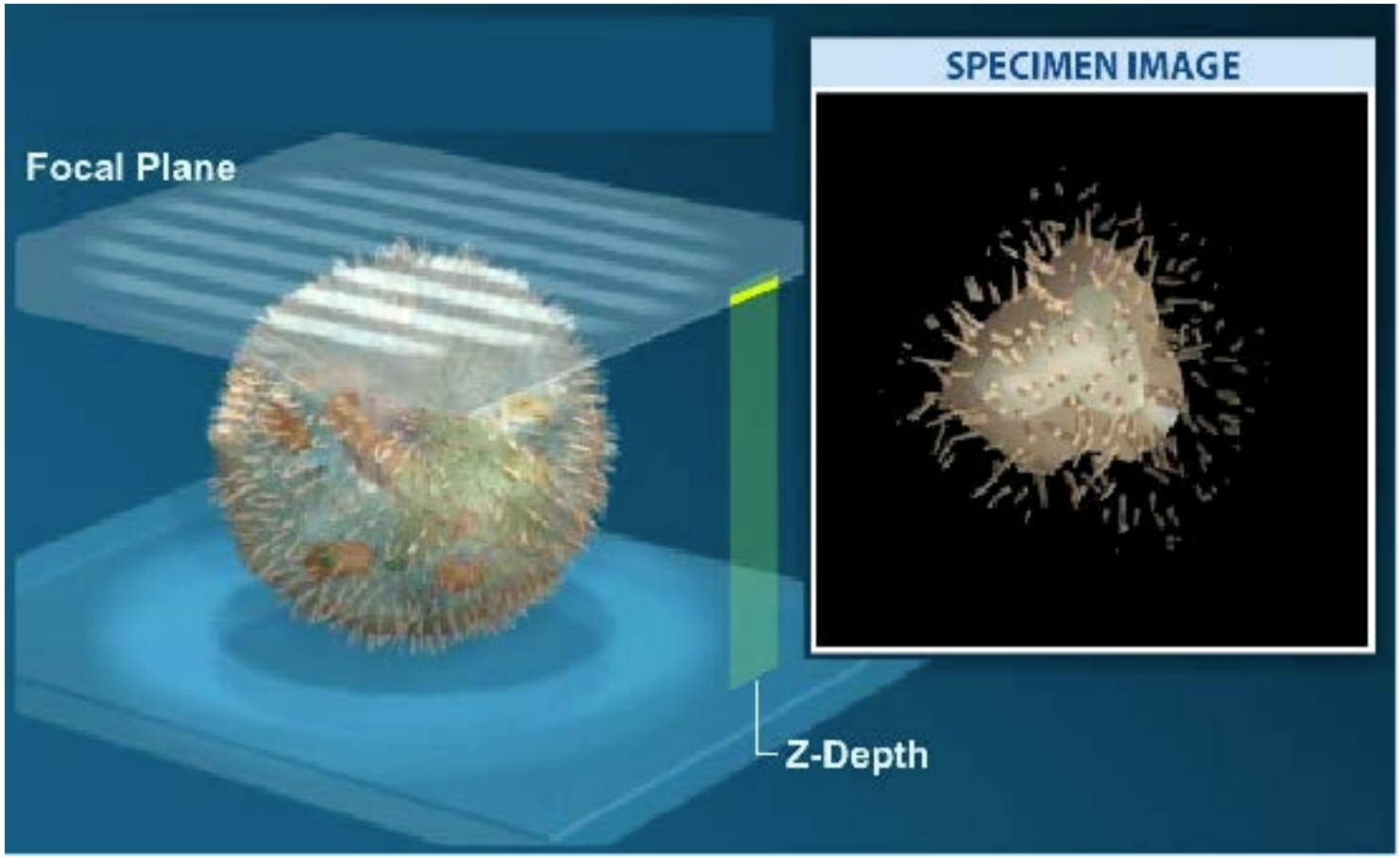
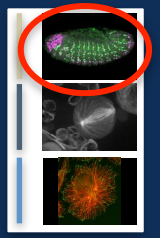
Optical section





Confocal enables 3D reconstruction

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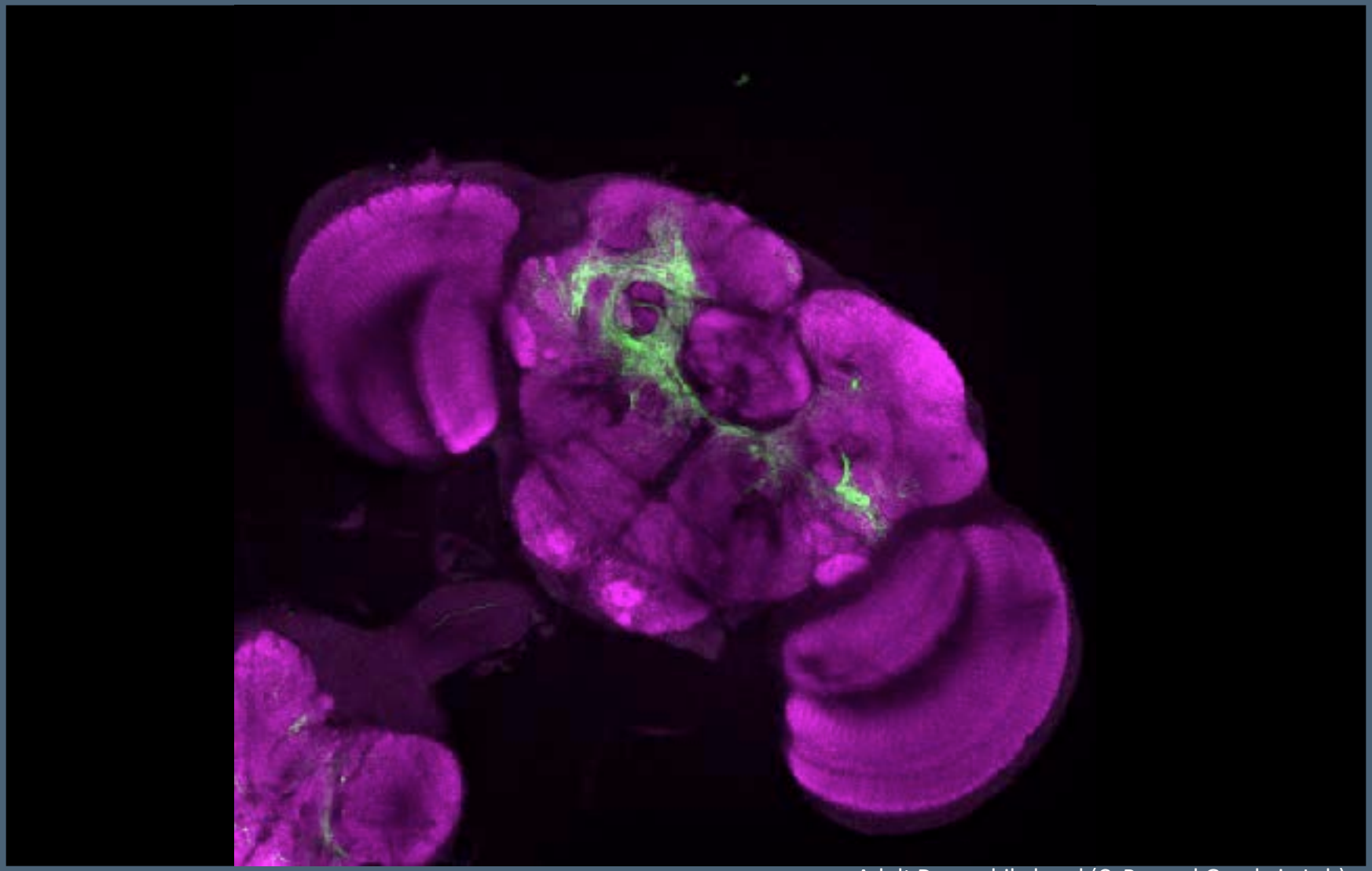
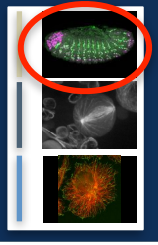
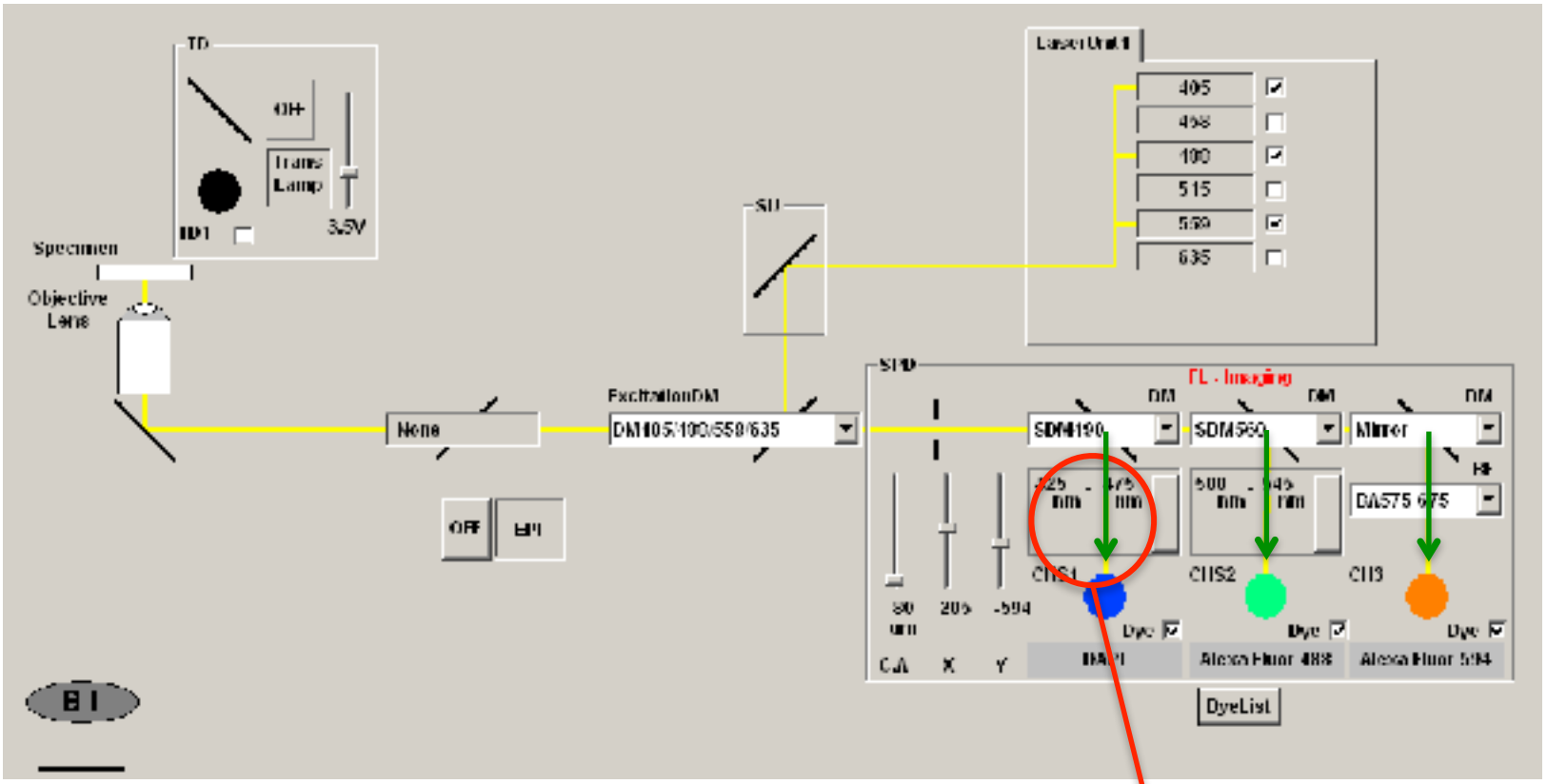
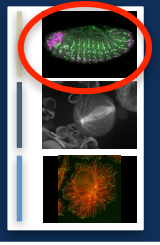


Adult Drosophila head (C. Rezeval Goodwin Lab)



Variable Detector Slit





Spectral Unmixing

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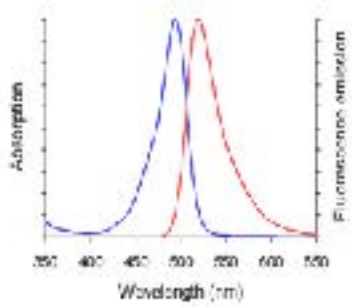
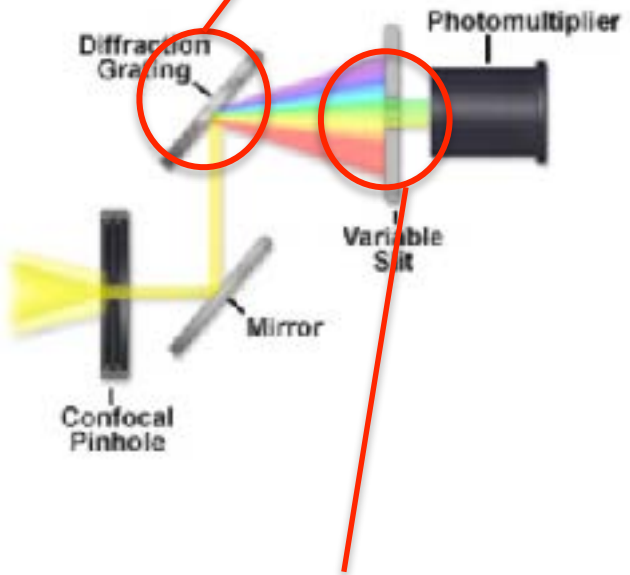
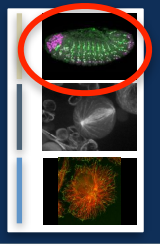


