Confocal Microscopy

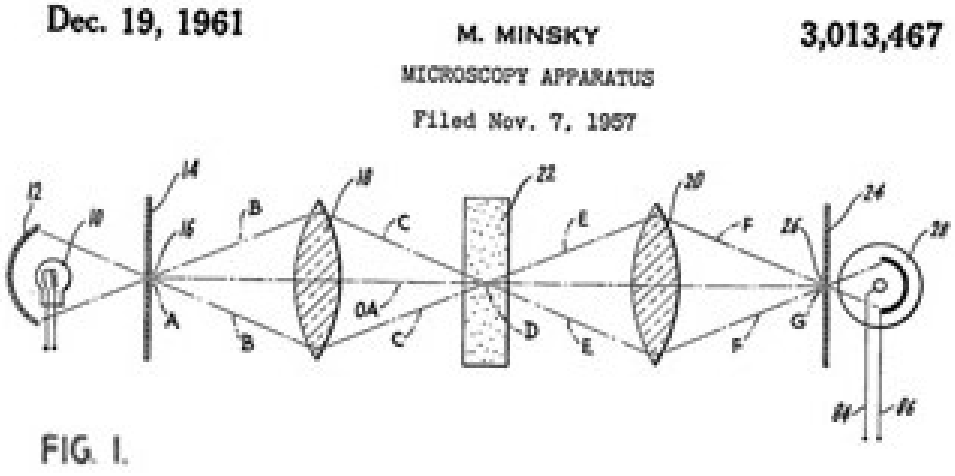
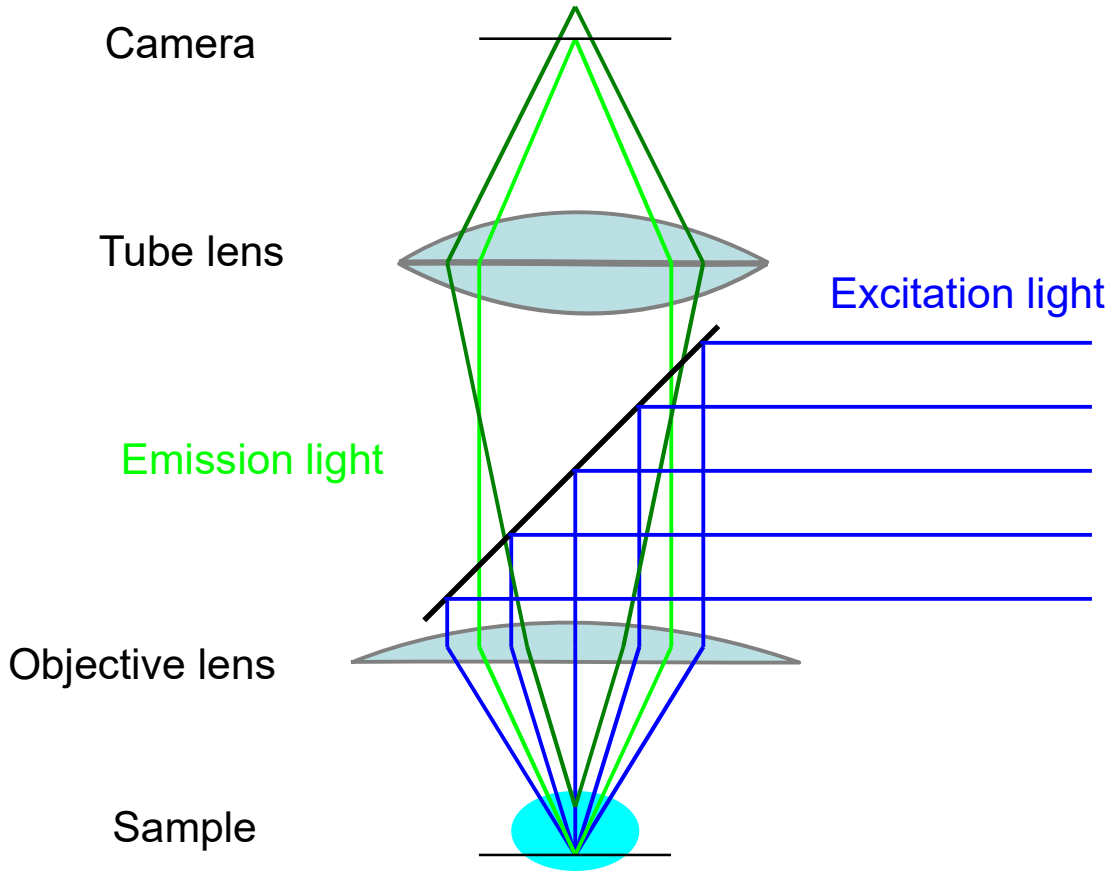


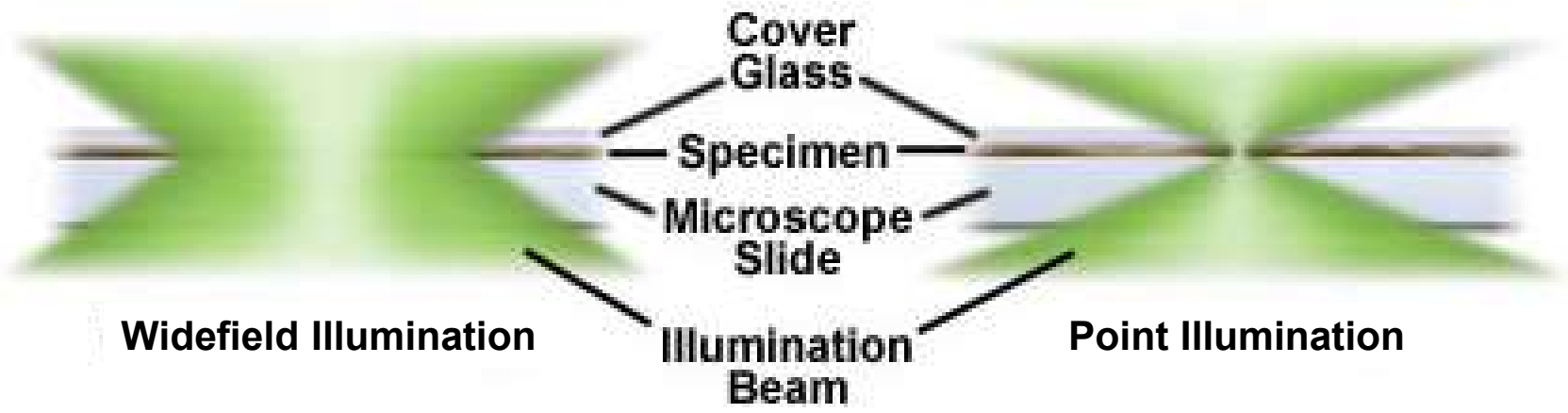
FIG. 1.

