Light Microscopy Core Facility
DUKE UNIVERSITY AND DUKE UNIVERSITY MEDICAL CENTER

CMB551 1A:

Microscopy and Image Analysis in Cell Biology

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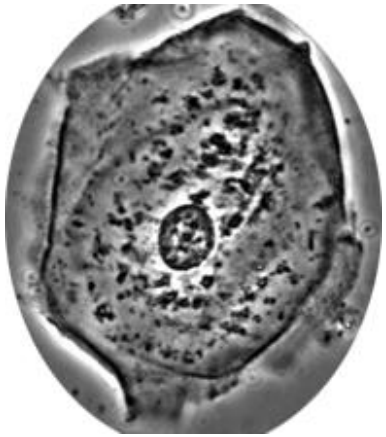
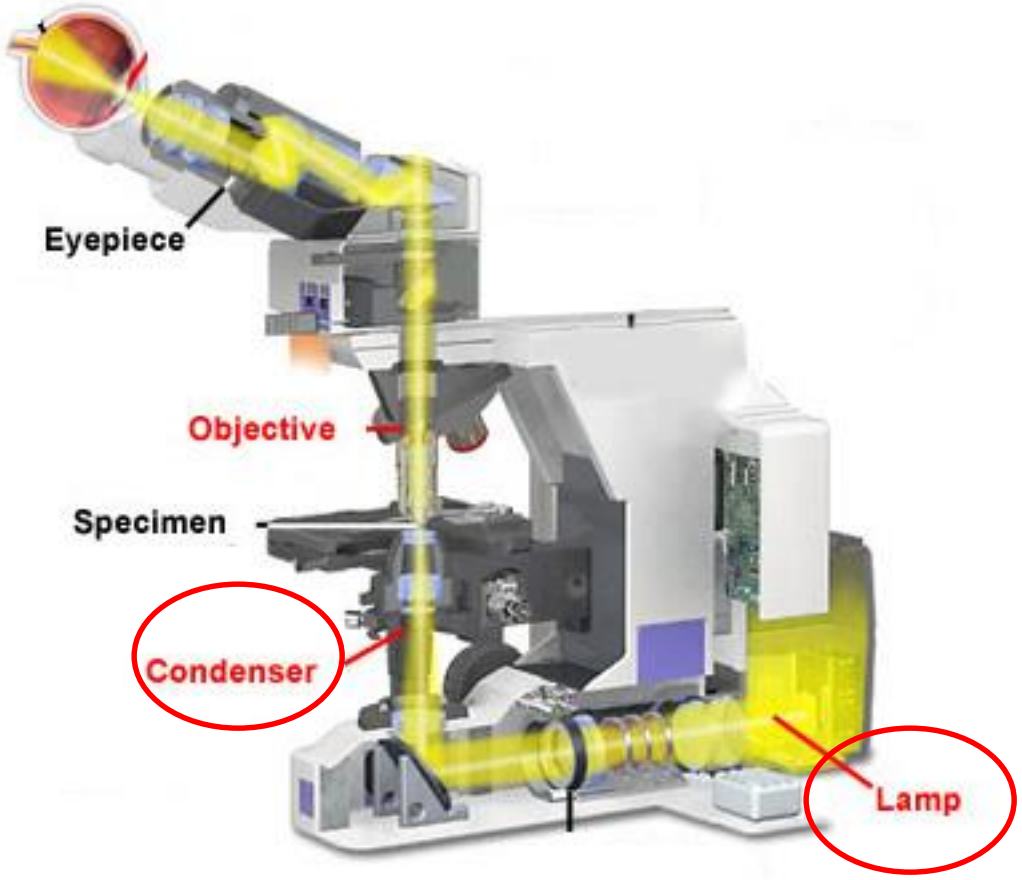
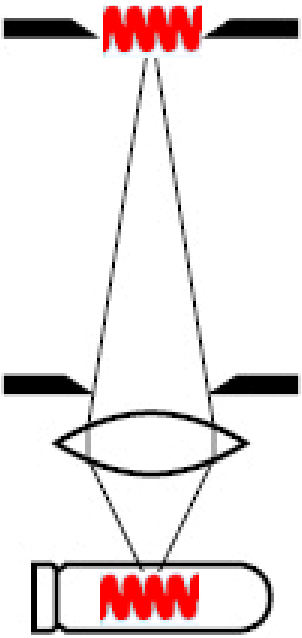
The insides of a microscope: transmitted light

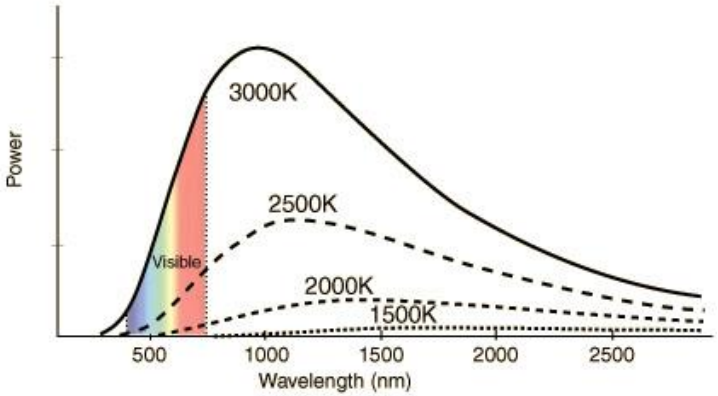


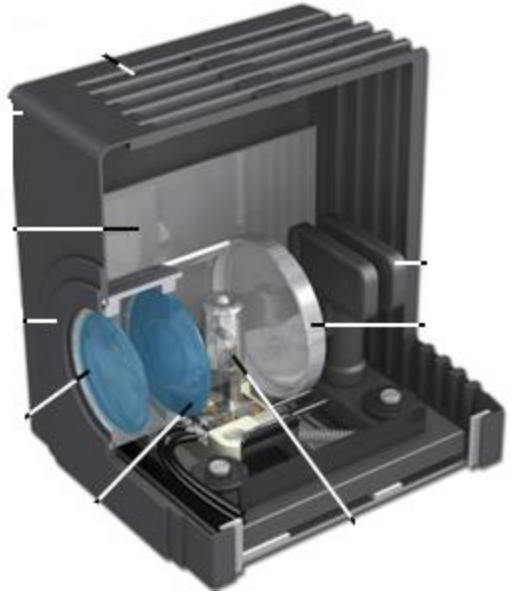


Adapted from http://www.microscopyu.com/articles/formulas/formulasconjugate.html

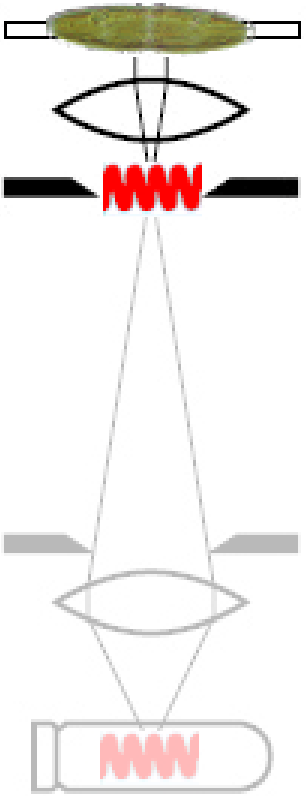
Light source: Halogen bulb







The condenser



The condenser collects the light and concentrates it onto to the specimen

May also have some special parts in for phase contrast or DIC etc

Kohler illumination

Bright and even illumination with good contrast and resolution

Optimal alignment of the condenser

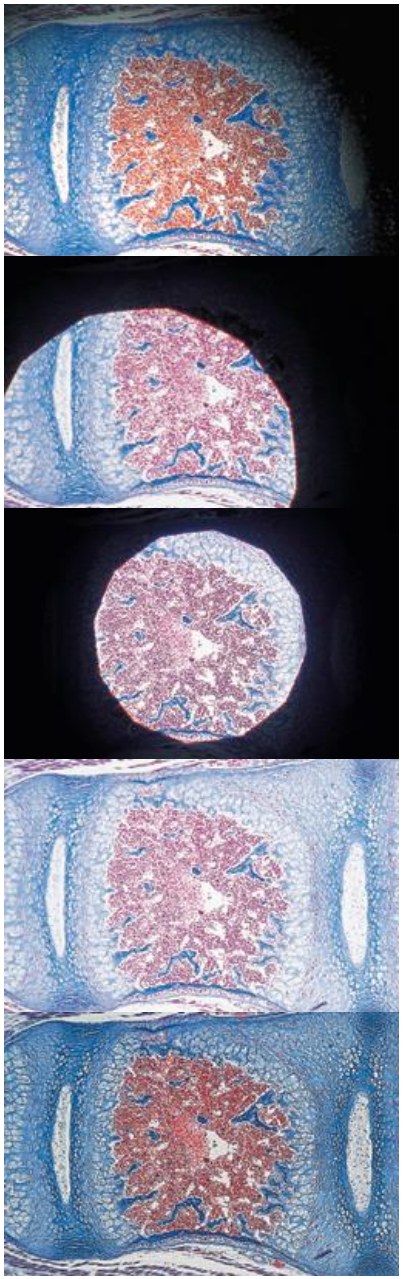
How do we do this alignment in practice?

http://www.microscopyu.com/tutorials/java/kohler/

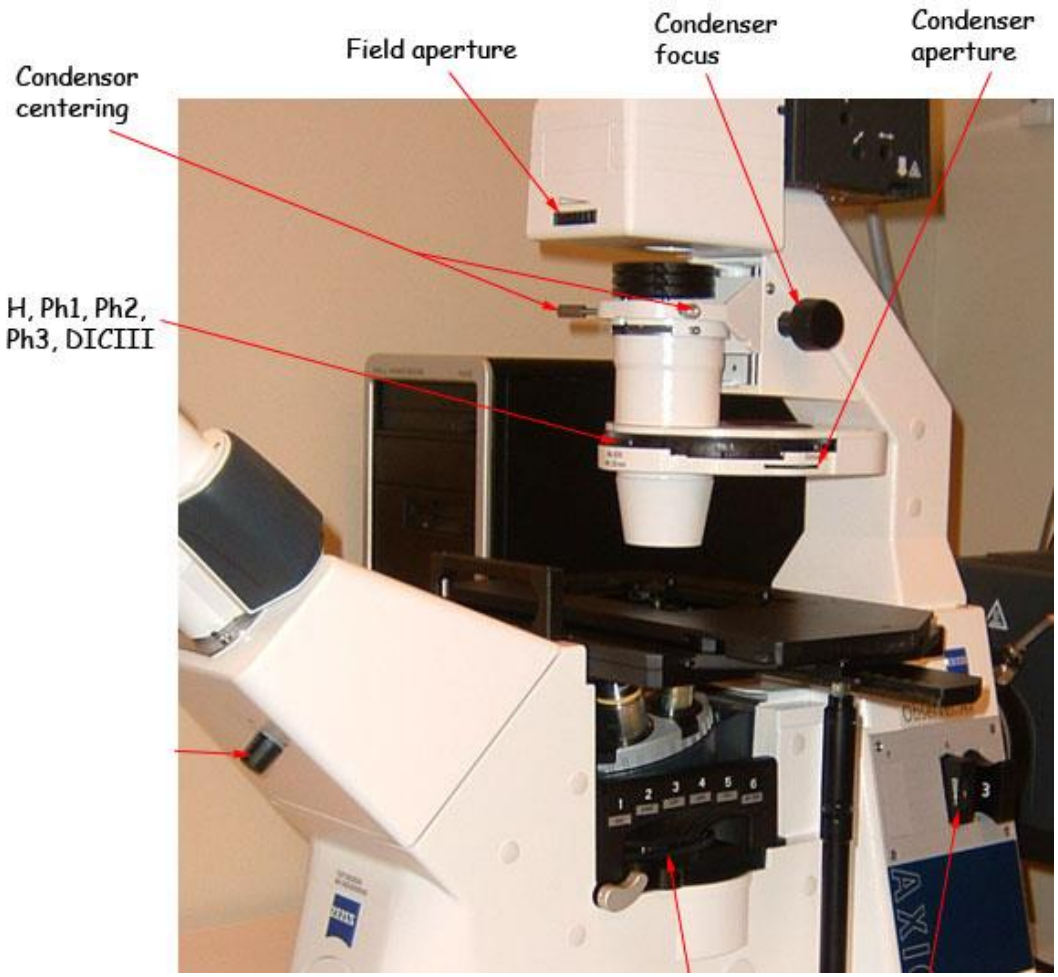
Kohler illumination in practice

Open the apertures and focus on your specimen as best you can in brightfield. Don't change the focus through out. You can adjust the lamp brightness at any stage

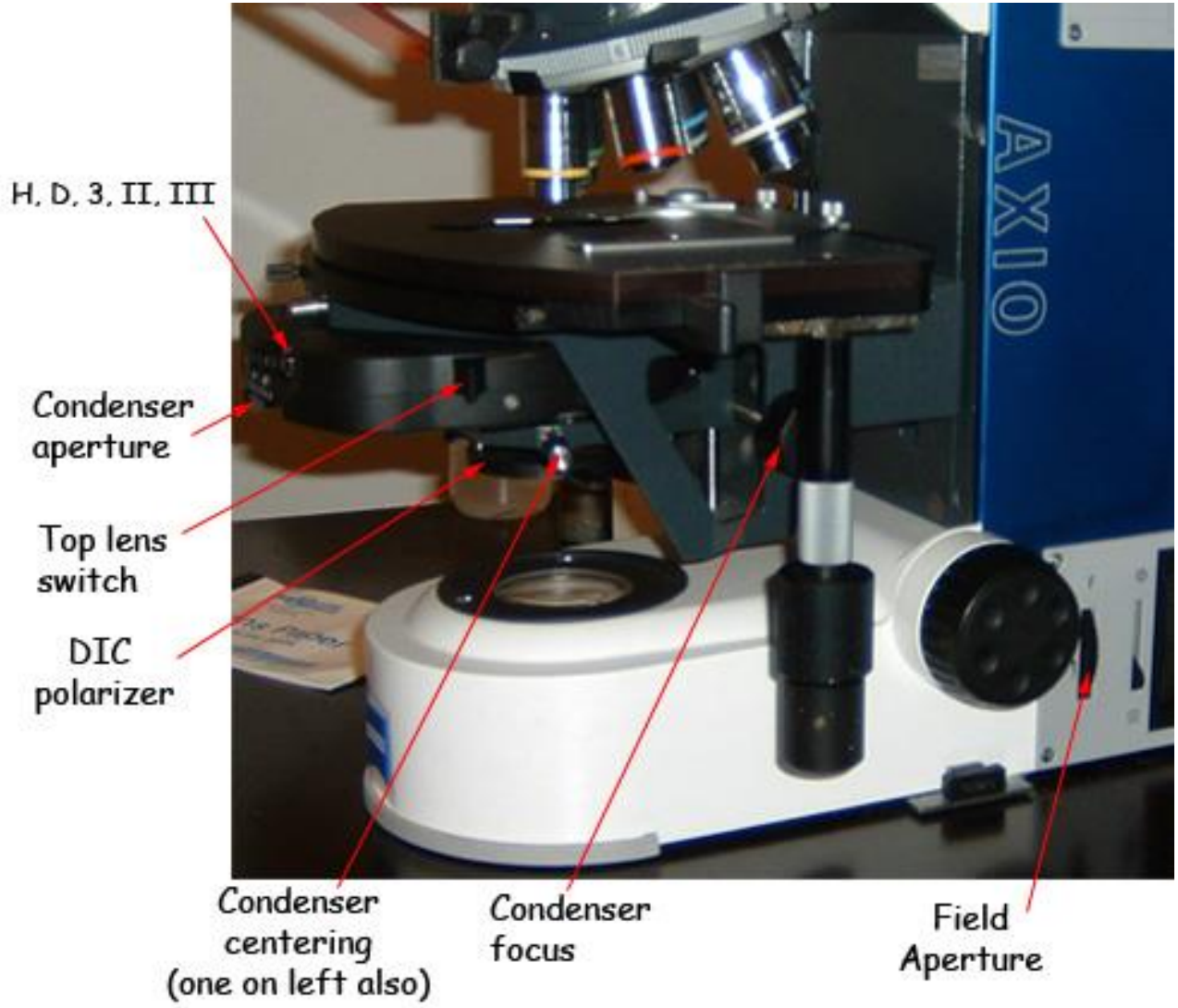
- Close down the field diaphragm/aperture so it becomes visible - you should see an octagon shaped aperture appear but it may be very blurry
- Focus the condenser with the knobs that raise/lower entire condenser - the octagon shaped field aperture should be made as sharp as possible
- Then center the condenser using the two centering pins
- Open the field diaphragm until it is just out of view – now the whole area is evenly illuminated
- Adjust the condenser aperture so the contrast of the image is good – you can do this empirically or remove the eyepiece and adjust so 2/3 to 5/6 of the pupil is filled



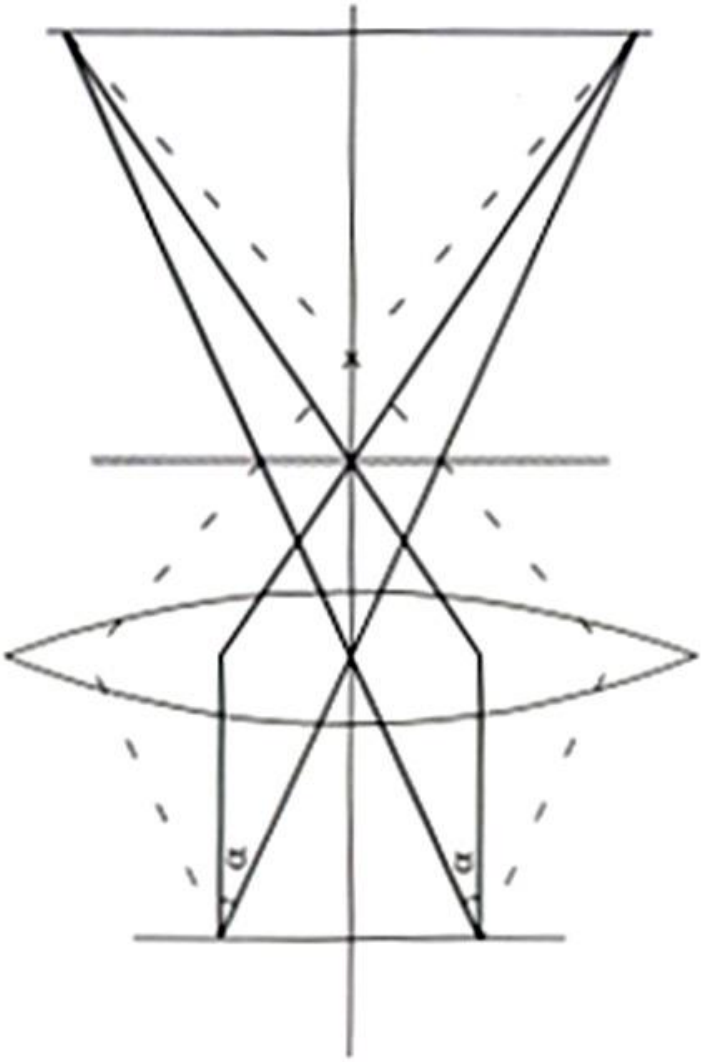
Components on an inverted scope



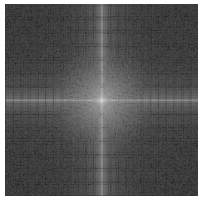
Components on an upright scope



Back focal plane of the objective



Image



Diffraction

Object

Conjugate planes

Image, object or field planes

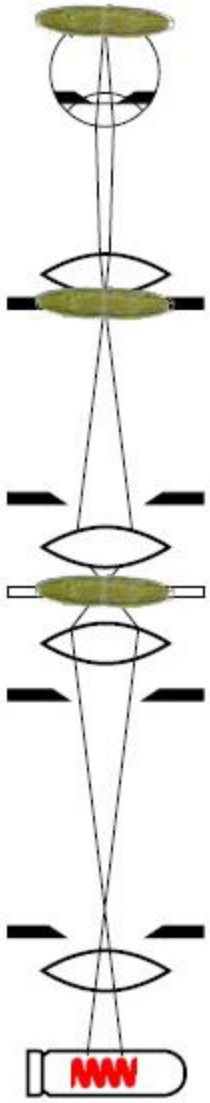
Illumination, aperture, or diffraction planes