Light emitted from
fluorophore as a spectrum

Defraction grating
separates wavelengths
over physical area



Variable slit lets through
only certain wavelengths



Spectral Unmixing

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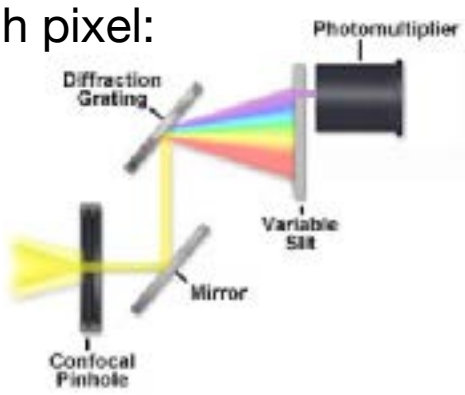
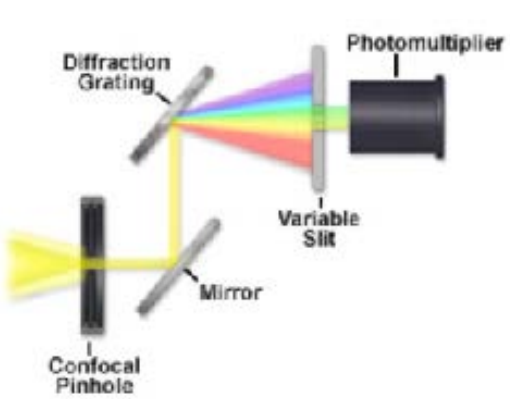
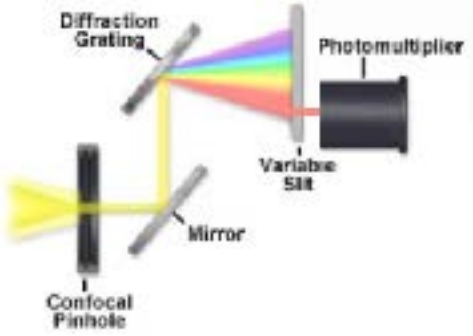
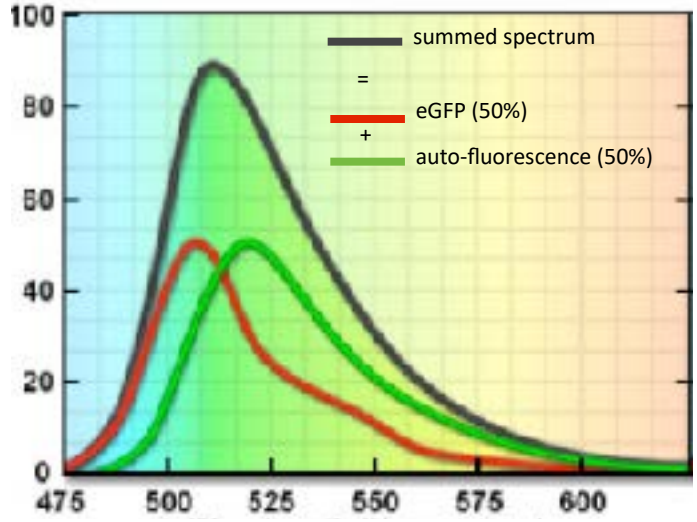
At each pixel:

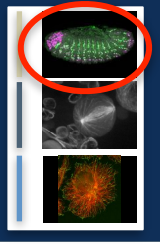








Match the summed spectrum with all possible summed combinations from a library
At each pixel you therefore know the proportion of each fluorophore present



Spectral Unmixing removal of autofluorescence

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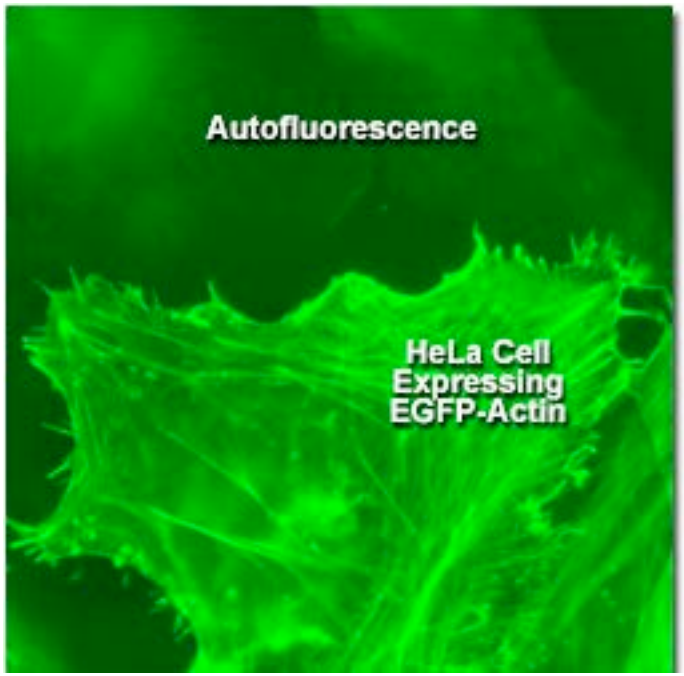
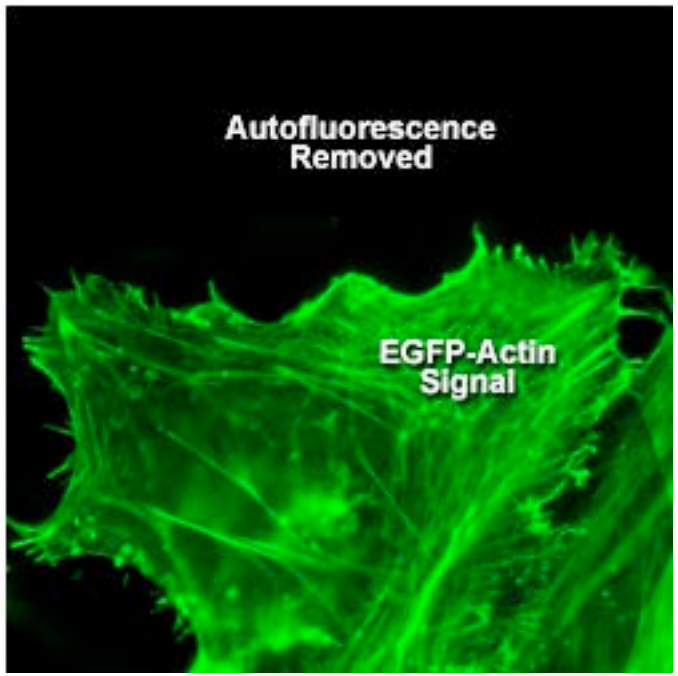
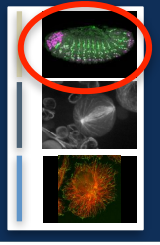




At each pixel:

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



PMT – Photon Multiplier Tube

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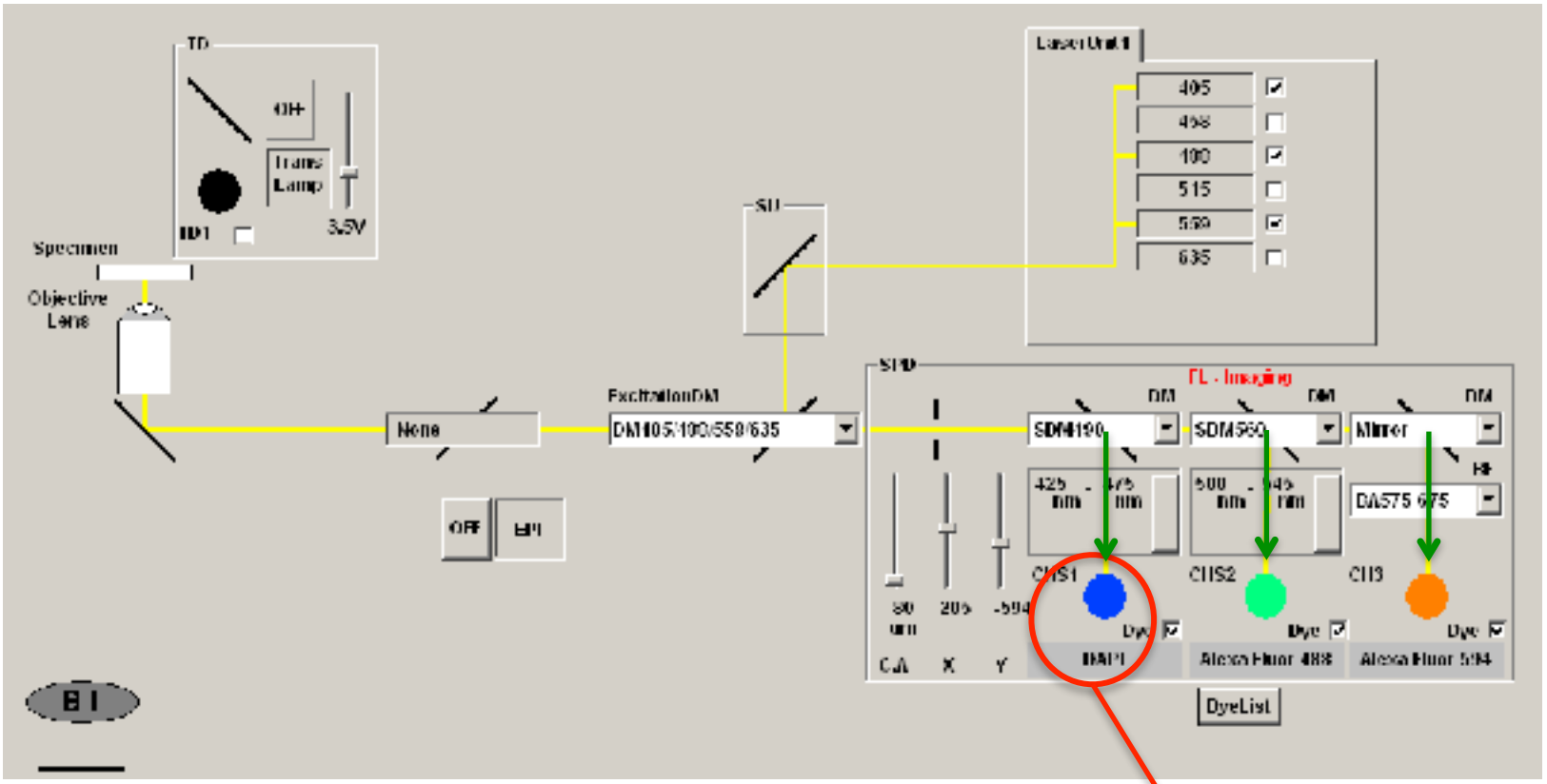
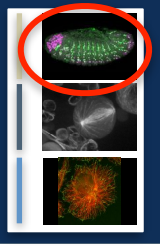
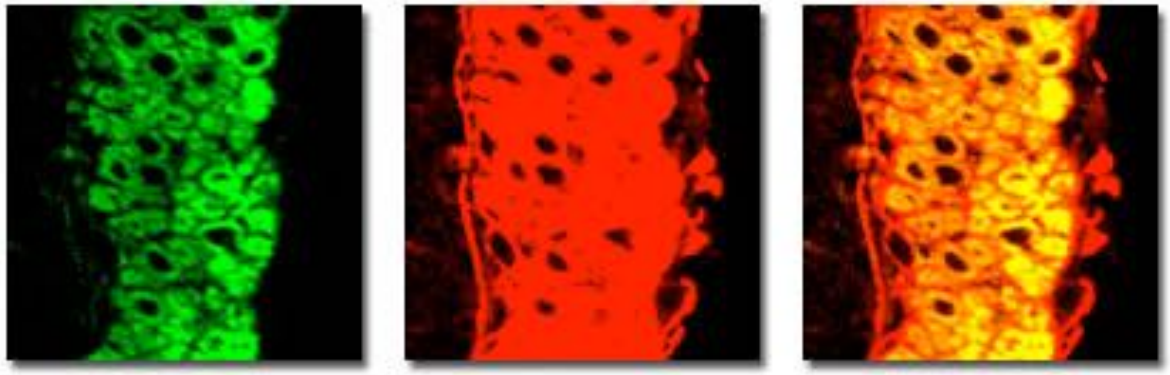


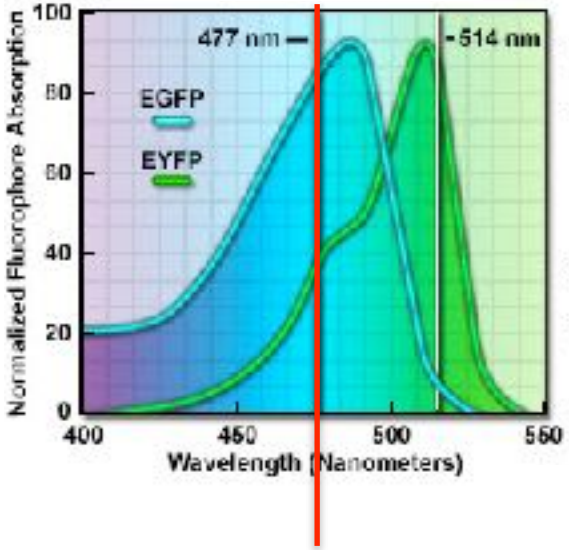
PMT detectors



‘bleed-through’

