Optical Sectioning II: How not to be seen

TIRF

Total Internal Reflection Microscopy

Excite just the fluorophores at the coverslip.

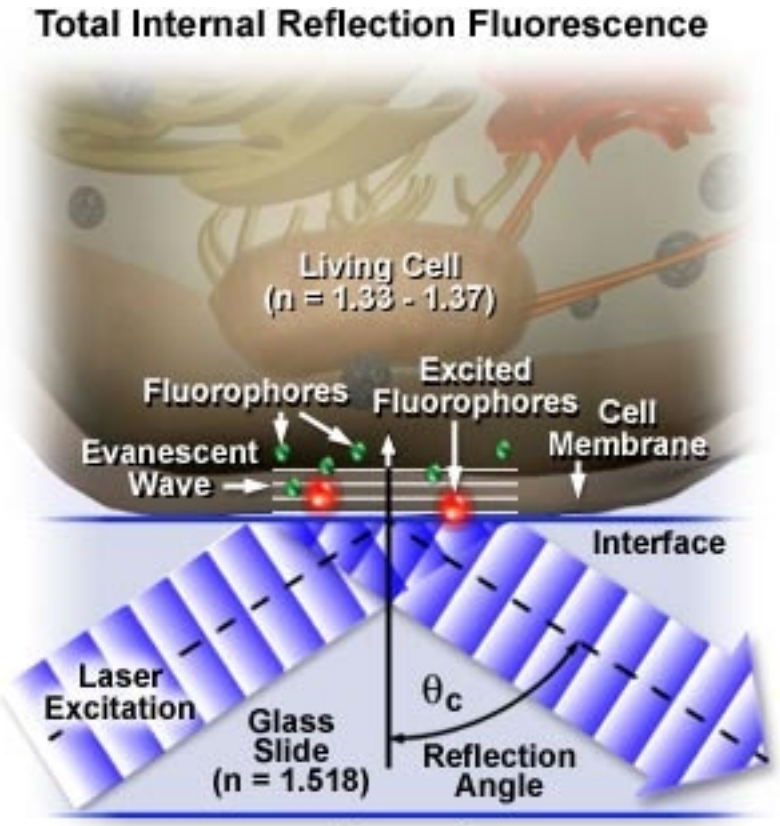


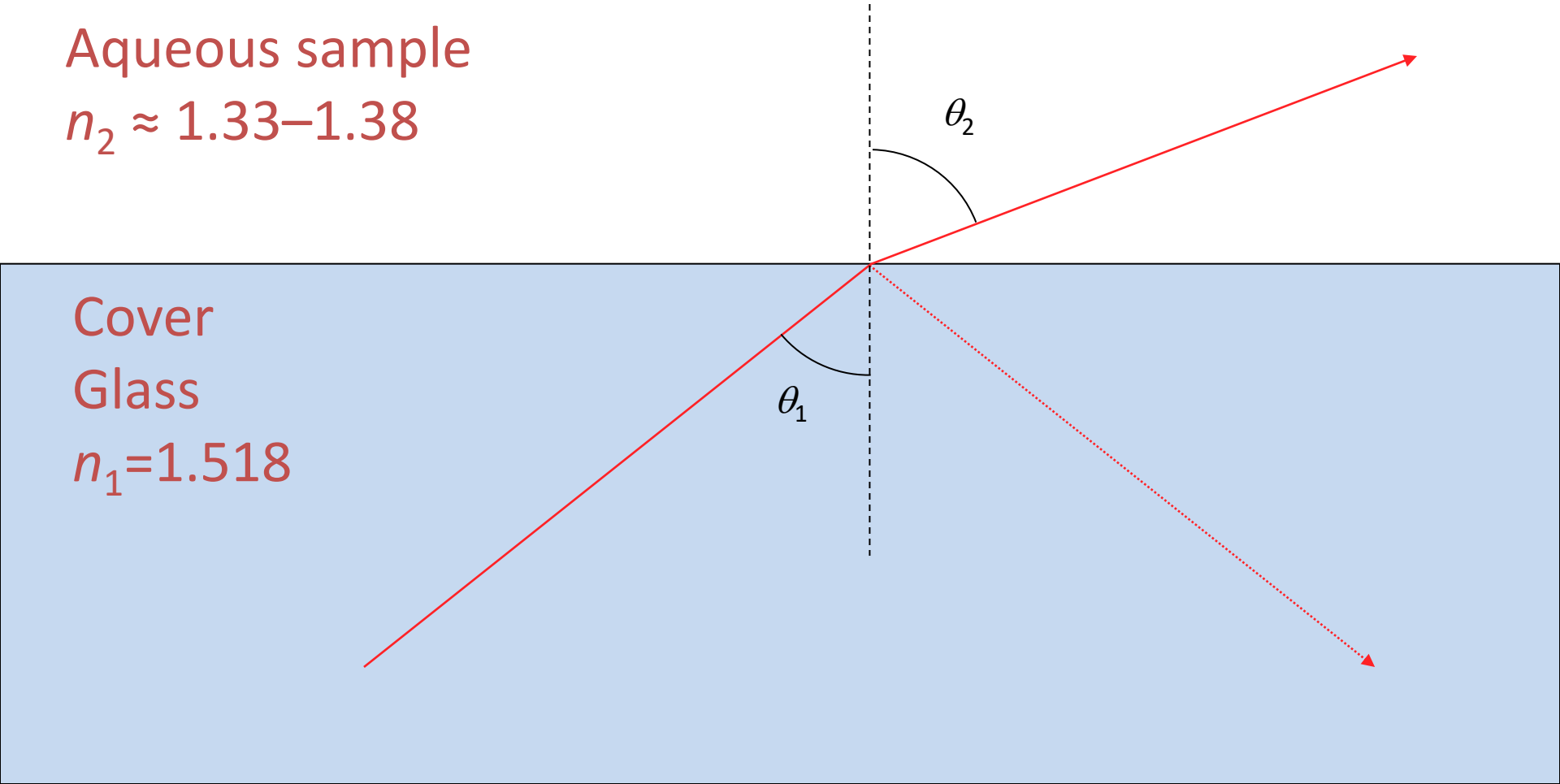
Figure 1

Total Internal Reflection

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

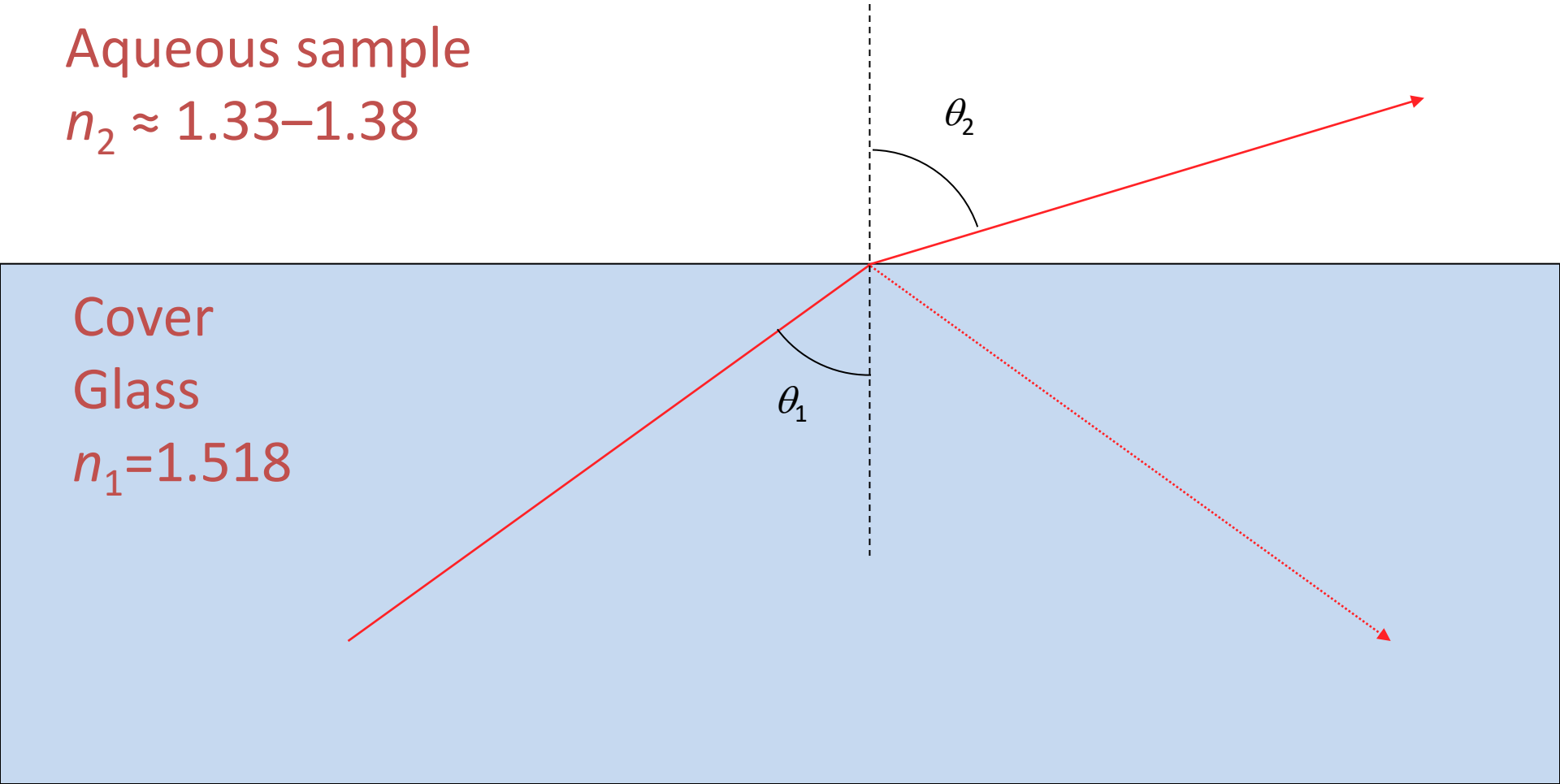
n_1=1.518

Total Internal Reflection

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

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

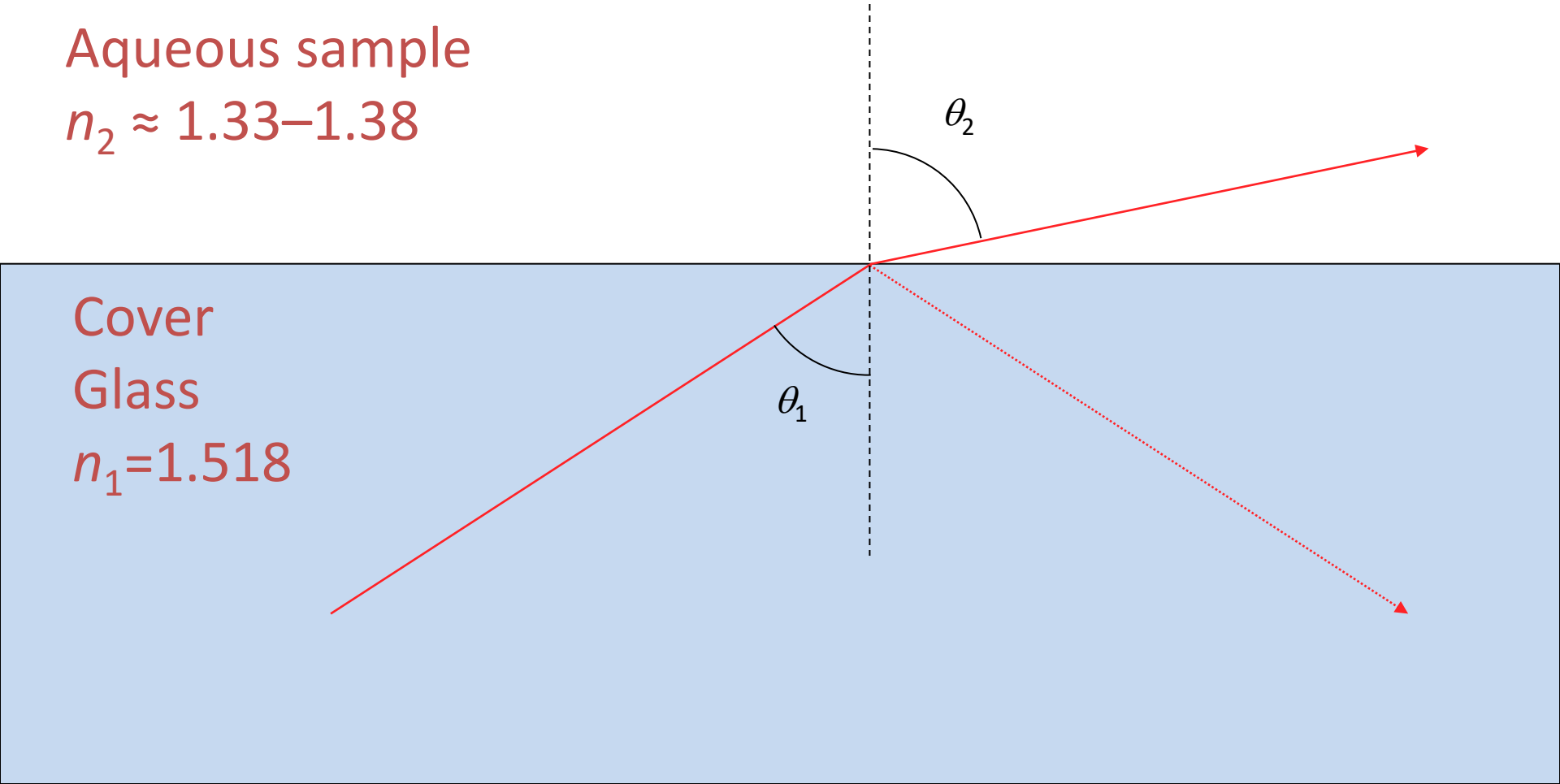
n_1=1.518

Total Internal Reflection

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

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

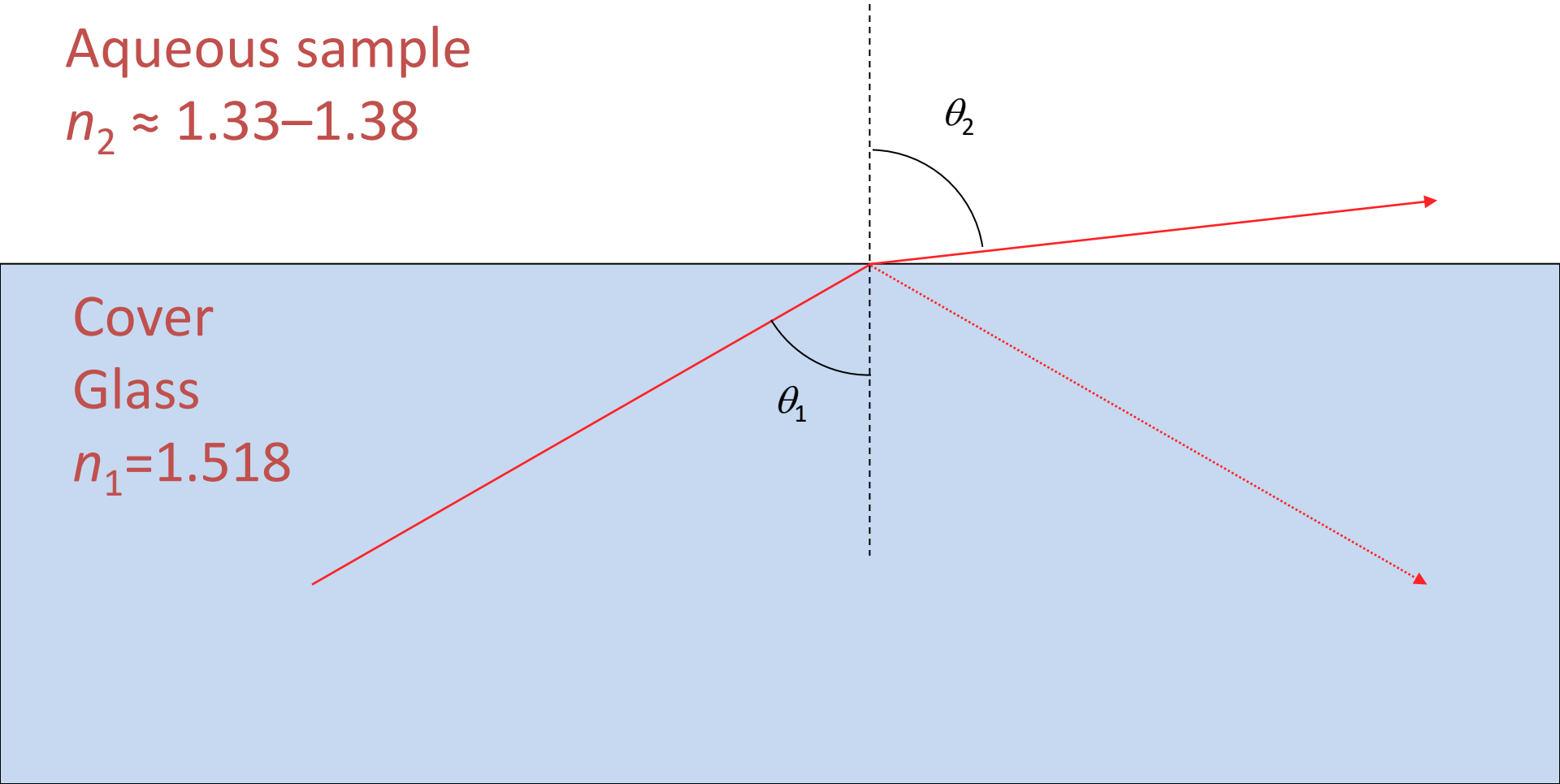
