



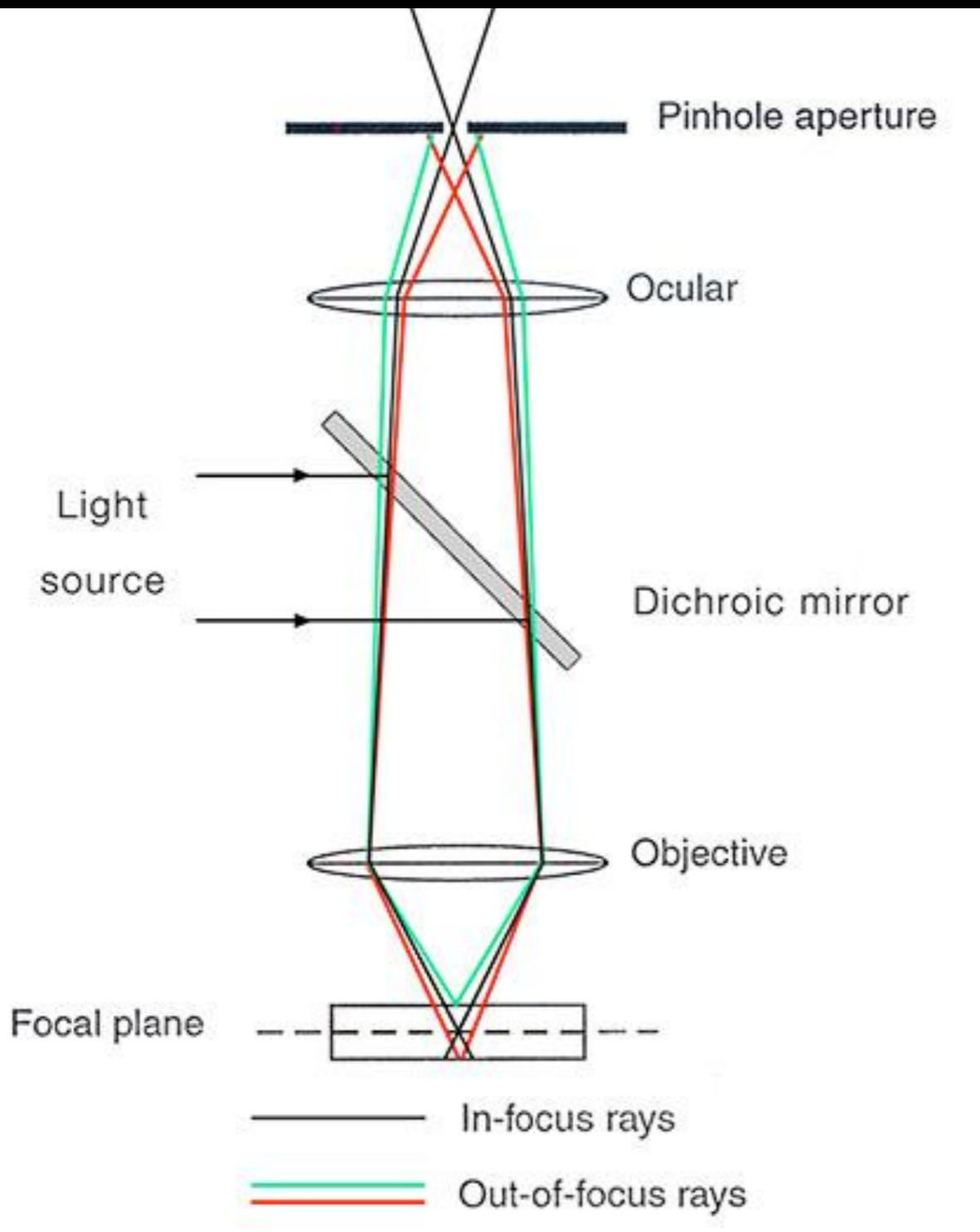
Scanning Microscopy

Confocal
Multi-photon
STED

Confocal Microscopy

- Laser beam scans the sample in x,y to excite the fluorophores at one spot at the time
- A pinhole then cuts out out-of-focus blur light
- No regular camera image - the “pixels” of the image are recorded one by one and combined

Confocal microscope



The light path in a confocal laser scanning microscope. Laser light excites fluorophores most efficiently in the focal point. High-intensity fluorescence from the focal plane is focussed on the pinhole aperture (black lines). Only a fraction of the lower-intensity fluorescence from out-of-focus passes the pinhole (red and green lines).

Pinhole is "confocal" with the sample (in the same focal plane)

Image built up spot by spot

Optical sectioning

- The pinhole cuts out the “out of focus blur light”

Resolution extension

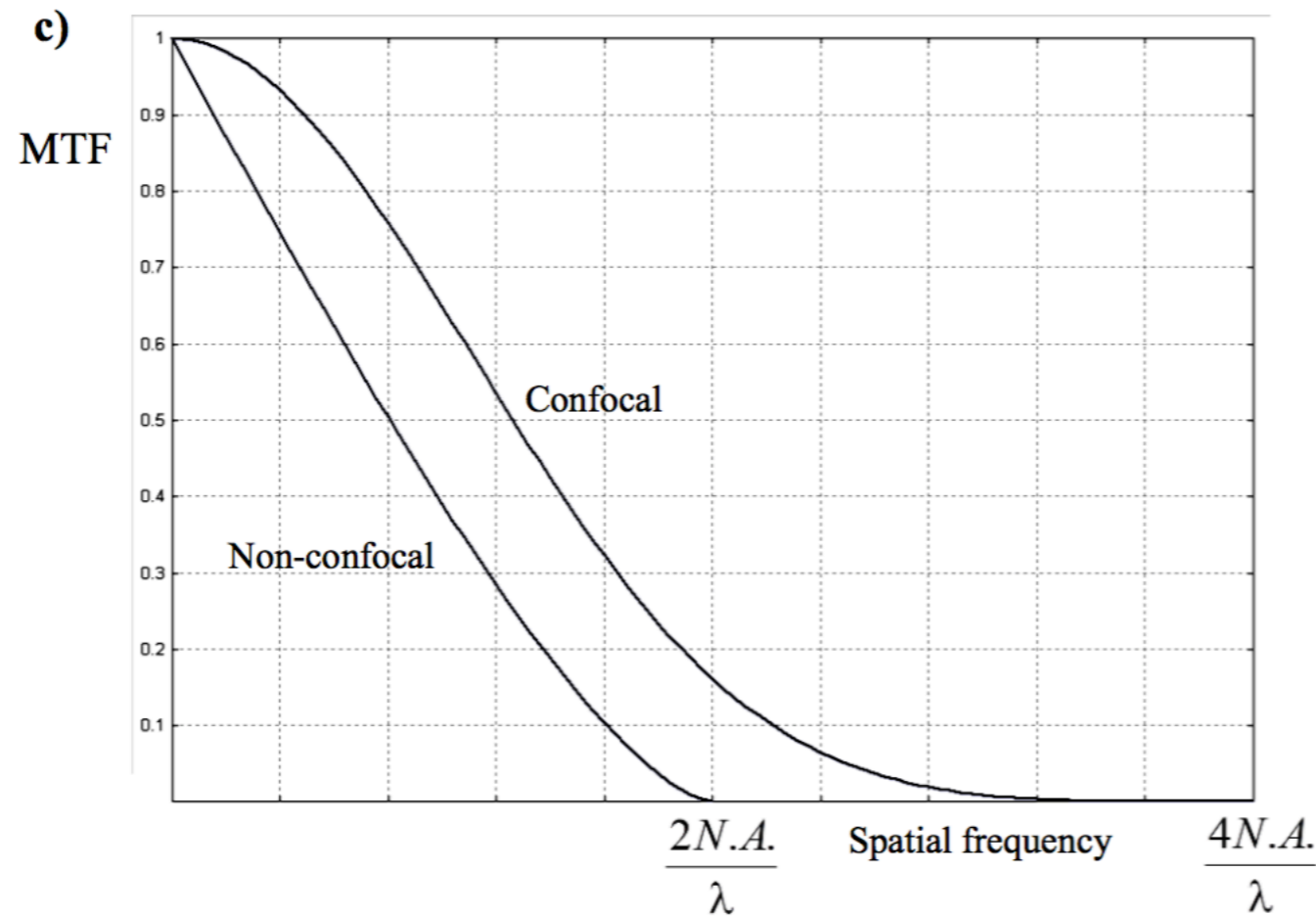
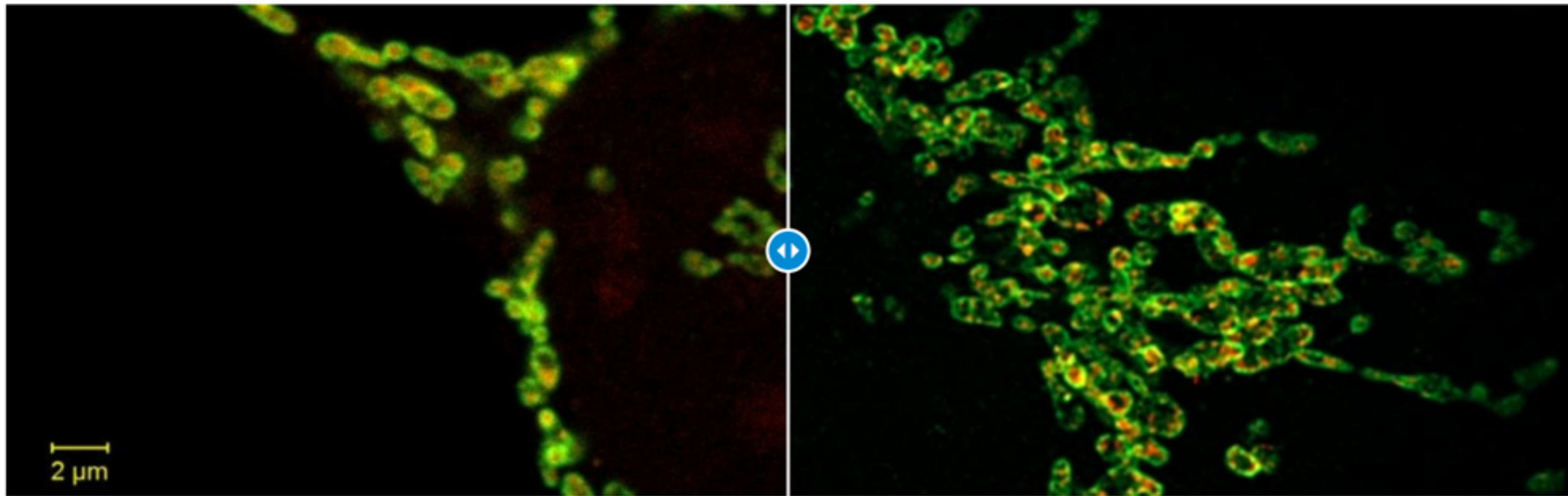


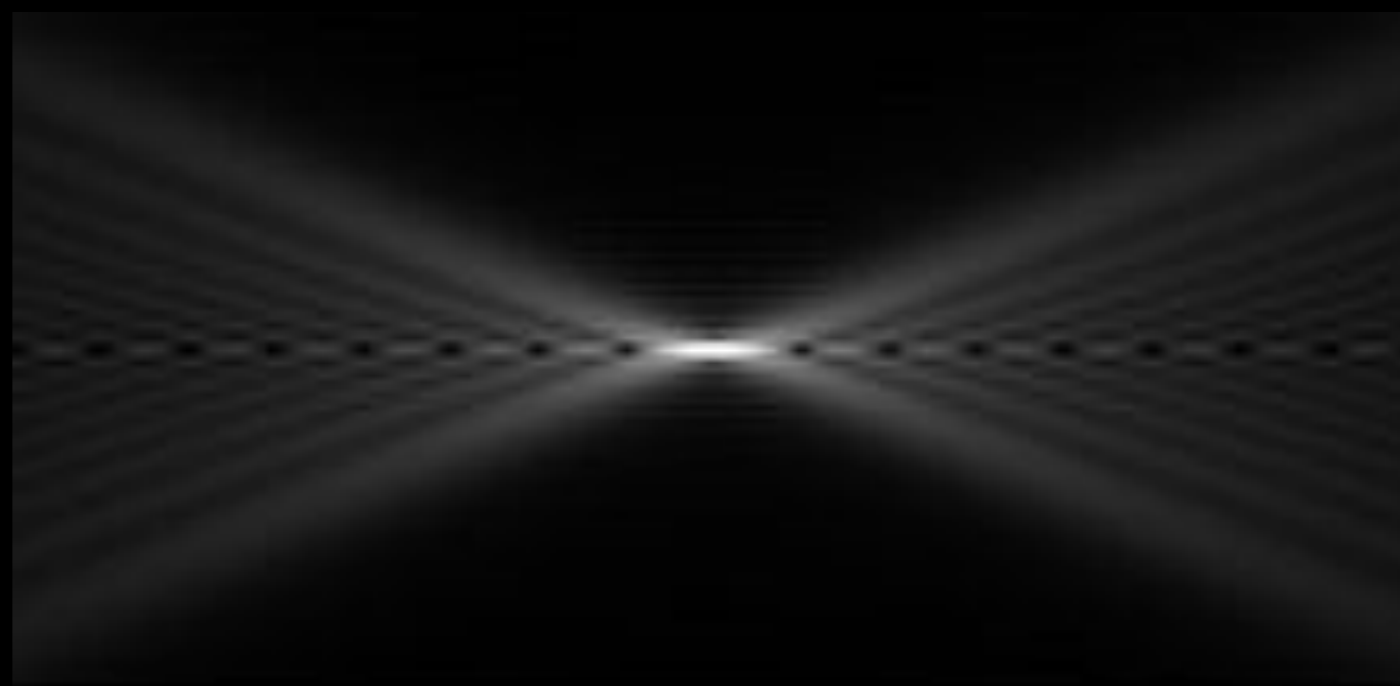
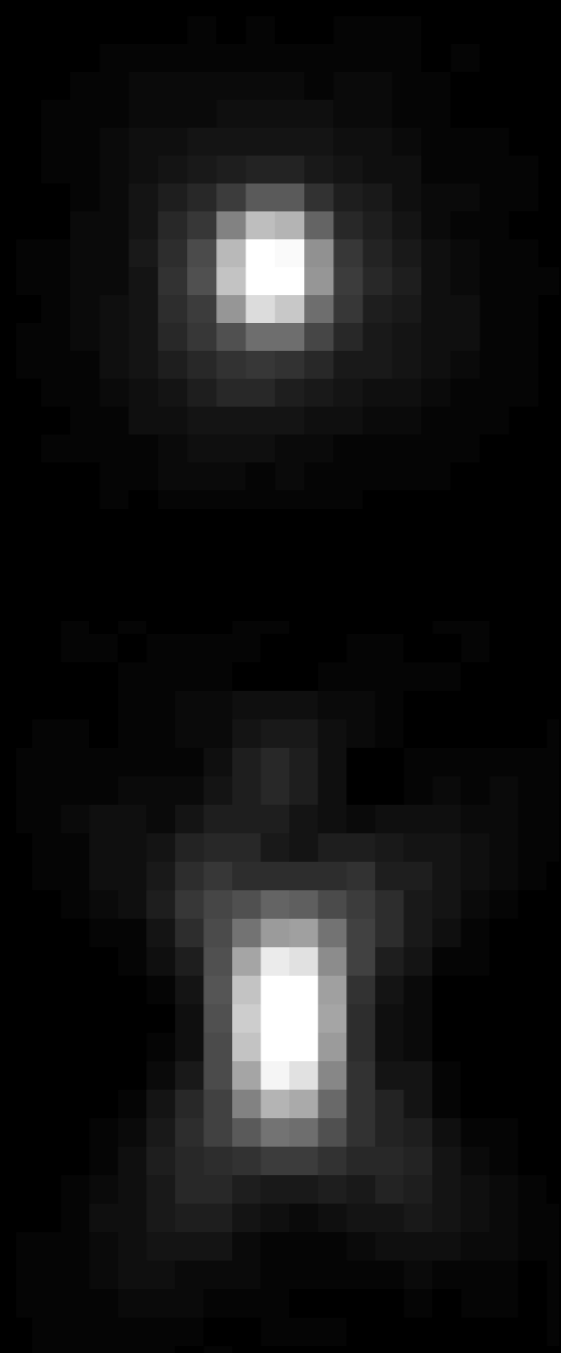
Fig. 22. MTF for confocal incoherent microscopy. a) The confocal MTF is the autocorrelation function of the non-confocal MTF. To calculate this function two rotationally symmetric versions of Fig. 8b (shaped as circus tents) are displaced a distance corresponding to v , multiplied, and the resulting volume is calculated. b) The area of integration when determining the volume (The circus tents are seen from above). c) Comparison of the confocal and non-confocal MTF curves. All spatial frequencies refer to the specimen plane.