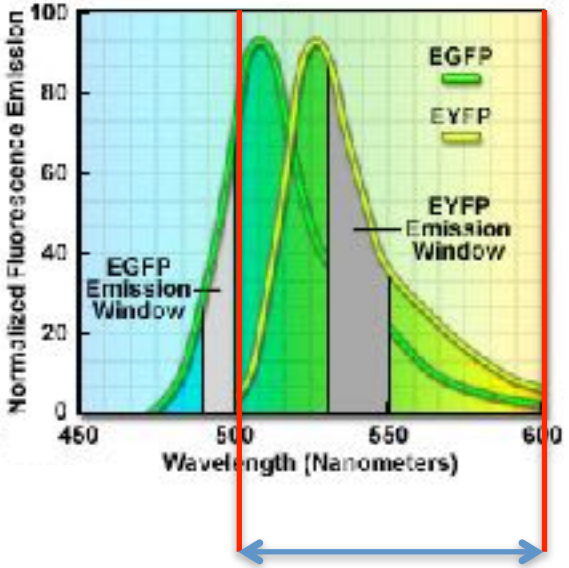


Absorption spectral profiles

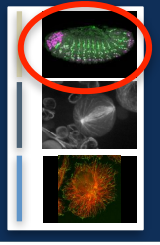


Excite at 477nm

Emission spectral profiles



overlapping emission



minimising‘bleed-through’ Variable Slits

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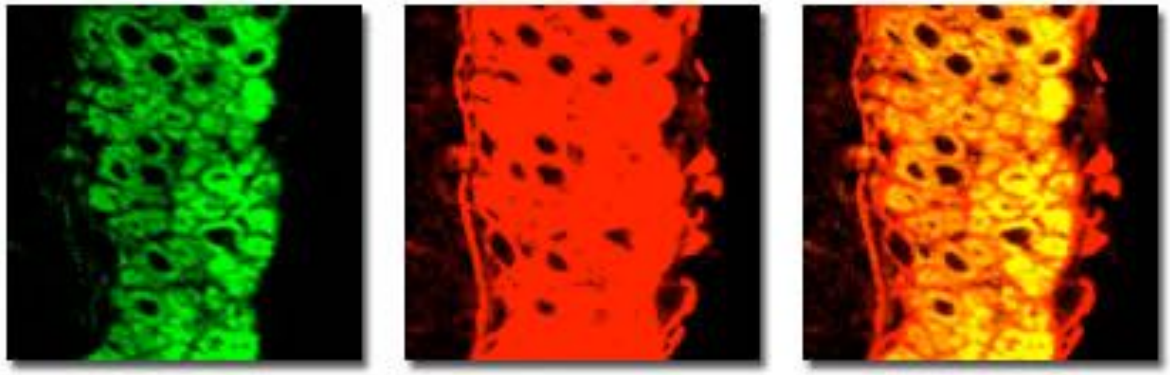
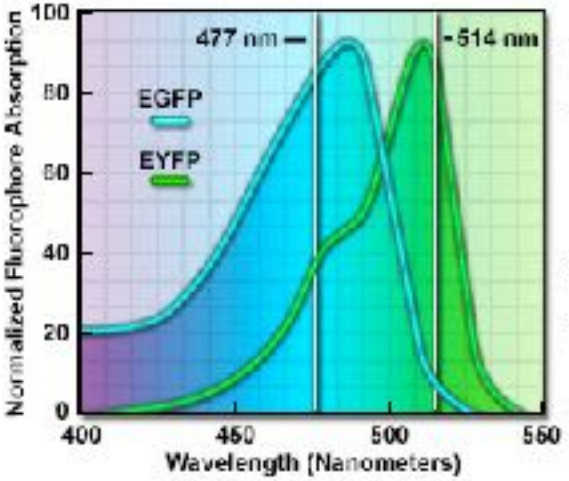
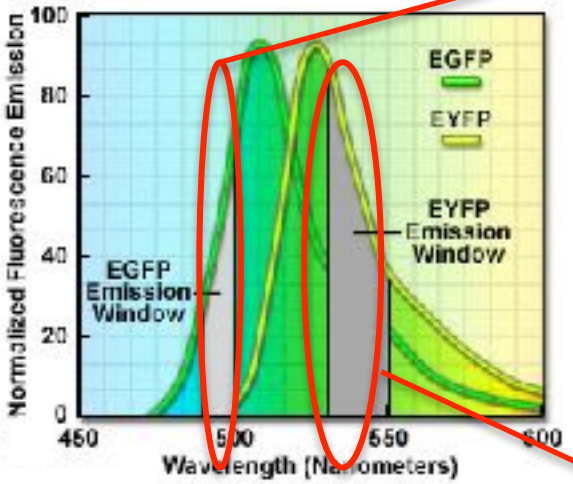
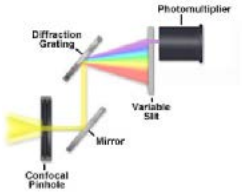


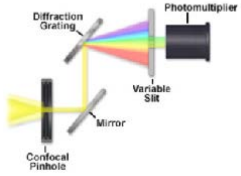
Absorption spectral profiles

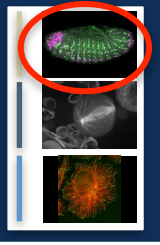


Emission spectral profiles

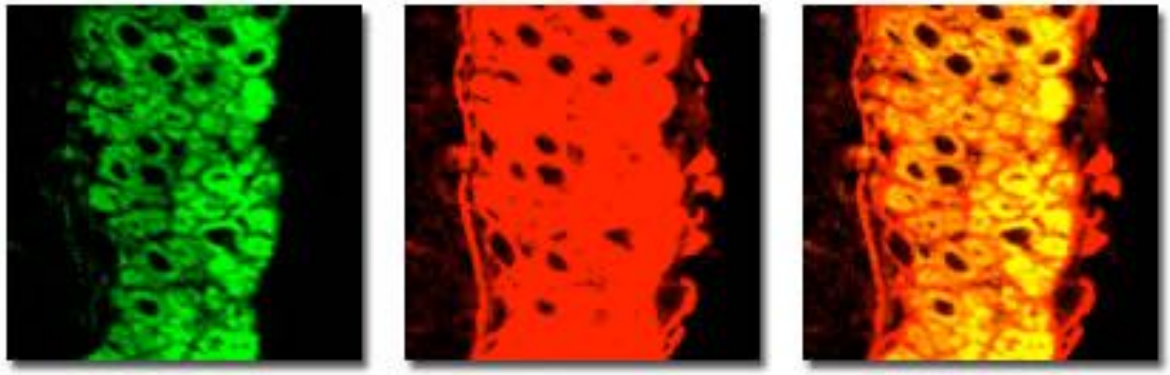




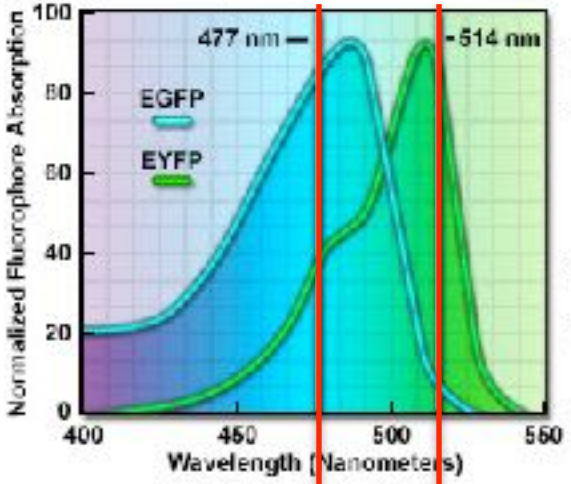




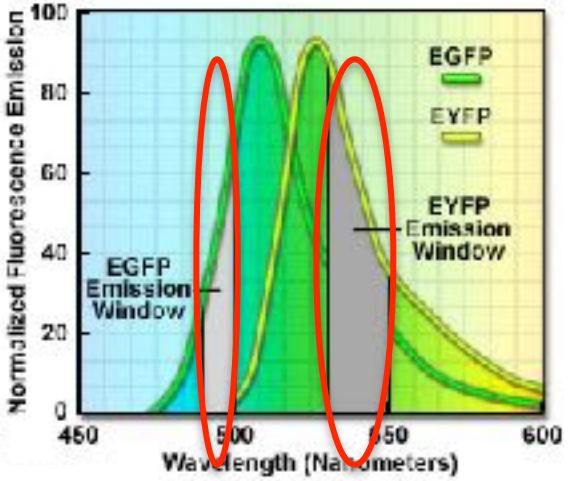
minimising‘bleed-through’ Sequential Scanning



Absorption spectral profiles



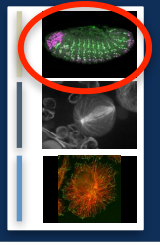
Emission spectral profiles



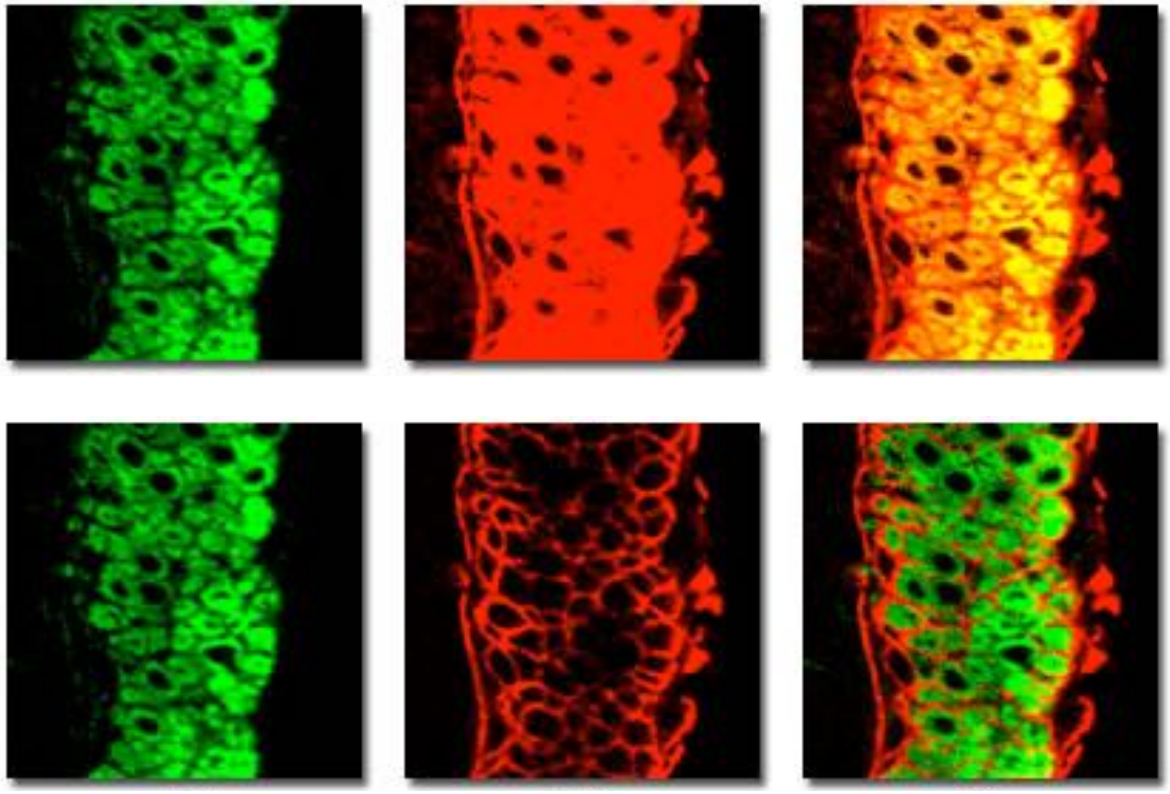
Excite at 477nm

Excite at 514nm

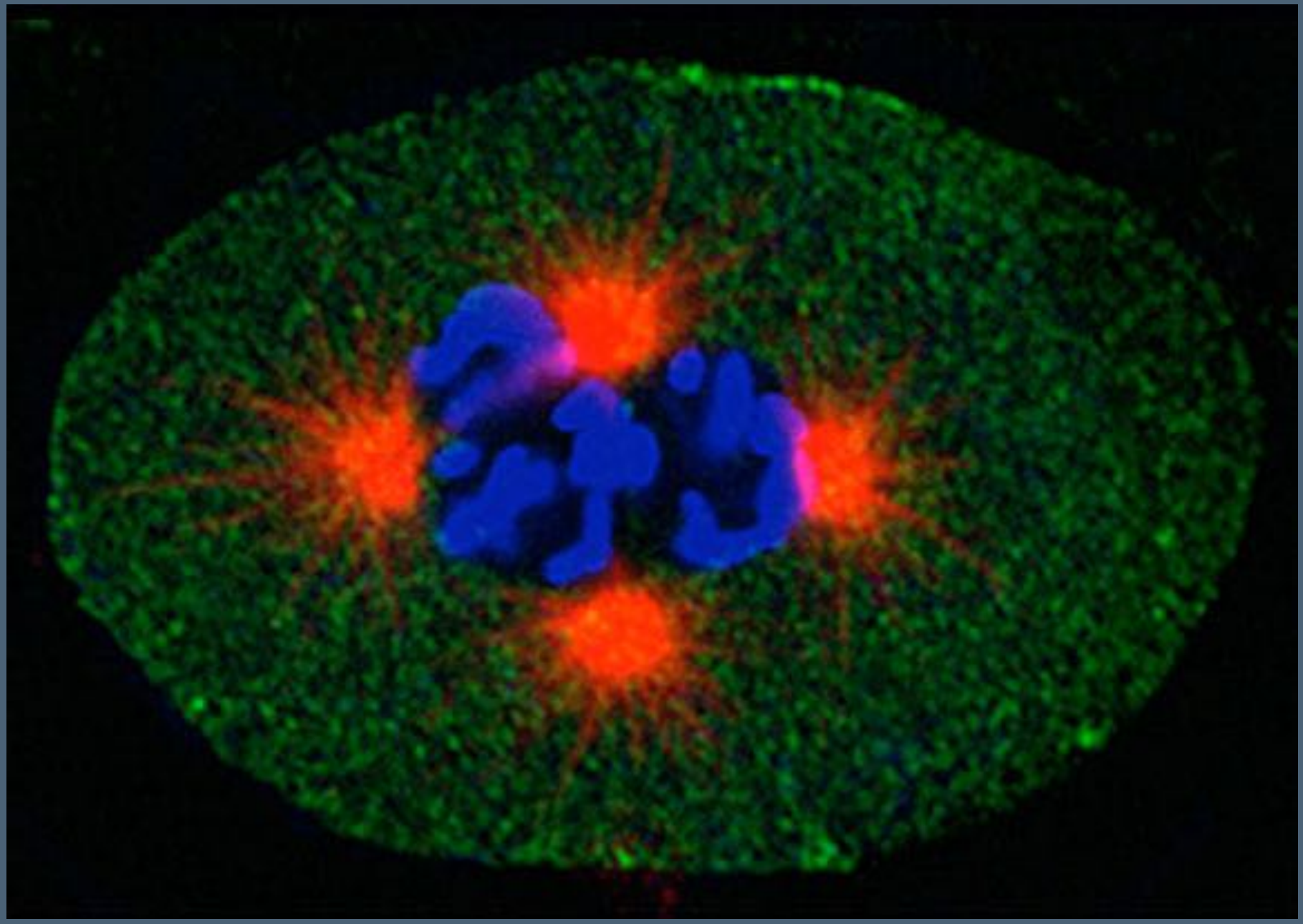
Temporal separation

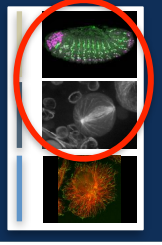


minimising ‘bleed-through’



Adjust detector slit widths
Use sequential scanning

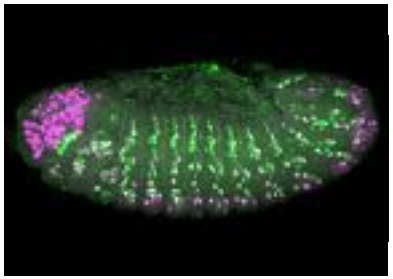




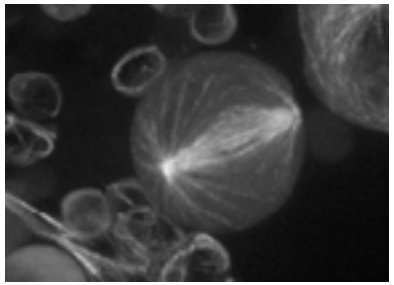
Confocal Microscopes

Confocal

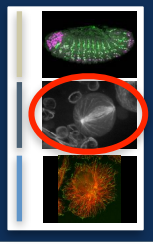
Laser Scanning



Spinning disc



Both are confocals



Spinning Disc Confocal

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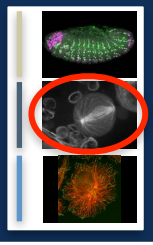
Great for live cell imaging

