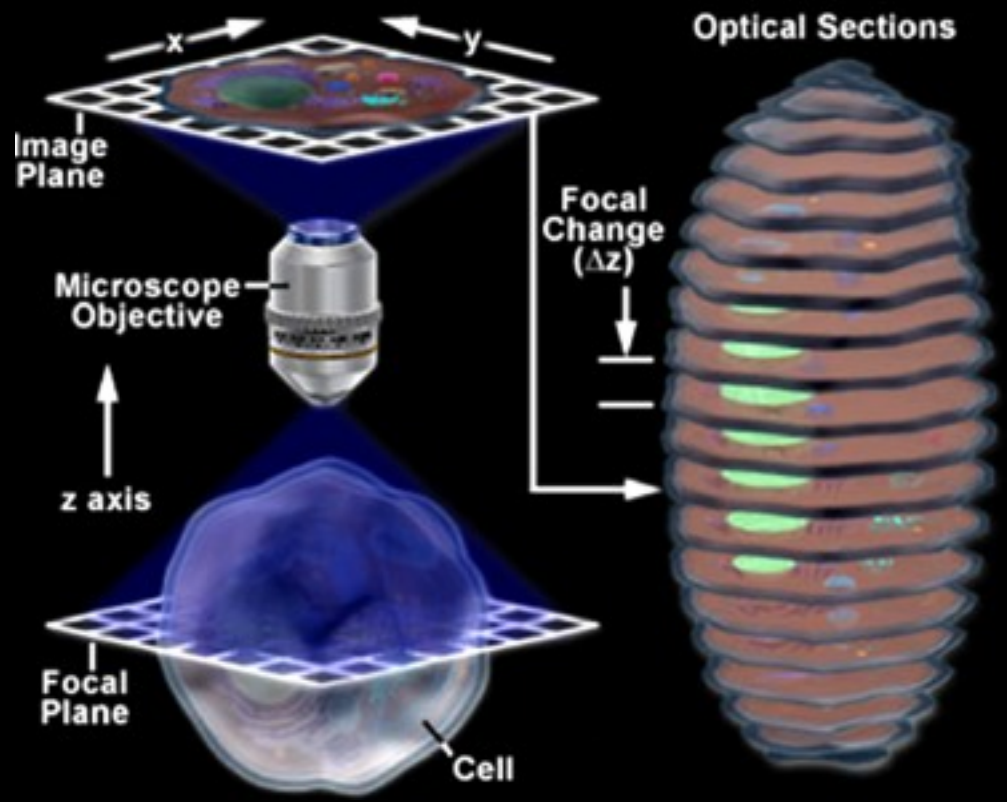
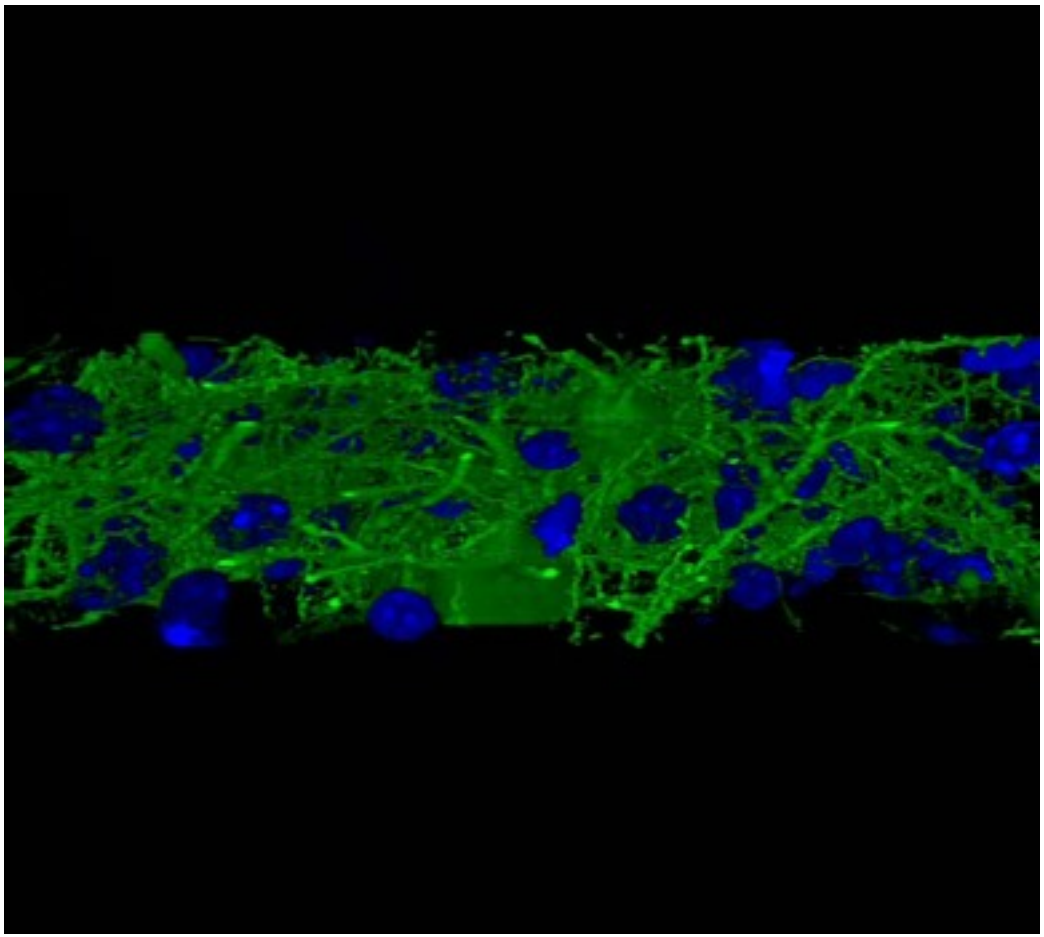
Optical Sectioning



Bo Huang
Pharmaceutical Chemistry

Approaches to 3D imaging

- Physical cutting
  - Technical difficulty
  - Highest resolution
  - Highest sensitivity
- Optical sectioning
  - Simple sample prep.
  - No physical damage
- Tomography



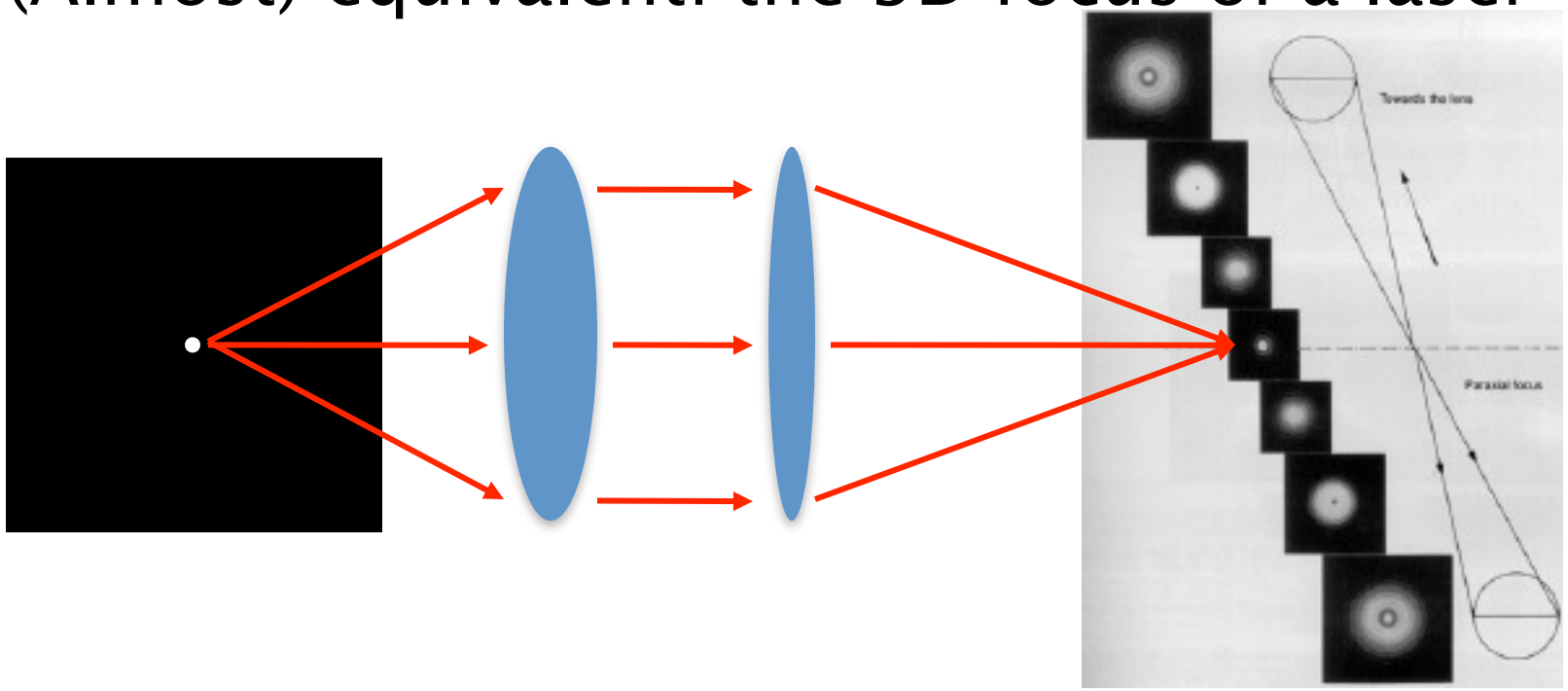
Micheva & Smith, Neuron (2007)

Optical sectioning

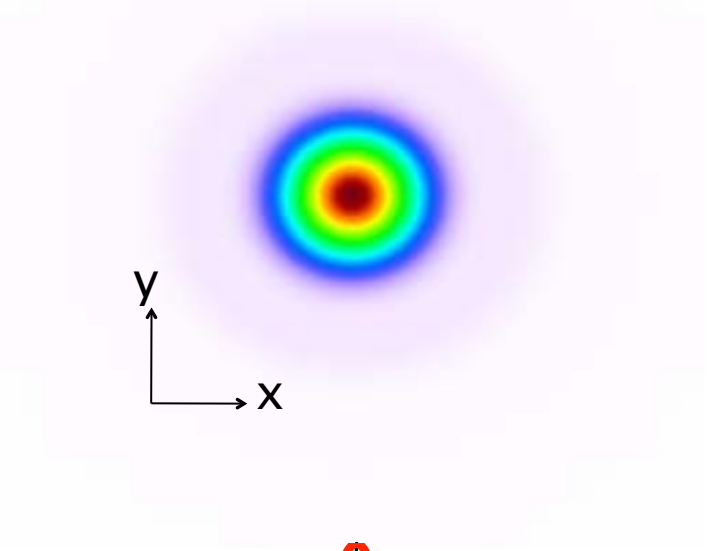
- Confocal microscopy principle
- Point scanning confocal
- Multi-point scanning confocal
  - Spinning disk
- Two-photon microscopy
- Total internal reflection fluorescence (TIRF)
- Other methods

The Point Spread Function (PSF)

- PFS: the three dimensional image of a point object
- (Almost) equivalent: the 3D focus of a laser



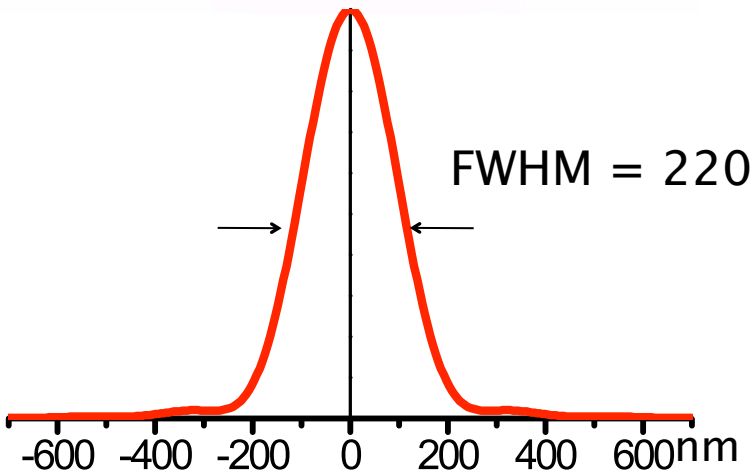
The lateral size of the PSF



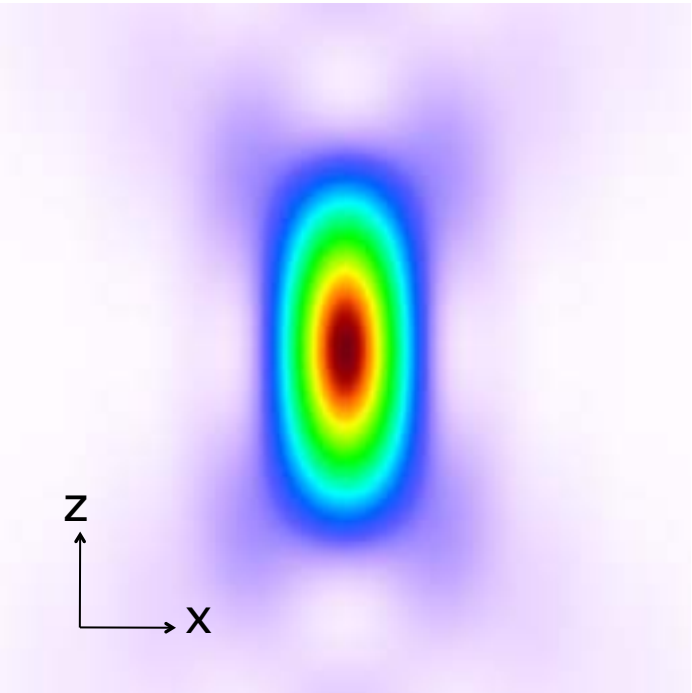
Lateral resolution $\approx \dfrac{0.61\lambda}{NA}$