Extended resolution

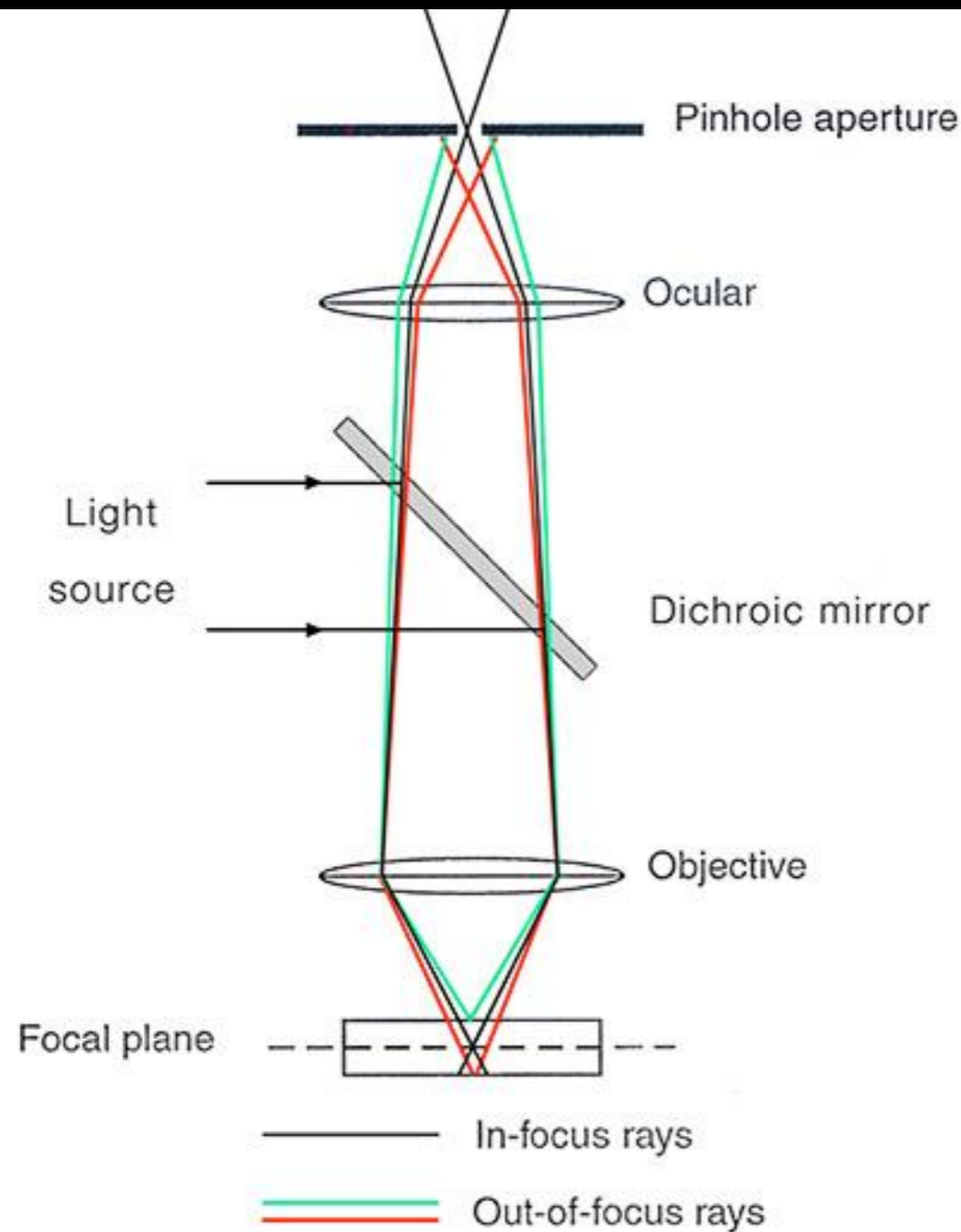


Capture smallest structures with Airyscan at 120 nm resolution

Confocal image (left) / Airyscan image (right). Cells labelled with TOMM20 – Alexa Fluor 488 (green) and TIMM – Alexa Fluor 568



Again...



The light path in a confocal laser scanning microscope. Laser light excites fluorophores most efficiently in the focal point. High-intensity fluorescence from the focal plane is focussed on the pinhole aperture (black lines). Only a fraction of the lower-intensity fluorescence from out-of-focus passes the pinhole (red and green lines).

cal" with the sample (in the same focal plane)

ot by spot

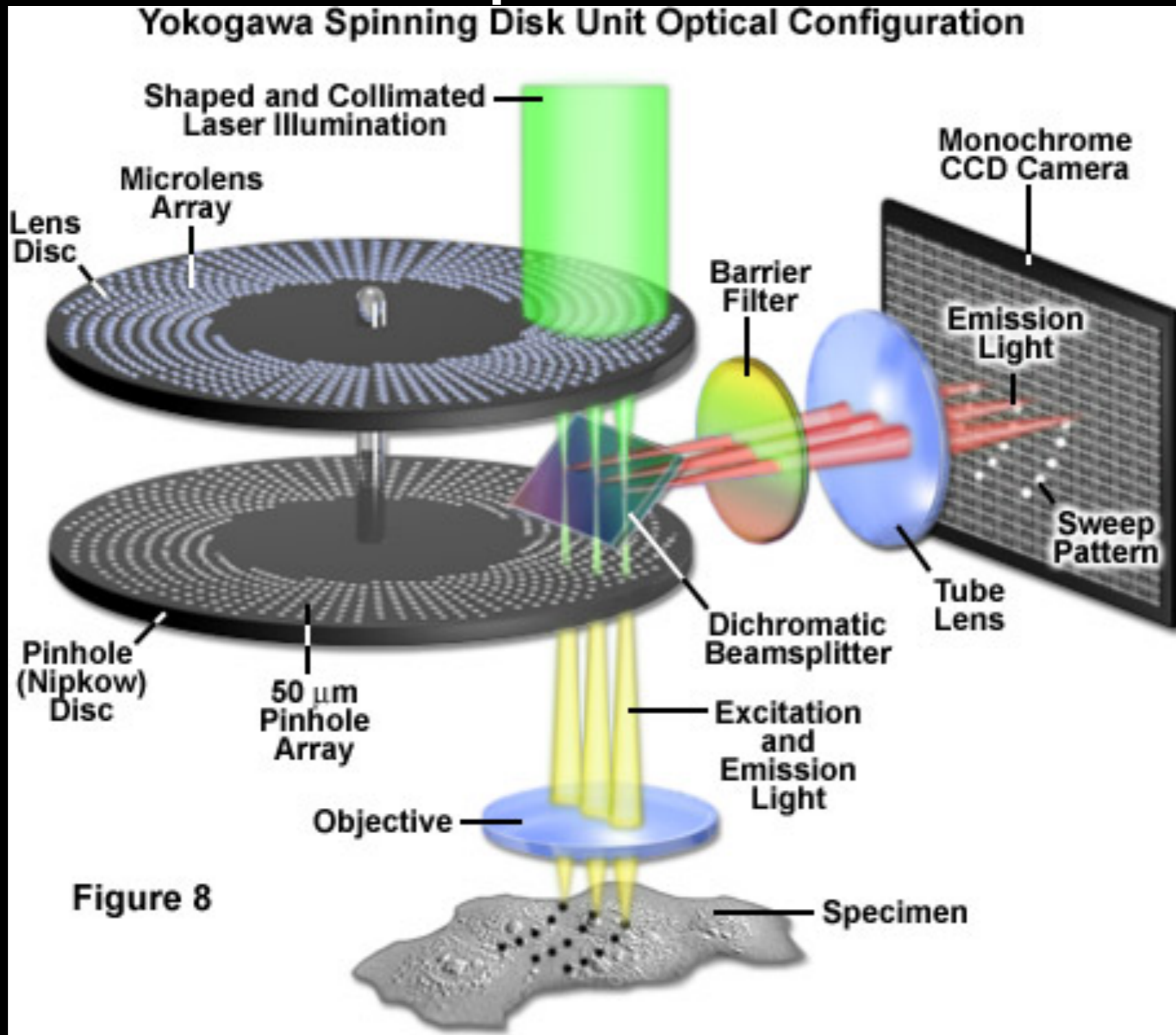
Scanning confocal is slow ~1 volume/s.

- Scanning confocal is slow - stuff moves
- Resonant scanners can scan super-fast but then the pixel dwell-time is very short and there is little signal and lots of noise

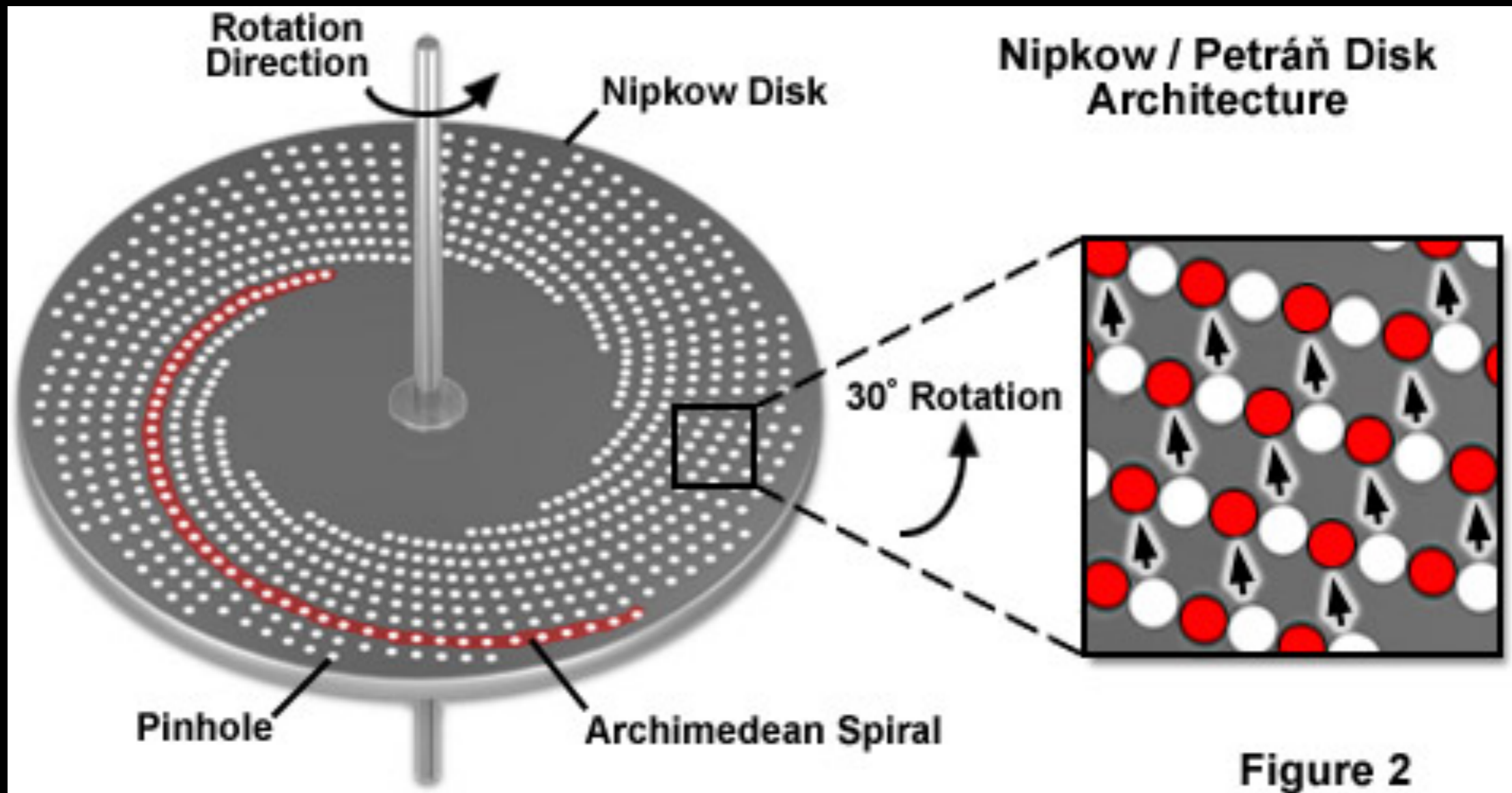
Just scan faster?

- Laser scanning confocal microscopy is generally limited in speed by the characteristics of the fast axis scan mirror. It is mechanically challenging to drive this mirror to oscillate at a speed that is compatible with video frame rates (30 frames per second). For example, using an image size of 512 lines (pixels) per frame, the galvanometer mirror must oscillate at just over 15,000 rotations per second. Therefore, standard video frame rates are only achievable using a resonant scanning device, where a specialized pair of mirrored galvanometer scanners is employed. A resonant scanner cannot be stopped nor adjusted for speed because it is driven by an oscillator at a predetermined frequency. However, this frequency is approximately 10 times higher than what can be achieved with fully controllable galvanometer scanners. By using a resonant scanner with a suitable resonance frequency, near-video rate image capture can be realized with a point scanning confocal microscope, however, at the expense of flexibility and often image quality

Multi-spot: faster!



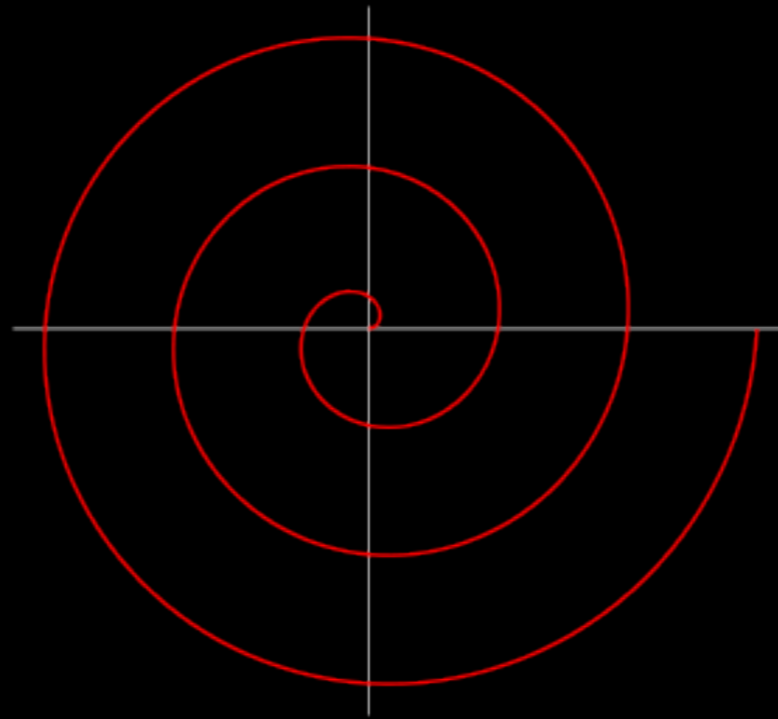
Spinning Disc



50-micrometer pinholes

5,000 or 10,000 rpm => image capture rate of 1,000 or 2,000 frames per second

Archimedean spiral

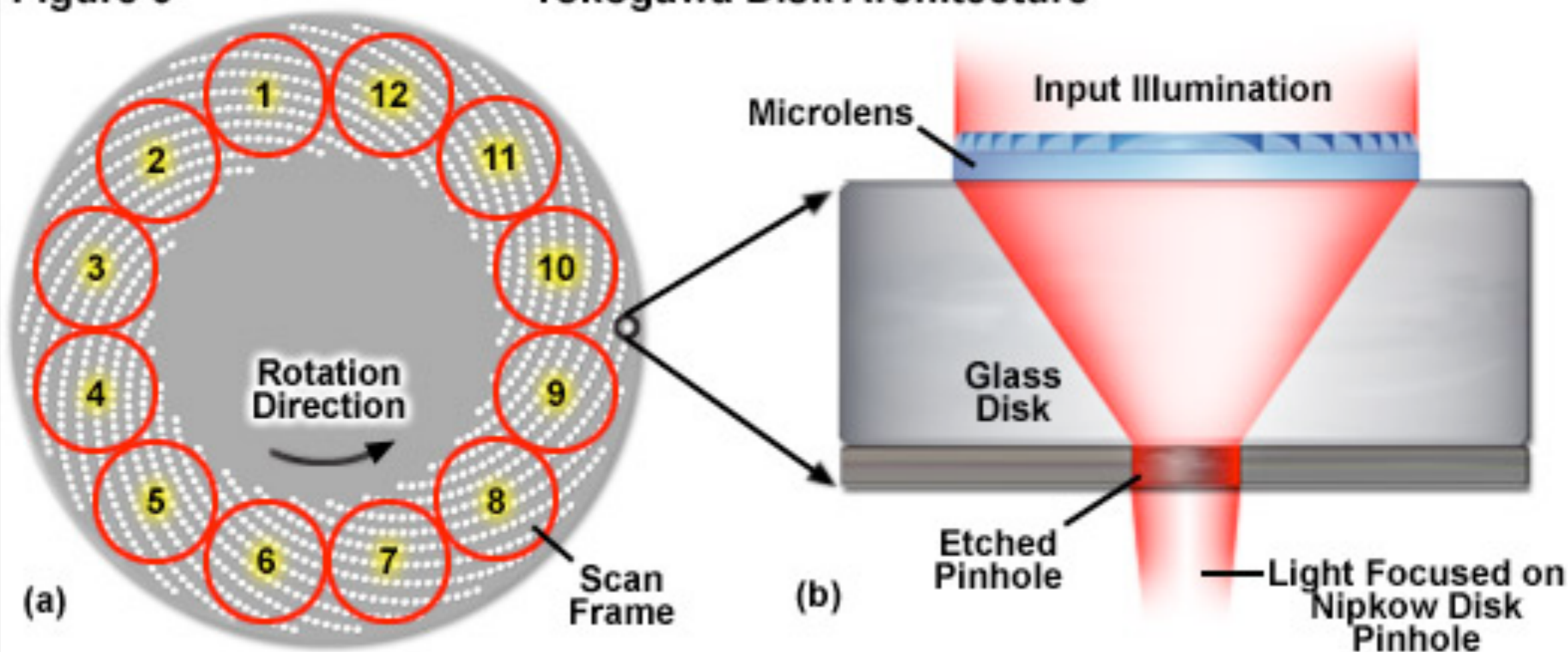


Archimedes, *Περὶ ἐλίκων (On Spirals)* ~225 B.C.