D = Retina of the eye

C = Intermediate image

B = Specimen plane

A = Field diaphragm



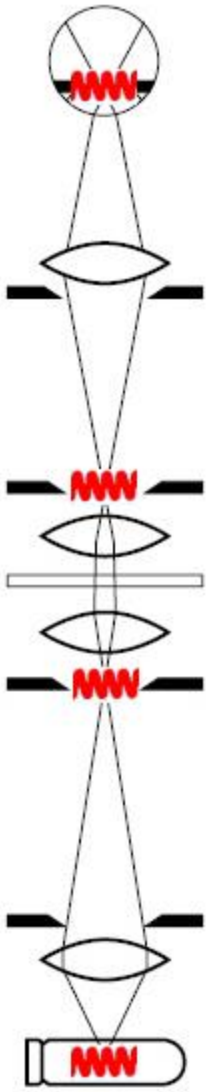
Eye

Eyepiece

Objective

Condenser

Lamp



4 = Pupil of the eye

3 = Objective pupil

2 = Condenser aperture

1 = Lamp filament

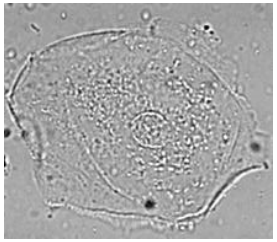
If that sounds useful to you please find a simple scope and try the alignment process

(or use this virtual scope)

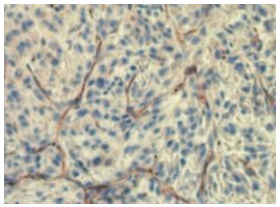
http://www.microscopyu.com/tutorials/java/kohler/

Contrast in transmitted light images

Cells don't absorb much light, there isn't much contrast based only on that



Staining isn't ideal

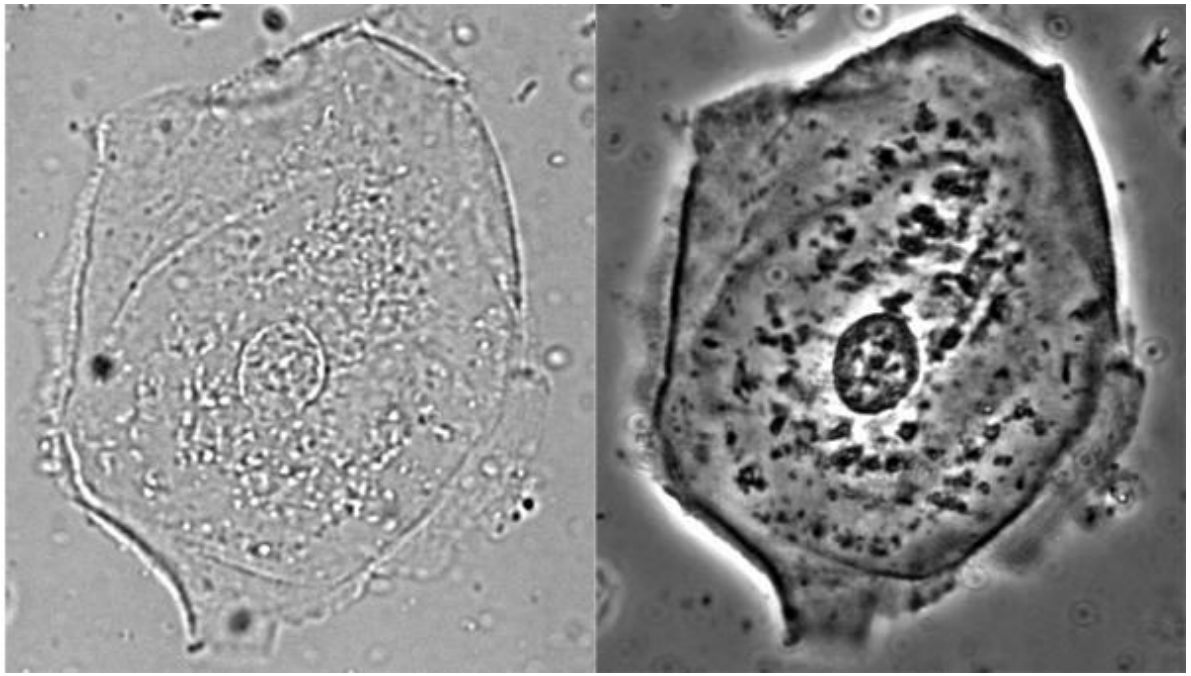


Many methods and variations to increase the contrast over brightfield. . .

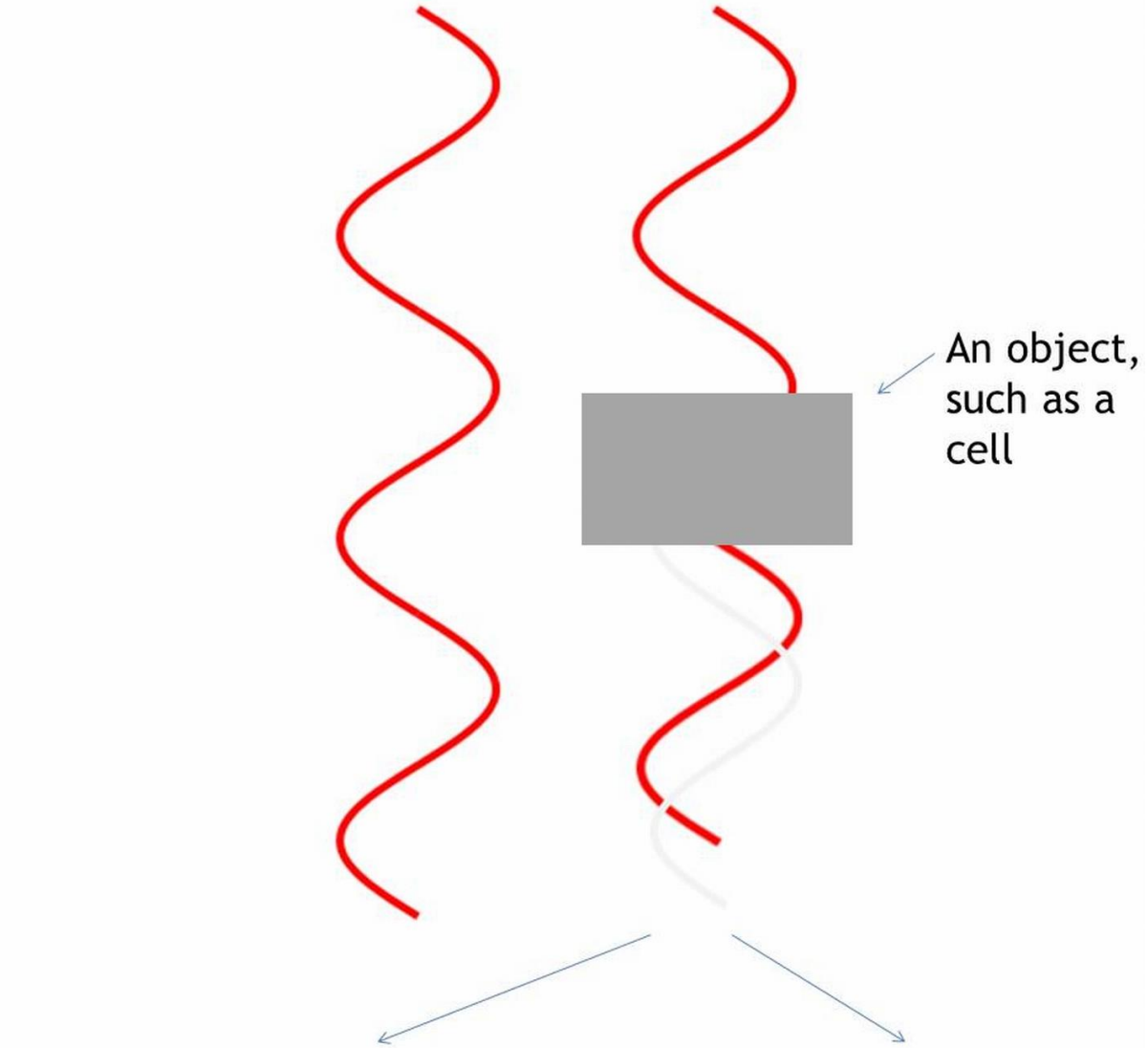
Phase contrast

Brightfield

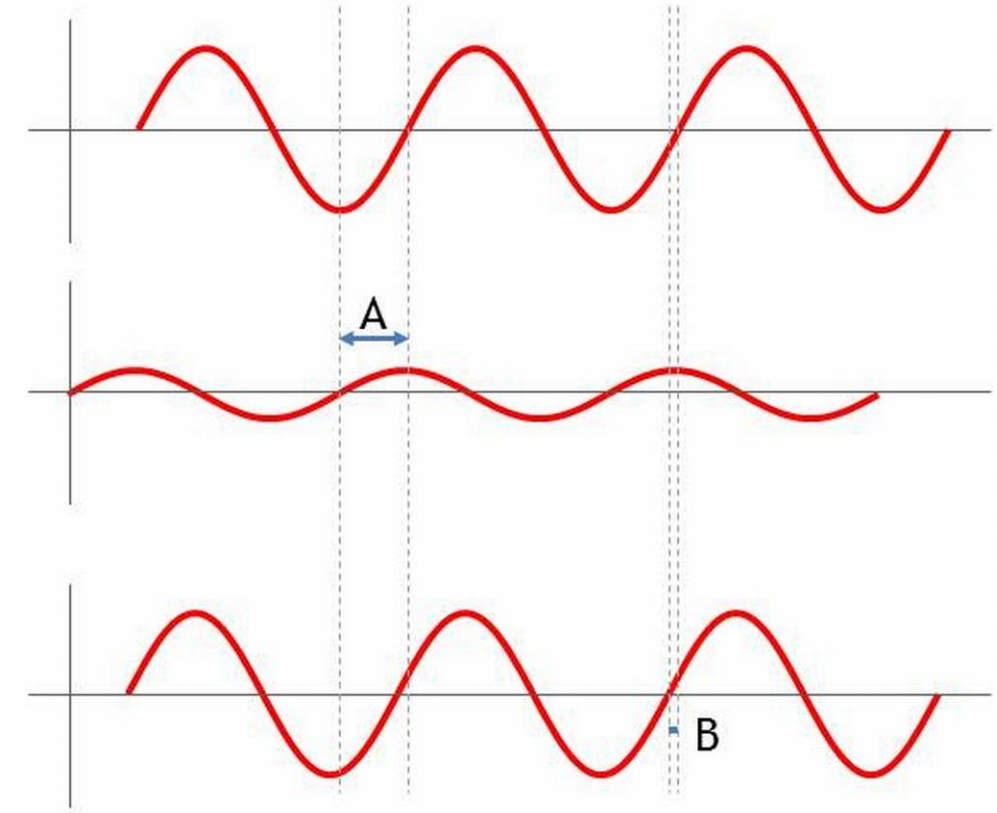
Phase



Simple to setup, good depth of field, copes with plastic (ideal for TC cells)


An object, such as a cell
Amplitude is unchanged by the object
The phase of the wave is shifted by the sample

. . . but not much contrast is produced

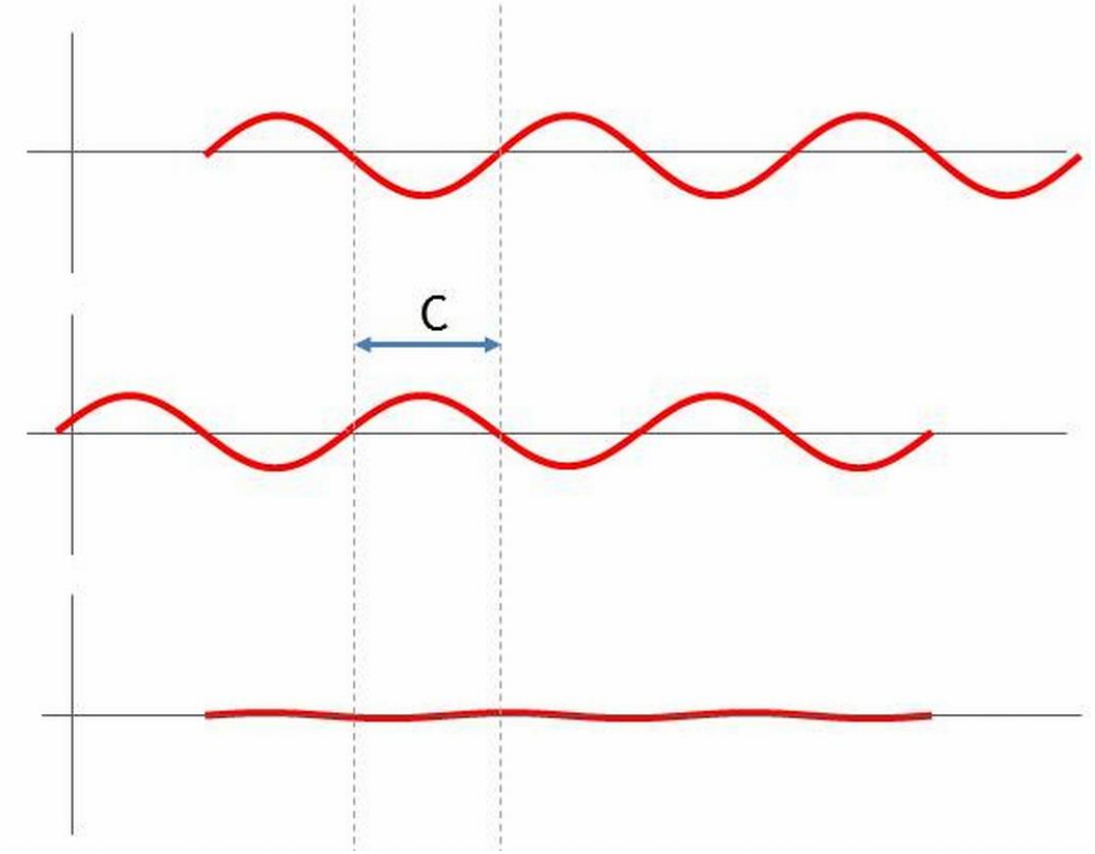


Surround wave

Diffracted wave-
Why is it smaller amplitude?

Combined wave

Much more contrast in this condition . . .

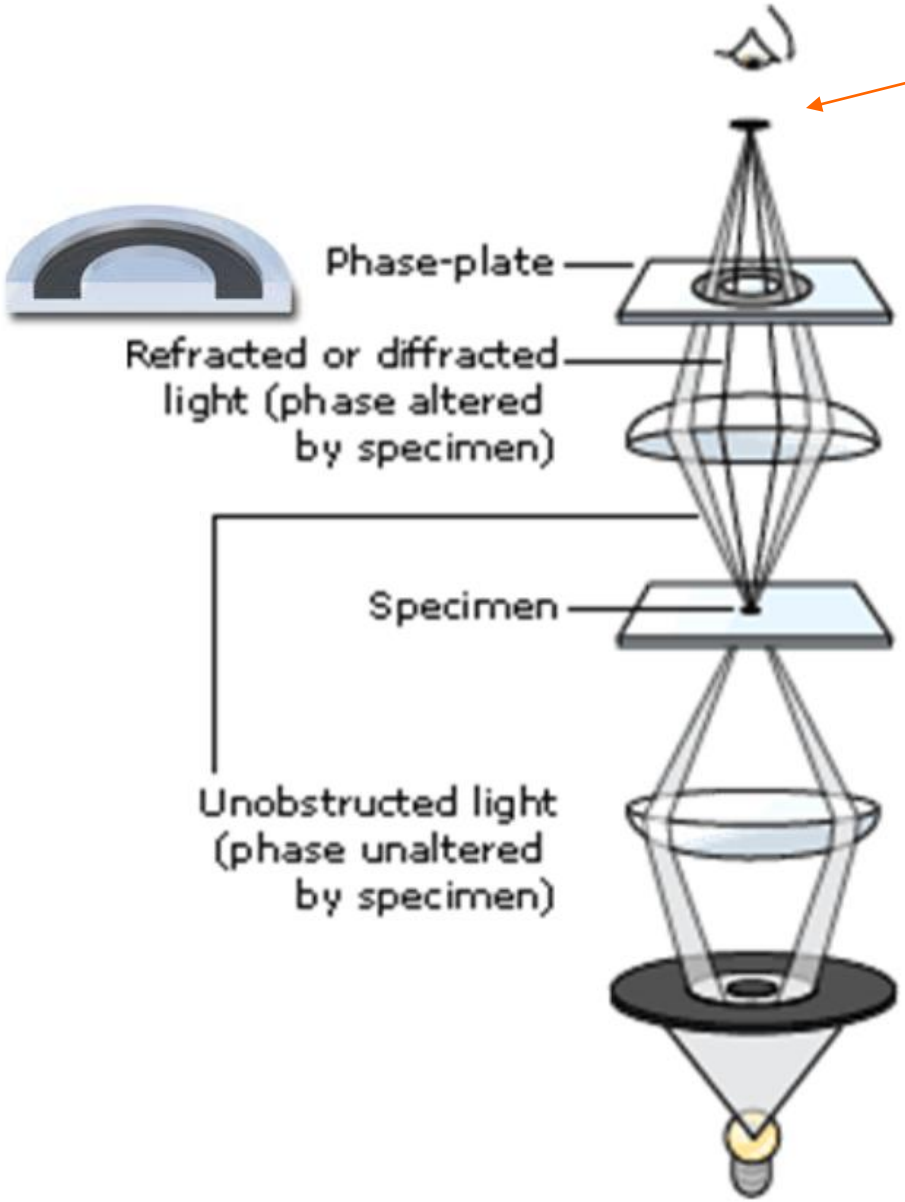


Surround wave

Diffracted wave-
Note the similar amplitude?

