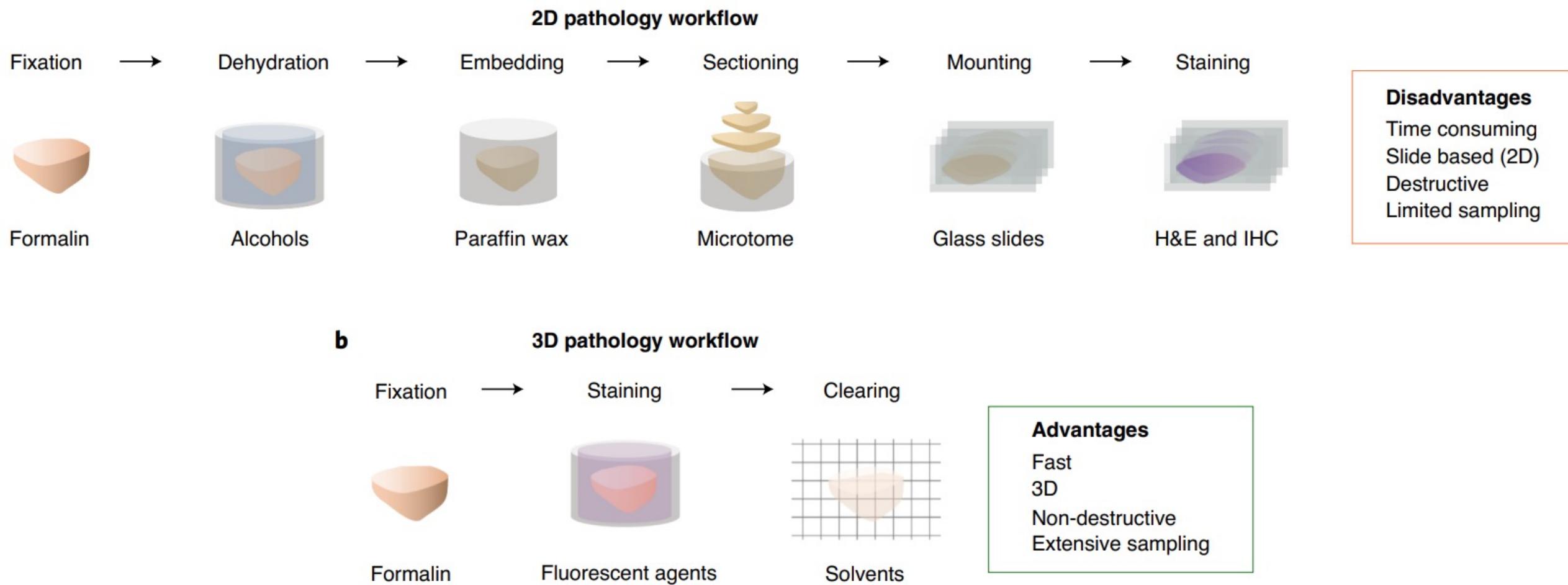


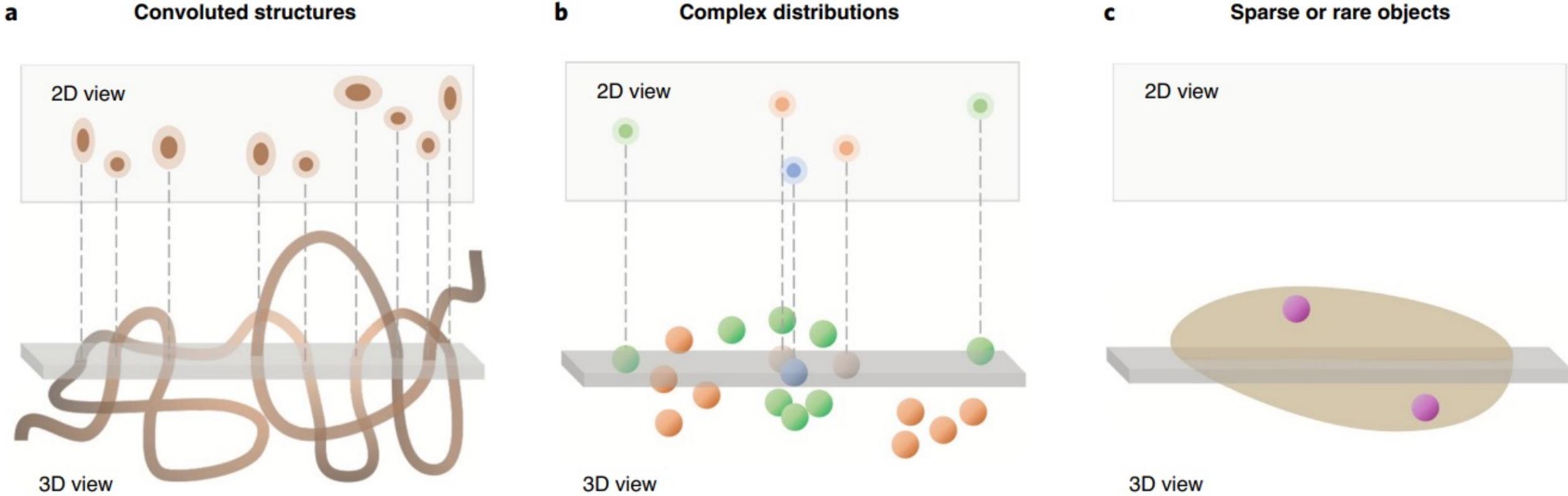
# 3D Imaging

Next-Generation Oncopathology  
Ryan Carelli, Loda Lab

# What is 3d pathology?



# Why 3d pathology?

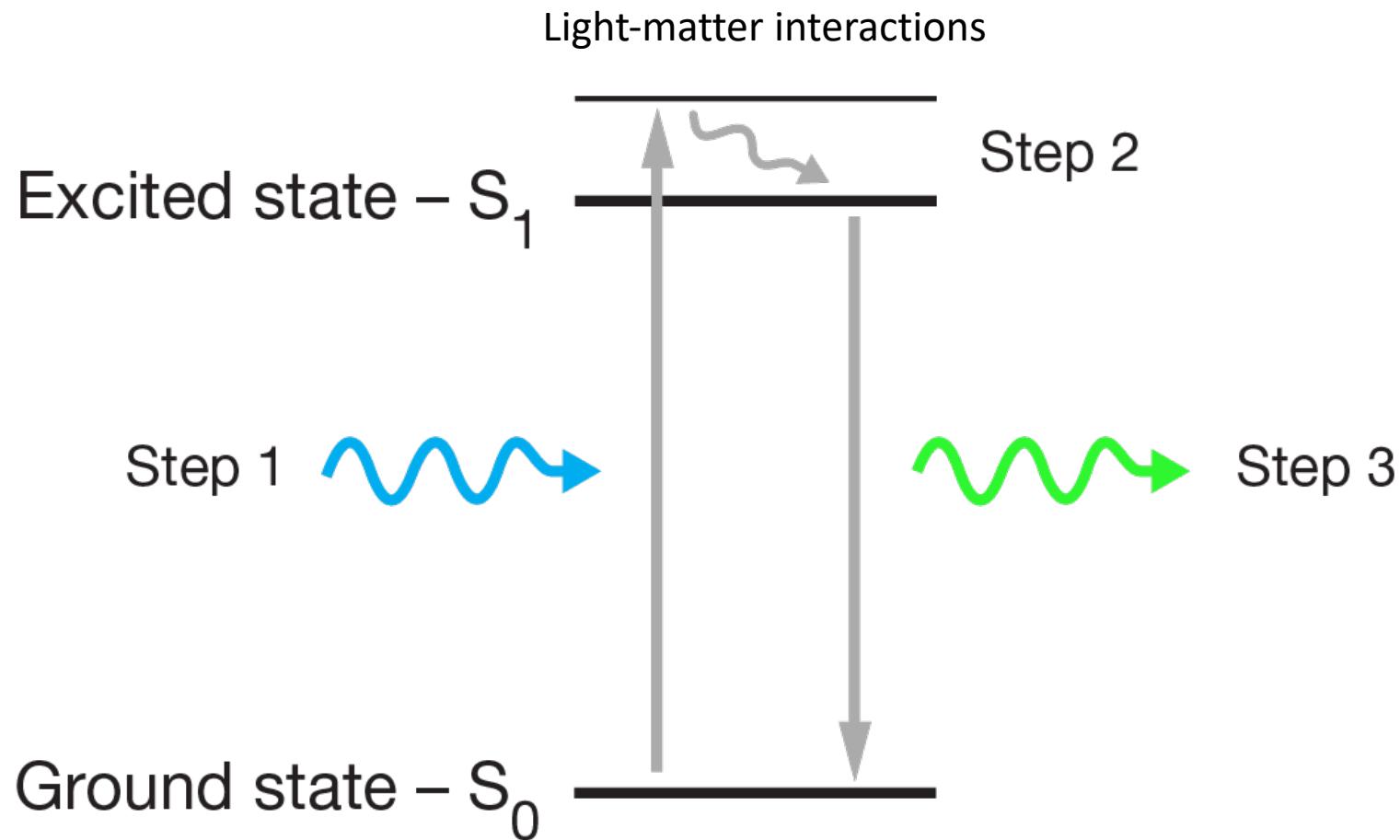


# Goals

1. Imaging fundamentals
2. Why is 3D imaging difficult?
3. Practical 3D imaging
4. Tissue Processing
5. 3D image analysis



