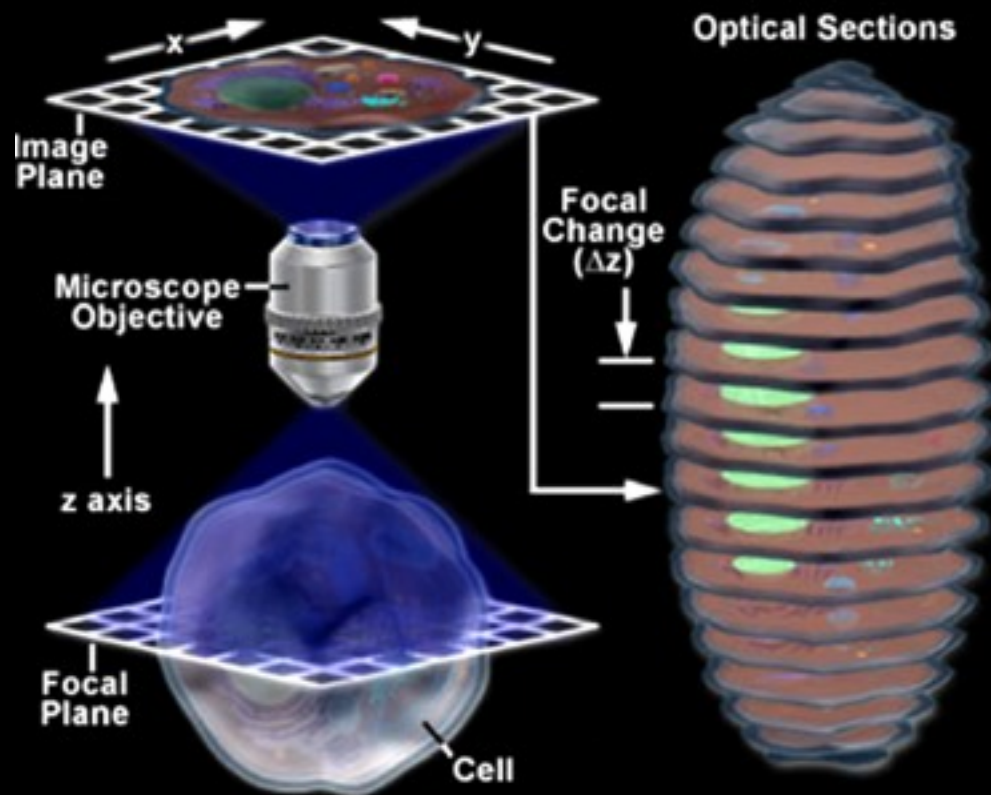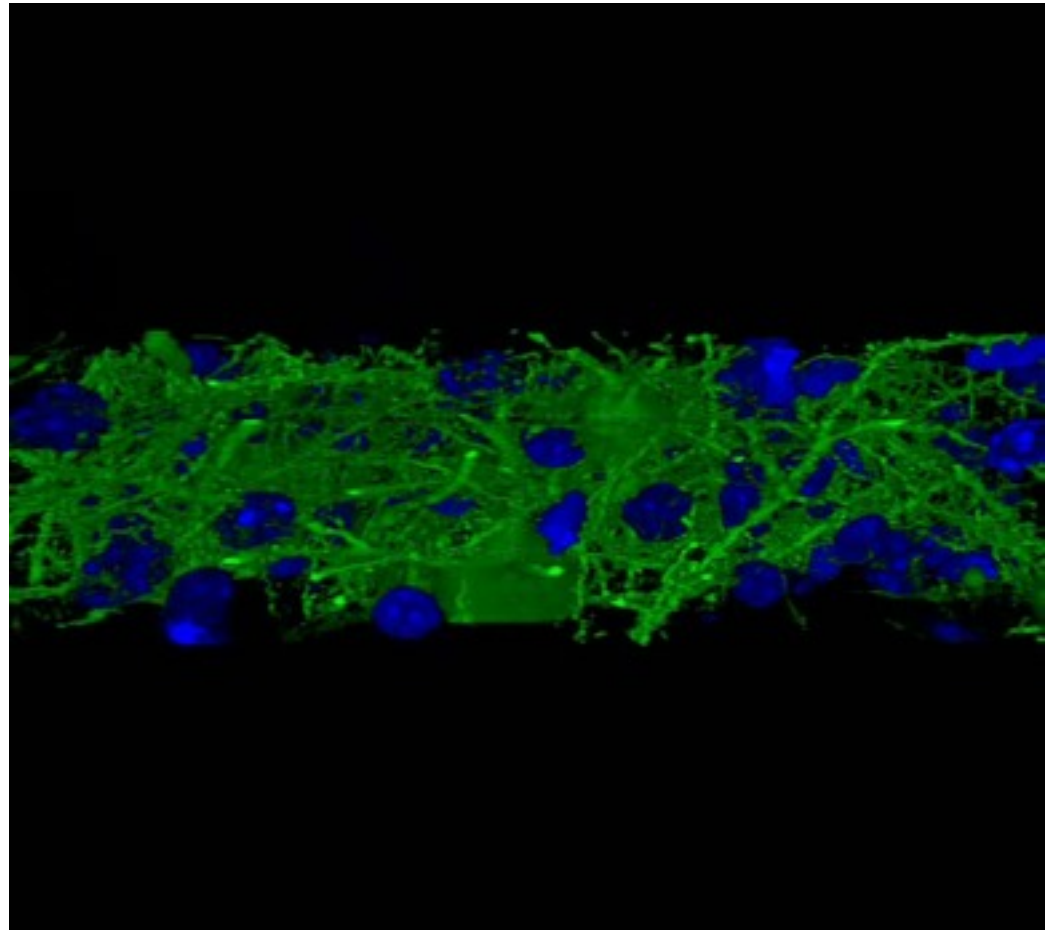# Optical Sectioning

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
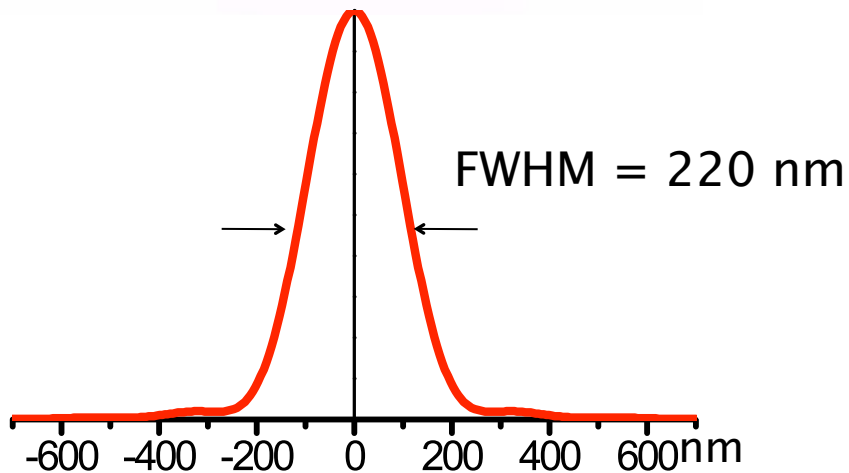
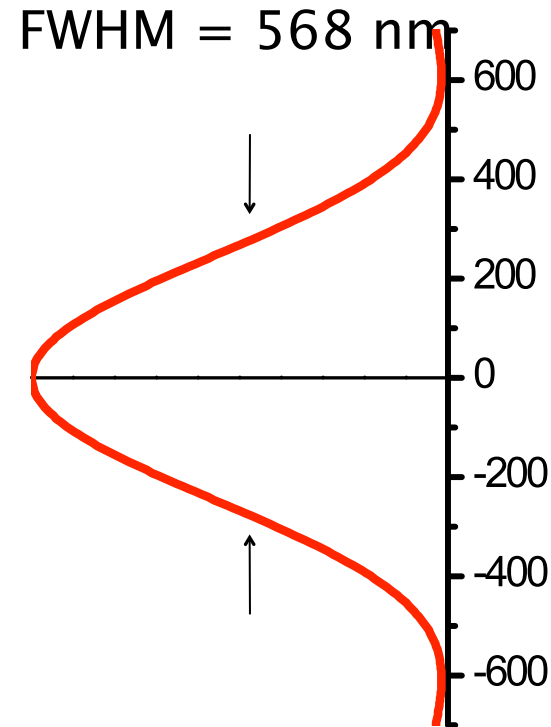- (Almost) equivalent: the 3D focus of a laser
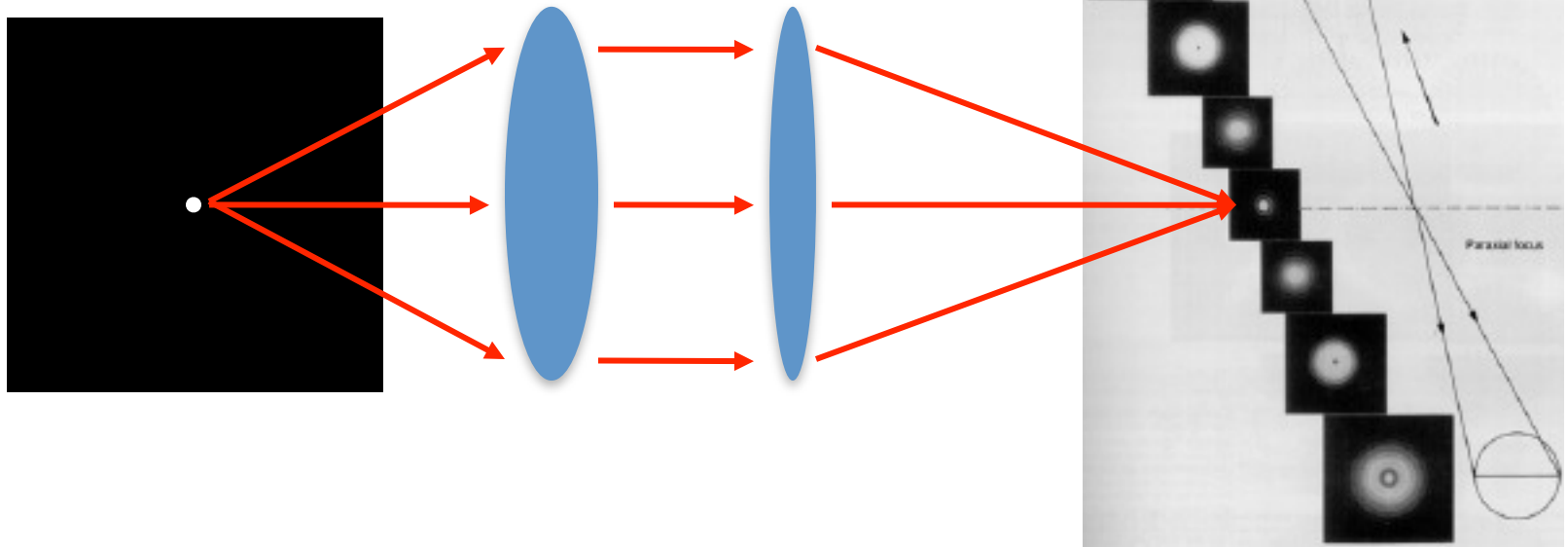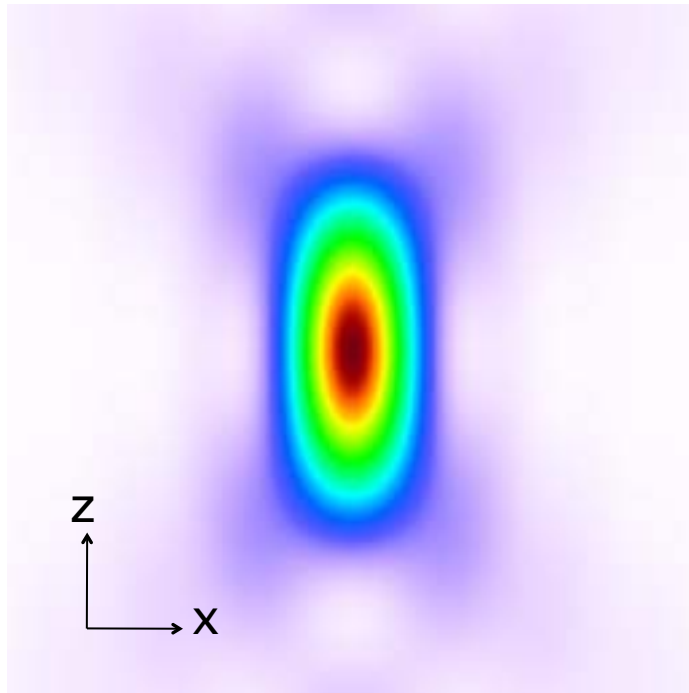
The lateral size of the PSF
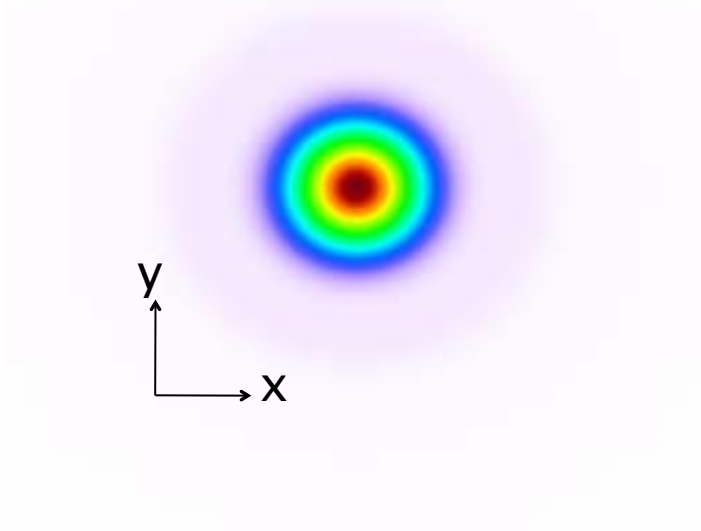
Lateral resolution $\approx$ $\dfrac{0.61\lambda}{NA}$