Combined wave

Phase contrast



Interference at BFP of objective

Objective

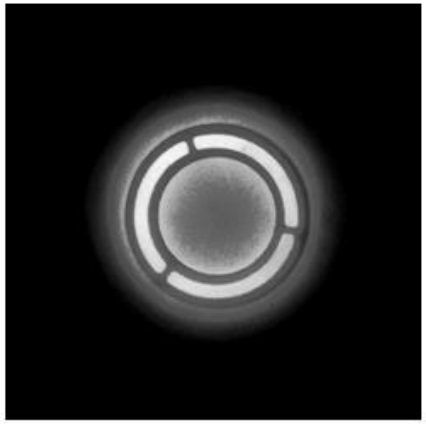
Scattered light has its phase shifted by 1/4λ

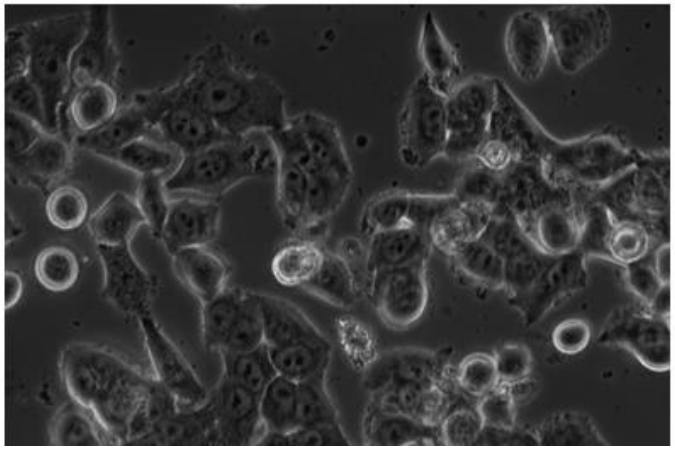
Condenser

Phase contrast alignment

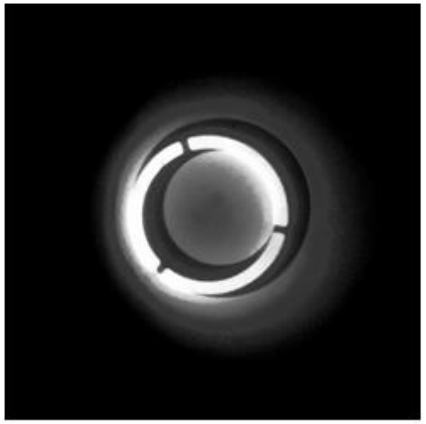
Kohler alignment with the condenser aperture fully open

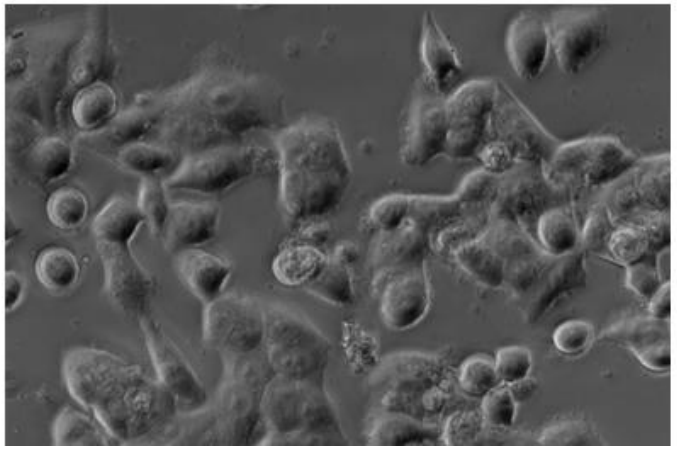
Aligned





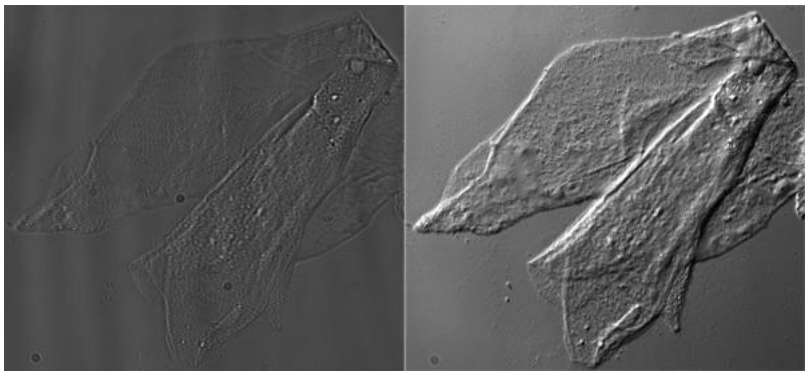
Misaligned





DIC (Normarski)

Brightfield DIC

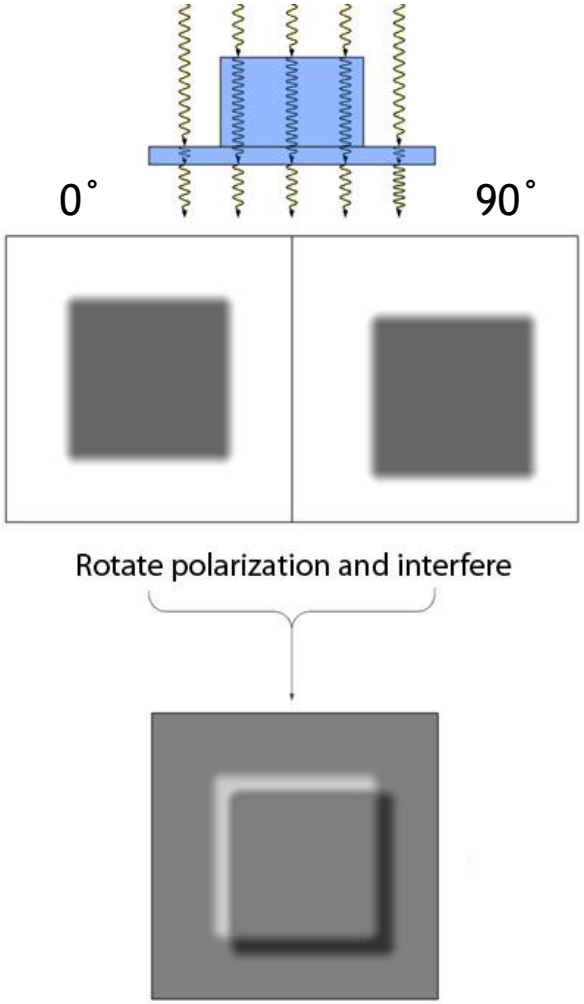


More complex and $$

Pseudo-3D

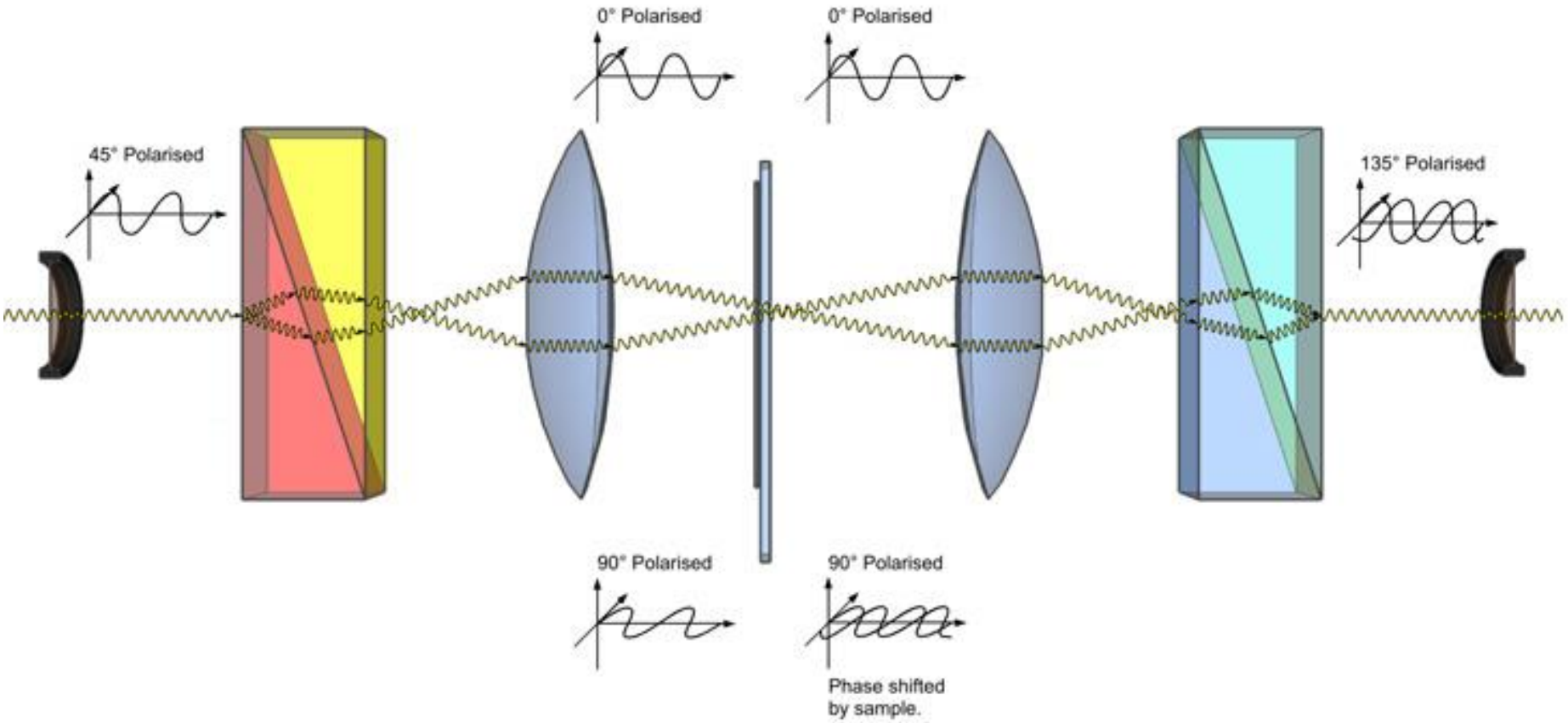
Two beams of different polarization pass through the sample and are recombined where they interfere and produce an image with high contrast

Differential Interference Contrast microscopy



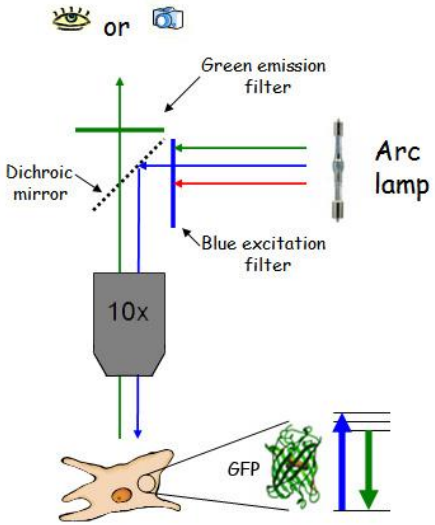
http://en.wikipedia.org/wiki/Differential_interference_contrast_microscopy

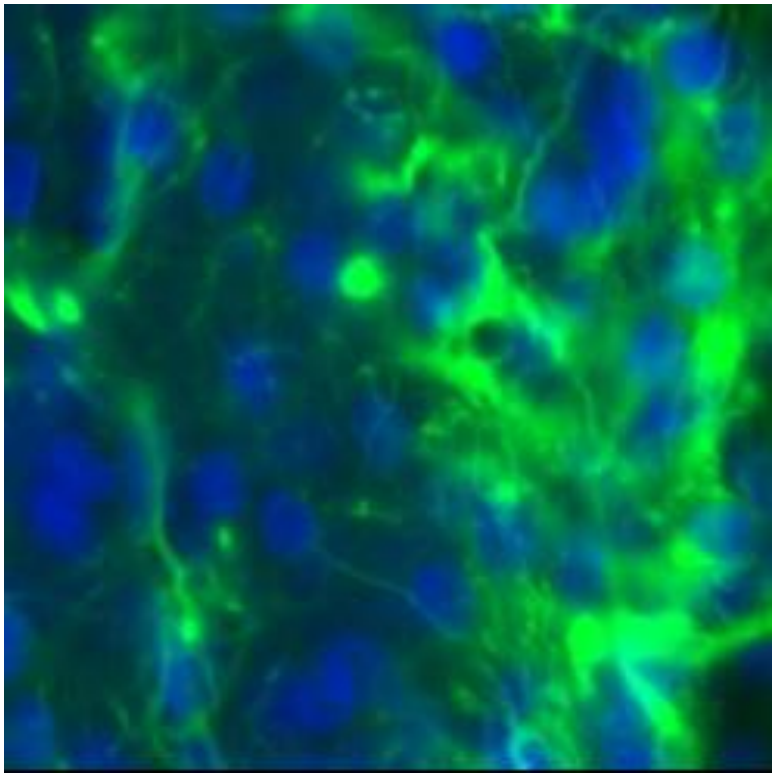
Differential Interference Contrast microscopy

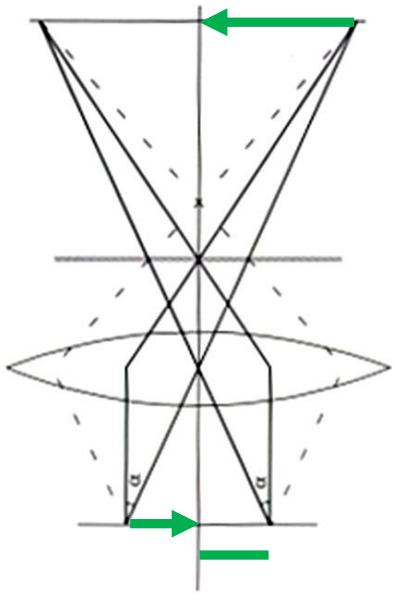


http://en.wikipedia.org/wiki/Differential_interference_contrast_microscopy

The problem with widefield microscopes and biology







Sectioning

Vibratome

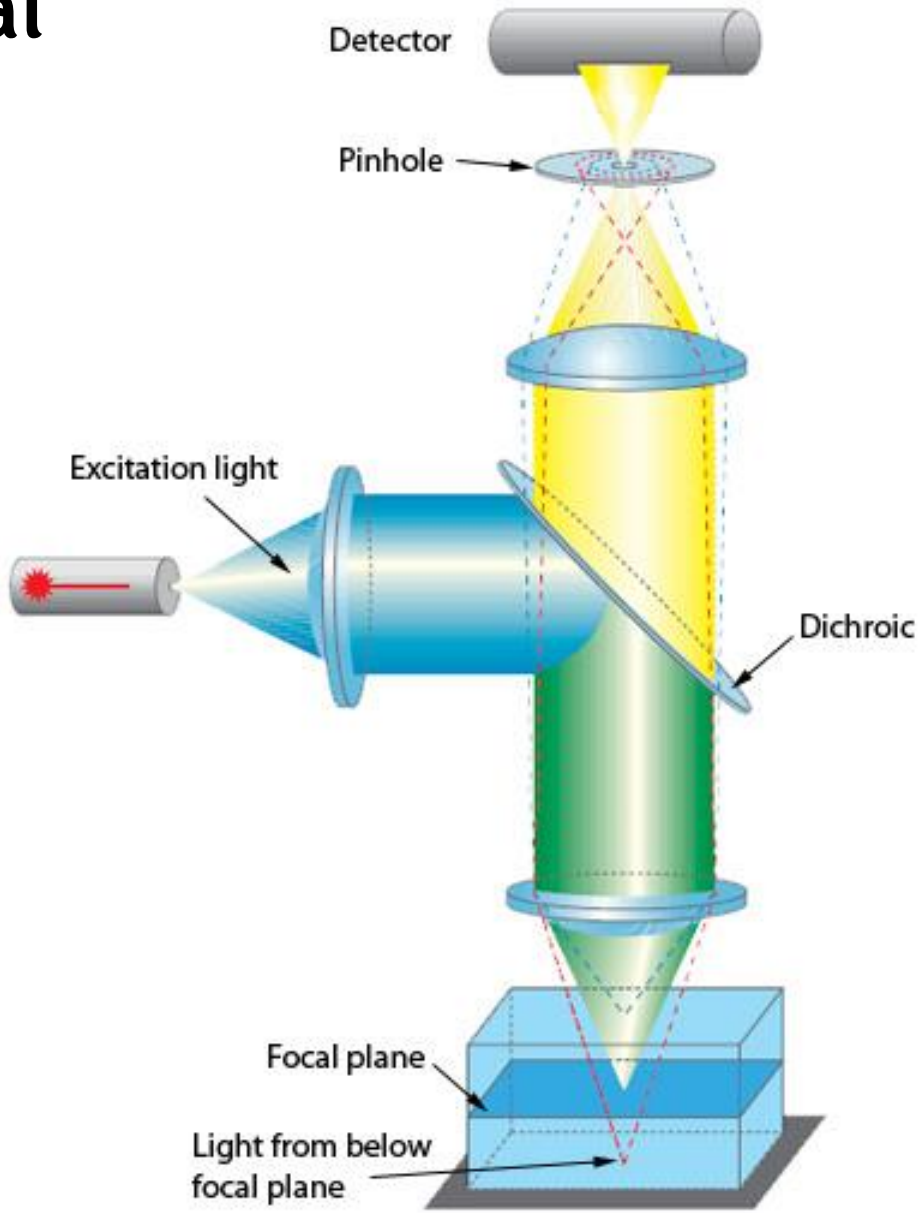
Microtome

Cryostat

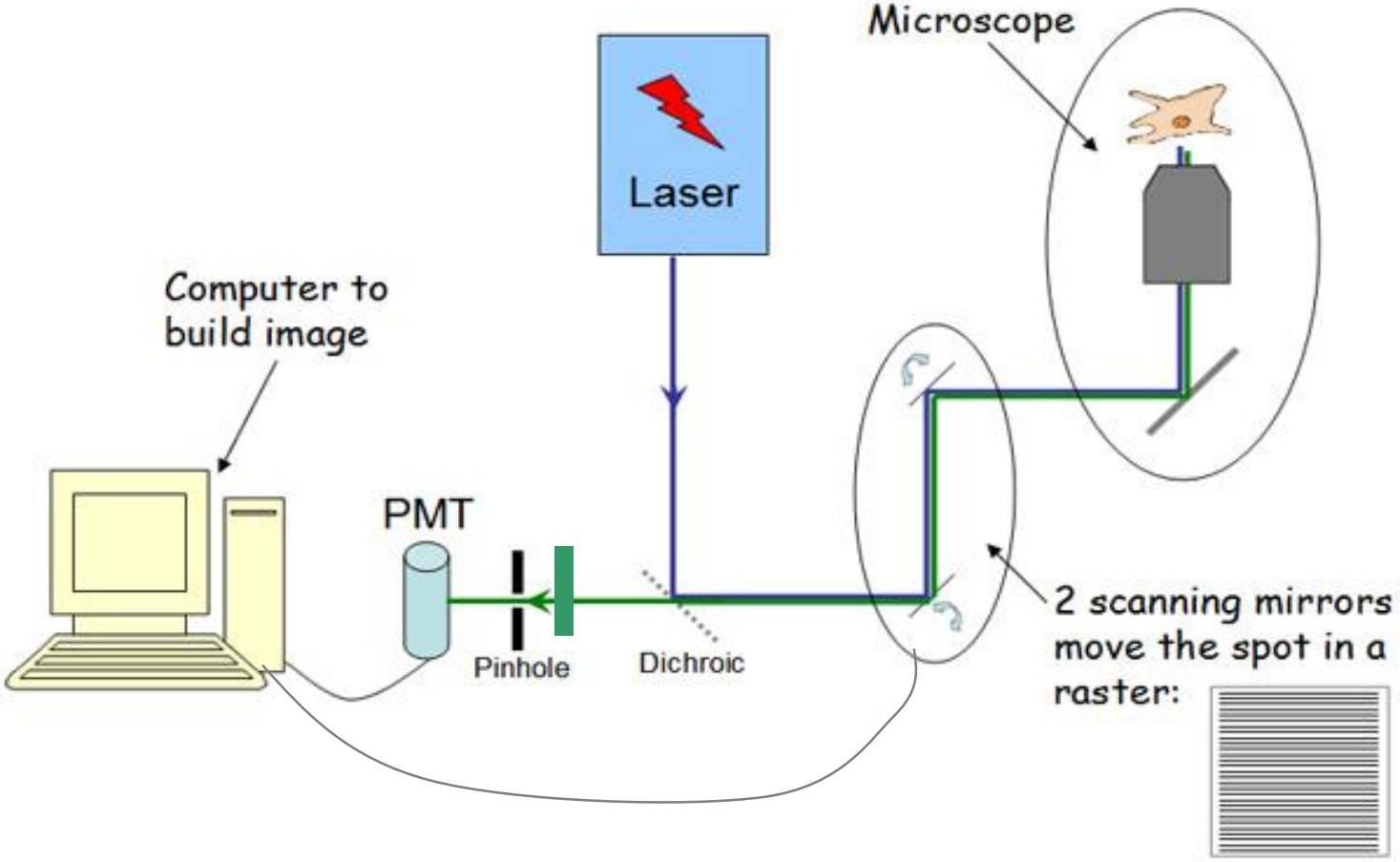
Non-physical sectioning – optical sectioning by . . .

