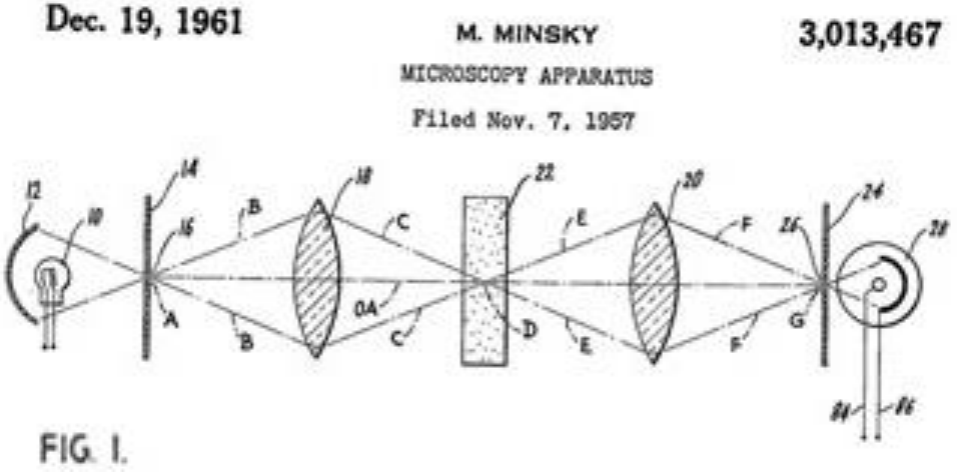
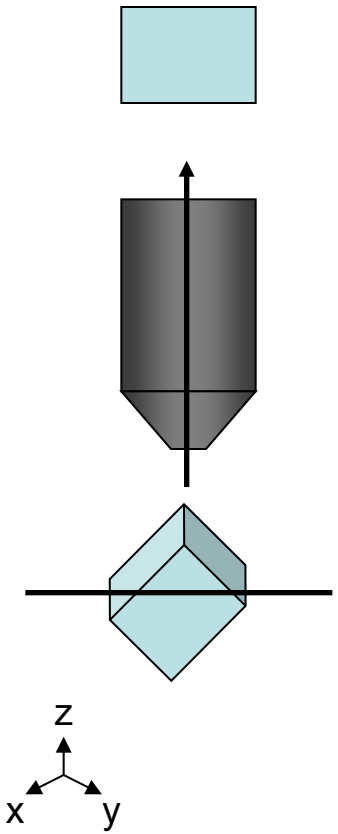
Confocal Microscopy

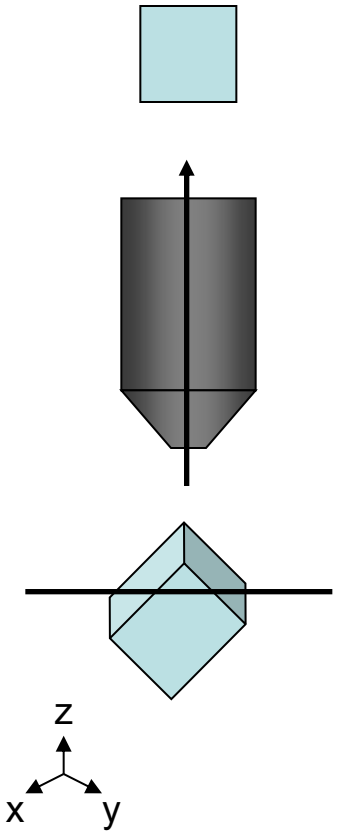
Kurt Thorn
NIC



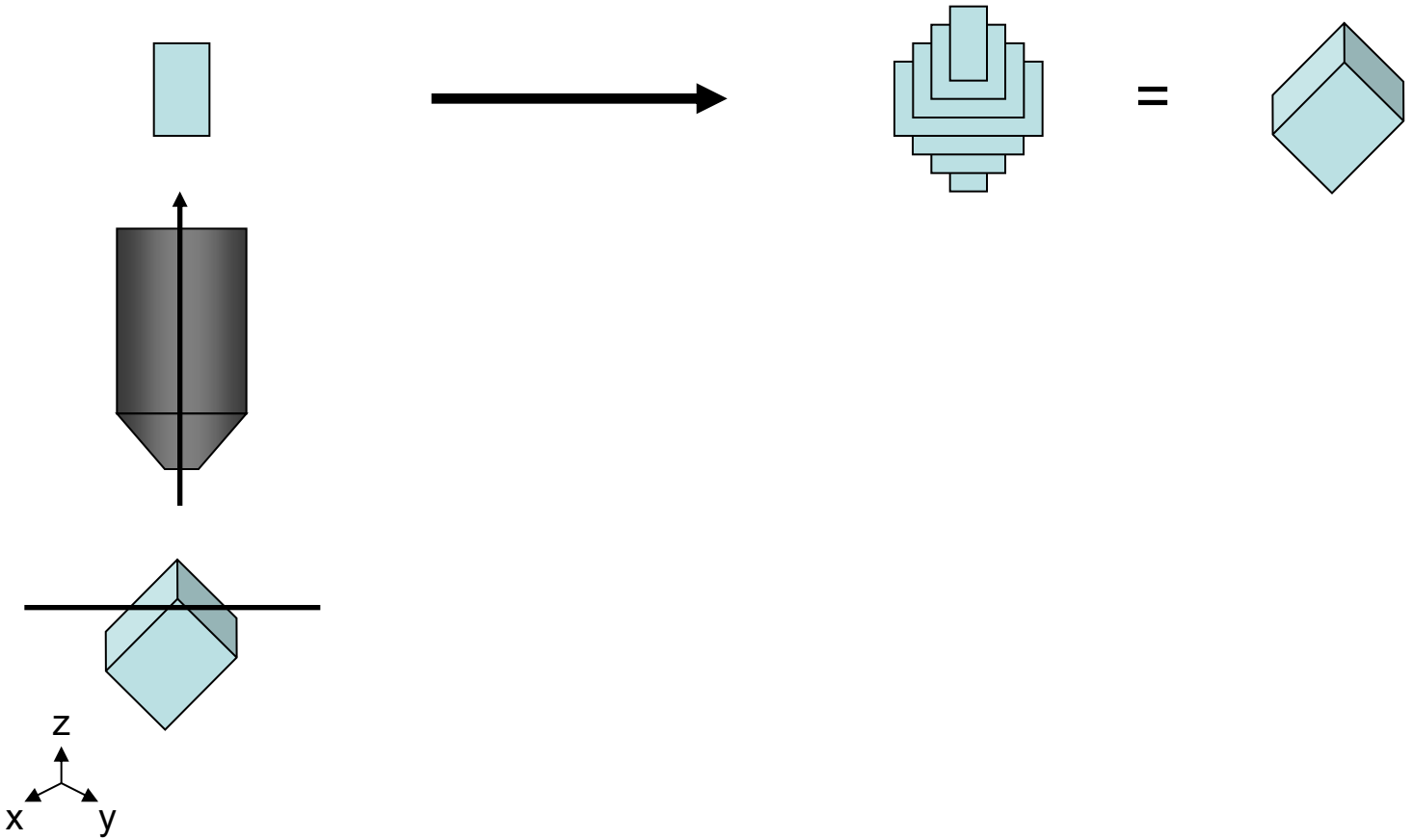
Optical Sectioning and 3D reconstruction



Optical Sectioning and 3D reconstruction



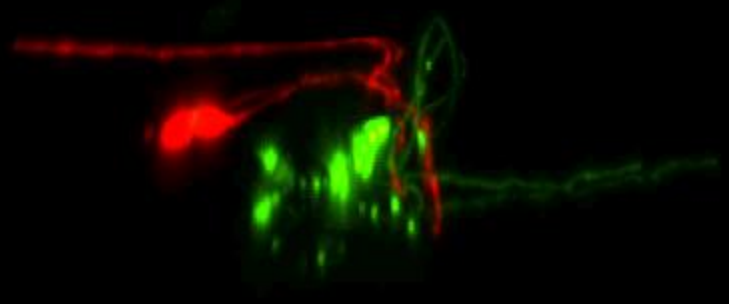
Optical Sectioning and 3D reconstruction



Optical Sectioning and 3D reconstruction

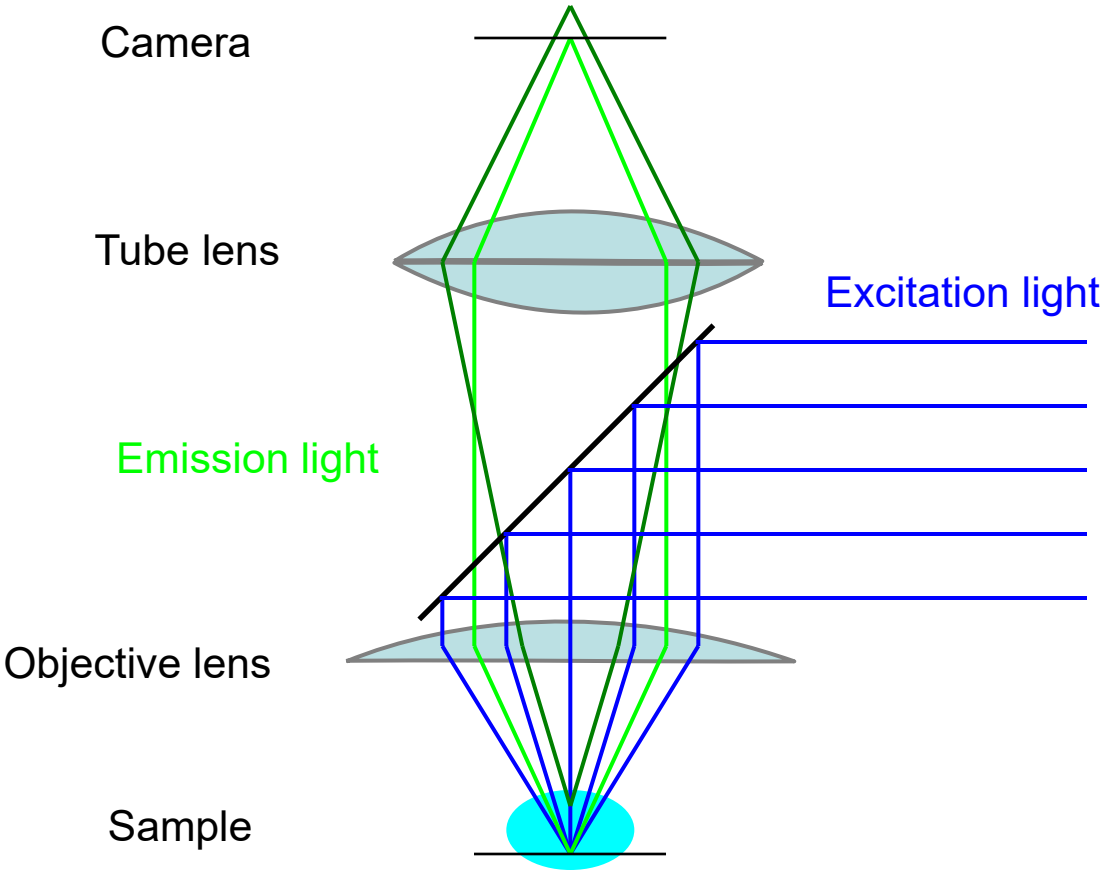
C. elegans with two different sensory neurons expressing GFP, DsRed
Swept-Field Confocal, 85 Z slices, 250 nm spacing

Optical Sectioning and 3D reconstruction



3D reconstruction

Fluorescence Illumination of a single point



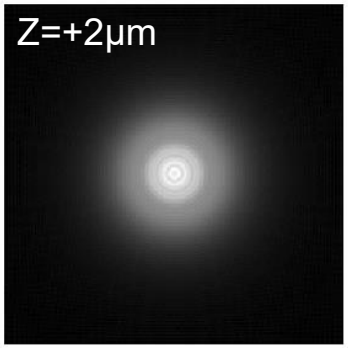
Problem – fluorescence is emitted along entire illuminated cone, not just at focus

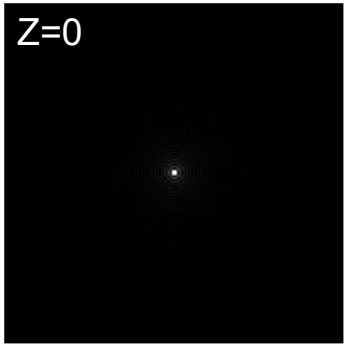
Widefield fluorescence imaging

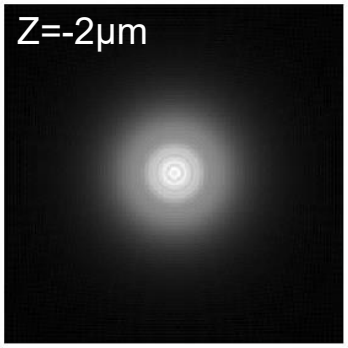
The Point Spread Function (PSF)

