

## Optical Sectioning II: How not to be seen

# TIRF

## Total Internal Reflection Microscopy

Excite just the fluorophores at the coverslip.

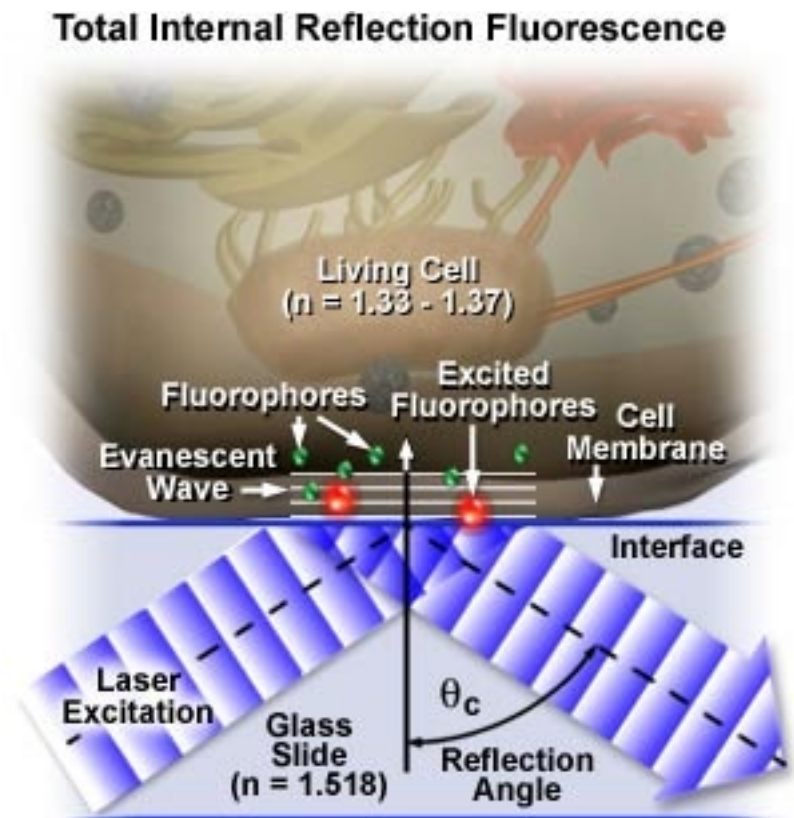


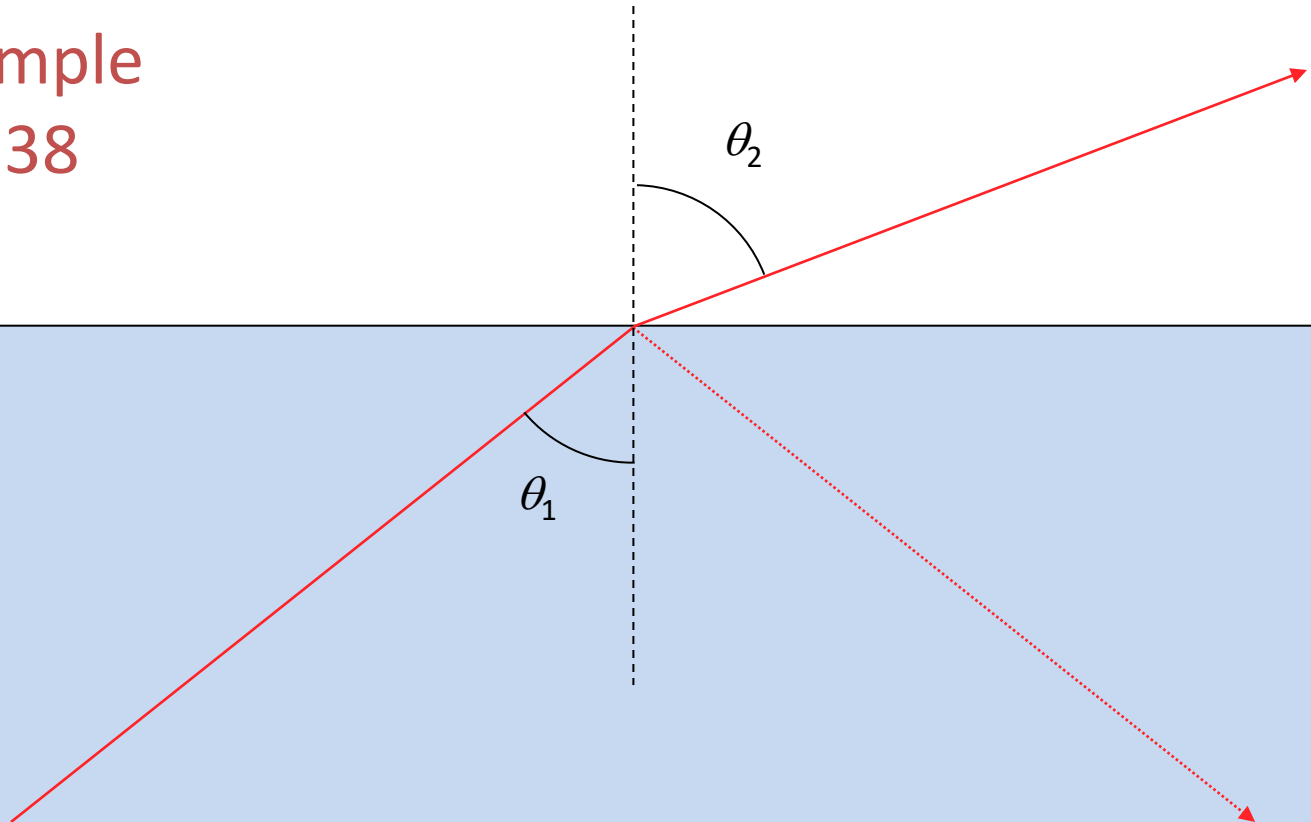
Figure 1

# Total Internal Reflection

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

Aqueous sample  
 $n_2 \approx 1.33\text{--}1.38$

Cover  
Glass  
 $n_1 = 1.518$

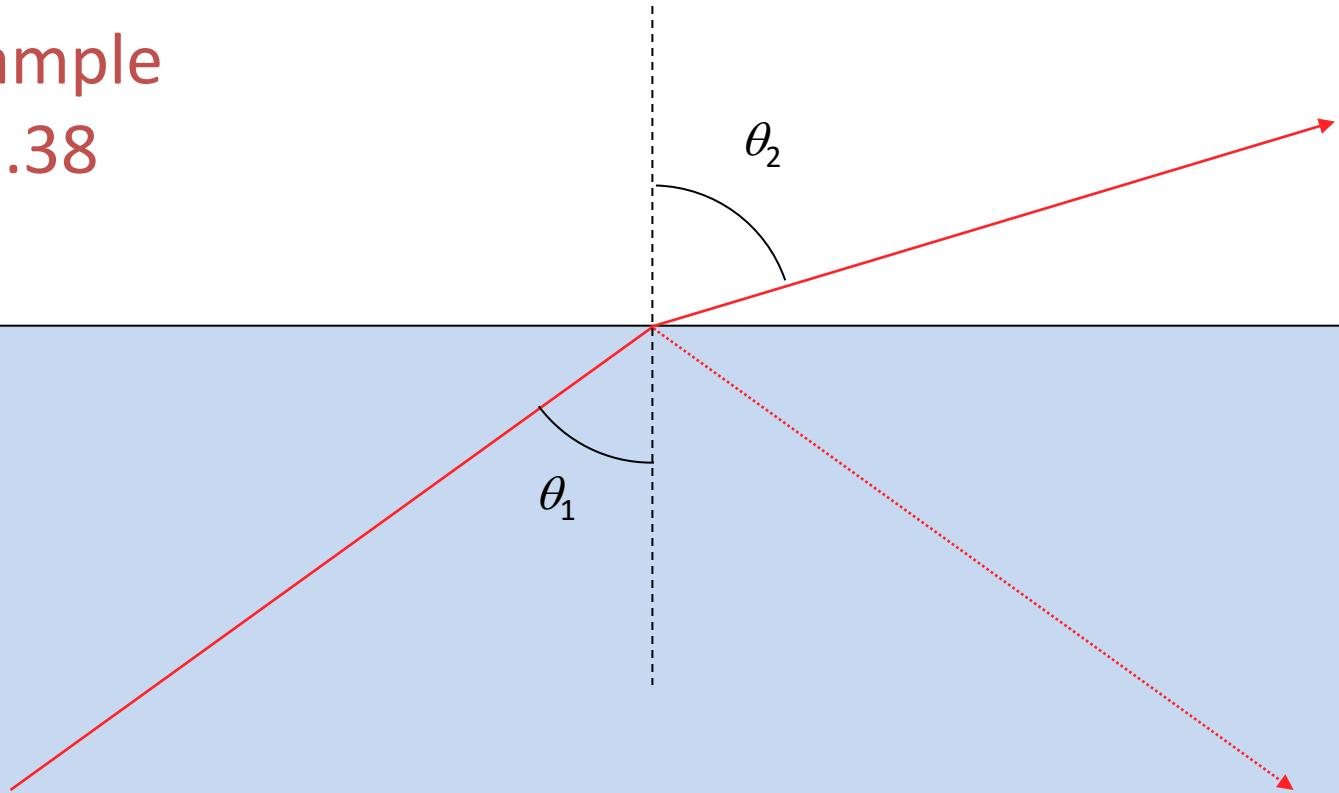


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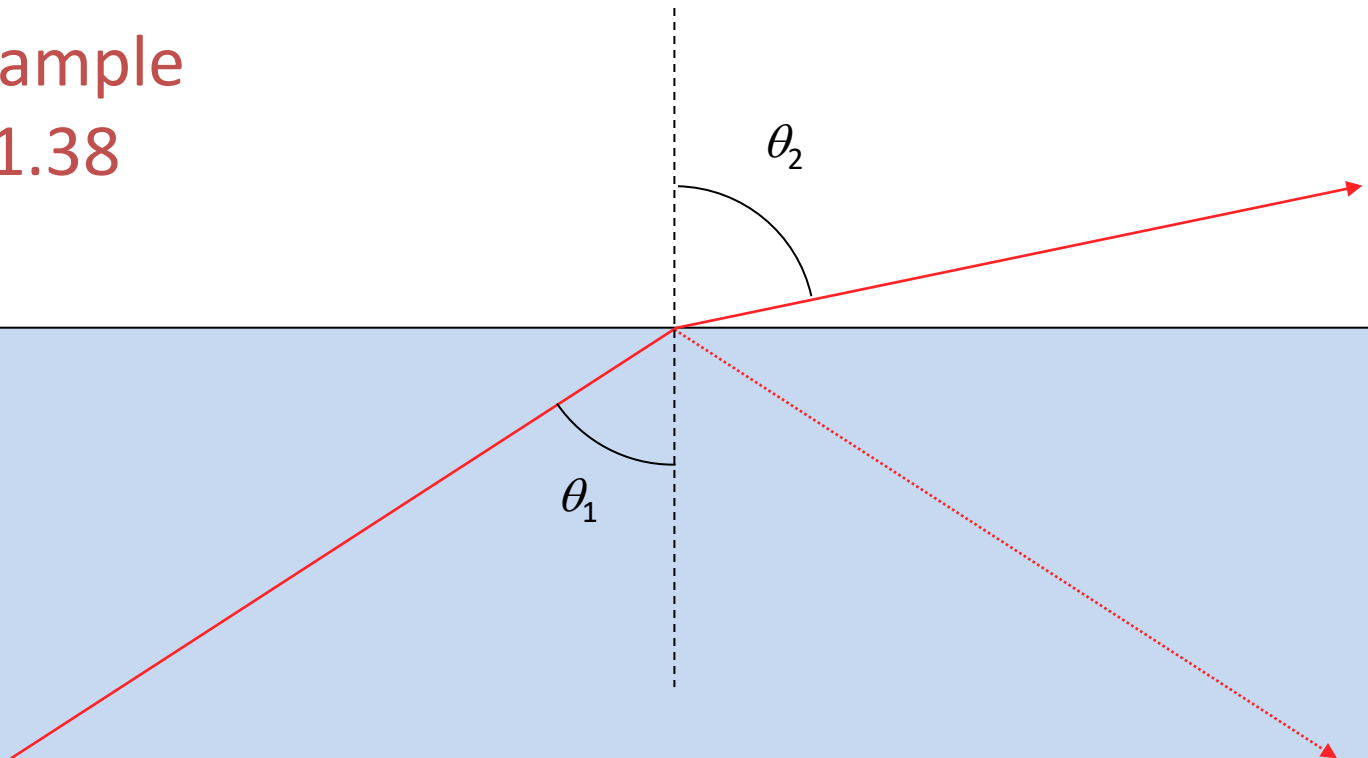


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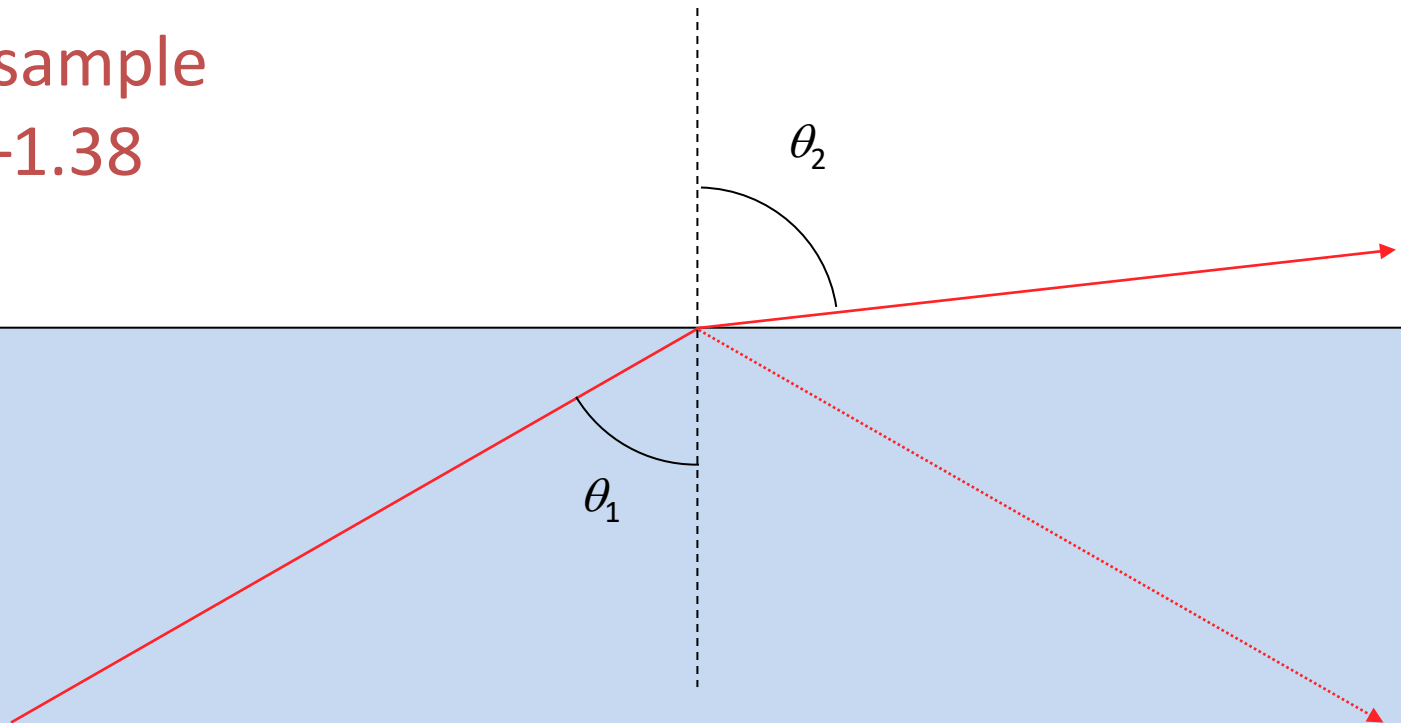