Confocal | Multiphoton | Spinning disk | TIRF | SPIM

The confocal principle



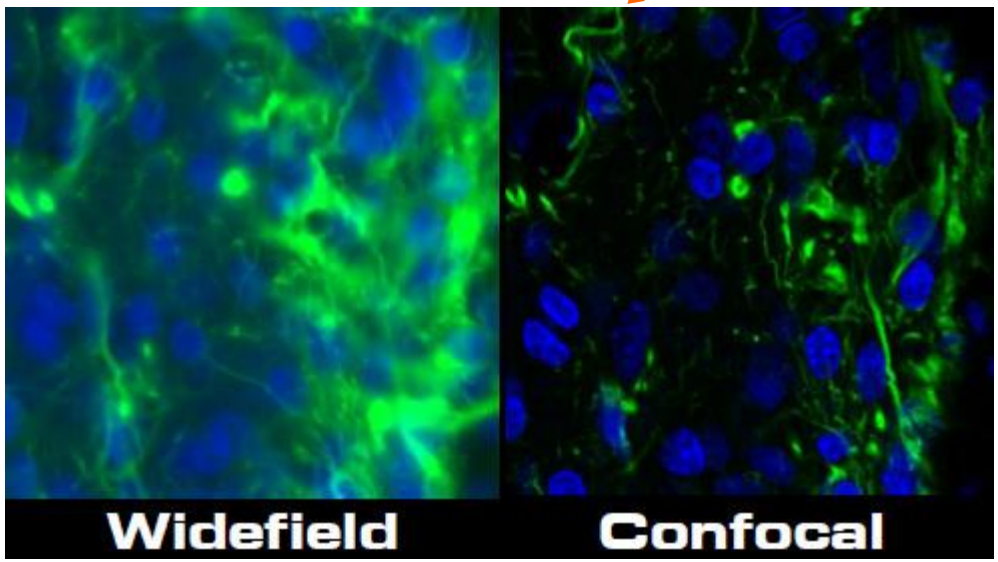
How a laser scanning confocal microscope works



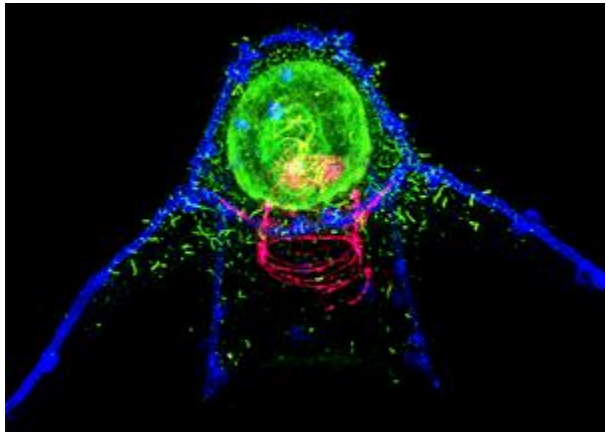
http://www.olympusfluoview.com/theory/index.html

The confocal advantage

Optical sectioning of thick samples

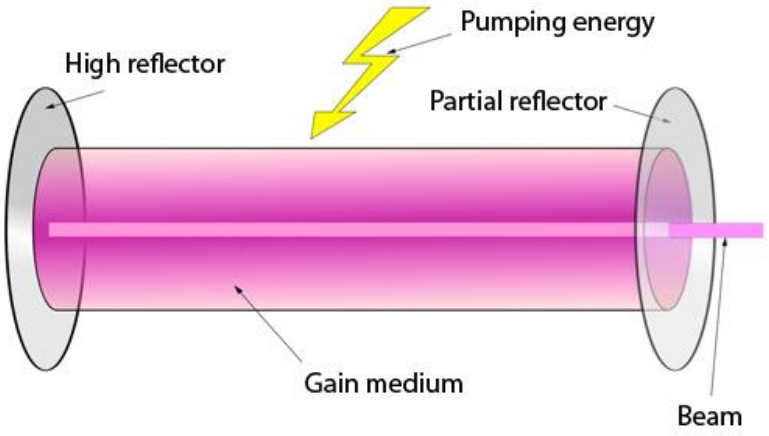


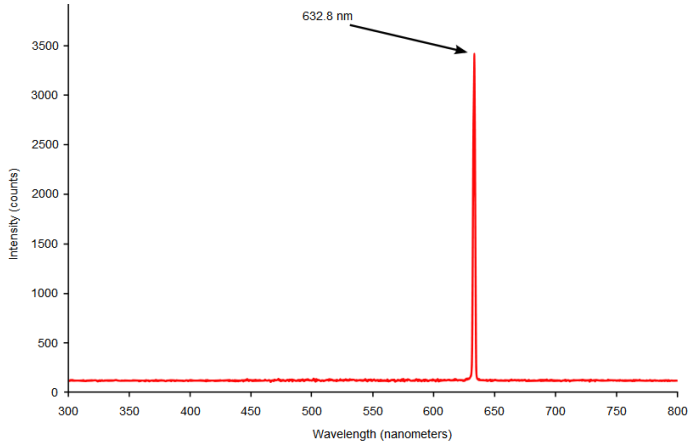
3D reconstruction



What a confocal looks like

LASERs are used for excitation





Ideal for point scanning:

- Narrow collimated beam, low divergence
- Powerful

http://www.olympusfluoview.com/theory/laserintro.html

Many lasers available . . .

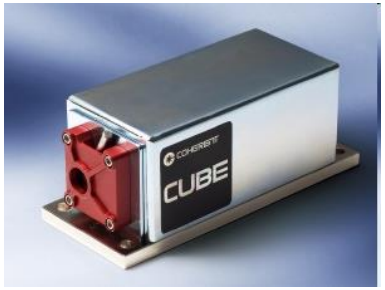
Gas lasers

(Kr/Ar 488 568 647)

Ar/Ar 458 488 514

HeNe 543 594 633

Diode lasers



405 488 561 635

(Color coding refers to the color of the fluorophore for which the laser line is most commonly used)

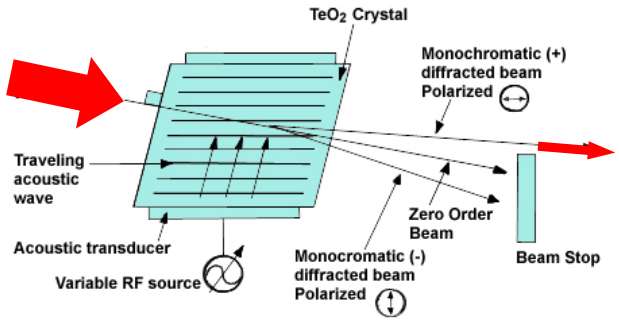
Adjusting the laser power

Relatively fixed output

Rapid control 0-100% T

Laser

AOTF

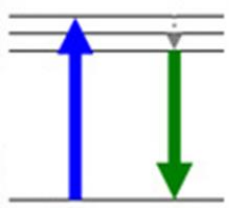


Laser

AOTF

λ selection and intensity control

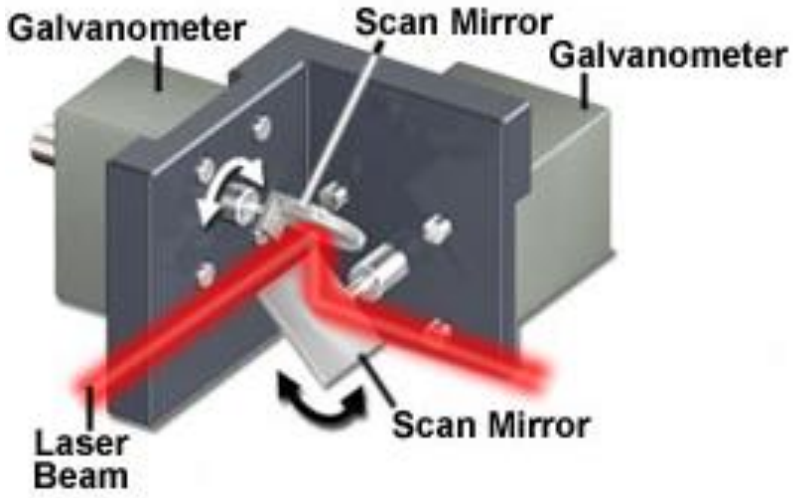
Fluorophore saturation



Widefield is normally in the linear range, the concentrated laser spot in confocal may not be.

Start low and increase to the minimum necessary

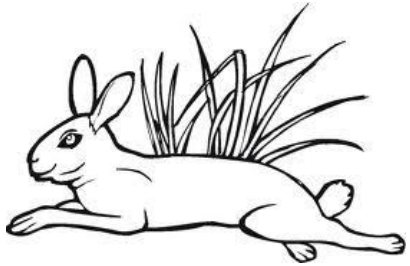
Scanning mirrors

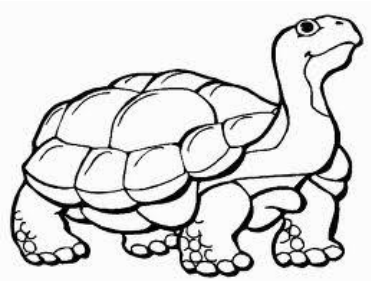


The relative position of the two mirrors can point the spot anywhere in the field

http://www.olympusconfocal.com/theory/confocalscanningsystems.html

Scan speed

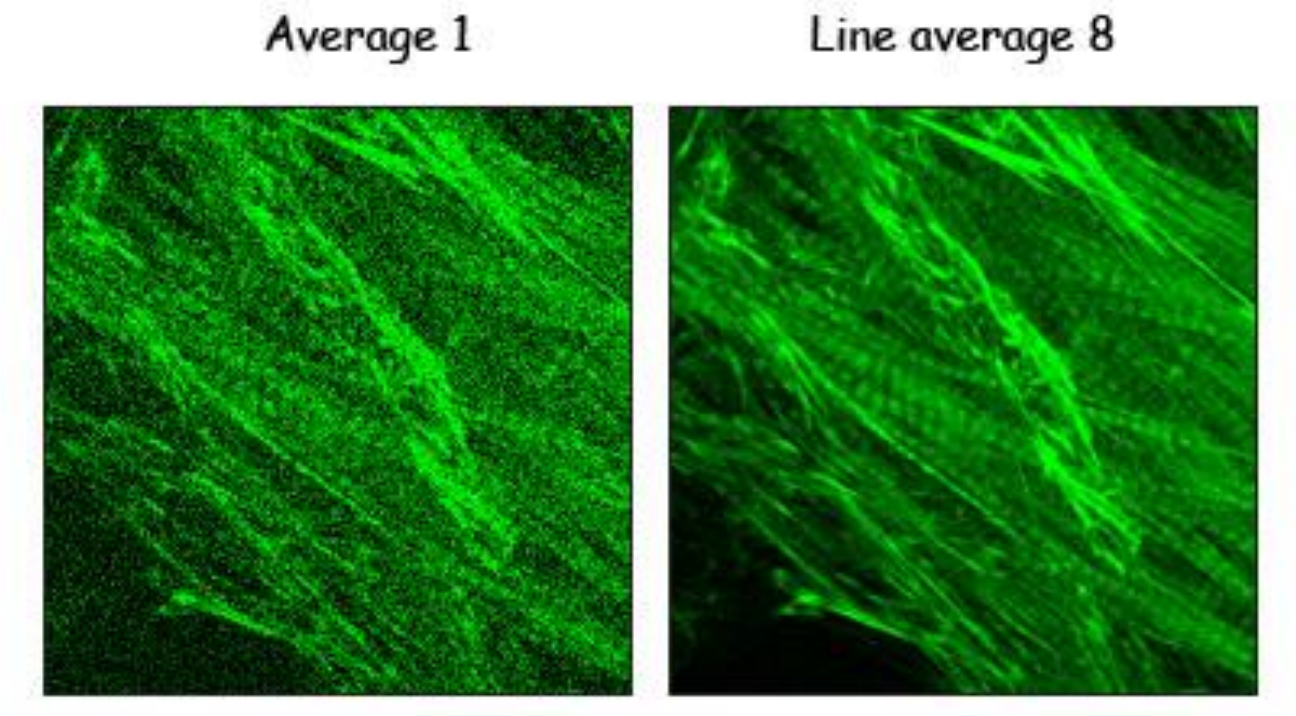




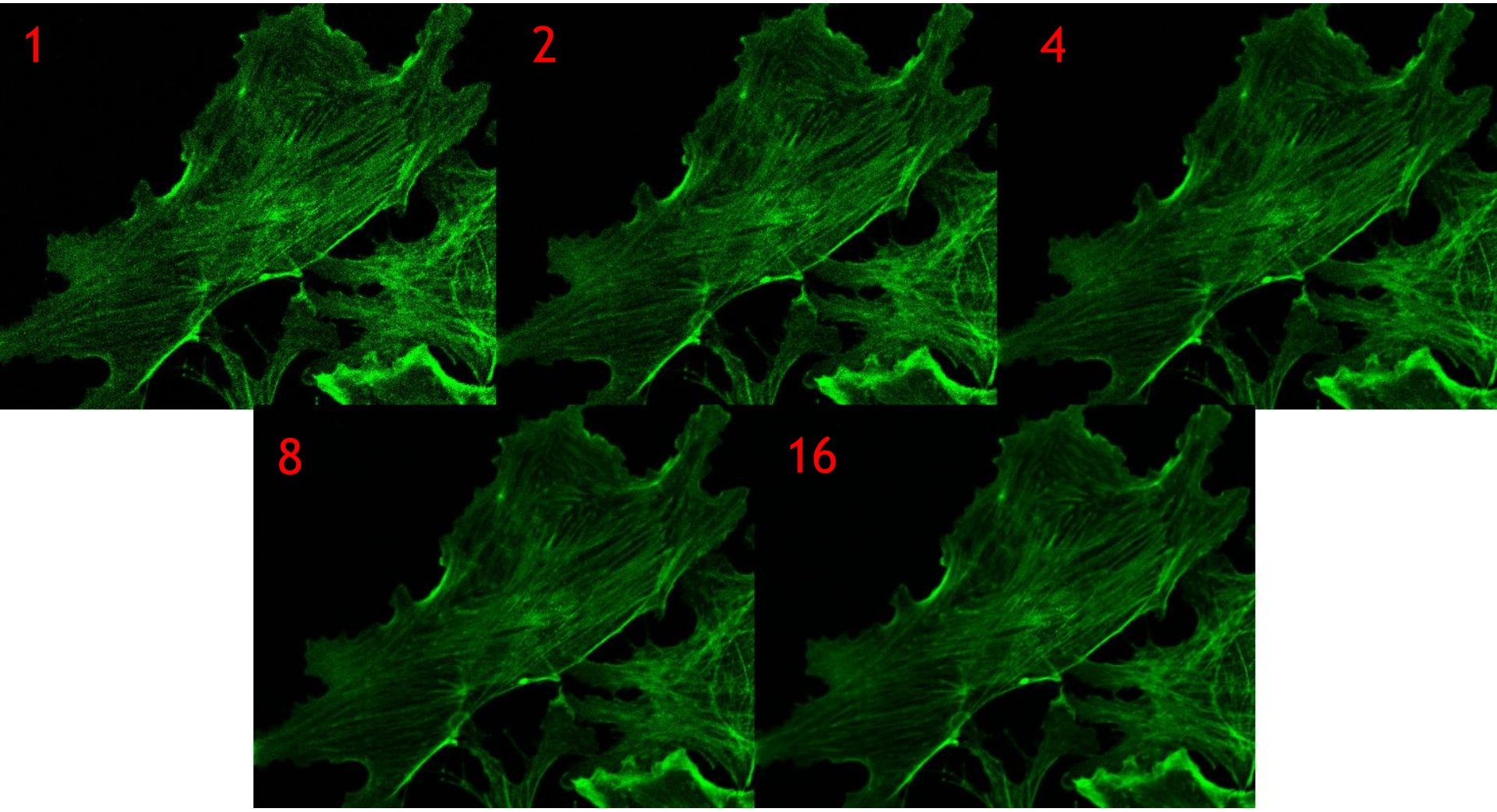
Fast scans	Slower scans
• Fast processes • Useful for focusing and adjustments – eg ≥1 fps	• More light gathered • Better images • More damage

Averaging

Scanning each pixel multiple times and averaging improves the noisy signal . . .



Averaging: how much do you need?