Example: 1.4 NA objective at 550 nm

$\Delta_{xy} \approx 240$ nm

FWHM = 220 nm

The axial size of the PSF

FWHM = 568 nm
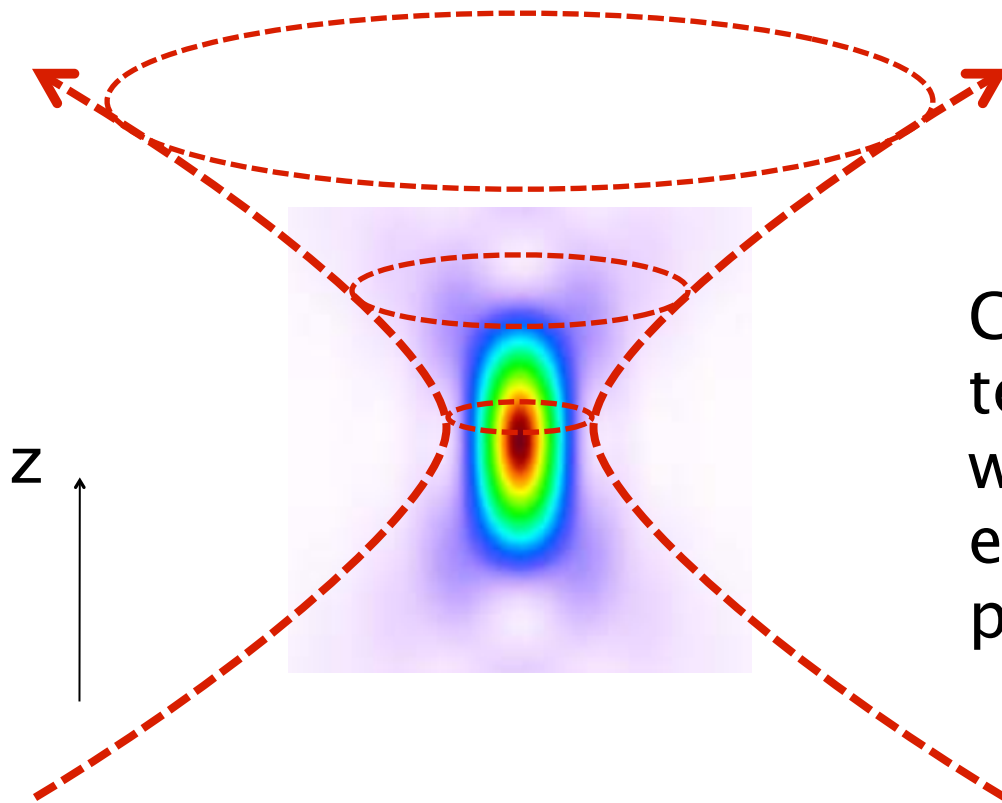
Axial resolution $\approx \dfrac{2n\lambda}{NA^2}$

(works only for low NA system)

Example: 1.4 NA objective at 550 nm

$\Delta_z \approx 850$ nm

z resolution ≠ z sectioning

z

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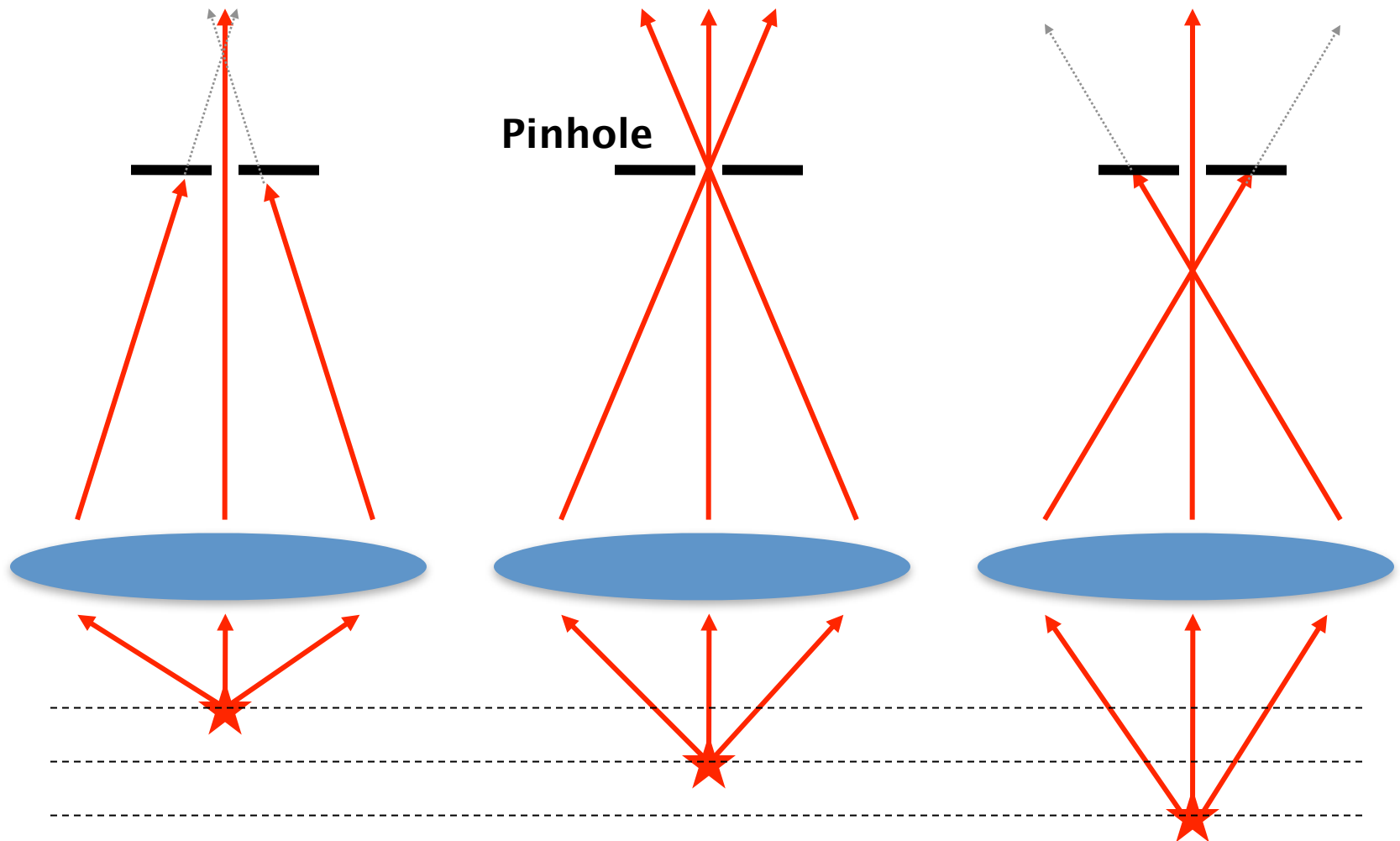
How to achieve optical

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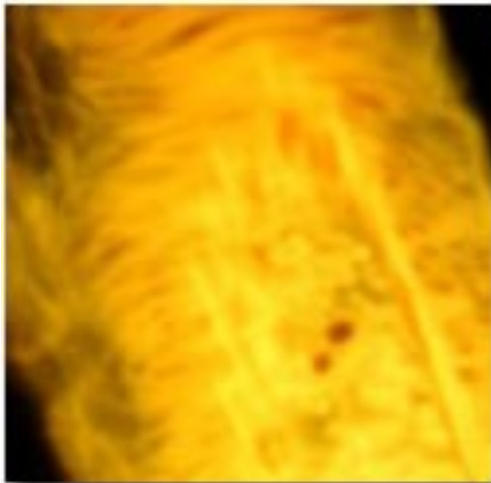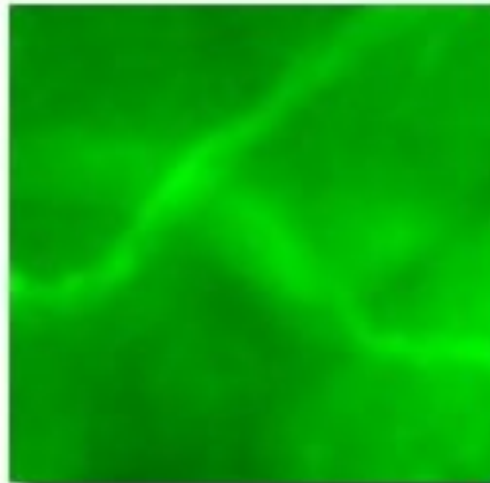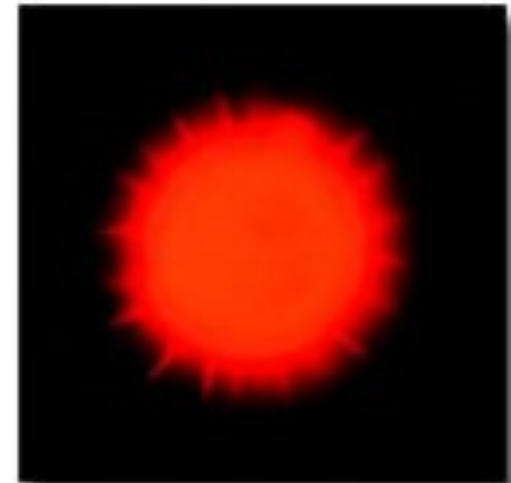 - Single plane illumination microscopy (SPIM)

z

Principle of confocal

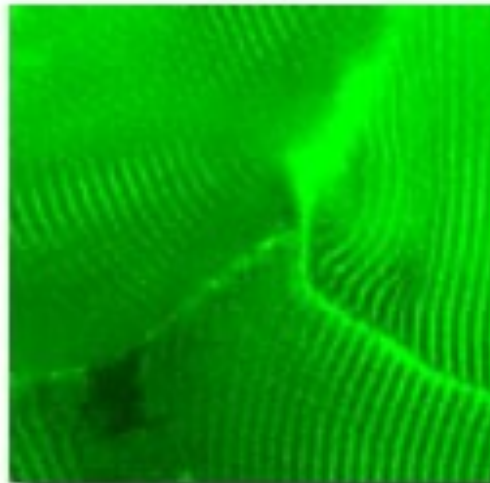

Pinhole

Confocal vs. widefield fluorescence microscopy
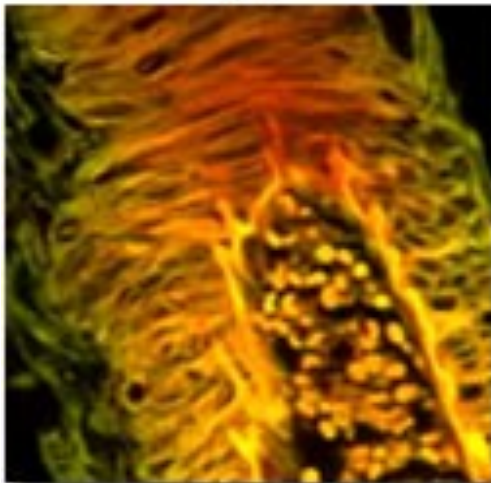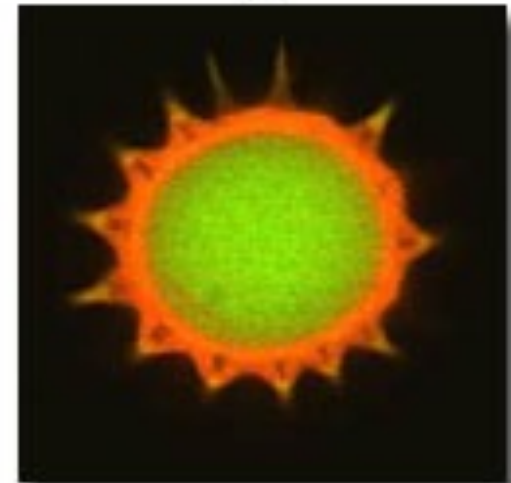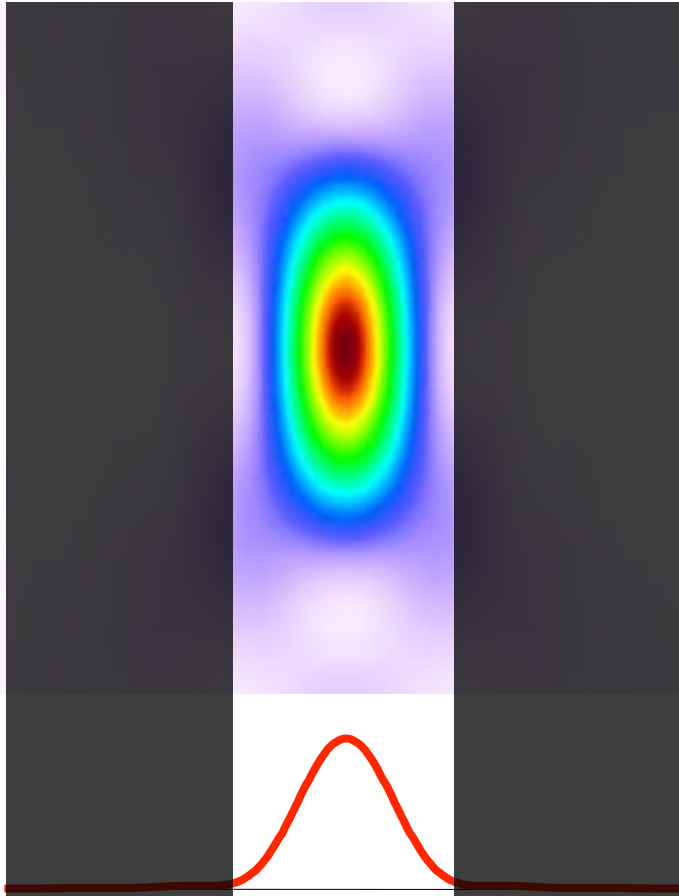
The pinhole size in confocal microscopy

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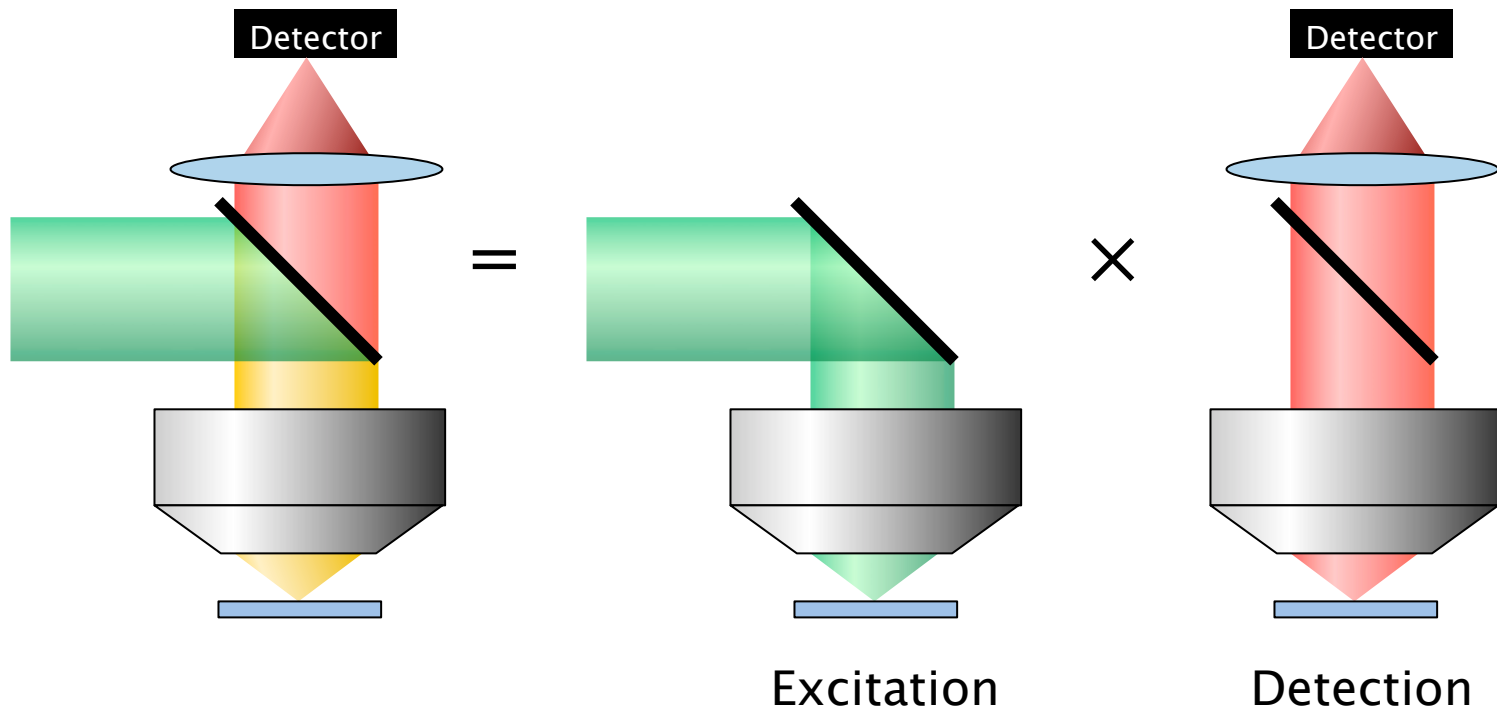
$$PSF_{Confocal} = PSF_{ex} \times PSF_{det}$$