- Nipkow disk is located in a conjugate image plane and a partial rotation of the disk scans the specimen with approximately 1000 individual light beams that can traverse the entire image plane in less than a millisecond. Thus, while a point-scanning confocal microscope illuminates and gathers intensity information serially (as discussed above), multiple pinholes enable the specimen to be sampled in parallel.

Figure 9

Yokogawa Disk Architecture



Unsynchronized Image Capture in Spinning Disk Microscopy

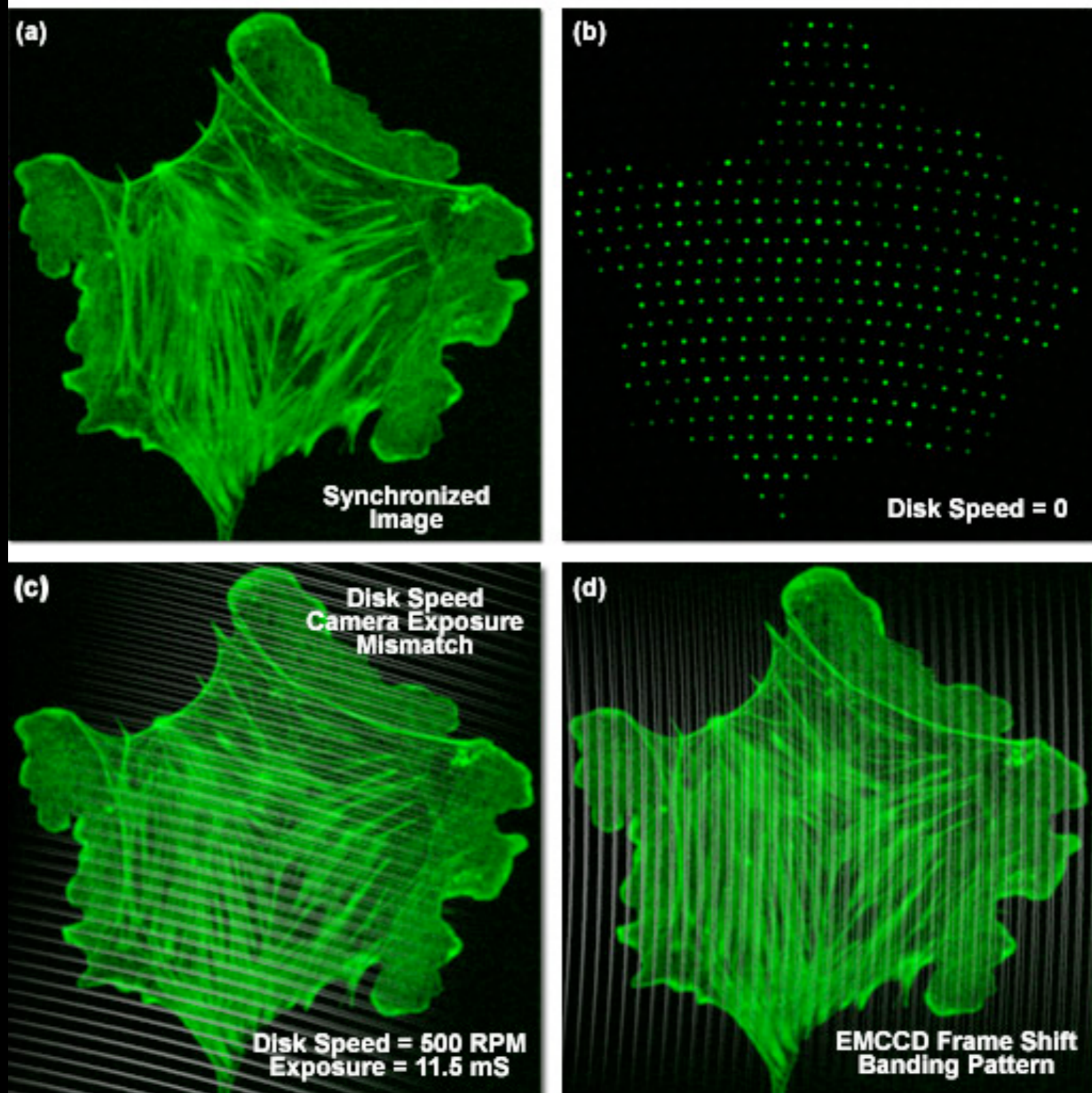
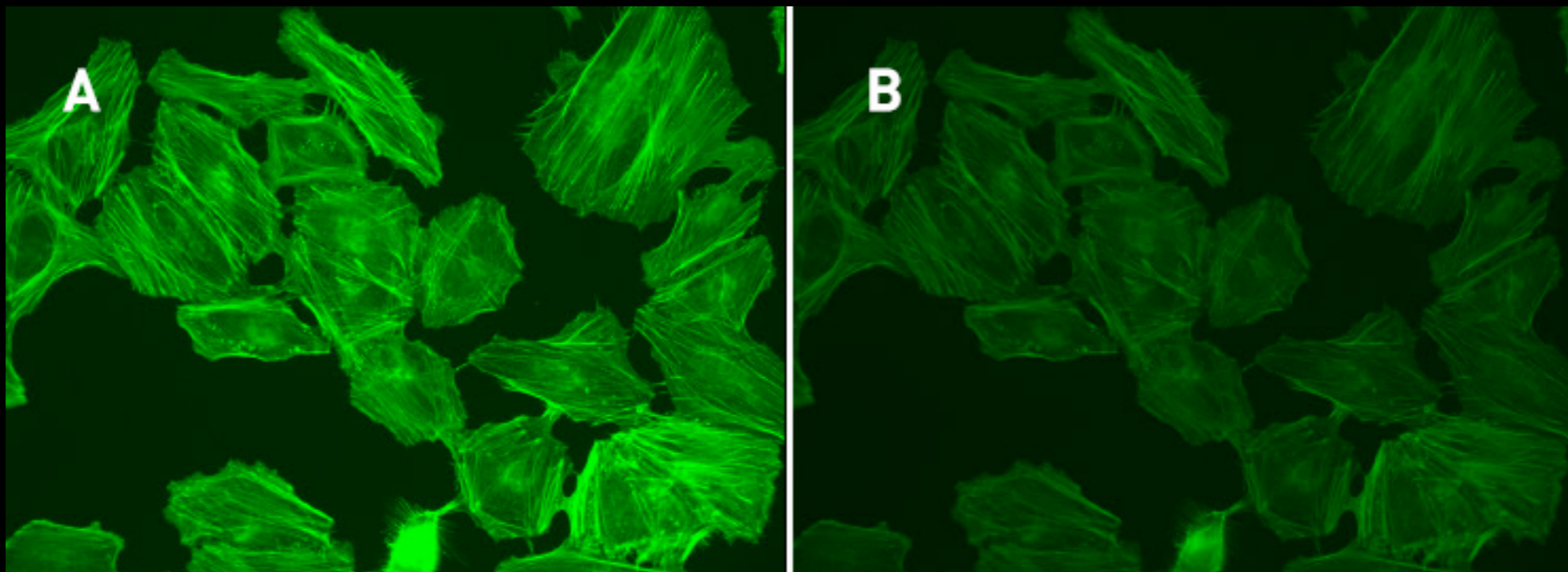


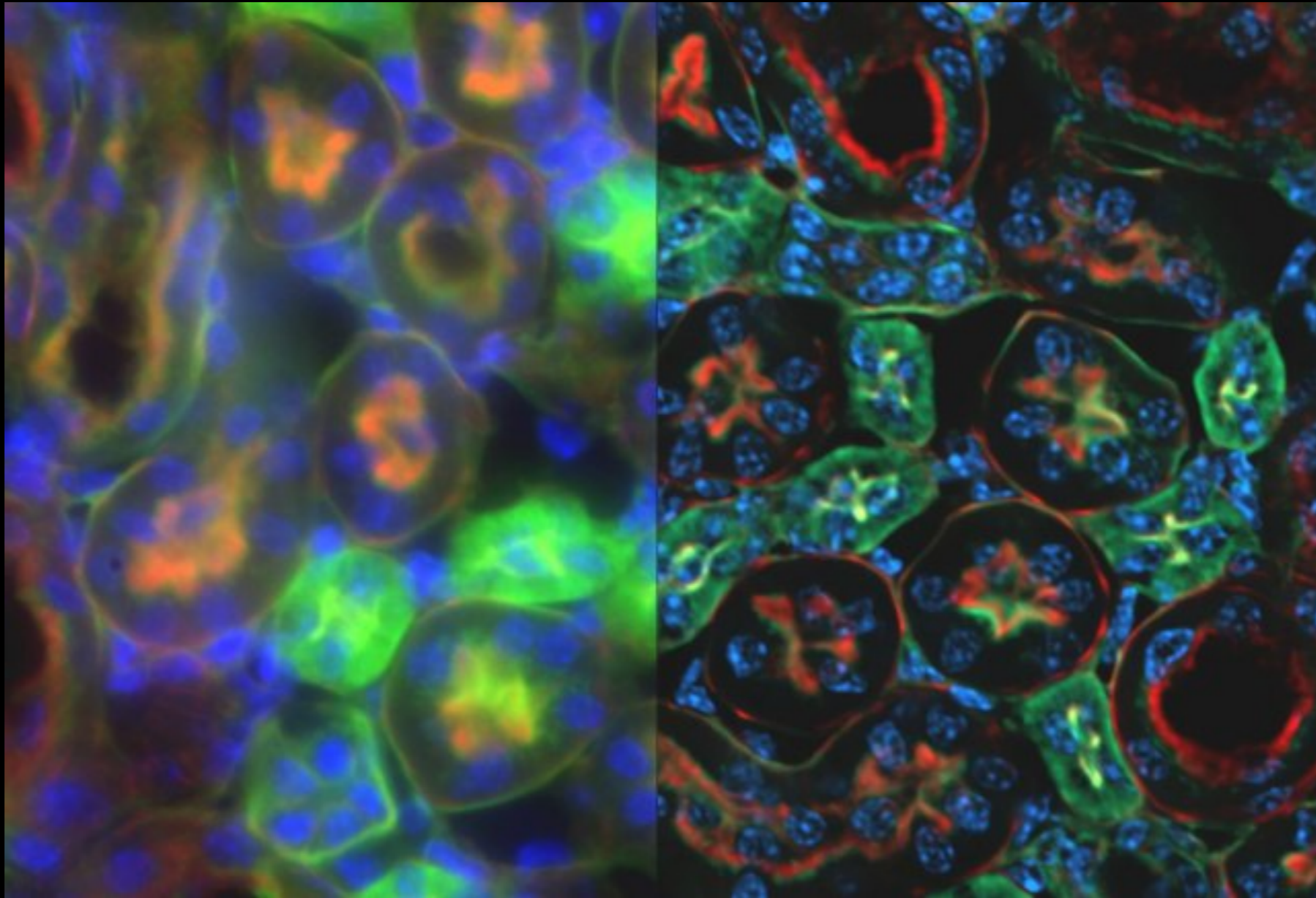
Figure 10

Lots and lots of light is thrown away

- Confocal microscopy is typically not very gentle
- Phototoxicity and photobleaching are major concerns