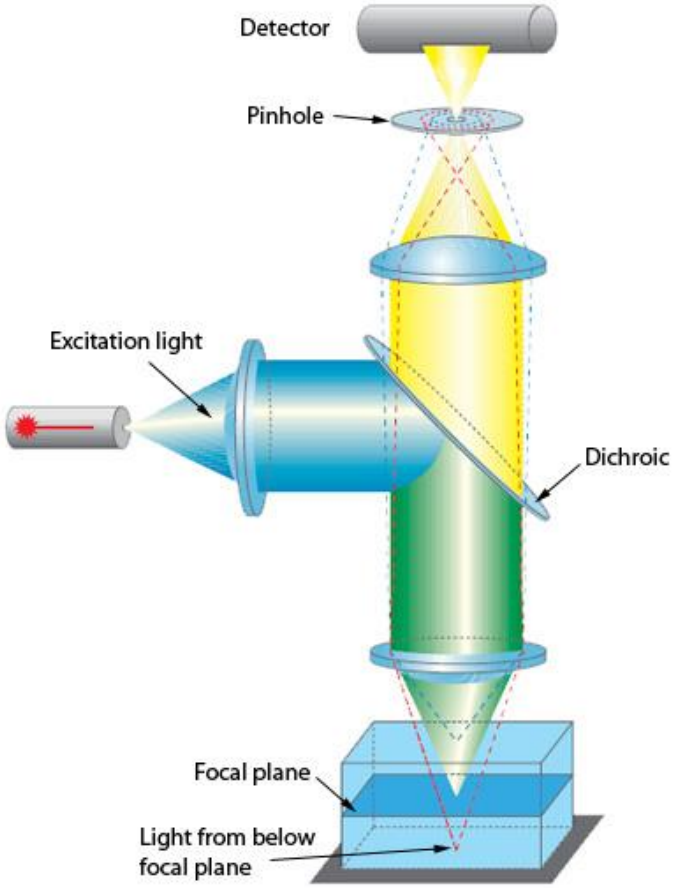
Decreasing rate of improvement, empirically determine a good balance between final SNR and time/damage in acquisition

Pinhole adjustment

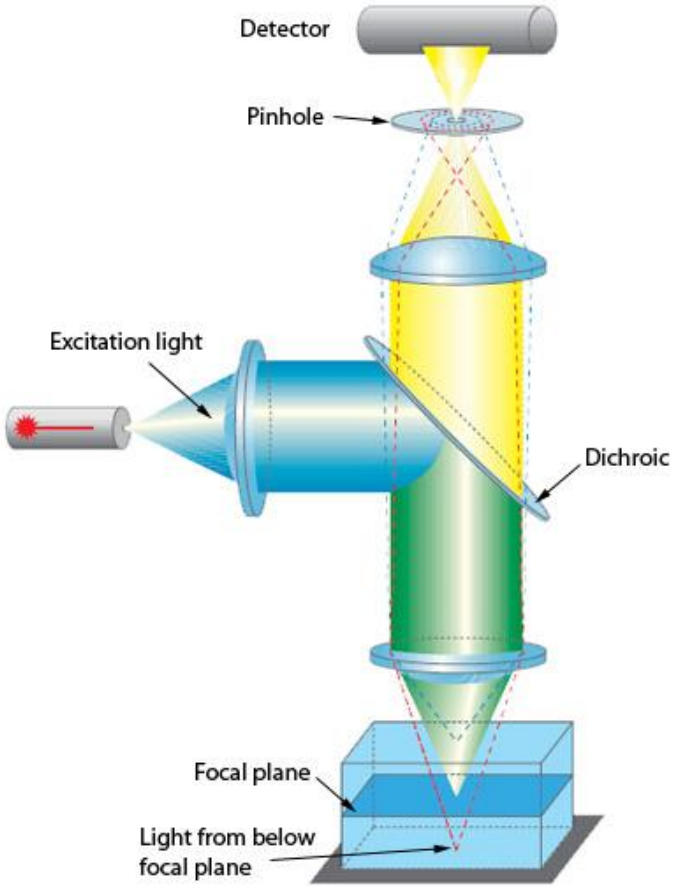
More light, thicker slice

More resolution

Axial (z) or lateral (xy) resolution



Is the pinhole . . .



A. A big waste of signal?

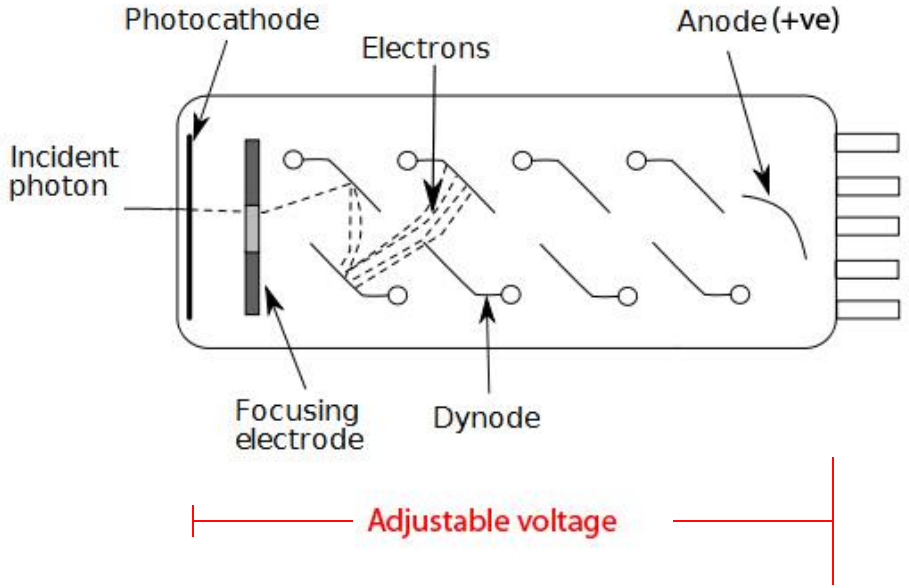
B. Only good as we didn't want that light?

C. A bit of both

Signal : Noise

Signal : background

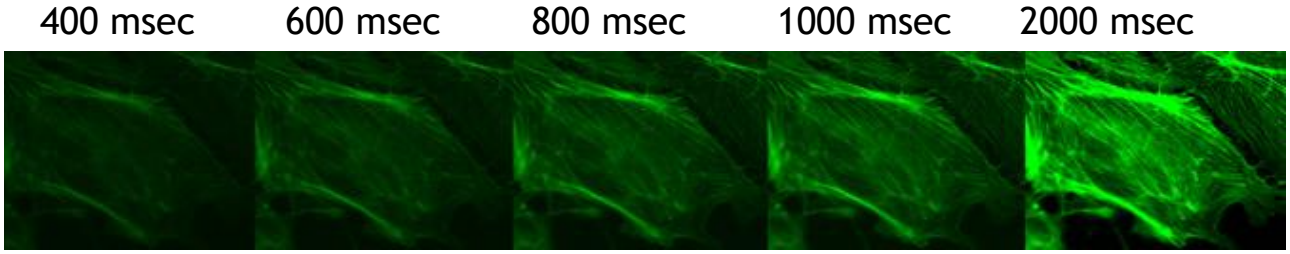
Photomultiplier tubes (PMTs) are used as detectors



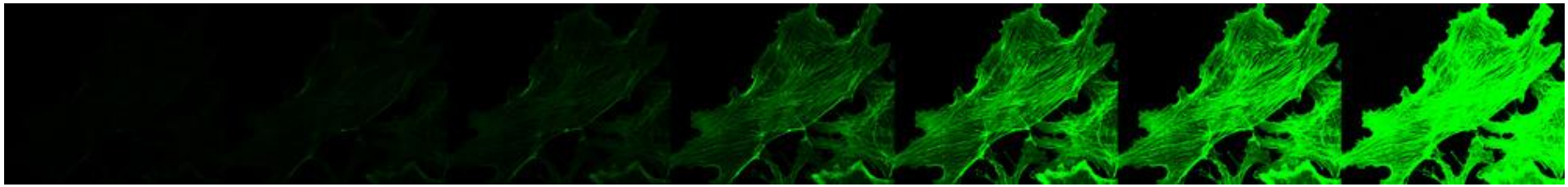
- Fast (good for scanning)
- Large collection area
- Good SNR
- Very large dynamic range (with gain&offset adjustment)
- Adequate dynamic range at a single gain&offset position
- **QE <30% (not as good as CCD)**

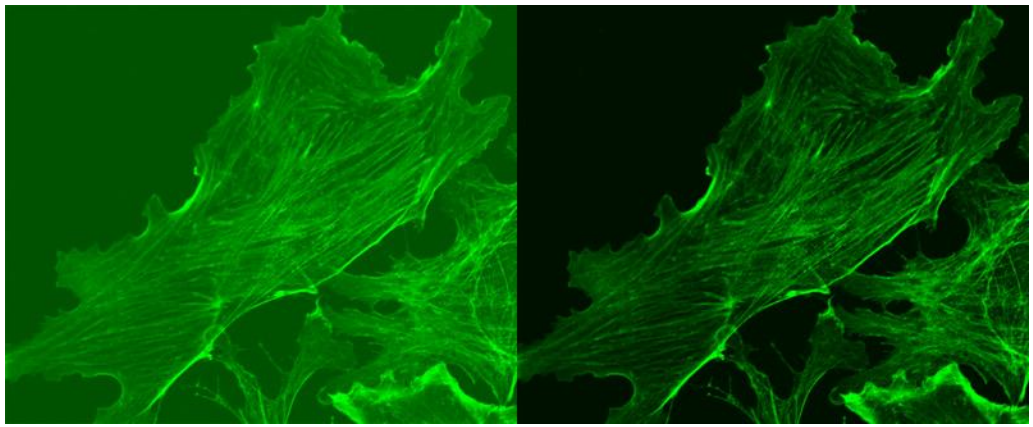
Gain and offset adjustments

Camera:



Confocal: Increasing gain (voltage on PMT)

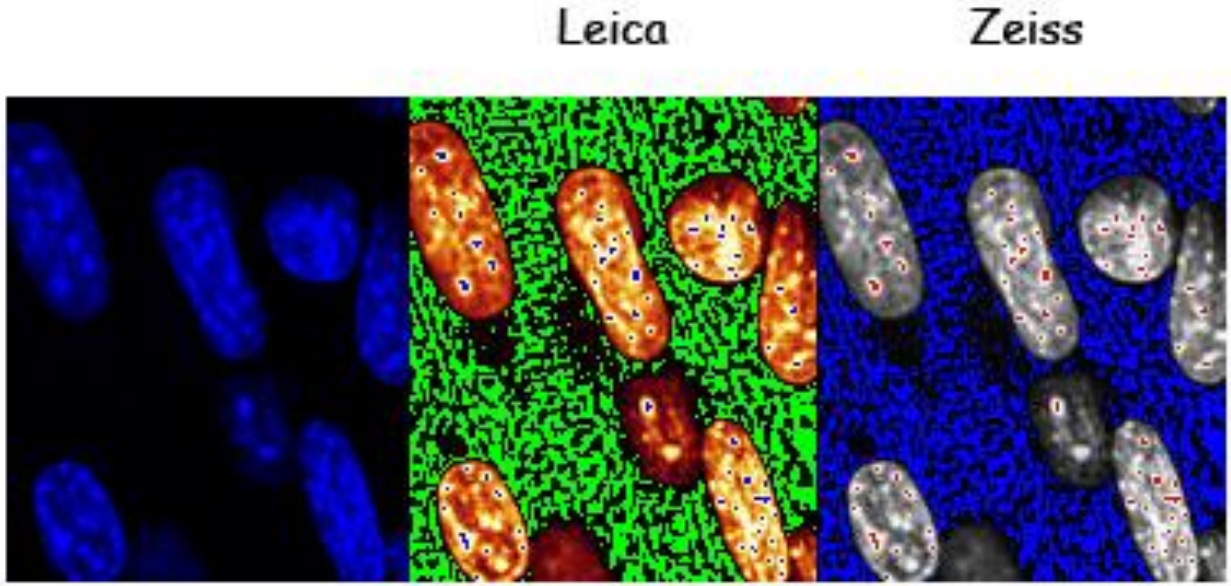




Offset to set the background to black

Optimal gain and offset

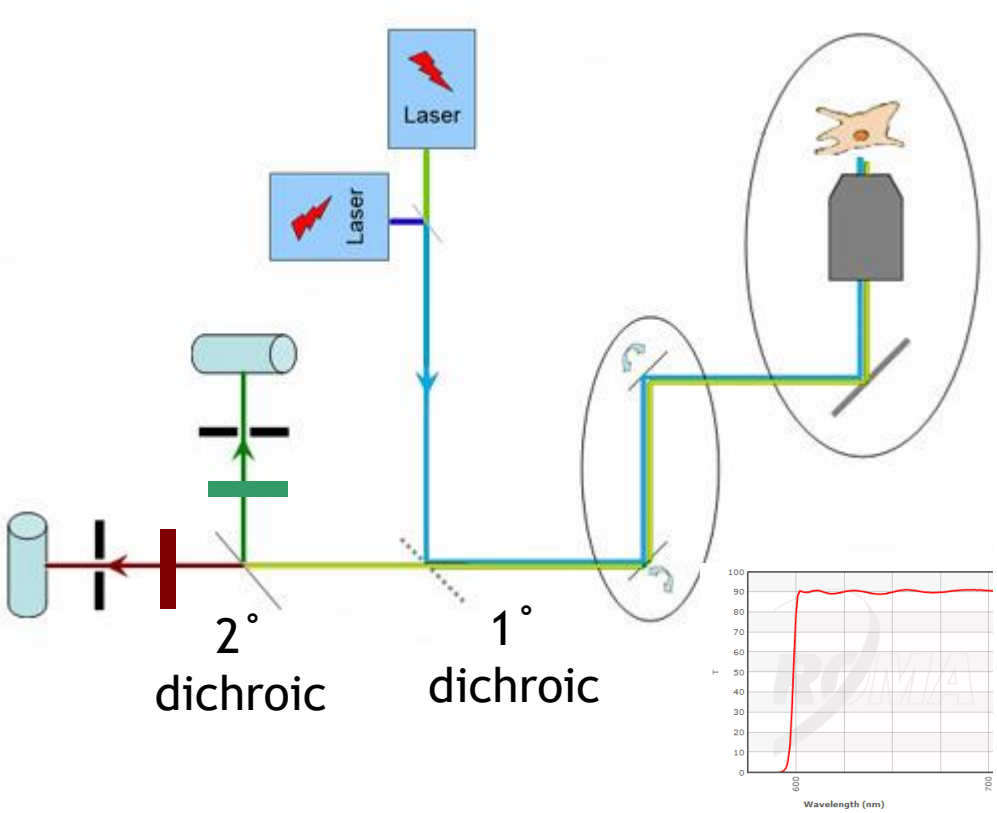
Confocals have special display modes to highlight saturated and 0 intensity pixels



Should you have no, a few or many saturated and zero intensity pixels?

Multi-channel confocals

Most confocals have several laser lines and PMTs



Why don't we in general do this with widefield systems?

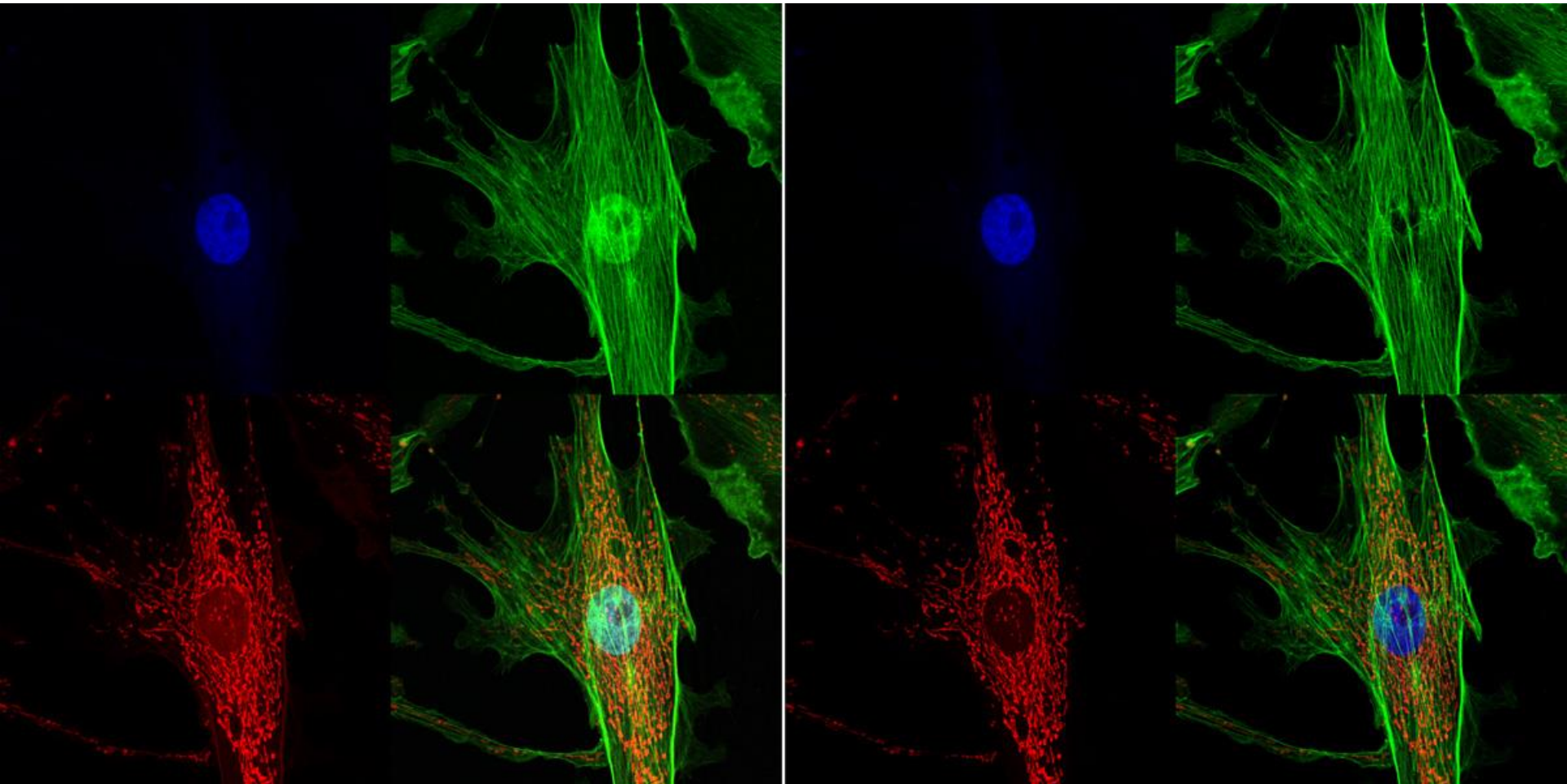
Simultaneous or sequential acquisition

Blue
Green
Red

Faster

Blue then Green then Red

Less bleedthrough



Line vs frame switching

Blue line1 then Green line1 then Red line1
Blue line2 then Green line2 then Red line2

Blue image then
Green image then
Red image

The AOTFs switch the laser light on and off very rapidly

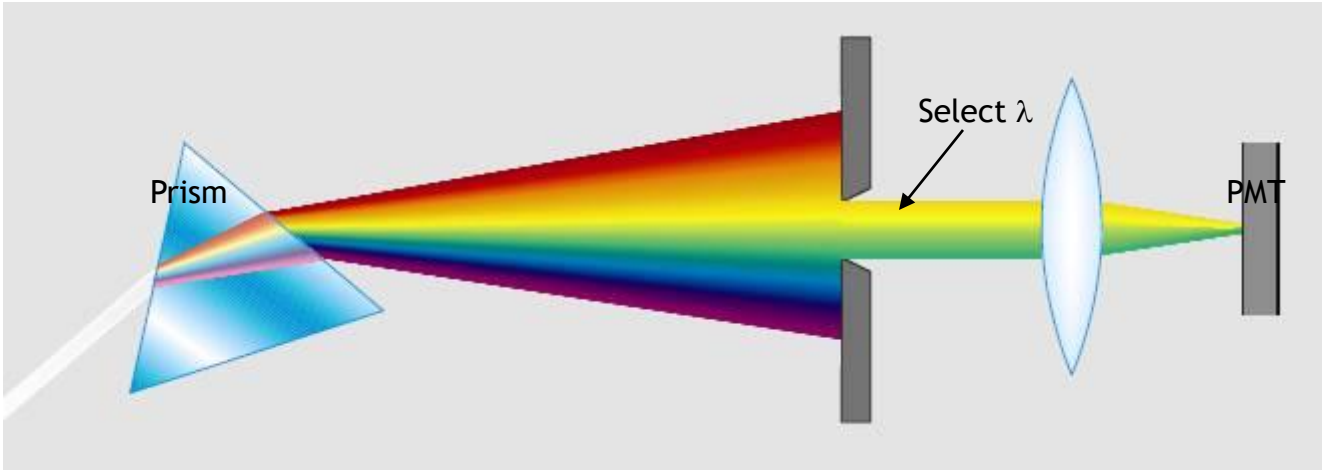
Good to see all the channels appearing together