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

The confocal volume
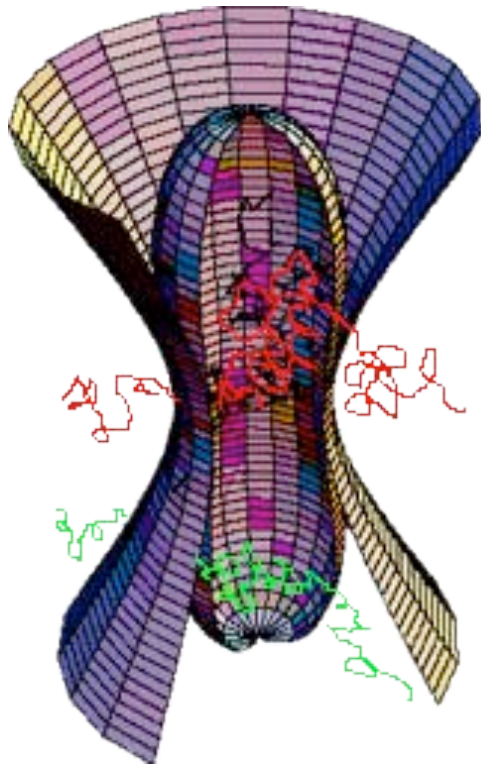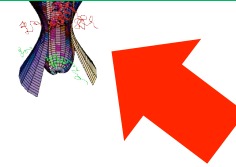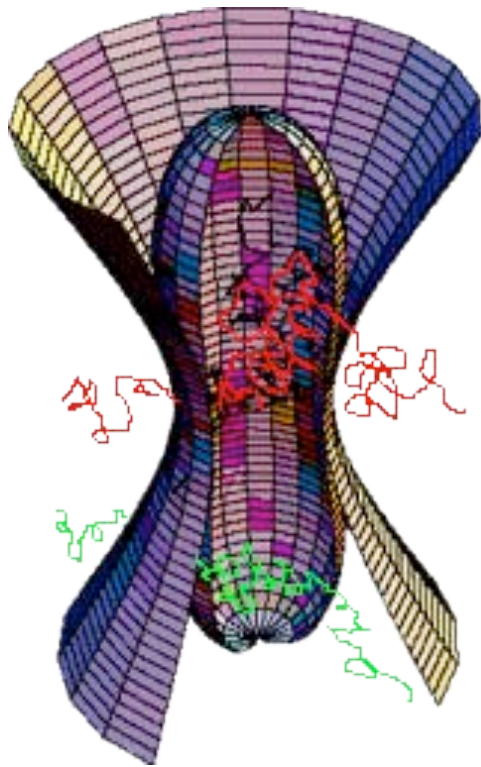

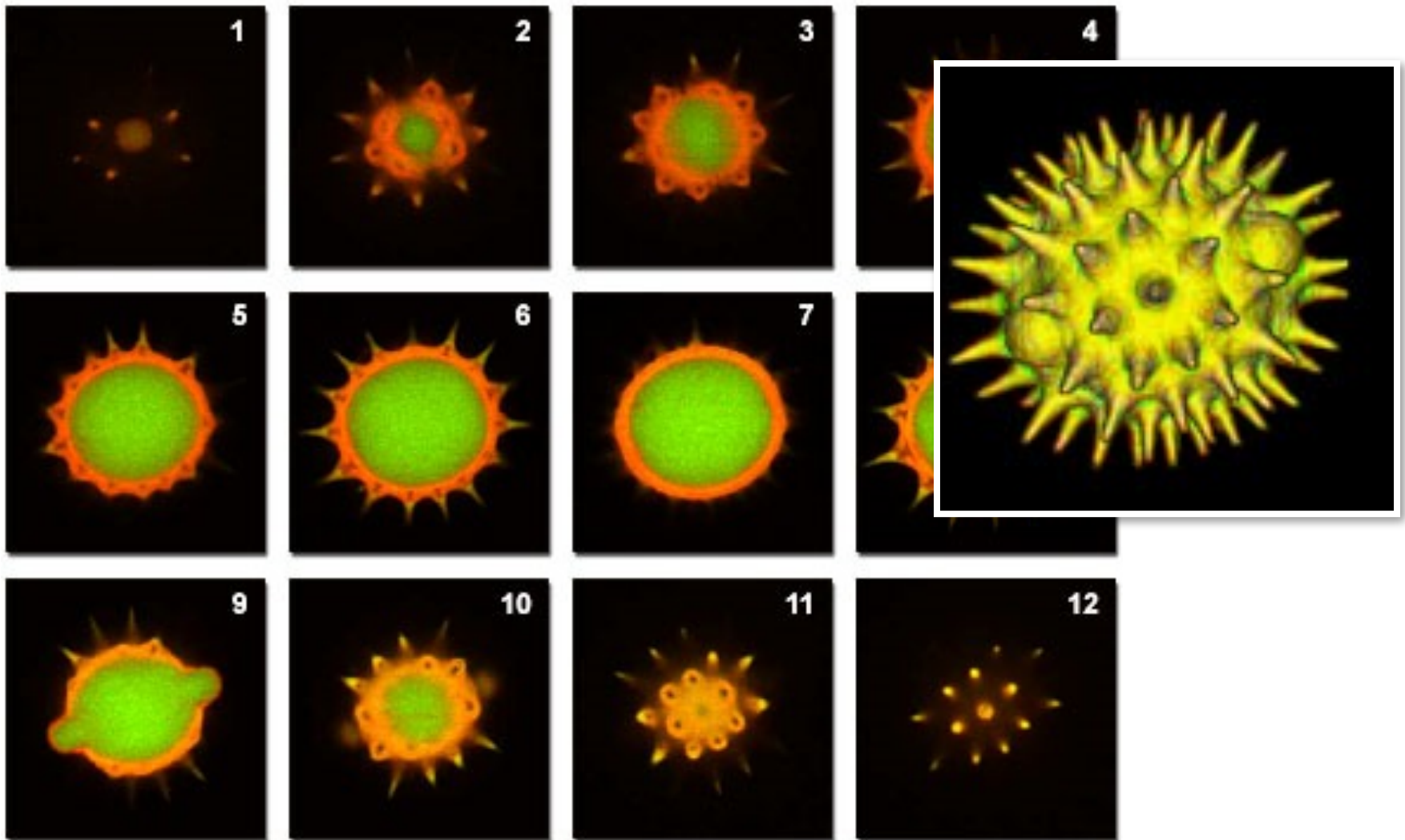Integrating PSF$_{confocal}$ over the entire space now gets a finite value

Diffraction limited volume:

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

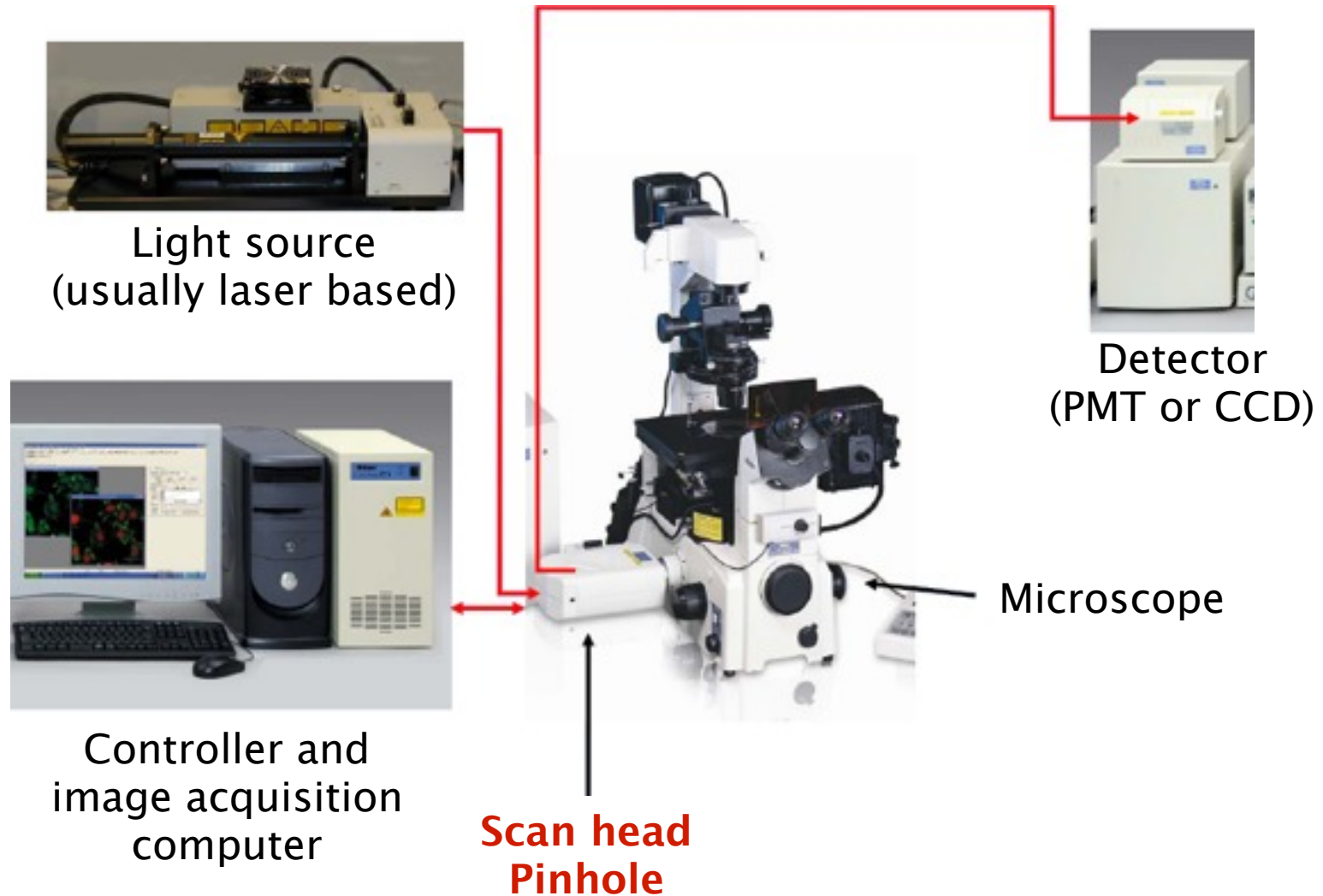# The sectioning ability of confocal microscopy

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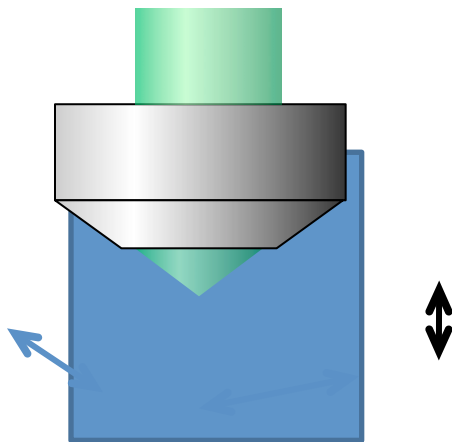the best confocal microscope, what is the fraction

Confocal sectioning and 3D reconstruction
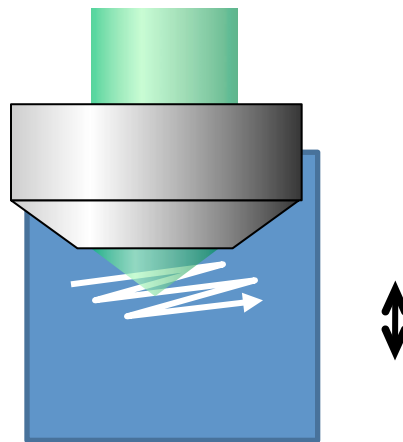
Components of a confocal

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
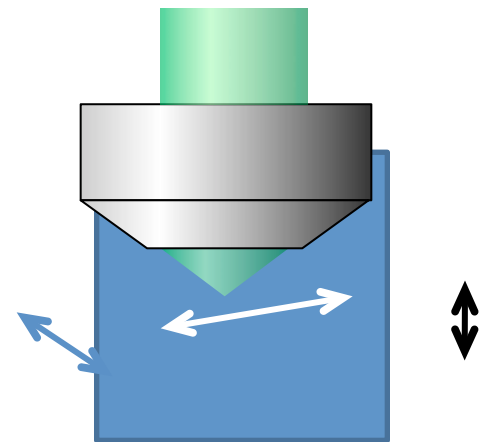
Galvanometer scanning /

