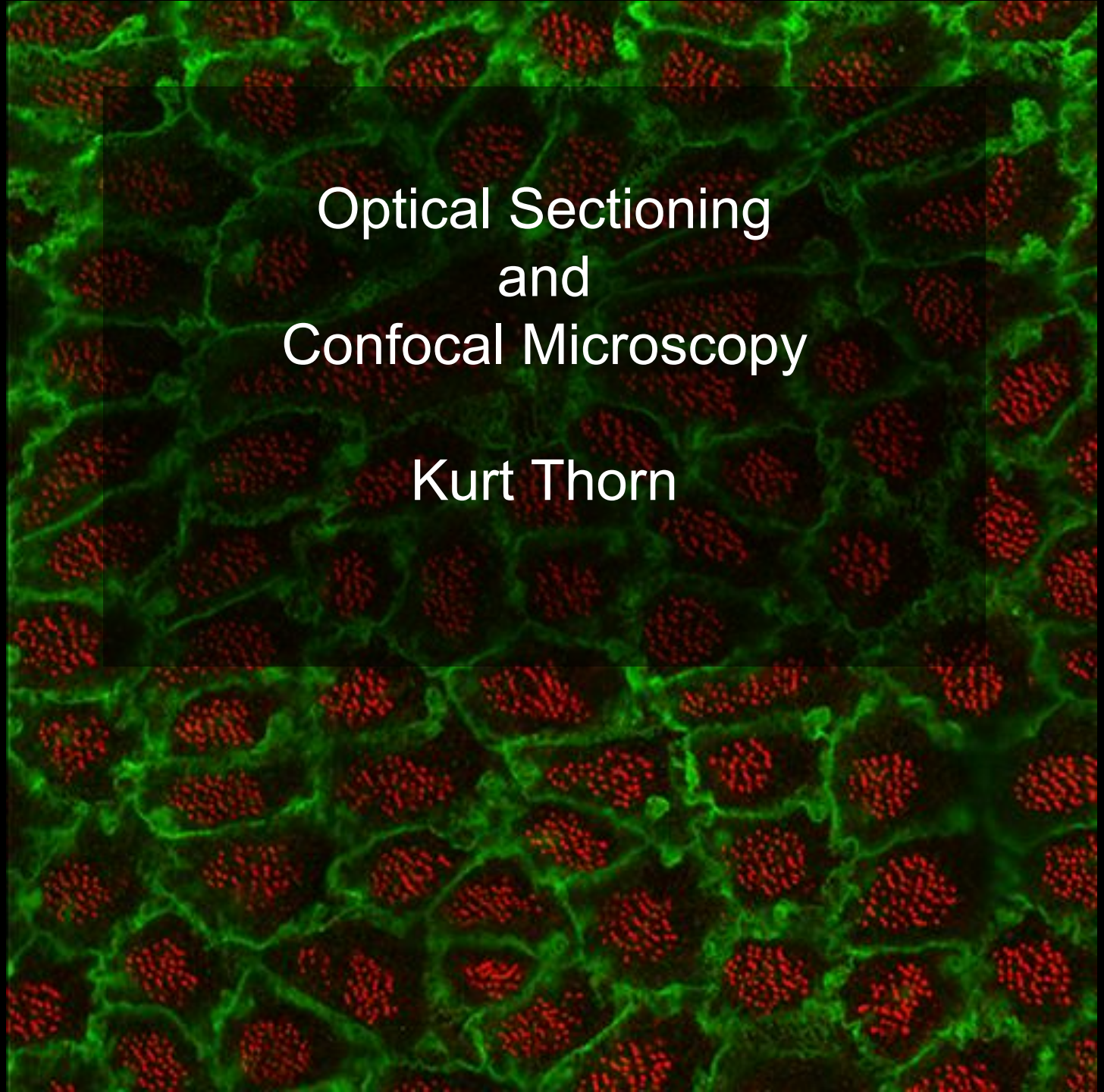
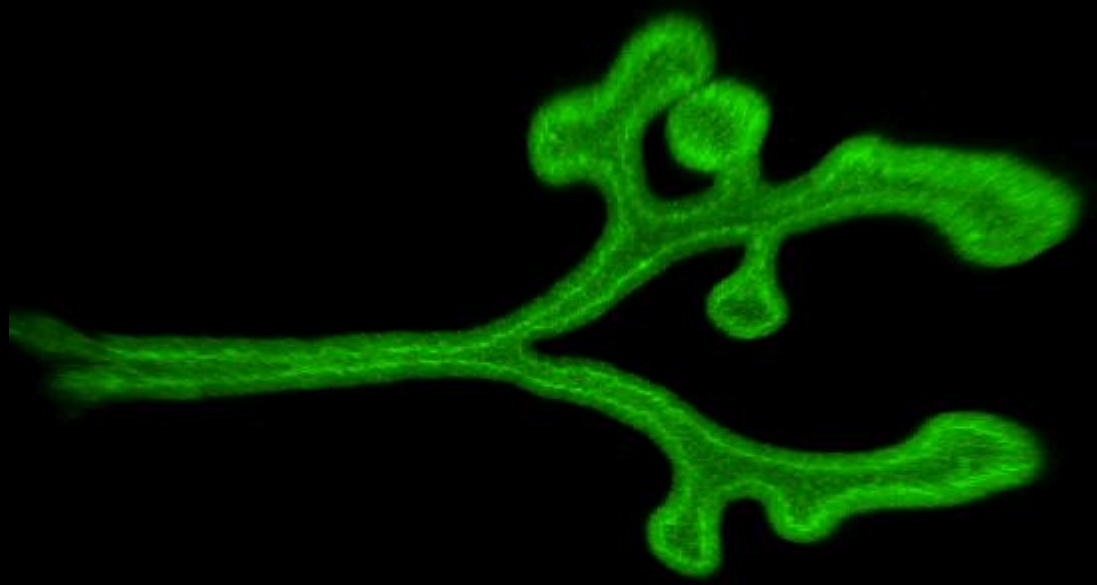
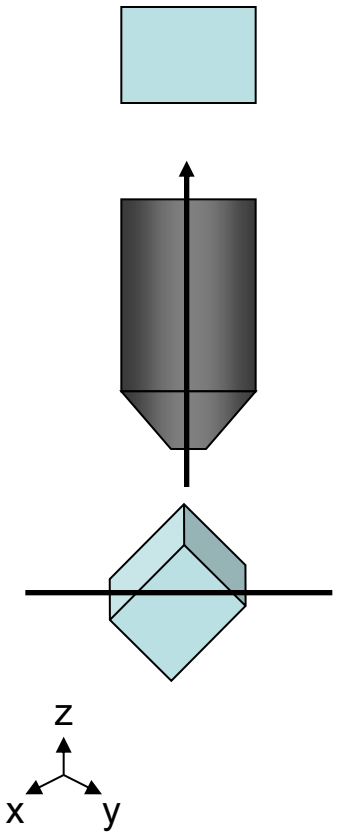

Optical Sectioning
and
Confocal Microscopy
Kurt Thorn

The goal: build 3D images of biological samples

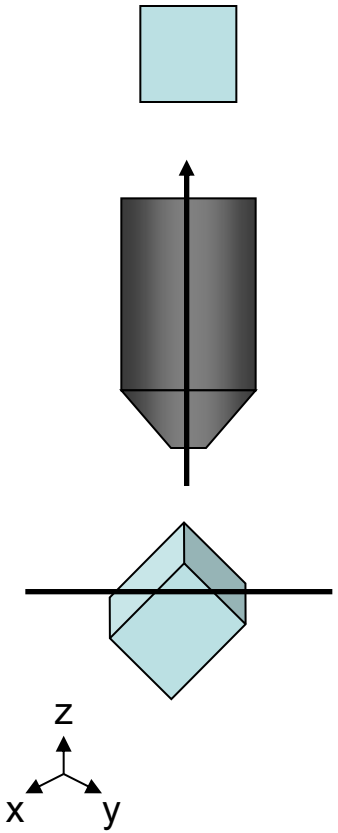


Embryonic mouse lung; Nan Tang, Martin lab

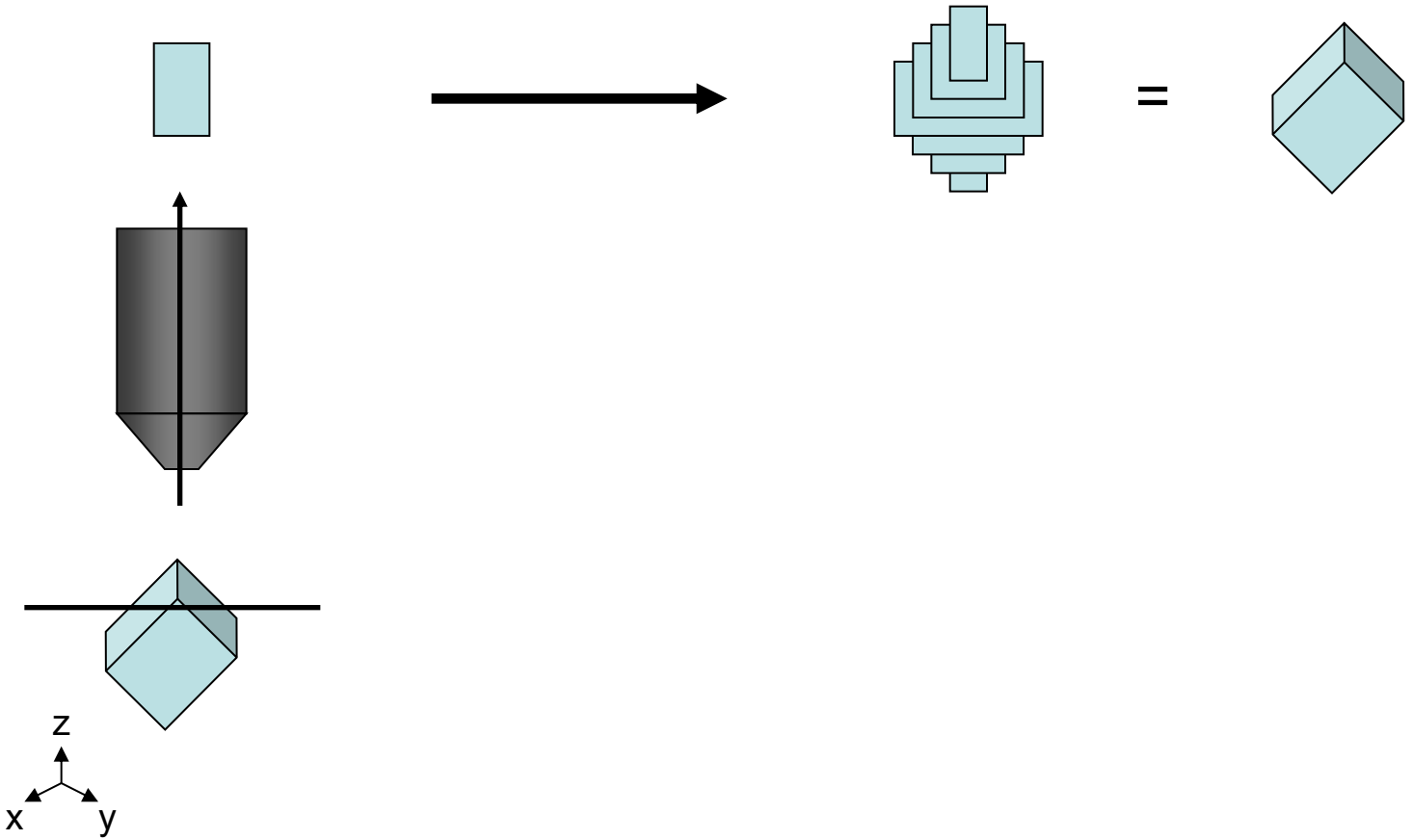
Optical Sectioning and 3D reconstruction



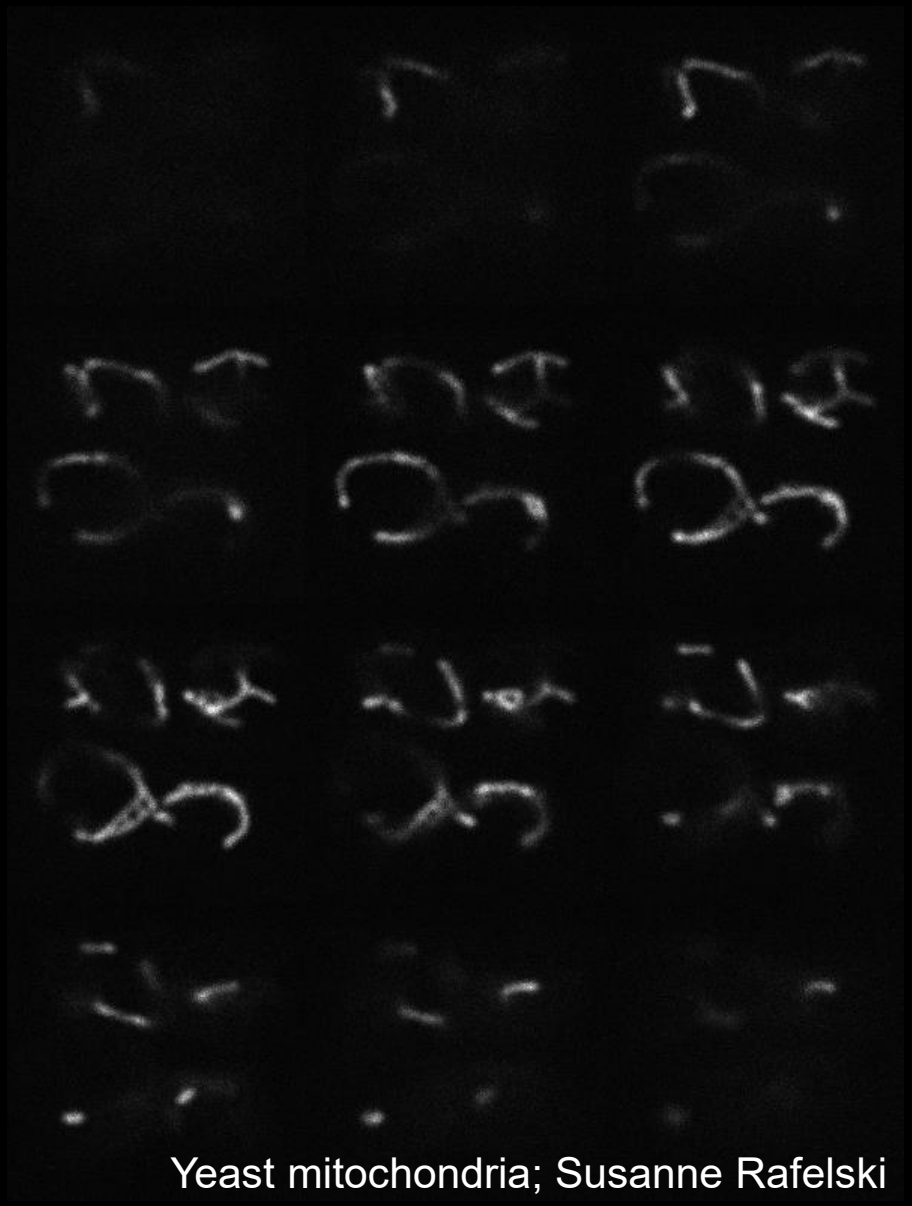
Optical Sectioning and 3D reconstruction

