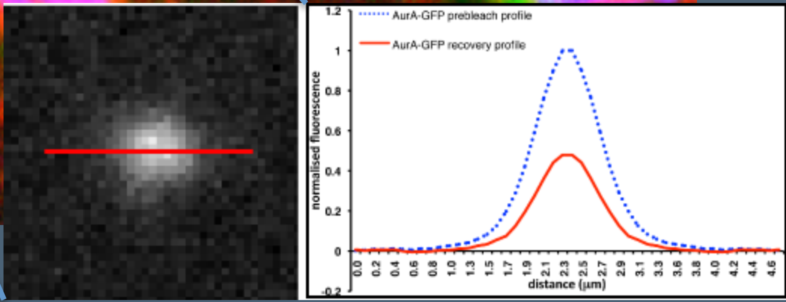
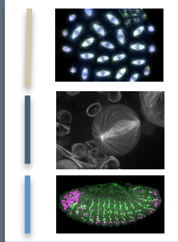
Confocal Microscopy

March 2013

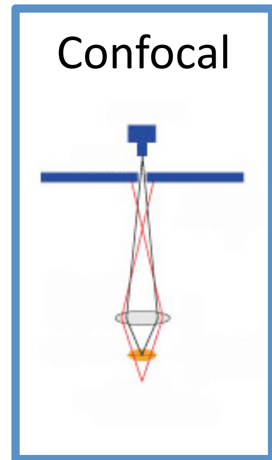
Micron
OXFORD


AurA-GFP prebleach profile
AurA-GFP recovery profile
normalised fluorescence
distance (µm)

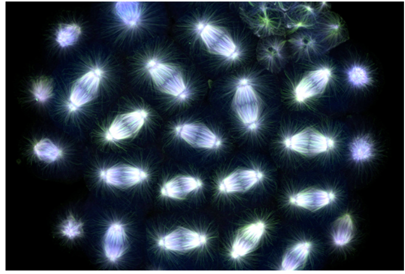


3 Flavours of Microscope

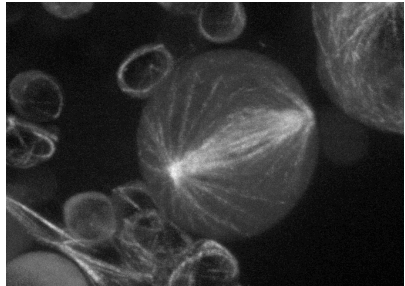
Confocal



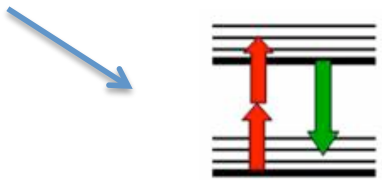
Laser Scanning



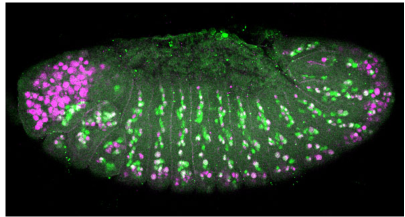
Spinning disc

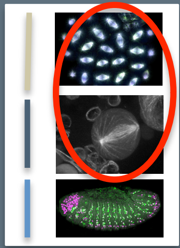


Problem:
Out of Focus
Light



2-Photon





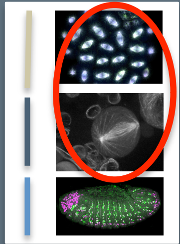
short History of Confocal Microscope

Confocal “concept’ patented by Marvin Minsky in 1957

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

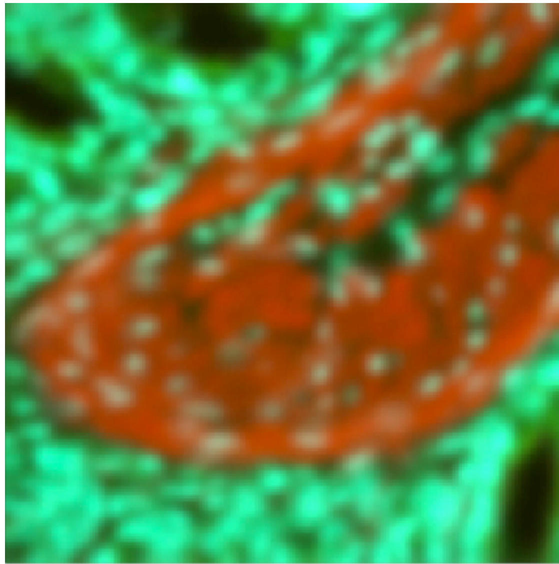
Brakenhoff, Stelzer developed “stage” scanning confocal in late 1970

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



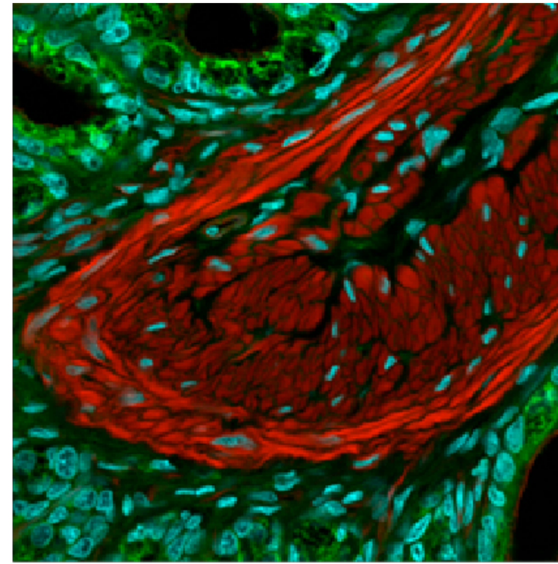
Comparison
Widefield Vs Confocal

Widefield

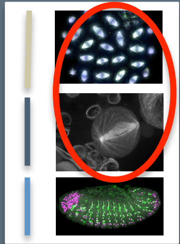


Out of focus light 'blurs' image

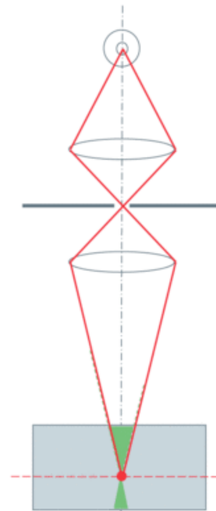
Confocal



Out of focus light is blocked

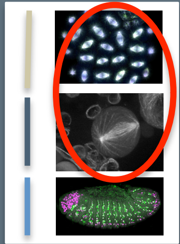


Principle of Confocal Microscopes Pinhole

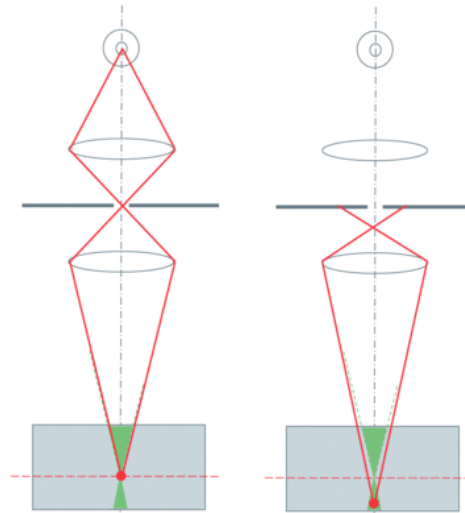


Pinhole diaphragm in the Conjugated focal plane = CONFOCAL

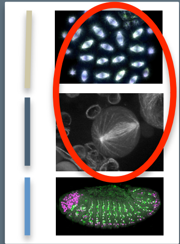
in focus light (from the optical section) passes through the pinhole and into the detector



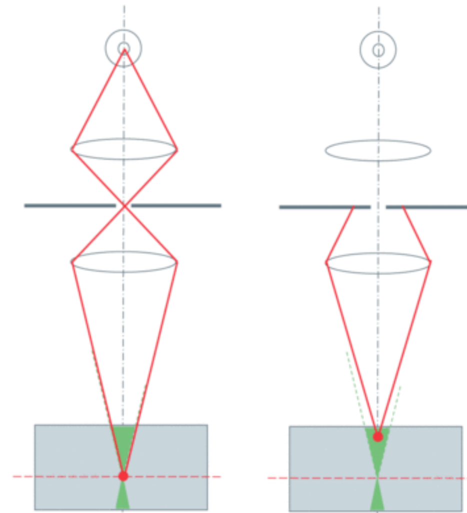
Pinhole – blocks out-of-focus light



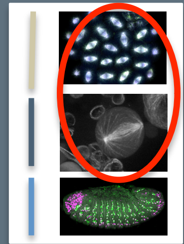
light from below the optical section crosses infront of the pinhole and doesn't pass through the pinhole aperture



Pinhole – blocks out-of-focus light

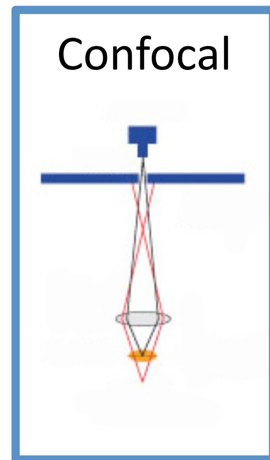


light from above the optical section also doesn't pass through the pinhole aperture

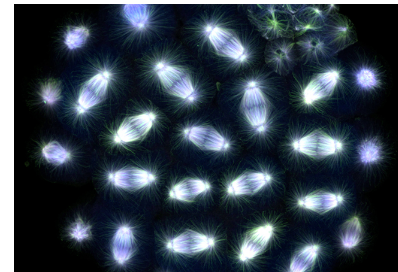


Confocal Microscopes

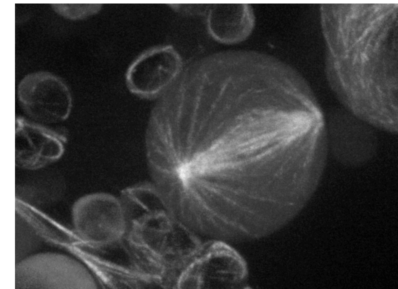
Confocal

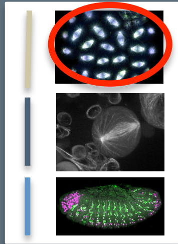


Laser Scanning



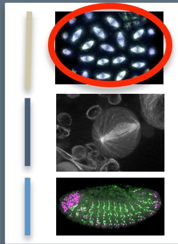
Spinning disc





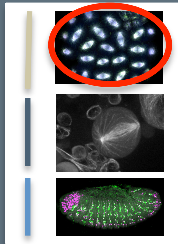
Laser Scanning Confocal Microscope

Laser Scanning Confocals are great to get ‘pretty’ images



Laser Scanning Confocal - components

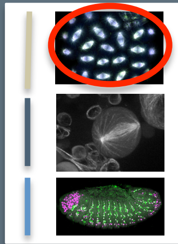
TD
OFF
Trans Lamp
TD1
3.5V
Specimen
Objective Lens
SU
LaserUnit1
405
458
488
515
559
635
ExcitationDM
OFF
EPI
SPD
FL - Imaging
DM
DM
DM
BF
BA575-675
425 nm - 475 nm
500 nm - 545 nm
CHS1
CHS2
CH3
80 um
205
-594
C.A
X
Y
Dye
Dye
Dye
DAPI
Alexa Fluor 488
Alexa Fluor 594
DyeList
B I



Laser Light Source

laser light source

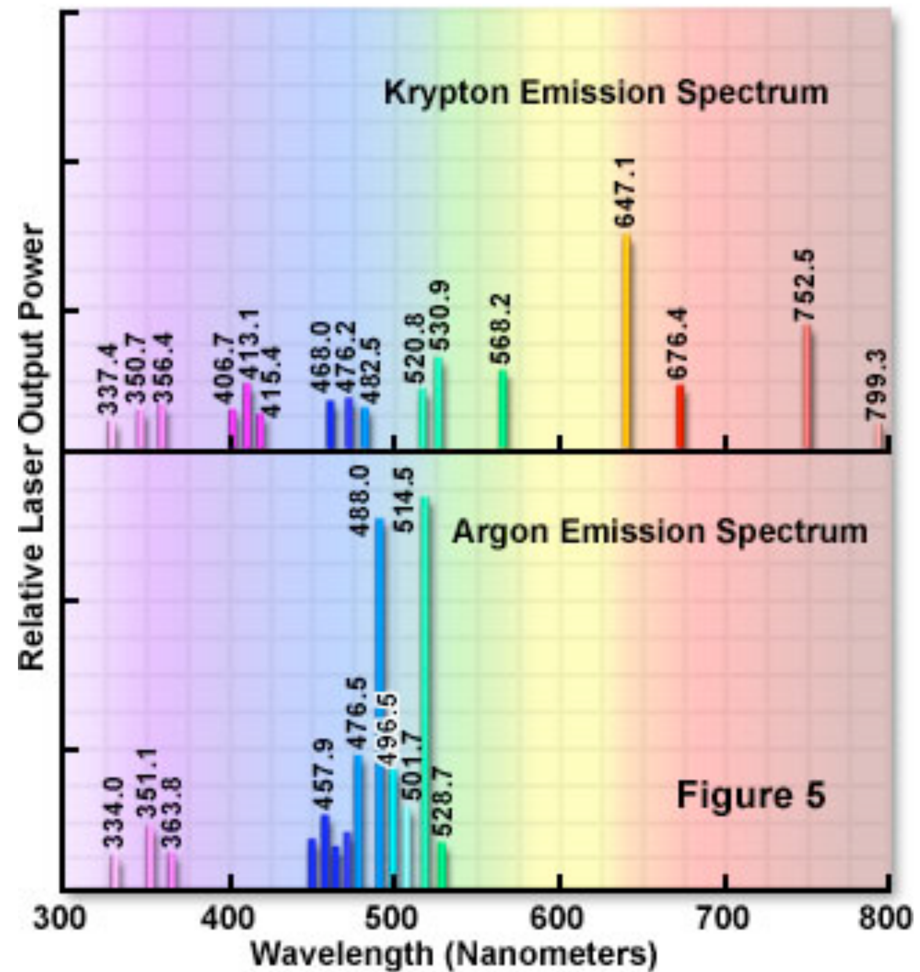
TD
OFF
Trans Lamp
TD1
3.5V
Specimen
Objective Lens
SU
LaserUnit1
405
458
488
515
559
635
None
ExcitationDM
DM405/488/559/635
OFF
EPI
SPD
FL - Imaging
DM
SDM490
SDM560
Mirror
BF
BA575-675
425 - 475 nm
500 - 545 nm
CHS1
CHS2
CH3
80 um
205
-594
C.A
X
Y
Dye
DAPI
Alexa Fluor 488
Alexa Fluor 594
DyeList
B I