Two mirrors for
x-y raster scan

Excitation

Detection
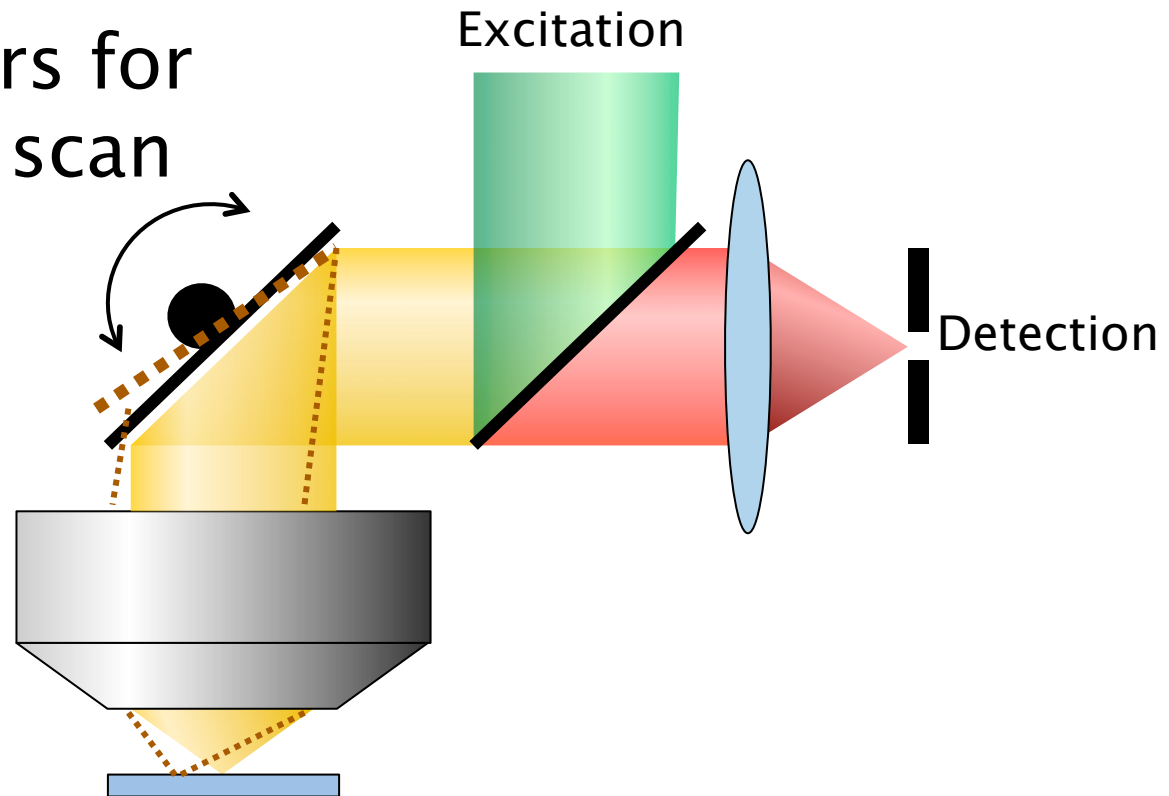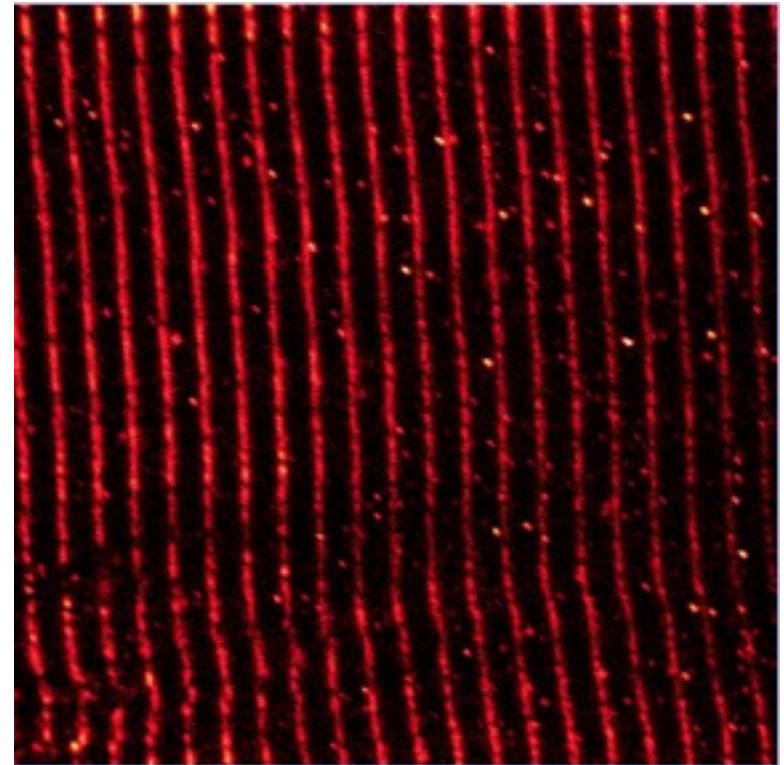
Averaging multiple scans to enhance S/N

1 frame

8-frame average

How to scan faster?

- Normal galvo mirror: several µs per pixel
  0.5 – 2 sec per image

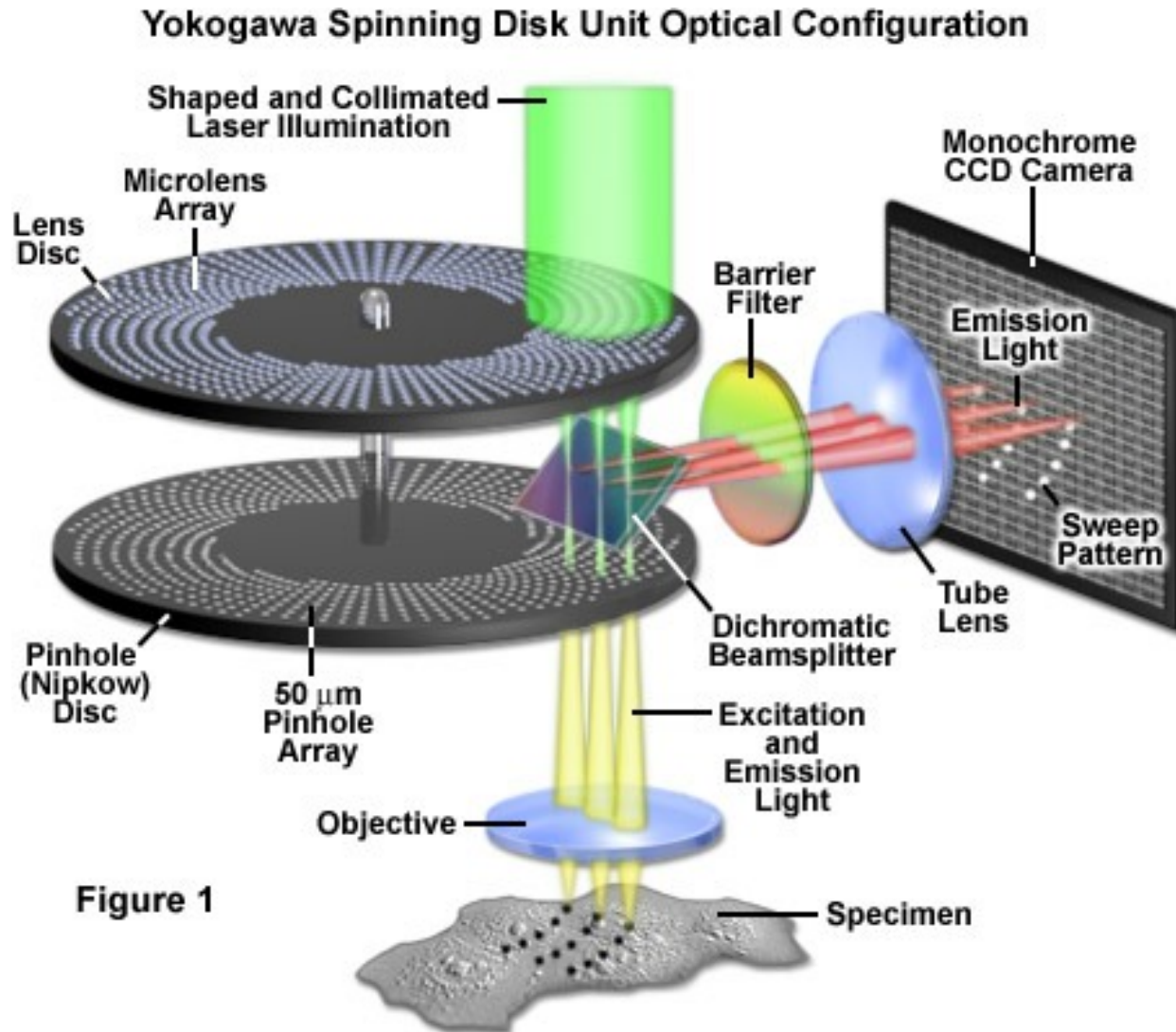- Resonance scanner: up to 30 fps

- The answer is:
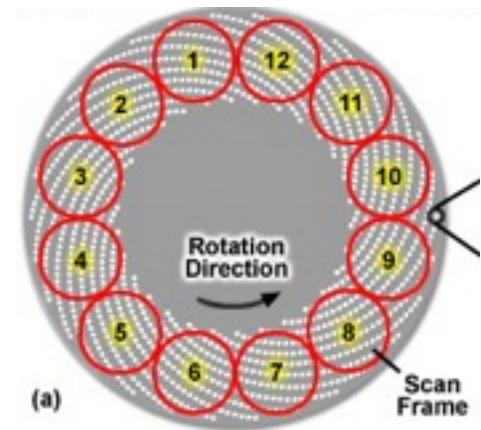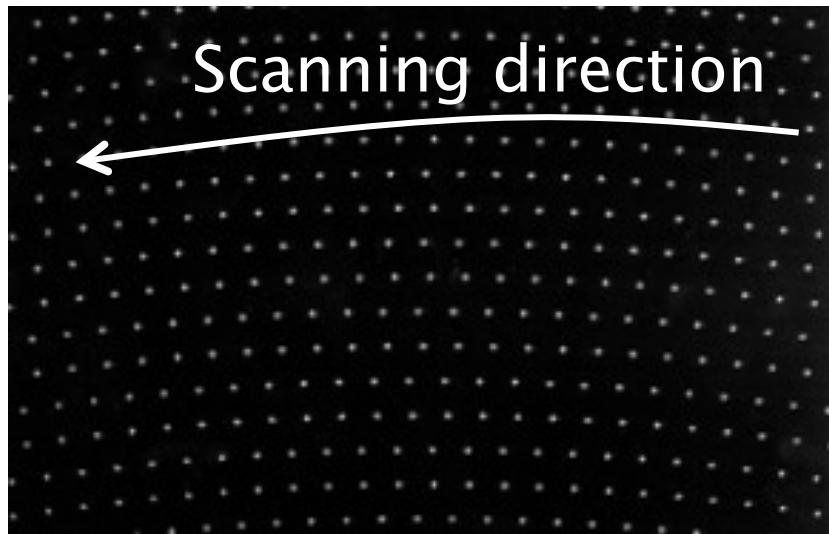
Spinning disk confocal

- Scanning with a rotating pinhole array
- CCD camera detection

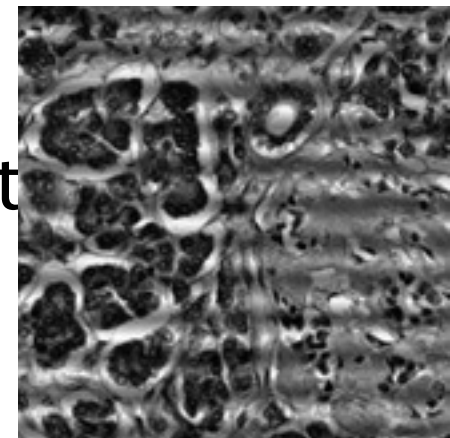# Spinning disk confocal

Yokogawa Spinning Disk Unit Optical Configuration

Figure 1

Spinning disk confocal
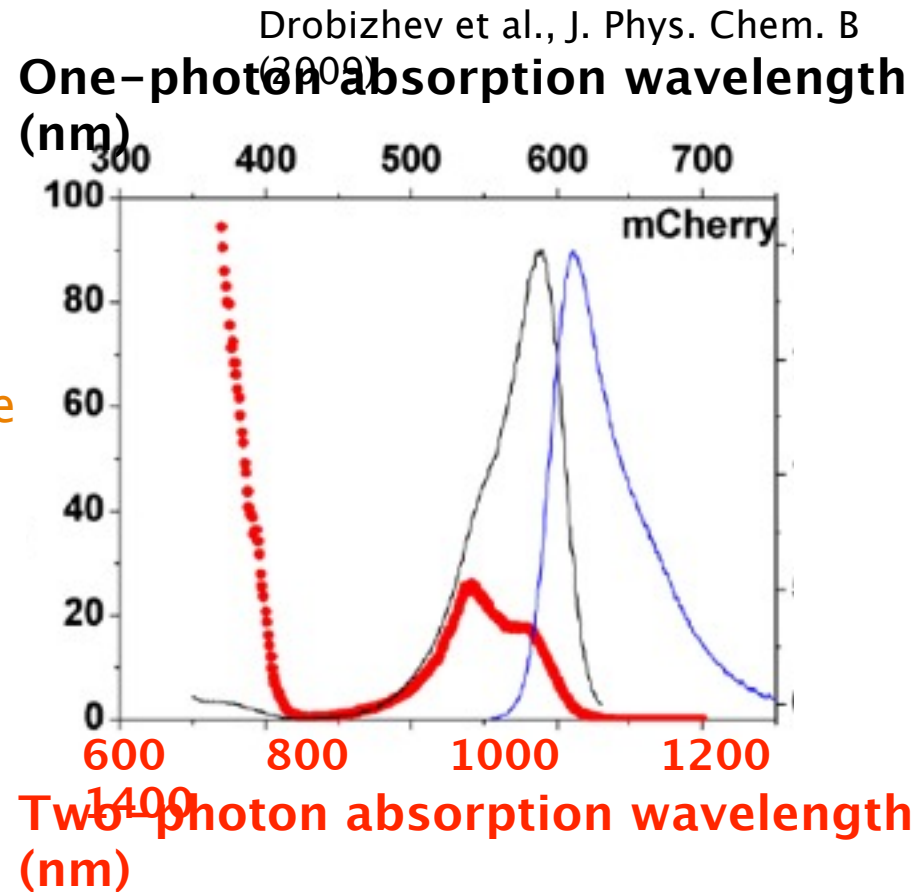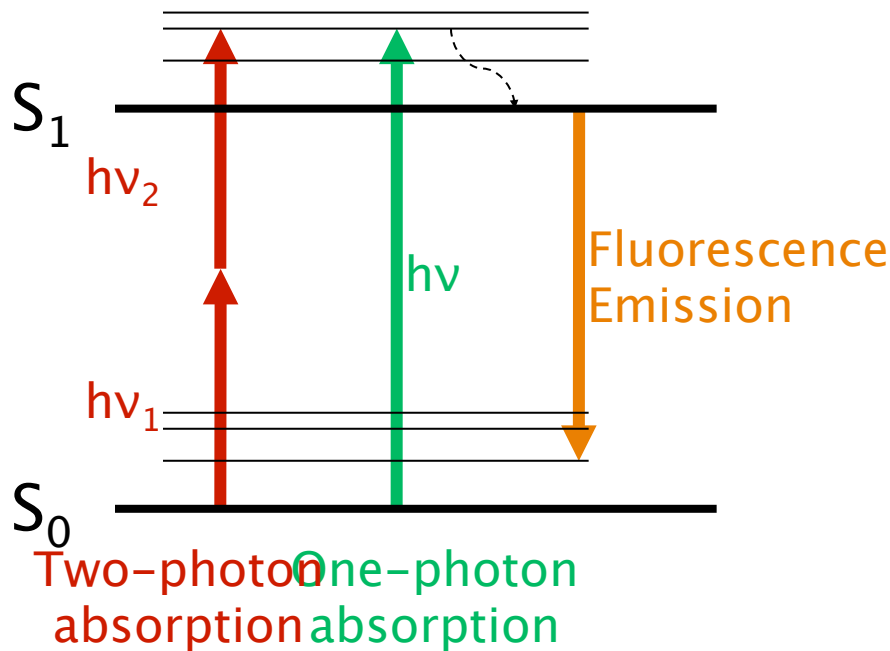

Scanning direction

Rotation Direction

Scan Frame

(a)

12 scanning frames per 360° rotation

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

Multi-photon microscopy

S_1

S_0

$h\nu_2$

$h\nu_1$

$h\nu$

Fluorescence
Emission

Two-photon
absorption

One-photon
absorption

One-photon absorption wavelength (nm)

300   400   500   600   700

100

80

60

40

20

0

mCherry

Two-photon absorption wavelength (nm)