Fluorescence Illumination of a single point



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

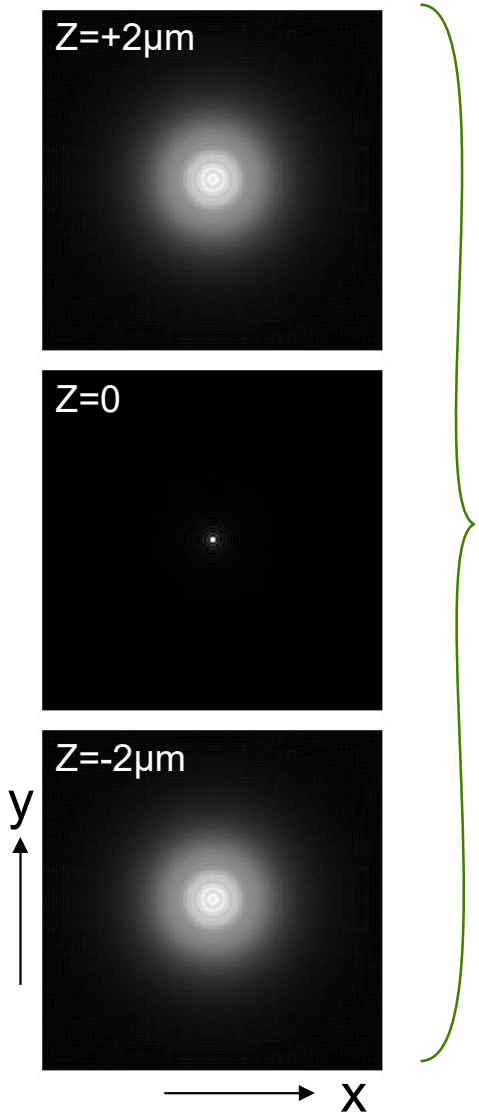
Widefield fluorescence imaging



The Point Spread Function (PSF)