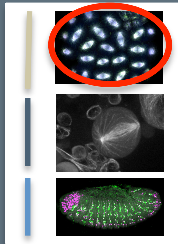


Laser Light Source

enables tighter control of fluorophores excited

Laser Emission Spectra

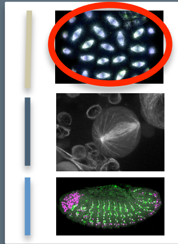




AOTF
Acousto-Optic Tunable Filter

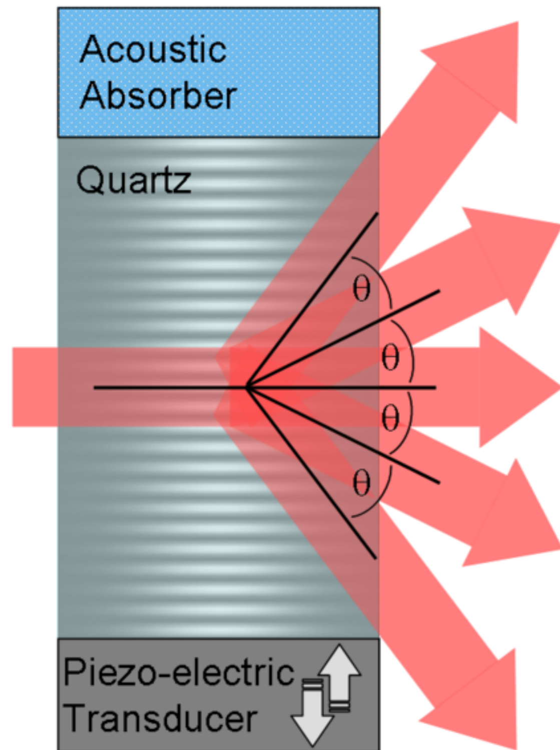
AOTF

TD
OFF
Trans Lamp
TD1
3.5V
Specimen
Objective Lens
SU
LaserUnit1
405
458
488
515
559
635
None
ExcitationDM
DM405/488/559/635
OFF
EPI
SPD
FL - Imaging
DM
SDM490
SDM560
Mirror
BF
BA575-675
425 - 475 nm
500 - 545 nm
CHS1
CHS2
CH3
80 um
205
-594
C.A
X
Y
Dye
DAPI
Alexa Fluor 488
Alexa Fluor 594
DyeList
B I



AOTF
Acousto-Optic Tunable Filter

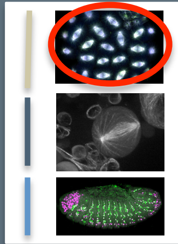
THEORY



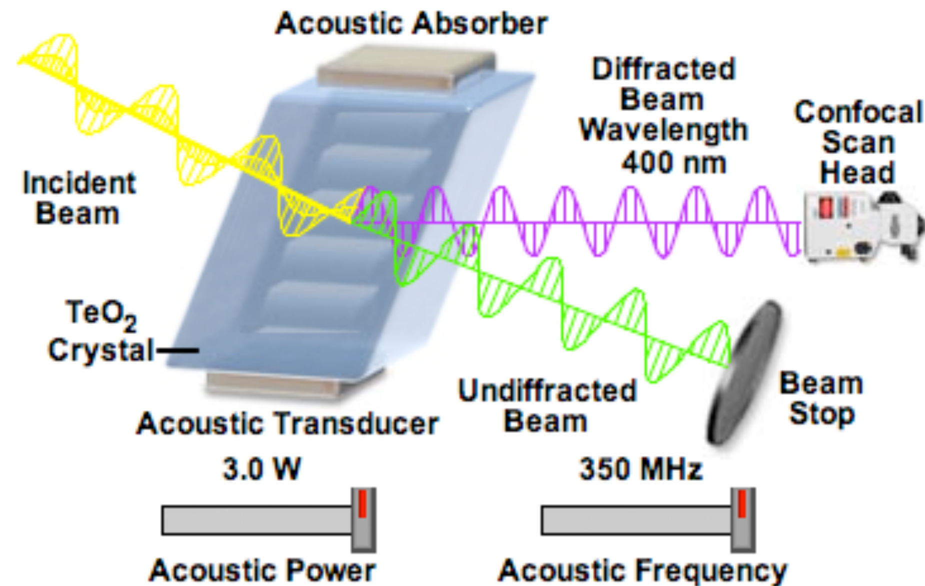
acousto-optic effect:

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

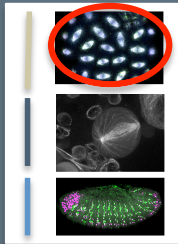
The wavelength of the diffracted light is dependent on the acoustic frequency in the quartz. By tuning the frequency of the acoustic wave, the desired wavelength of the optical wave can be diffracted acousto-optically.



AOTF
Acousto-Optic Tunable Filter



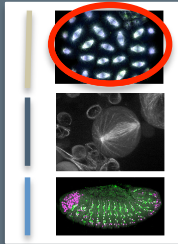
Quick On/Off of lasers
Very fast changes between excitation wavelengths



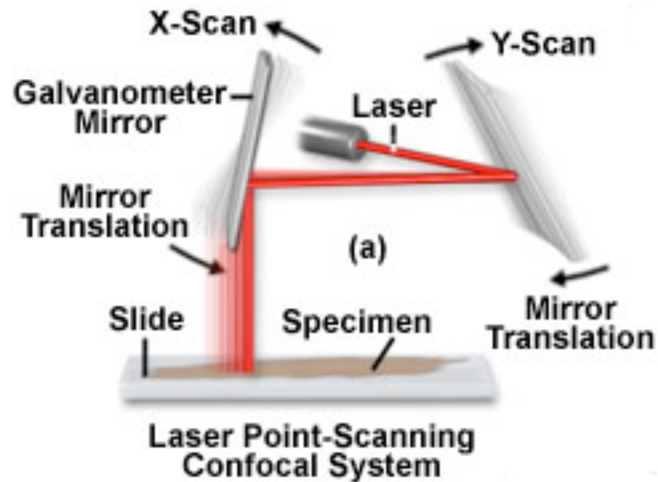
Galvo Scanning Mirrors

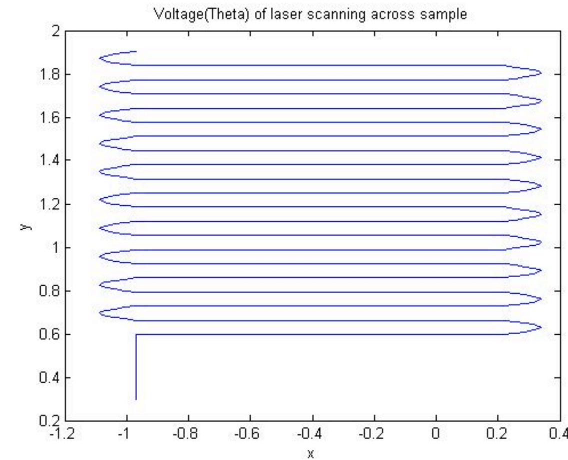
TD
OFF
Trans Lamp
TD1
3.5V
LaserUnit1
405
458
488
515
559
635
SU
Specimen
Objective Lens
None
ExcitationDM
DM405/488/559/635
SPD
FL - Imaging
DM
SDM490
SDM560
Mirror
BF
BA575-675
425 - 475 nm nm
500 - 545 nm nm
CHS1
CHS2
CH3
80 um
205
-594
C.A
X
Y
Dye
DAPI
Alexa Fluor 488
Alexa Fluor 594
DyeList
OFF
EPI
B I

Galvo Scanning Mirrors

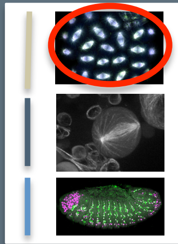


Galvo Scanning Mirrors





Sample excited at one point at a time
Relatively slow

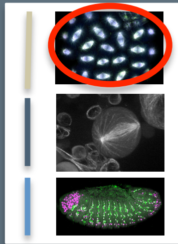


Adjustable Pinhole

AOTF

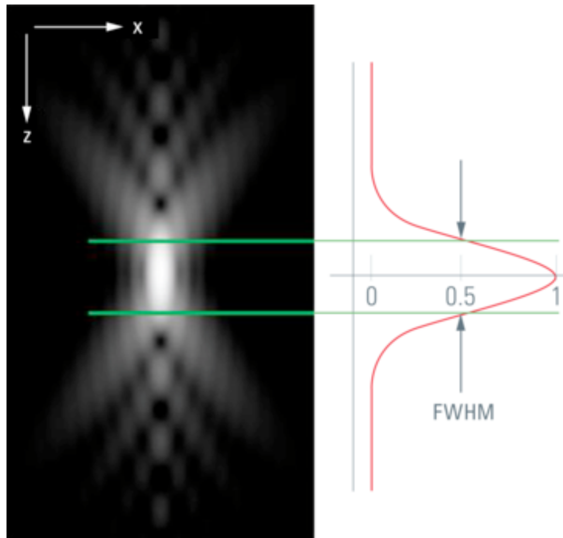
TD
OFF
Trans Lamp
TD1
3.5V
SU
LaserUnit1
405
458
488
515
559
635
Specimen
Objective Lens
None
ExcitationDM
DM405/488/559/635
OFF
EPI
SPD
FL - Imaging
DM
SDM480
SDM560
Mirror
BF
BA575-675
425 - 475 nm
500 - 545 nm
CHS1
CHS2
CH3
80 um
205
-594
C.A
X
Y
Dye
DAPI
Alexa Fluor 488
Alexa Fluor 594
DyeList
B I

pinhole



THEORY

Pinhole – Optical Sectioning



FWHM=Full Width Half-Maximum

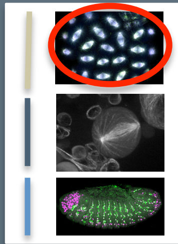
Shorter the wavelength the thinner the optical section

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

Diameter of the pinhole
Larger pinhole thicker section

The higher the NA.
the thinner the section

Weak signal > open pinhole > more light but thicker section



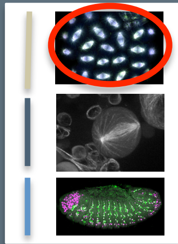
Confocal enables 3D reconstruction

Optical section

Focal Plane

SPECIMEN IMAGE

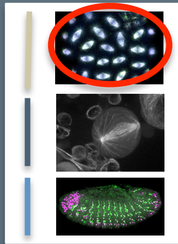
Z-Depth



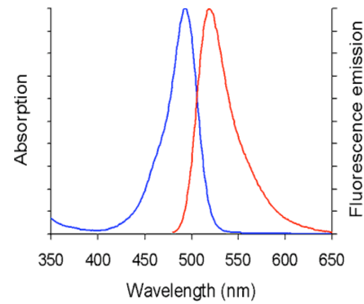
Variable Detector Slit

TD
OFF
Trans Lamp
TD1
3.5V
Specimen
Objective Lens
SU
LaserUnit1
405
458
488
515
559
635
None
ExcitationDM
DM405/488/559/635
OFF
EPI
SPD
FL - Imaging
DM
SDM490
SDM560
Mirror
BF
BA575-675
425 nm - 475 nm
500 nm - 545 nm
CHS1
CHS2
CH3
80 um
205
-594
C.A
X
Y
Dye
DAPI
Alexa Fluor 488
Alexa Fluor 594
DyeList
BI

variable detector slit

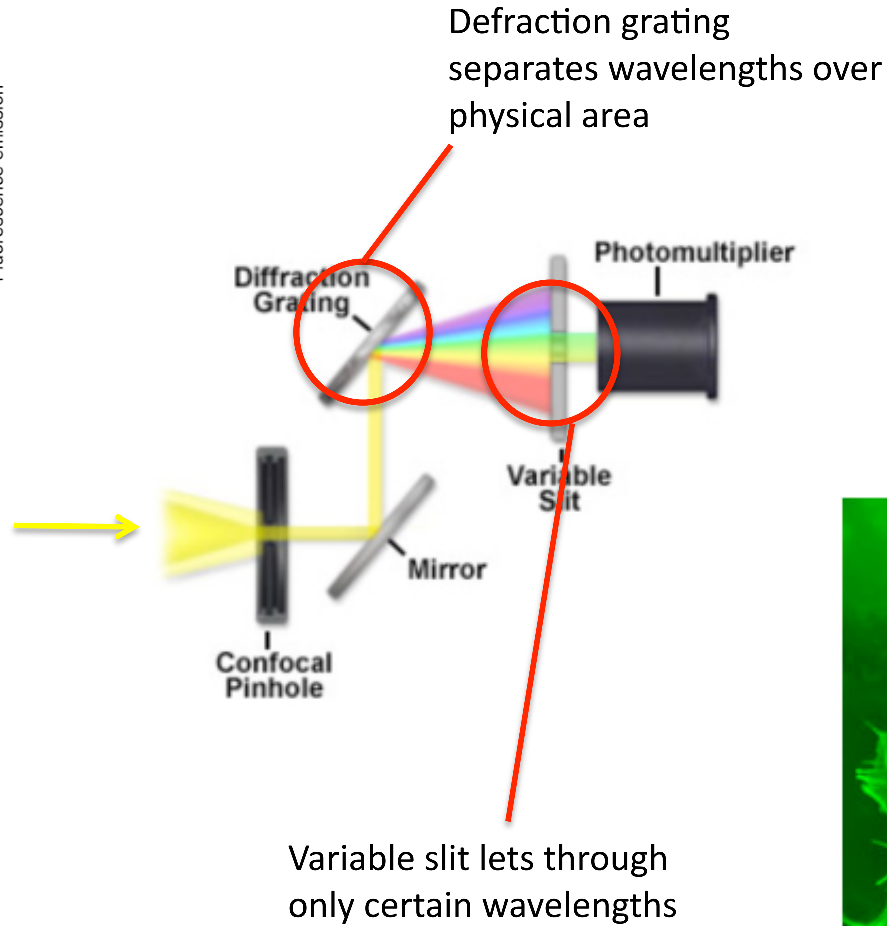


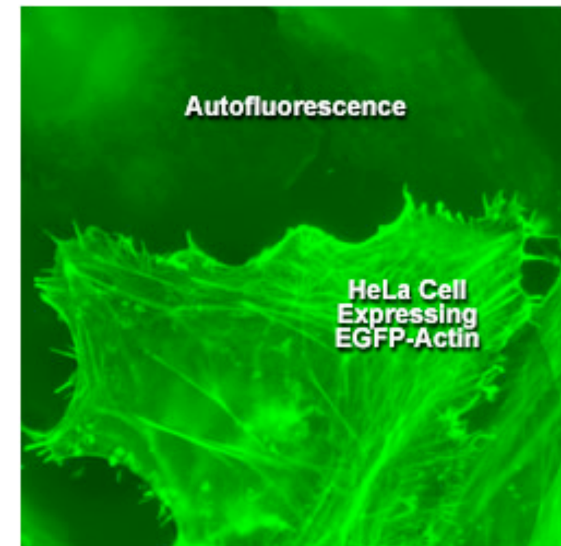
Spectral Unmixing



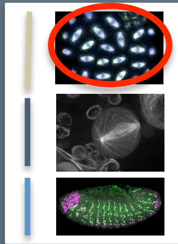
Defraction grating separates wavelengths over physical area