600   800   1000   1200   1400

Two-photon excitation: Fluorescence $\propto I^2$
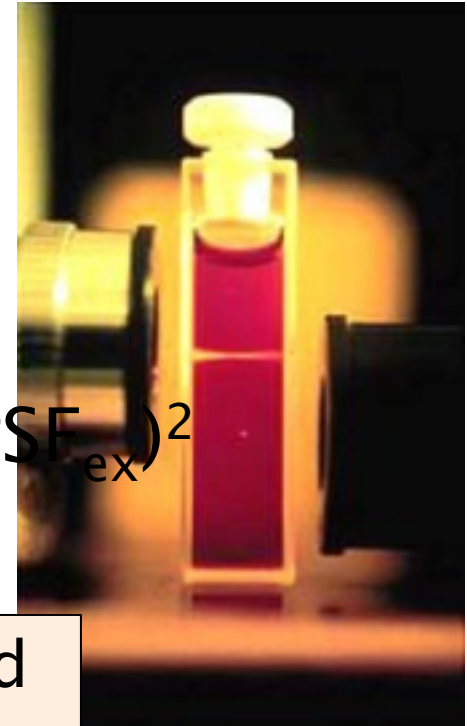
Optical sectioning without a

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

⬇

$$\text{PSF}_{2P.ex} = (\text{PSF}_{1P.ex})^2$$

Recall:

$$\text{PSF}_{Confocal} = \text{PSF}_{ex} \times \text{PSF}_{det} \approx (\text{PSF}_{ex})^2$$

⬇

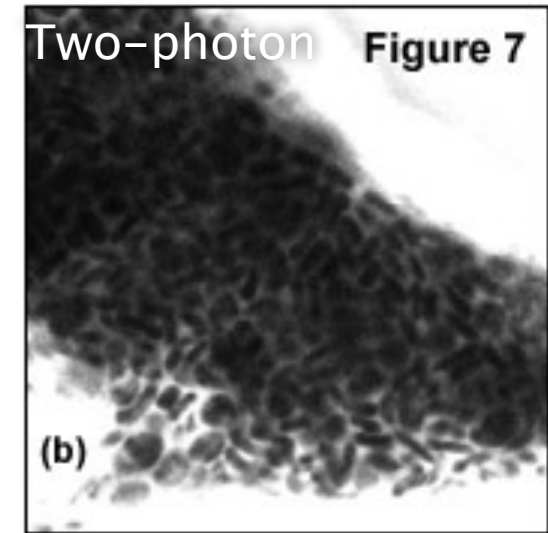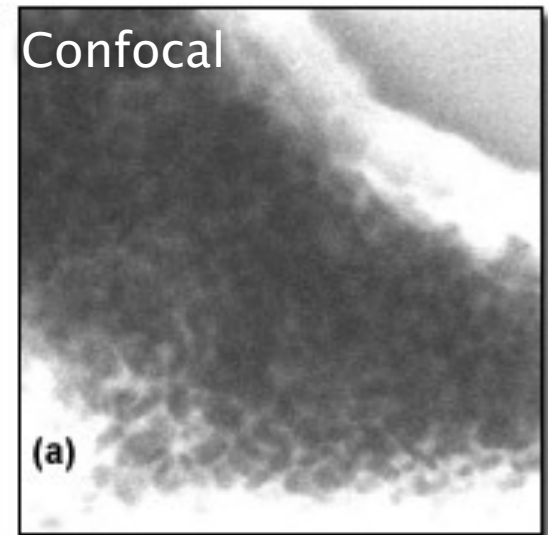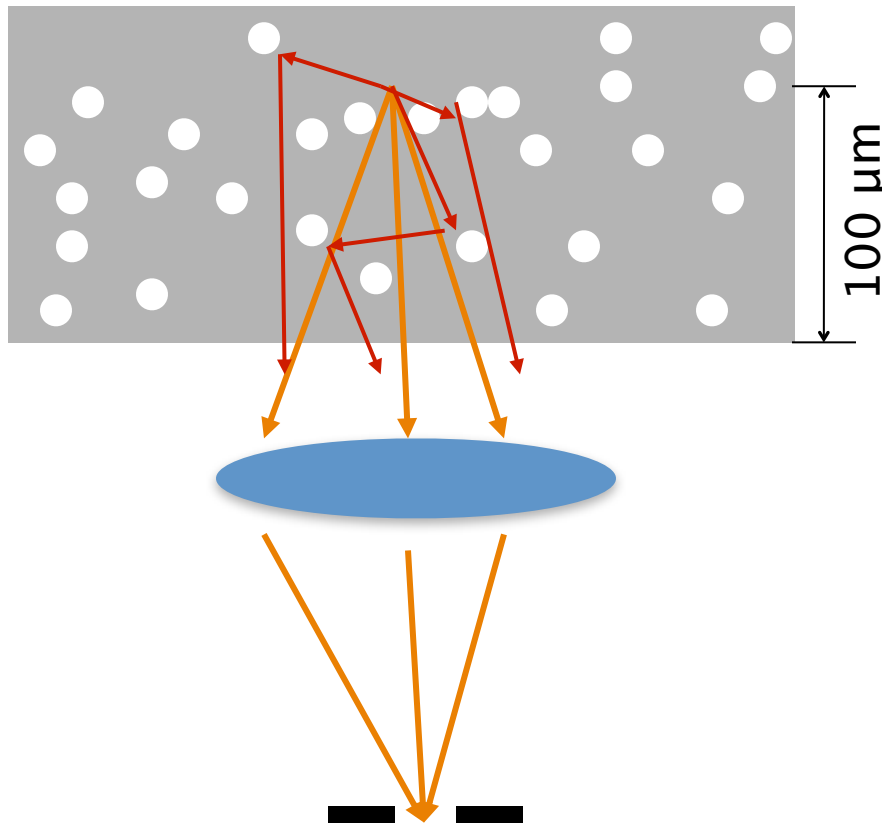Two-photon excitation is localized to the laser focal point

Deep tissue imaging

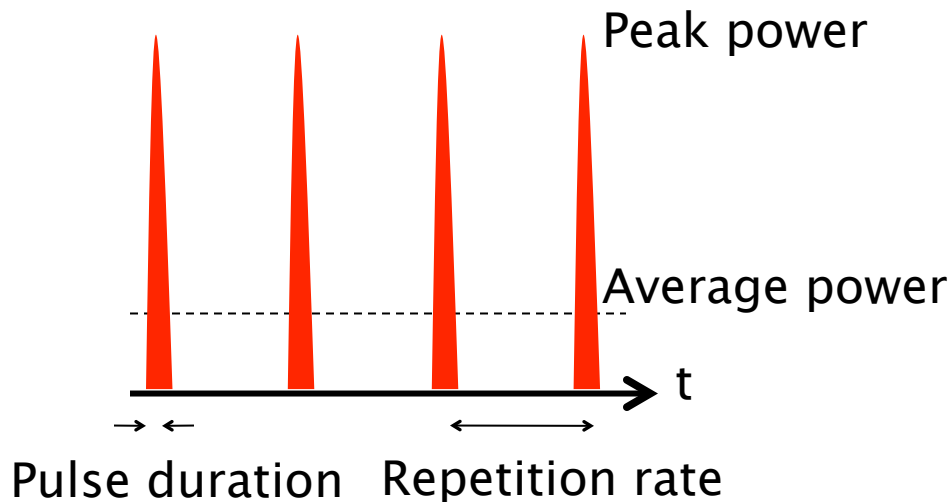Tissue scattering and absorption decrease confocal performance

100 μm

Confocal

(a)

Two-photon   Figure 7

(b)

Ultrafast laser source for 2PE

Continuous wave (CW) laser

t

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

Pulsed laser

Peak power

Average power

t

Pulse duration   Repetition rate
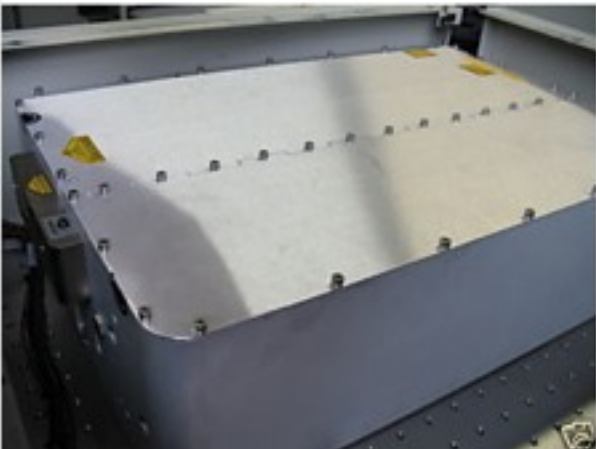
Ti:Sapphire laser, 100 fs, 80 MHz

10 mW avg ≈ 1.2 kW peak

Ultrafast laser source for 2PE

Spectra Physics Mai Tai femtosecond laser New Diodes

Item condition: Used

Price: US $65,000.00   Buy It Now

or

Best Offer: Make Offer

Add to Watch list
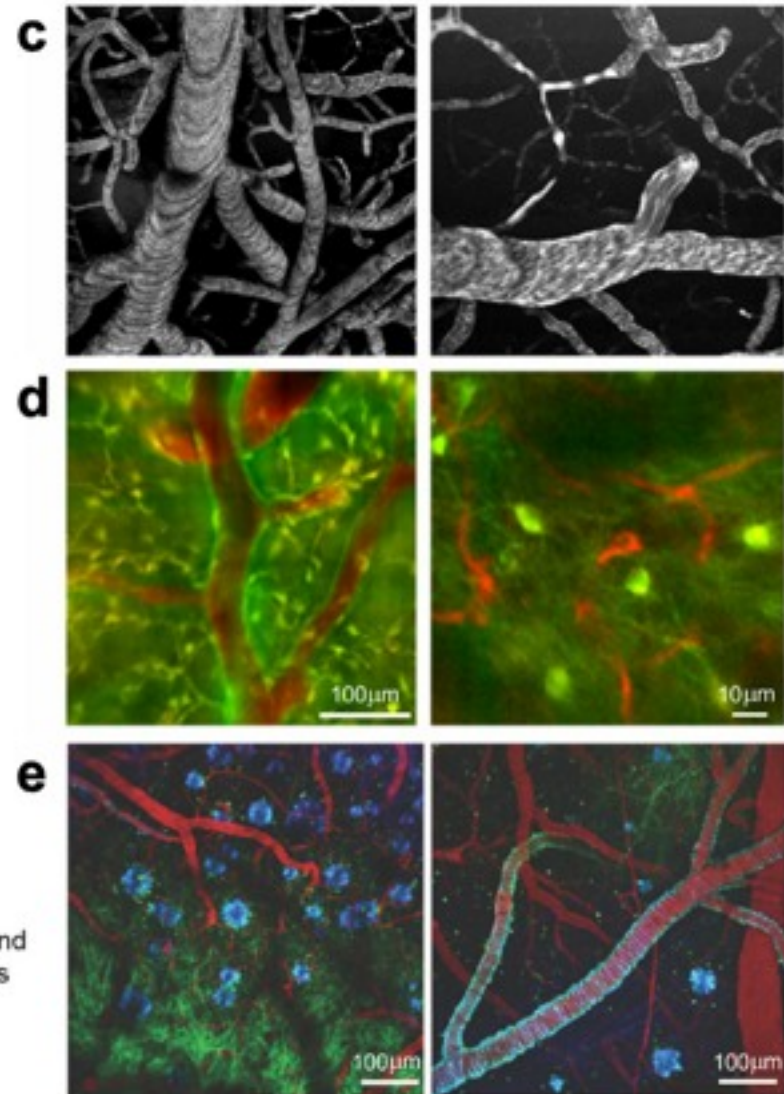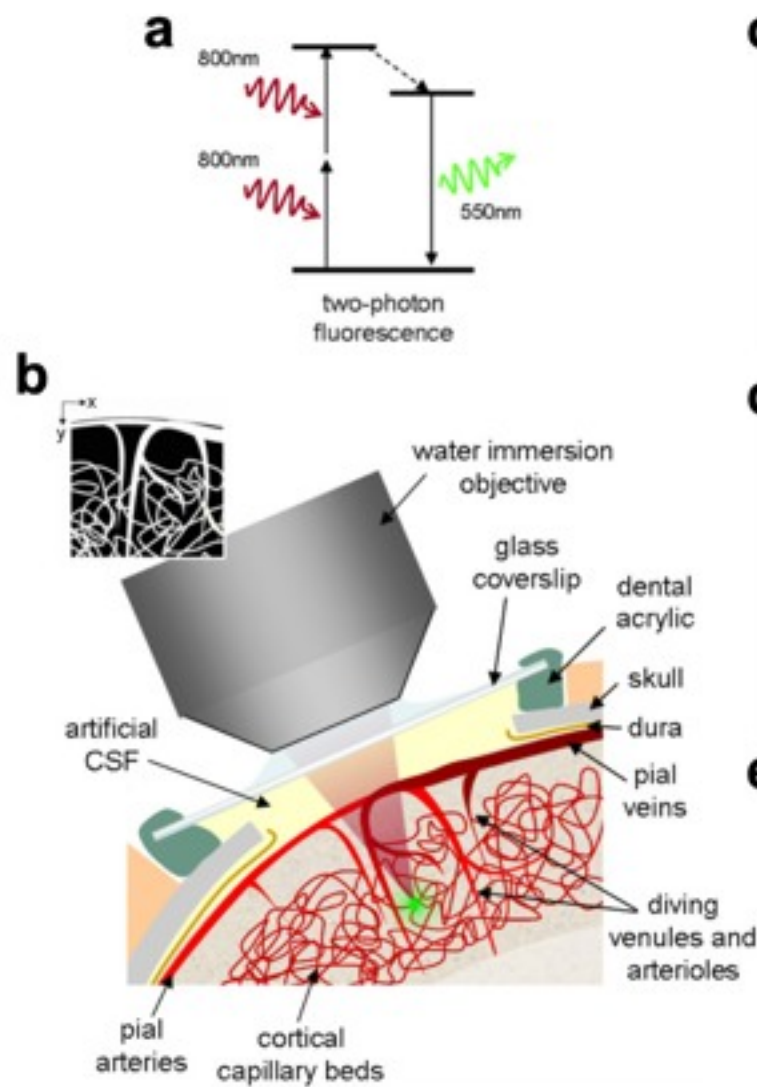
Shipping: Freight - See shipping details
| See all details
Estimated delivery time varies for freight shipping.