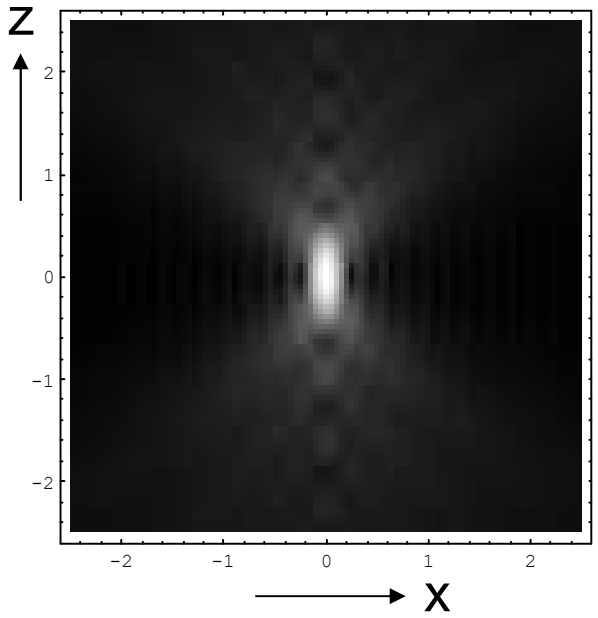
The image of a point object

2D PSF
for different defocus

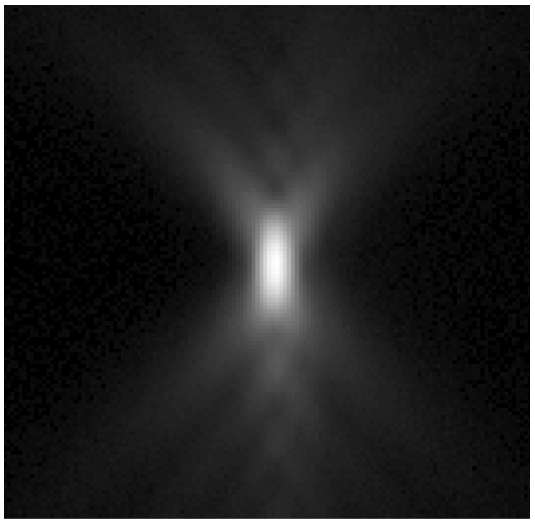


3D PSF

Calculated



Measured



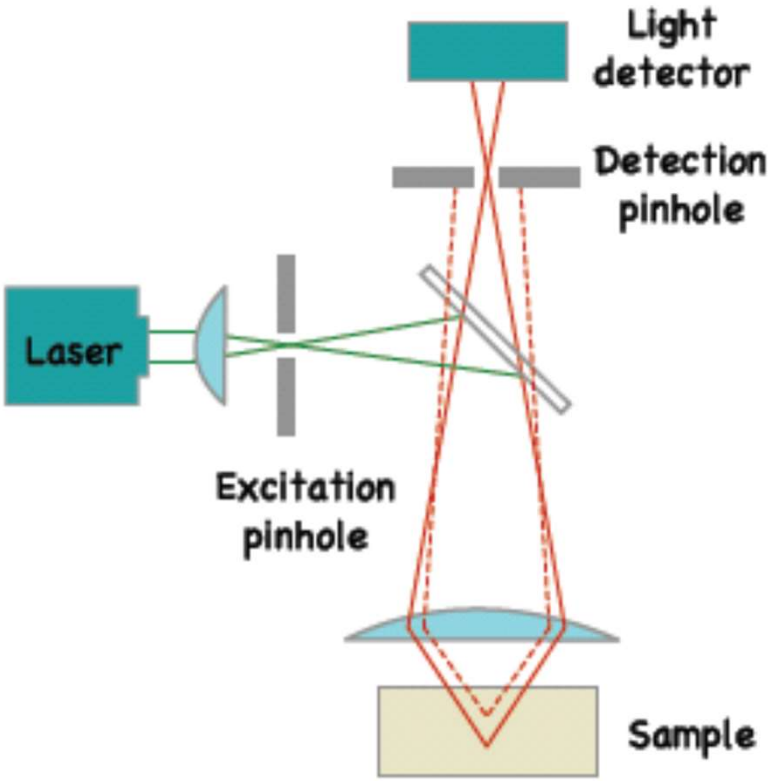
3D fluorescence microscopy

Acquire a "focal series" (stack) of images

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

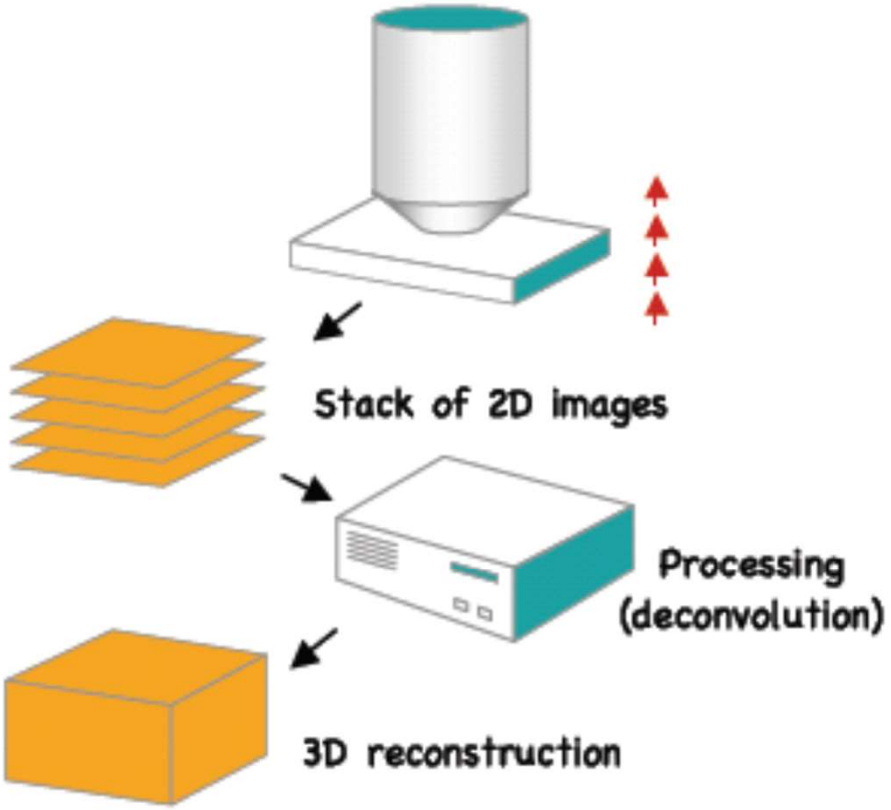
Approach 1

Physically exclude the blur
Example: confocal microscopy



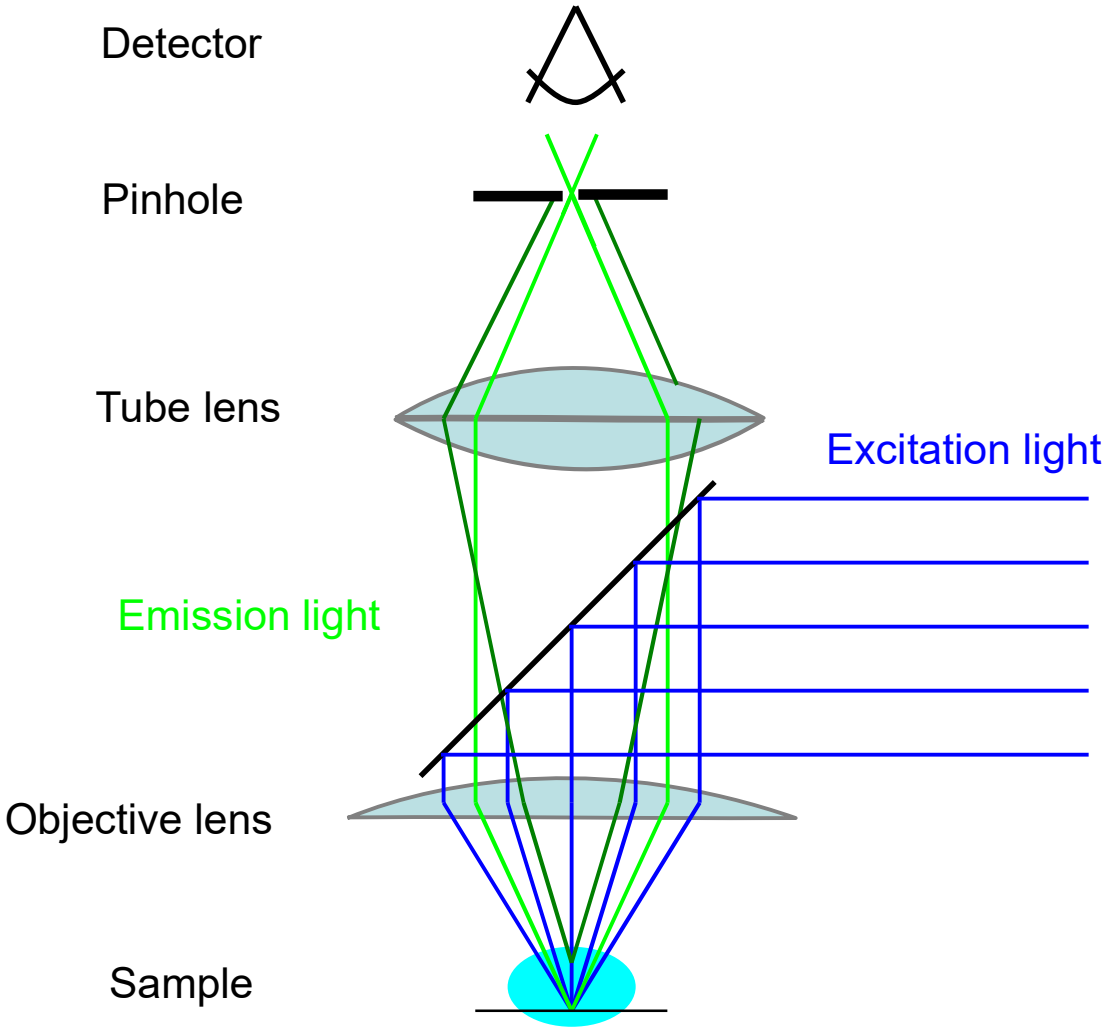
Approach 2

Remove the blur computationally



How well can this be done?

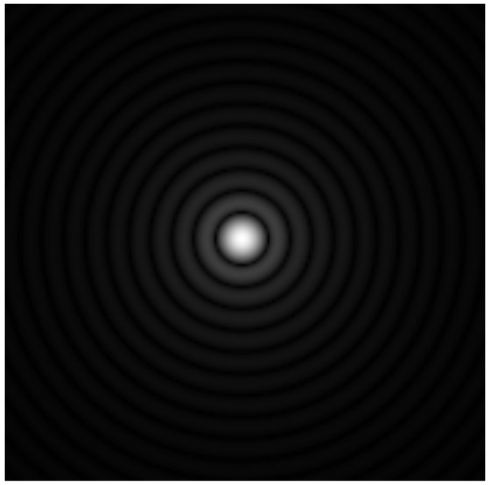
The confocal microscope



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

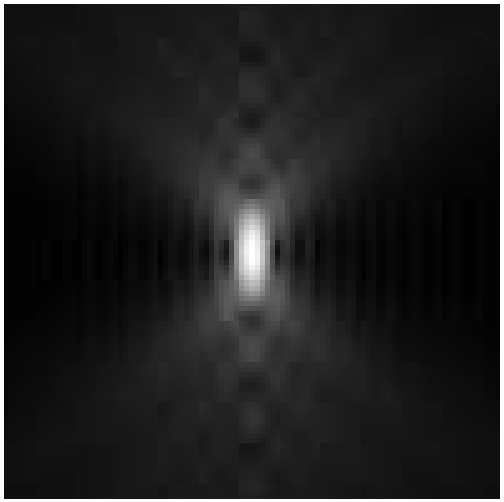
How big should your pinhole be?