# How does immunofluorescence work? (Jablonski)



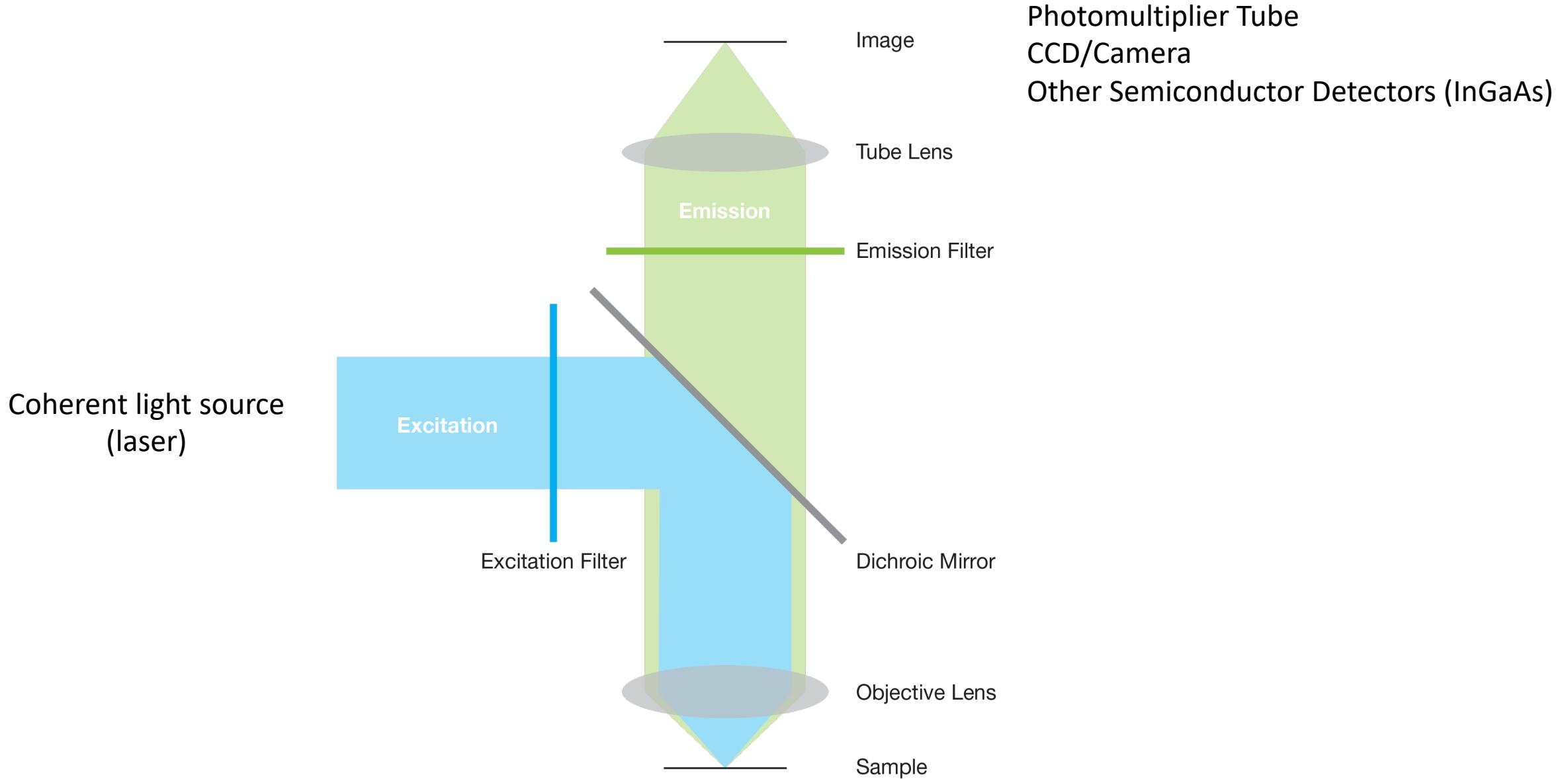
$$E = hf$$

$$= \frac{hc}{\lambda}$$

Planck-Einstein,  
Wave-Particle property of light

Planck Nobel Prize 1918  
Einstein Nobel Prize 1921

# A simple microscope configuration



# How do we collect light? Objectives and NA

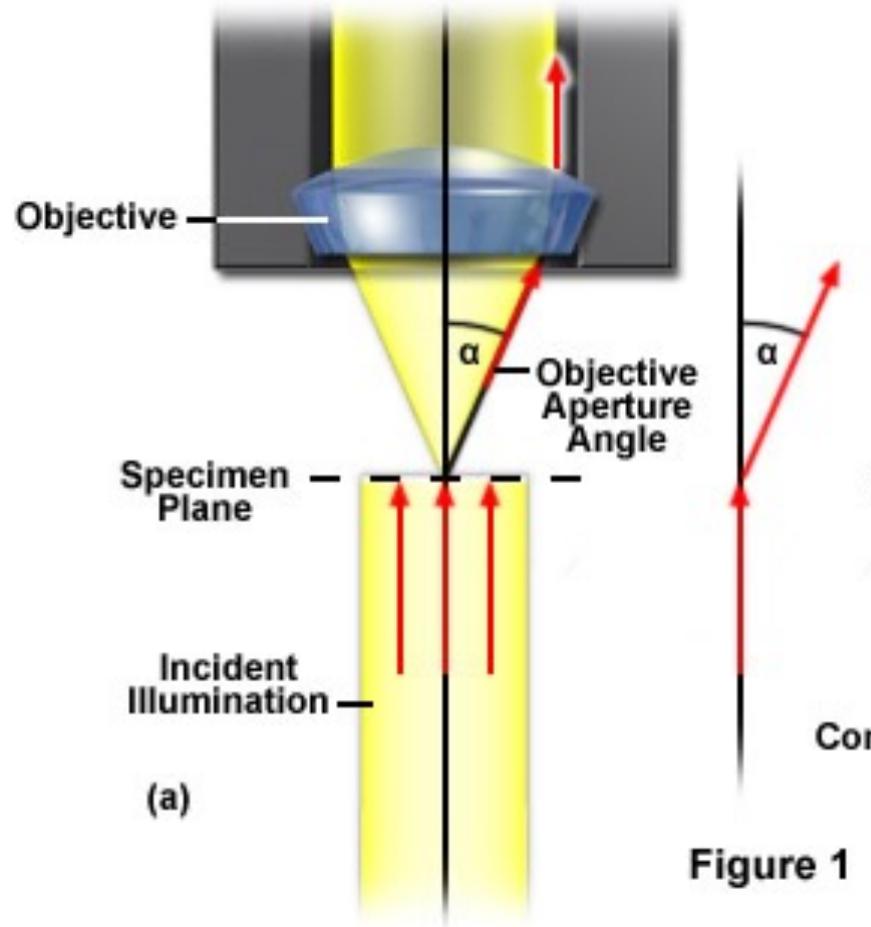
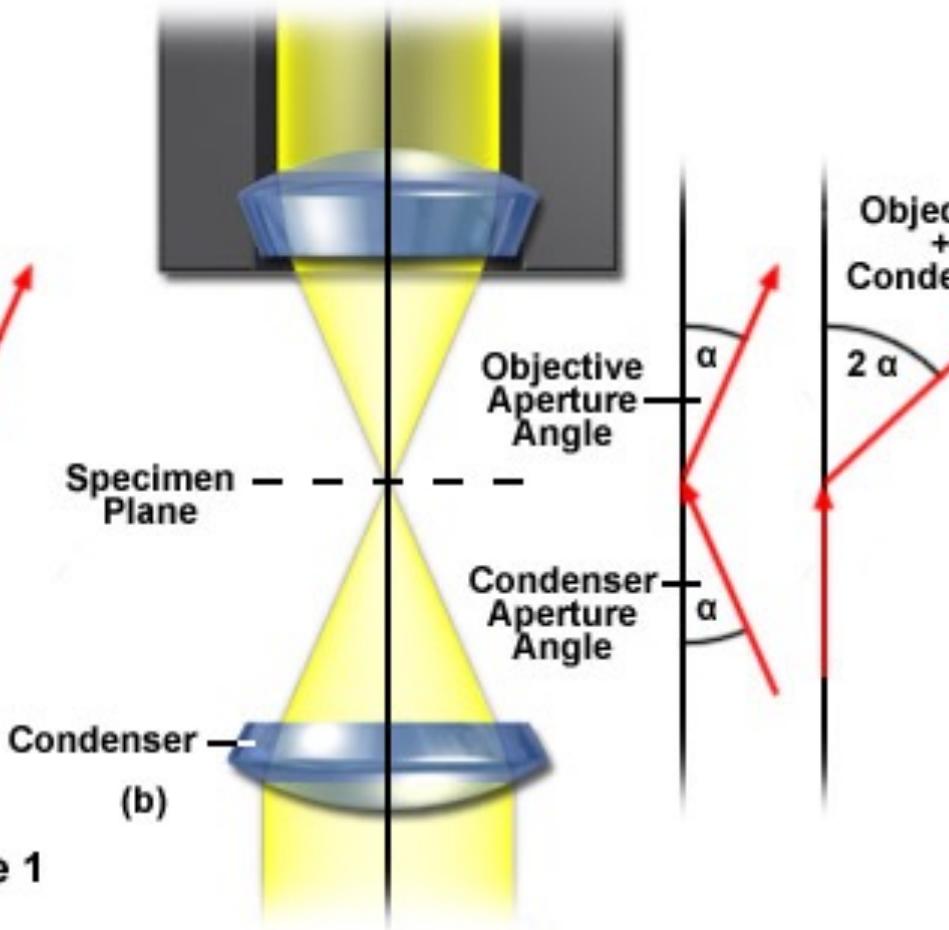


Figure 1

(a)



(b)

$$NA = n \sin(\alpha)$$

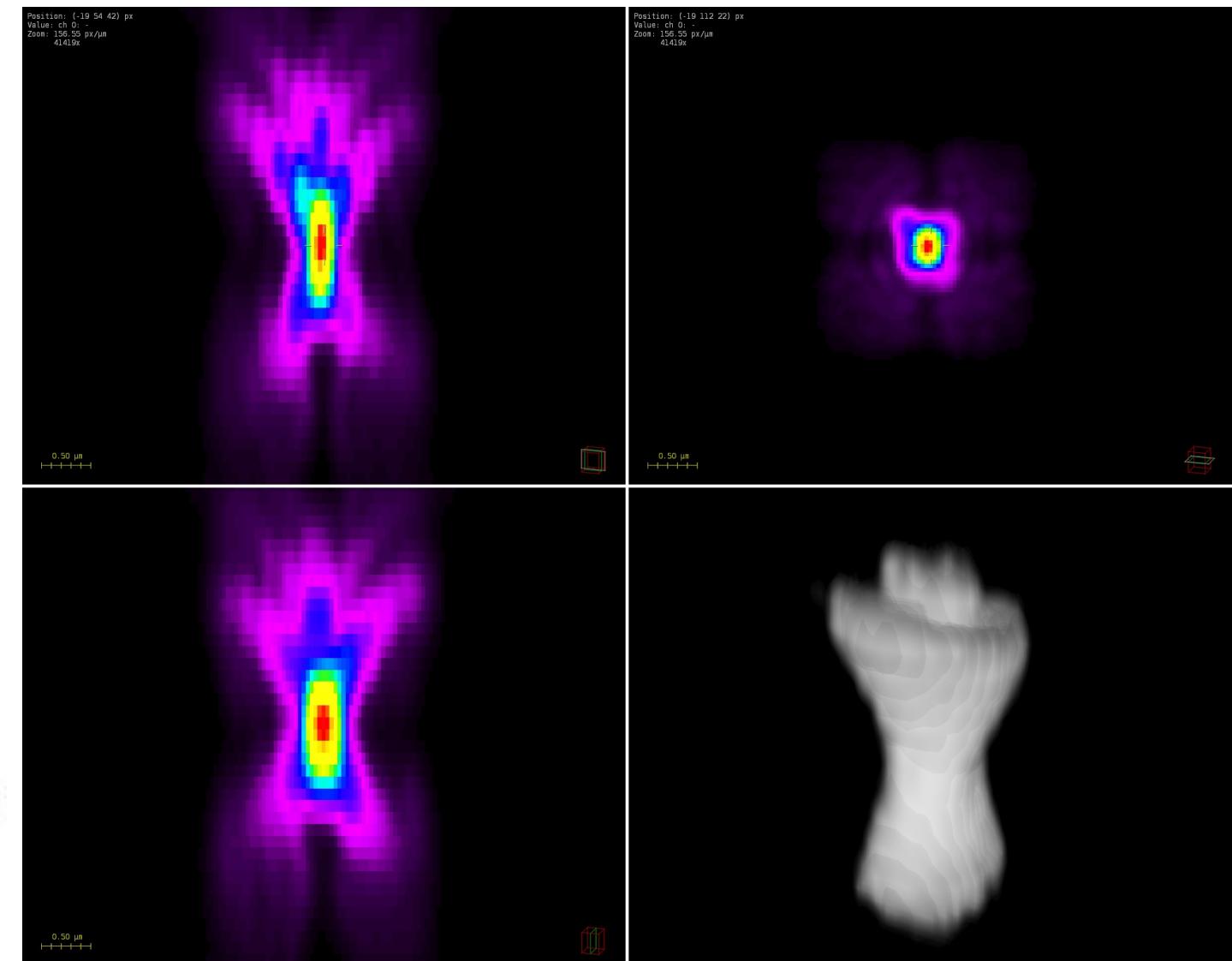
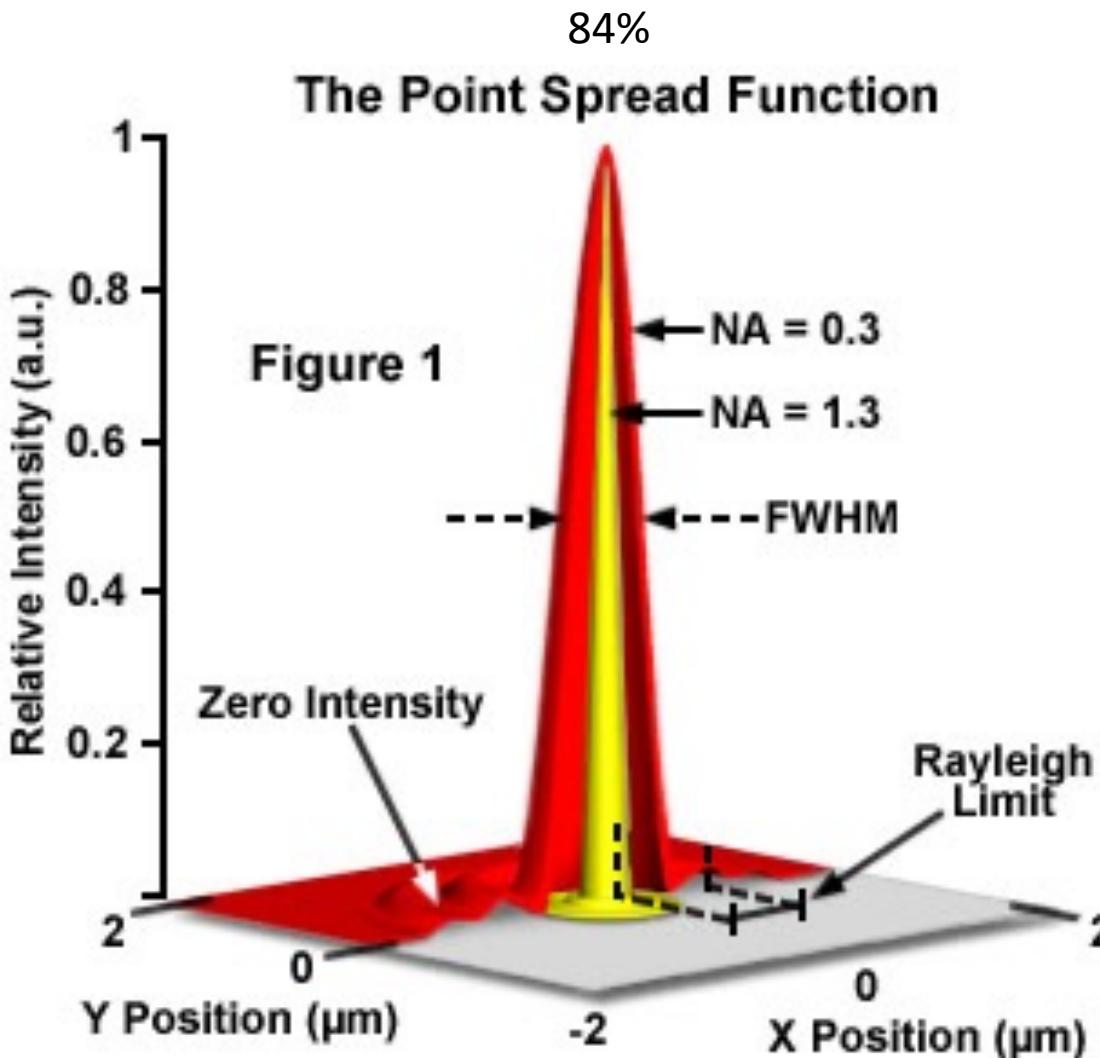
Abbe Diffraction Limit (1873)

$$\text{In xy: } d = \frac{\lambda}{2NA}$$

$$\text{In z: } d = \frac{2\lambda}{NA^2}$$

Limiting Cases

# How exactly does low NA impact our image quality?



# If we want to fully utilize available resolution, how far apart should our measurements be?

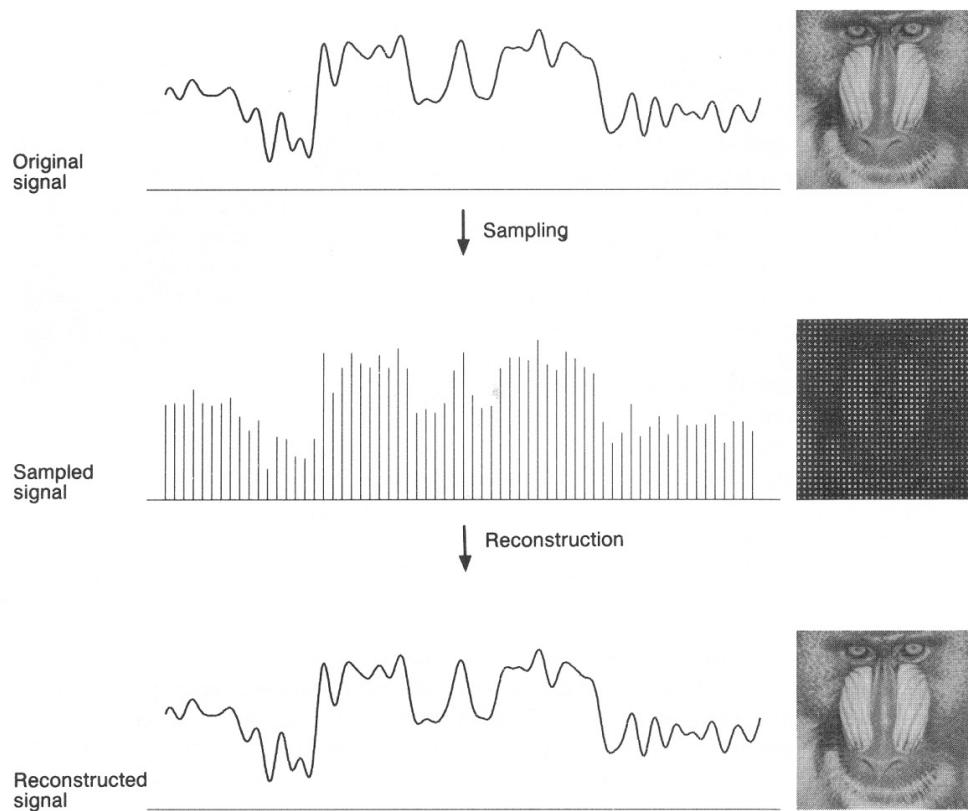
- (Shannon, Nyquist) If a function  $f(t)$  contains no frequencies higher than  $B$  hertz, it is completely determined by giving its ordinates at a series of points spaced  $\frac{1}{2B}$  seconds apart.
- Restated for Imaging  
(Nyquist Sampling Criterion) If an image  $f(x)$  contains no features smaller than  $\lambda$  wavelength, it is completely determined by sampling at a series of points space  $\frac{1}{2}\lambda$  apart.

This provides an optimal pixel-size criterion.

# If we want to fully utilize available resolution, how far apart should our measurements be?

Restated for imaging

If an image  $f(x)$  contains no features smaller than  $\lambda$  wavelength, it is completely determined by sampling at a series of points space  $\frac{1}{2}\lambda$  apart.