The image of a point object

2D PSF for different defocus

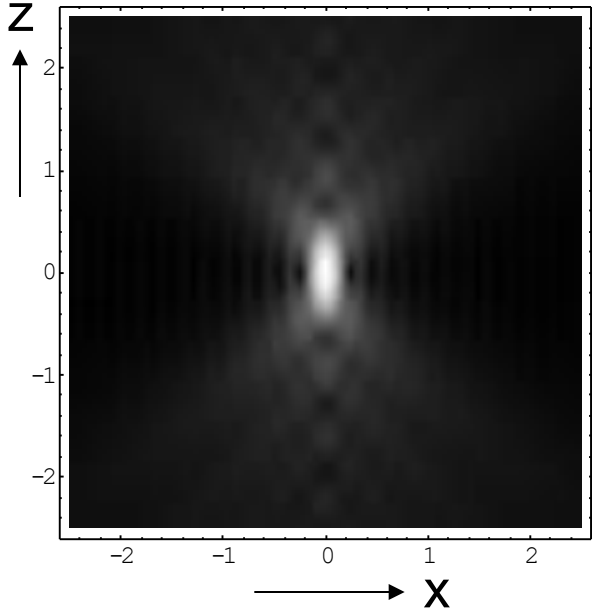




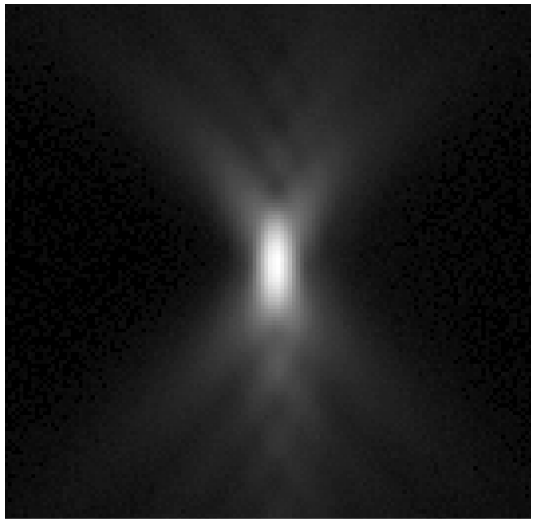


3D PSF

Calculated



Measured



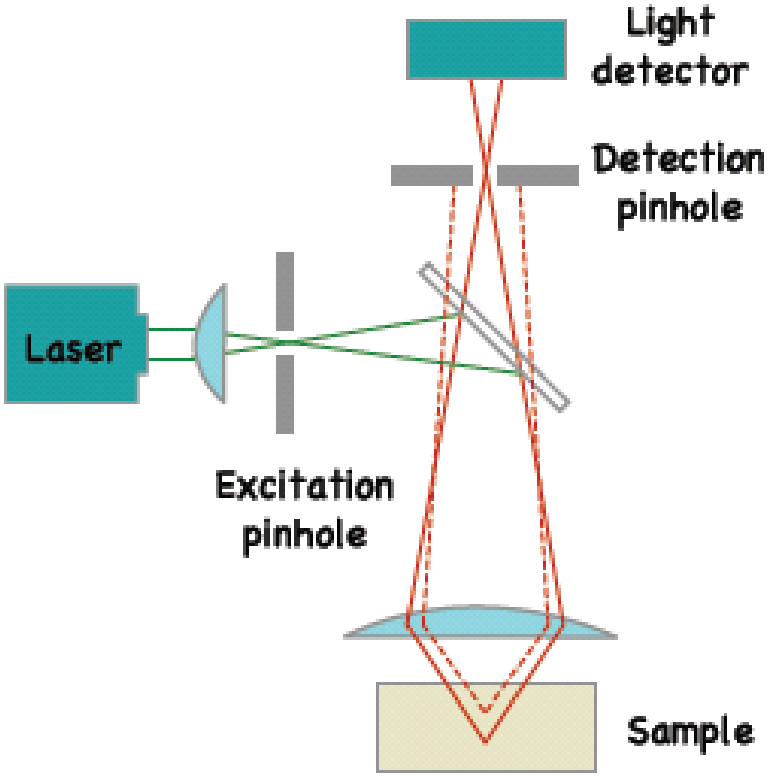
3D fluorescence microscopy

Acquire a "focal series" (stack) of images

Problem:
Each image contains out-of-focus blur from other focal planes

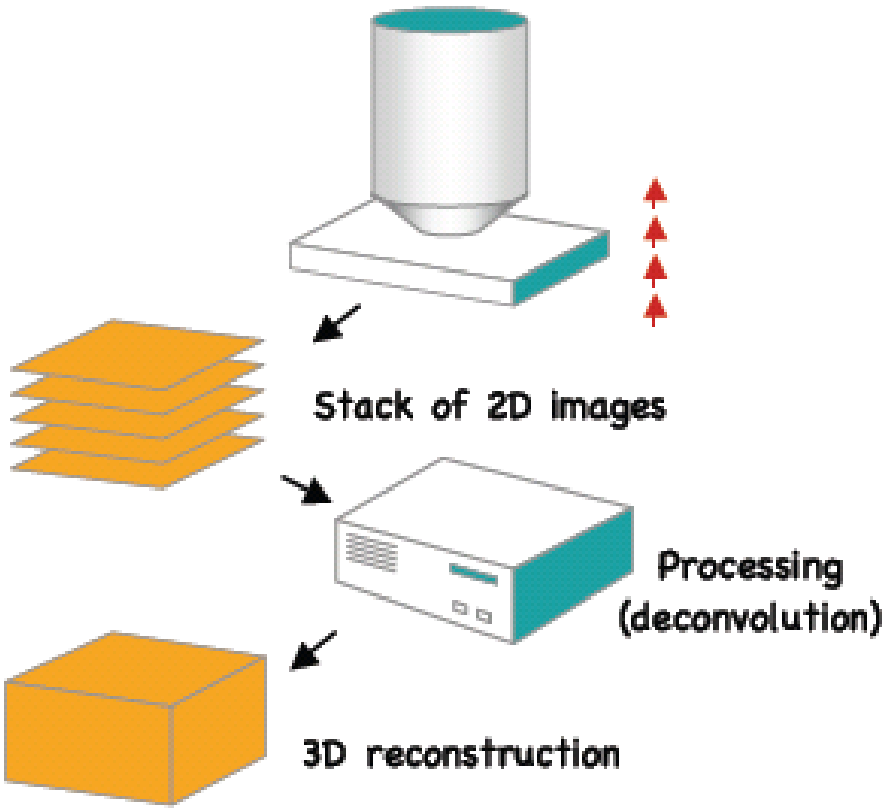
Approach 1

Physically exclude the blur
Example: confocal microscopy



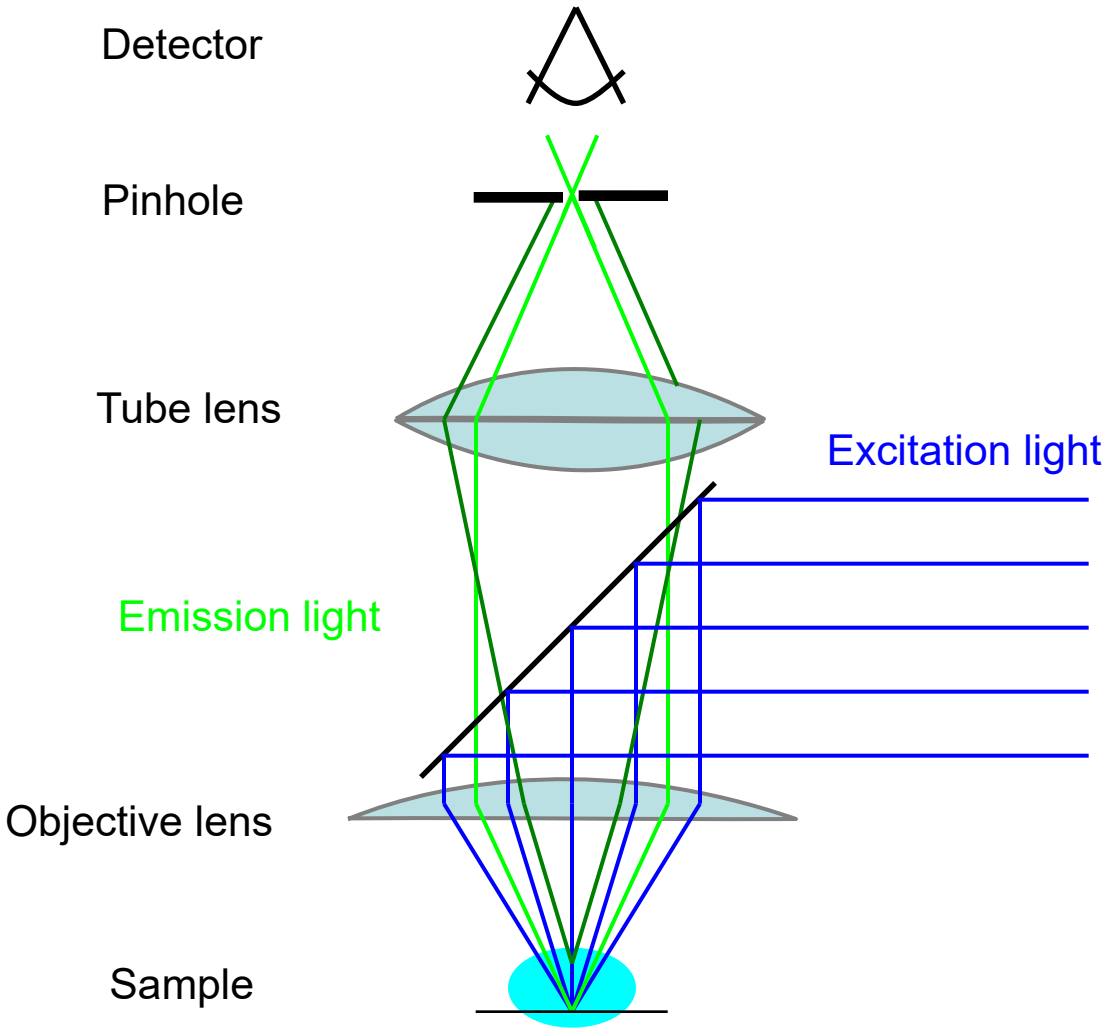
Approach 2

Remove the blur computationally



How well can this be done?

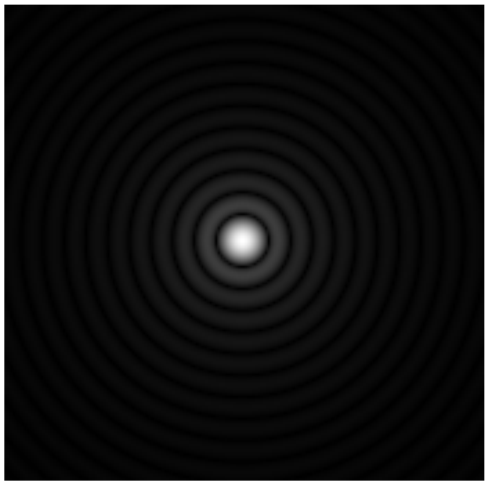
The confocal microscope



Scan excitation spot point-by-point to build up image

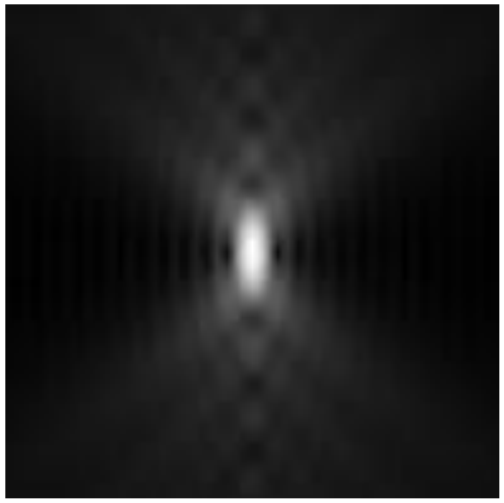
How big should your pinhole be?

Resolution is limited by the point-spread function

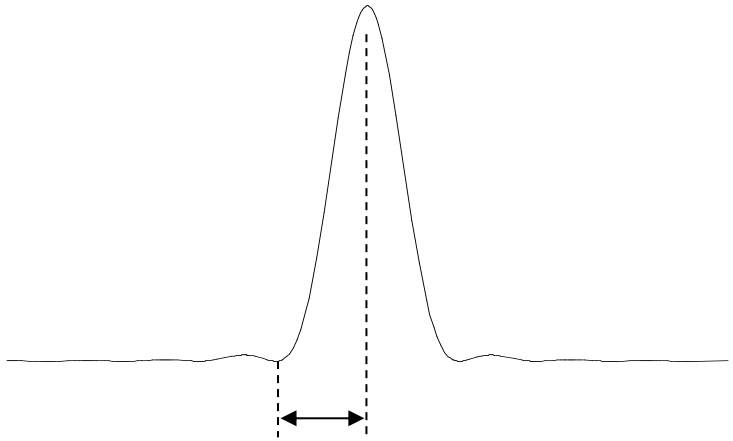


Y

X



Z

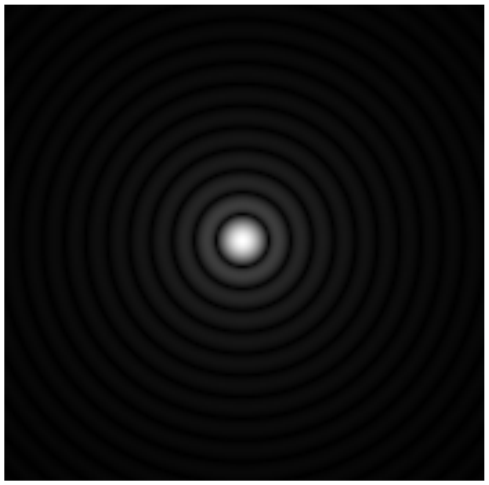


Airy disk radius

≈ 0.61 λ /*NA*

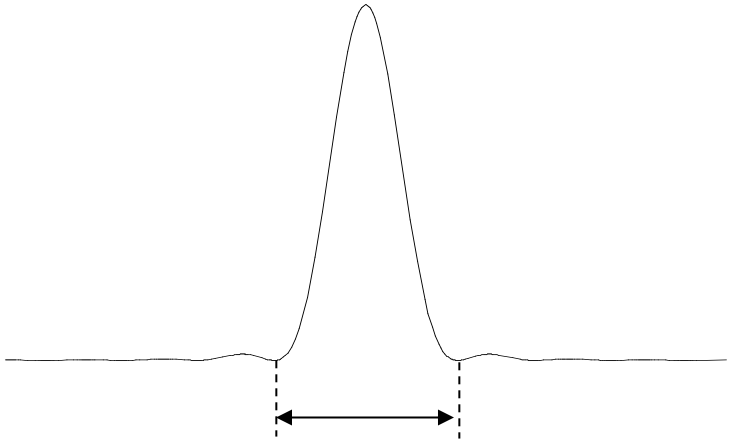
How big should your pinhole be?

Want pinhole to pass entire Airy disk



Y

X



Airy disk diameter
≈ 1.22 λ /NA

Width of point spread function at pinhole:
Airy disk diameter × magnification of lens

How big should your pinhole be?