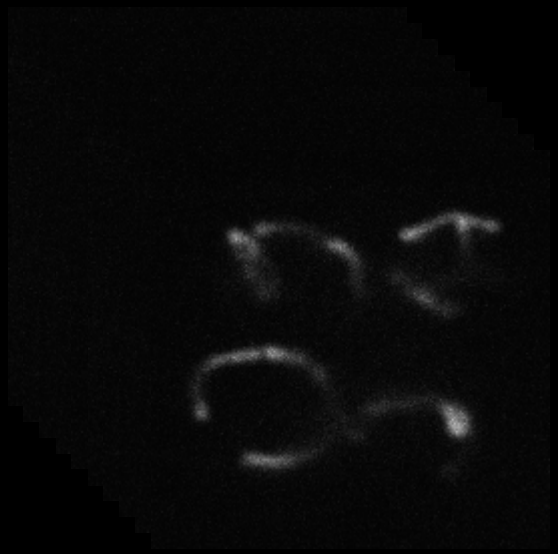


Optical Sectioning and 3D reconstruction



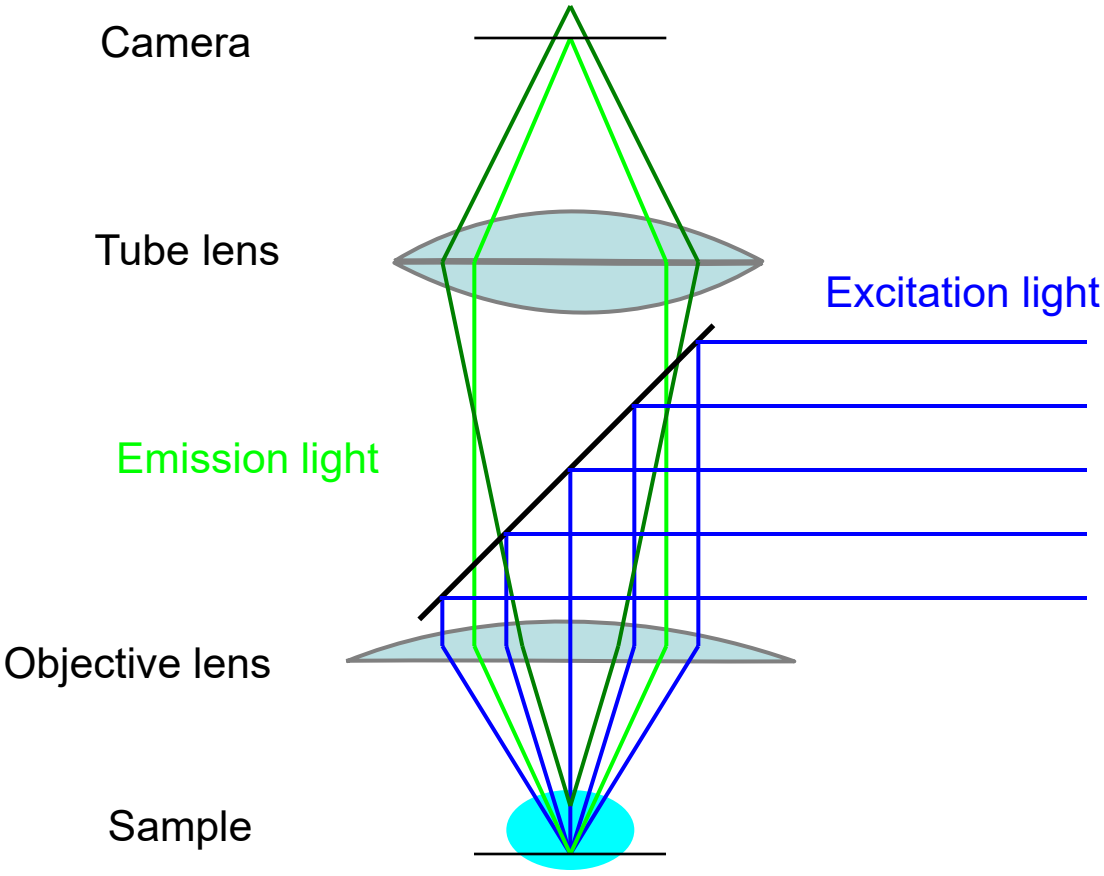
A series of optical sections





Yeast mitochondria; Susanne Rafelski

Fluorescence Illumination of a single point



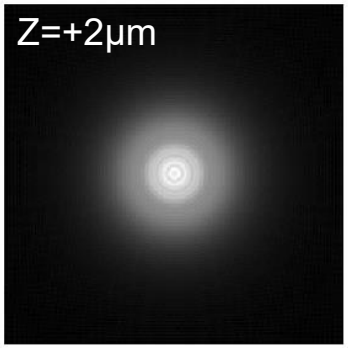
Problem – fluorescence is emitted along entire illuminated cone, not just at focus

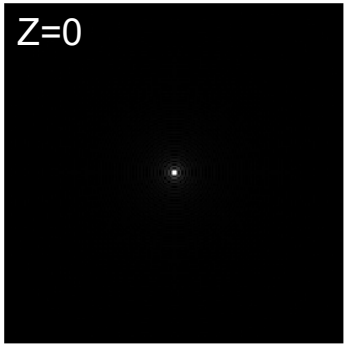
Widefield fluorescence imaging

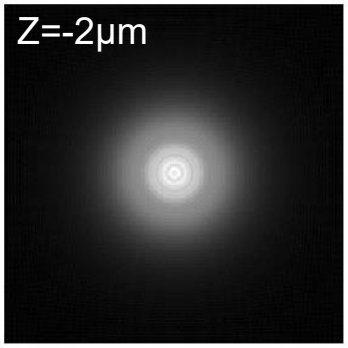
The Point Spread Function (PSF)

The image of a point object

2D PSF for different defocus

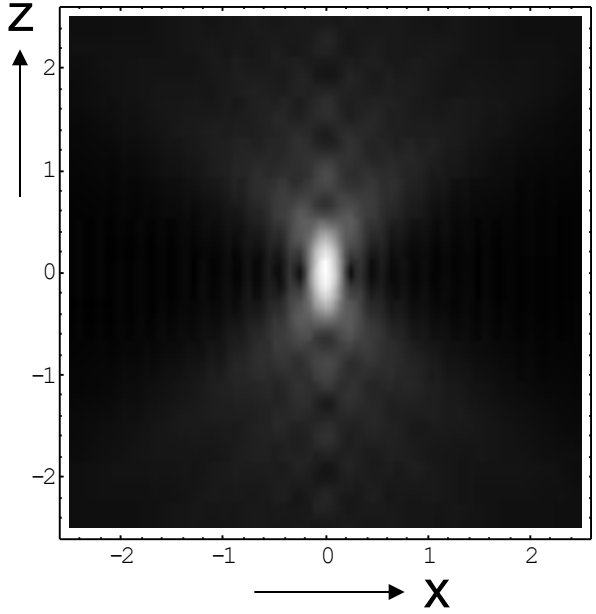




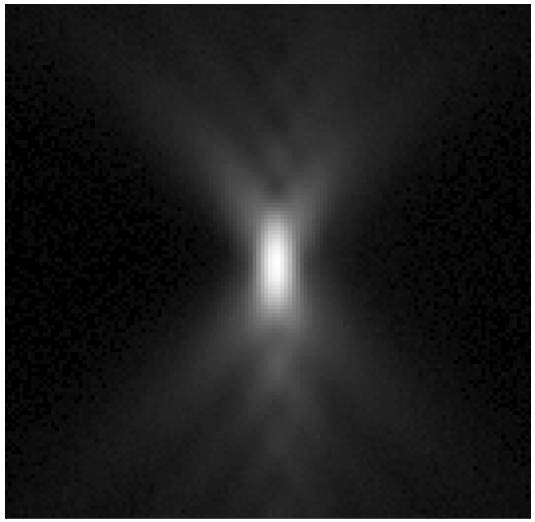


3D PSF

Calculated



Measured

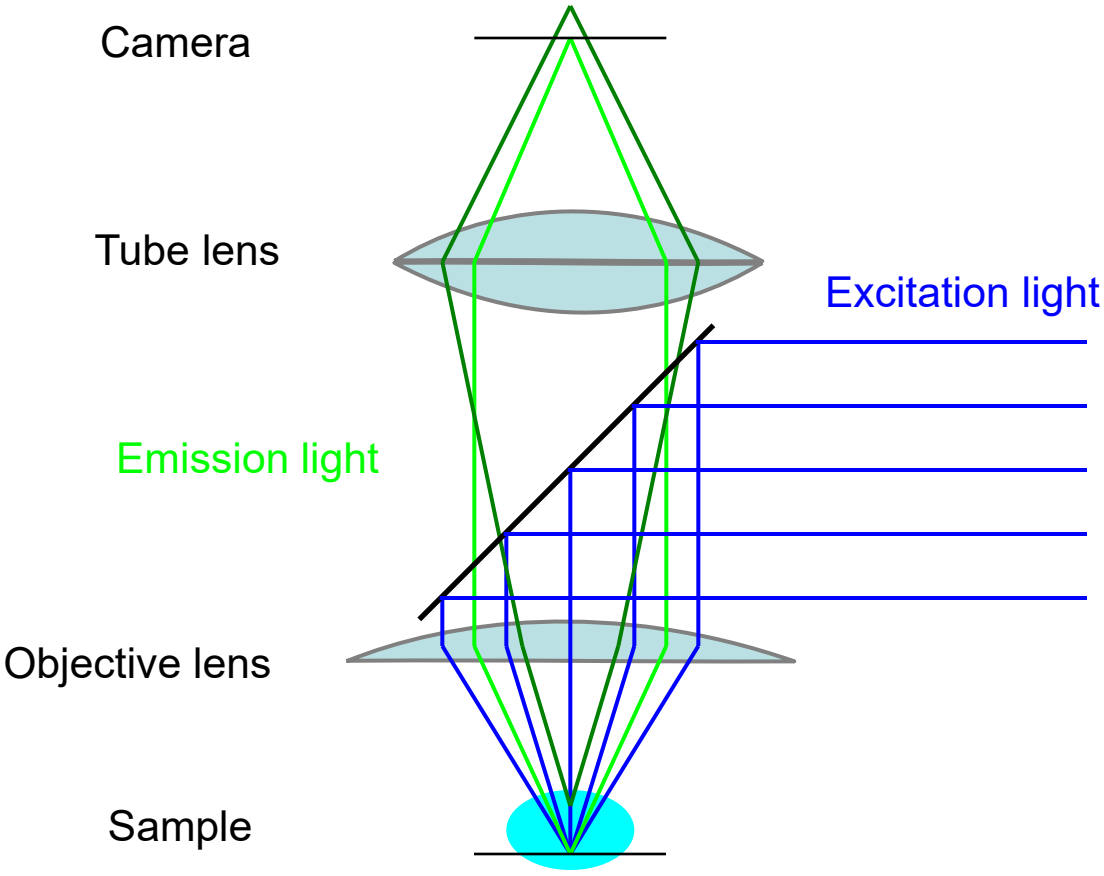


The Problem of Out-of-Focus Light

Three Options

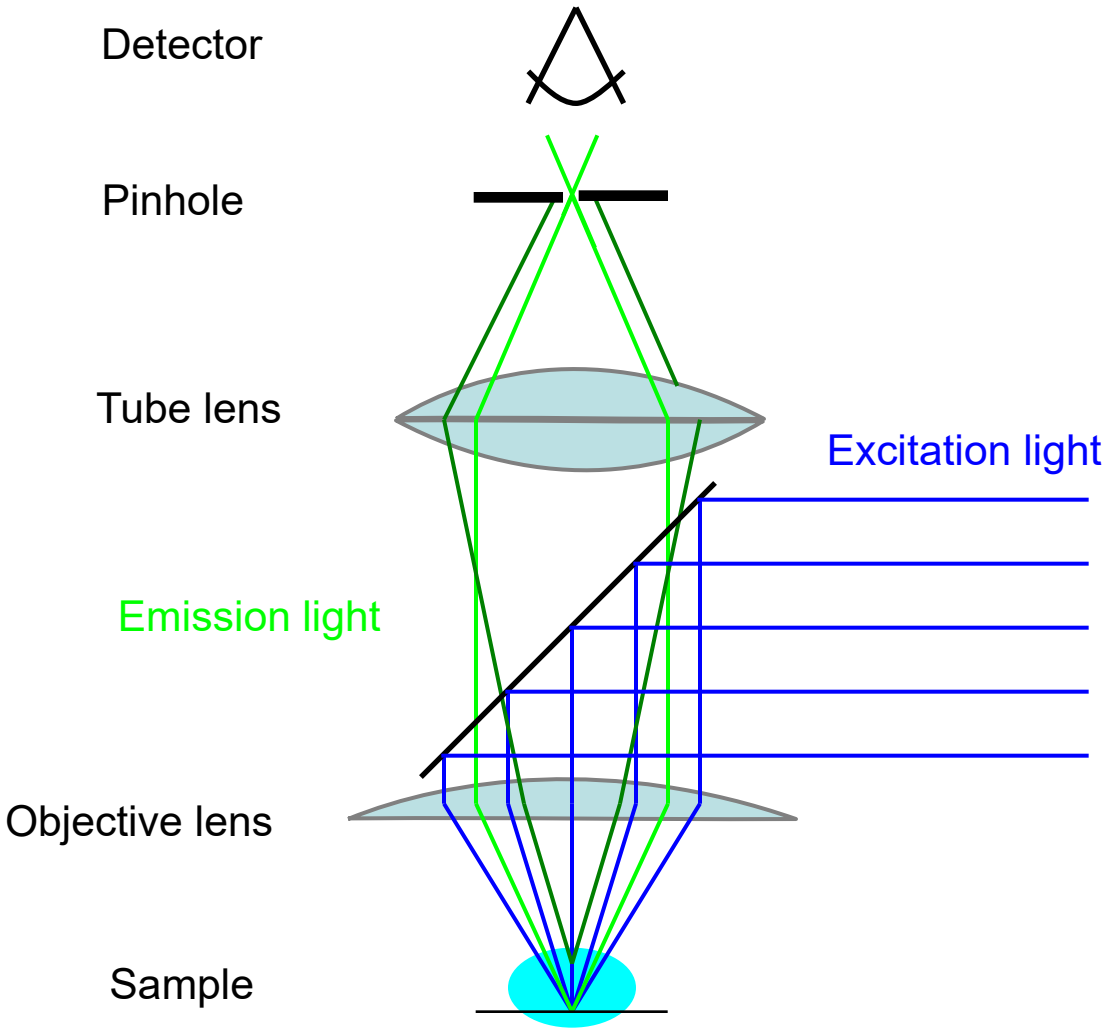
- Prevent it from reaching the detector
  - Confocal microscopy
- Prevent it from being excited
  - Two-photon, light sheet microscopy, TIRF
- Remove it after the fact (computationally)
  - Deconvolution

Fluorescence Illumination of a single point



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

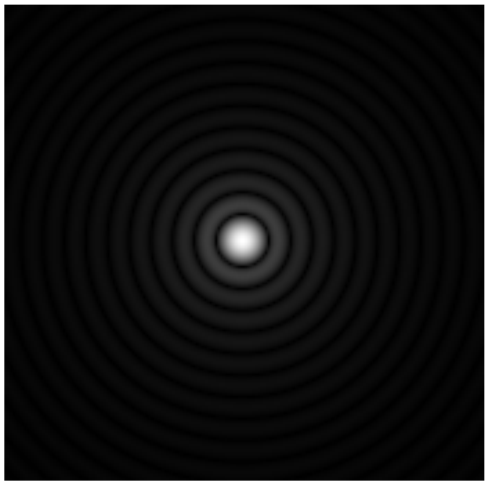
The confocal microscope



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

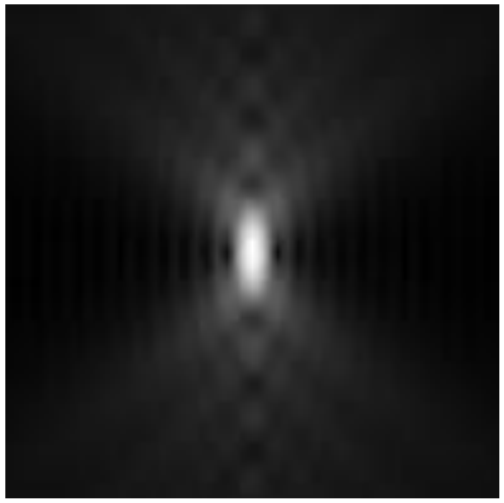
How big should your pinhole be?