Resolution is limited by the point-spread function

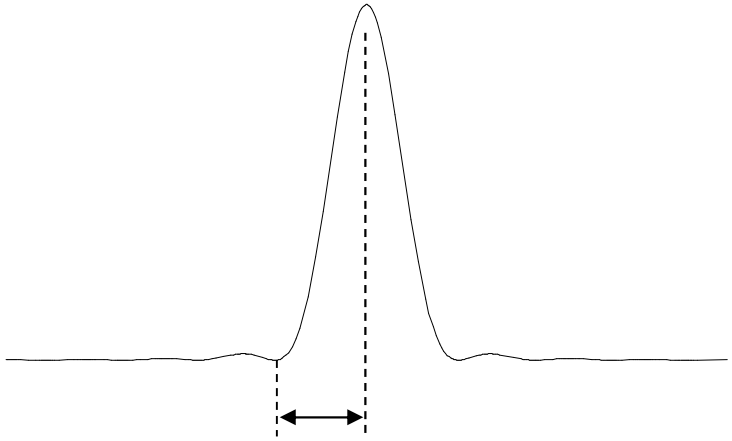


Y

X



Z

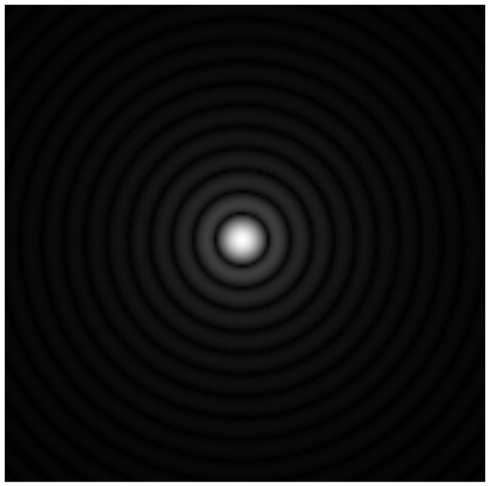


Airy disk radius

≈ 0.61 λ /*NA*

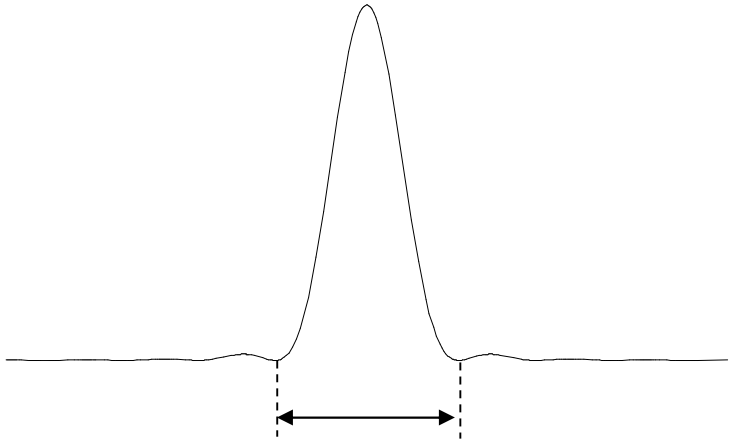
How big should your pinhole be?

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

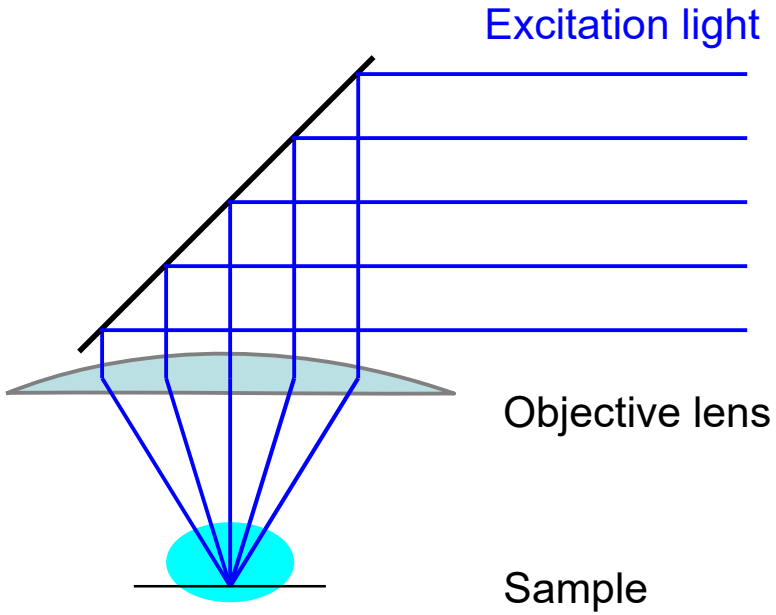
- All are substantially larger than Airy Disk for low magnification lenses.
  - On spinning disk, can use 1.5x magnification changer

Light sources

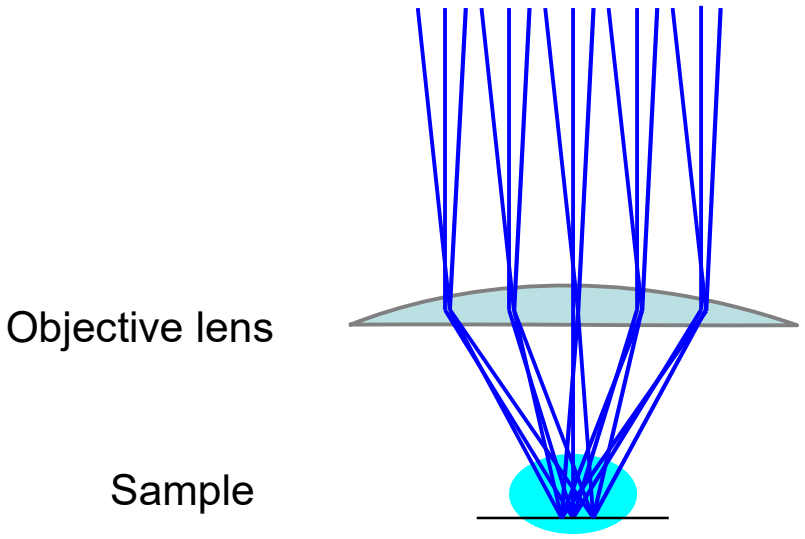
Excitation light must be focused to a diffraction limited spot