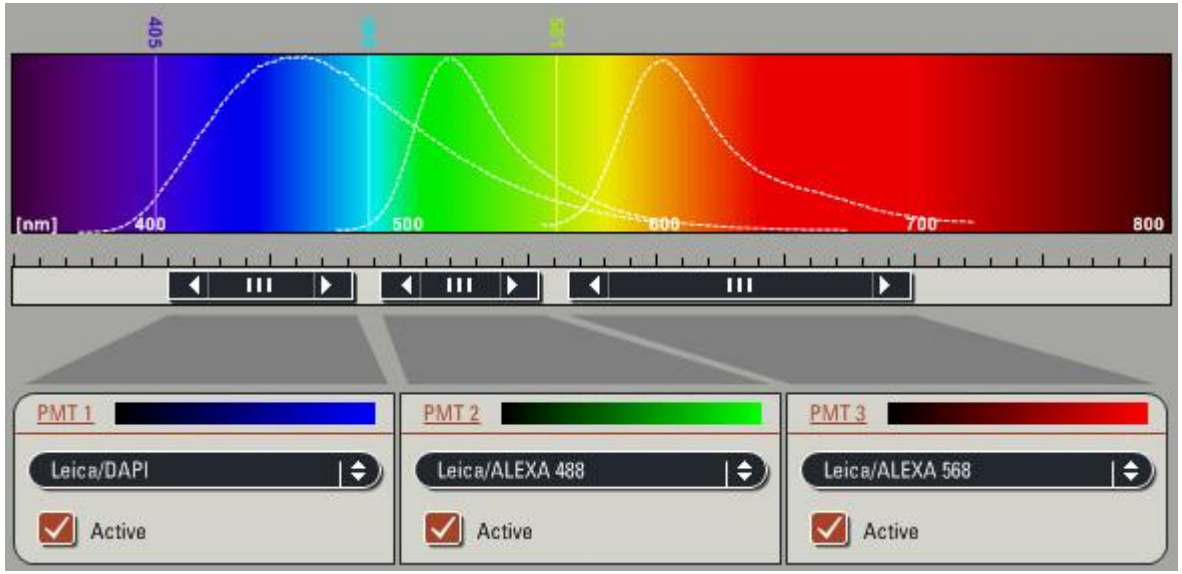
Good when something physical happens between colours (eg change dichroic)

Use this when you have more channels than PMTs

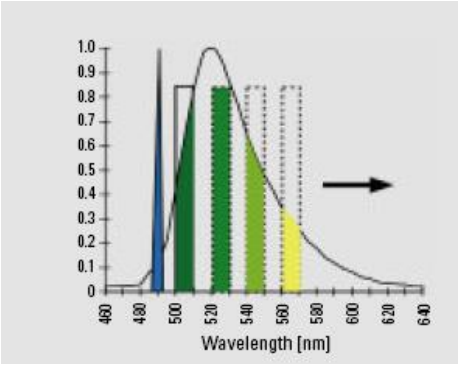
Spectral imaging: serial

Leica spectral detector



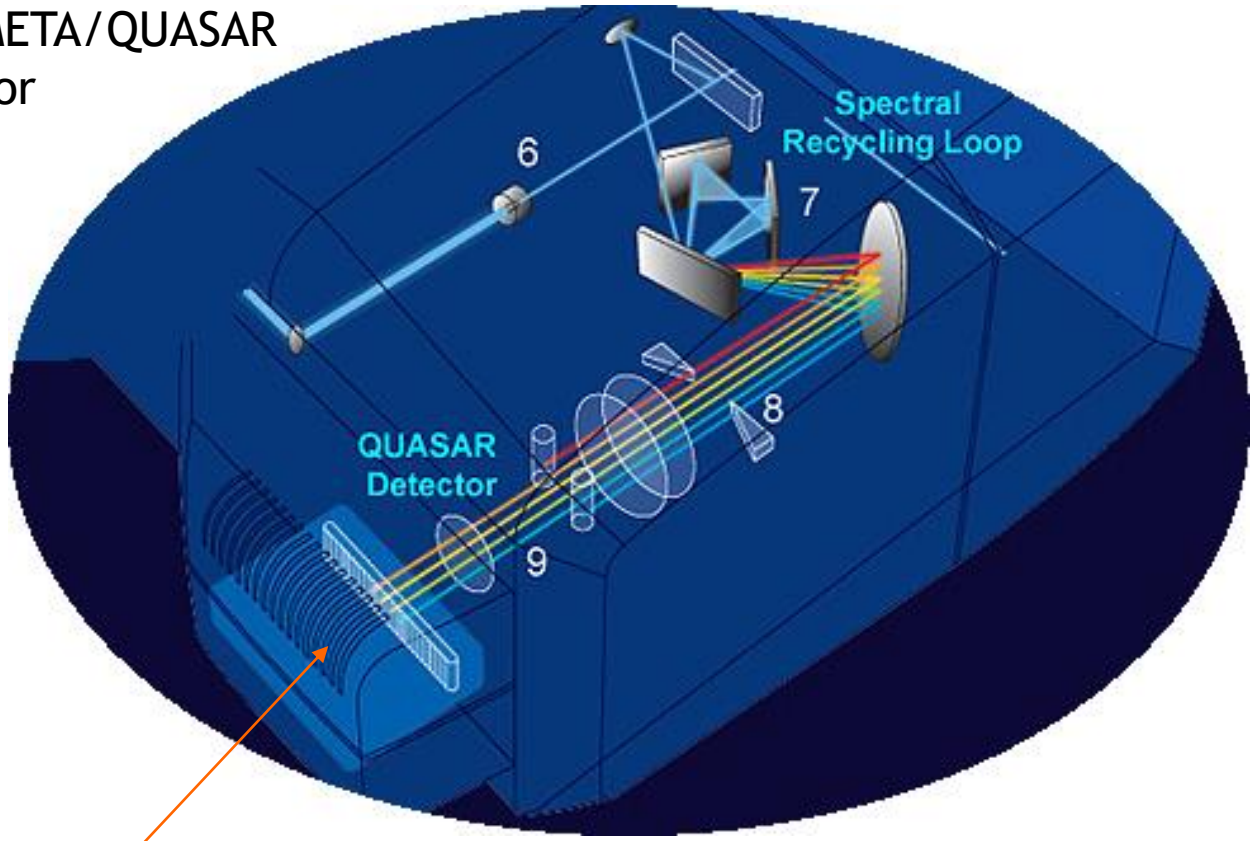


Lamda-scan over time



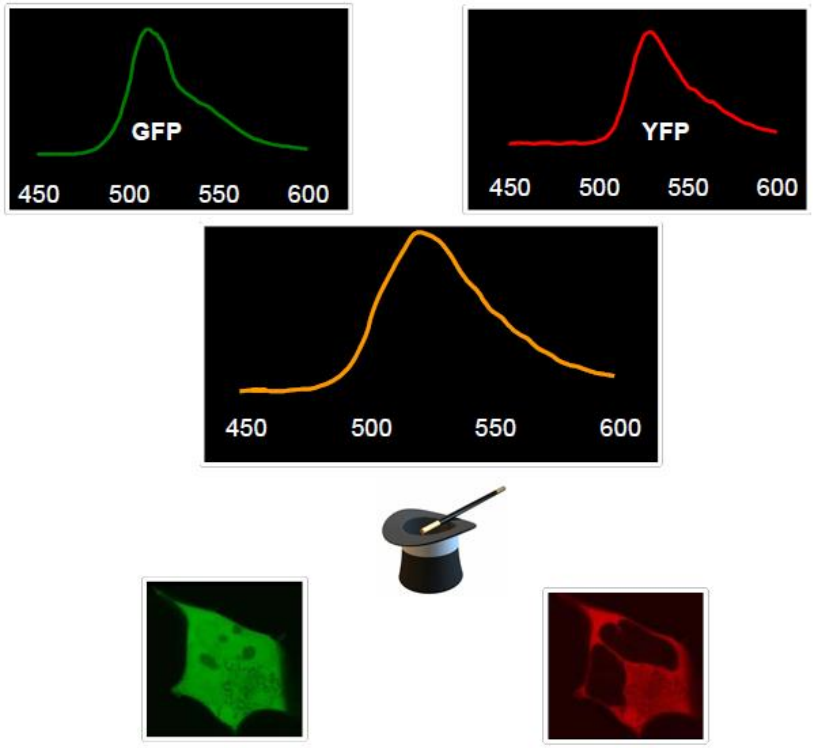
Spectral imaging: parallel

Zeiss META/QUASAR detector



Array of 32 PMTs

Unmixing overlapping signals



Reference spectra (or ACE)

Overlapping signals

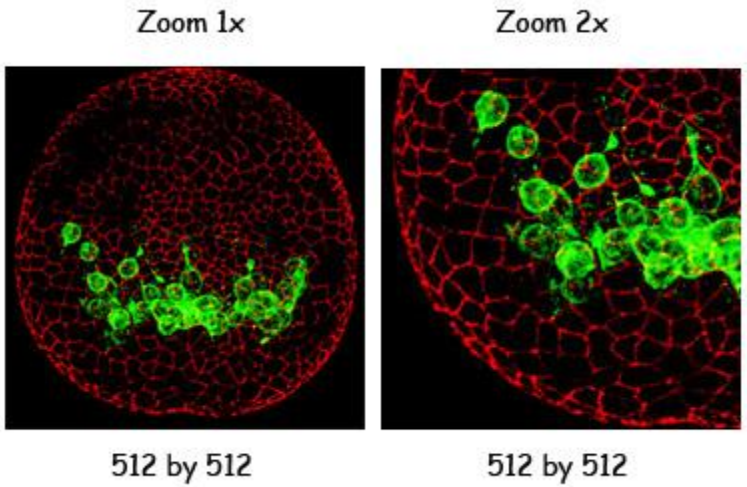
Separate signals

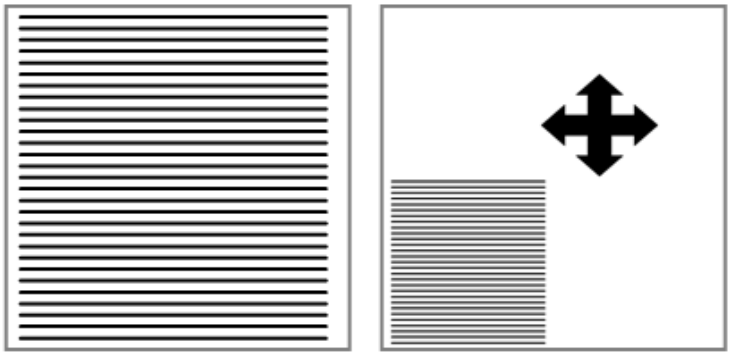
Ideally, avoid having to do this. But maybe . . .

- You've only GFP-YFP mice
- You have a strong autofluorescence (eg chloroplasts)
- You need many colours

Scan area: zoom

The area swept by the galvo mirrors can be adjusted . . .

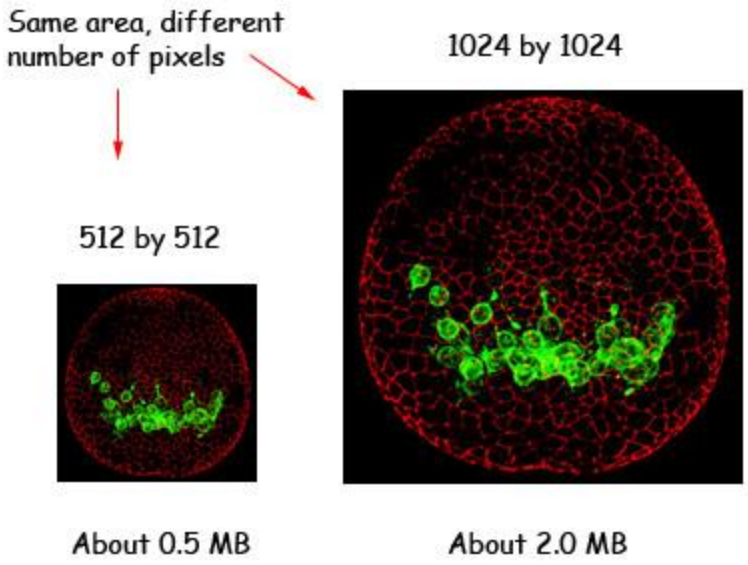




Is this meaningful zoom or just digital zoom? . . .

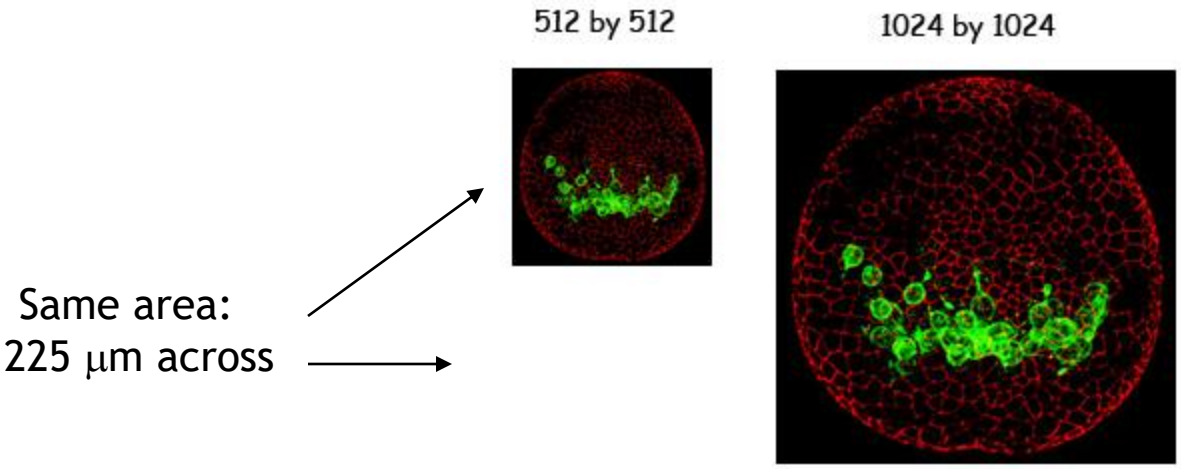
Scan area and number of pixels

Any particular frame can have different numbers of pixels. . .



Zoom and number of pixels are obviously related

How many pixels do I need in my image?



225 μm/512
= 440 nm

225 μm/1024
= 220 nm

The most possible, right?

The Leica SP5 goes up to 8K by 8K, shall we have 64 Mpx for every scan?

How many pixels do I need in my image for the best resolution?

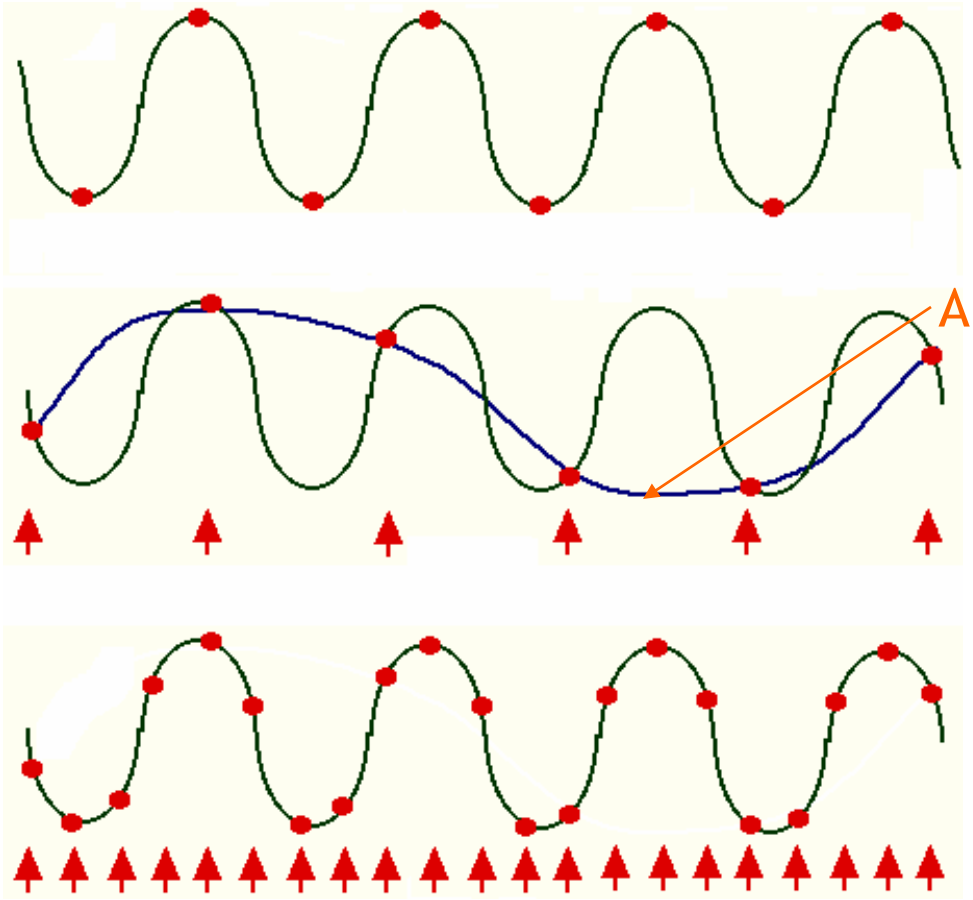
Nyquist sampling theorem: Sample at twice the resolution

$$\text{Resolution} = 0.61\,\lambda / \text{NA}$$

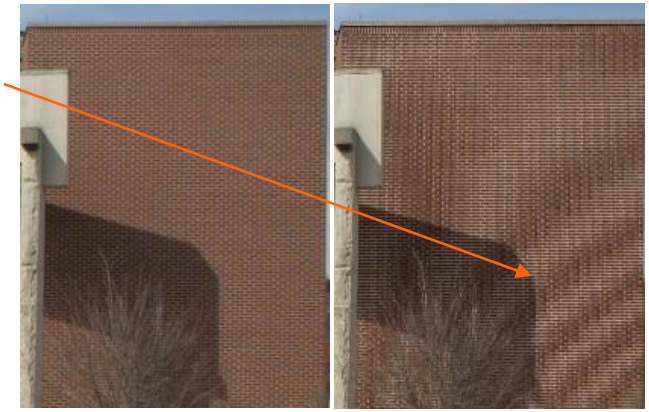
Increasing number of pixels per area

Signal under-sampled	Signal Well sampled	Signal Over-sampled
Not capturing all the resolution of the system	Just right, The Nyquist rate	Not gaining any more resolution, more bleaching waste of time and disk space