Light emitted from fluorophore as a spectrum

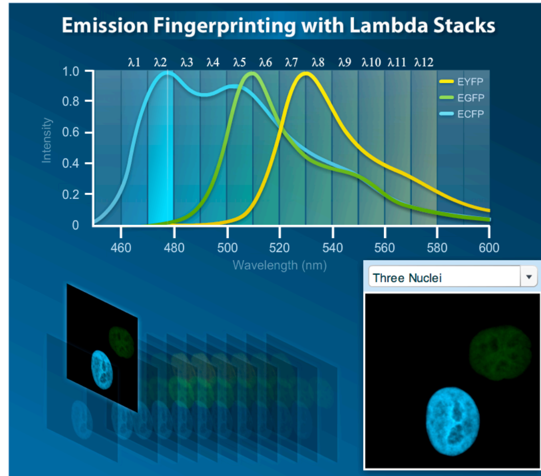


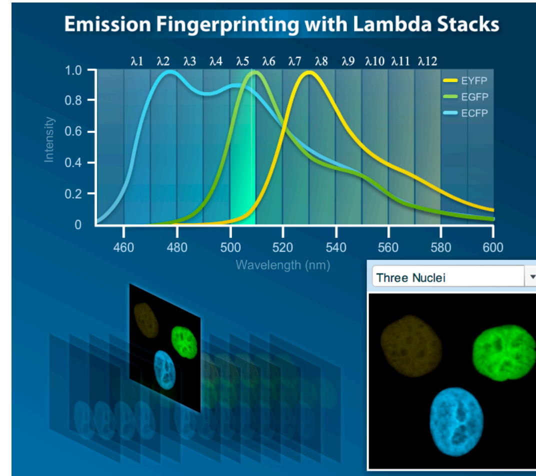


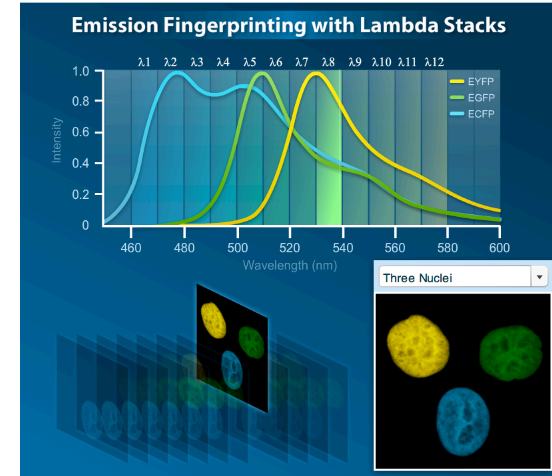
Variable slit lets through only certain wavelengths

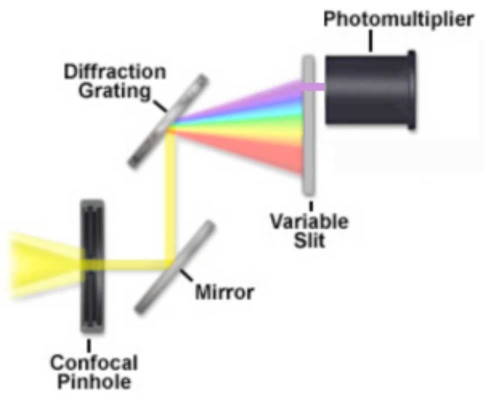


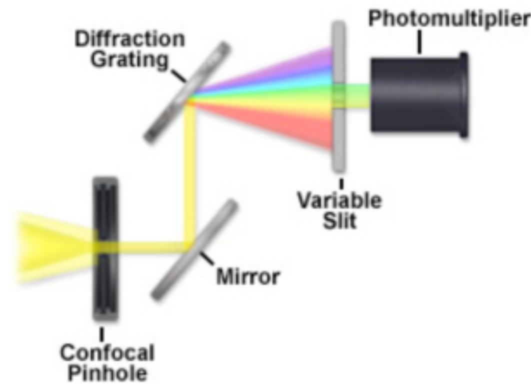
Spectral Unmixing

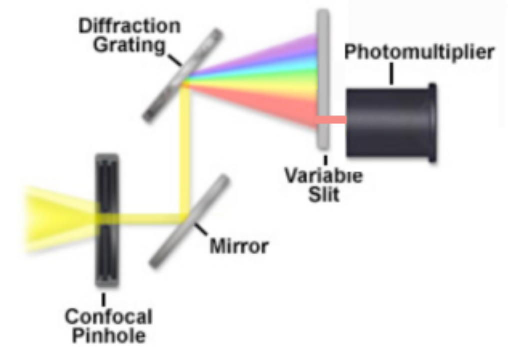


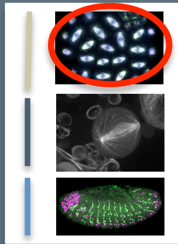




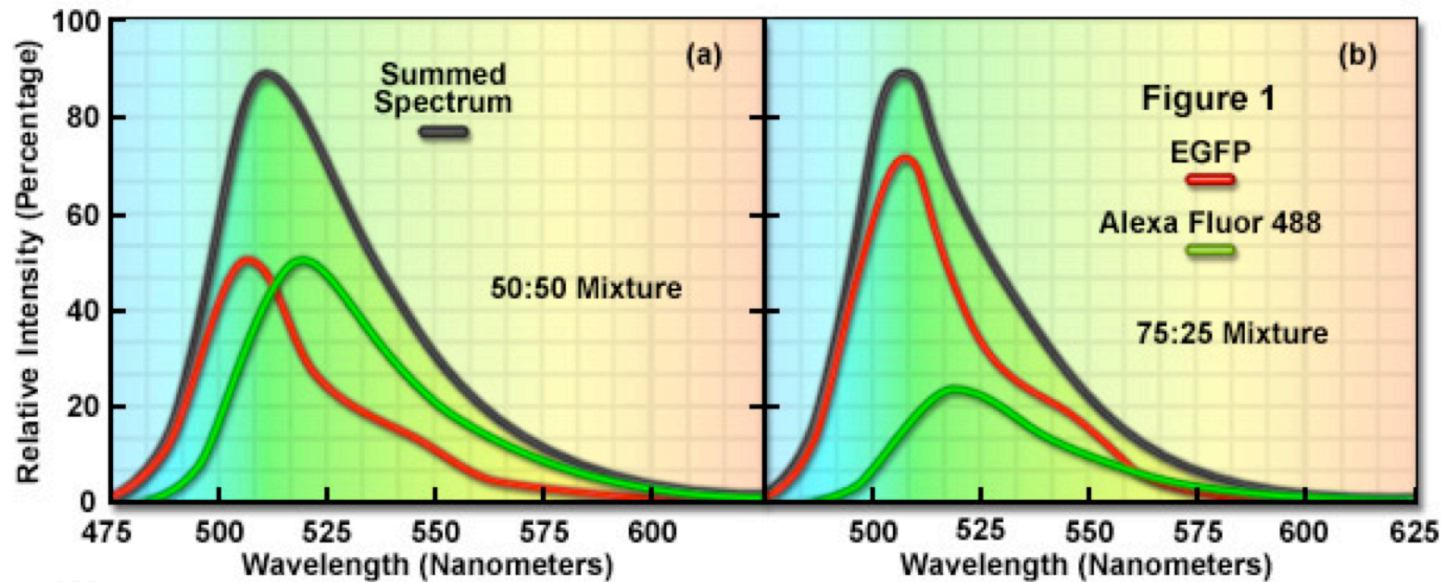






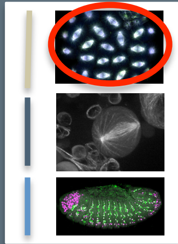


Spectral Unmixing

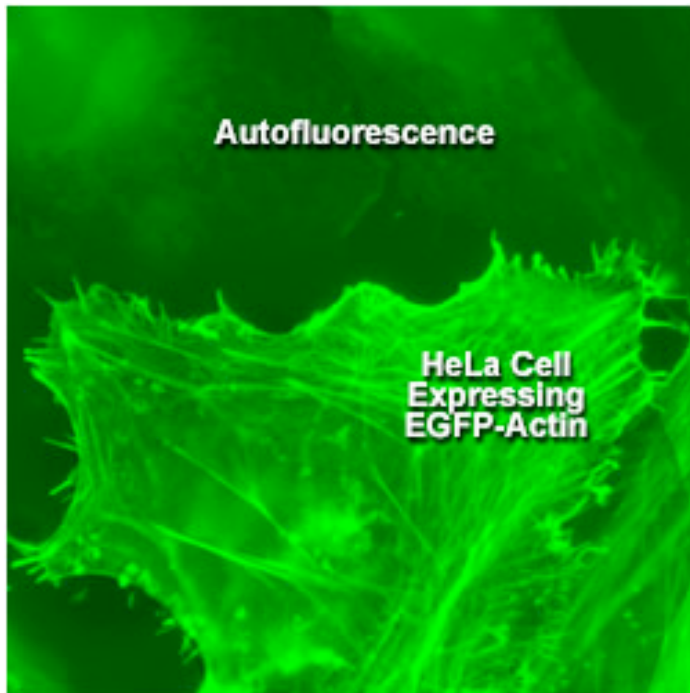


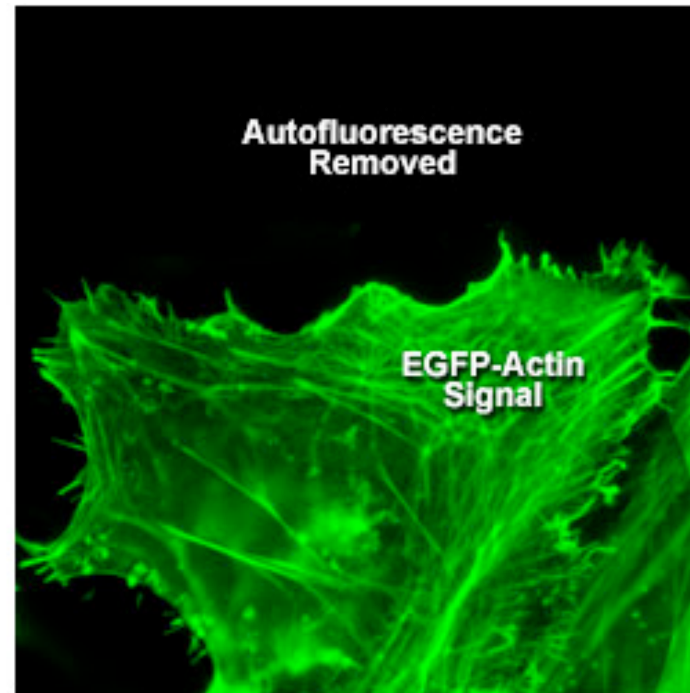
At each pixel:

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



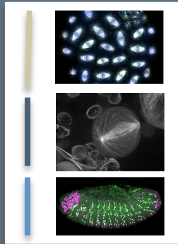
Spectral Unmixing
removal of autofluorescence





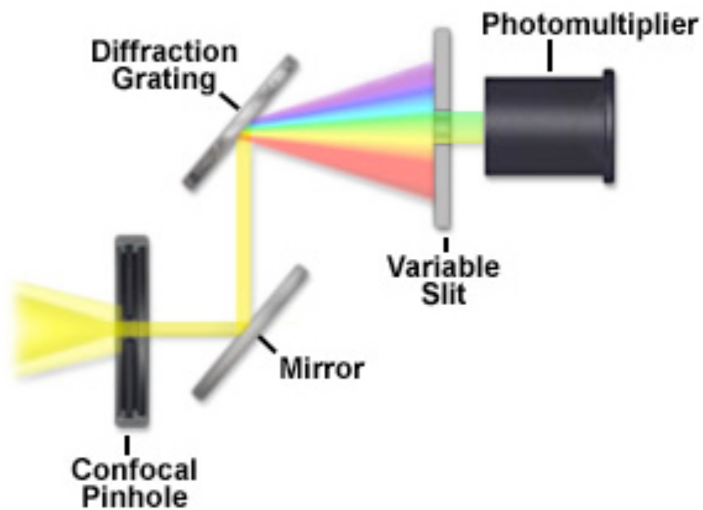
At each pixel:

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

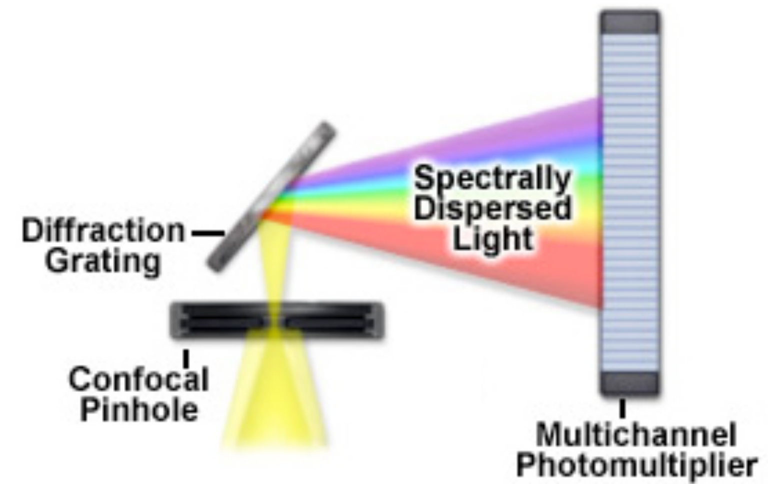


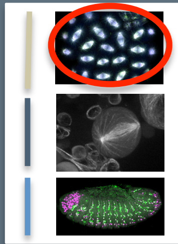
Spectral Unmixing

Olympus FV1000



Zeiss 510 META

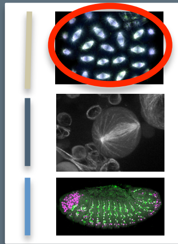




PMT – Photon Multiplier Tube

TD
OFF
Trans Lamp
TD1
3.5V
Specimen
Objective Lens
SU
LaserUnit1
405
458
488
515
559
635
None
OFF
EPI
ExcitationDM
DM405/488/559/635
SPD
FL - Imaging
DM
SDM490
SDM560
Mirror
BF
BA575-675
425 nm - 475 nm
500 nm - 545 nm
80 um
205
-594
C.A
X
Y
CHS1
CHS2
CH3
Dye
DAPI
Alexa Fluor 488
Alexa Fluor 594
DyeList
B I

PMT detectors

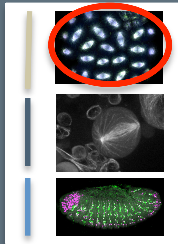


PMT – Photon Multiplier Tube

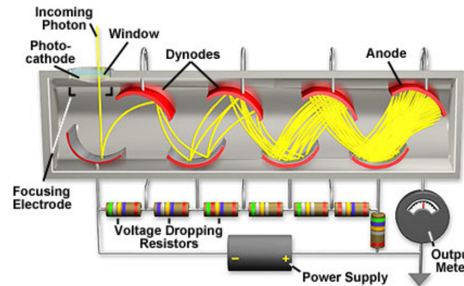
Incoming Photon
Window
Photo-cathode
Dynodes
Anode
Focusing Electrode
Voltage Dropping Resistors
Power Supply
Output Meter