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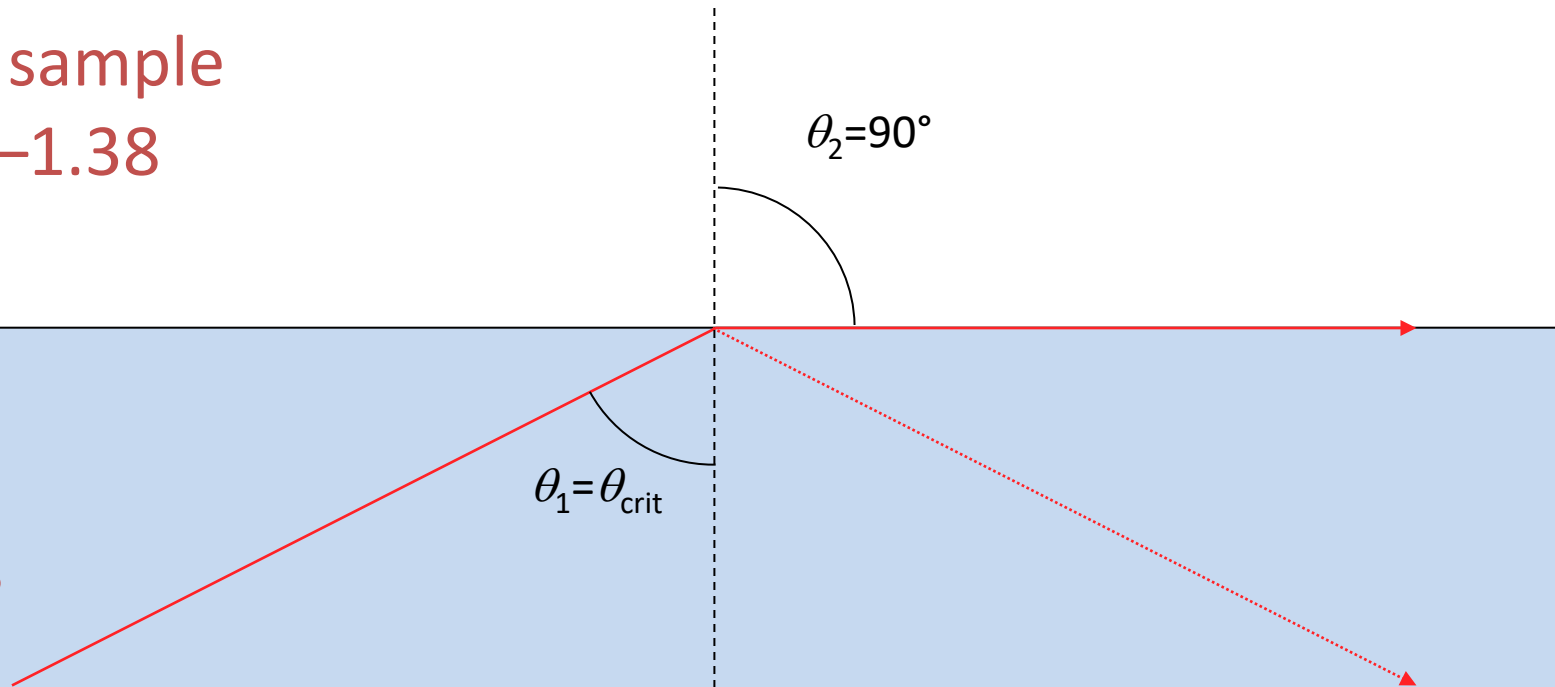


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Cover  
Glass  
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$$\sin(\theta_{\text{crit}}) = n_2 / n_1$$

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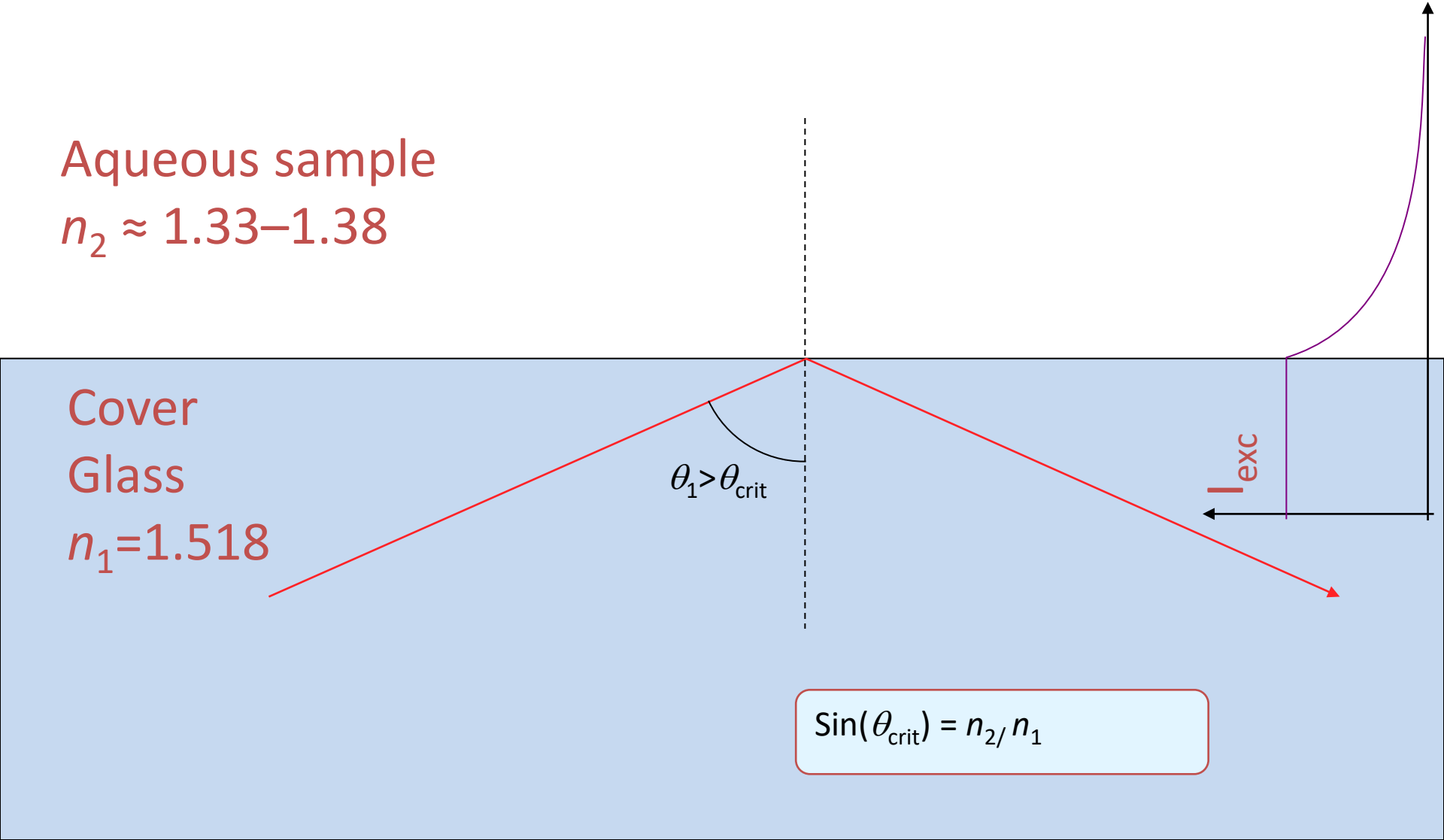
Aqueous sample  
 $n_2 \approx 1.33\text{--}1.38$

Cover  
Glass  
 $n_1 = 1.518$

$$\theta_1 > \theta_{\text{crit}}$$

$I_{\text{exc}}$

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



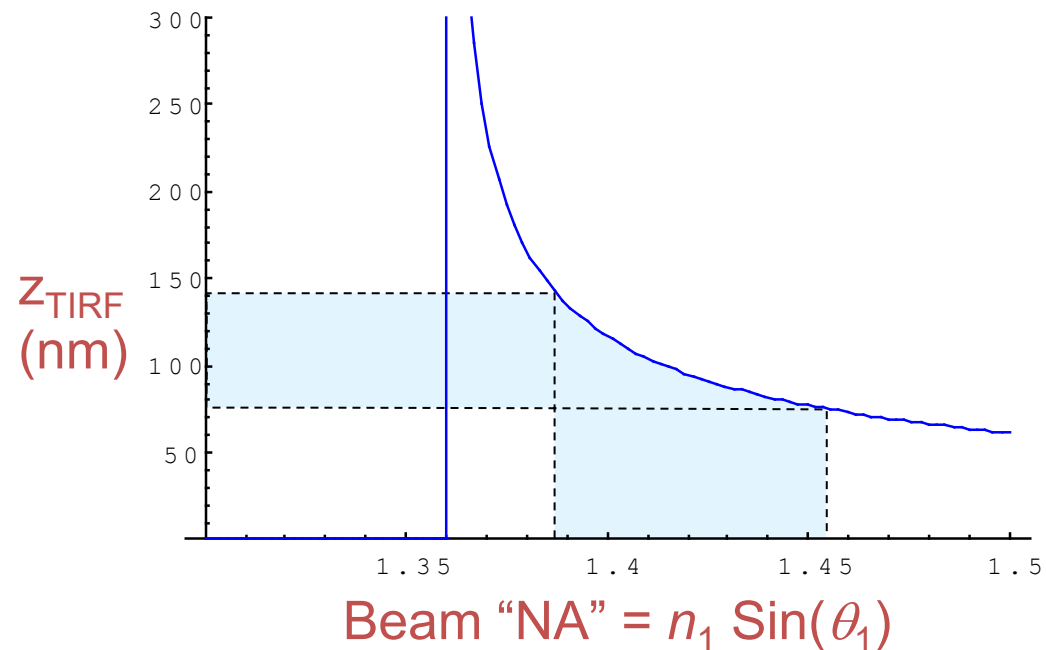
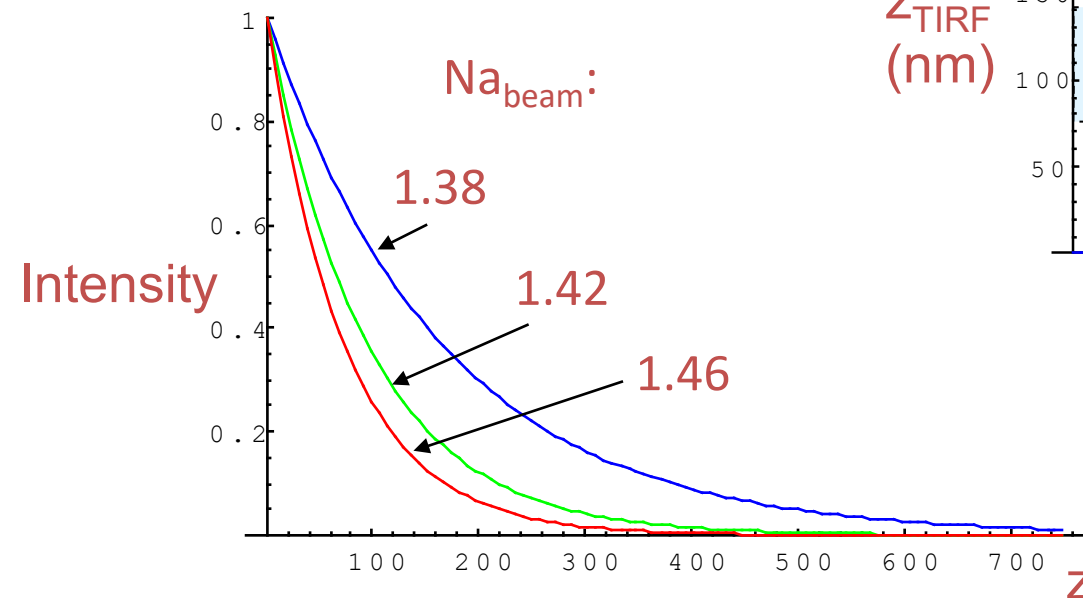


# Total Internal Reflection

## Decay length vs. angle

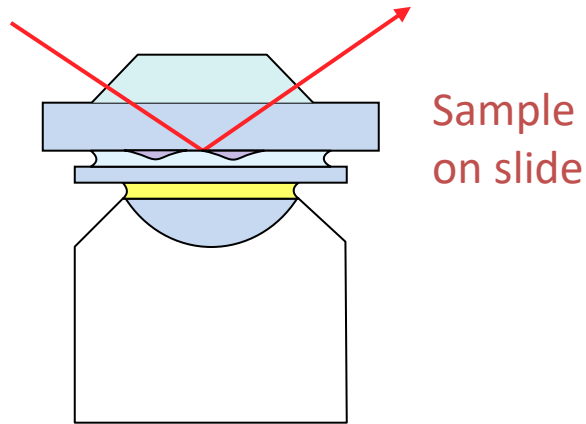
$$I \propto e^{-z/z_{TIRF}} = e^{-z \frac{4\pi}{\lambda} \sqrt{n_2^2 - n_1^2 \sin^2(\theta_1)}}$$

Typical TIRF depth  
 $\approx 75\text{--}150\text{ nm}$



# Two forms of TIRF microscopy

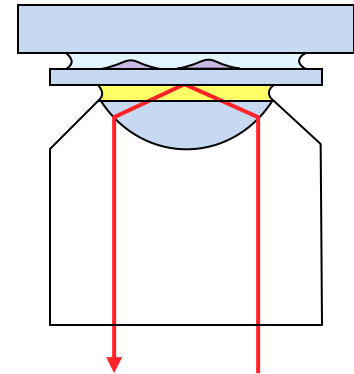
## Prism coupled



- 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

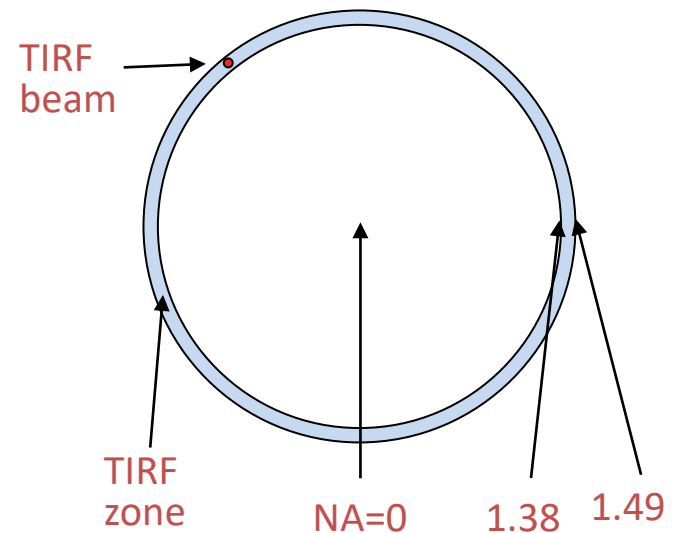


- Easy to align
- Excitation light in emission path  
⇒ Vulnerable to autofluorescence in the optics
- Requires very high NA