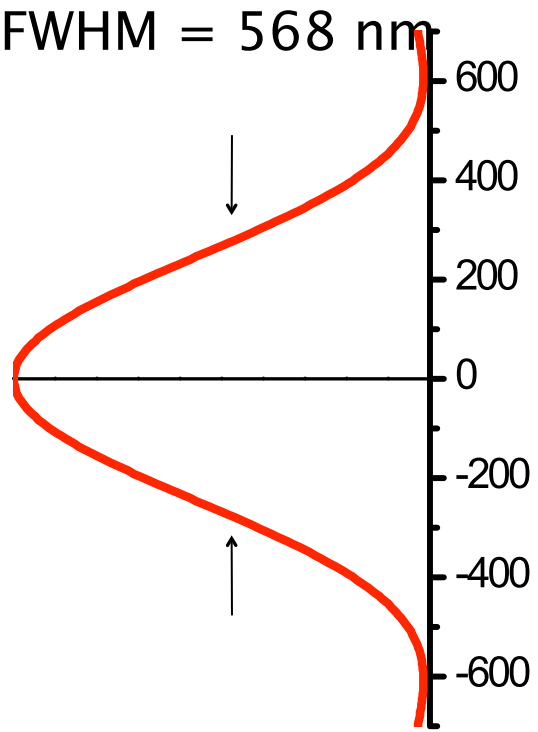
Example: 1.4 NA objective at 550 nm

$\Delta_{xy} \approx 240$ nm



The axial size of the PSF





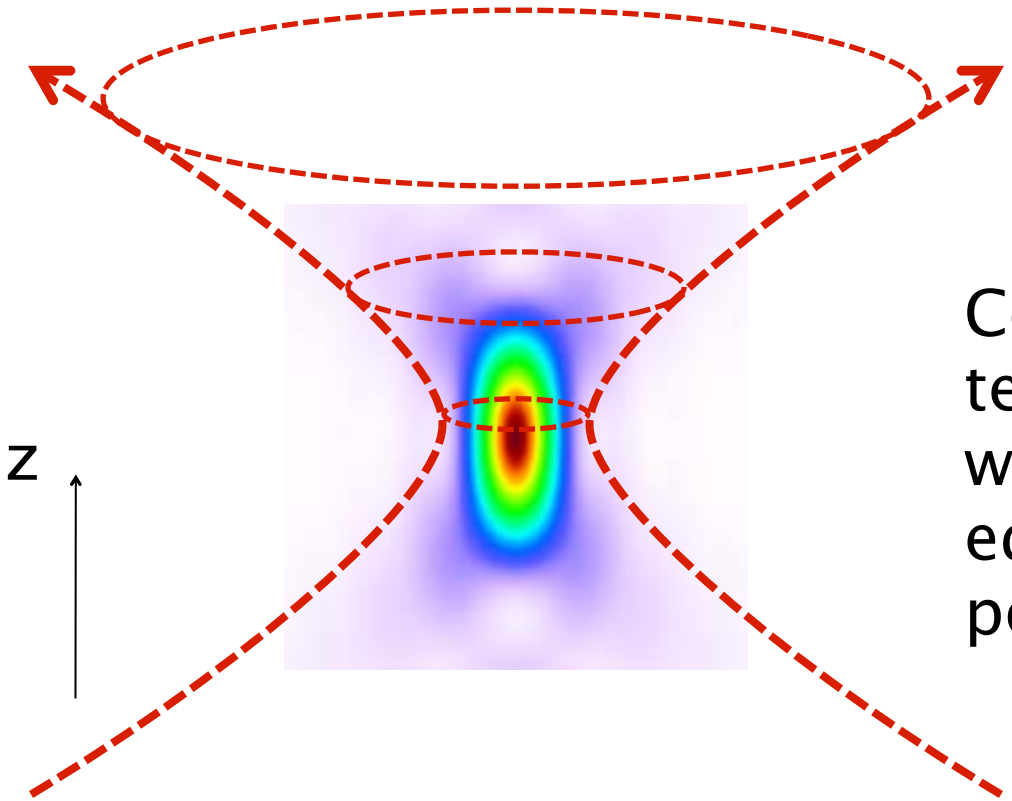
Axial resolution $\approx \frac{2n\lambda}{NA^2}$

(works only for low NA system)

Example: 1.4 NA objective at 550 nm

$\Delta_z \approx 850$ nm

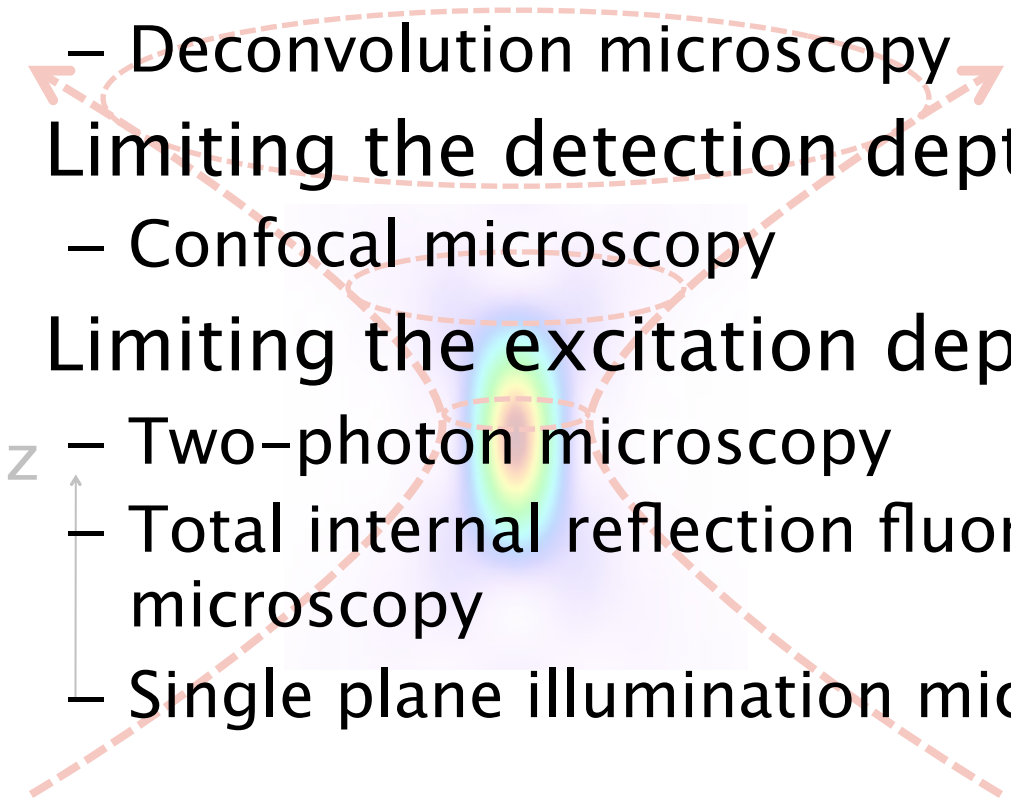
z resolution ≠ z sectioning



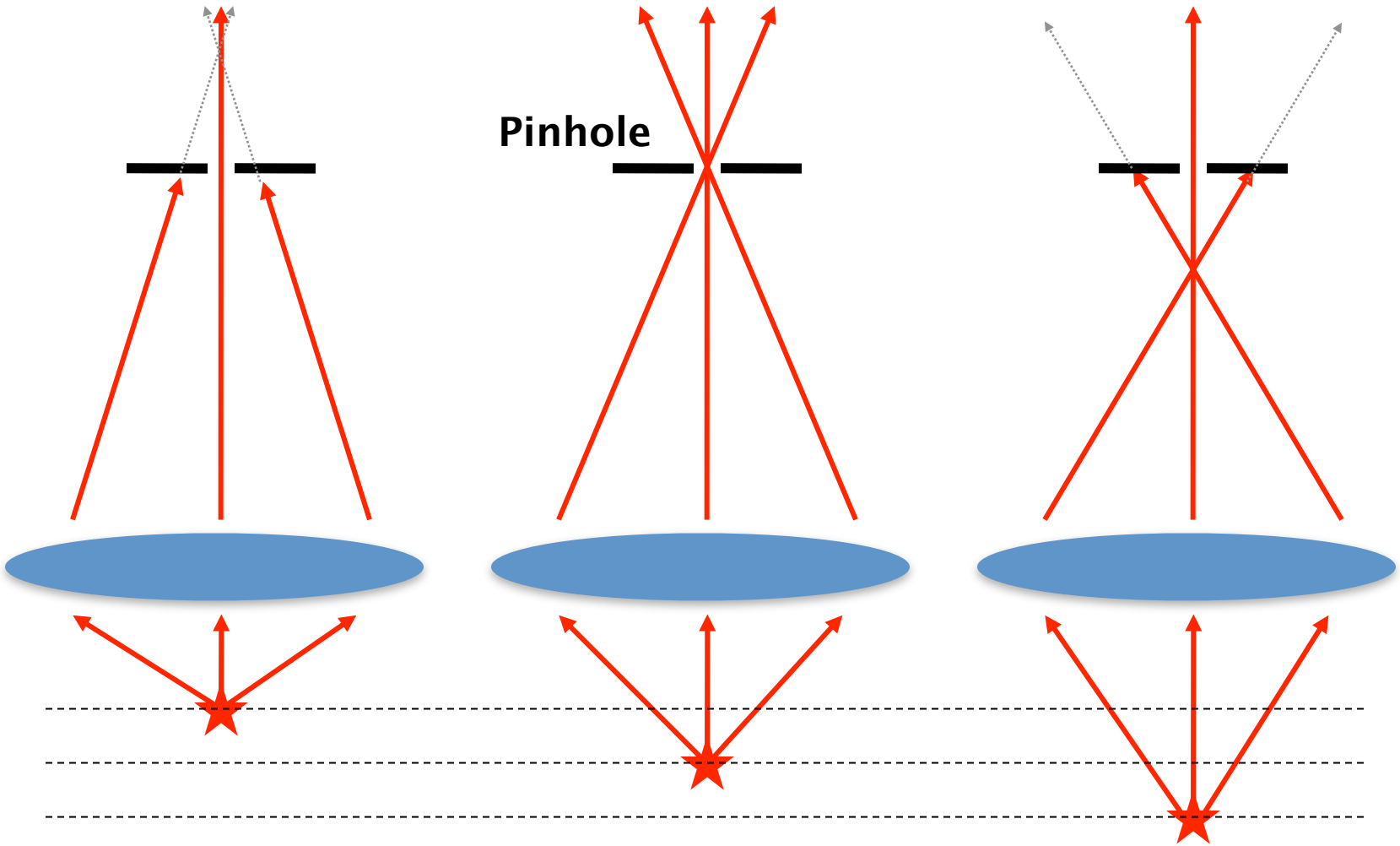
Conservation of energy tells that the detector will collect the signal equally well at any z position

How to achieve optical

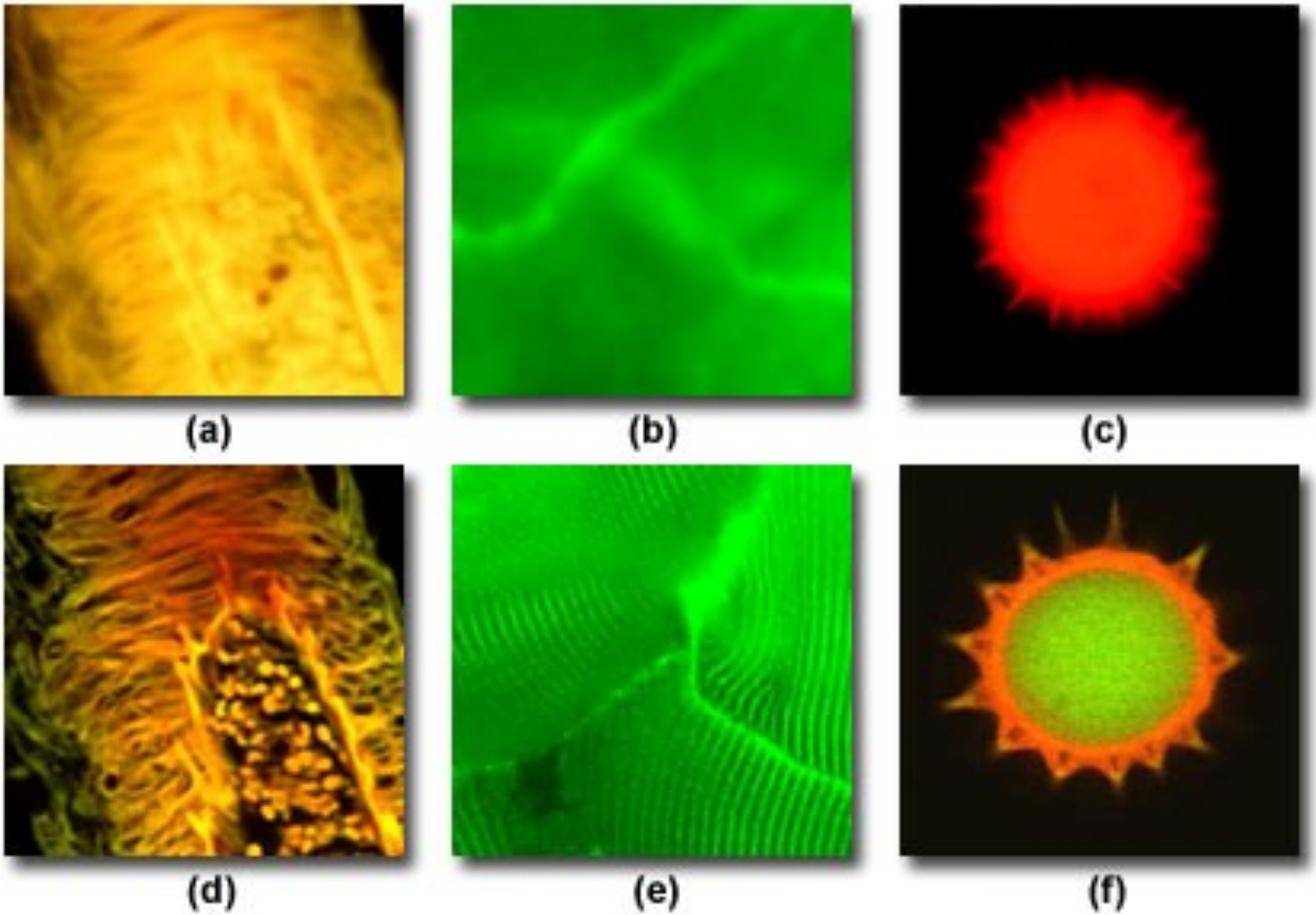
- Mathematics
  - Deconvolution microscopy
- Limiting the detection depth
  - Confocal microscopy
- Limiting the excitation depth
  - Two-photon microscopy
  - Total internal reflection fluorescence (TIRF) microscopy
  - Single plane illumination microscopy (SPIM)