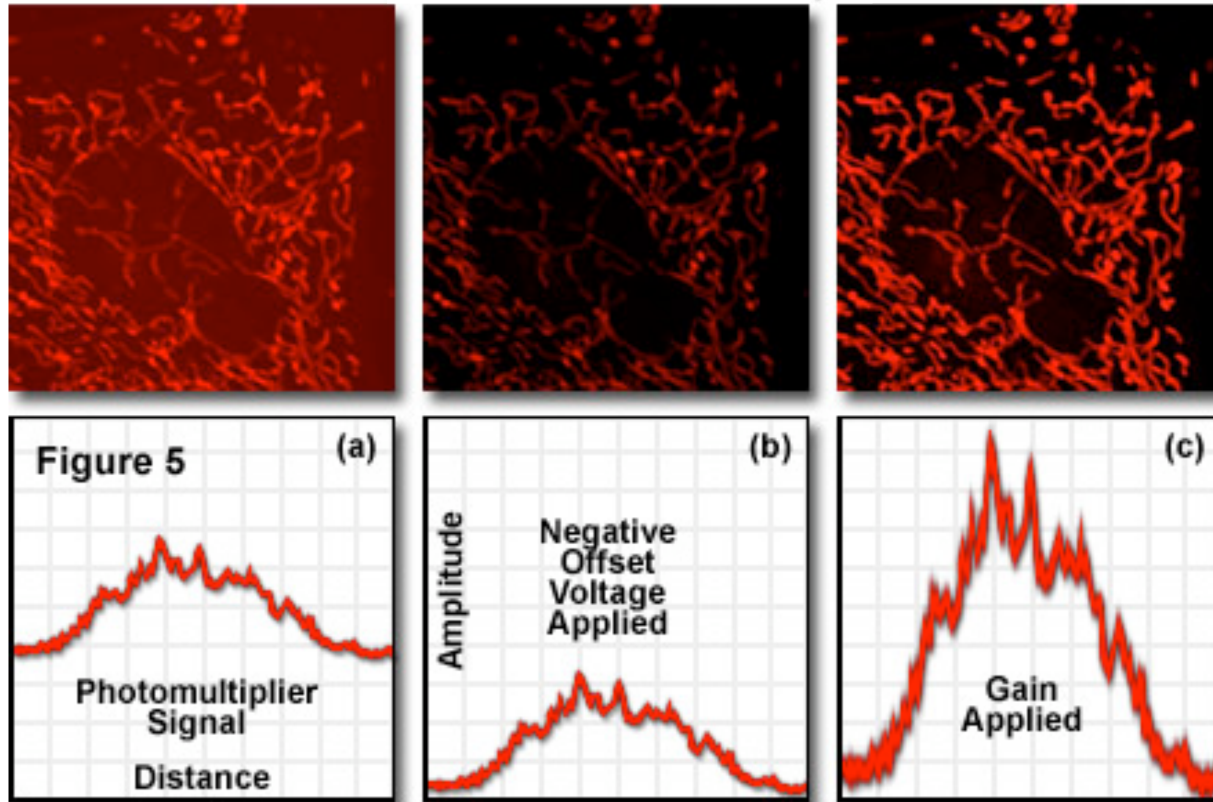
Very Low Noise

Huge Signal Amplification (~1x10)

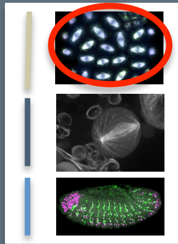


PMT – Photon Multiplier Tube
Adjusting Gain and Offset

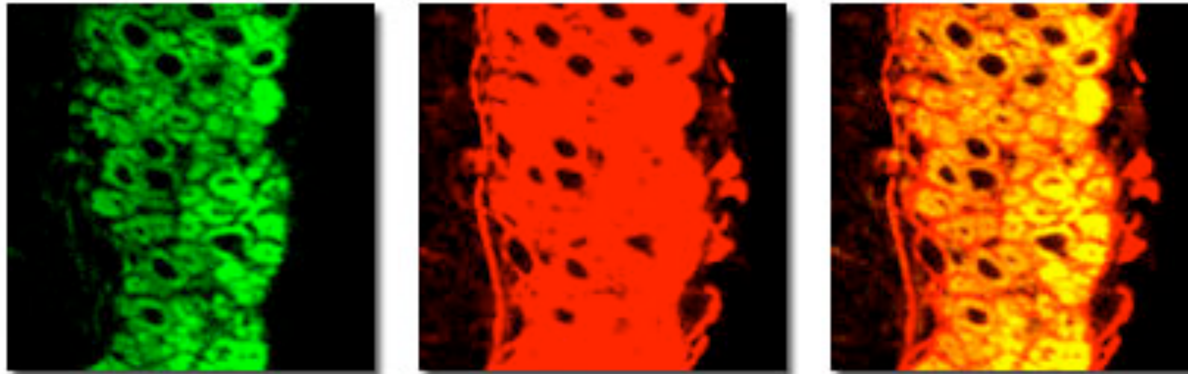


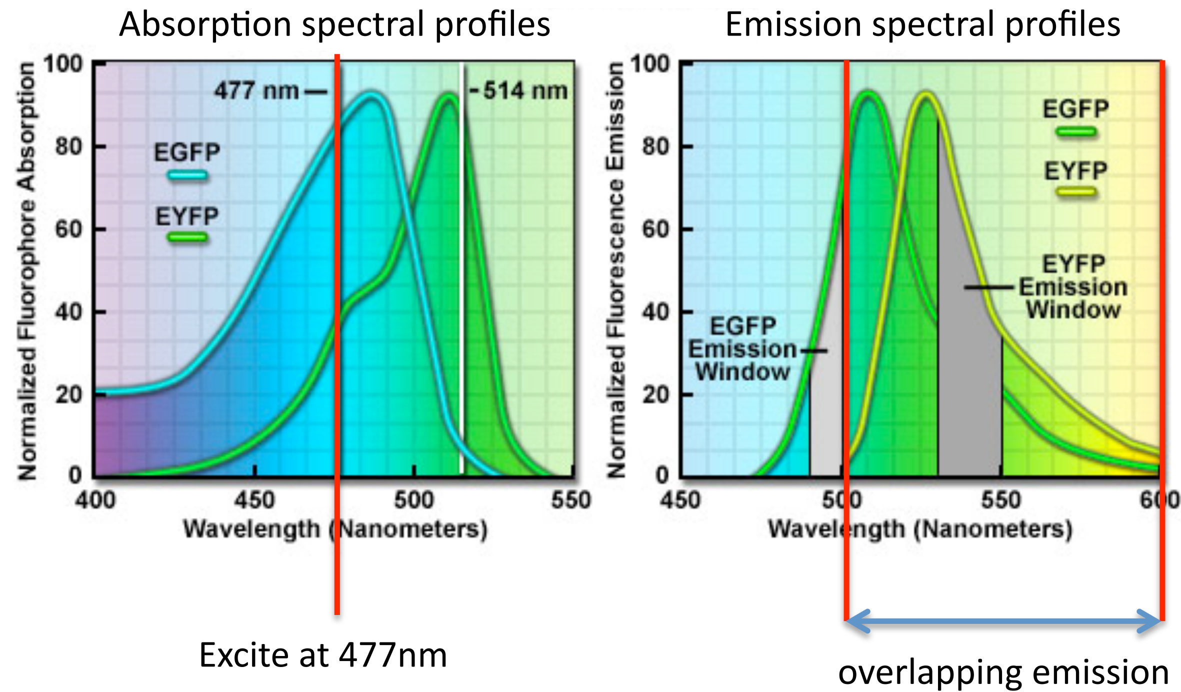


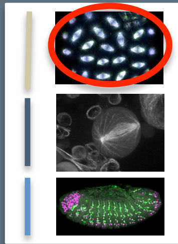
Beware - this is how your image will be saved!



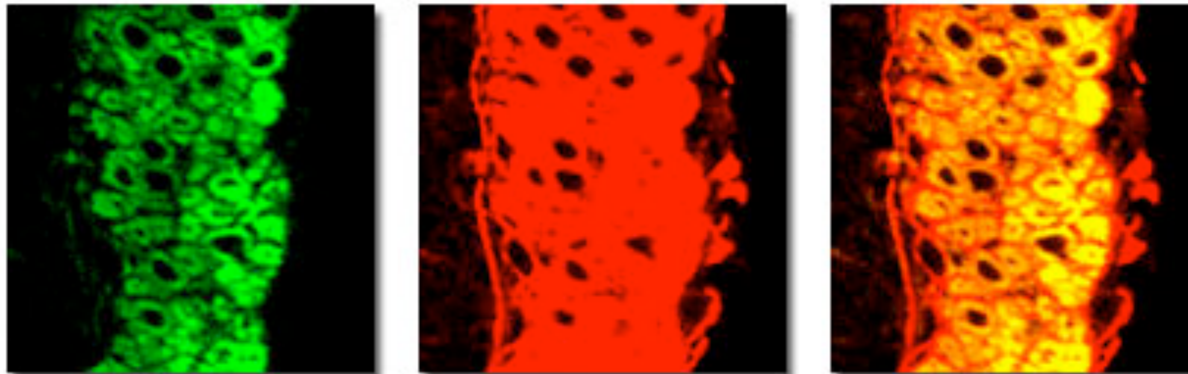
‘bleed-through’

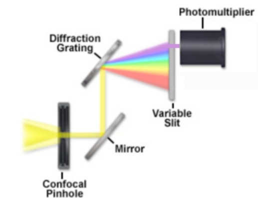


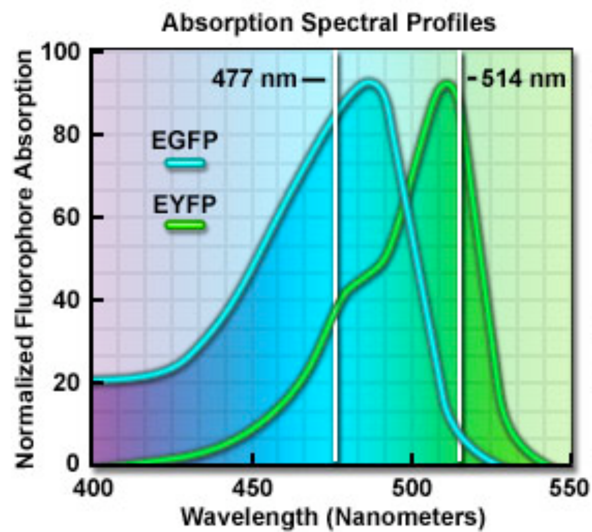


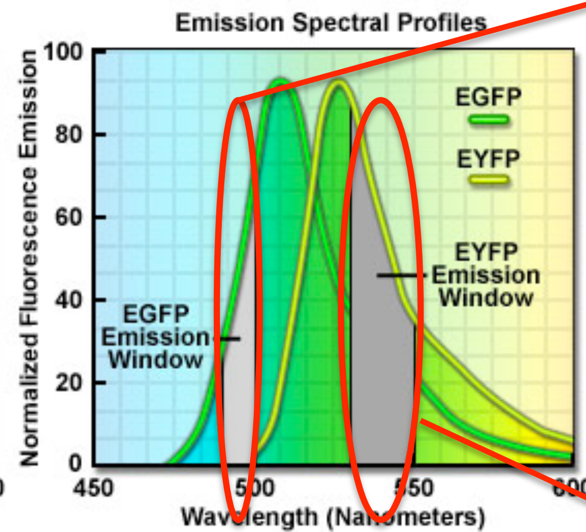


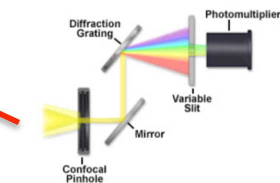
minimising 'bleed-through' Variable Slits