# TIRF Objectives



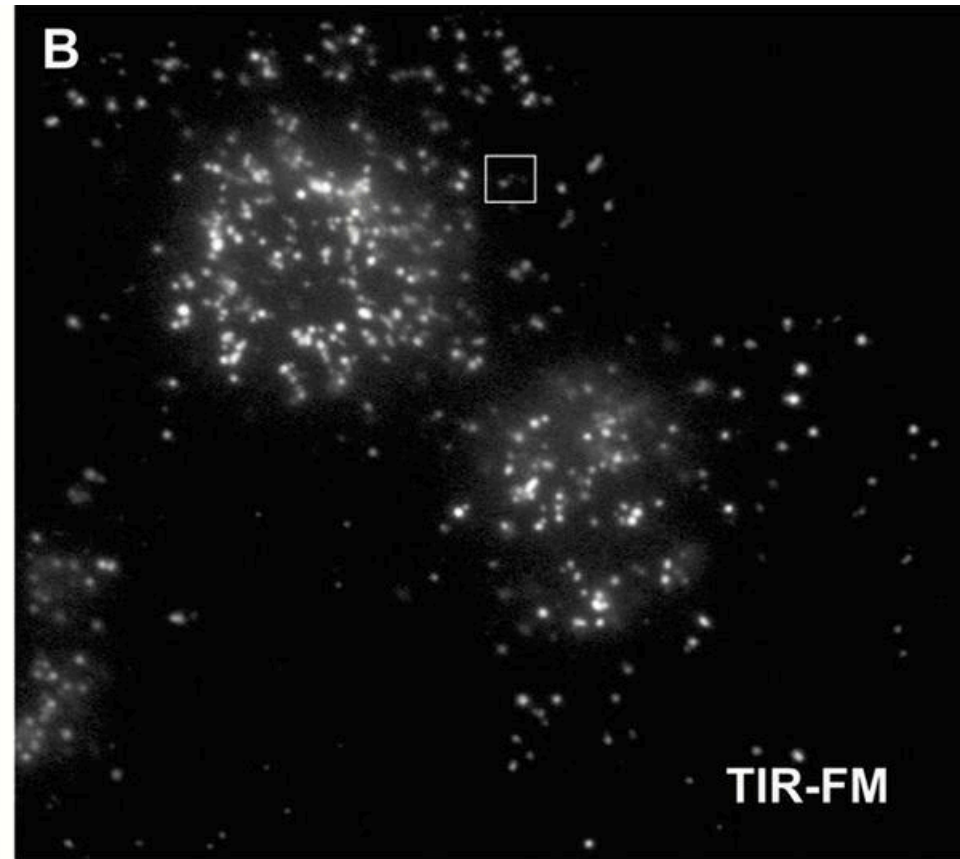
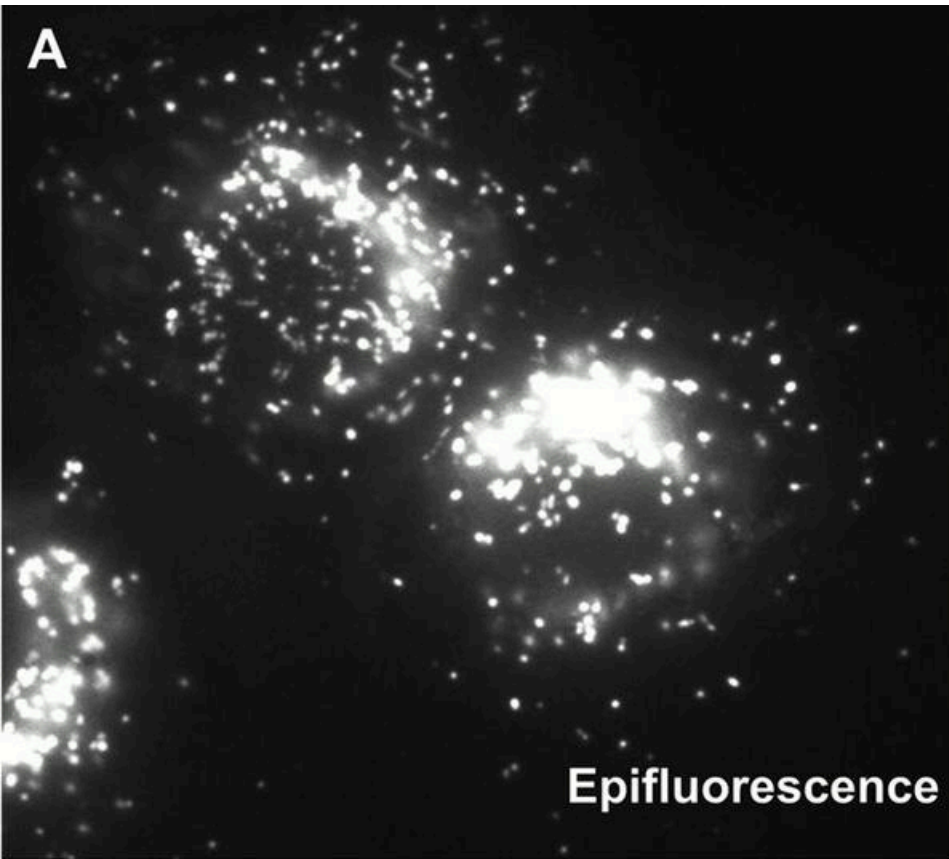
Back focal plane  
 $r \propto n \sin(\theta) \sim \text{"NA"}$



Typical NA 1.45–1.49

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

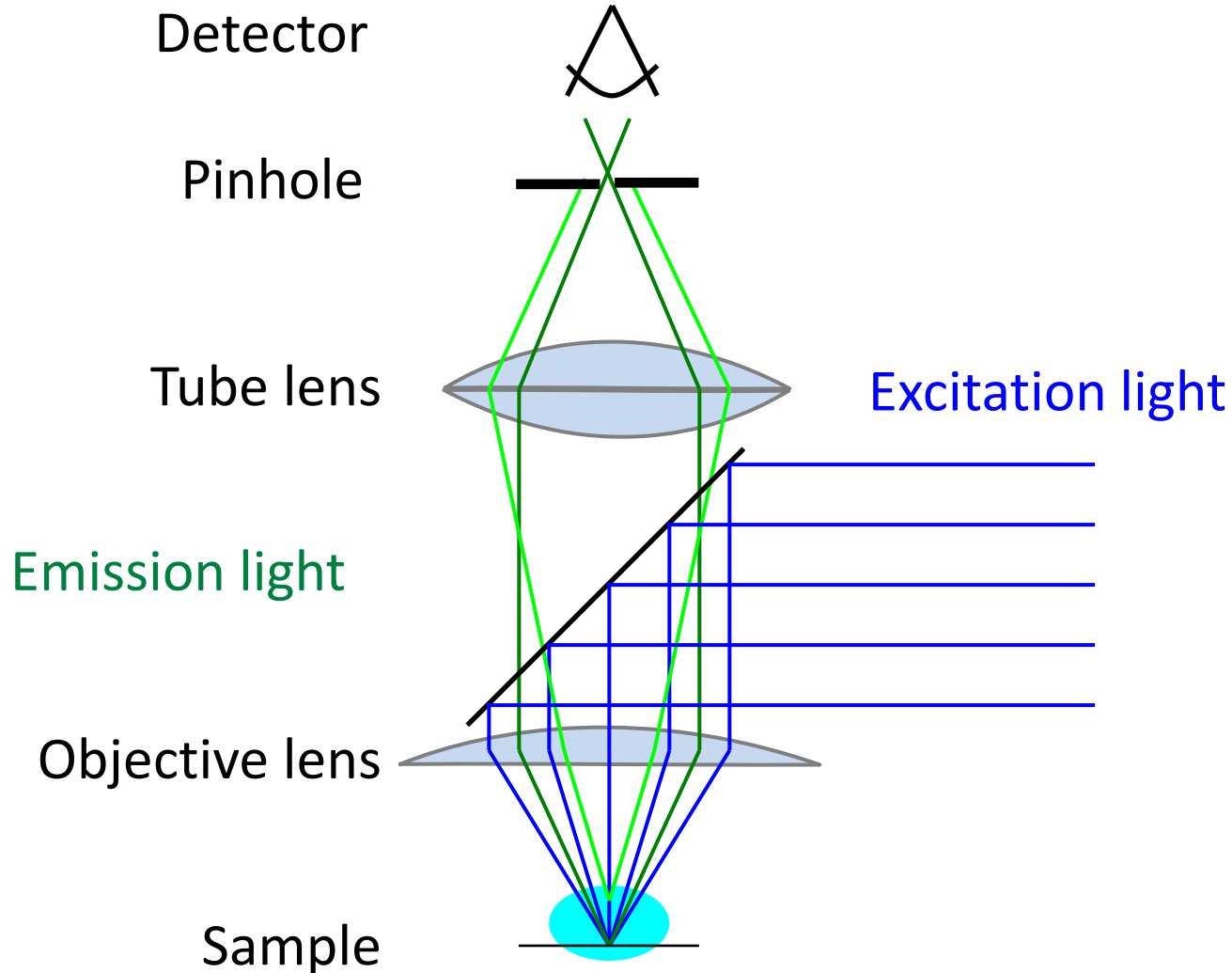
# Epifluorescence vs. TIRF



Jaiswal et al 2002; cells loaded with FITC-dextran

# The Confocal Microscope, Redux

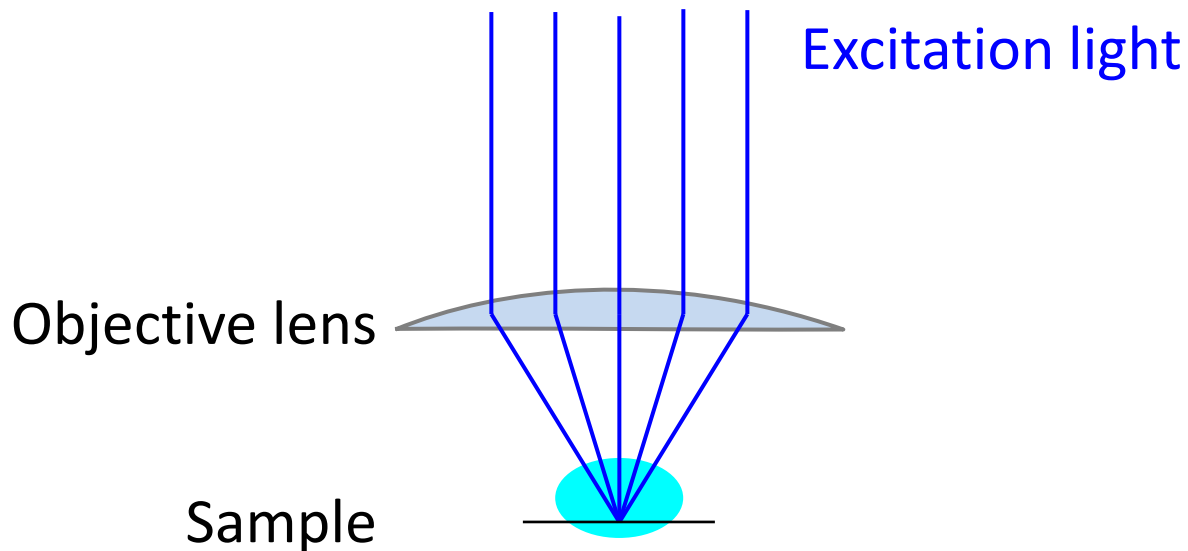
What limits the imaging depth?



# The Confocal Microscope

What limits the imaging depth?

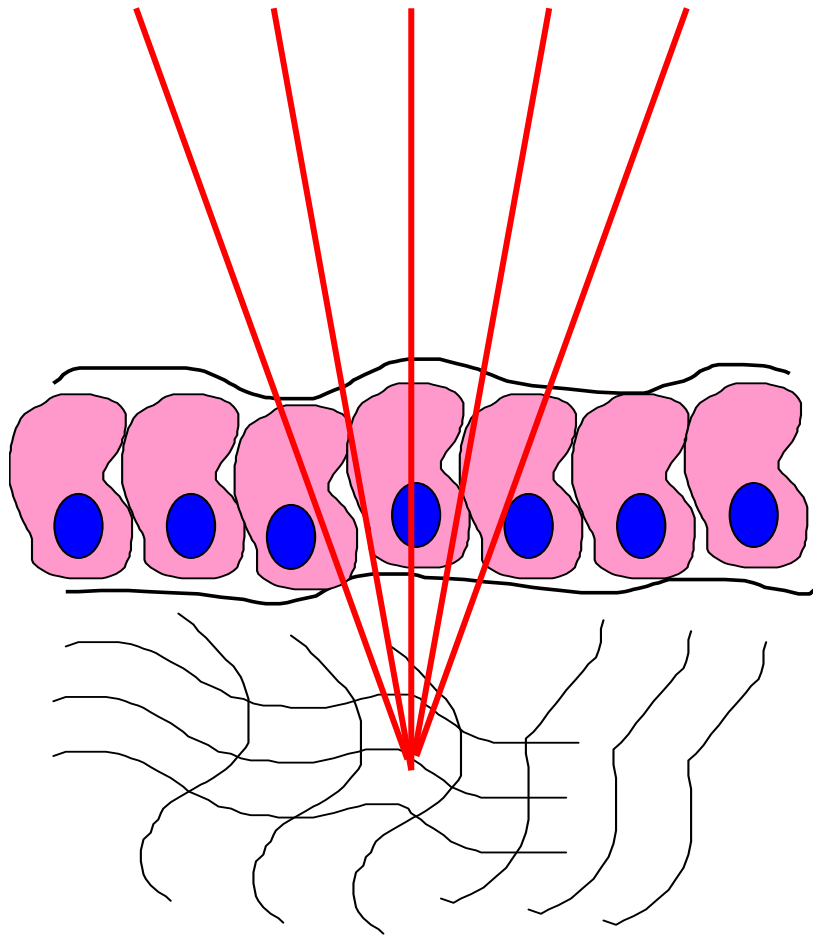
1. Physical limitations – objective working distance
2. Tissue penetration depth