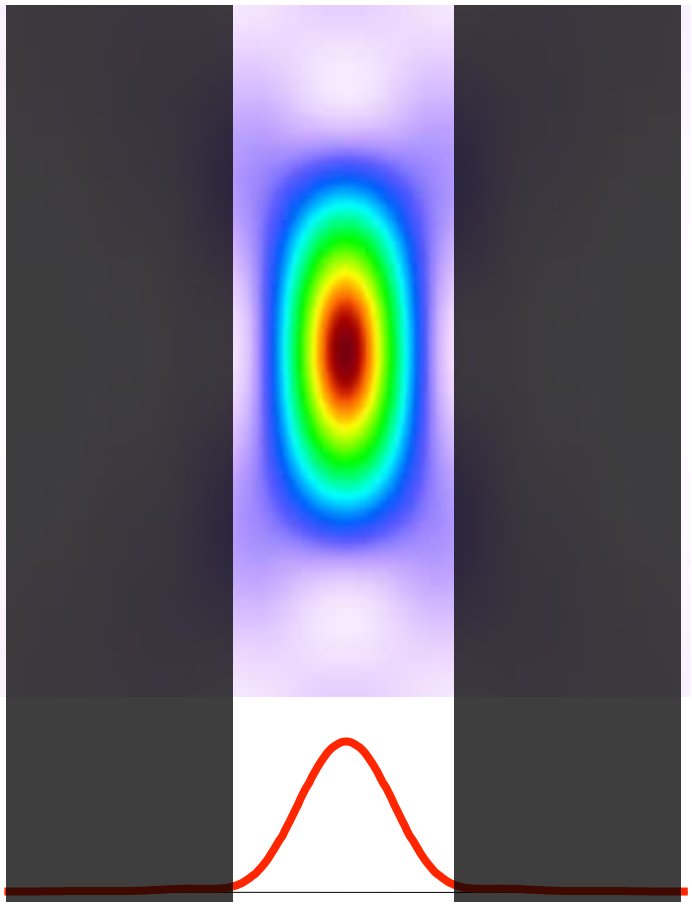
Principle of confocal



Confocal vs. widefield fluorescence microscopy



The pinhole size in confocal microscopy



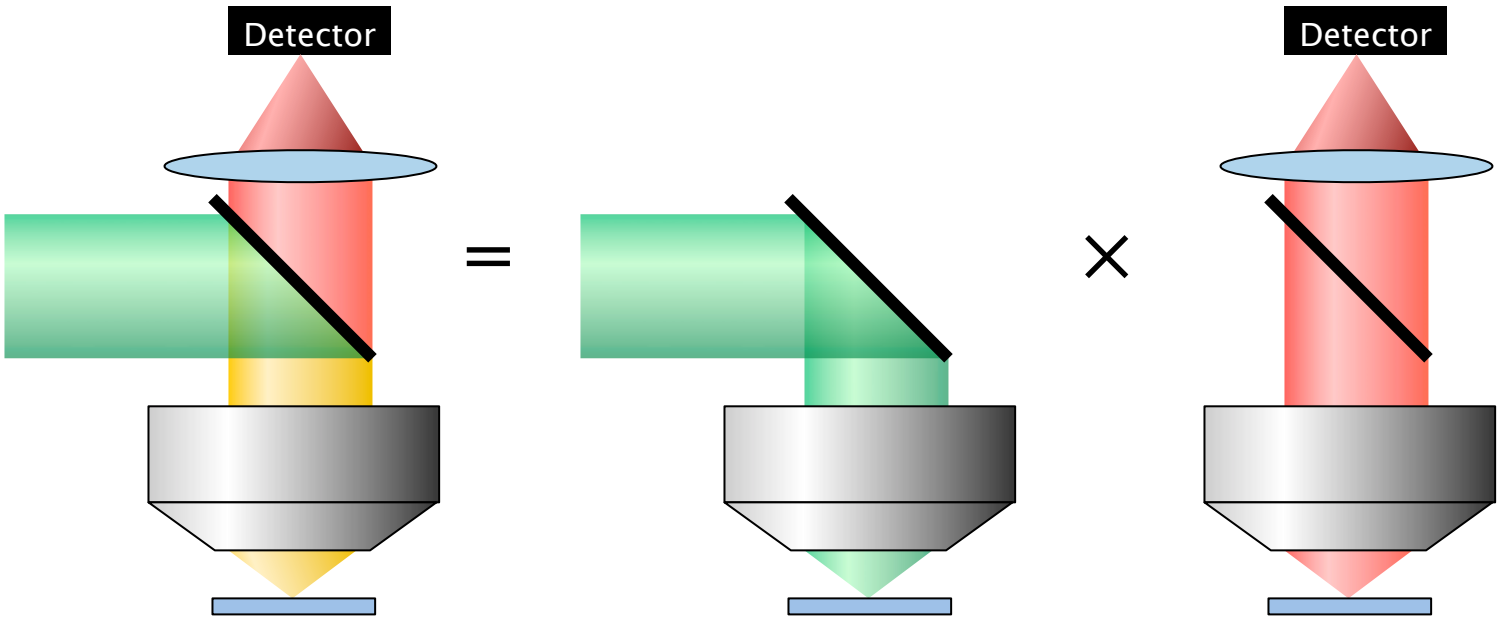
Detection efficiency (PSF_{det})

$$= PSF \otimes Pinhole$$

Pinhole size should be related to PSF width:

- 100x / 1.4 NA 220 nm x 100 = 22 µm
- 40x / 1.3 NA 235 nm x 40 = 9.4 µm
- 20x / 0.75 NA 407 nm x 20 = 8.1 µm
- 10x / 0.45 NA 678 nm x 10 = 6.8 µm

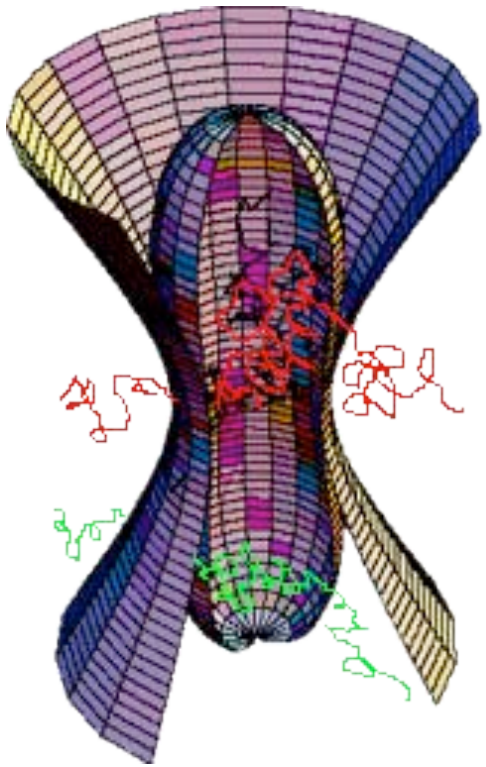
The PSF of confocal microscopy



$PSF_{Confocal} = PSF_{ex} \times PSF_{det}$

In the case of infinitely small pinhole: $PSF_{Confocal} \approx PSF_{ex}^{2}$

The confocal volume

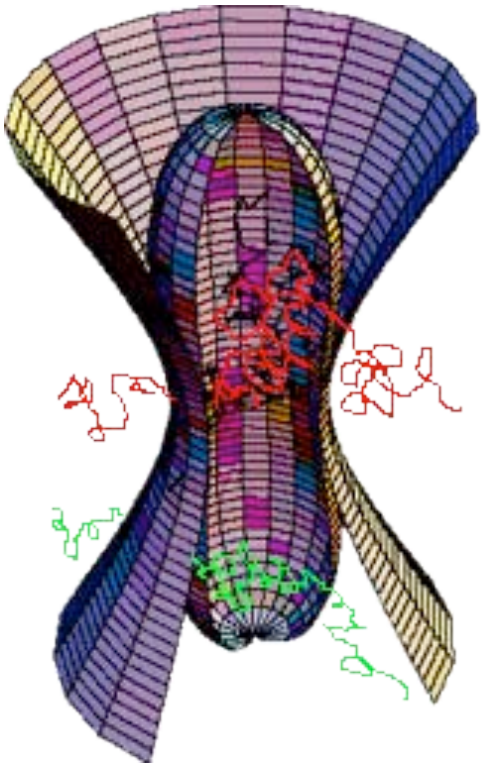


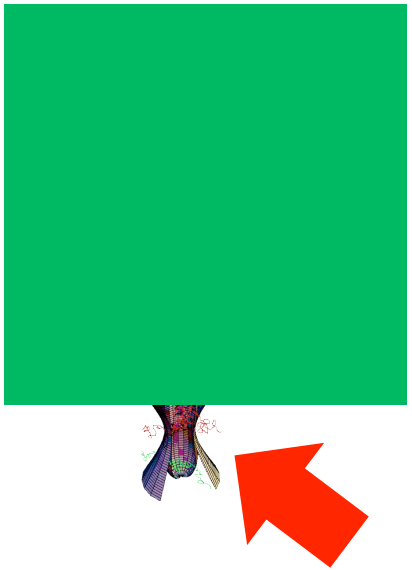
Integrating $PSF_{confocal}$ over the entire space now gets a finite value

Diffraction limited volume:

$\Delta_{xy} \approx 250$ nm, $\Delta_z \approx 600$ nm

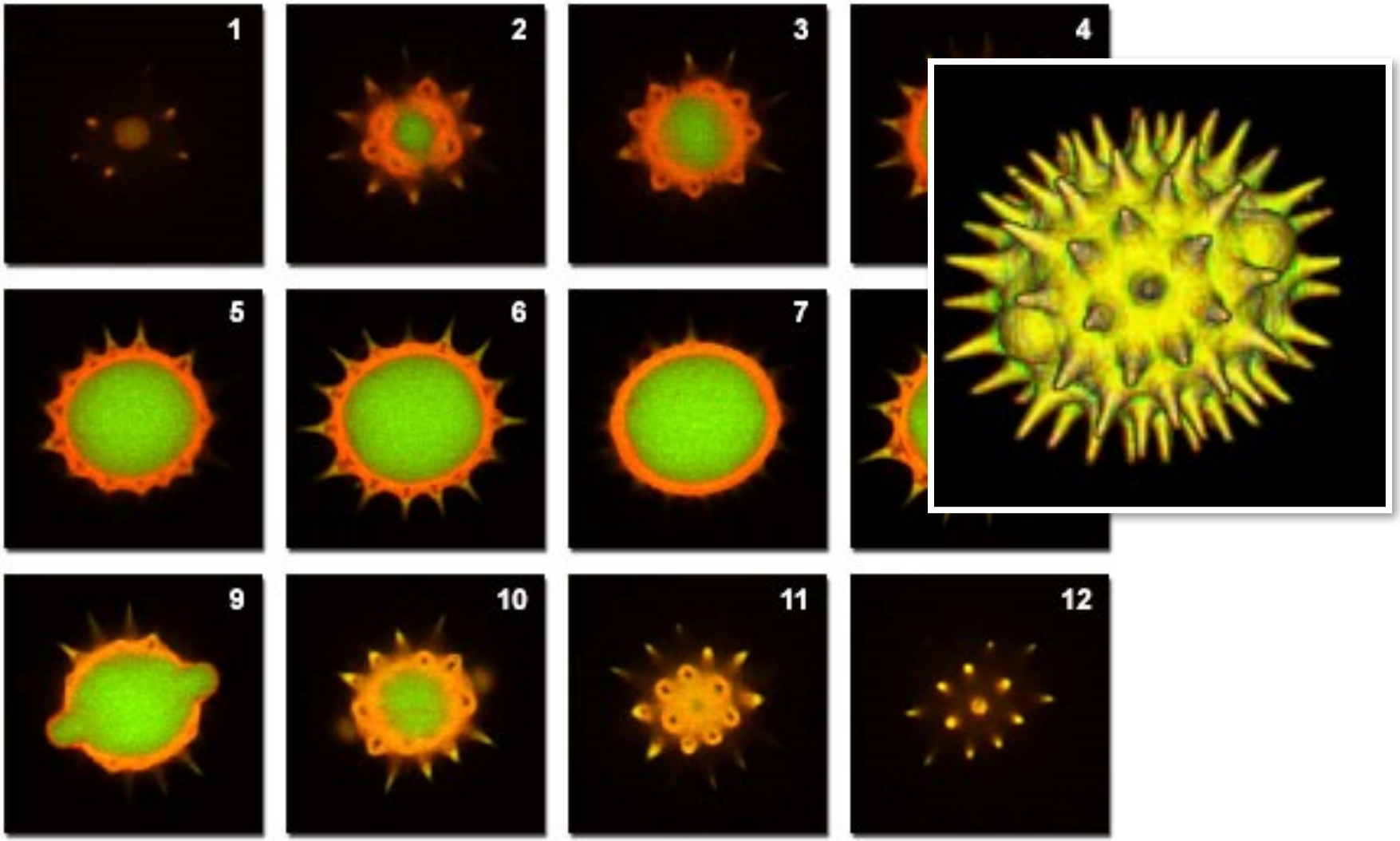
The sectioning ability of confocal microscopy





Guess:
When imaging a thick homogenous sample with
the best confocal microscope, what is the fraction

Confocal sectioning and 3D reconstruction



Components of a confocal

Light source
(usually laser based)

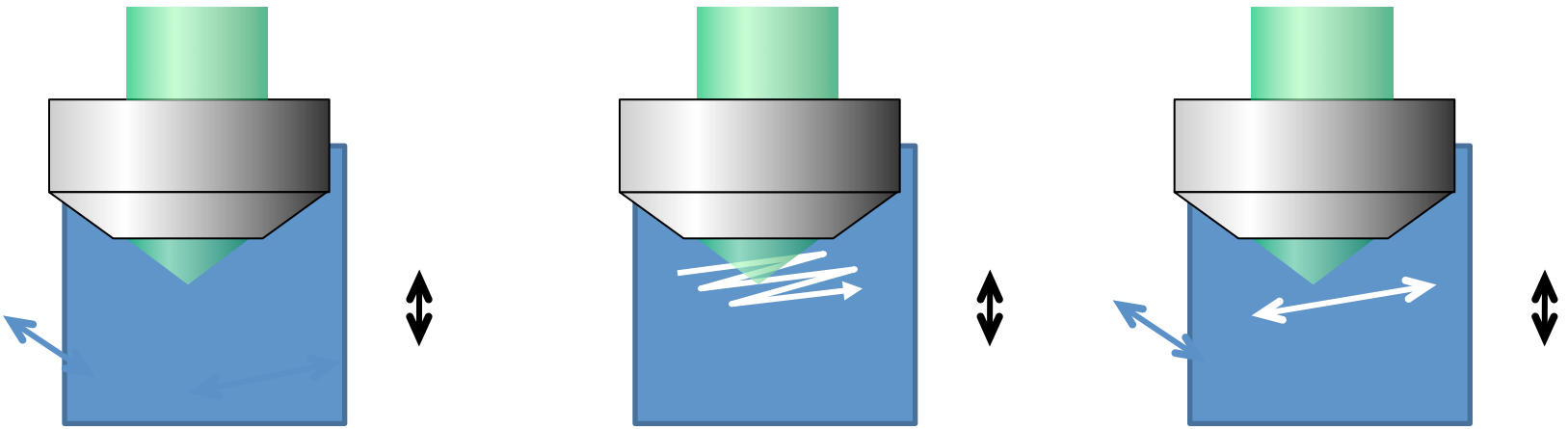
Detector
(PMT or CCD)