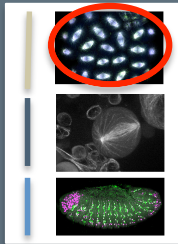


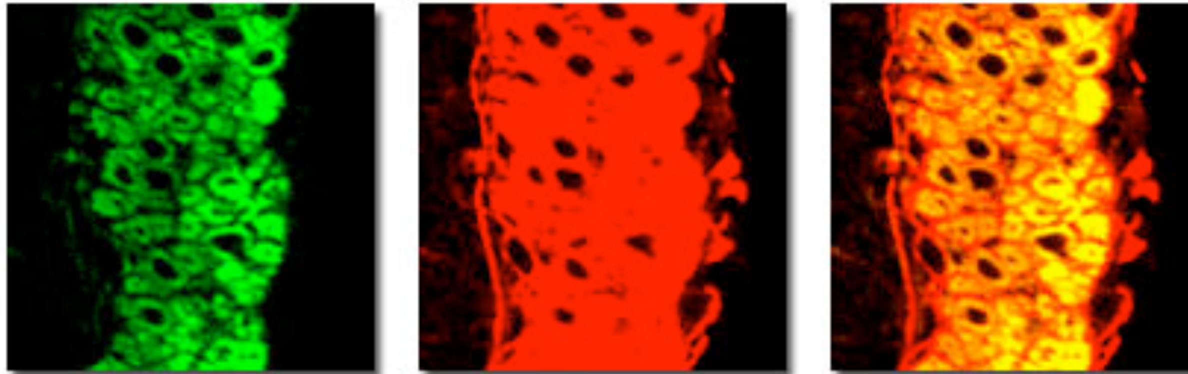


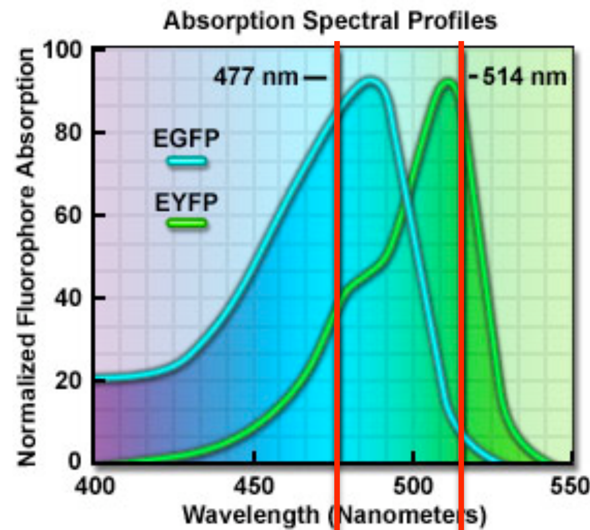


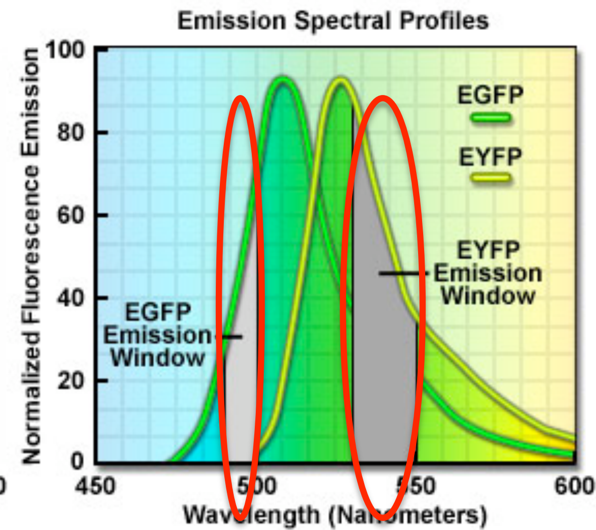


minimising 'bleed-through' Sequential Scanning





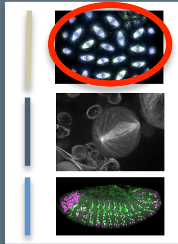




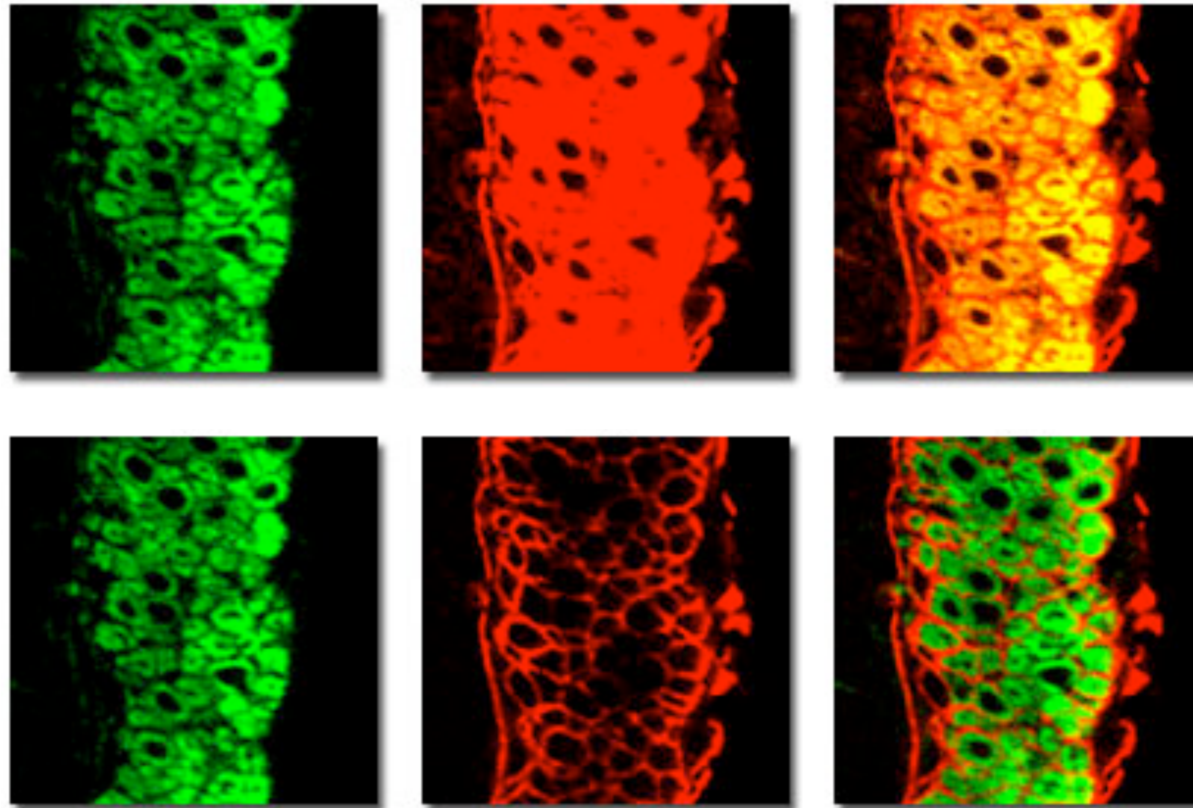
Excite at 477nm

Excite at 514nm

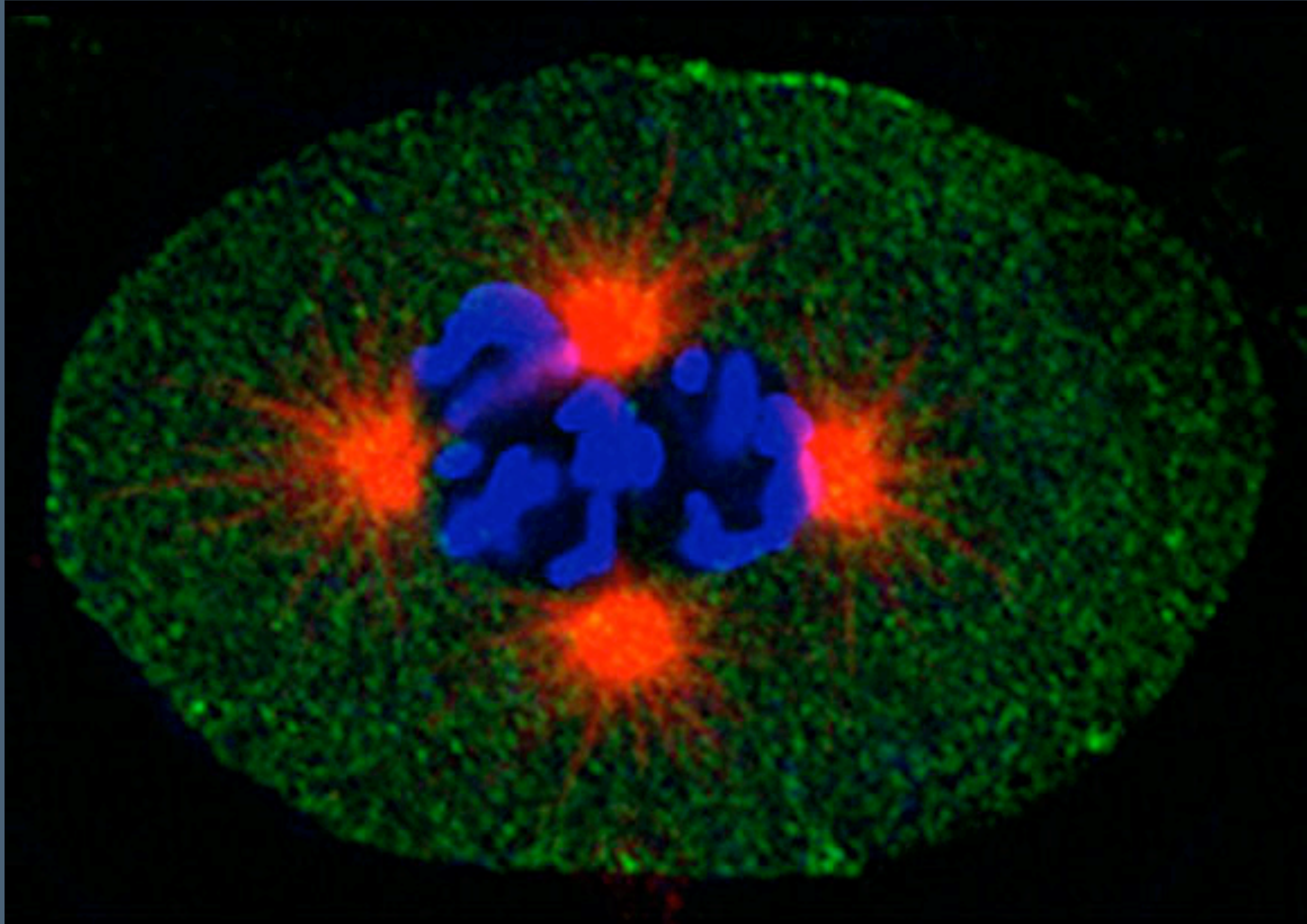
Temporal separation

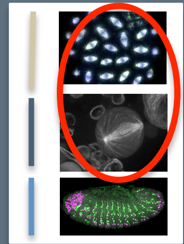


minimising 'bleed-through'



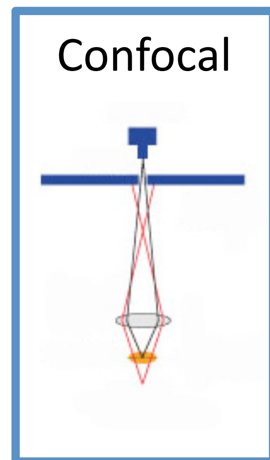
Adjust detector slit widths
Use sequential scanning



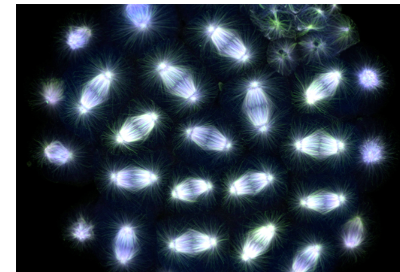


Confocal Microscopes

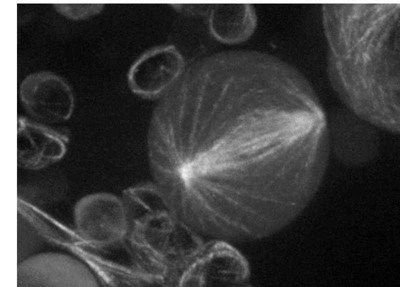
Confocal



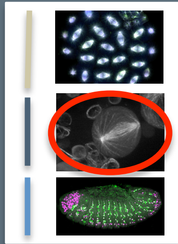
Laser Scanning



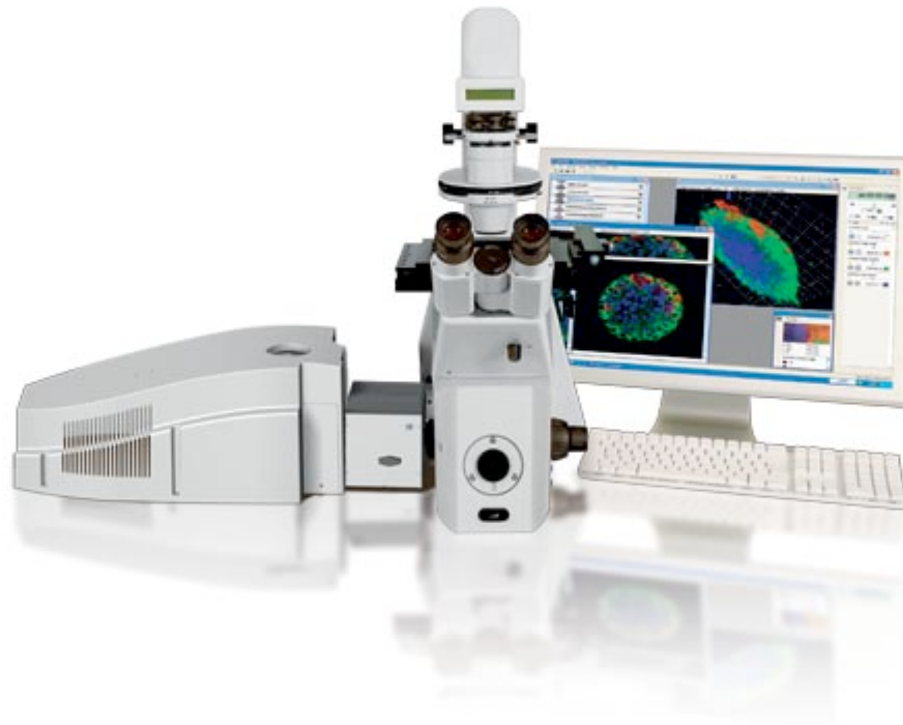
Spinning disc



Both are confocals

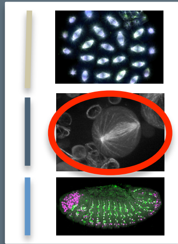


Spinning Disc Confocal



Great for live cell imaging

Can collect 2000 images per second



Yokogawa CSU-X1 Spinning Disc

Micro lens Array

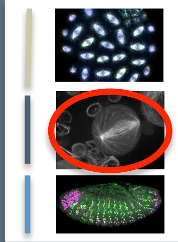
Nipkow Disk

Drive Motor

Beamsplitter

To CCD camera
(Lecture 10)

Sample



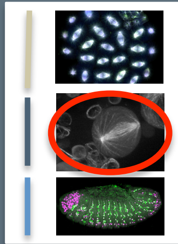
Yokogawa CSU-X1
Spinning Disc

Nipkow Disk

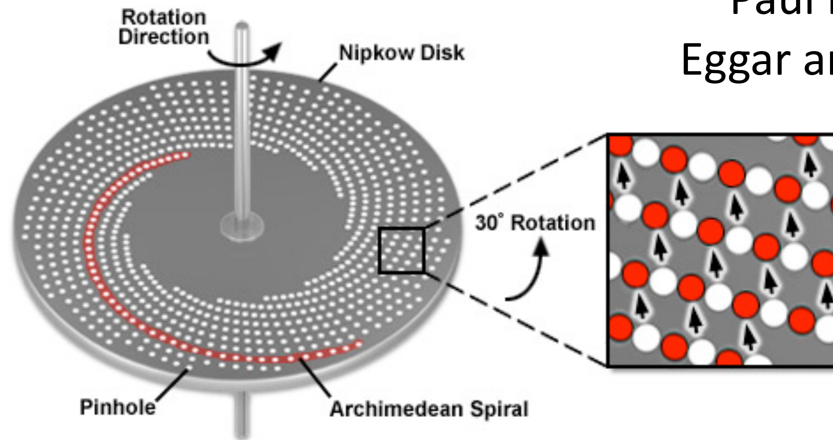
Drive Motor

Beamsplitter

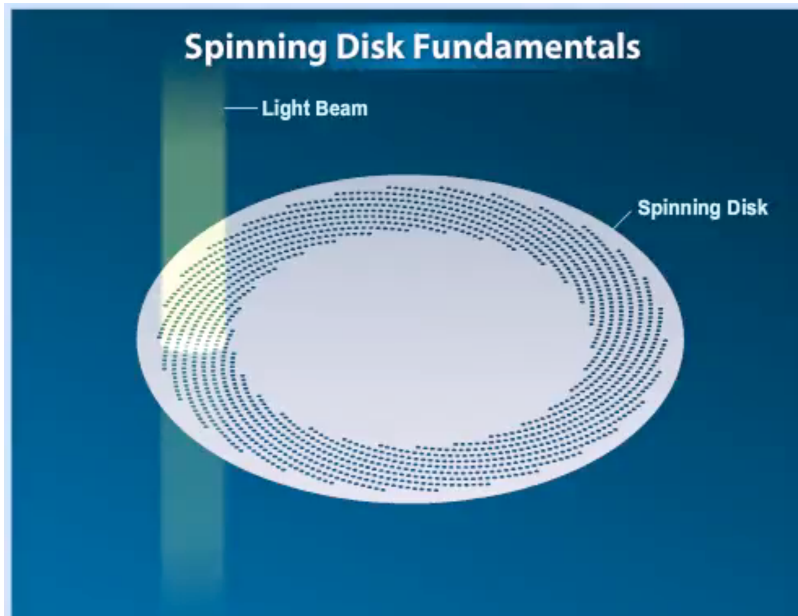
Sample



The Nipkow Disk

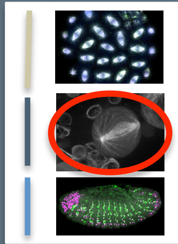


Paul Nipkow, 1884
Eggar and Petran, 1967

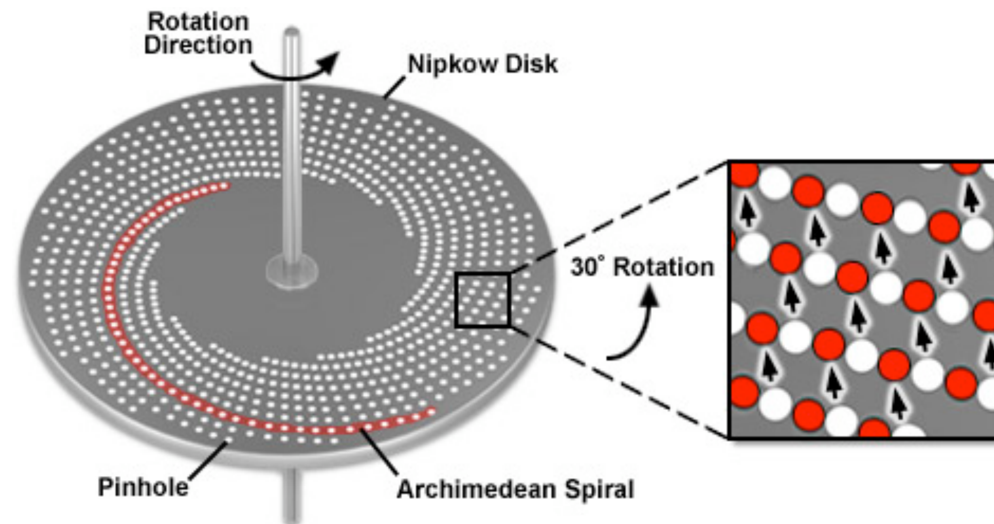


Approx. 1000 pinholes

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

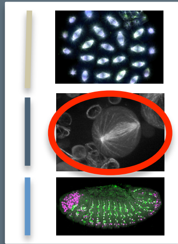


The Nipkow Disk

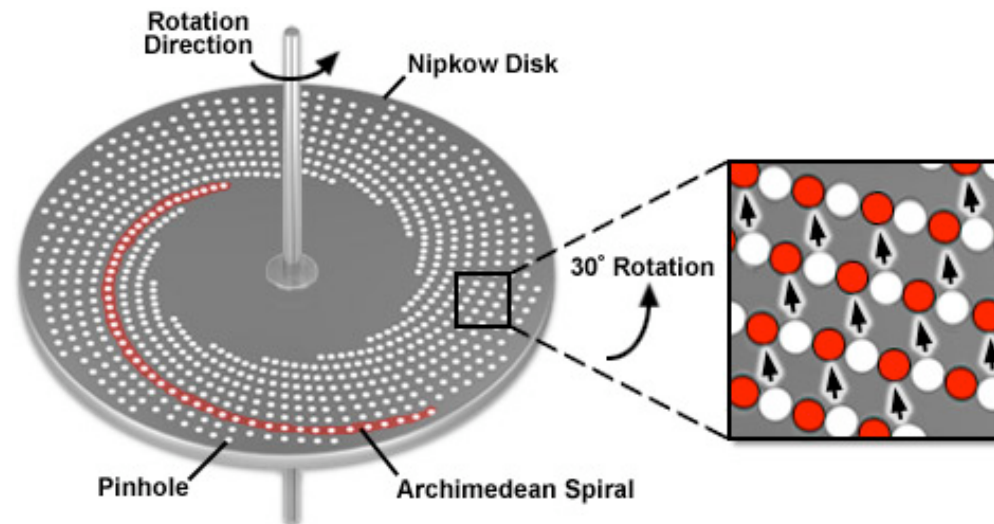


Larger pinholes - brighter image, but less “confocal”