Can collect many images per second



Yokogawa CSU-X1 Spinning Disc

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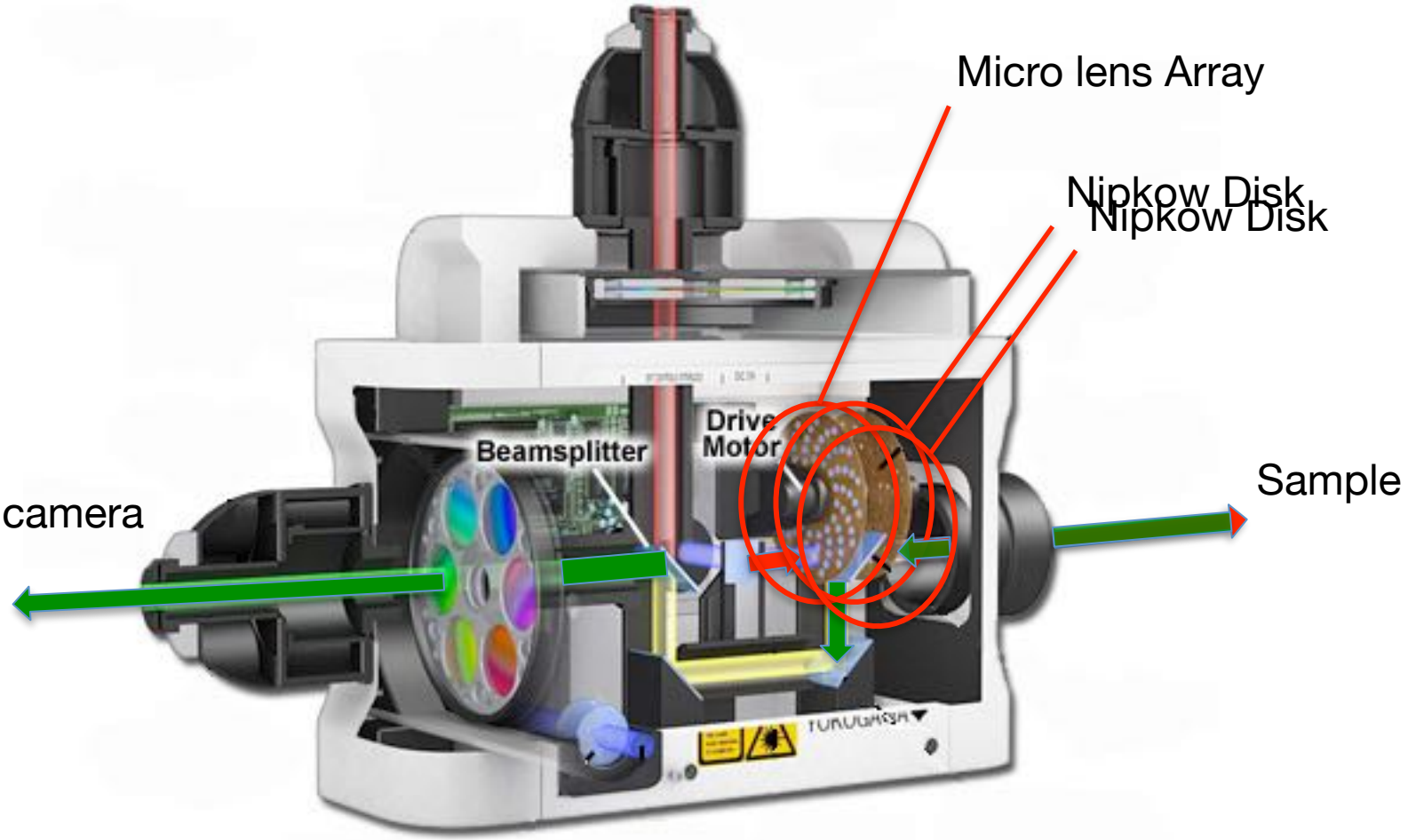

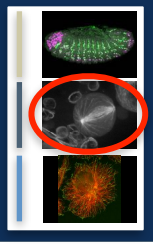

Yokogawa CSU-X1 Spinning Disc

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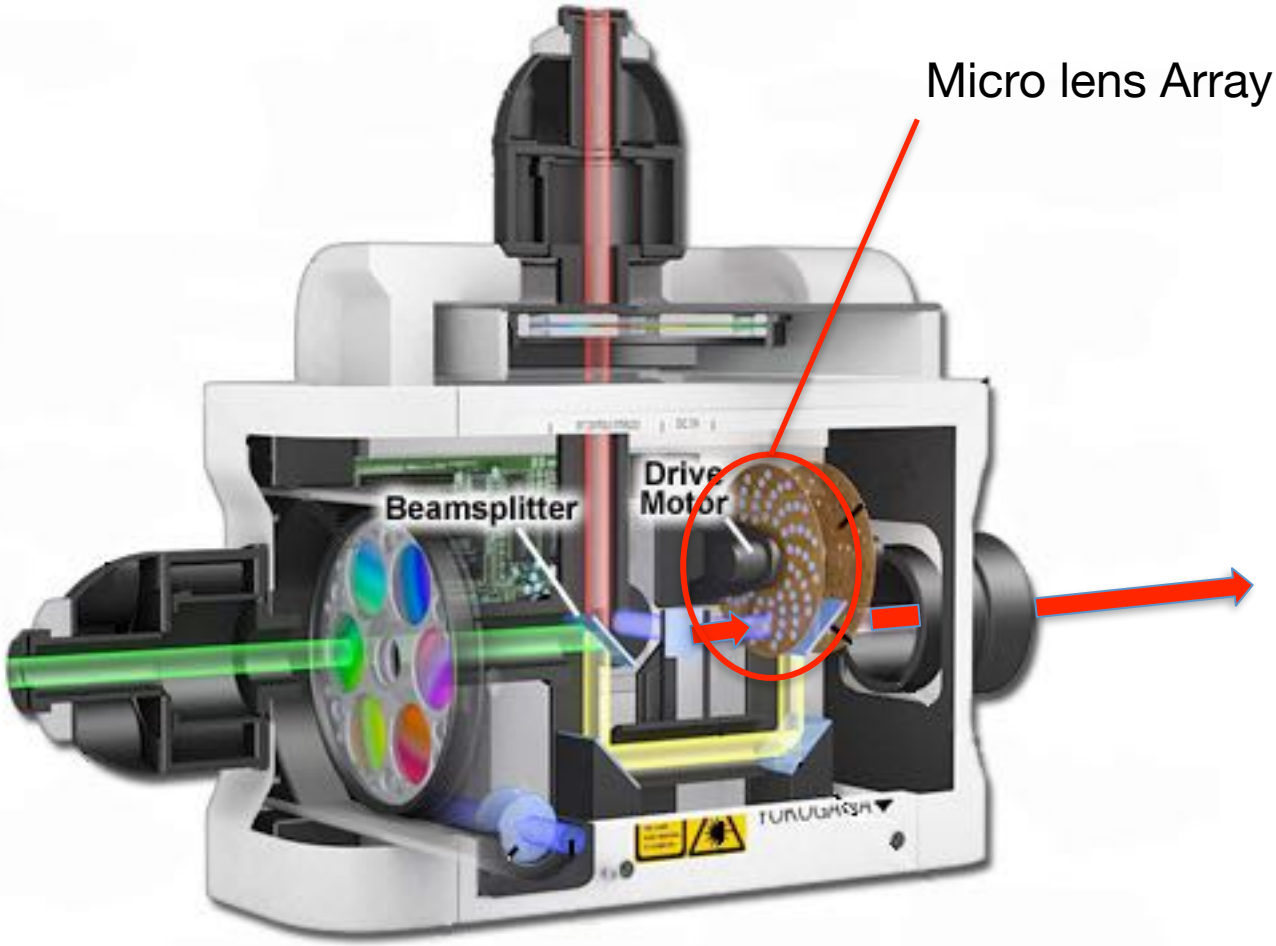

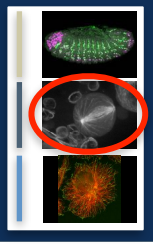

Yokogawa Spinning Disc Confocal Microlens

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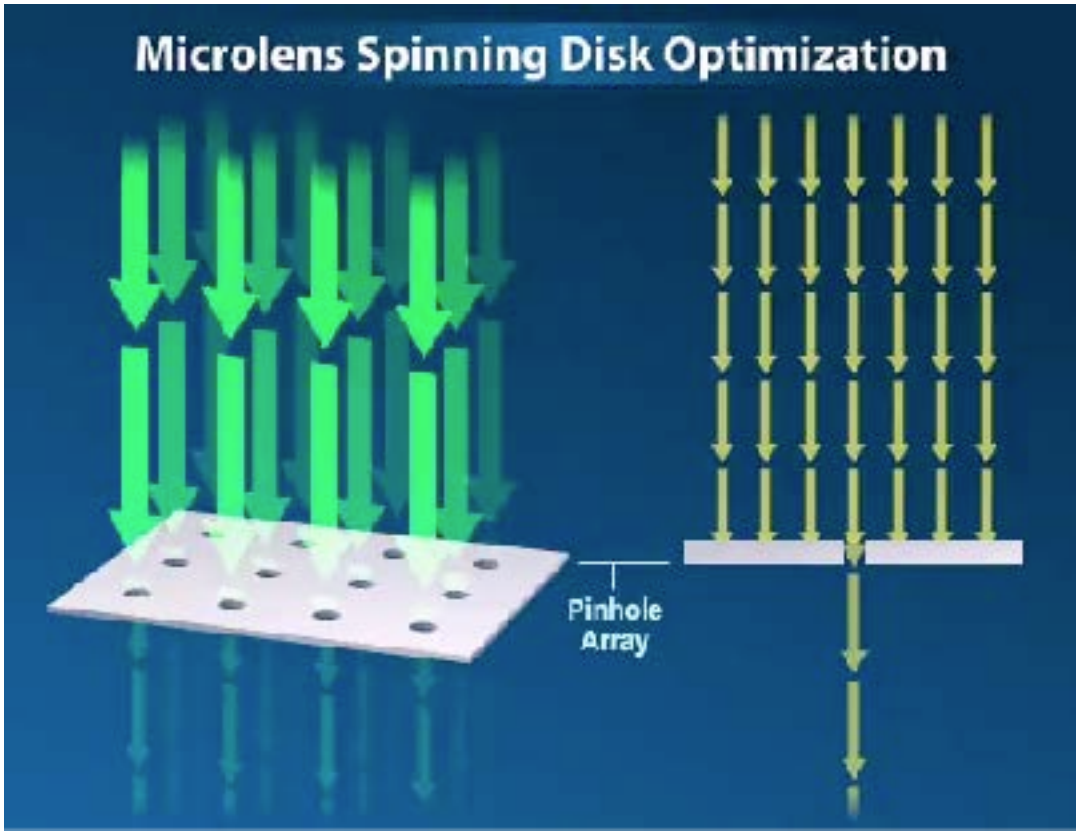
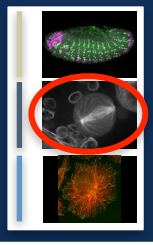


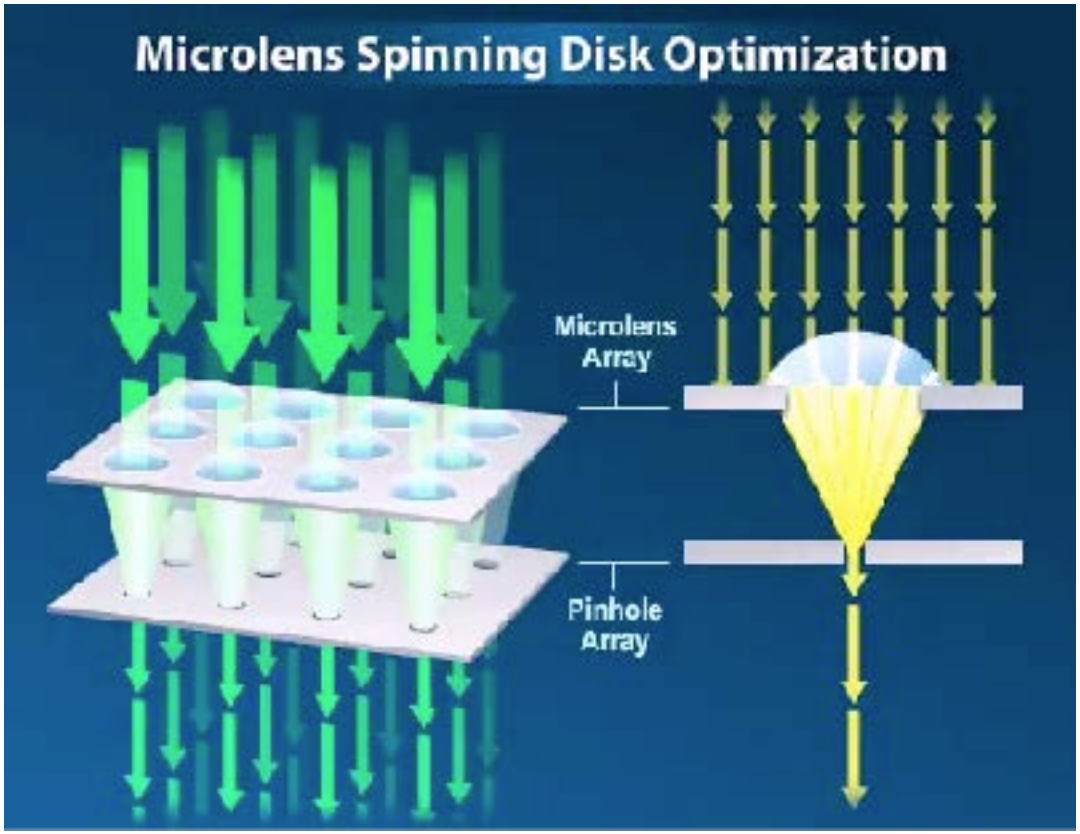
Just a pinhole array – optimised for ‘confocality’ and ‘crosstalk’