- Width of point spread function at pinhole =
  Airy disk diameter × magnification of lens = 1 Airy unit
  = resolution of lens × magnification of lens × 2

  - 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 44 μm
  - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 19 μm
  - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 16 μm
  - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 14 μm

Pinhole size

- C1si: 30, 60, 100, 150 μm
- Spinning Disk: 50 μm

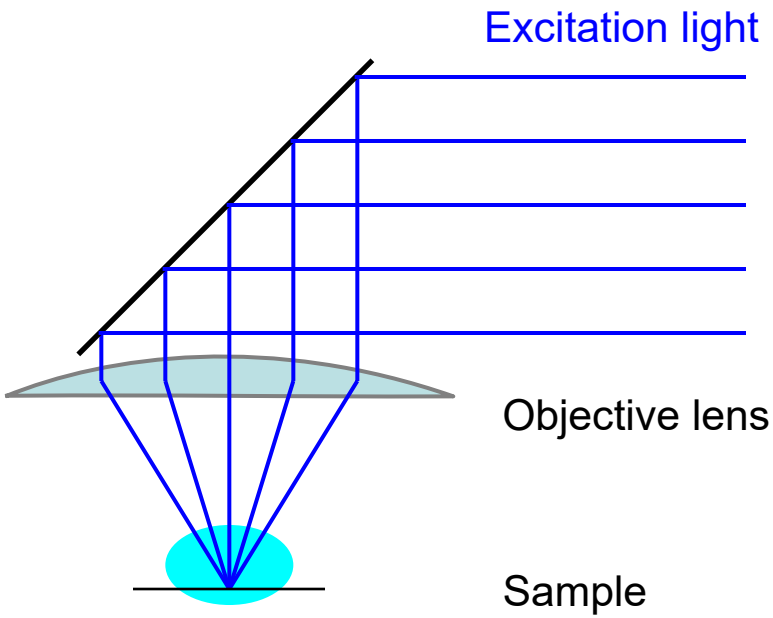
- All are substantially larger than Airy Disk for low magnification lenses.
  - On spinning disk, can use 1.5x magnification changer

Light sources

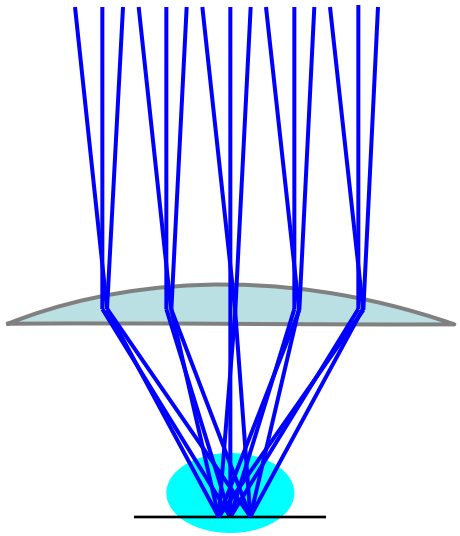
Excitation light must be focused to a diffraction limited spot

Could be done with an arc lamp and pinhole – but very inefficient

Enter the laser:
Perfectly collimated and high power



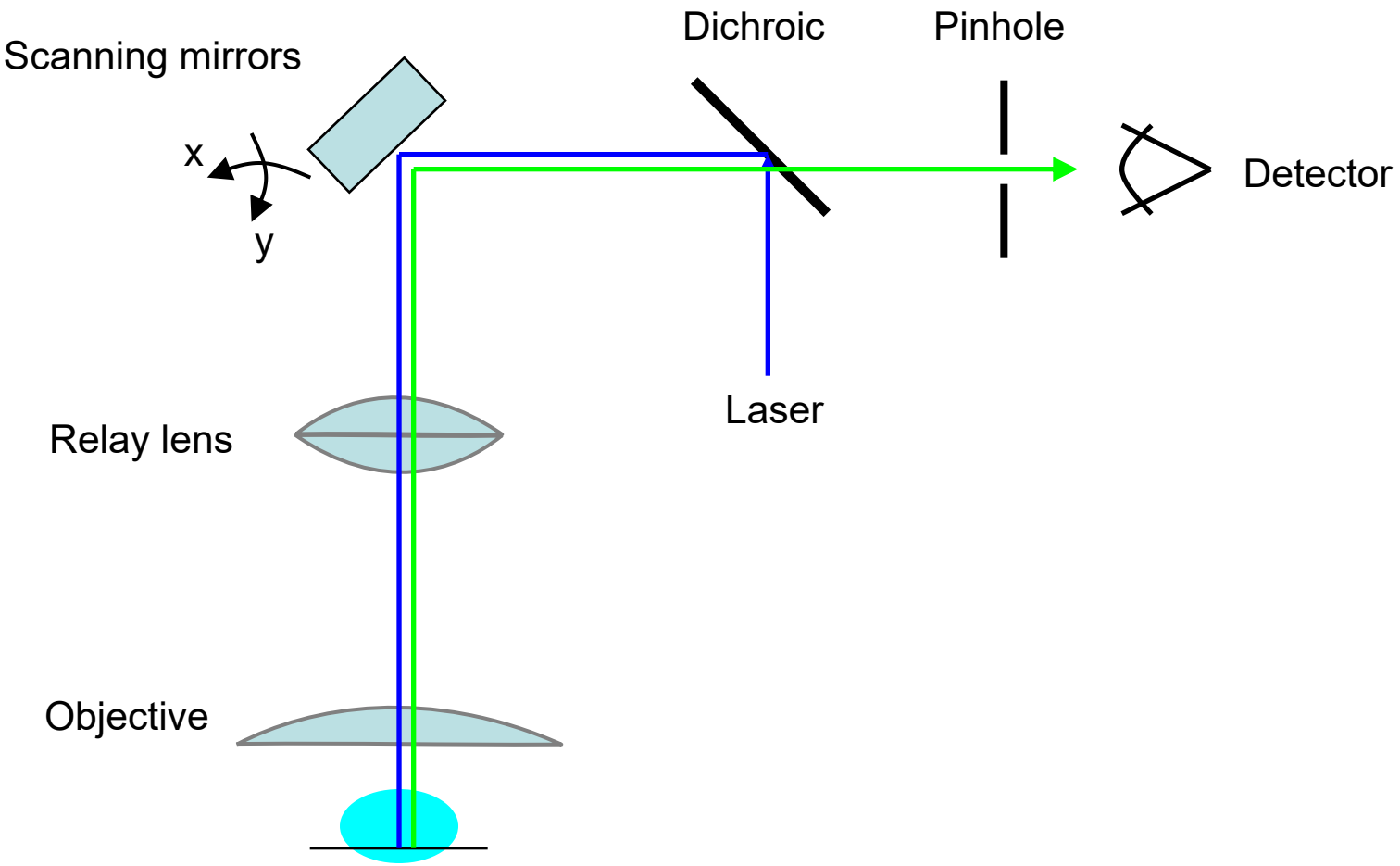
Scanning



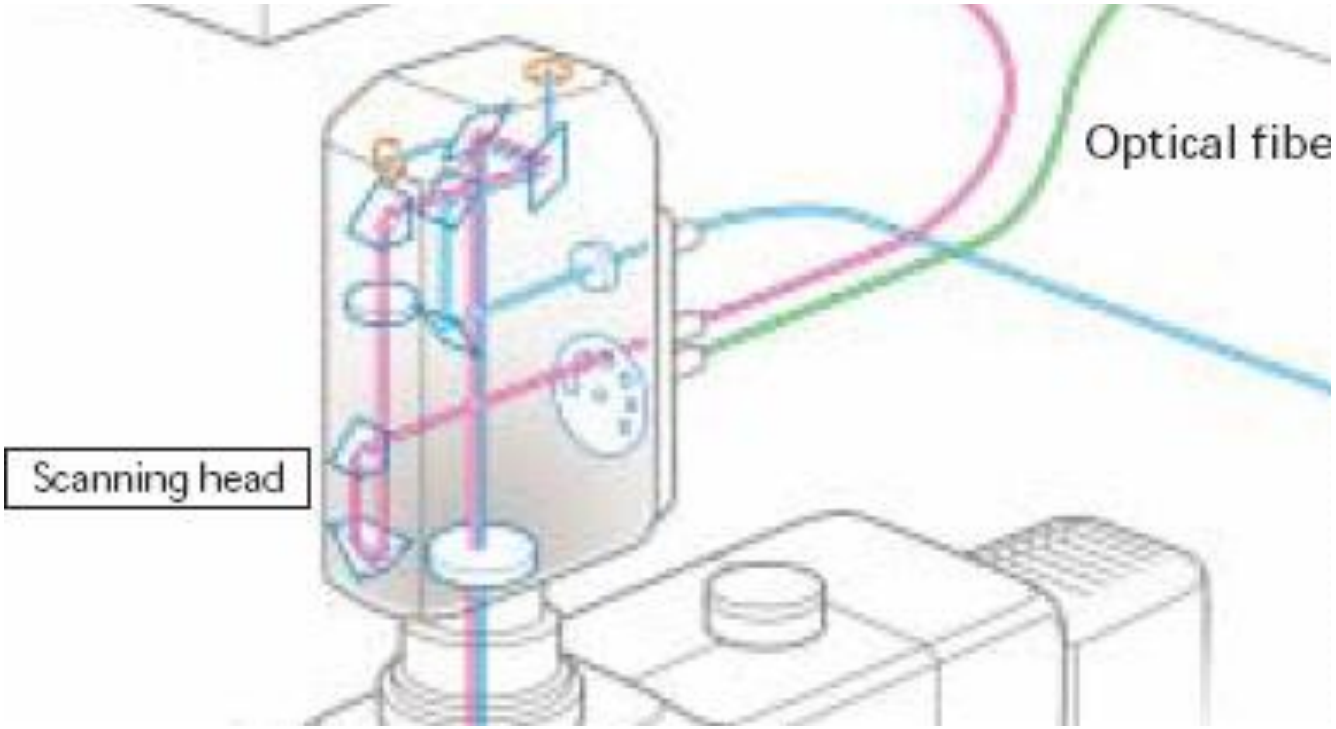
Changing entrance angle of illumination moves illumination spot on sample

The emission spot moves, so we have to make sure pinhole is coincident with it

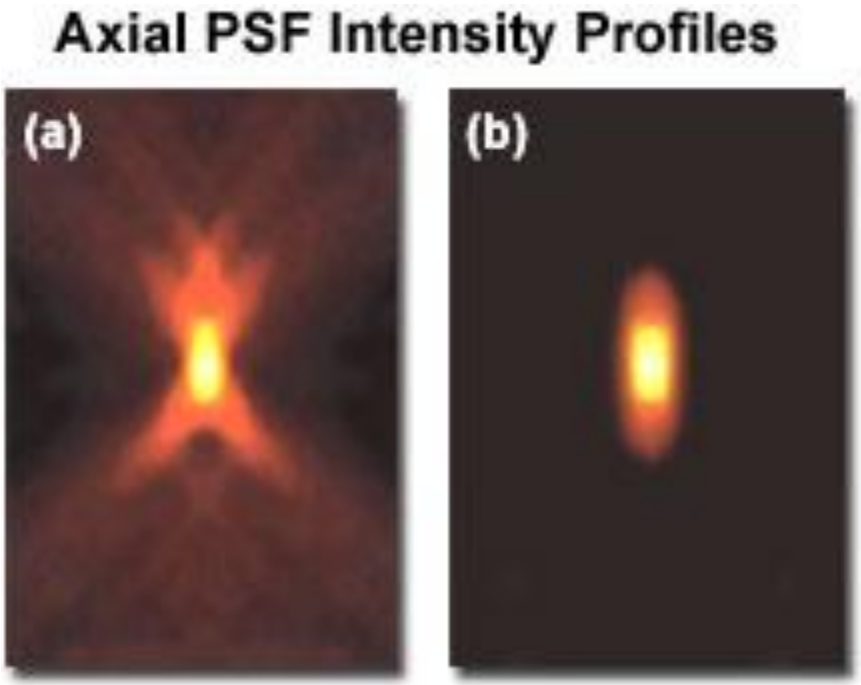
Confocal optical path



C1si internals

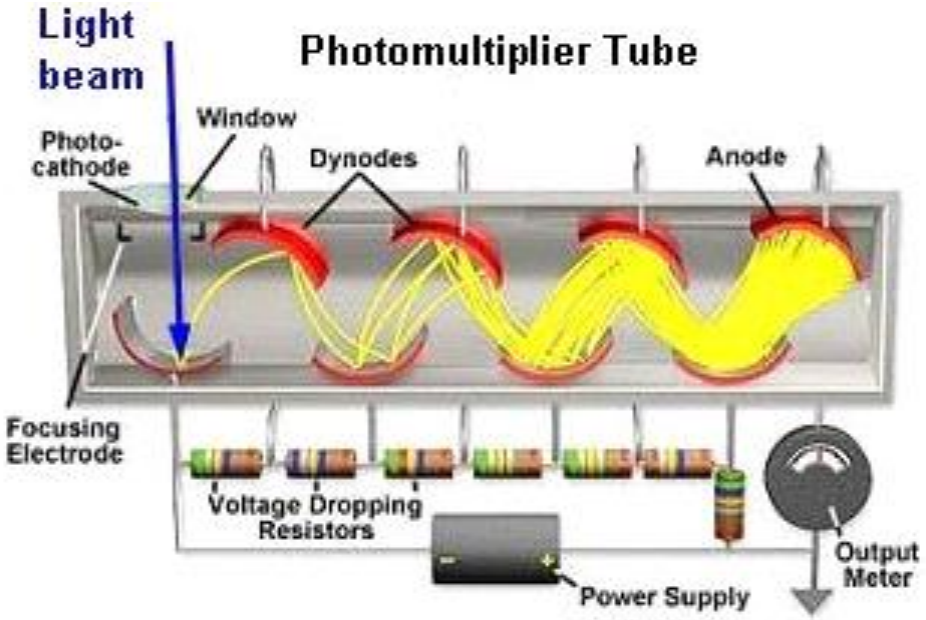


What do you get?