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



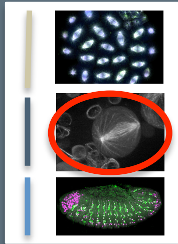
The Nipkow Disk



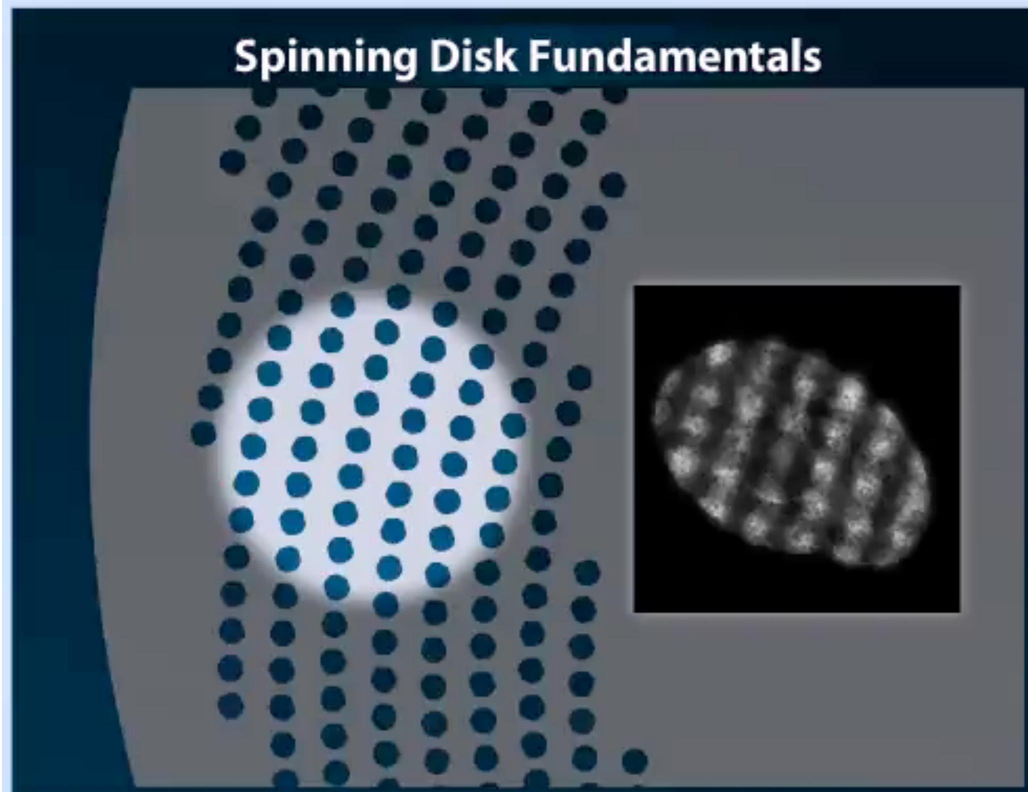
Constant Battle:

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

Pinhole Spacing Typically = 2.5um apart



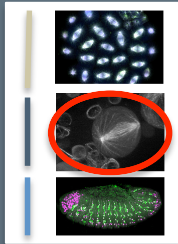
Spinning Disc Confocal



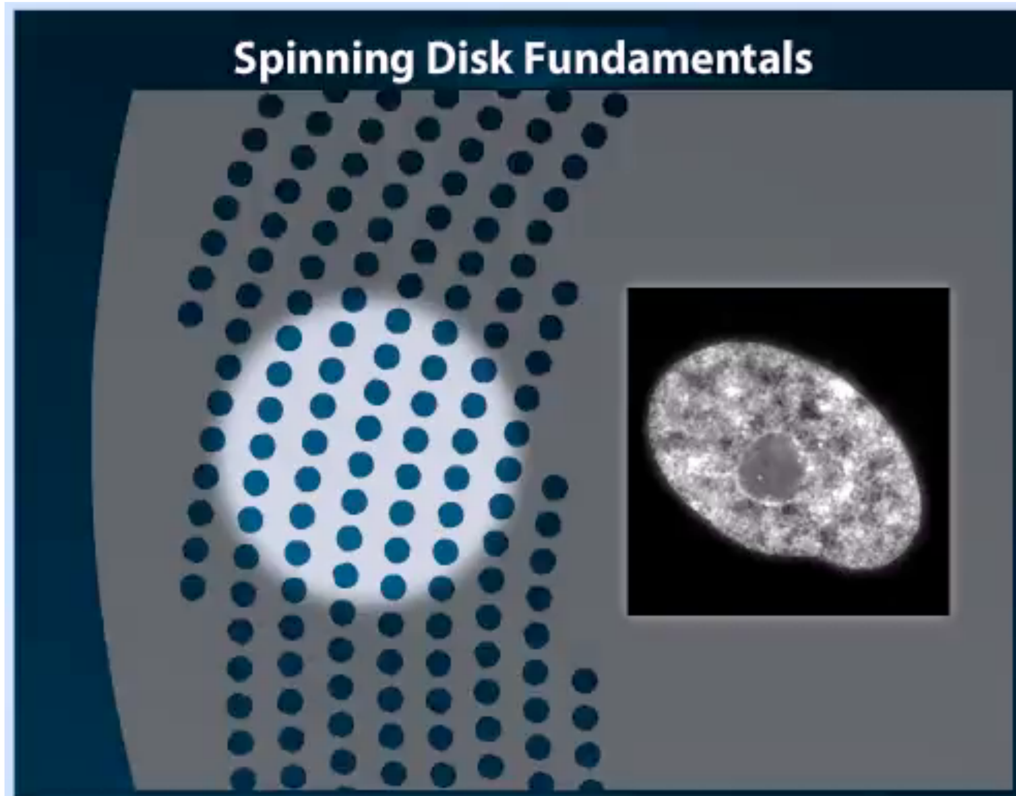
Disc spins to illuminate sample

spin speed – disc spinning too slowly

you see the pinholes on the sample

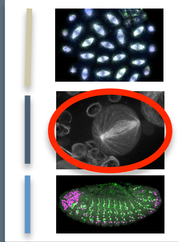


Spinning Disc Confocal



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

Disc speed must carefully match camera frame rate

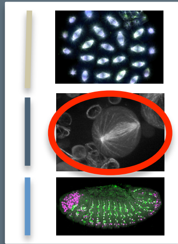


Yokogawa CSU-X1
Spinning Disc

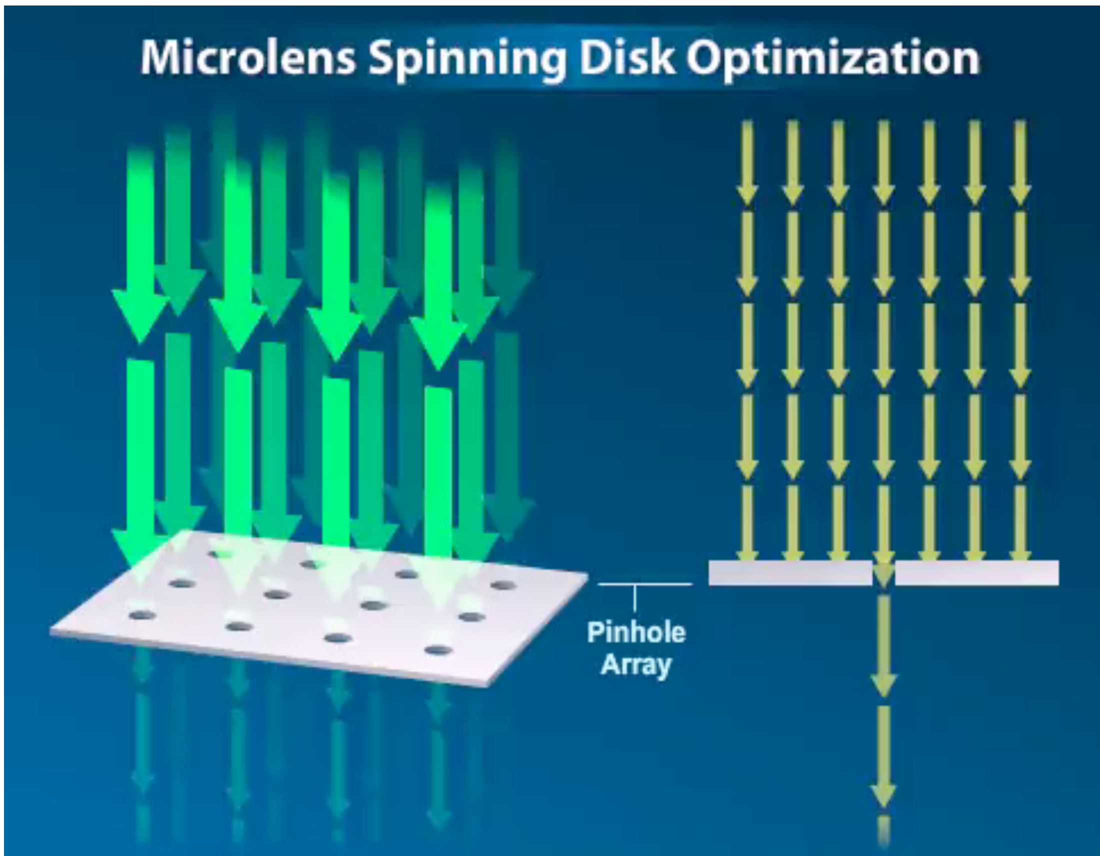
Micro lens Array

Drive Motor

Beamsplitter



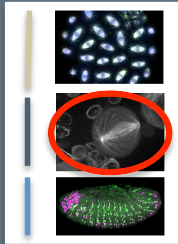
Yokogawa Spinning Disc Confocal Microlenses



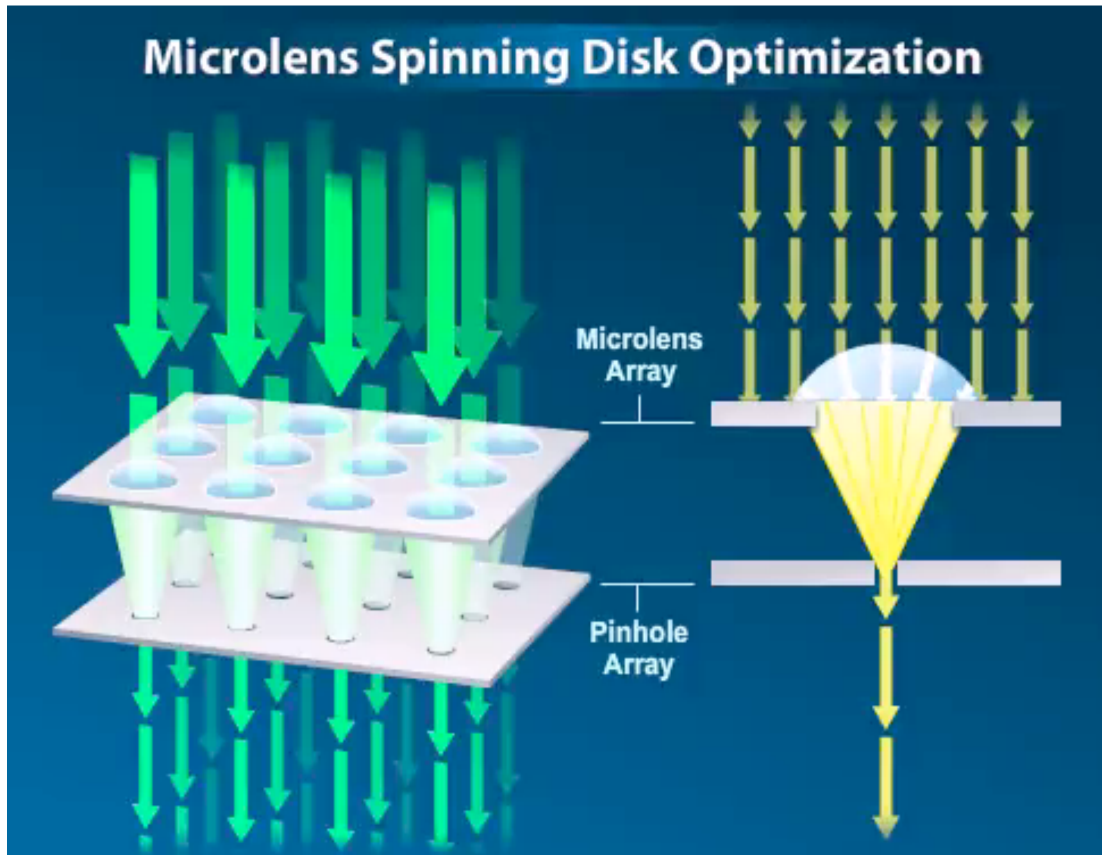
just a pinhole array – Optimised for 'cofocality' and 'crosstalk'

too much light is blocked from reaching the specimen

Only 4% light passes through disc



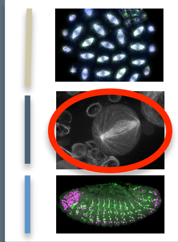
Yokogawa Spinning Disc Confocal Microlenses