Returns: No Returns Accepted

eBay Buyer Protection
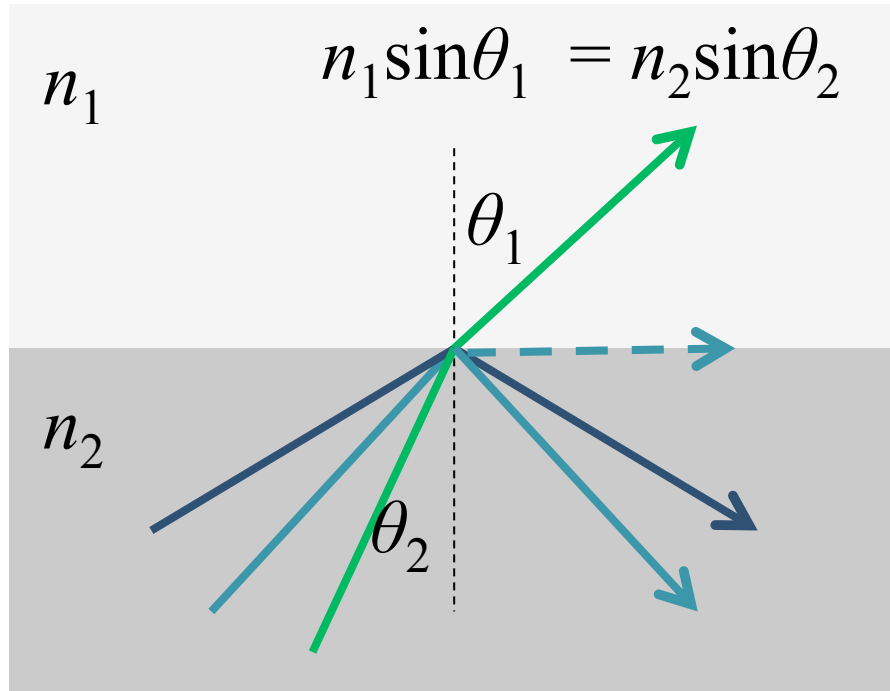eBay will cover your purchase price plus original shipping.
Learn more

Two–photon microscopy of in vivo brain function

Kherlopian, et al., BMC Systems Biology (

# Total internal reflection fluorescence microscopy

$$n_1\sin\theta_1 = n_2\sin\theta_2$$

n_1

θ_1

n_2

θ_2

Total internal reflection:

$$\theta_1 = 90° \; \blacktriangleright$$

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

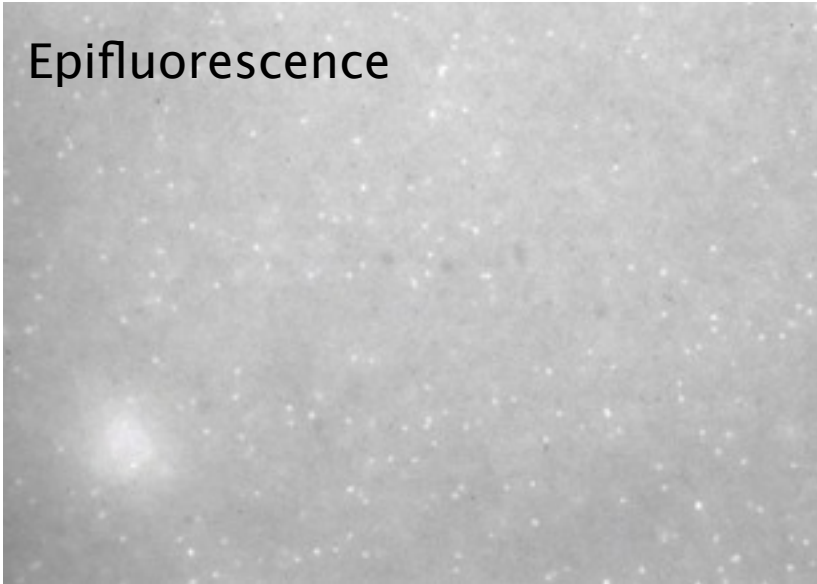$$n_1 = 1.33 \text{ (water)}, \; n_2 = 1.52 \text{ (glass)}, \; \theta_c = 61°$$

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.
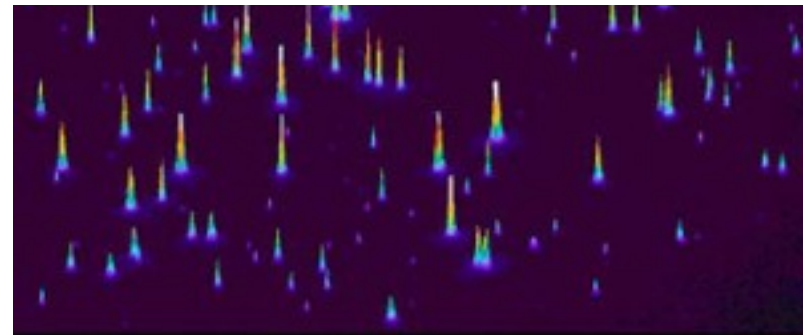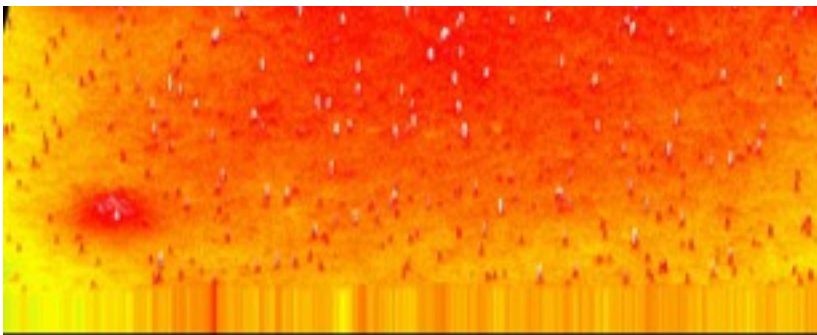
- The penetration depth is a function of the wavelength and the incident angle.
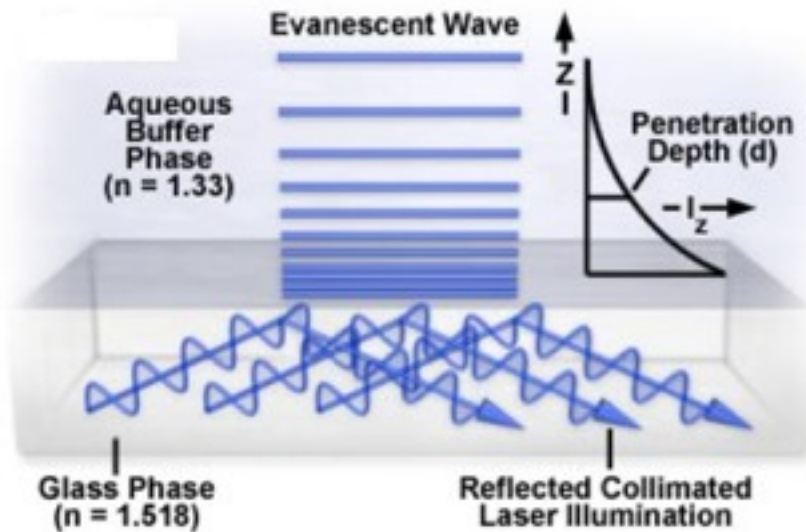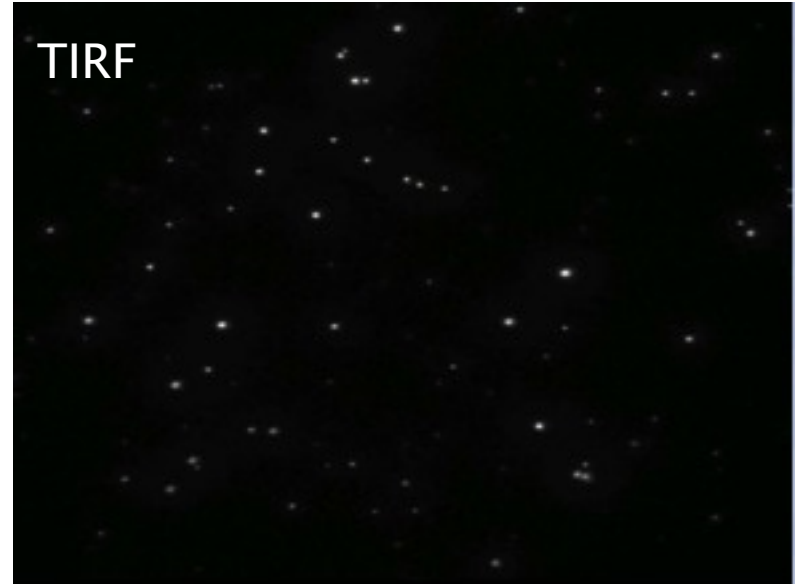
TIRM improves S/N for surface
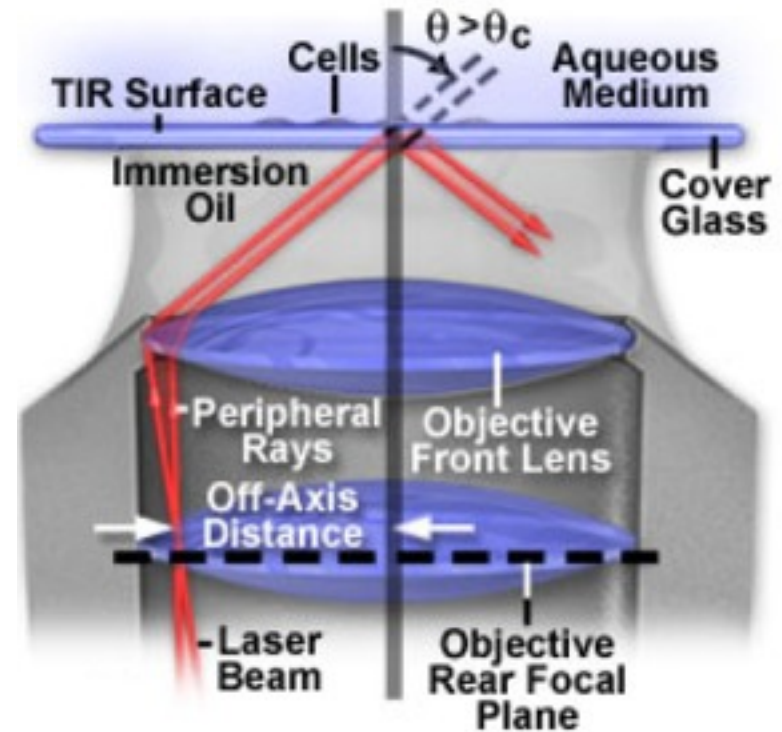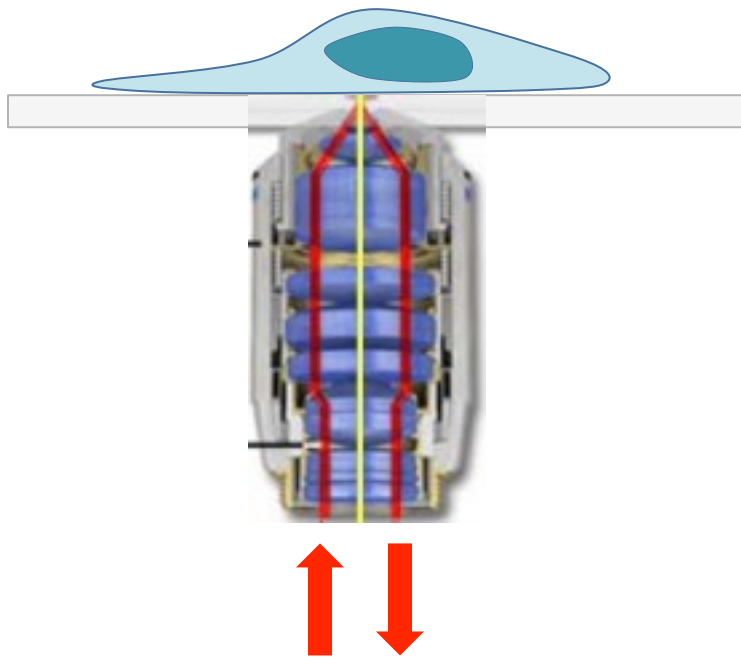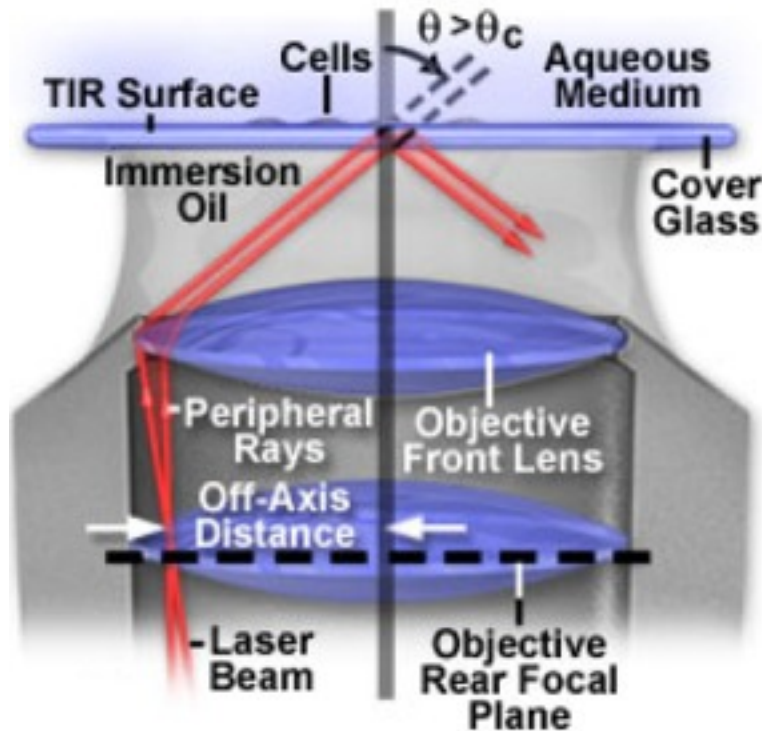

Epifluorescence

TIRF

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

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

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

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

$$\mathrm{NA} \geq n_{\mathrm{water}} \approx 1.33$$
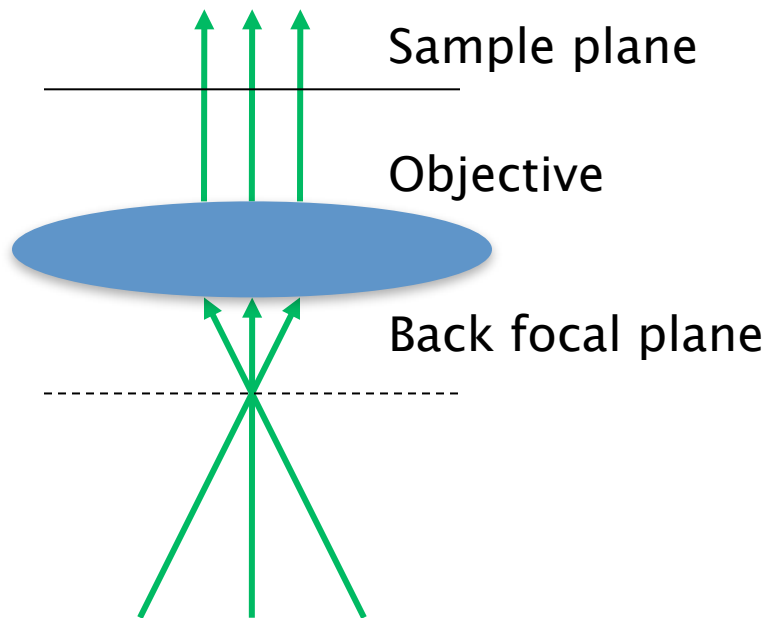
TIRF compatible objectives