n_1=1.518

Total Internal Reflection

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

Aqueous sample

$n_2 \approx 1.33–1.38$



Cover Glass

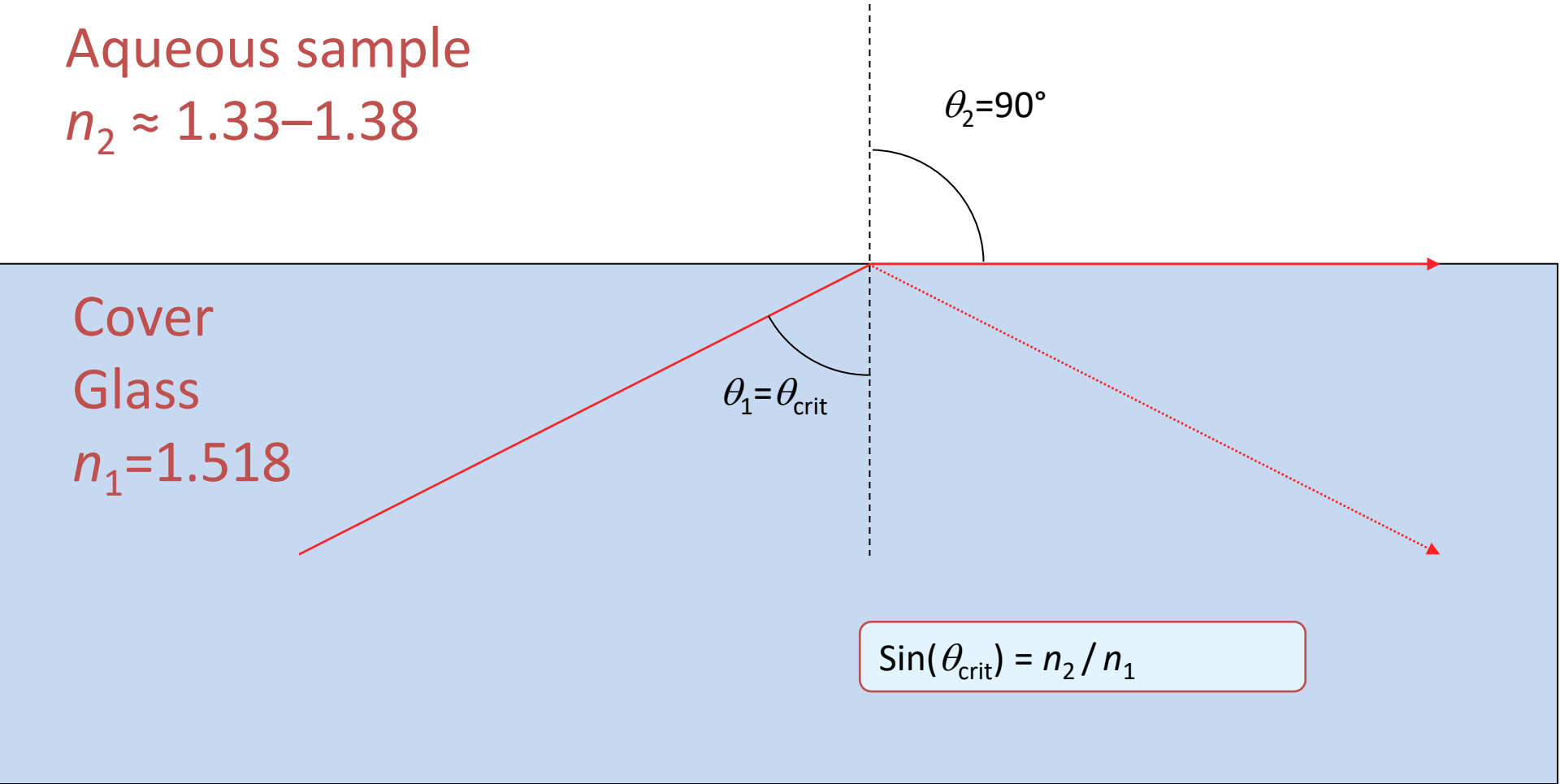
n_1=1.518

Total Internal Reflection

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

Aqueous sample

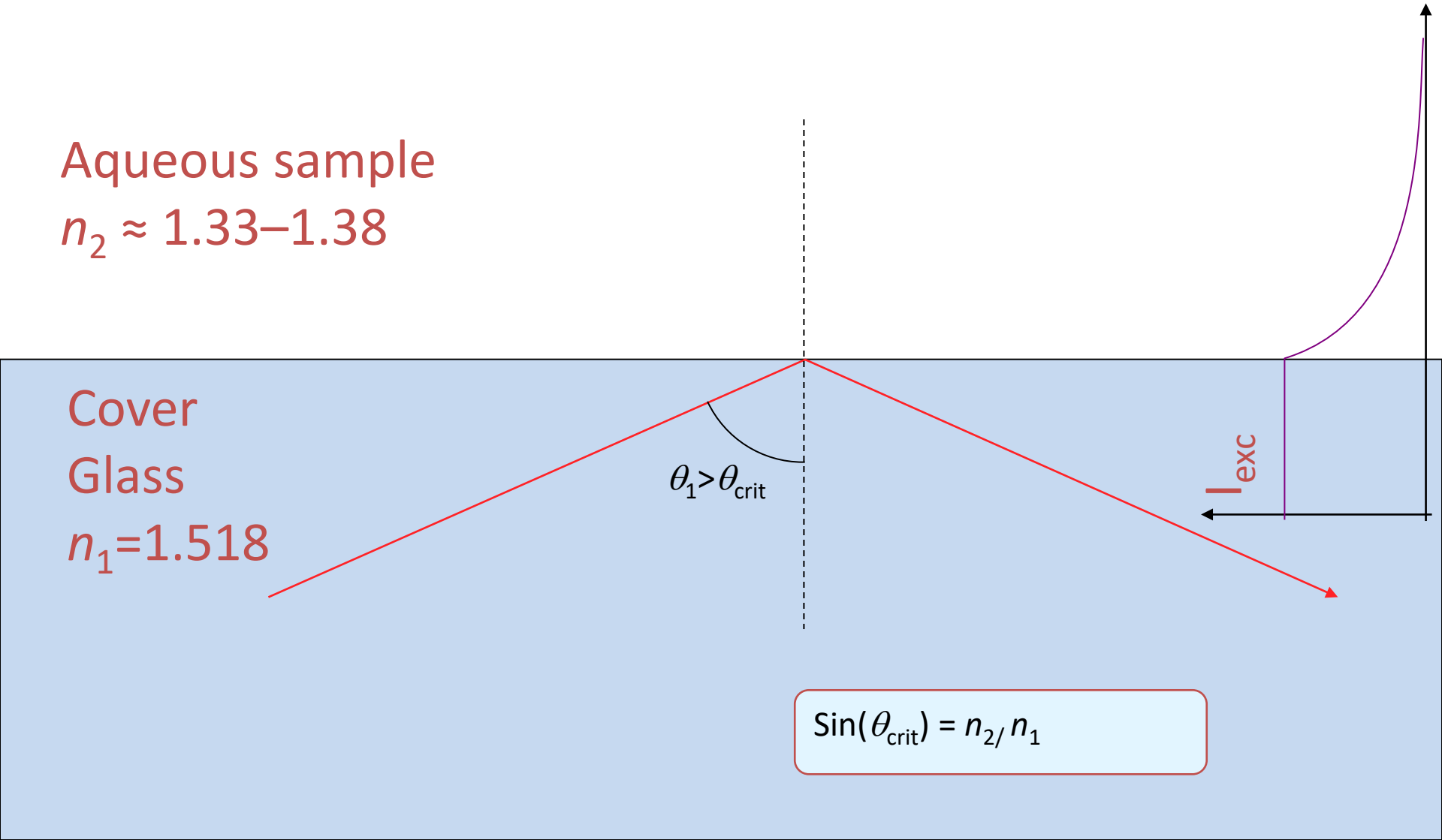
$n_2 \approx 1.33–1.38$



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

Total Internal Reflection

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

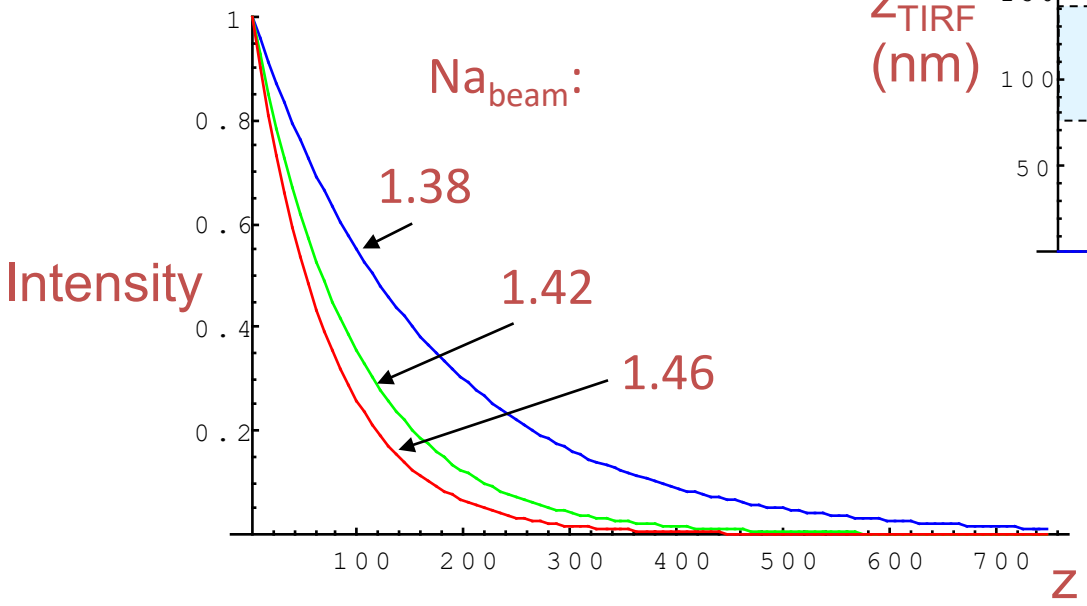


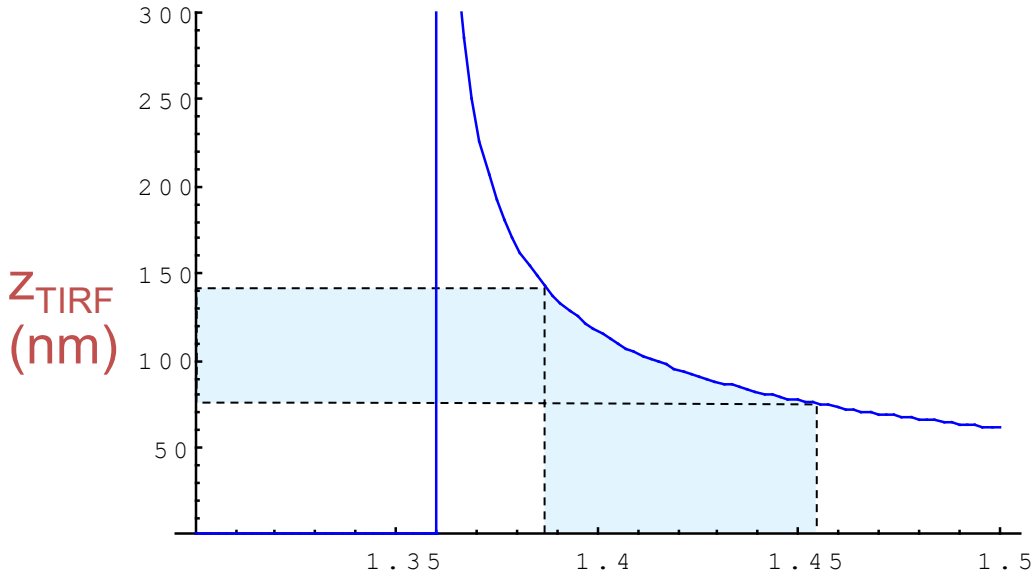
Total Internal Reflection

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

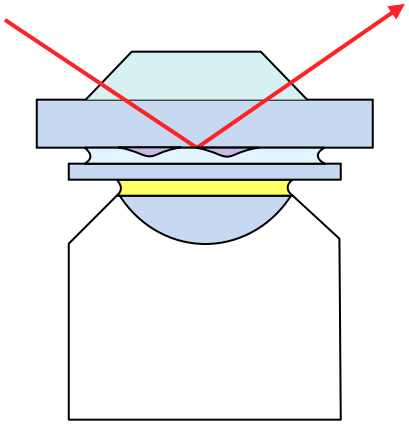




Beam "NA" = n_1 Sin(θ_1)