Controller and
image acquisition
computer

Microscope

Scan head
Pinhole

Point scanning confocal



Stage scan

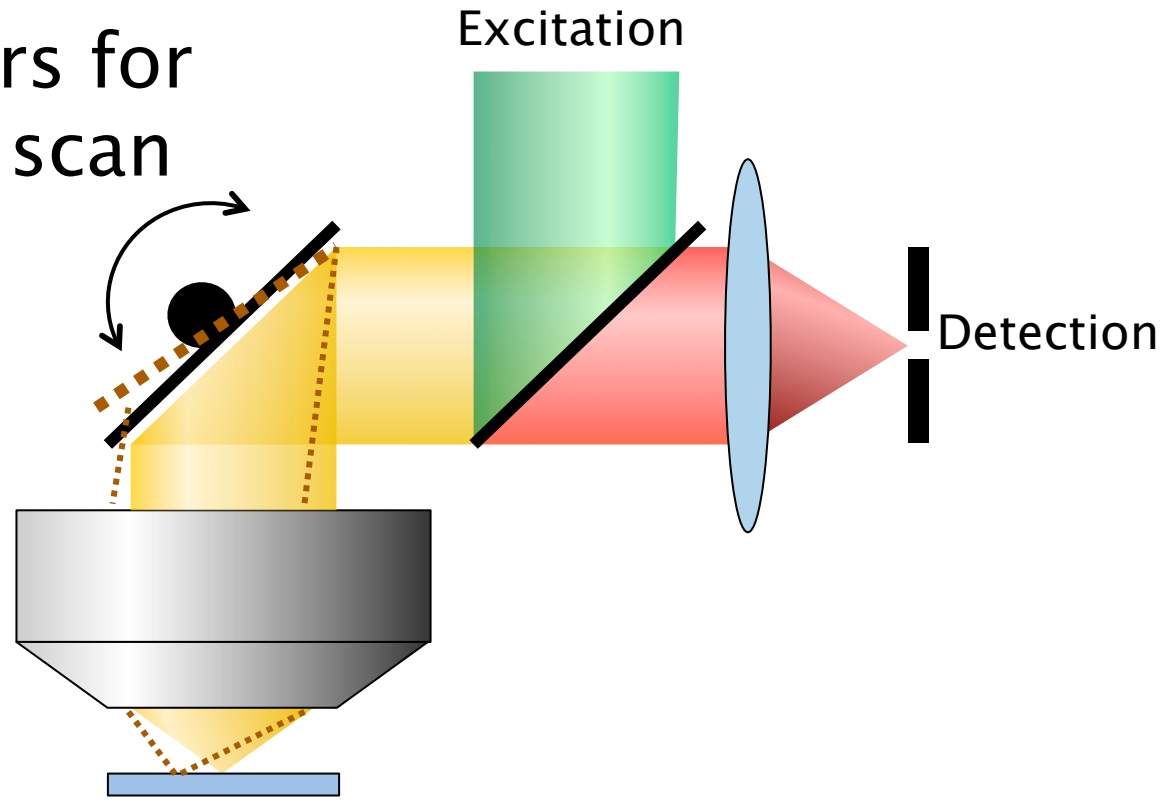
Laser scan

Hybrid

Simple but slow

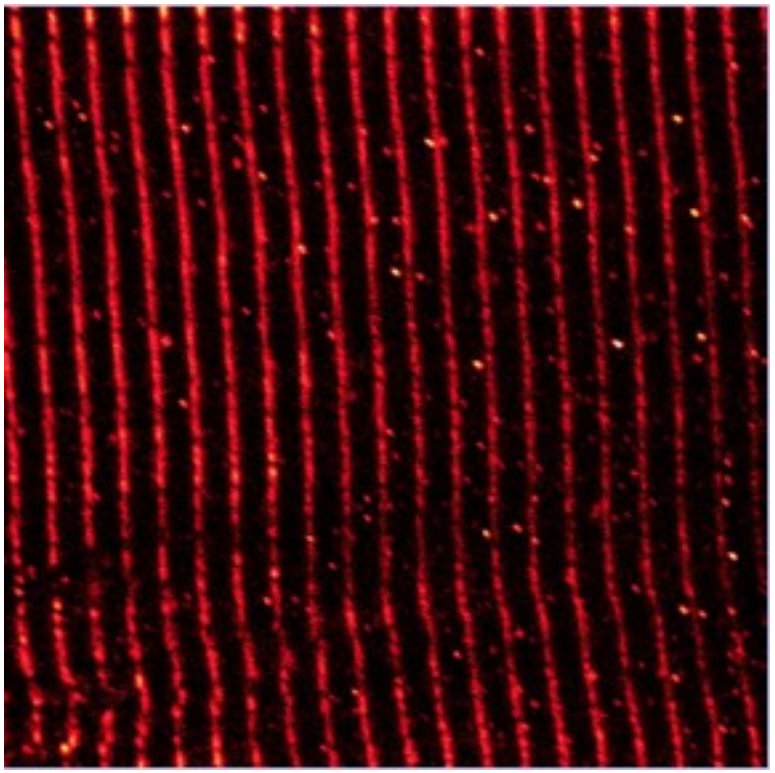
Galvanometer scanning /

Two mirrors for
x-y raster scan



Averaging multiple scans to enhance S/N

1 frame



8-frame average

How to scan faster?

- Normal galvo mirror: several µs per pixel
  0.5 – 2 sec per image
- Resonance scanner: up to 30 fps
- The answer is:

Spinning disk confocal

- Scanning with a rotating pinhole array
- CCD camera detection

Spinning disk confocal

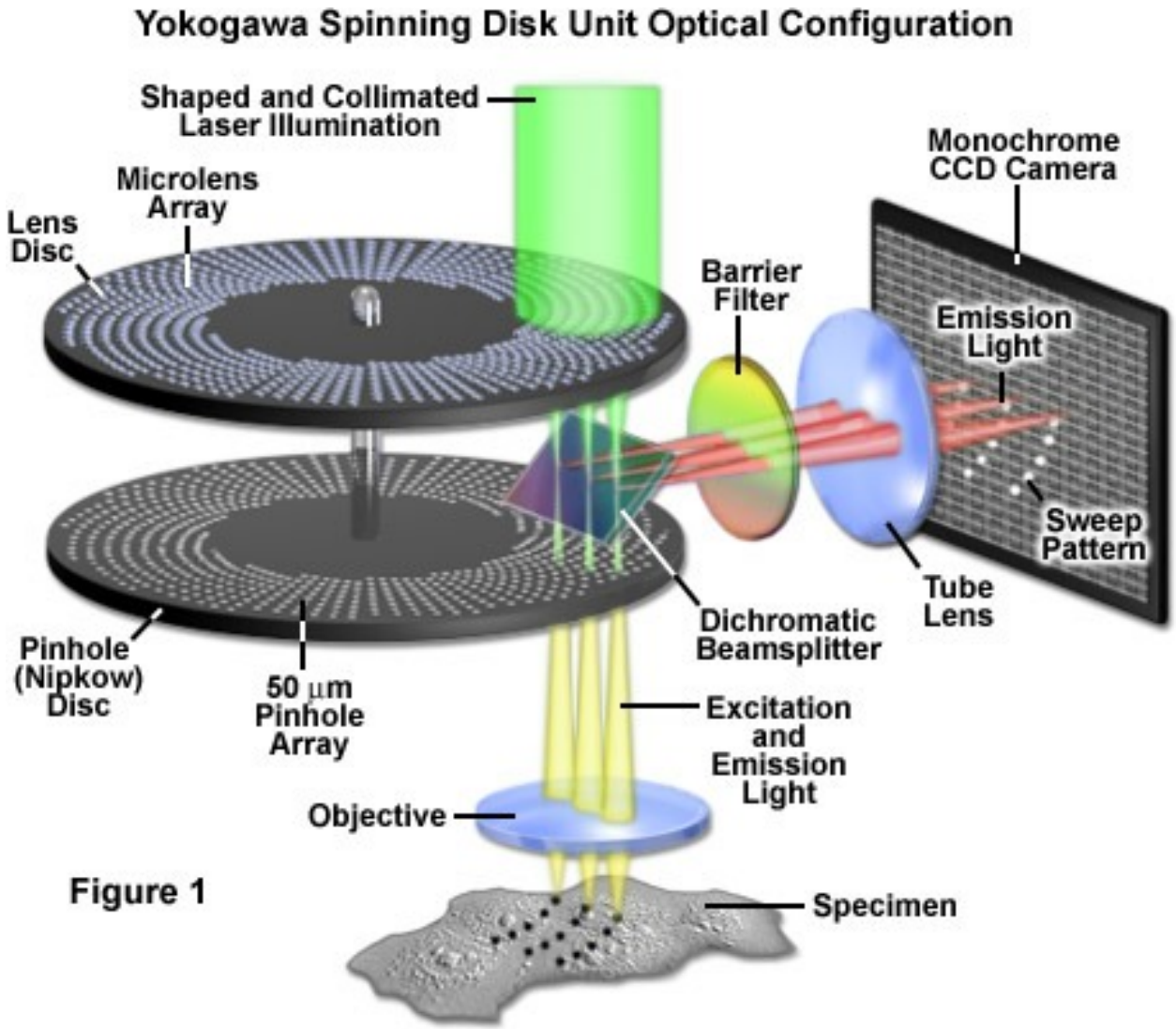
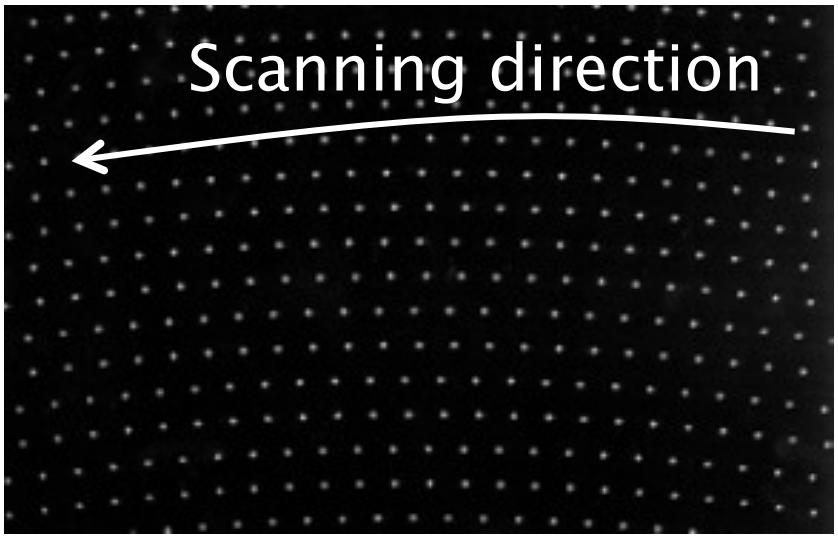
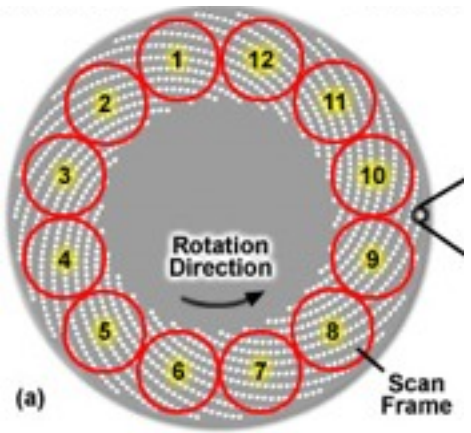


Figure 1

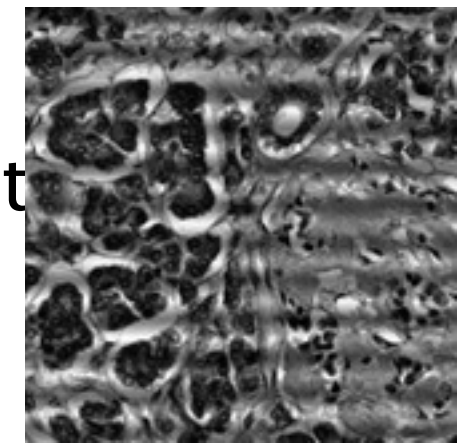
Spinning disk confocal



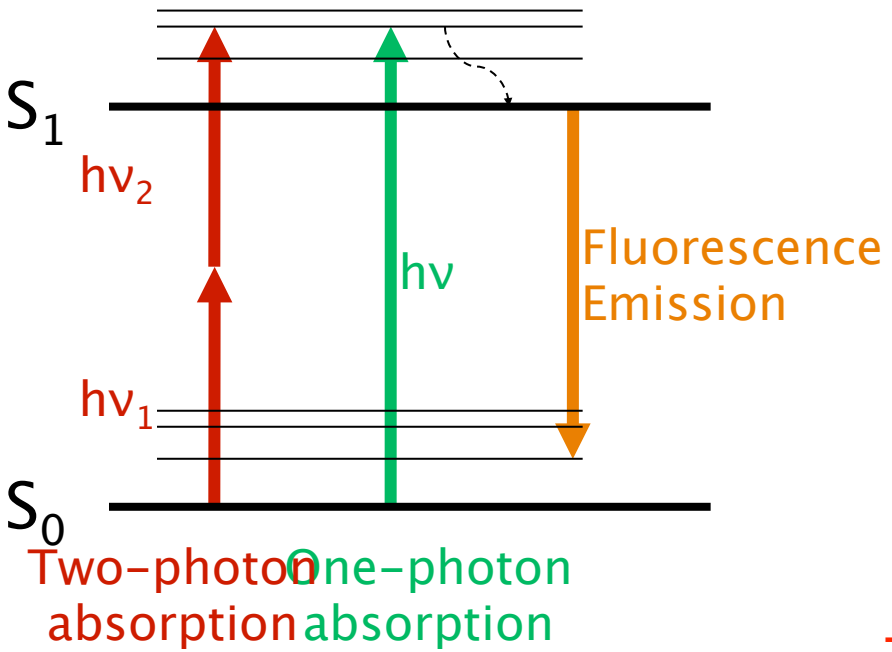


12 scanning frames per 360° rotation

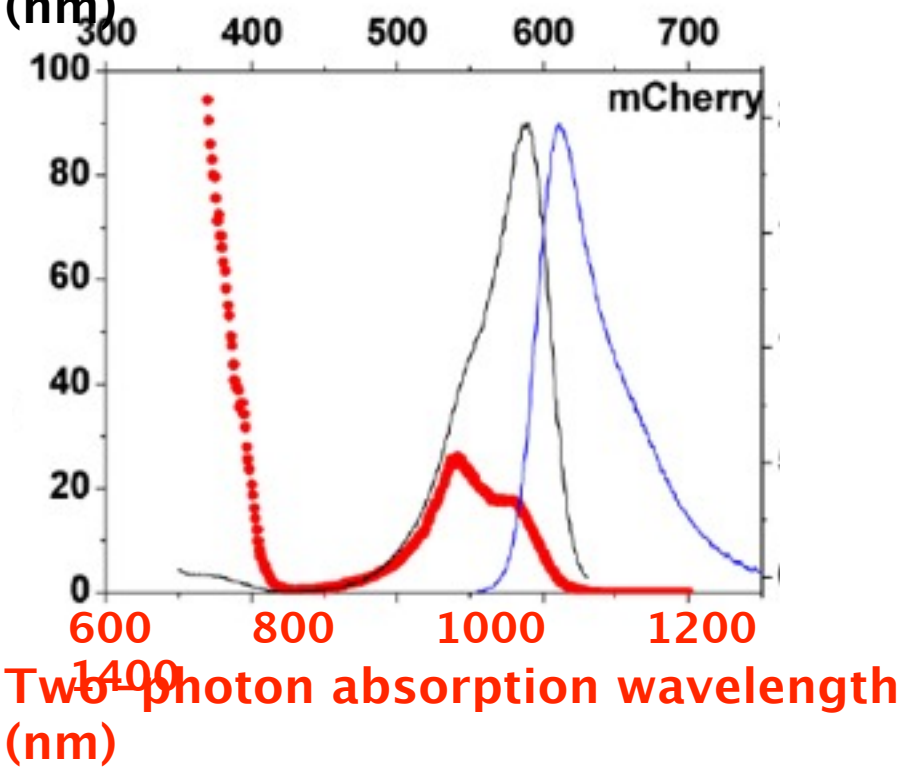
- Up to 2000 frames / sec
- The disk rotation must match t camera frame rate



Multi-photon microscopy



Drobizhev et al., J. Phys. Chem. B (2009)



Two-photon excitation: Fluorescence $\propto I^2$

Optical sectioning without a

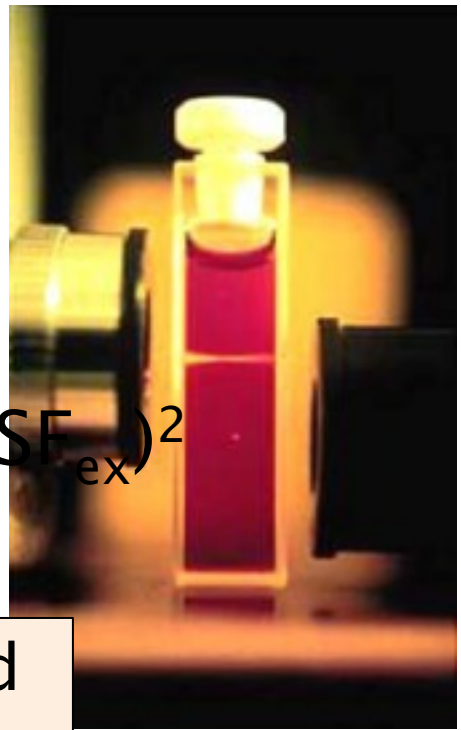
$$\text{Fluorescence} \propto I^2$$

$$PSF_{2P.ex} = (PSF_{1P.ex})^2$$

Recall:

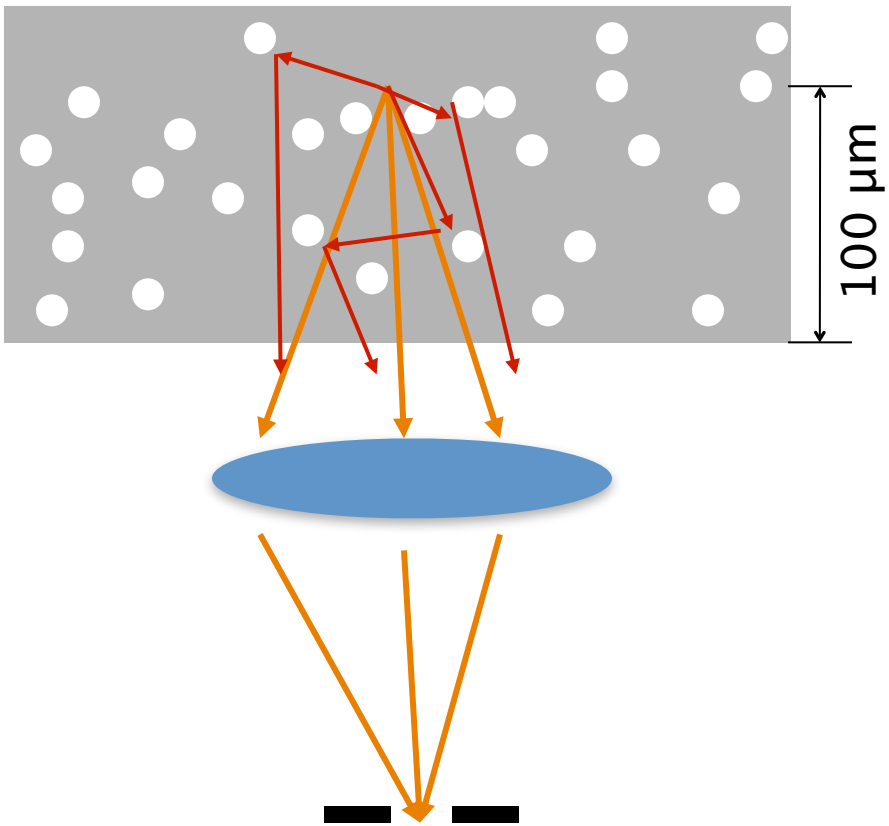
$$PSF_{Confocal} = PSF_{ex} \times PSF_{det} \approx (PSF_{ex})^2$$

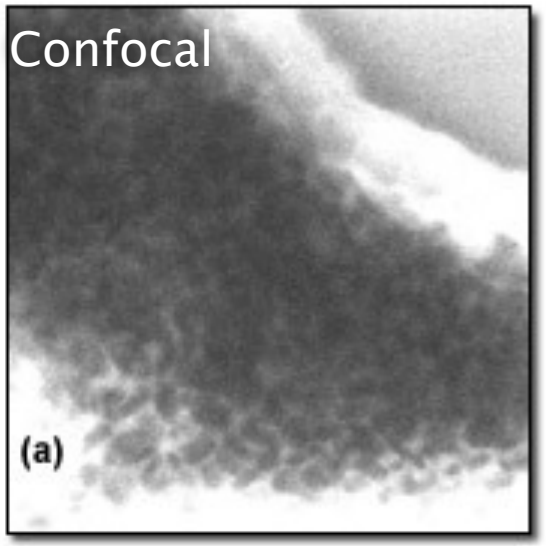
Two-photon excitation is localized to the laser focal point

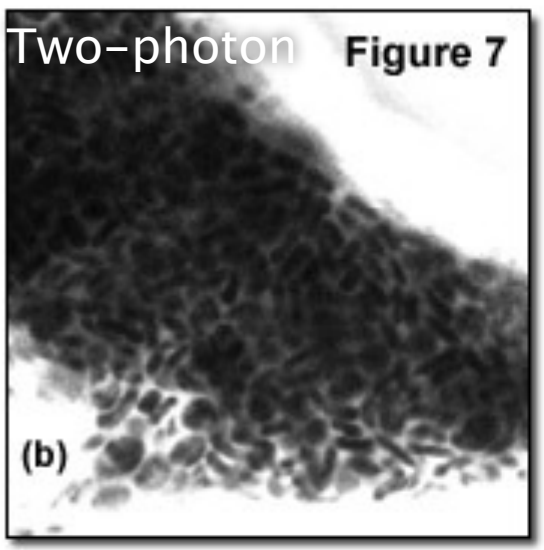


Deep tissue imaging

Tissue scattering and absorption decrease confocal performance





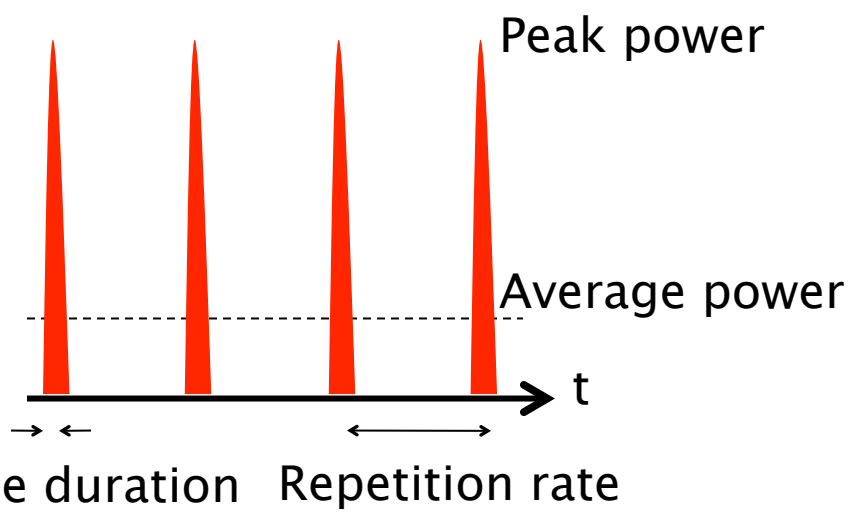


Ultrafast laser source for 2PE

Continuous wave (CW) laser

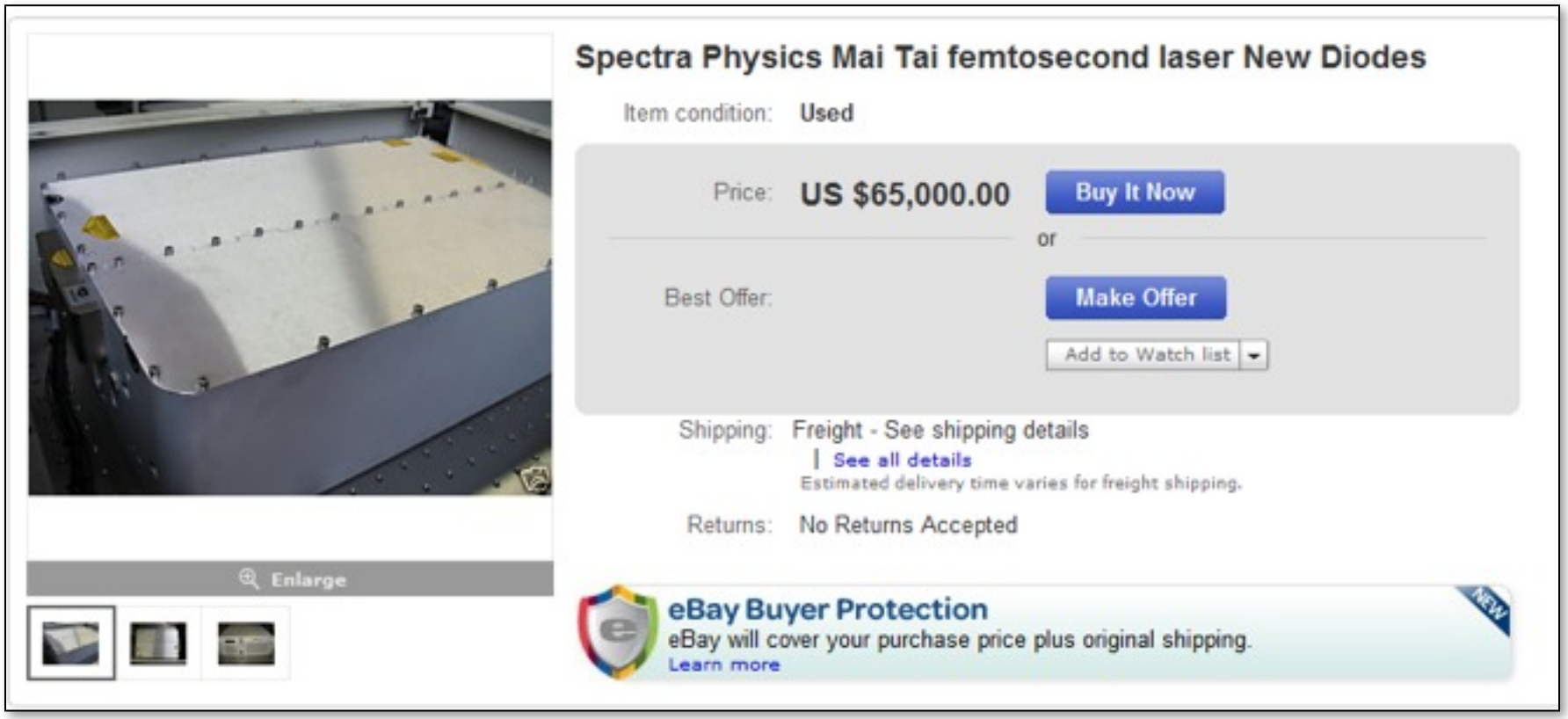
To see reasonable 2PE with CW laser:
~ 1W power