Deconvolution



Multi photon microscopy

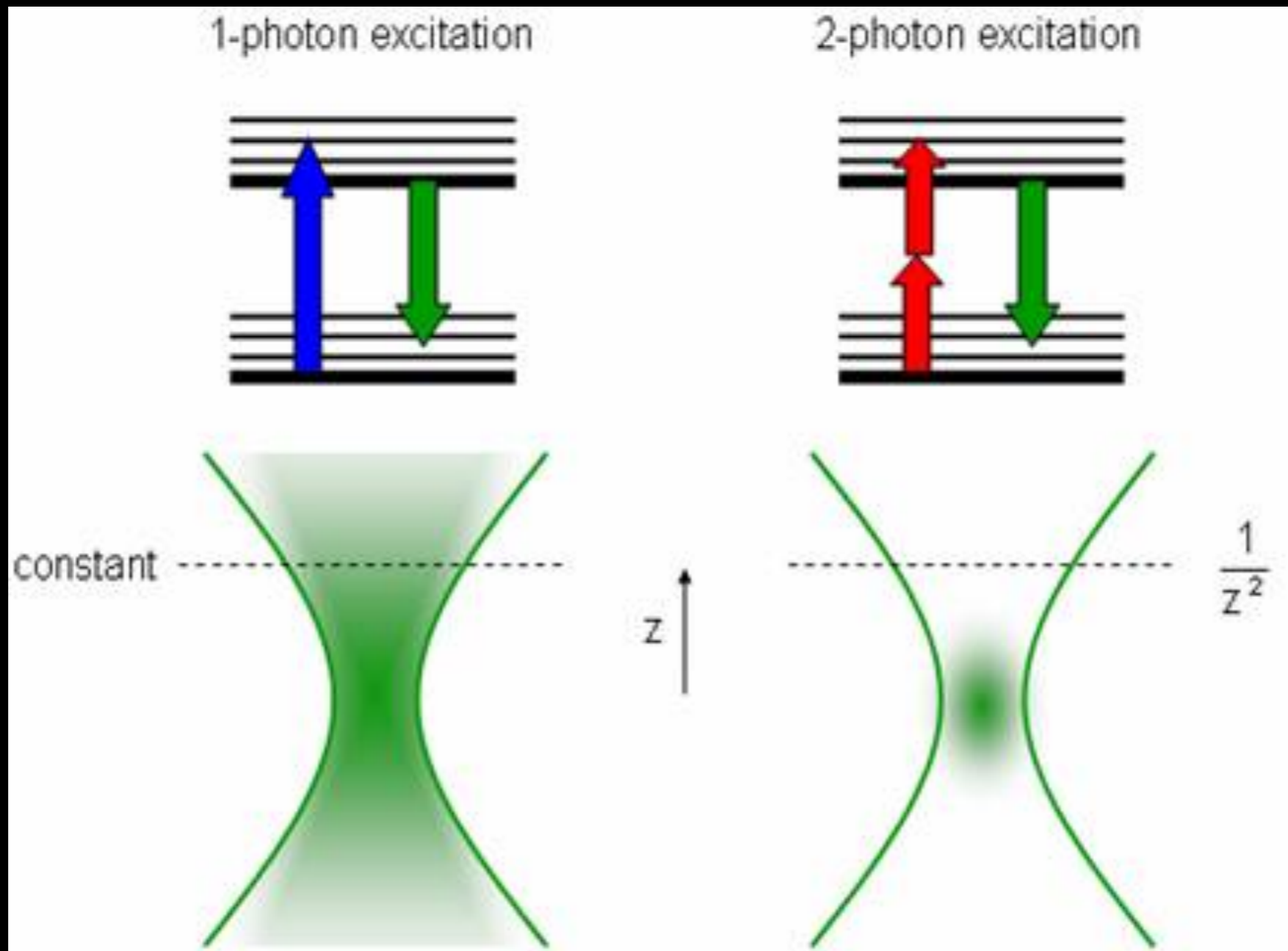


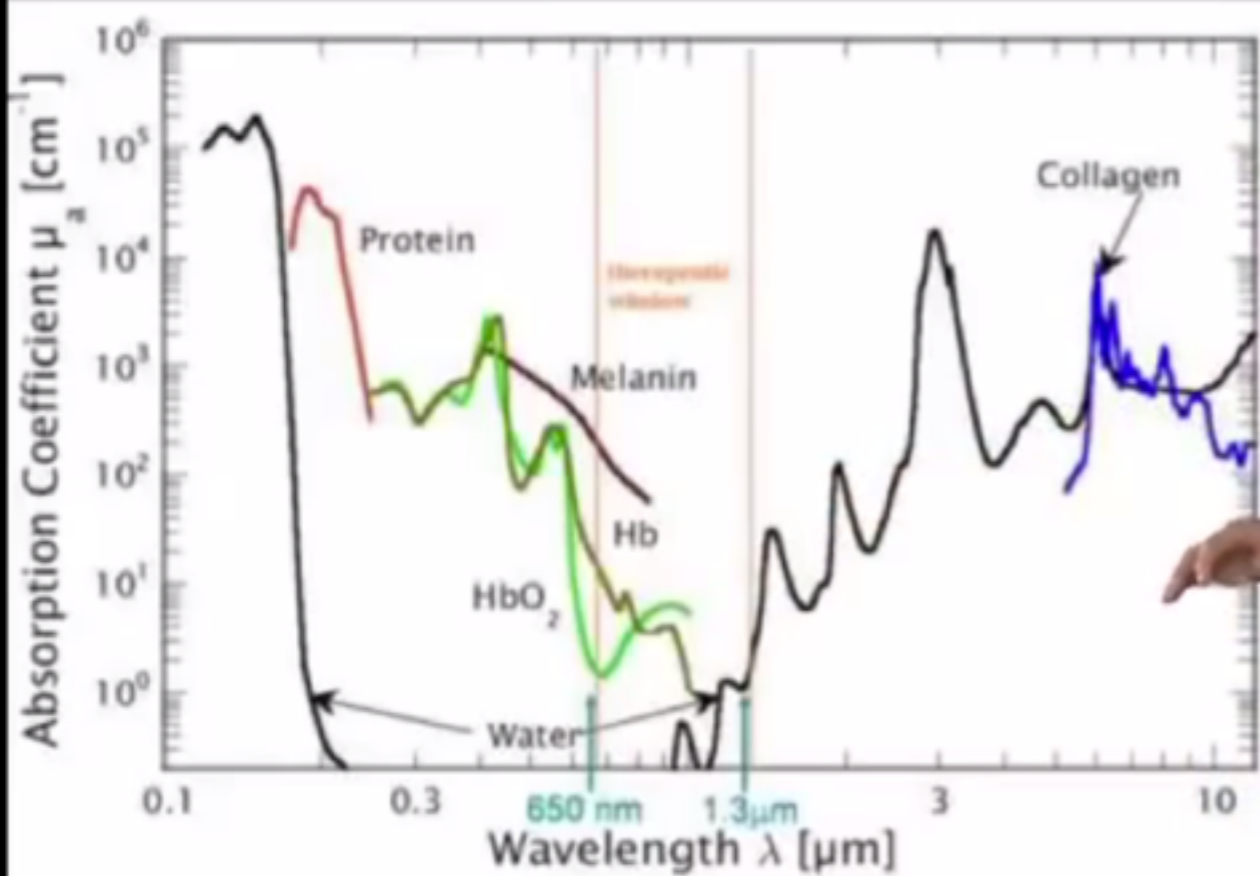
Illustration:
Jerome Mertz

Live animals,
deeper than 200 microns

Did you ever put a flashlight in your mouth?



Absorption of common biological molecules



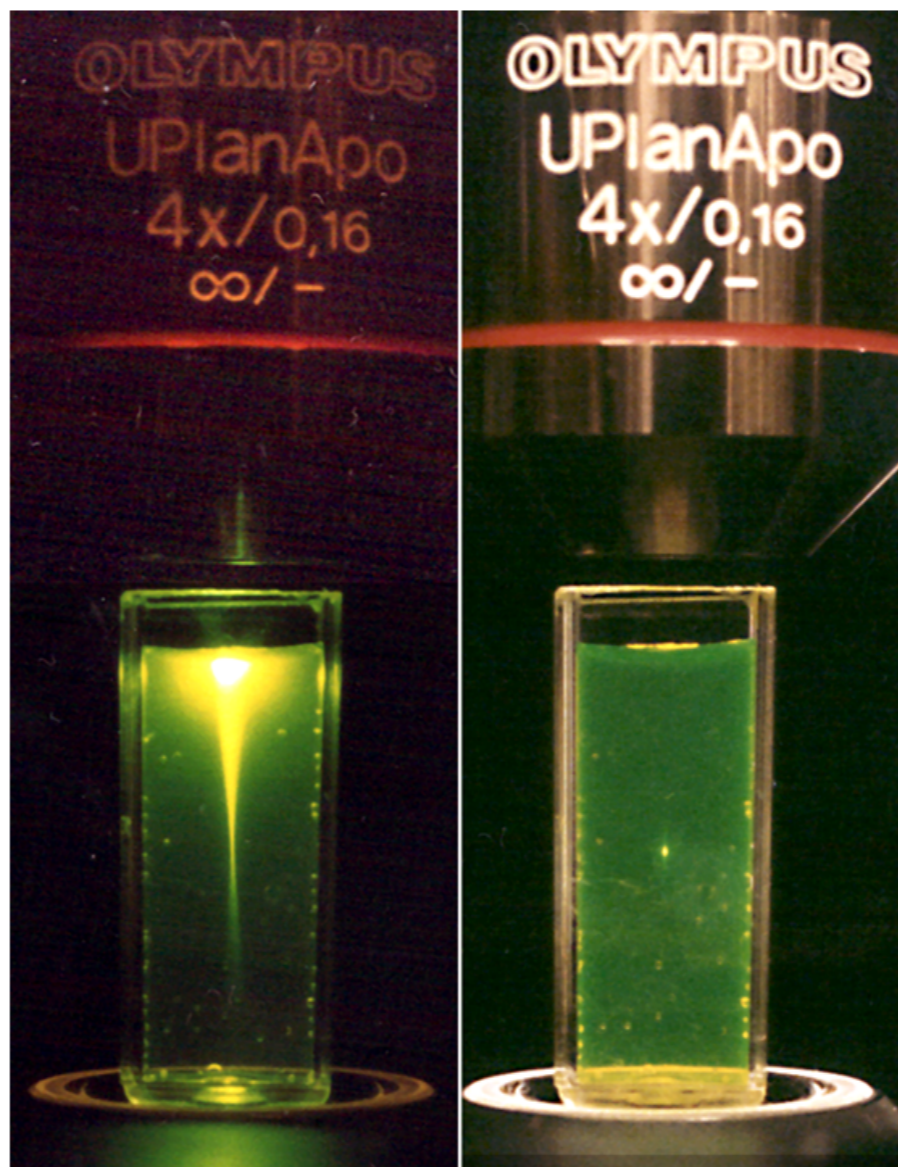
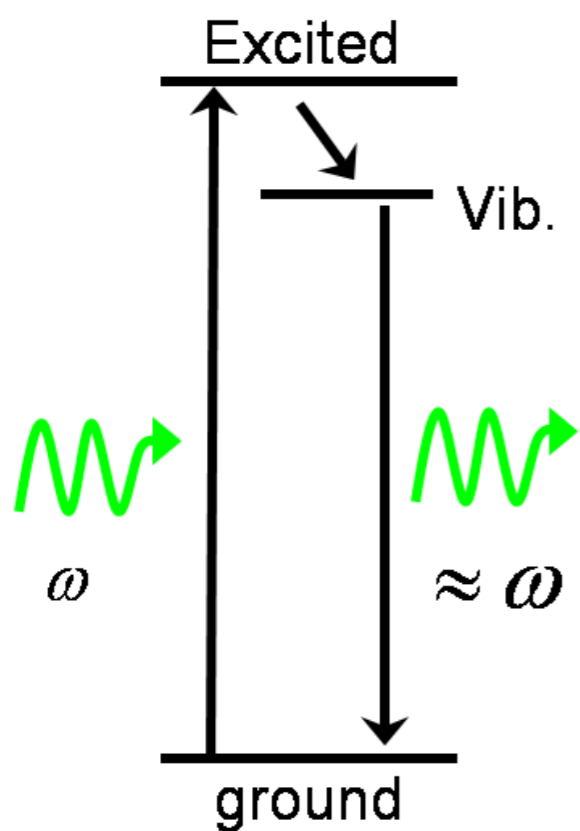
From <http://www.chem.duke.edu/~wwarren/tissueimaging.php>

iBiology.org



One-photon
fluorescence

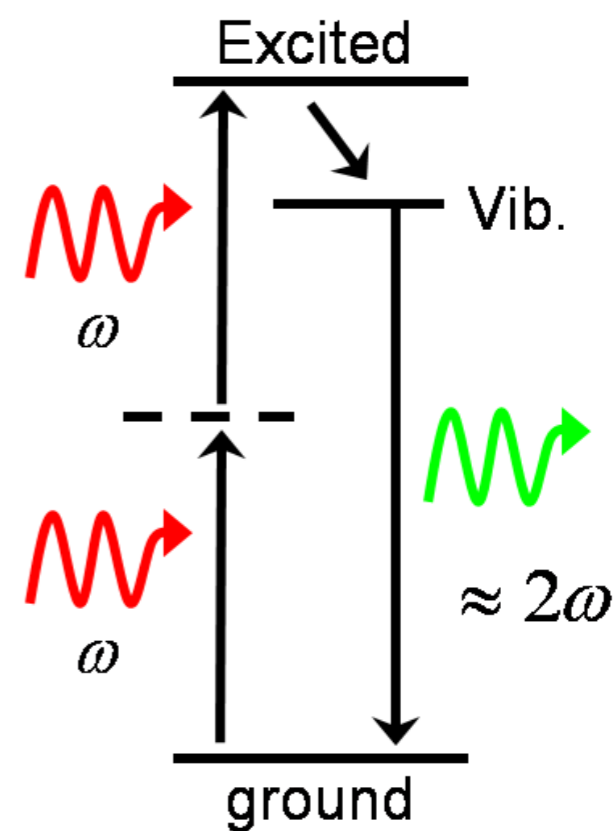
$$\text{Signal} \propto I$$

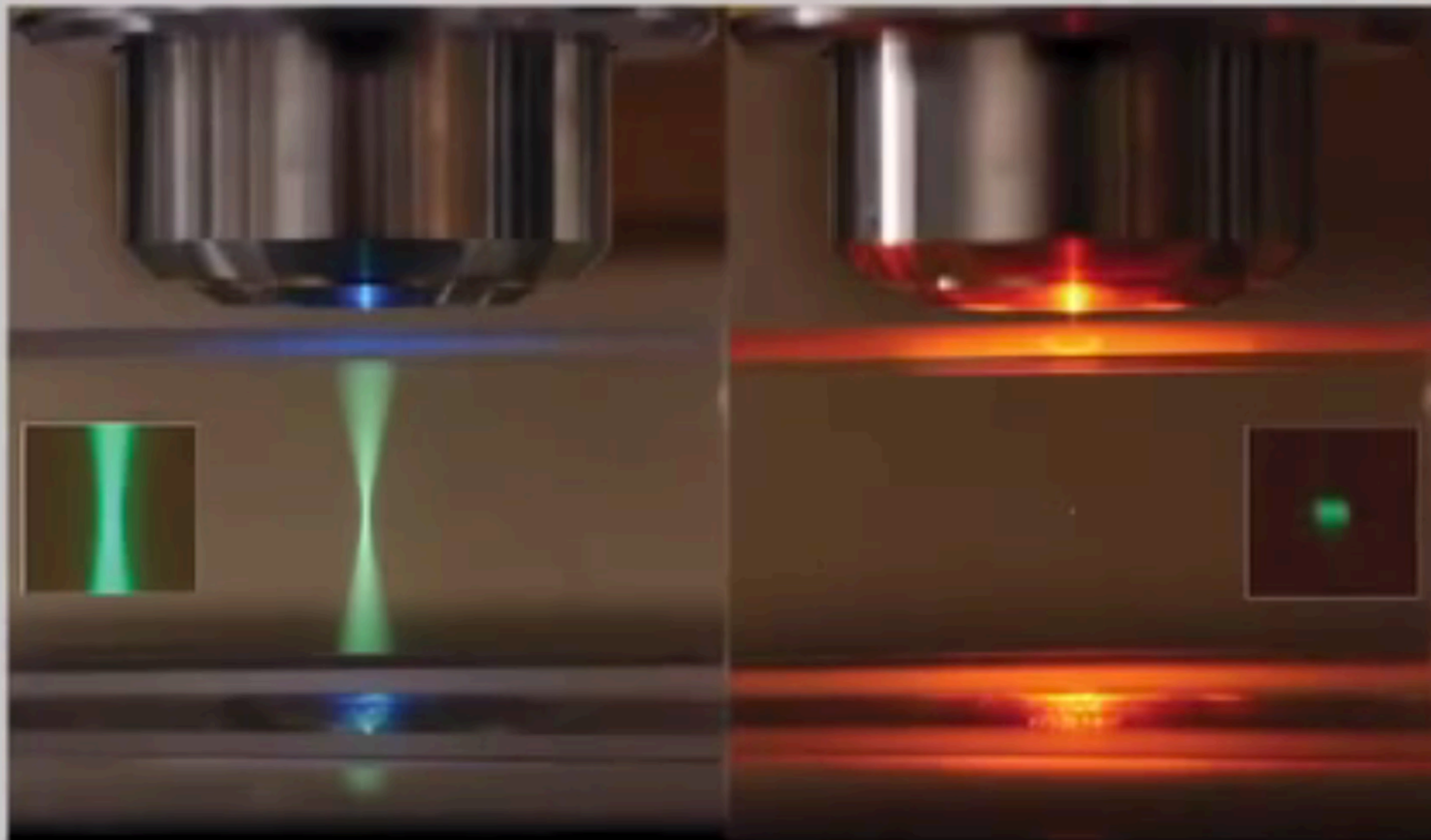


488 nm excitation 900 nm pulsed
excitation

Two-photon
fluorescence

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





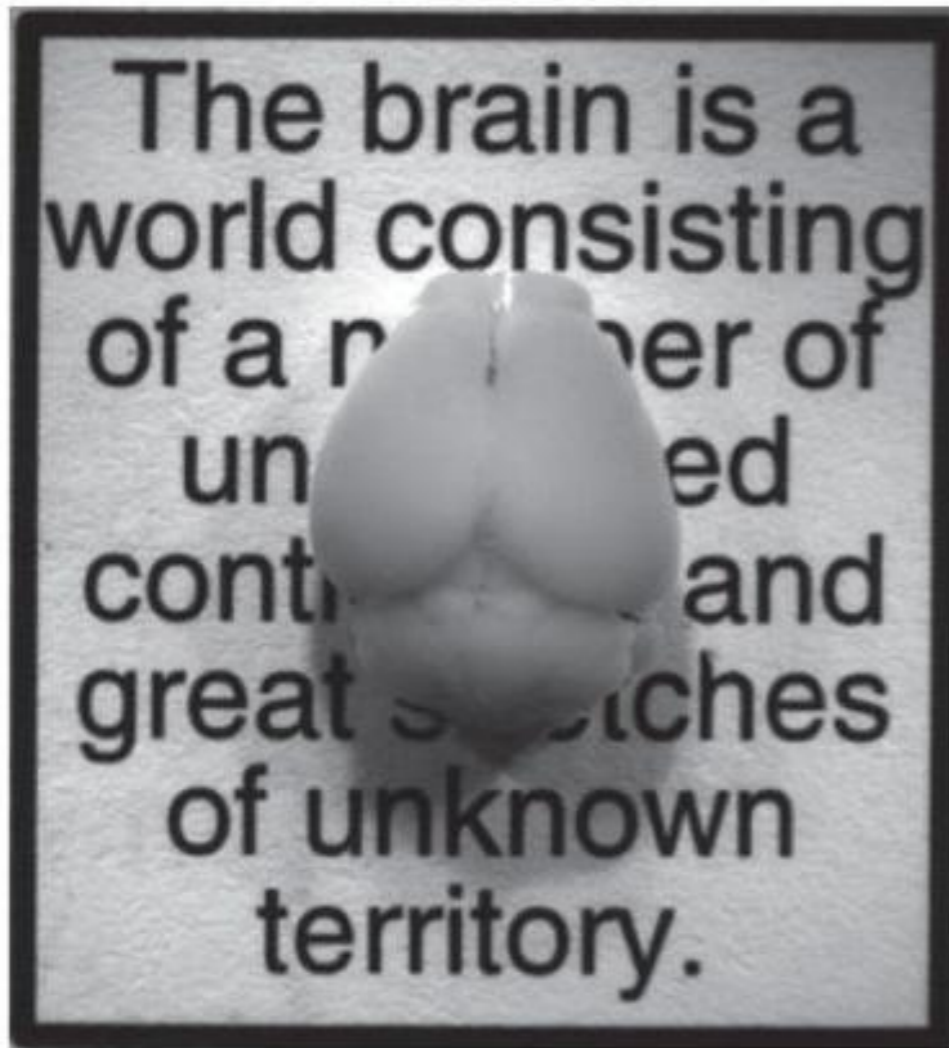
The scanning blue (488nm) laser excites an entire column of sample.

