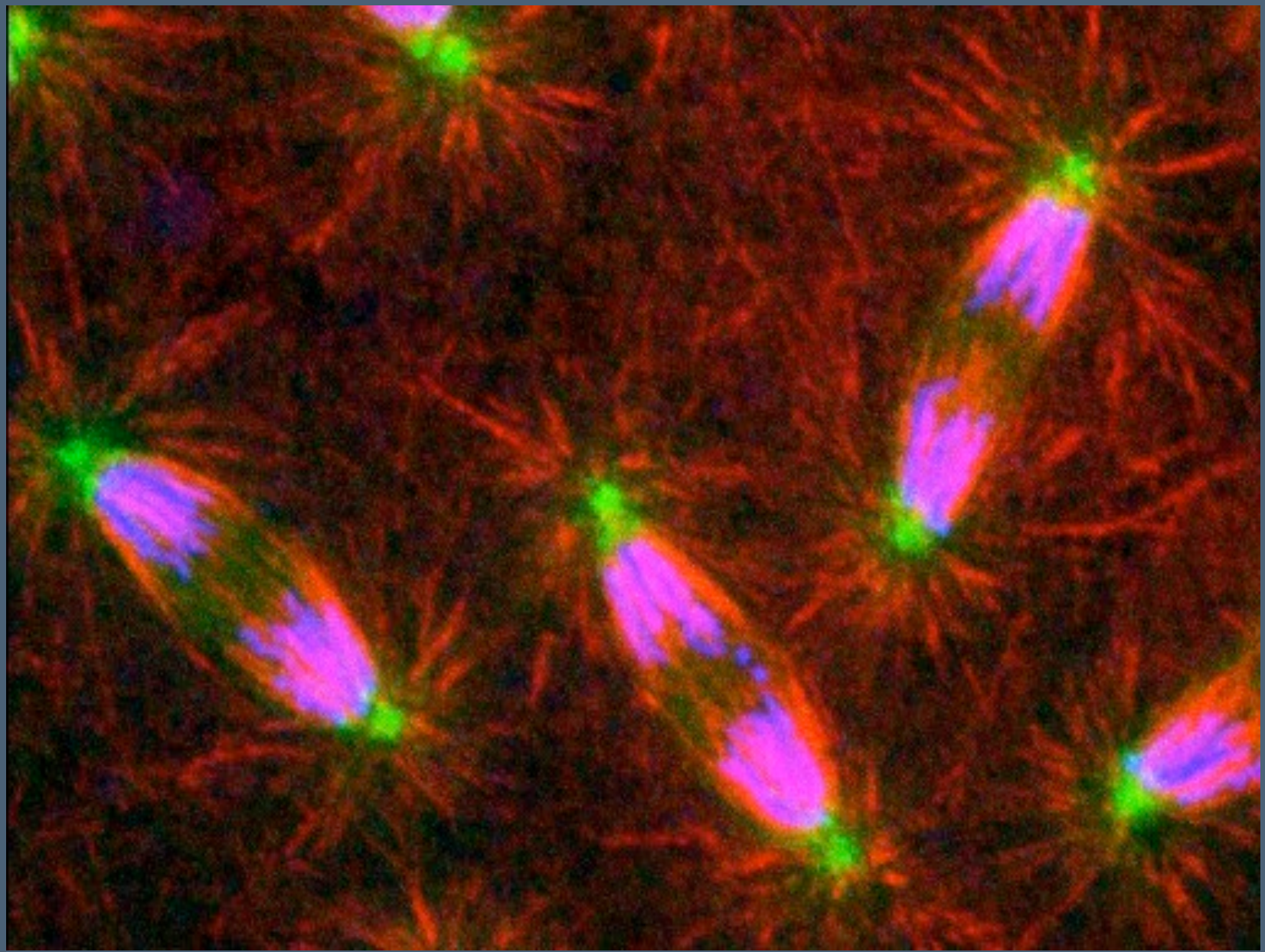
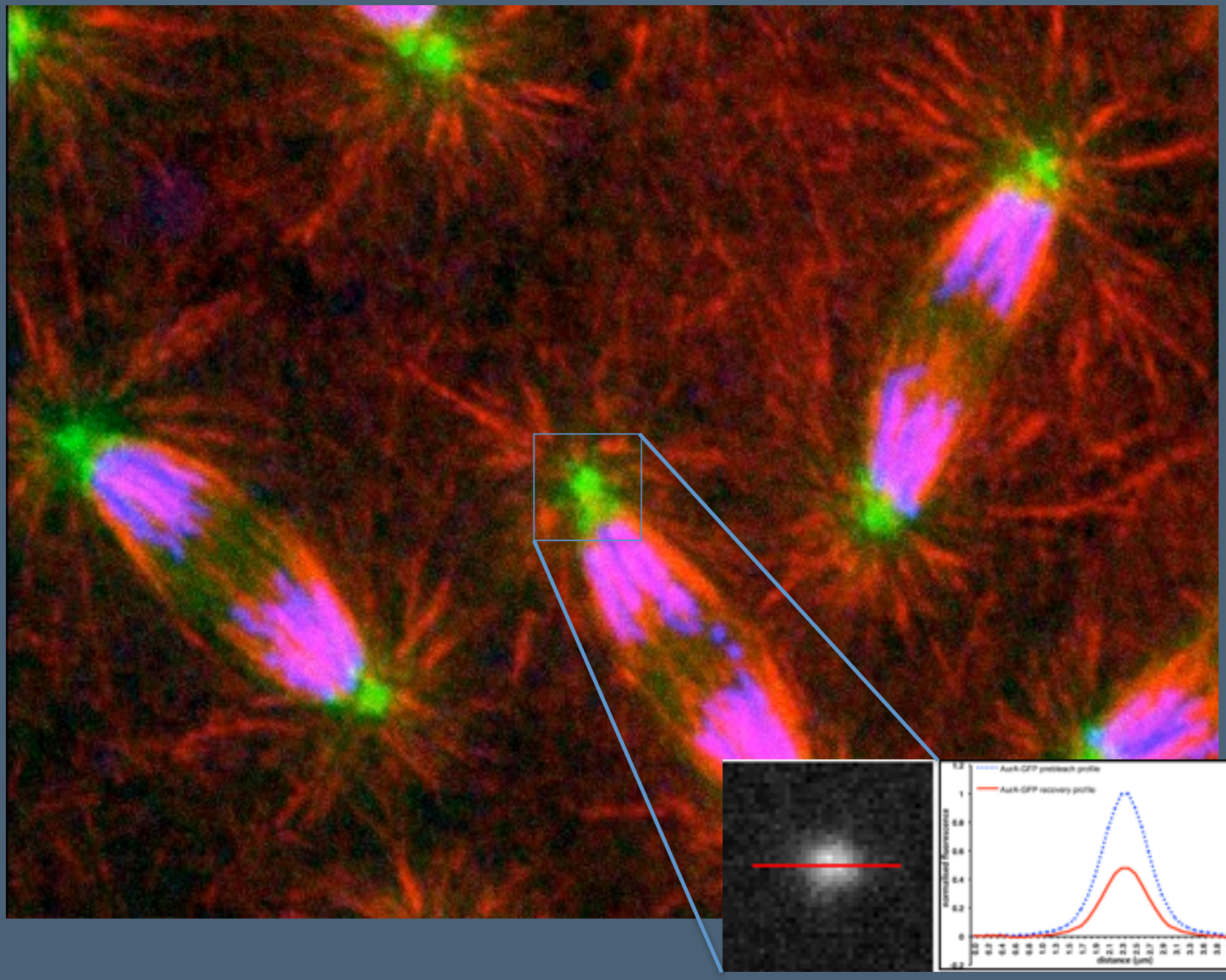
Confocal Microscopy

(Increasing contrast and resolution using optical sectioning)
Lecture 7

November 2016




Aux/k-GFP prebleach profile
Aux/k-GFP recovery profile
normalized fluorescence
distance (µm)

3 Flavours of Microscope

Problem:
Out of Focus
Light

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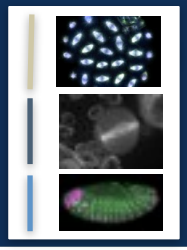

3 Flavours of Microscope

Problem:
Out of Focus
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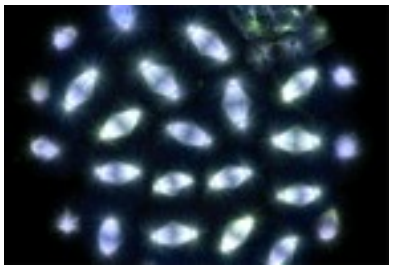
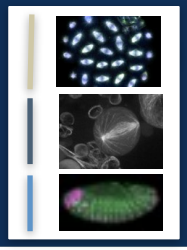
Confocal

Laser
Scanning





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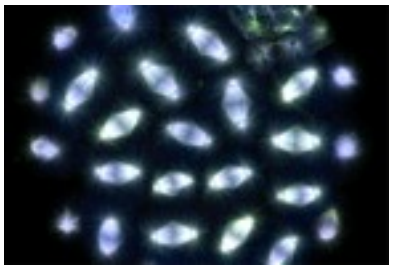
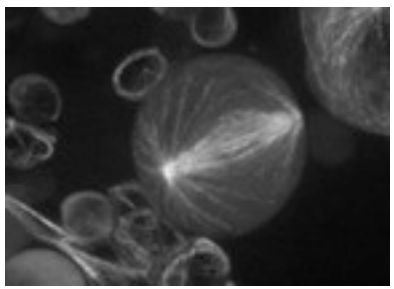
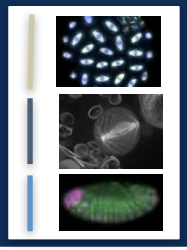
Spinning disc







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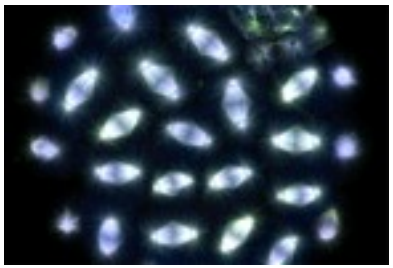

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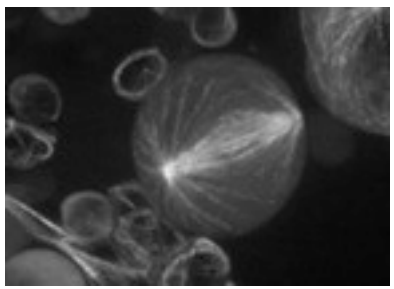

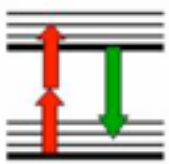

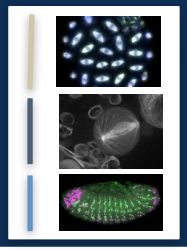

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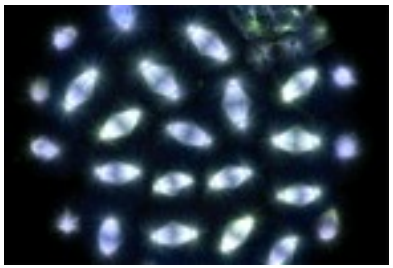

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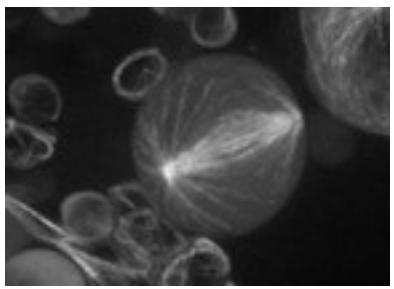
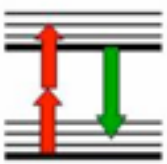
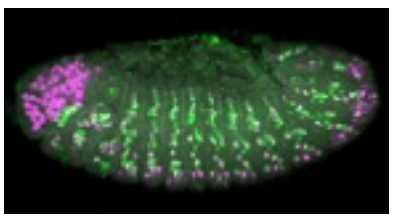
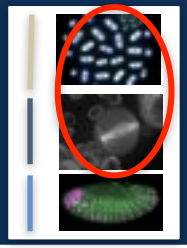


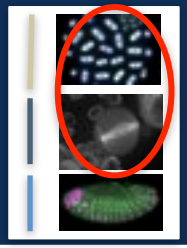


2-Photon





short History of Confocal Microscope



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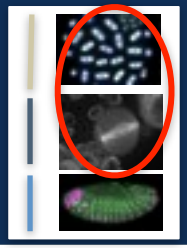

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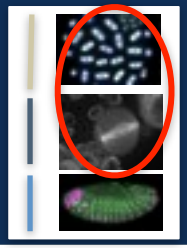

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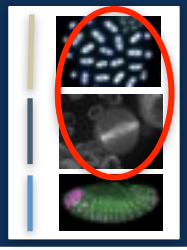
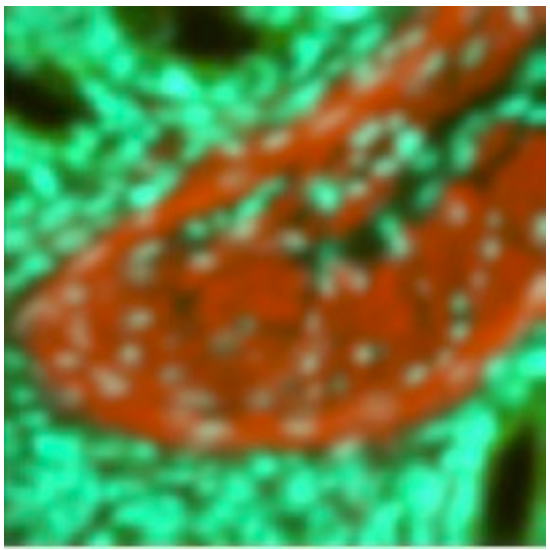
White, Amos and Wilson developed the MRC500 point scanning confocal
-Marketed commercially in 1987



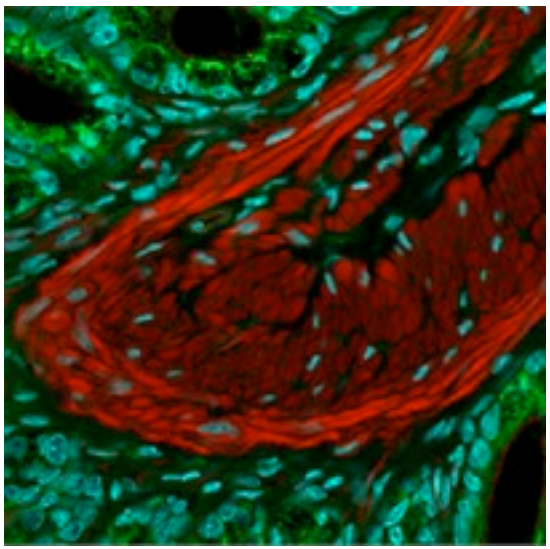
Comparison
Widefield Vs Confocal

Widefield

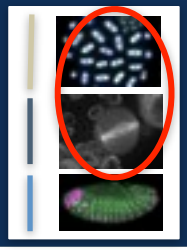


Out of focus light ‘blurs’ image

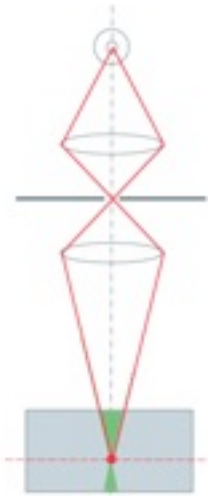
Confocal



Out of focus light is blocked

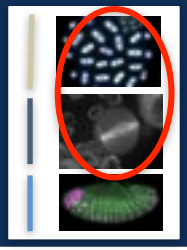


Principle of Confocal Microscopes Pinhole

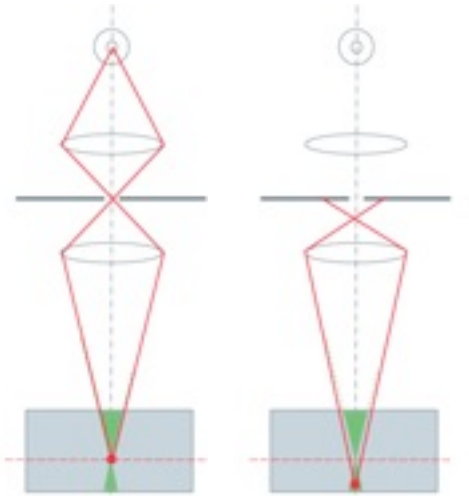


Pinhole diaphragm in the Conjugated focal plane = CONFOCAL

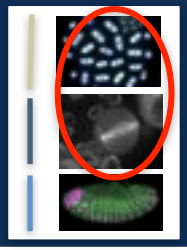
in focus light (from the optical section) passes through the pinhole and into the detector



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



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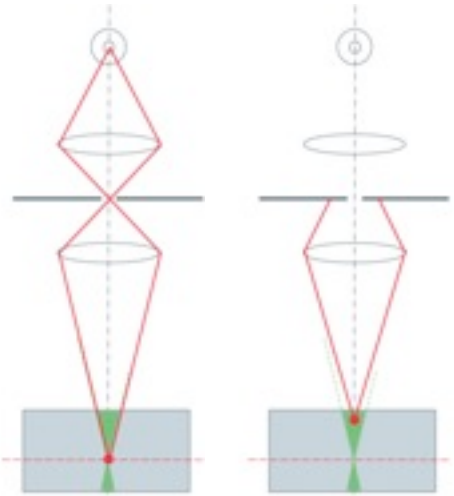
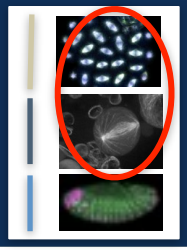
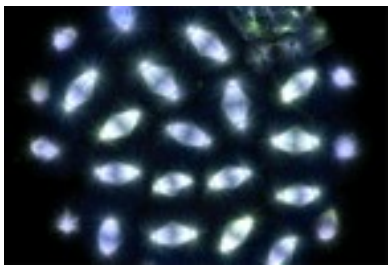


light from above the optical section also doesn't pass through the pinhole aperture

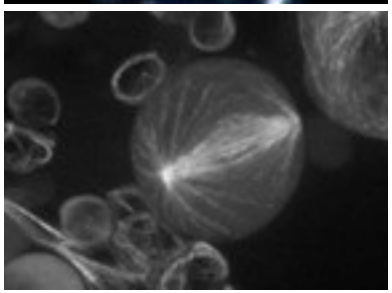


Confocal Microscopes

Laser Scanning



Spinning disc

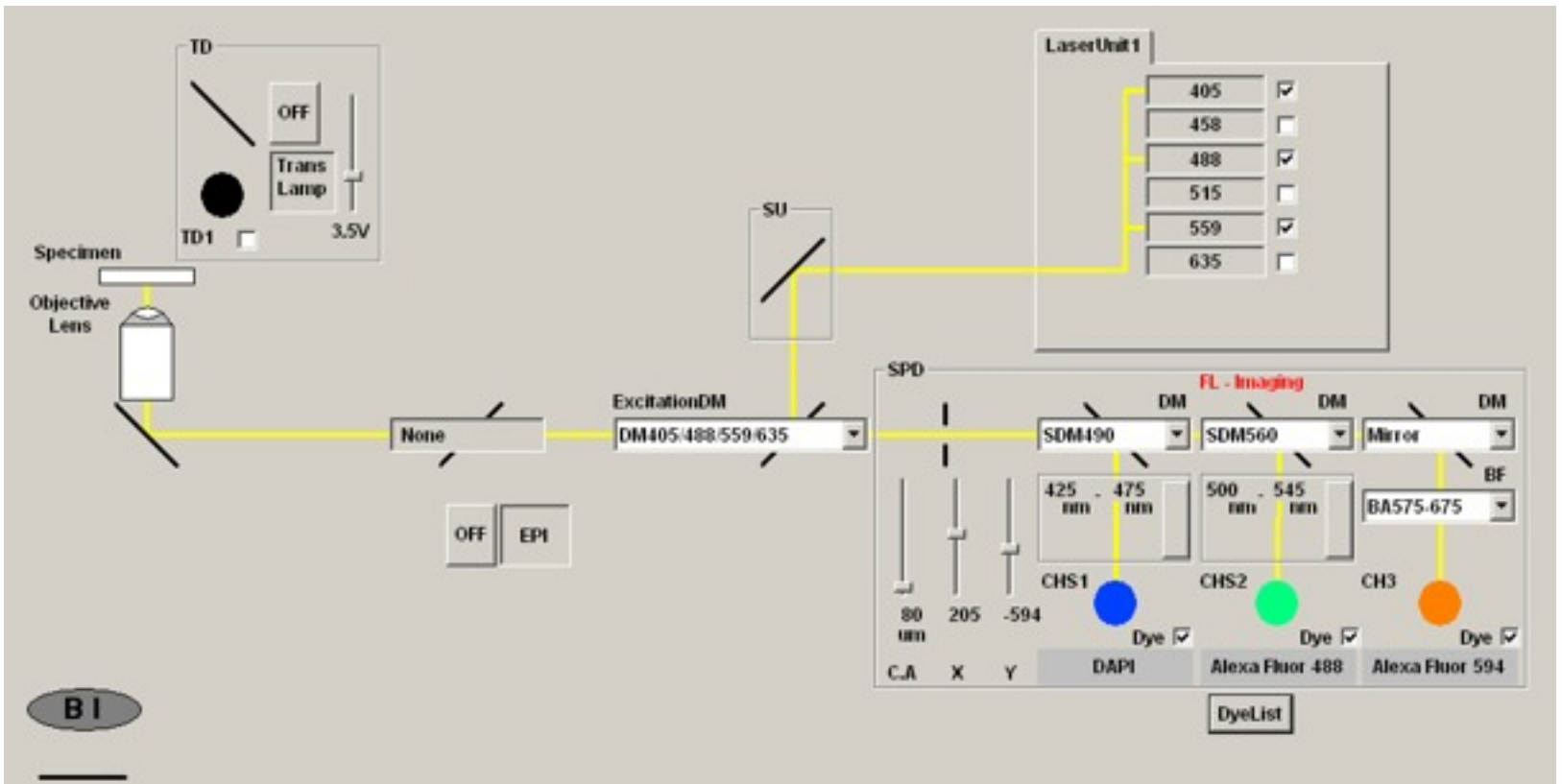


Laser Scanning Confocal

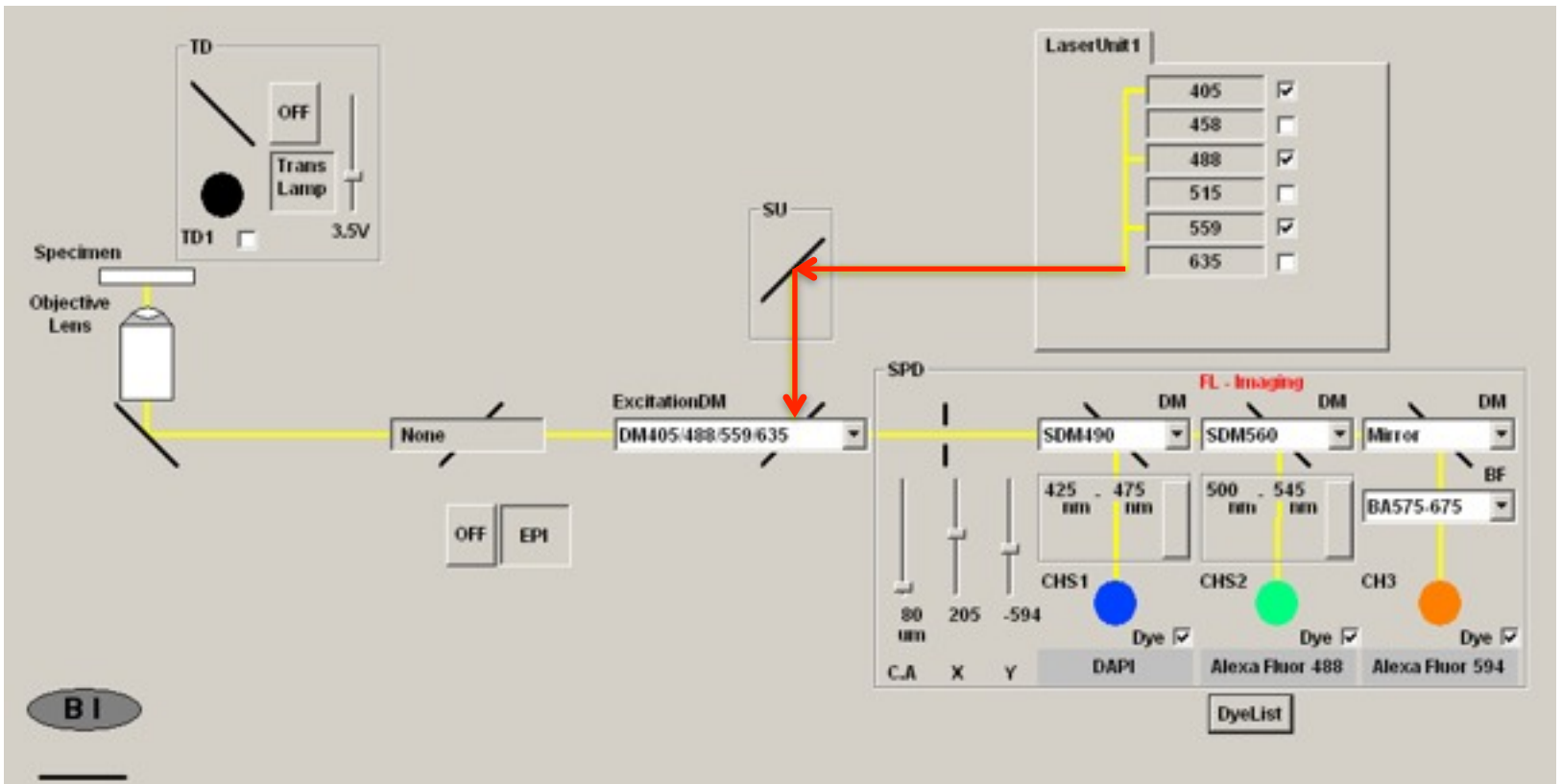
Laser Scanning Confocal

Laser Scanning Confocals are great to get ‘pretty’ images

Laser Scanning Confocal



Laser Scanning Confocal



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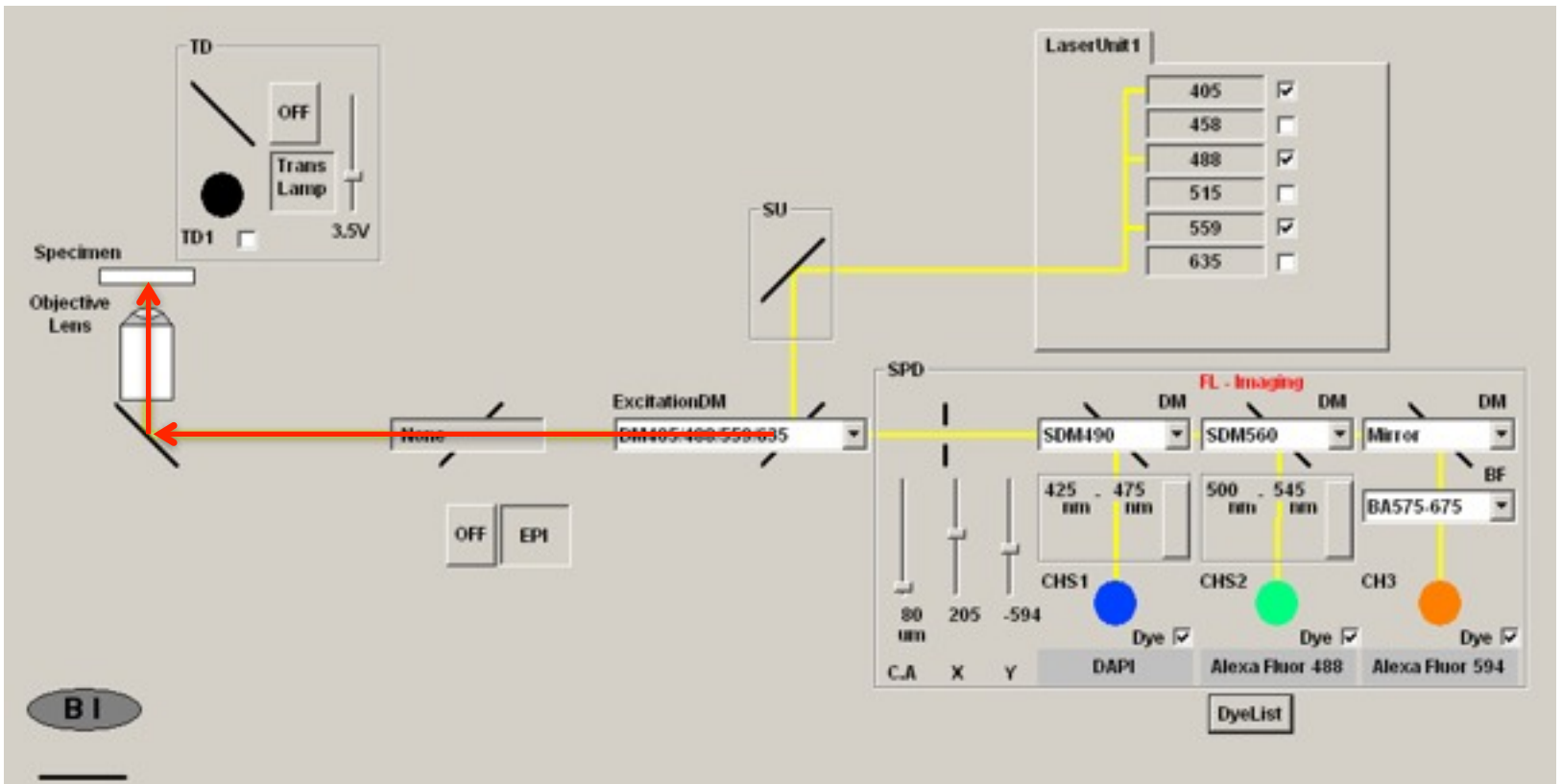

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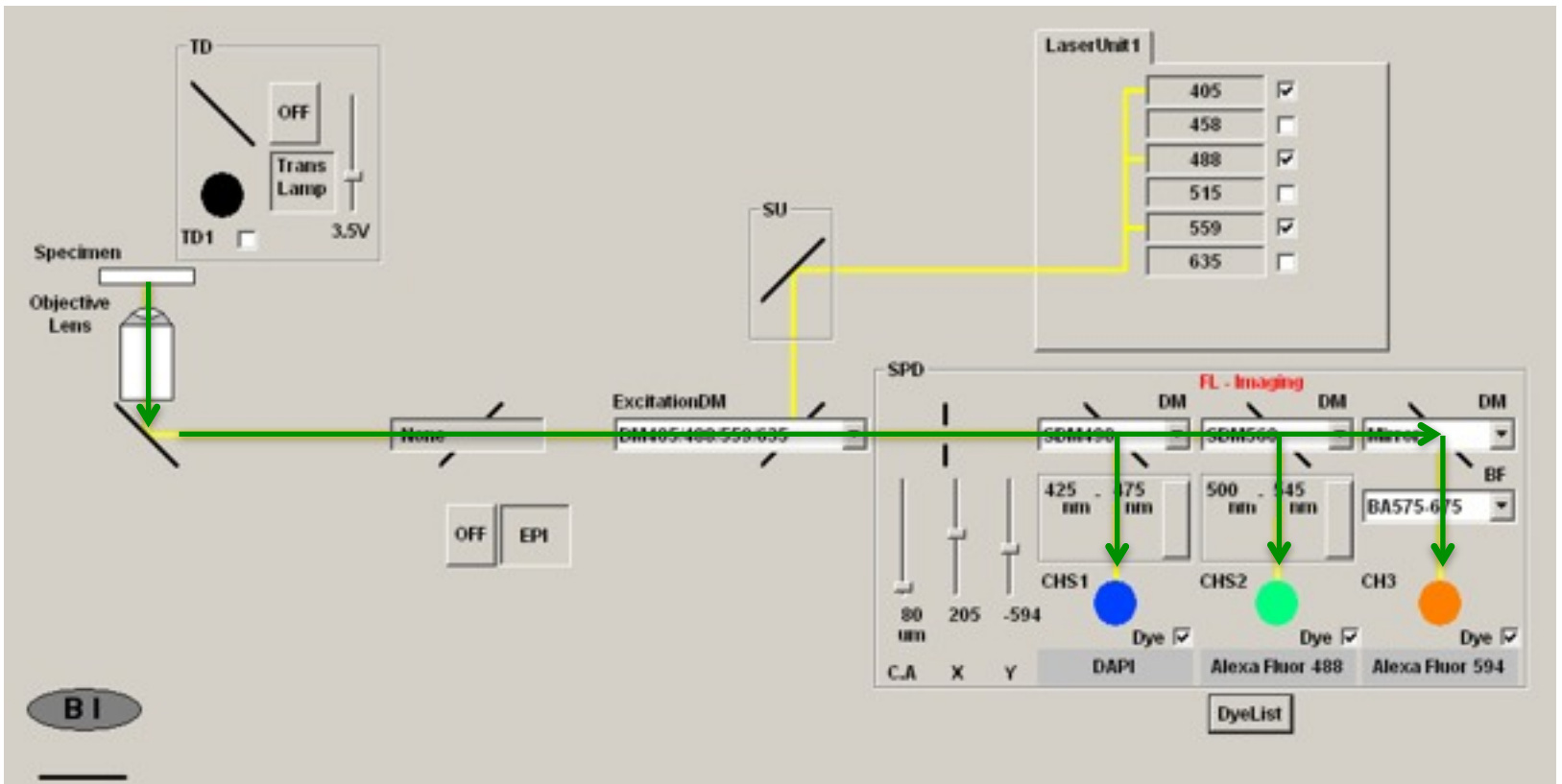

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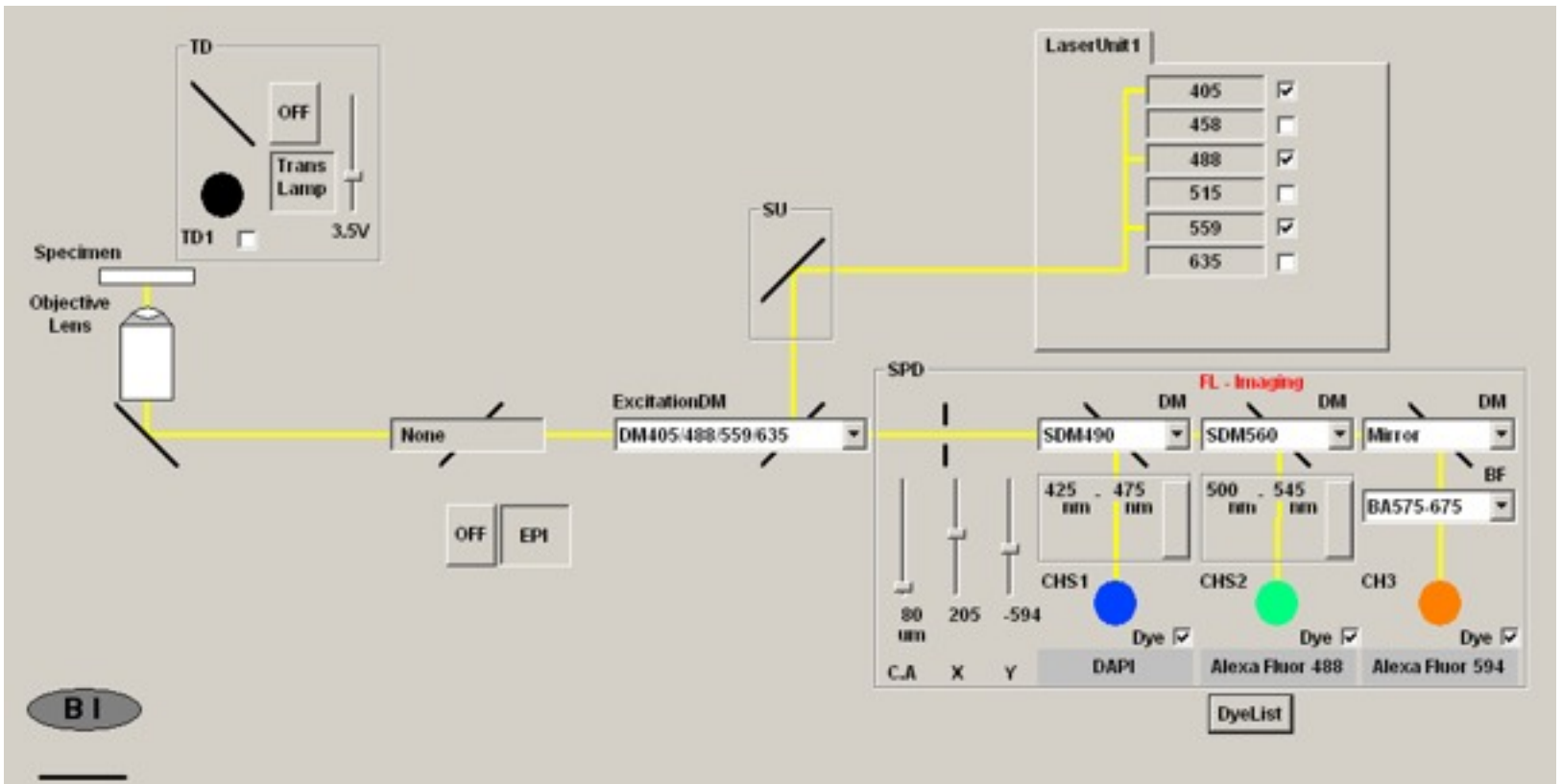
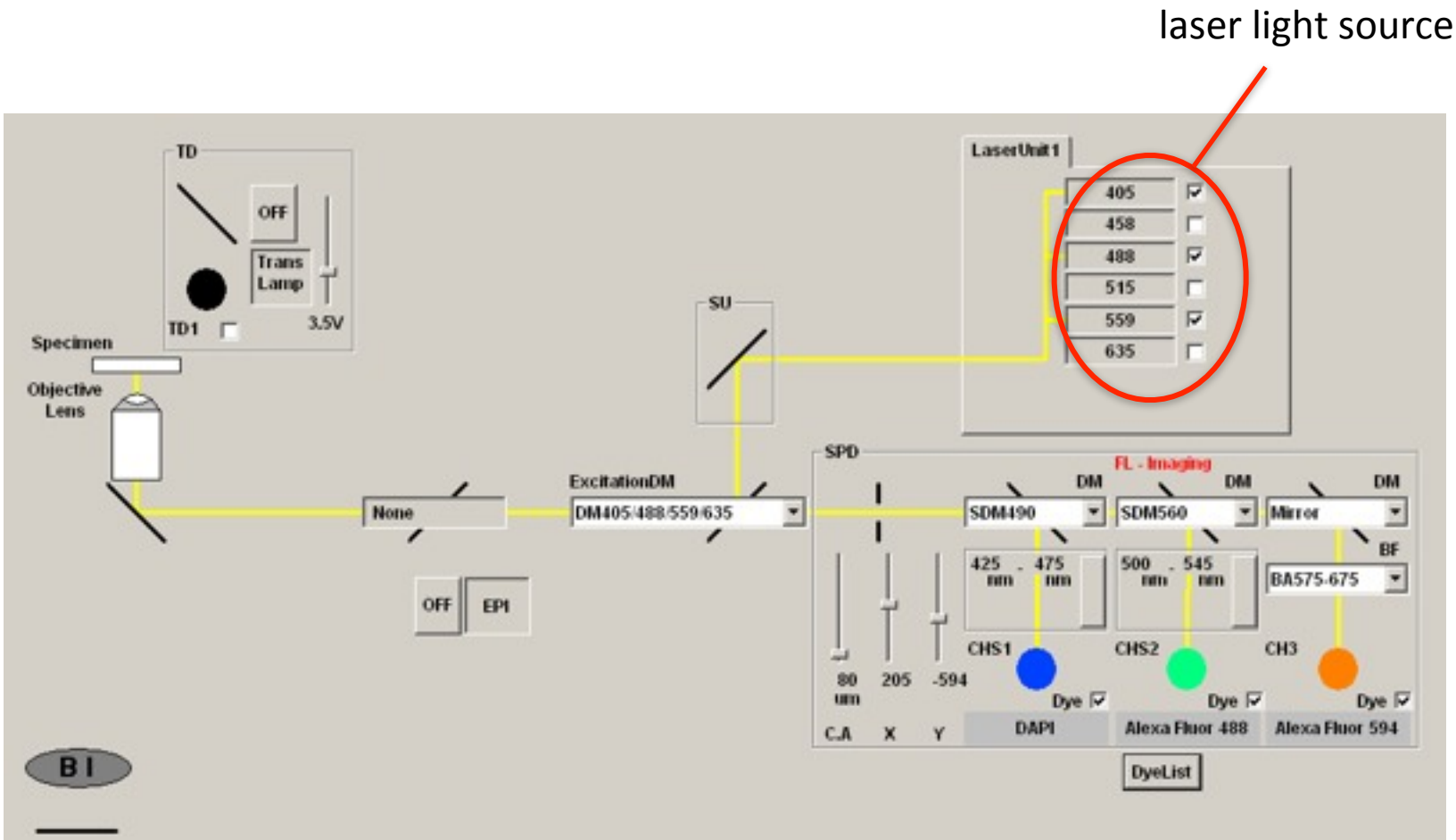