micro-lens array increase the light reaching the specimen

Typically 56% light passes through disc

Yokogawa
Spinning Disc Confocal



Yokogawa Spinning Disk

Light Direction

Microlens Array

Lens

CCD

Dichromatic Mirror

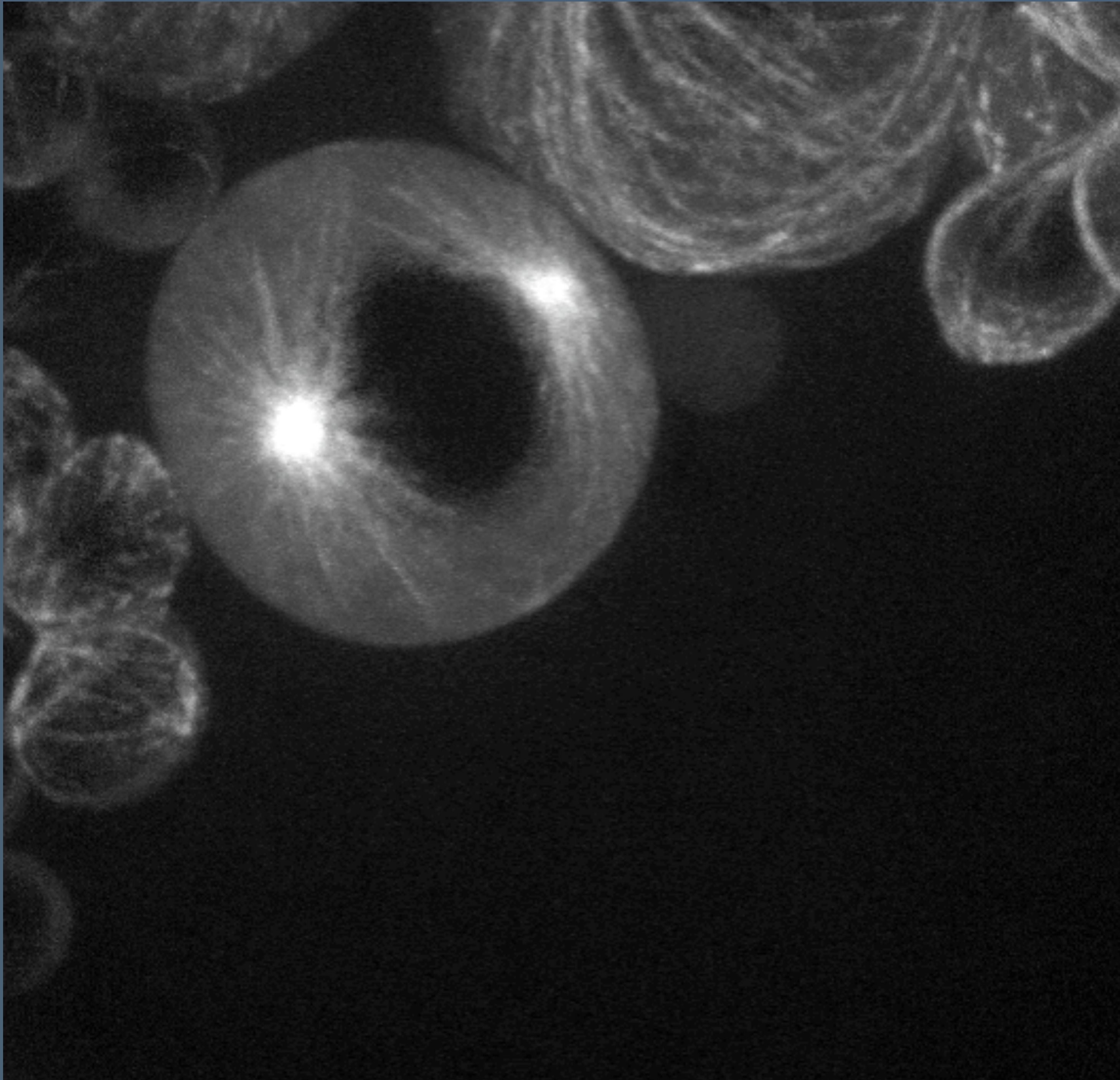
Pinhole Array

Objective

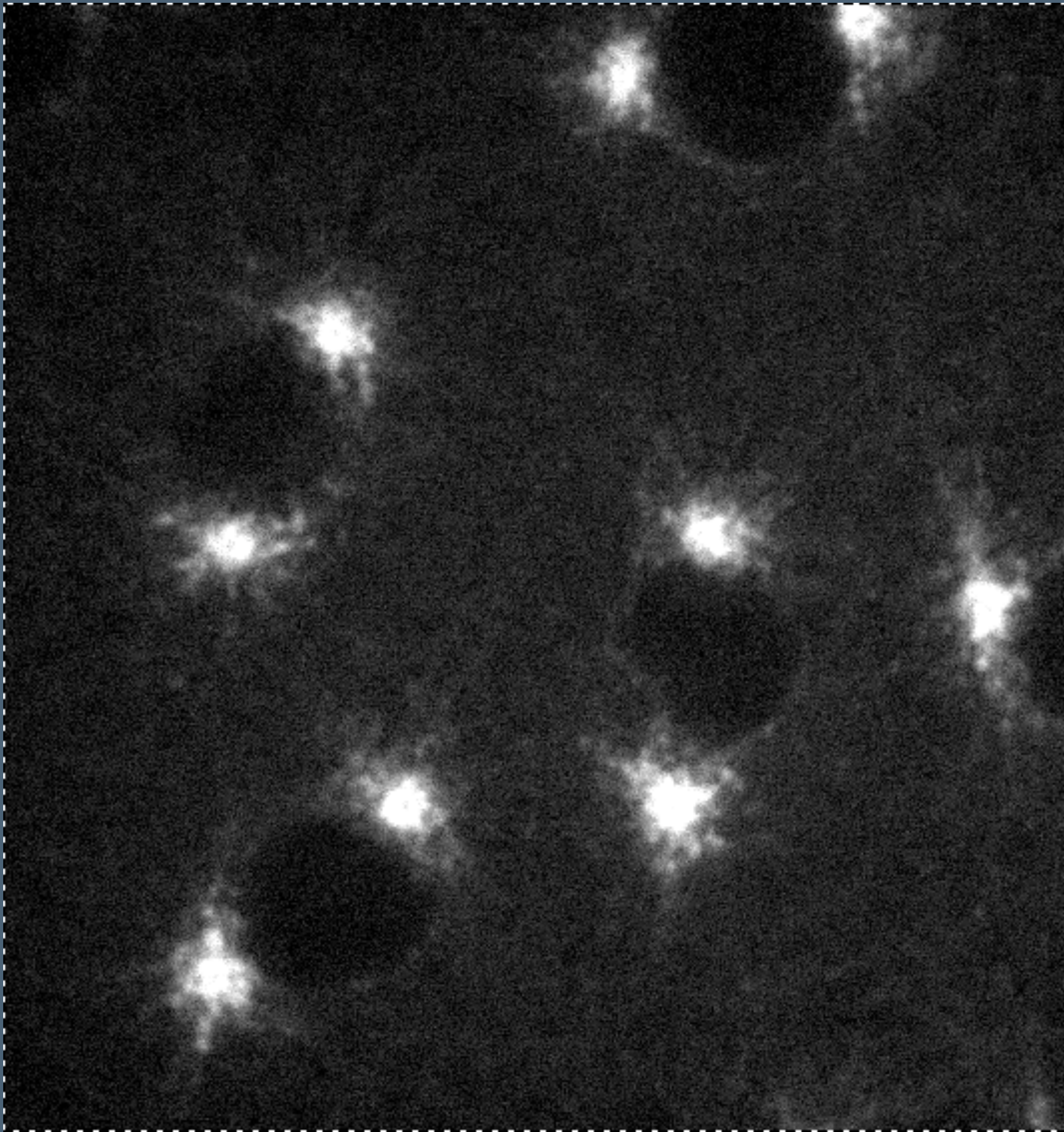
Slide

CCD

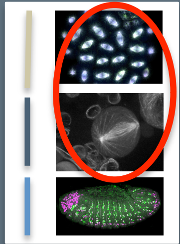
SPECIMEN



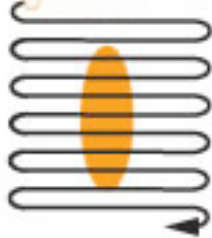
Cell division in brain stem cells (neuroblasts), Raff Lab

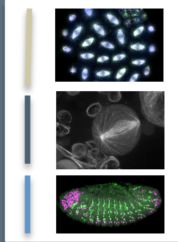


MT binding protein in *Drosophila* embryo, Raff Lab



Point Scanning Vs Spinning Disc

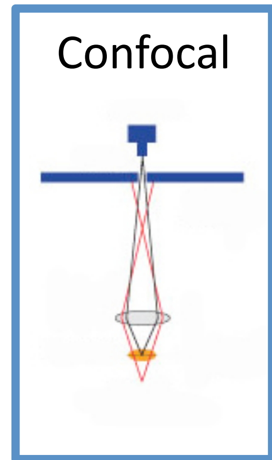
	Point Scanning	Spinning Disc
		
Speed	Slow (secs)	Fast (msecs)
Sensitivity	OK	OK
Flexibility	Good	Poor
Bleaching	Poor	Good
Pretty Pictures	Unbeatable!	Pretty damn good!
Pretty Movies	Good – if process slow	Unbeatable!



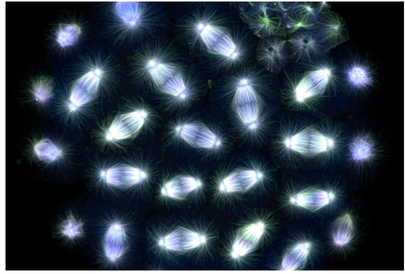
3 Flavours of Microscope

Problem:
Out of Focus
Light

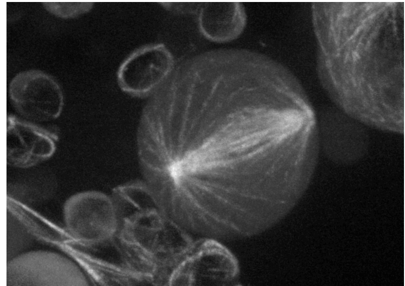
Confocal

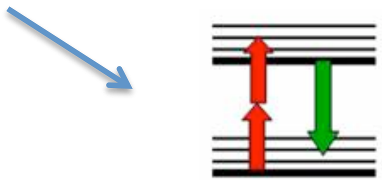


Laser
Scanning

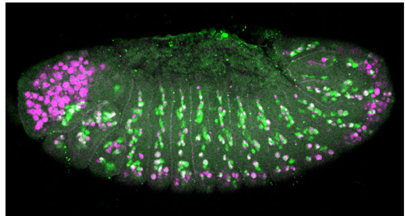


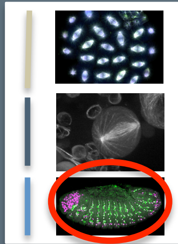
Spinning
disc





2-Photon



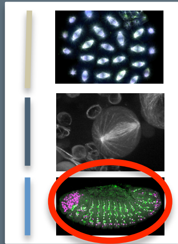


2-photon Microscope

Not a 'confocal'

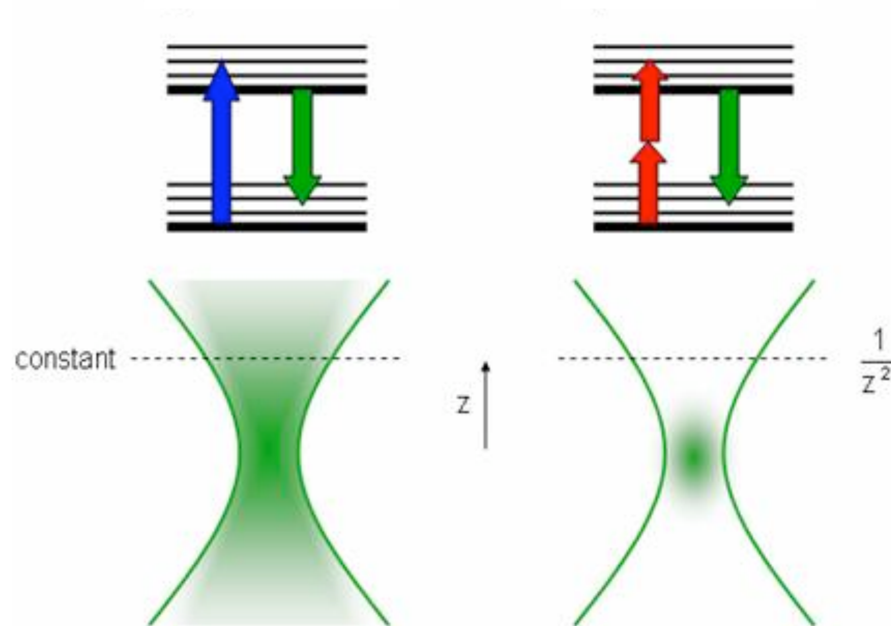
for imaging deeper into thick specimens

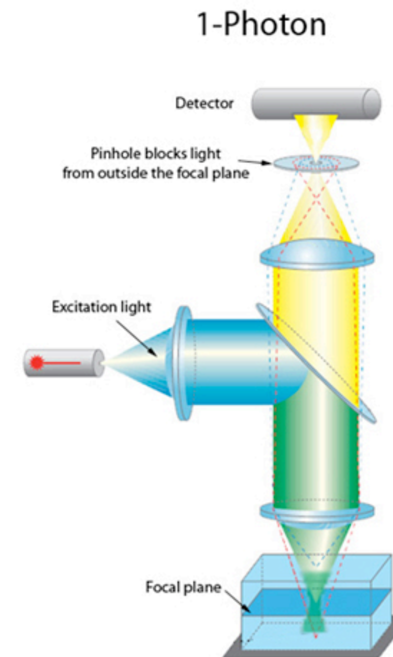
less damaging to biological samples

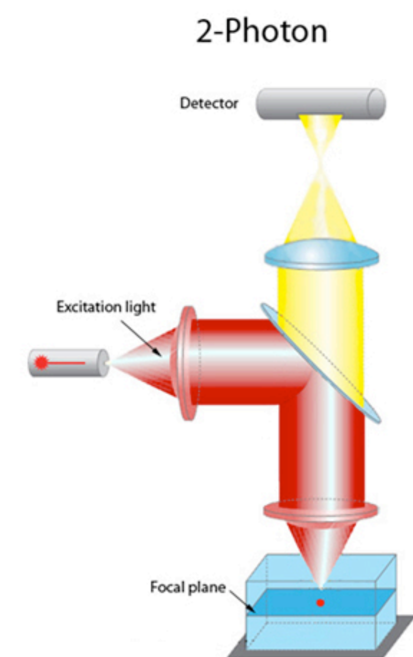


Confocal Vs 2-photon

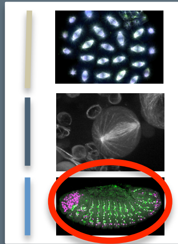
1 Photon Excitation   2 Photon Excitation