Two forms of TIRF microscopy

Prism coupled

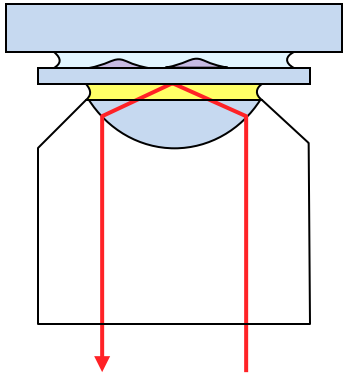


Sample on slide

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

Through the objective

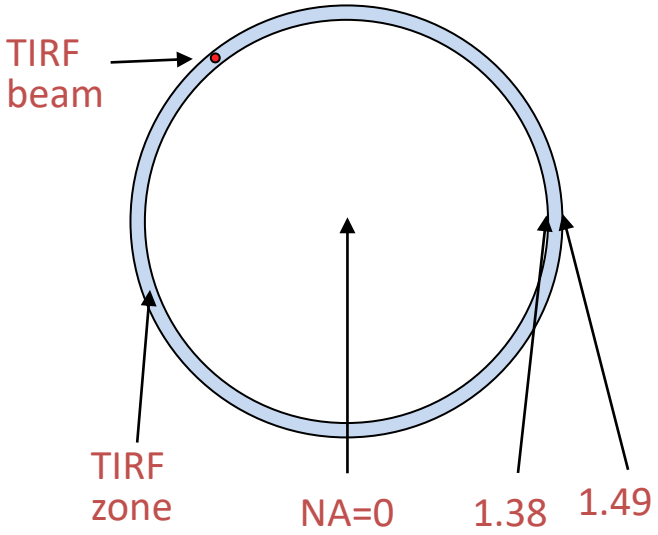
Sample on cover glass



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

TIRF Objectives

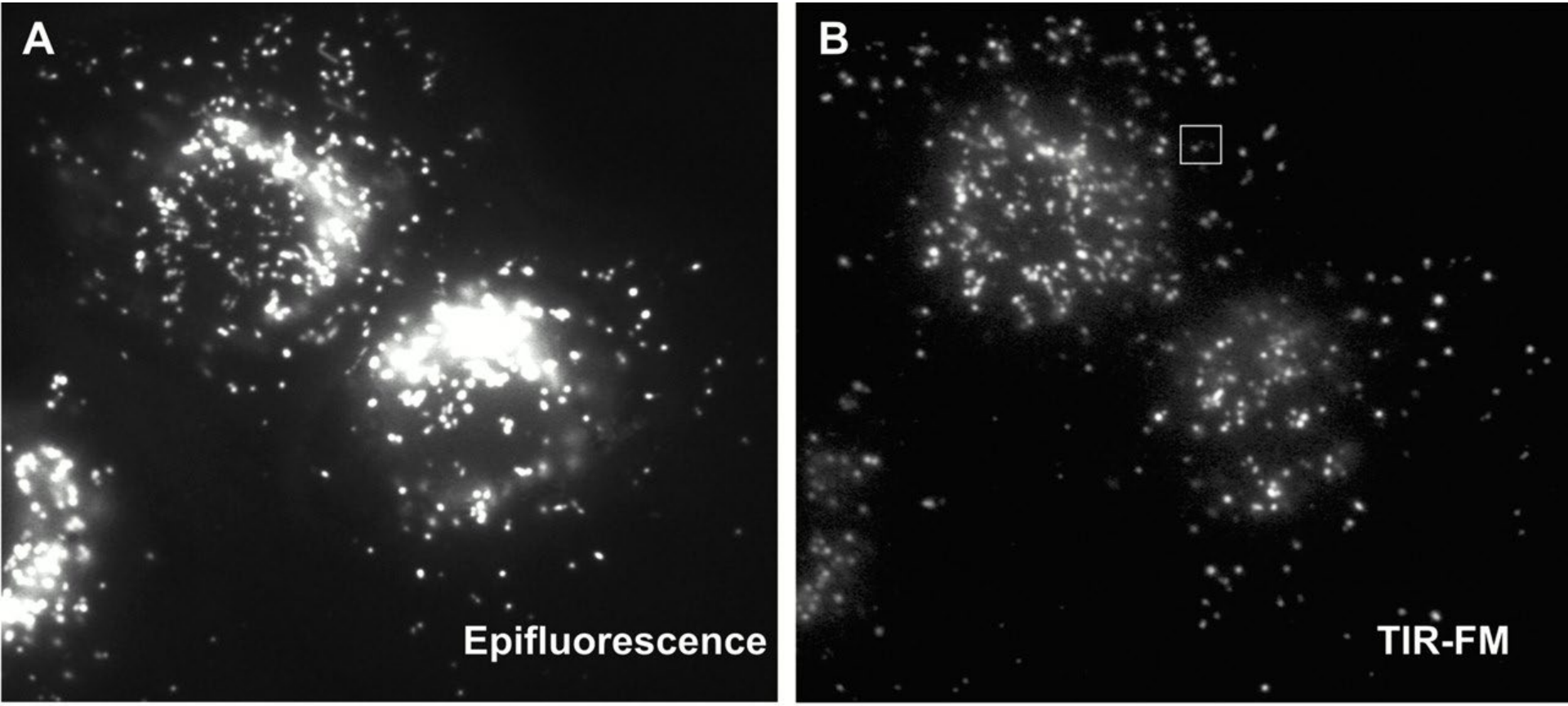
Back focal plane
$r \propto n \sin(\theta) \sim$ "NA"



Typical NA 1.45–1.49

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

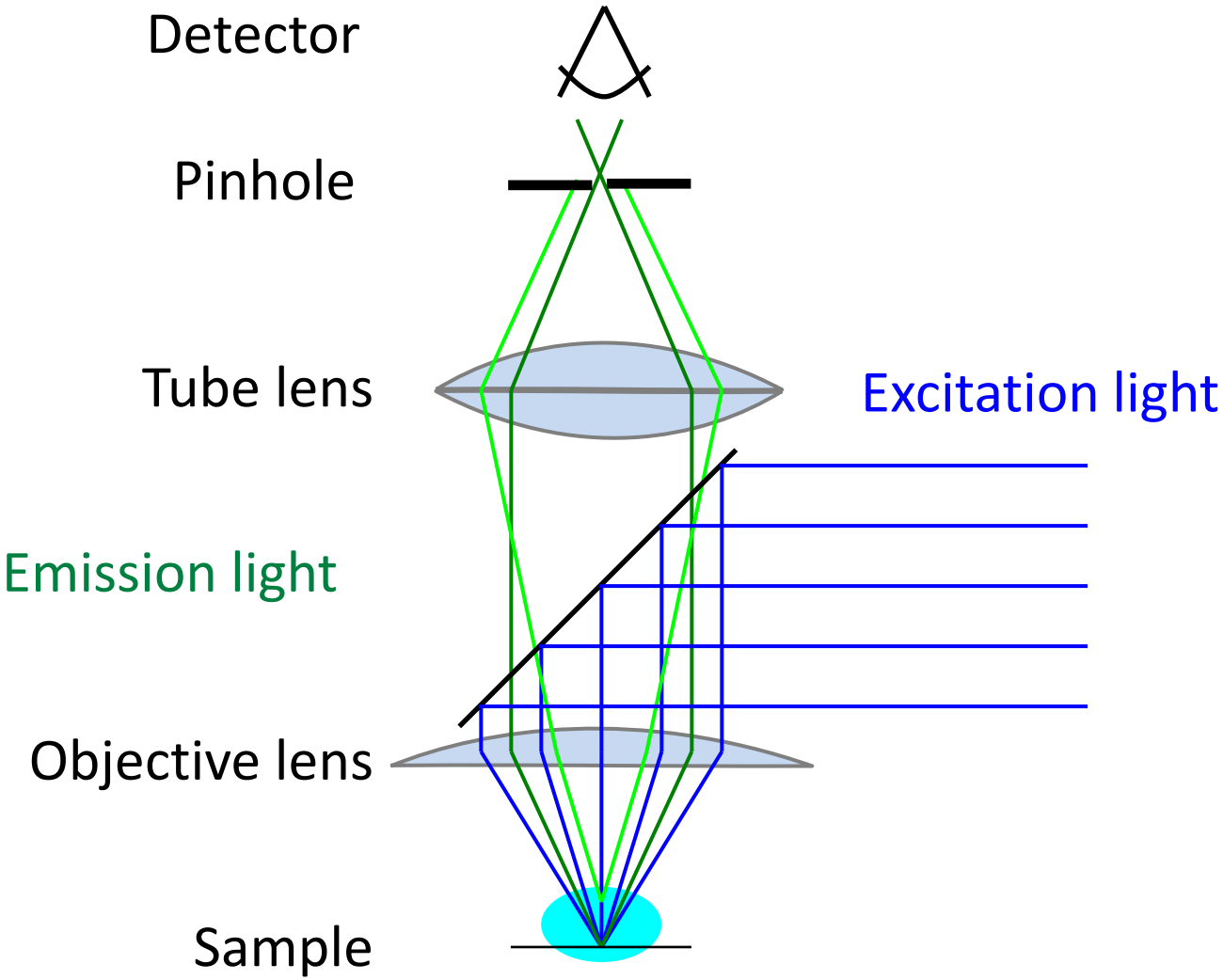
Epifluorescence vs. TIRF



Jaiswal et al 2002; cells loaded with FITC-dextran

The Confocal Microscope, Redux

What limits the imaging depth?

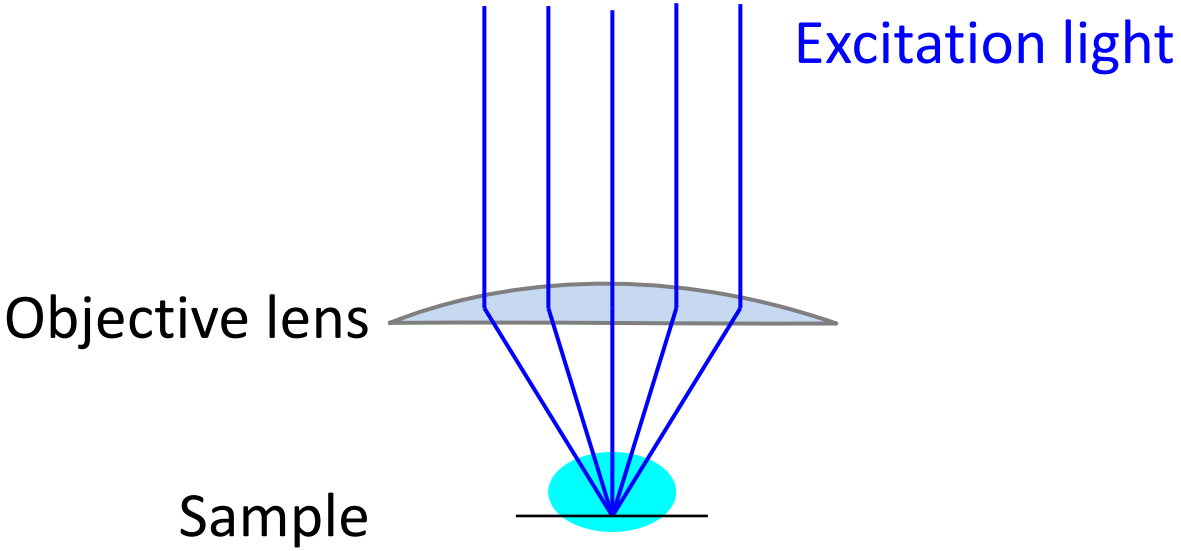


The Confocal Microscope

What limits the imaging depth?

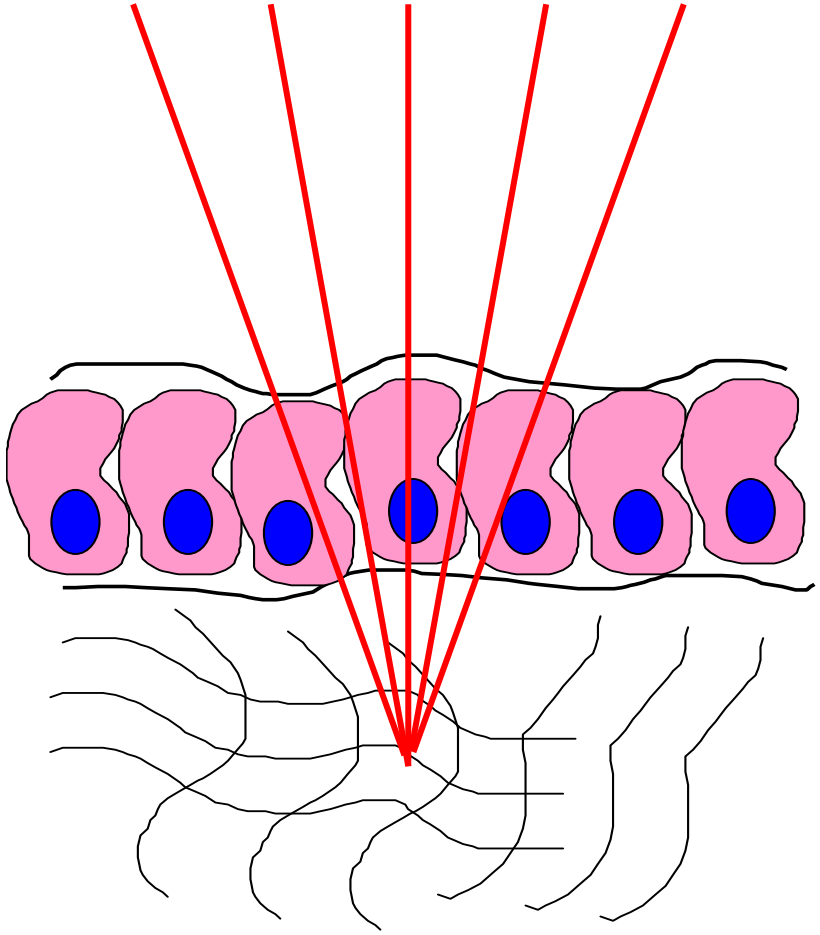
1. Physical limitations – objective working distance

2. Tissue penetration depth



What limits tissue penetration depth?