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

Enter the laser:
Perfectly collimated and high power



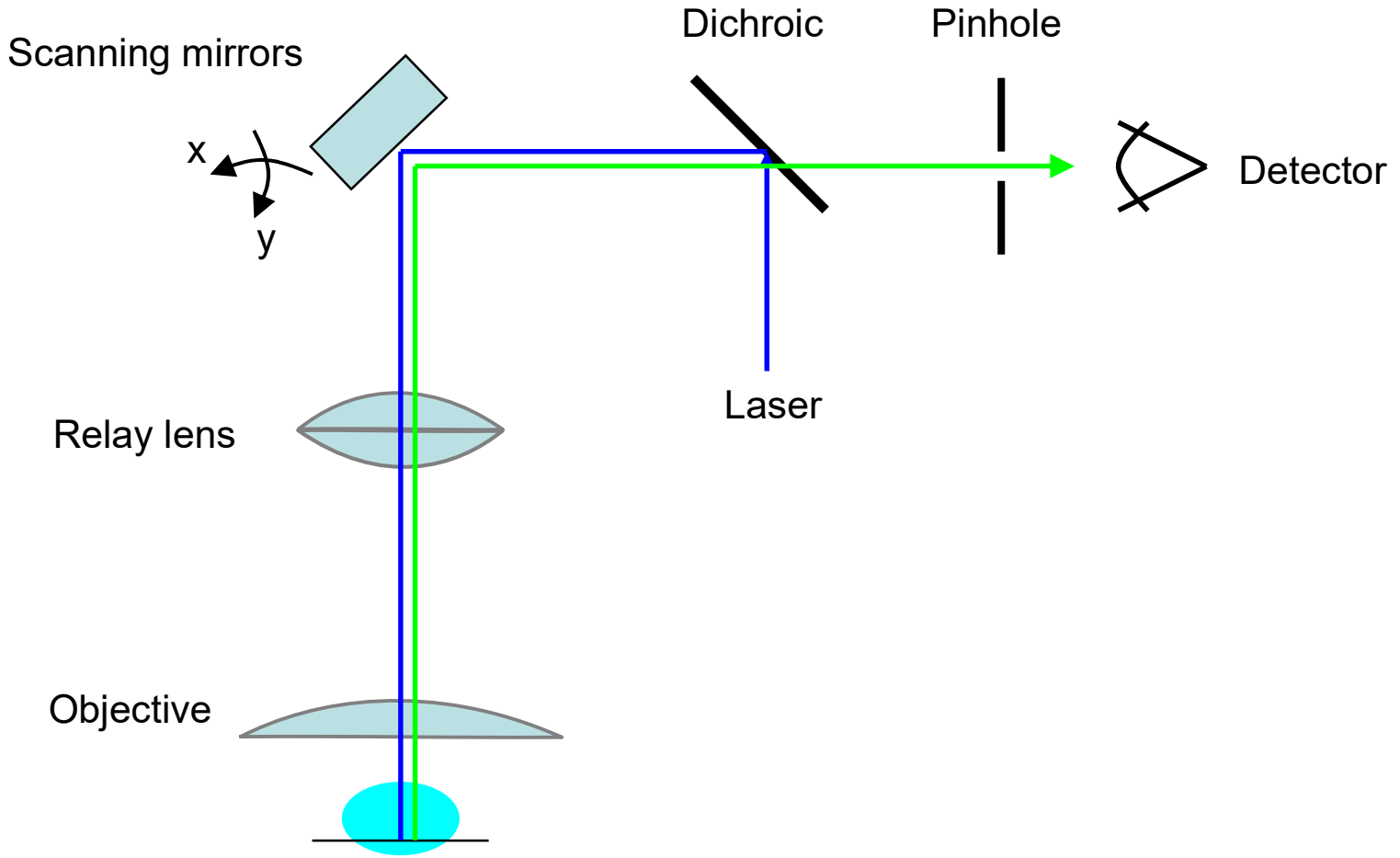
Scanning



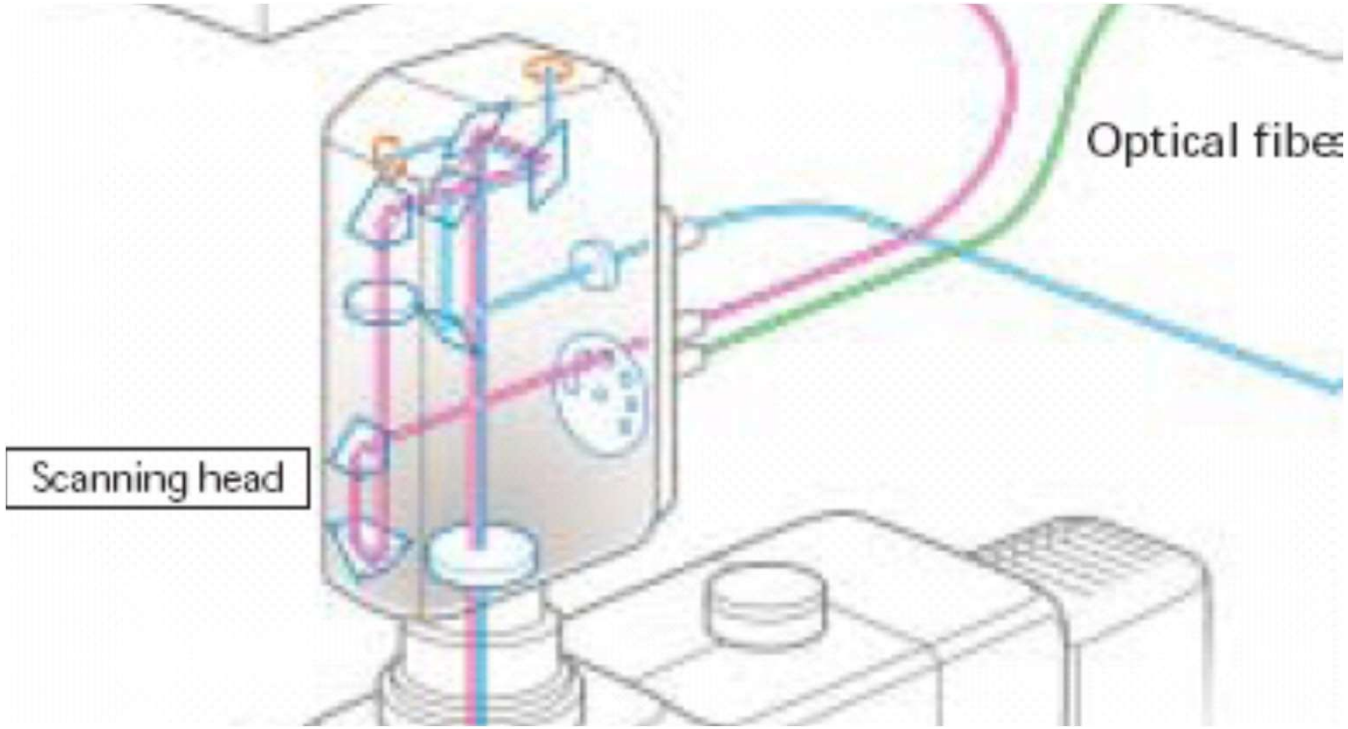
Changing entrance angle of illumination moves illumination spot on sample