Detectors - PMTs

- Must be fast – confocal beam spends only a few μs on each pixel
  - Photomultiplier tubes



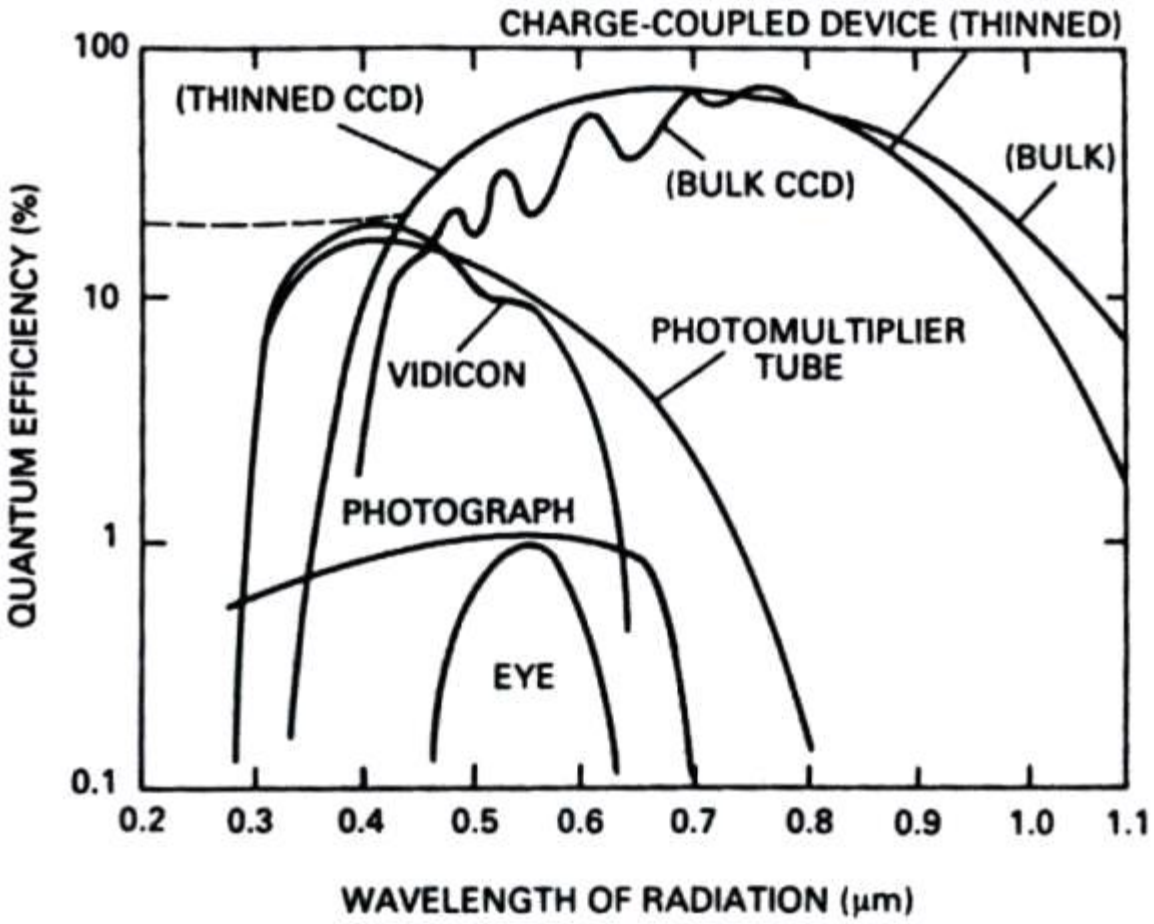
Pulse width for single photon
~ 10-100ns