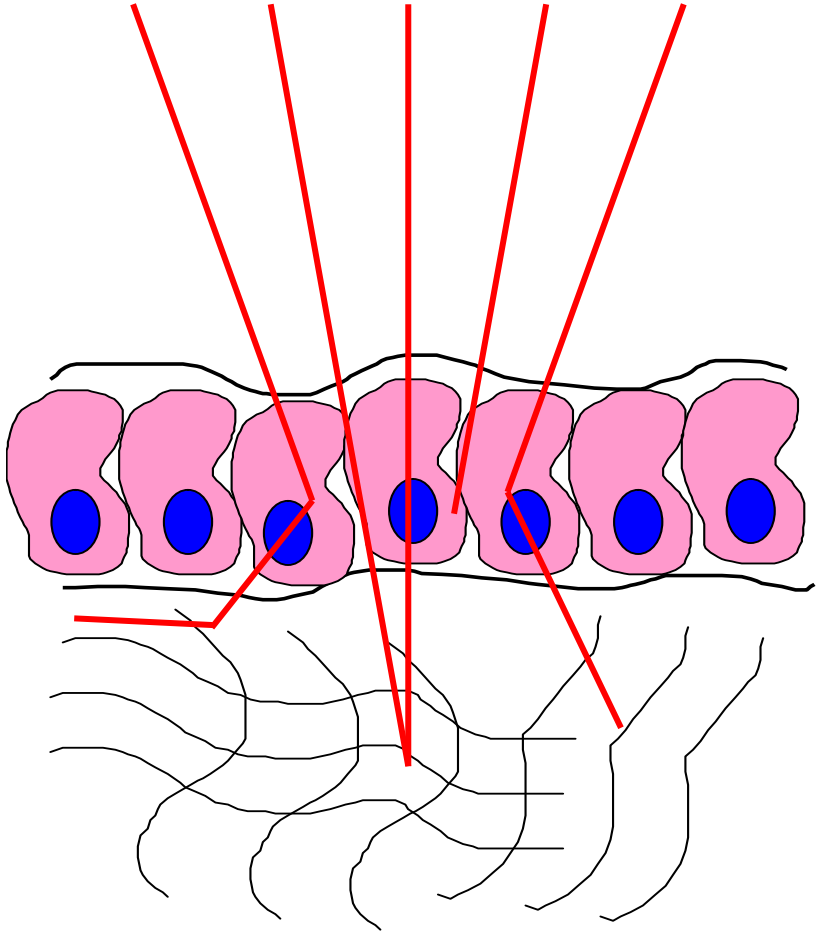
Ideal Case



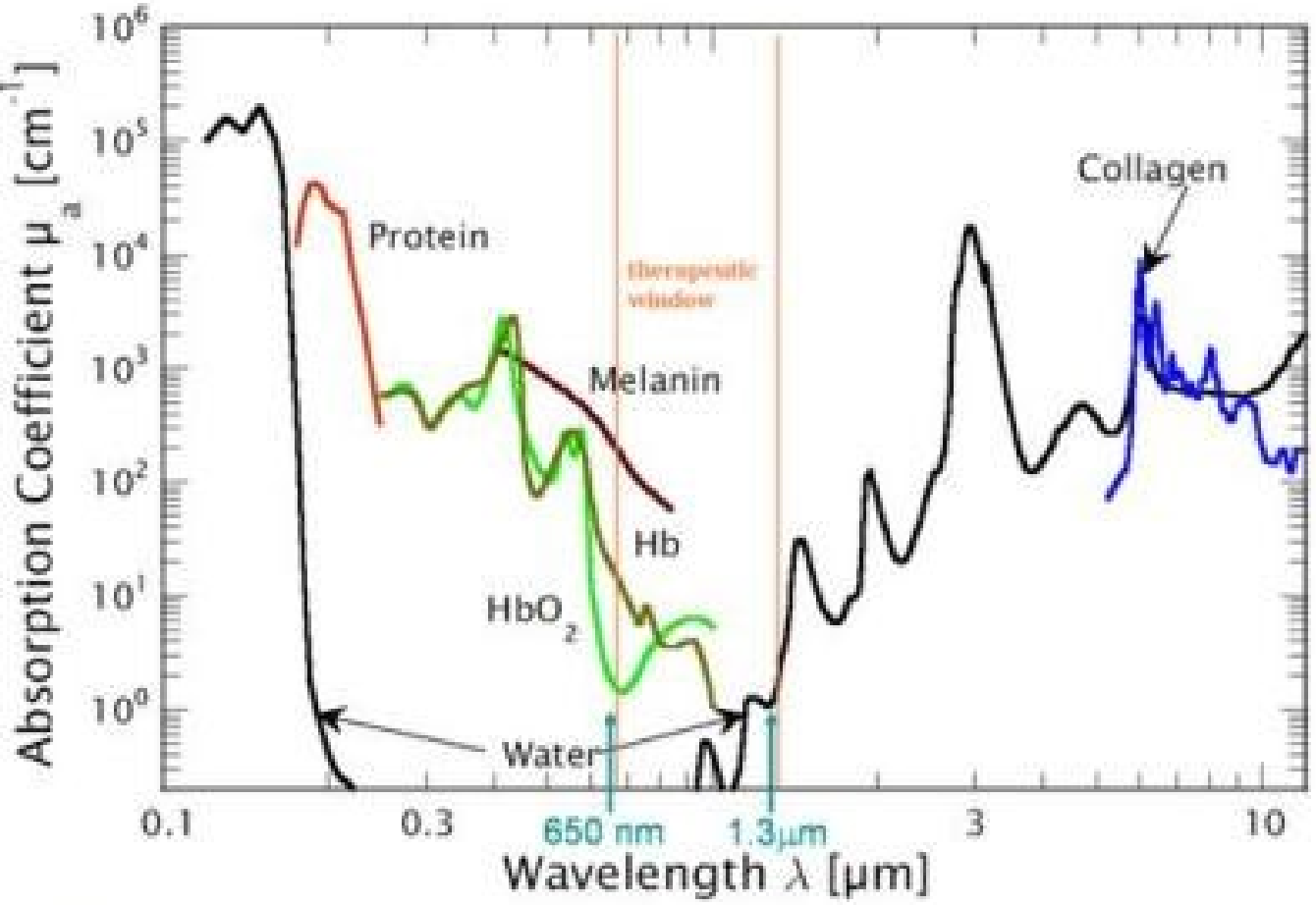
What limits tissue penetration depth?

Absorption

Scattering



Absorption of common biological molecules



From http://www.chem.duke.edu/~wwarren/tissueimaging.php

Scattering

- The amount of light scattered scales as $1/\lambda^4$ (Rayleigh scattering)

- Imaging in the near-infrared minimizes both absorption and scattering

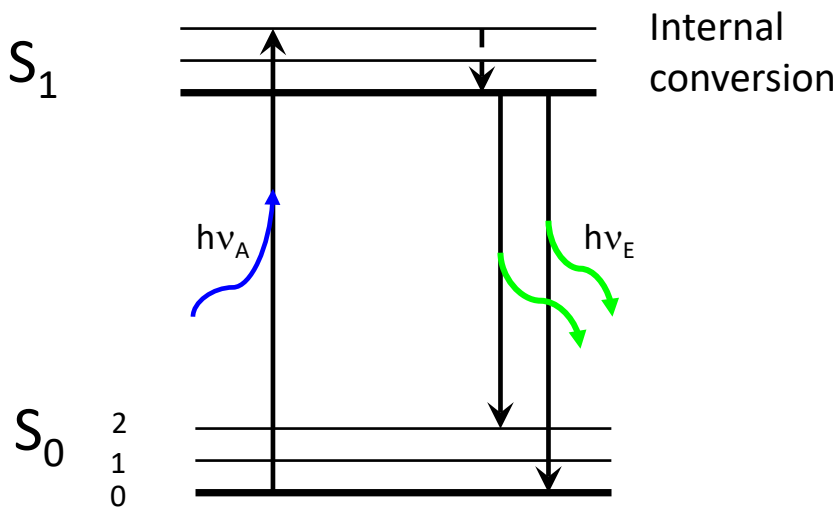
Imaging in the Infrared

- 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

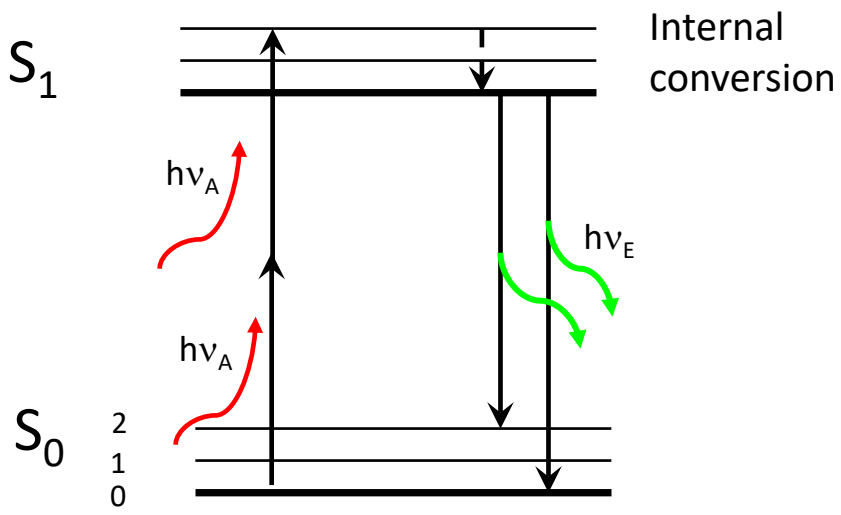
Conventional (one-photon) excitation

Jabłoński Diagram



Two-photon excitation

Jabłoński Diagram

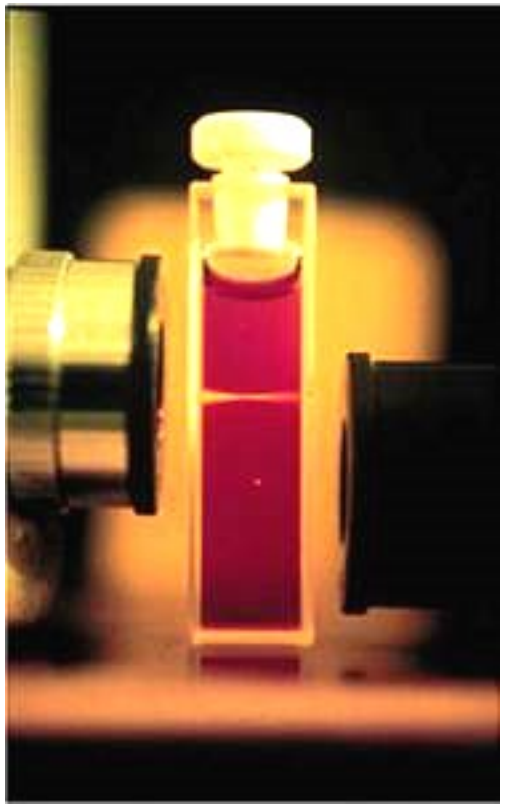


Both photons must arrive nearly simultaneously (< 1 fs)

Two-photon excitation

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

1P

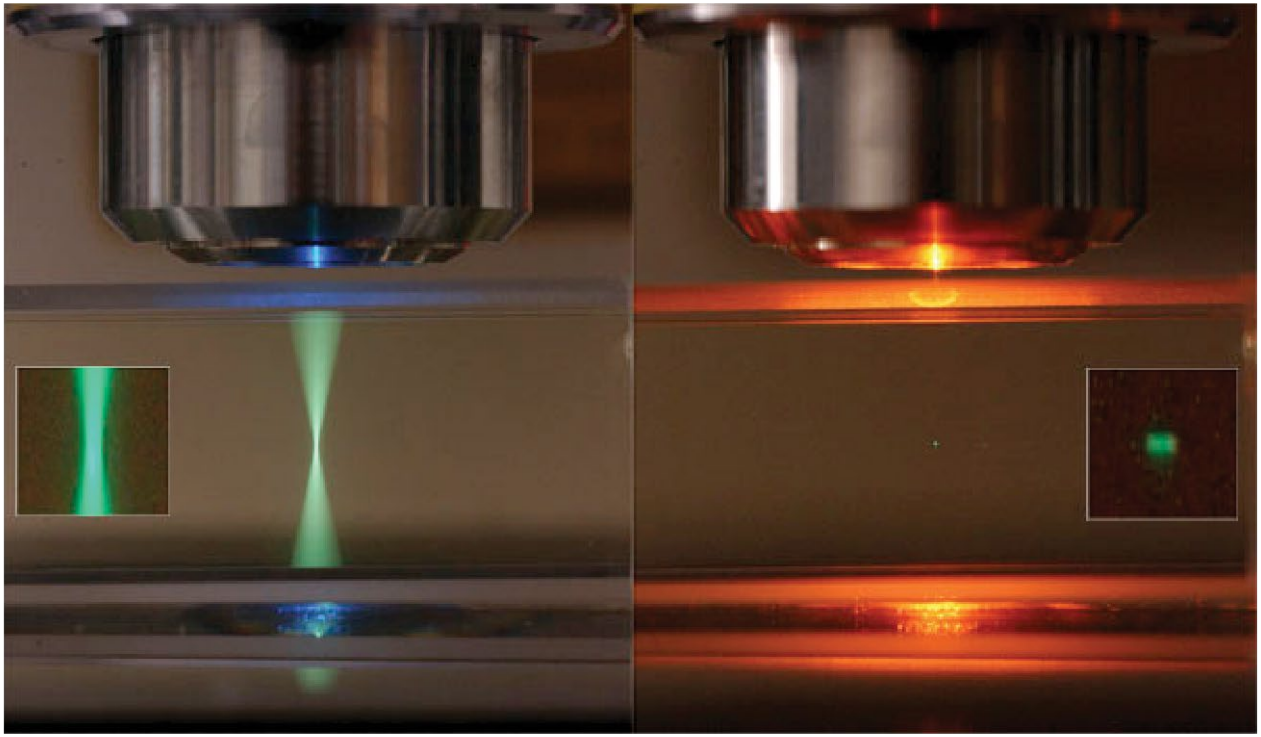


2P

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.

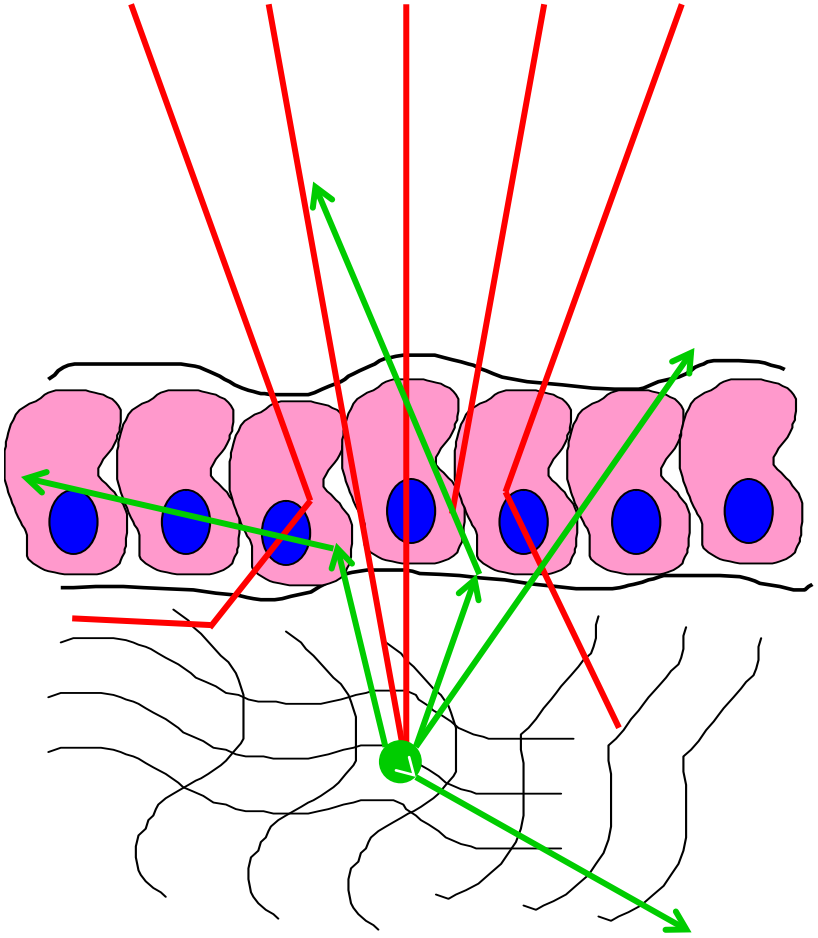
Image by Steve Ruzin and Holly Aaron, UC Berkeley

Two-Photon excitation: no out of focus light

- In confocal, the focal volume is defined by a point of light × 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



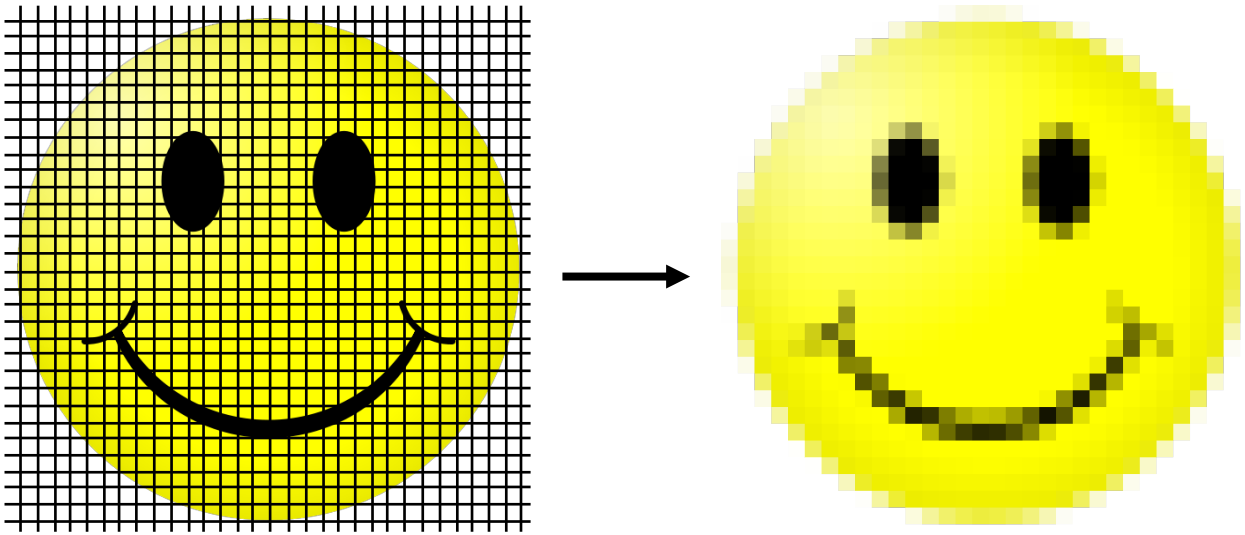
Tissue Absorption and Scattering, revisited