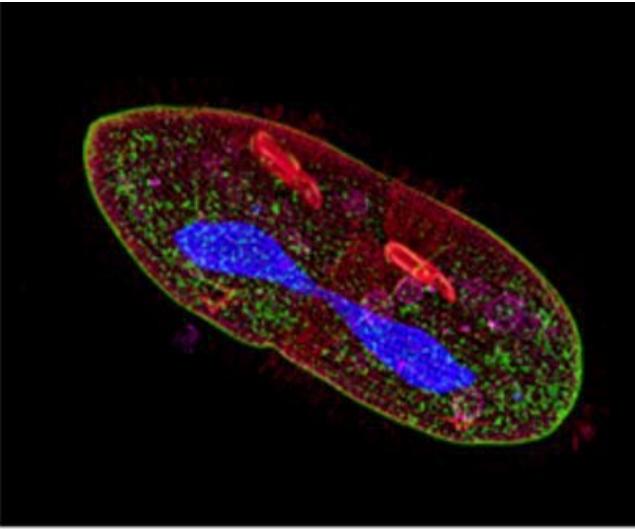
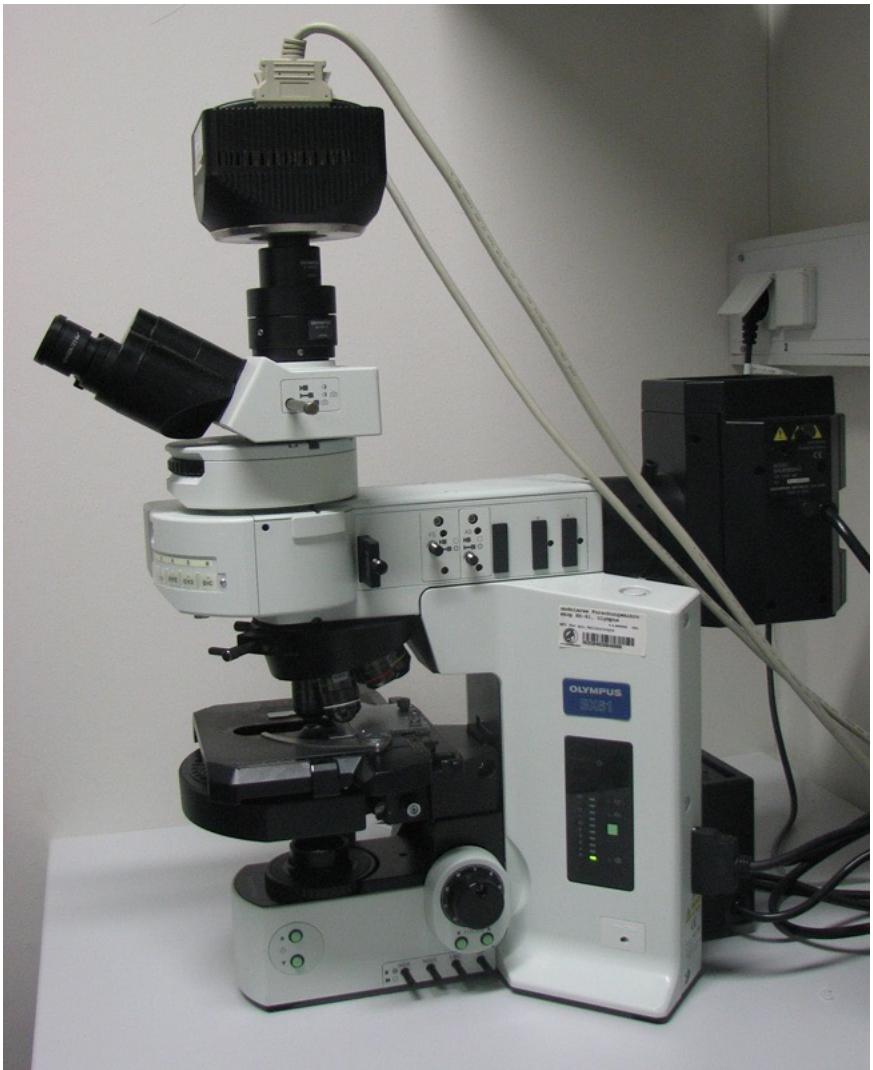
If our laser is 488nm (green) with 0.7 NA objective, our resolution is:

$$\frac{\lambda}{2NA} \quad x/y: 488/(2*0.7) = 348 \text{ nm}$$

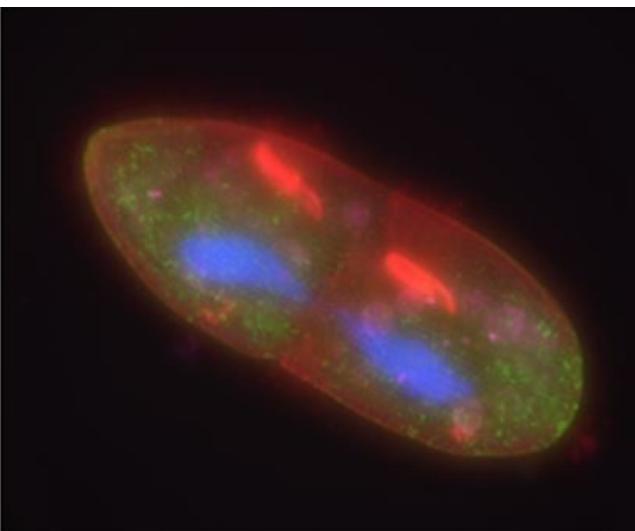
$$\frac{2\lambda}{NA^2} \quad z: (2*488)/(0.7^2) = 1991 \text{ nm}$$

And our image is an of 174nm x 174nm x 996nm pixels

# So you bring your sample to the microscope...



Cut on the slide



Why does image quality deteriorate in 3d?

Intact (3d)

# Why does image quality deteriorate in 3d?

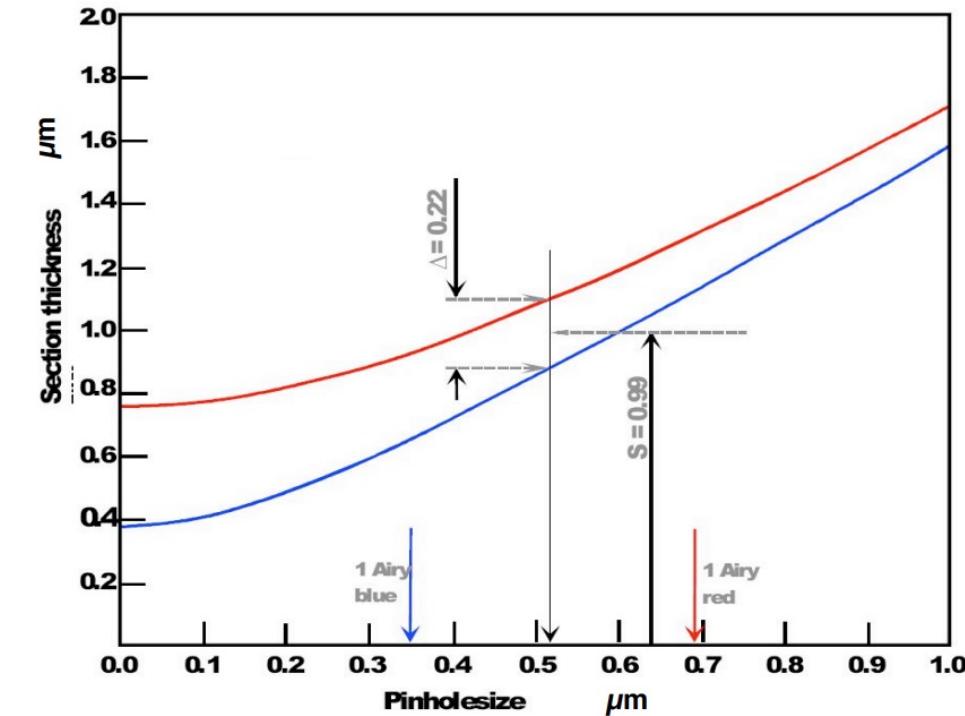
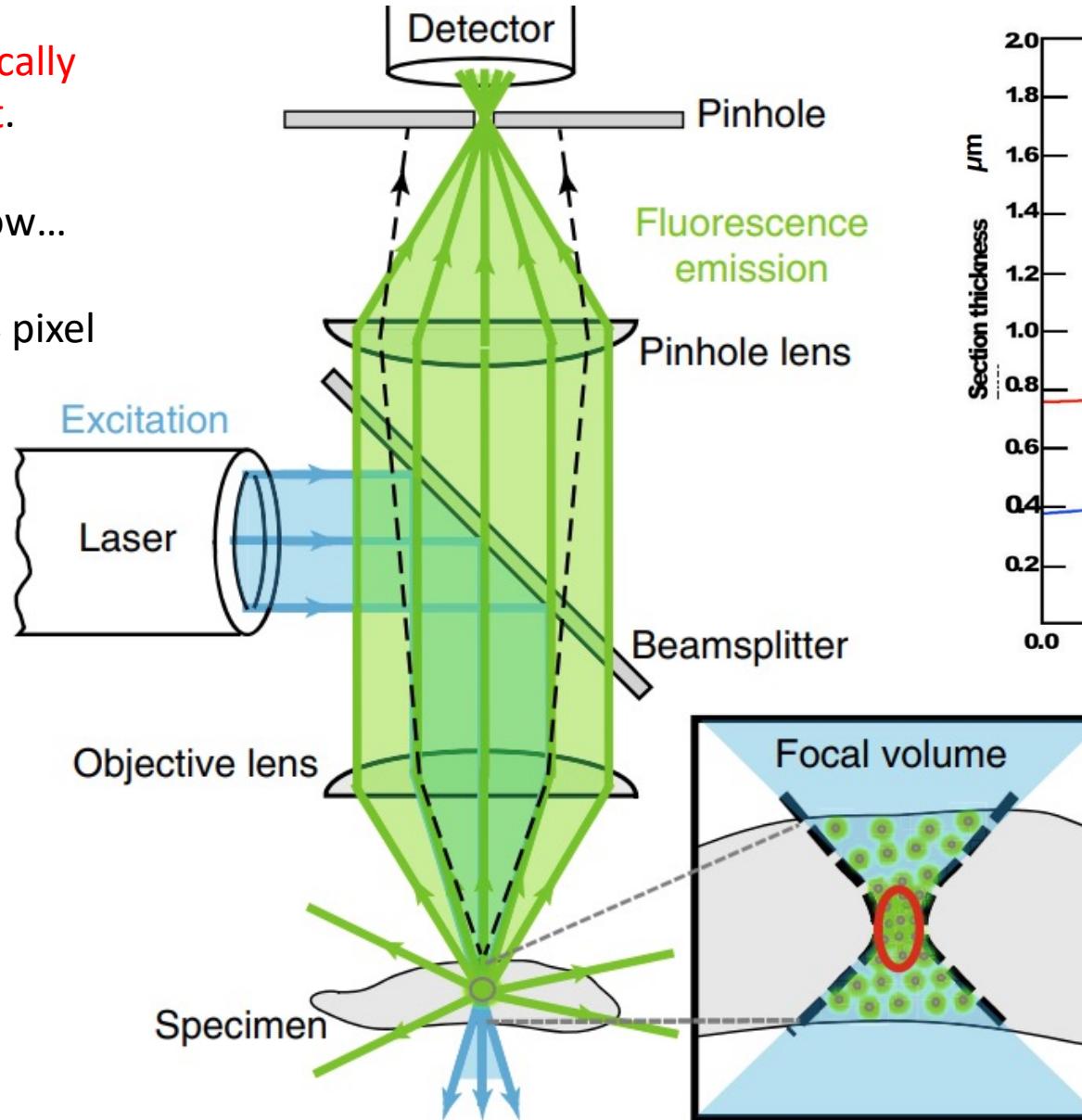
1. Out of focus light
2. Absorption and Scattering
3. Autofluorescence

# Point-Scanning Confocal Microscopy

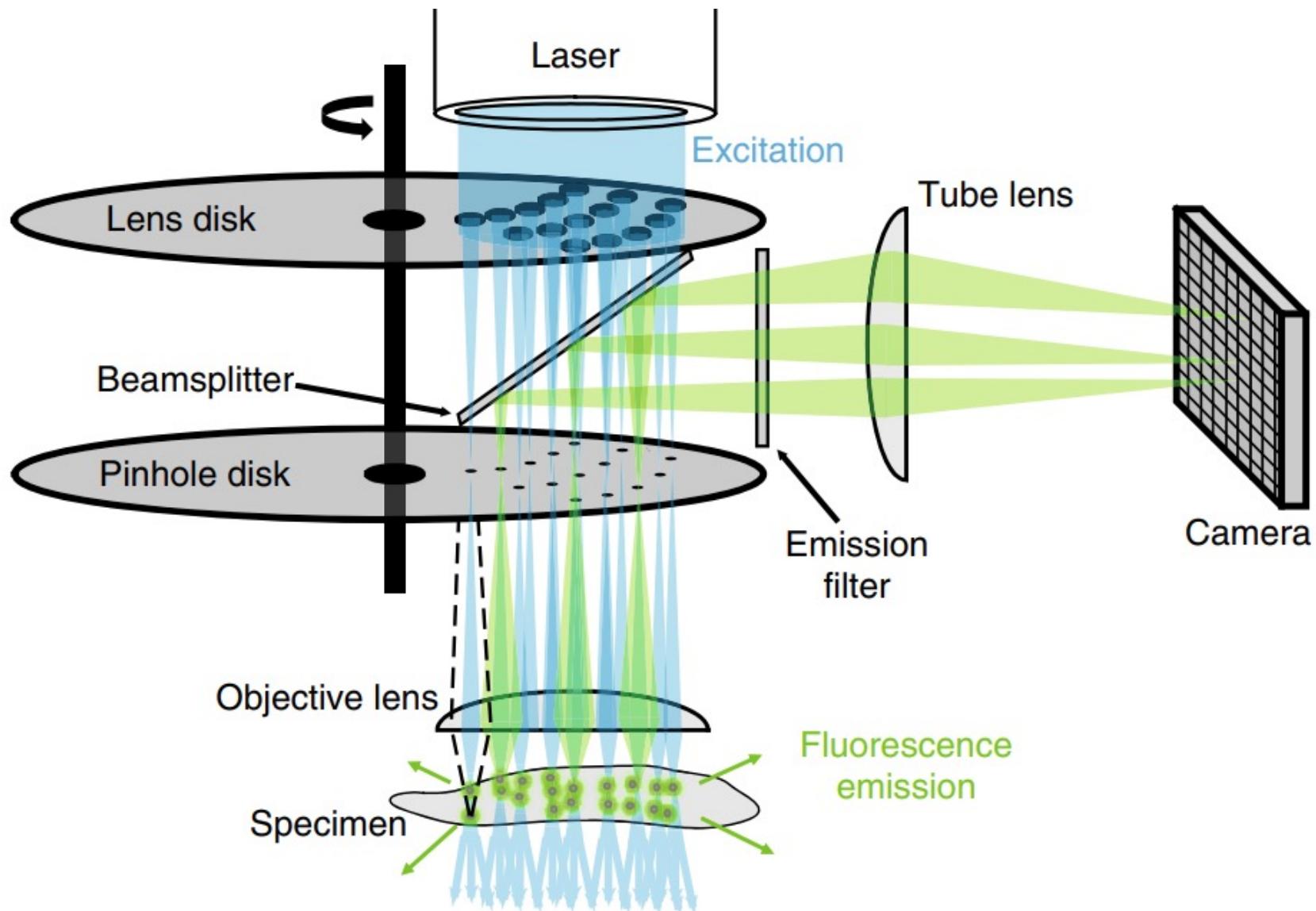
This configuration **physically** rejects out of focus light.

But point scanning is slow...