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

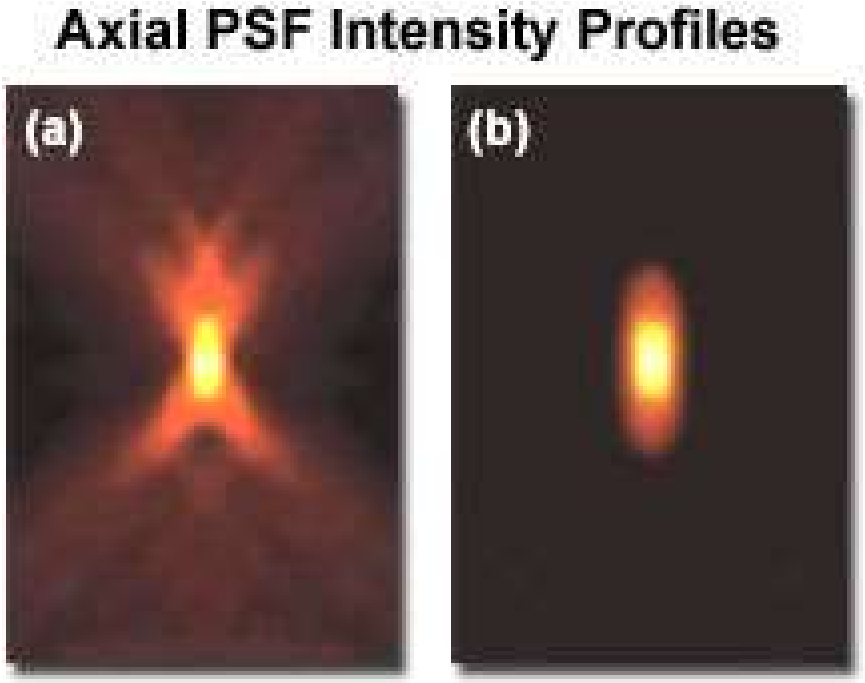
Confocal optical path

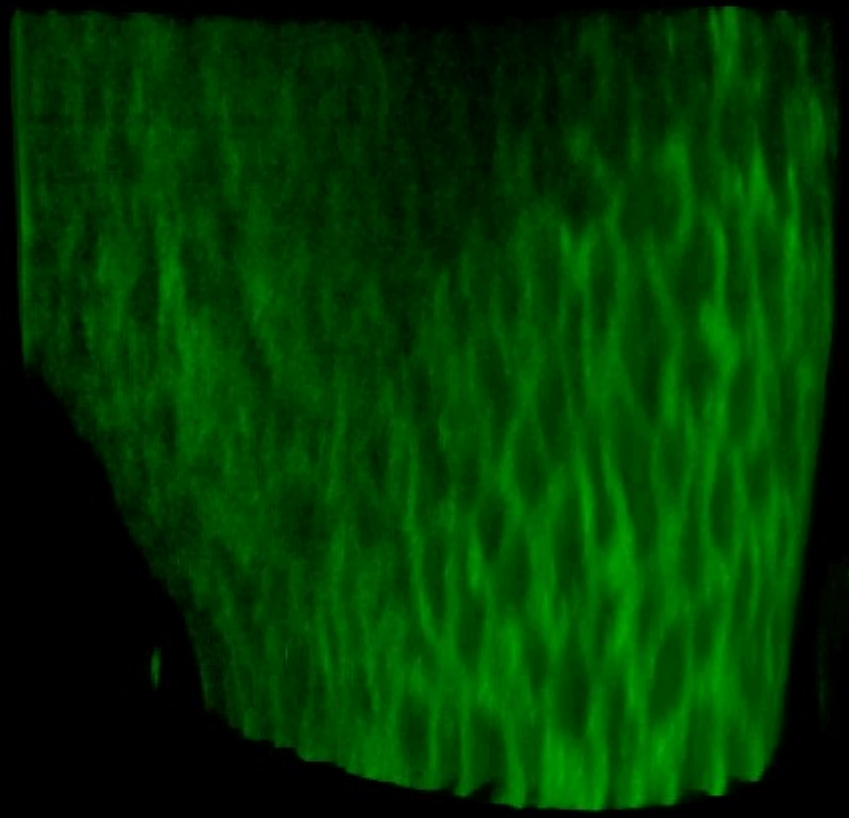


C1si internals



What do you get?