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

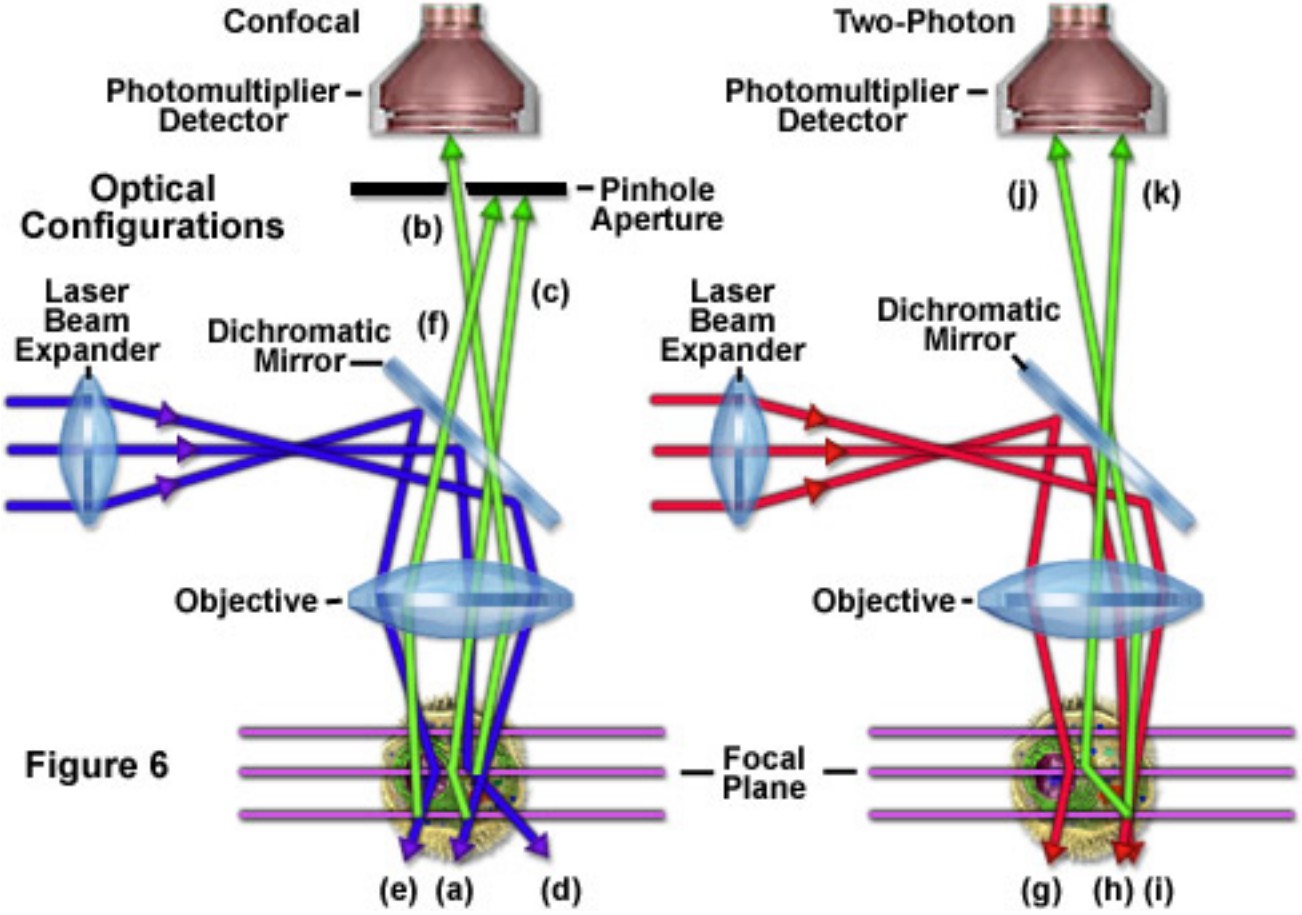
- Because emitted light all comes from focus, we don't need to image it.
- Collect all emission light, regardless of path, and assign it to focus
- Don't use the pinhole: Non-descanned detection

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



http://www.microscopyu.com

A home-built two-photon microscope

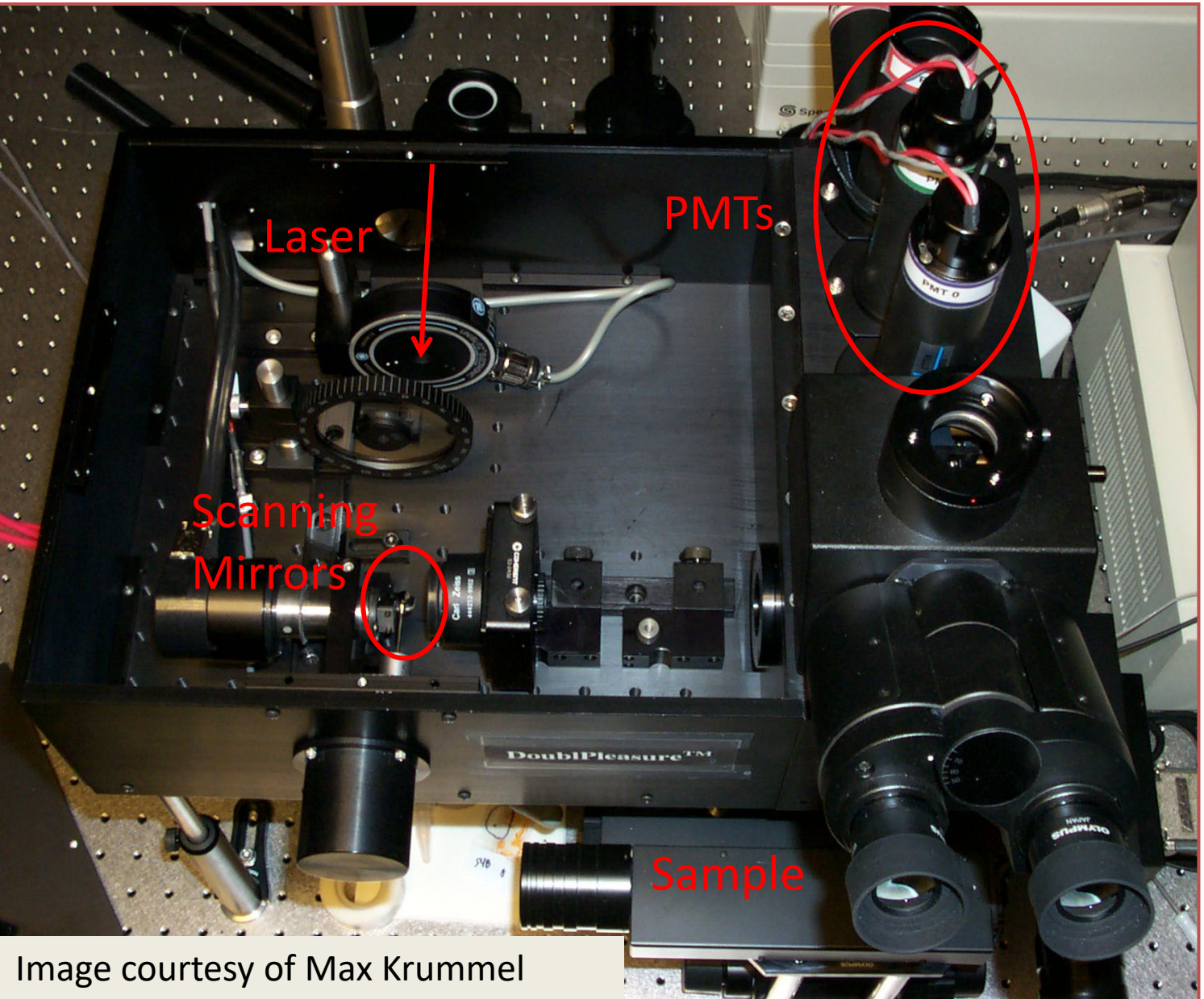
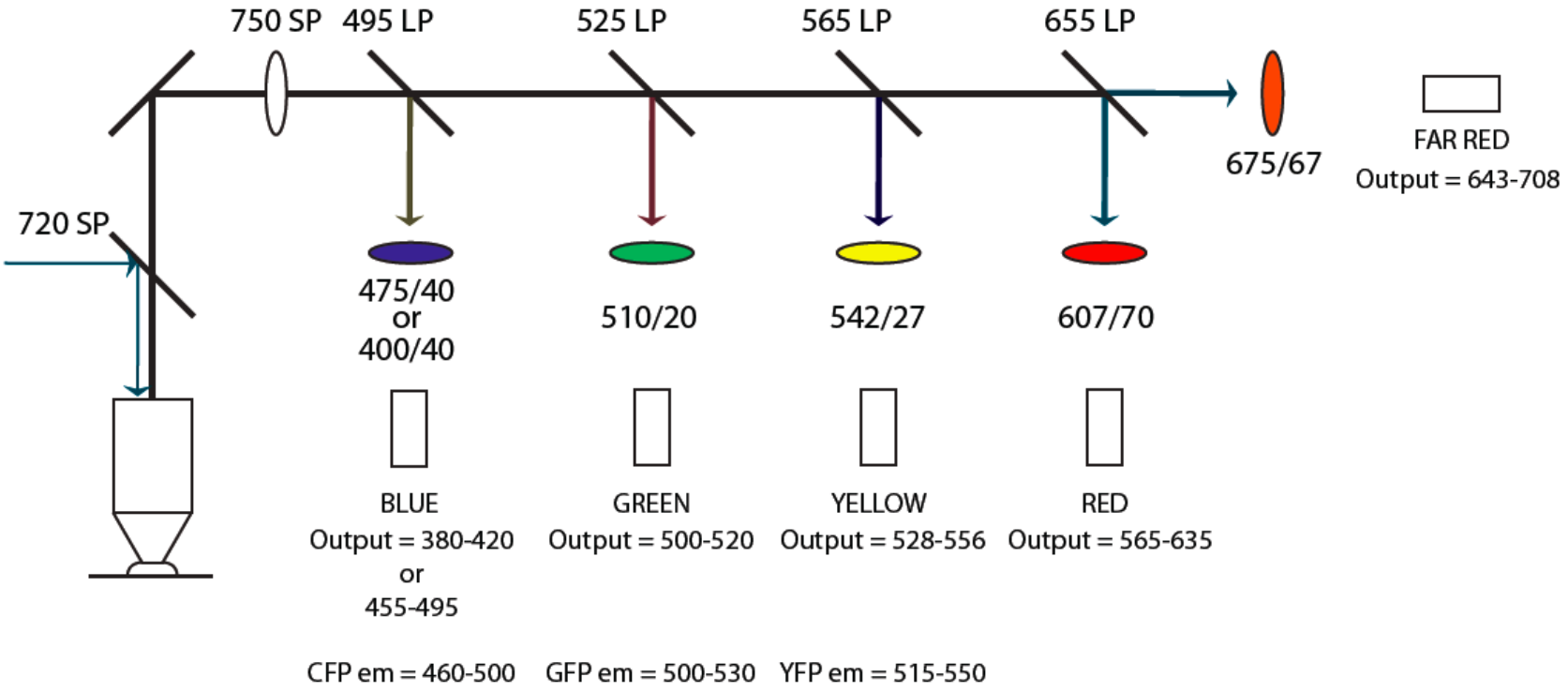


Image courtesy of Max Krummel

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

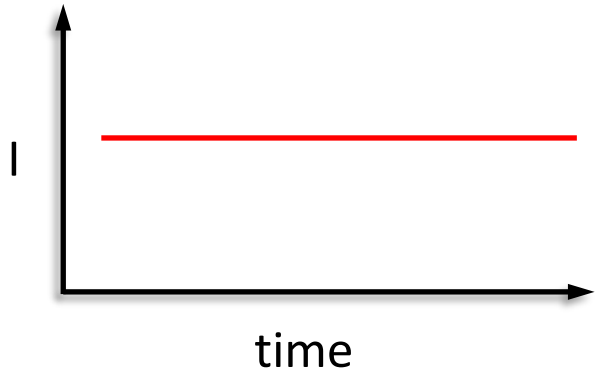


Lasers for two-photon excitation

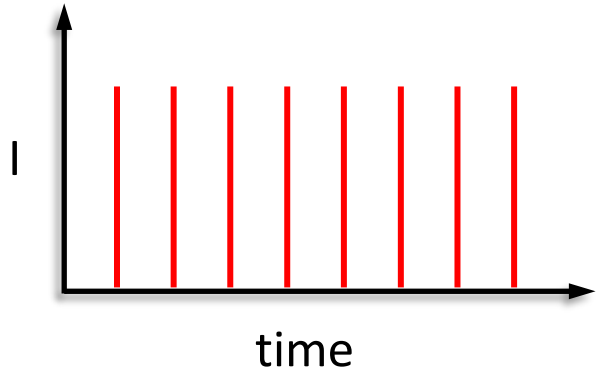
- Emission intensity depends on the square of excitation intensity.
- We want high excitation power.
- Use pulsed laser to get high peak power but normal average power.