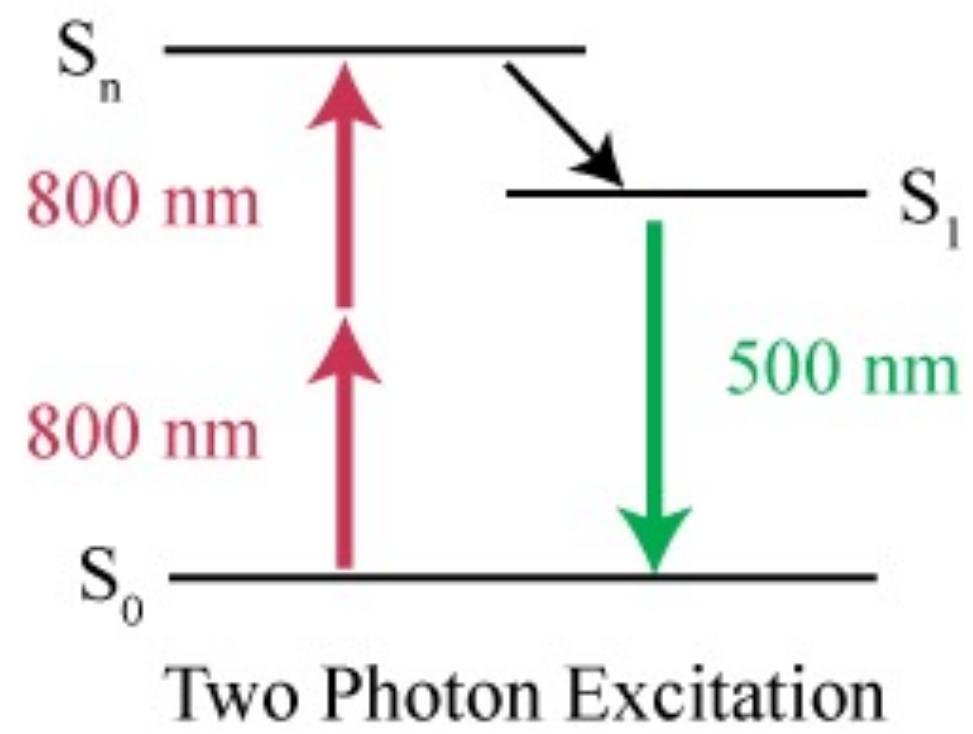
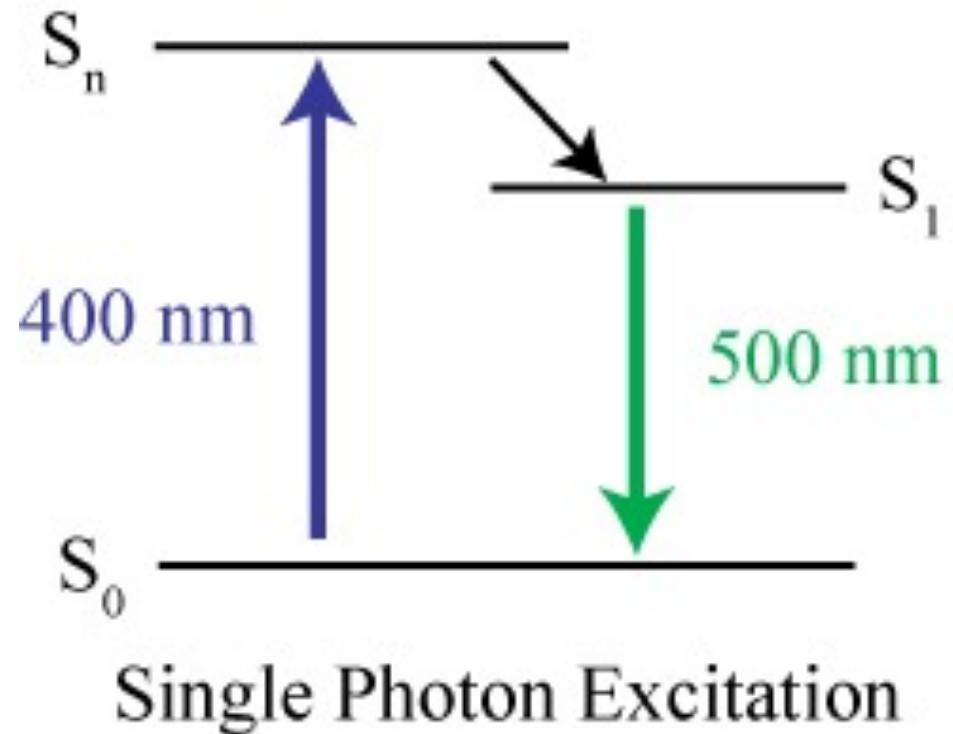
1 second for 1024x1024 pixel field of view



# Spinning Disk Confocal Microscopy



# Two-Photon Microscopy

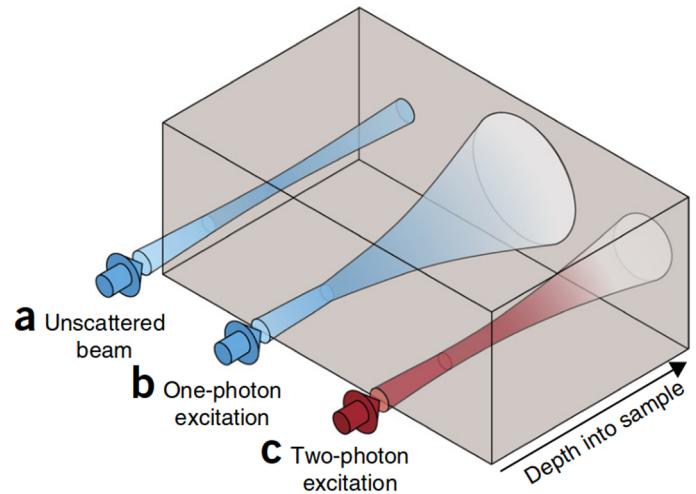


Photons must arrive within one femtosecond!

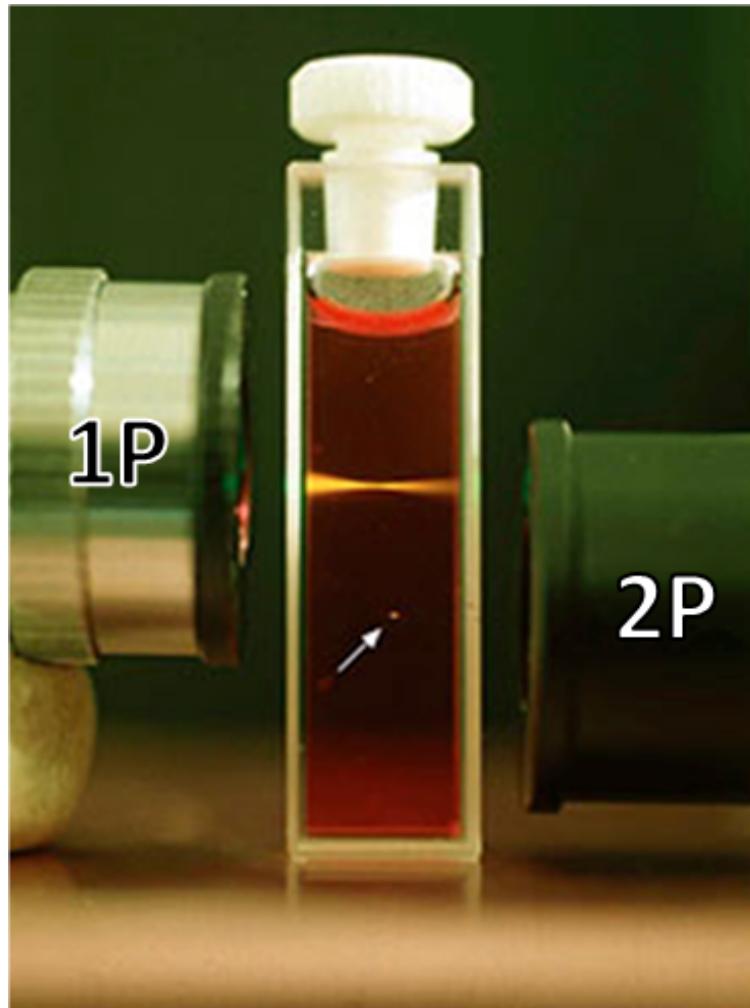
# Two-Photon Microscopy

a) Rayleigh Scattering Cross-section

$$\sigma_s = \frac{2\pi^5}{3} \frac{d^6}{\lambda^4} \left( \frac{n^2 - 1}{n^2 + 2} \right)^2$$



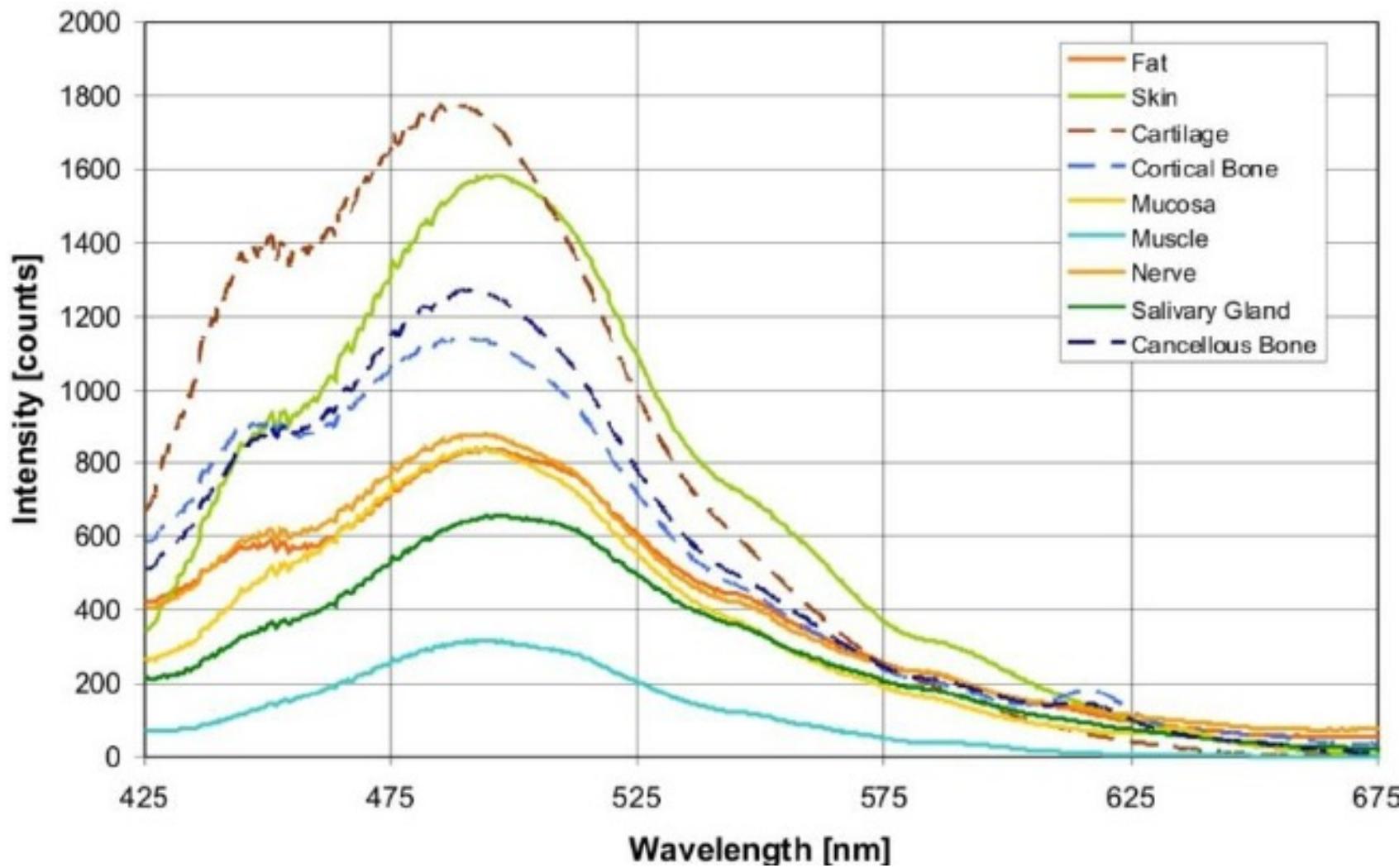
b) Nonlinear response and Localized Excitation



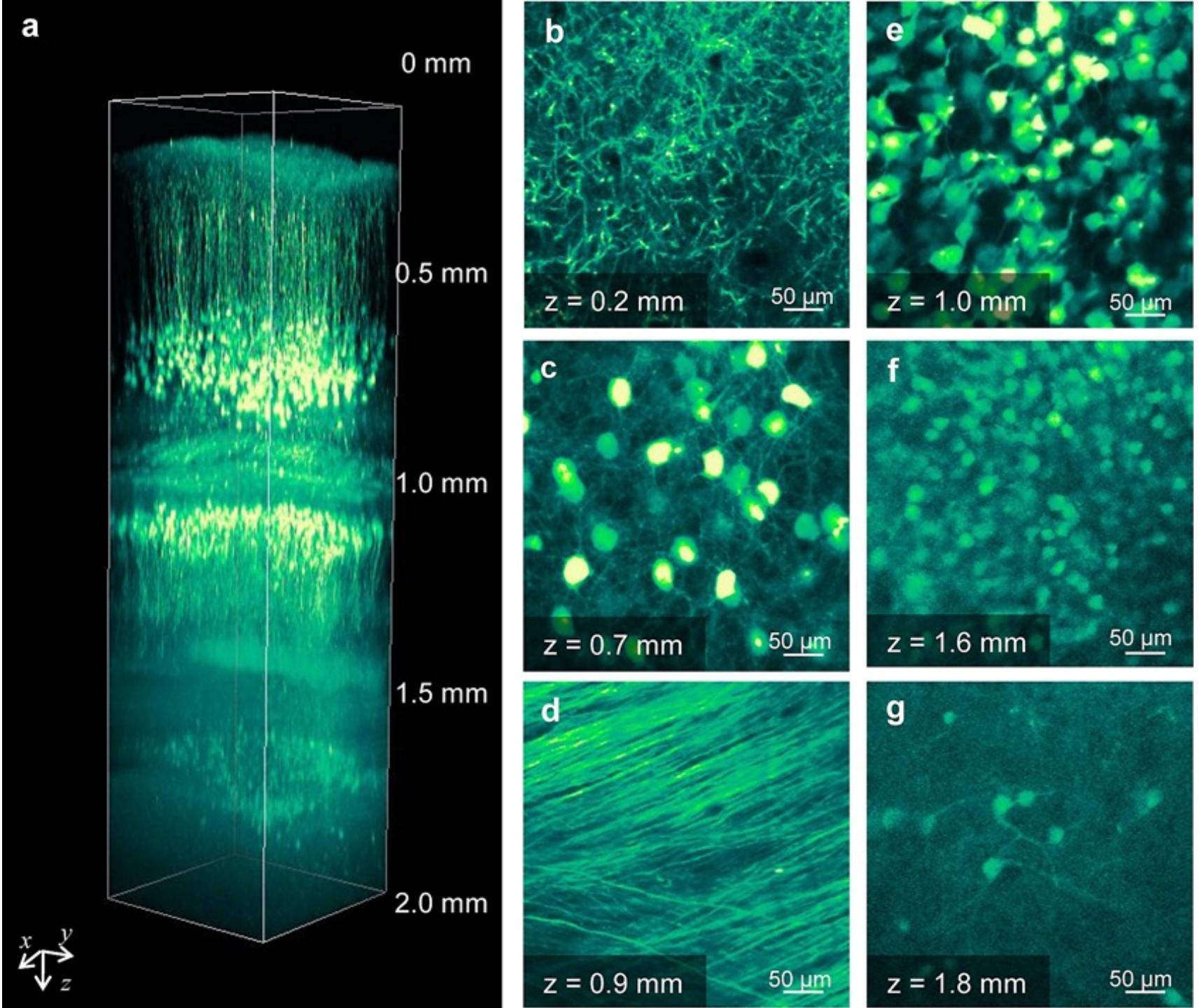
$$N_{abs} = \int_V dV \delta C(r, t) I^2(r, t)$$

# Two-Photon Microscopy

c) Autofluorescence is reduced at longer wavelengths (although this trades off with resolution)