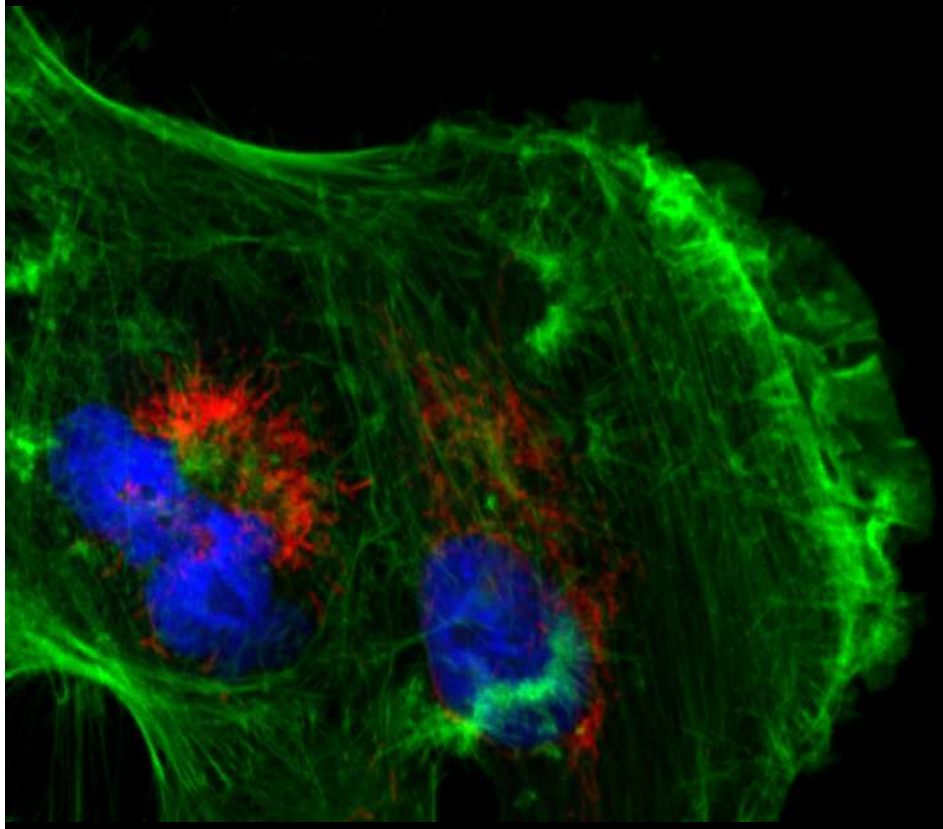


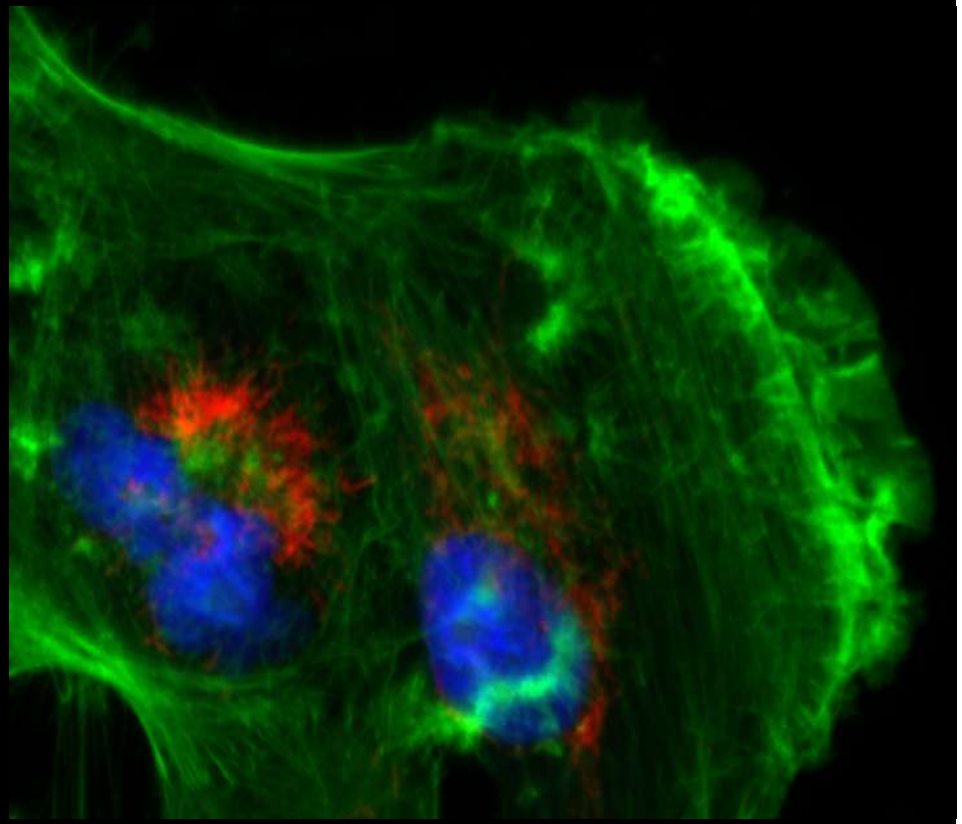
Laser-Scanning
Confocal

E11.5 mouse lung
stained for E-cadherin

Nan Tang, Martin lab

Confocal vs. Widefield



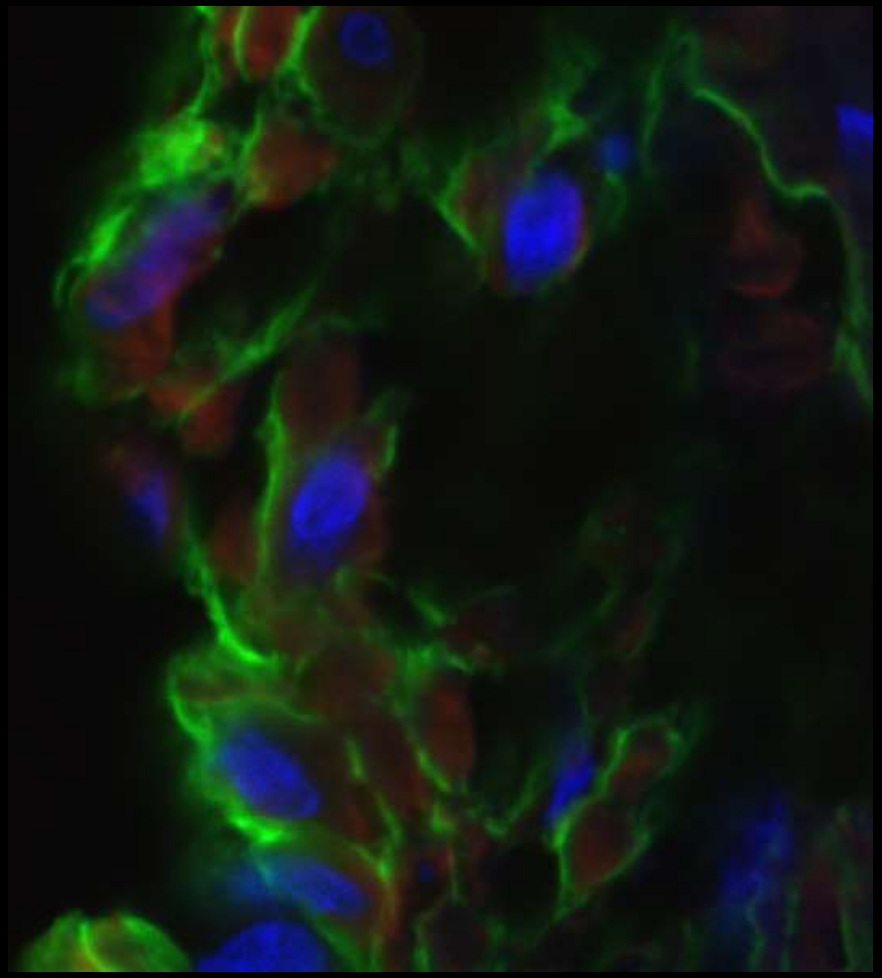


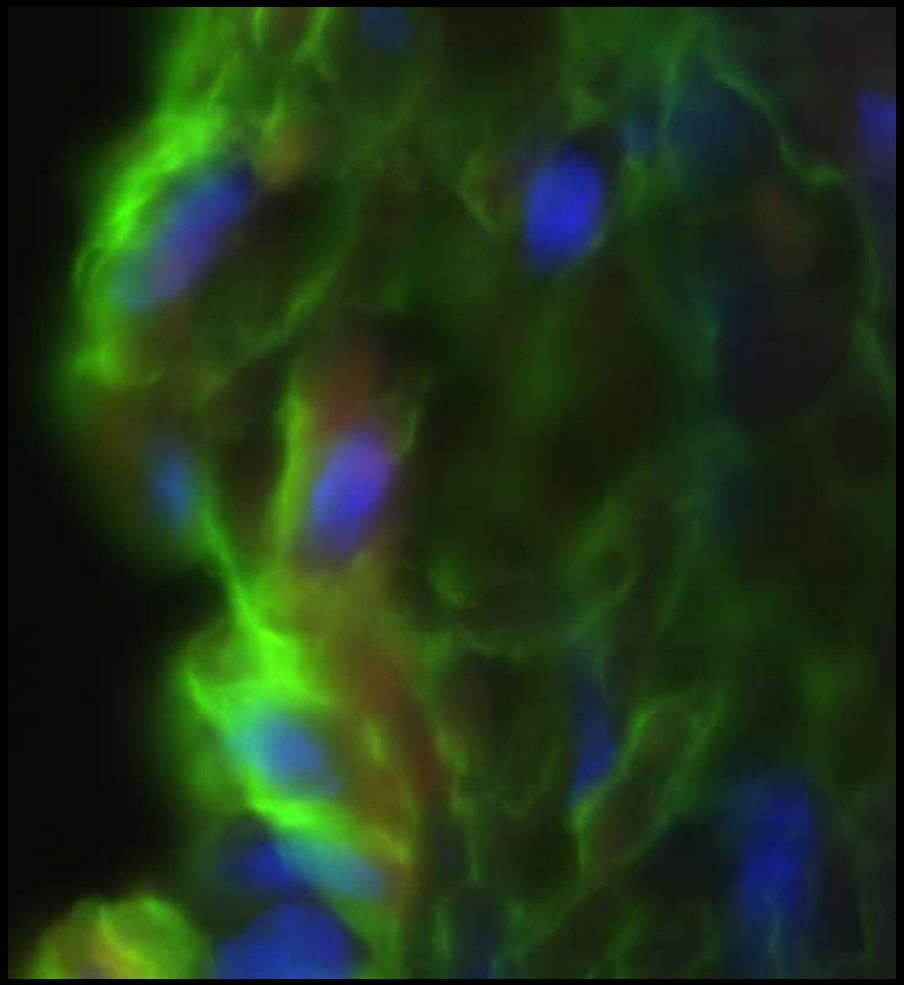
Confocal

Widefield

Tissue culture cell with 60x / 1.4NA objective

Confocal vs. Widefield



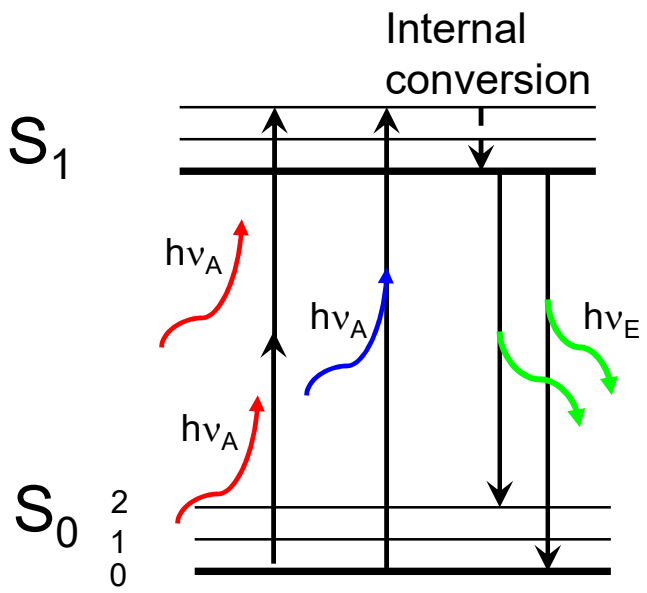


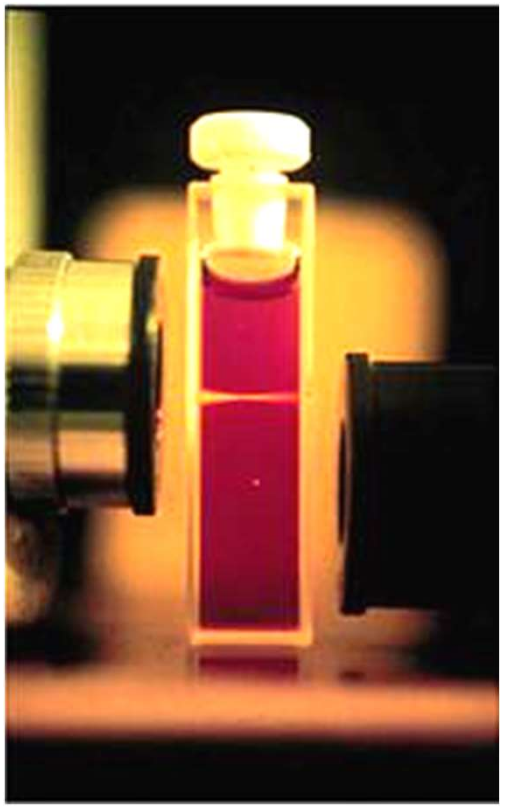
Confocal

Widefield

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