Too much light is blocked from reaching the specimen

Only 4% light passes through disc

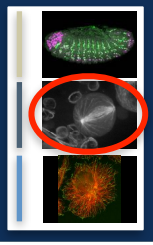


Yokogawa Spinning Disc Confocal Microlens



Micro-lens array increase the light reaching the specimen

Typically 56% light passes through disc



Yokogawa CSU-X1 Spinning Disc

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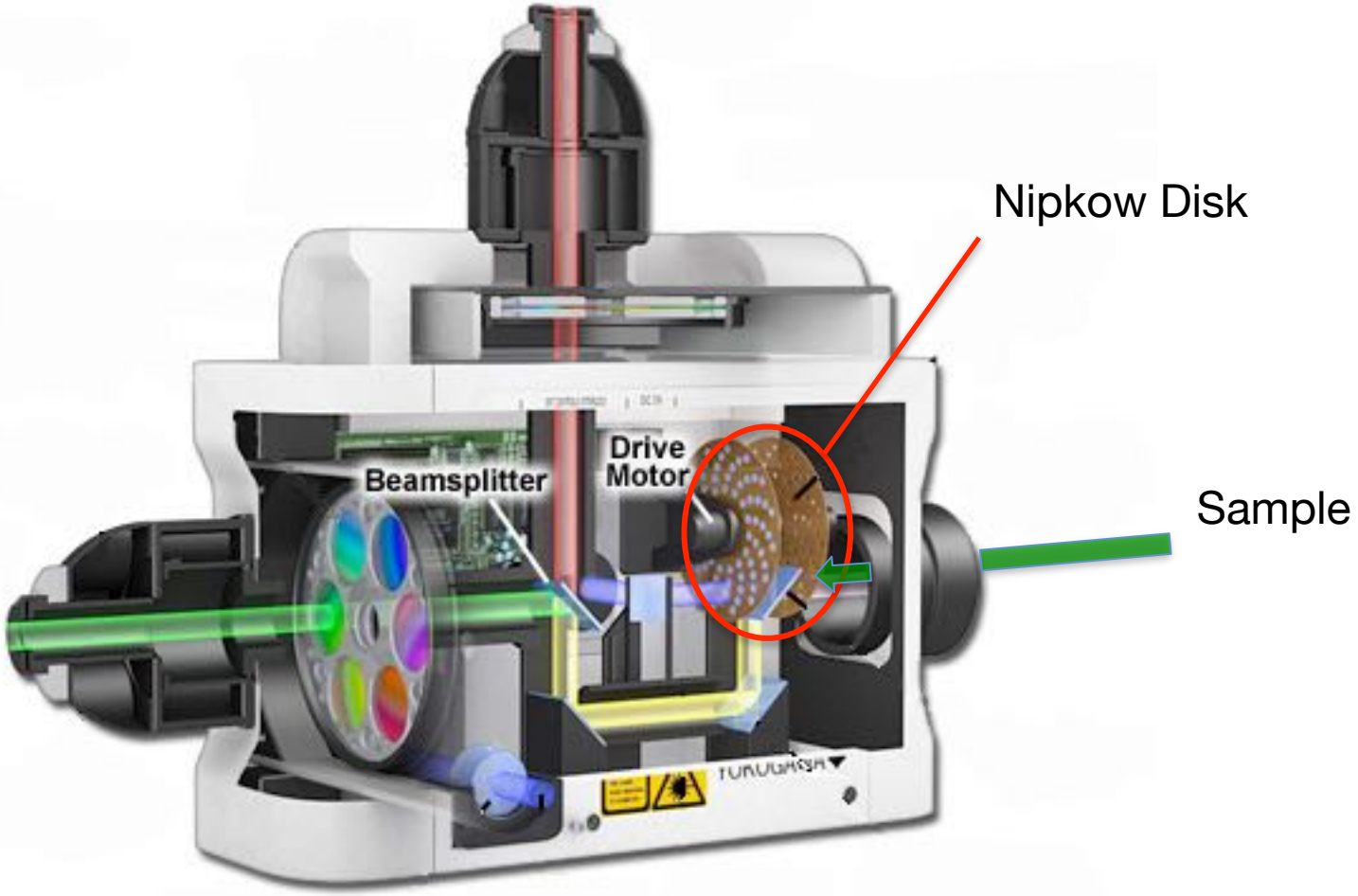
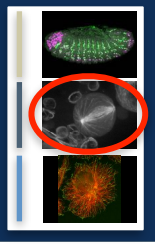
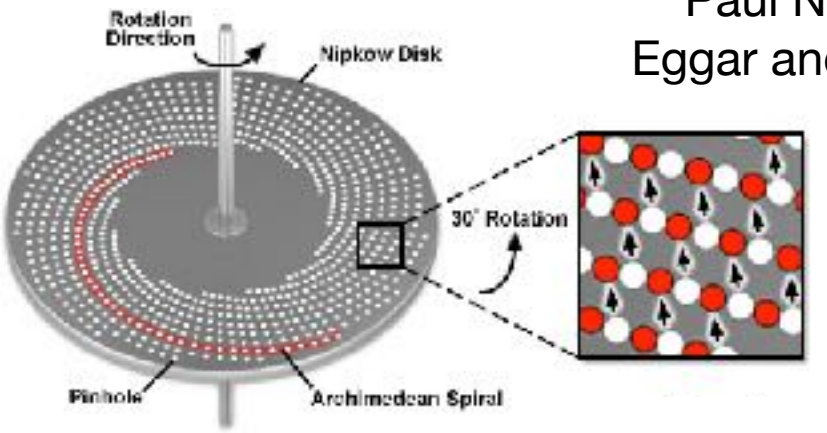
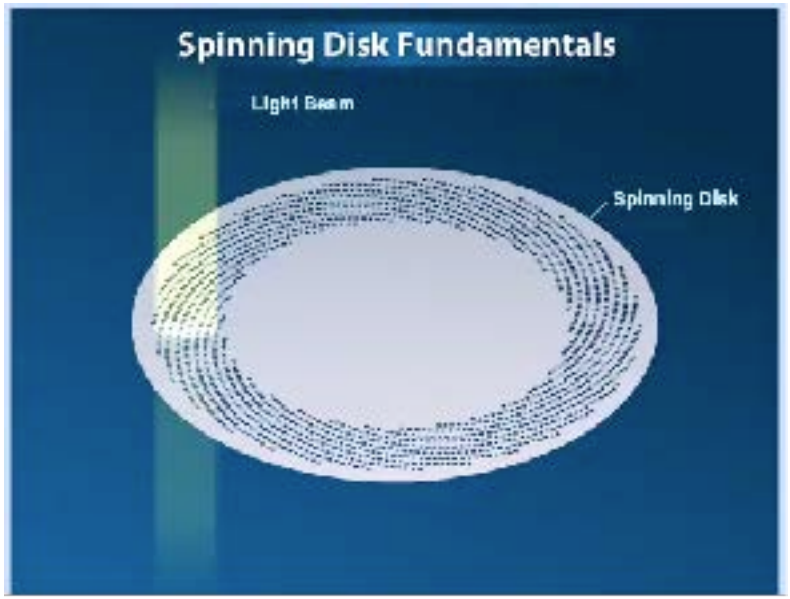




The Nipkow Disk

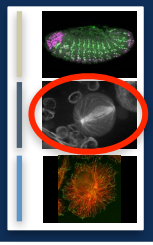


Paul Nipkow, 1884
Eggar and Petran, 1967



Approx. 1000 pinholes

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



The Nipkow Disk

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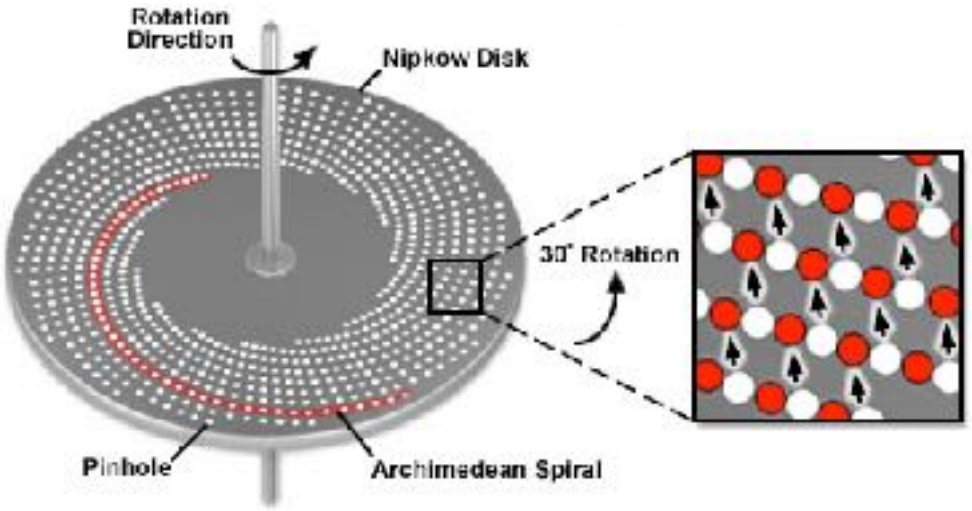
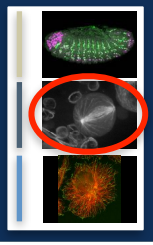


Larger pinholes - brighter image, but less “confocal”

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



The Nipkow Disk

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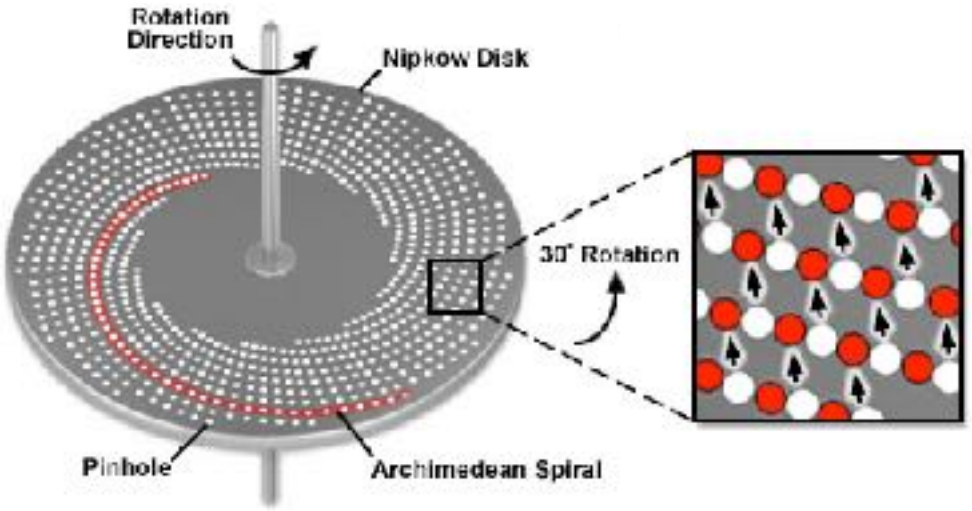
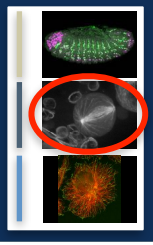


Constant Battle:
Smaller spacing - more light gets through, but “crosstalk”