# What limits tissue penetration depth?

Ideal Case

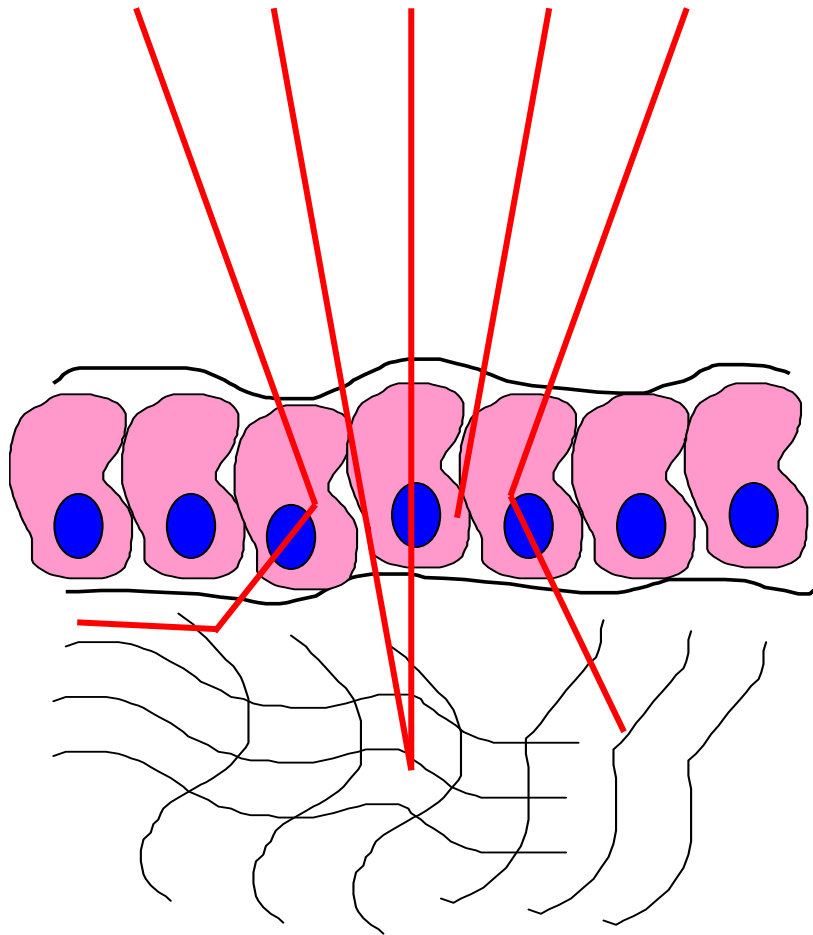




# What limits tissue penetration depth?

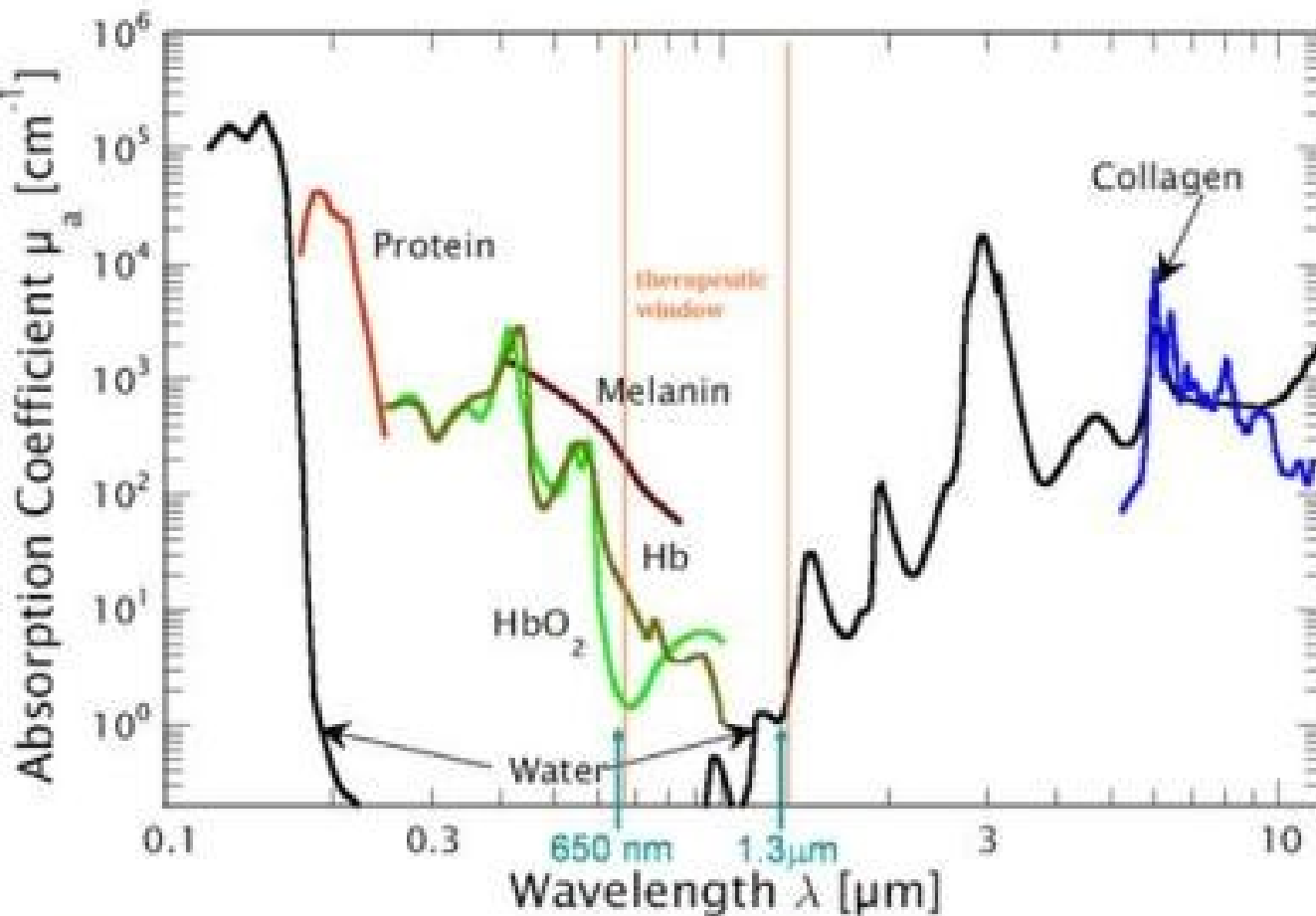
Absorption

Scattering





# Absorption of common biological molecules



# Scattering

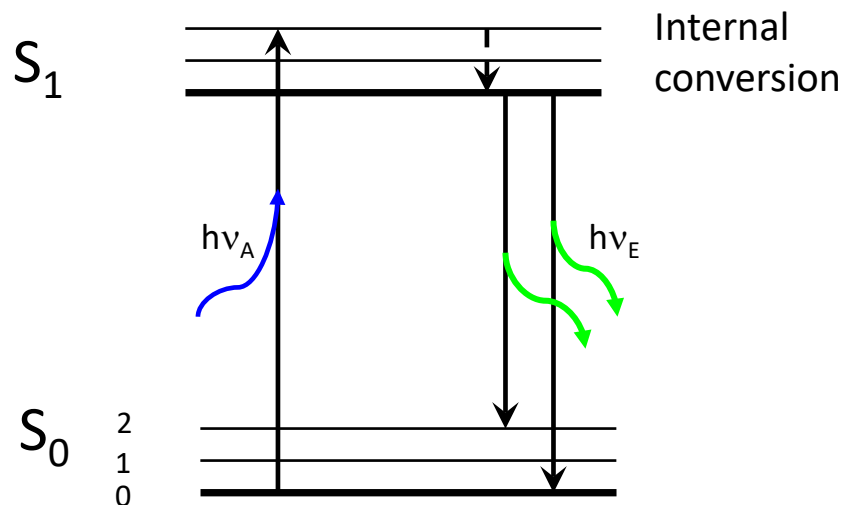
- The amount of light scattered scales as  $1/\lambda^4$  (Rayleigh scattering)
- Imaging in the near-infrared minimizes both absorption and scattering

# Imaging in the Infrared

- Can use infrared excited dyes  
(commonly used for whole-animal imaging)
- Or: use two photons to do the work of one and image standard dyes in the infrared

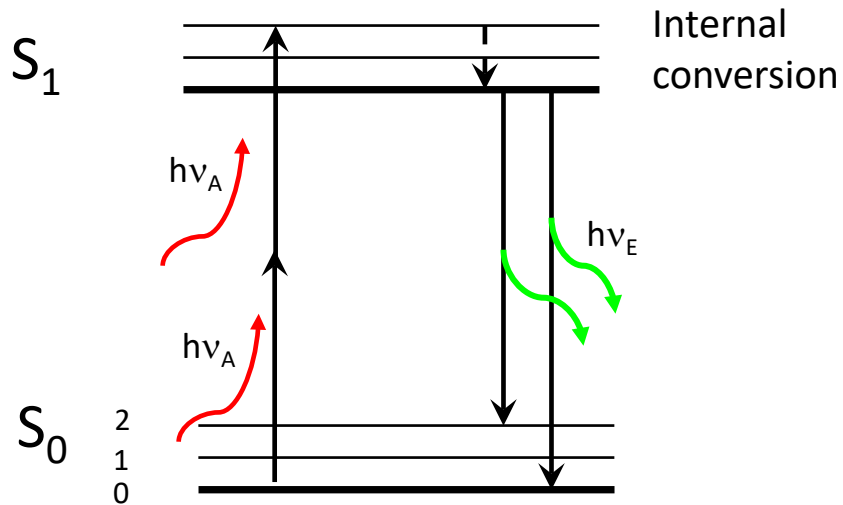
# Conventional (one-photon) excitation

## Jabłoński Diagram



# Two-photon excitation

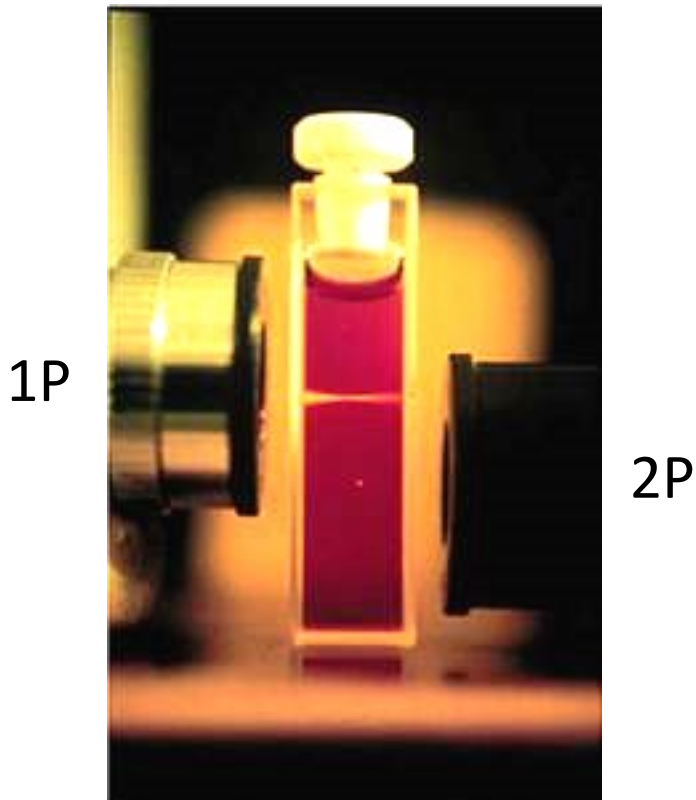
## Jabłoński Diagram



Both photons must arrive nearly simultaneously ( $< 1$  fs)

# Two-photon excitation

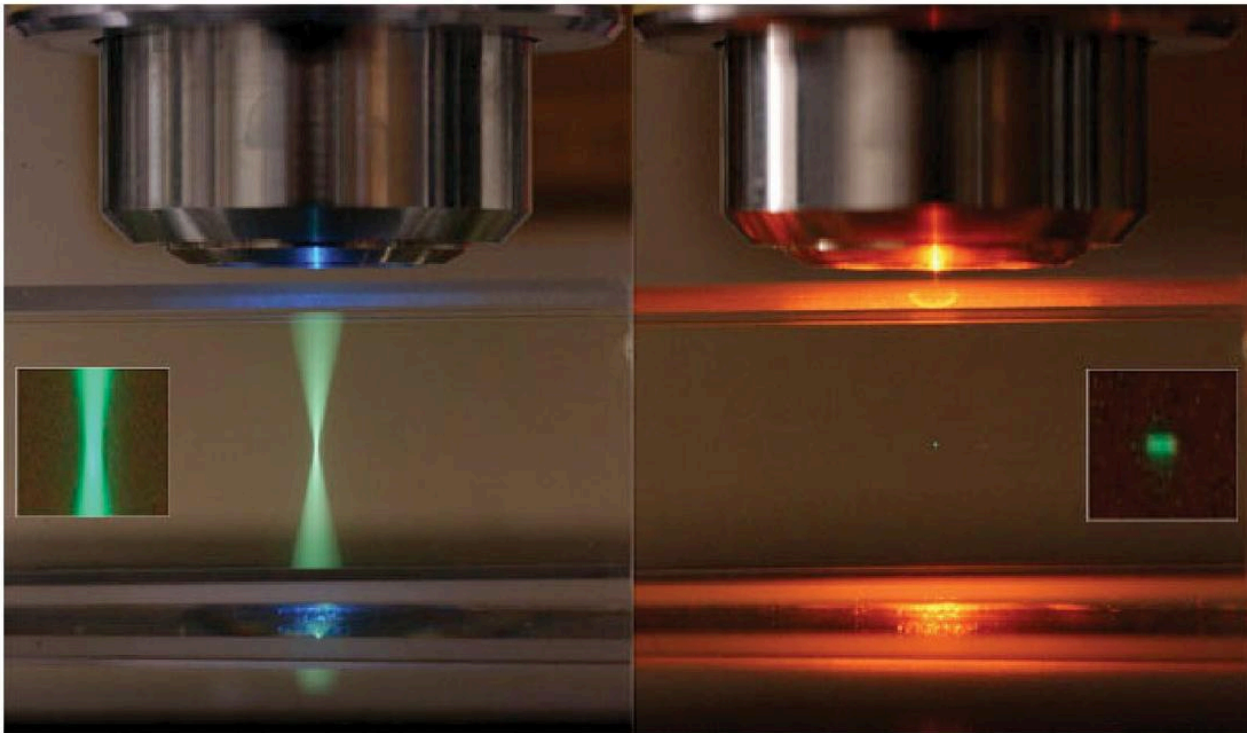
Requiring near-simultaneous photon arrival  
means fluorescence intensity depends on  
square of excitation intensity



Brad Amos, MRC, Cambridge

# Two-Photon excitation

No out-of-focus light



*The scanning blue (488nm) laser  
excites an entire column of sample.*

*The scanning IR pulse laser (Zeiss NLO system)  
excites only a small spot of sample.*

*Image by Steve Ruzin and Holly Aaron, UC Berkeley*

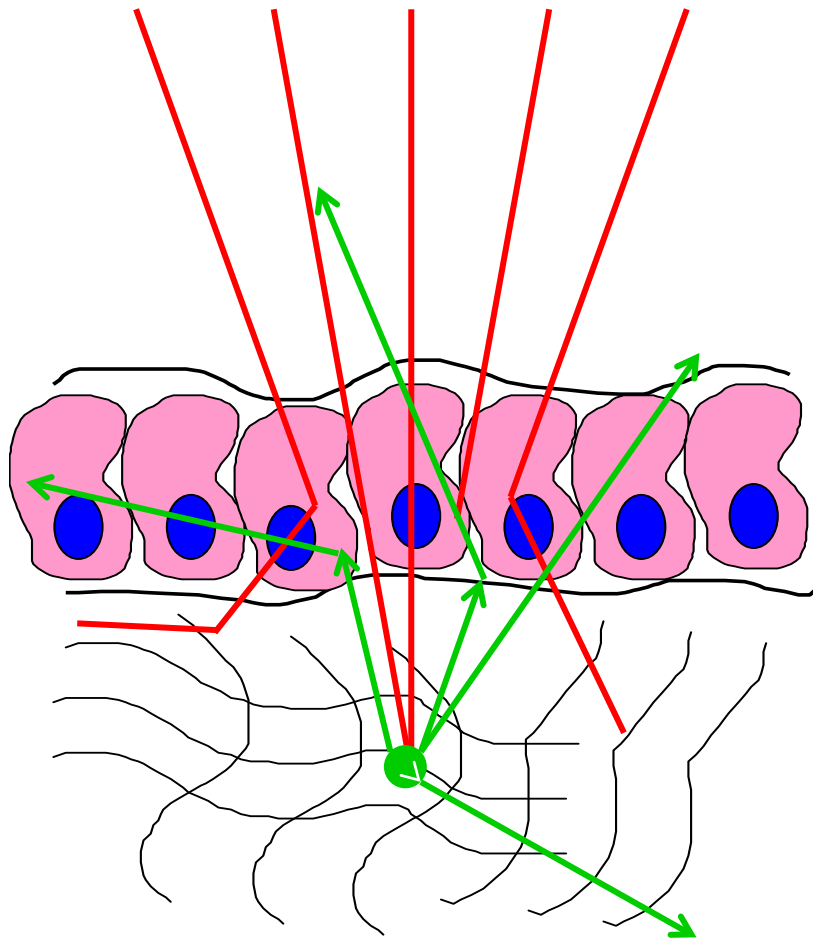
# Two-Photon excitation: no out of focus light

- In confocal, the focal volume is defined by a point of light  $\times$  a detection pinhole
- In two-photon excitation, the focal volume is defined by a point of light times itself.
- Two-photon microscopy can achieve the same point-spread function as confocal, *without a pinhole*.