- ## 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
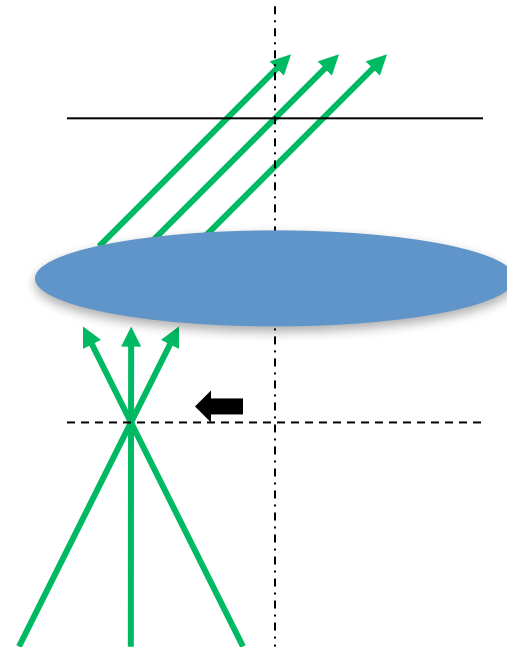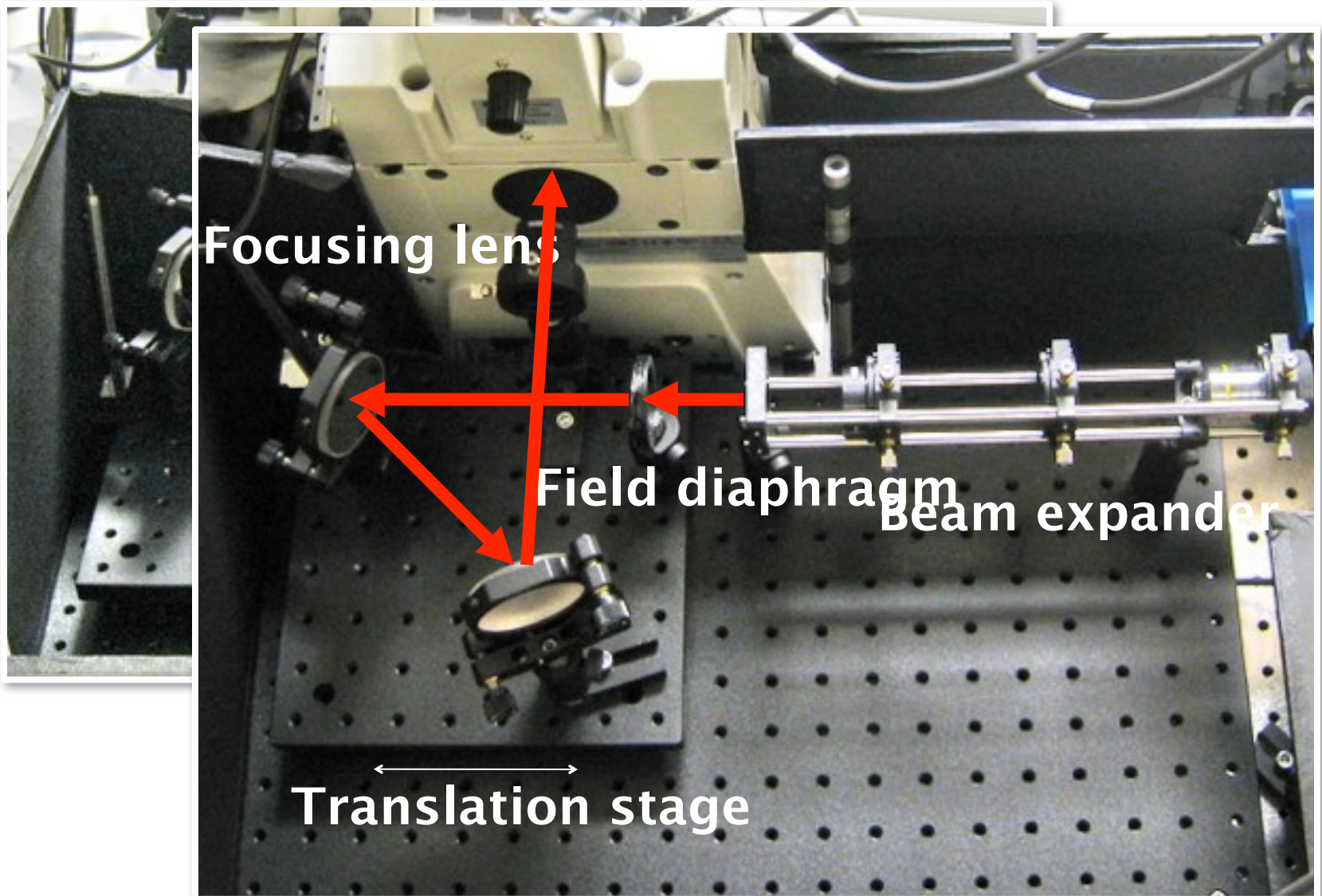 - Sapphire coverglass
  - Toxic oil…

TIRF illuminator

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

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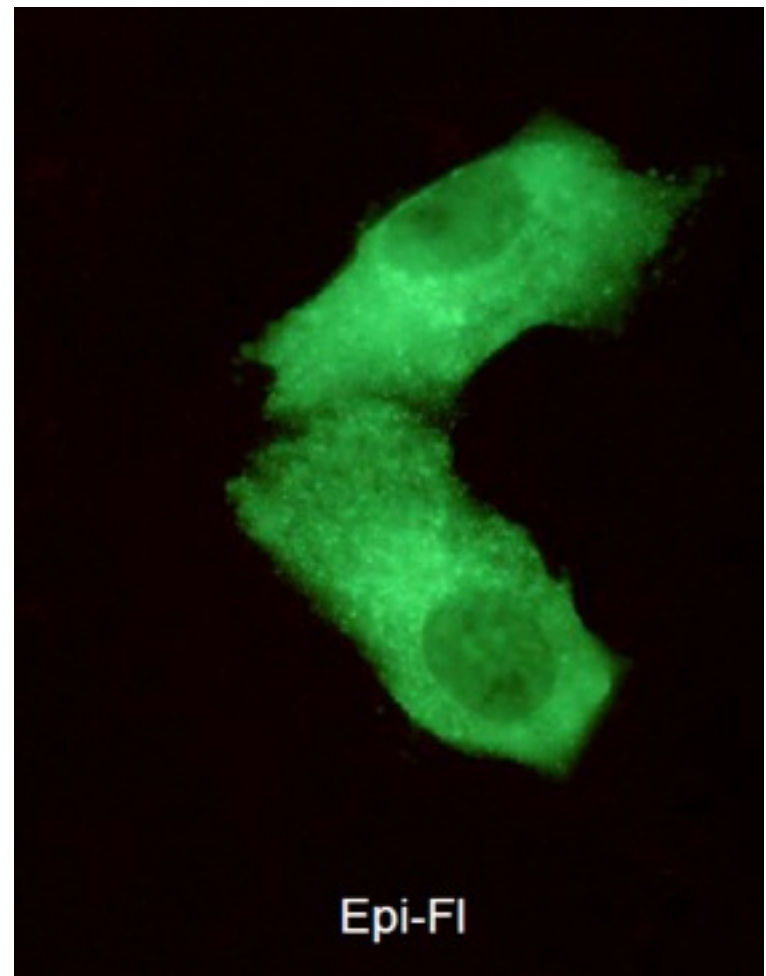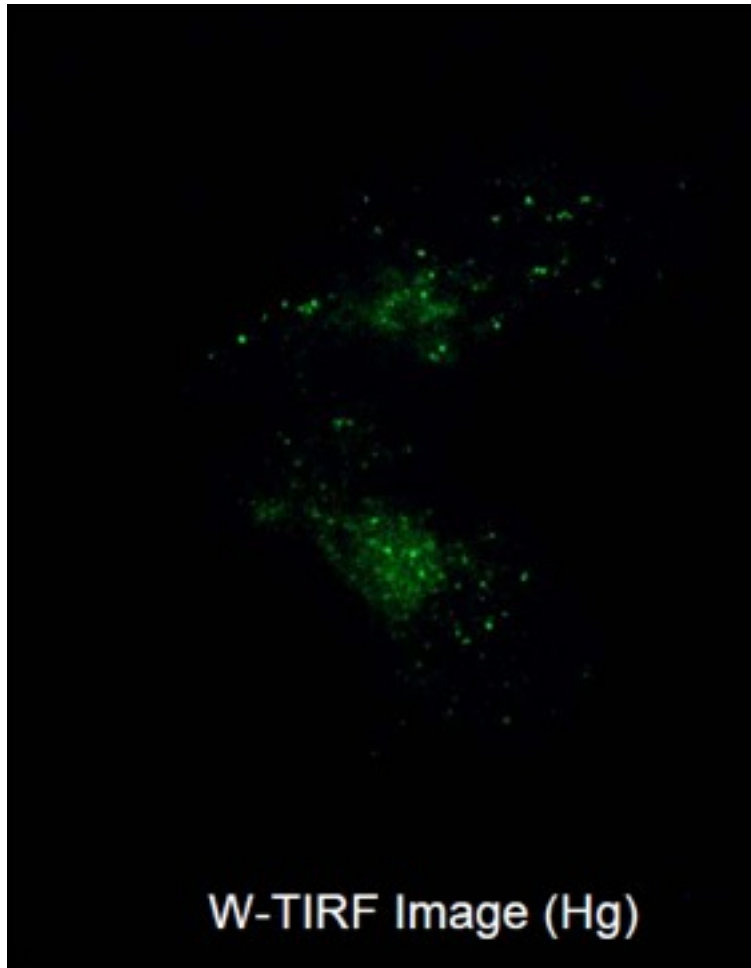edge of the objective
back–aperture

Sample plane

Objective

Back focal plane

A home-built TIRF illuminator
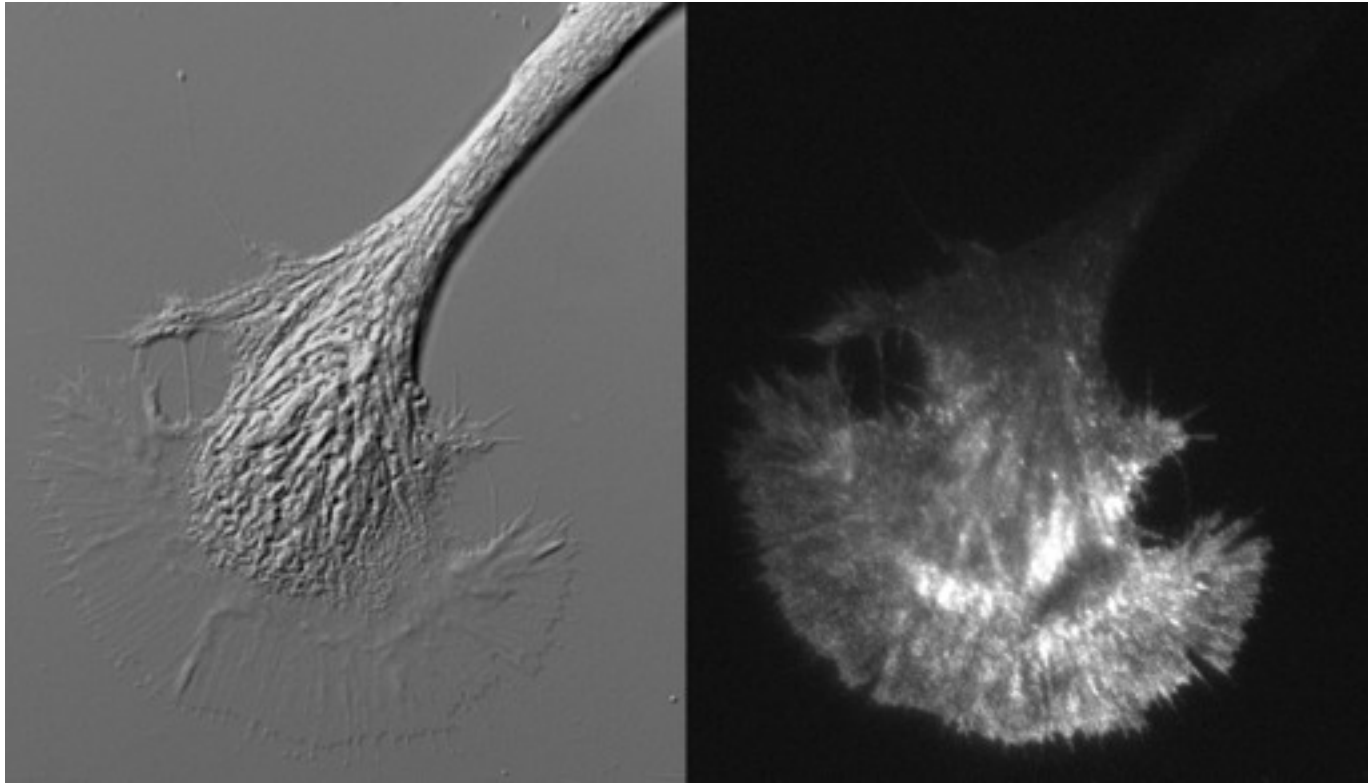
A commercial TIRF

COS cells expressing GFP

W-TIRF Image (Hg)

Epi-Fl

Dan Axelrod, Univ of Michigan

Alternating Epi/TIRF

clath, EF, pH7.4
clath, Epi, pH5.5
Tfnr-phl, Epi, ppH5.5

1.0 μm

-28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28

time (s)