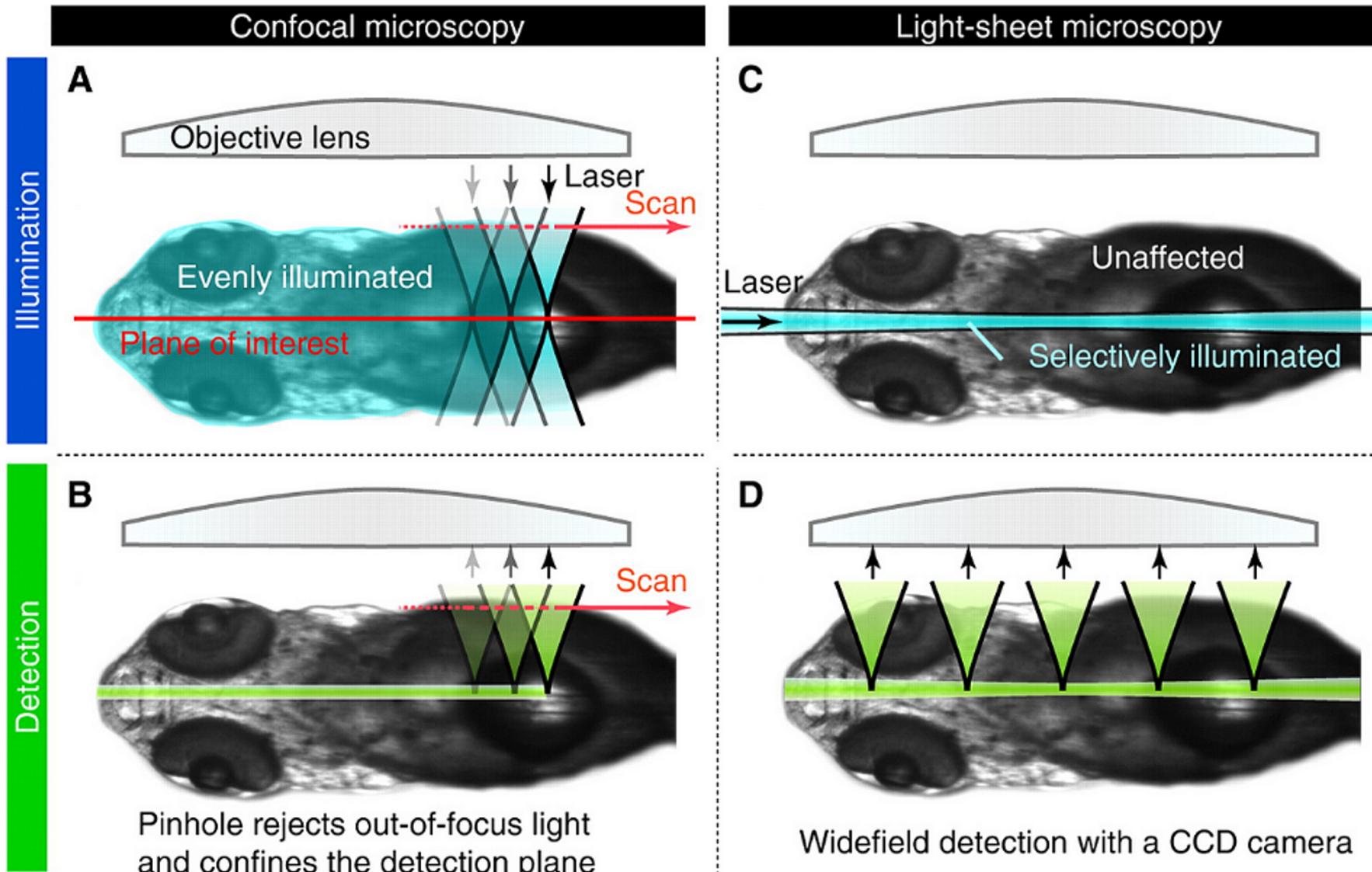


Two photon excitation with  
NA=1.05 and additional  
tissue processing.

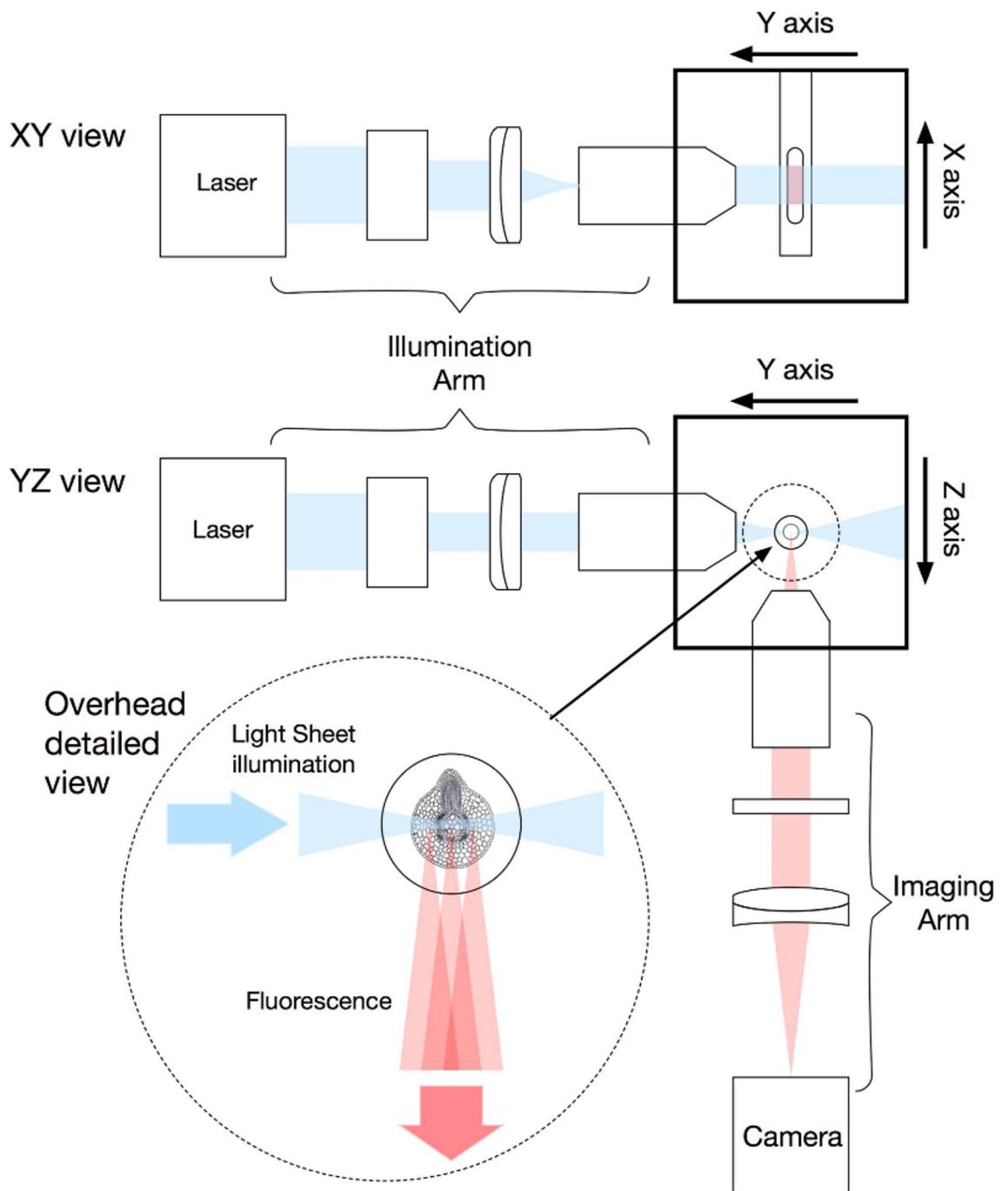
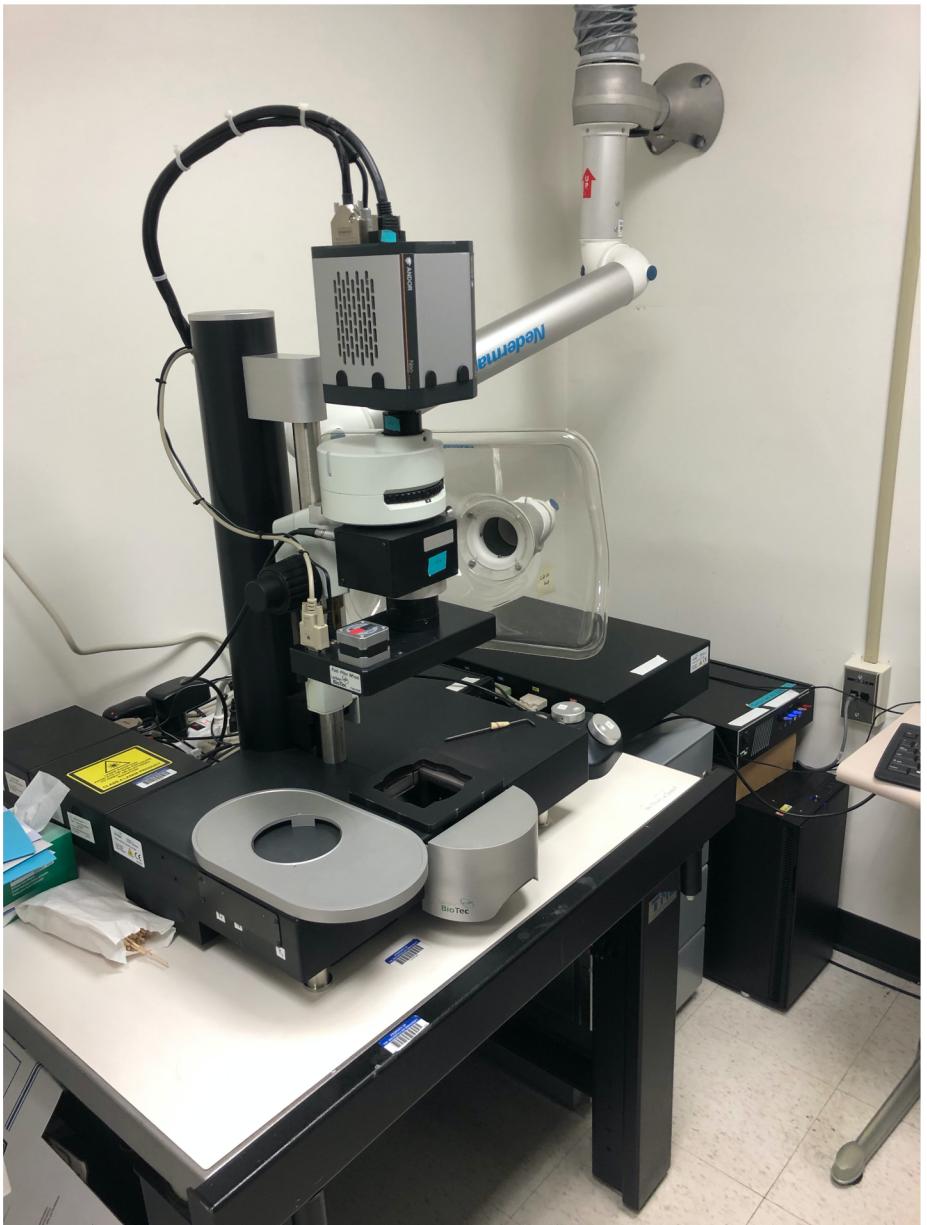


# Light Sheet Microscopy

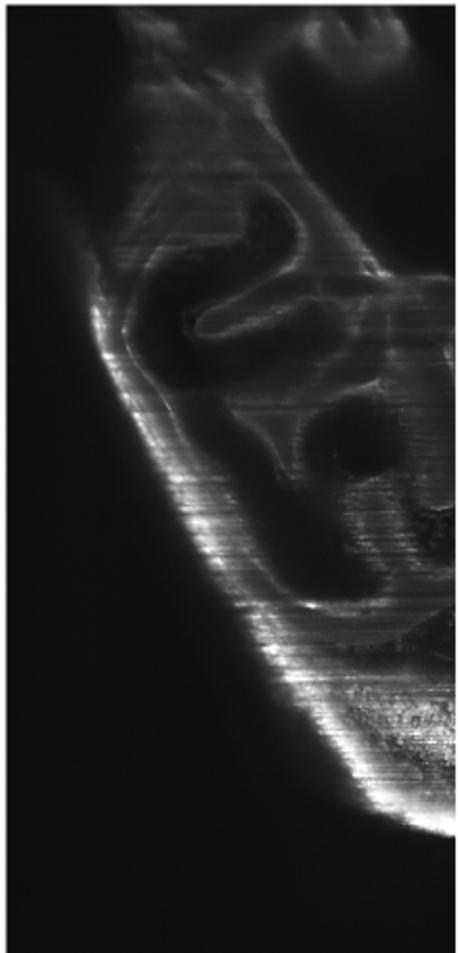
Only illuminate in-focus regions of tissue  
Excitation scattering is entirely removed