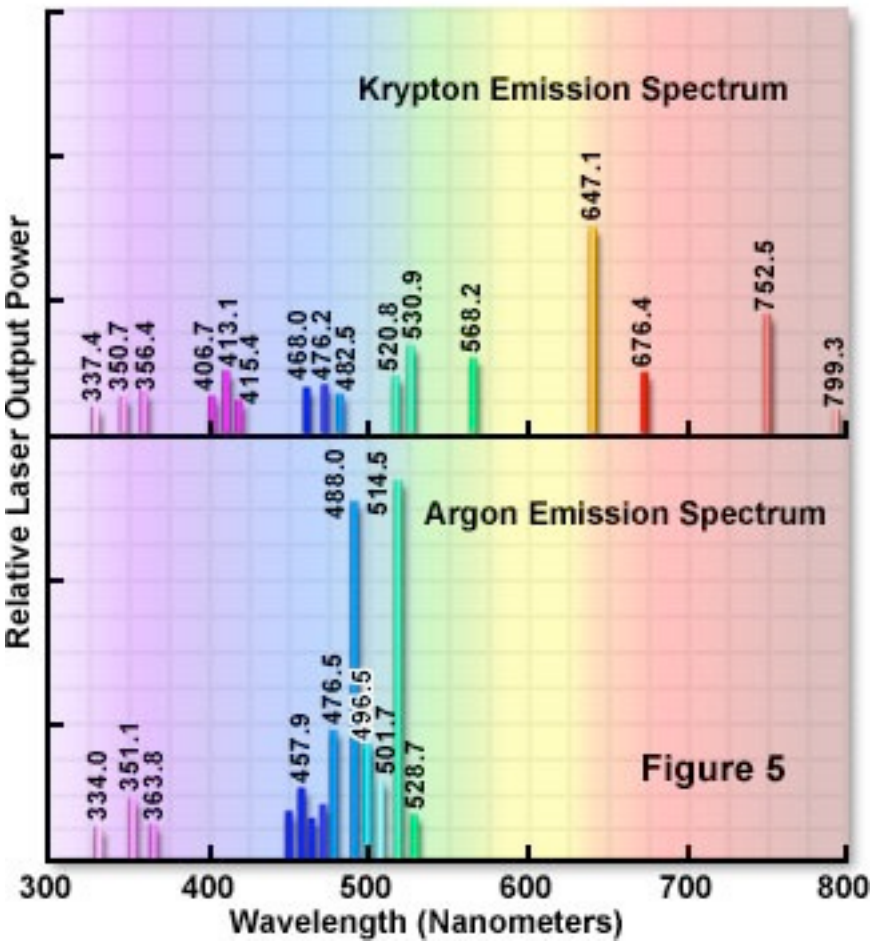
Laser Light Source



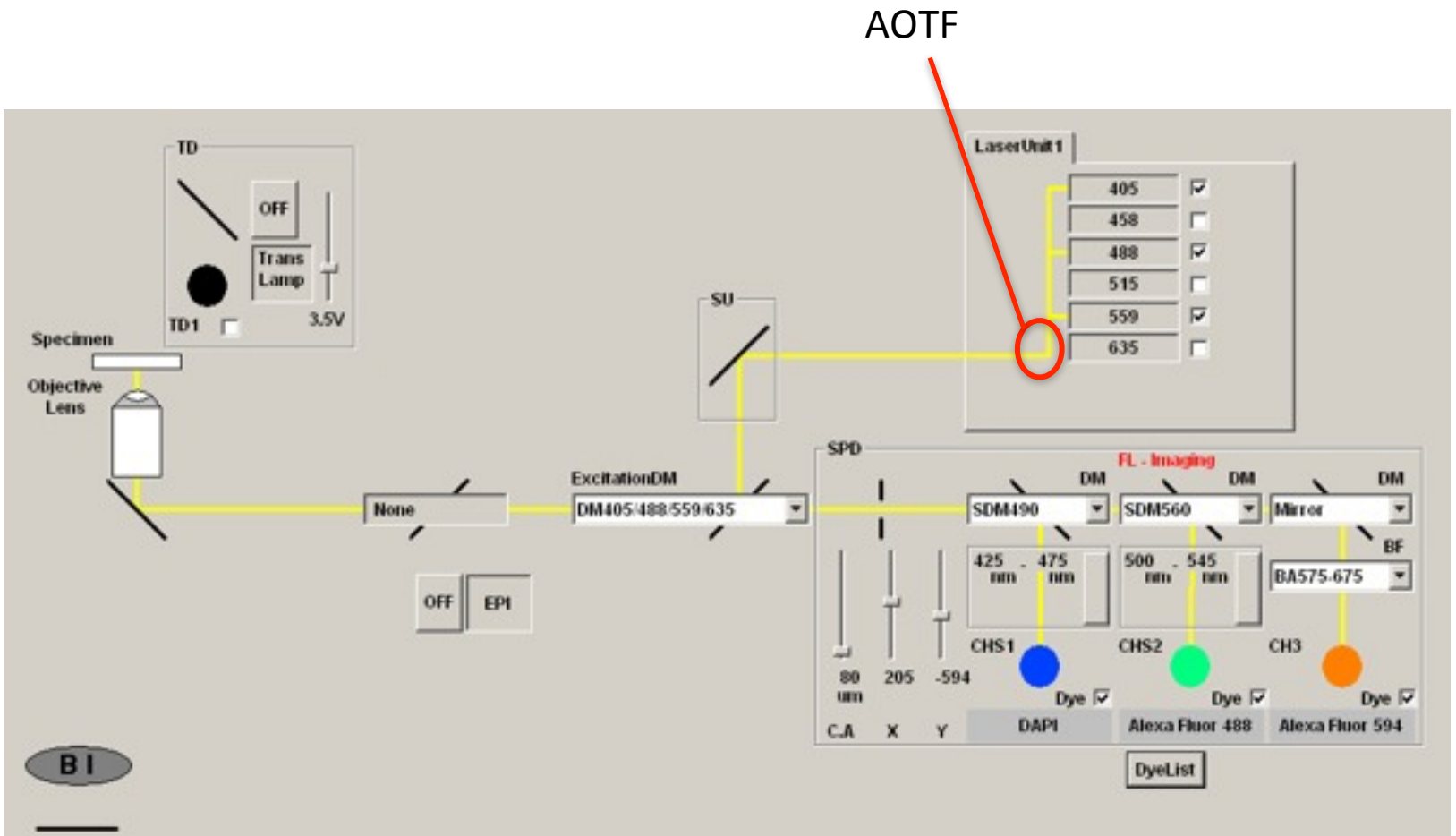
Laser Light Source

enables tighter control of fluorophores excited

Laser Emission Spectra



AOTF
Acousto-Optic Tunable Filter



THEORY

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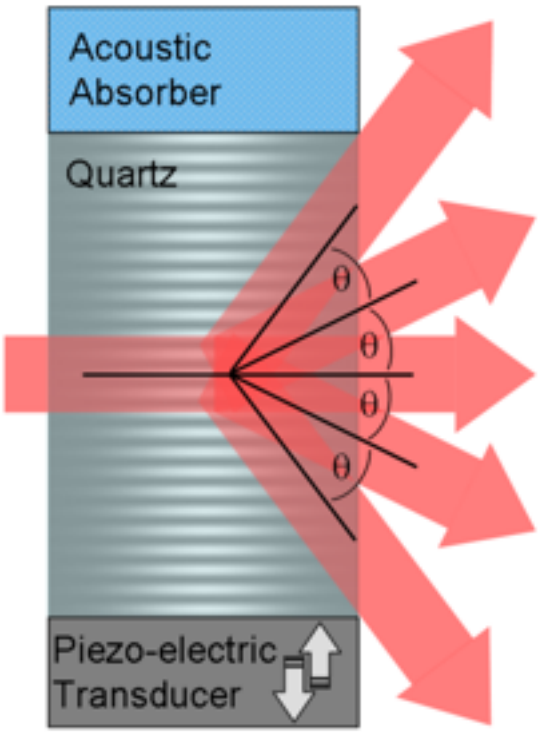

acousto-optic effect:

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.

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Acousto-Optic Tunable Filter

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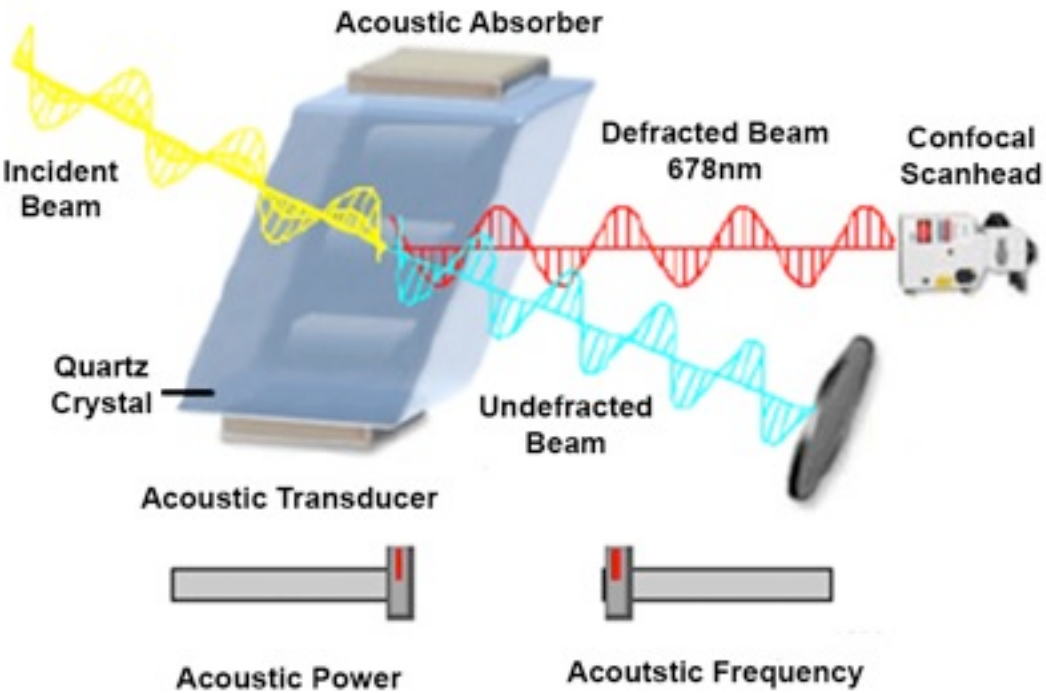

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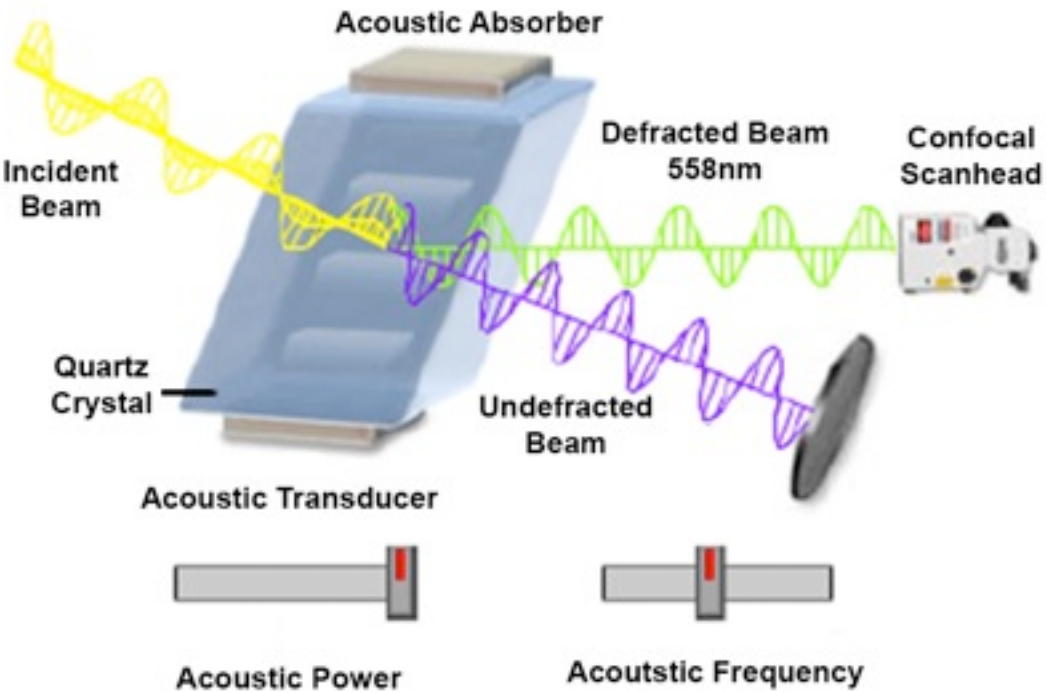

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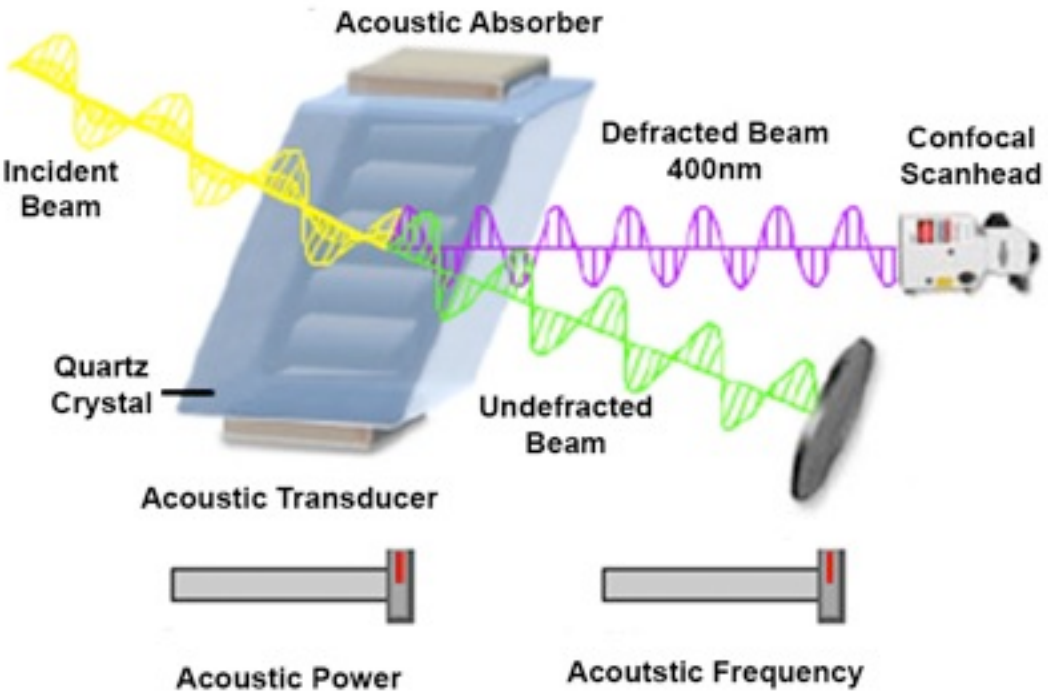

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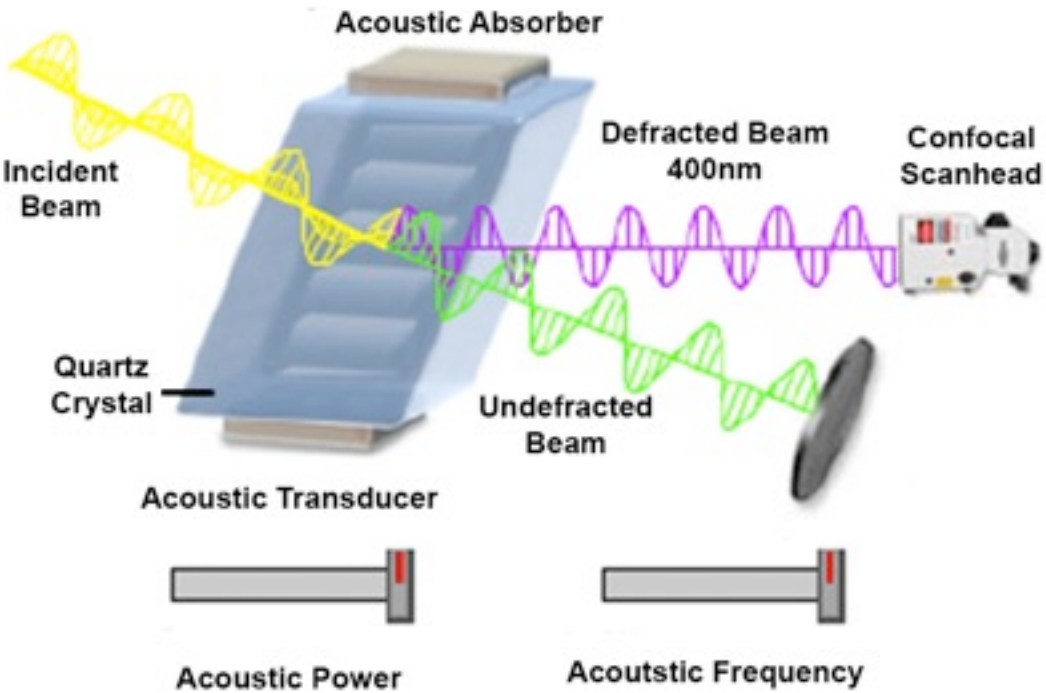
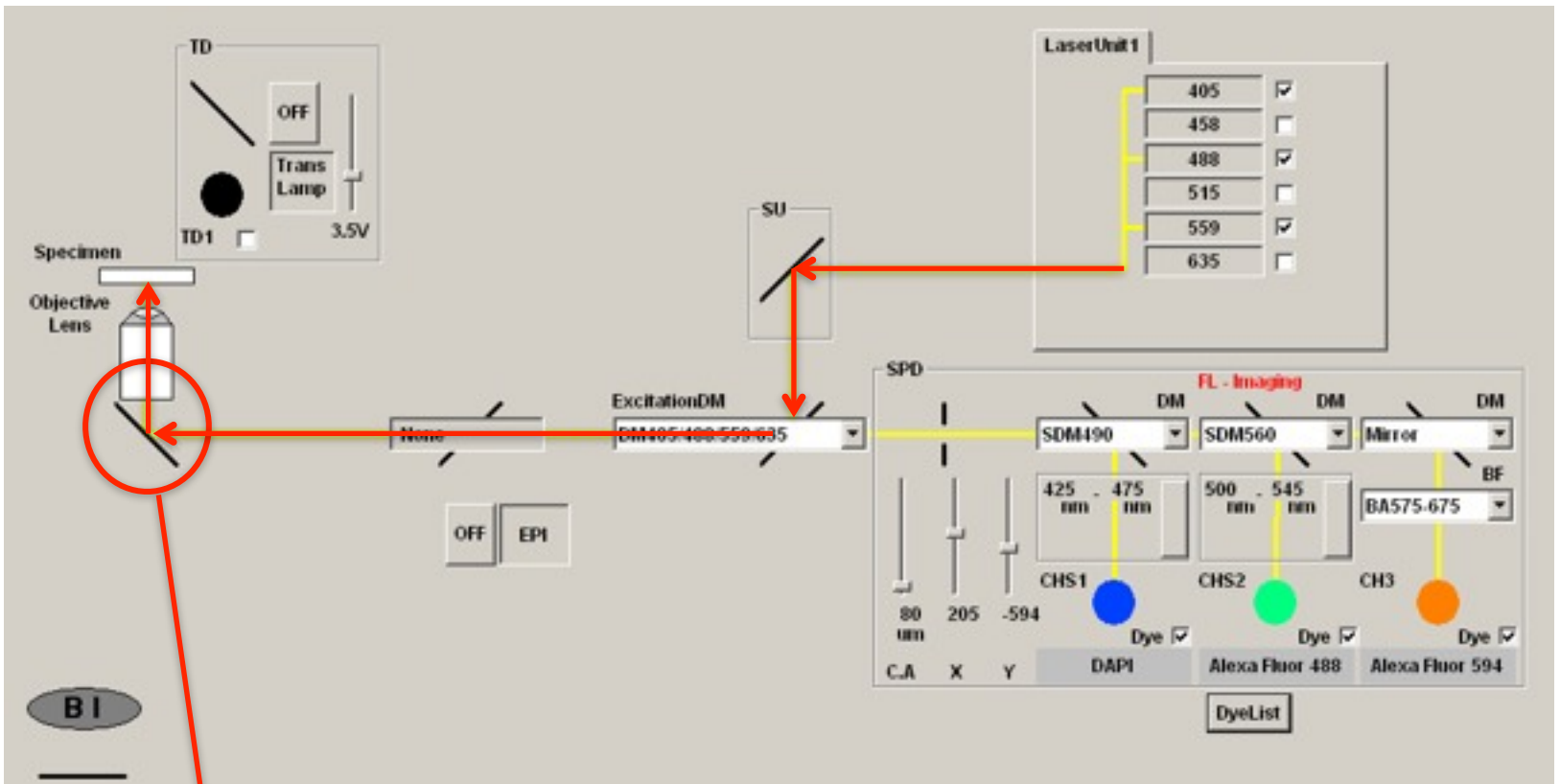


Quick On/Off of lasers

Very fast changes between excitation wavelengths

Galvo Scanning Mirrors



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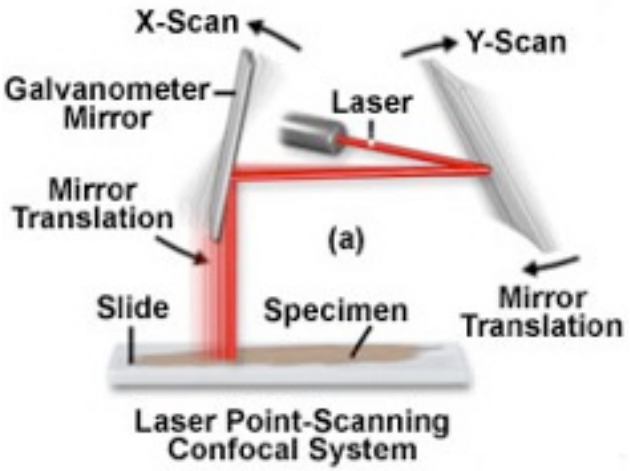
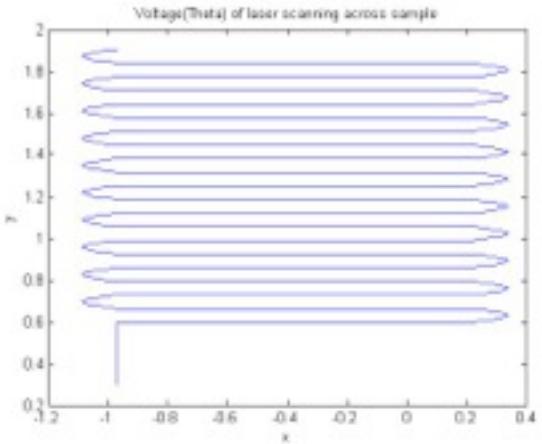
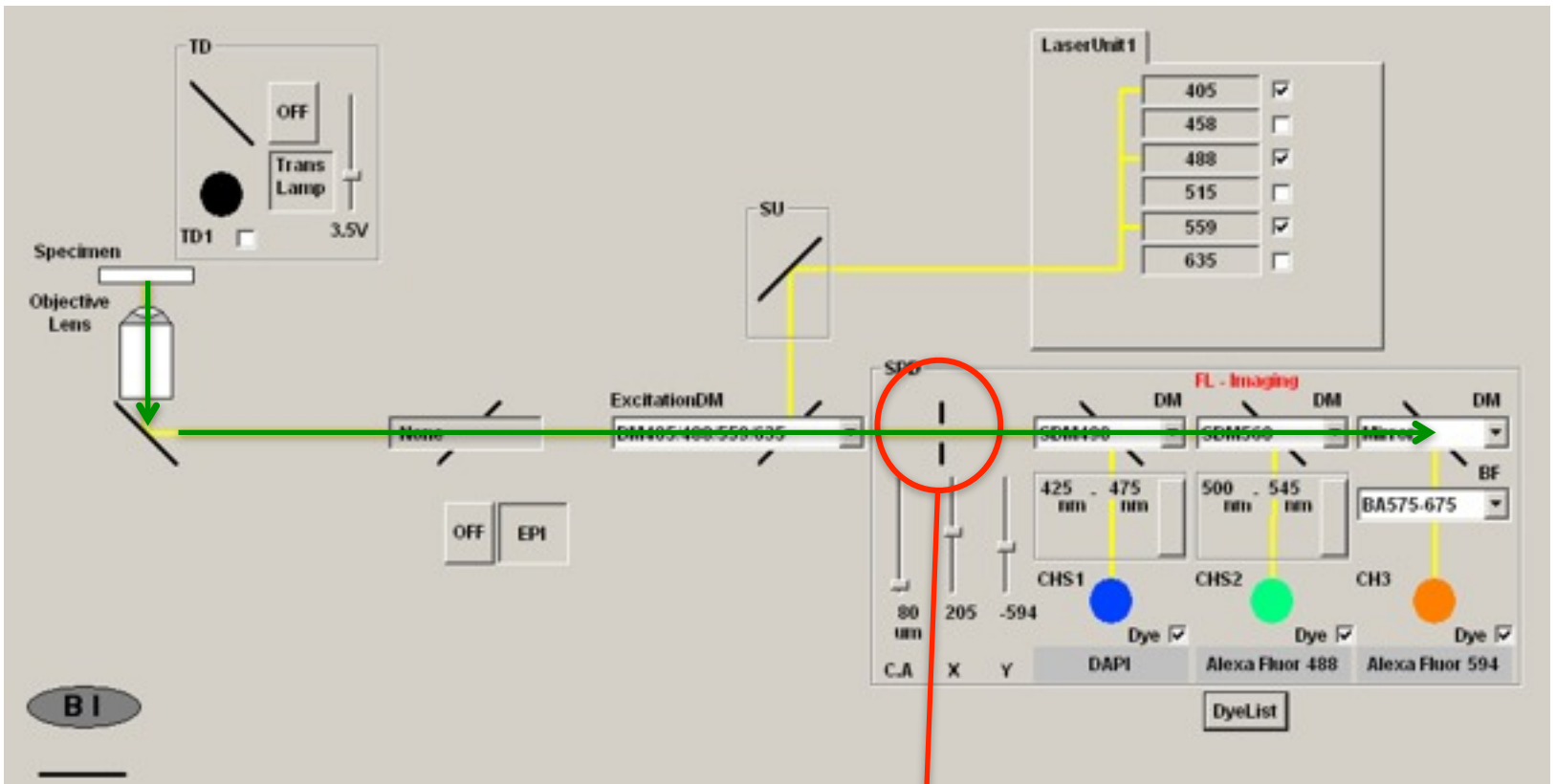




Sample excited at one point at a time
Relatively slow

Adjustable Pinhole

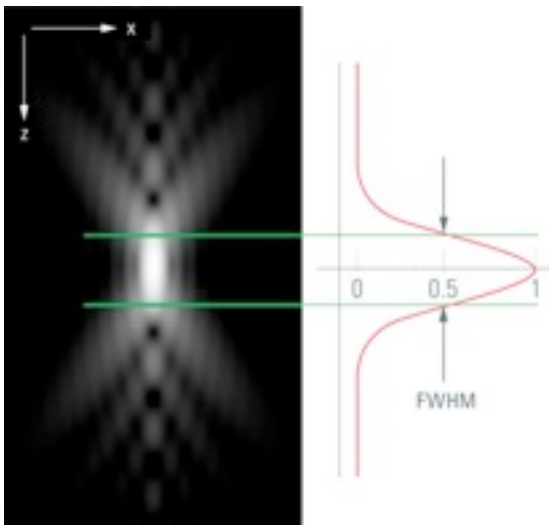
AOTF



pinhole

THEORY

Pinhole – Optical Sectioning



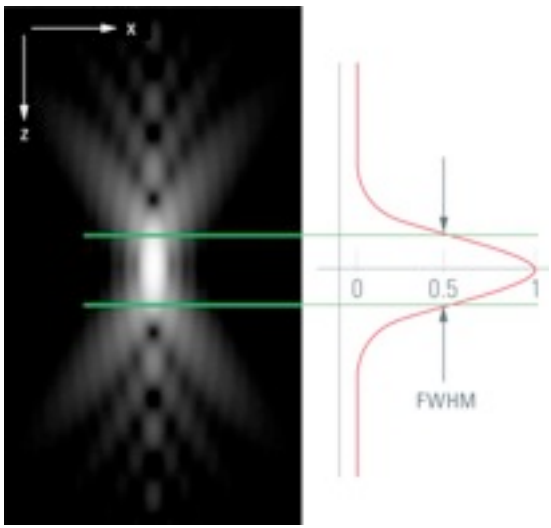
$$FWHM_{axial} = \sqrt{\left(\frac{\lambda_{exc} \cdot n}{NA^2}\right)^2 + \left(\frac{n \cdot \sqrt{2} \cdot PH}{NA}\right)^2}$$

FWHM=Full Width Half-Maximum

THEORY

Pinhole – Optical Sectioning

Shorter the wavelength the thinner the optical section



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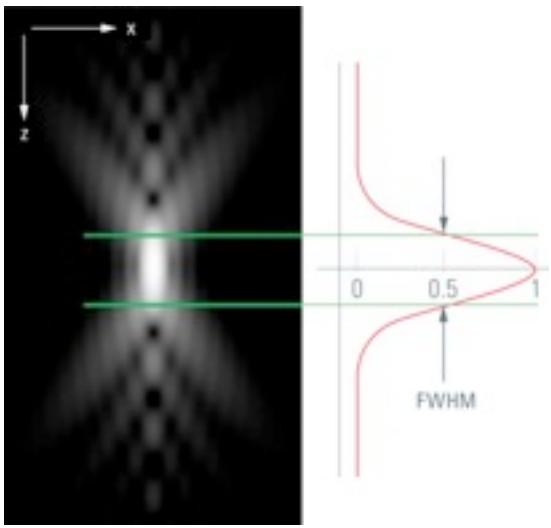
Diameter of the pinhole: Smaller pinhole thinner optical section



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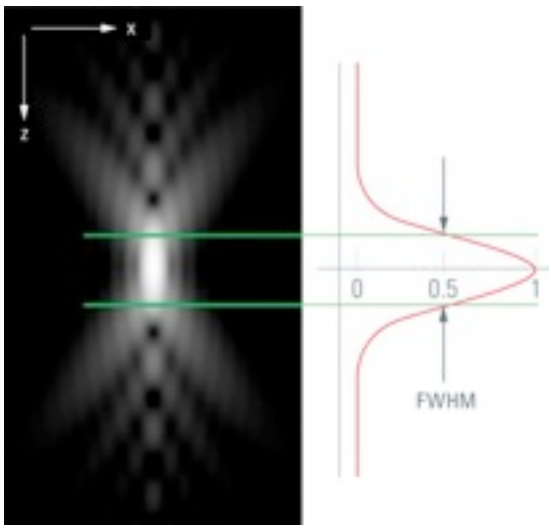
Diameter of the pinhole: Smaller pinhole thinner optical section



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

FWHM=Full Width Half-Maximum

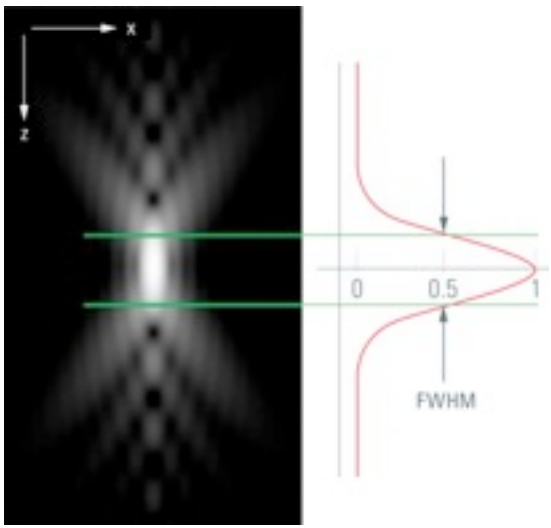
The higher the NA.
the thinner the section

THEORY

Pinhole – Optical Sectioning

Shorter the wavelength the thinner the optical section

Diameter of the pinhole: Smaller pinhole thinner optical section



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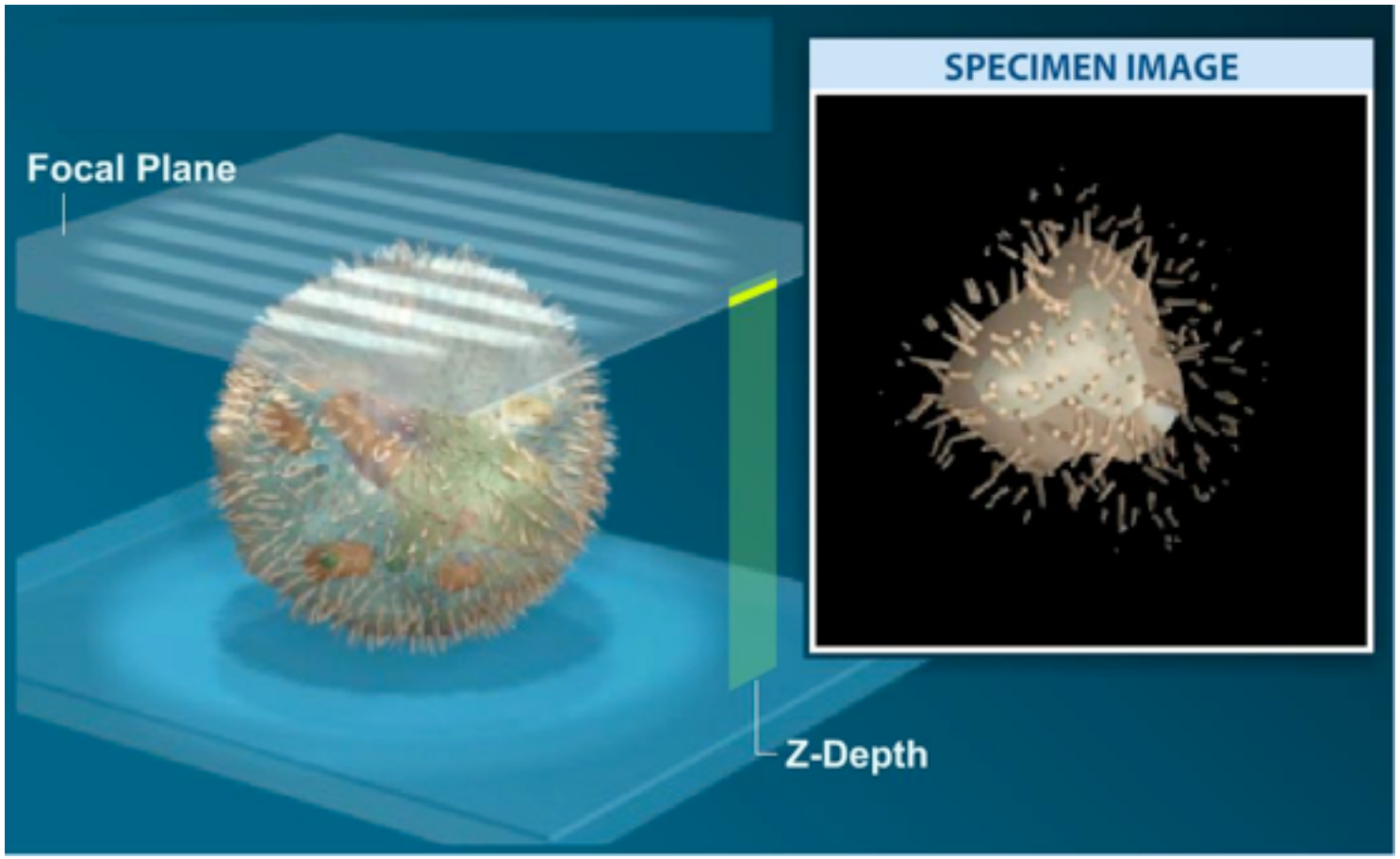
FWHM=Full Width Half-Maximum