Resolution is limited by the point-spread function

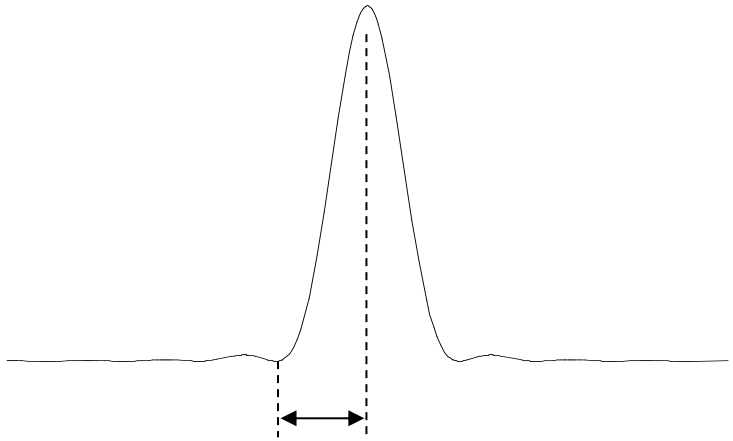


Y

X



Z

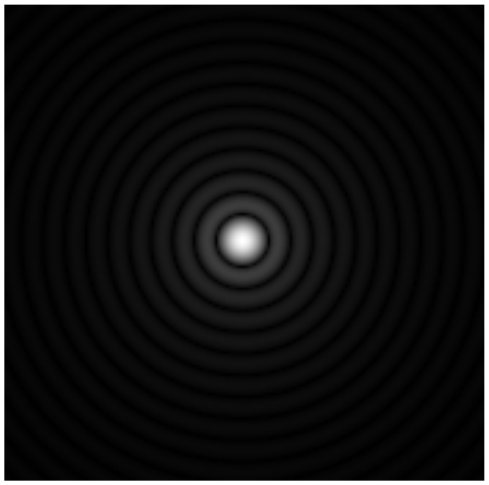


Airy disk radius

≈ 0.61 λ /*NA*

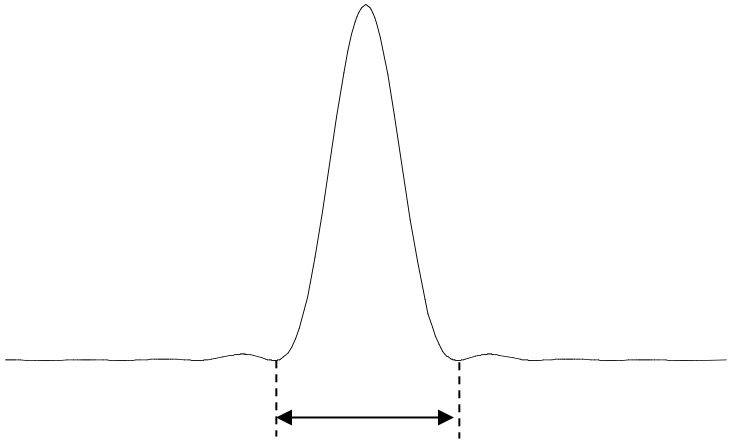
How big should your pinhole be?

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

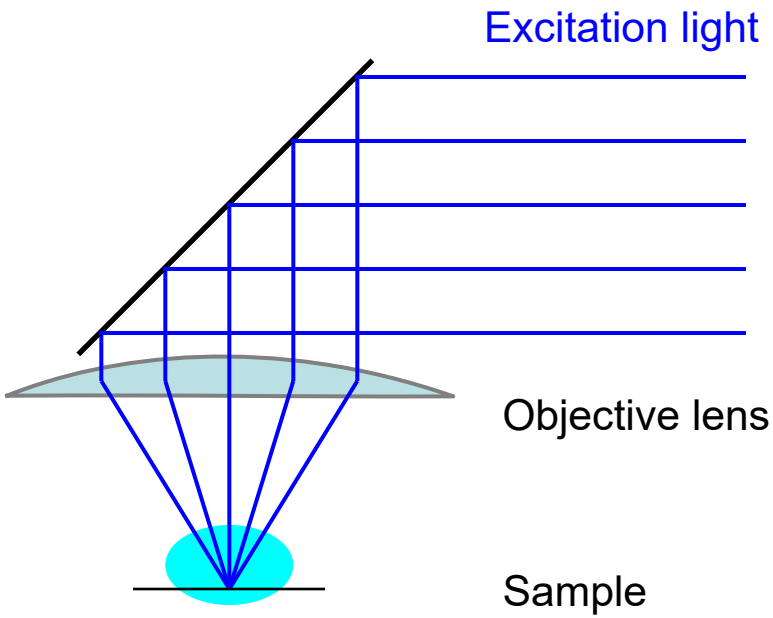
  - 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 44 μm
  - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 19 μm
  - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 16 μm
  - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 14 μm

Light sources

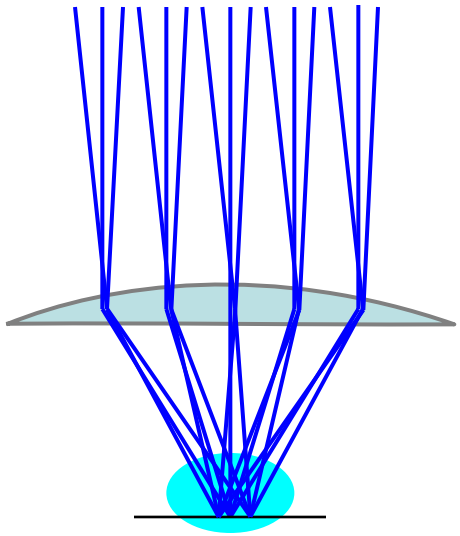
Excitation light must be focused to a diffraction limited spot

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

Enter the laser:
Perfectly collimated and high power



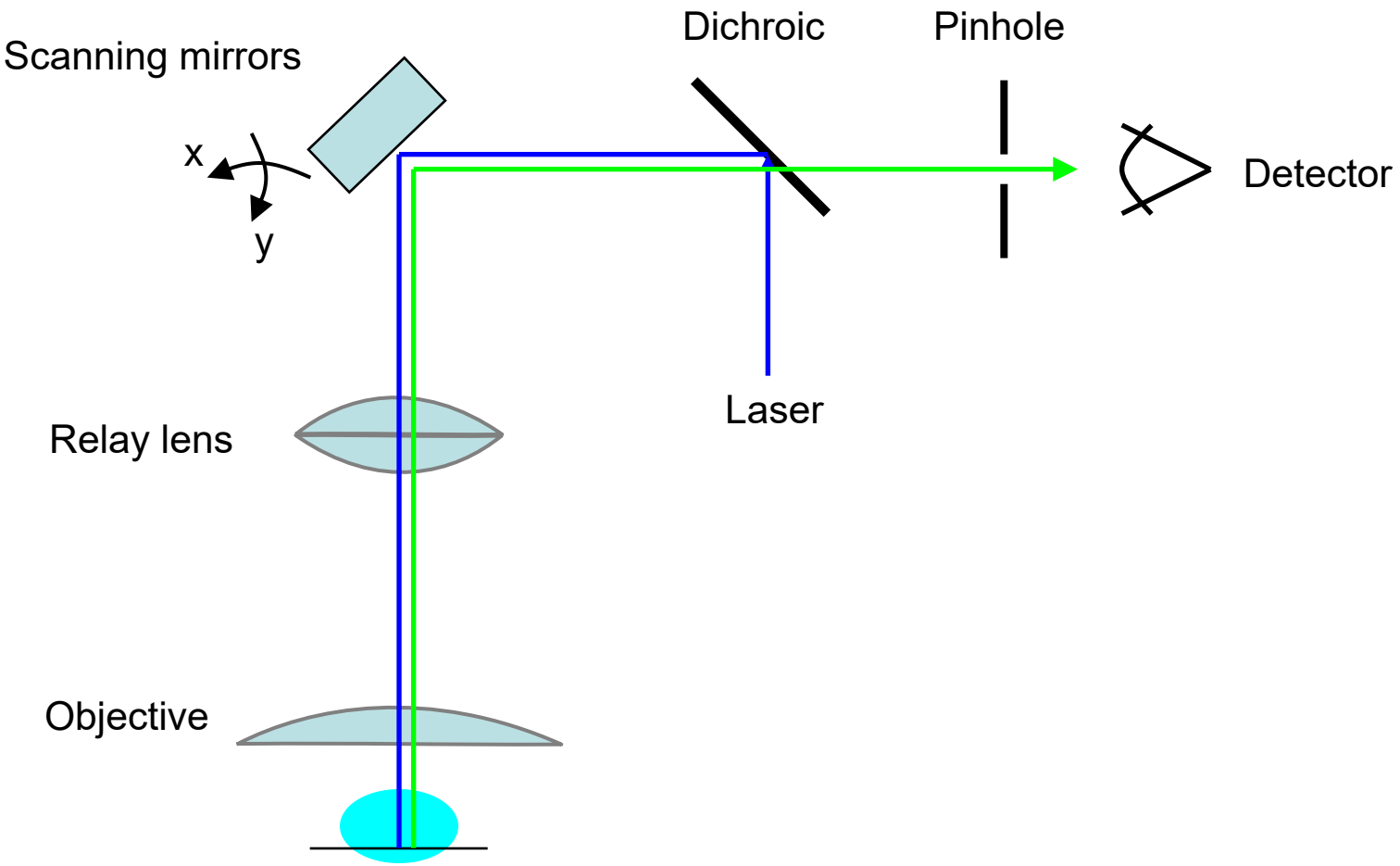
Scanning



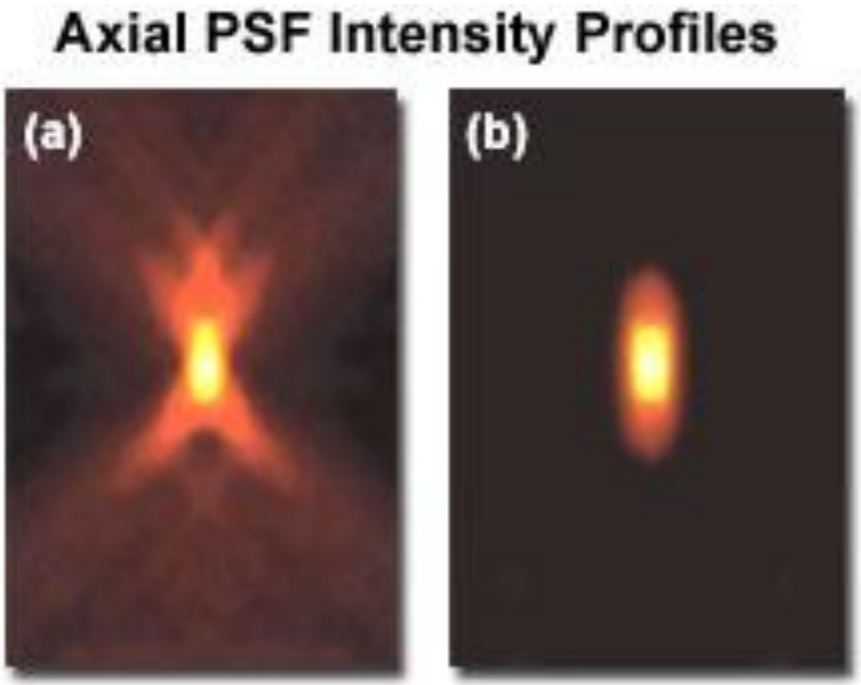
Changing entrance angle of illumination moves illumination spot on sample

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

Confocal optical path

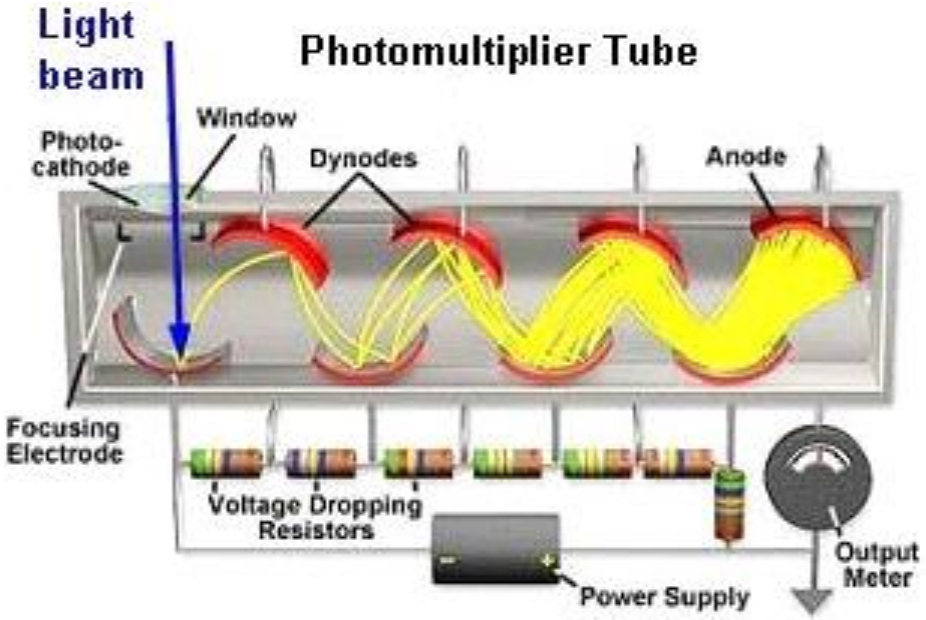


What do you get?



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

- Peak QE for standard PMT: ~30%
- Front-illuminated CCD: ~60%
- Back-illuminated CCD: ~95%
- GaAsP PMTs: ~40-50%

However, GaAsP PMTs can be damaged by exposure to too much light.