# Tissue Absorption and Scattering, revisited

Focus is only point in sample to emit light:  
all emission comes from focus





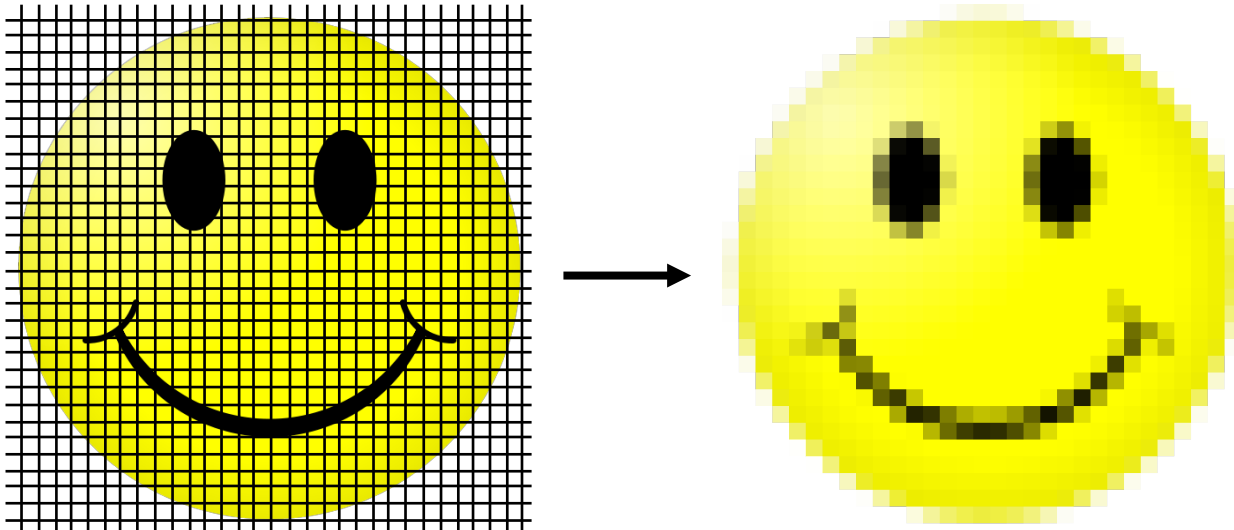
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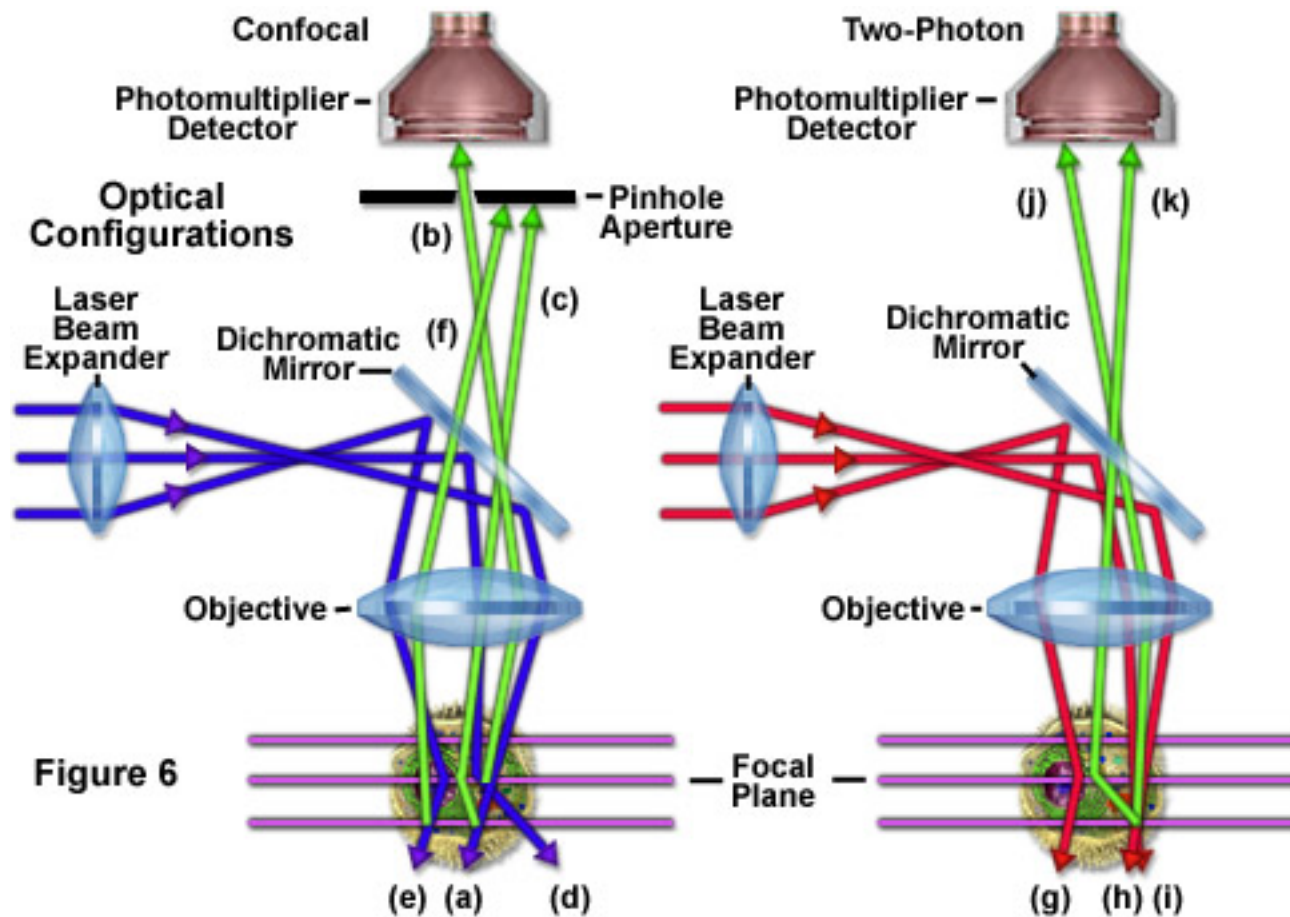
- Because emitted light all comes from focus, we don't need to image it.
- Collect all emission light, regardless of path, and assign it to focus
- Don't use the pinhole: Non-descanned detection

# Image by raster scanning

Create image by scanning laser point-by-point over sample and recording intensity at each spot.



# The most sensitive two-photon microscopes use non-descanned detectors



# A home-built two-photon microscope

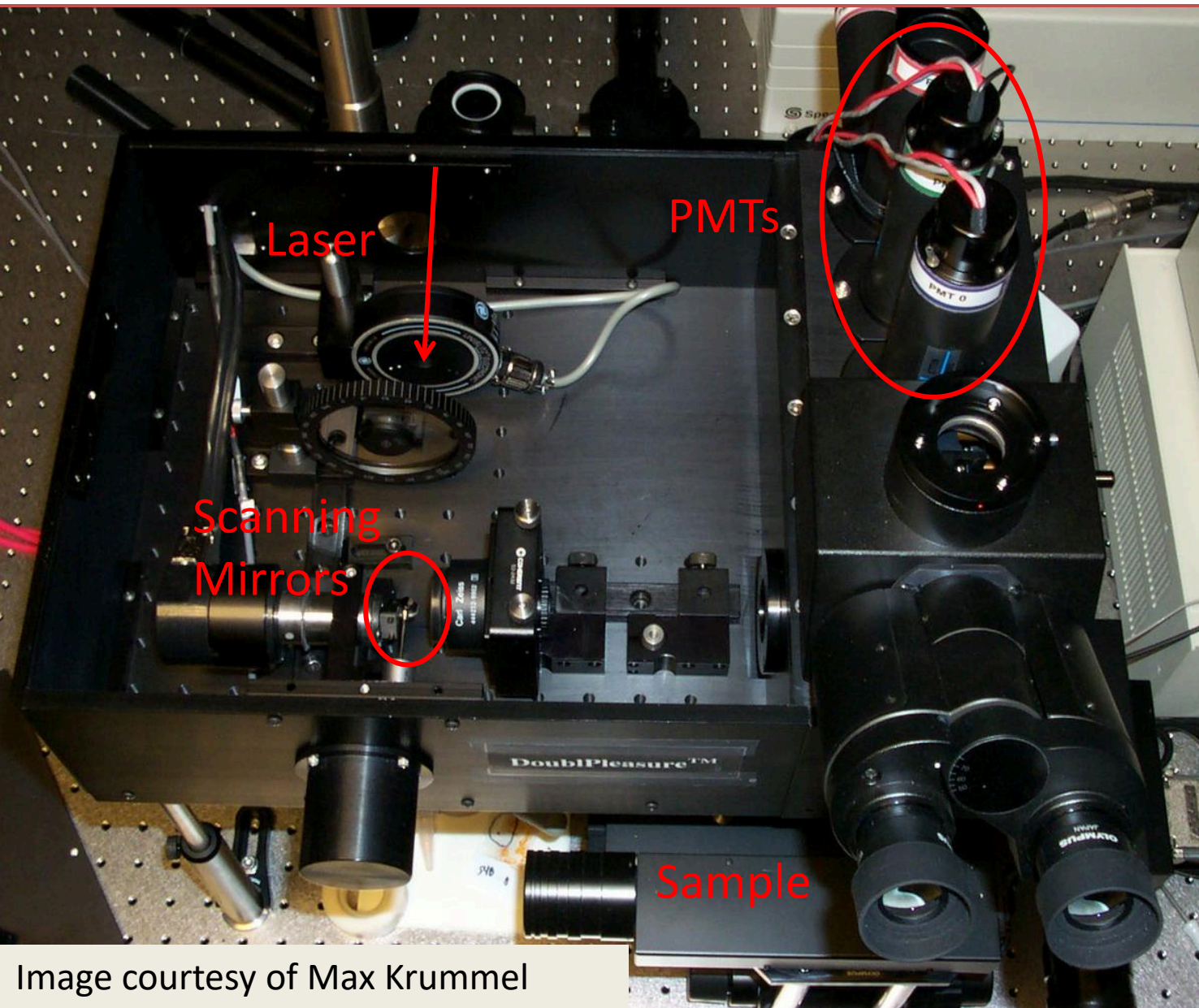
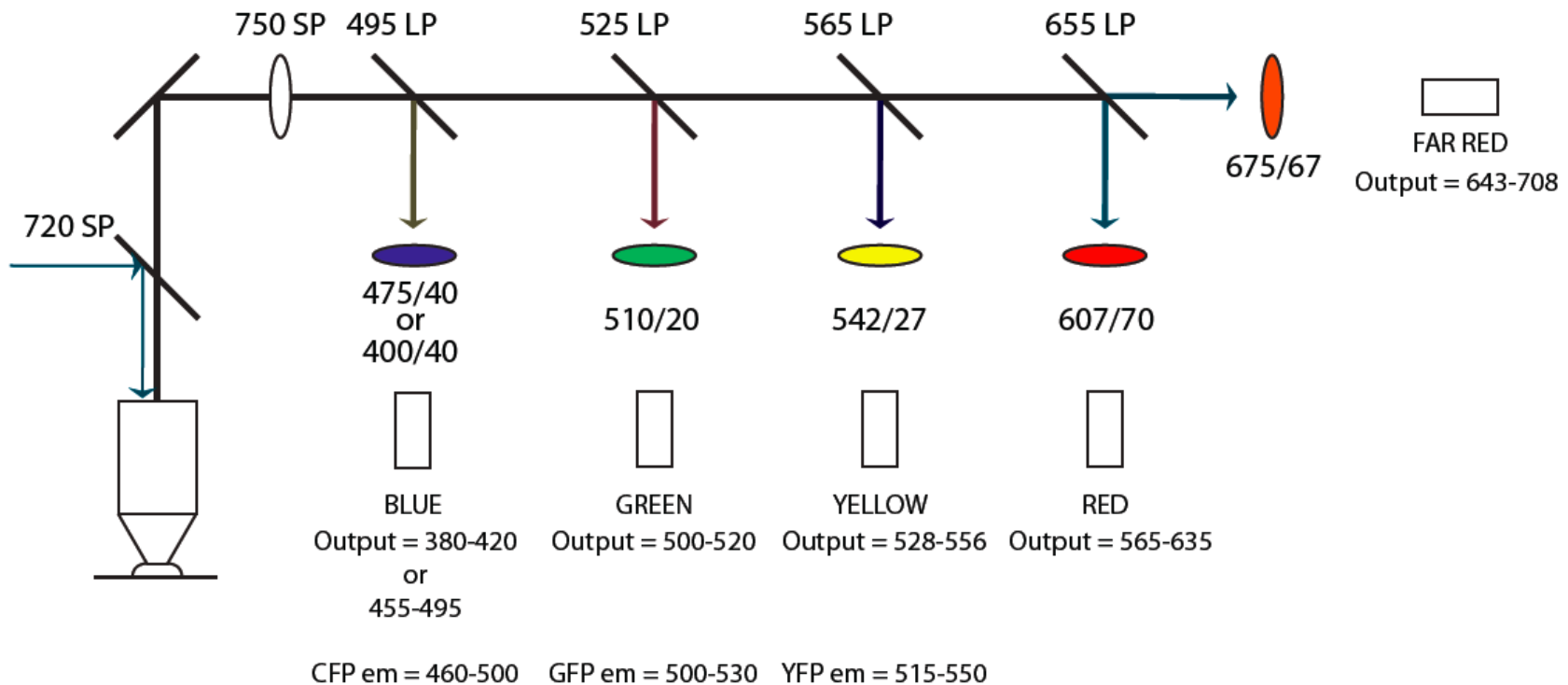


Image courtesy of Max Krummel

# Two photon optics are simple and allow multiple channels to be collected simultaneously





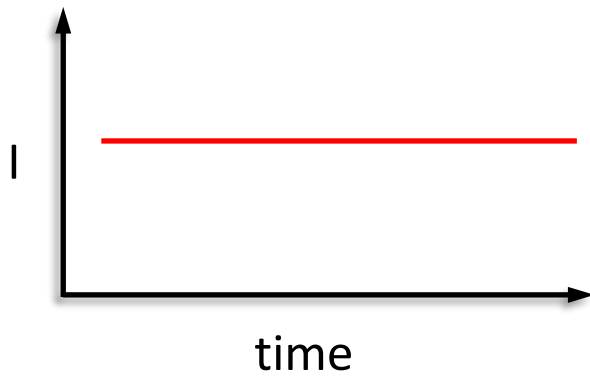
# Lasers for two-photon excitation

- Emission intensity depends on the square of excitation intensity.
- We want high excitation power.
- Use pulsed laser to get high peak power but normal average power.

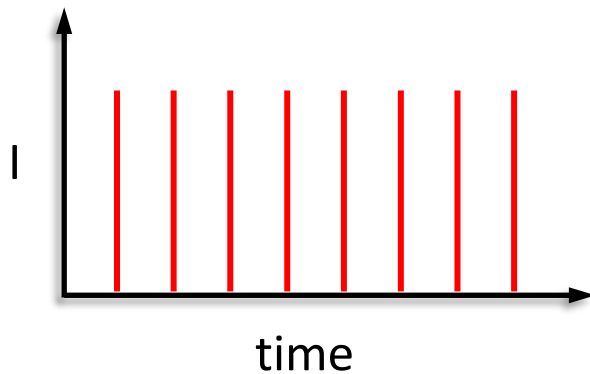


# Lasers for two-photon excitation

Typical CW laser



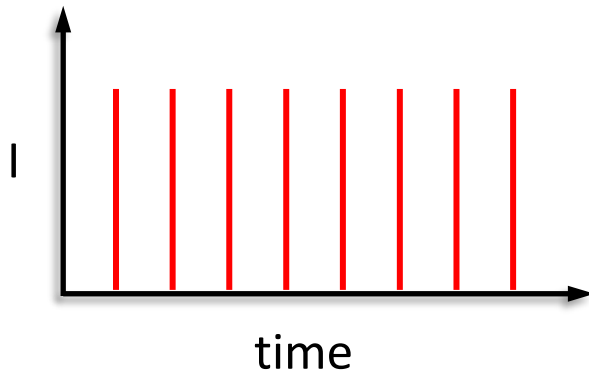
Pulsed laser





# Lasers for two-photon excitation

Pulsed laser



Typical Ti-Sapphire laser for two-photon microscopy (Newport Mai Tai):

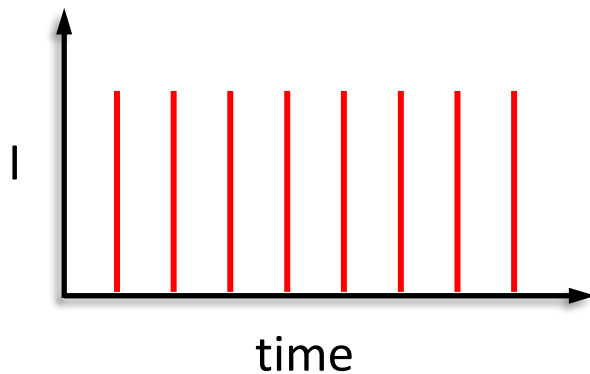
Pulse width: 80 fs

Repetition rate: 80 MHz

Time between pulses: 12.5 ns

# Lasers for two-photon excitation

Pulsed laser



80 fs pulse every 12.5 ns:

The laser is only 'on'  $\sim 1/150\,000^{\text{th}}$  of the time

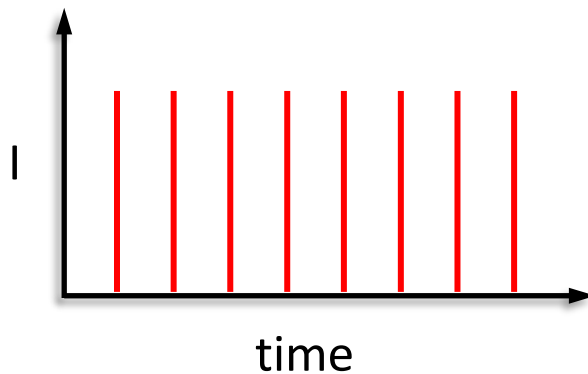
→ 1W average power results in

150 000W peak power

Cost: \$100 000 – \$200 000

# Ti-Sapphire lasers for two-photon excitation

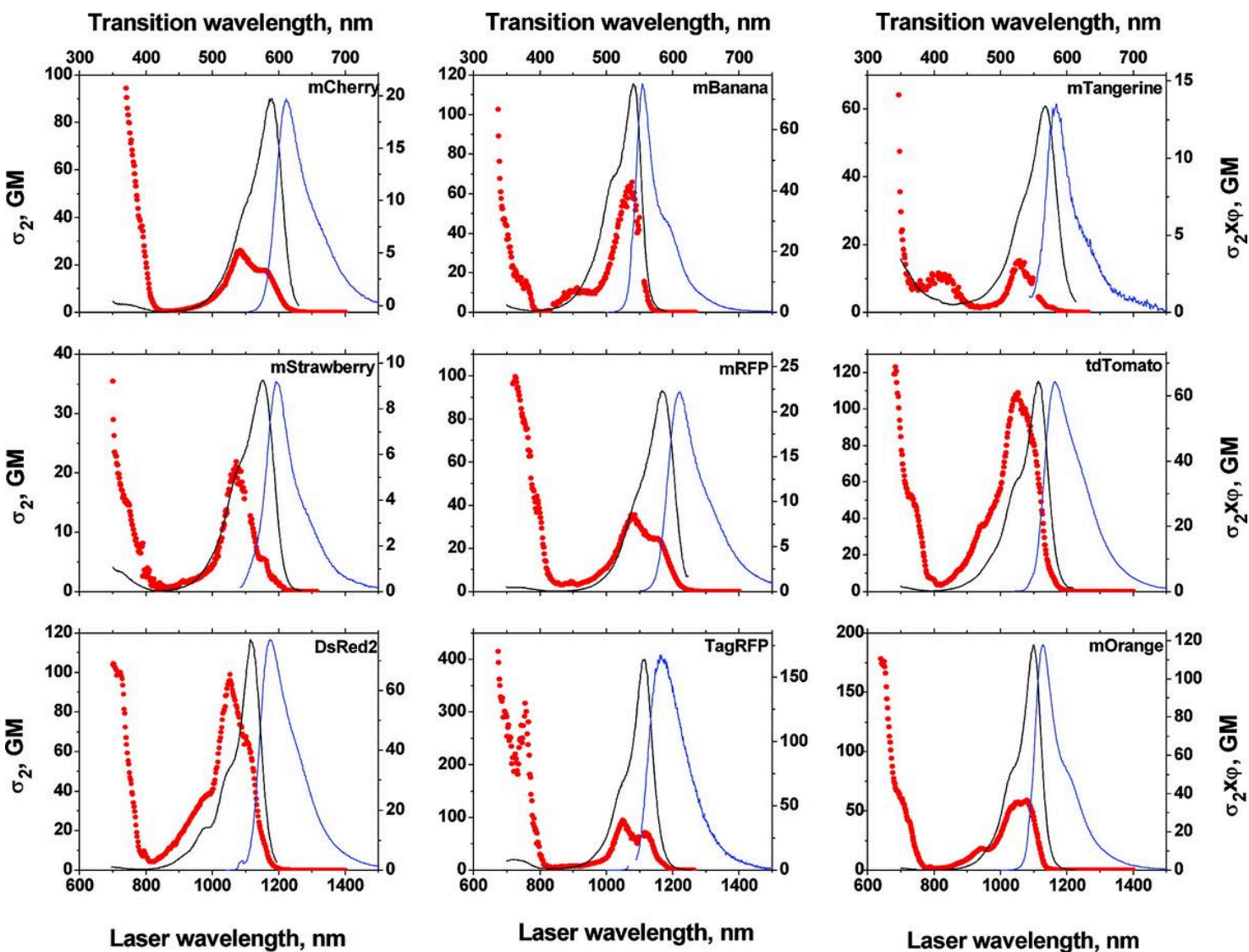
Pulsed laser



Ti-Sapphire lasers are tunable:

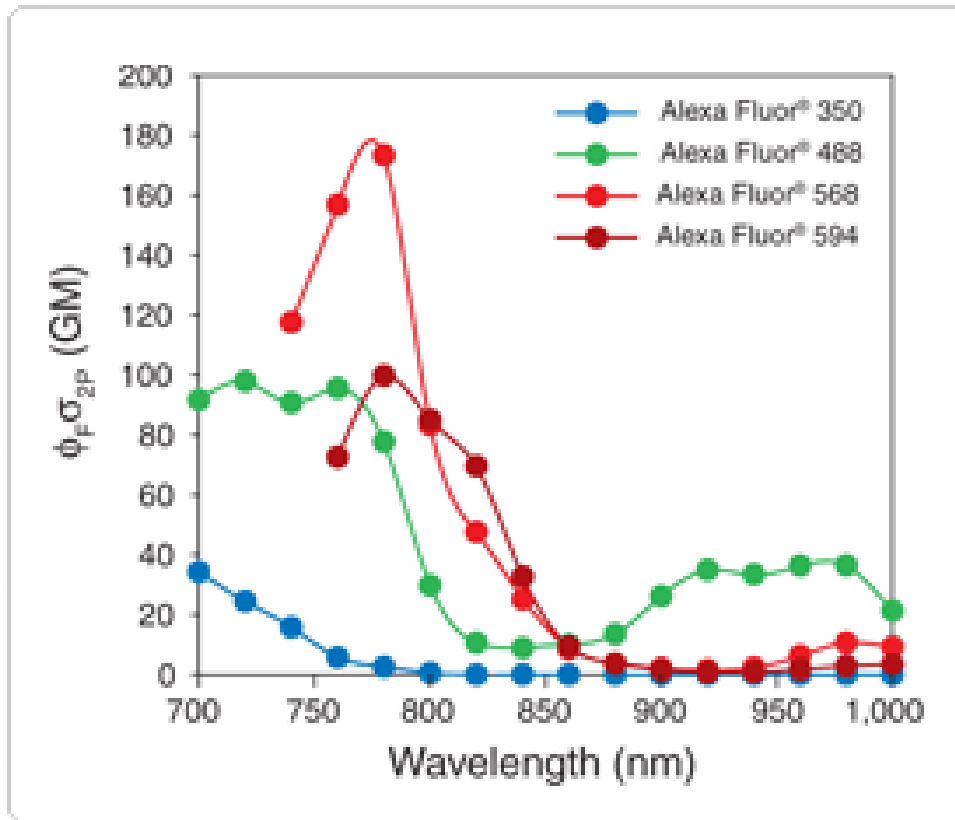
Tuning range  $\sim 700 - 1000$  nm

# Two-photon excitation spectra



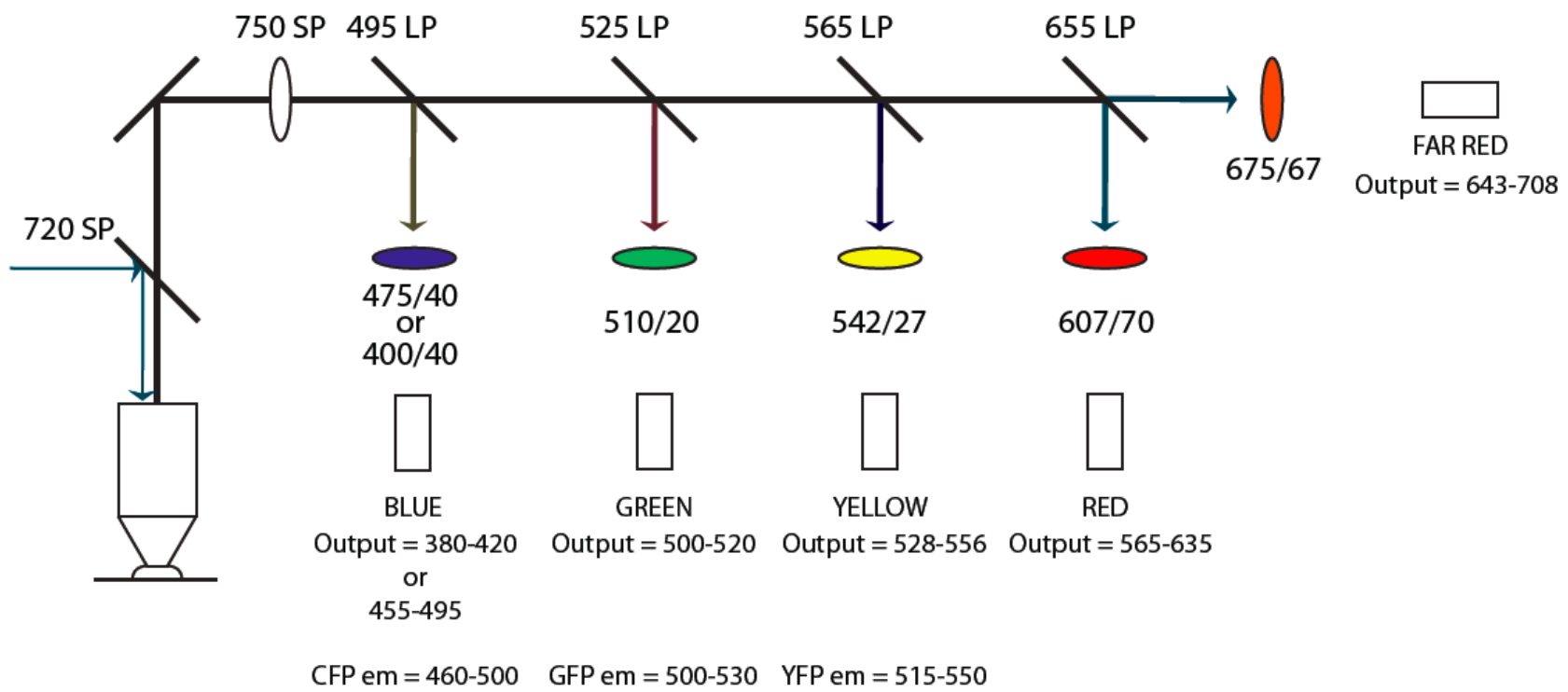
# Two-photon excitation spectra

Not twice the 1-photon excitation spectrum



Broadness of spectrum means one wavelength can excite multiple dyes

# Collect multiple emission channels to take advantage of broad excitation spectra



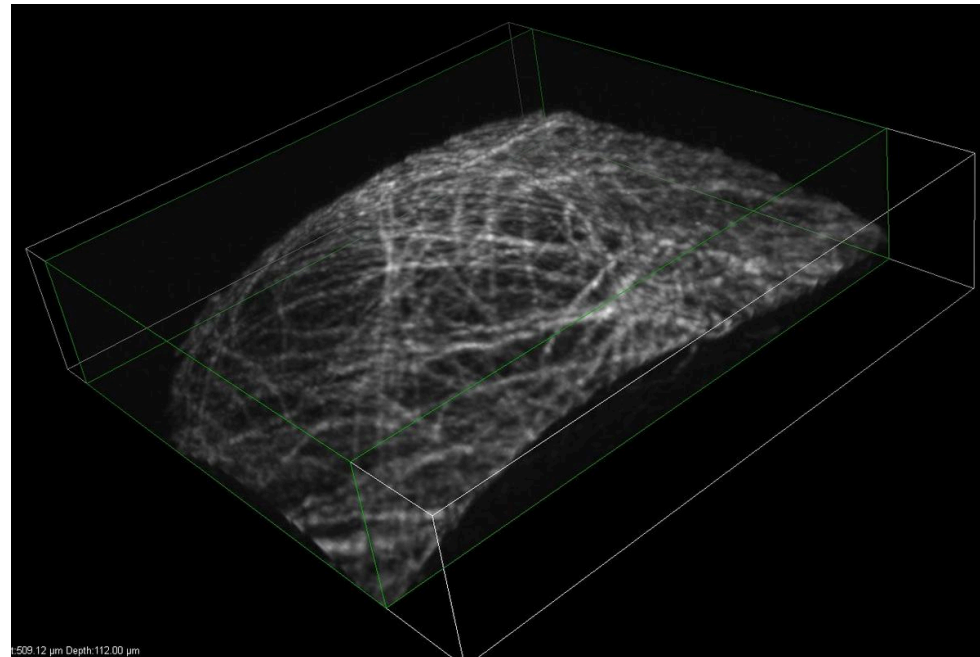
# Second Harmonic Generation

Some anisotropic molecules frequency-double light and generate emission at  $\frac{1}{2}$  the incident wavelength

Collagen I and IV

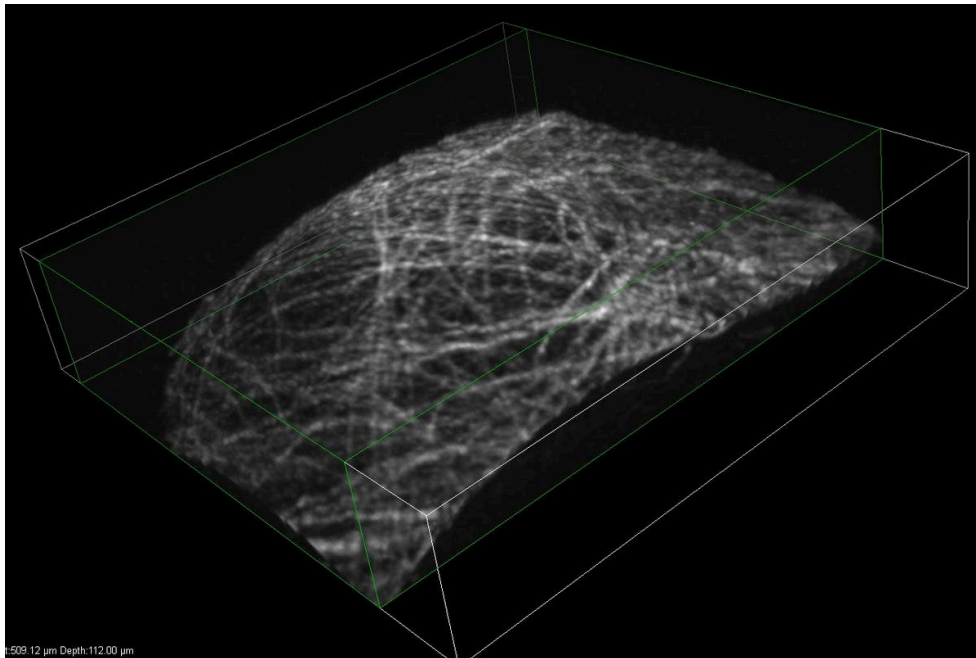
Cellulose

Some membrane dyes



# Second Harmonic Generation

This is a nonlinear process, and so its brightness scales as the square of the excitation light intensity too.