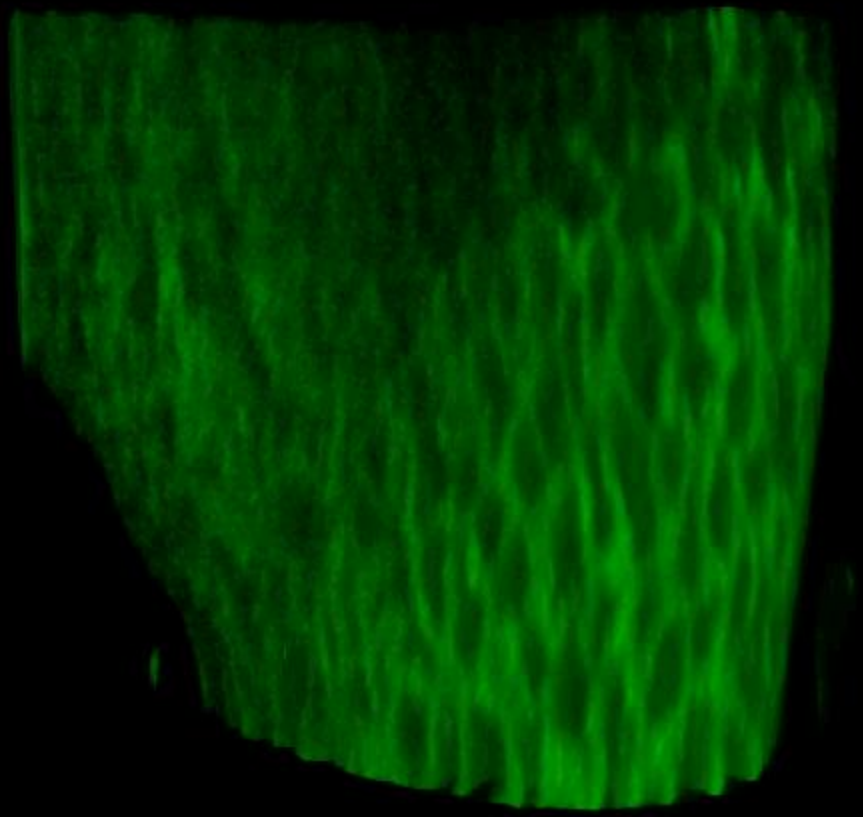
Very linear

Very high gain

~ 0 read noise

Detectors - PMTs



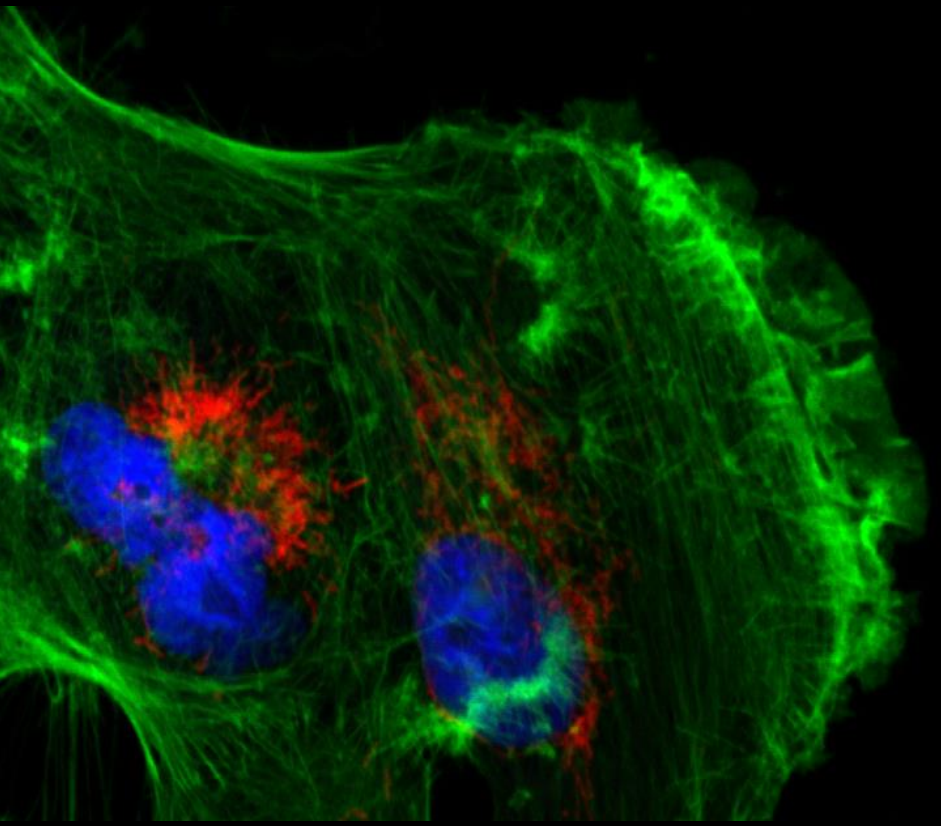


Laser-Scanning
Confocal

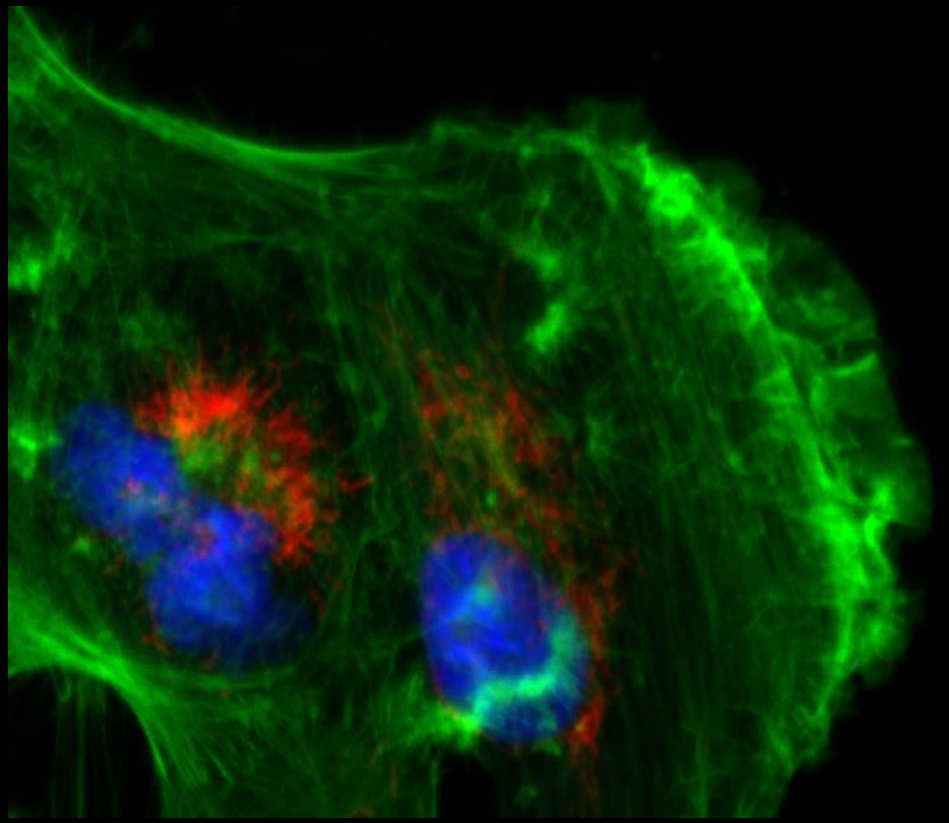
E11.5 mouse lung
stained for E-cadherin

Nan Tang, Martin lab

Confocal vs. Widefield



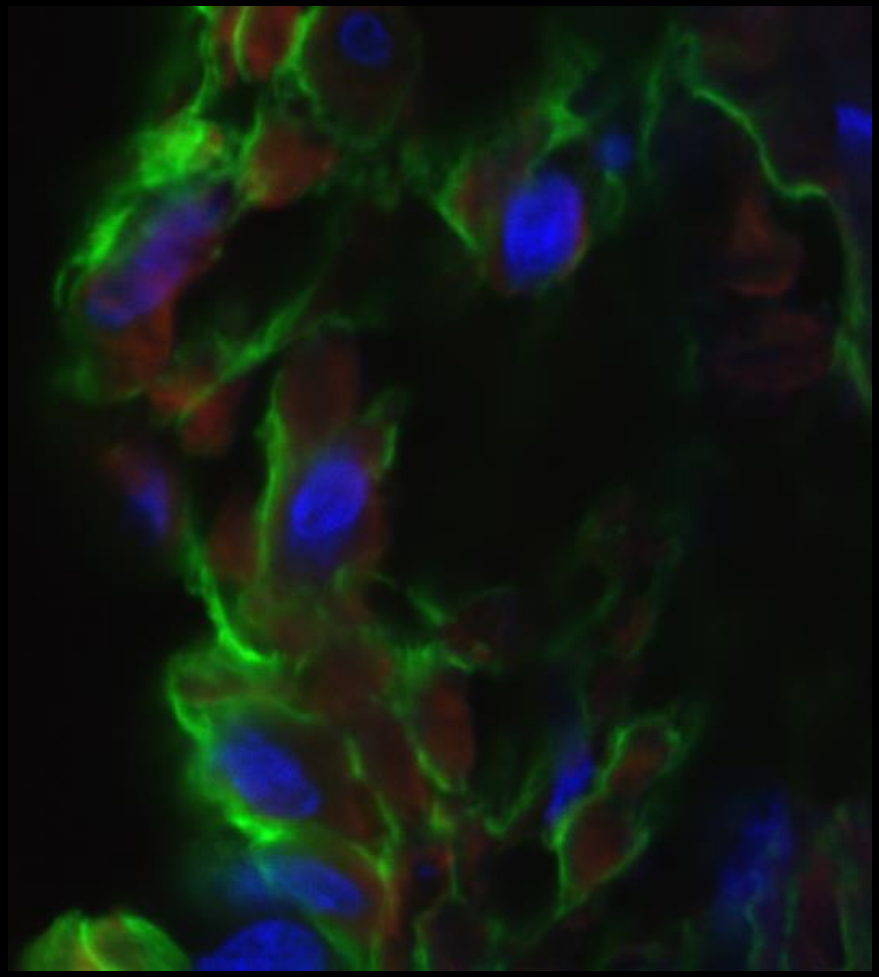
Confocal



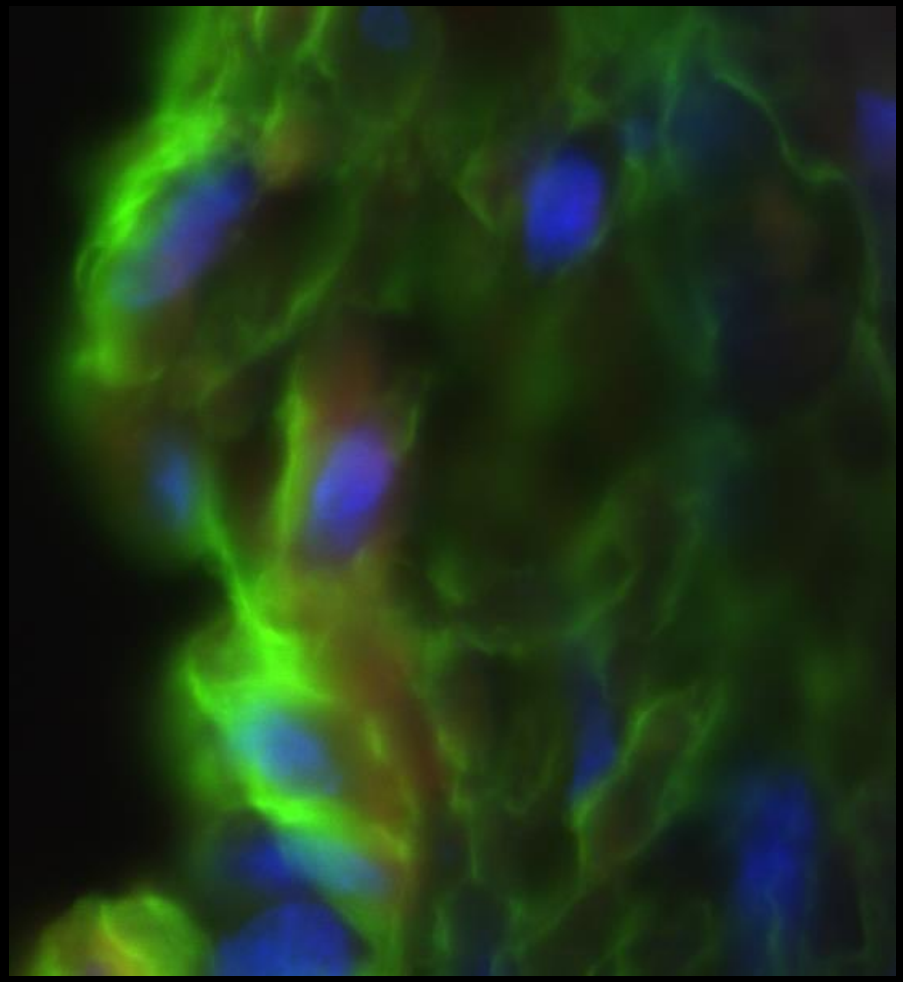
Widefield

Tissue culture cell with 60x / 1.4NA objective

Confocal vs. Widefield



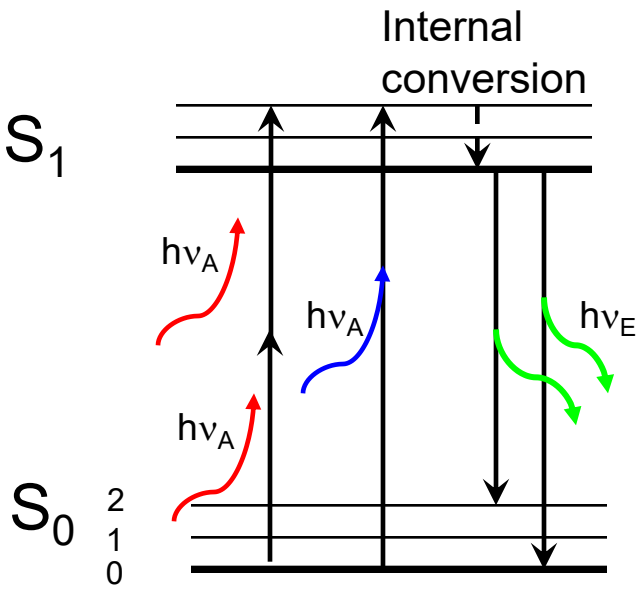
Confocal

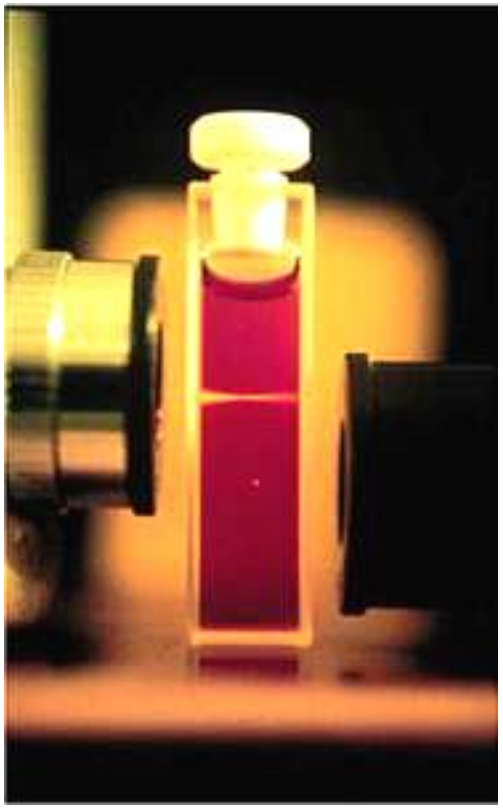


Widefield

20 μm rat intestine section recorded with 60x / 1.4NA objective

Multi-photon excitation

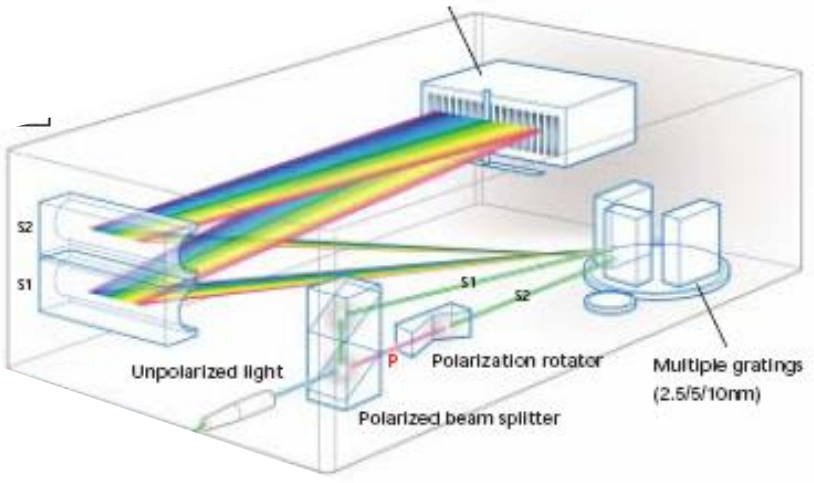




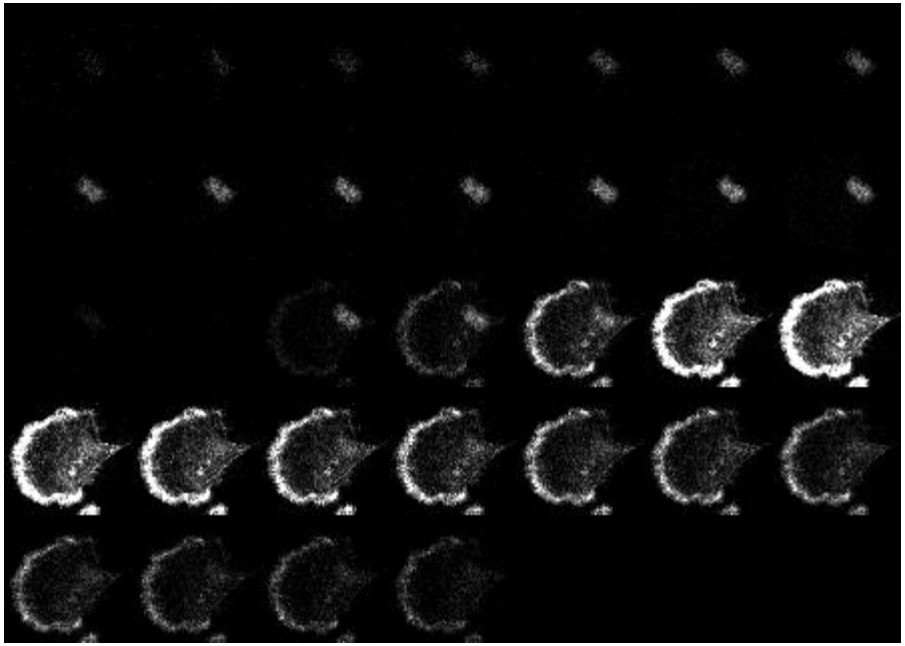
Brad Amos, MRC, Cambridge

Multi-photon excitation does not excite out-of-focus light, so you can get rid of pinhole