The higher the NA.
the thinner the section

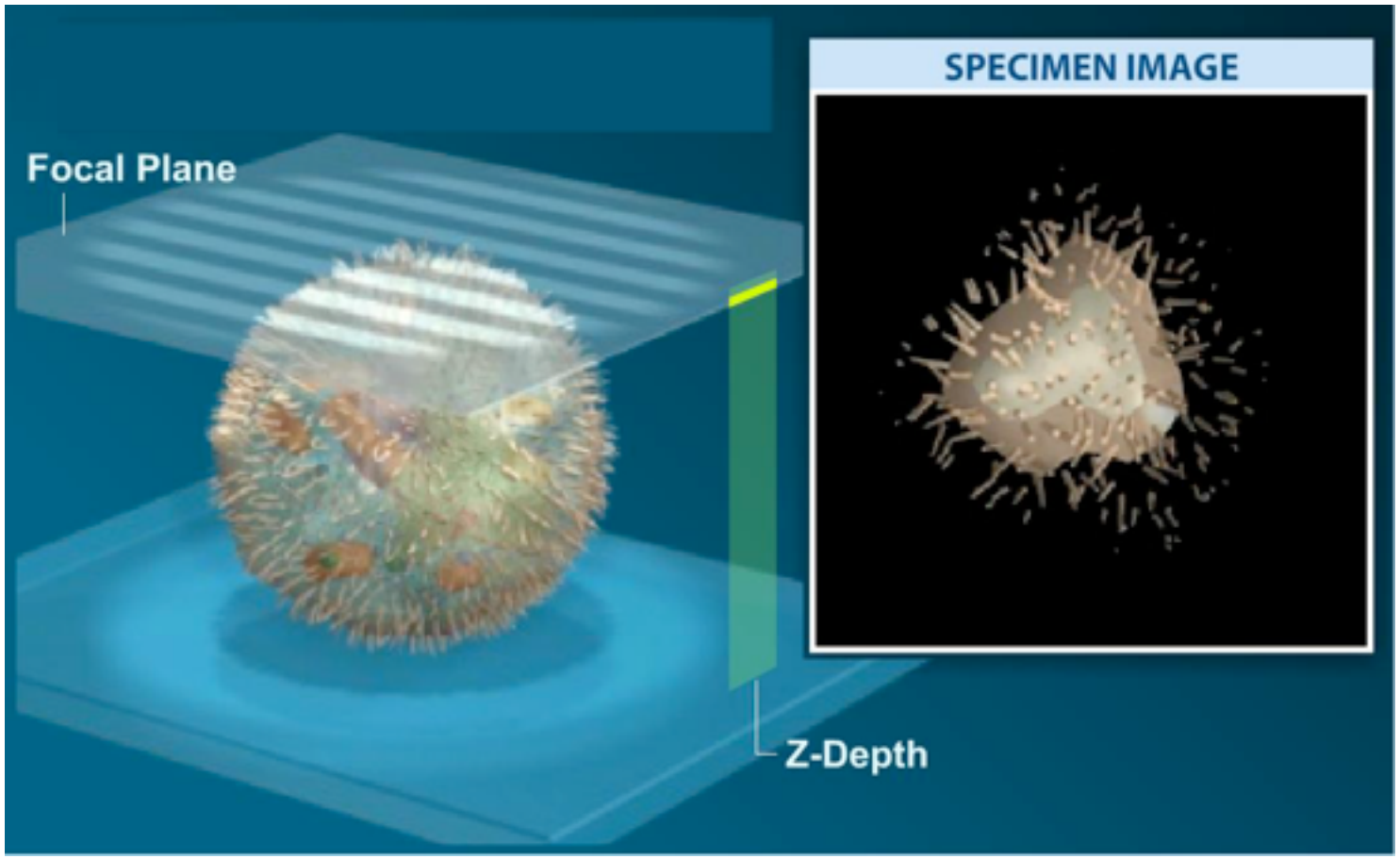
Weak signal > open pinhole > more light but thicker section

Confocal enables 3D reconstruction

Optical section



Confocal enables 3D reconstruction

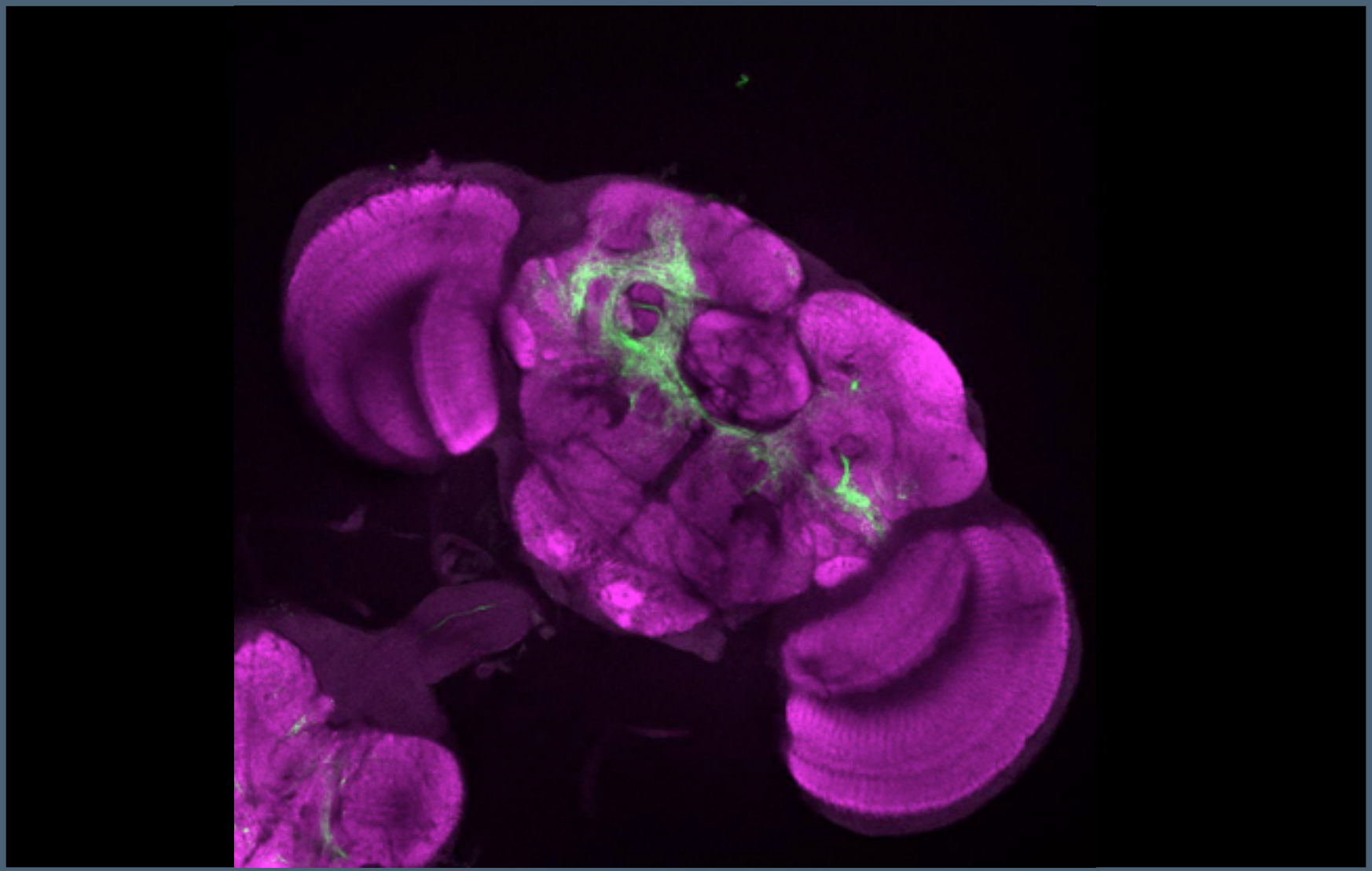


Confocal enables 3D reconstruction

Confocal enables 3D reconstruction

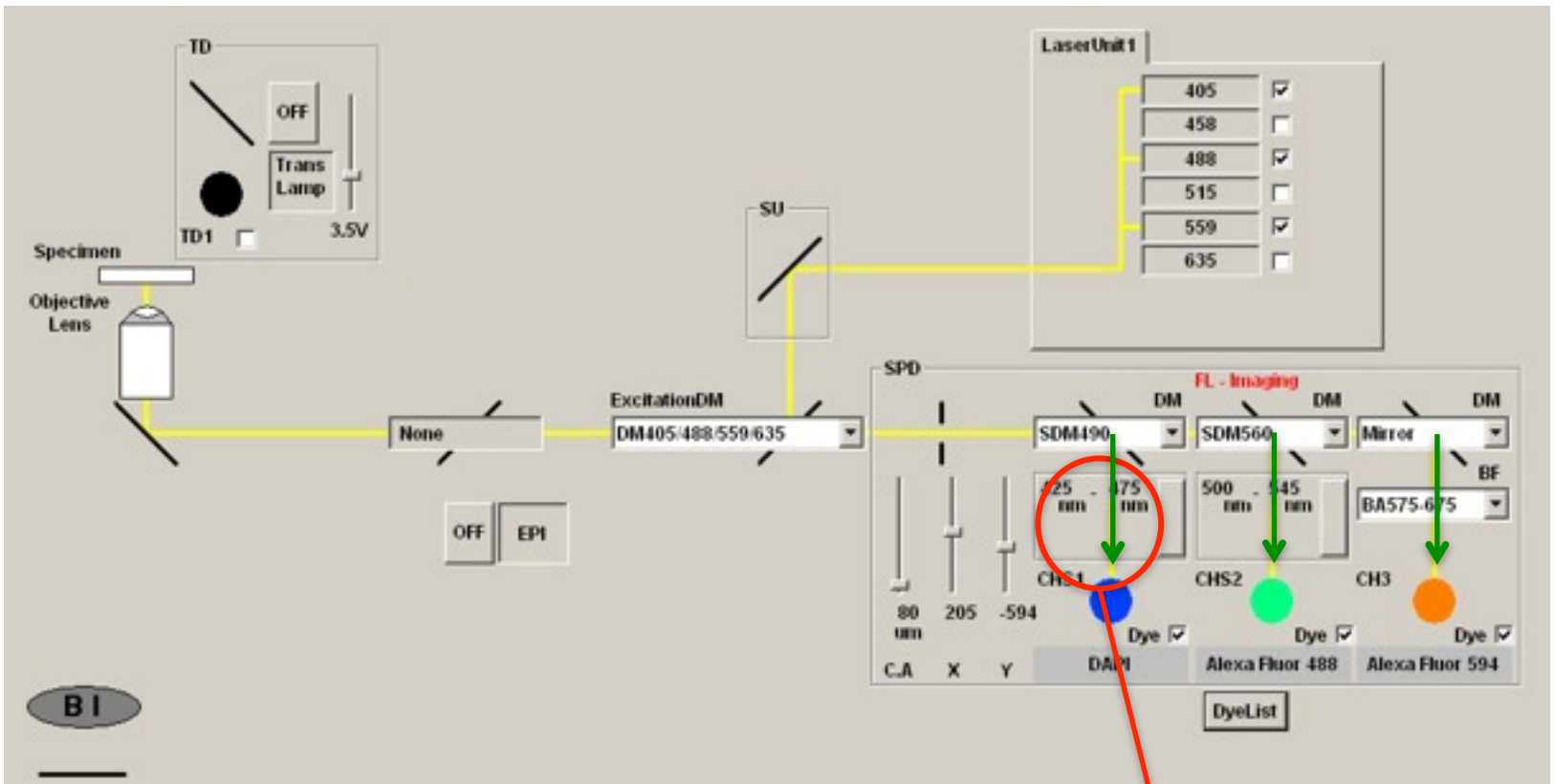
Adult Drosophila head (C. Rezeval Goodwin Lab)

Confocal enables 3D reconstruction



Adult Drosophila head (C. Rezeval Goodwin Lab)

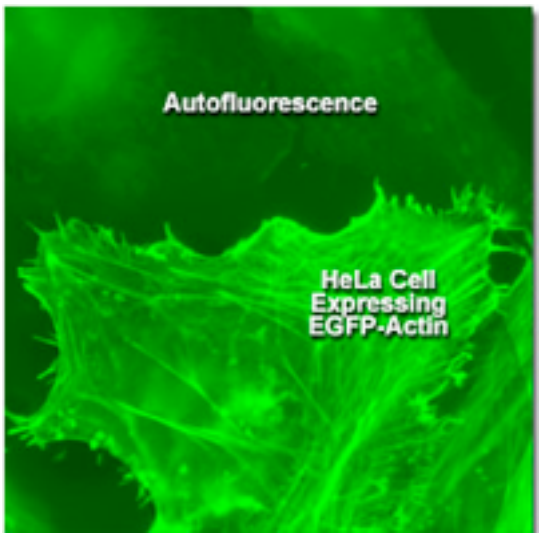
Variable Detector Slit



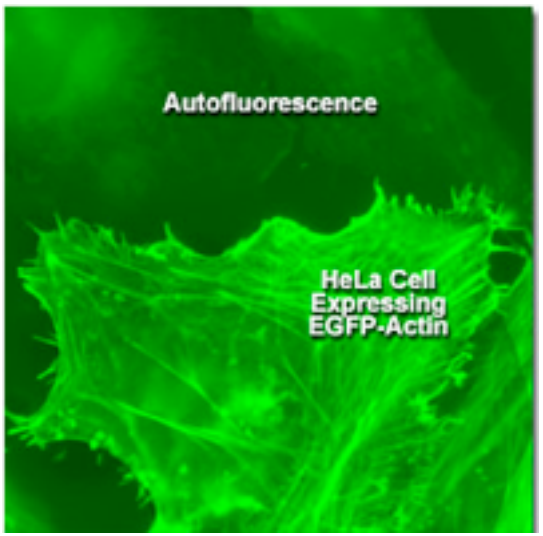
variable detector slit

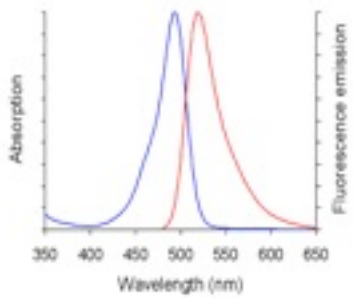
Spectral Unmixing

Spectral Unmixing



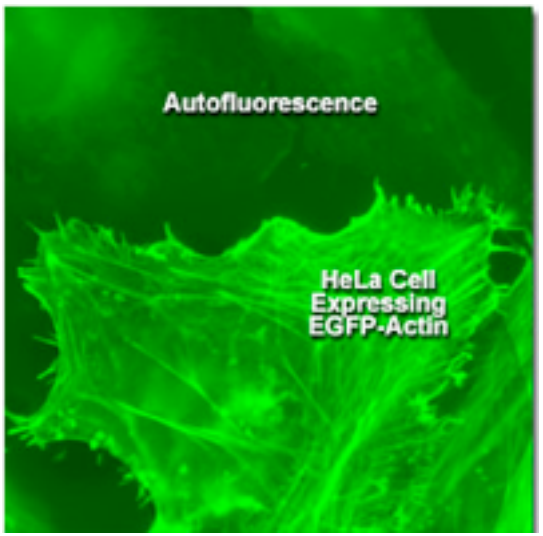
Spectral Unmixing

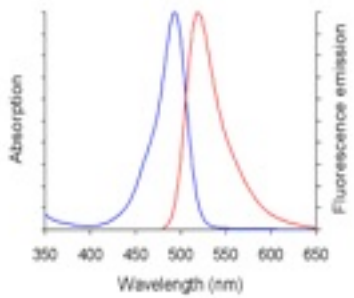




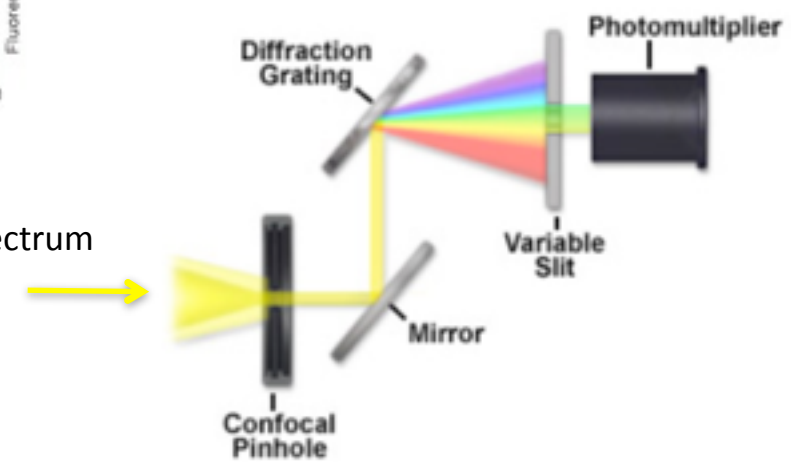
Light emitted from fluorophore as a spectrum

Spectral Unmixing

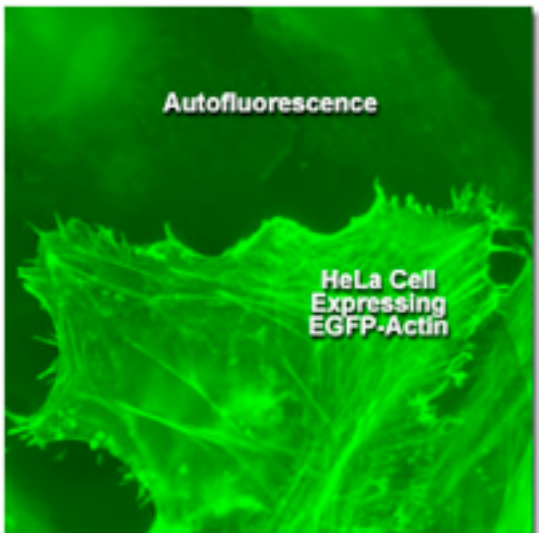


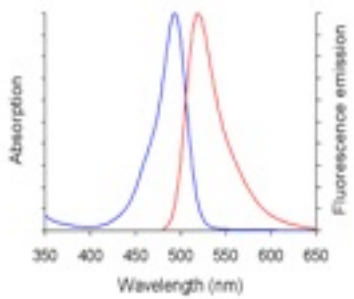


Light emitted from fluorophore as a spectrum



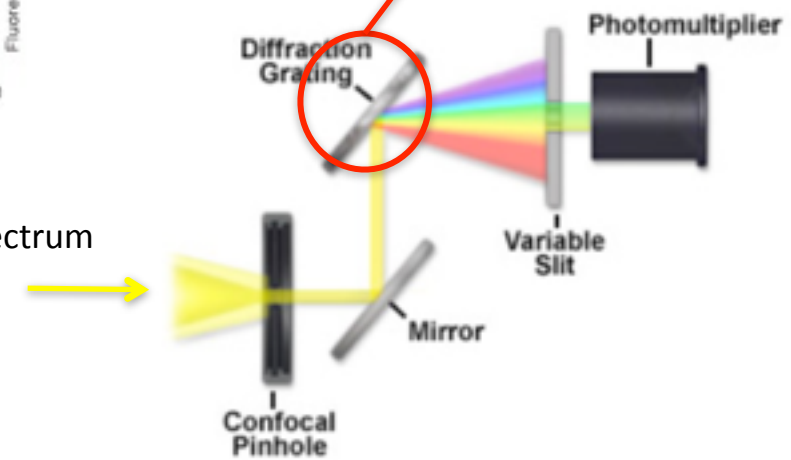
Spectral Unmixing



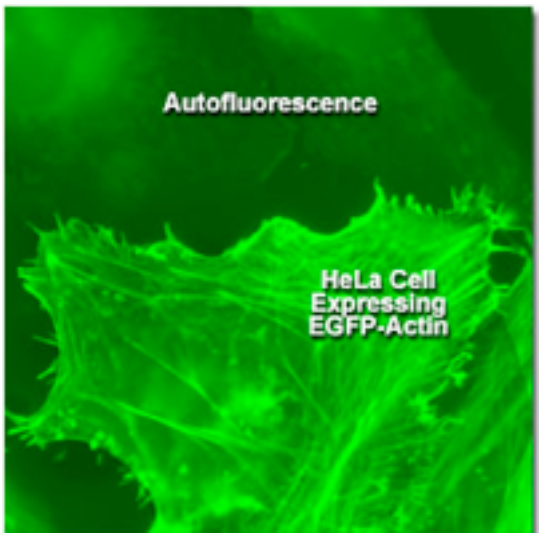


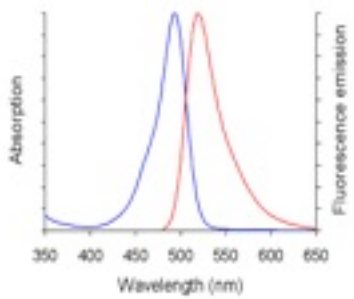
Light emitted from fluorophore as a spectrum

Defraction grating separates wavelengths over physical area



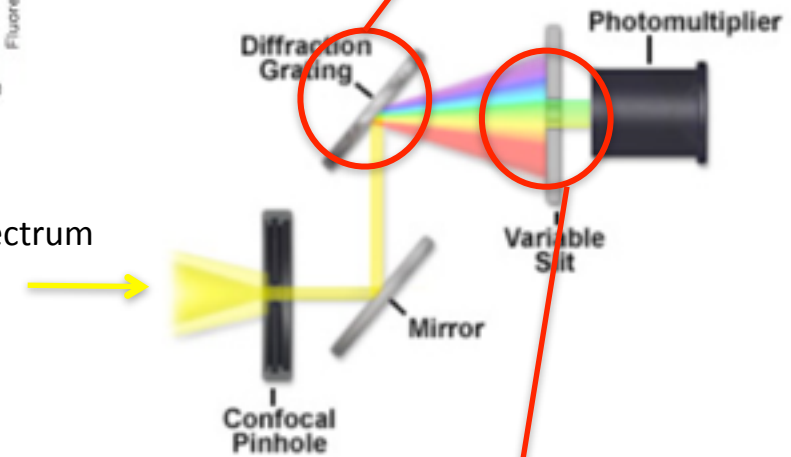
Spectral Unmixing





Light emitted from fluorophore as a spectrum

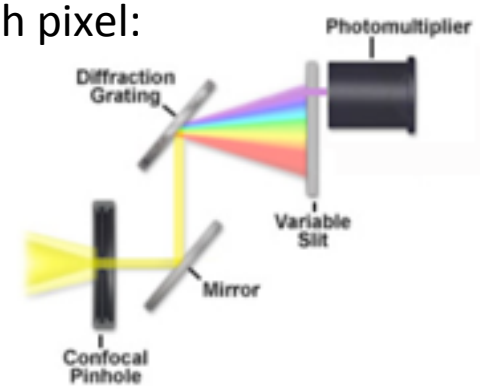
Defraction grating separates wavelengths over physical area

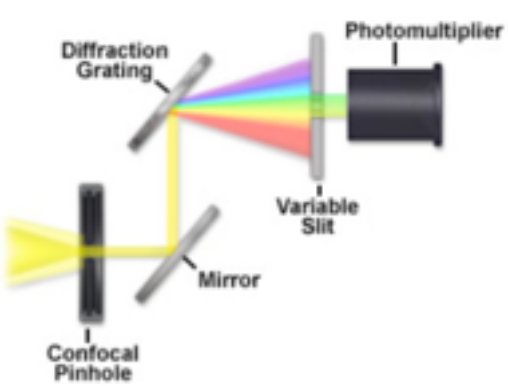


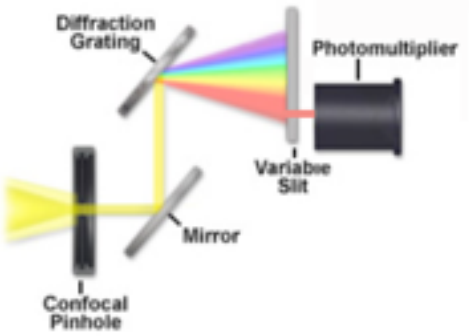
Variable slit lets through only certain wavelengths

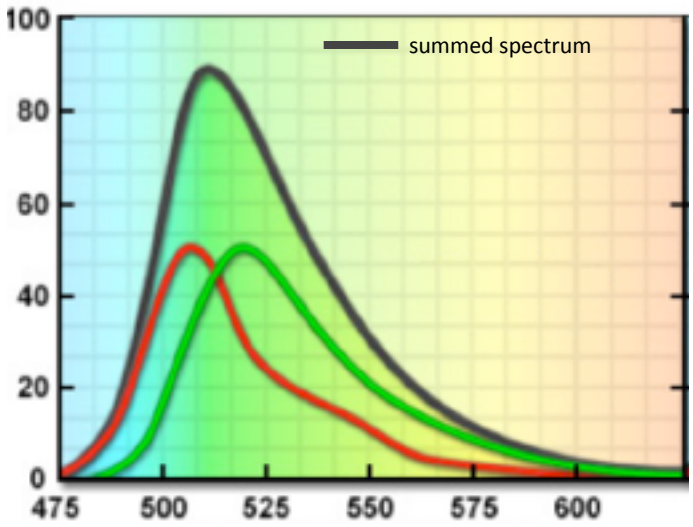
Spectral Unmixing

At each pixel:



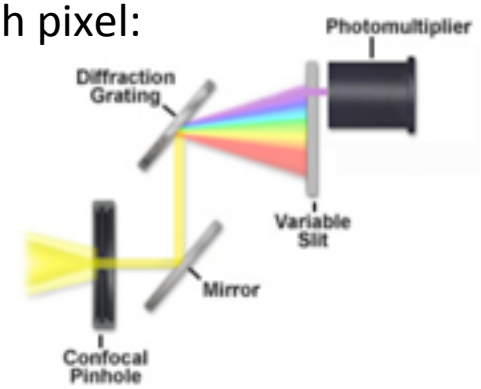


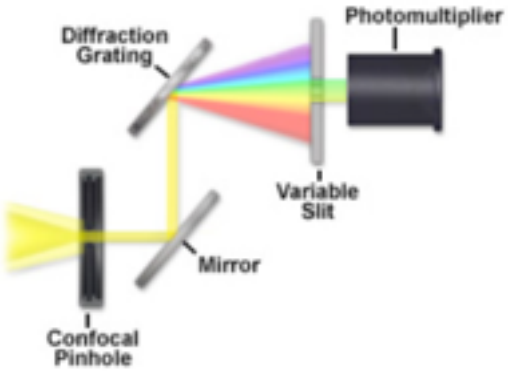


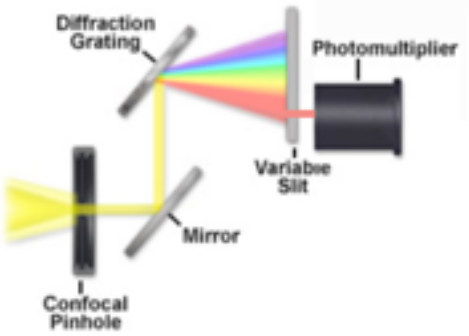


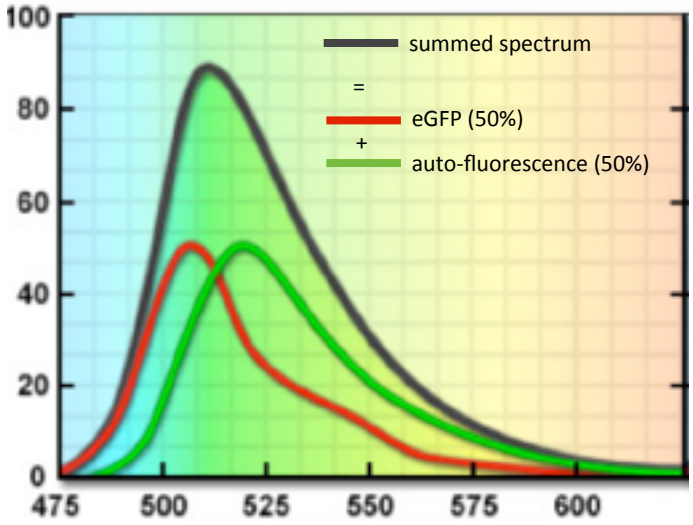
Spectral Unmixing

At each pixel:



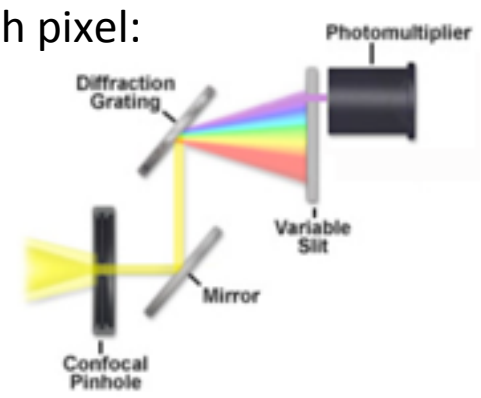


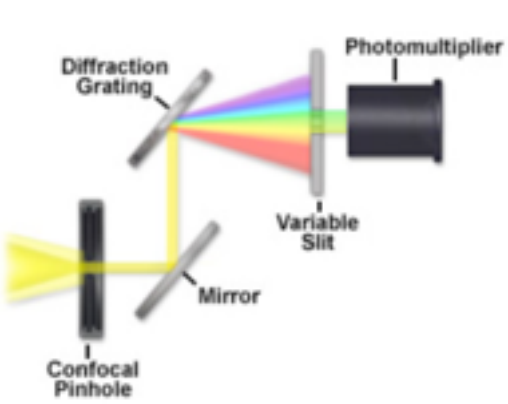


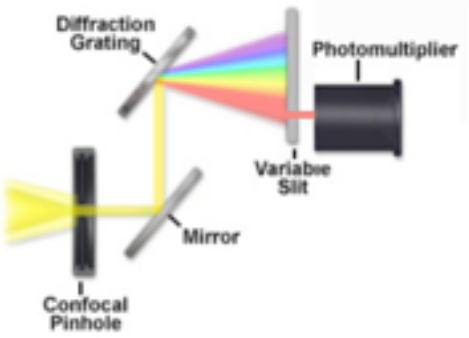


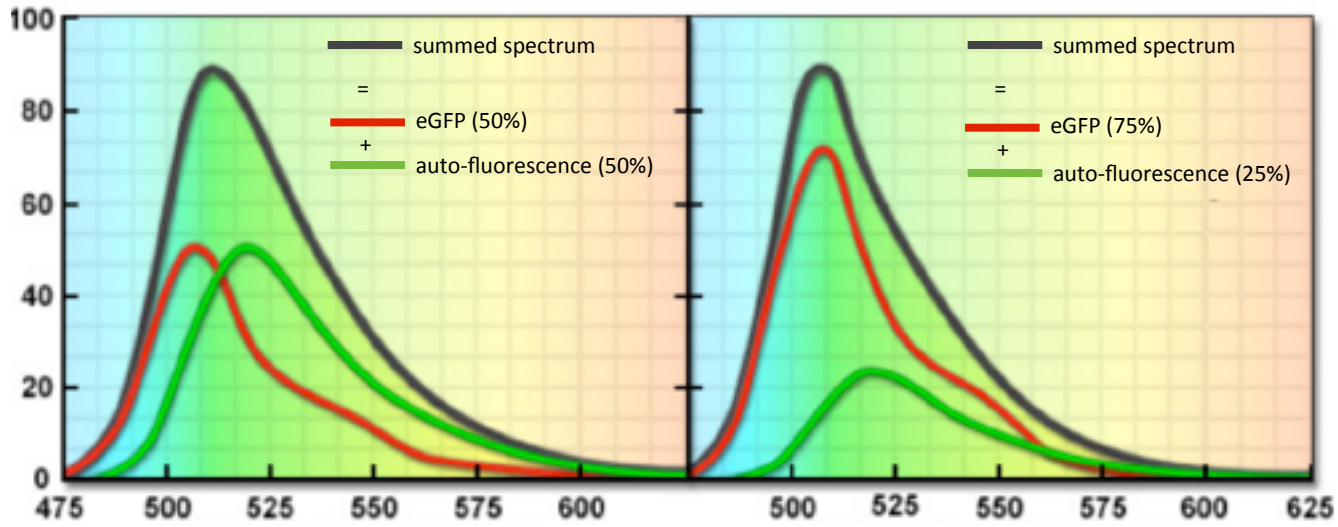
Spectral Unmixing

At each pixel:



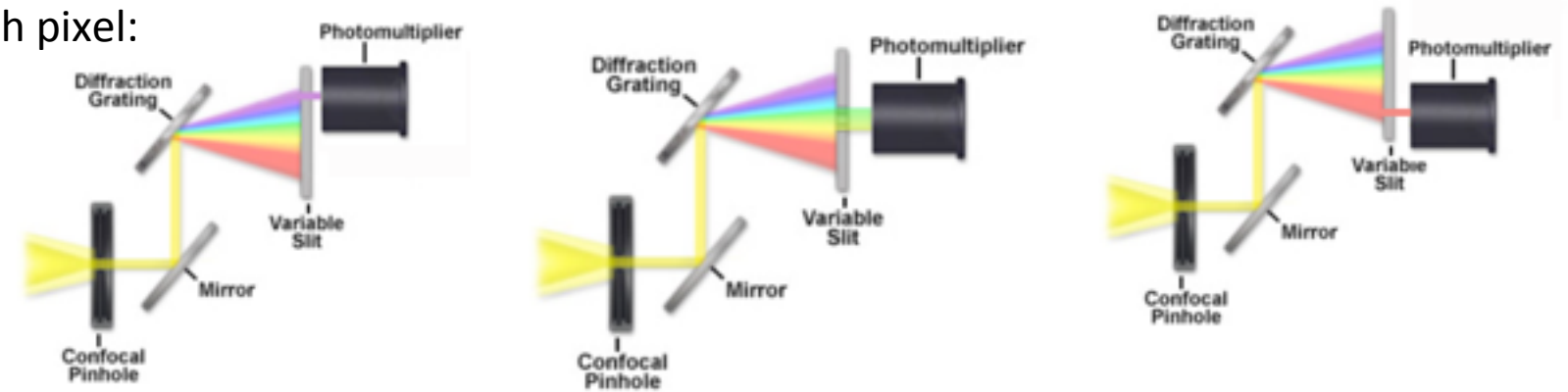


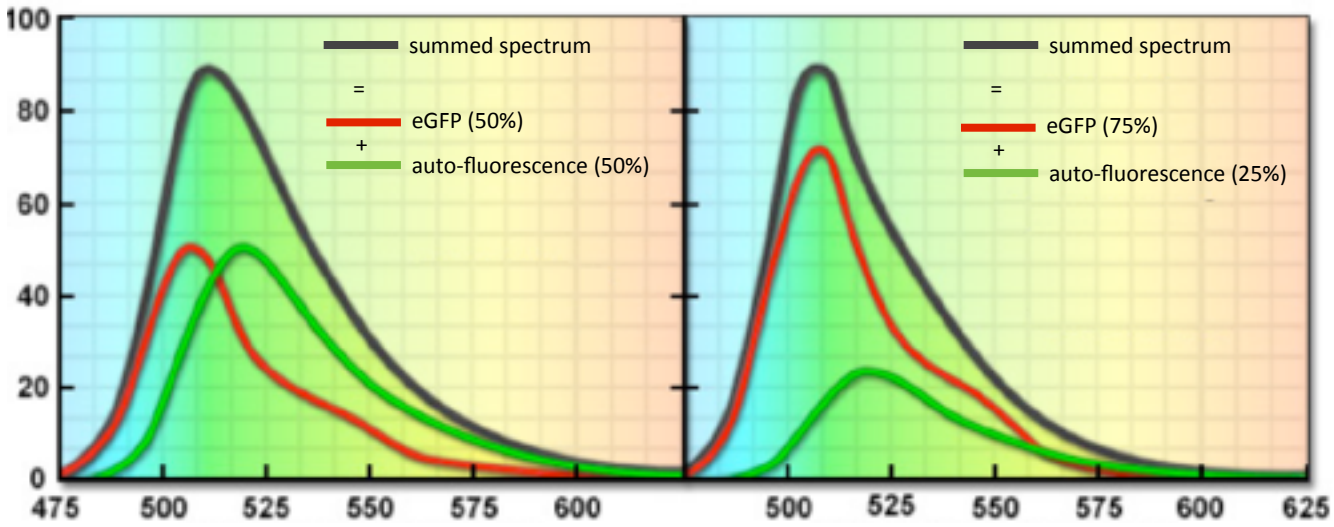




Spectral Unmixing

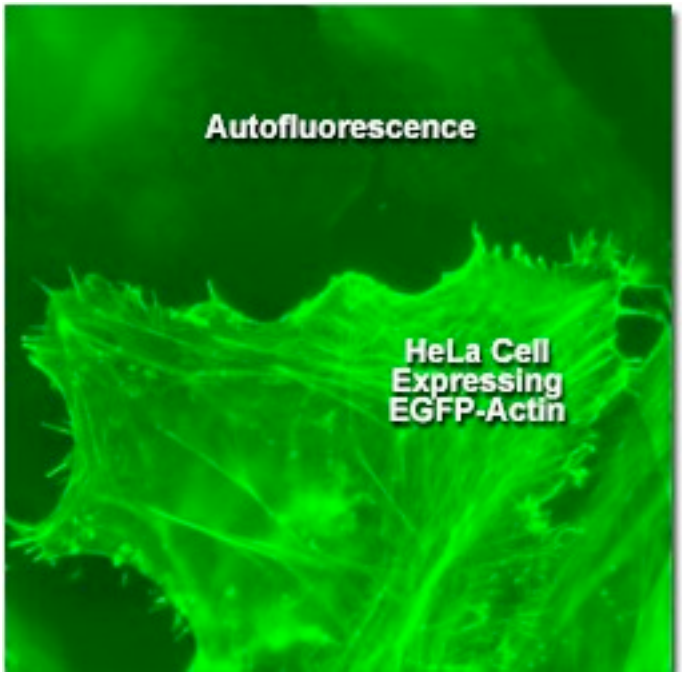
At each pixel:

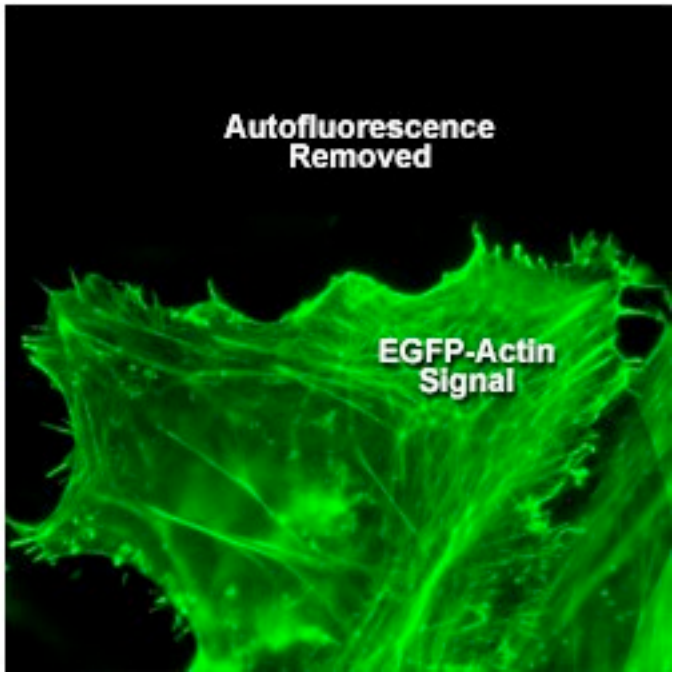




Match the summed spectrum with all possible summed combinations from a library
At each pixel you therefore know the proportion of each fluorophore present