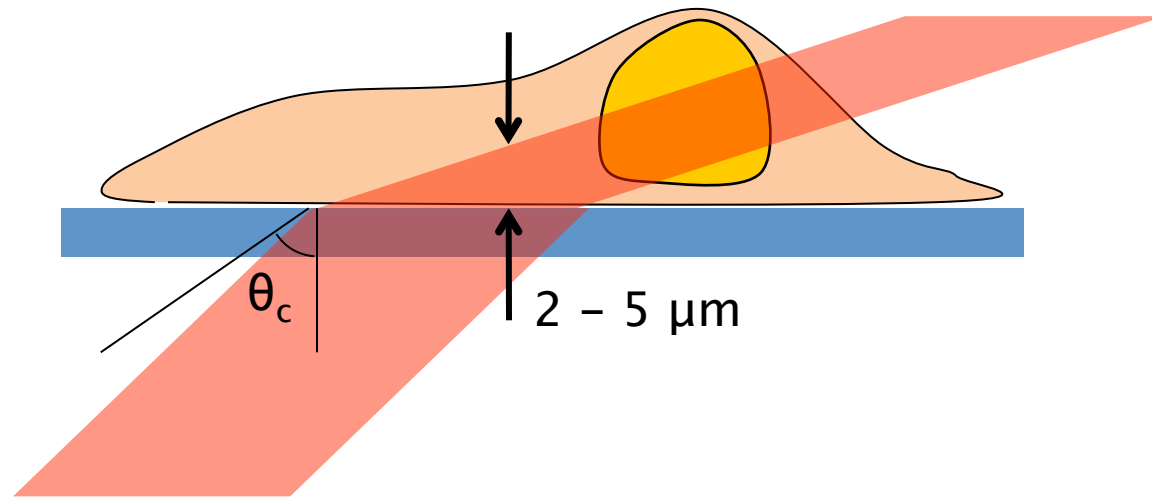Merrifield et al., Cell 2005
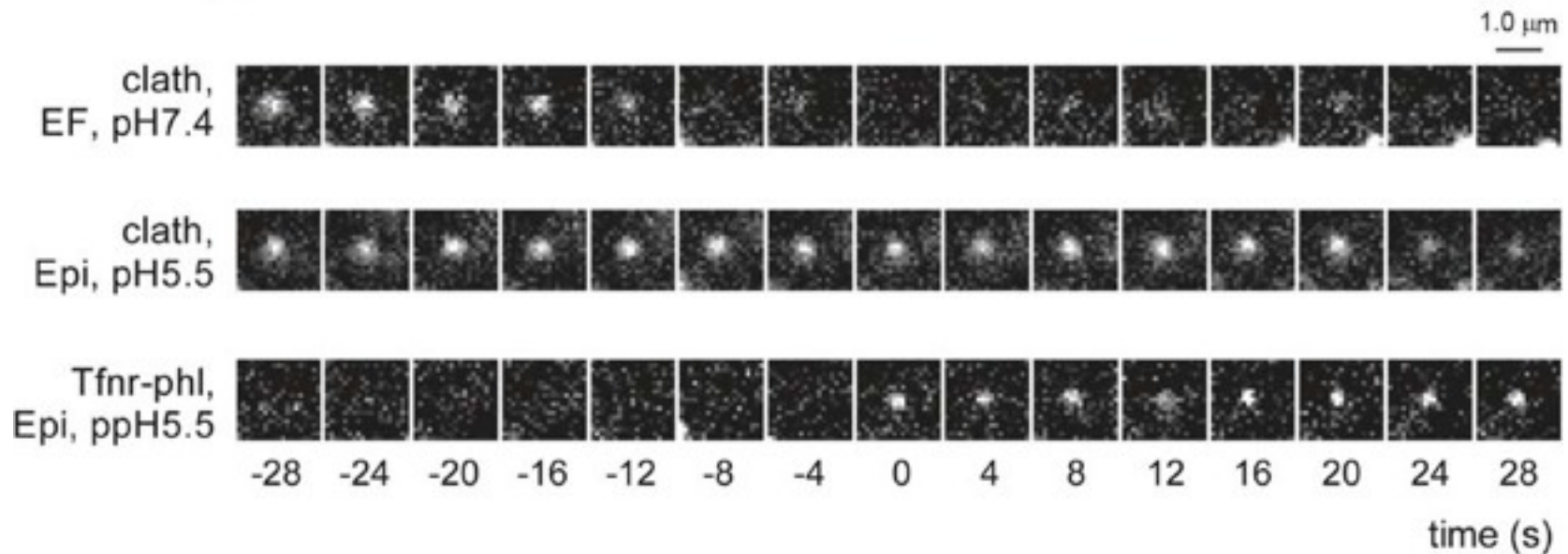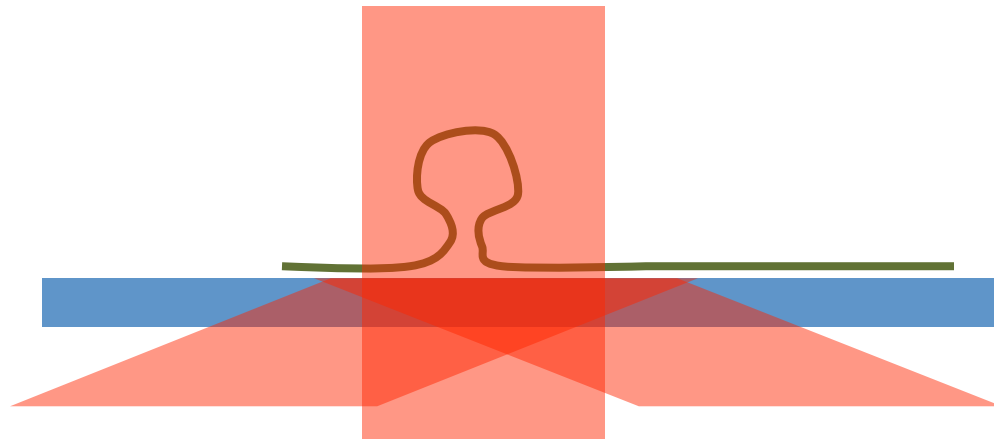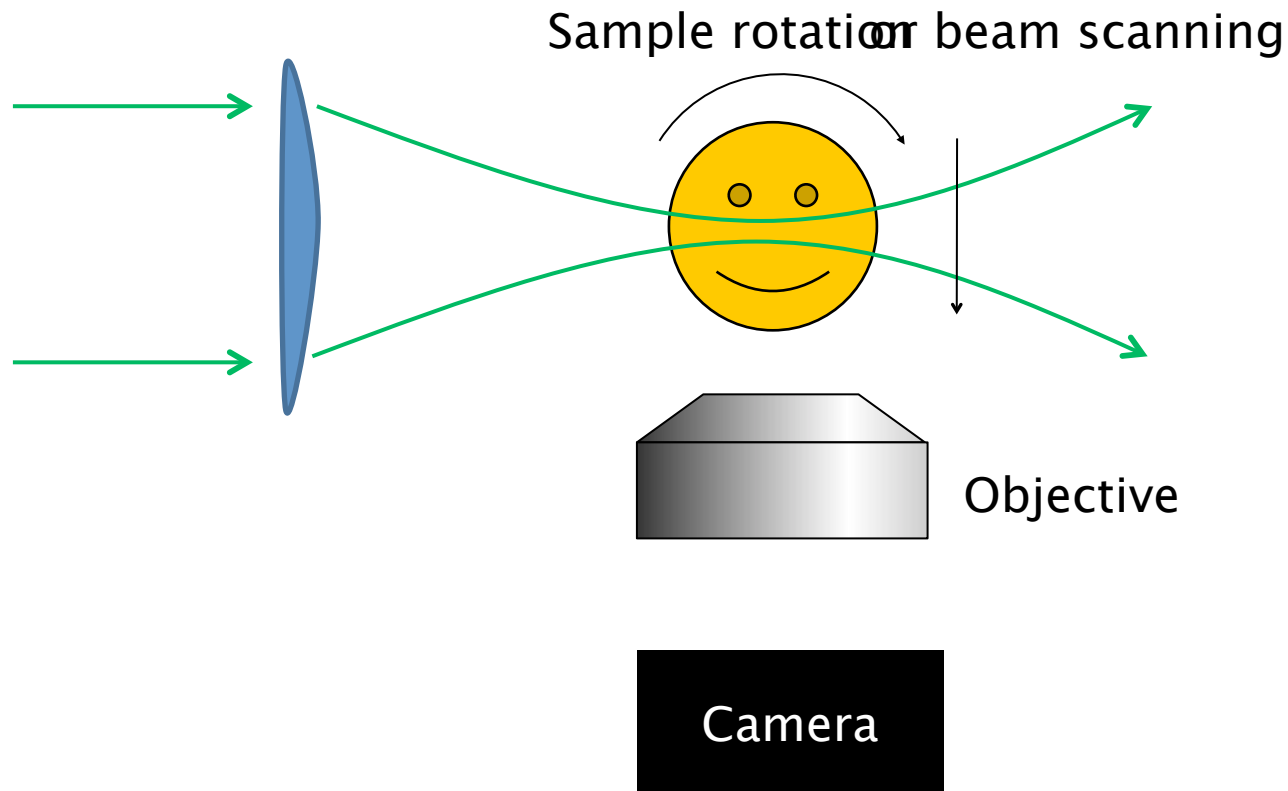
Aplysia growth cone

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.

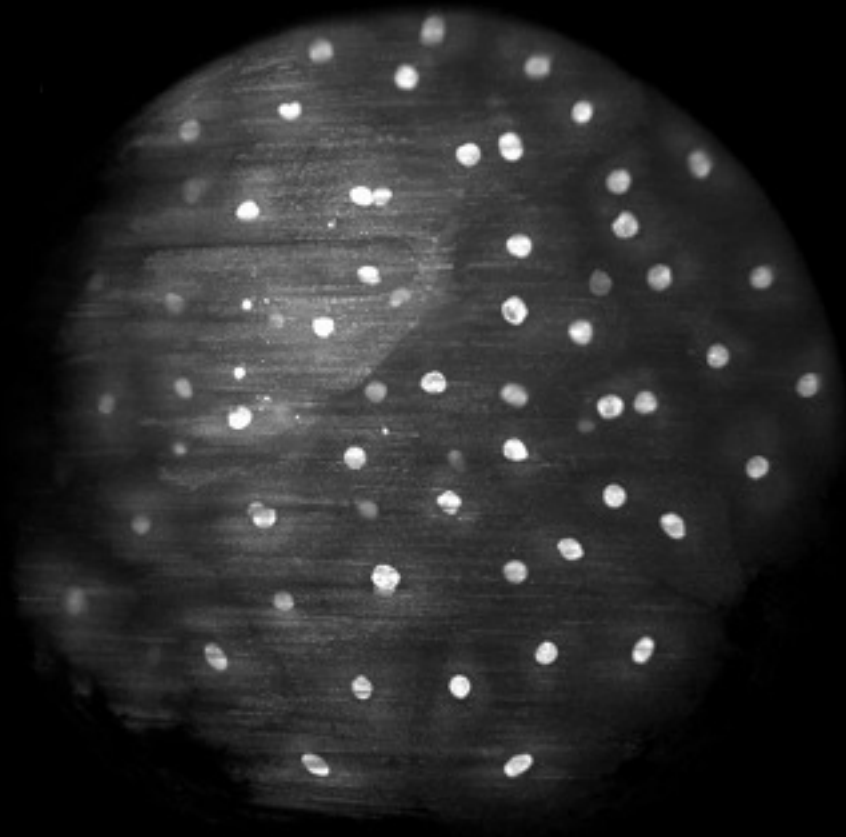# Single-plane illumination microscopy (SPIM)

Sample rotation or beam scanning
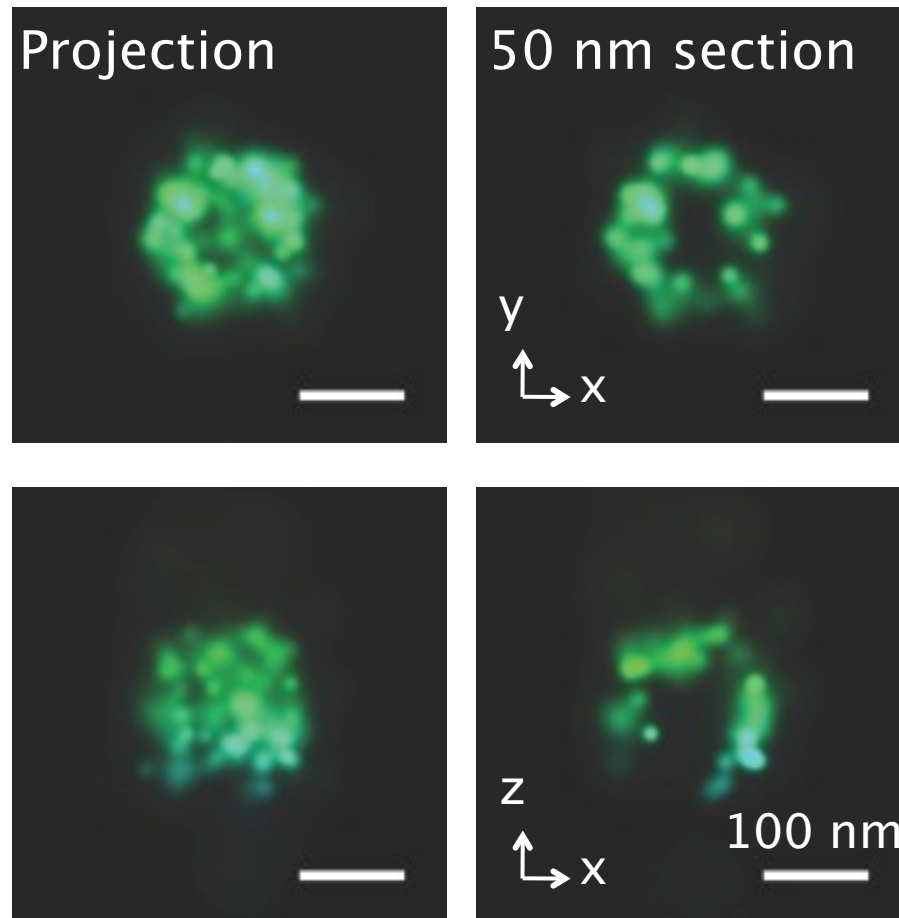
Objective

Camera

Zebra fish embryo development imaged by SPIM
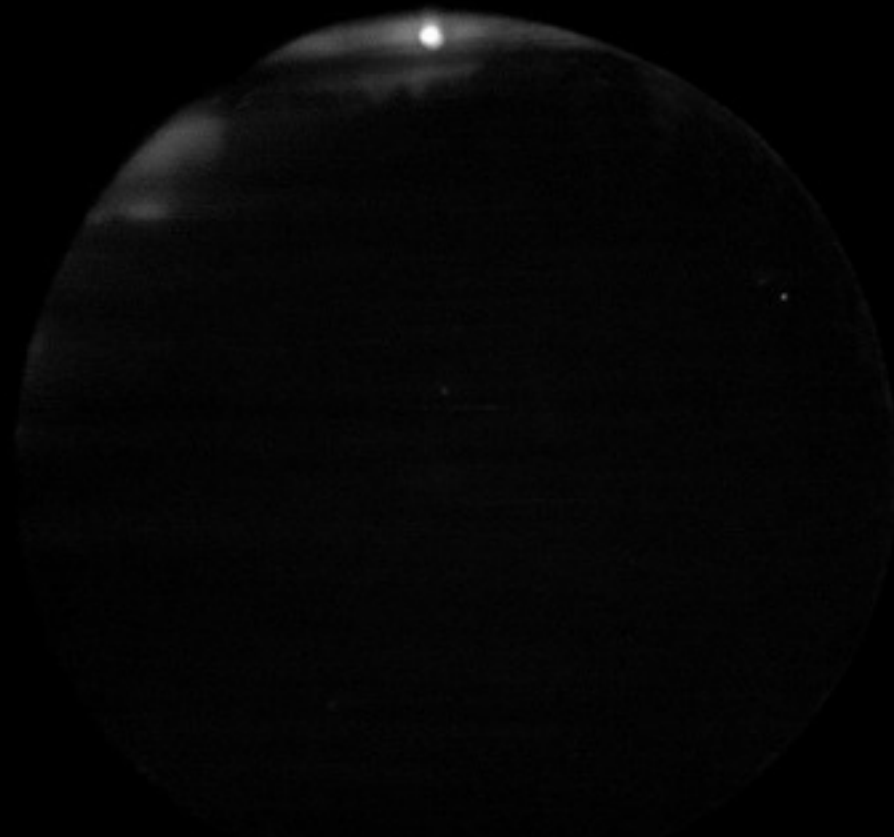
Keller et al., Science (200

Super-resolution microscopy

3D STORM image of a clathrin-coated pit

Now the tools are in your hands!