Detectors - APDs

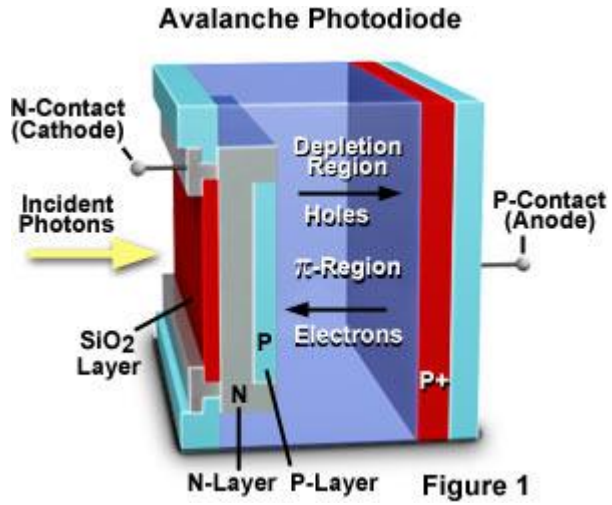


Figure 1

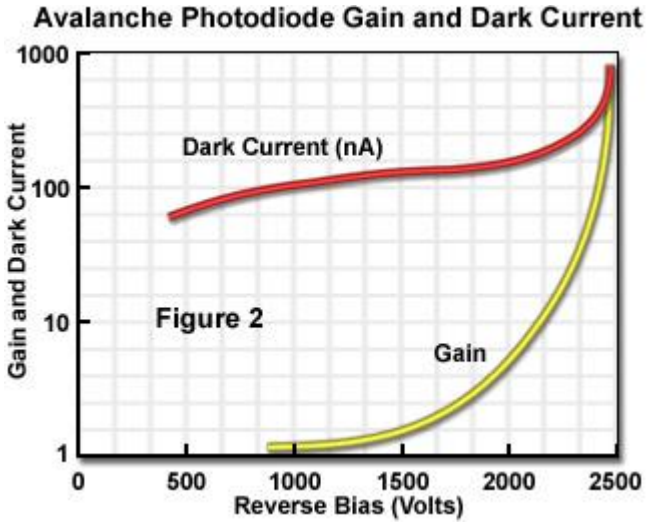
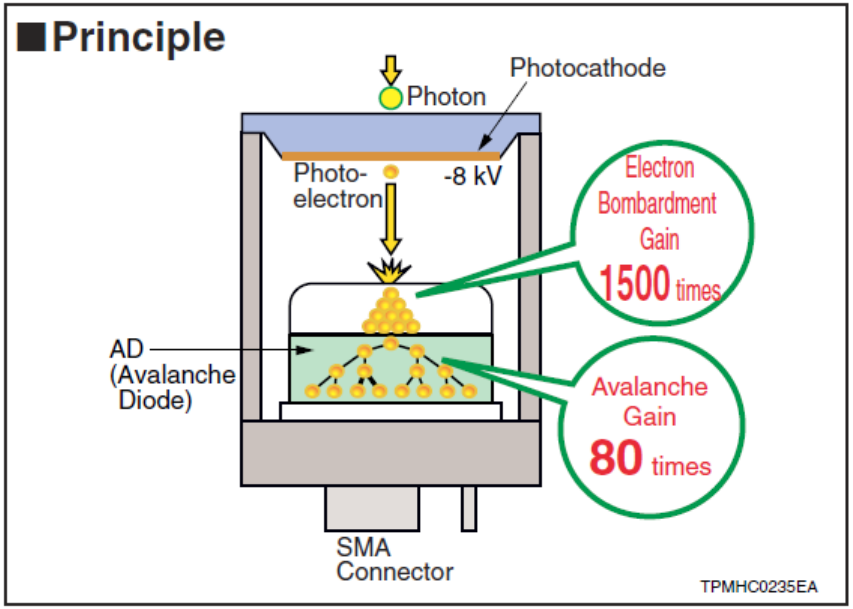


Figure 2

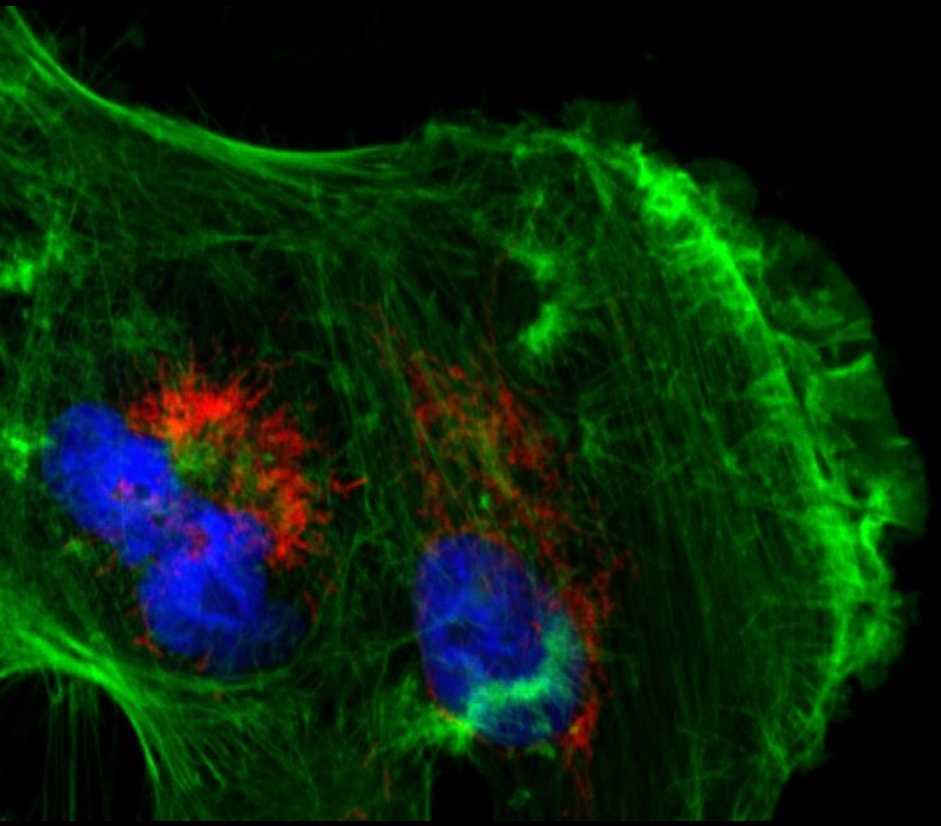
- Photons produce photoelectrons
- Photoelectrons are amplified by collisional ejection of electrons
- Small dynamic range

Hybrid Detectors

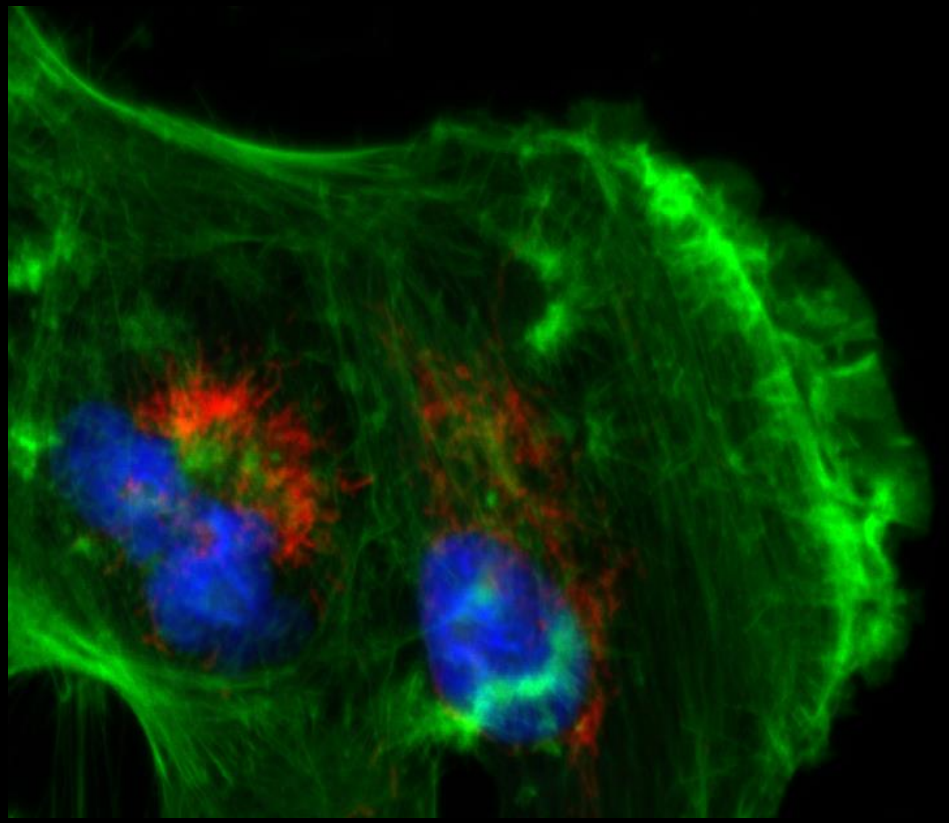


- GaAsP photocathode bombards an APD
- Very high gain
- Excellent for single photon counting
- Damaged by excessive light