Pinhole Spacing Typically = 2.5um apart



Yokogawa Spinning Disc Confocal

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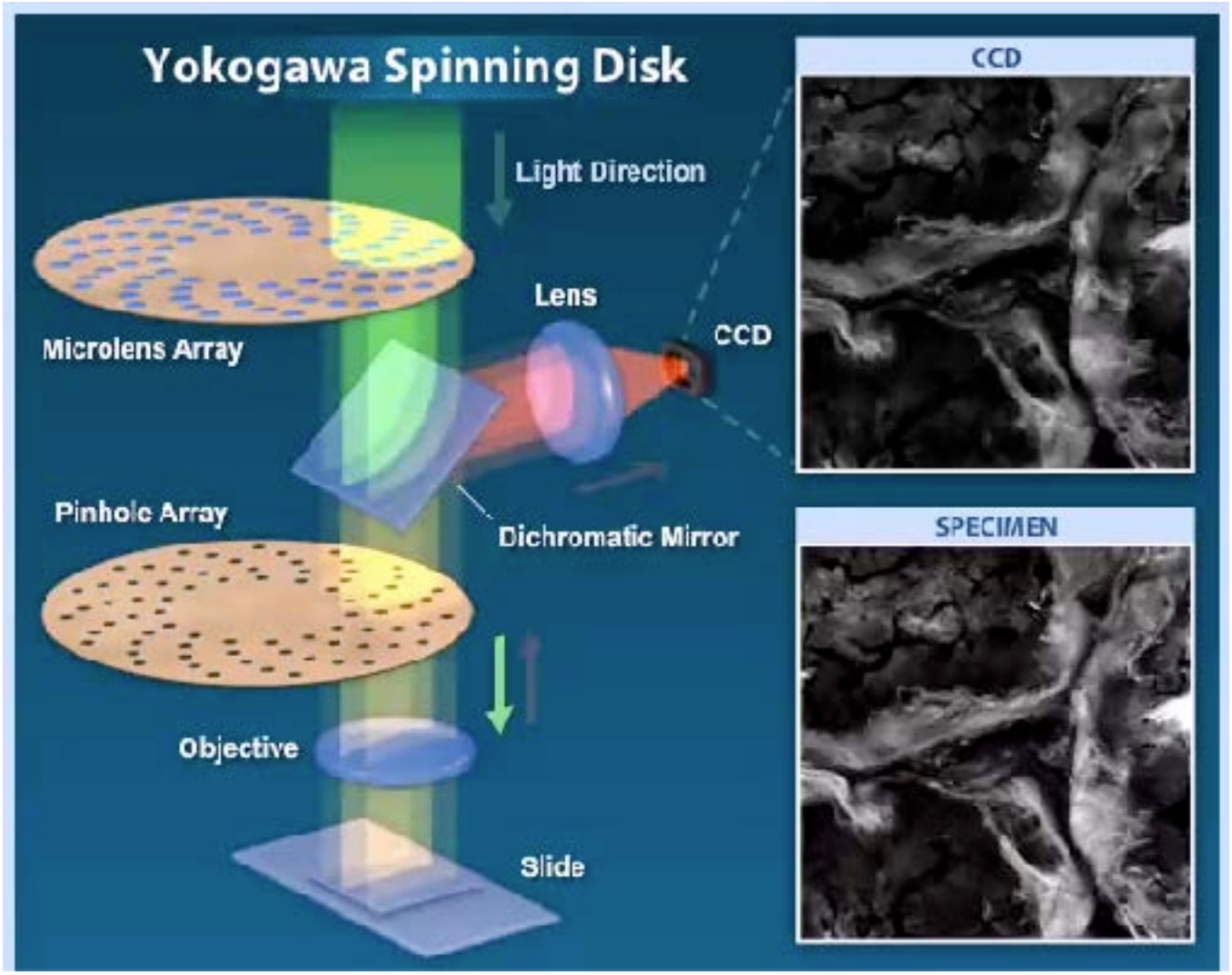
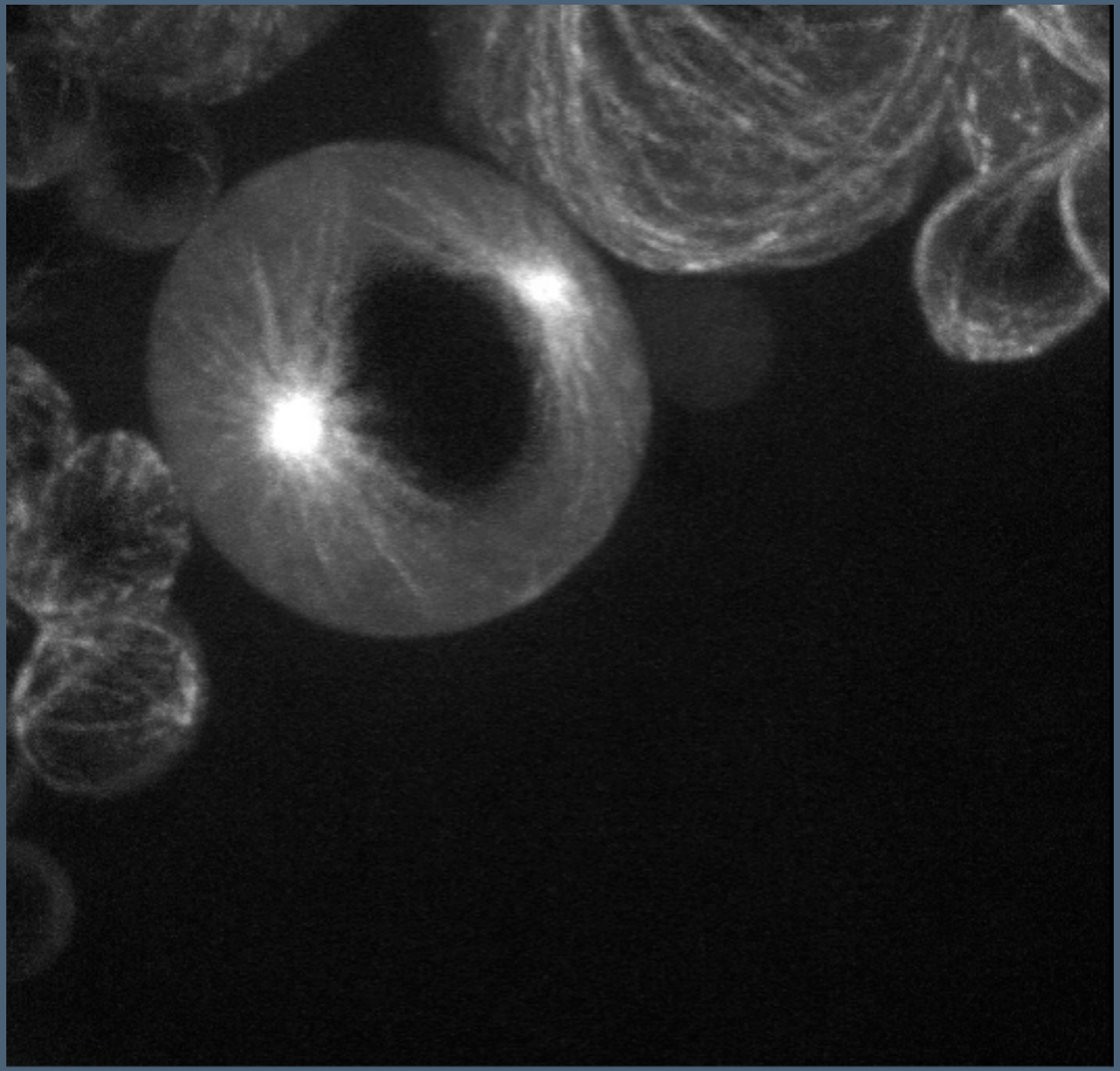
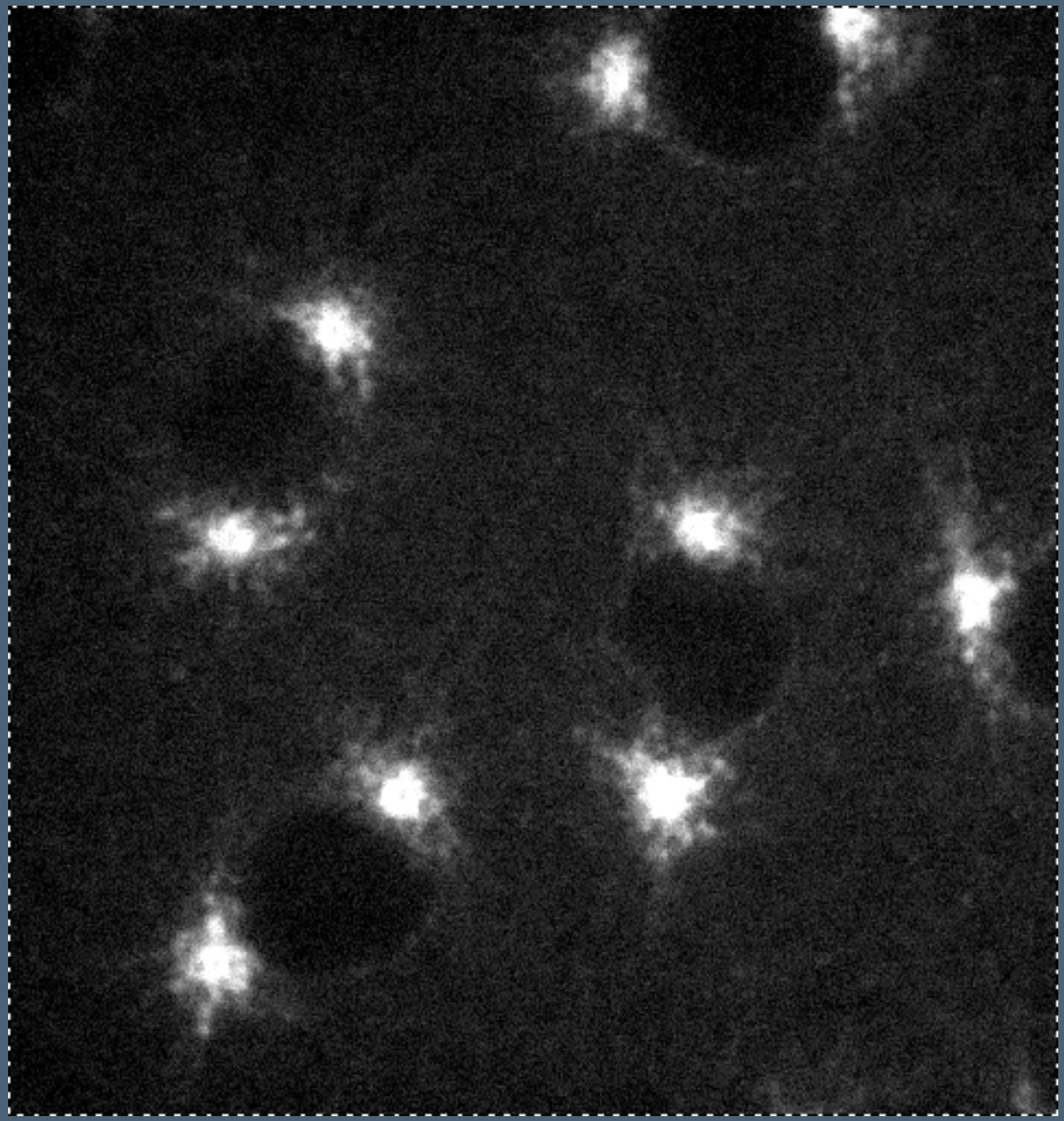
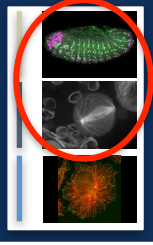




Cell division in brain stem cells (neuroblasts), Raff Lab



MT binding protein in *Drosophila* embryo, Raff Lab



Point Scanning Vs Spinning Disc

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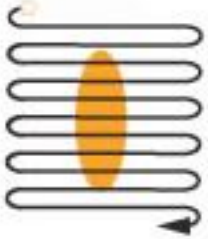
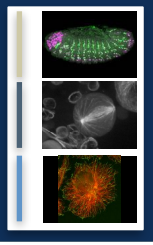
	Point Scanning	Spinning Disc
		
Speed	Slow (secs)	Fast (msecs)
Sensitivity	OK	OK
Flexibility	Good	Poor
Bleaching	Poor	Good
Pretty Pictures	Unbeatable!	Pretty damn good!
Pretty Movies	Good – if process slow	Unbeatable!



3 Flavours of Microscope

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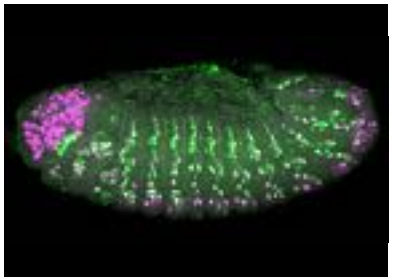
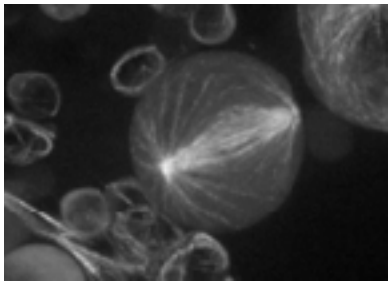
Problem:
Out of Focus
Light

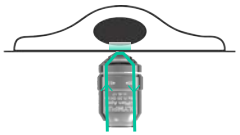
Confocal

Laser
Scanning

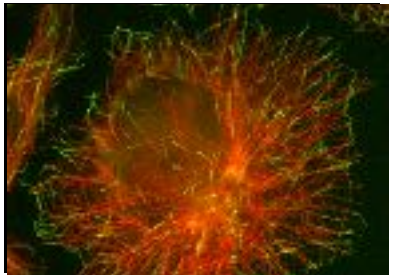


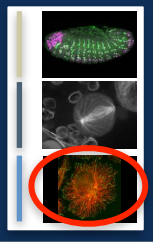
Spinning
disc





TIRF





TIRF
Total Internal Reflection Fluorescnece

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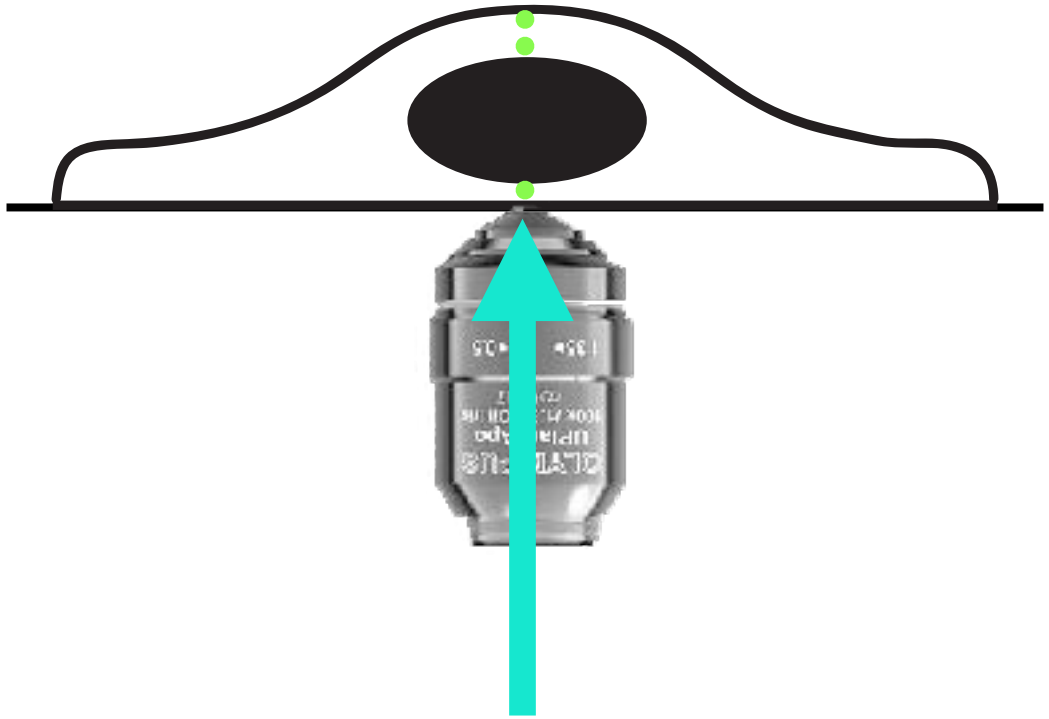

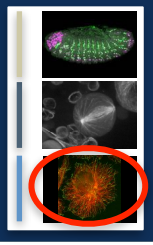

TIRF
Total Internal Reflection Fluorescnece

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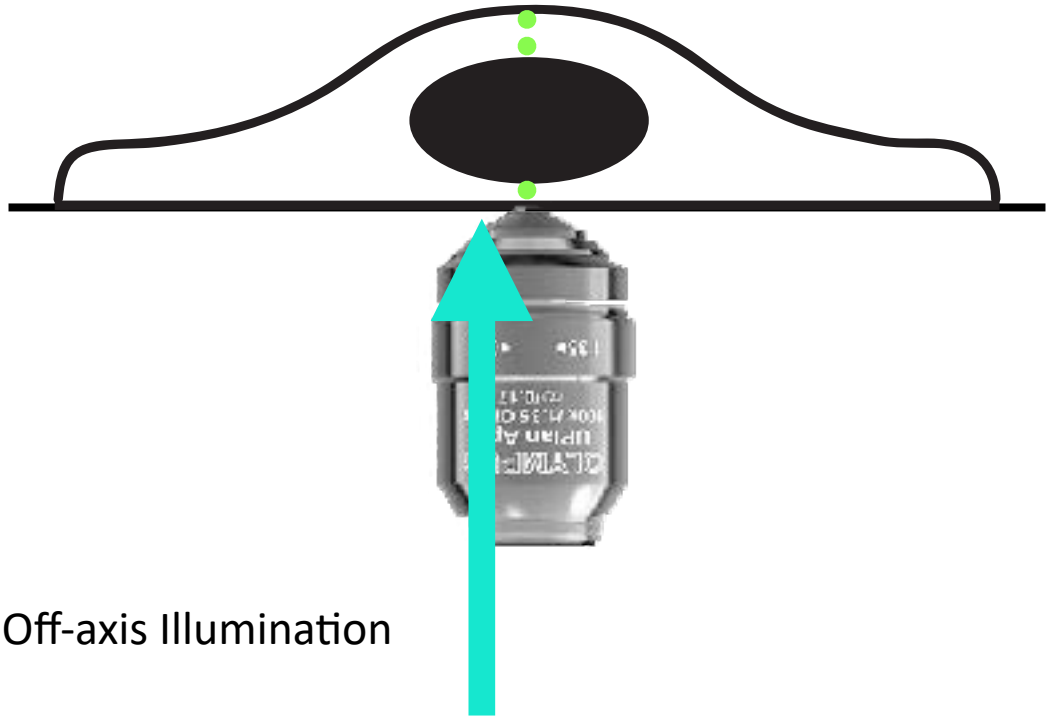

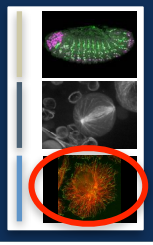

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Total Internal Reflection Fluorescnece