Pulsed laser

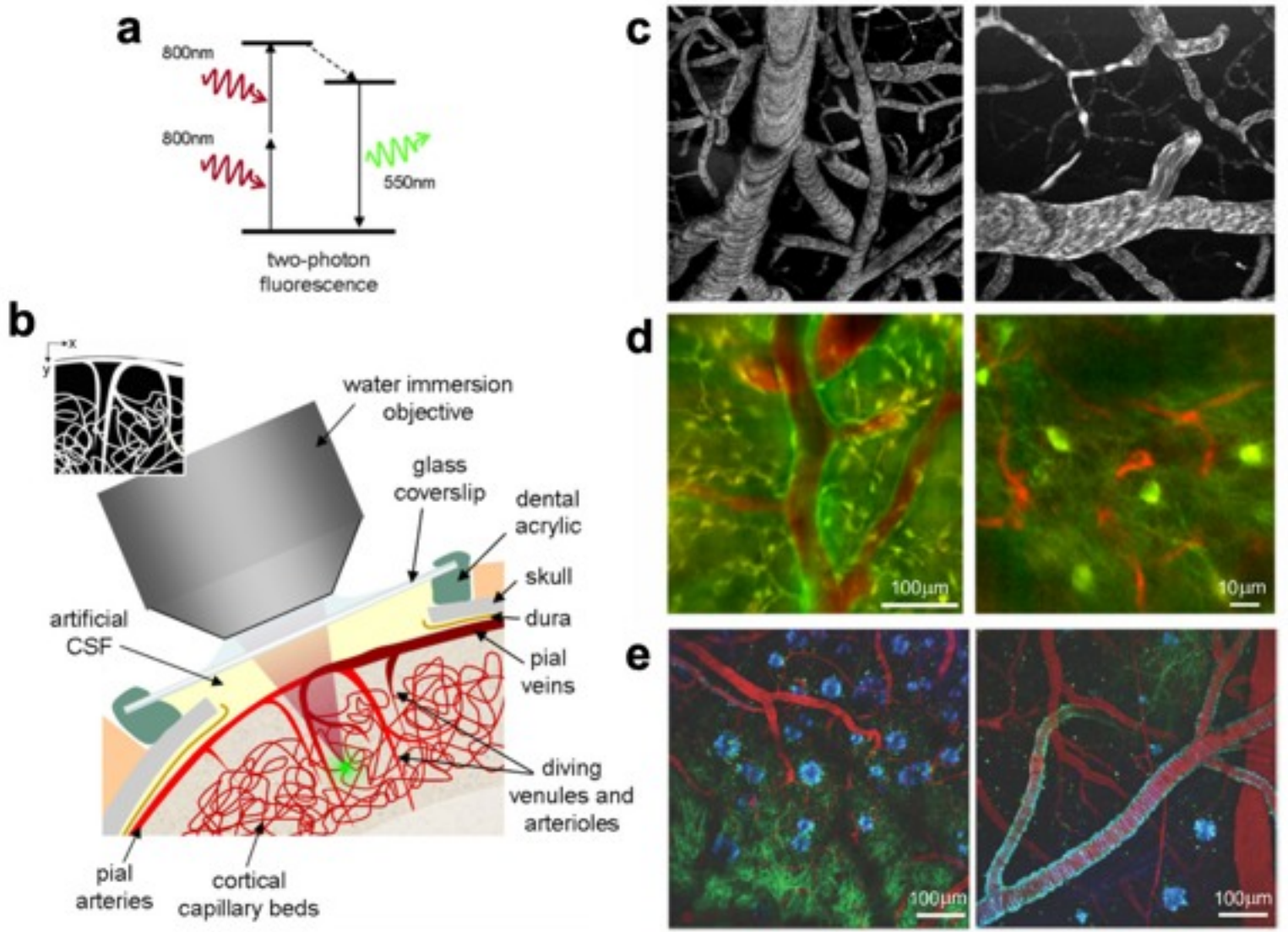


Ti:Sapphire laser, 100 fs, 80 MHz
10 mW avg ≈ 1.2 kW peak

Ultrafast laser source for 2PE

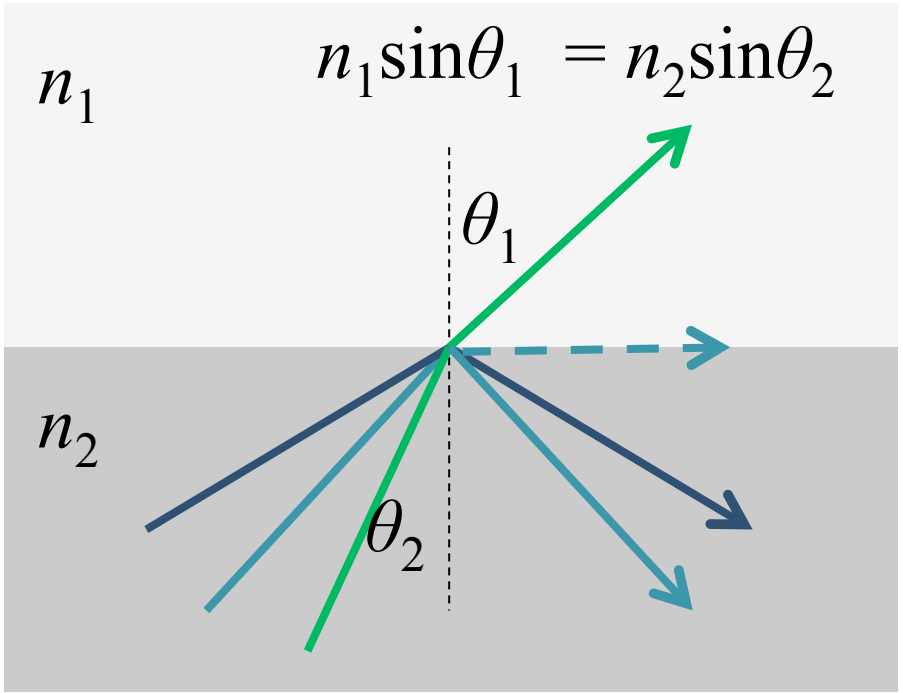


Two-photon microscopy of in vivo brain function



Kherlopian, et al., BMC Systems Biology (

Total internal reflection fluorescence microscopy



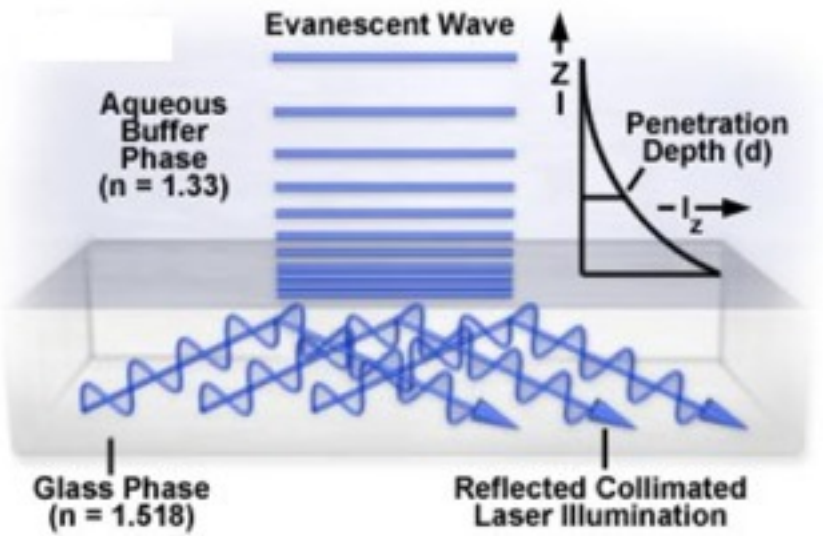
Total internal reflection:

$\theta_1 = 90°$ ➔

$\sin\theta_2 \equiv \sin\theta_c = n_1/n_2$

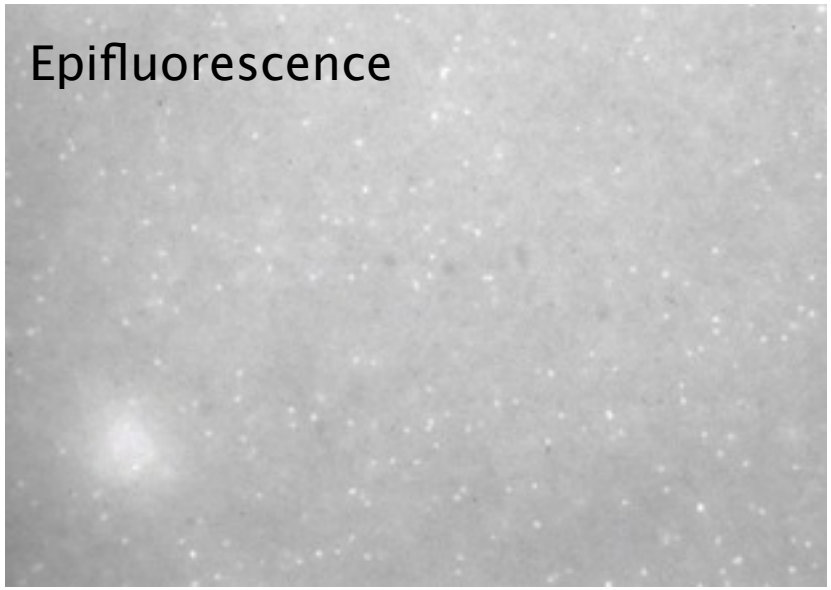
$n_1 = 1.33$ (water), $n_2 = 1.52$ (glass), $\theta_c = 61°$

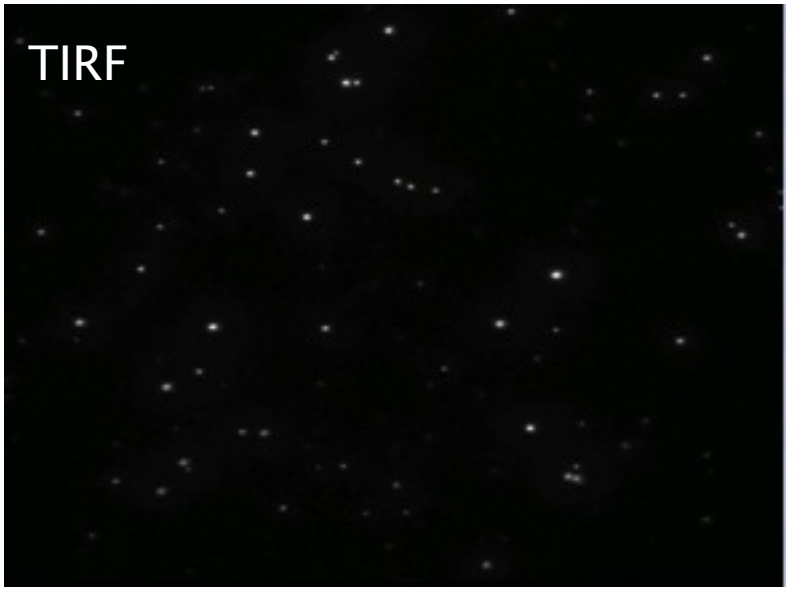
The evanescent wave in TIR

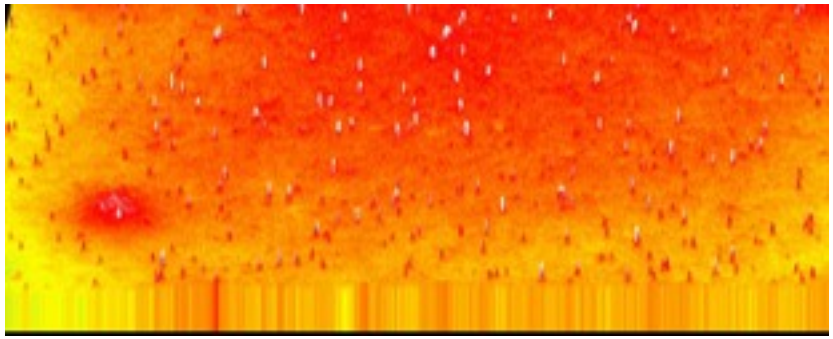


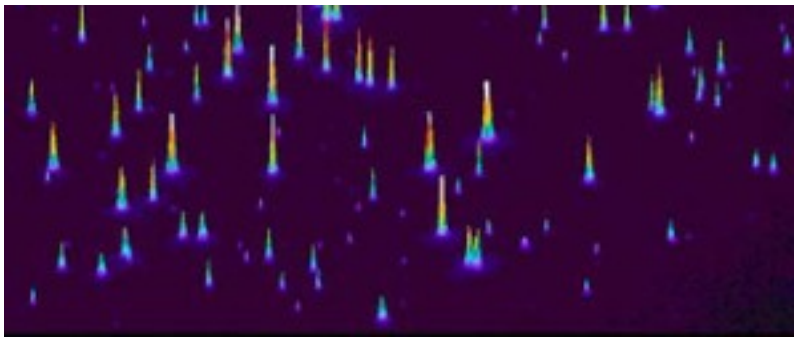
- The energy of the evanescent wave is localized near the interface.
- The strength of the evanescent wave field decreases exponentially.
- The penetration depth is a function of the wavelength and the incident angle.

TIRM improves S/N for surface









What are the applications of

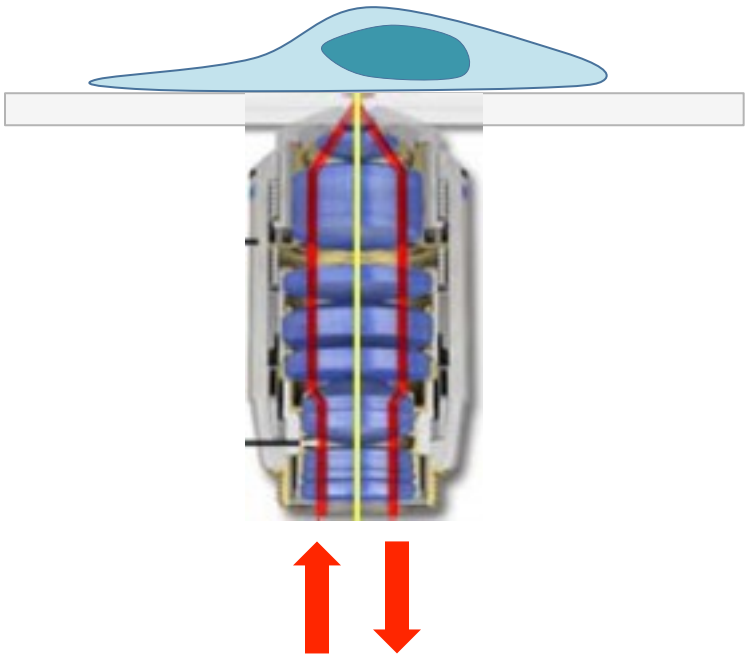
- Organization of plasma membrane proteins
- Dynamics of plasma membrane lipids

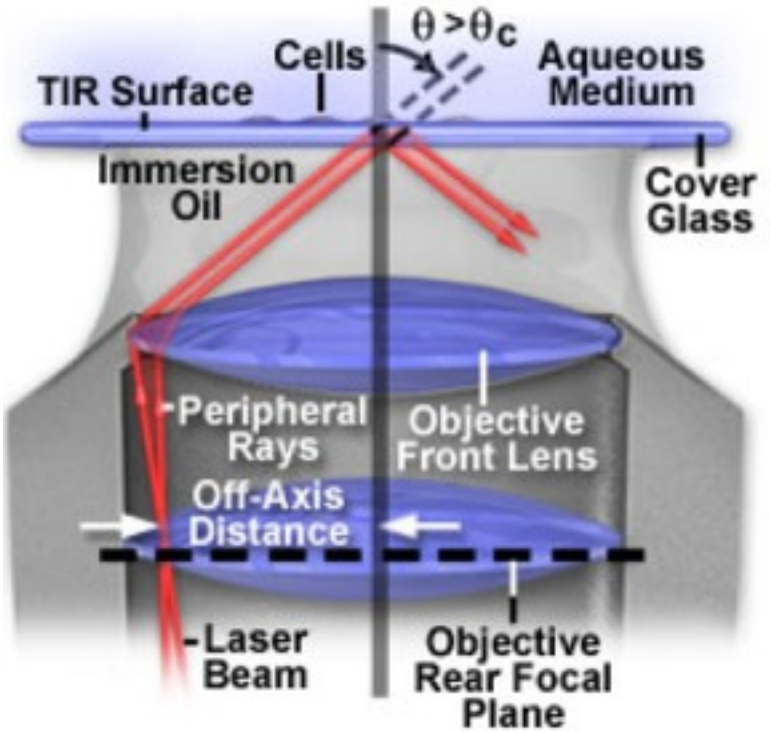
- Endocytosis and exocytosis
- Focal adhesion
- Growth cone migration

- Imaging in supported lipid bilayers
- In vitro reconstituted cytoskeleton filaments

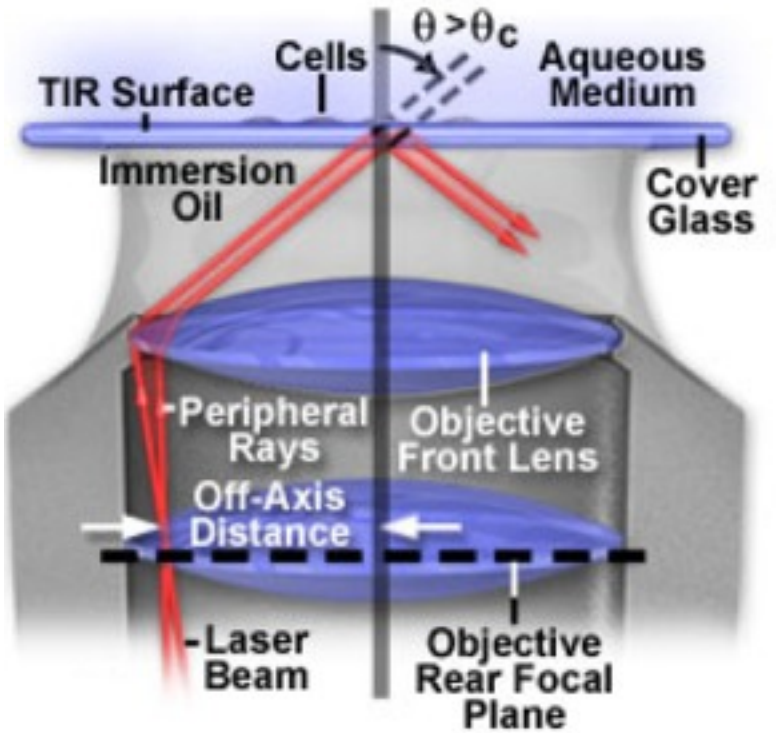
- Single molecule imaging

Through-the-objective TIRF





Requirement for TIRF



$$\mathrm{NA} = n_{\mathrm{glass}} \sin\theta_{\mathrm{max}}$$

$$n_{\mathrm{water}} = n_{\mathrm{glass}} \sin\theta_{\mathrm{c}}$$

$$\theta_{\mathrm{max}} \geq \theta_{\mathrm{c}}$$

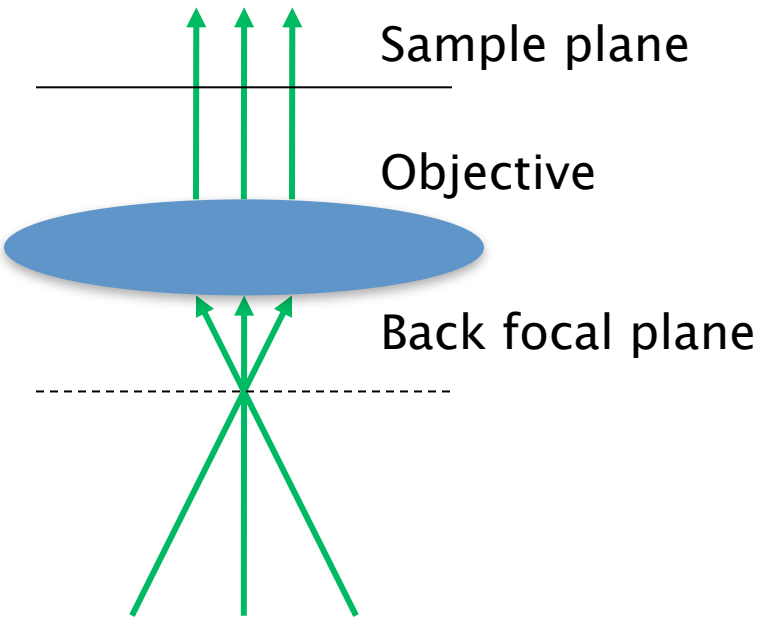
$$\mathrm{NA} \geq n_{\mathrm{water}} \approx 1.33$$