Spectral Unmixing removal of autofluorescence

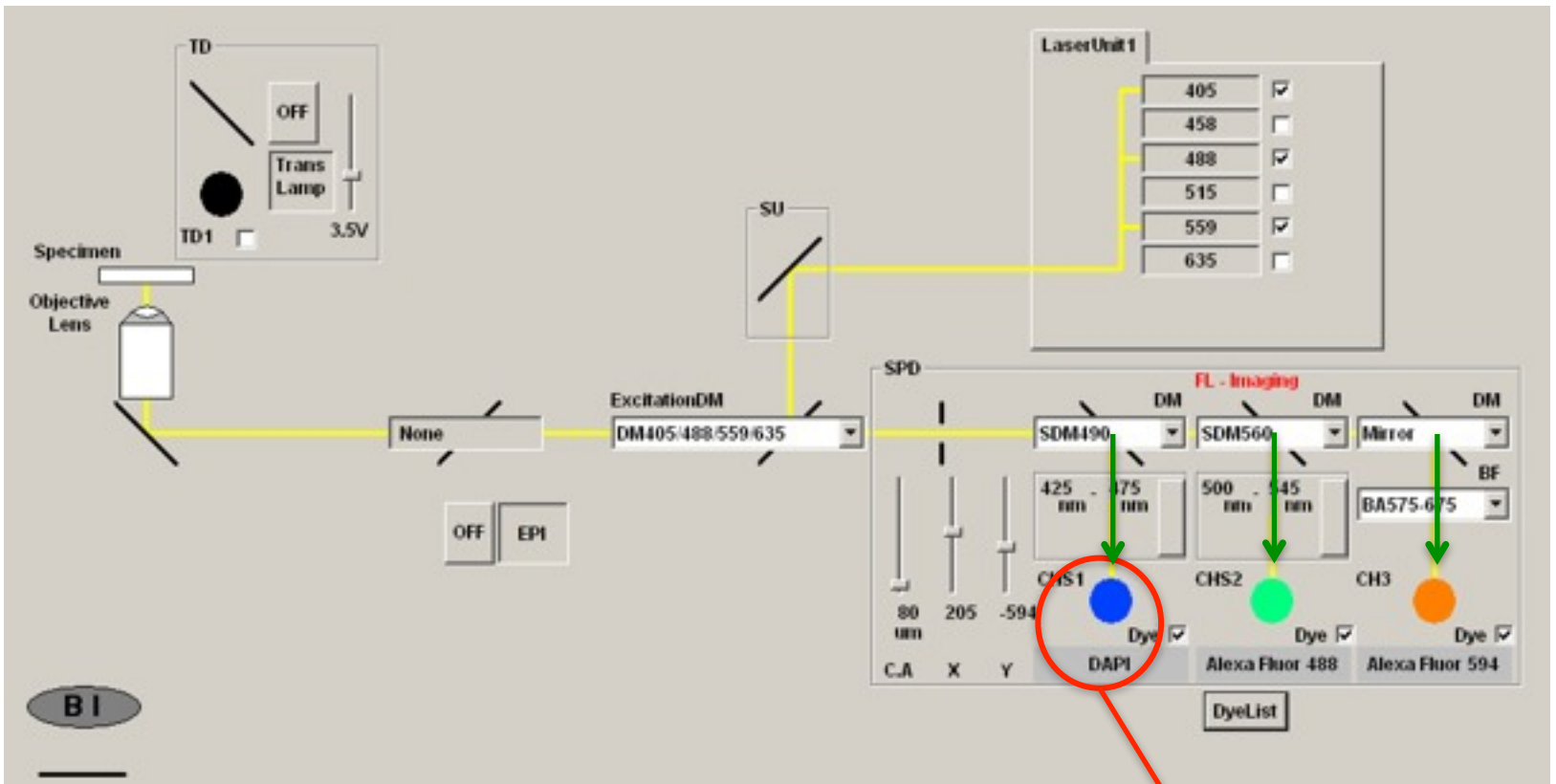




At each pixel:

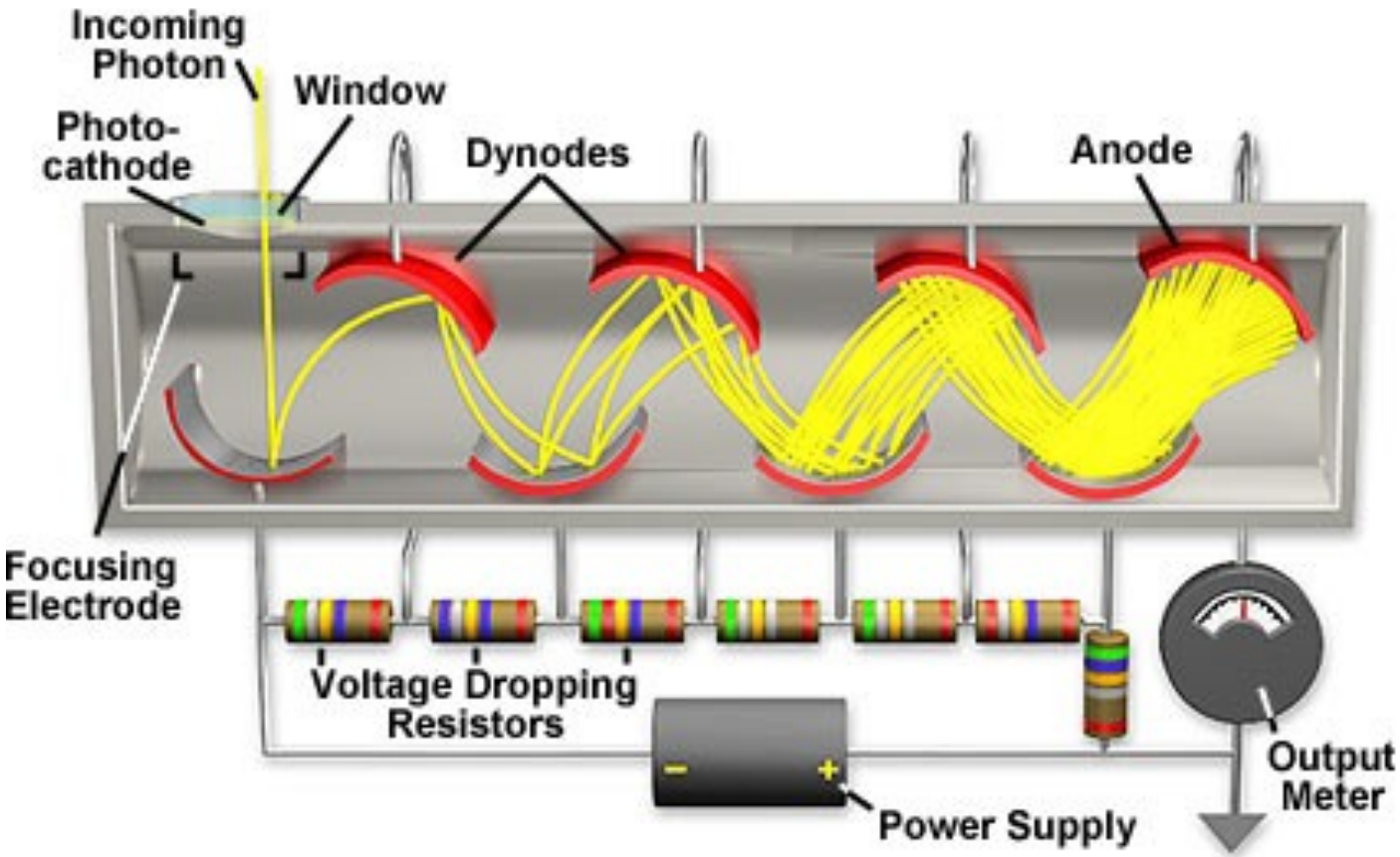
Calculate the proportion of the pixel is due to autofluorescence.
Subtract the autofluorescence from the ‘true’ GFP value.

PMT – Photon Multiplier Tube

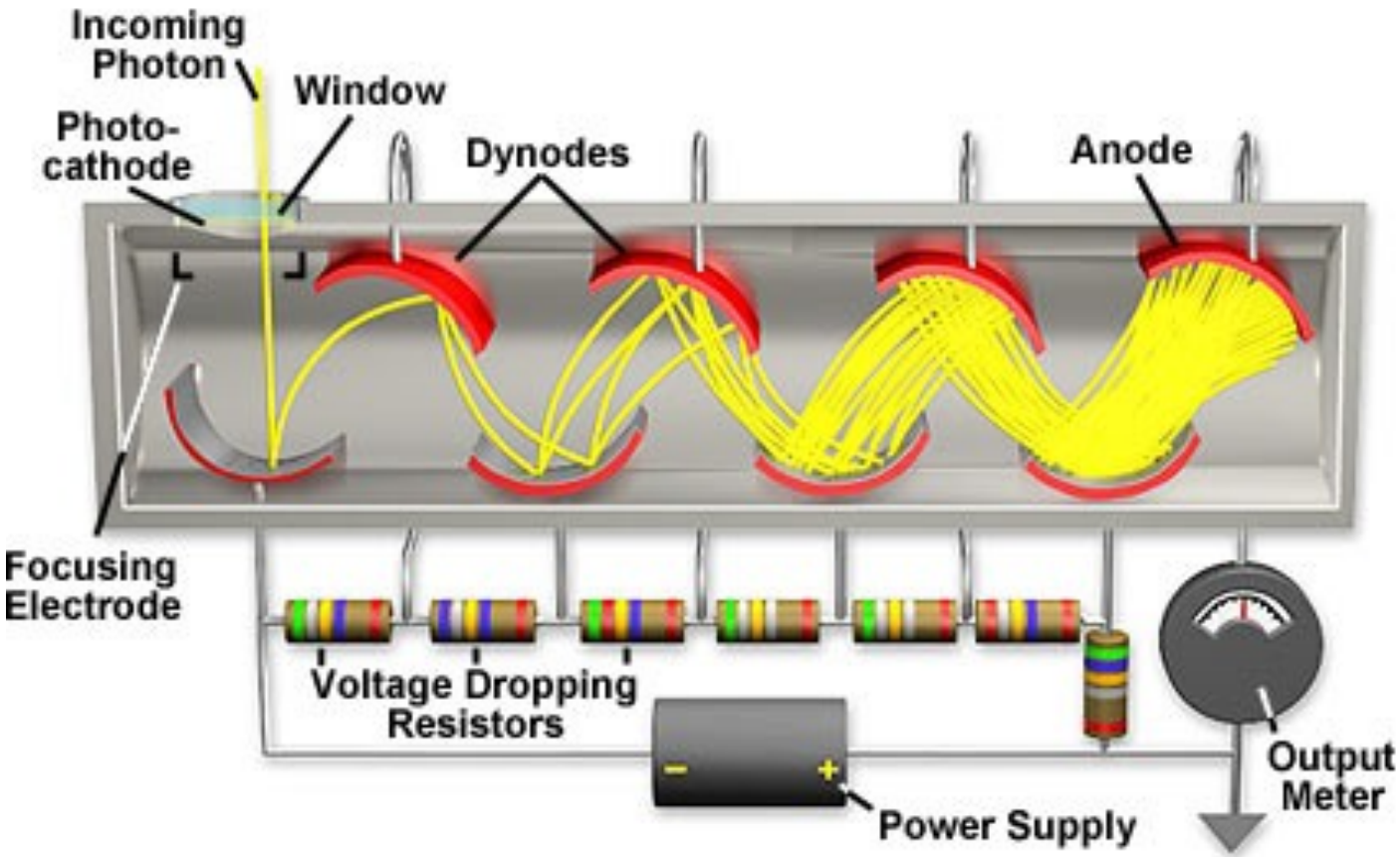


PMT detectors

PMT – Photon Multiplier Tube

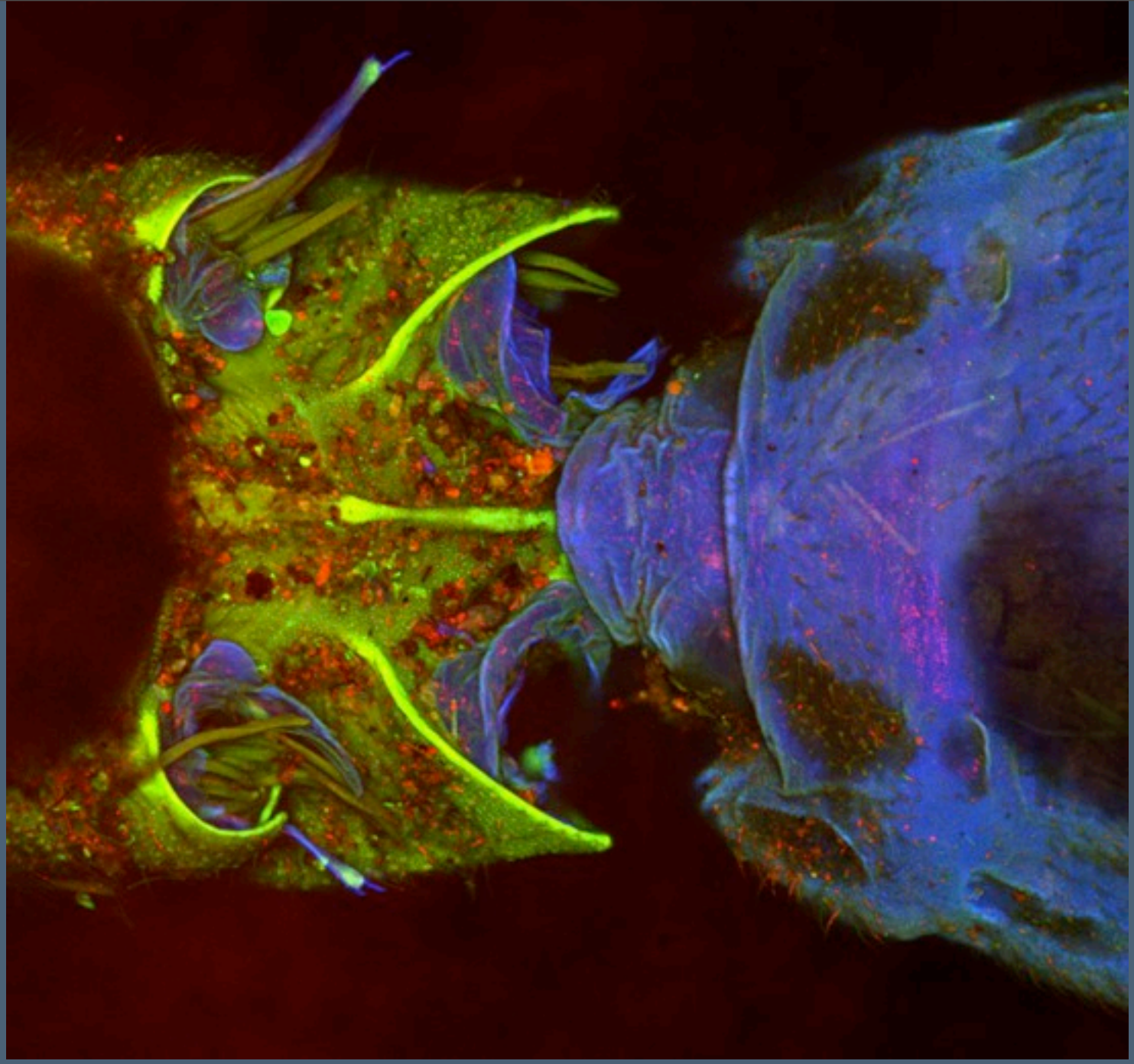


PMT – Photon Multiplier Tube



Very Low Noise

Huge Signal Amplification (~1x10)



insect autofluorescence

‘Airy-Scan’ technology

THEORY

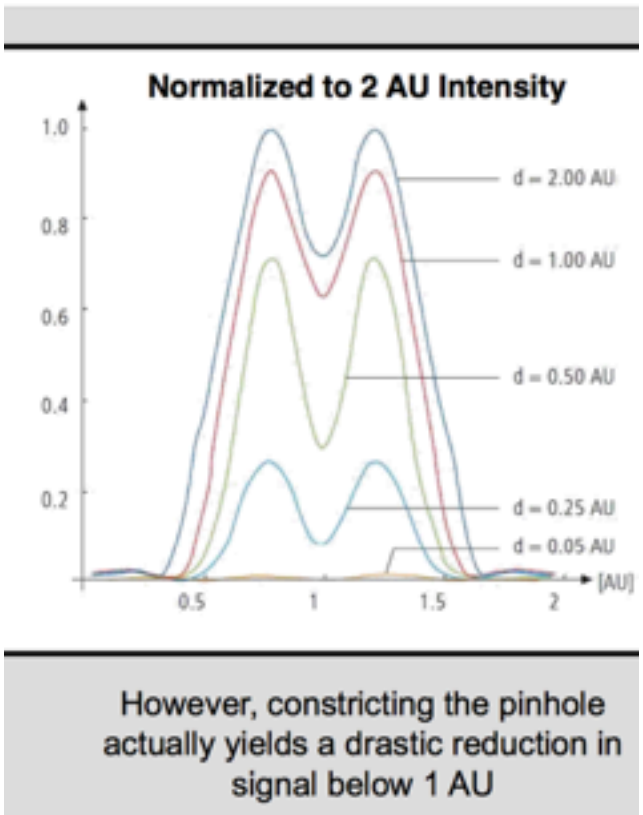
‘Airy-Scan’ technology

Small Pinhole, signal loss but resolution gain..

THEORY

‘Airy-Scan’ technology

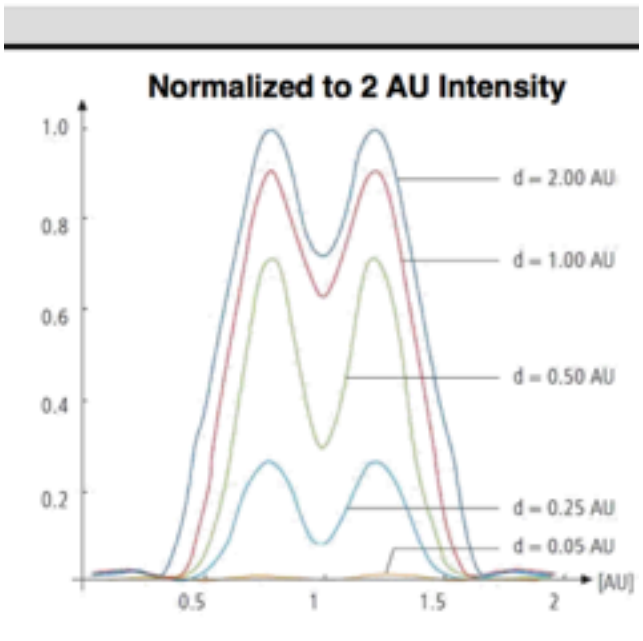
Small Pinhole, signal loss but resolution gain..



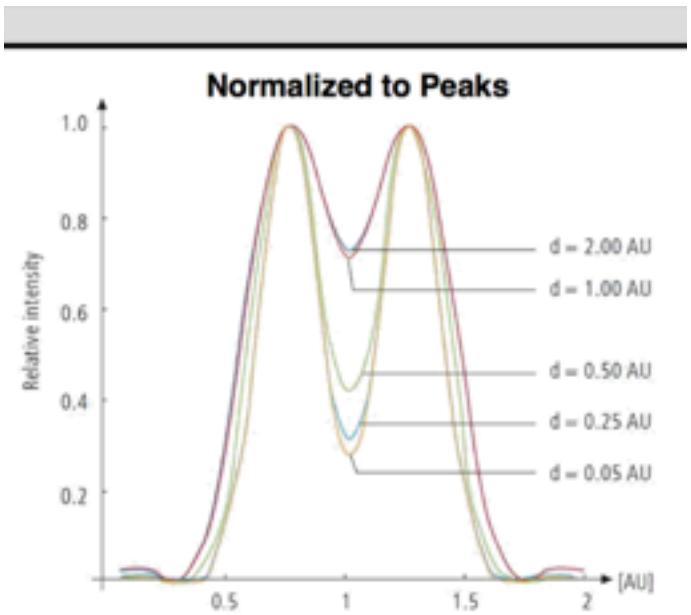
THEORY

‘Airy-Scan’ technology

Small Pinhole, signal loss but resolution gain..



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

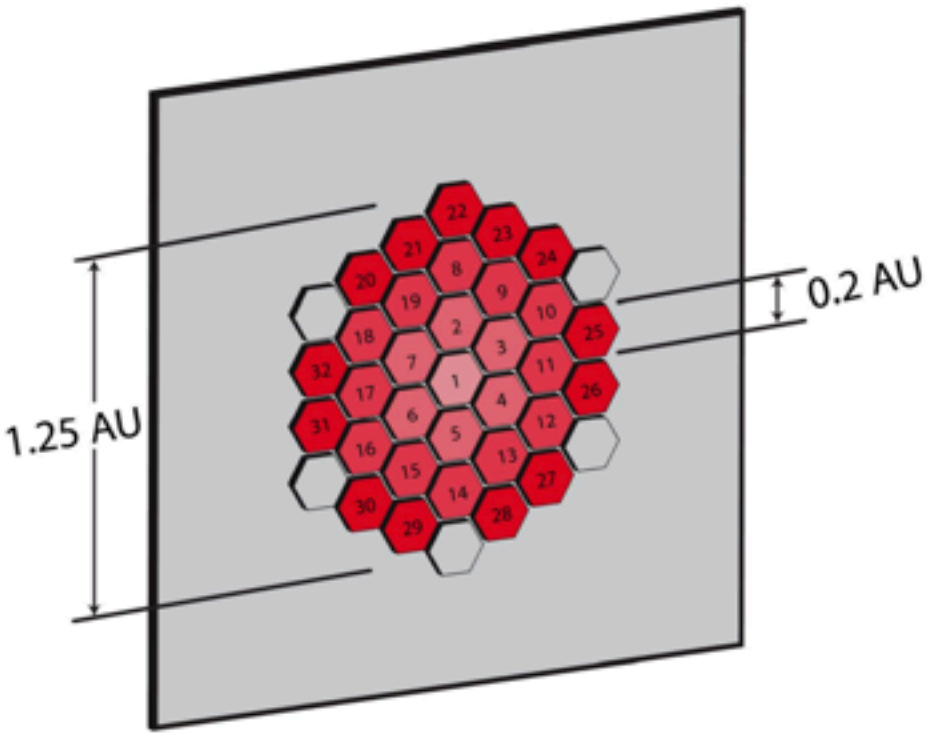


Small pinhole diameters lead to improved resolution steadily until about 0.2 AU, results in deeper dips between two objects

THEORY

‘Airy-Scan’ technology

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

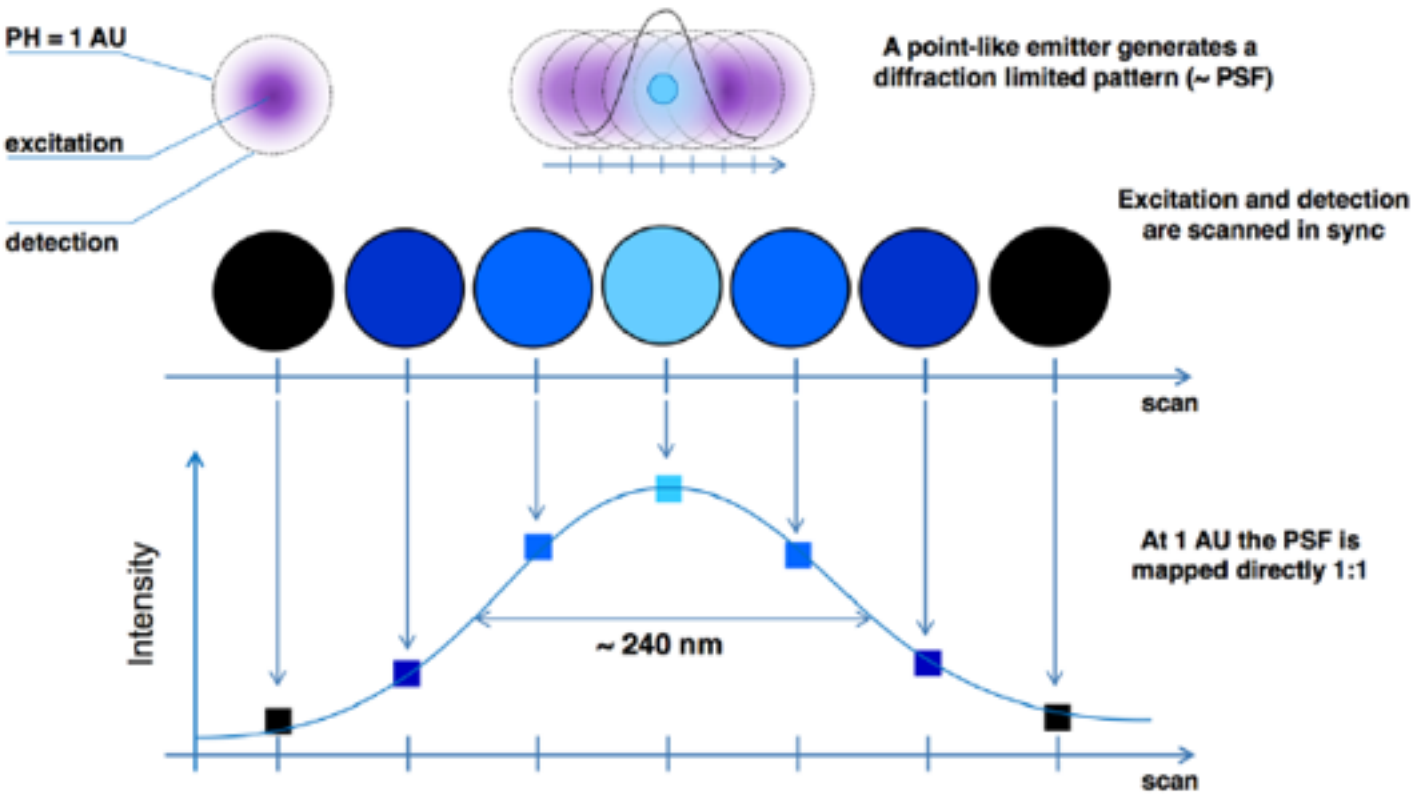


- 32 GaAsP detectors in hexagonal lattice
- Each detector approximately 0.2 AU in diameter
- Total detection area approximately 1.25 AU in diameter
- **Simultaneous improvement in resolution and signal**

THEORY

‘Airy-Scan’ technology

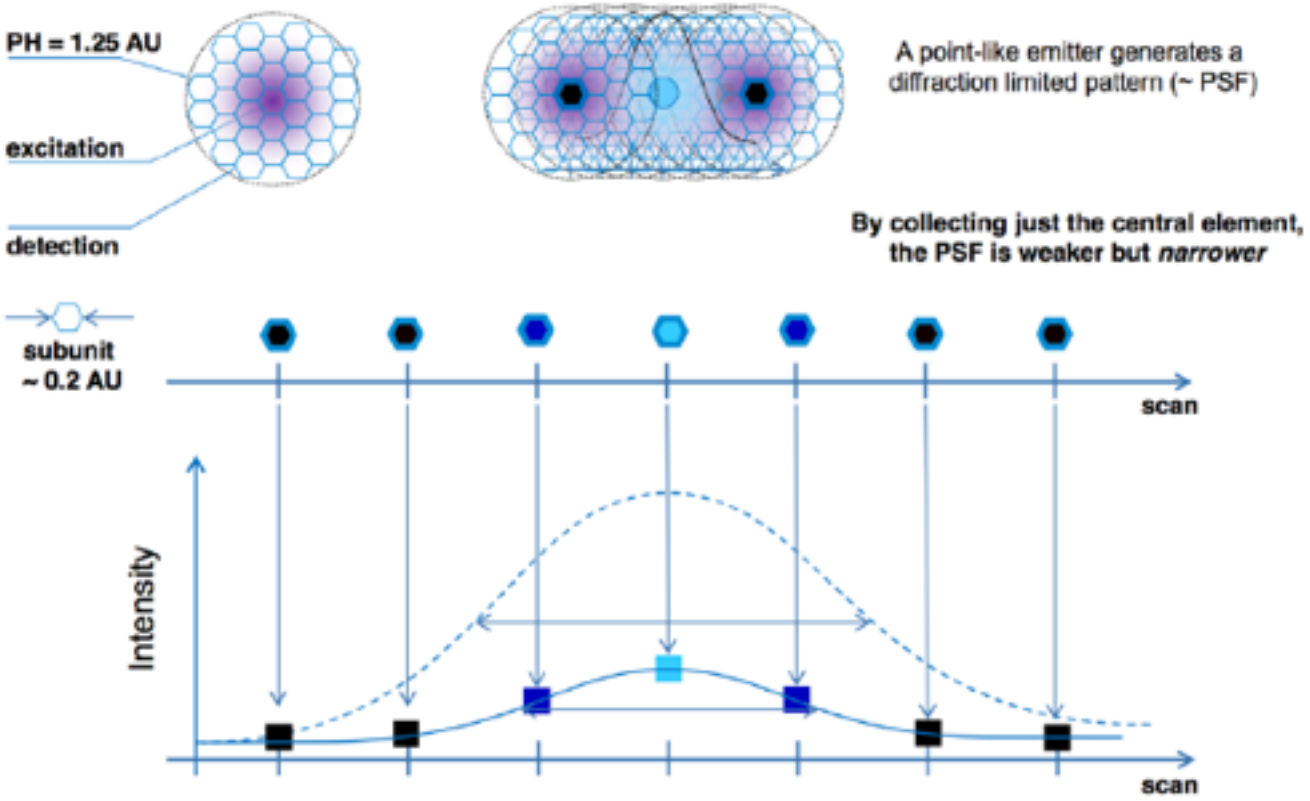
point of light scanned with 1AU ‘standard’ detector



THEORY

‘Airy-Scan’ technology

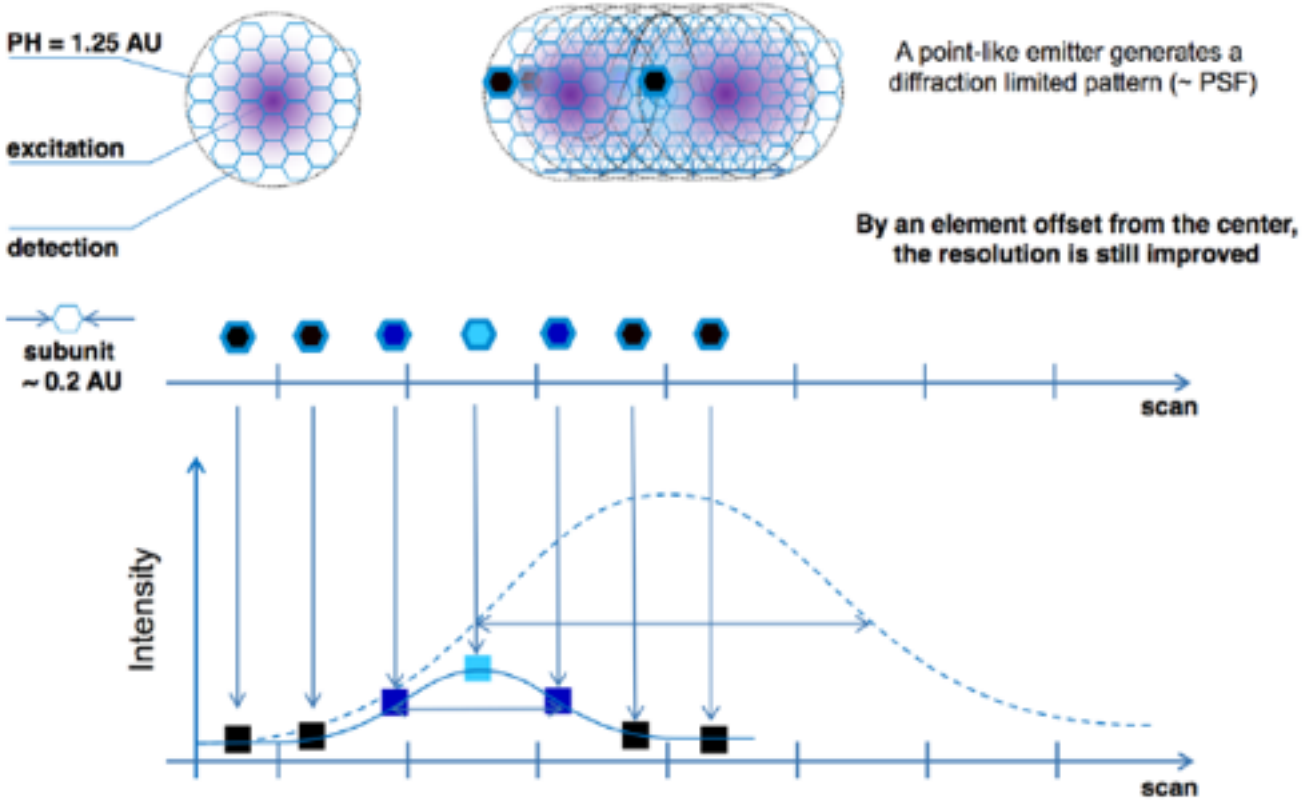
point of light scanned with 0.2AU ‘Airyscan’ detector
>increased resolution



THEORY

‘Airy-Scan’ technology

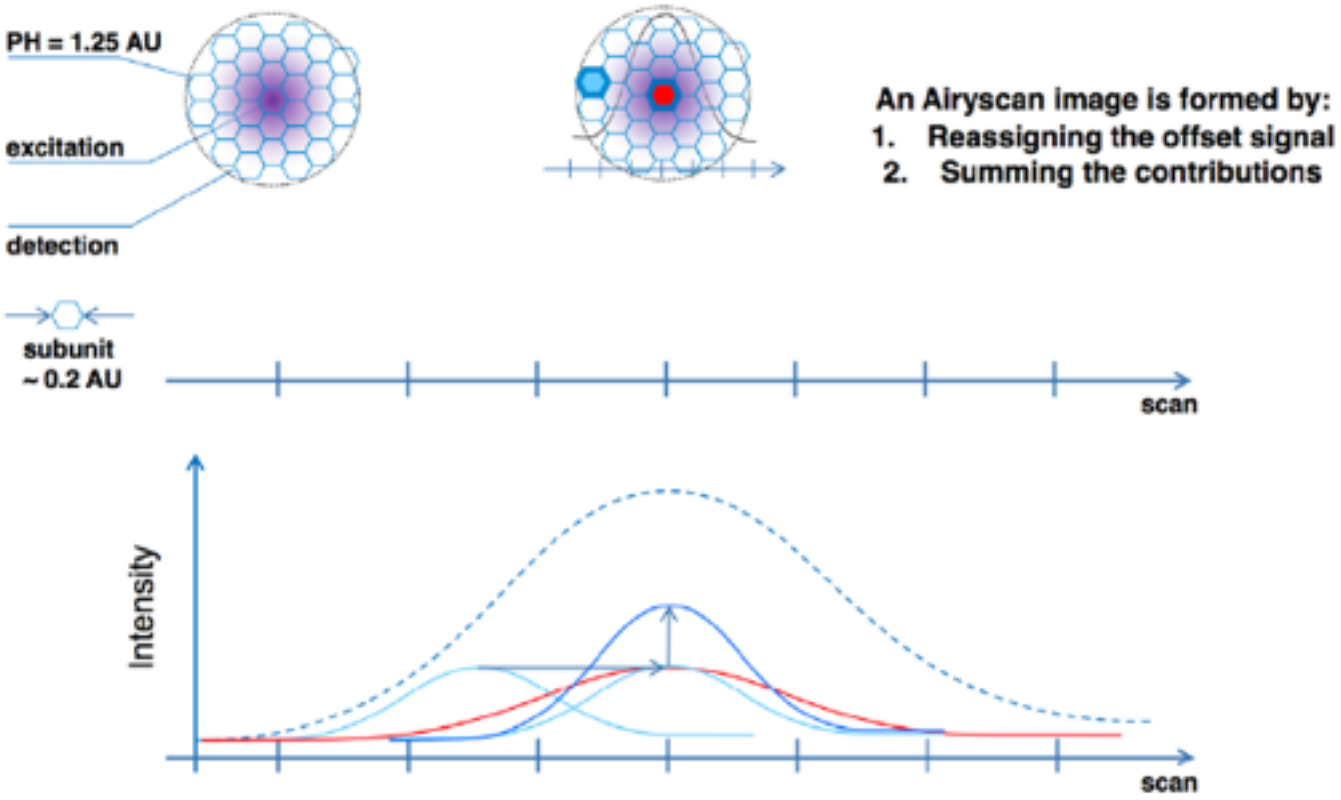
each 0.2AU ‘Airyscan’ detector provides
>increased resolution