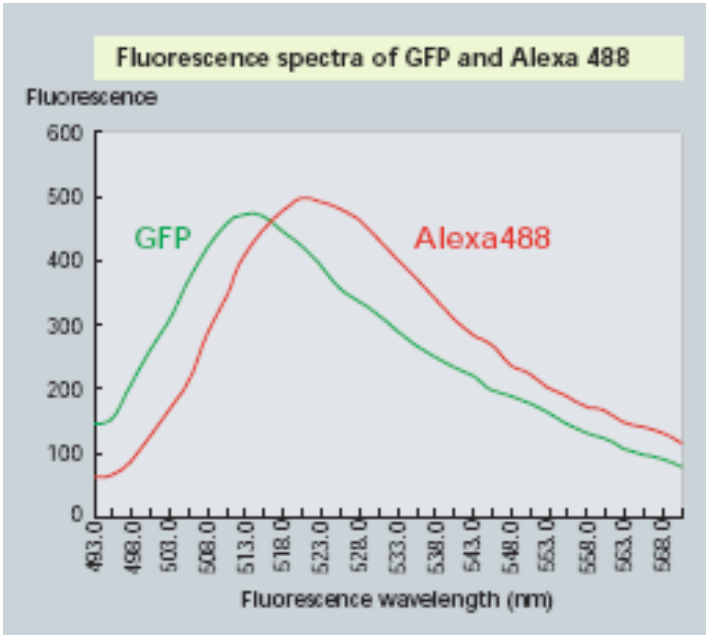
Spectral Detection

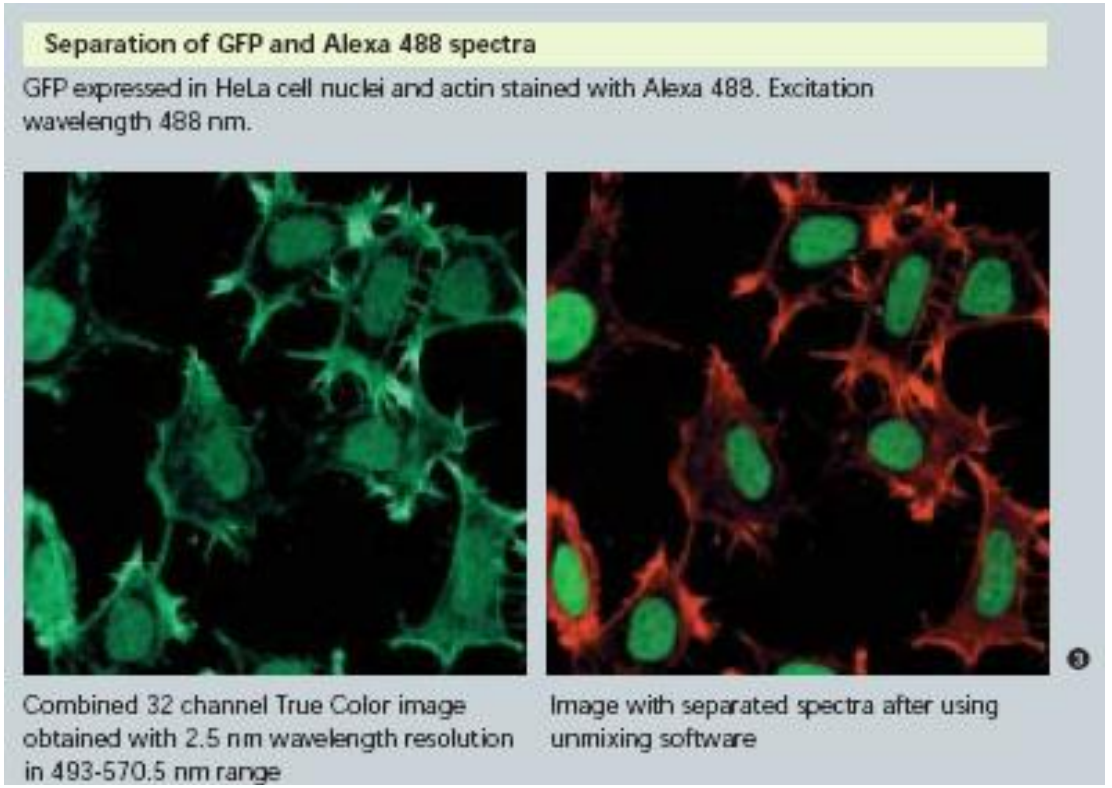


Montage of 32 channels of sample stained with DAPI and Alexa 488



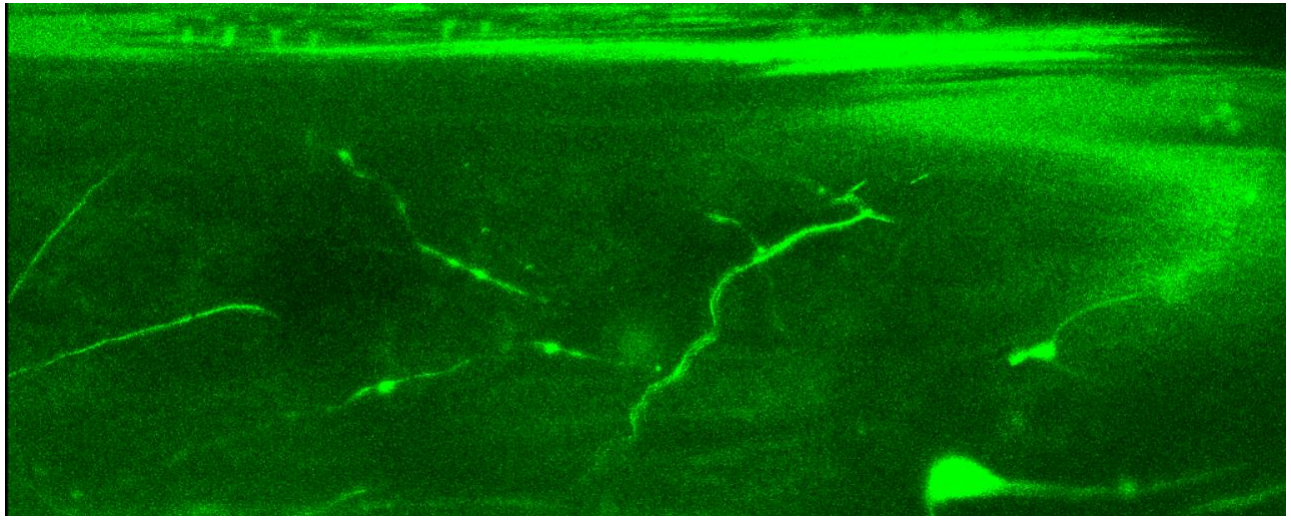
Spectral Confocal



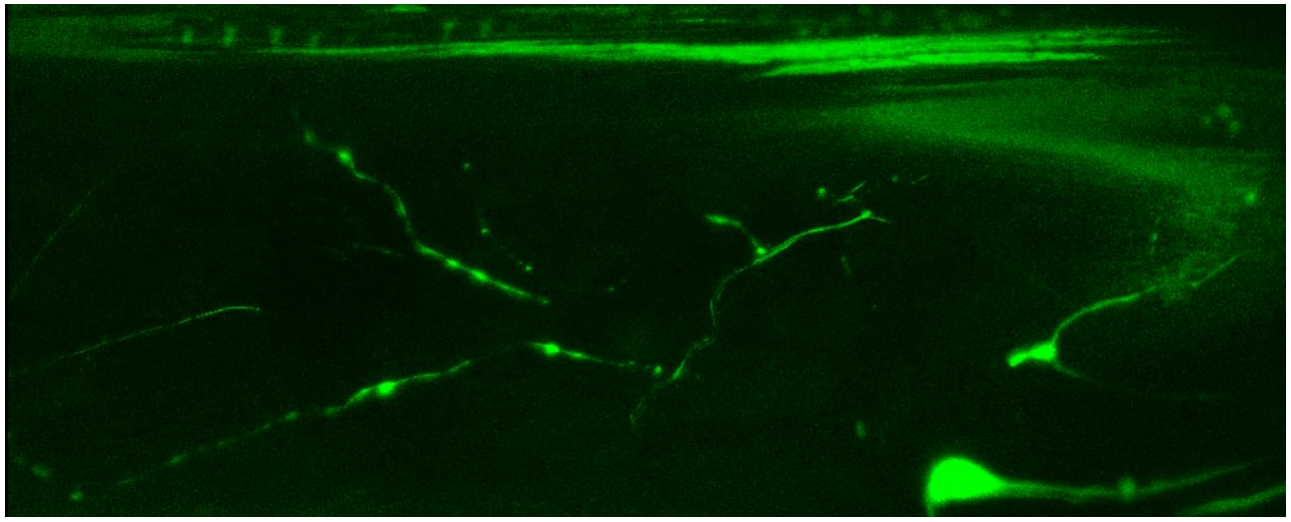


Autofluorescence removal

Drosophila embryo with neuronal GFP staining – Jan lab

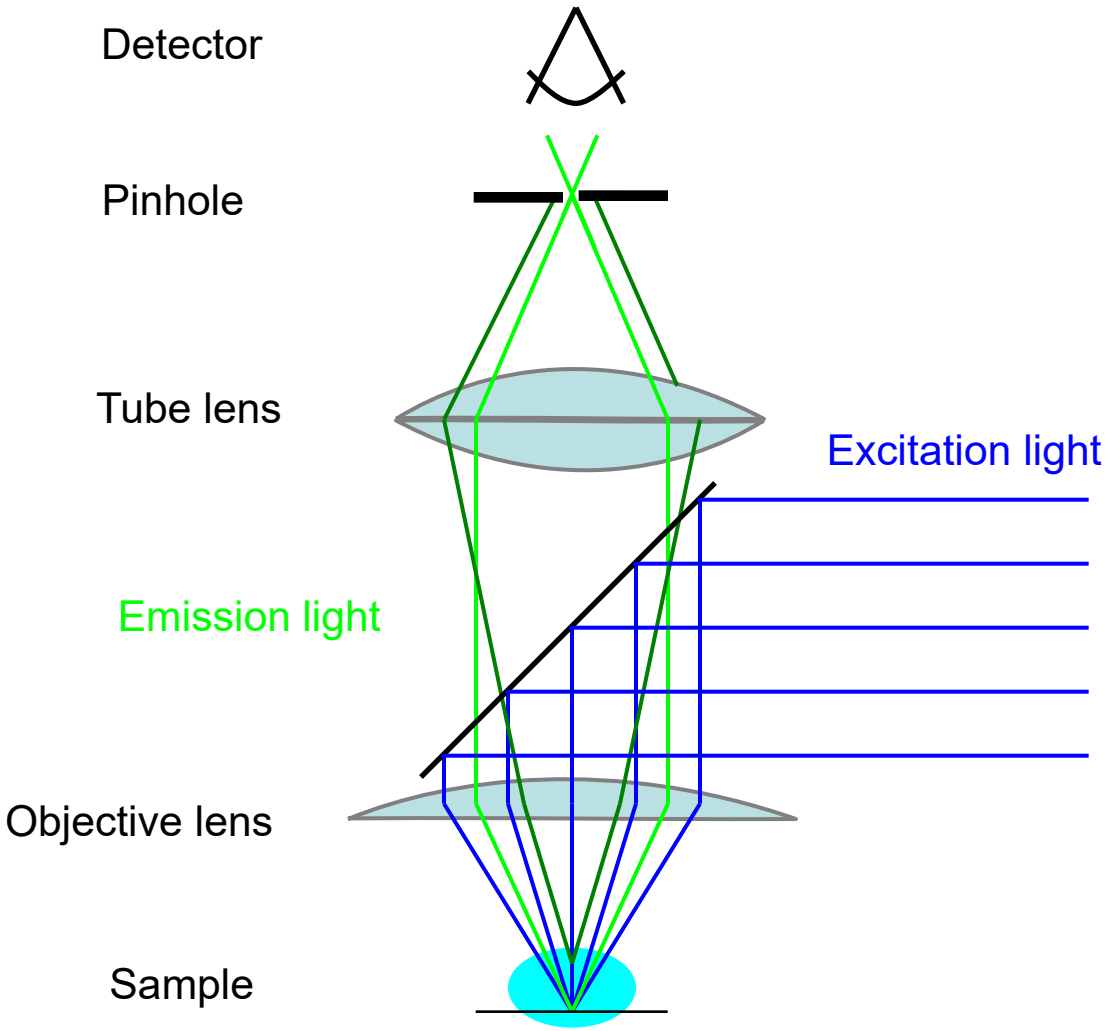


Best single channel image



After autofluorescence removal by linear unmixing

The confocal microscope

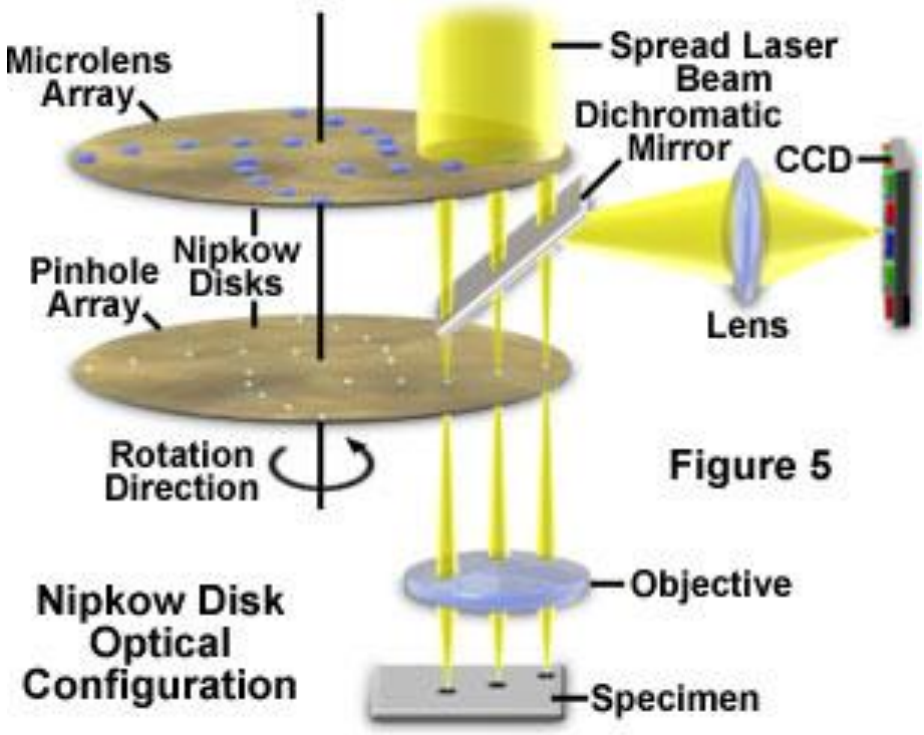


Scan excitation spot point-by-point to build up image

Problems:
Slow (~1 sec to acquire an image)
Low light efficiency (due to use of PMT as detector)

Solution:
Use multiple pinholes and a camera

A Solution: Spinning Disk Confocal



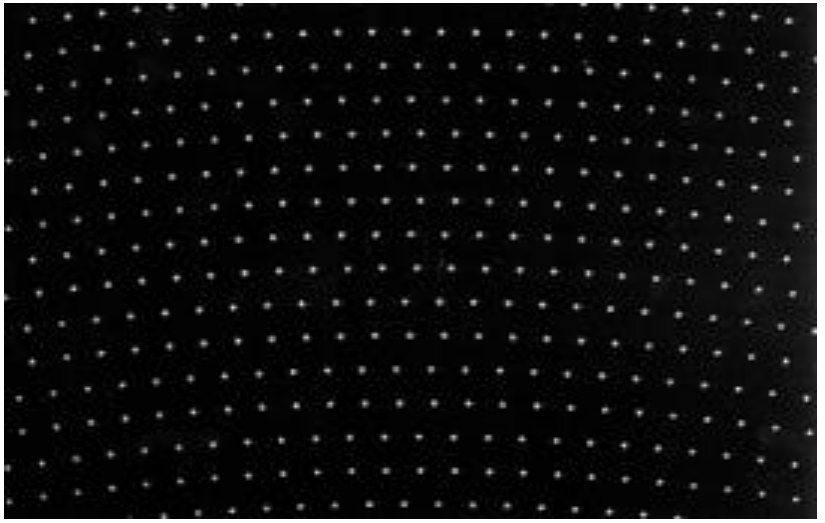


Image with many pinholes at once, so fast

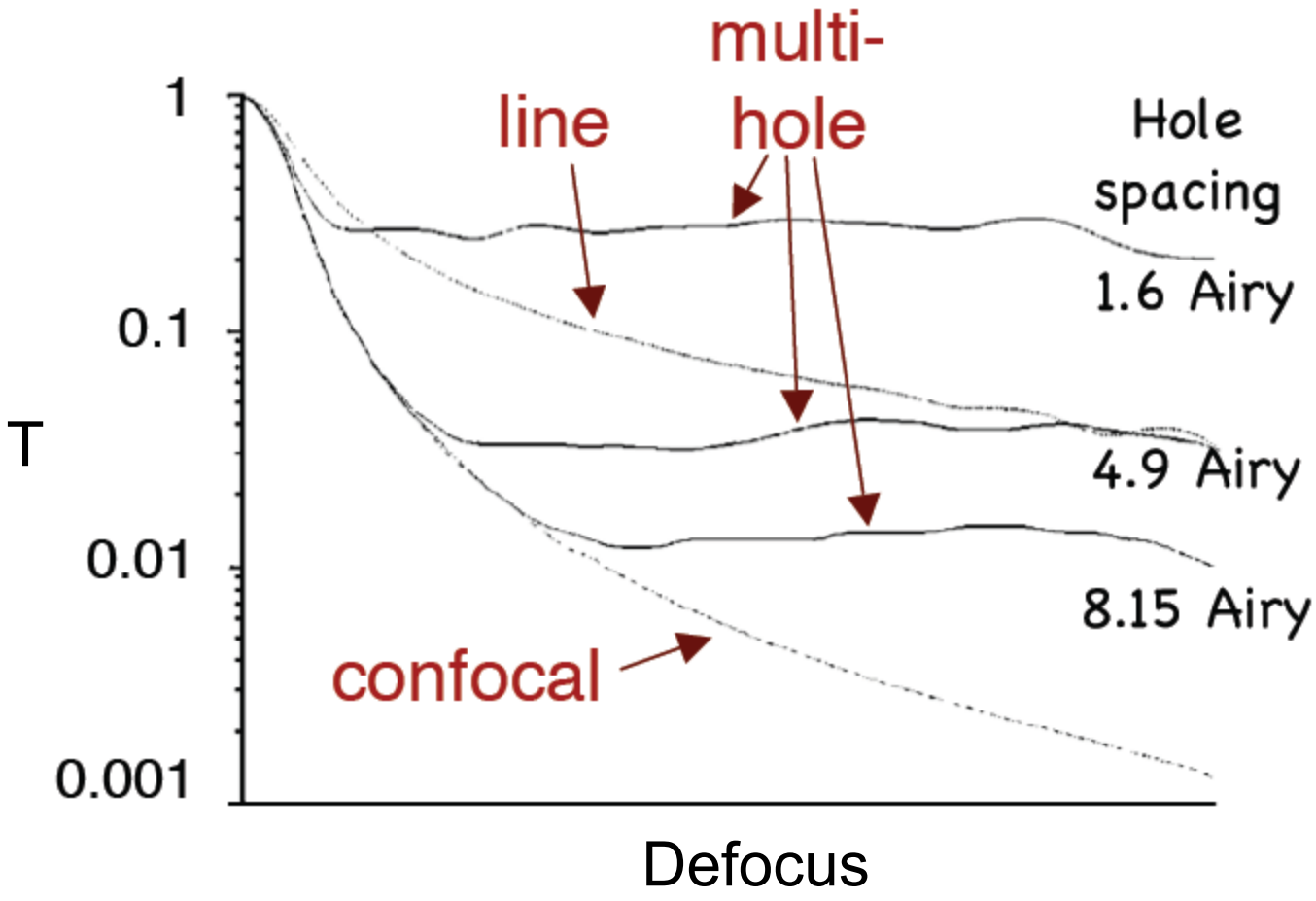
Use CCD as detector, so much higher QE

Pros/Cons of spinning disk

- Fast – multiple points are illuminated at once
- Photon efficient – high QE of CCD
- Gentler on live samples – usually lower laser power

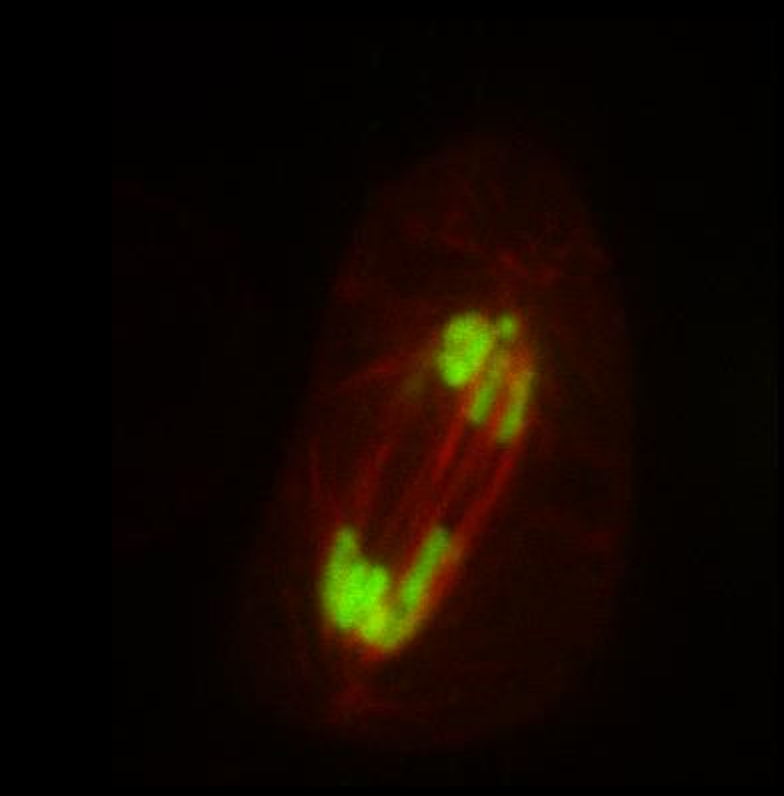
- Fixed pinhole – except in swept-field
- Small field of view (usually)
- Crosstalk through adjacent pinholes limits sample thickness