Multi-photon excitation

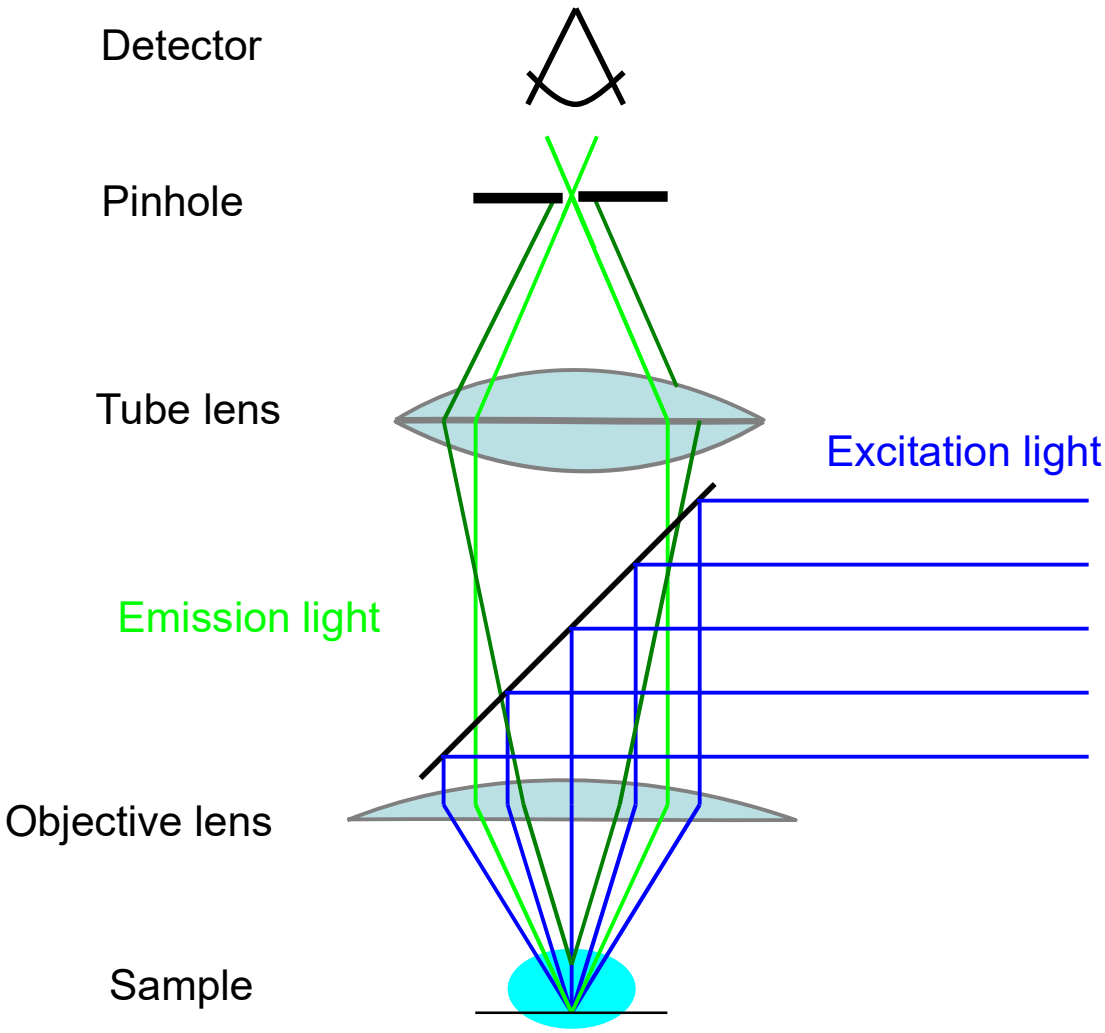




Brad Amos, MRC, Cambridge

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

The confocal microscope

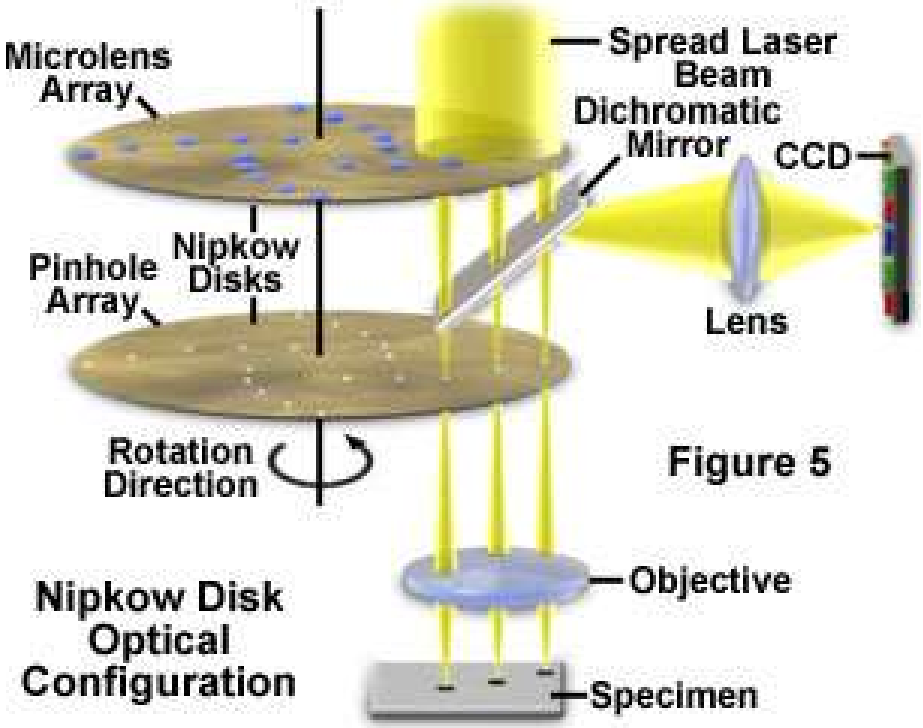


Scan excitation spot point-by-point to build up image

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



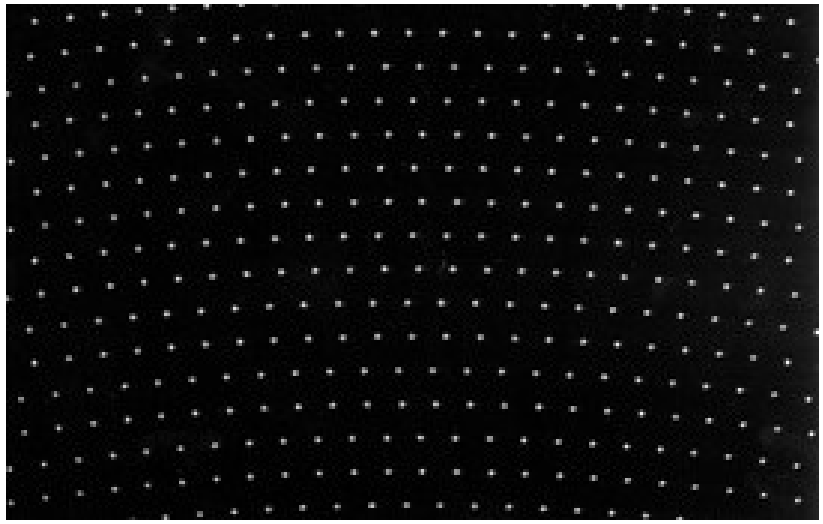


Image with many pinholes at once, so fast

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

Which imaging technique should I use?