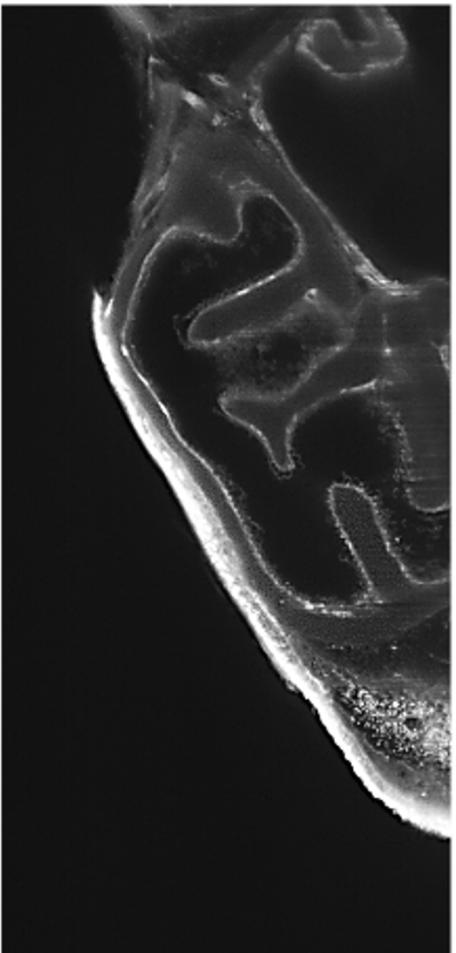
# Light Sheet Microscopy



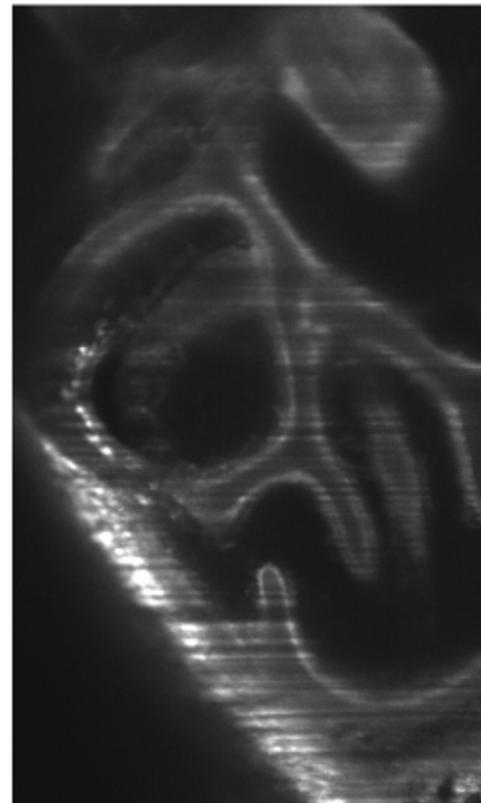
# Triple-Sheet Light Sheet Microscopy



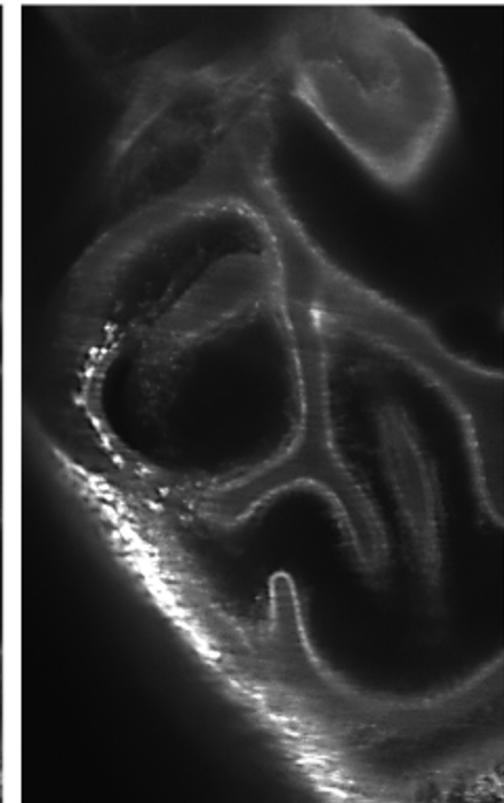
single light sheet



triple light sheet

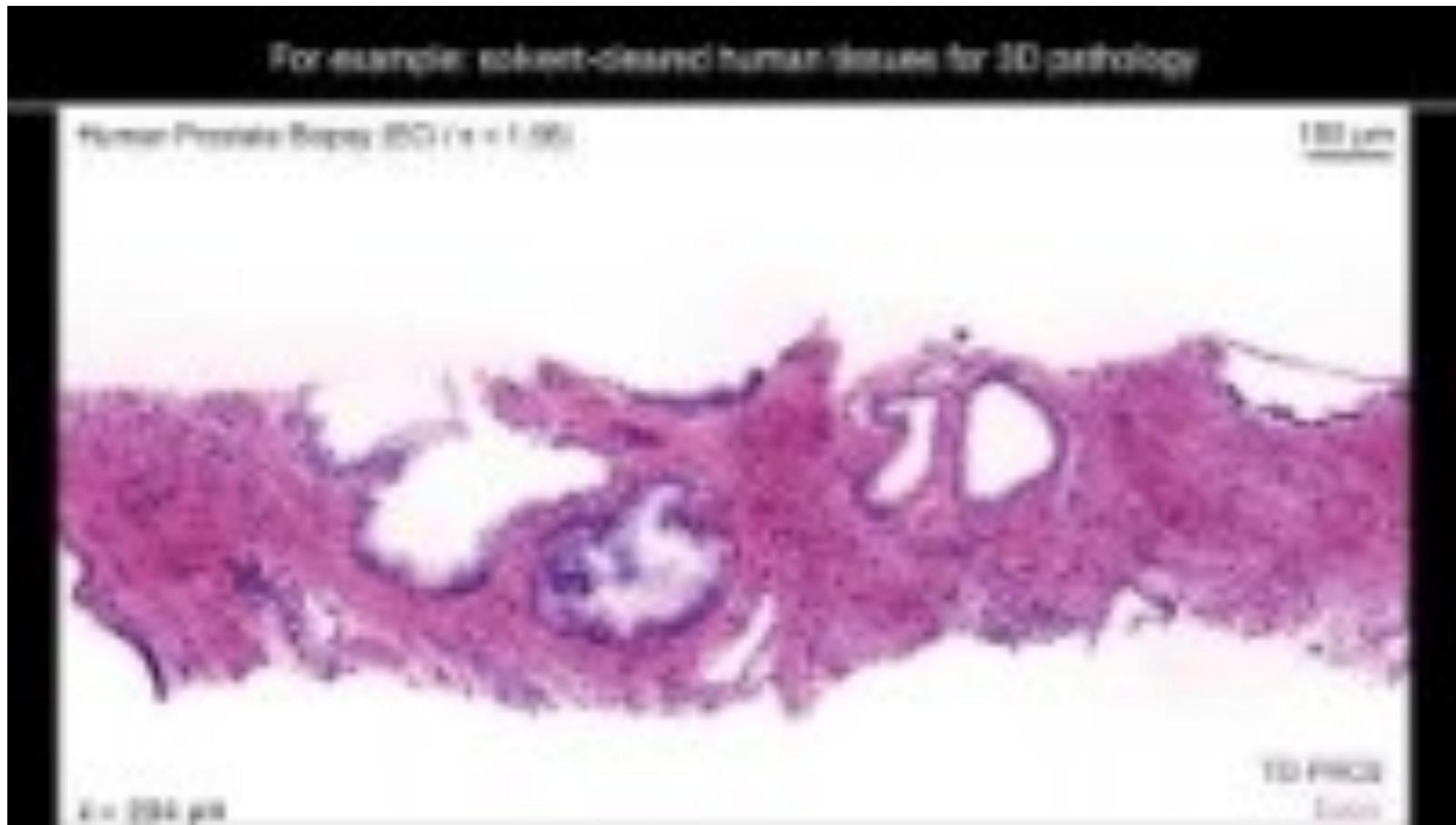


single light sheet

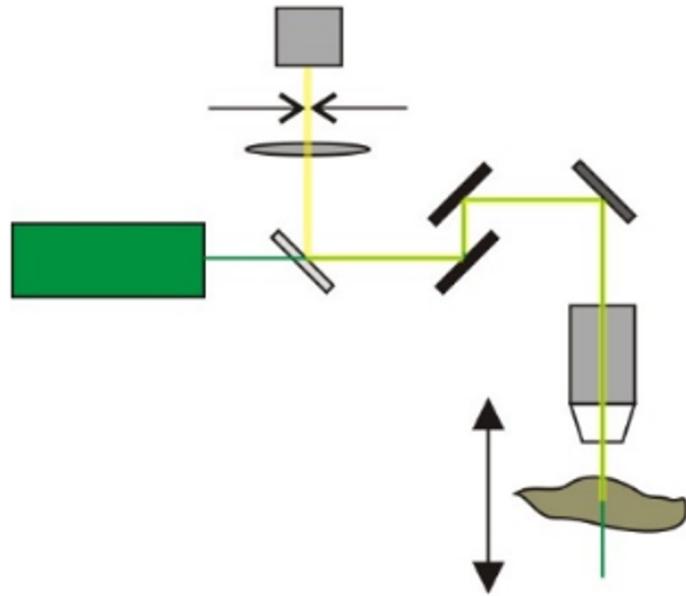


triple light sheet

# Open Top Lightsheet Configuration (Lightspeed)



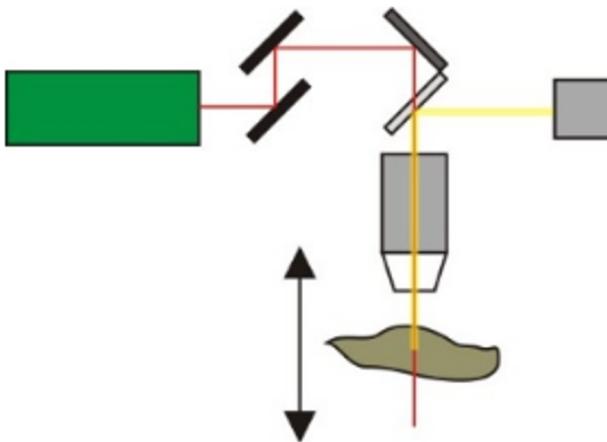
# Summary (Optics)



50 μm

## Confocal Microscope

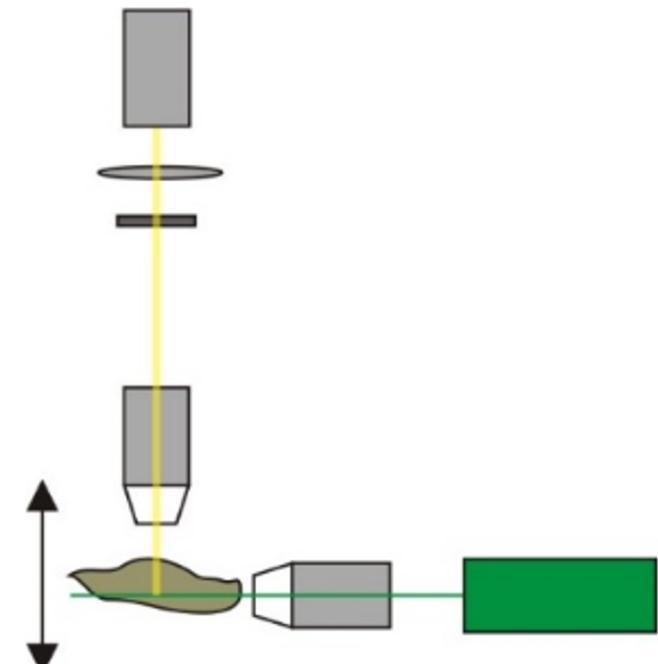
- + 3D resolution
- High bleaching rate
- Penetration depth
- Slow



1-2 mm

## 2-Photon Microscope

- + 3D resolution
- + Low bleaching rate
- + Penetration depth
- Slow



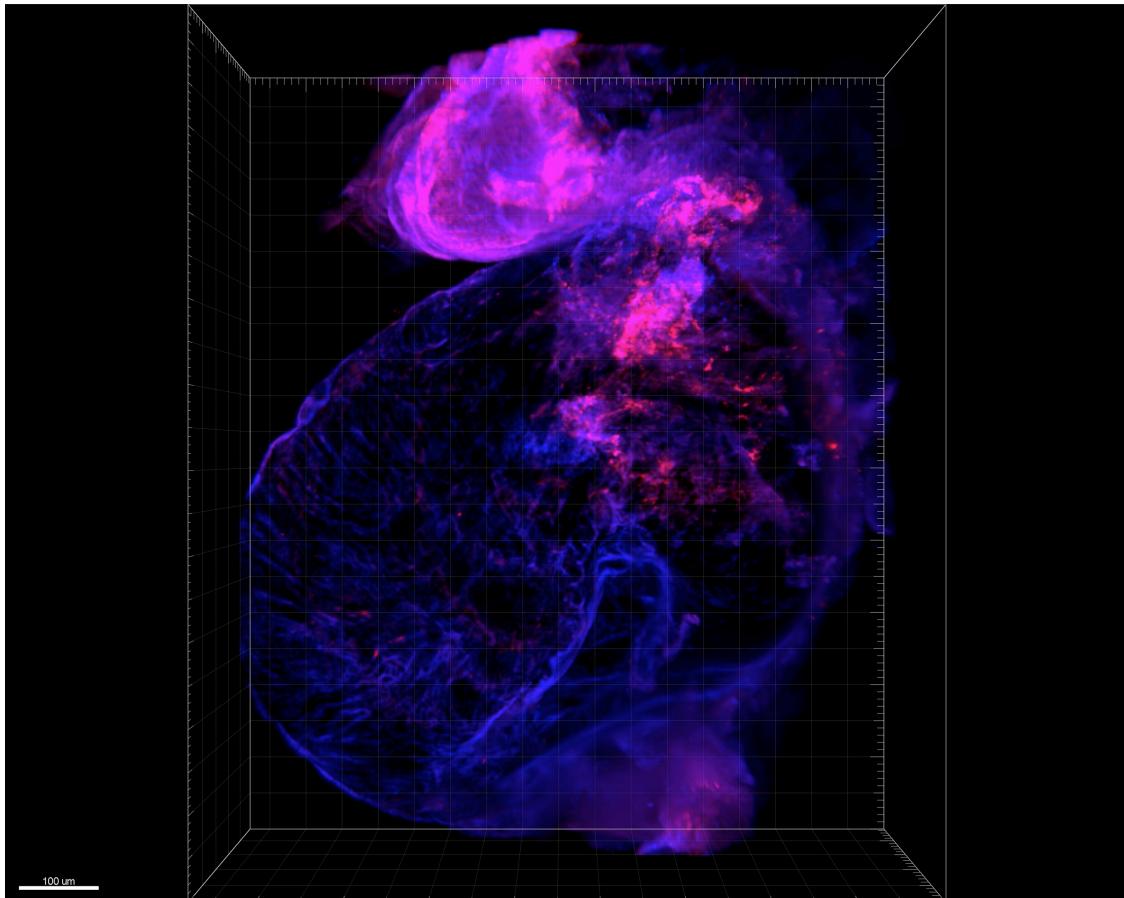
Many mm

## Light Sheet Microscope

- + 3D resolution
- + Low bleaching rate
- + Penetration depth
- + Fast

## Light Sheet

Neuroendocrine marker (CHGA) in MYCN+; RB1  
KO; Pten KO mouse model



5μm slices in z

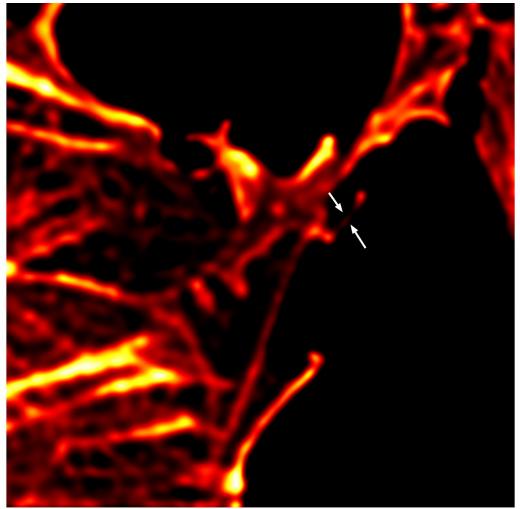
## Confocal

Tumor-stroma organoid  
co-culture system

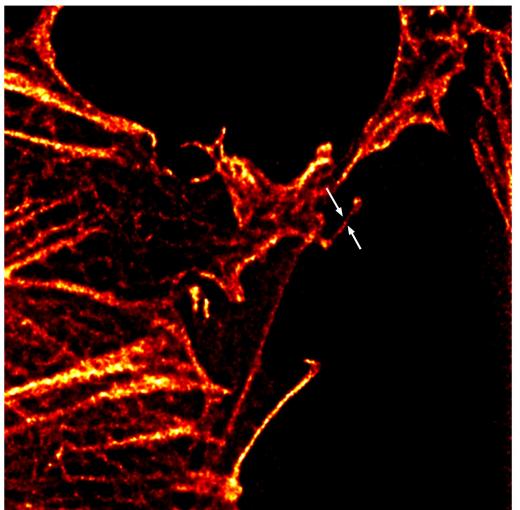


.5μm slices in z

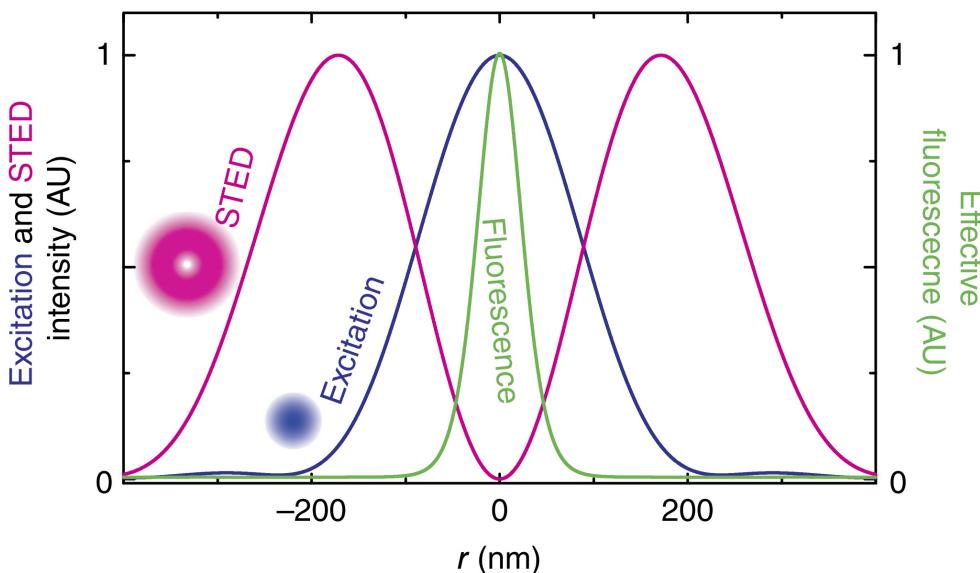
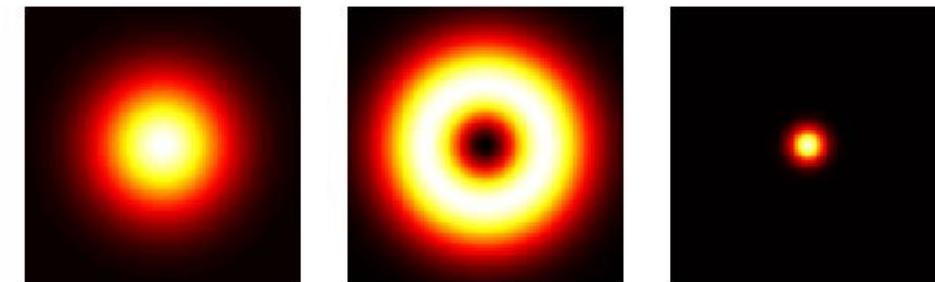
# Stimulated Emission Depletion (Super-Resolution) Microscopy