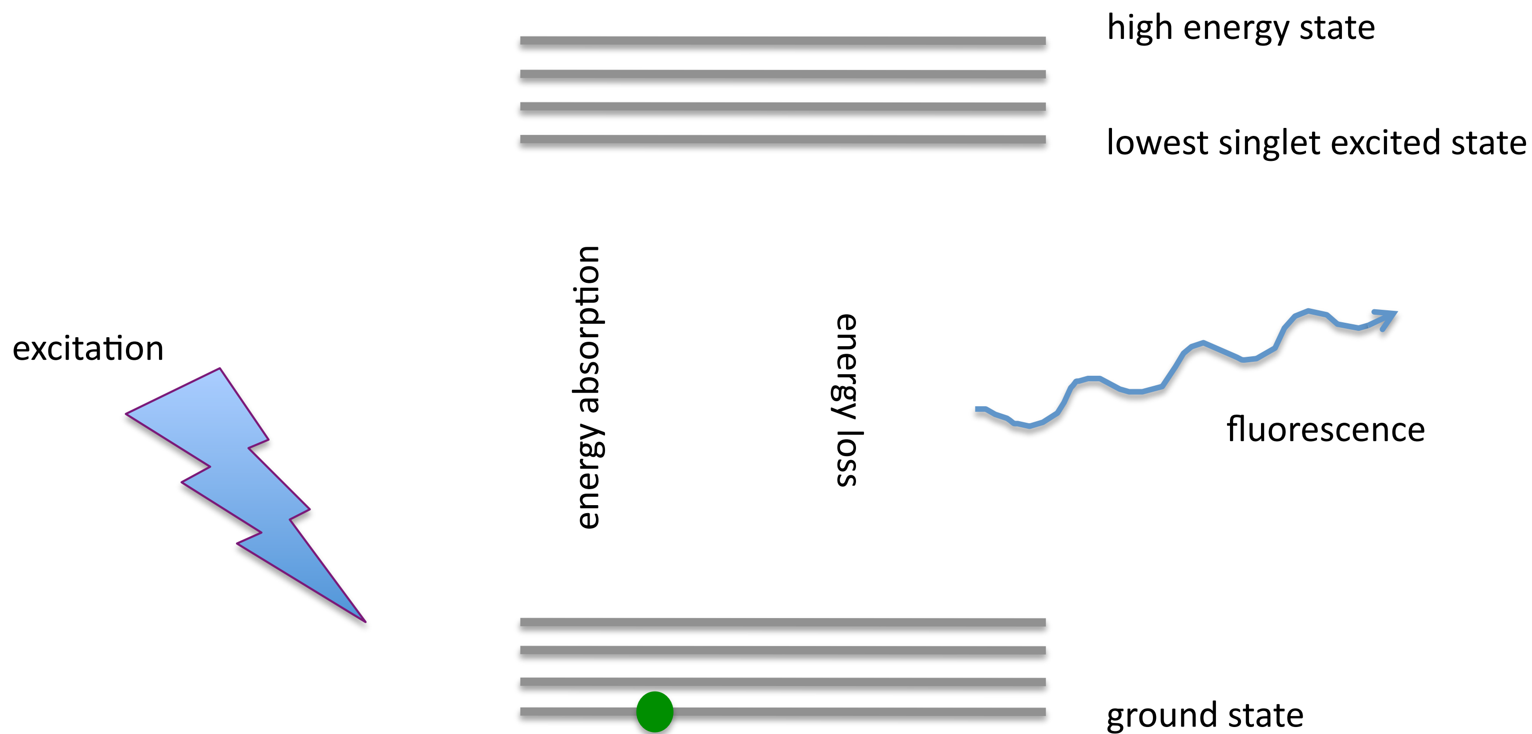


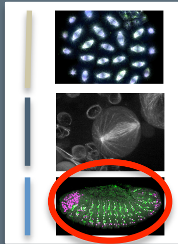
There is no out of focus light



1 Photon Excitation

THEORY

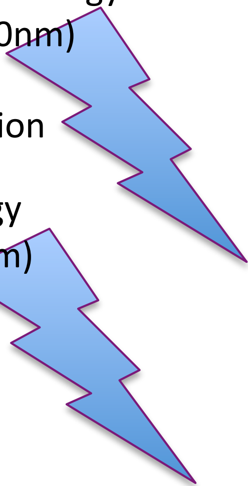


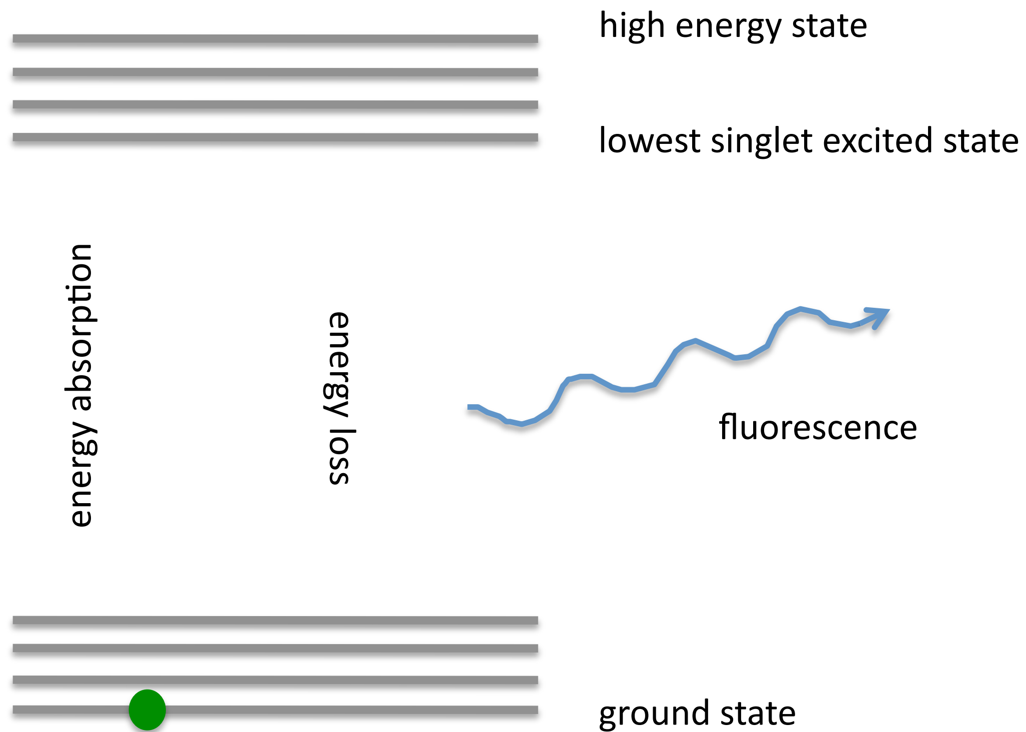


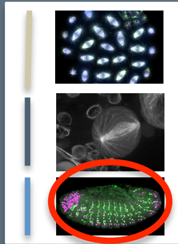
2 Photon Excitation

THEORY

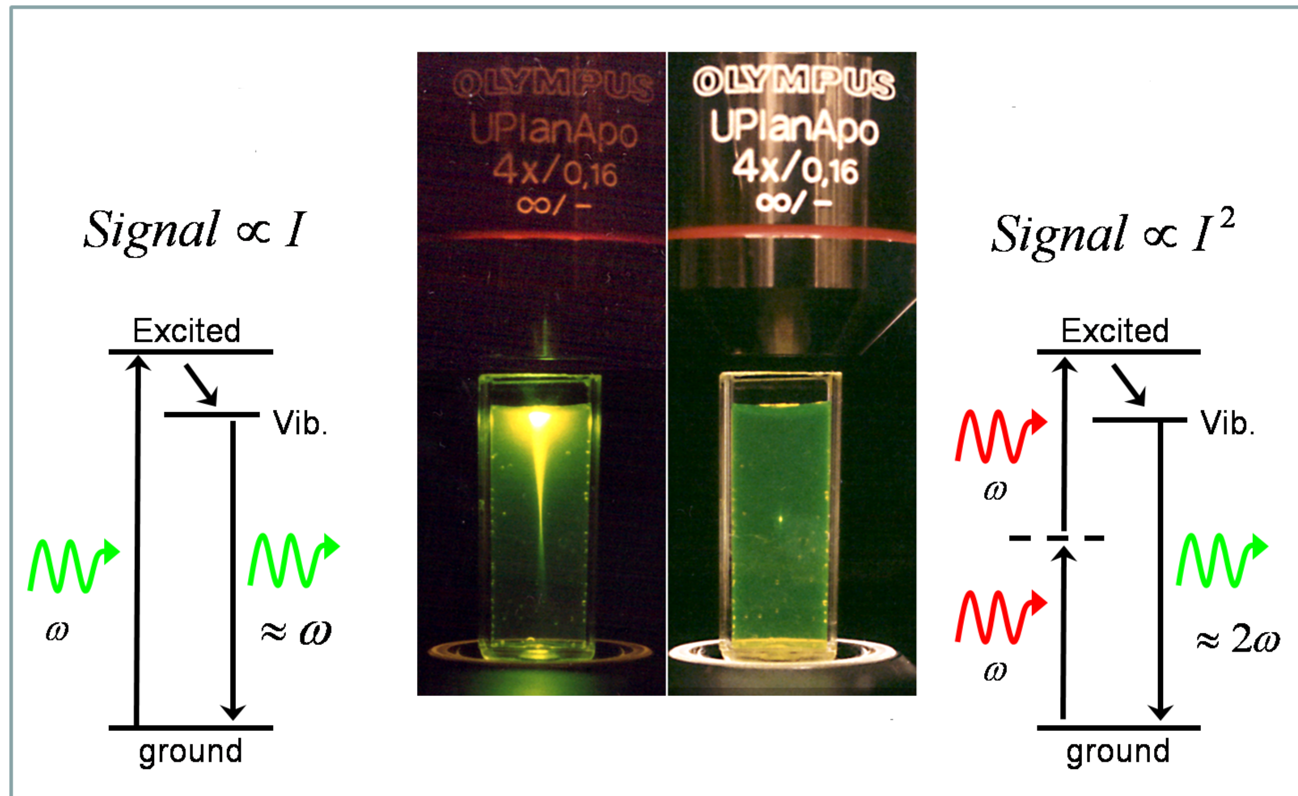
Almost simultaneous





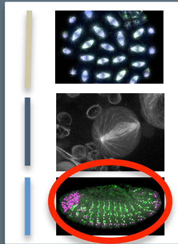


Principle of 2-photon Microscope

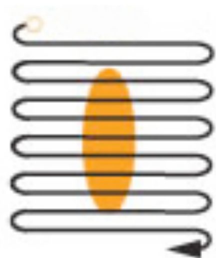


Near simultaneous, two photon event highly unlikely, only really possible a focal point

Tightly focused excitation



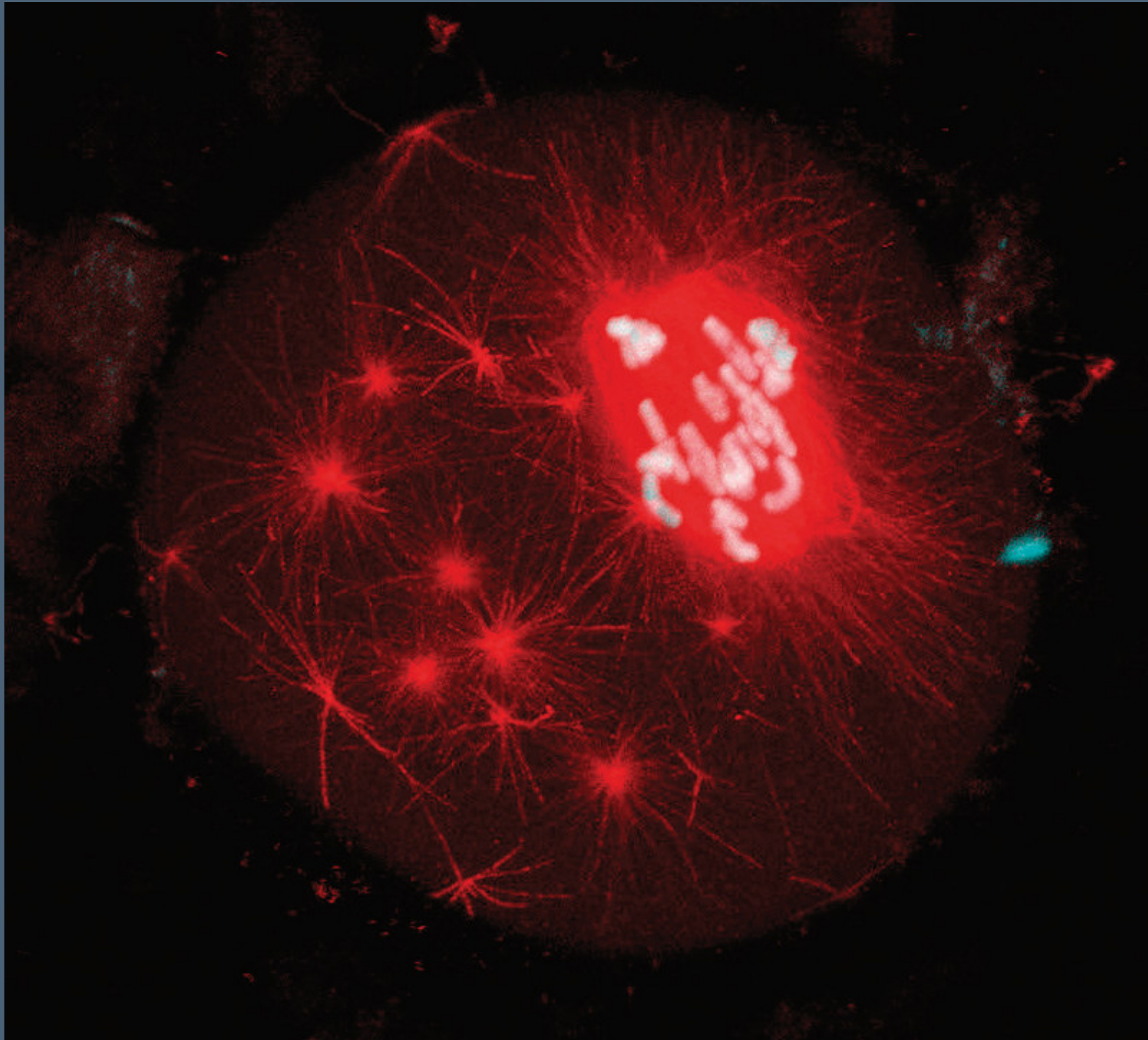
2-photon Microscope



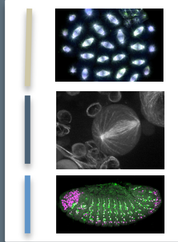
Pulsed excitation laser is then scanned across the sample.

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.



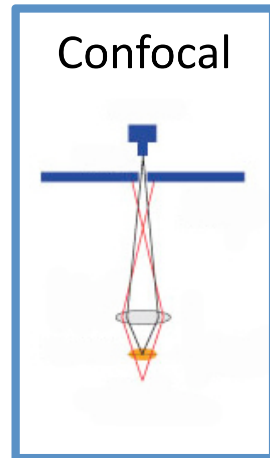
Spindle formation in mouse ooctye, labelled with Hoechst, Alexa 680. M Schuh. EMBL, Heidelberg, Germany



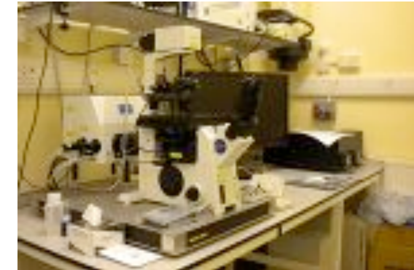
3 Flavours of Microscope

Problem:
Out of Focus
Light

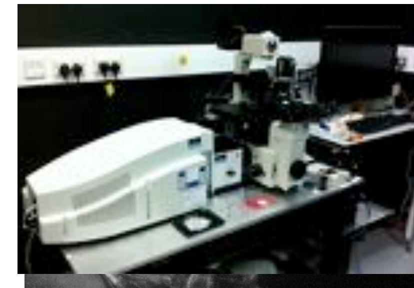
Confocal



Laser Scanning



Spinning disc



2-Photon

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