THEORY

‘Airy-Scan’ technology

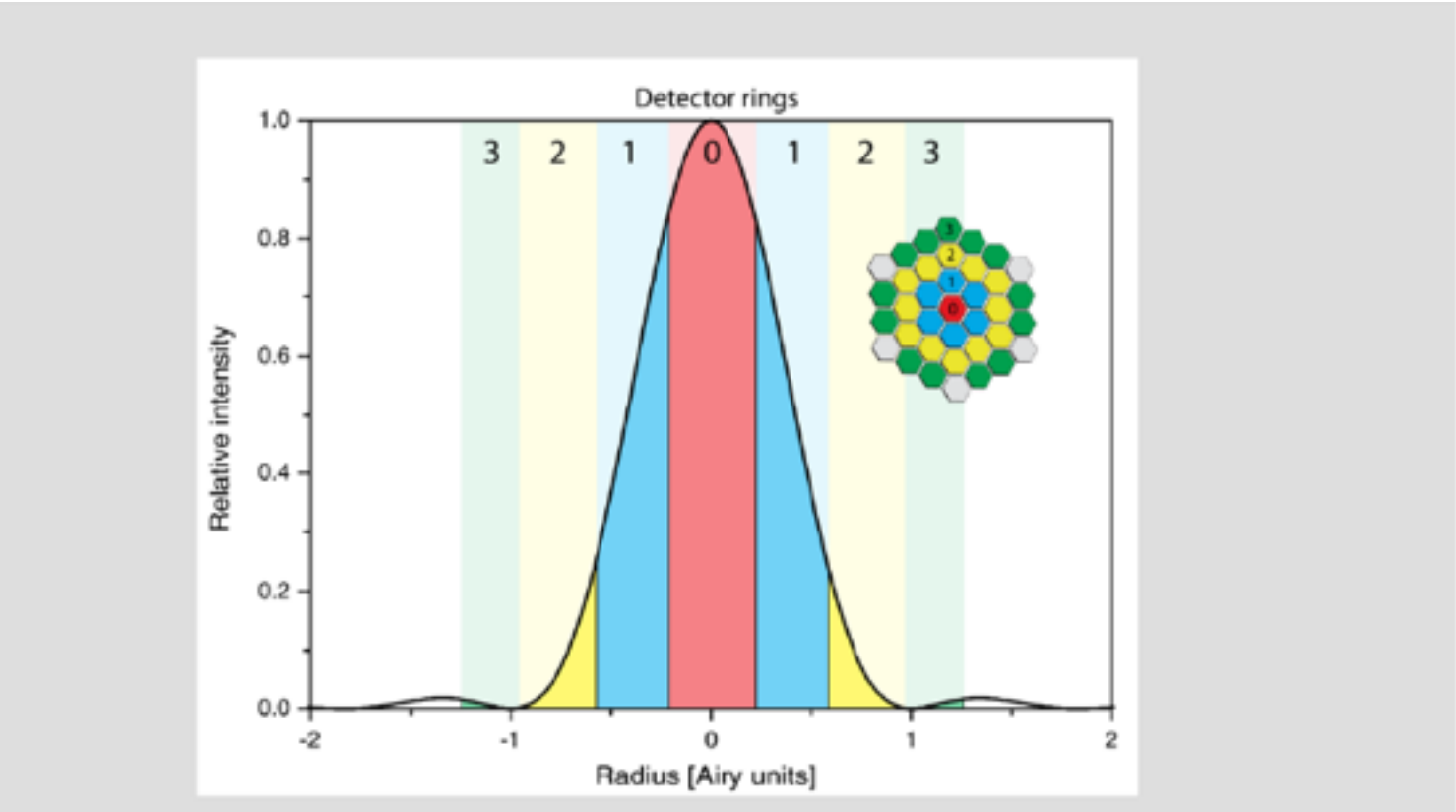
each 0.2AU ‘Airyscan’ detector info
is reassigned and summed

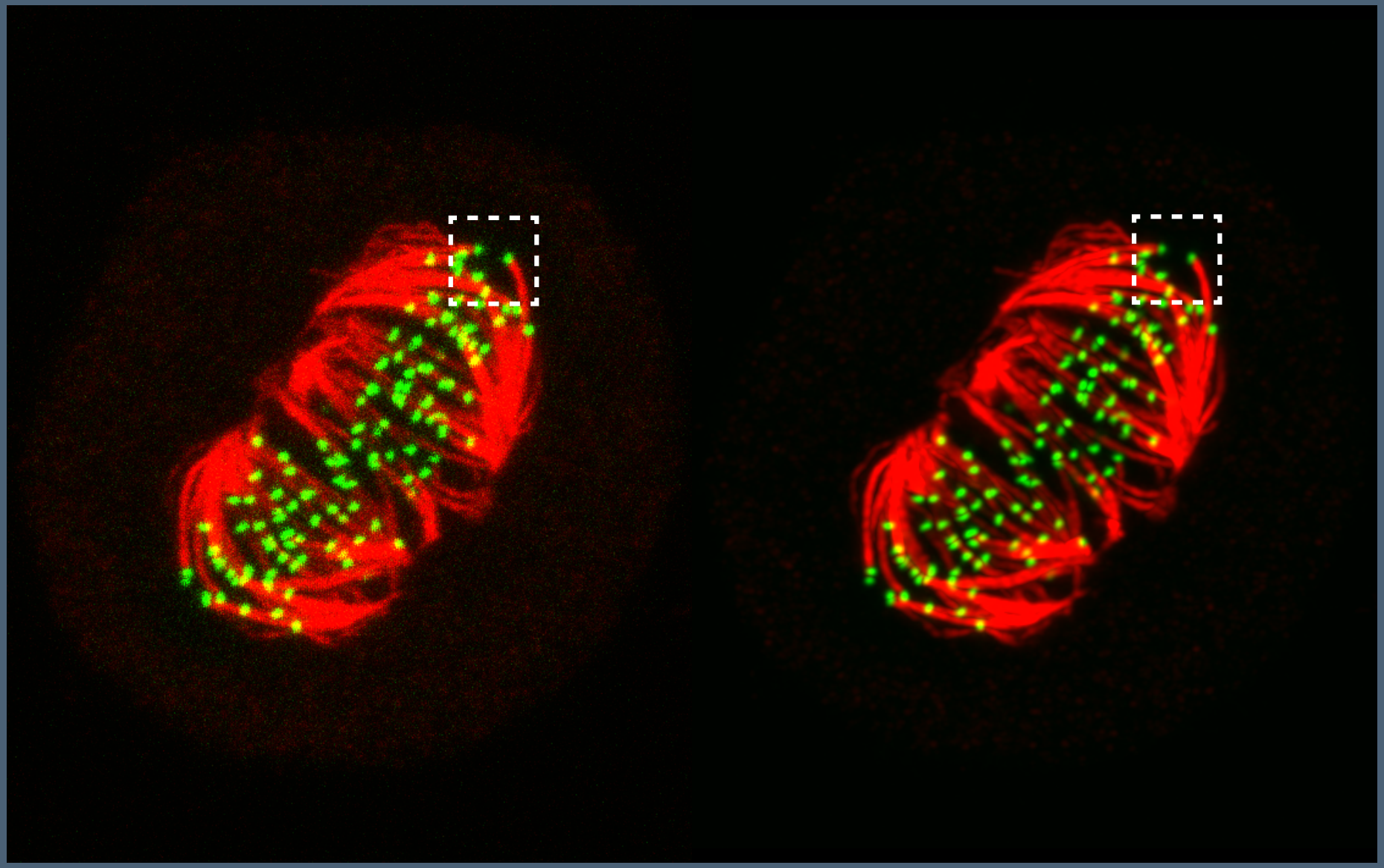


THEORY

‘Airy-Scan’ technology

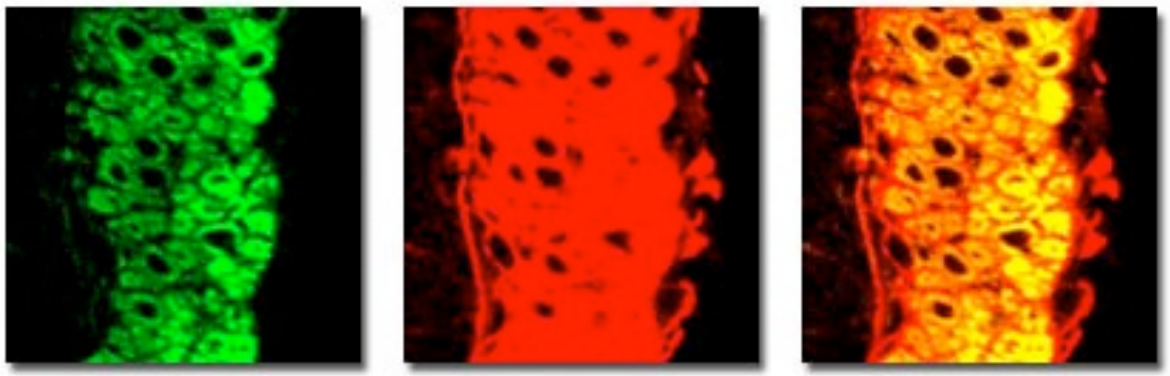
effective PSF is now smaller.. > increased resolution (1.4x - 1.7x)



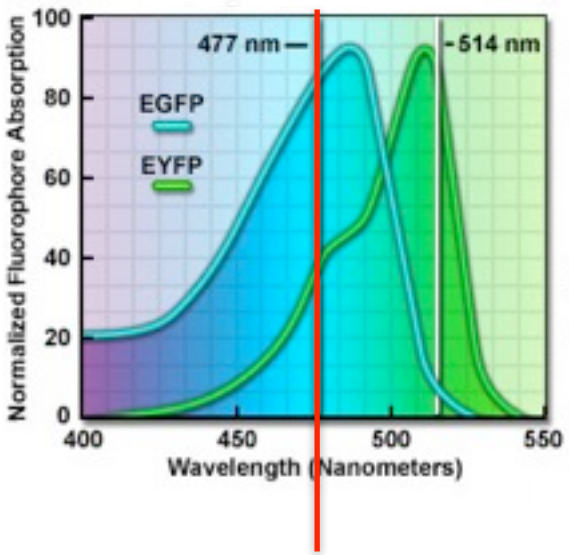


kinetochores (James Banecroft, Gruneberg Lab)

‘bleed-through’

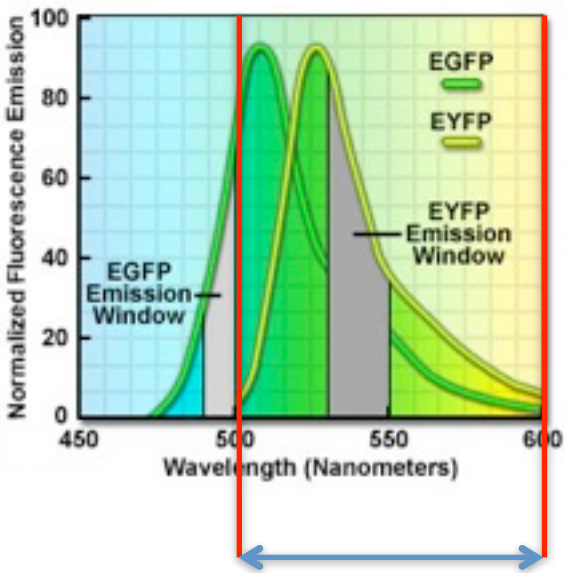


Absorption spectral profiles



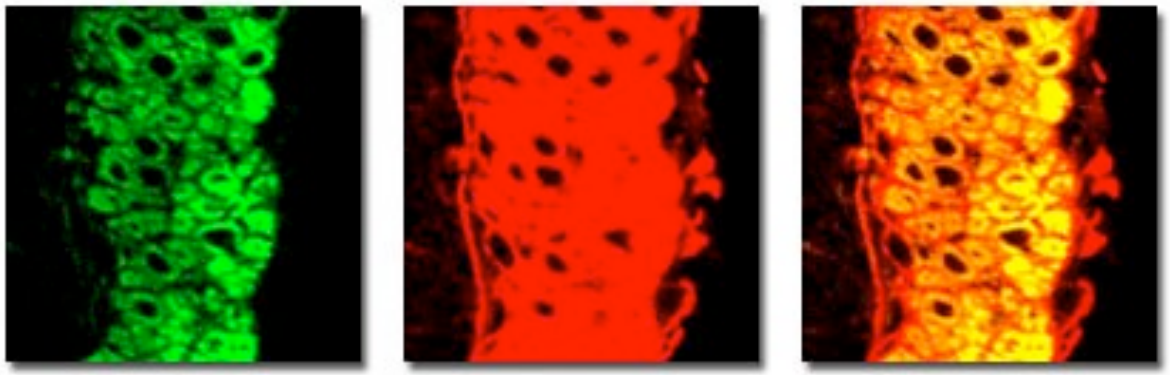
Excite at 477nm

Emission spectral profiles

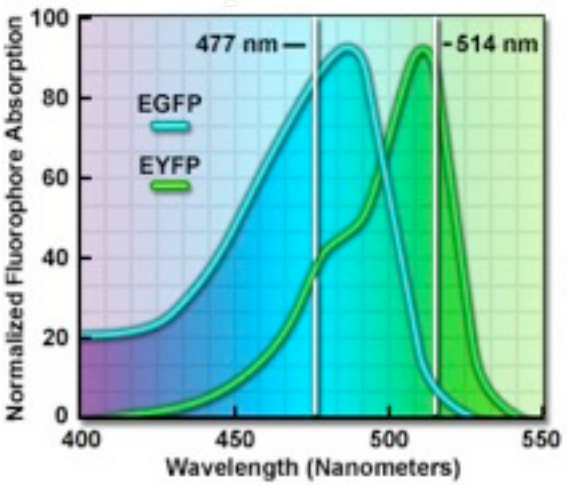


overlapping emission

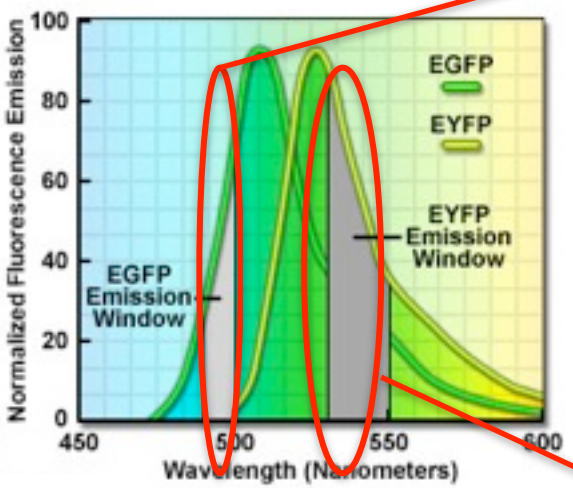
minimising‘bleed-through’ Variable Slits

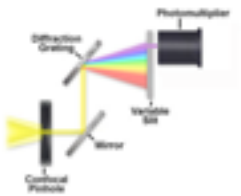


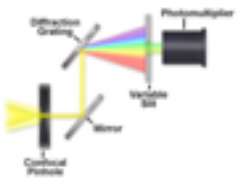
Absorption spectral profiles



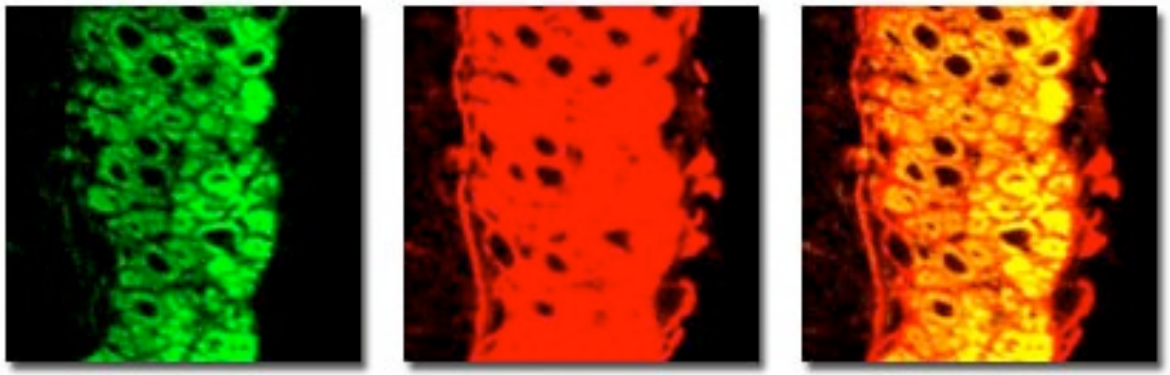
Emission spectral profiles



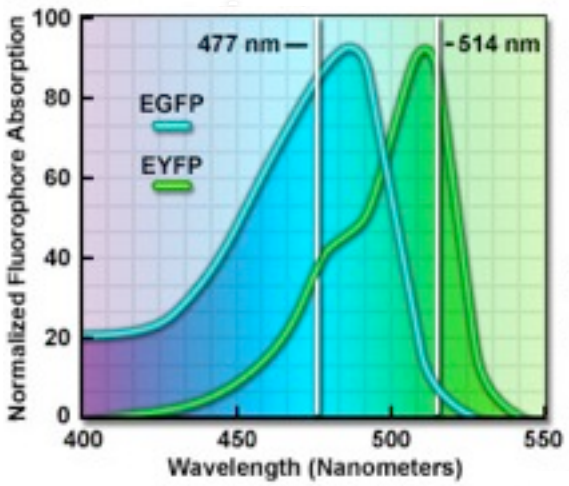




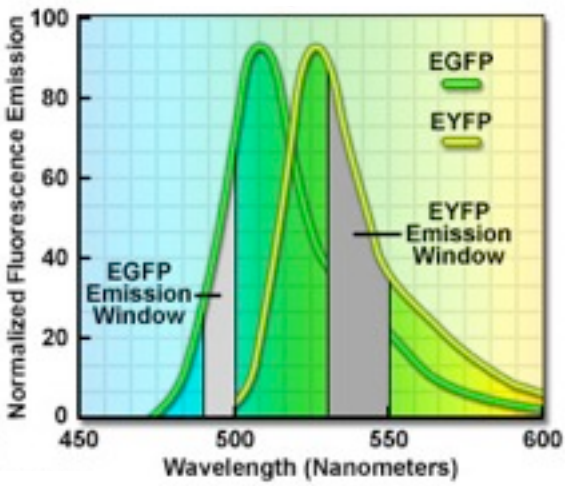
minimising‘bleed-through’ Sequential Scanning



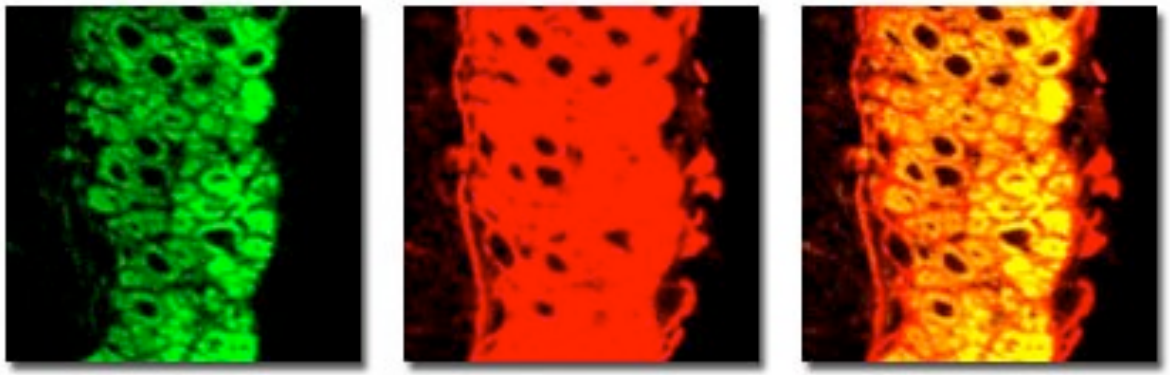
Absorption spectral profiles



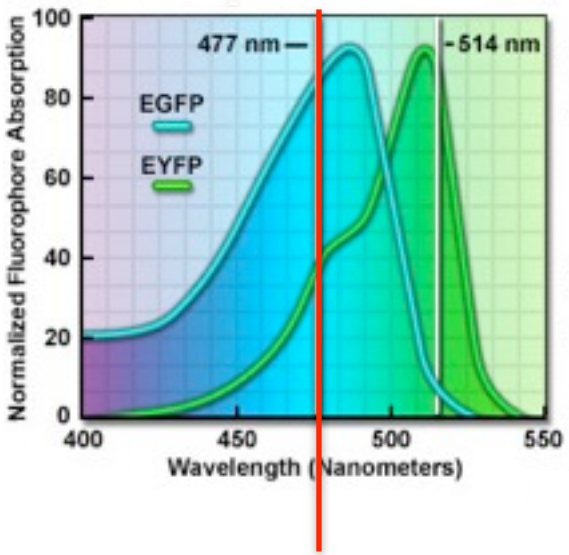
Emission spectral profiles



minimising‘bleed-through’ Sequential Scanning

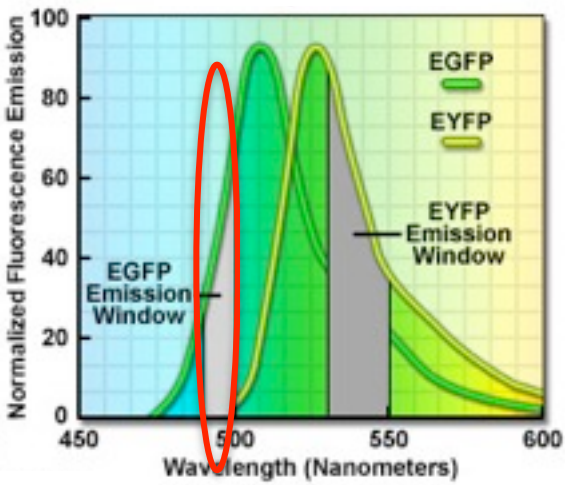


Absorption spectral profiles

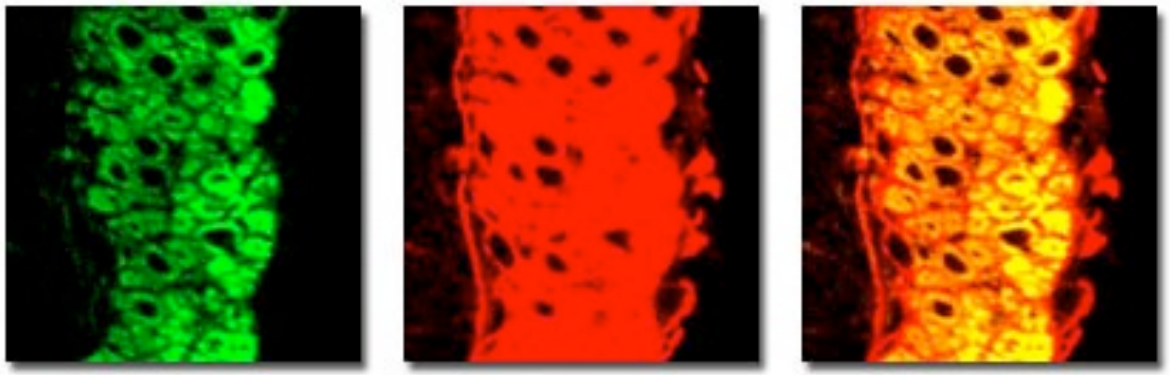


Excite at 477nm

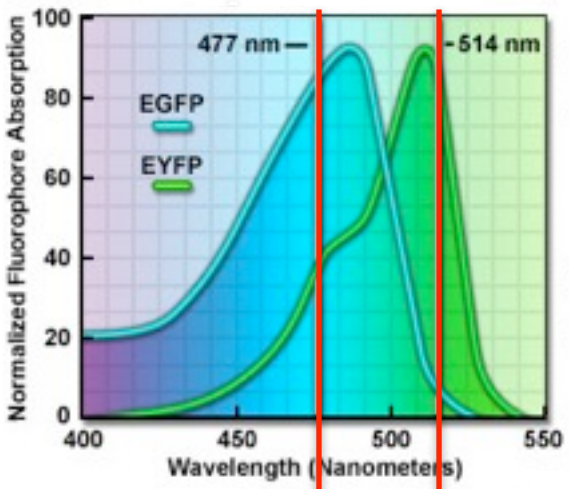
Emission spectral profiles



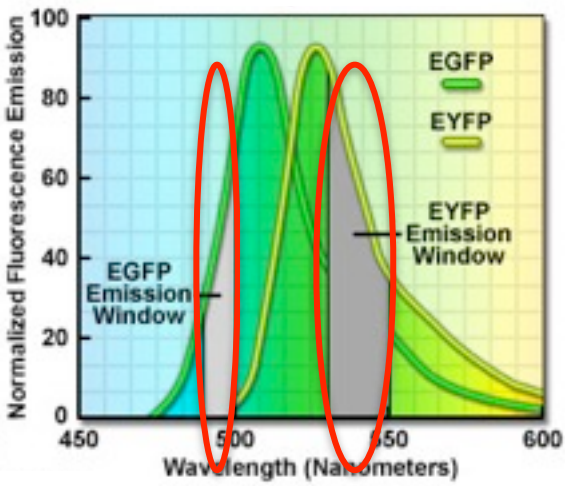
minimising‘bleed-through’ Sequential Scanning



Absorption spectral profiles



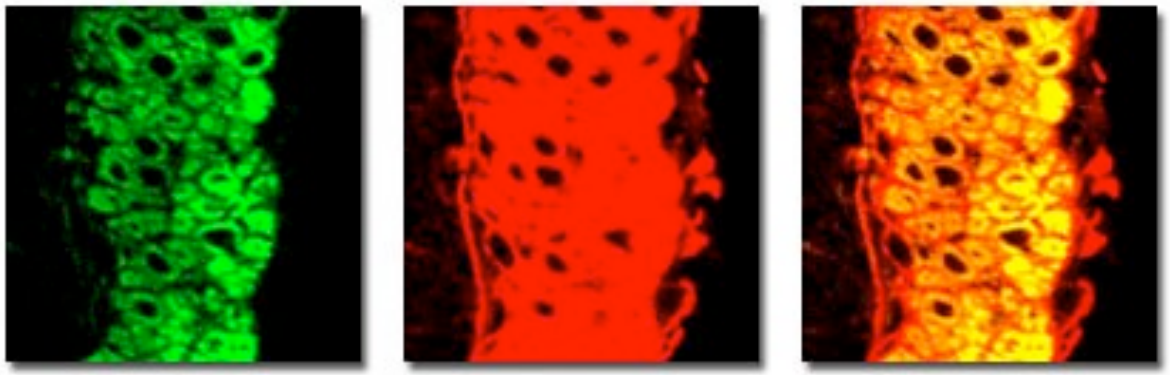
Emission spectral profiles



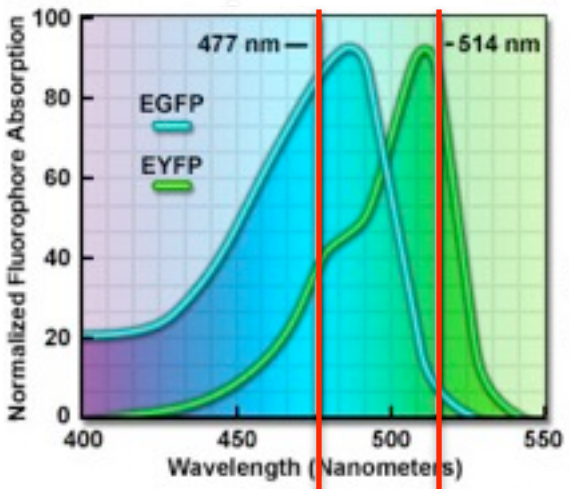
Excite at 477nm

Excite at 514nm

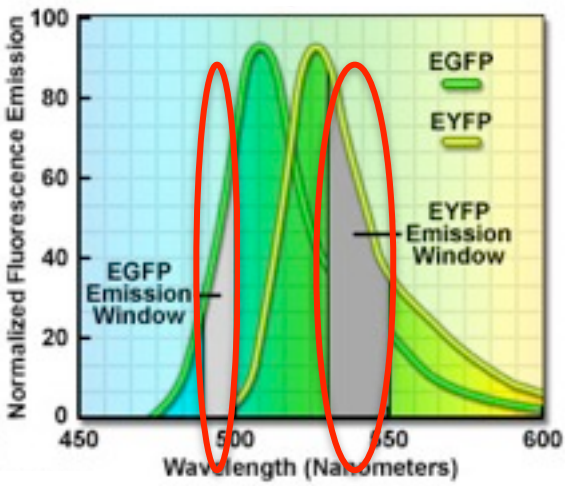
minimising‘bleed-through’ Sequential Scanning



Absorption spectral profiles



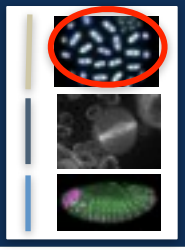
Emission spectral profiles



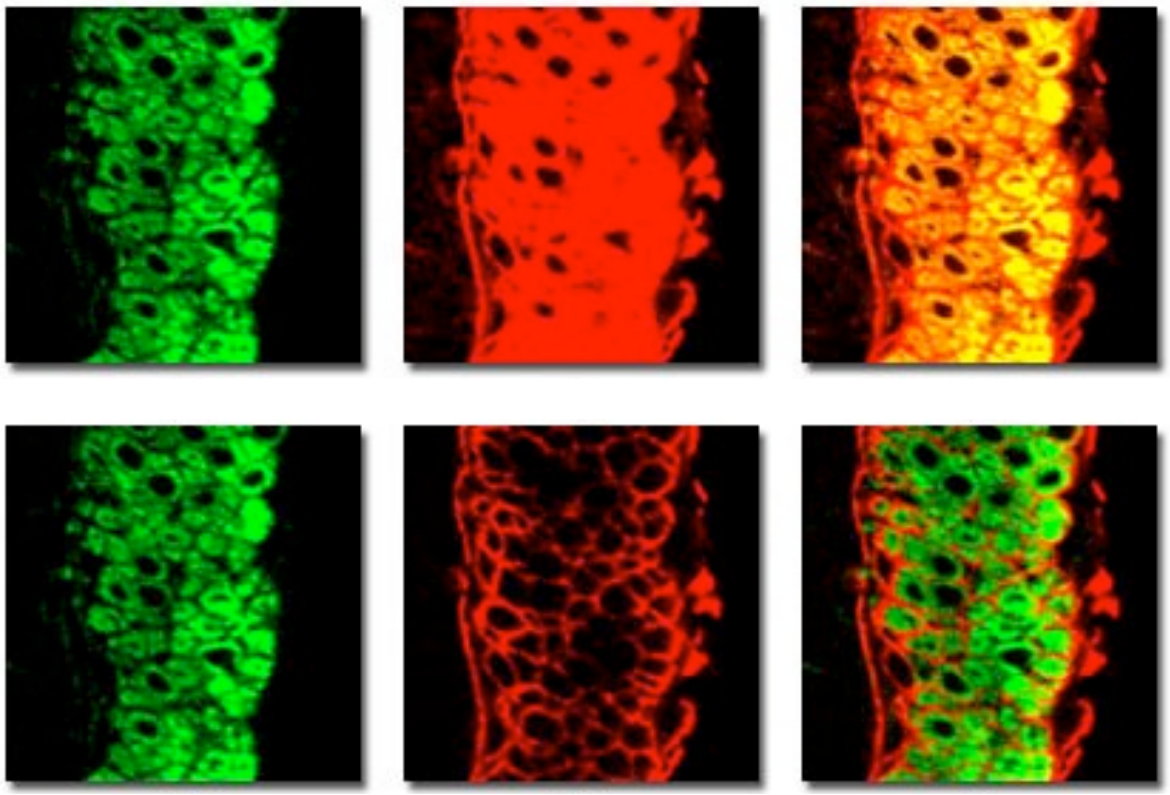
Excite at 477nm

Excite at 514nm

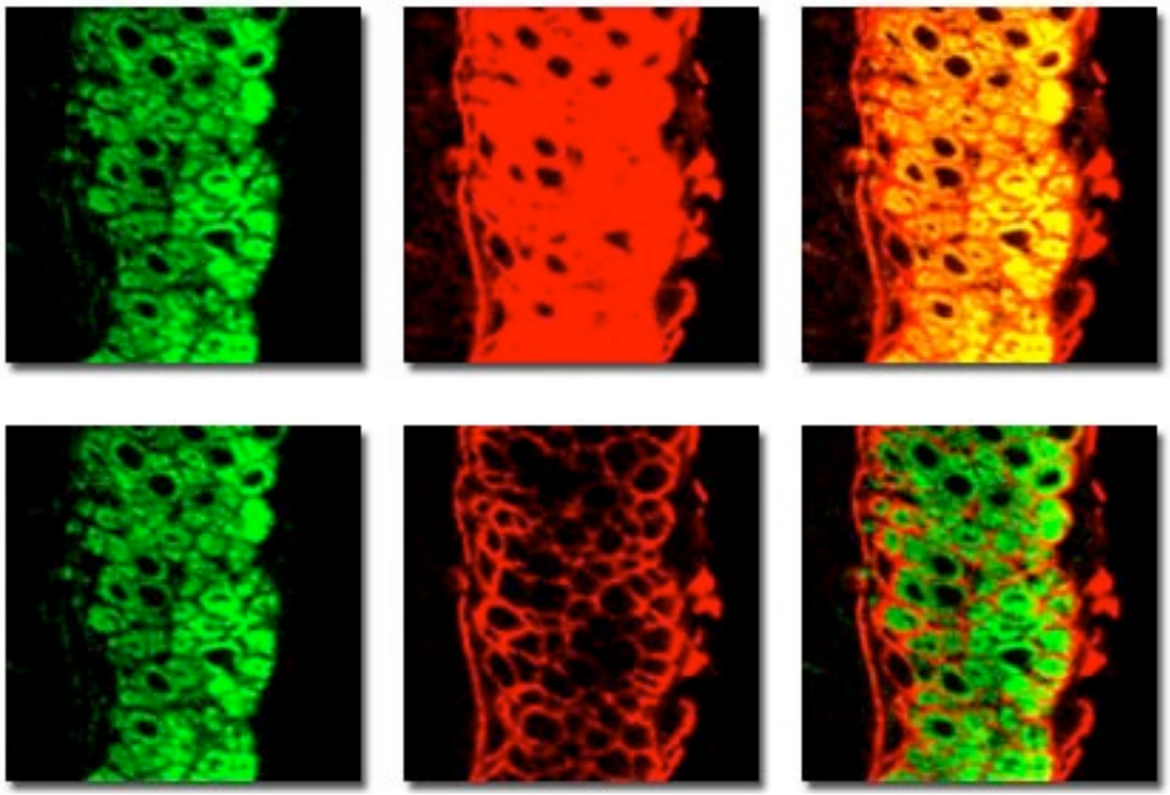
Temporal separation



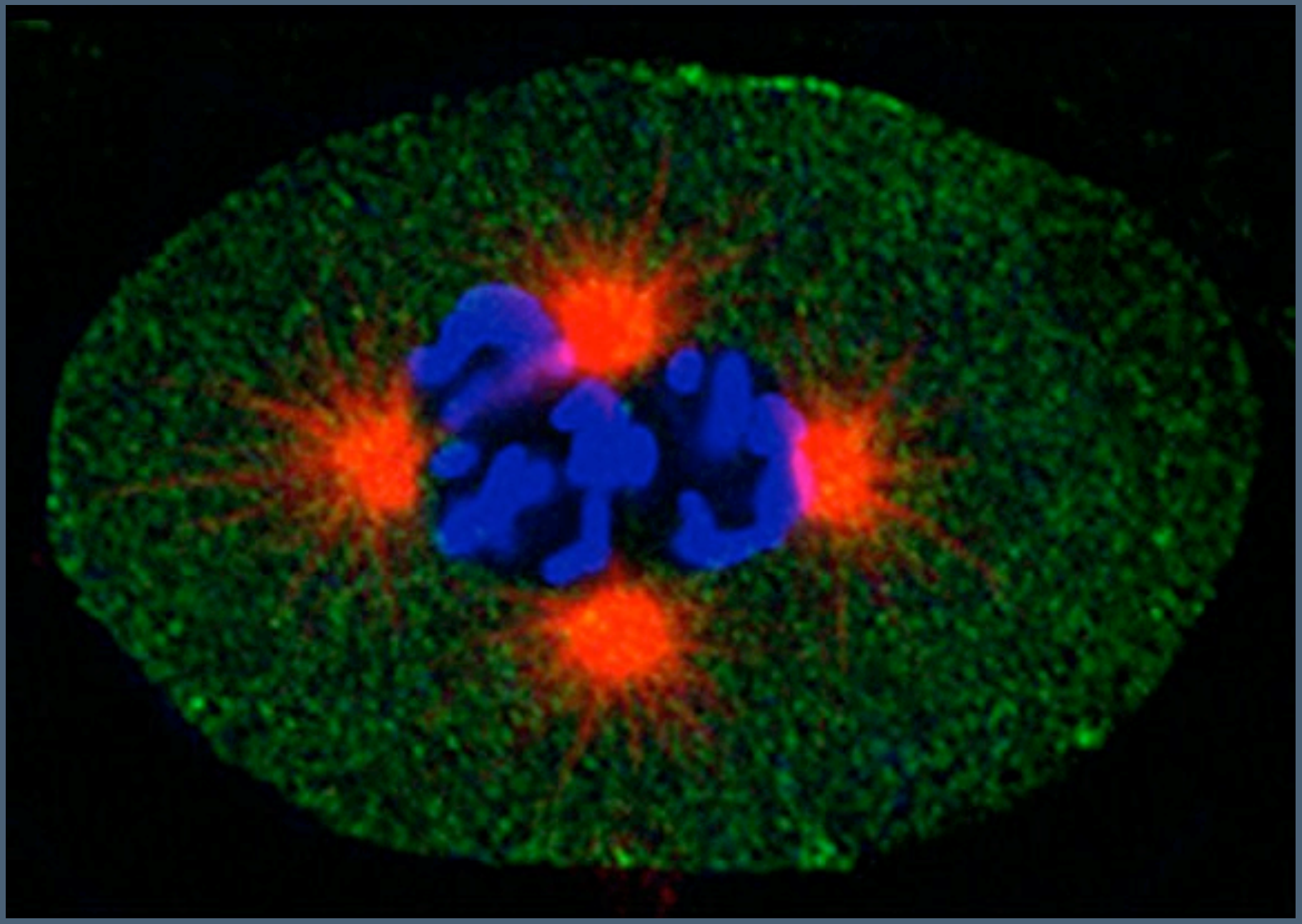
minimising ‘bleed-through’

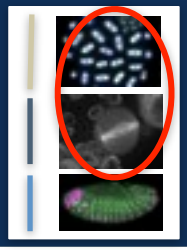


minimising ‘bleed-through’