Confocal vs. Widefield



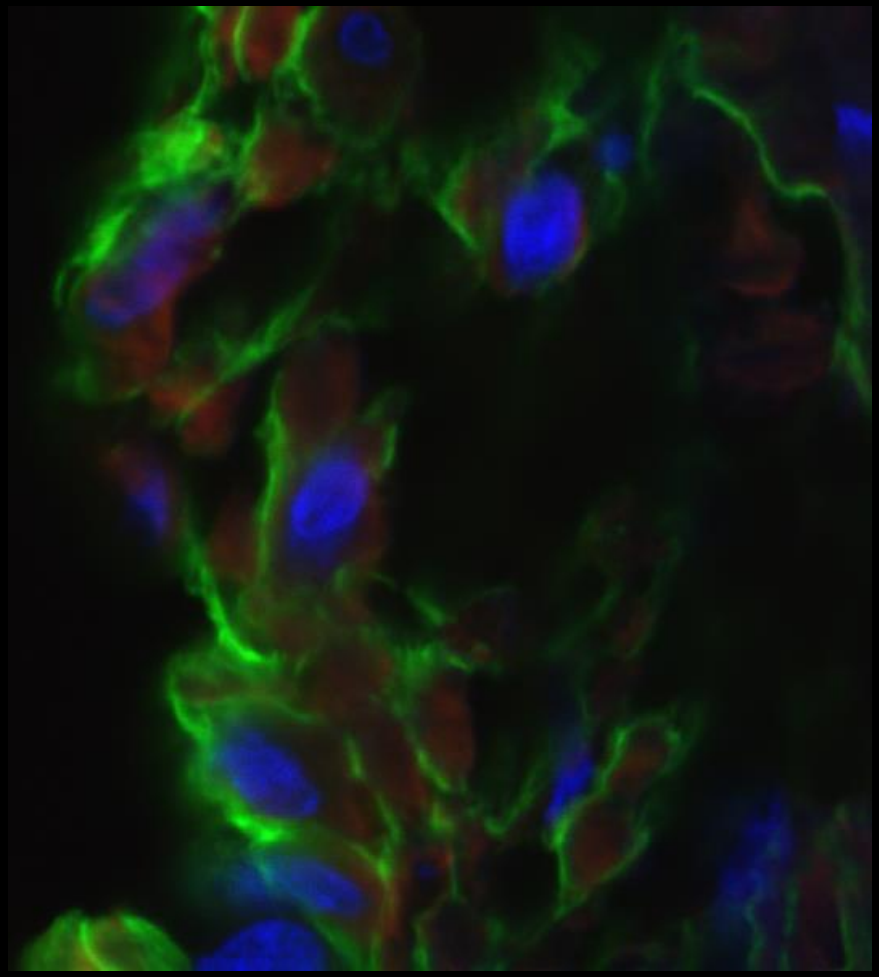
Confocal



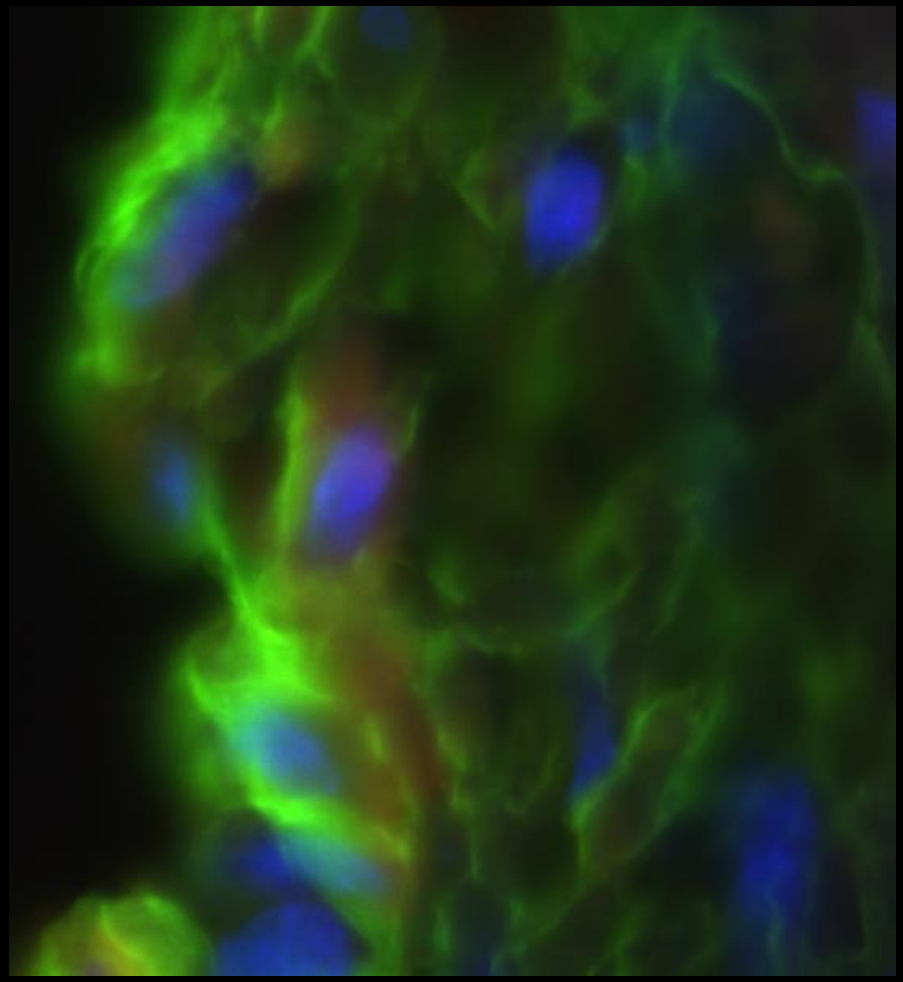
Widefield

Tissue culture cell with 60x / 1.4NA objective

Confocal vs. Widefield



Confocal

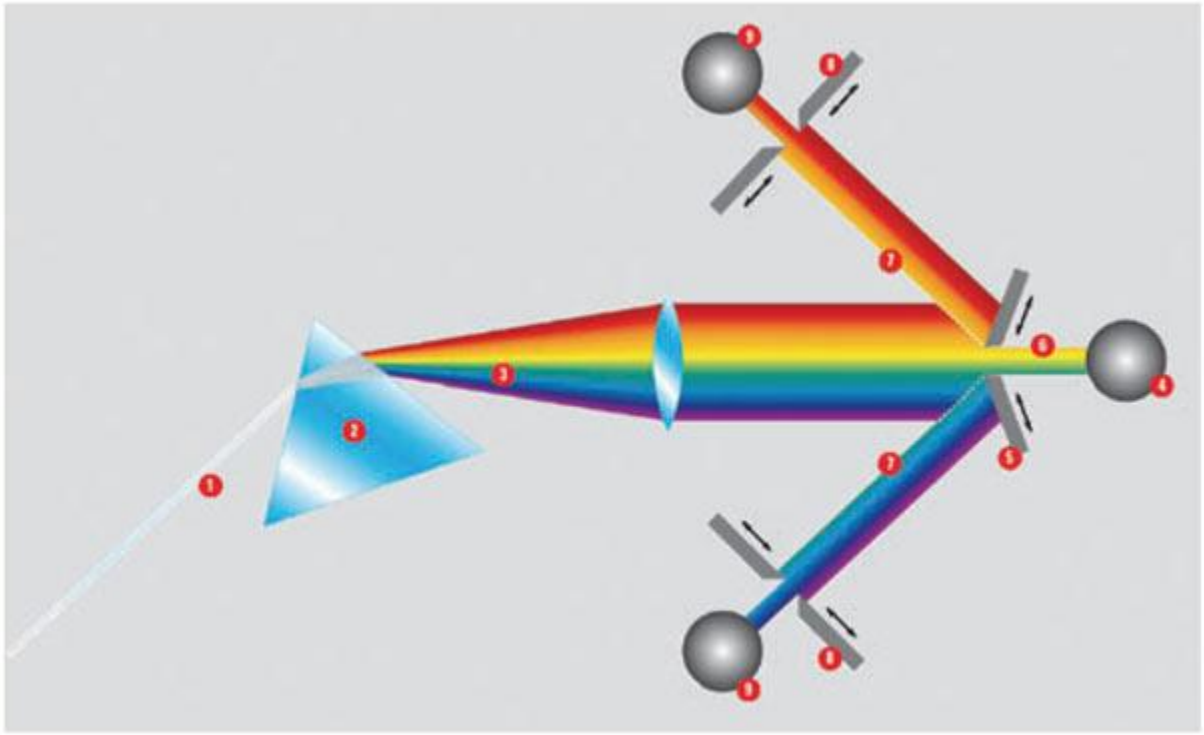


Widefield

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

Doing more with your photons

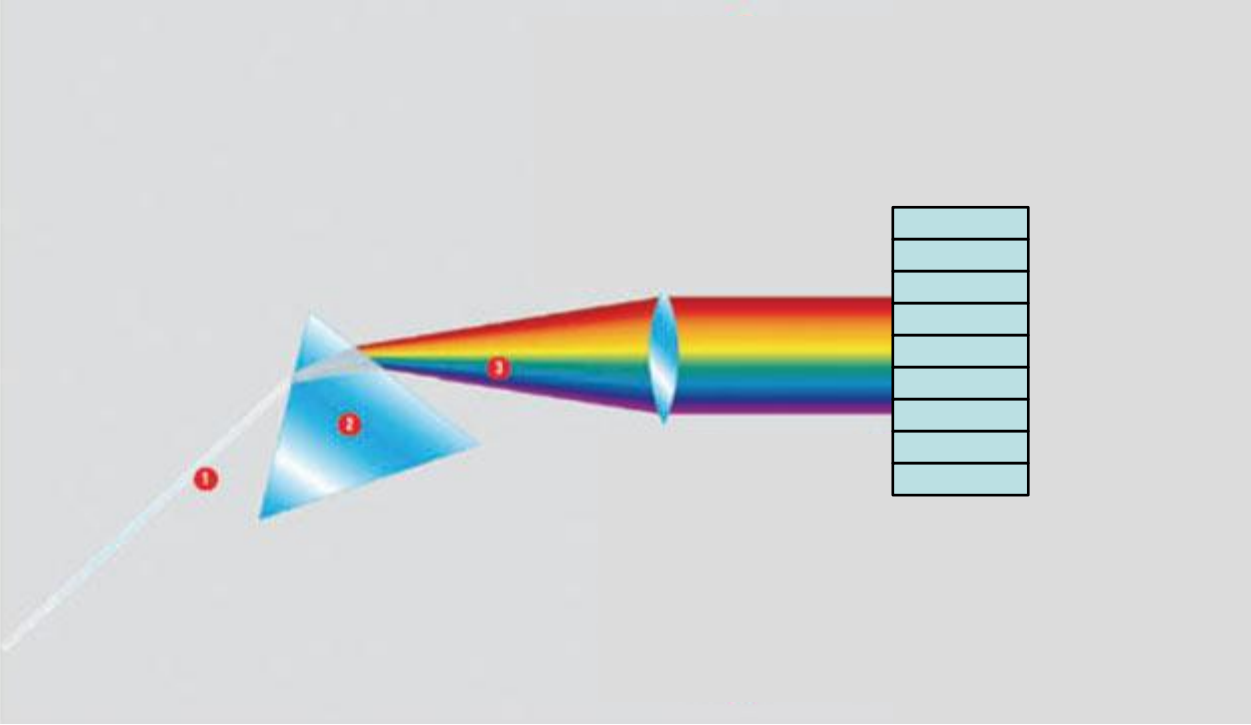
Spectral Detection



Prism + Adjustable Slits: Leica SP microscopes

Doing more with your photons

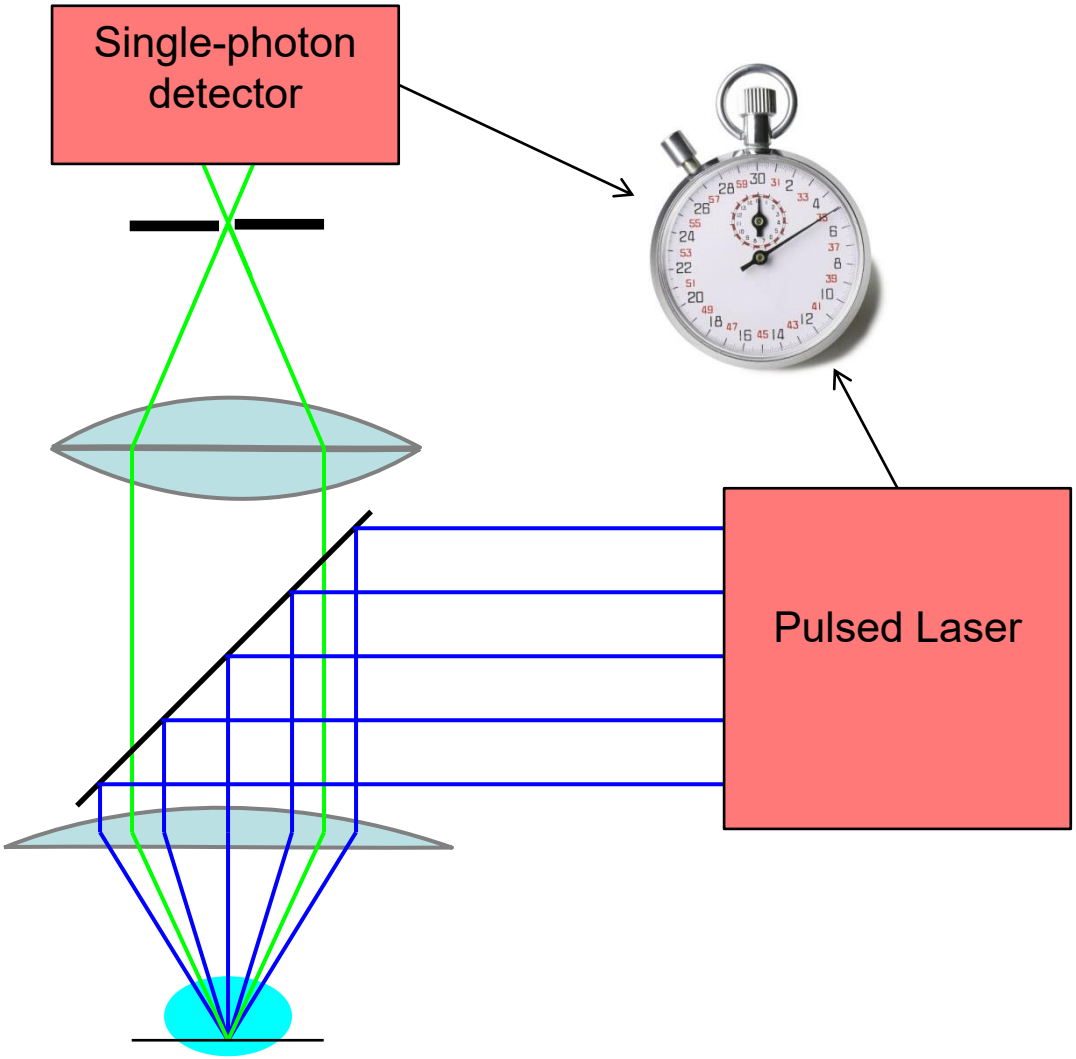
Spectral Detection



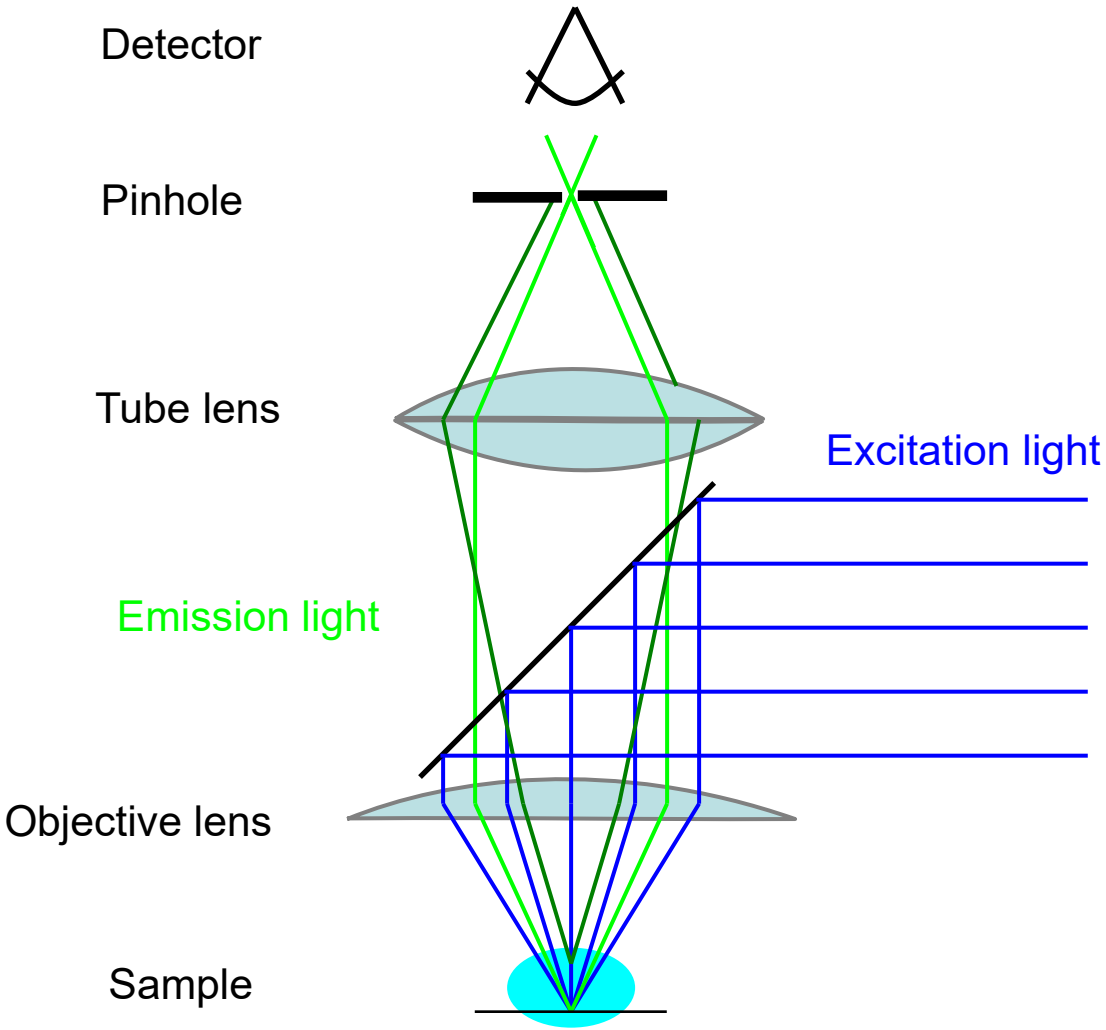
Prism + PMT-array: Zeiss and Nikon

Doing more with your photons

Time-Correlated Single Photon Counting



The confocal microscope: Drawbacks

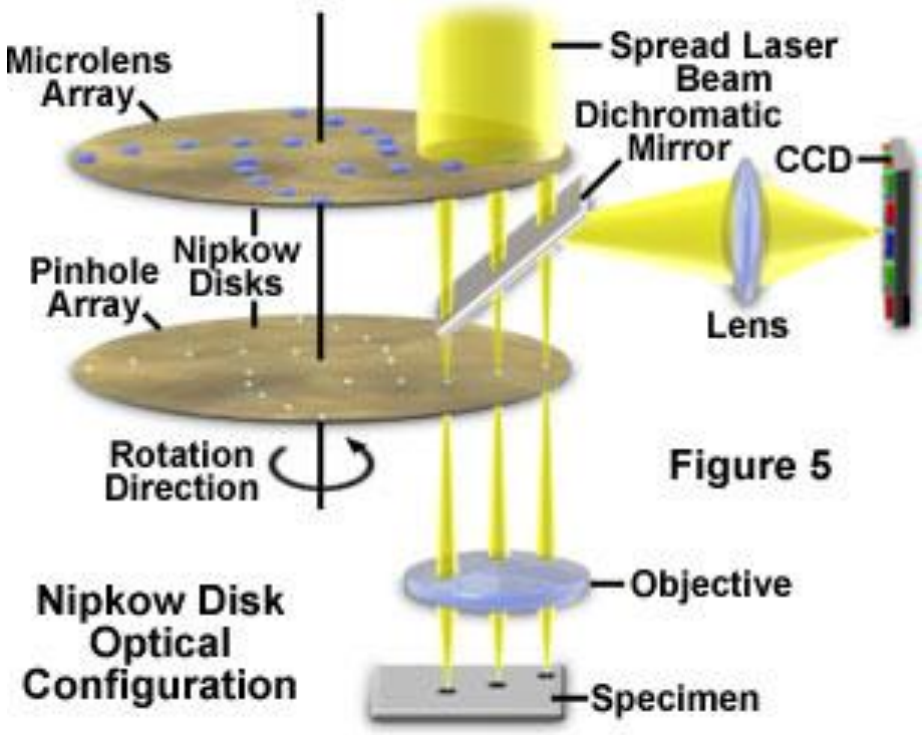


Scan excitation spot point-by-point to build up image

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



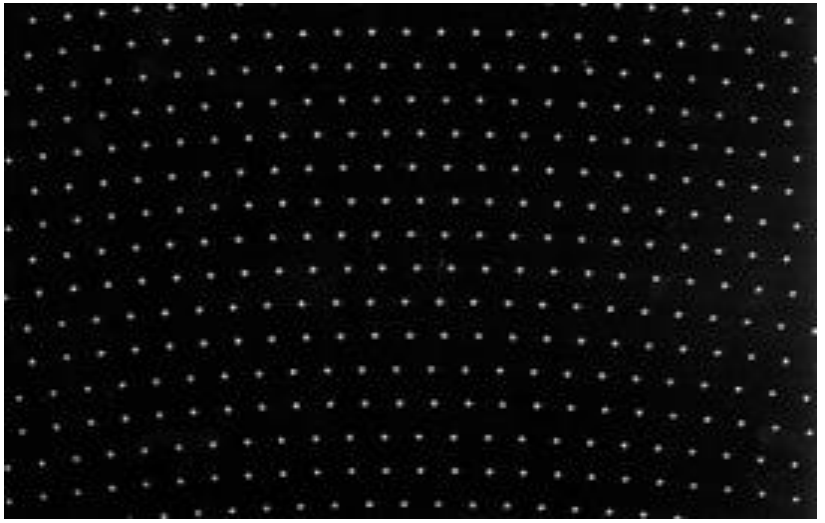
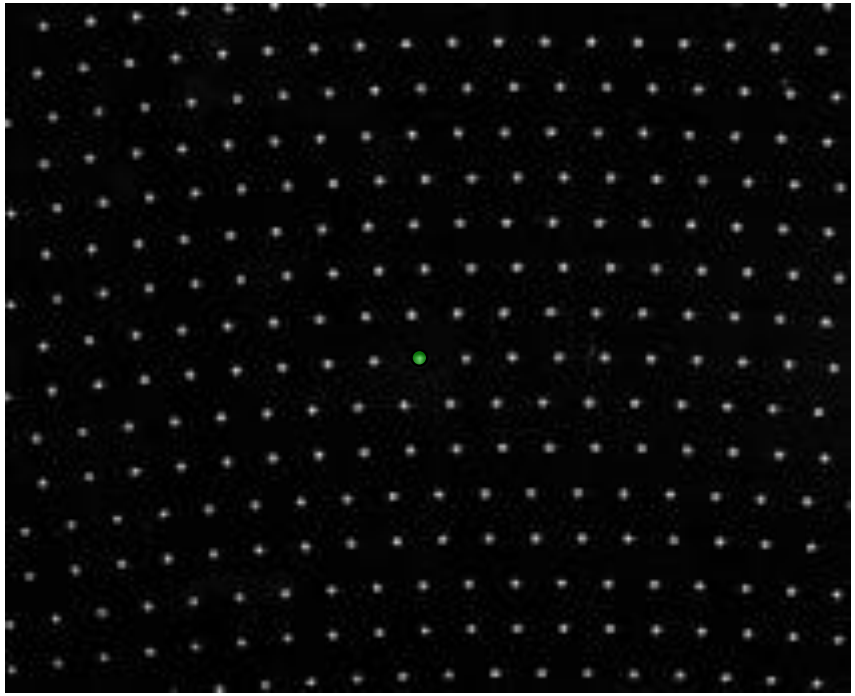