Confocal

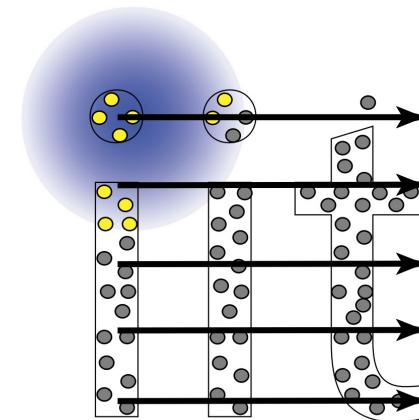


STED

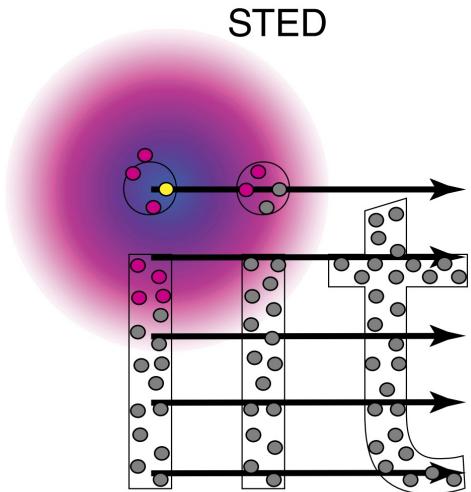


First excite, then deexcite ("stimulated emission") with a precise pulse laser

Conventional

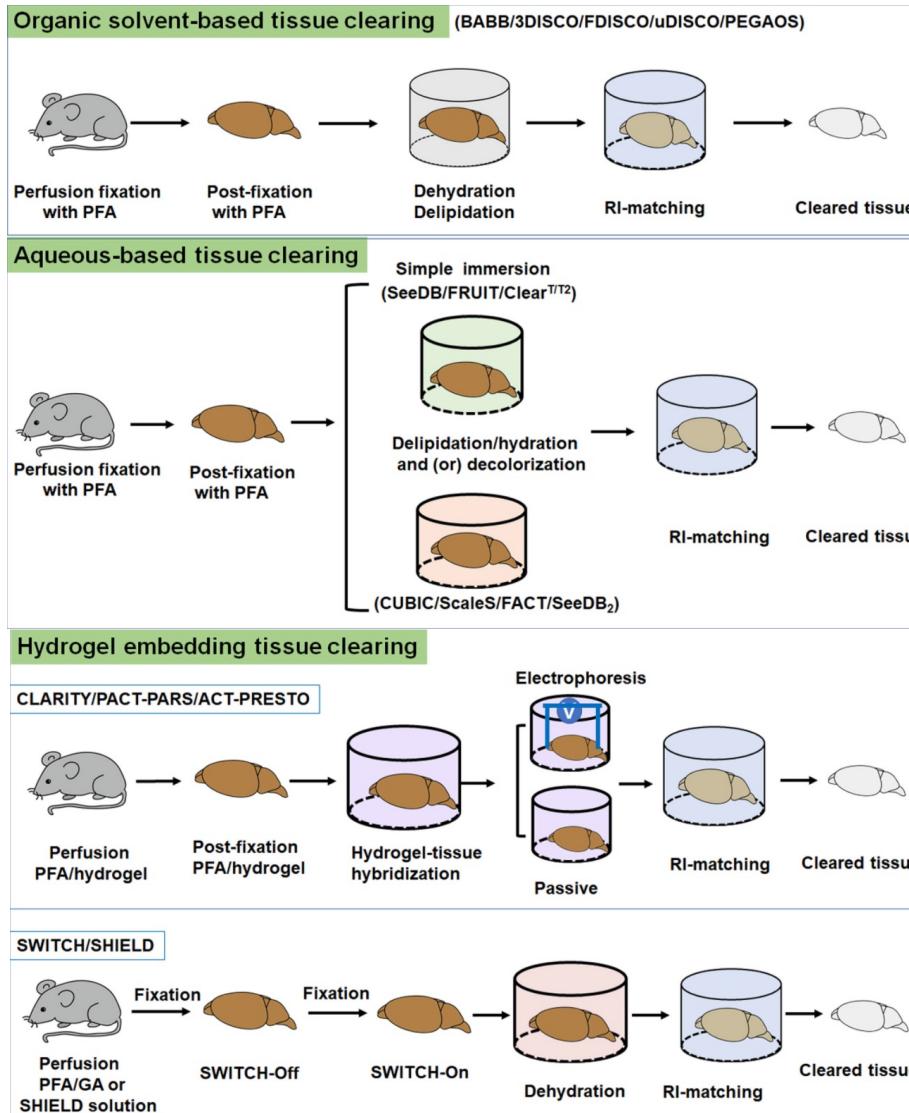


STED

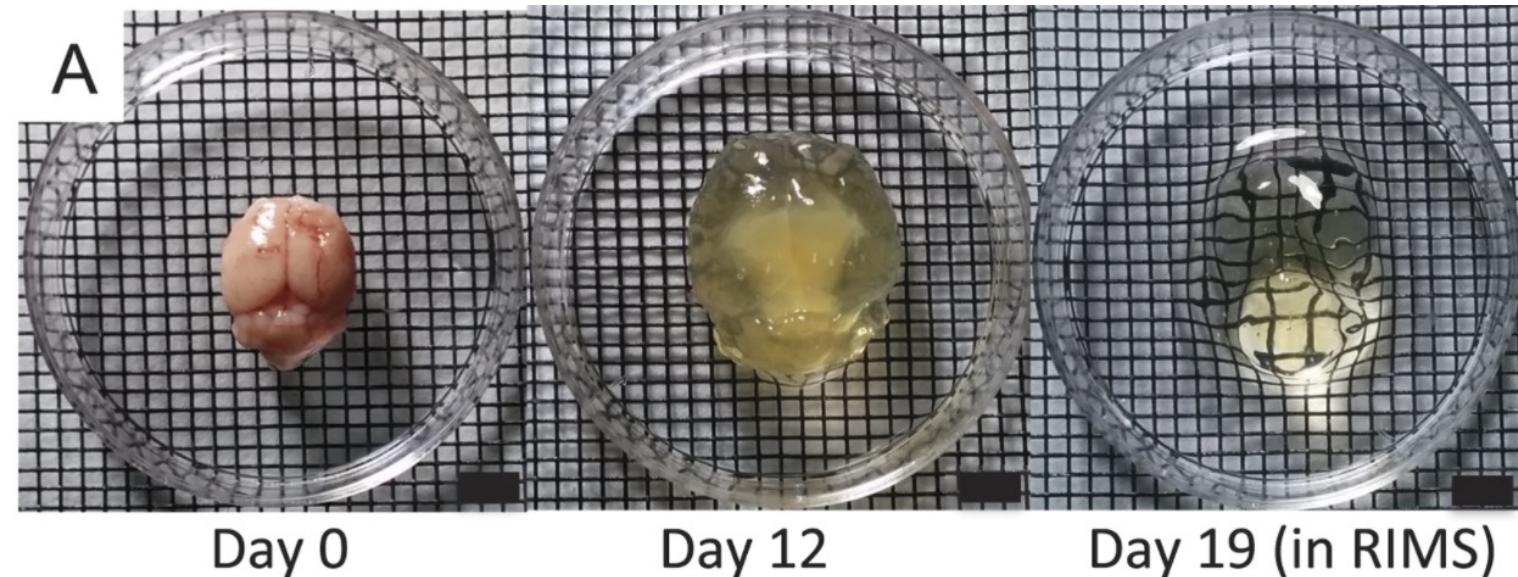


# Tissue Clearing

Replace water, lipids, etc with high RI solution.



Light is scattered by ribosomes, nuclei, nucleoli, mitochondria, lipid droplets, membranes, myelin, cytoskeletal components, and extracellular matrix components such as collagen and elastin.



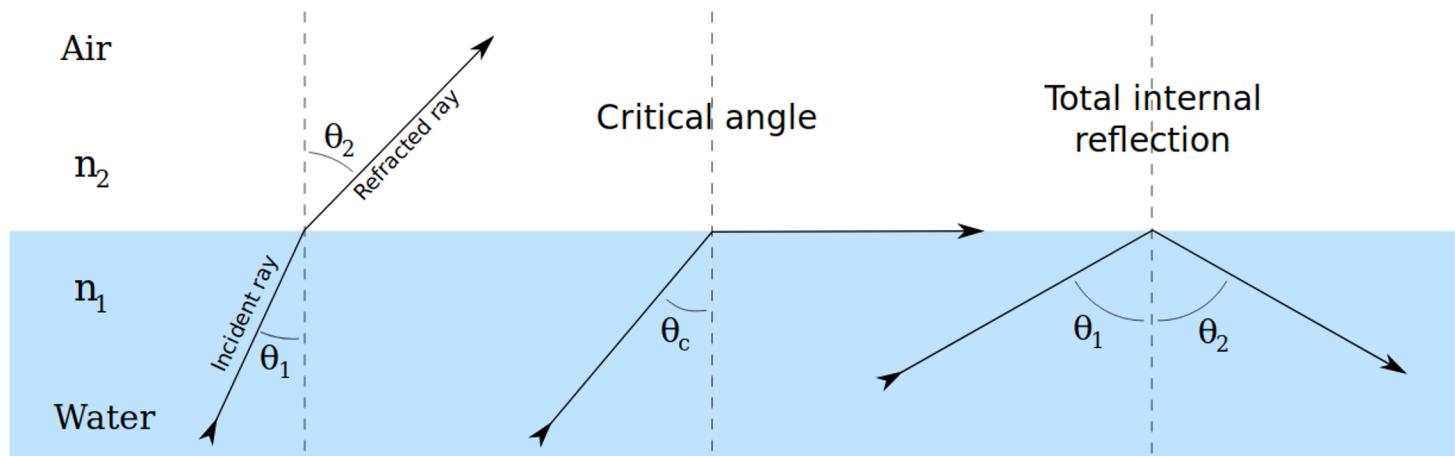
Published: 30 May 2013

## CLARITY for mapping the nervous system

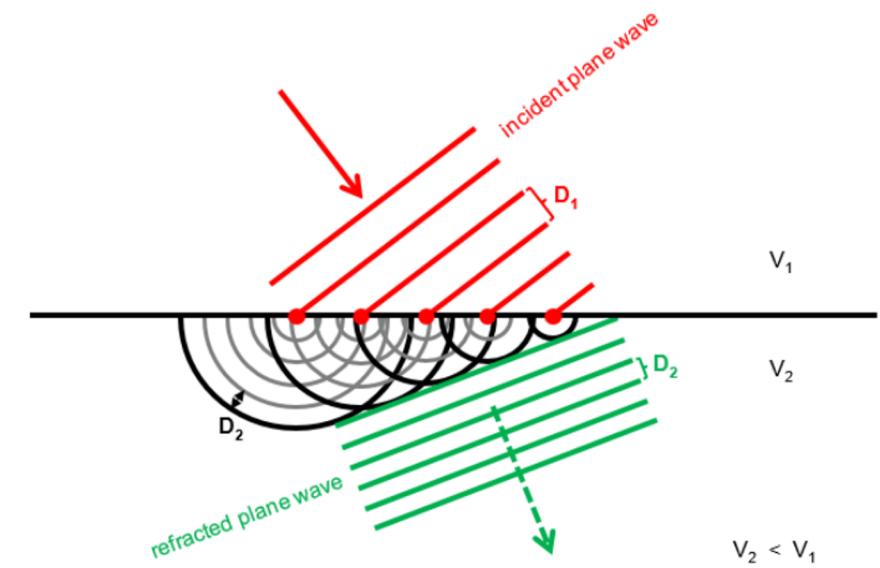
Kwanghun Chung & Karl Deisseroth

Nature Methods **10**, 508–513 (2013) | Cite this article

# Refractive Index Matching



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

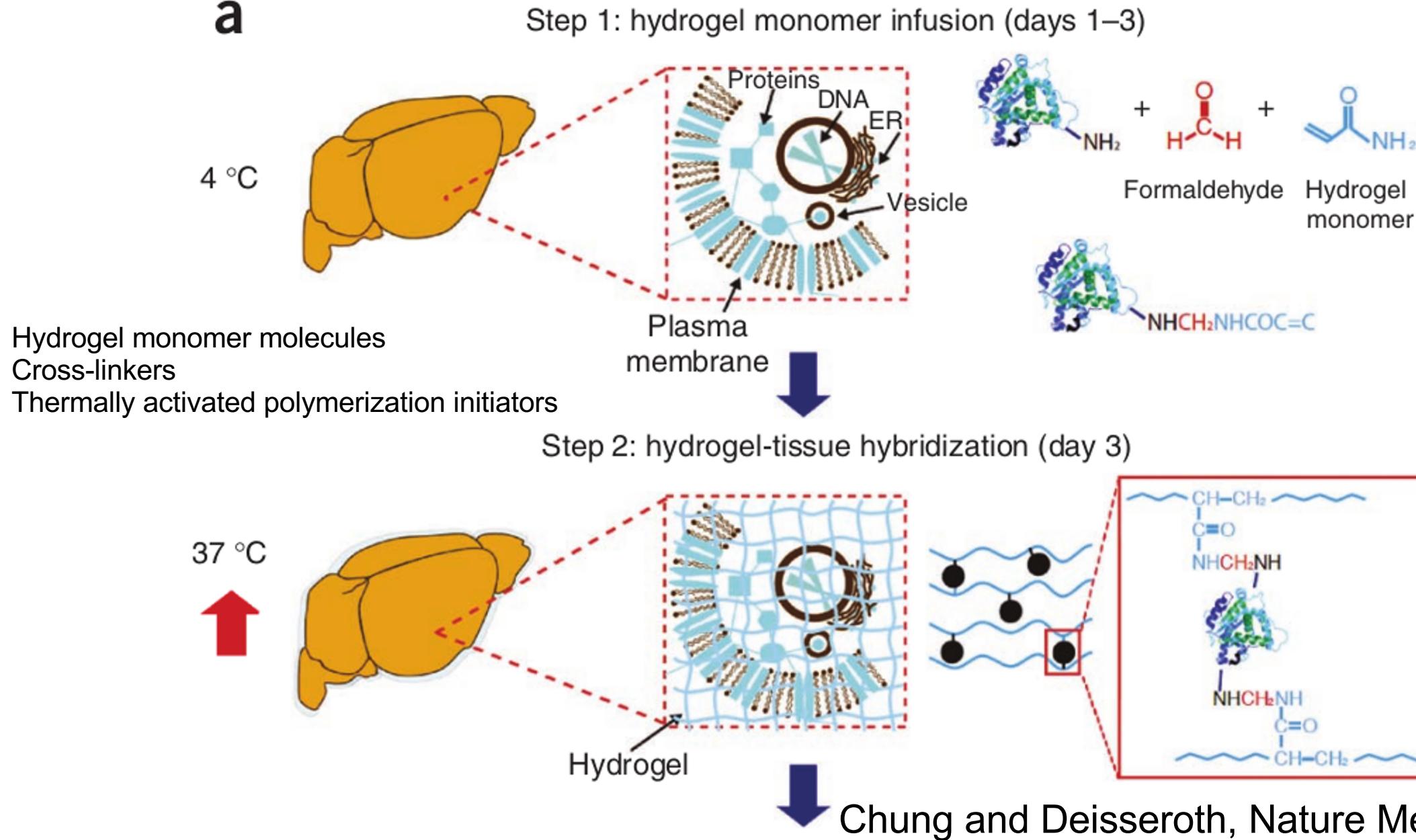


Refraction is a consequence of different speed of light in different materials.