Lasers for two-photon excitation

Typical CW laser

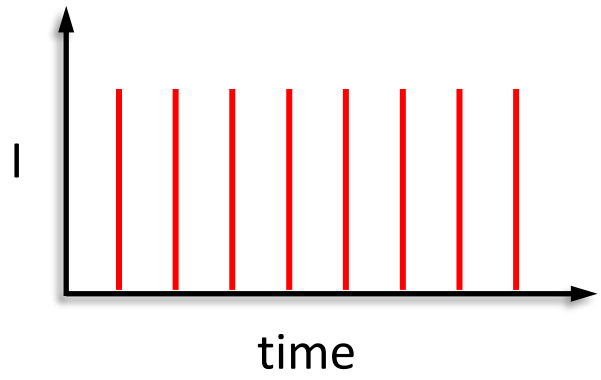


Pulsed laser



Lasers for two-photon excitation

Pulsed laser



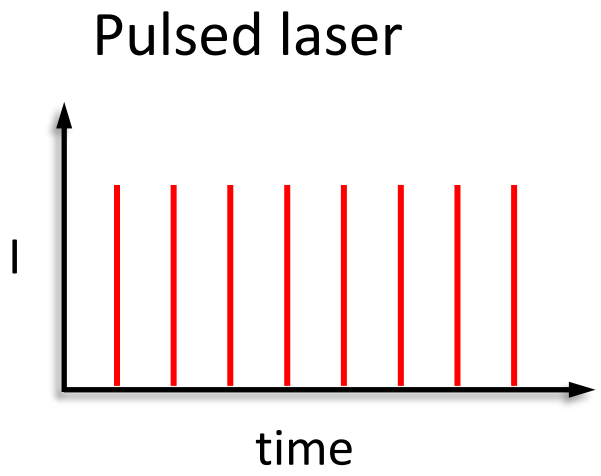
Typical Ti-Sapphire laser for two-photon microscopy (Newport Mai Tai):

Pulse width: 80 fs

Repetition rate: 80 MHz

Time between pulses: 12.5 ns

Lasers for two-photon excitation



80 fs pulse every 12.5 ns:

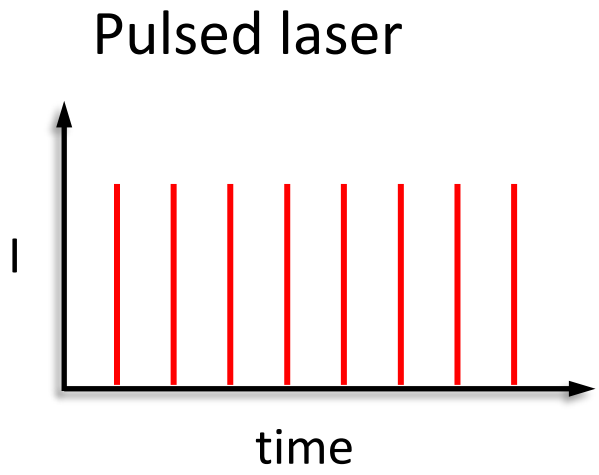
The laser is only ‘on’ ~1/150 000th of the time

→ 1W average power results in

150 000W peak power

Cost: $100 000 – $200 000

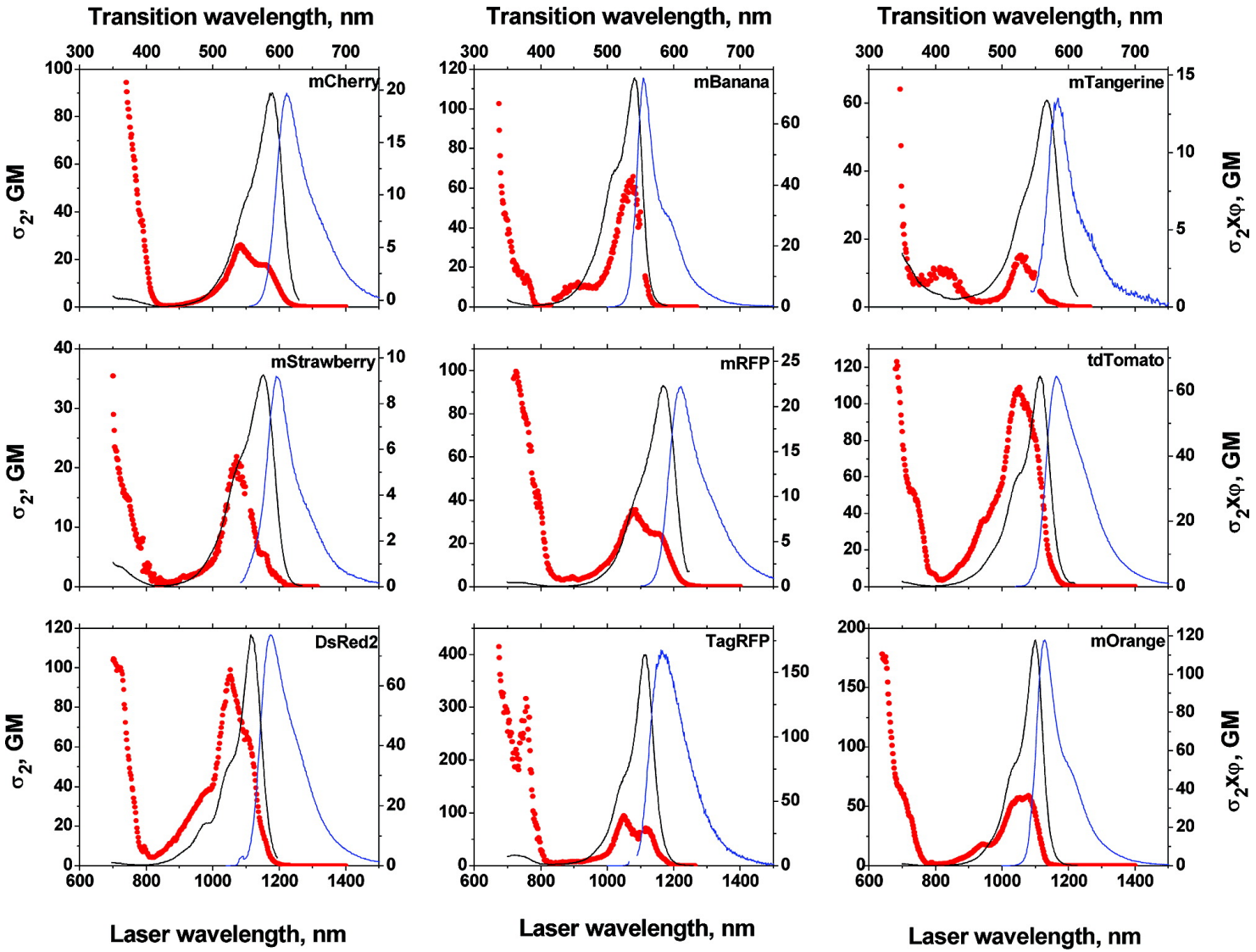
Ti-Sapphire lasers for two-photon excitation



Ti-Sapphire lasers are tunable:

Tuning range ~700 – 1000 nm

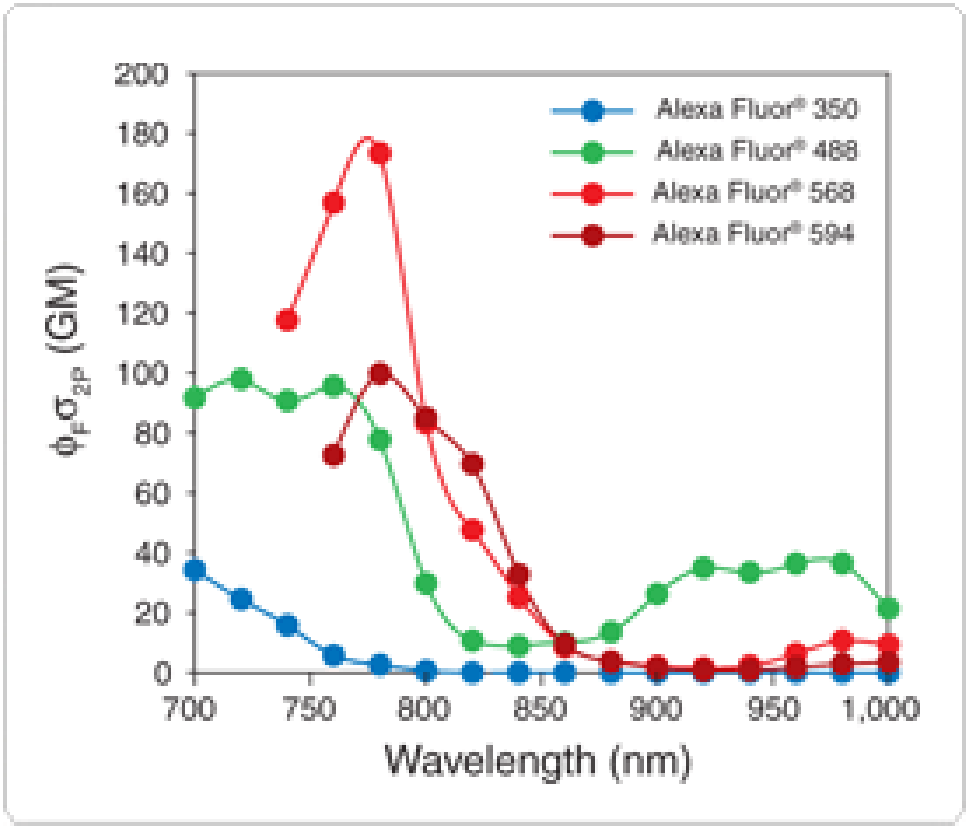
Two-photon excitation spectra



Drobizhev et al. J. Phys. Chem. B 2009

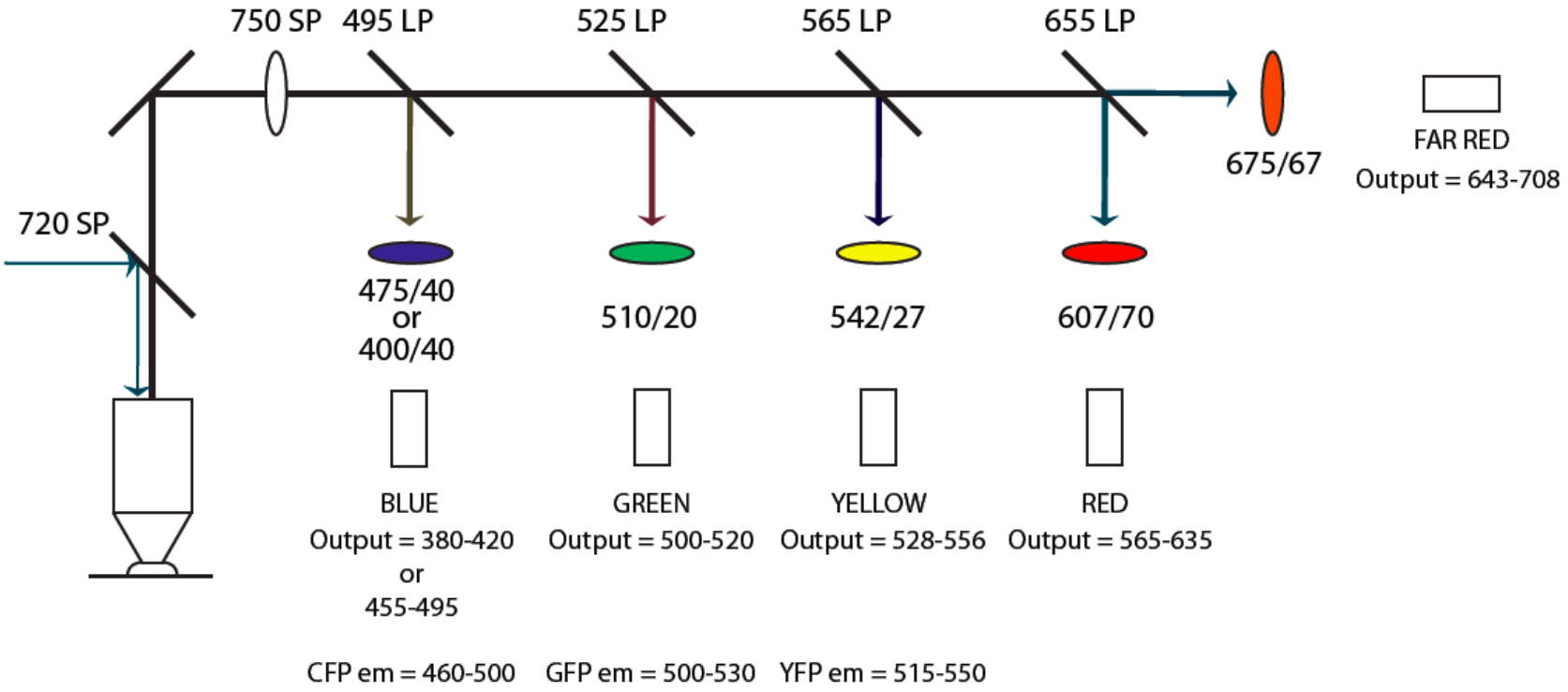
Two-photon excitation spectra

Not twice the 1-photon excitation spectrum



Broadness of spectrum means one wavelength can excite multiple dyes

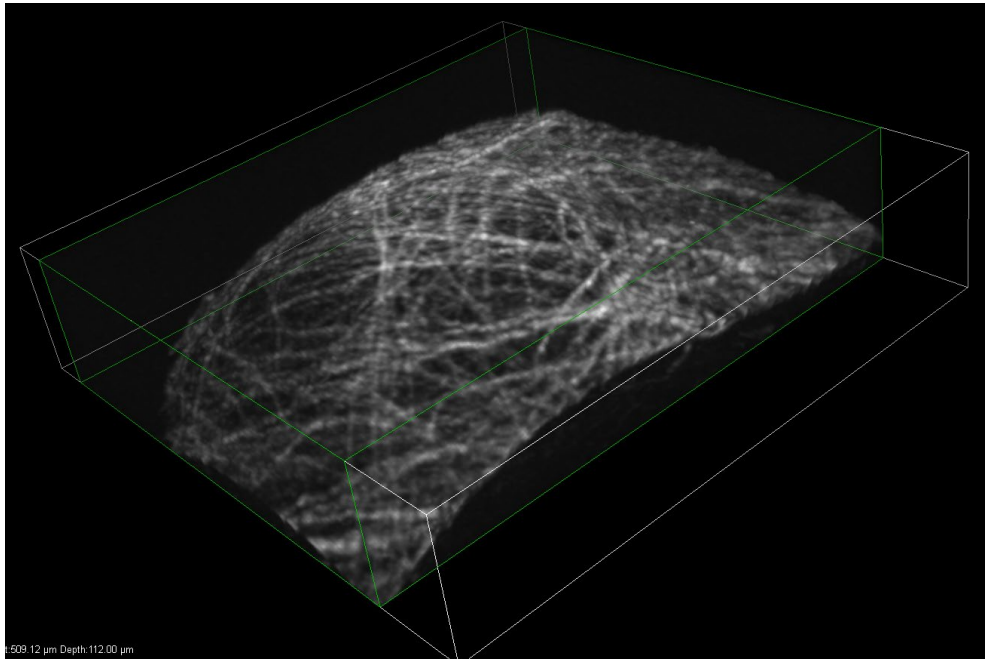
Collect multiple emission channels to take advantage of broad excitation spectra



Second Harmonic Generation

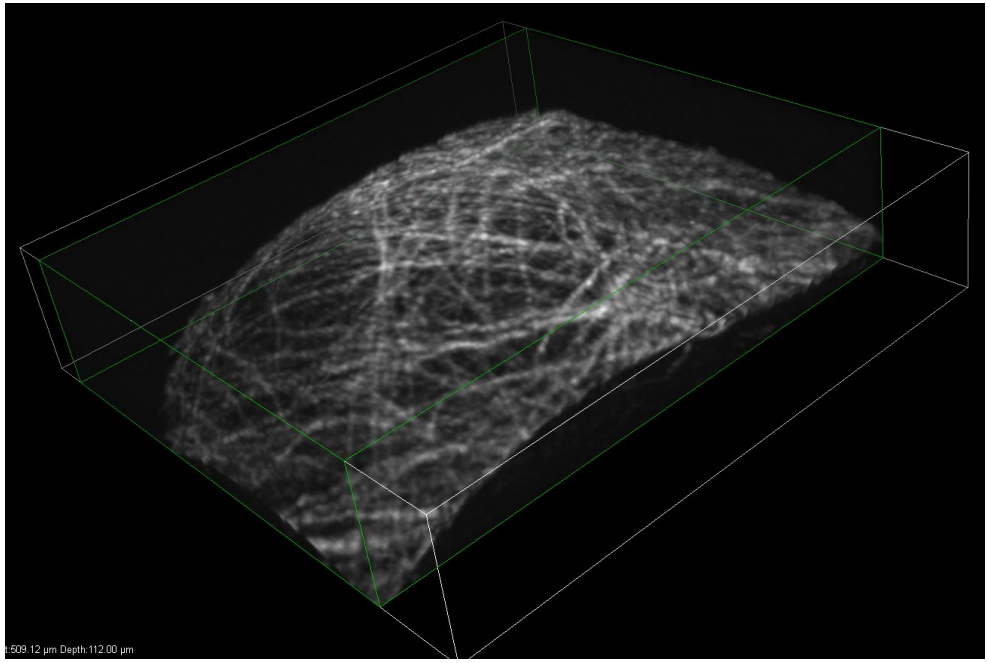
Some anisotropic molecules frequency-double light and generate emission at ½ the incident wavelength