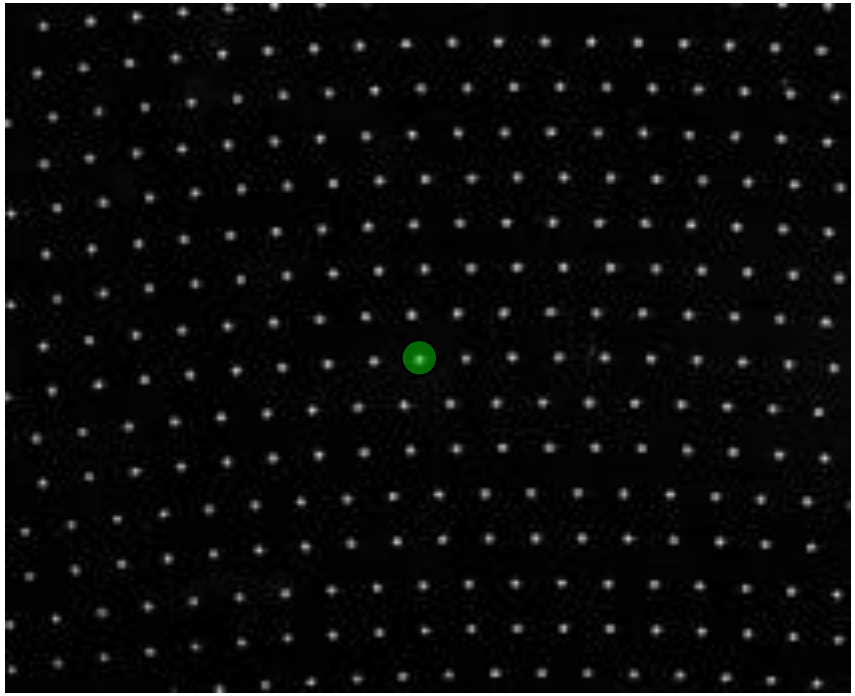
Image with many pinholes at once, so fast

Use CCD as detector, so much higher QE

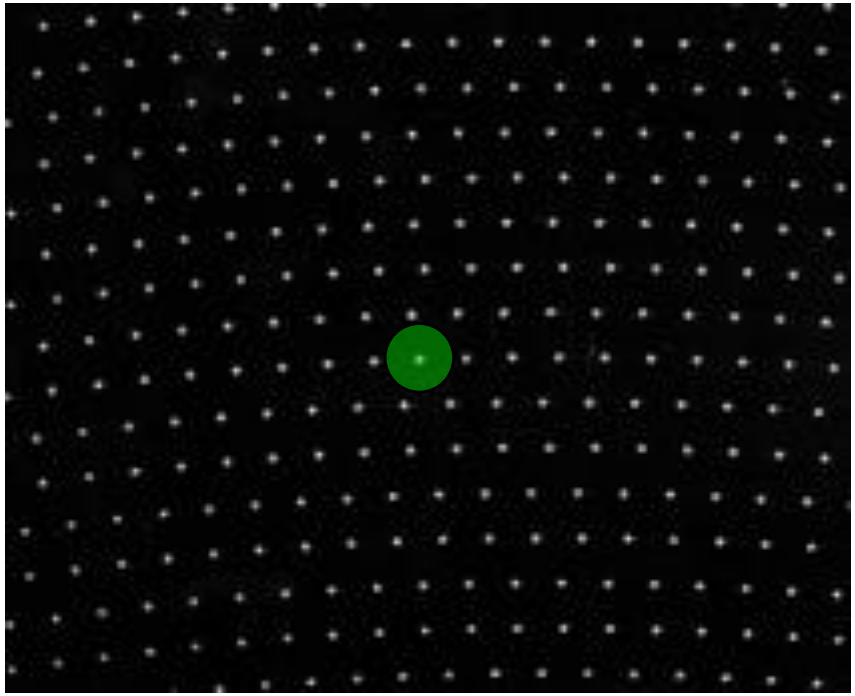
The downside to the spinning disk



The downside to the spinning disk

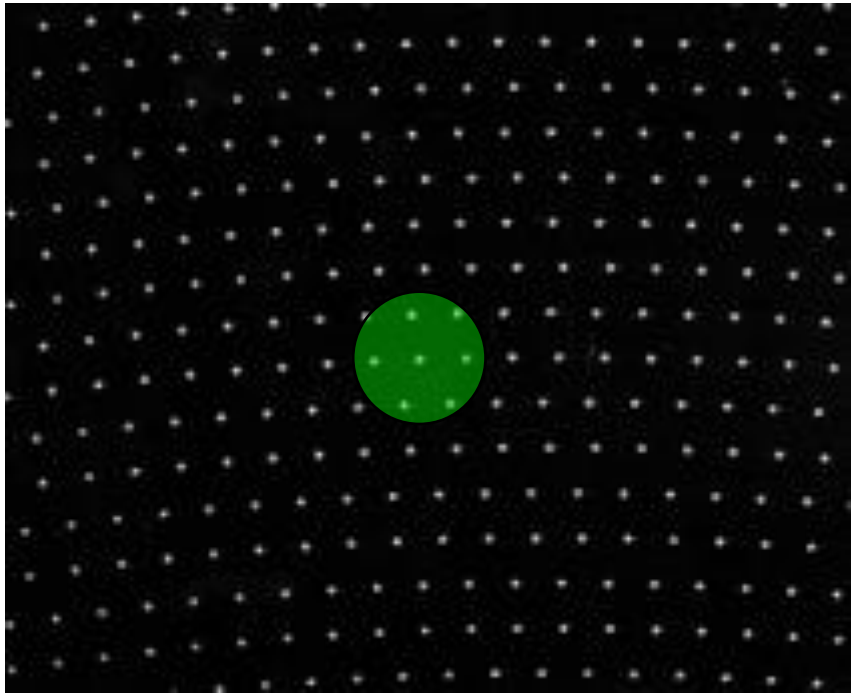


The downside to the spinning disk



The downside to the spinning disk

Limited out of focus rejection

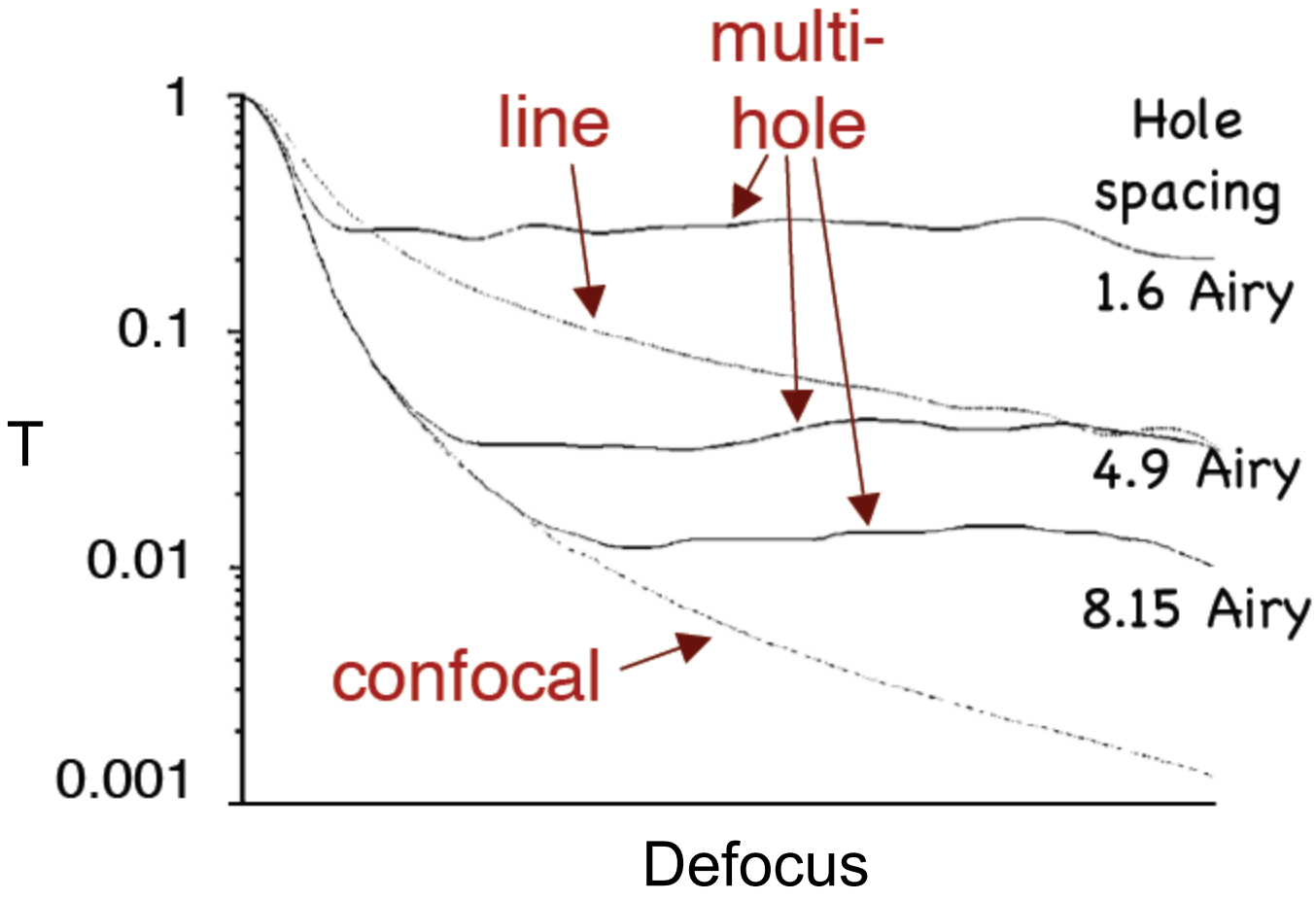


Pros/Cons of spinning disk

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

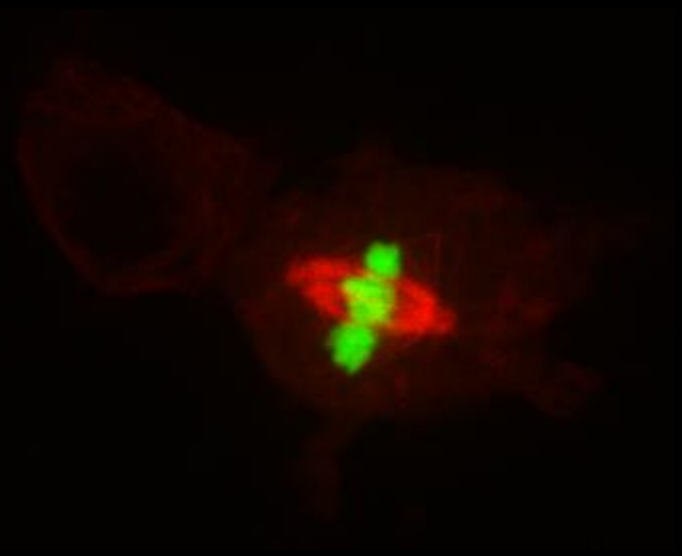
- Fixed pinhole size (usually)
- Small field of view (usually)
- Crosstalk through adjacent pinholes limits sample thickness

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

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

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

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

TIRF

Total Internal Reflection Microscopy

Excite just the fluorophores at the coverslip.