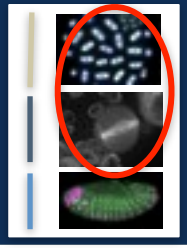


Adjust detector slit widths
Use sequential scanning

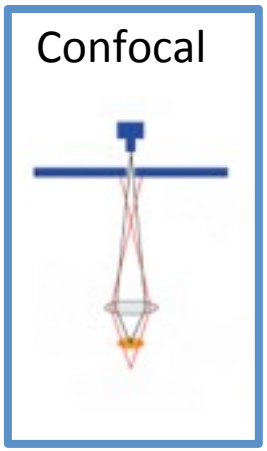


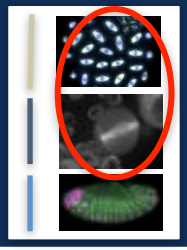


Confocal Microscopes



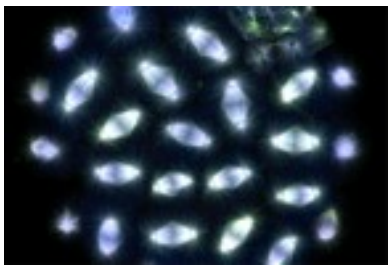
Confocal Microscopes

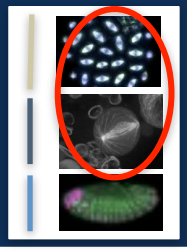




Confocal Microscopes

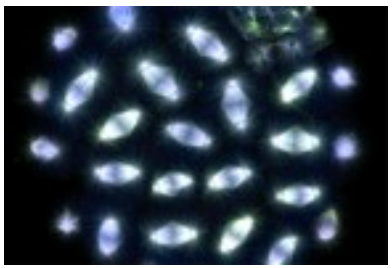
Laser Scanning



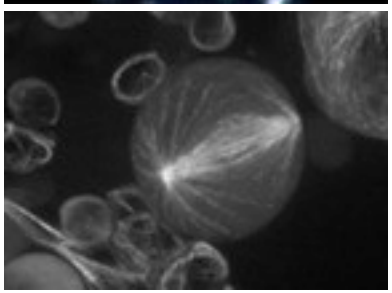


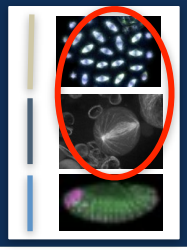
Confocal Microscopes

Laser Scanning



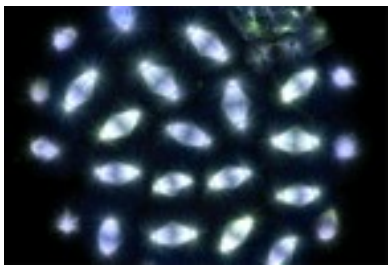
Spinning disc



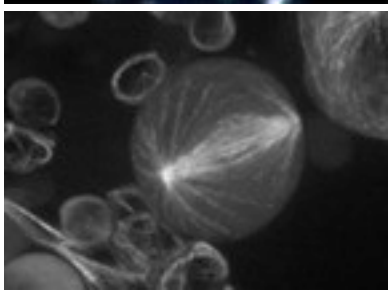


Confocal Microscopes

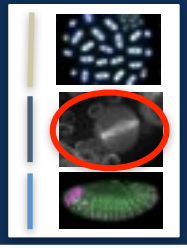
Laser Scanning



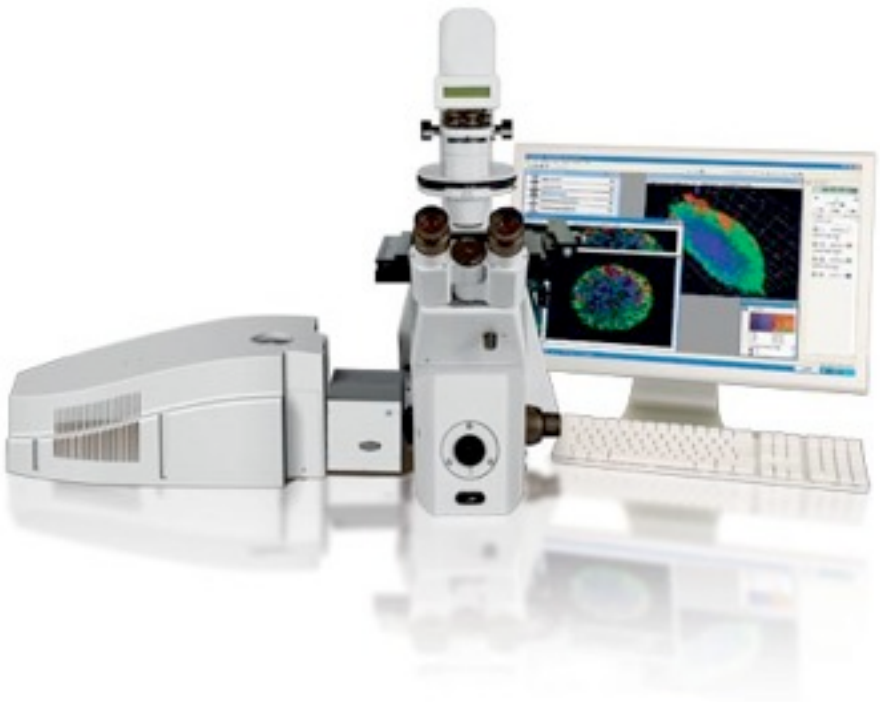
Spinning disc



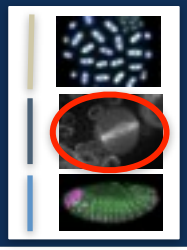
Both are confocals



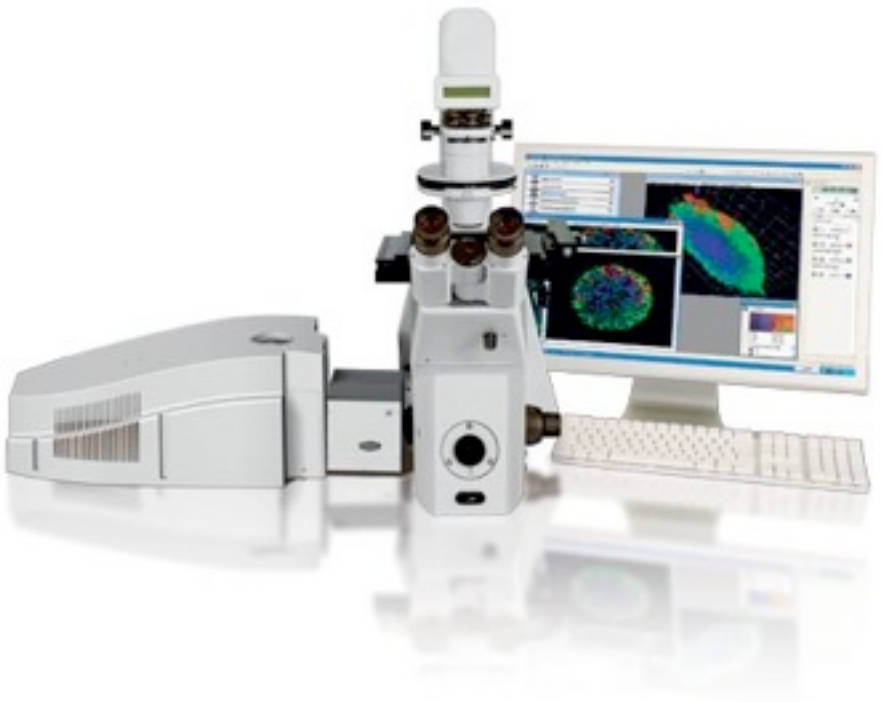
Spinning Disc Confocal



Great for live cell imaging

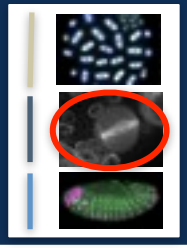


Spinning Disc Confocal

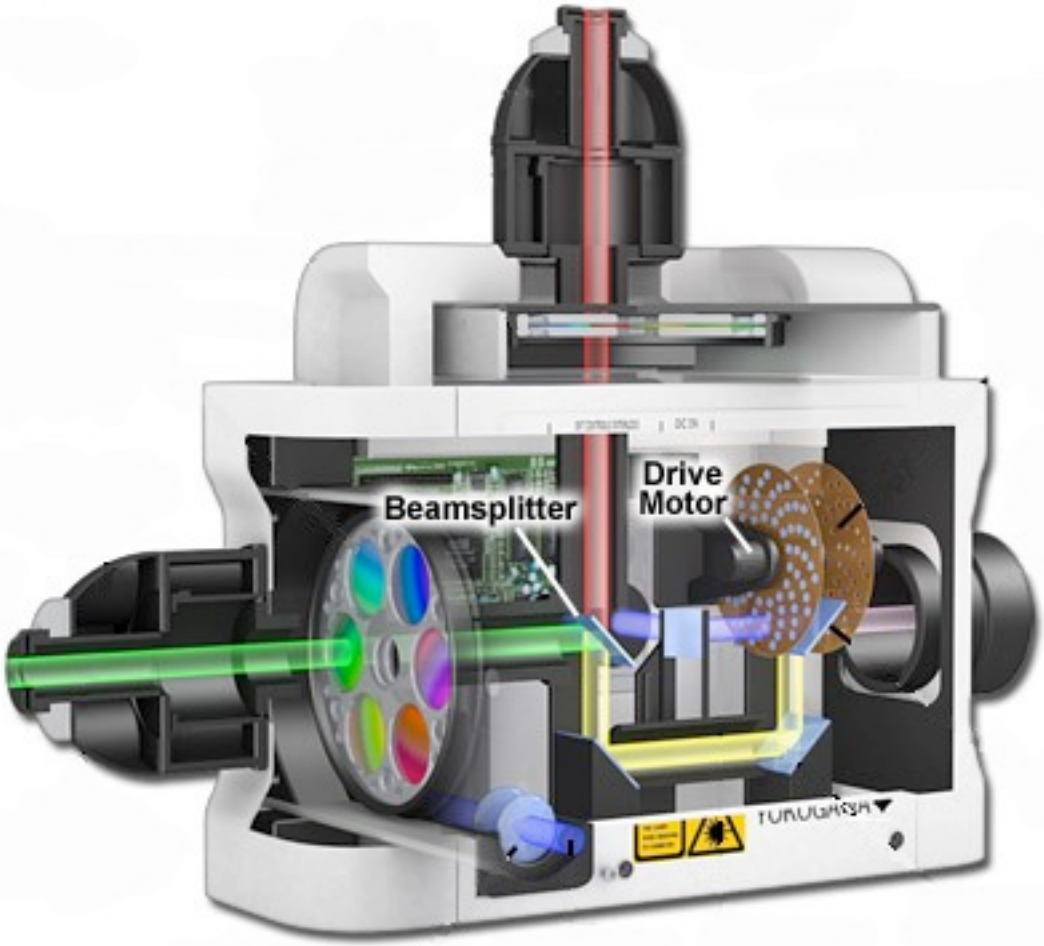


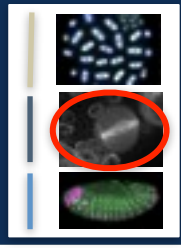
Great for live cell imaging

Can collect many images per second

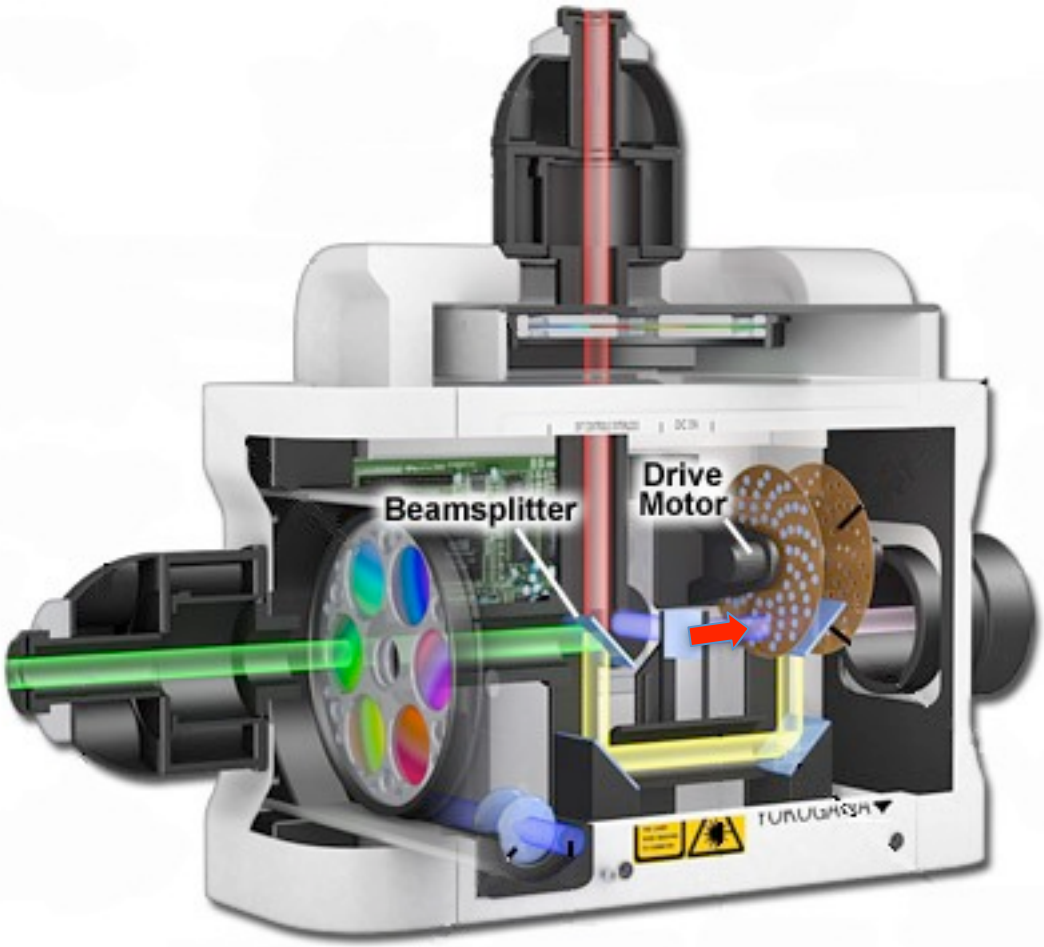


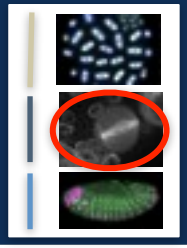
Yokogawa CSU-X1



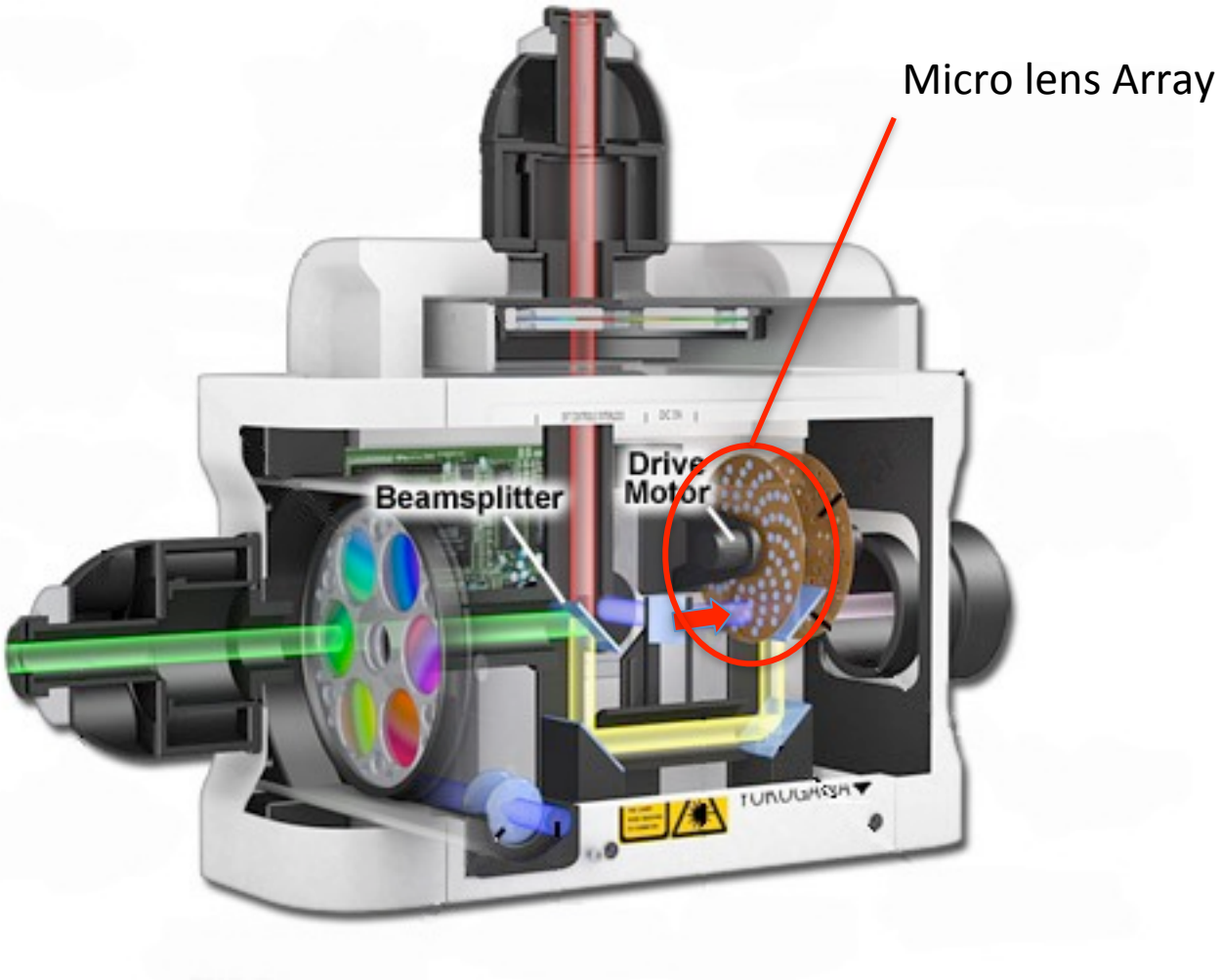


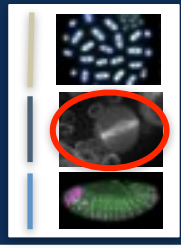
Yokogawa CSU-X1



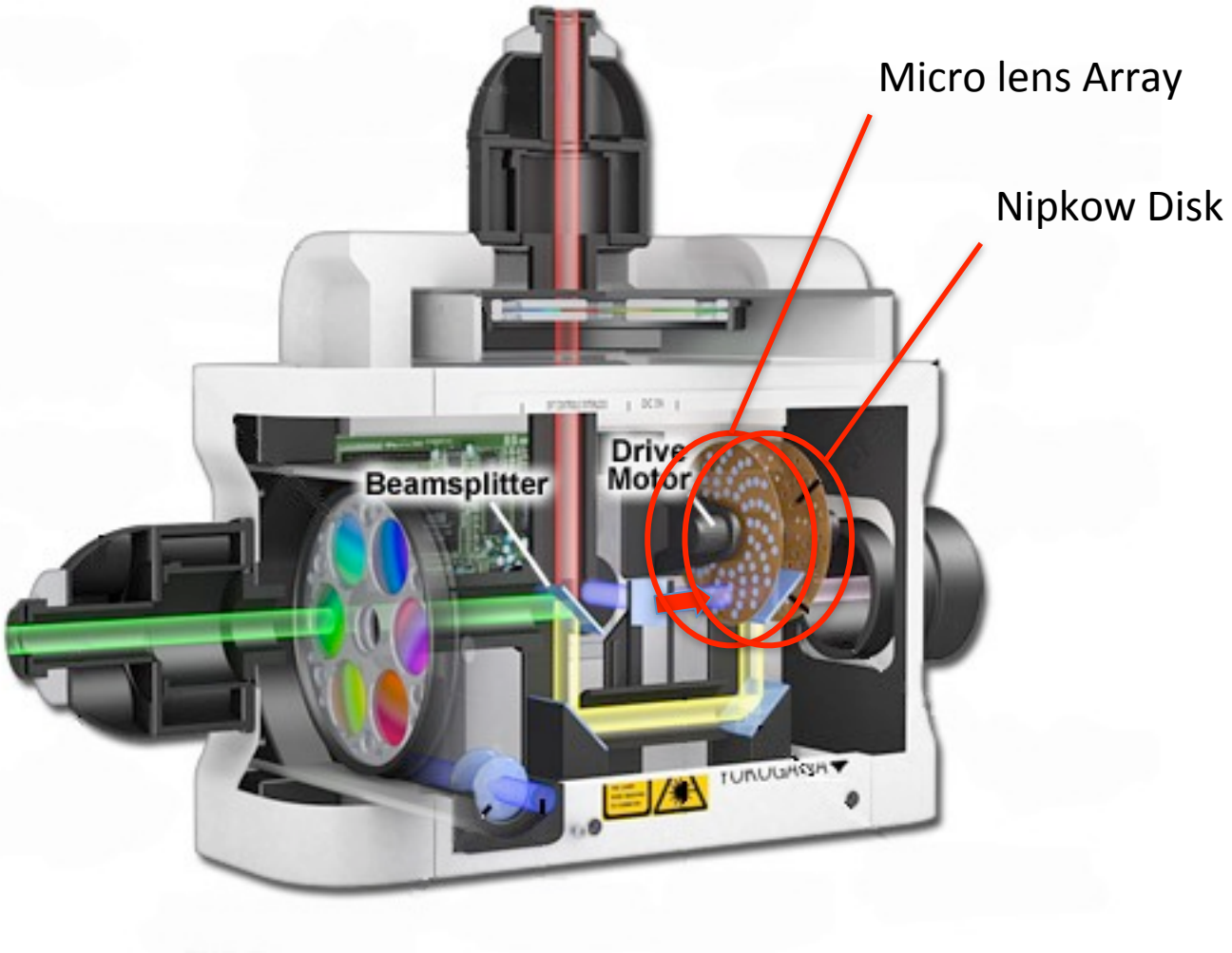


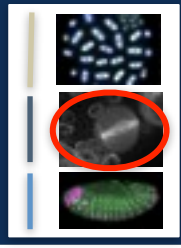
Yokogawa CSU-X1



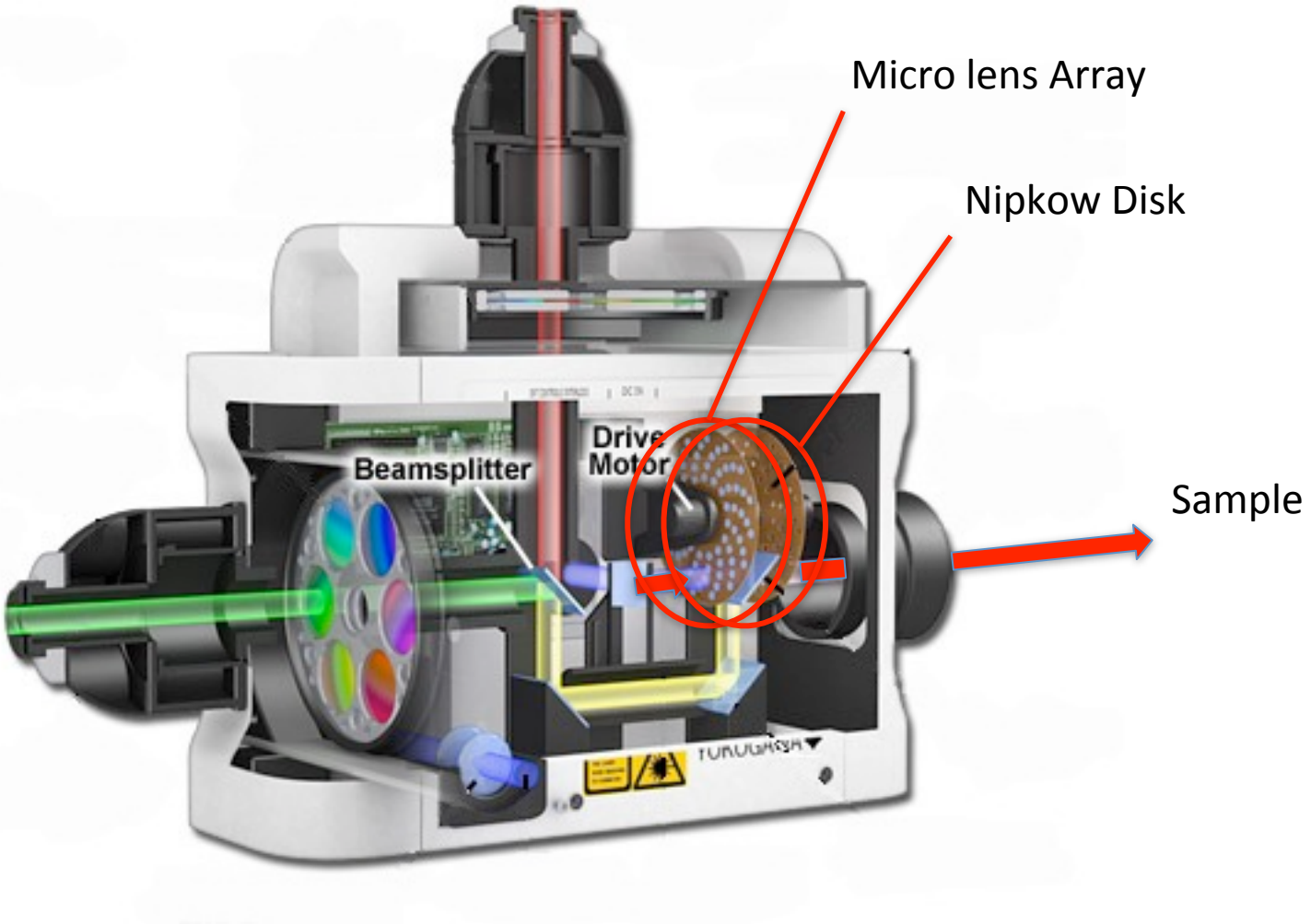


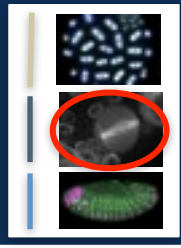
Yokogawa CSU-X1



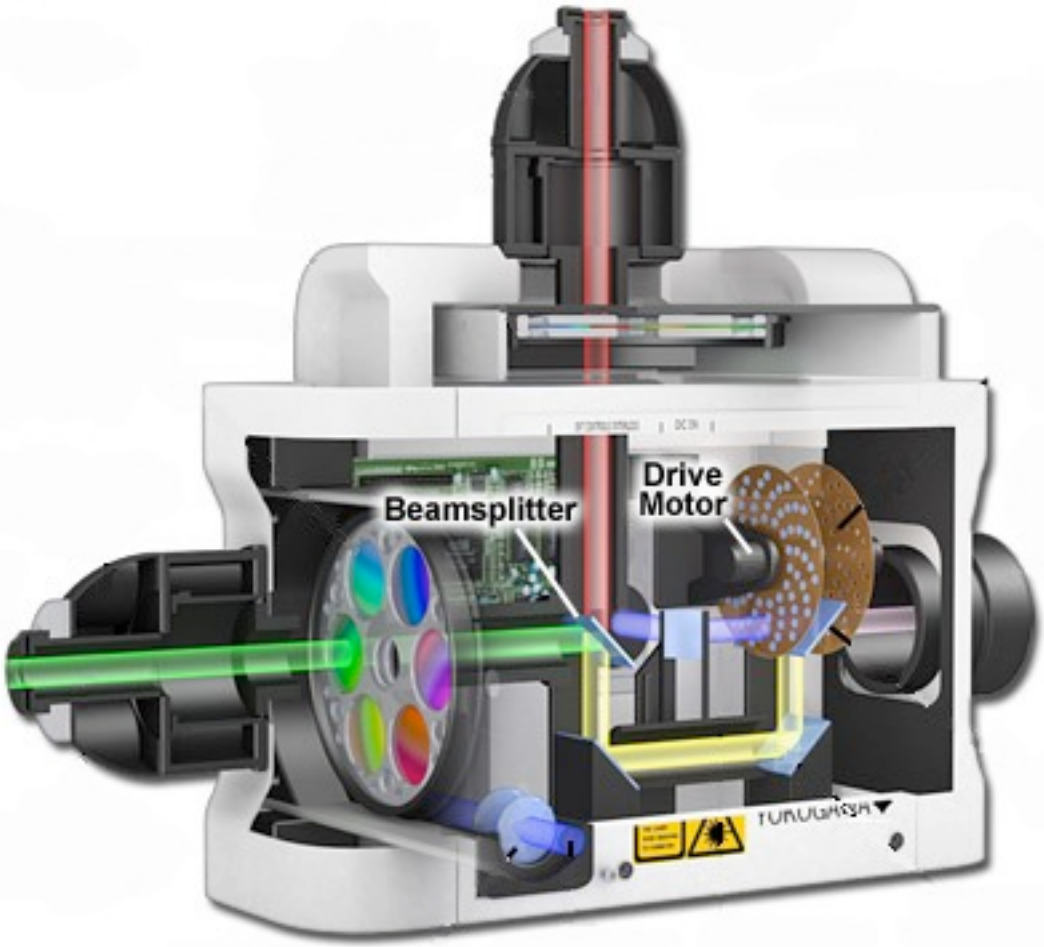


Yokogawa CSU-X1

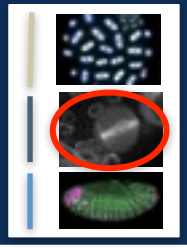




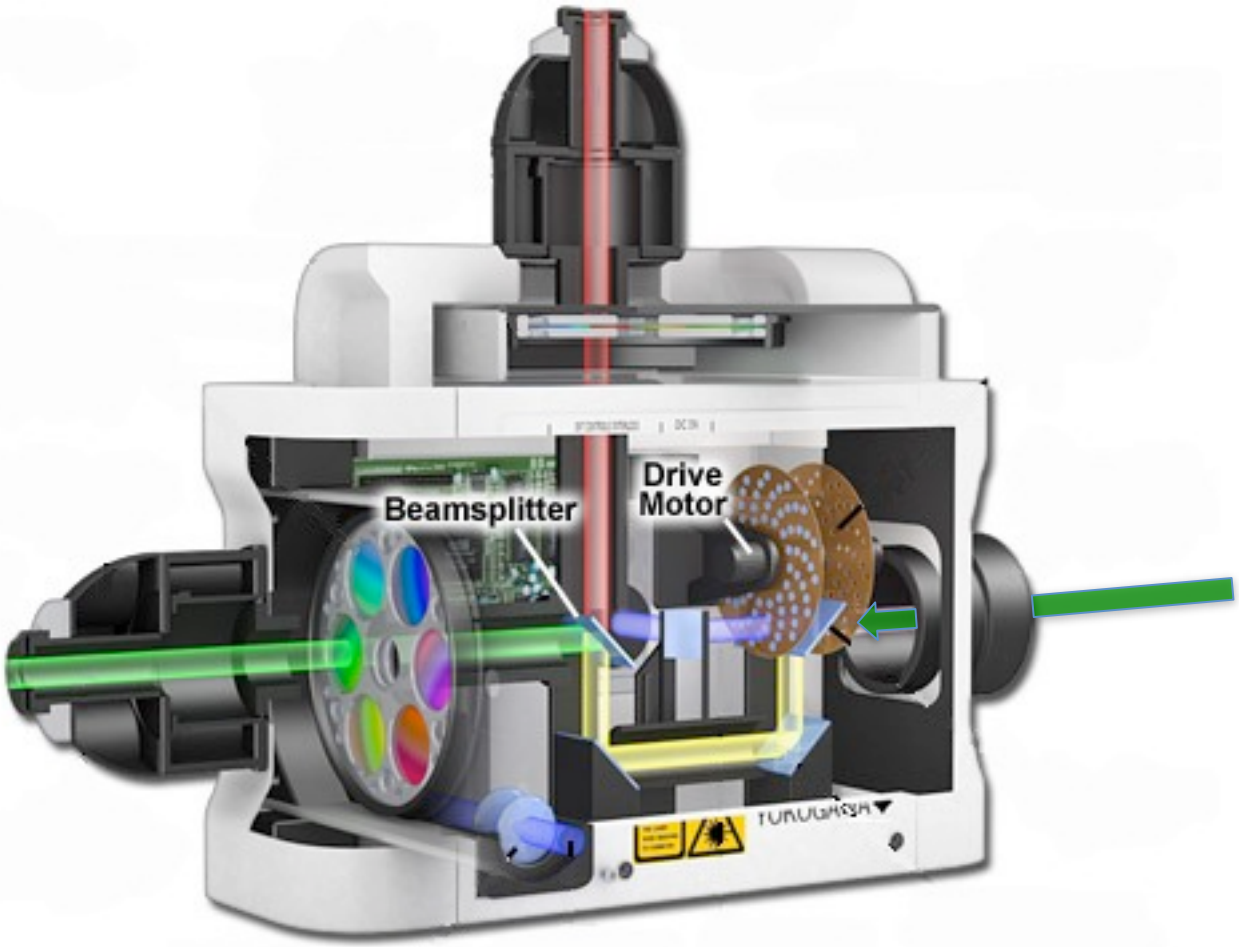
Yokogawa CSU-X1

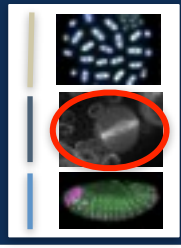


Sample

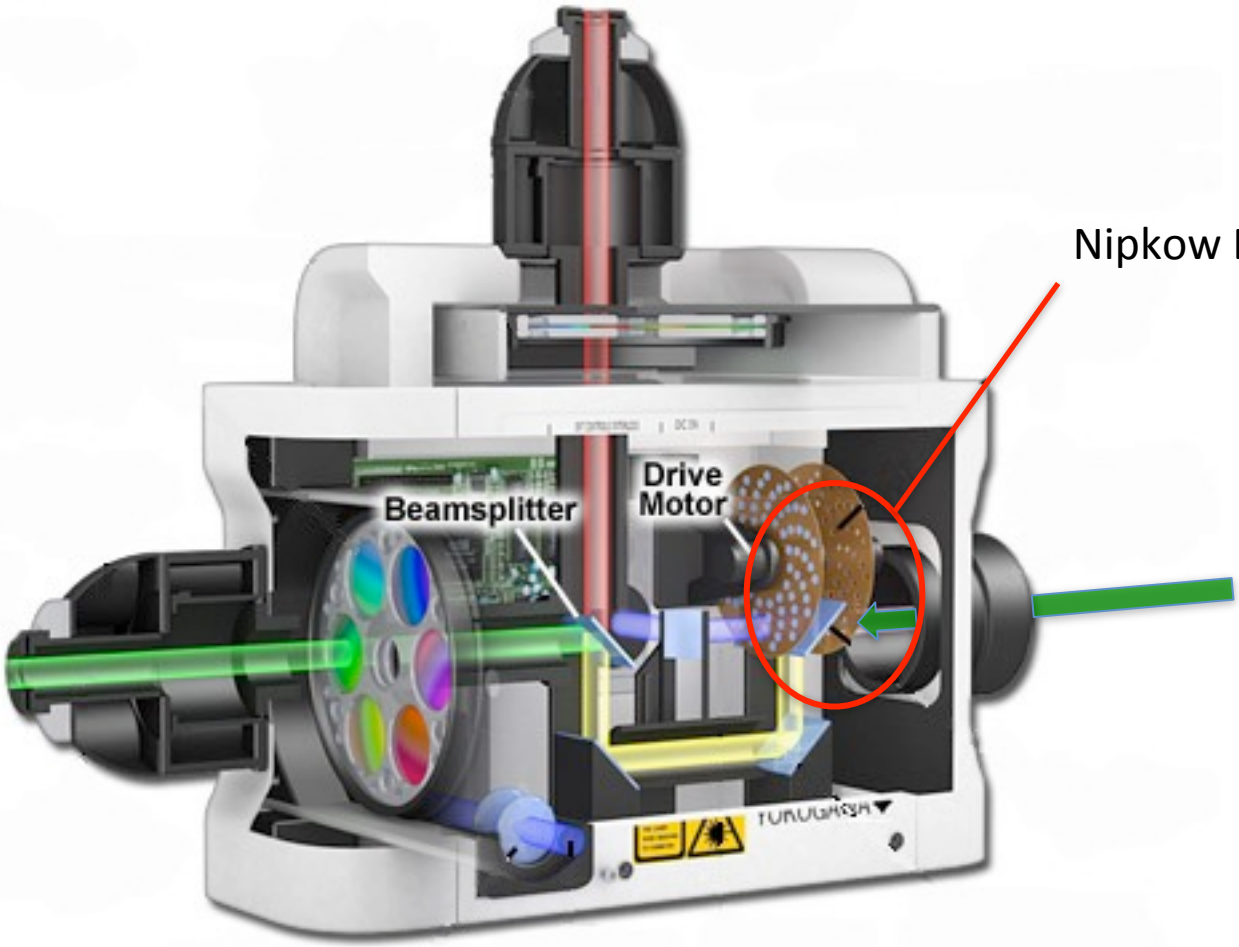


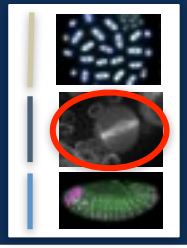
Yokogawa CSU-X1



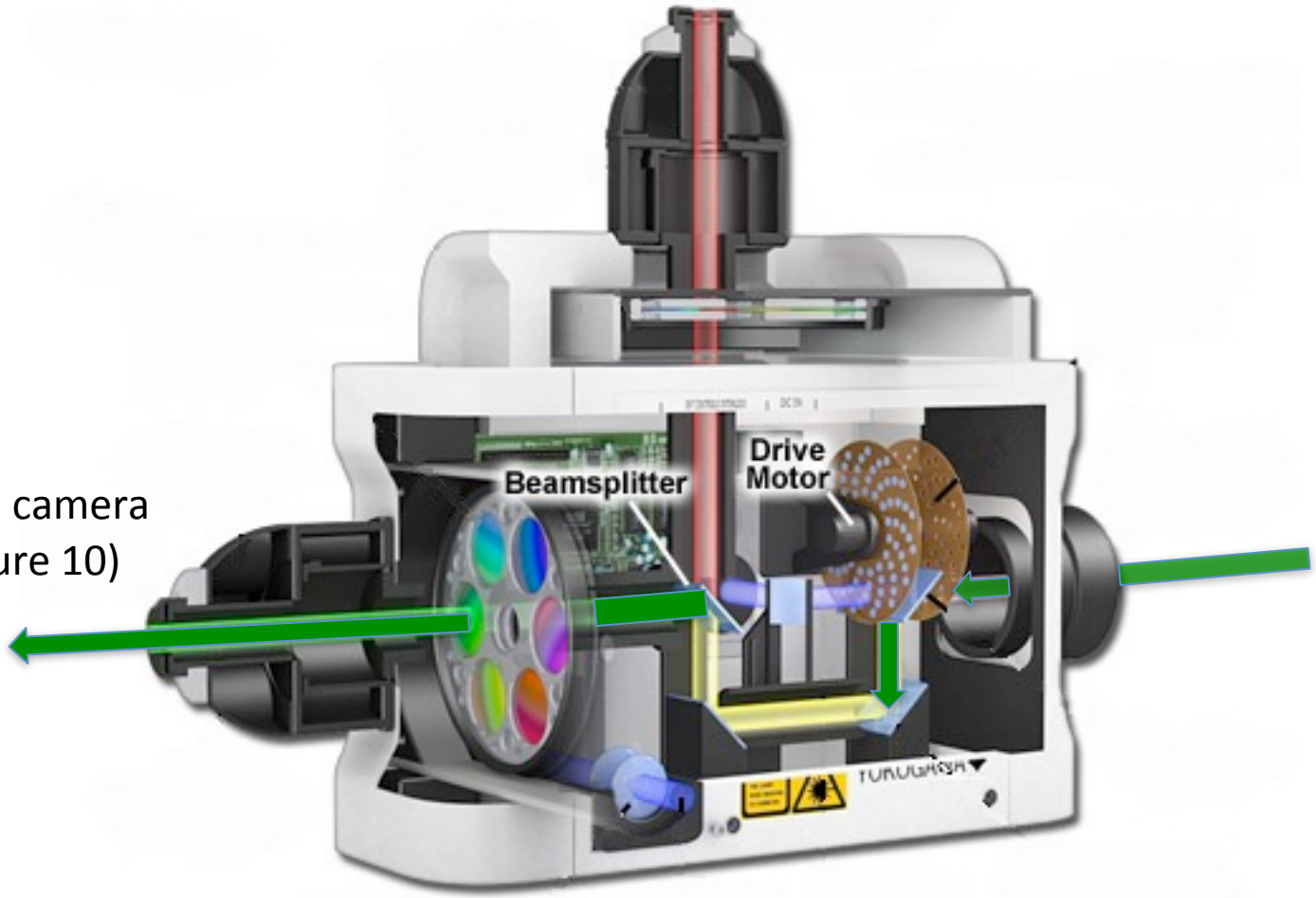


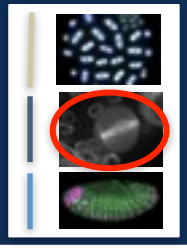
Yokogawa CSU-X1



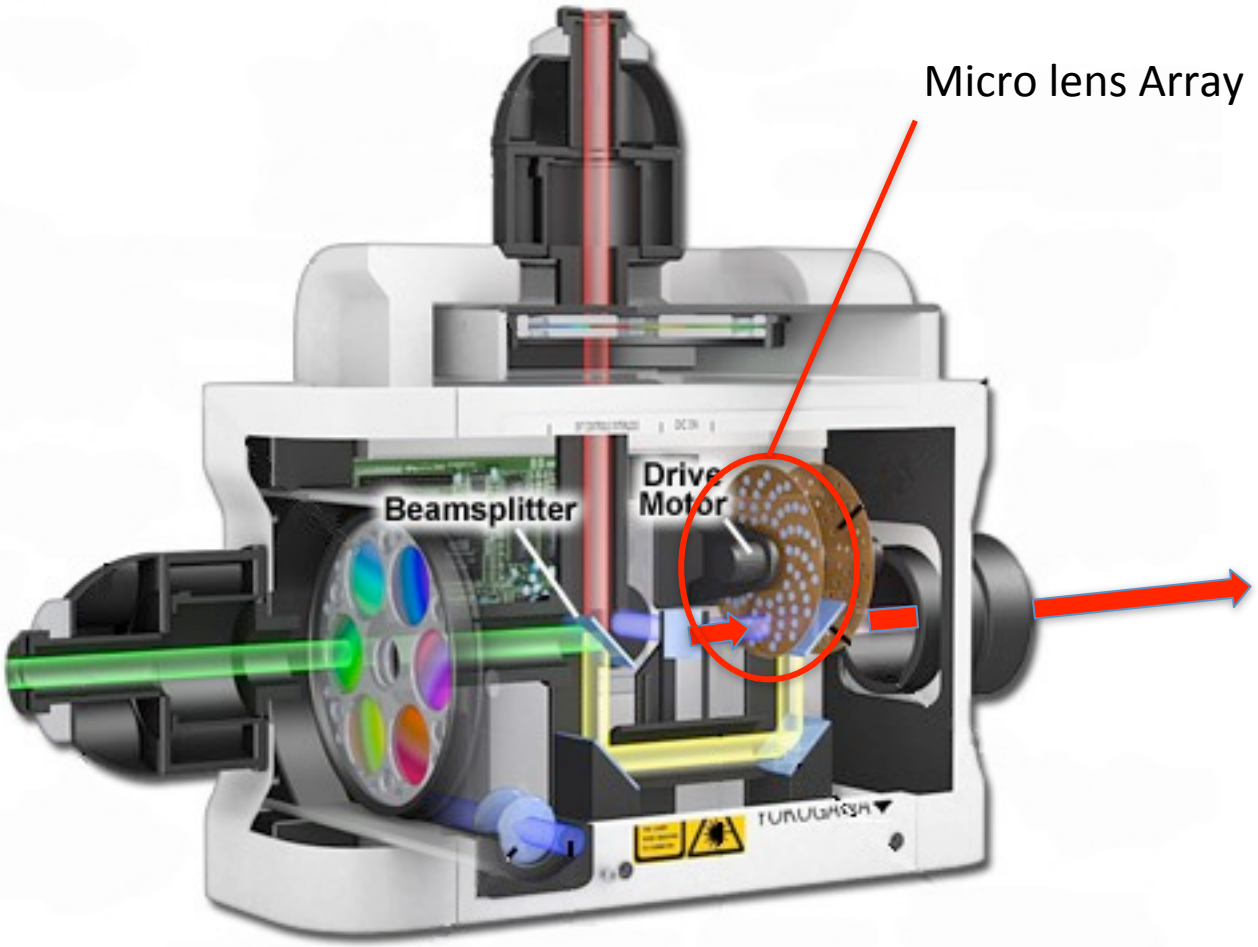


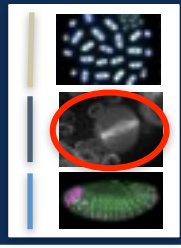
Yokogawa CSU-X1



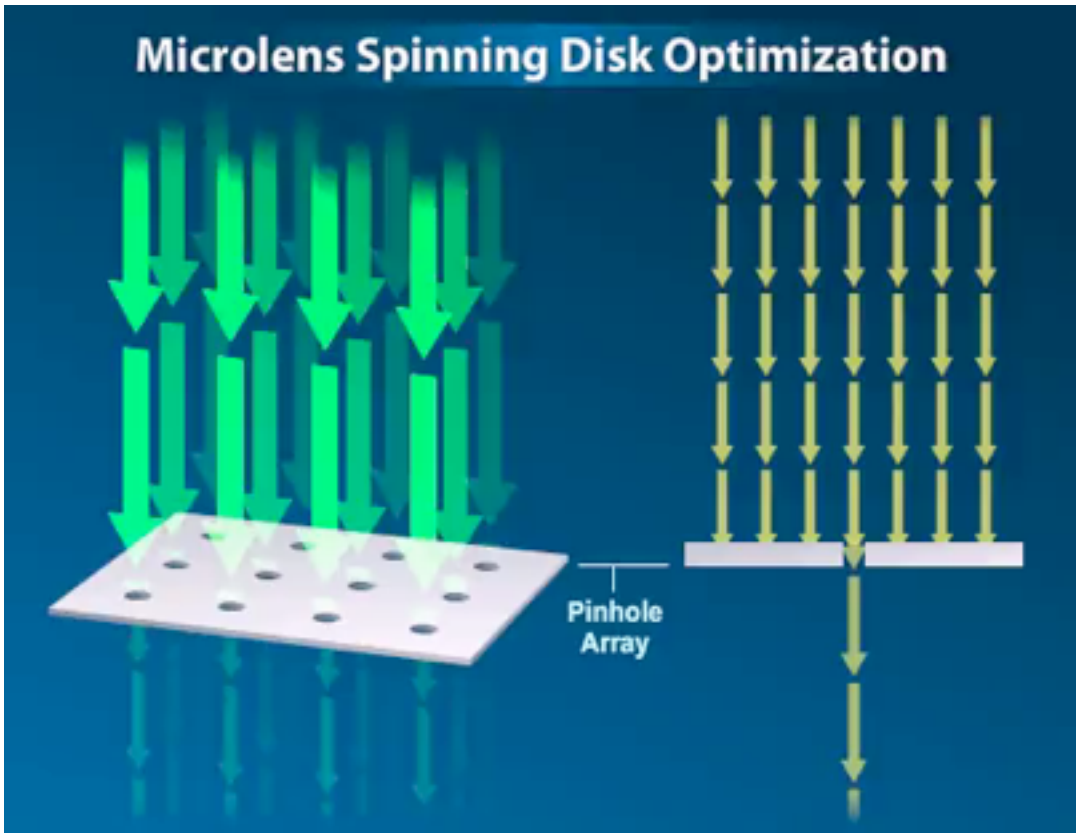


Yokogawa CSU-X1



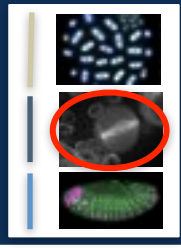


Yokogawa Spinning Disc Confocal

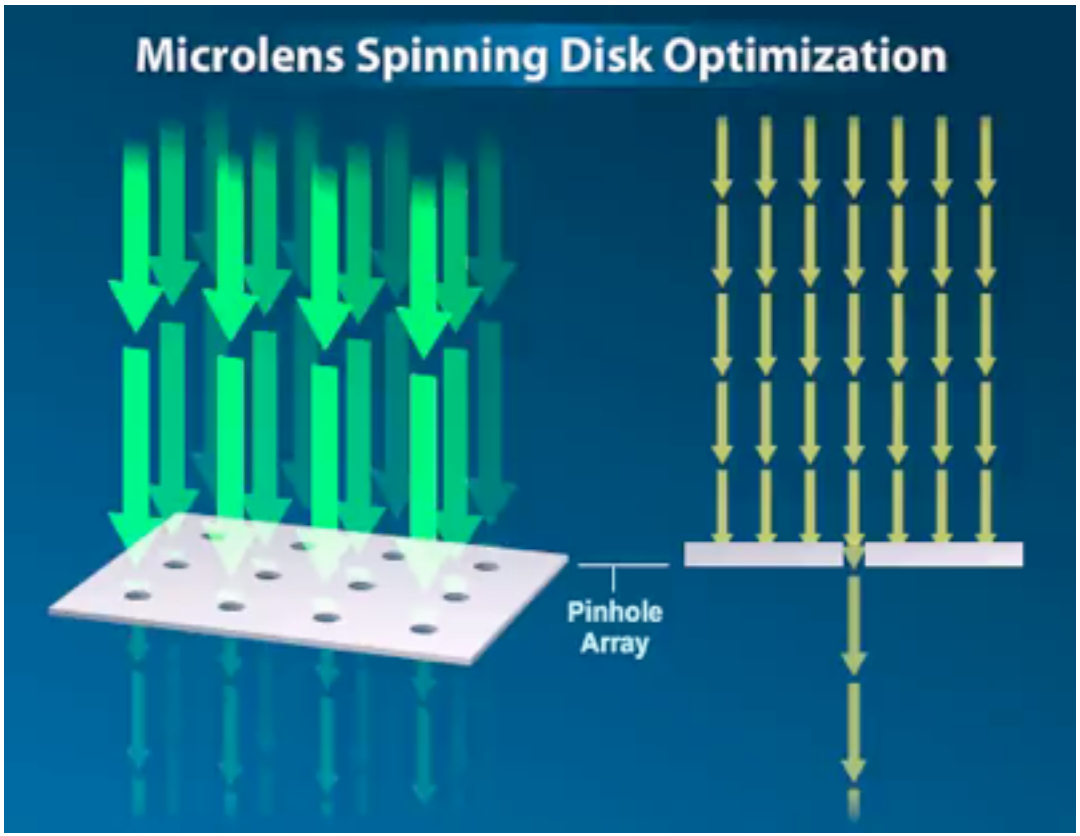


just a pinhole array –
Optimised for ‘cofocality’