Out-of-focus rejection



Wolleschensky et al. J. Biomed. Opt. 11(6) 2006

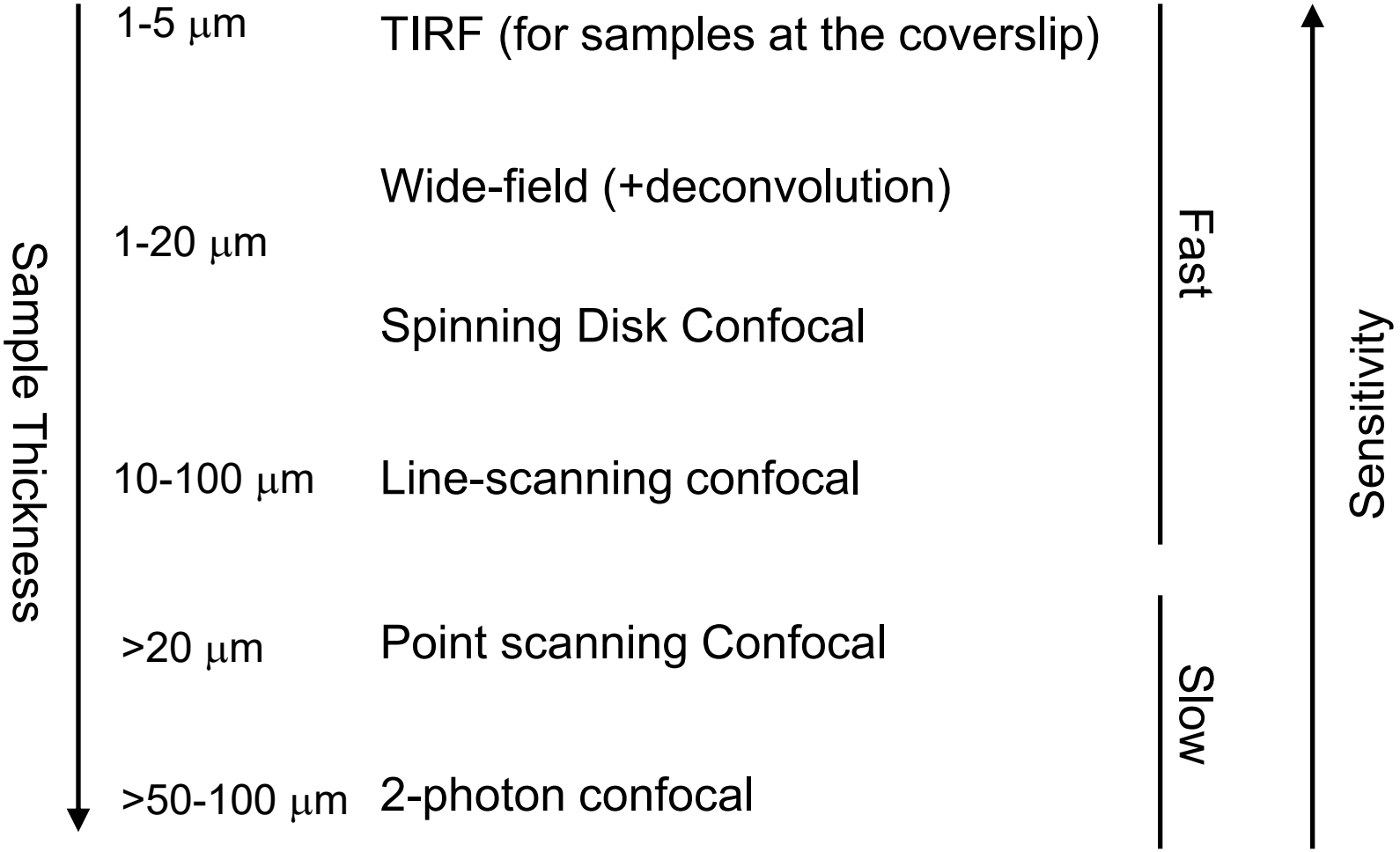
Examples



Drosophila S2 cell expressing
GFP-H2B and mCherry-tubulin
(Nico Stuurman and Ron Vale)

S. cerevisiae expressing a
mitochondrially targeted RFP,
Susanne Rafelski, Marshall lab

Which imaging technique should I use?



When to use confocal?

- Confocal is not a magic bullet
  - It is extremely wasteful of photons
  - Laser-scanning confocal is 100 – 200-fold less sensitive than widefield
  - Spinning-disk confocal is ~4-fold less sensitive than widefield

When to use confocal?

- Confocal is not a magic bullet
  - It is extremely wasteful of photons
  - High laser power generally result in more photobleaching and photodamage.
- For thin specimens, widefield epifluorescence is better – especially with deconvolution
- Confocal excels with thick, heavily stained specimens

When to use confocal?

- What is thick?
  - A good rule of thumb is 10 × the depth of field of the objective
  - 100x / 1.4 NA: d.o.f. 0.66μm
  - 20x / 0.75 NA: d.o.f 2.3 μm
- Sample preparation is KEY for imaging thick specimens
  - Confocal does not fix scattering, refractive index mismatch, or everything else that can go wrong – it only removes out of focus light

Sample preparation

- For fixed samples: match refractive index of mounting media to immersion oil.
  - Mount in immersion oil itself, BABB, benzyl alcohol/glycerol, 2,2'-thiodiethanol, or other high-RI mounting medium
- Clearing to remove lipids and other scattering substances is also important

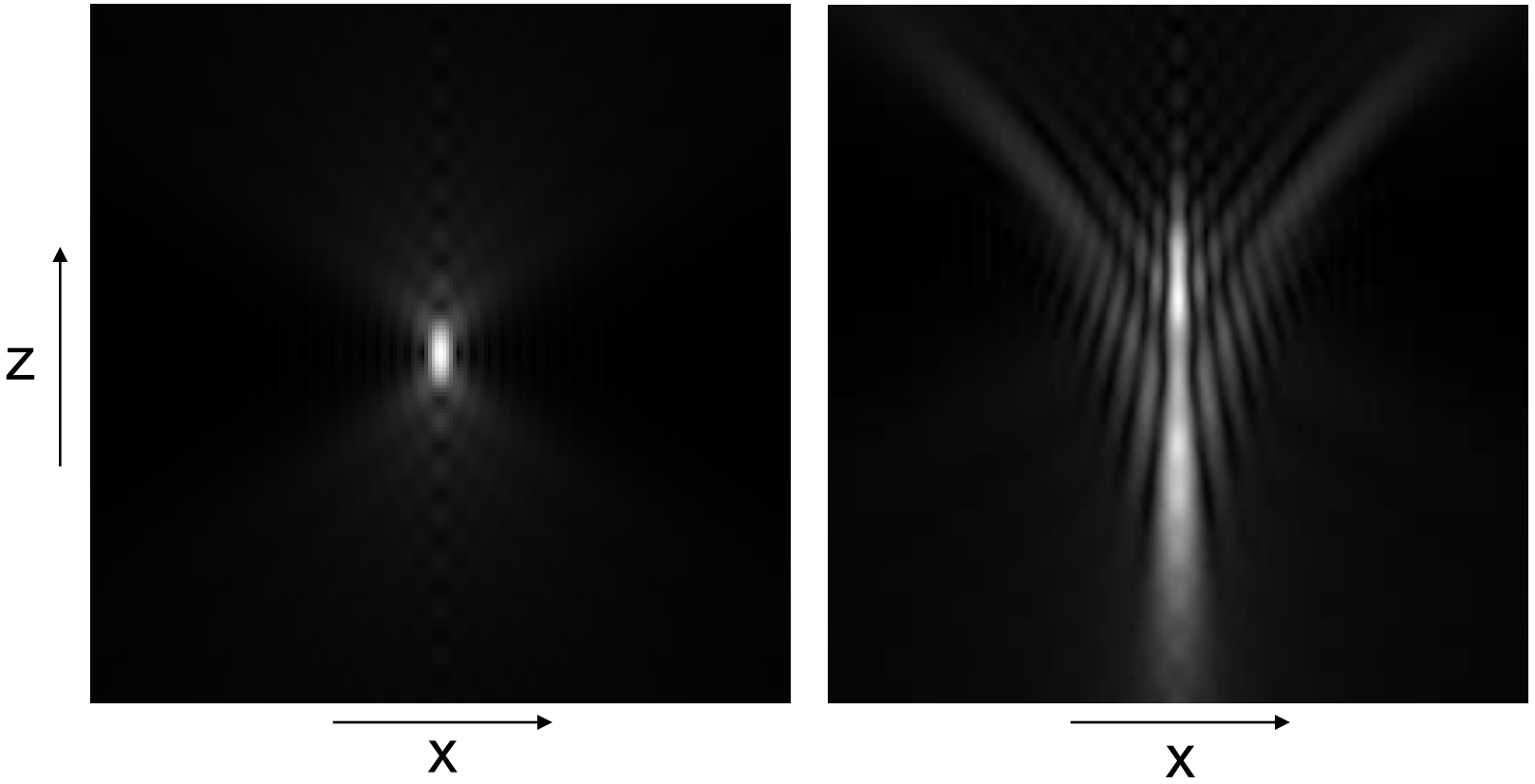
- For live samples, use water immersion lenses

Spherical Aberration

Point spread functions

Ideal

1 wave of spherical ab



Sources of Spherical Aberration

Design compromises

Manufacturing tolerances

Immersion fluid index error

Temperature variation

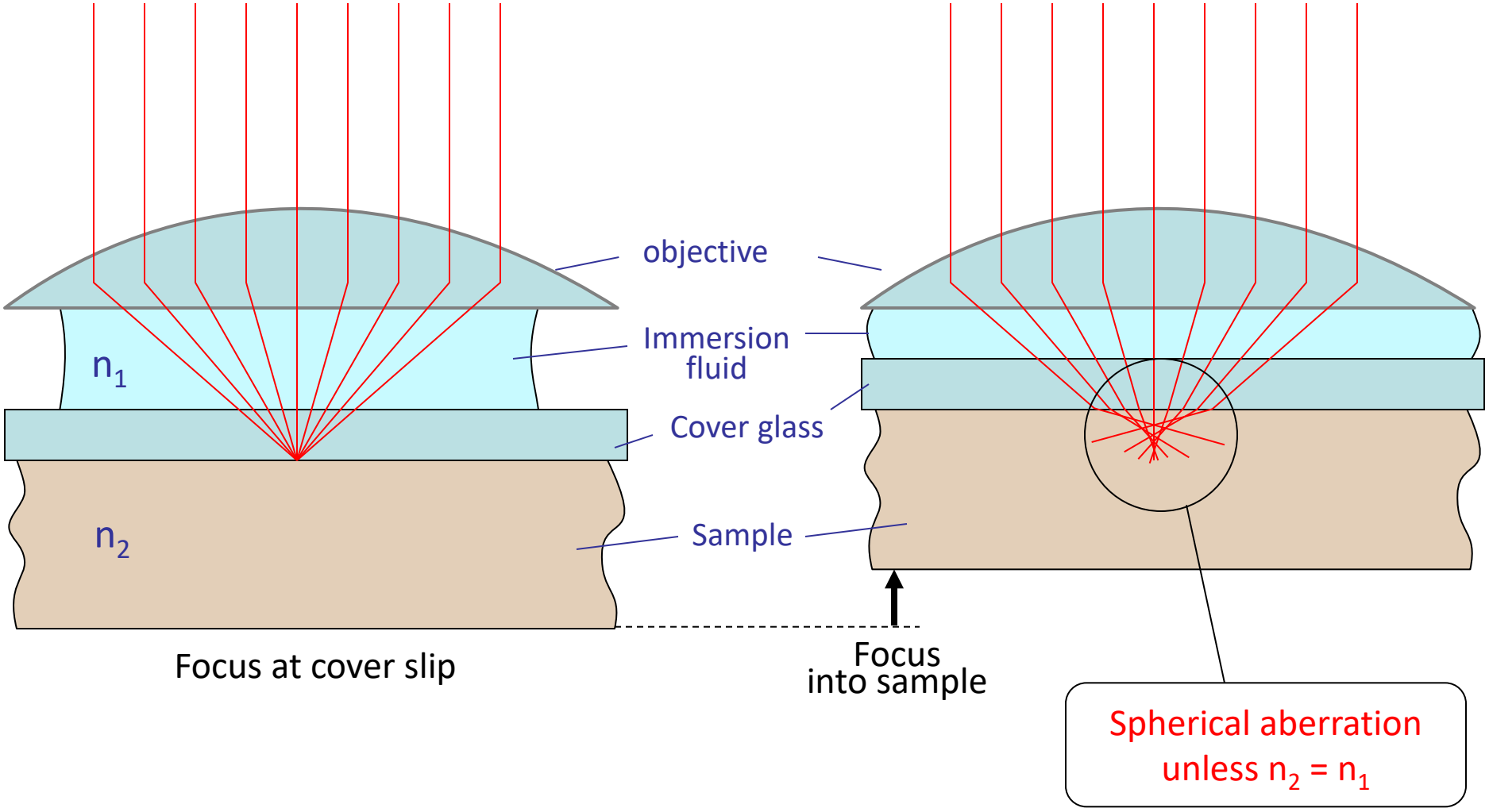
Cover slip thickness

(high-NA objectives except oil immersion)