We will submerge our objective refractive index matching solution so  $n_1=n_2$ . CLARITY uses  $n=1.45$  (air is 1, water 1.33, oil 1.52).

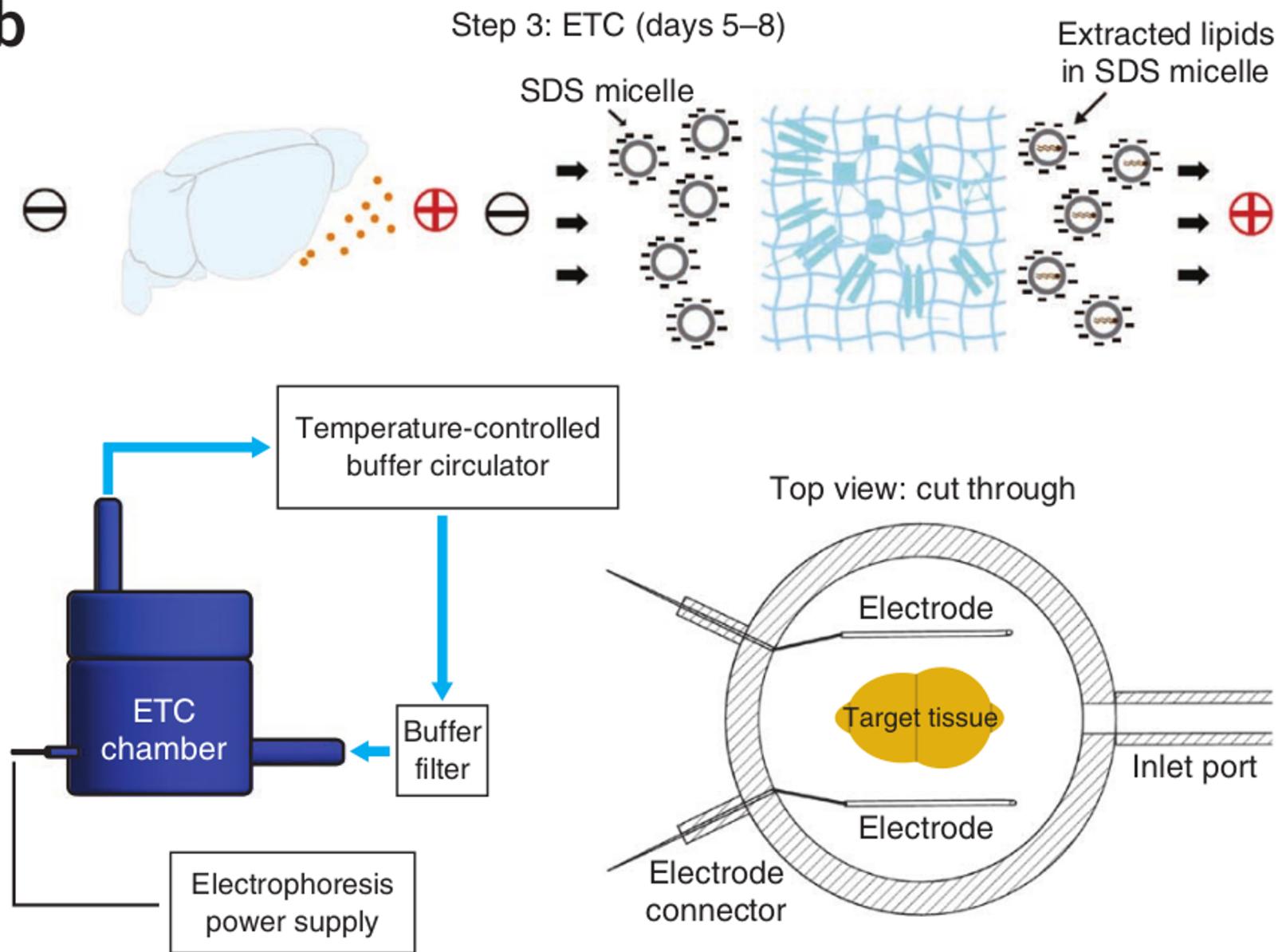
# CLARITY Protocol

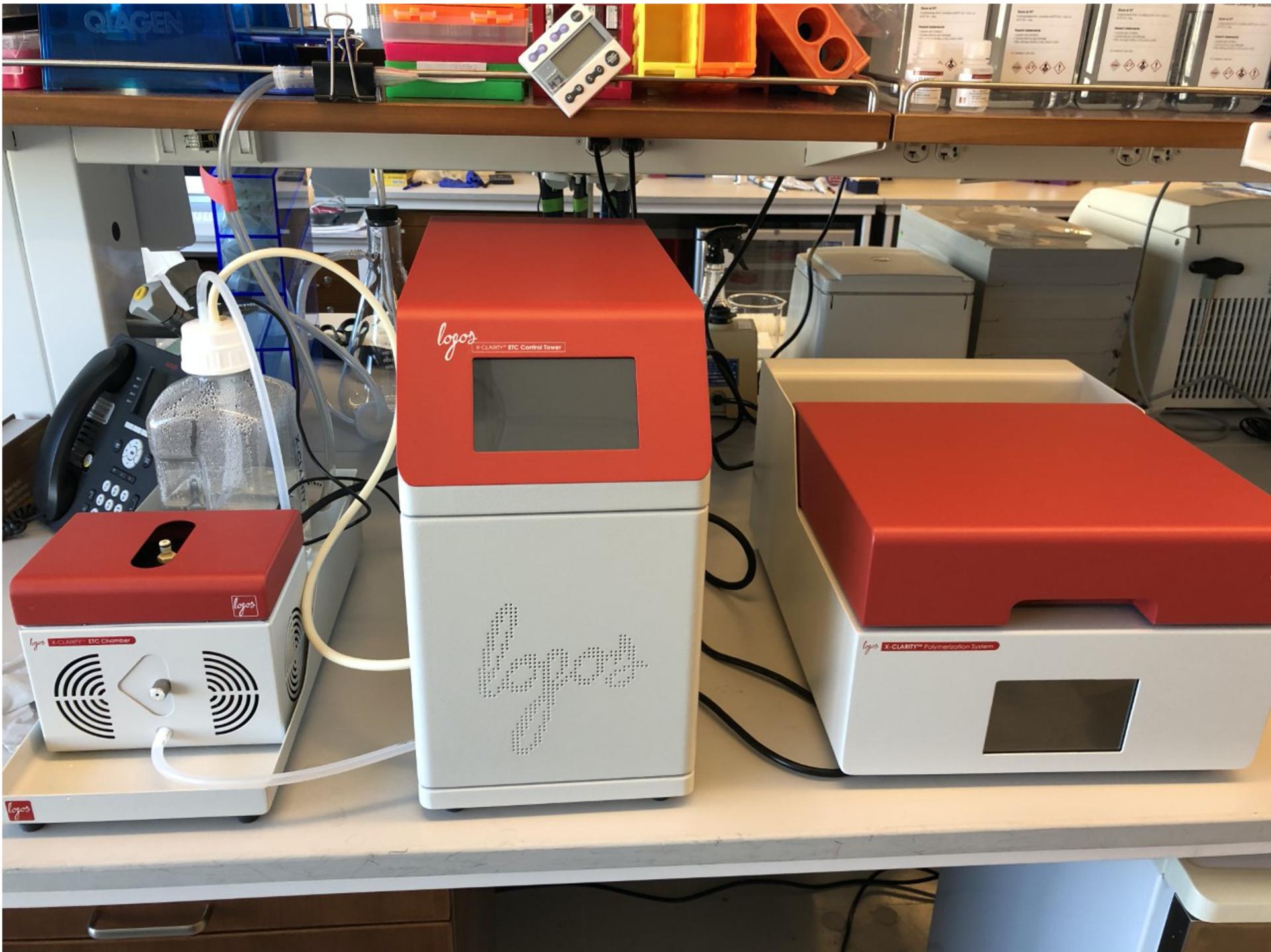
a



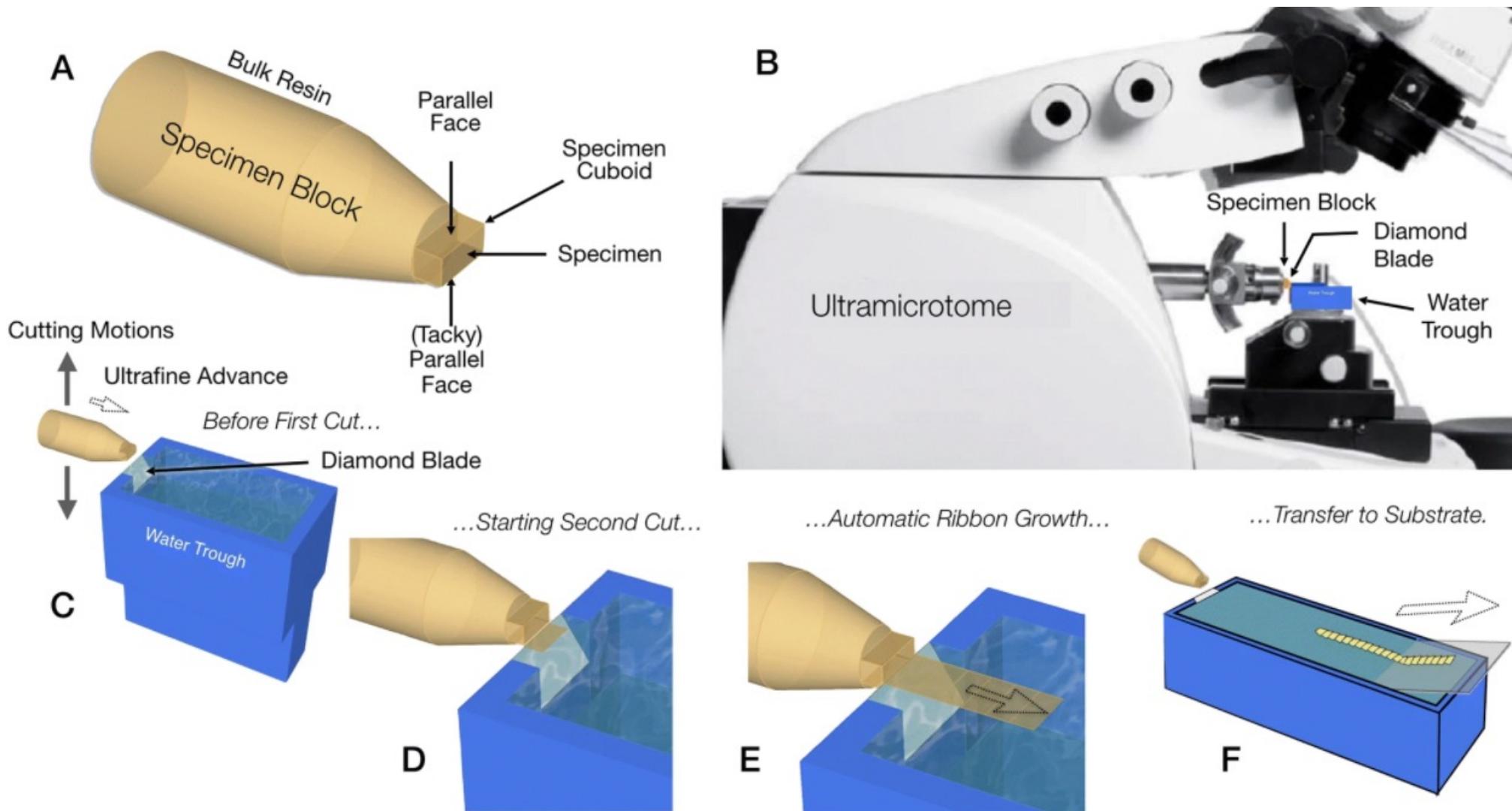
# CLARITY Protocol

b

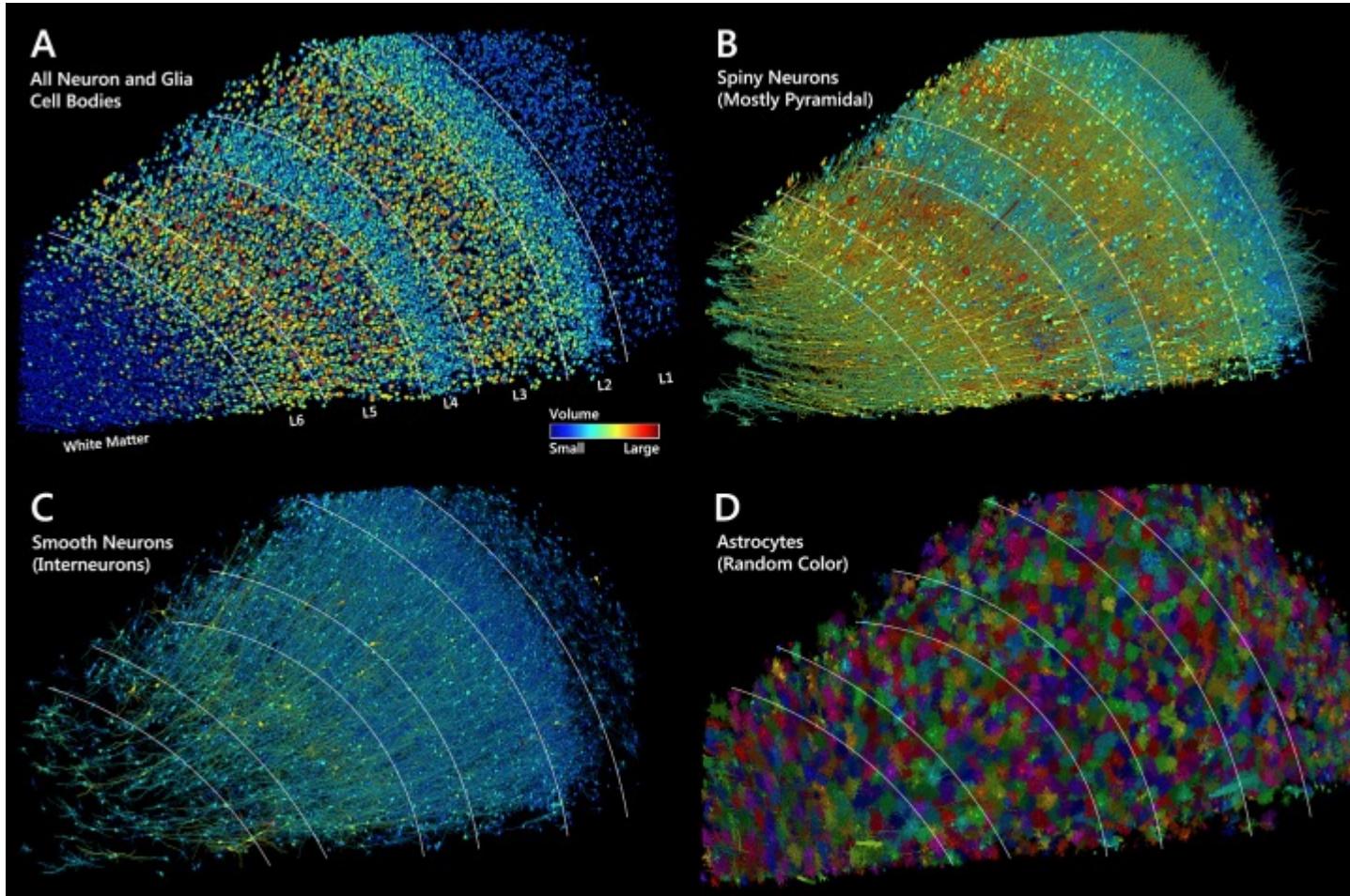




# Array Tomography



# Array Tomography (Example)



1mm<sup>3</sup> from temporal lobe of cerebral cortex  
Cut into 5000 slices @ 30nm each  
Imaged on electron microscope

50,000 cells  
130 million synaptic connections

[dataset](#)

[embeddings](#)