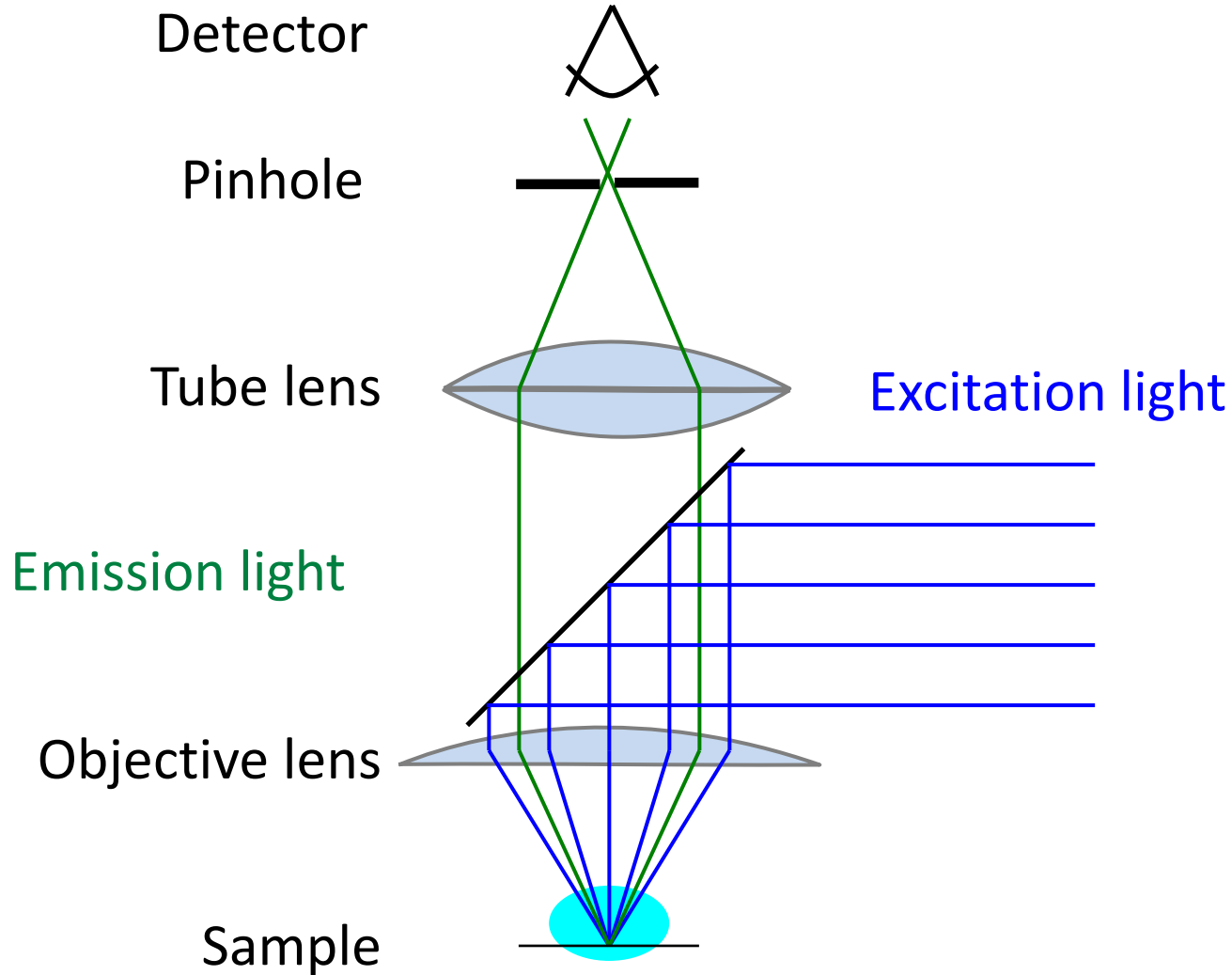




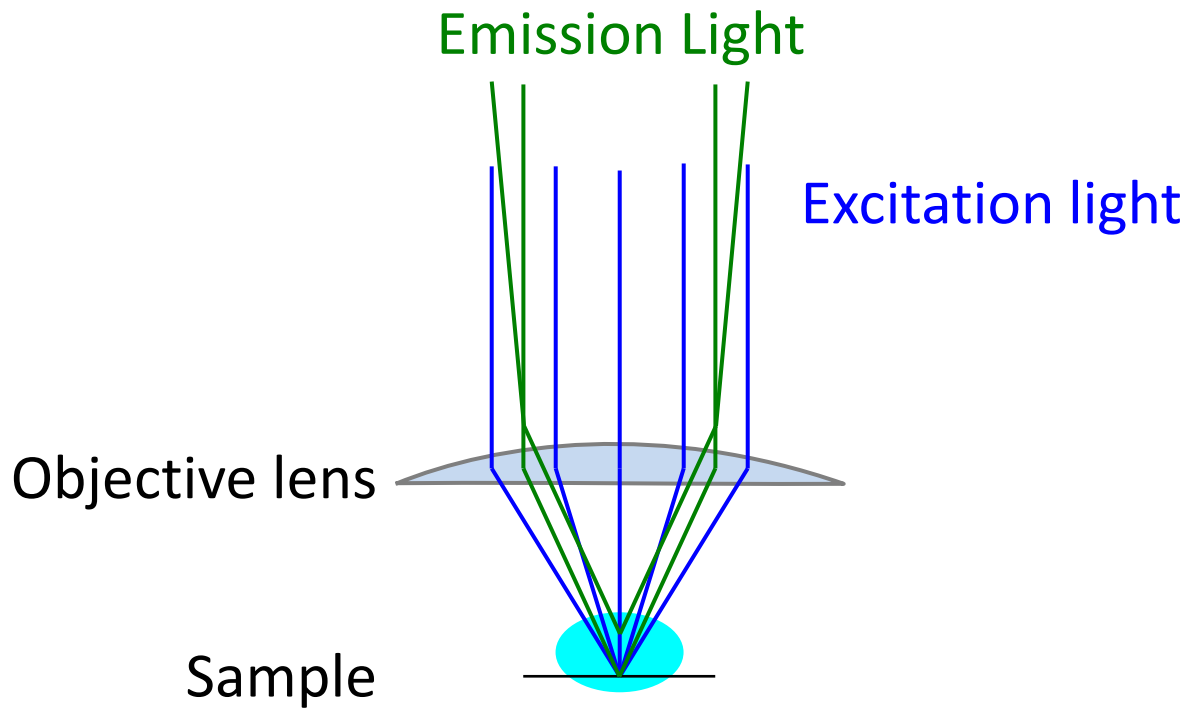
# When to use Two Photon Microscopy?

- Thick samples – 200  $\mu\text{m}$  – several mm
- Live samples – fixed samples can often be cleared or cut
- Excellent for imaging cellular detail inside of live animals

# The Problem With Epi-Illumination

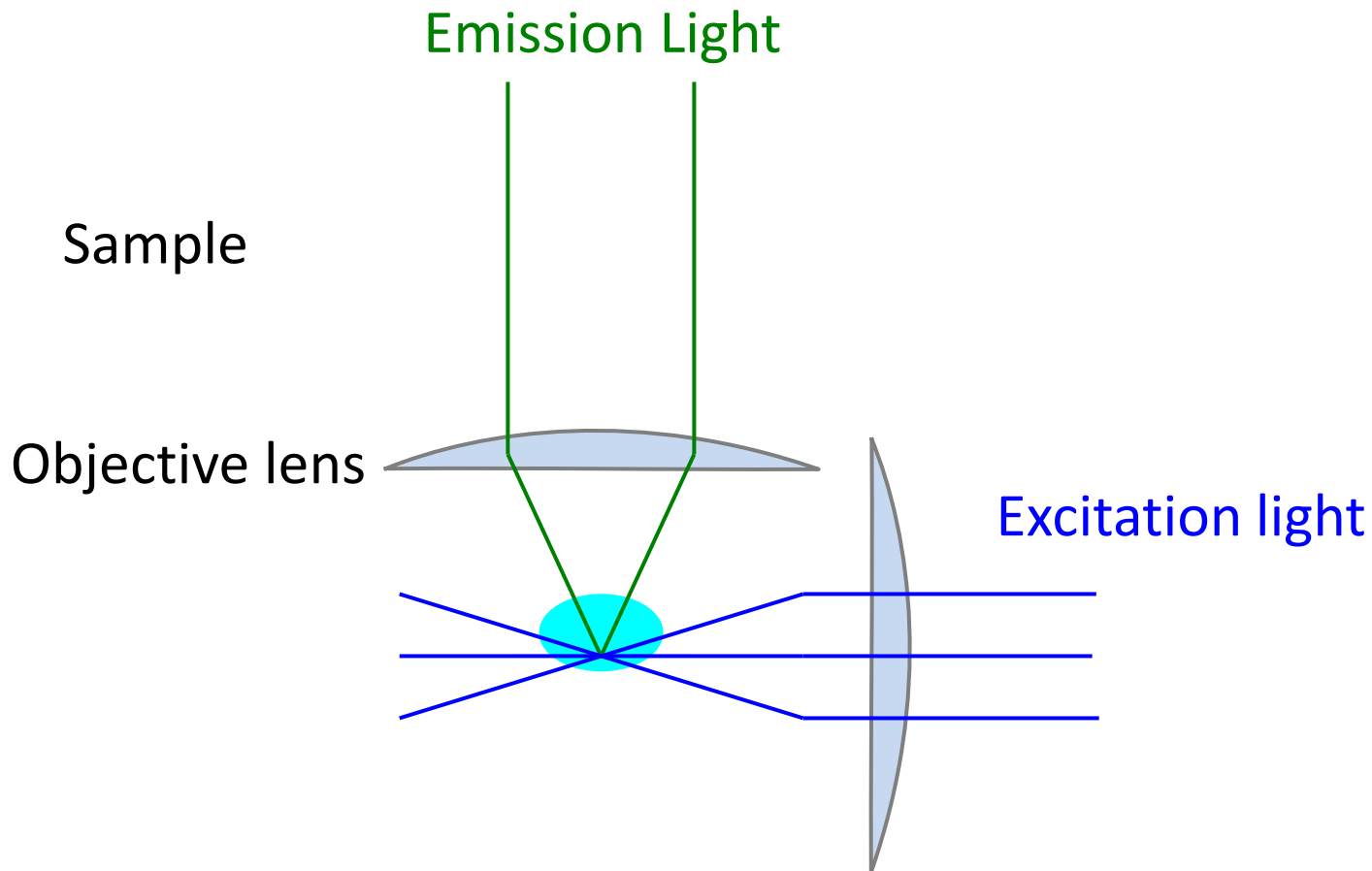


# The Problem with Epi-Illumination



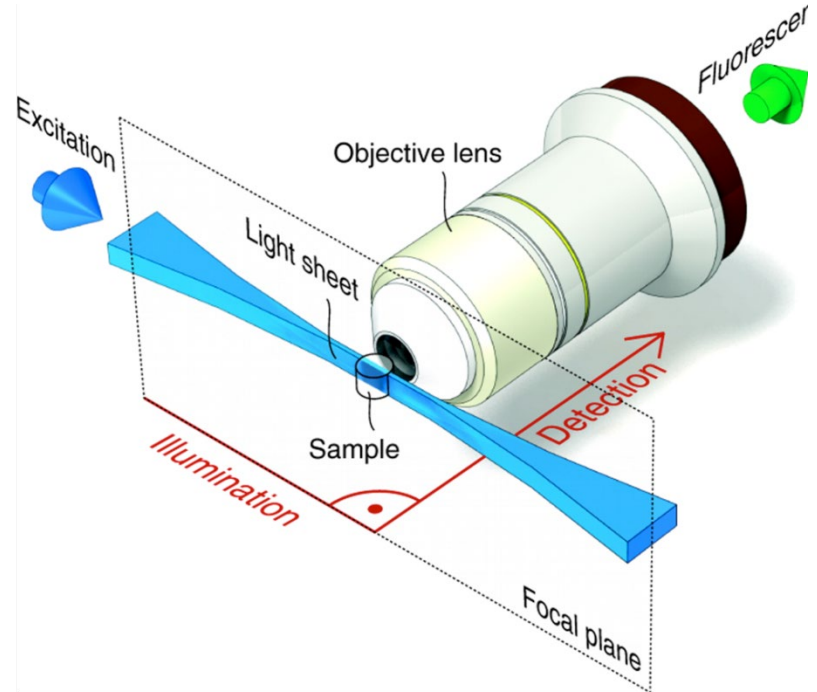
# Selective Plane Illumination or Light Sheet Microscopy

Illuminate with a plane of light at  $90^\circ$  to the detection objective



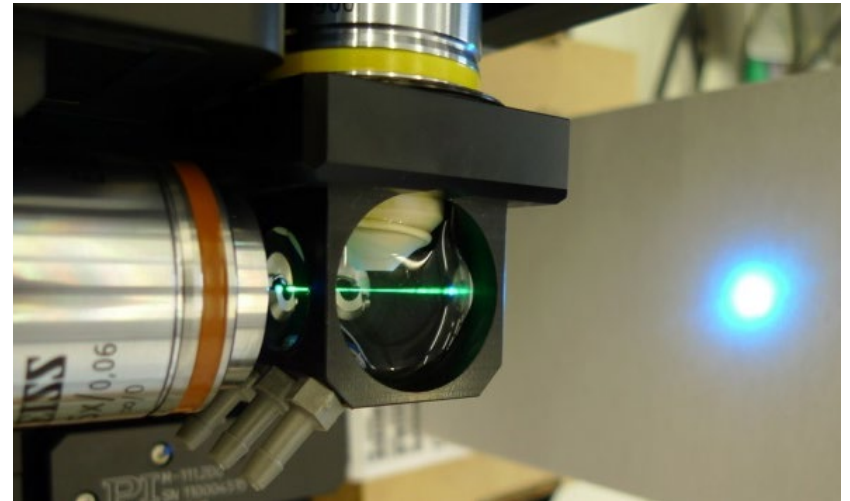
# Single Plane Illumination Microscopy

- Illuminate with a very thin light sheet
- Image at a 90 degree angle from illumination
- Optically sections the sample, illuminating a whole xy plane at once
- Move the sample in Z or rotate to create 3D images