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

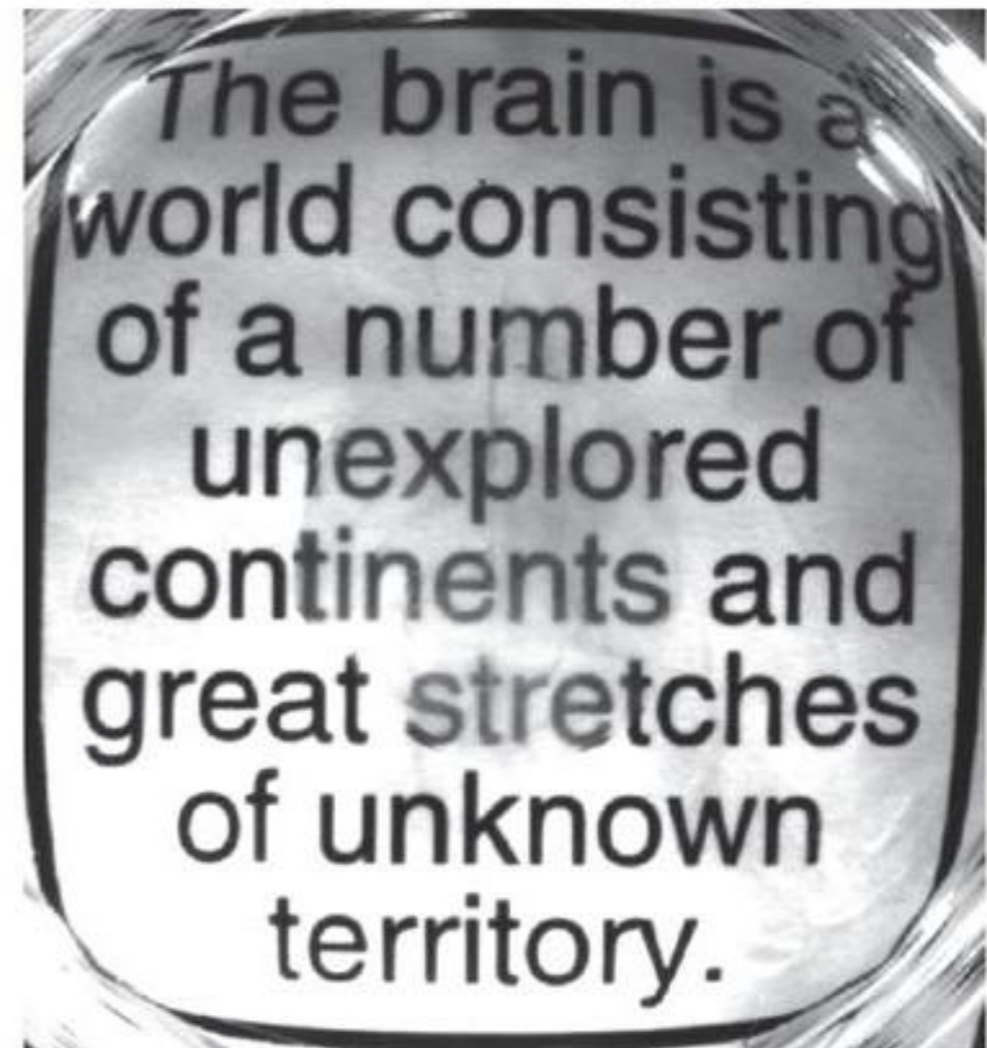
Steve Rubin and Holly Aaron, UC Berkeley

Tissue clearing

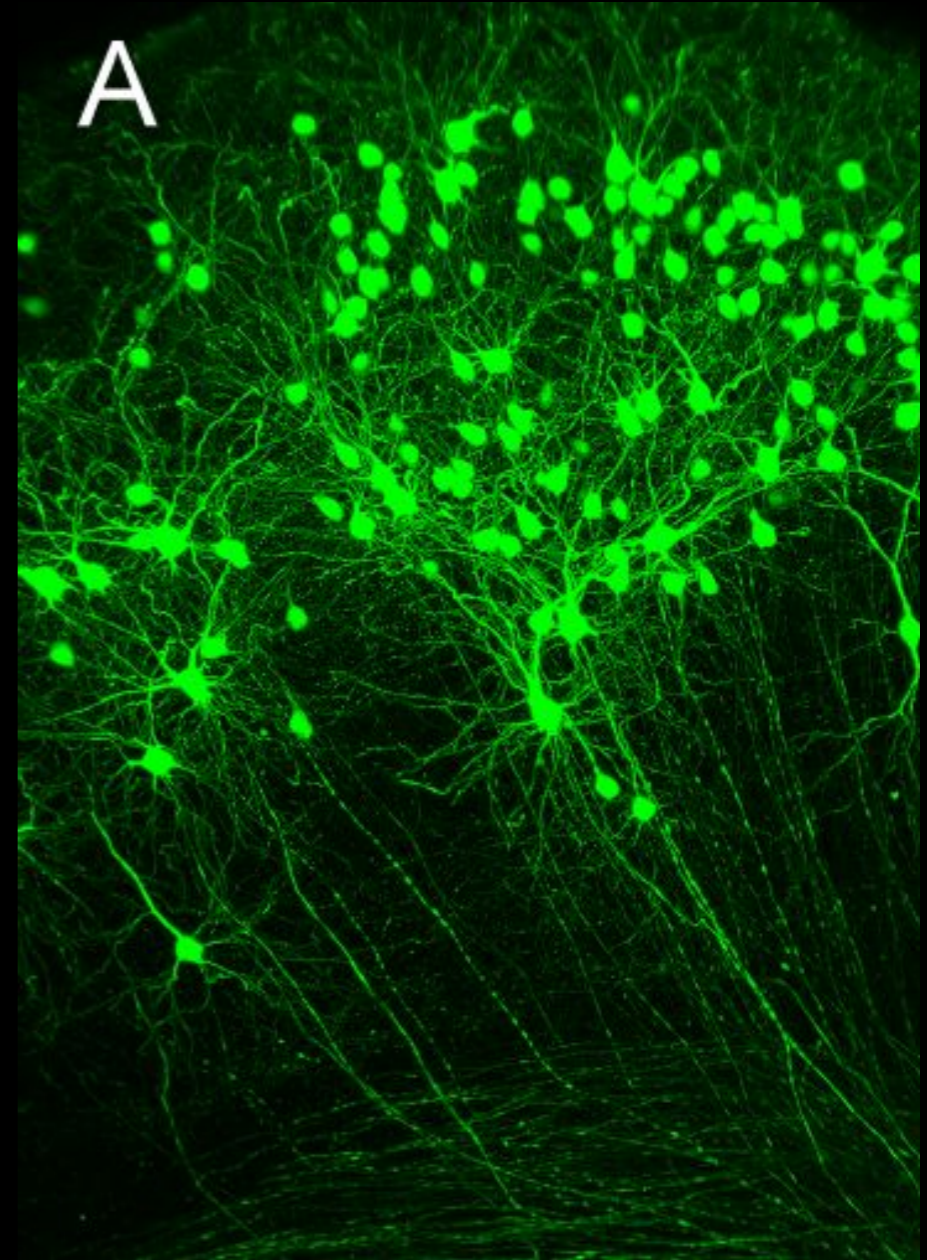
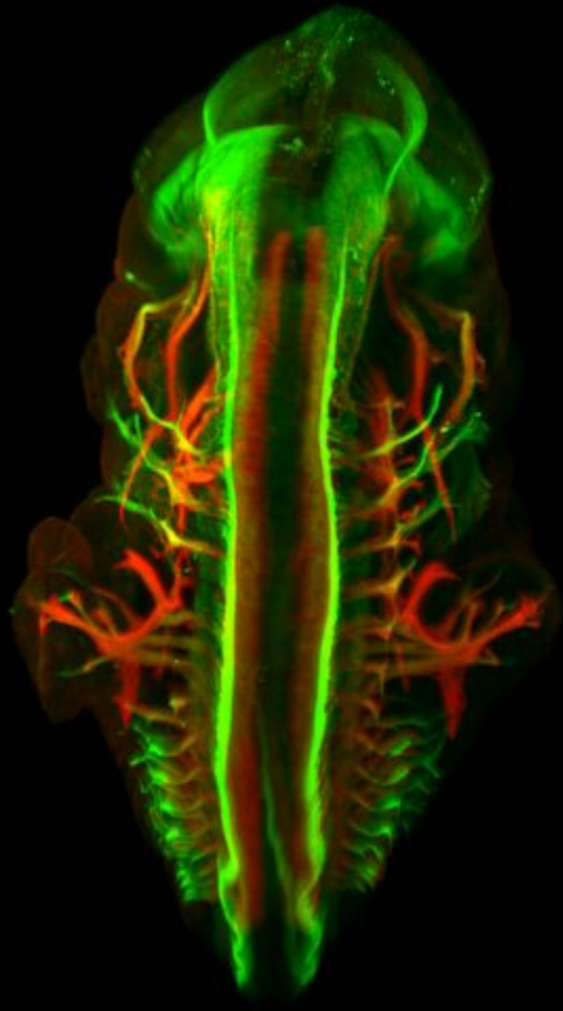
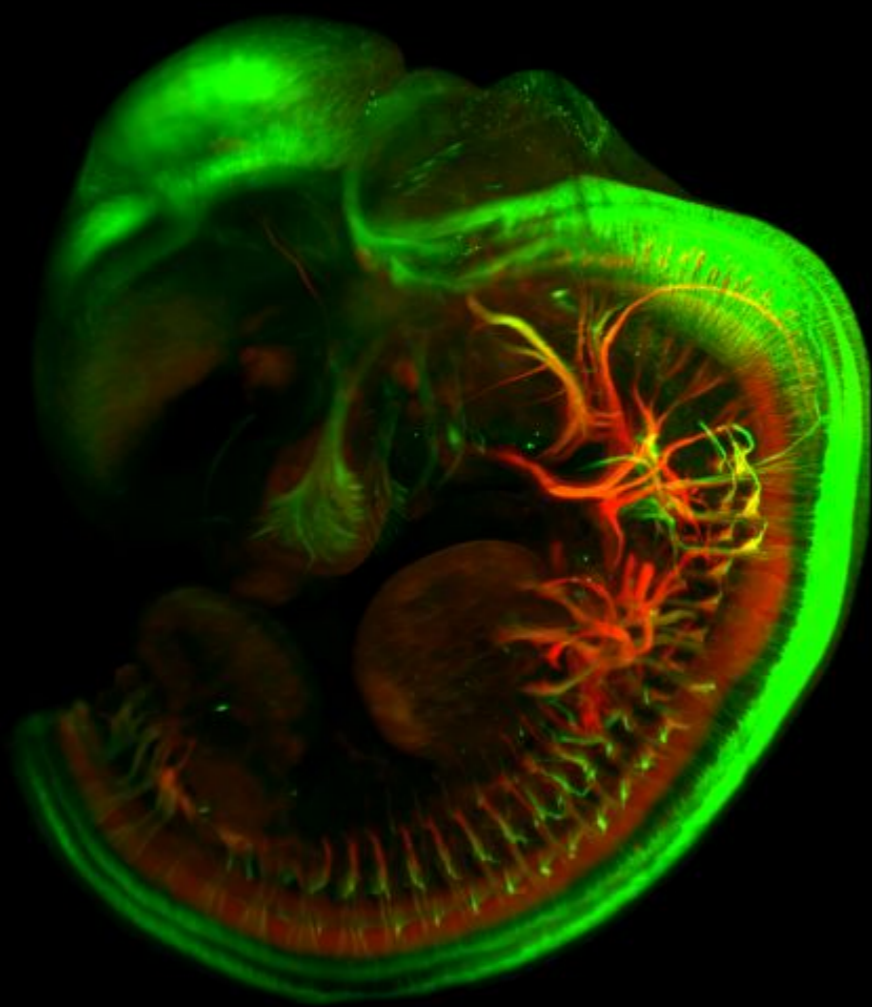
Before



After CLARITY

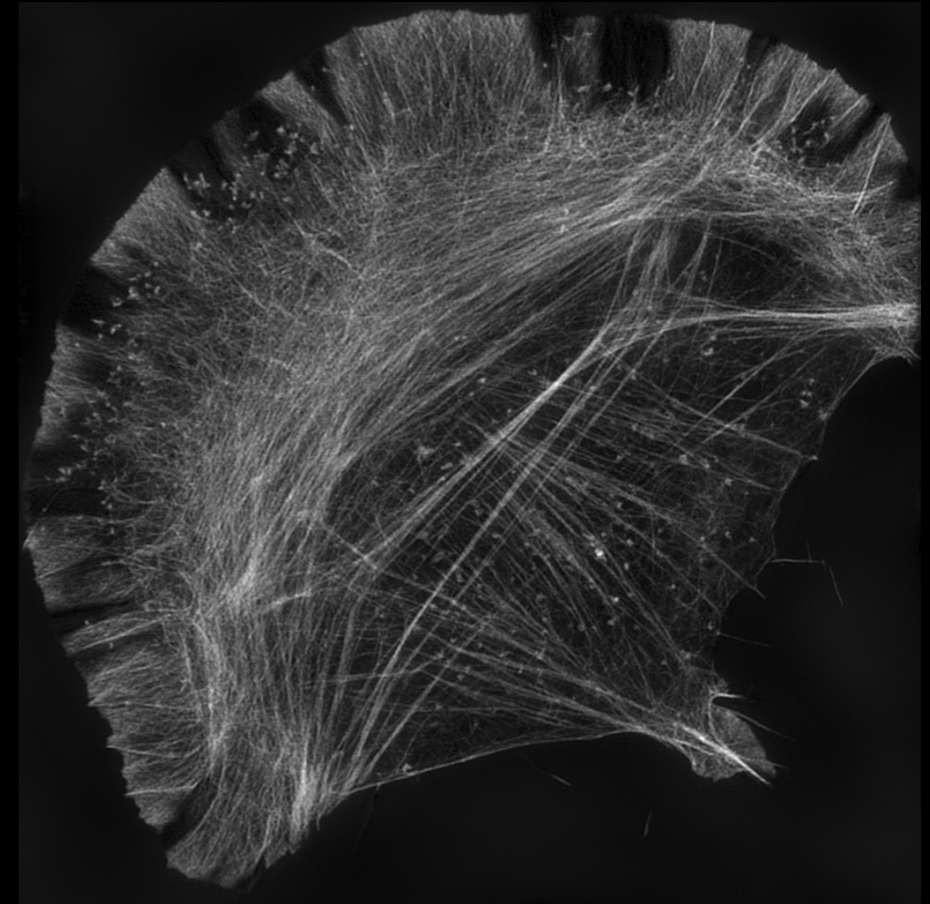


“iDisco” tissue clearing



Nicolas Ranier, Rockefeller University, Mouse embryo neurons (left) and cortex (right “A”)