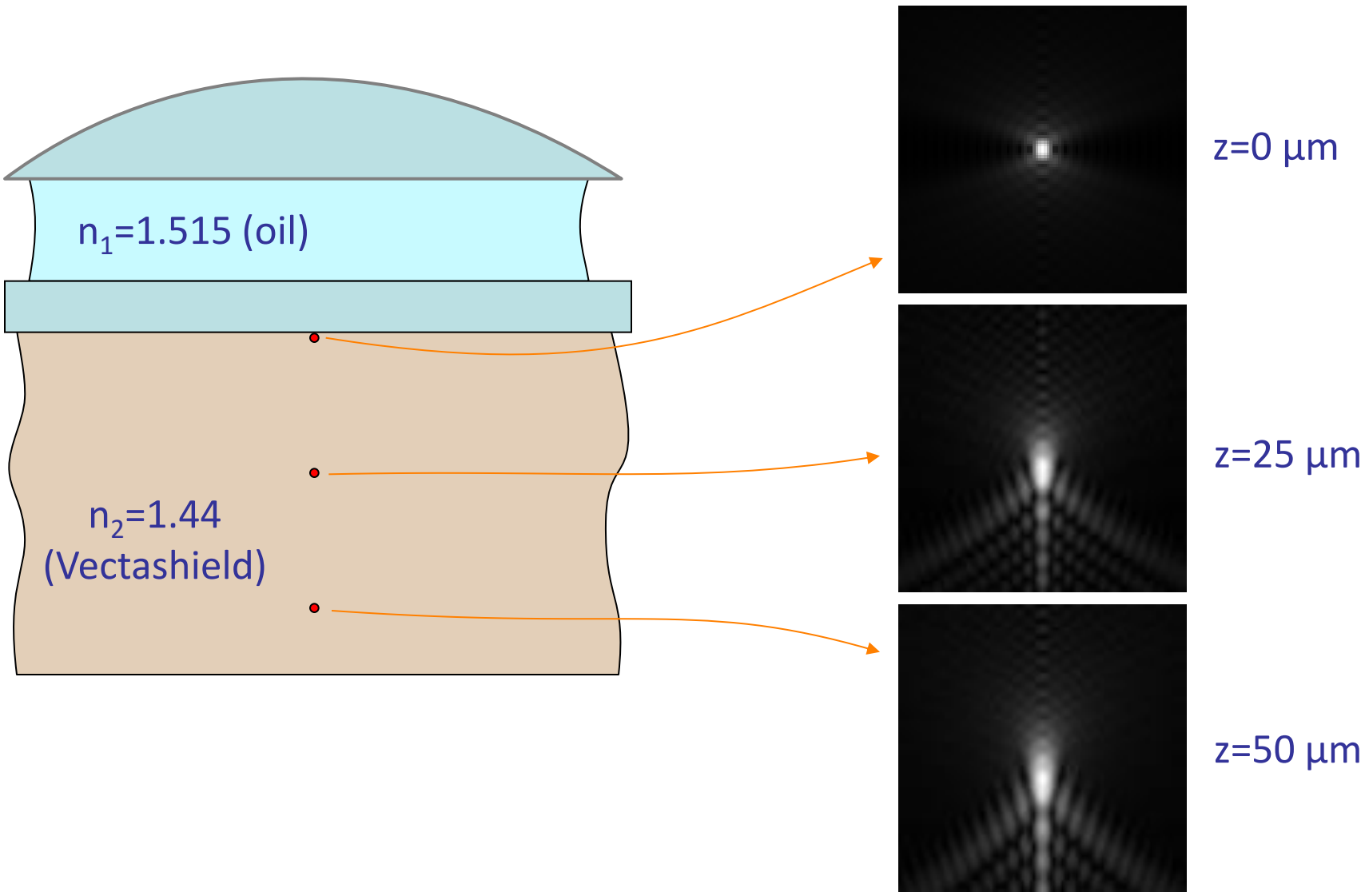
Correction collar setting

Sample refractive index mismatch

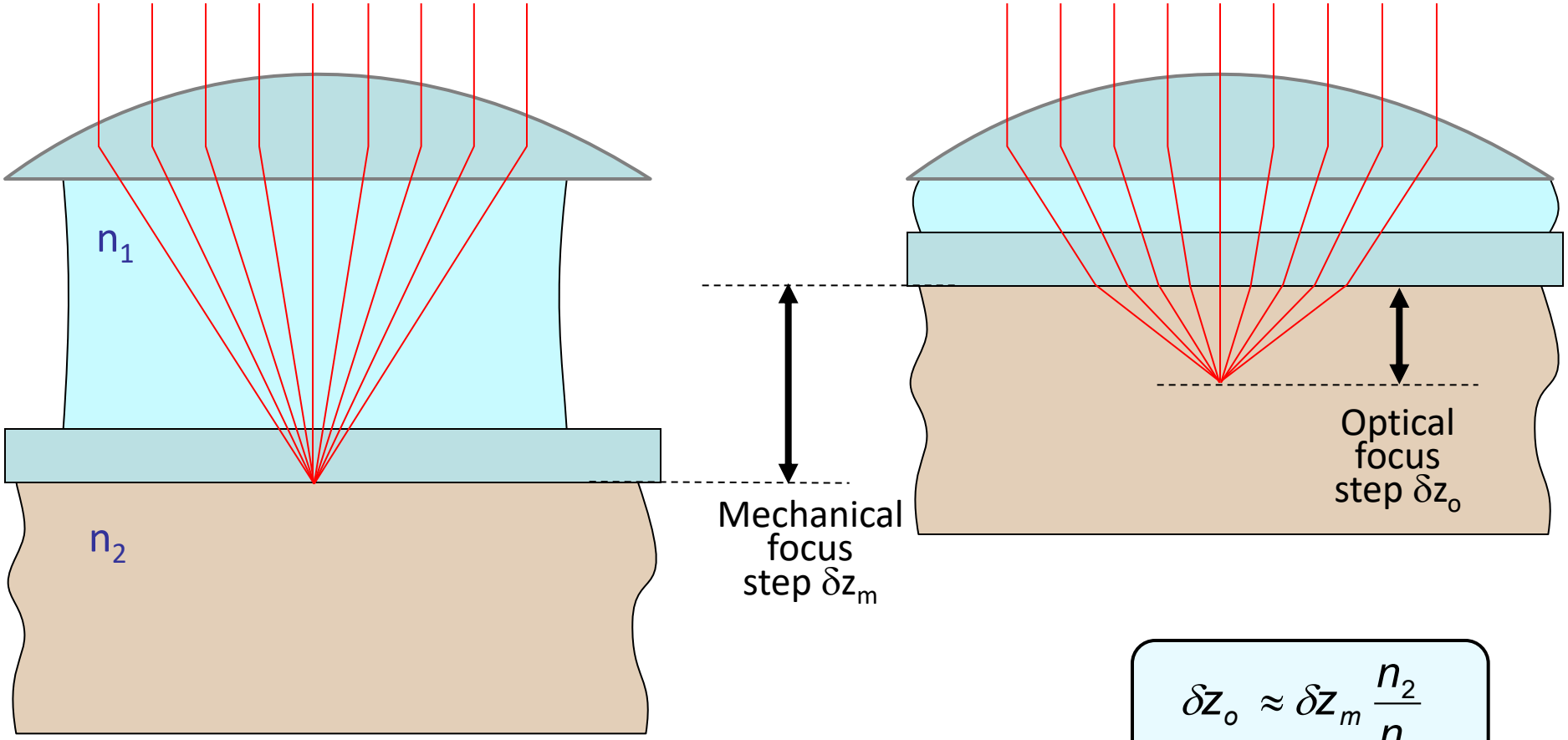
Index Mismatch
& Spherical Aberration



Index Mismatch
& Spherical Aberration



Index Mismatch & Axial Scaling



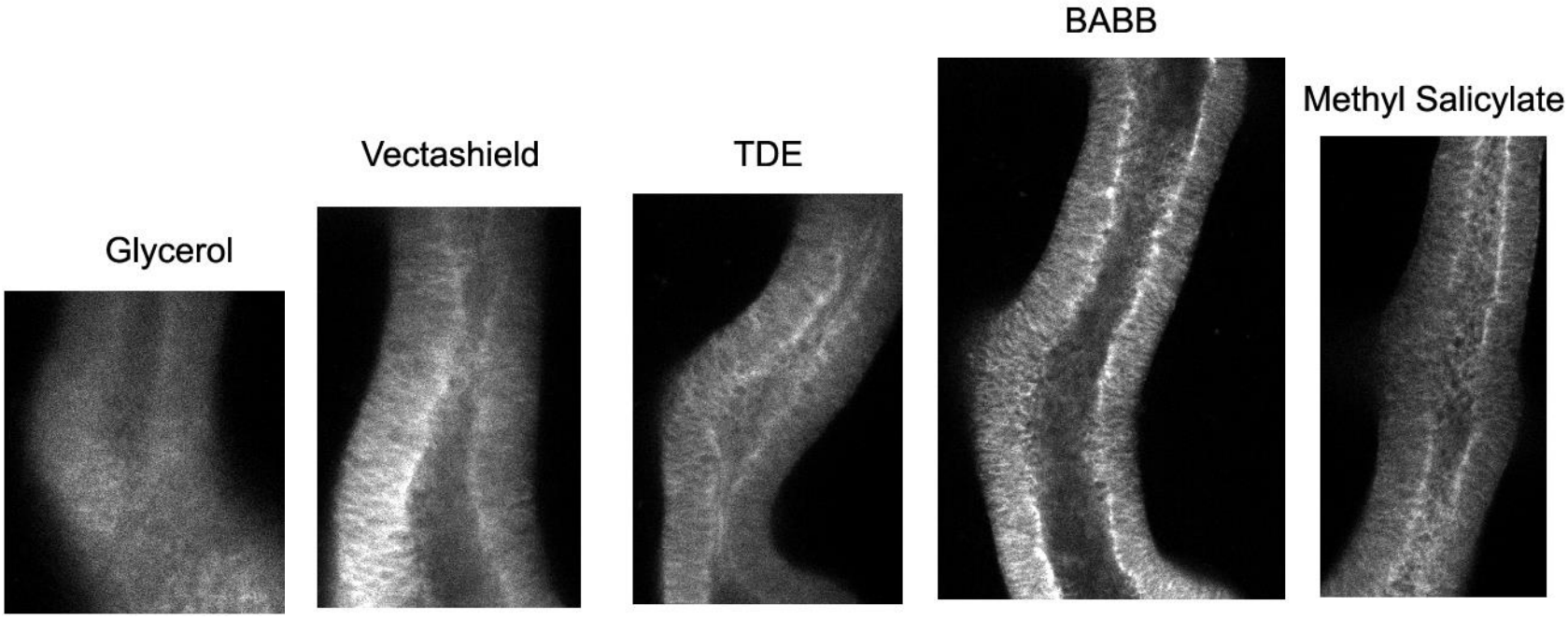
$$\delta z_o \approx \delta z_m \frac{n_2}{n_1}$$

If there is index mismatch,
your z pixel size is not what you think

Getting Rid of Spherical Aberration

- Use correct and consistent cover slips
- Pick a room with stable temperature ≈ 20°C
- Adjust correction collar
- If no collar, adjust immersion medium index
- **Use an objective that is matched to the mounting medium:**
  - For aqueous samples, use a water immersion objective
  - For fixed samples viewed with oil immersion objectives, ideally use a mounting medium with index ≈ 1.515
  - For fixed samples in commercial media, ideally use a glycerol immersion objective

Sample Preparation

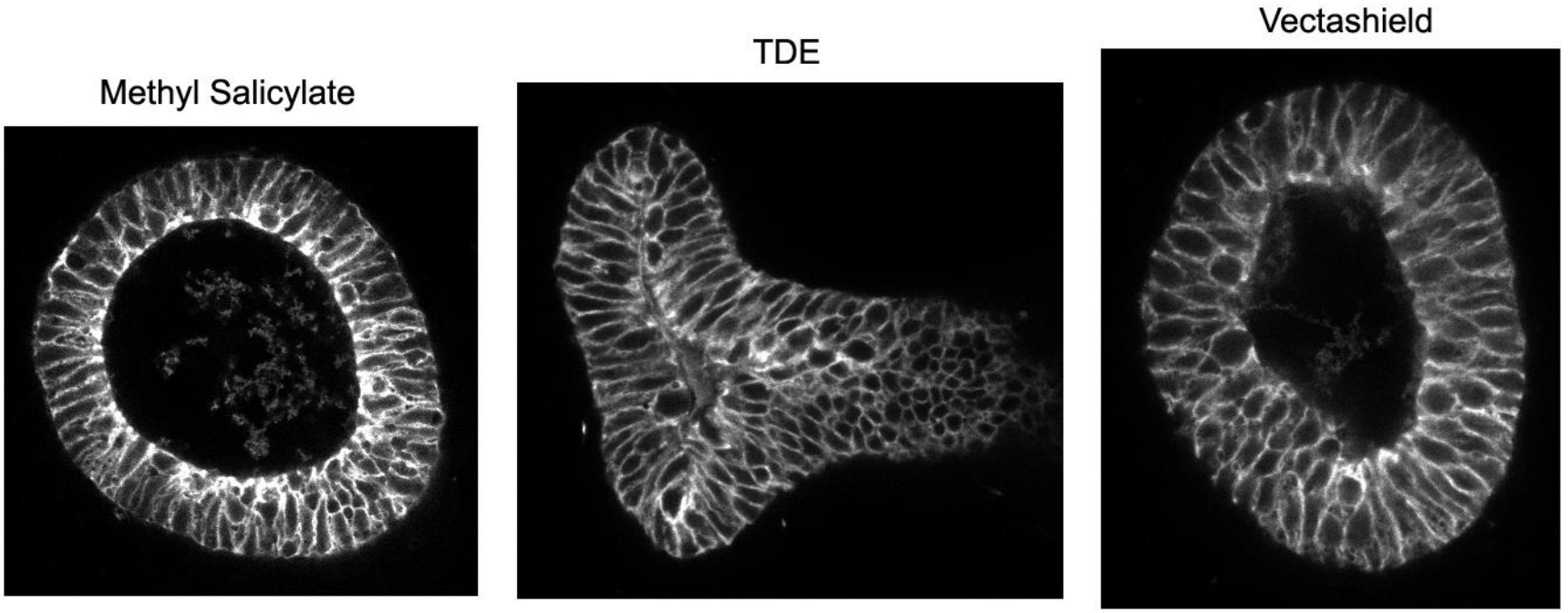


Samples imaged with 20x / 0.75 air objective on spectral confocal

Sections acquired ~ 50 μm into tissue

Embryonic mouse lungs; samples from Nan Tang, Martin Lab

Sample Preparation



Samples imaged with 40x / 1.3 oil objective on spectral confocal

Sections acquired ~ 50 μm into tissue

Embryonic mouse lungs; samples from Nan Tang, Martin Lab

Clearing and mounting summary

- Both clearing and refractive index matching are important.
- BABB and methyl salicylate clear very well and give best image depth, but may disrupt cell morphology
- TDE preserves cell morphology and also allows for decent imaging depth

Slides can be downloaded from:

http://nic.ucsf.edu/dokuwiki/doku.php?id=presentations

Resources

http://www.microscopyu.com

http://micro.magnet.fsu.edu

James Pawley, Ed. “Handbook of Biological Confocal Microscopy, 3rd ed.”

Murray JM et al., J. Microsc. 2007, 228: p.390-405

Acknowledgements

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