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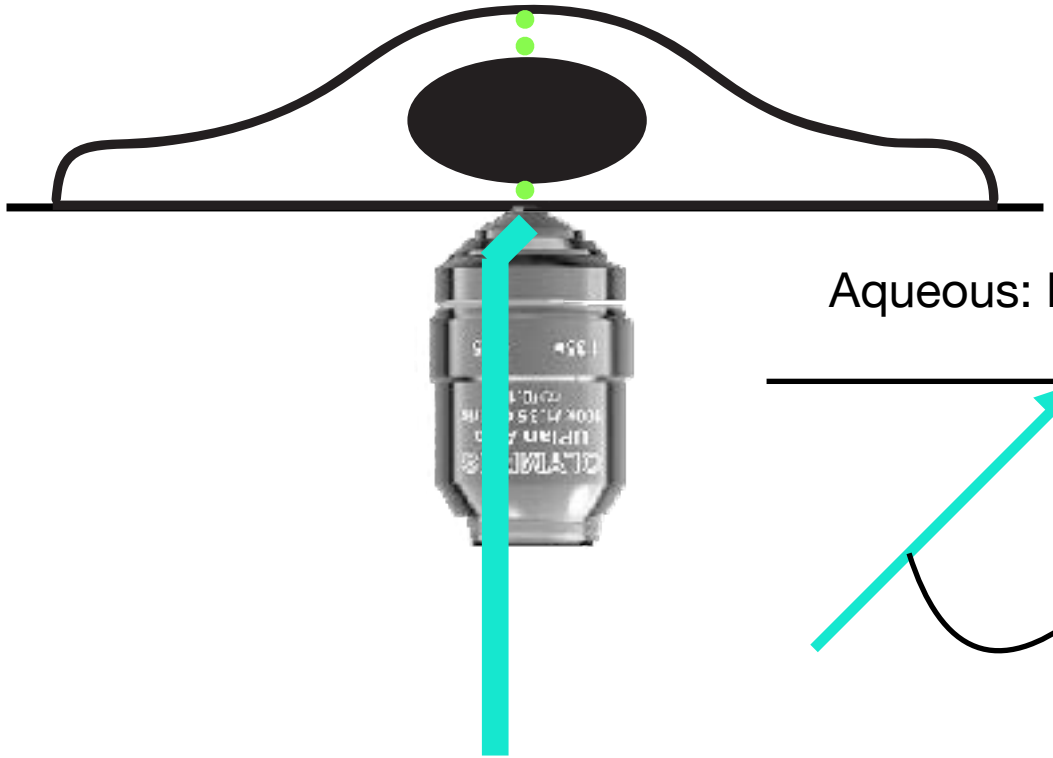

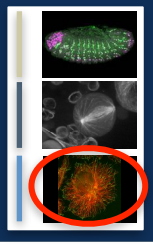

TIRF
Total Internal Reflection Fluorescnece

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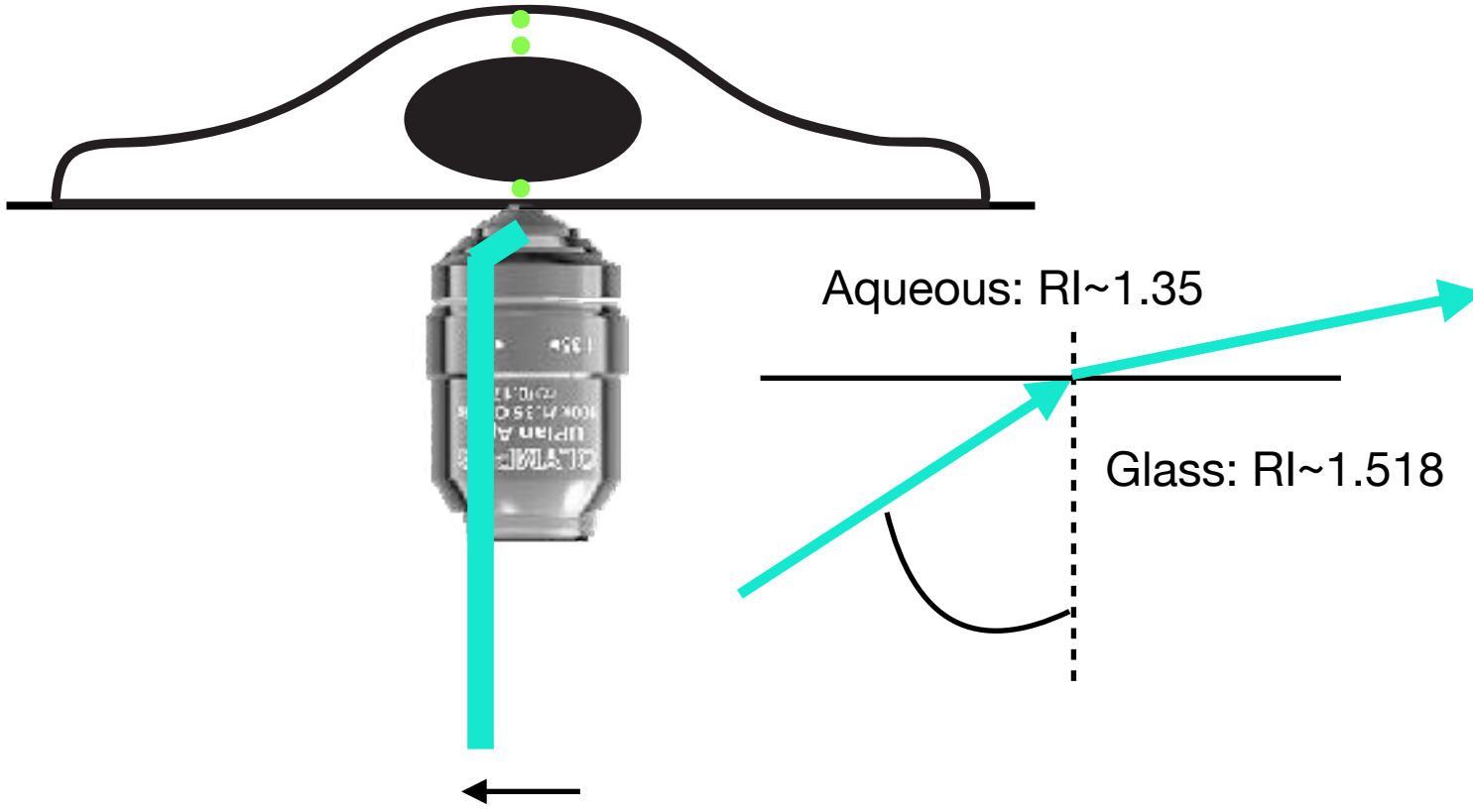
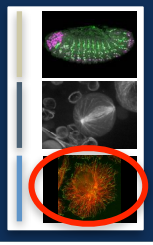


As illumination becomes more off axis, the angle the light strikes the interface becomes shallower



TIRF
Total Internal Reflection Fluorescnece

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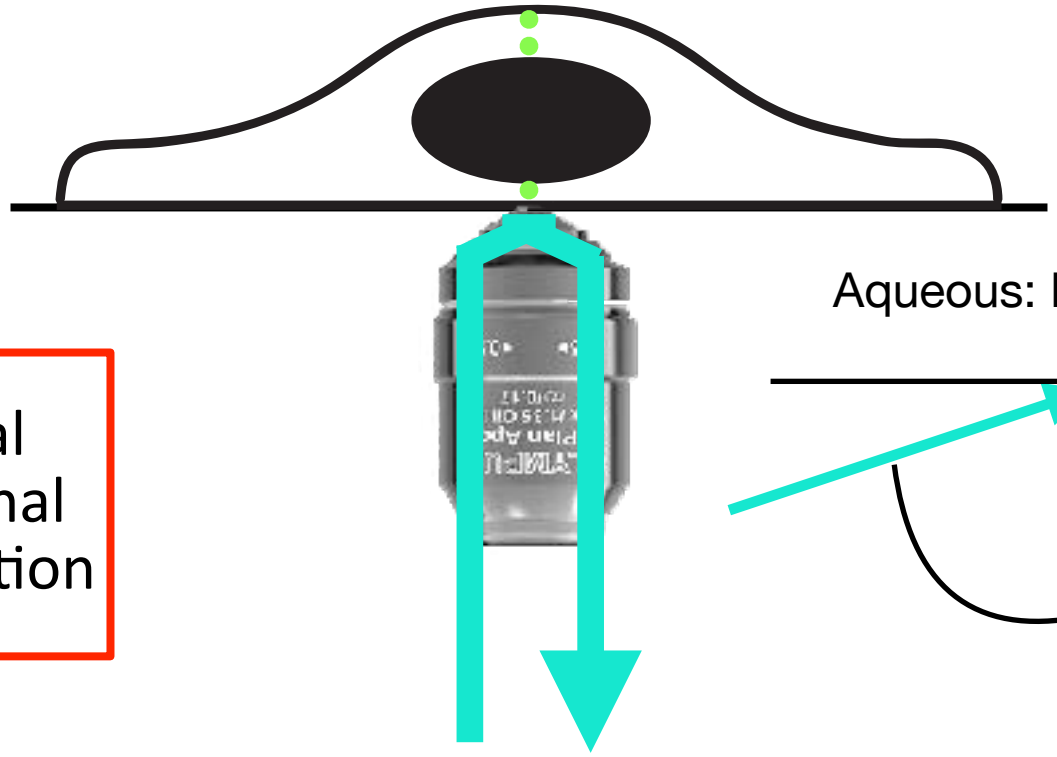
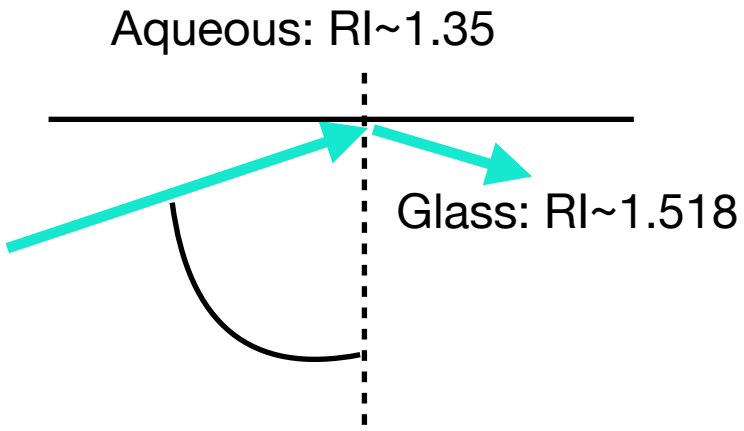
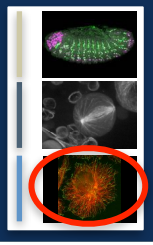
Total Internal Reflection





Critical angle



TIRF
Total Internal Reflection Fluorescence

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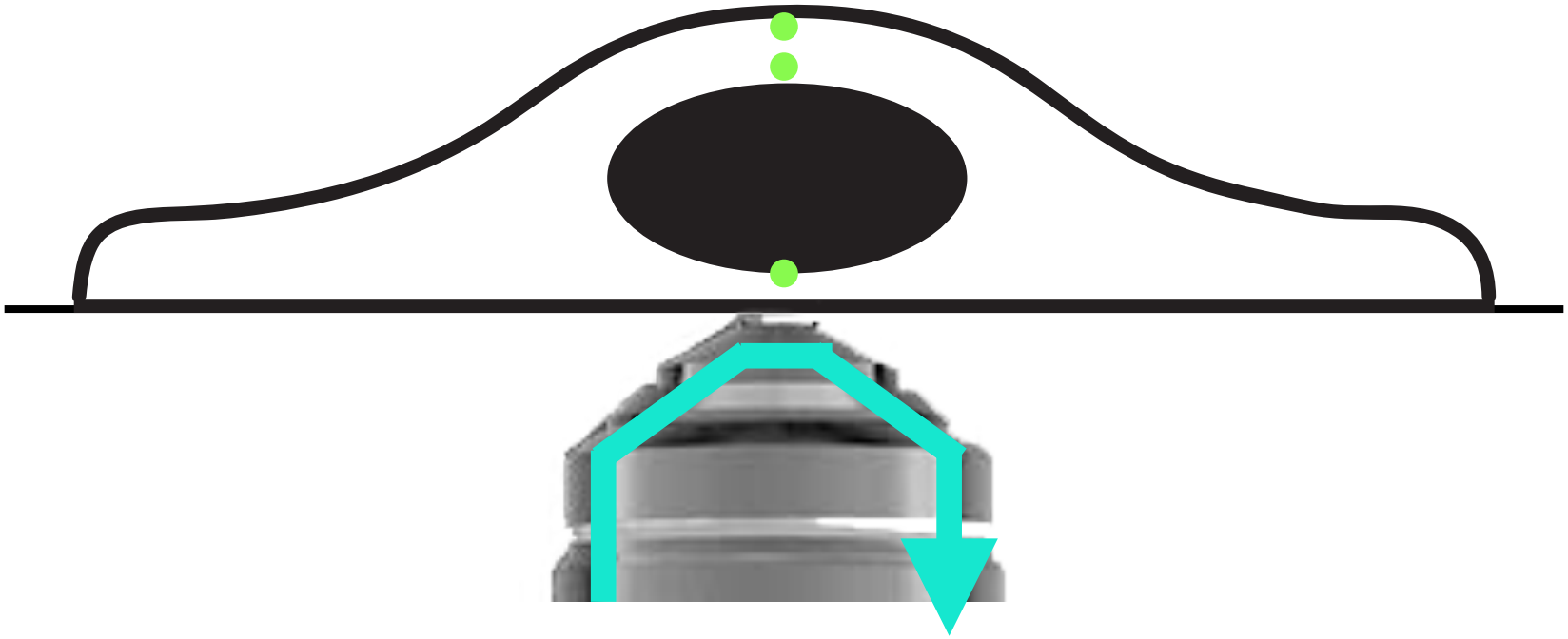