SIM



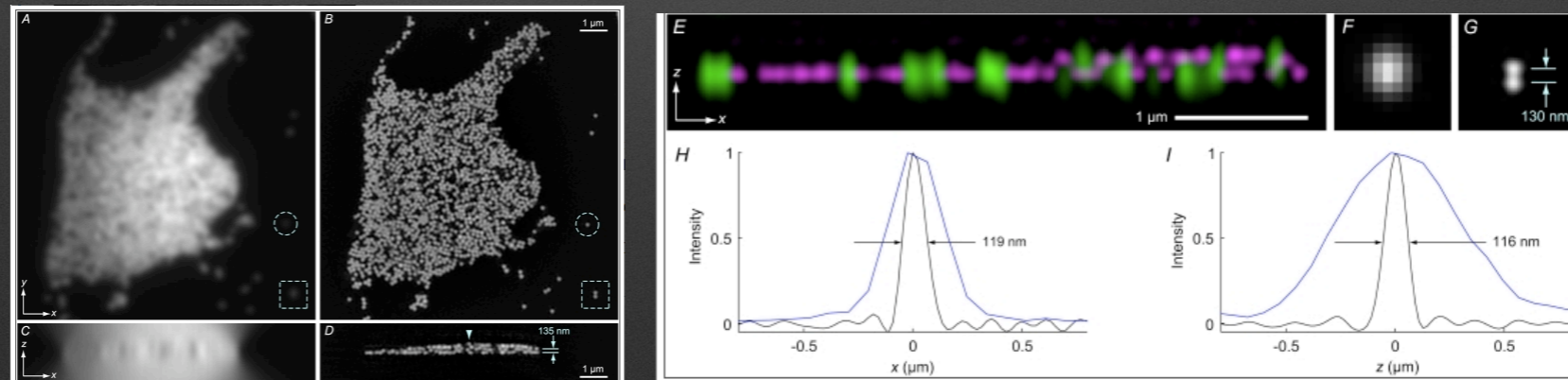
- “Confocal done right”
- Optical sectioning
- Two-fold resolution extension
- Wide-field imaging
- IMAGE RECONSTRUCTION NECESSARY

Assumptions

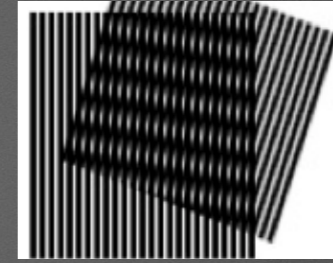
- An axiom is only valid under specific conditions
- The diffraction limited is defined by three conditions:
 - Limited NA
 - Uniform illumination
 - Linear responses
- Change these conditions and the limit **does not apply!**
 - Super-resolution microscopy resolves smaller than 200 nm

Break Axiom 1: Limited NA

- Ideally, we should extend the NA to image all the way around the sample
 - Sample mounting is a major challenge
 - Dual objective is one approach:
examples: 4 pi imaging, I5S imaging



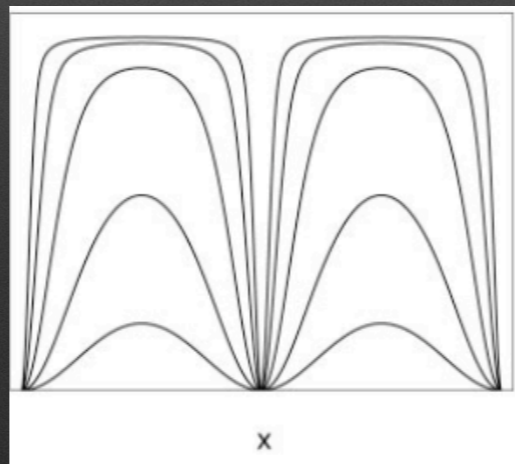
Break Axiom 2: Uniform illumination



- Use patterned illumination to create effects that let us extract more information from the sample
 - SIM uses striped light
 - More complicated patterns are also possible
 - Confocal microscopy and STED are other examples of using non-uniform illumination to break the diffraction limit

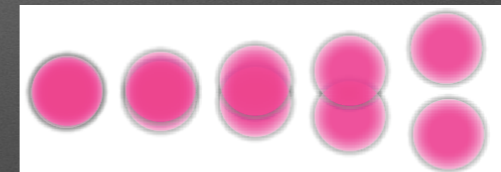
Break Axiom 3: Linear processes

- Introduce non-linearities
 - Can be done using saturation, two-photon imaging, or switchable probes
- This is the same principle used in STED



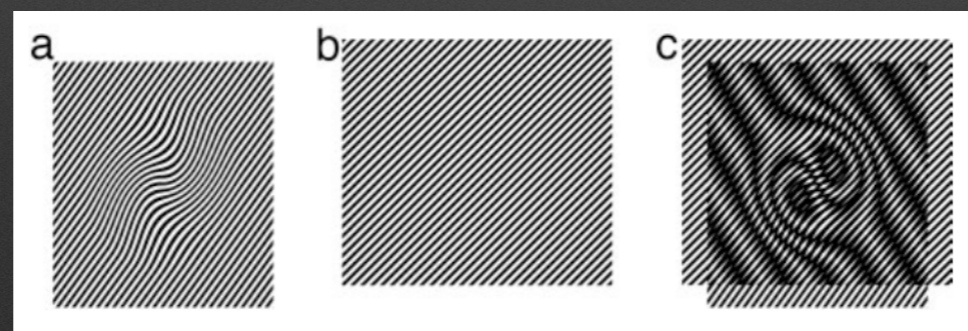
Resolution *versus* Localization

- Localization precision is much higher than resolution
 - **Localization:** Given that there is only one point, where is the center of the blurry point?
 - **Resolution:** In an unknown sample, resolve infinite number of points
- Localization: PALM / STORM
 - Use switchable fluorophores to imagine one point of the sample at the time, then combine the information to a super-resolution image (tens of nanometers)



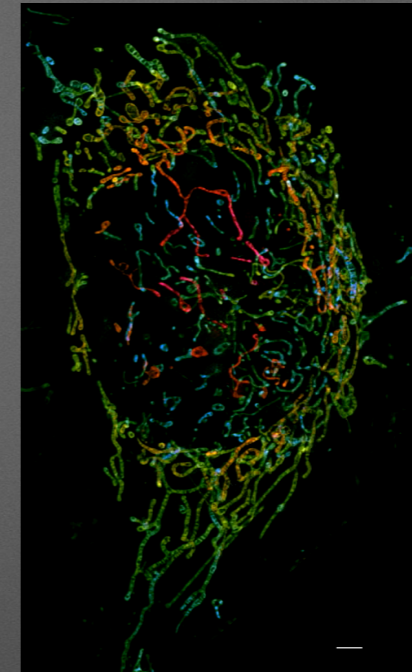
Structured Illumination Microscopy (SIM)

- Structured Illumination Microscopy uses non-uniform illumination to create Moire fringes from very small structures in the sample
- The fringes are big and can be imaged
- We are moving information into the observable region



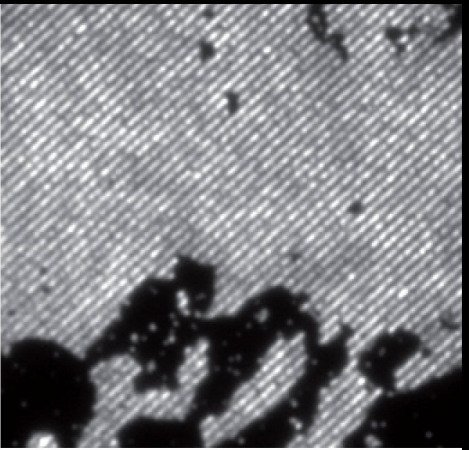
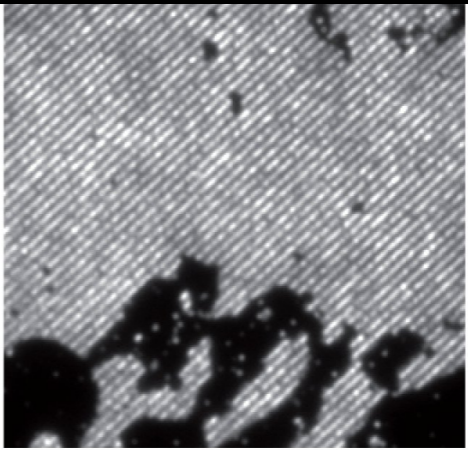
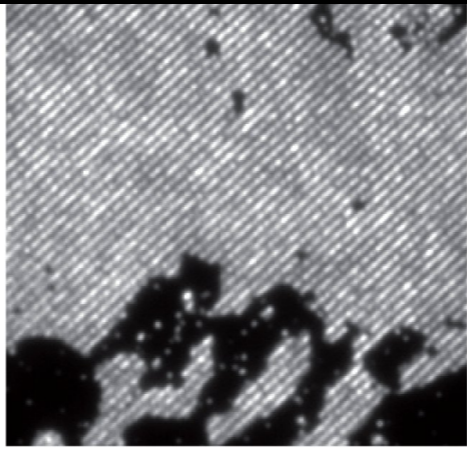
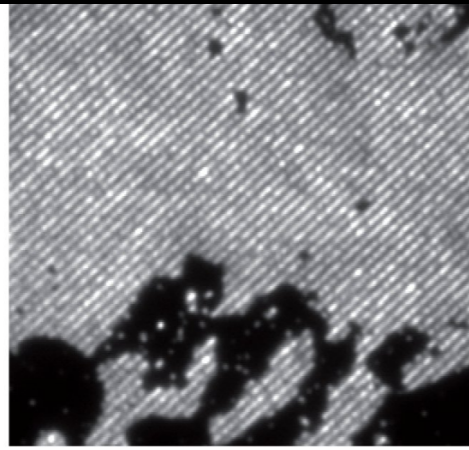
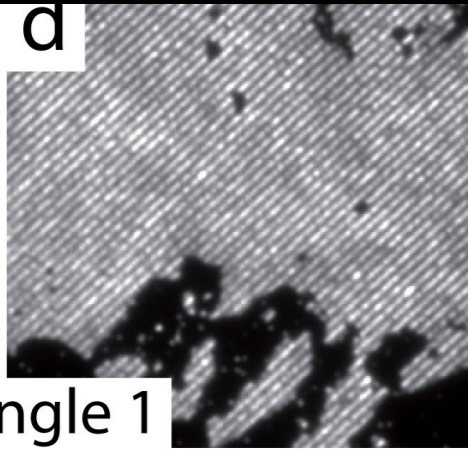
SIM

- Truly super-resolution
- Relatively mild light-dose
- Relatively fast
- Commercially available as “OMX”, Zeiss “Elyra” and n-SIM
- Linear SIM does not require special probes
- Non-linear SIM has theoretically unlimited resolution

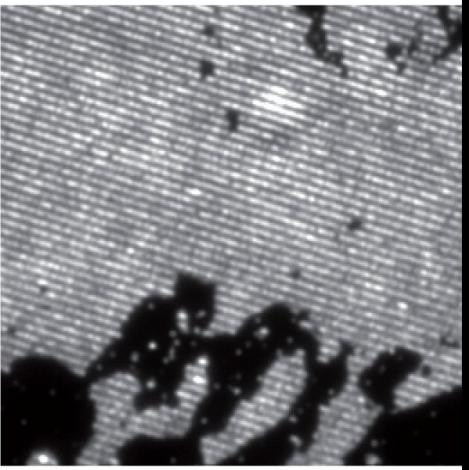
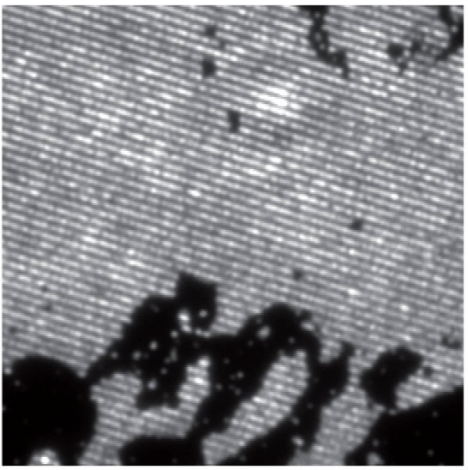
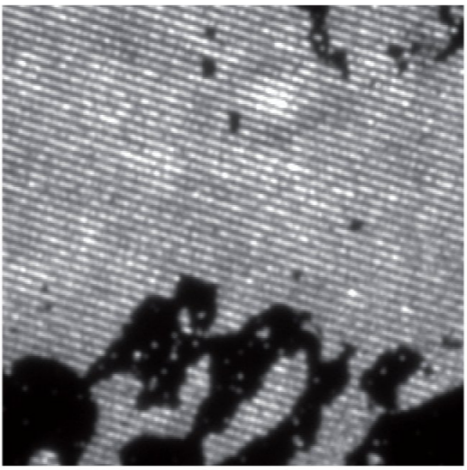
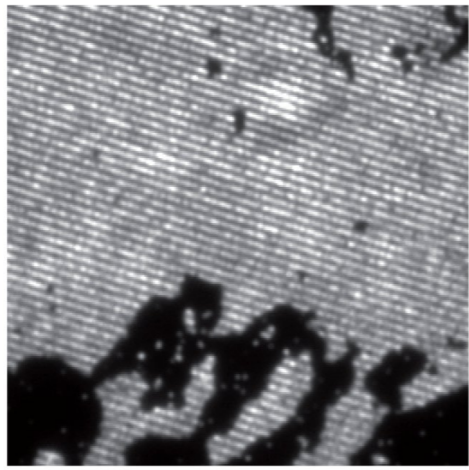
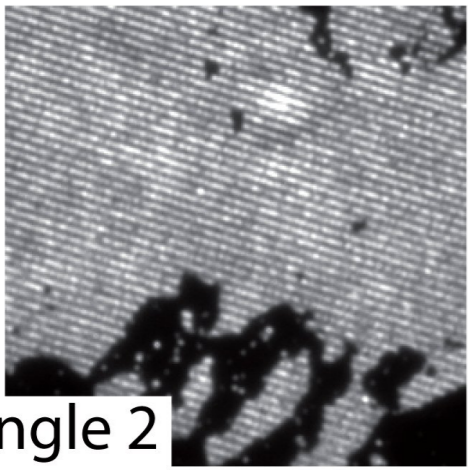


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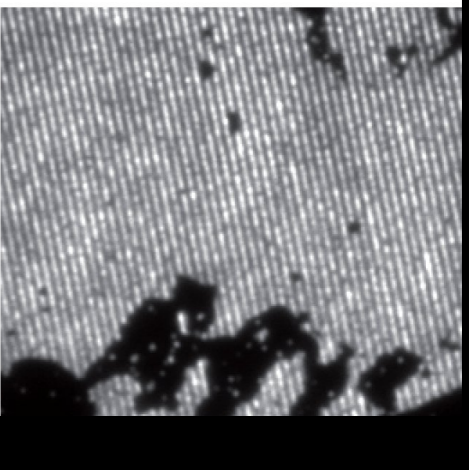
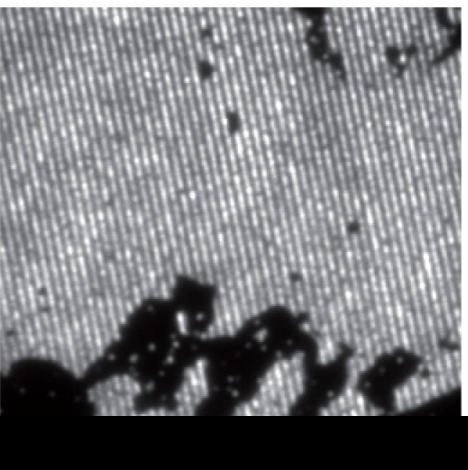
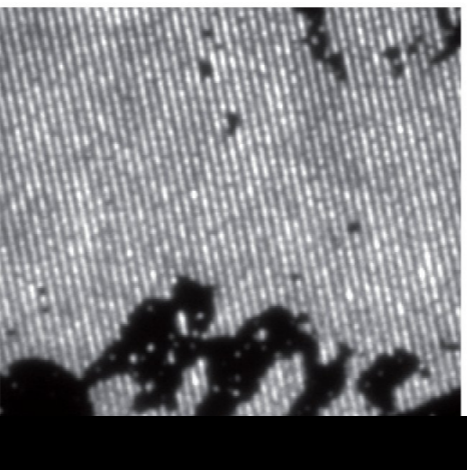
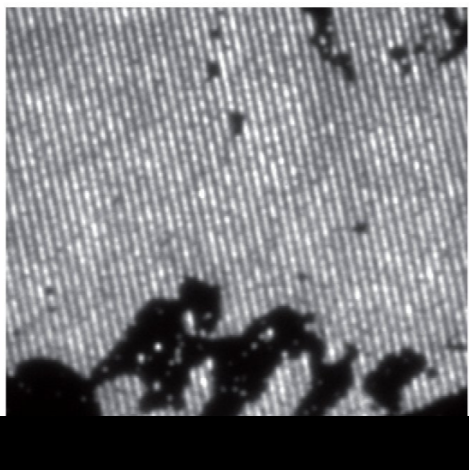
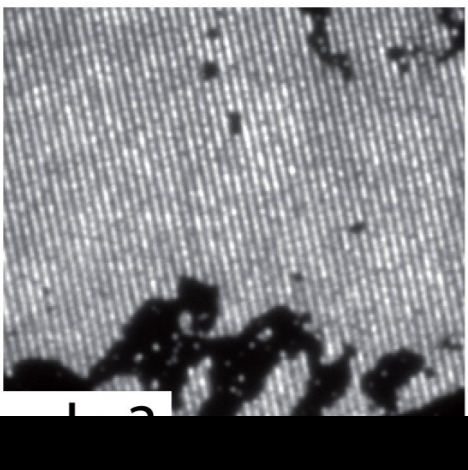
Angle 1