and ‘crosstalk’

too much light is blocked
from reaching the specimen



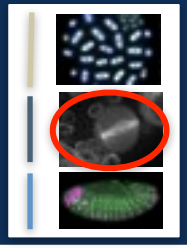
Yokogawa Spinning Disc Confocal



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

just a pinhole array –
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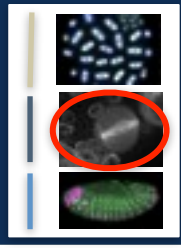
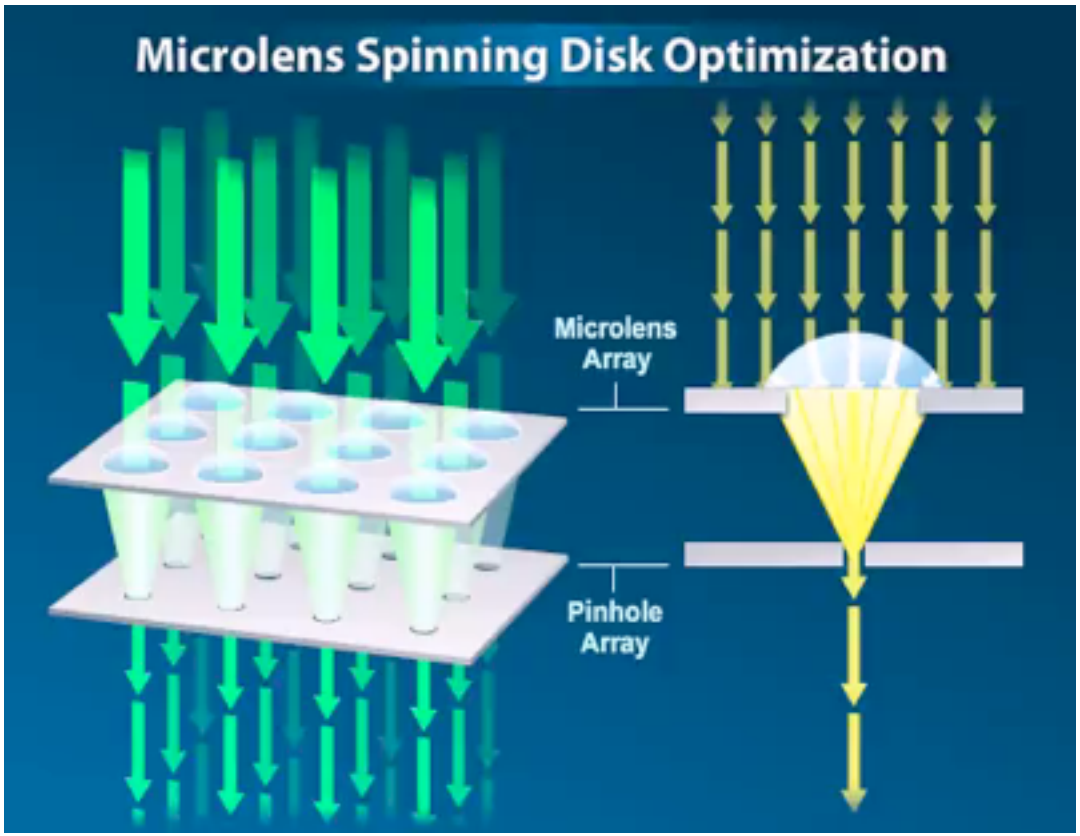
too much light is blocked
from reaching the specimen

Only 4% light passes through disc

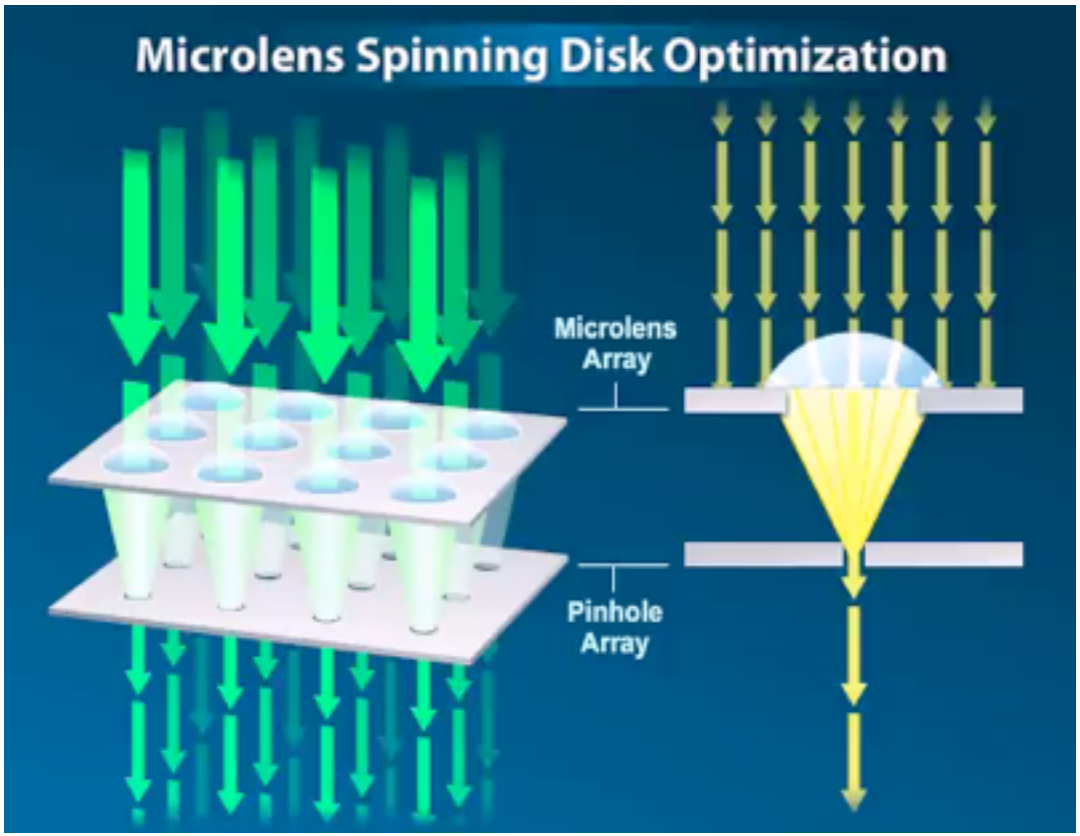


Yokogawa Spinning Disc Confocal



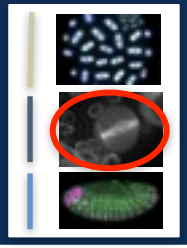
micro-lens array increase
the light
reaching the specimen

Yokogawa Spinning Disc Confocal



micro-lens array increase
the light
reaching the specimen

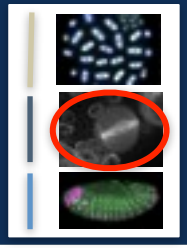
Typically 56% light passes through disc



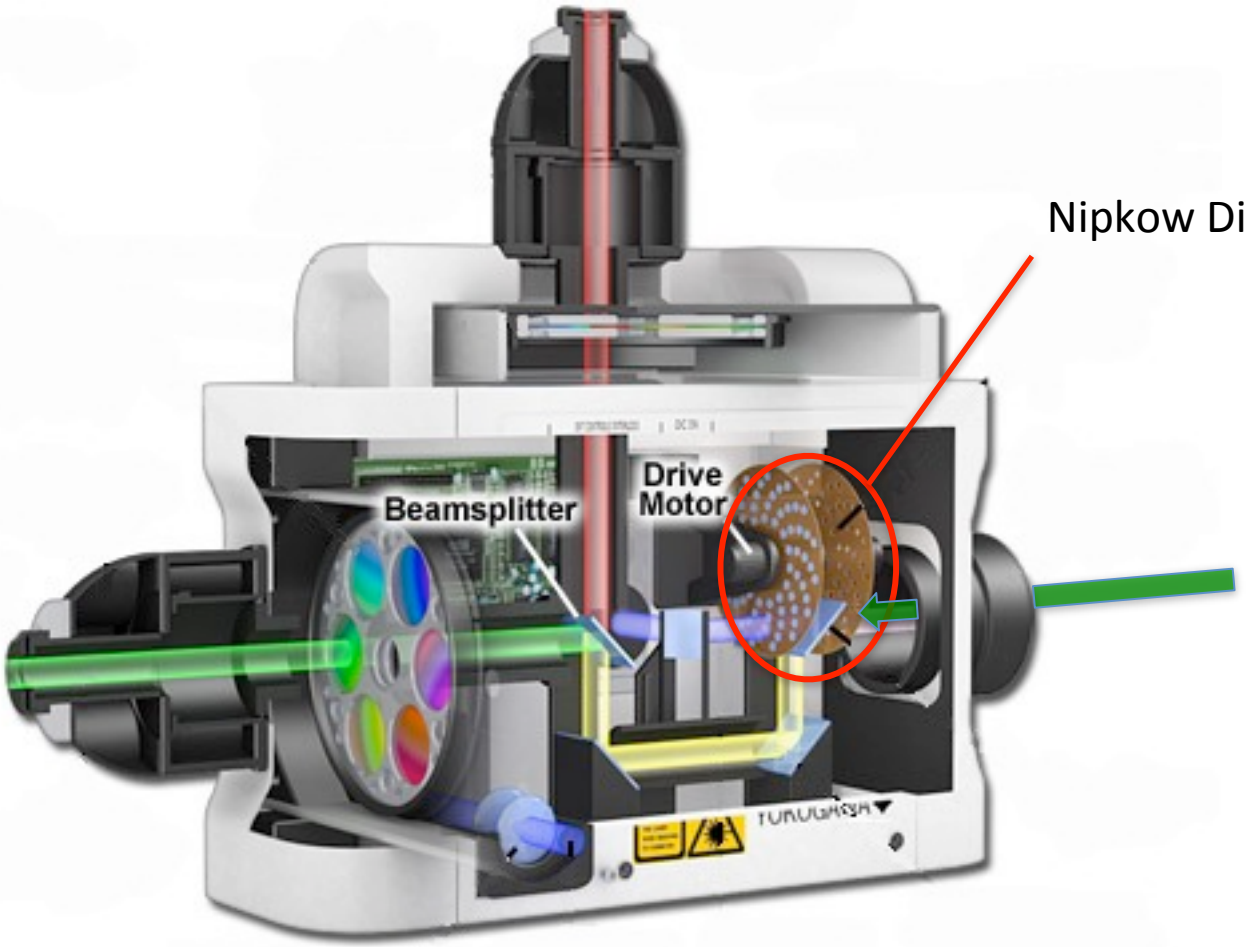
Yokogawa Spinning Disc Confocal

micro-lens array increase
the light
reaching the specimen

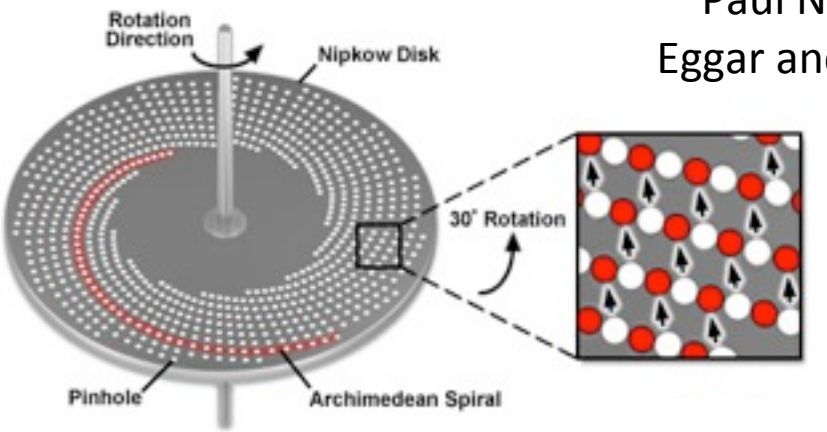
Typically 56% light passes through disc



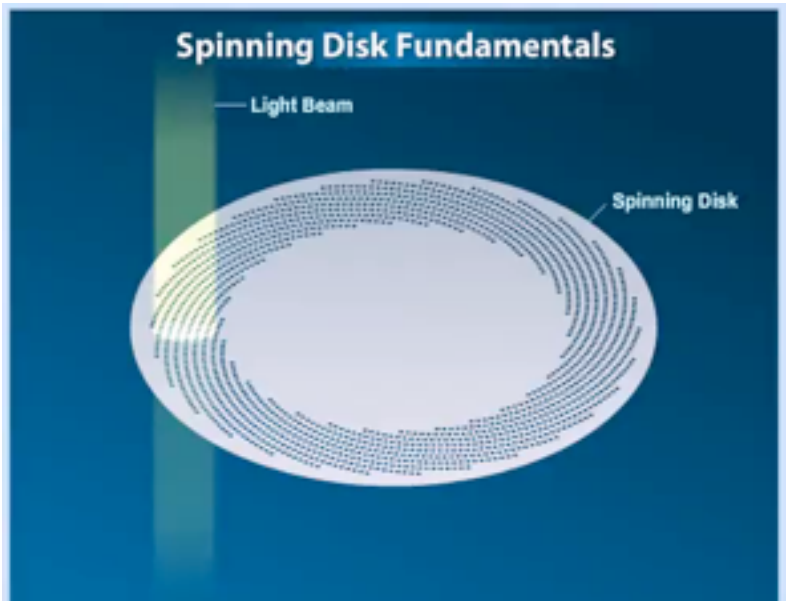
Yokogawa CSU-X1



The Nipkow Disk



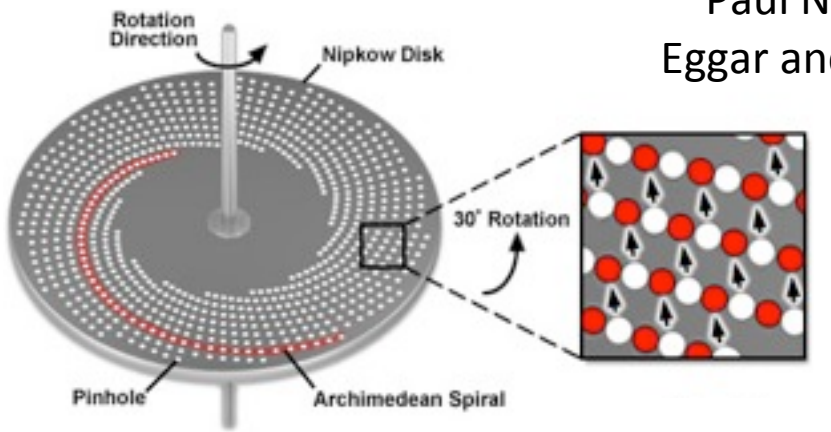
Paul Nipkow, 1884
Eggar and Petran, 1967



Approx. 1000 pinholes

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

The Nipkow Disk



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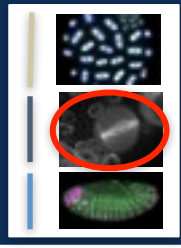
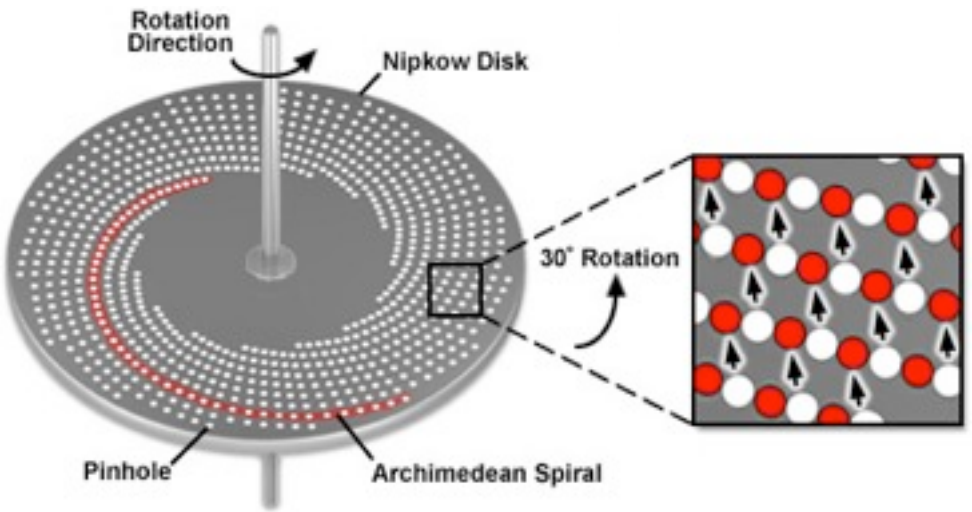
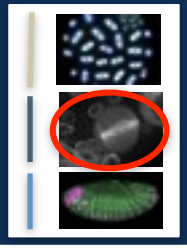


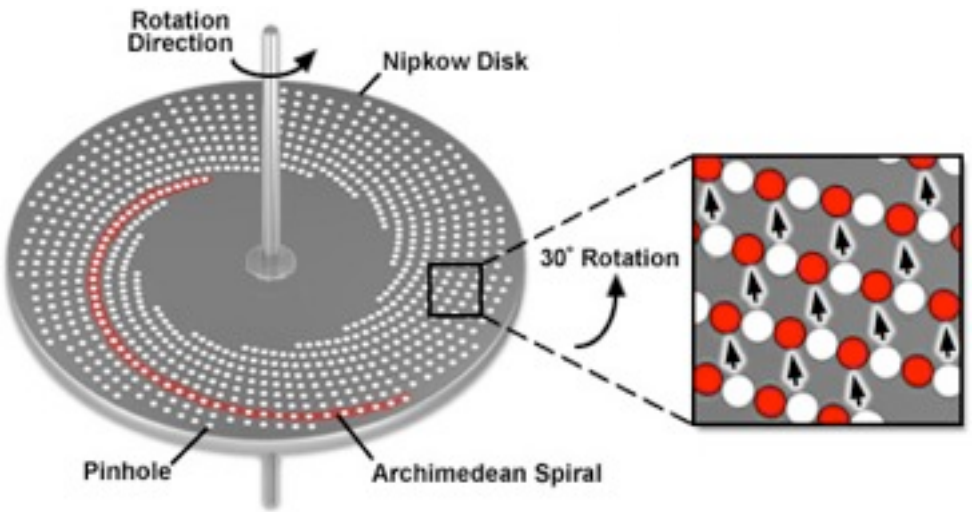
The Nipkow Disk



Larger pinholes - brighter image, but less “confocal”

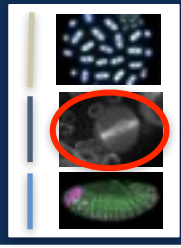


The Nipkow Disk

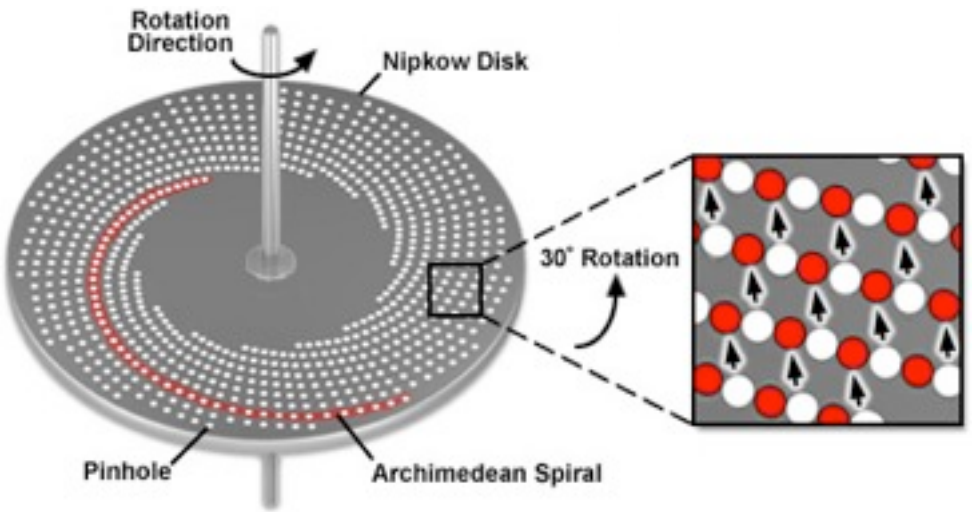


Larger pinholes - brighter image, but less “confocal”

Pinholes fixed size: Typically = 50um



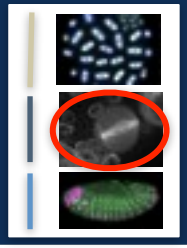
The Nipkow Disk



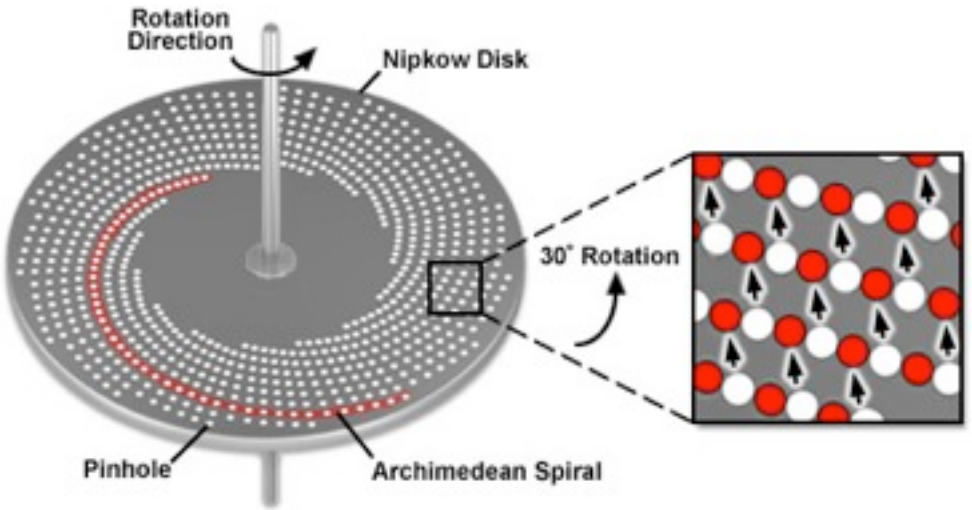
Larger pinholes - brighter image, but less “confocal”

Pinholes fixed size: Typically = 50um

(optimised for biology)

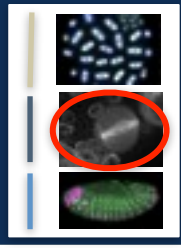


The Nipkow Disk

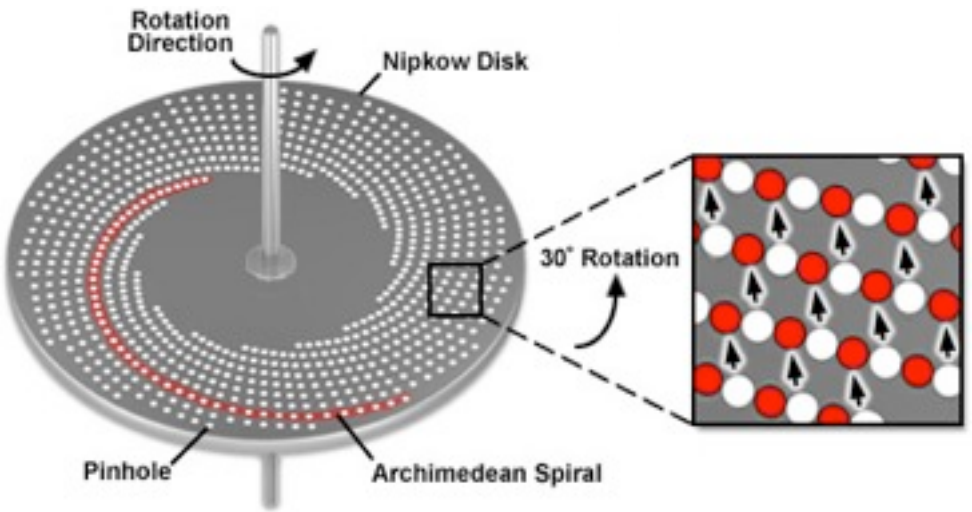


Constant Battle:

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



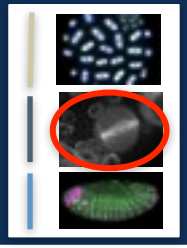
The Nipkow Disk



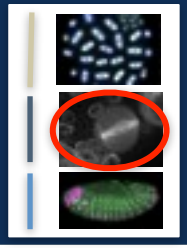
Constant Battle:

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

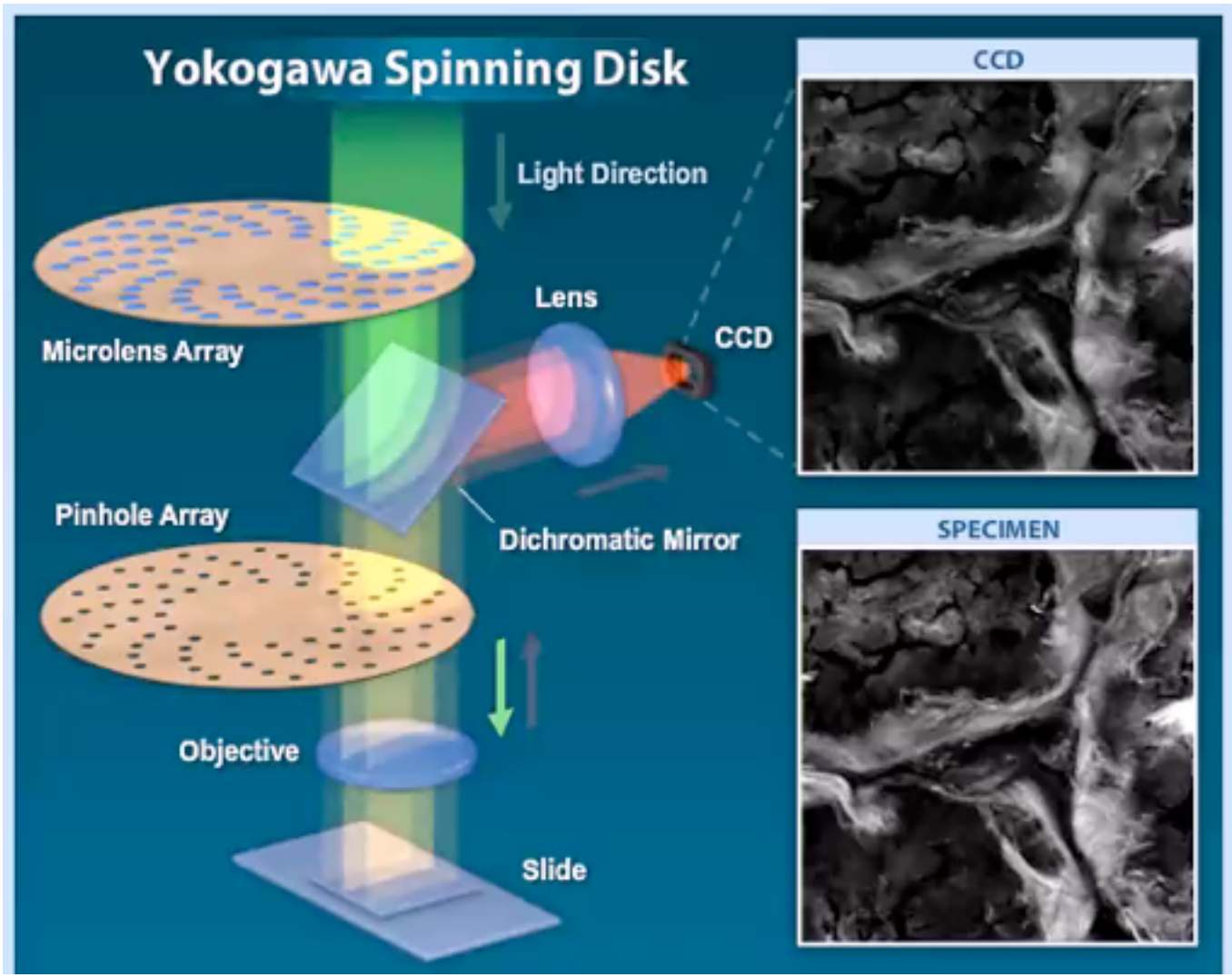
Pinhole Spacing Typically = 2.5um apart

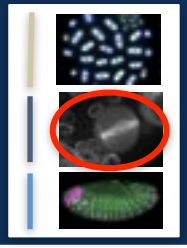


Yokogawa



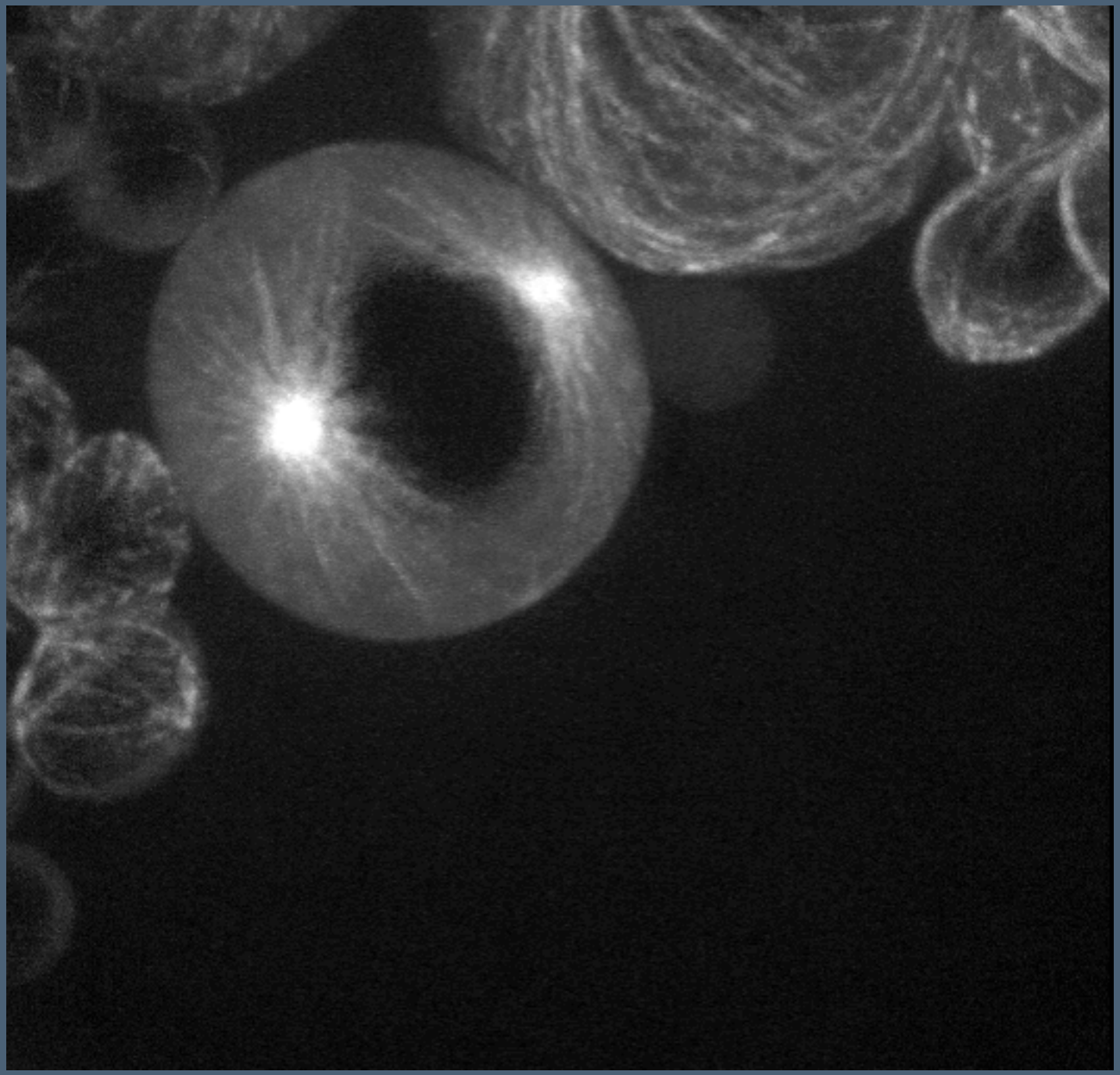
Yokogawa


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



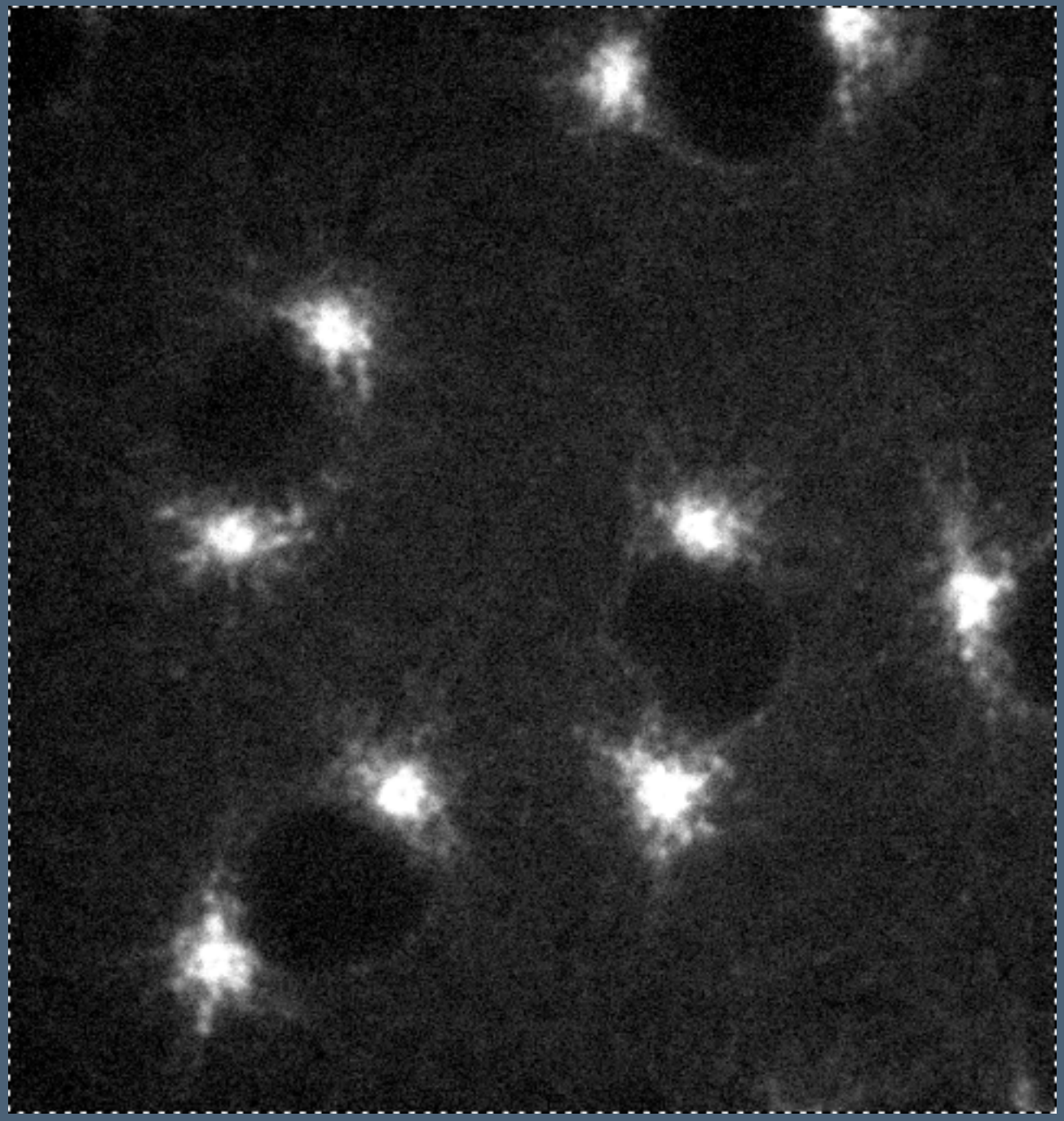
Yokogawa

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



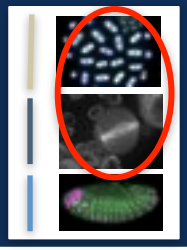
Cell division in brain stem cells (neuroblasts), Raff Lab

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



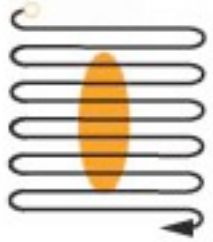
MT binding protein in *Drosophila* embryo, Raff Lab

MT binding protein in *Drosophila* embryo, Raff Lab

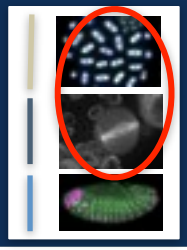


Point Scanning Vs Spinning Disc

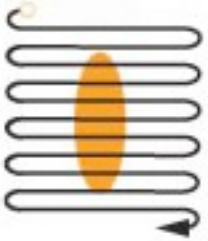
Point Scanning

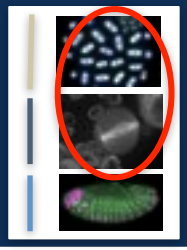


Spinning Disc

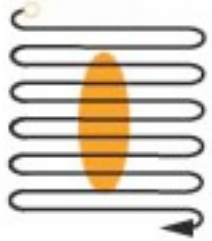


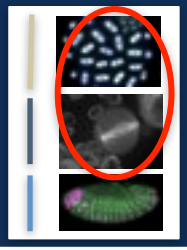
Point Scanning Vs Spinning Disc

	Point Scanning	Spinning Disc
		
Speed	Slow (secs)	Fast (msecs)

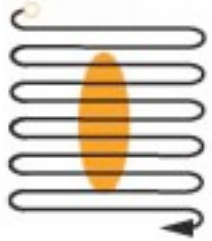


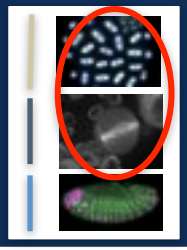
Point Scanning Vs Spinning Disc

	Point Scanning	Spinning Disc
		
Speed	Slow (secs)	Fast (msecs)
Sensitivity	OK	OK

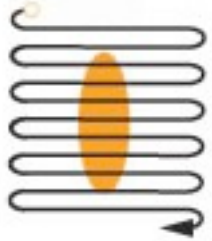


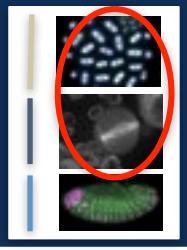
Point Scanning Vs Spinning Disc

	Point Scanning	Spinning Disc
		
Speed	Slow (secs)	Fast (msecs)
Sensitivity	OK	OK
Flexibility	Good	Poor

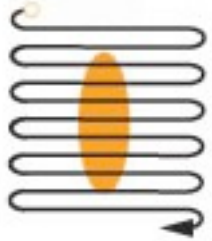


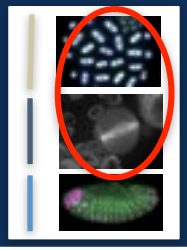
Point Scanning Vs Spinning Disc

	Point Scanning	Spinning Disc
		
Speed	Slow (secs)	Fast (msecs)
Sensitivity	OK	OK
Flexibility	Good	Poor
Bleaching	Poor	Good

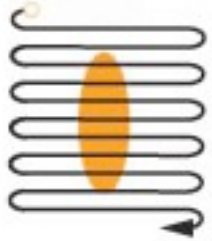


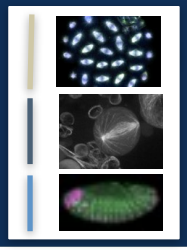
Point Scanning Vs Spinning Disc

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



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!



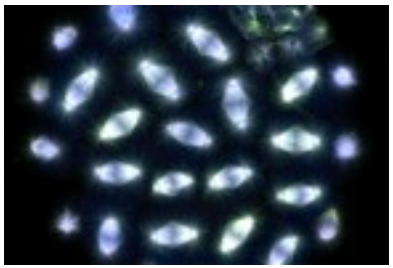
3 Flavours of Microscope

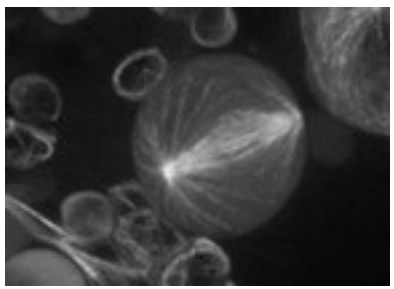
Problem:
Out of Focus
Light

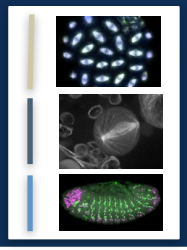
Confocal

Laser
Scanning

Spinning disc





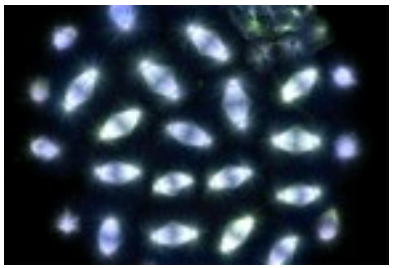


3 Flavours of Microscope

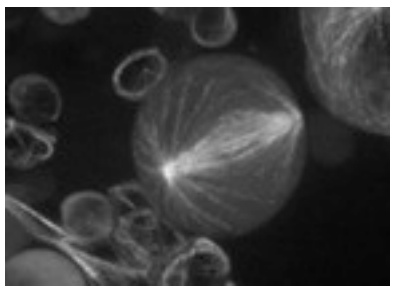
Problem:
Out of Focus
Light

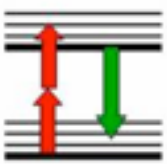
Confocal

Laser
Scanning

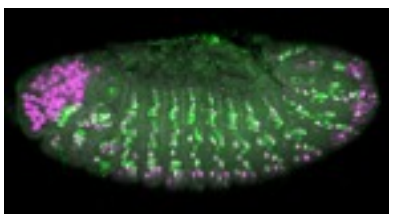


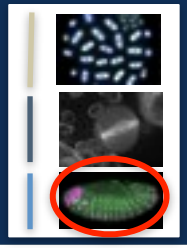
Spinning disc





2-Photon



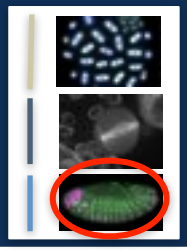


2-photon Microscope

Not a ‘confocal’

for imaging deeper into thick specimens

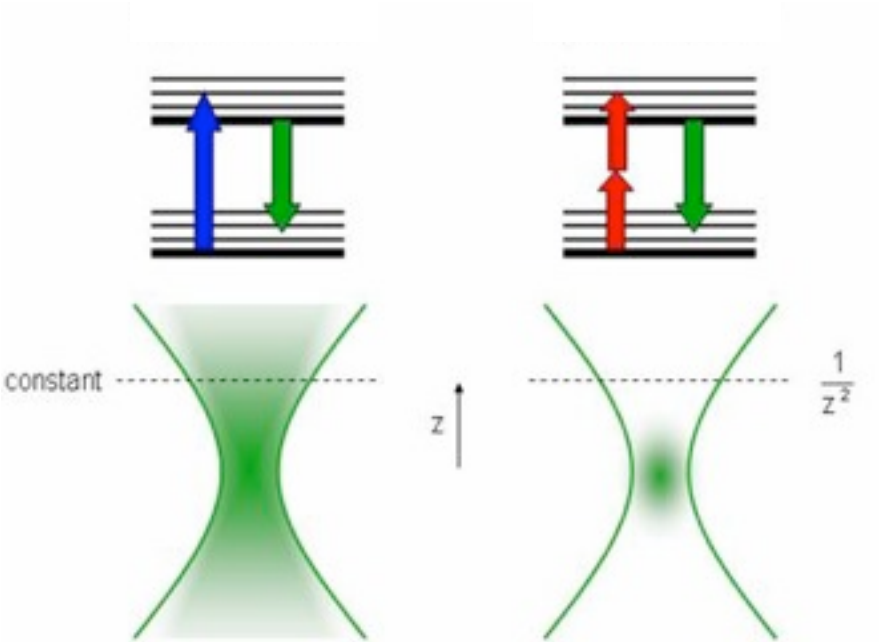
less damaging to biological samples

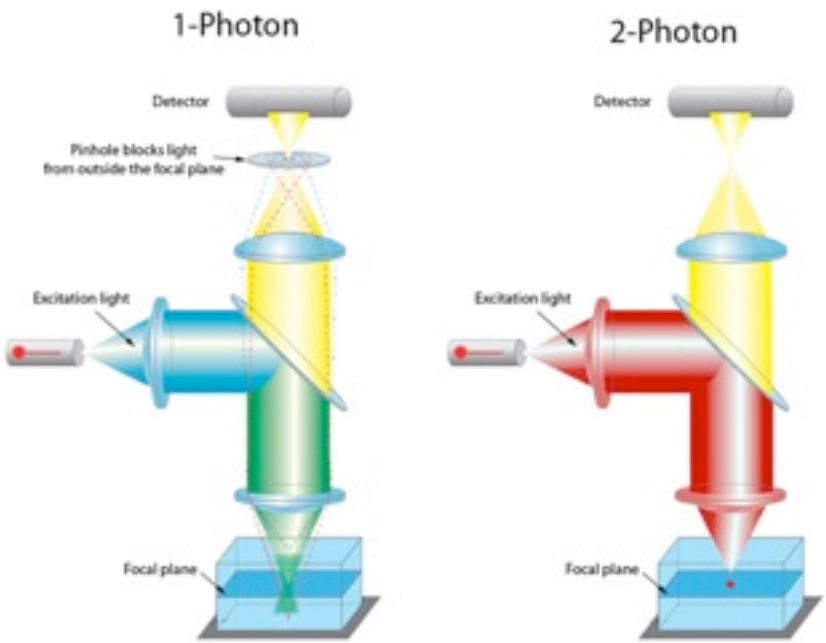


Confocal Vs 2-photon

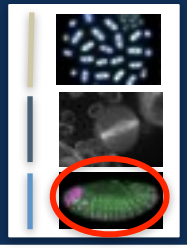
1 Photon Excitation

2 Photon Excitation





There is no out of focus light



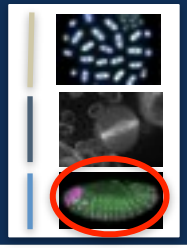
THEORY

1 Photon Excitation

high energy state

lowest singlet excited state

ground state



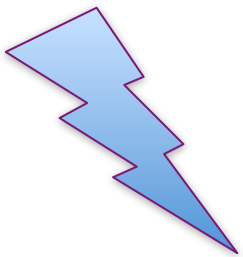
THEORY

1 Photon Excitation

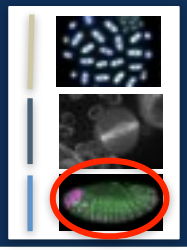
high energy state

lowest singlet excited state

excitation



ground state



THEORY

1 Photon Excitation

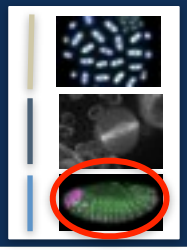
high energy state

lowest singlet excited state

energy absorption

excitation

ground state



THEORY

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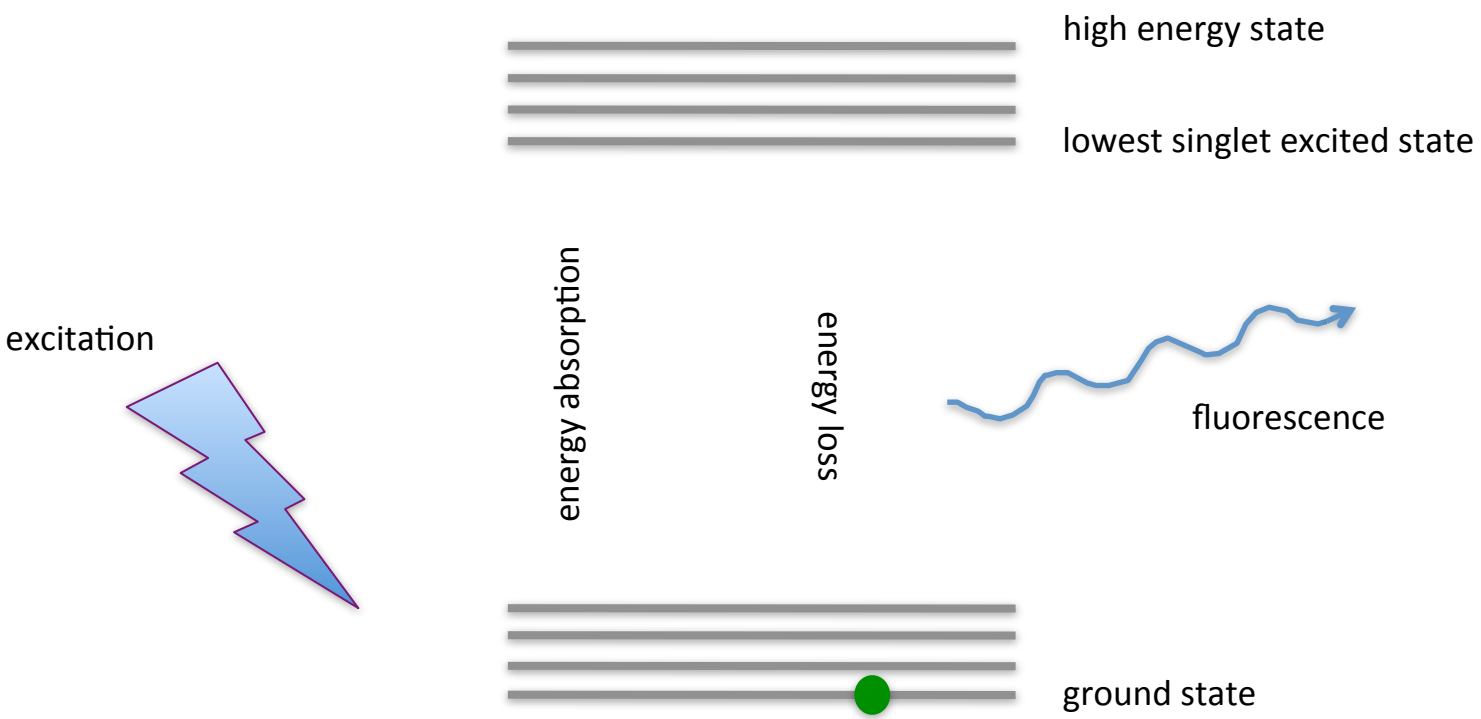
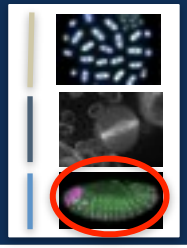




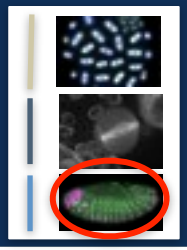
THEORY

2 Photon Excitation

high energy state

lowest singlet excited state

ground state



THEORY

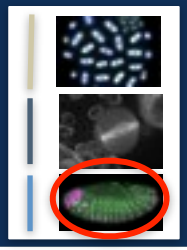
2 Photon Excitation

high energy state

lowest singlet excited state

Low energy (IR ~700nm) Pulsed excitation

ground state



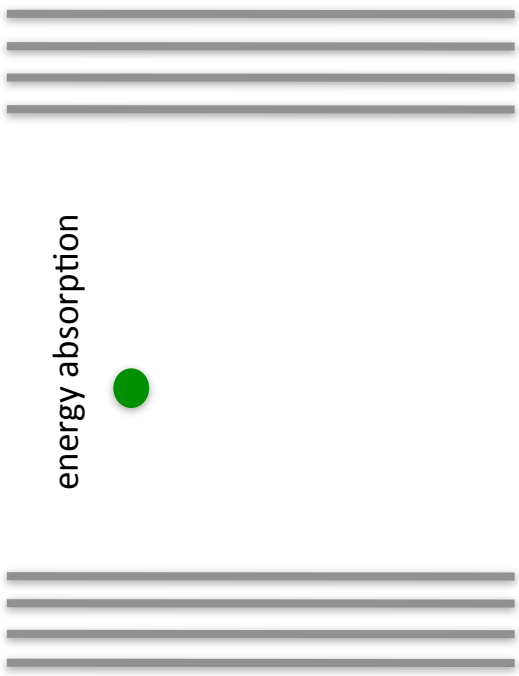
THEORY

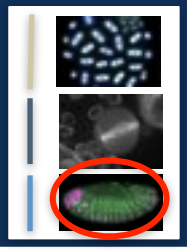
2 Photon Excitation

Almost simultaneous

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

Low energy (IR ~700nm) Pulsed excitation





THEORY

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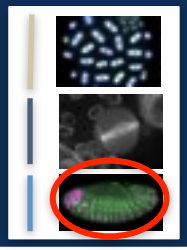


THEORY

2 Photon Excitation

Almost simultaneous

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

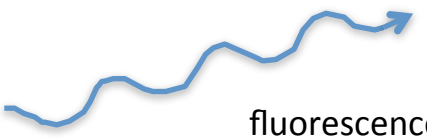
Low energy (IR ~700nm) Pulsed excitation

high energy state

lowest singlet excited state

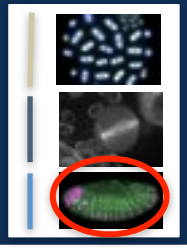
energy absorption

energy loss

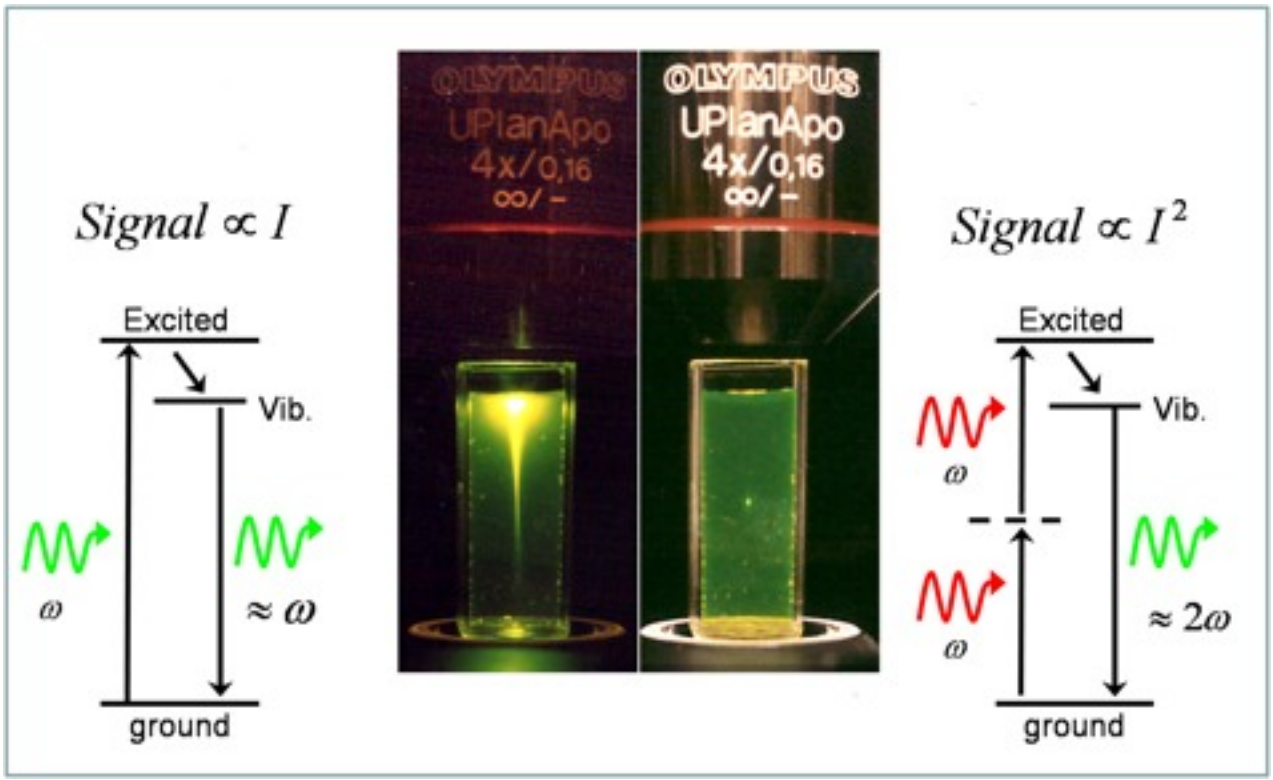


fluorescence

ground state

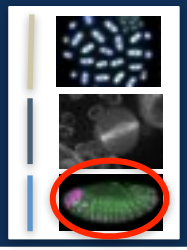


Principle of 2-photon Microscope

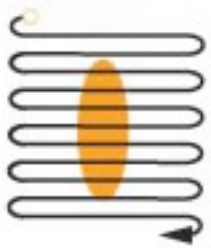


Near simultaneous, two photon event highly unlikely, only really possible a focal point

Tightly focused excitation



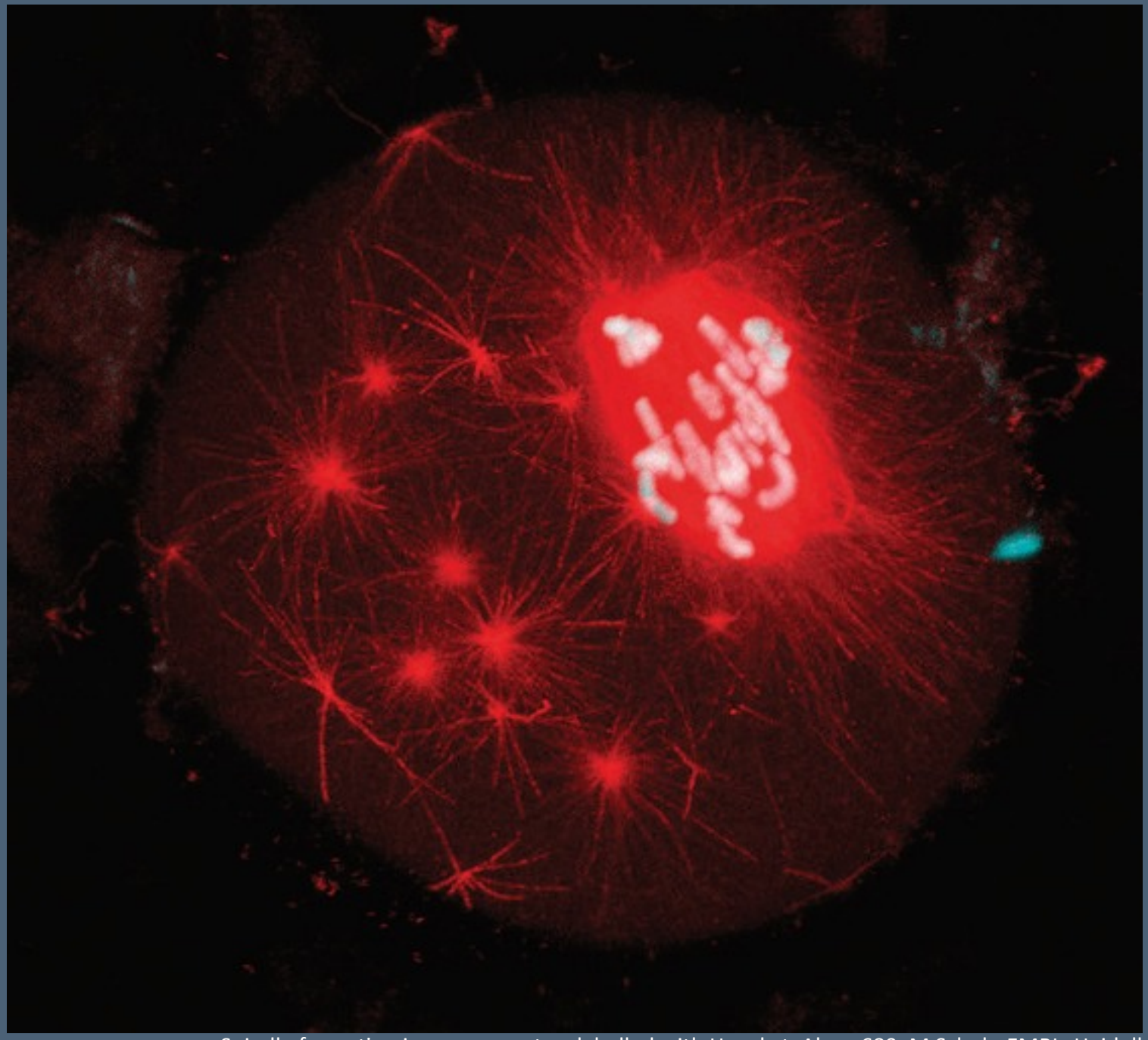
2-photon Microscope



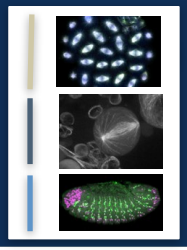
Pulsed excitation laser is then scanned across the sample.

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

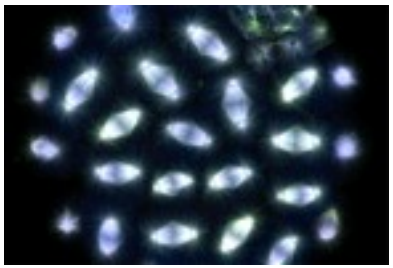


3 Flavours of Microscope

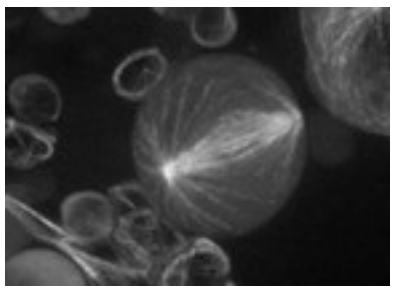
Problem:
Out of Focus
Light

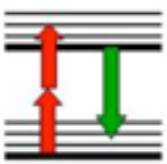
Confocal

Laser
Scanning

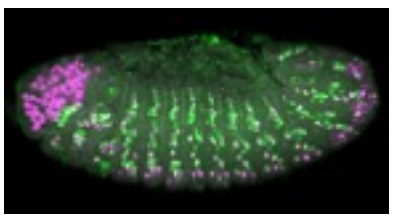


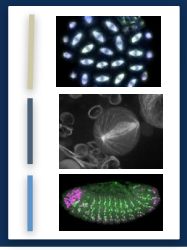
Spinning disc





2-Photon





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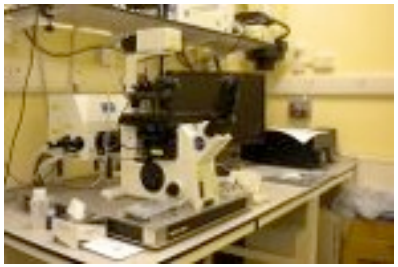
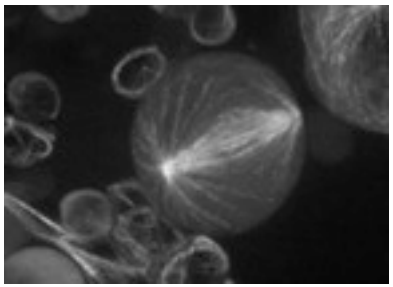
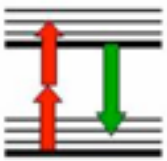
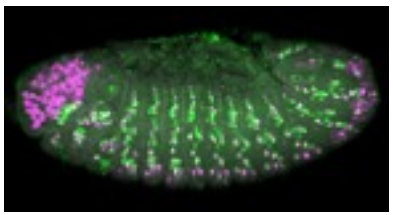


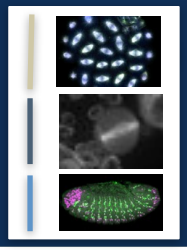
Spinning disc





2-Photon





3 Flavours of Microscope

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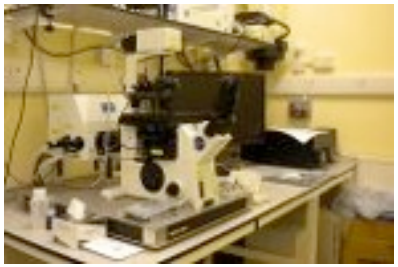

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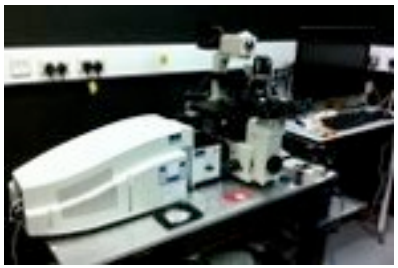

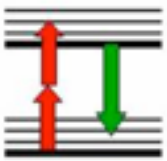

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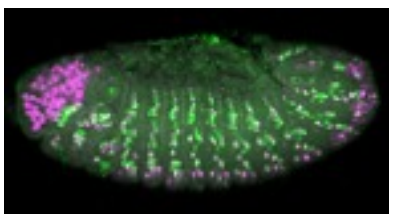

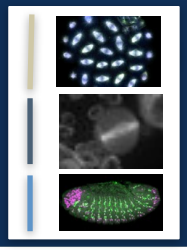

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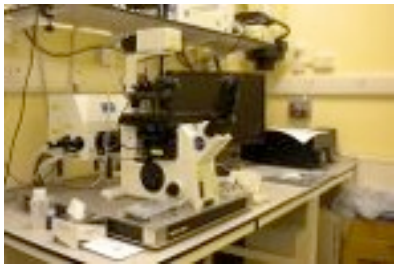

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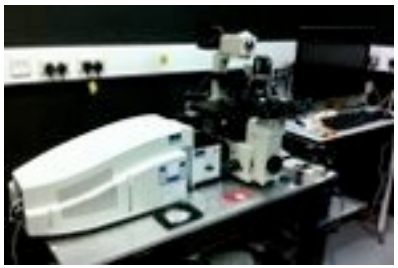

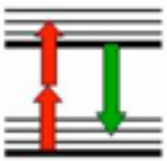

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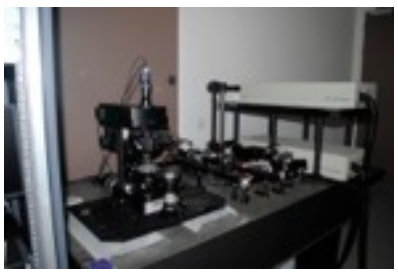


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