TIRF compatible objectives

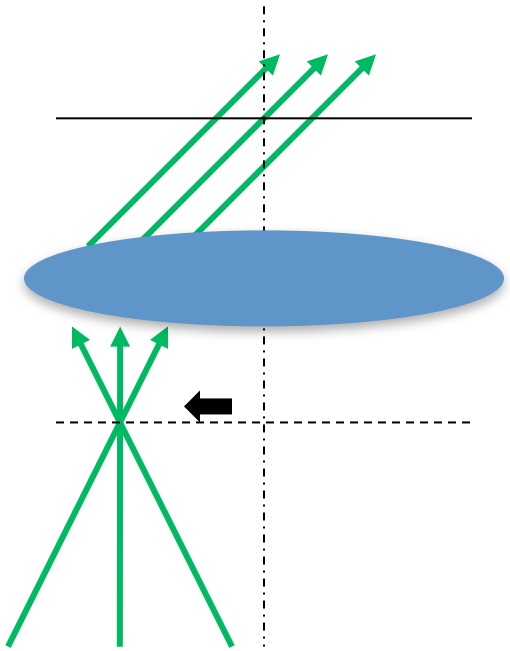
- 1.40 NA
  - "Barely enough" for laser TIRF
- 1.45 / 1.49 NA "TIRF" objective
  - More homogenous illumination field
  - Higher efficiency for lamp TIRF
  - Compromised image quality
- 1.65 NA
  - Sapphire coverglass
  - Toxic oil…

TIRF illuminator

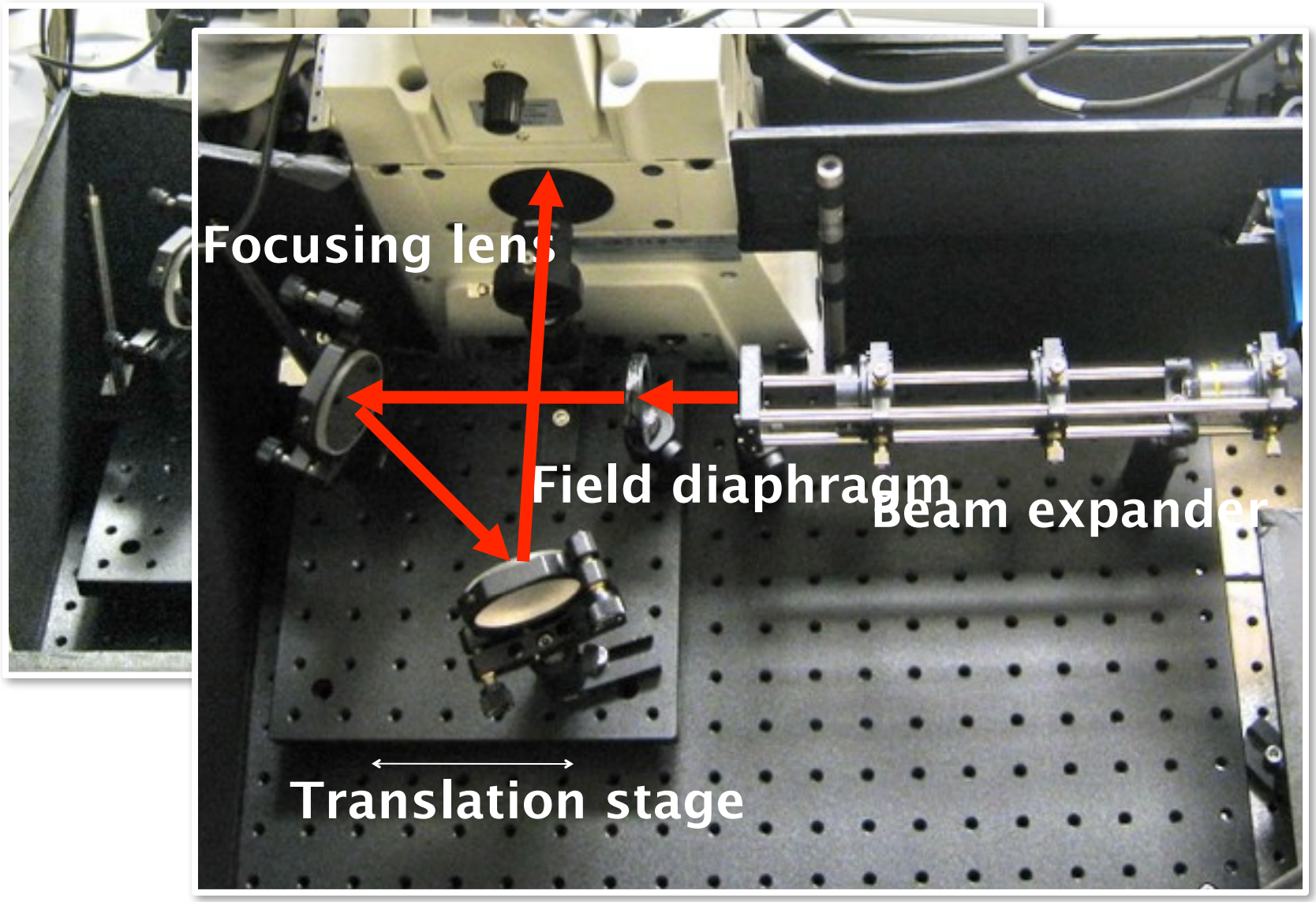
Focusing the light to the back-focal-plane of the objective