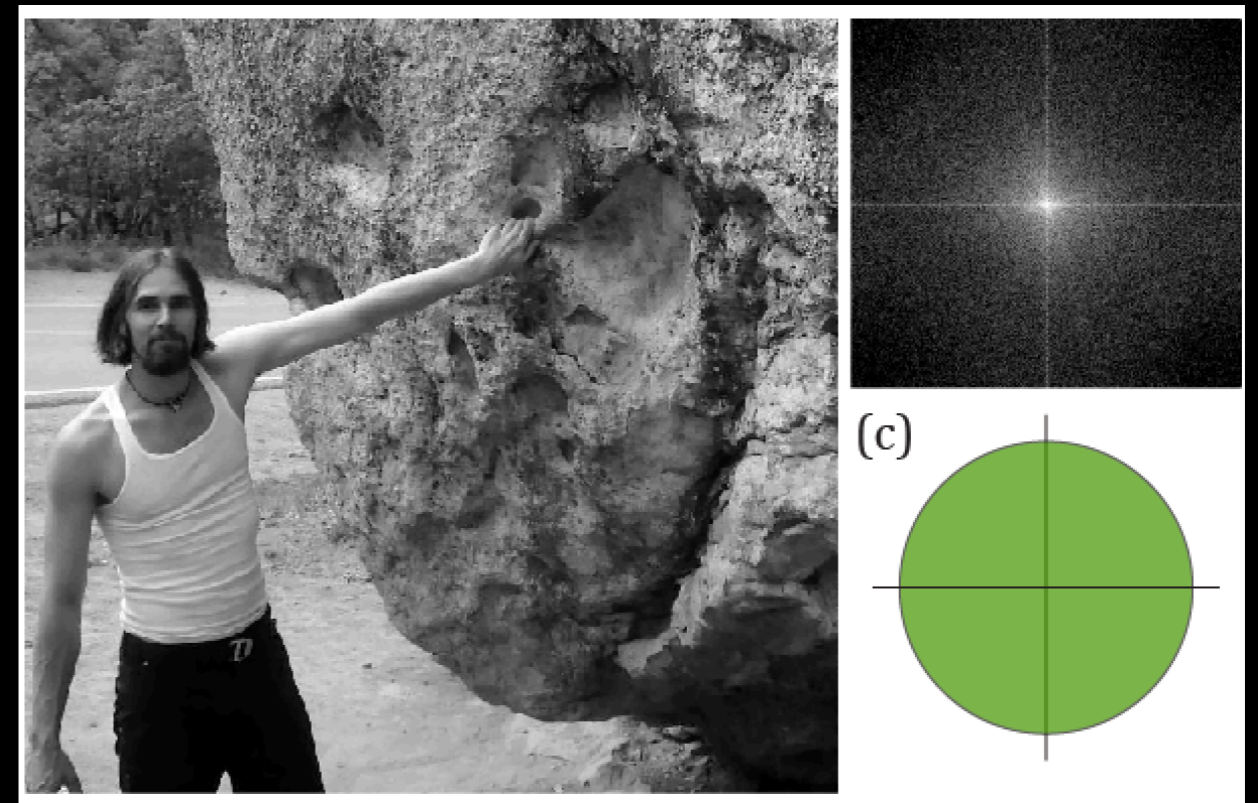
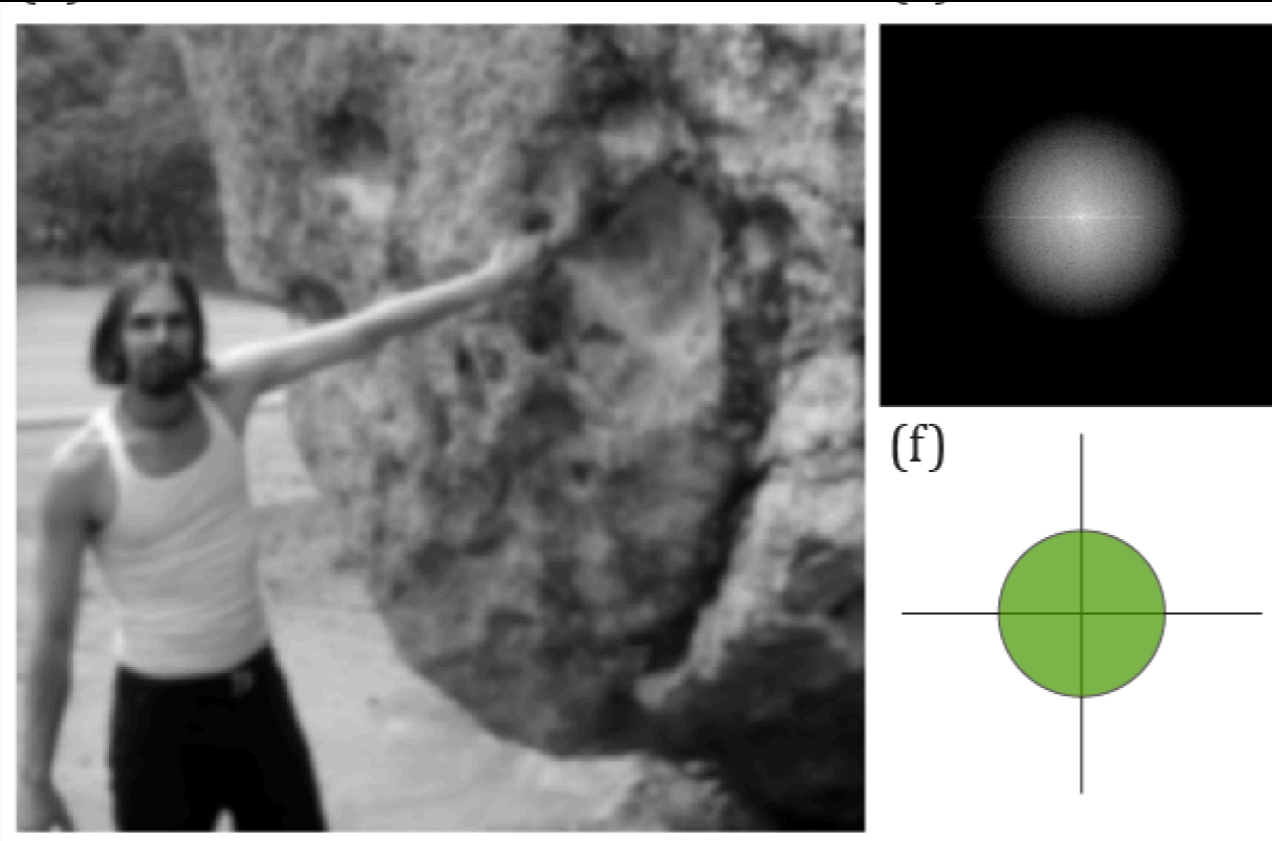
Angle 2



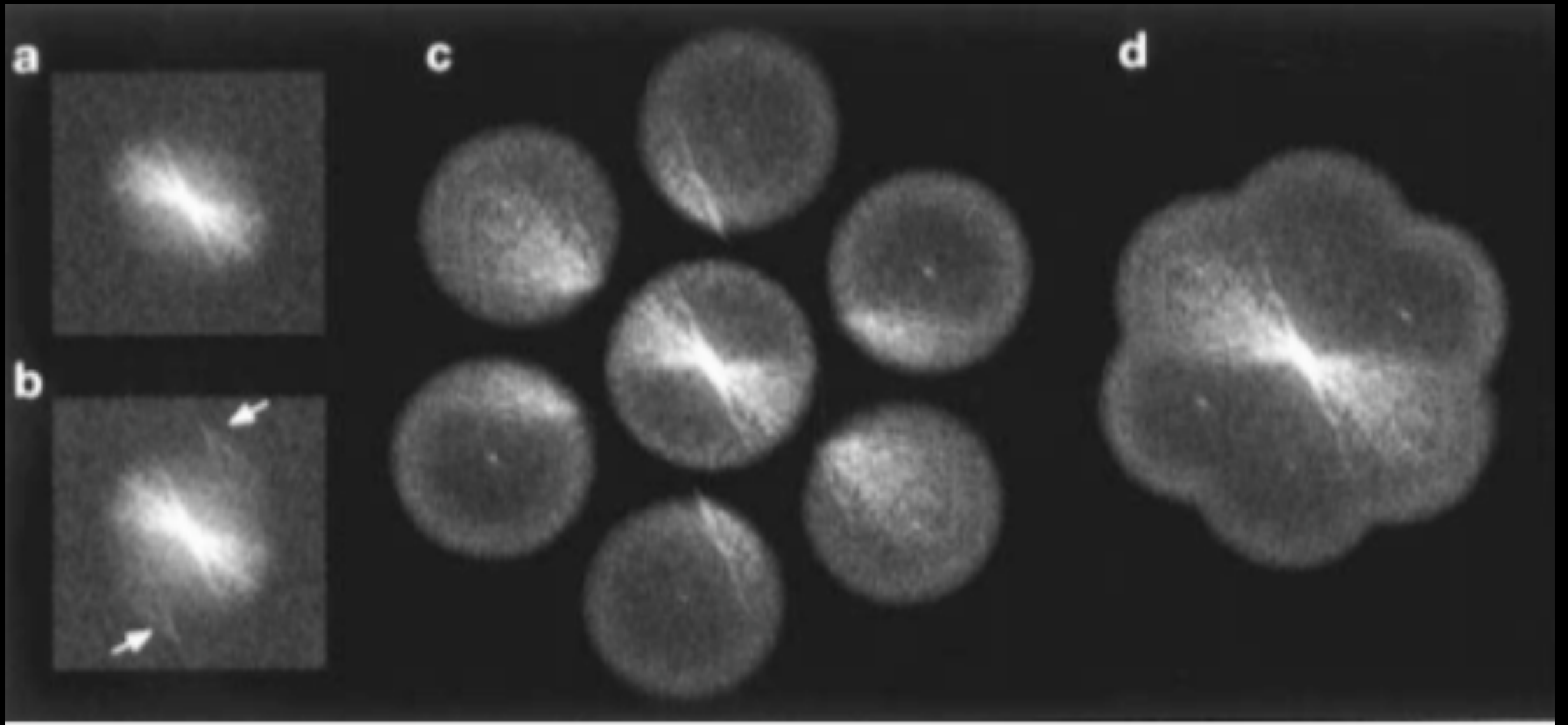
Angle 3

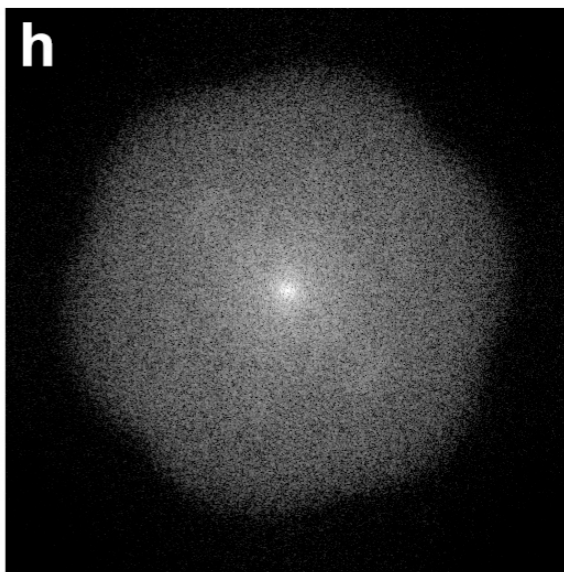
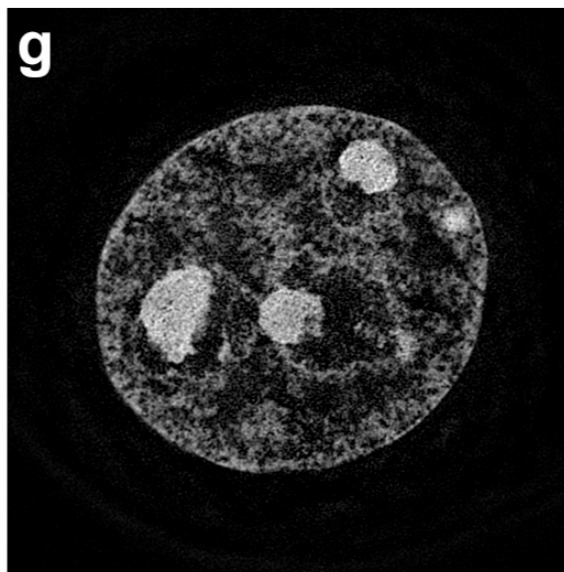
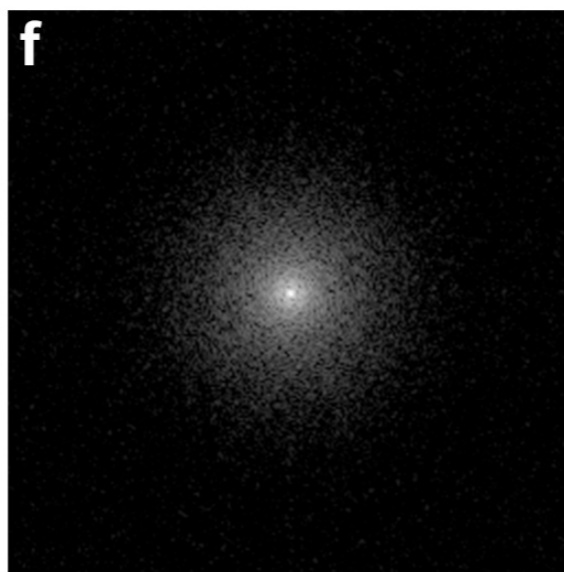
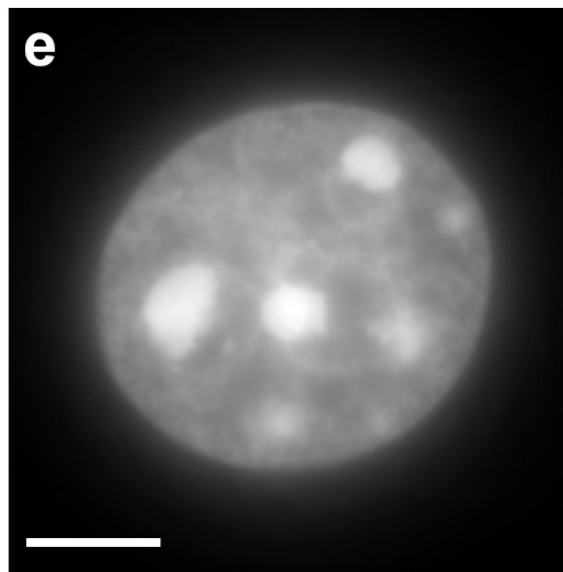
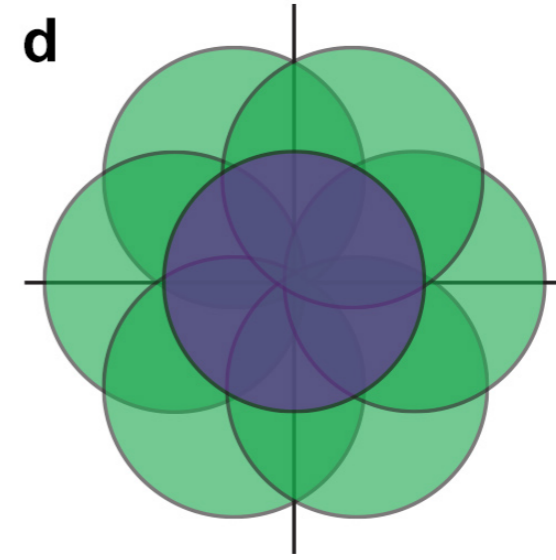
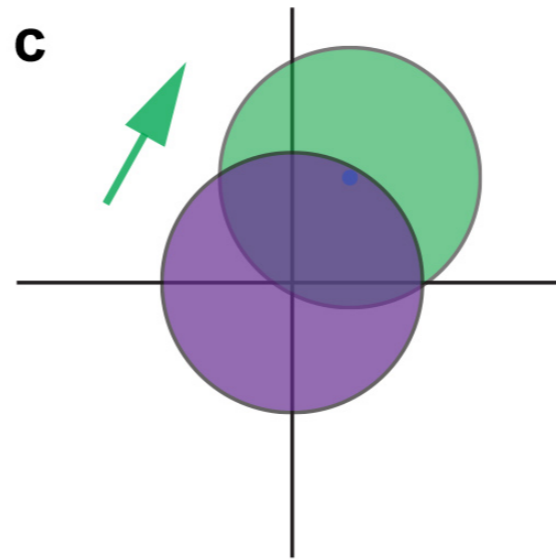
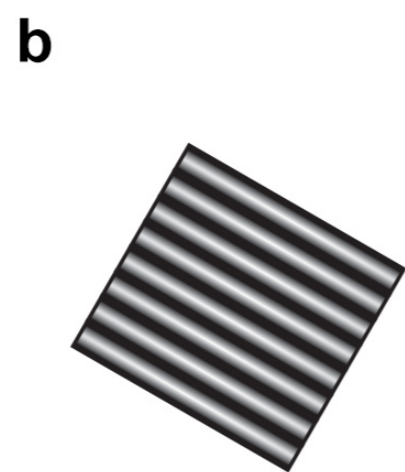
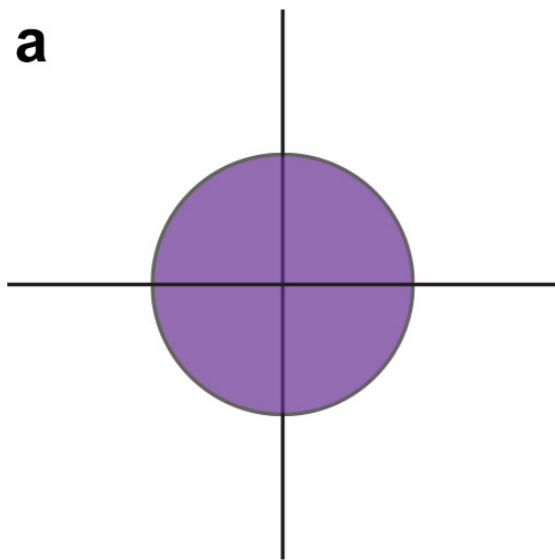