Sampling rate



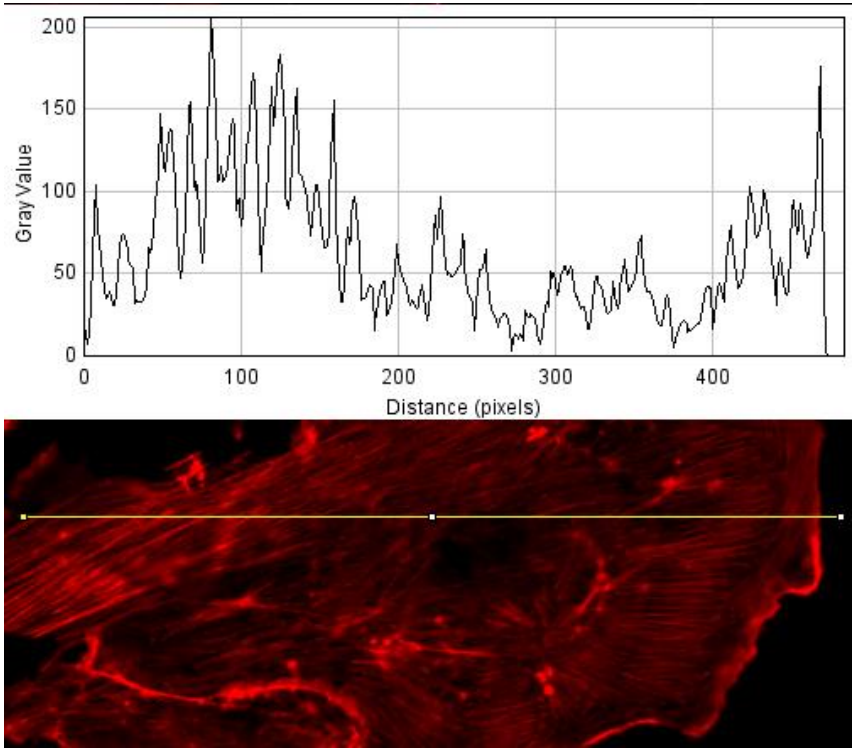
Aliasing

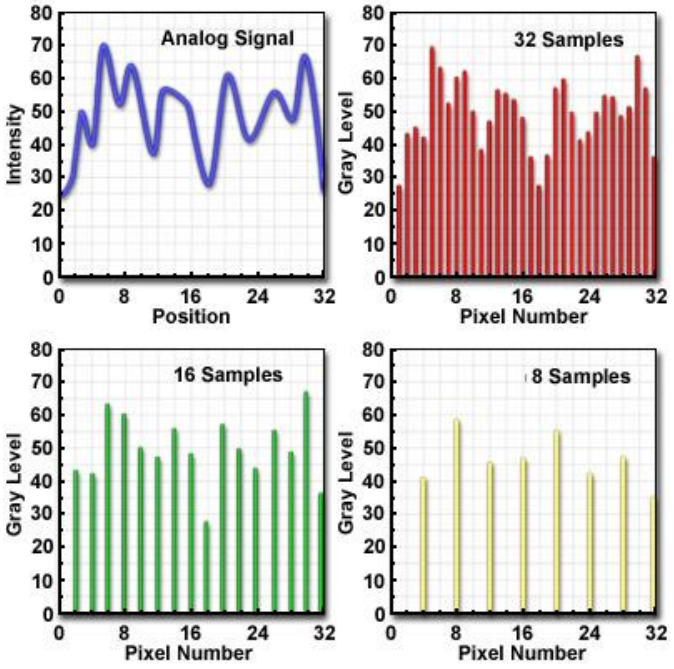


Over-sampled

How do frequencies relate to resolution?

But I don’t image wavy green lines or brick walls





Our object has a spatial frequency in the distribution of the contrast we are imaging

Nyquist calculator for microscopy

https://svi.nl/NyquistCalculator

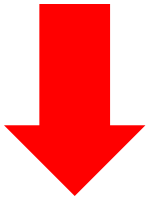
Q

Nyquist rate and PSF calculator

Results

This is the parameter list used in this calculation:

Parameter	Value
Microscope type	Confocal
Numerical aperture	1.4
Excitation wavelength	488
Emission wavelength	520
Number of excitation photons	1
Lens immersion refractive index	1.515



Consider this principle and a few more subtle factors

A

The optical axis lays along z. Your Nyquist sampling is:

> x: 43 nm
> y: 43 nm
> z: 130 nm
> — Set your zooms and scanning steps so that each pixel covers a x-y area

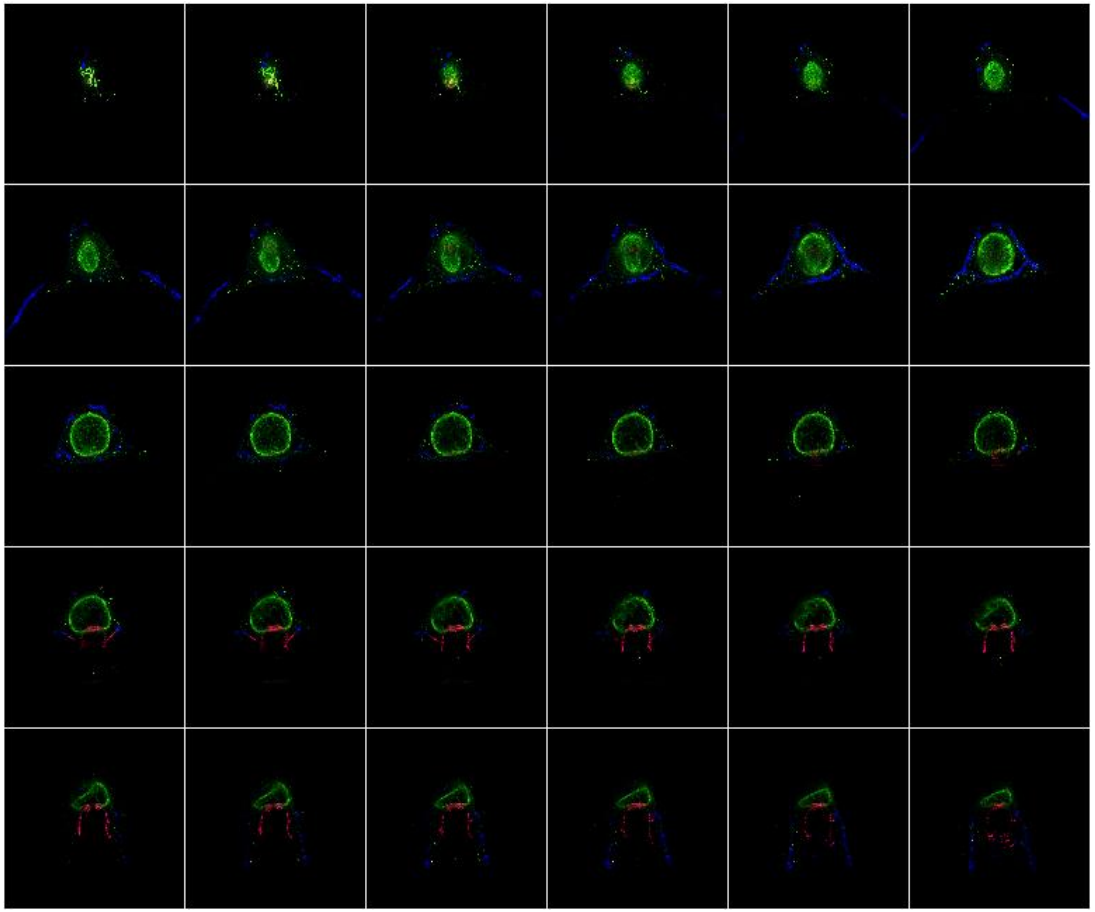
Do I really need to listen to Nyquist?

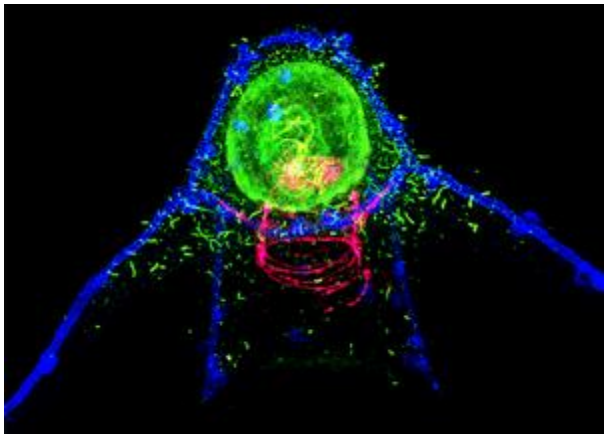
- You might not always be seeking the best resolution
- You might need to under-sample for speed, phototoxicity . . .
- Over-sampling is effectively averaging

But optimal sampling is important and beneficial in many cases

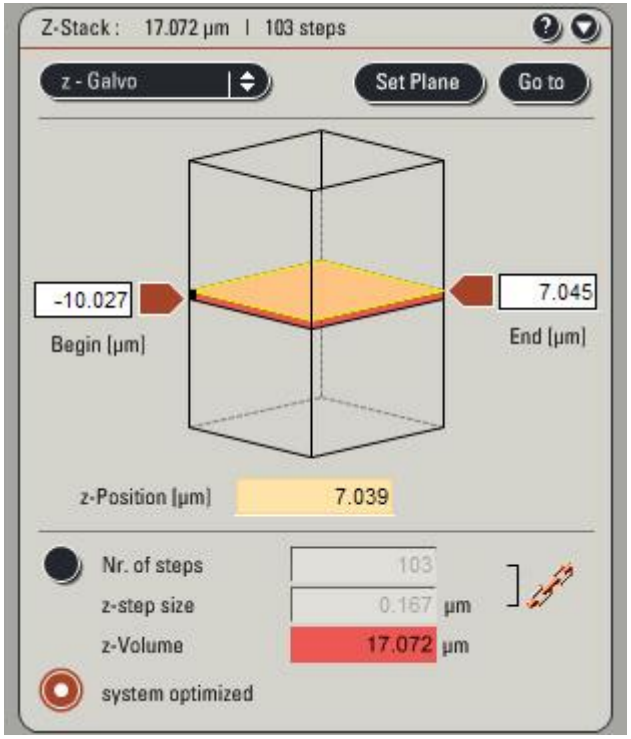
3D acquisition

Confocals are good for acquiring stacks of images because of the optical sectioning ability





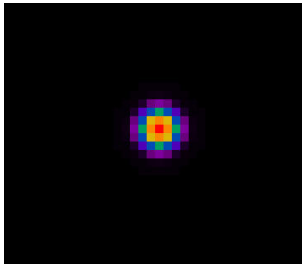
Z-stack acquisition control



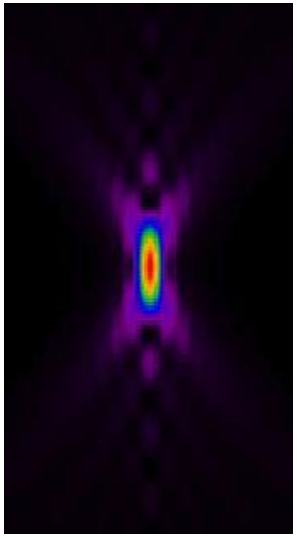
The point-spread function

Calibration of the microscope,
the 3D image of a point source

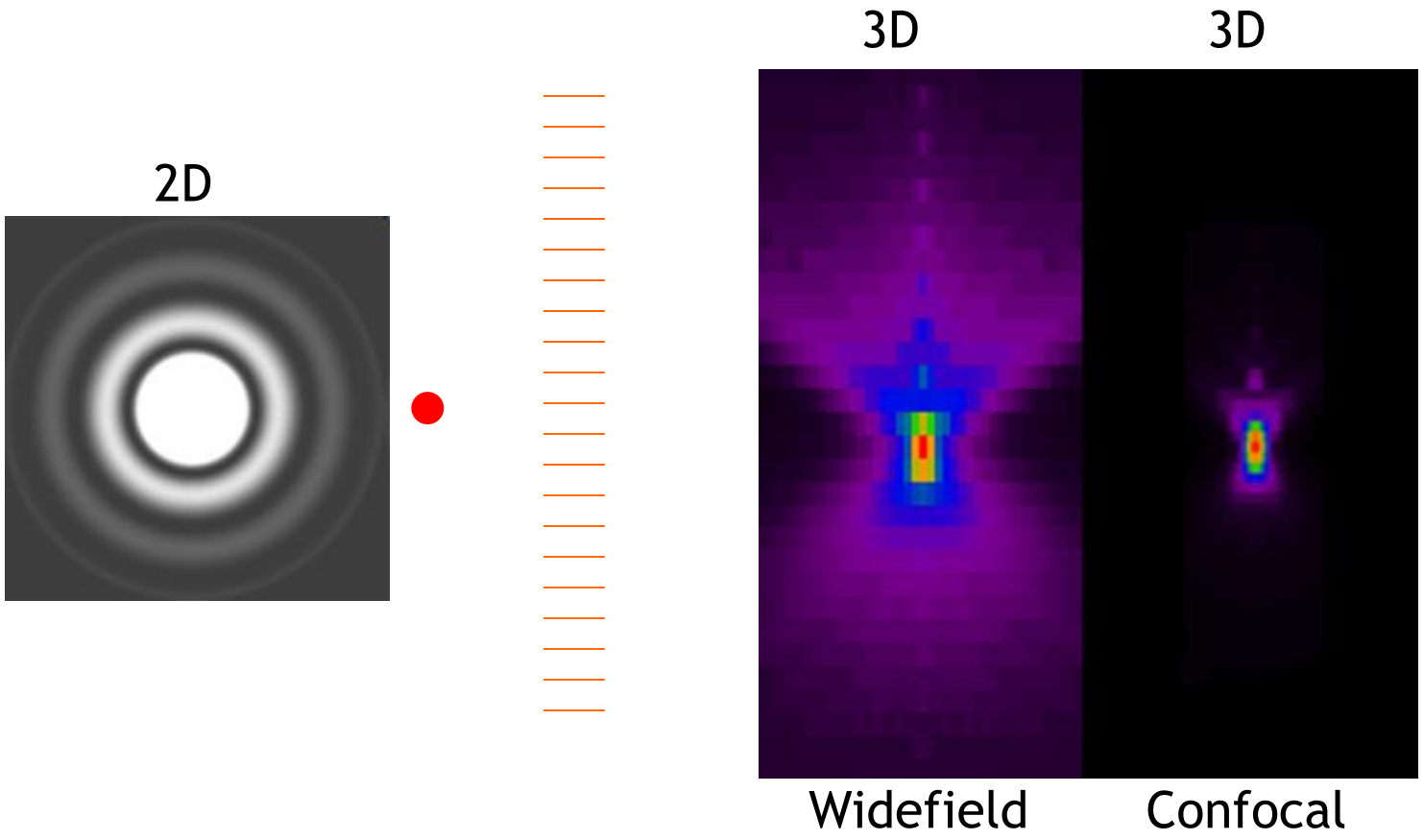
XY



XZ

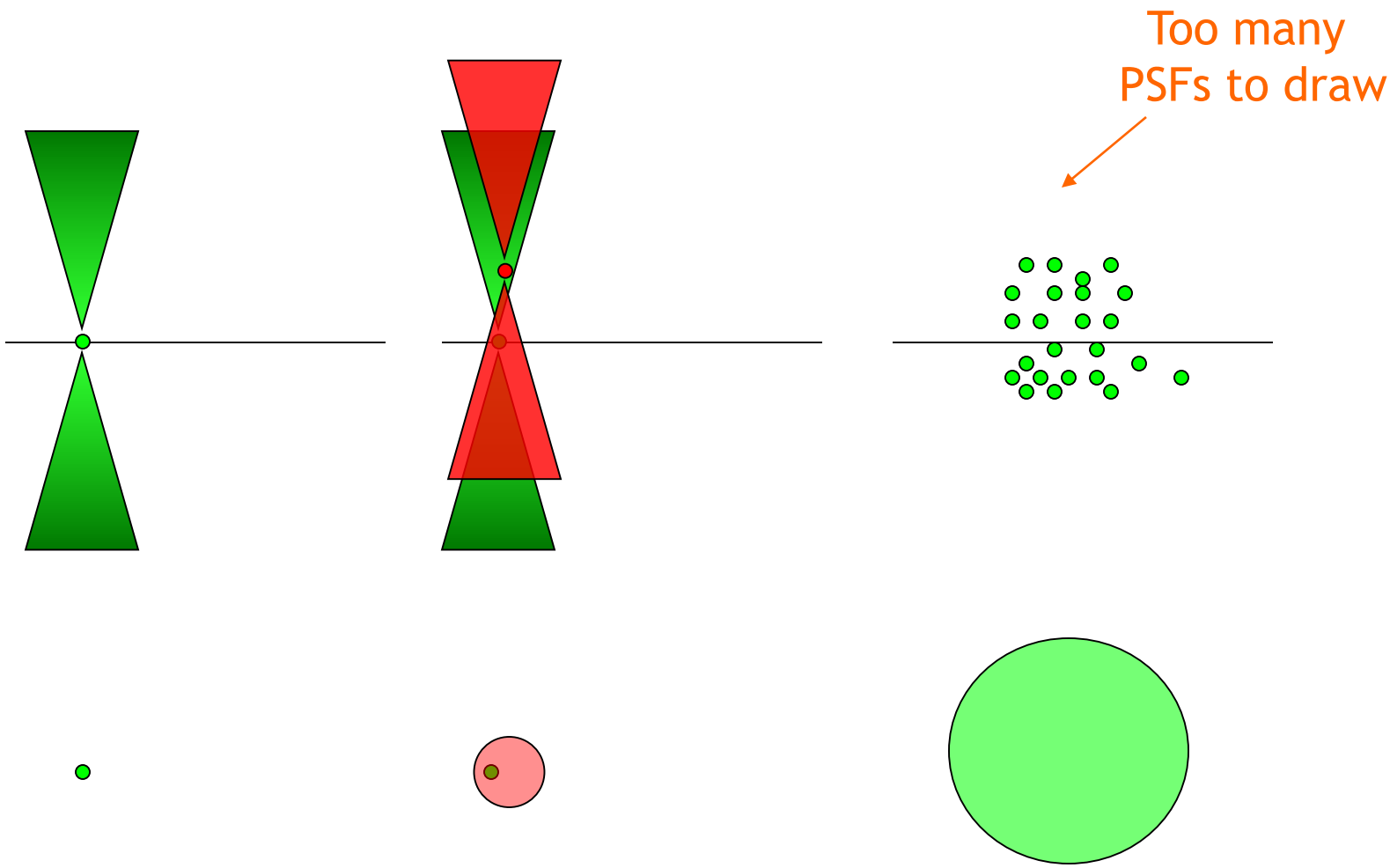


Point spread function



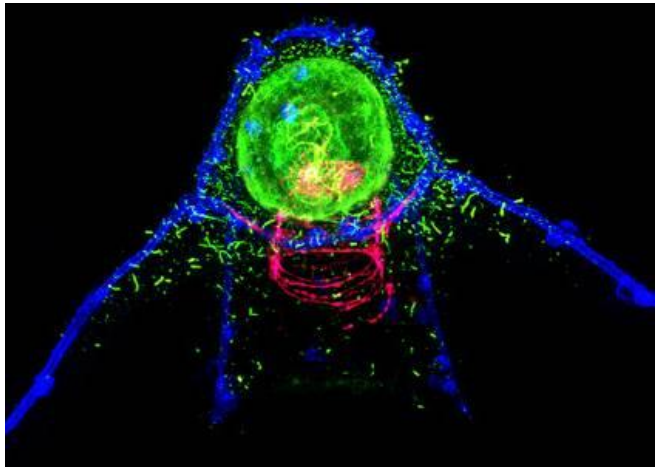
FWHM for 63x/1.4 NA
is about 200 * 500 nm