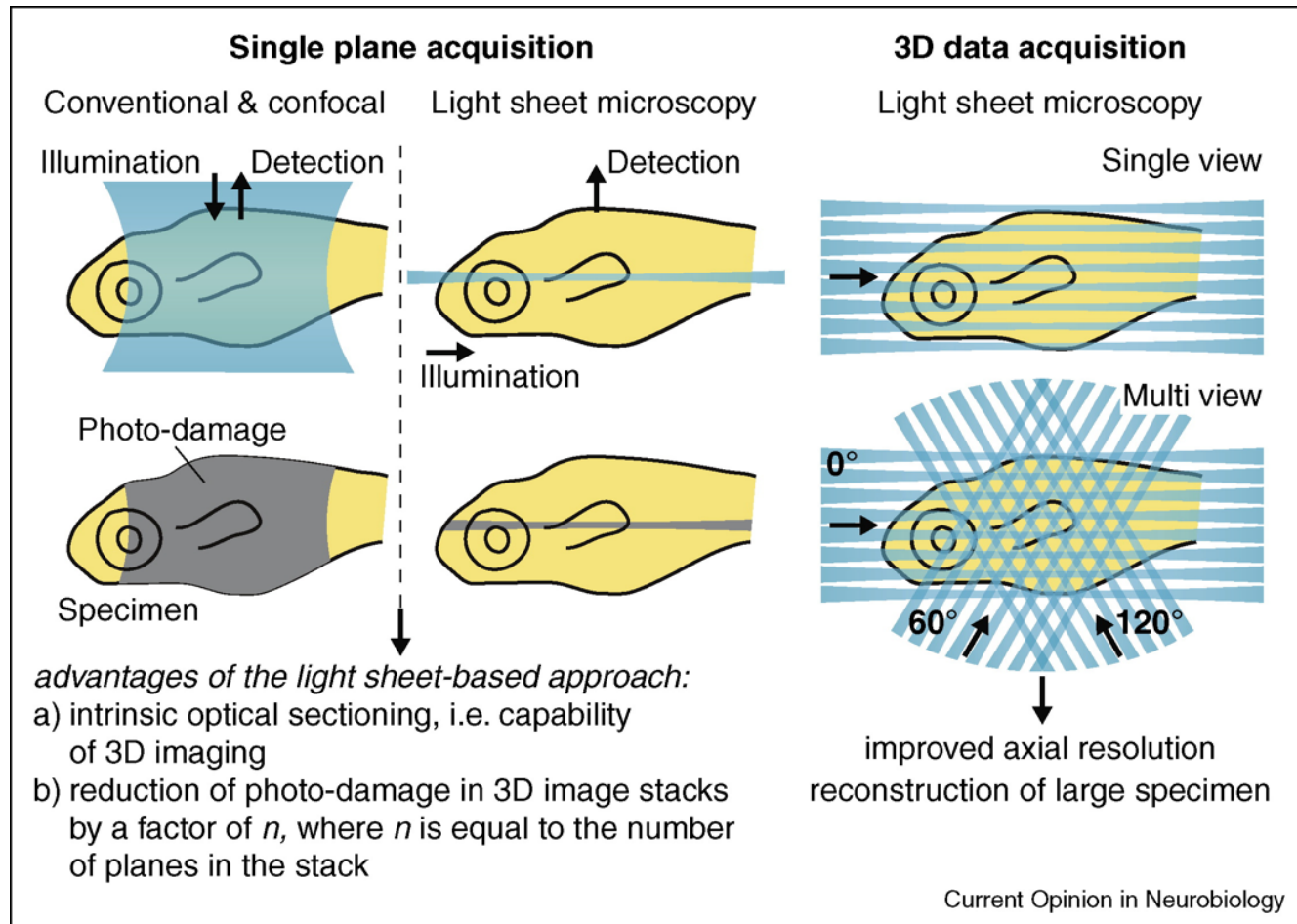


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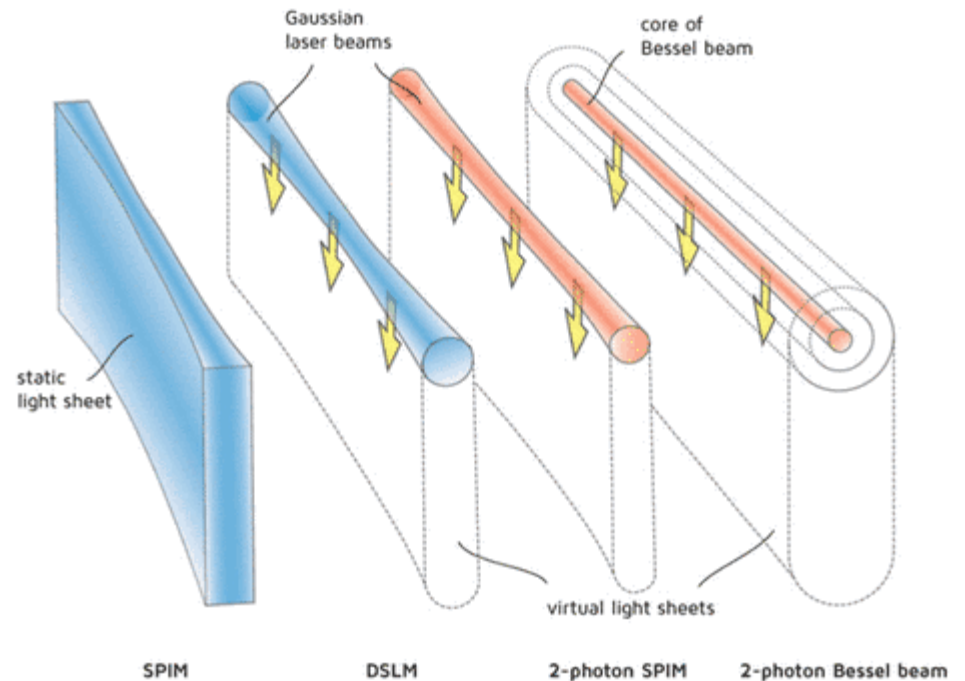


# SPIM Advantages



# Generating the Light Sheet

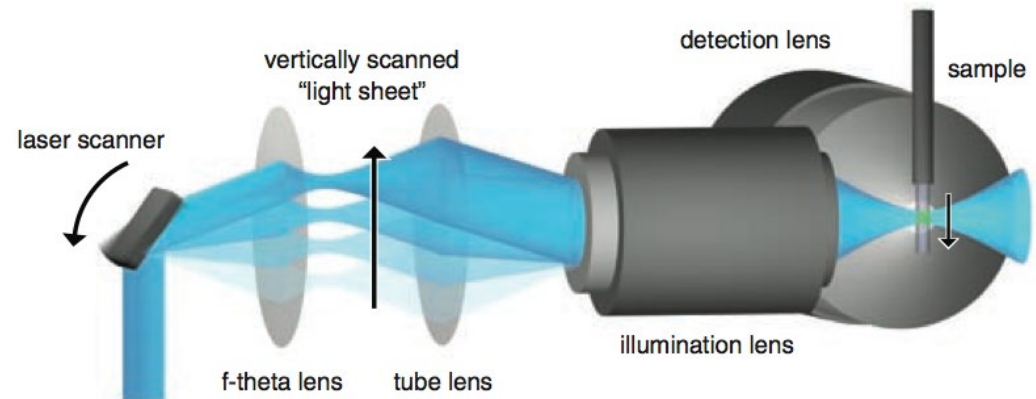
- Cylindrical lens
- Gaussian beam plus scanning (DSLM)
- Bessel beam plus scanning





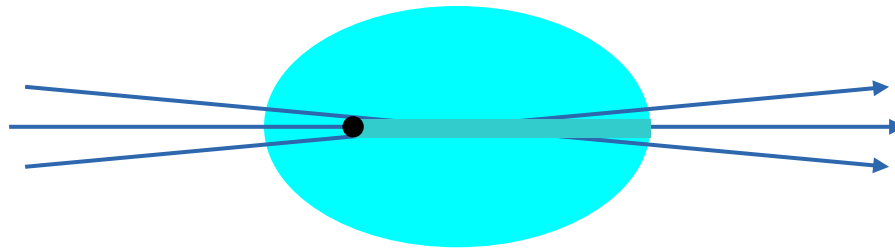
# Generating the Light Sheet

- Cylindrical lens
- Gaussian beam plus scanning (DSLM)
- Bessel beam plus scanning



# A Problem: Absorption and Scattering

Solution: Image from Multiple Directions



SPIM

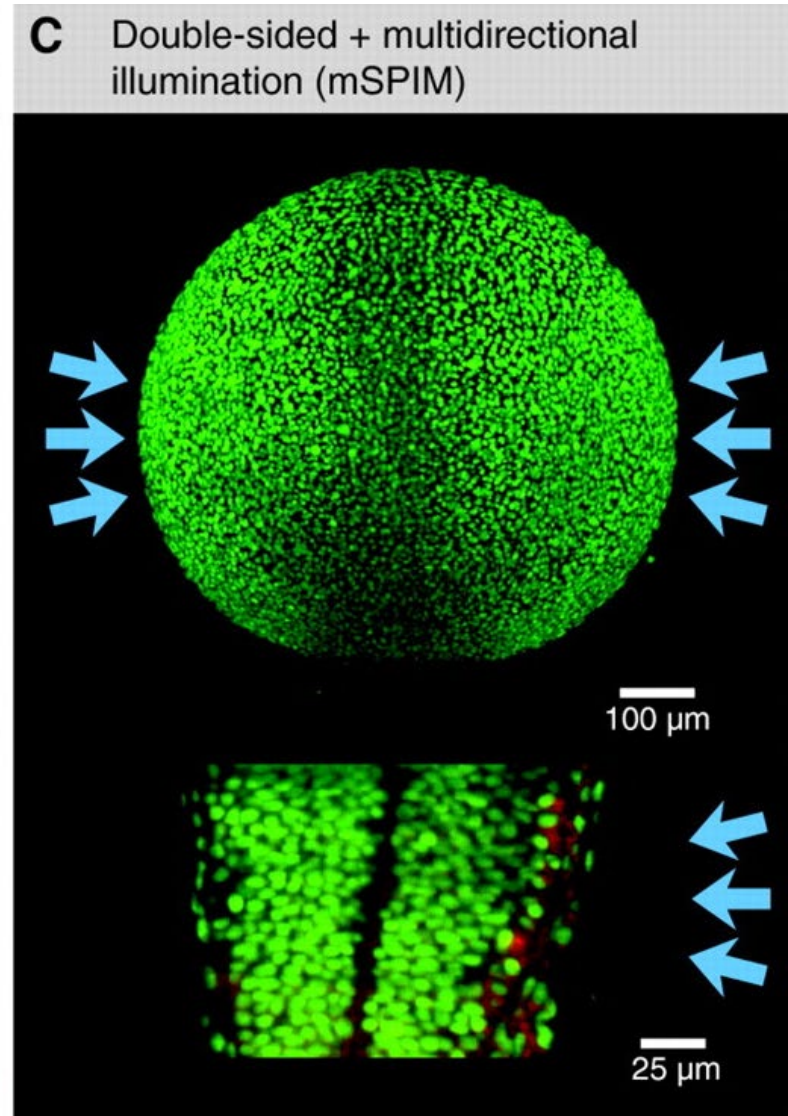
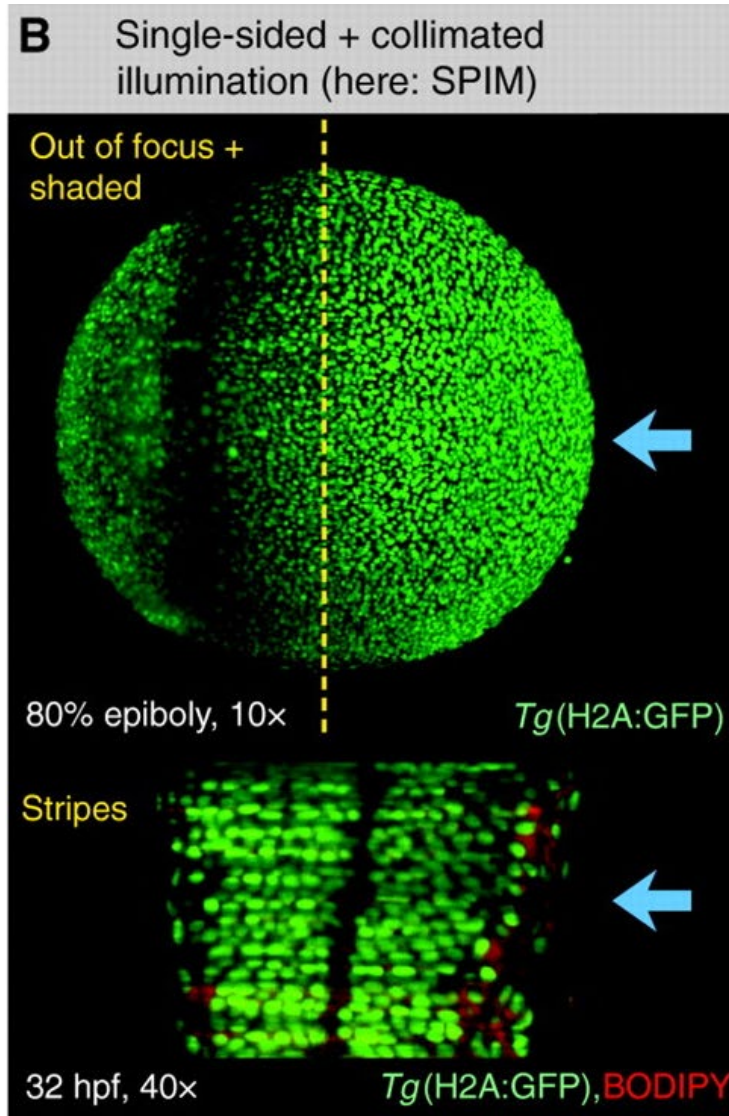


mSPIM



Four-lens SPIM  
(MuVi-SPIM, SiMView)

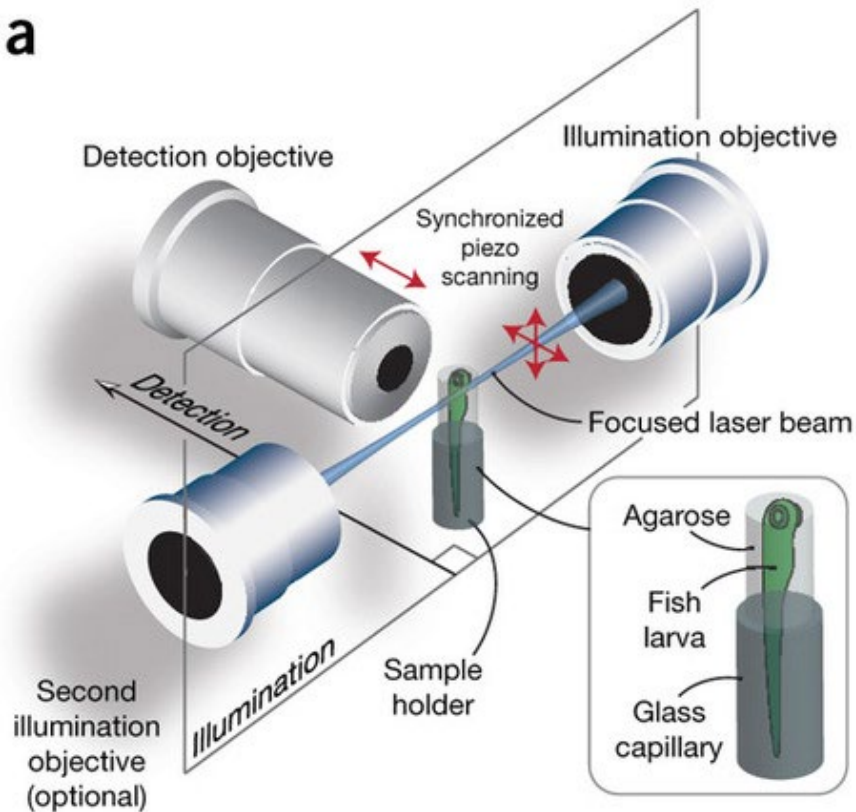
# Multidirectional SPIM (mSPIM)



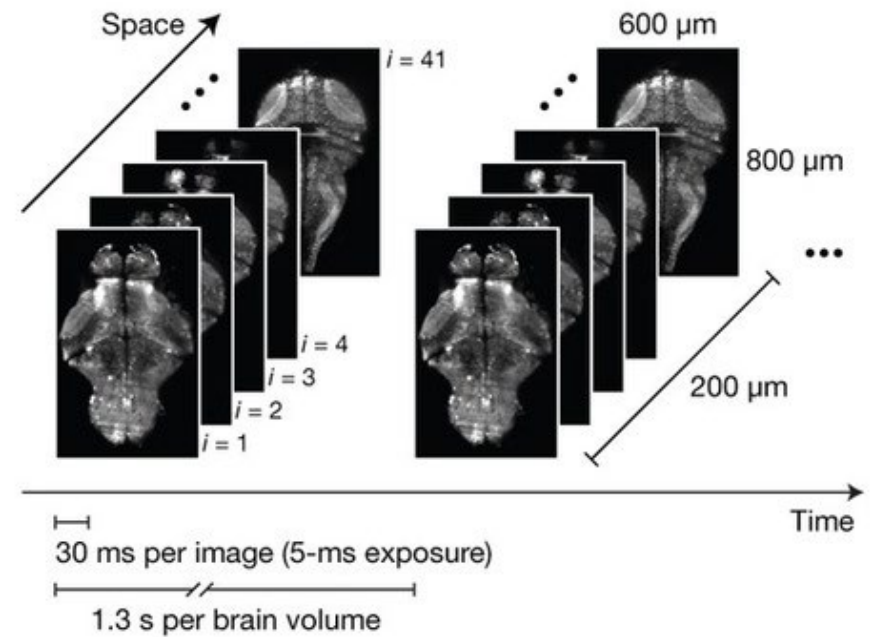
# Ultrafast Light Sheet Imaging of Zebrafish

Capture  $800 \times 600 \times 200 \mu\text{m}$  at  $0.65 \times 0.65 \times 5 \mu\text{m}$  resolution

**a**



**b**





# Imaging of GCaMP5 Zebrafish