Translate the light to the edge of the objective back-aperture

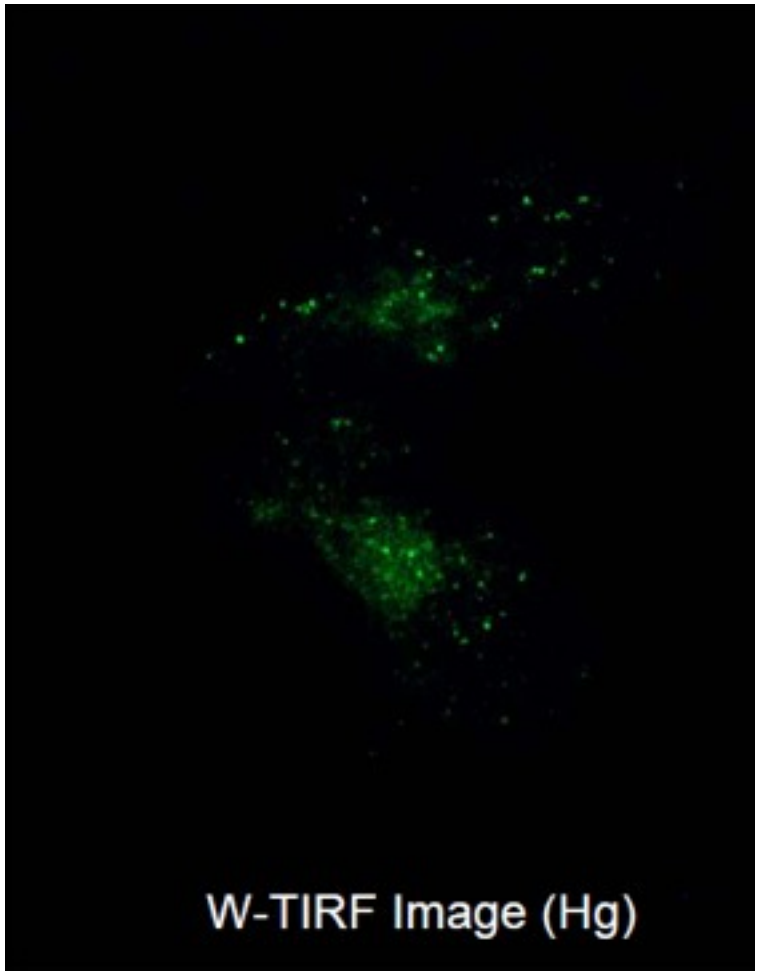


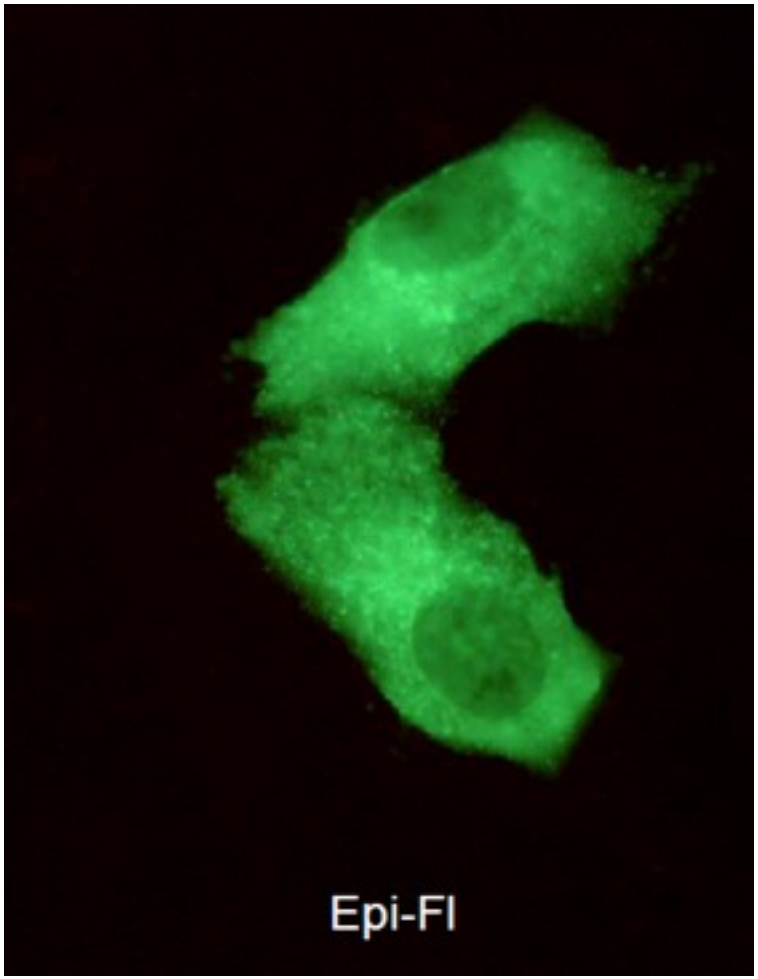
A home-built TIRF illuminator



A commercial TIRF

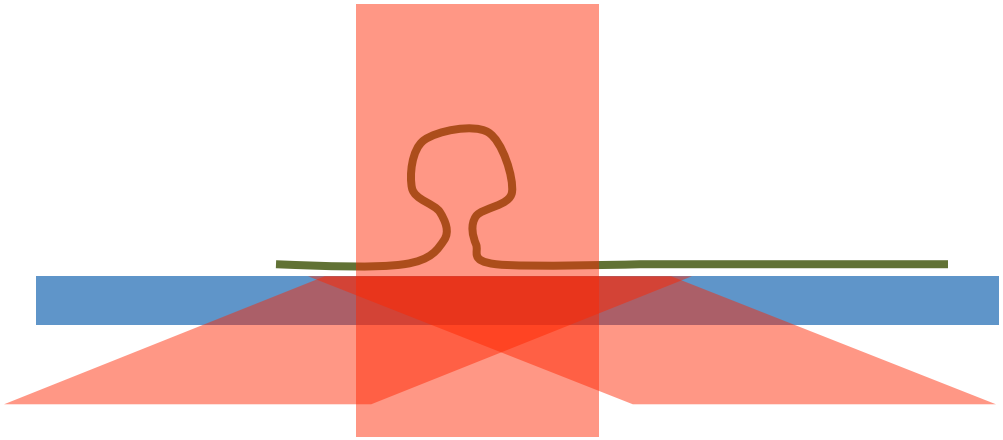
COS cells expressing GFP

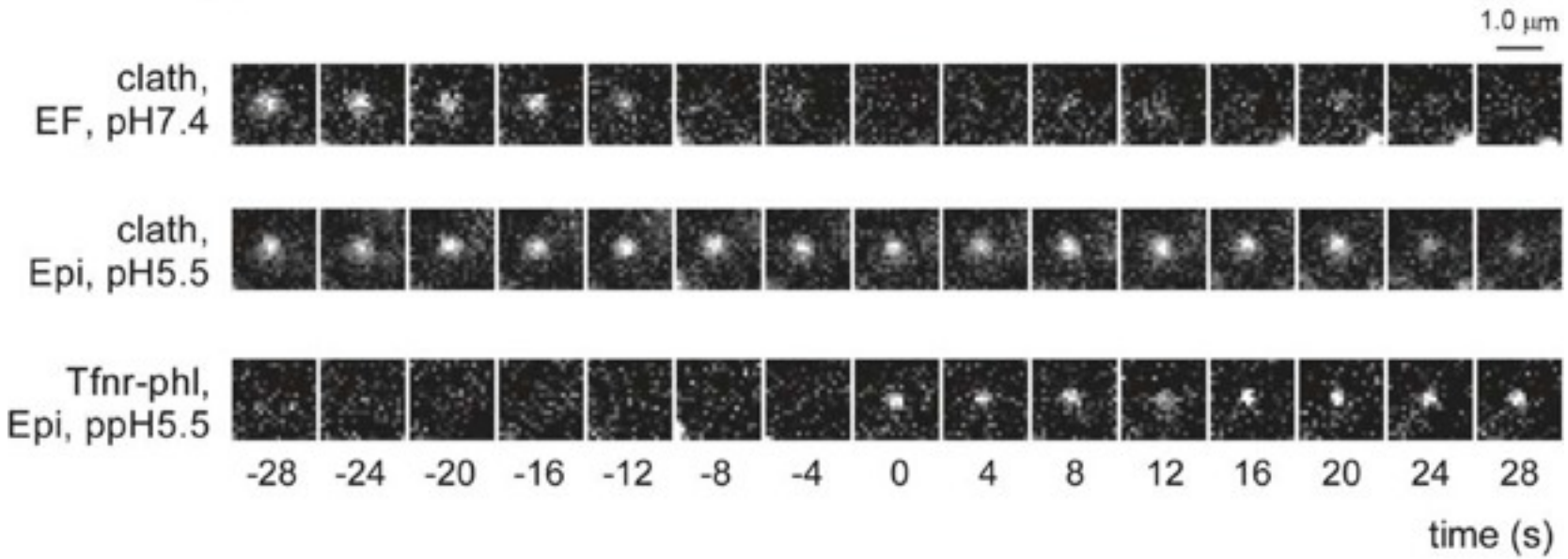




Dan Axelrod, Univ of Michigan

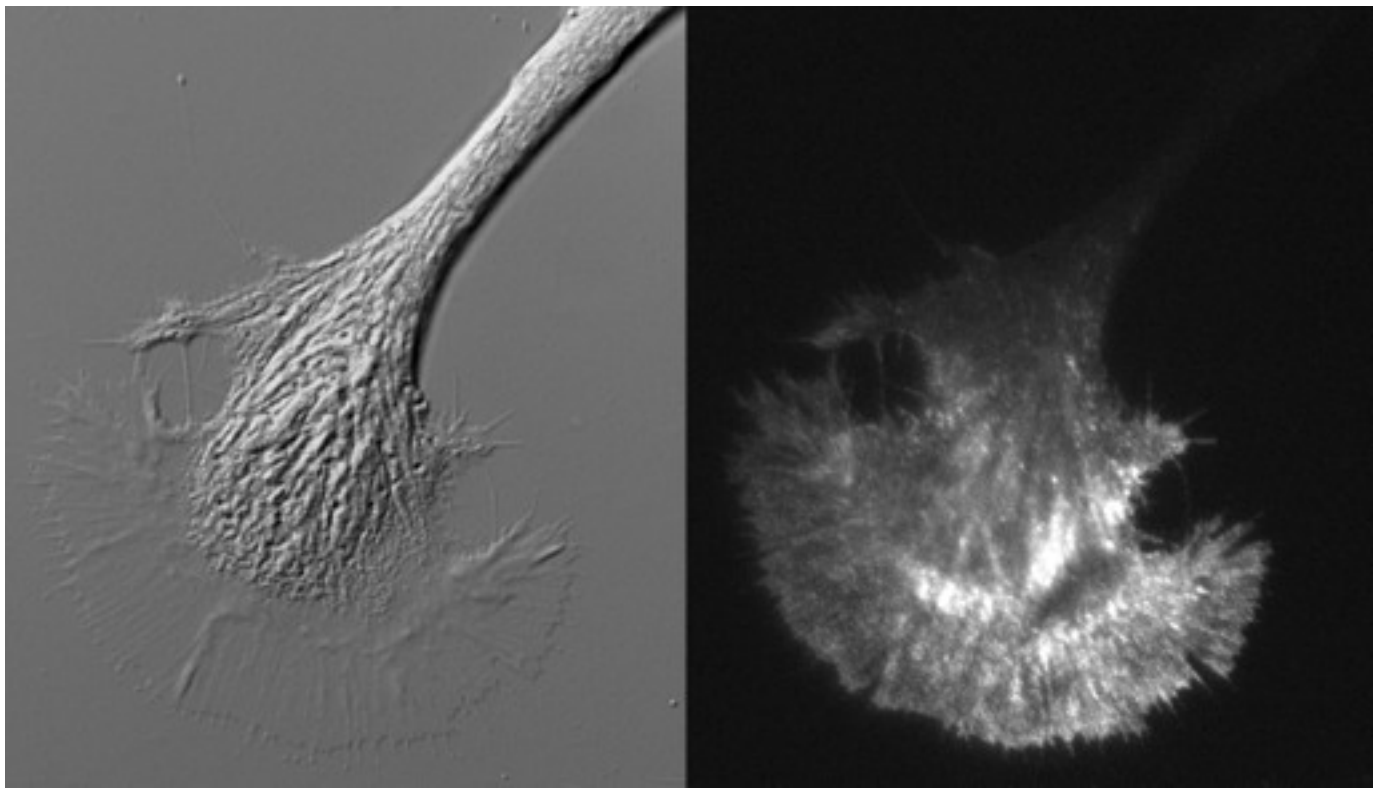
Alternating Epi/TIRF





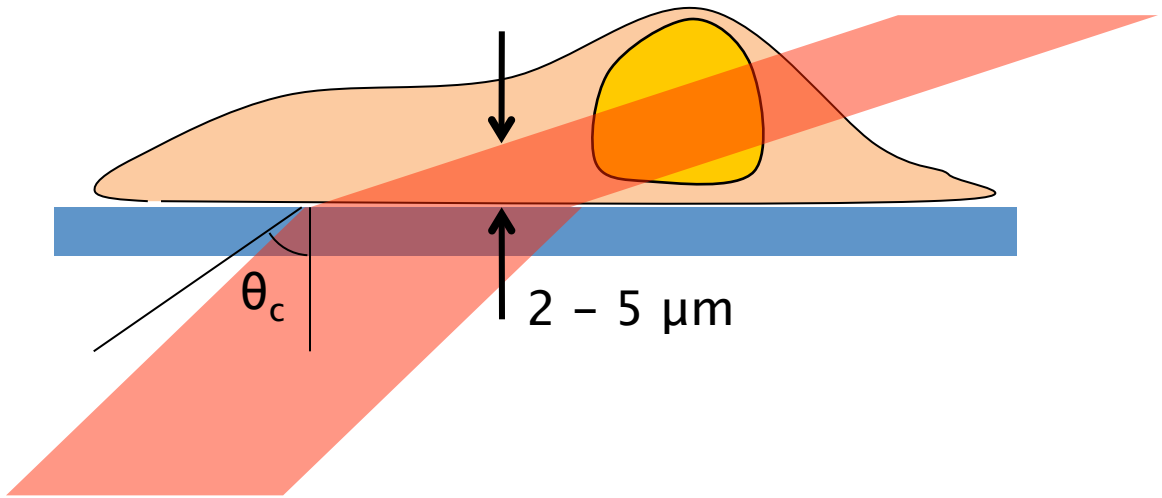
Merrifield et al., Cell 2005

Aplysia growth cone



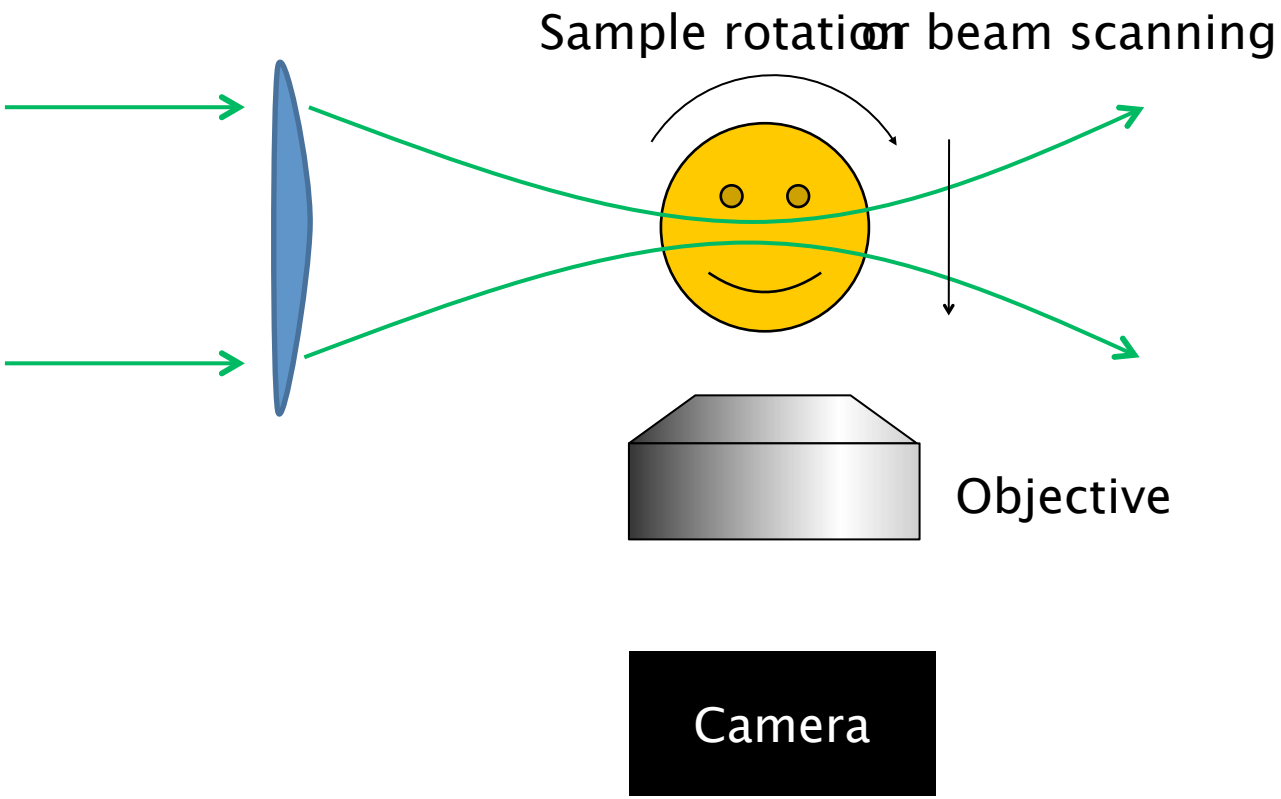
Andy Schaefer, Paul Forscher, Yale Univ.

Oblique angle illumination: the "gray zone"

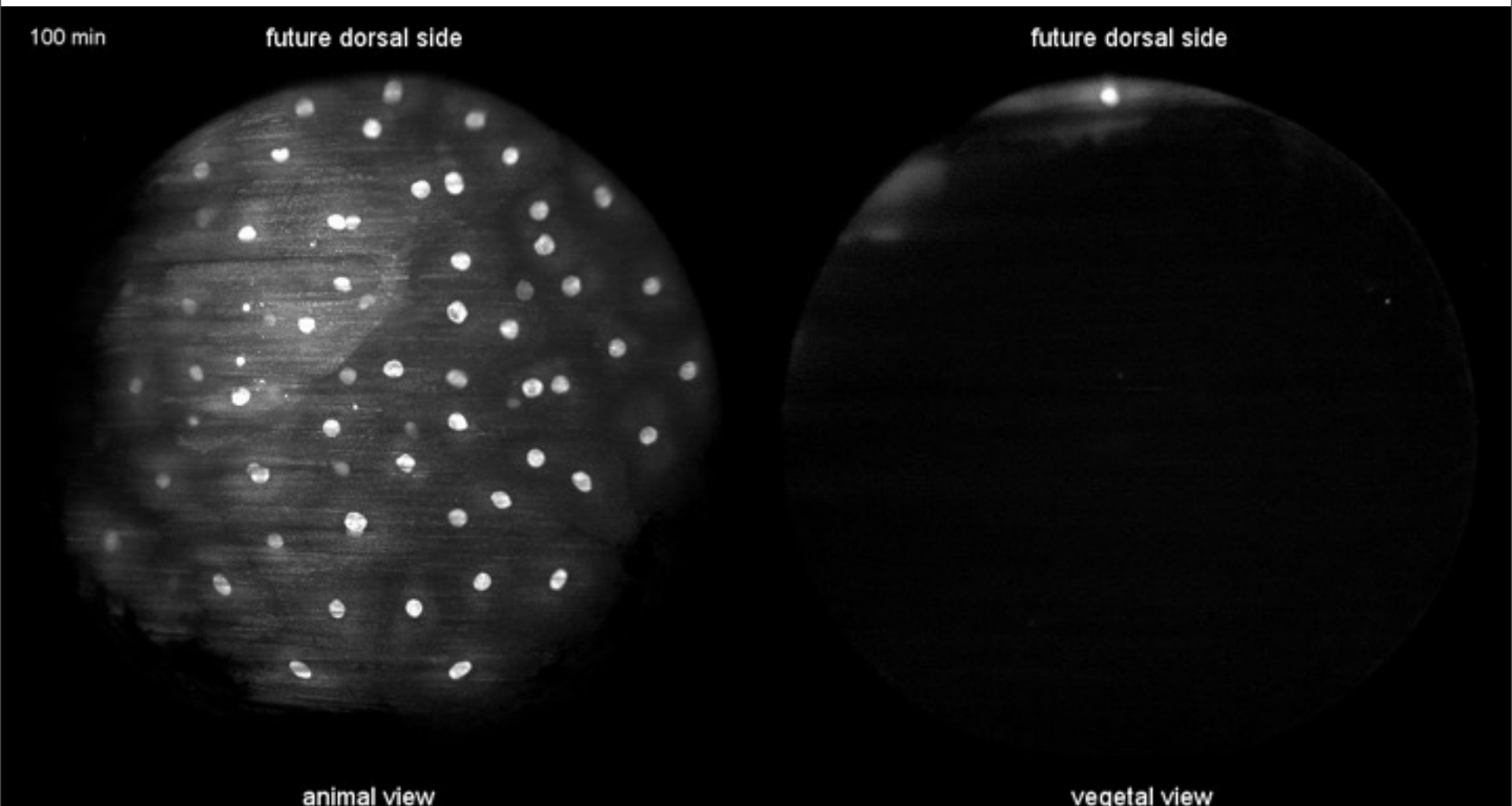


An incident angle close but smaller than the critical angle can improve the signal from near-surface objects.

Single-plane illumination microscopy (SPIM)

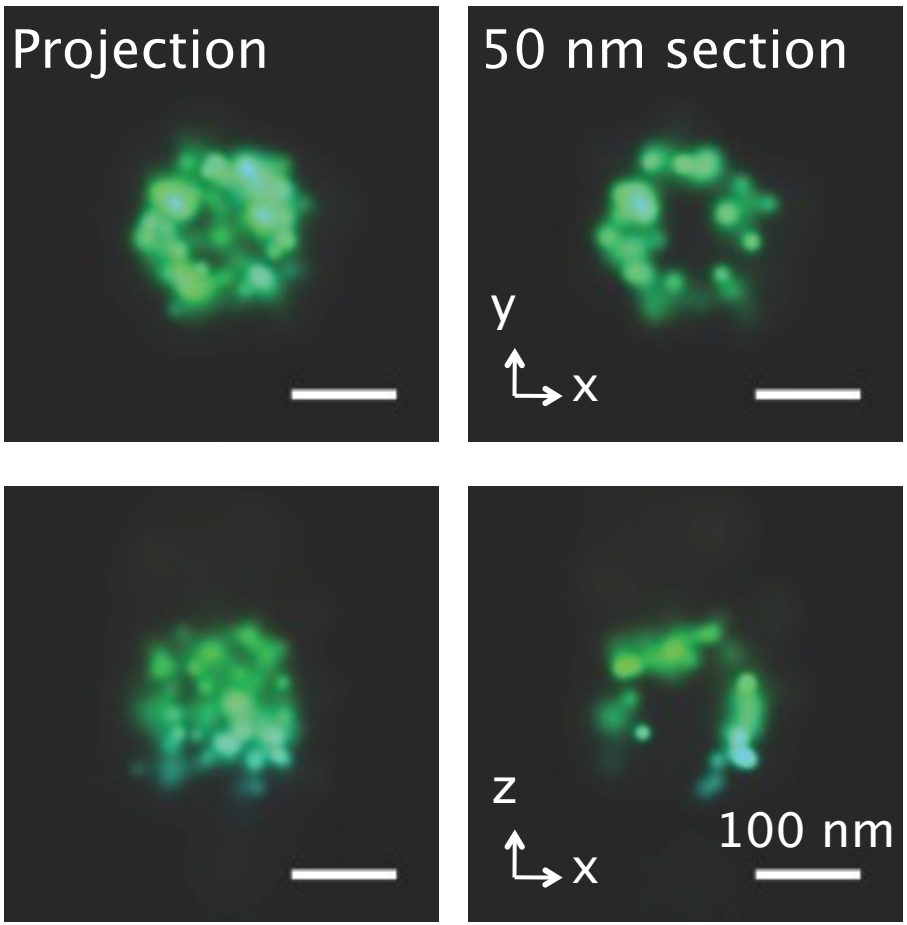


Zebra fish embryo development imaged by SPIM



Keller et al., Science (200

Super-resolution microscopy



3D STORM image of a clathrin-coated pit

Wu et al., Nat Cell Biol (20

Now the tools are in your hands!