Blur degradation in 3D wf images

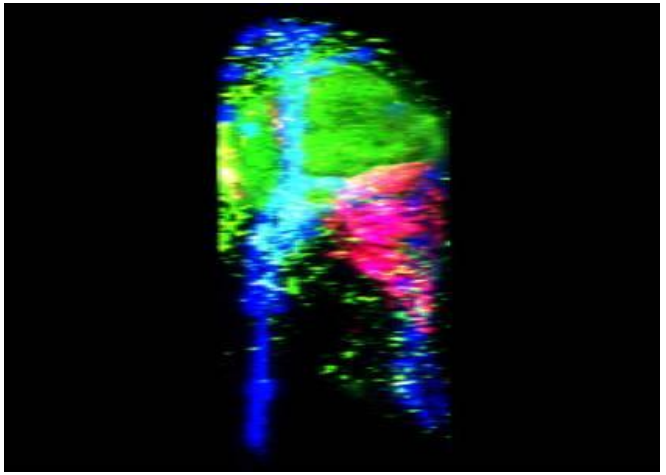


Lateral and axial resolution

XY



YZ

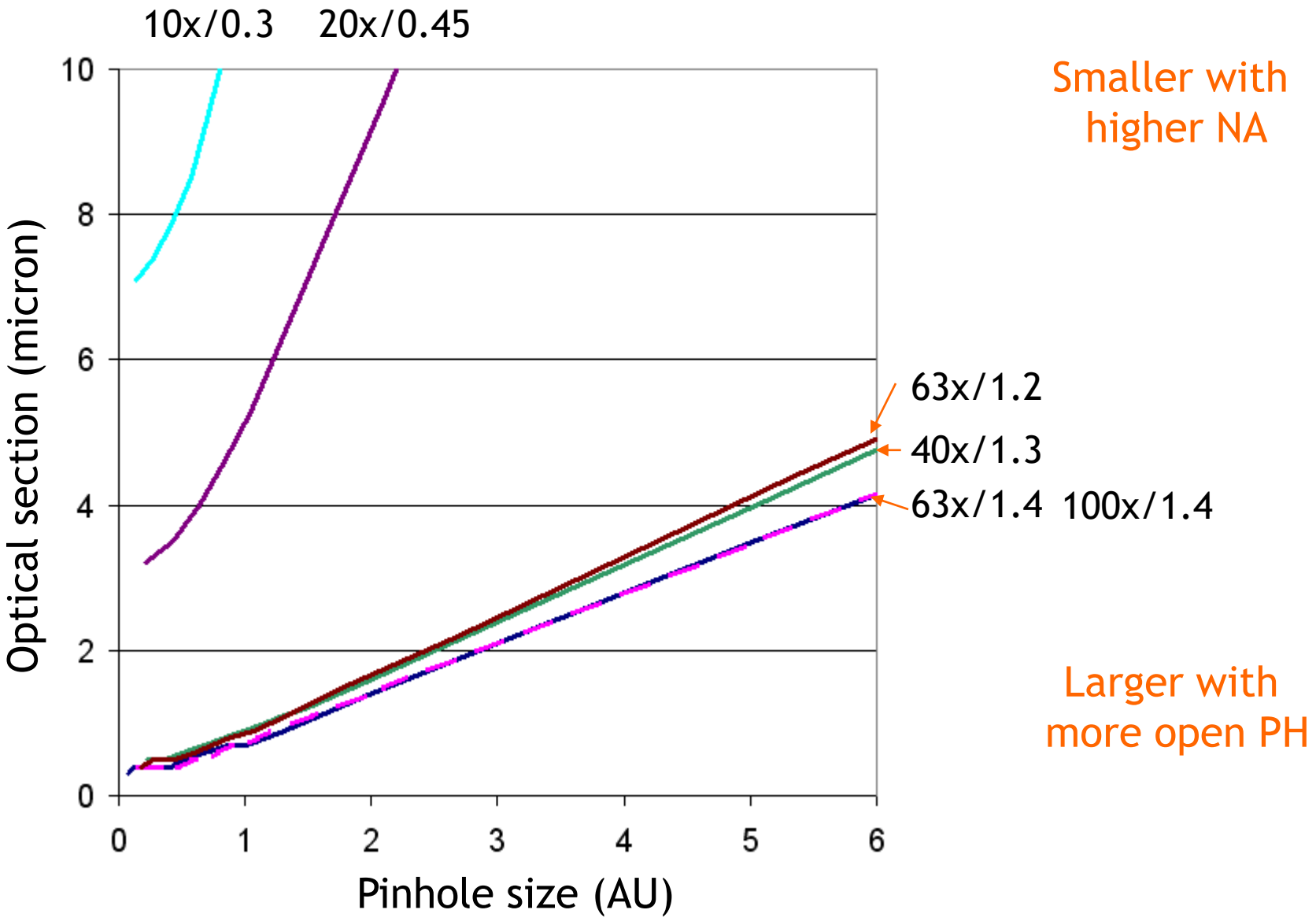


$r_{xy} \sim 0.61\ \lambda\ /\ NA$

$r_z \propto \lambda\ /\ NA^2$

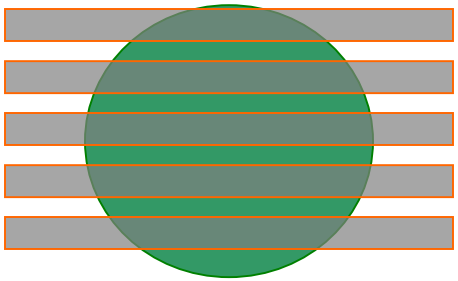
Resolution is always worse in Z than XY

Optical section thickness

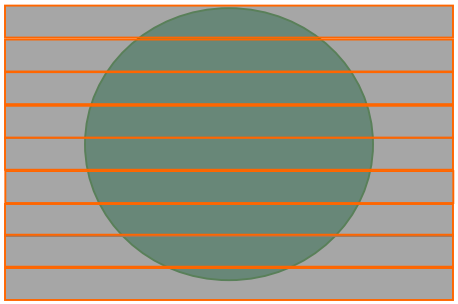


Sampling in the z-axis

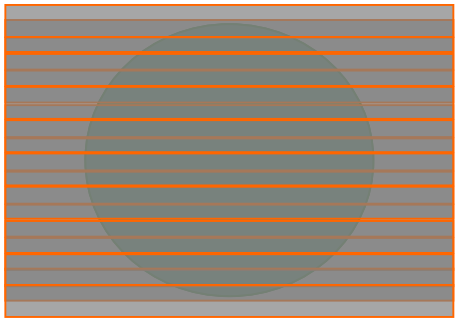
The same principle as in XY



Some regions not imaged

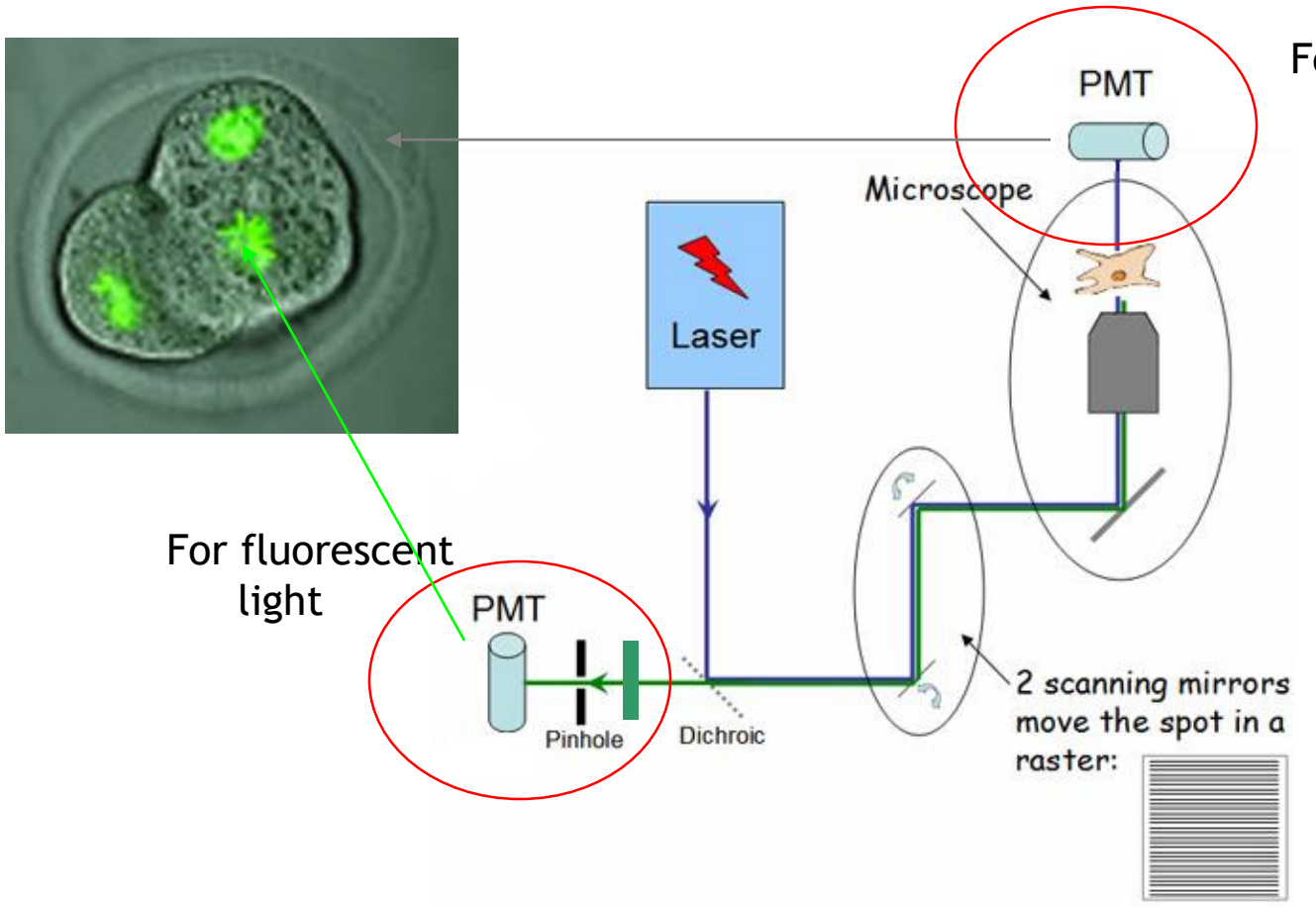


Covered



Covered and well sampled

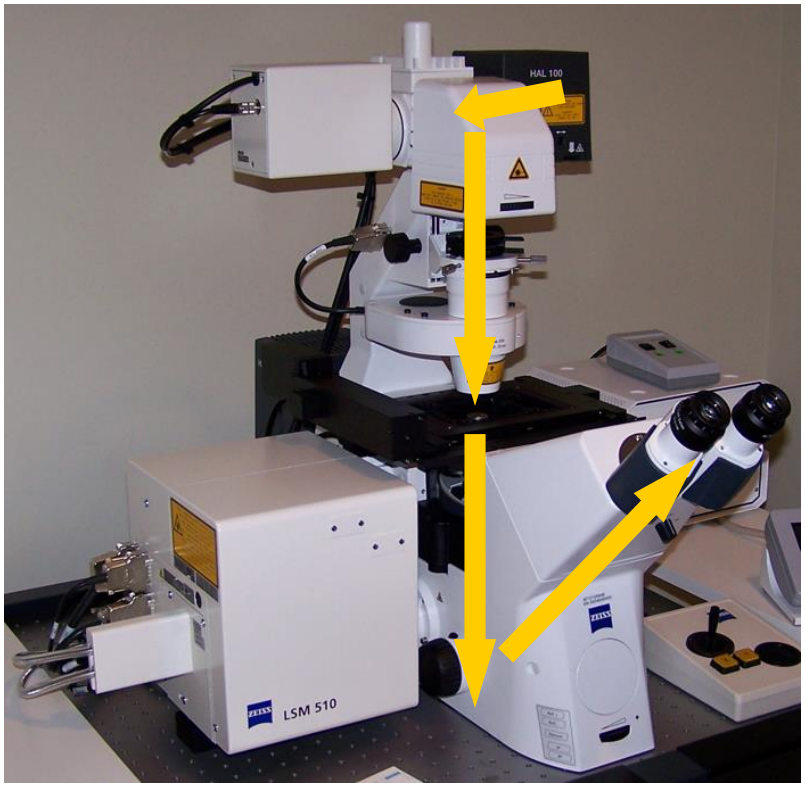
Transmitted image



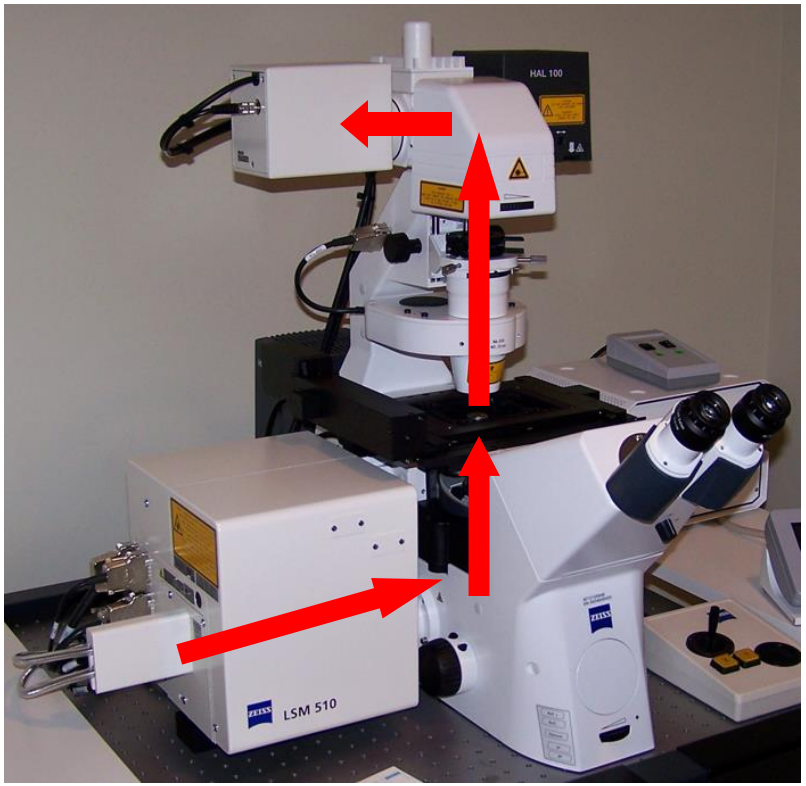
It is NOT confocal (why?), beware of the overlay

Setting up a transmitted image

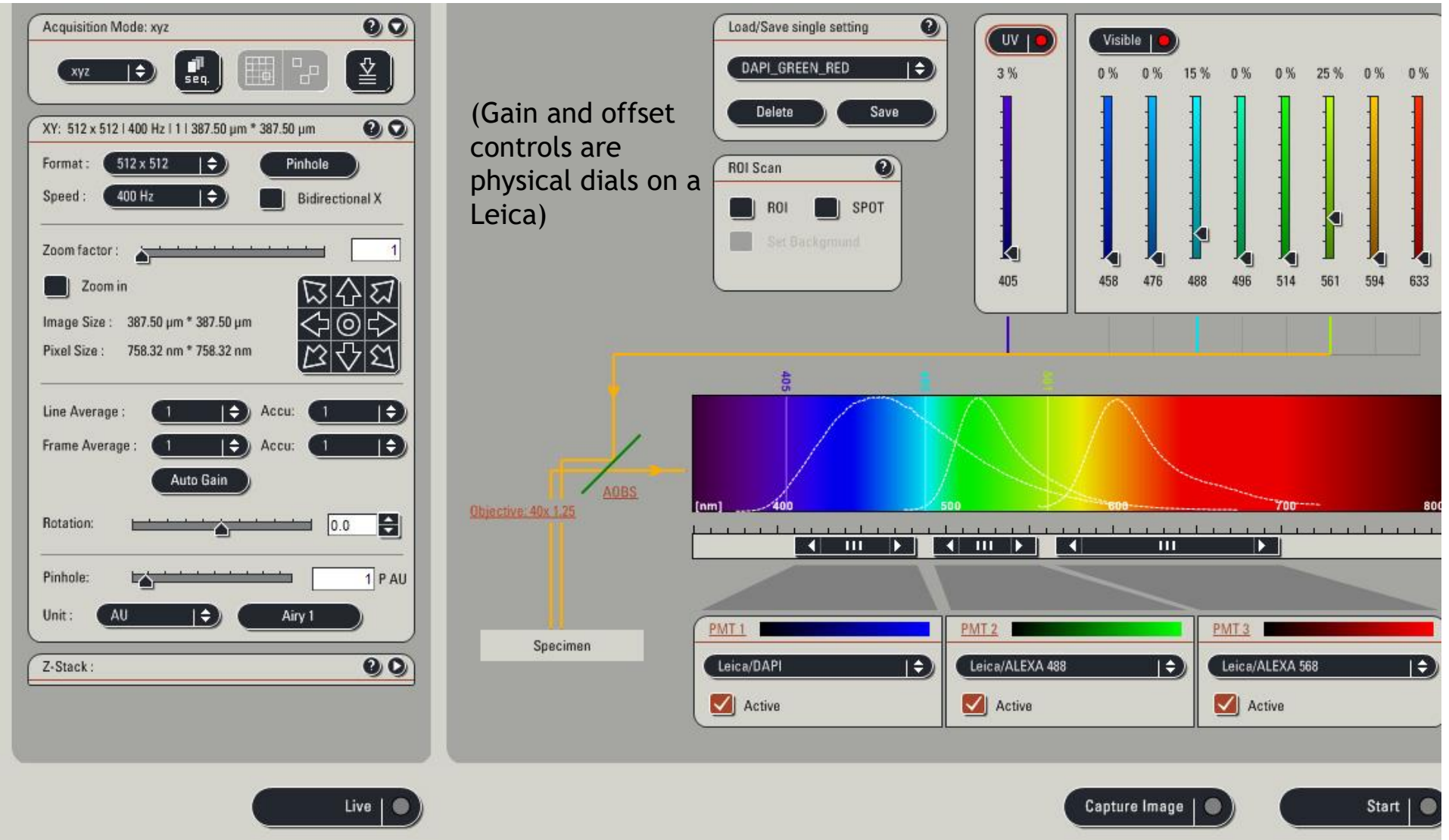
1. Setup Kohler TL



2. Confocal forms a TL image



You can use whichever lasers you are using for fluorescence, but it tends to be best with far-red



http://www.olympusfluoview.com/java/confocalsimulator/index.html