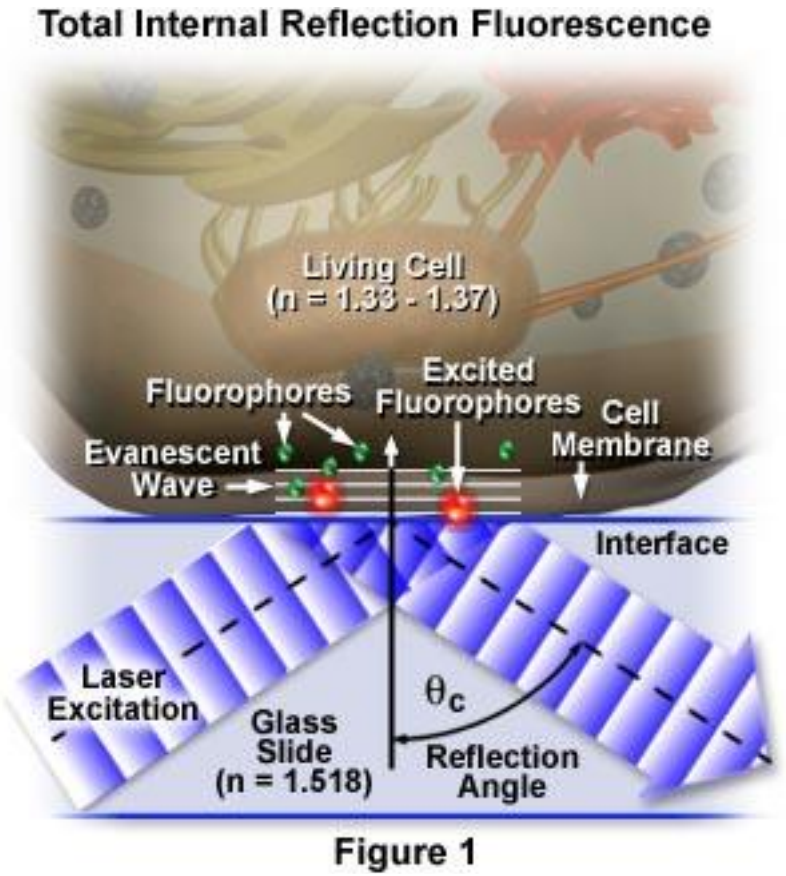


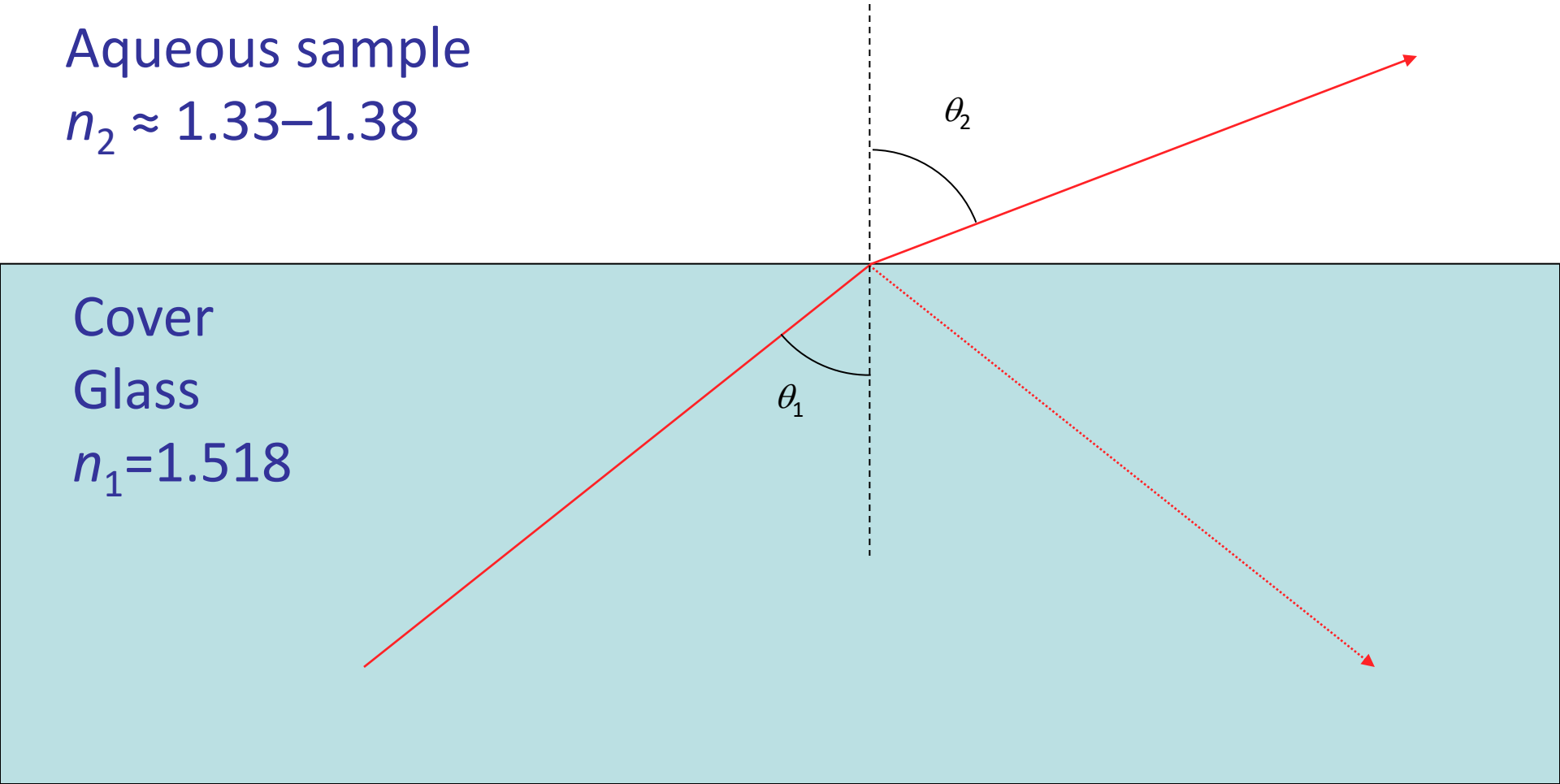
Figure 1

Total Internal Reflection

Snell's Law: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

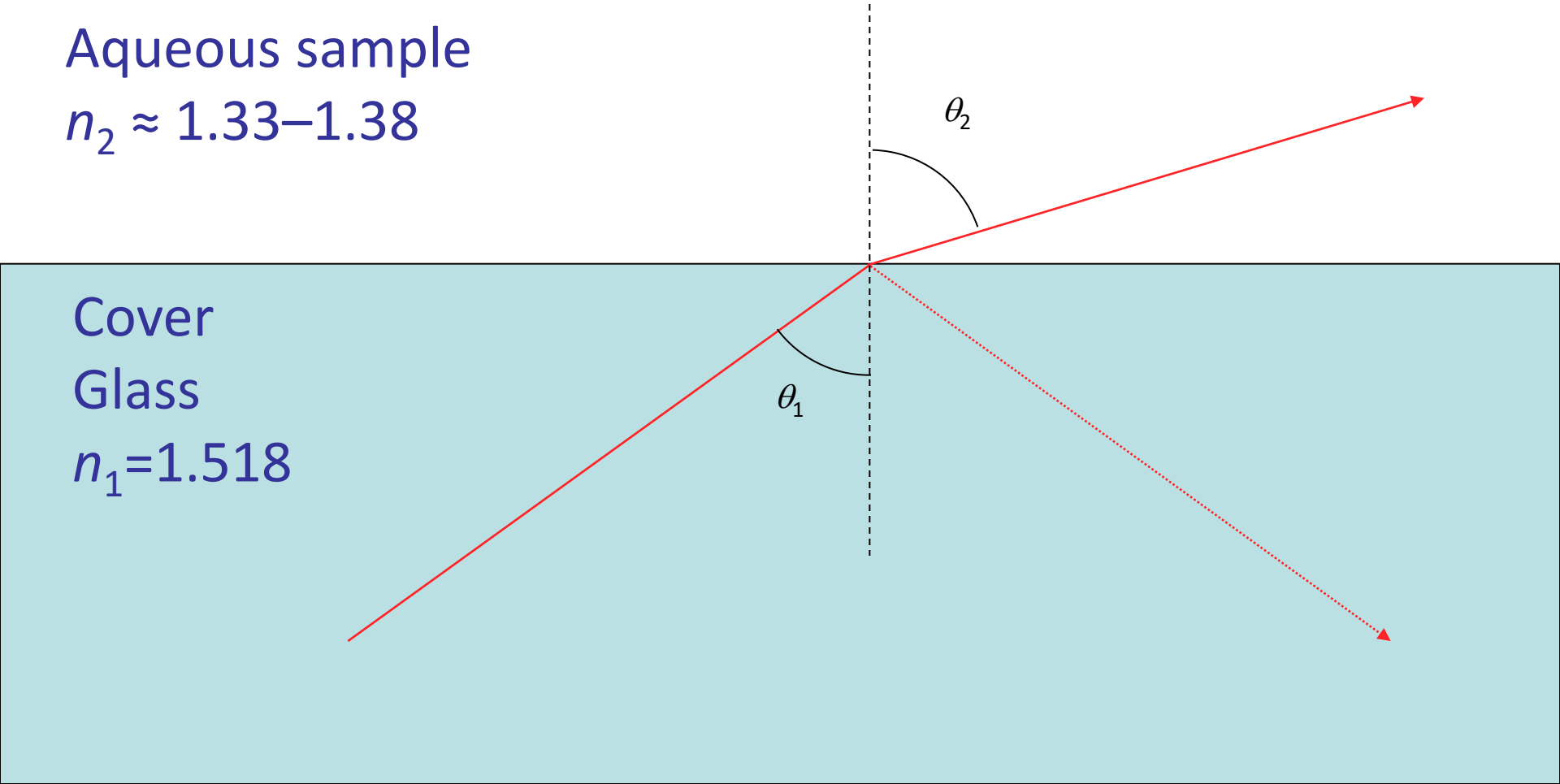
n_1=1.518

Total Internal Reflection

Snell's Law: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

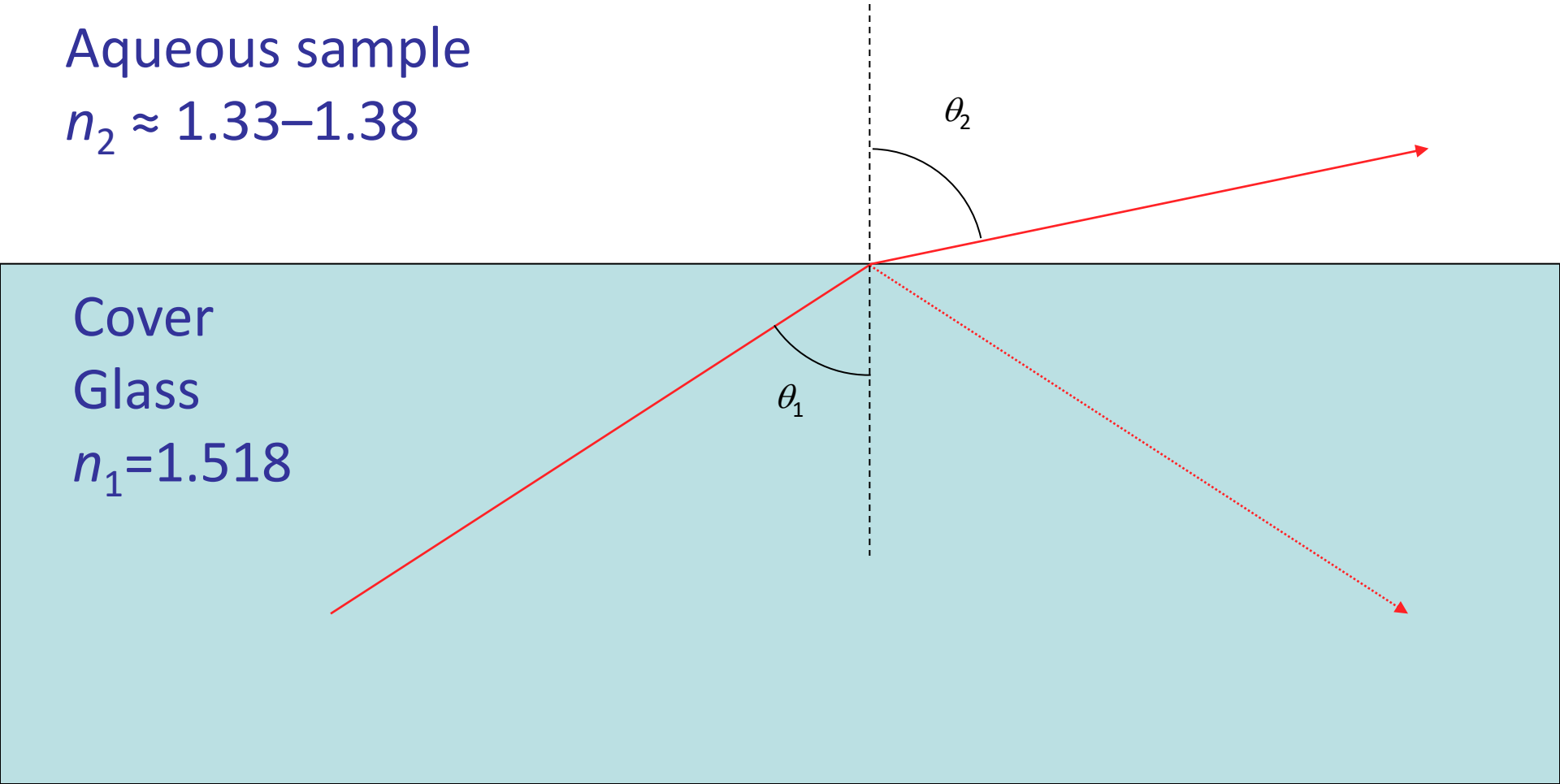
n_1=1.518

Total Internal Reflection

Snell's Law: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

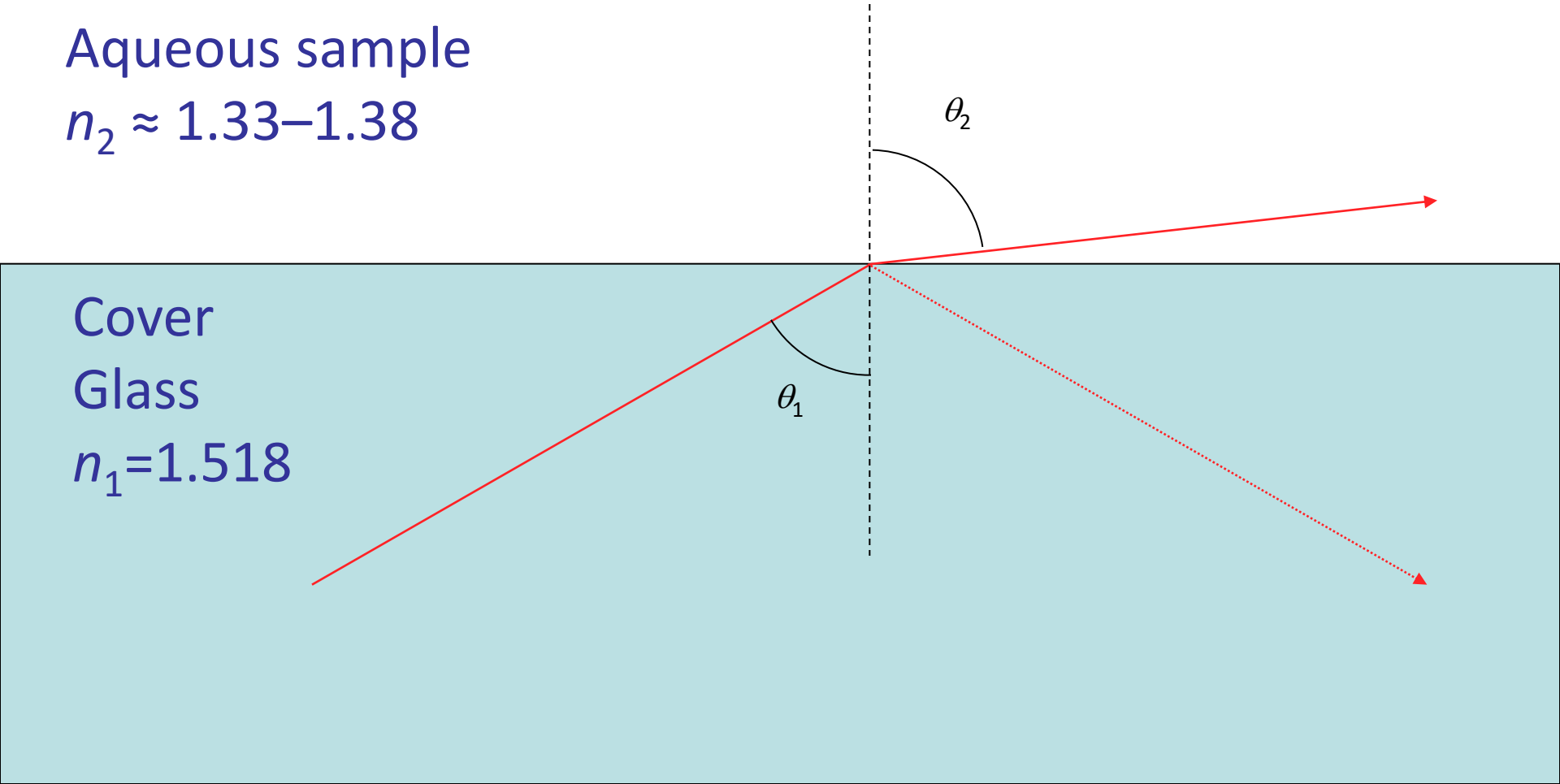
n_1=1.518

Total Internal Reflection

Snell's Law: $n_1 \text{Sin}(\theta_1) = n_2 \text{Sin}(\theta_2)$

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

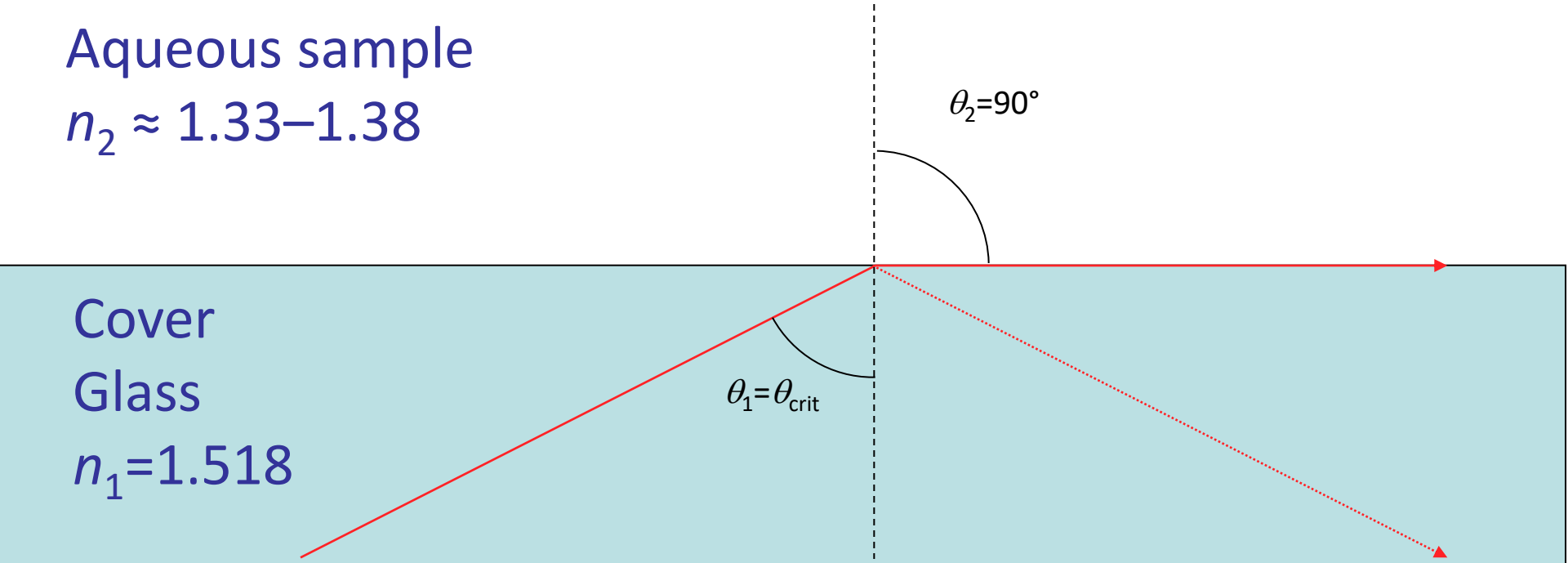
n_1=1.518

Total Internal Reflection

Snell's Law: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

n_1=1.518

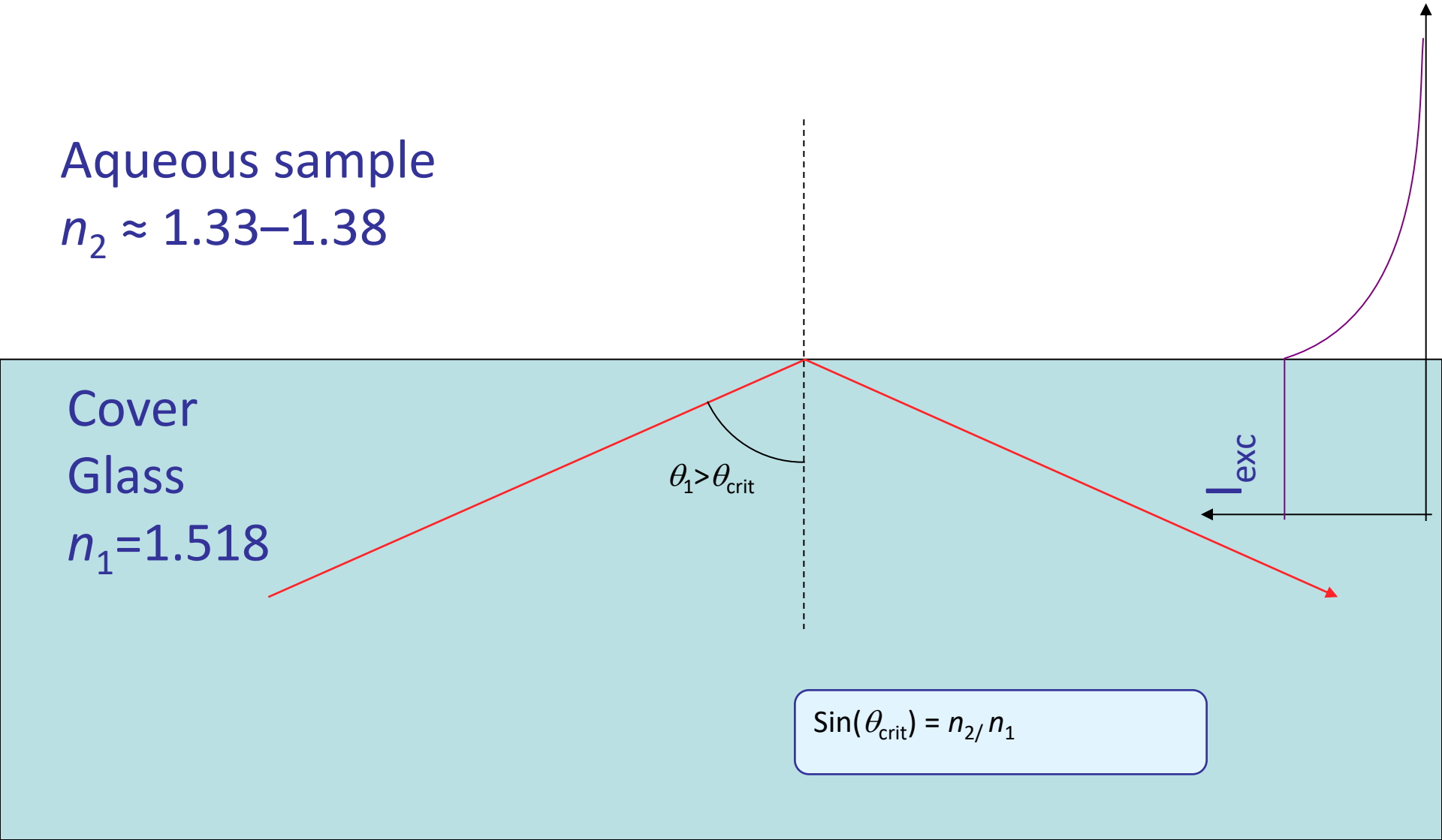
$\sin(\theta_{crit}) = n_2 / n_1$

Total Internal Reflection

Snell's Law: $n_1 \text{Sin}(\theta_1) = n_2 \text{Sin}(\theta_2)$

Aqueous sample

$n_2 \approx 1.33–1.38$



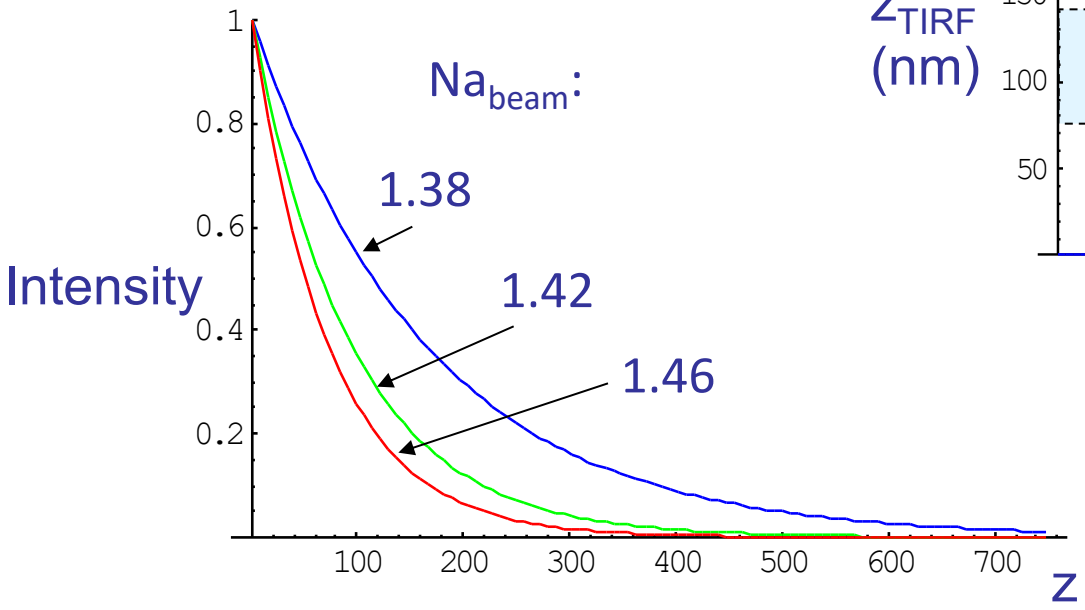
$\text{Sin}(\theta_{crit}) = n_{2/} n_1$

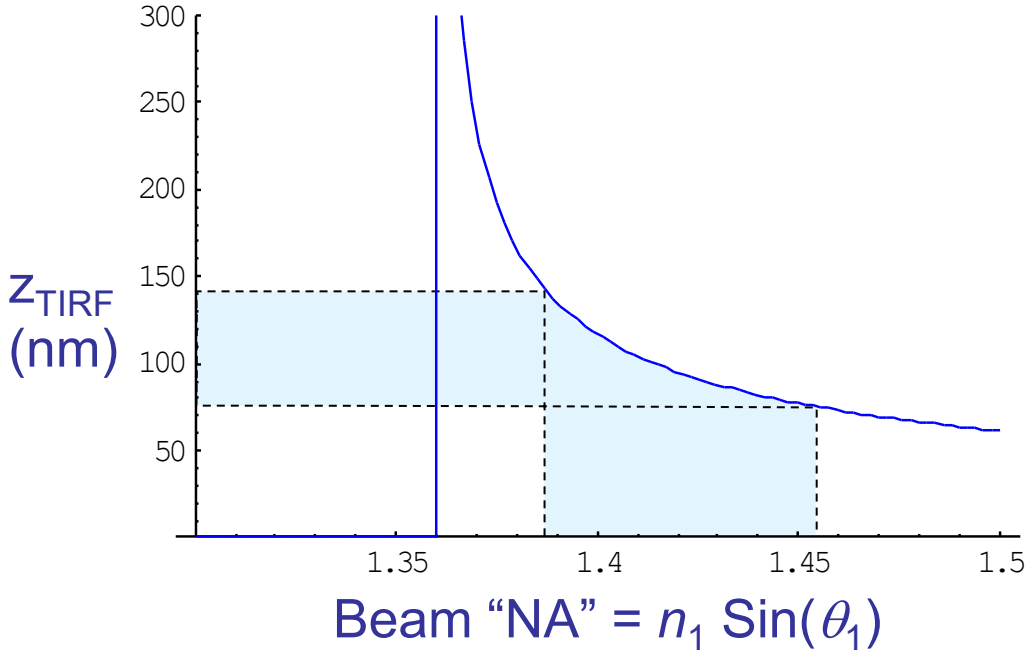
Total Internal Reflection

Decay length vs. angle