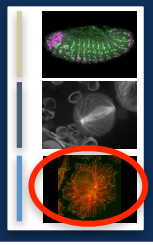

TIRF
Total Internal Reflection Fluorescnece

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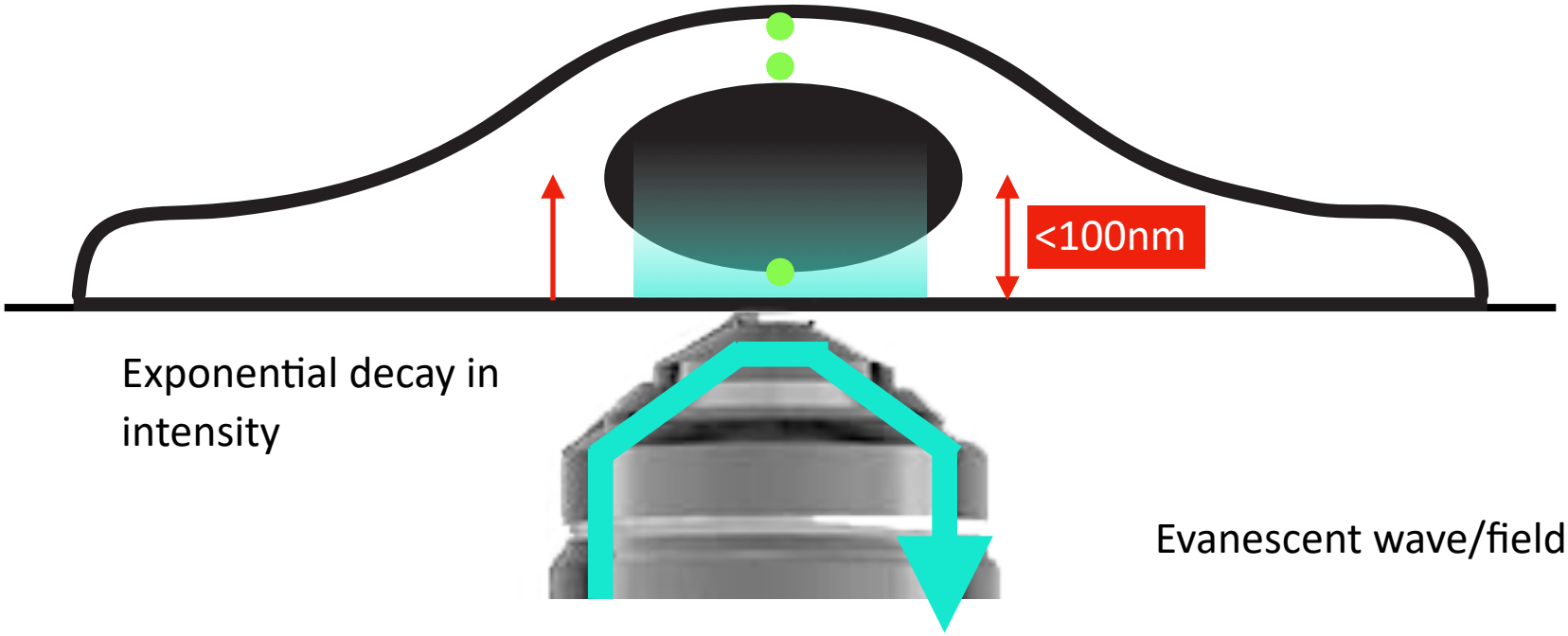

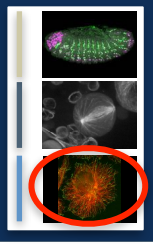

TIRF
Total Internal Reflection Fluorescnece

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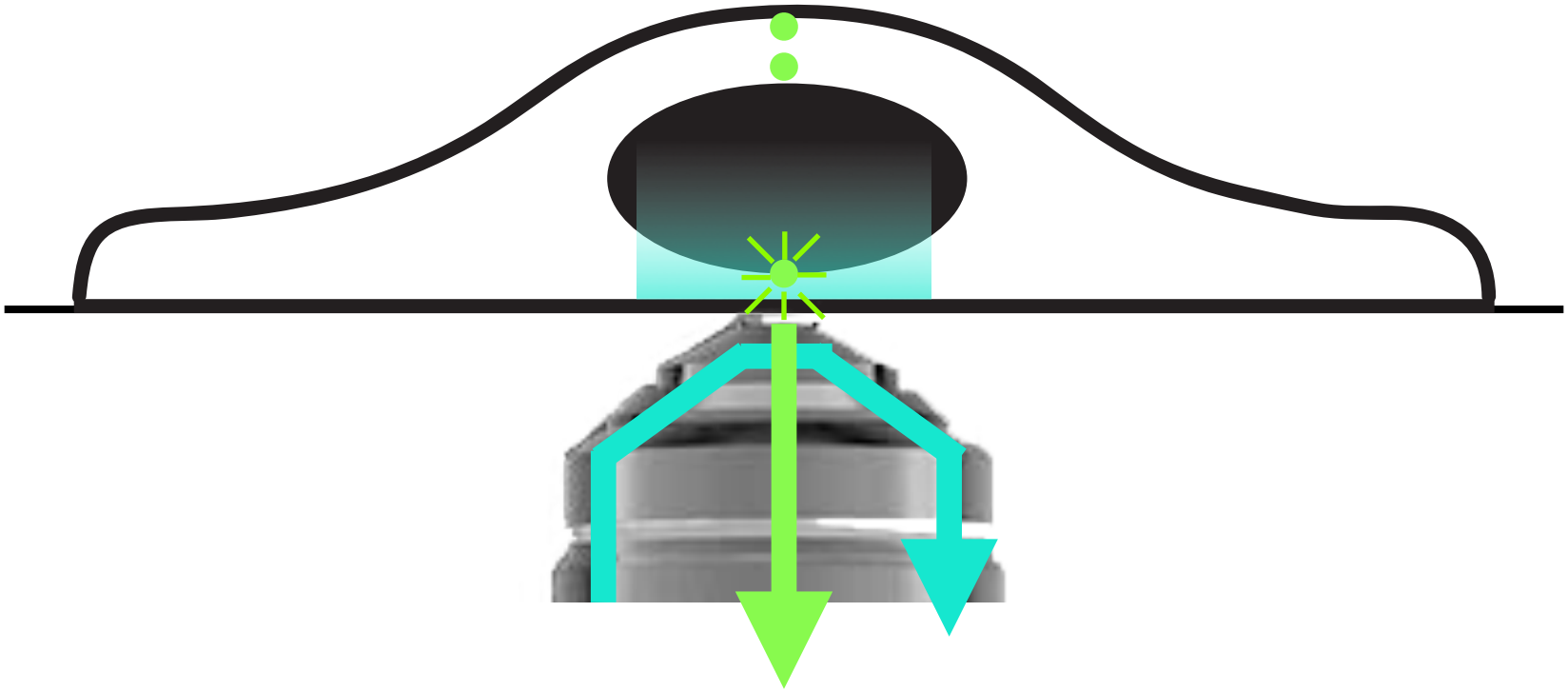
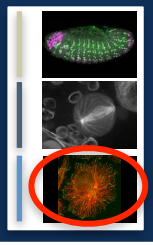


Only fluorophores very close to the coverslip are excited and fluoresce



TIRF
Total Internal Reflection Fluorescnece

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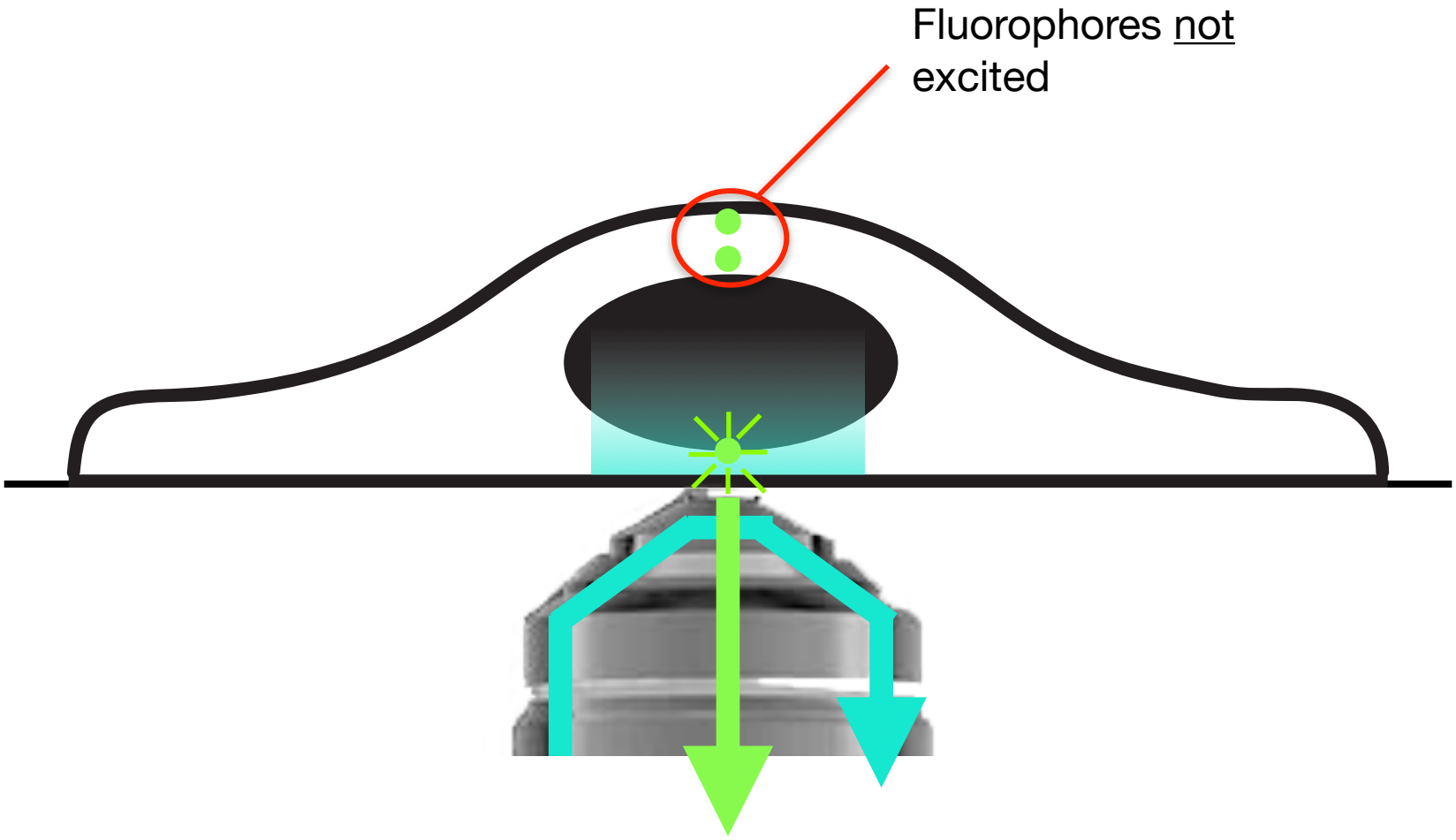
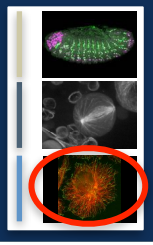


Very low background = very high signal to noise

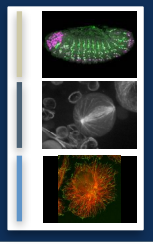


TIRF
Typical uses

Very thin samples - ie bacteria

In vitro samples - MTs on coverslip

In vivo events very close to coverslip where imaging requires v. Low background (ie PALM, single particle tracking - MT + ends)



Dealing with out of focus light

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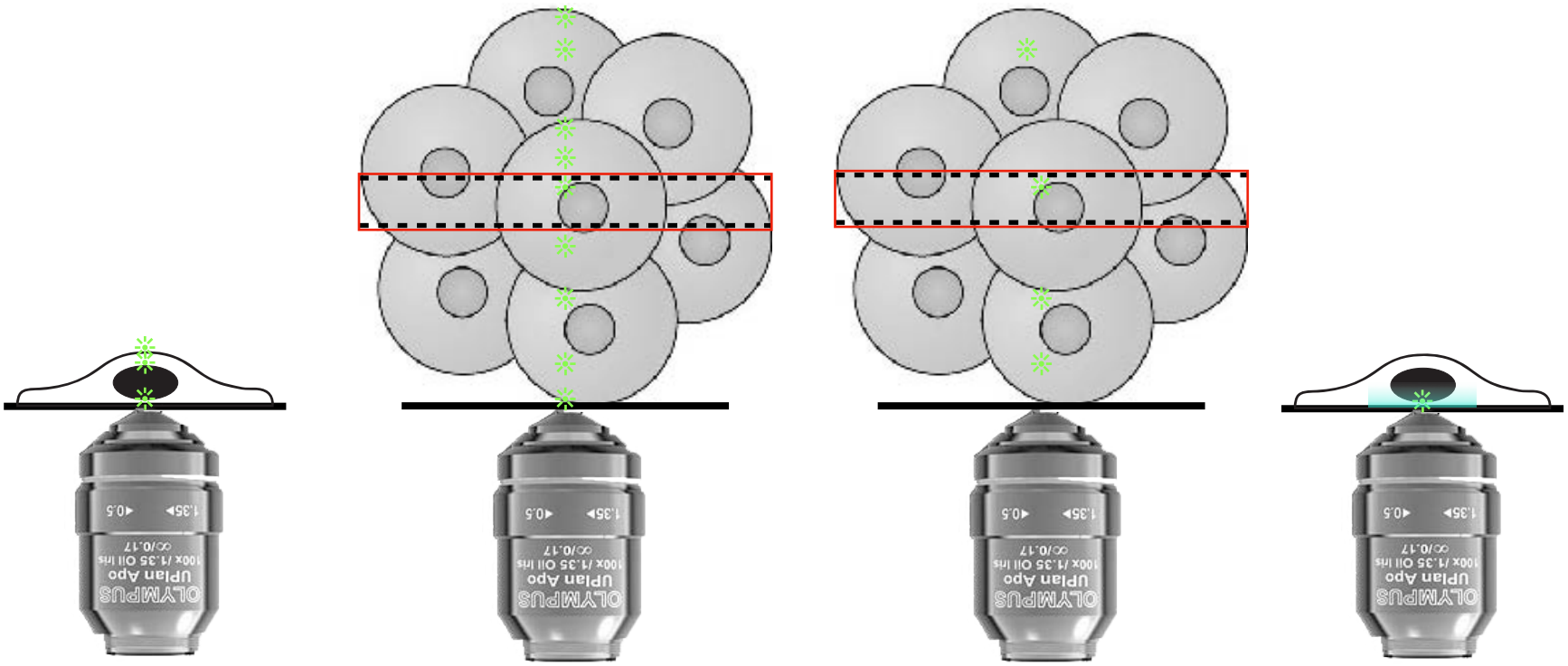


Widefield

OK for thin samples

Point scanning confocal

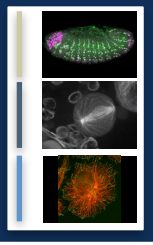
For brightly stained thick samples - that don't move quickly

Spinning disc confocal

For bright live thick samples - that do move quickly

TIRF

For imaging very close to coverslip



3 Flavours of Microscope

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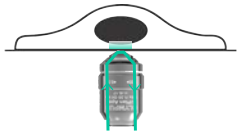
Problem:
Out of Focus
Light

Confocal

Laser
Scanning

Spinning disc



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