Collagen I and IV
Cellulose
Some membrane dyes



Second Harmonic Generation

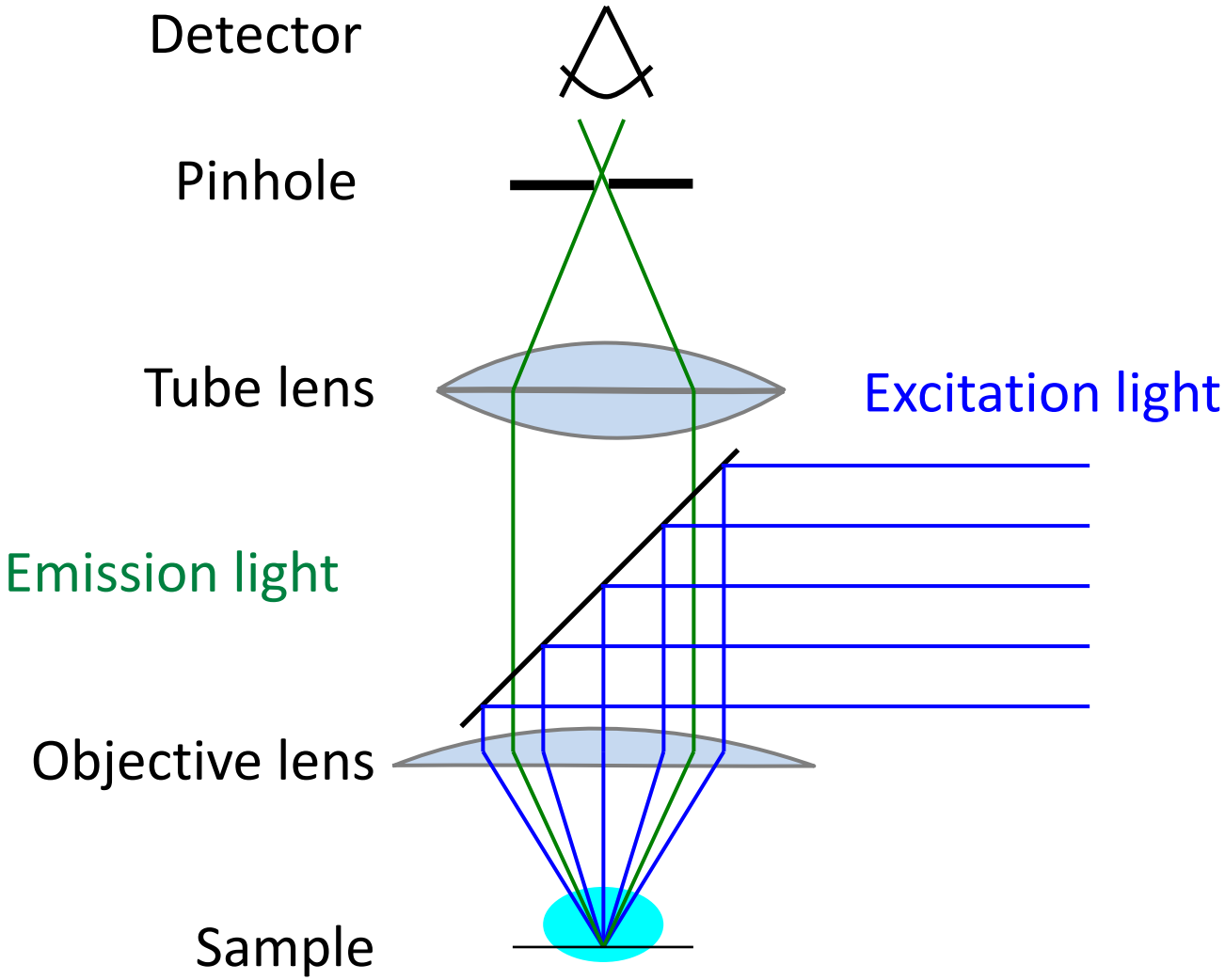
This is a nonlinear process, and so its brightness scales as the square of the excitation light intensity too.



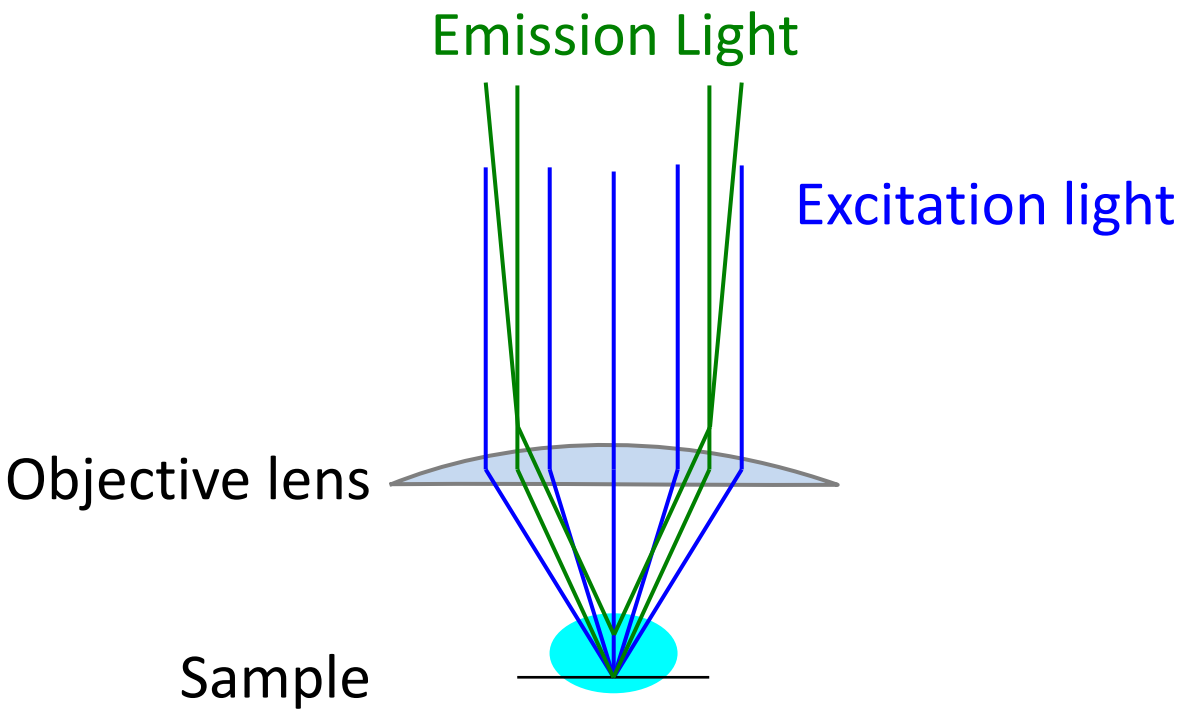
When to use Two Photon Microscopy?

- Thick samples – 200 μm – 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

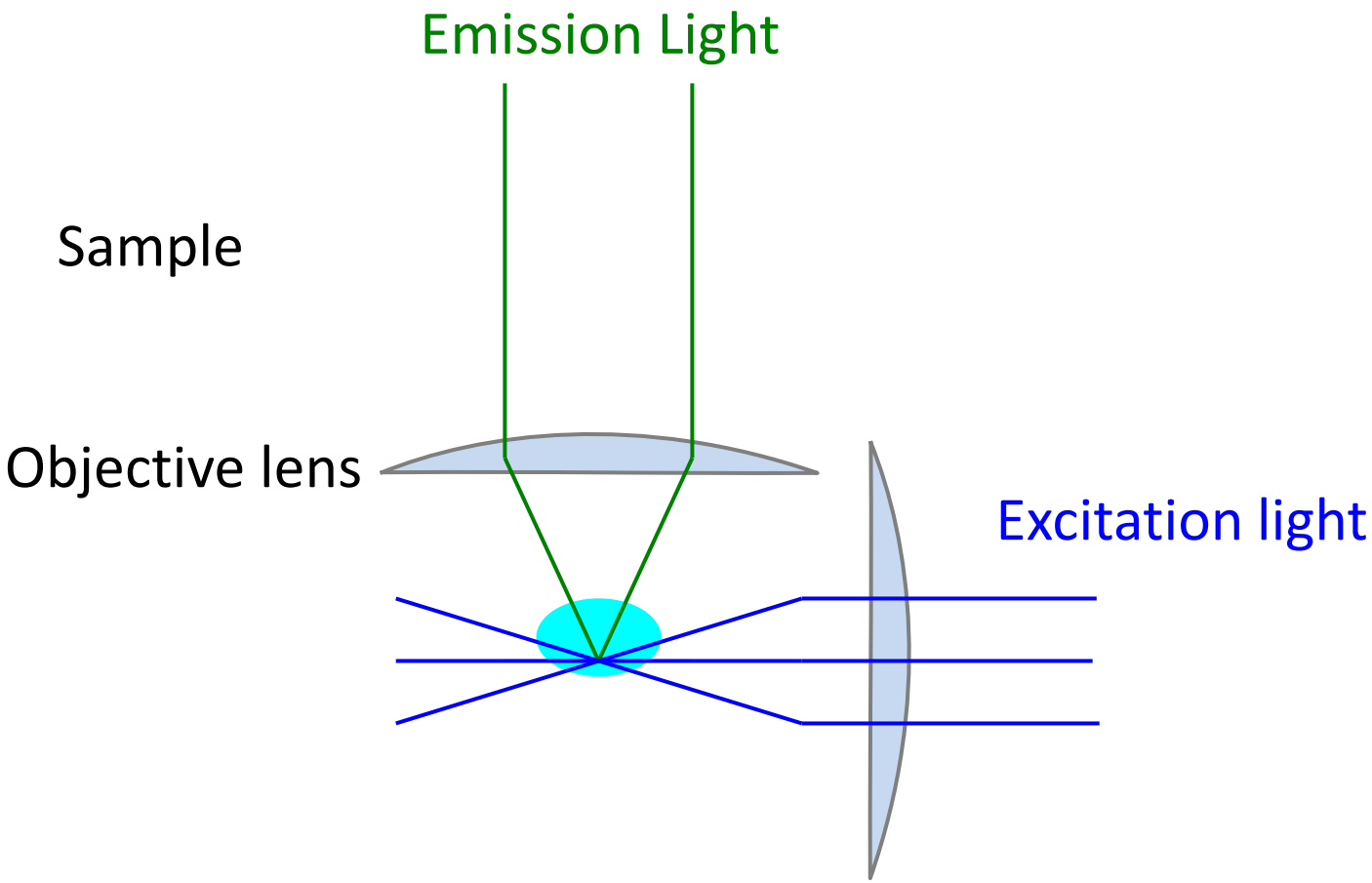


The Problem with Epi-Illumination



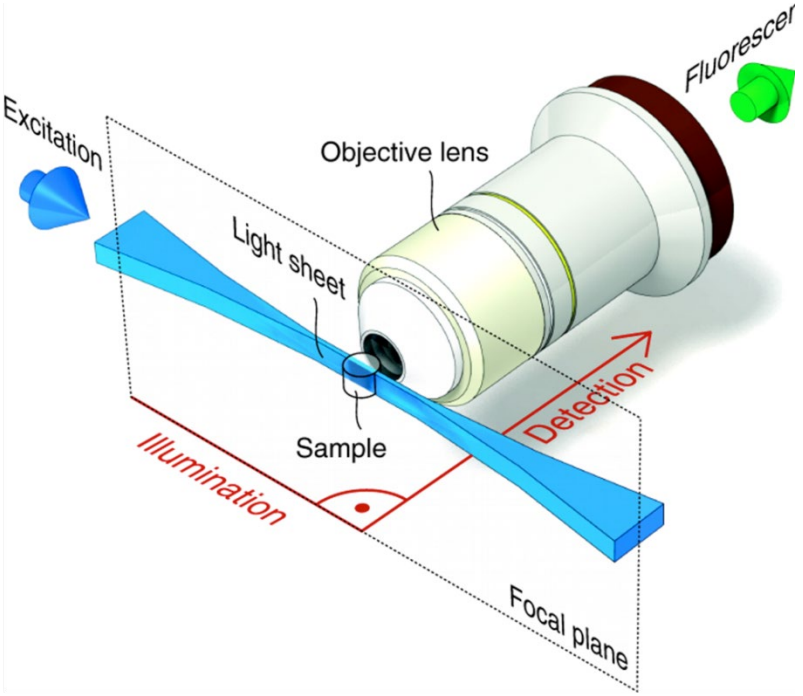
Selective Plane Illumination or Light Sheet Microscopy

Illuminate with a plane of light at 90° 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



Jan Huisken
2009

Single Plane Illumination Microscopy

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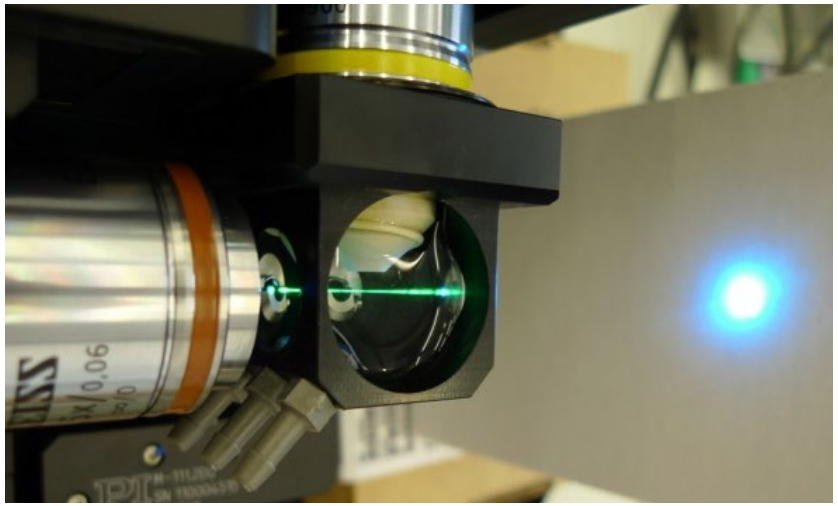
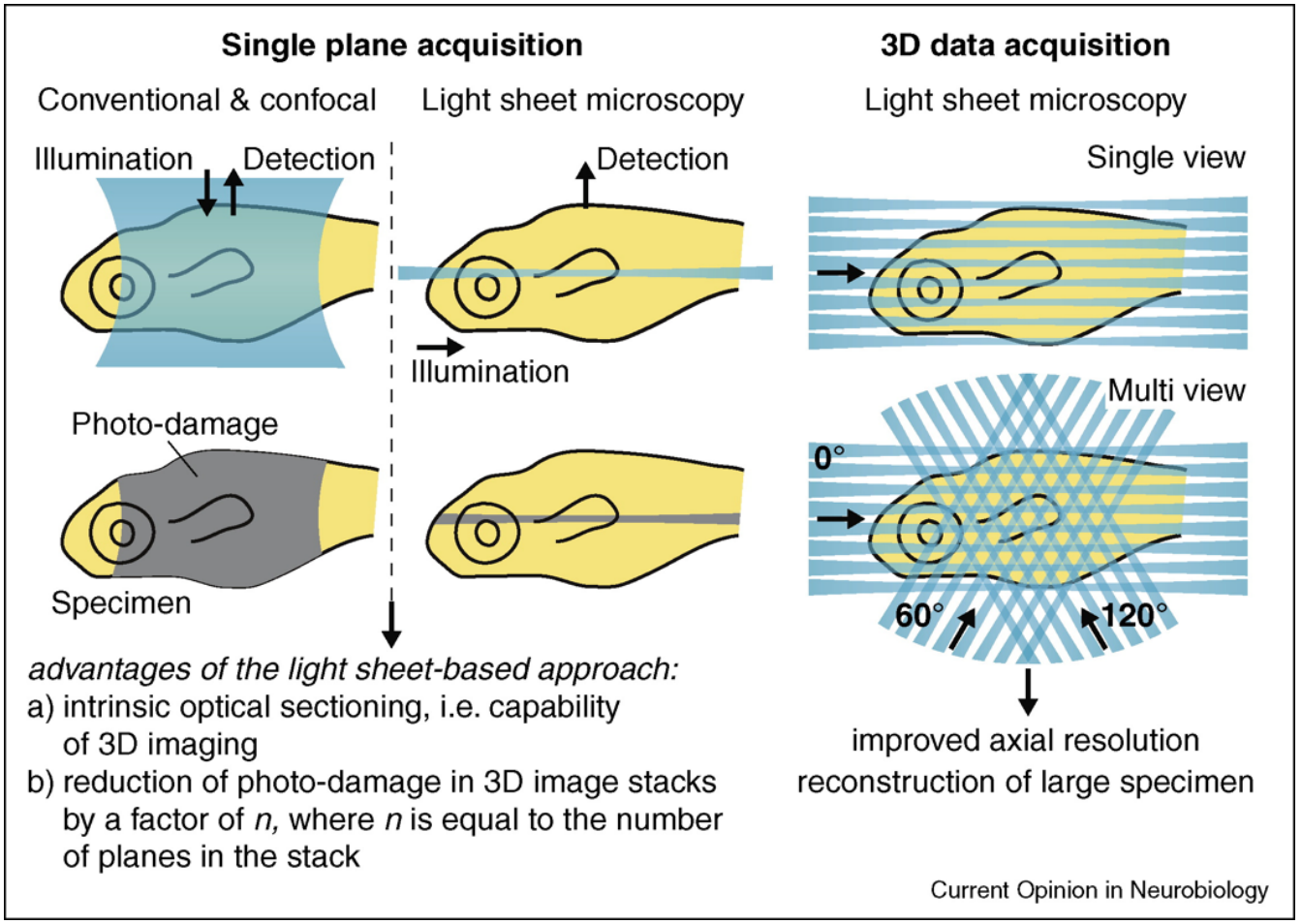