$$I \propto e^{-z/z_{TIRF}} = e^{-z\frac{4\pi}{\lambda}\sqrt{n_2{}^2 - n_1{}^2 \,\mathrm{Sin}^2(\theta_1)}}$$

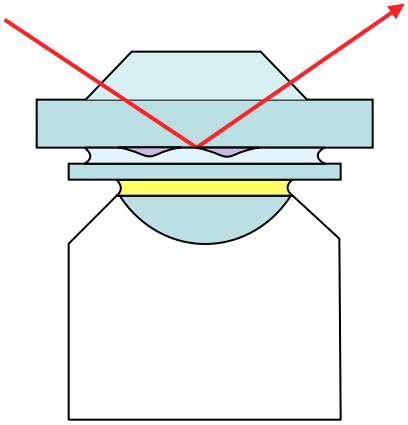
Typical TIRF depth
≈ 75–150 nm





Two forms of TIRF microscopy

Prism coupled

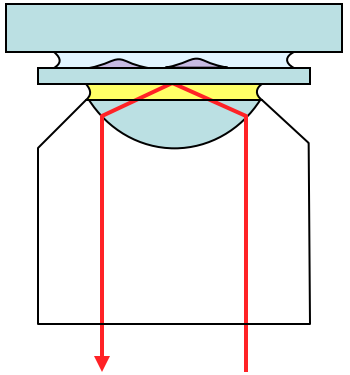


Sample on slide

- No excitation light in emission path
  ⇒ Very low background (if quartz slide)
- Needs separate, external beam path
  ⇒ Harder to align

Through the objective

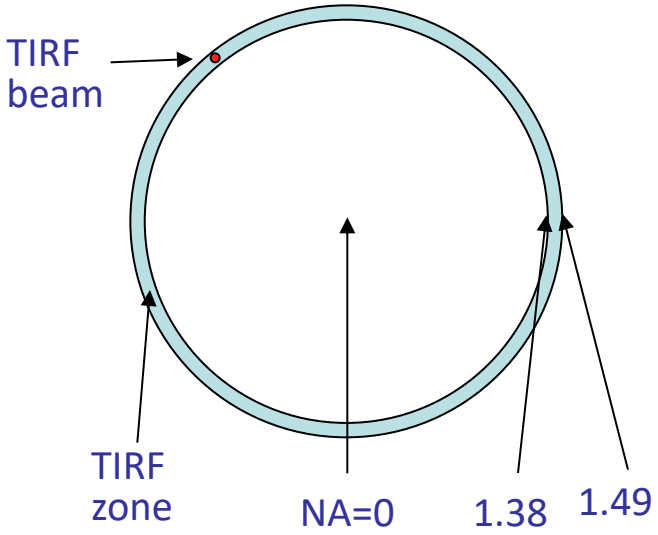
Sample on cover glass



- Easy to align
- Excitation light in emission path
  ⇒ Vulnerable to autofluorescence in the optics
- Requires very high NA

TIRF Objectives

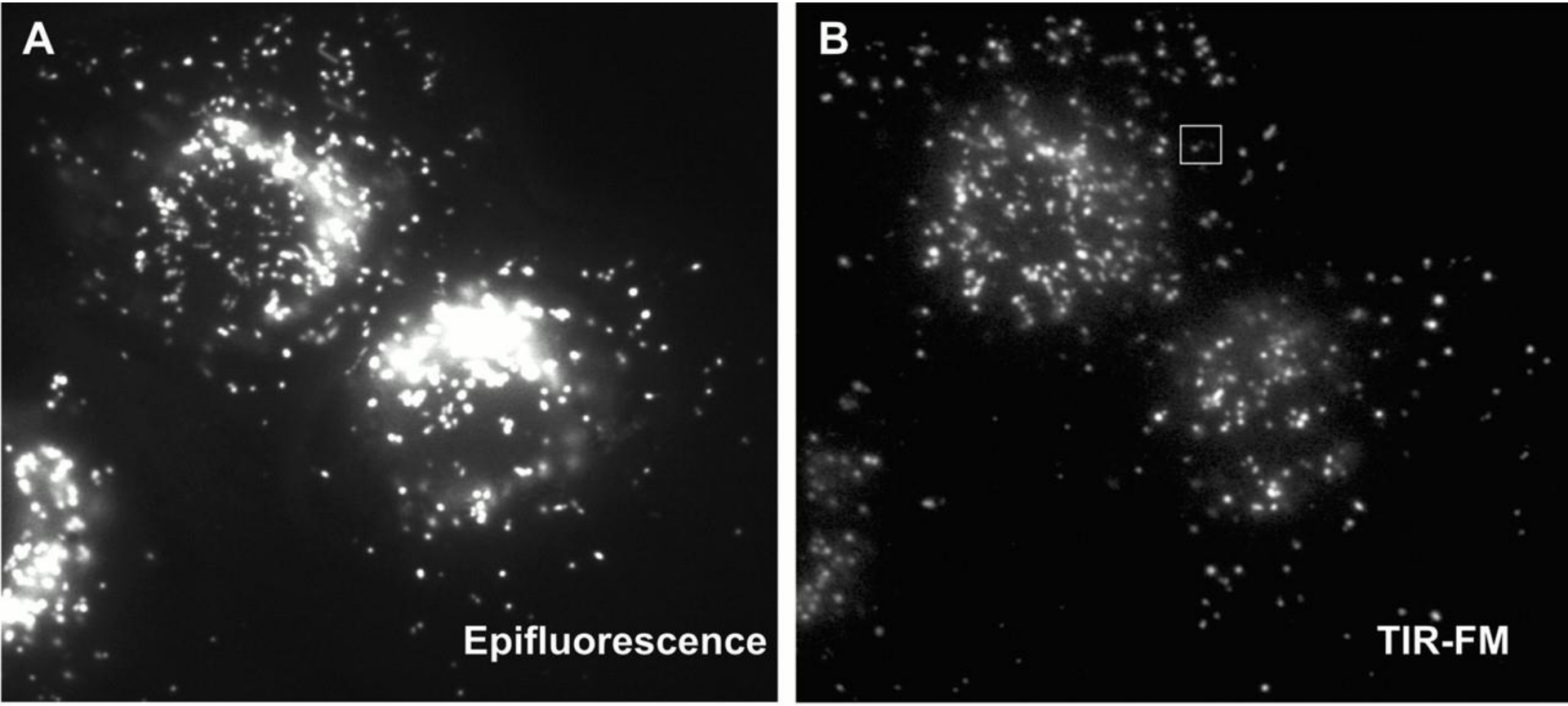
Back focal plane
$r \propto n \sin(\theta)$ ~ "NA"



Typical NA 1.45–1.49

Extreme example: Olympus NA 1.65
Requires special high-index cover glass
and (volatile, toxic) immersion fluid

Epifluorescence vs. TIRF



Jaiswal et al 2002; cells loaded with FITC-dextran

Resources

http://www.microscopyu.com

http://micro.magnet.fsu.edu

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

http://www.hamamatsu.com/resources/pdf/etd/PMT_handbook_v3aE.pdf

Acknowledgements

Steve Ross, Mats Gustafsson