Anna Reade

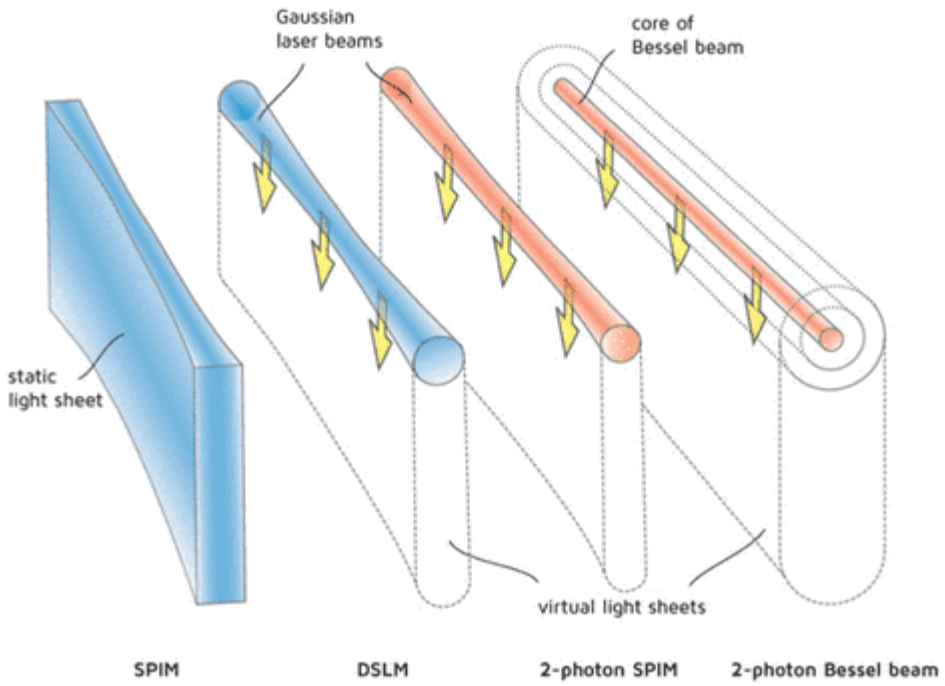
SPIM Advantages



Keller and Steltzer Curr. Op. Neurobiol., 2008

Generating the Light Sheet

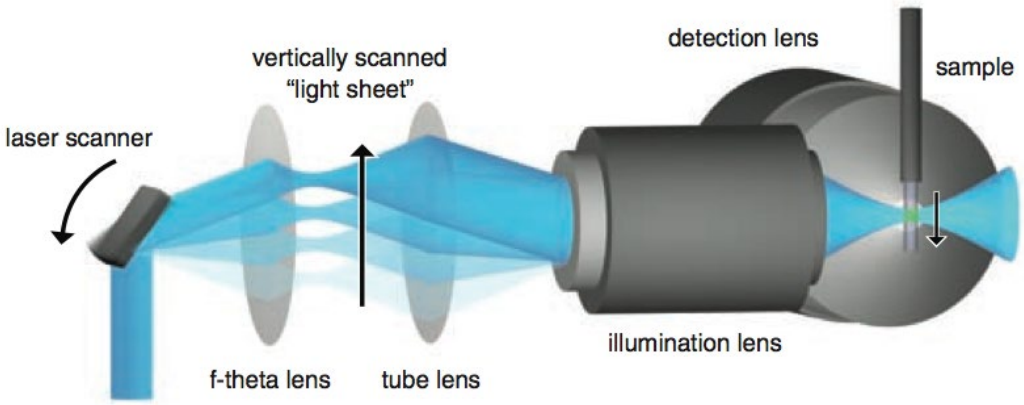
- Cylindrical lens
- Gaussian beam plus scanning (DSLM)
- Bessel beam plus scanning



Jan Husiken

Generating the Light Sheet

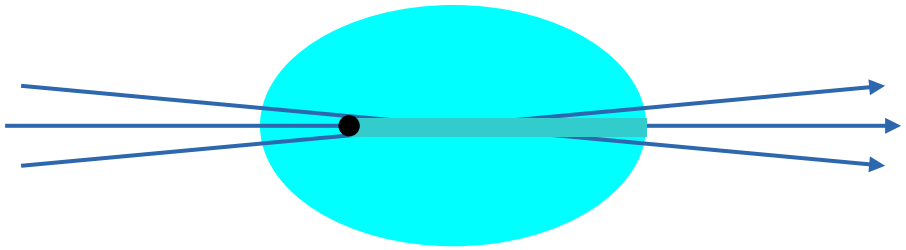
- Cylindrical lens
- Gaussian beam plus scanning (DSLM)
- Bessel beam plus scanning

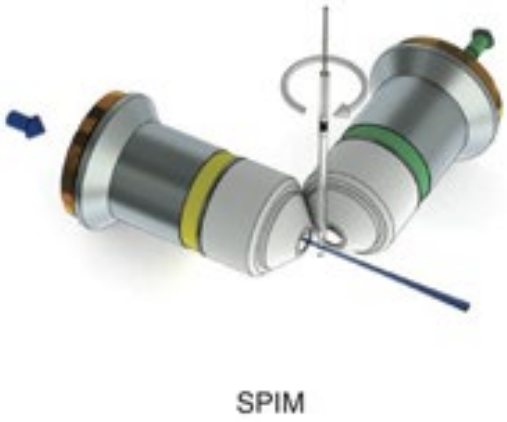


Keller

A Problem: Absorption and Scattering

Solution: Image from Multiple Directions





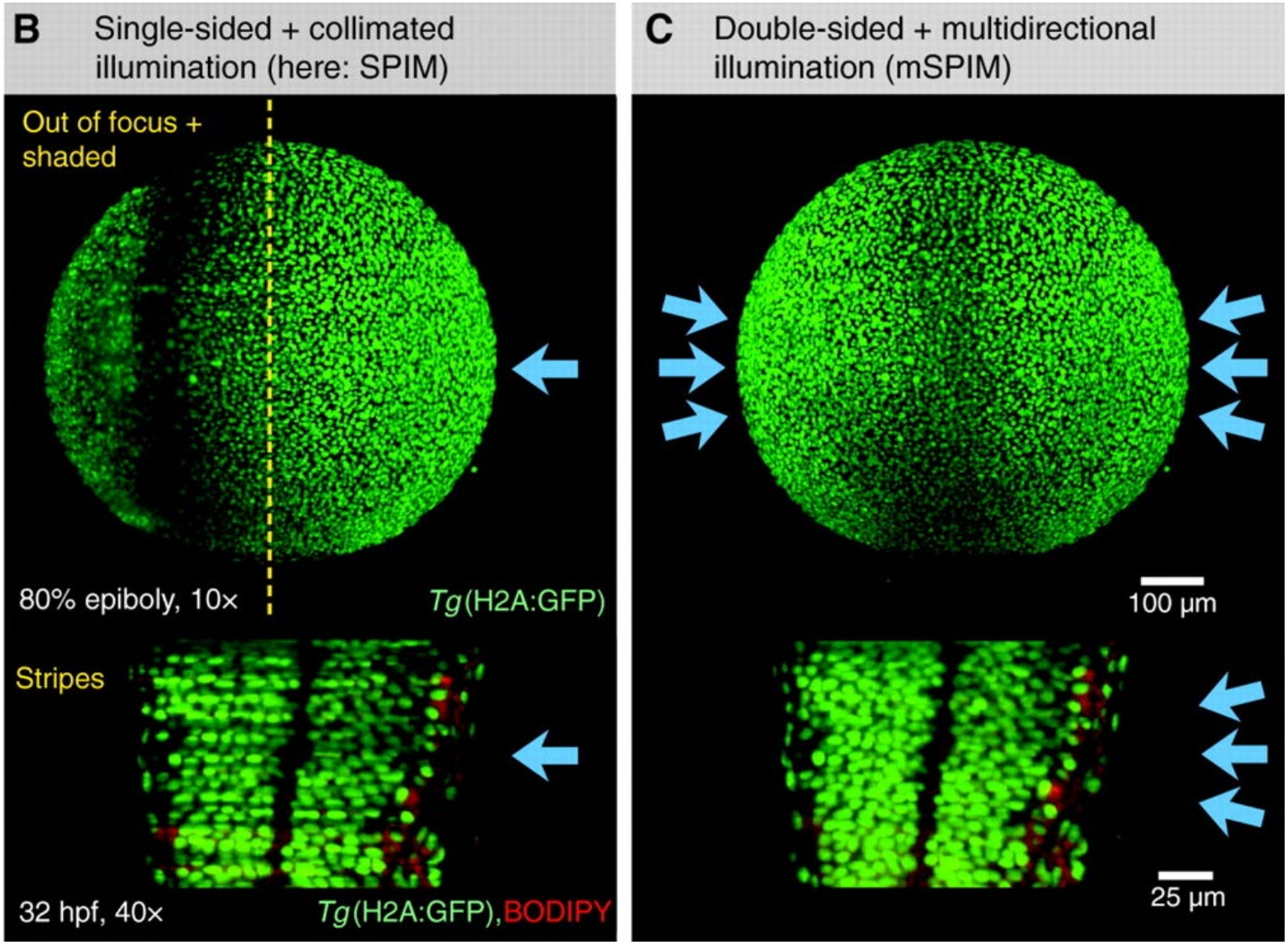
SPIM

mSPIM

Four-lens SPIM
(MuVi-SPIM, SiMView)

Jan Huisken

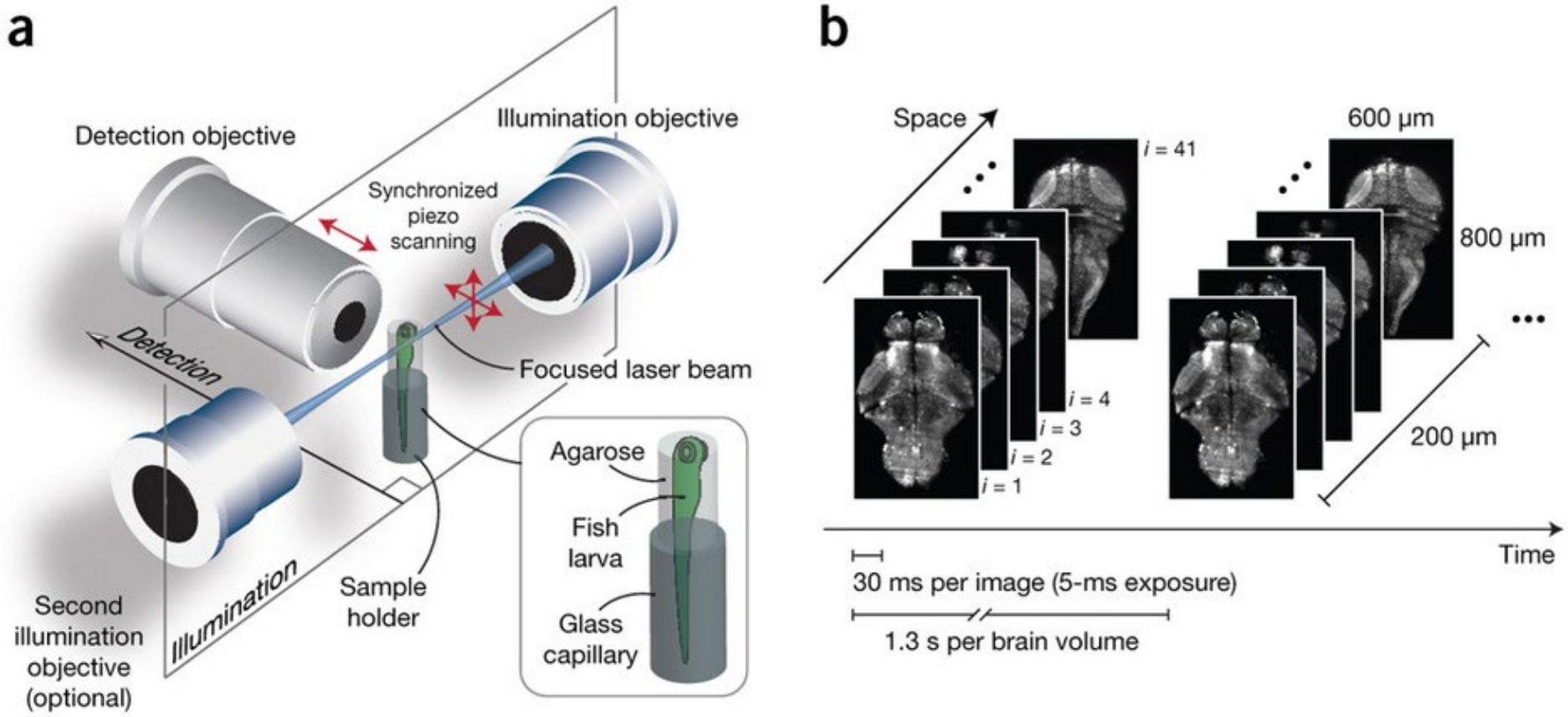
Multidirectional SPIM (mSPIM)



Huisken and Stanier, Development 2009

Ultrafast Light Sheet Imaging of Zebrafish

Capture 800 x 600 x 200 μm at 0.65 x 0.65 x 5 μm resolution



Ahrens et al. Nat. Meth. 2013

Imaging of GCaMP5 Zebrafish