Remember Fourier space



SIM reconstruction

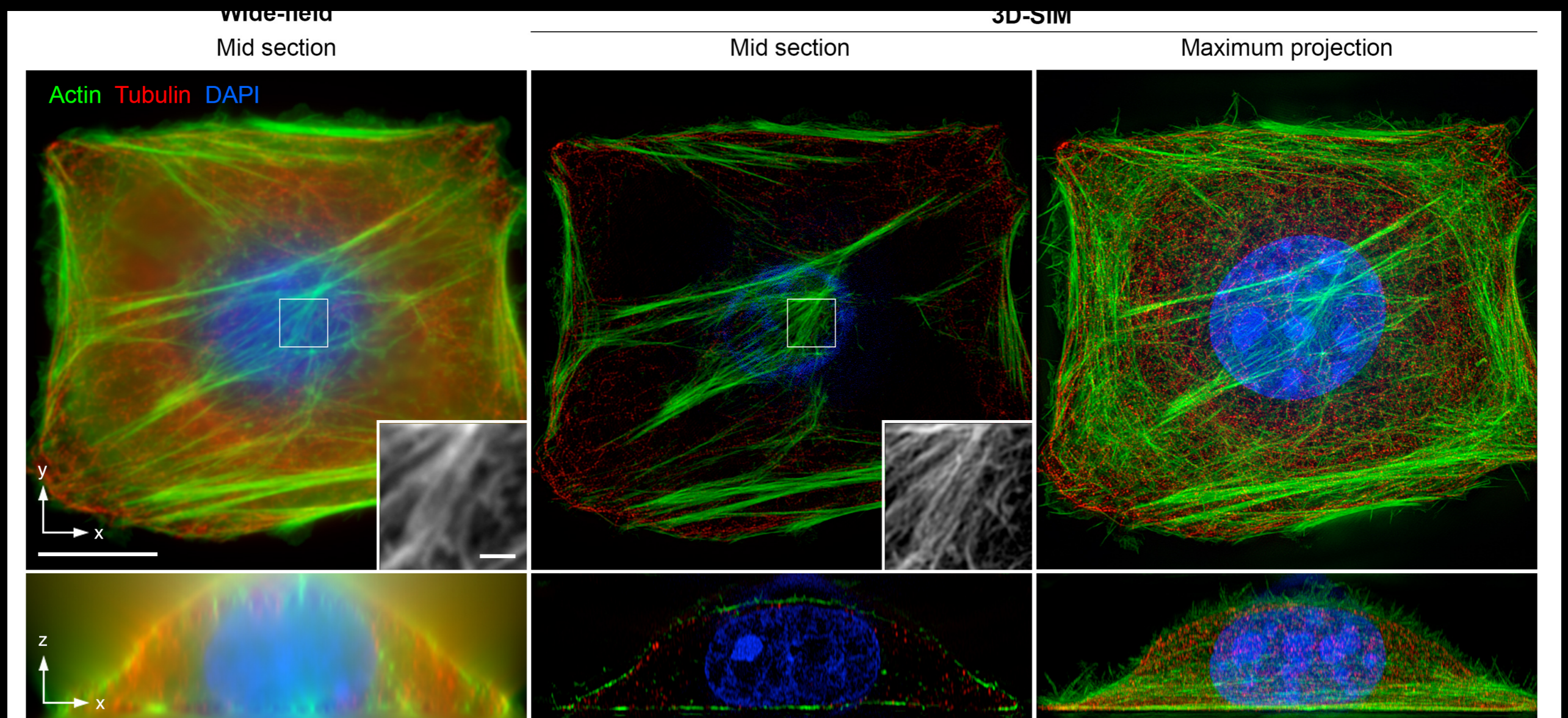




SIM-check

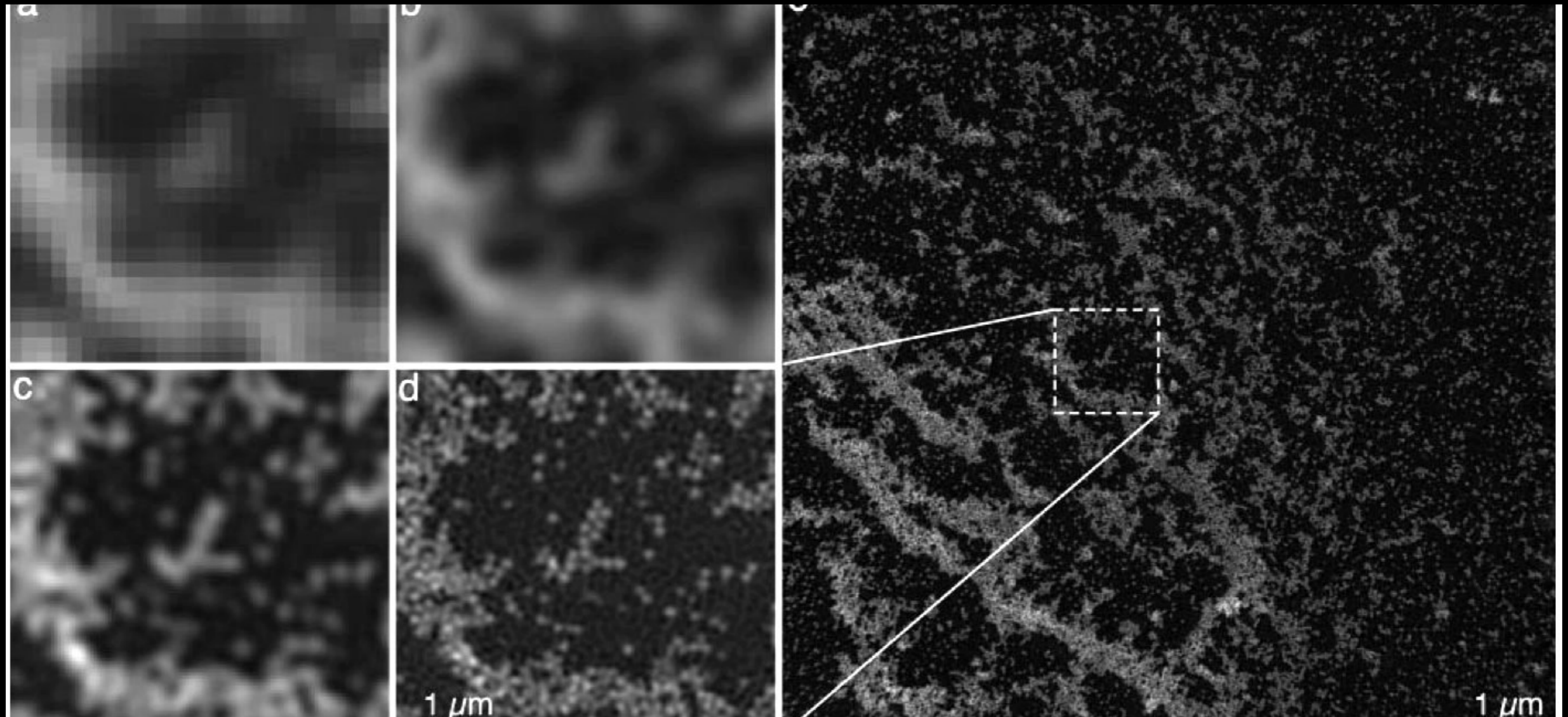
- SIM reconstruction is very reliable when done “right”
 - Linear, quantitative, no guessing
- Data processing is demanding
 - SIM check for ImageJ is now available to check your data analysis!

Optical sectioning



Data by Lothar Schermelleh, Micron Oxford

NL-SIM on fluorescent beads



NL-SIM in a cell

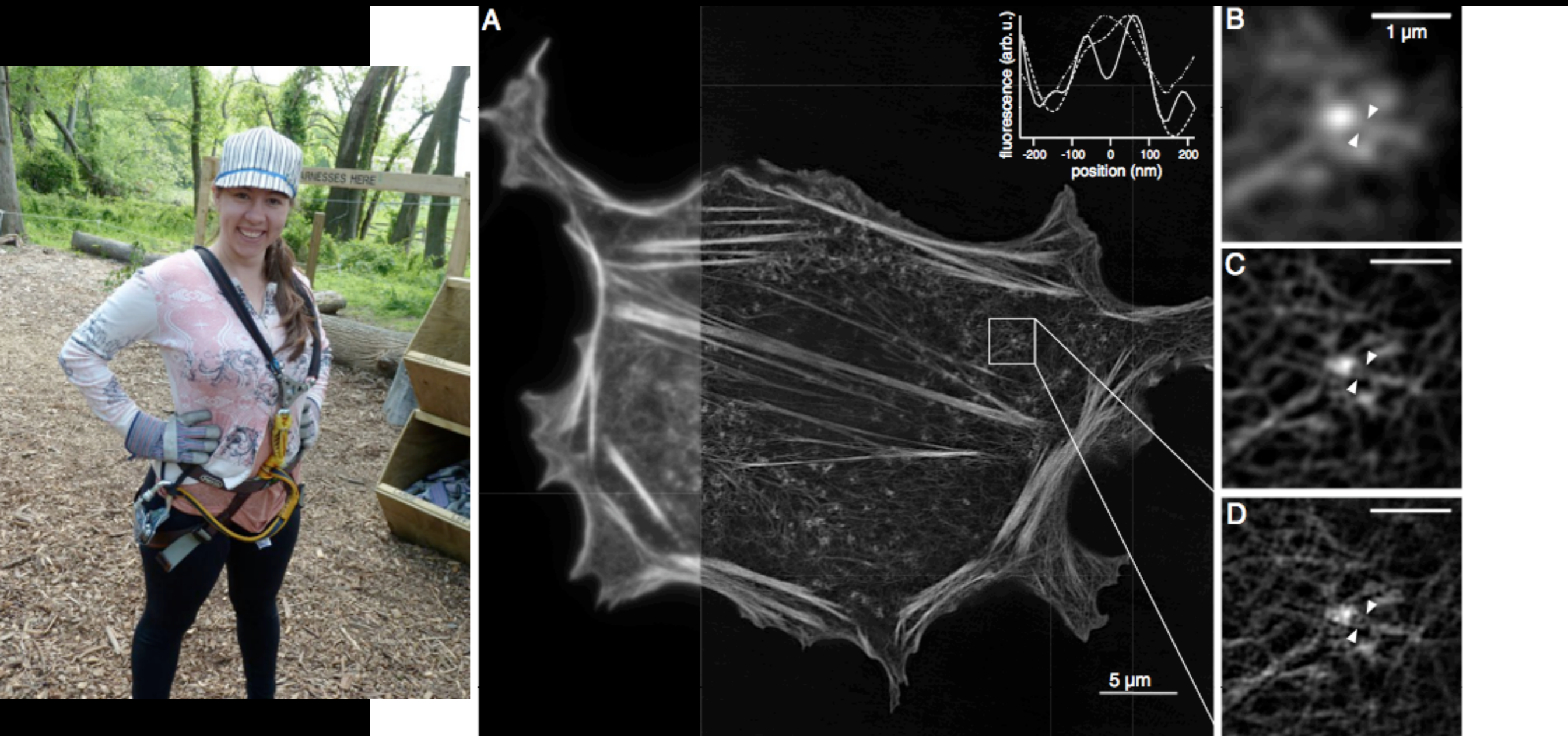
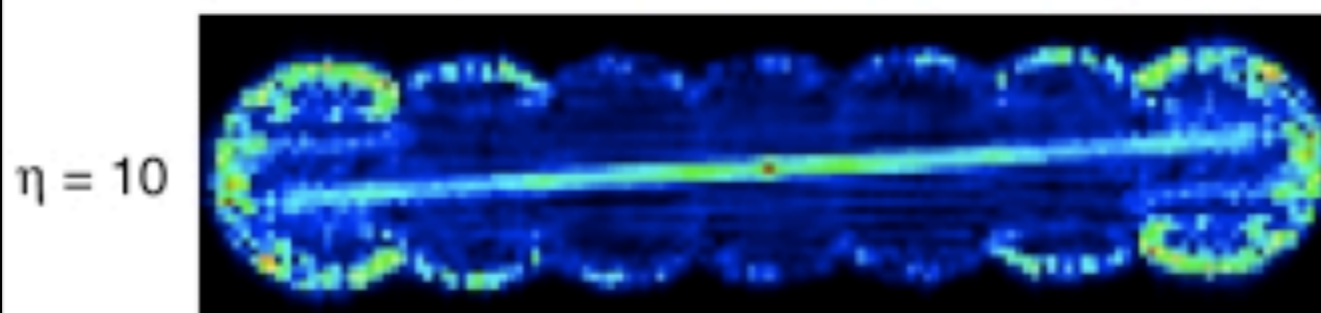
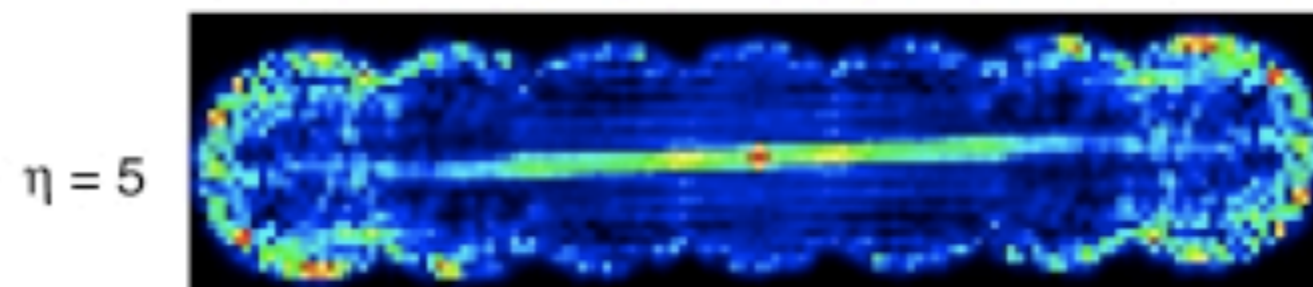
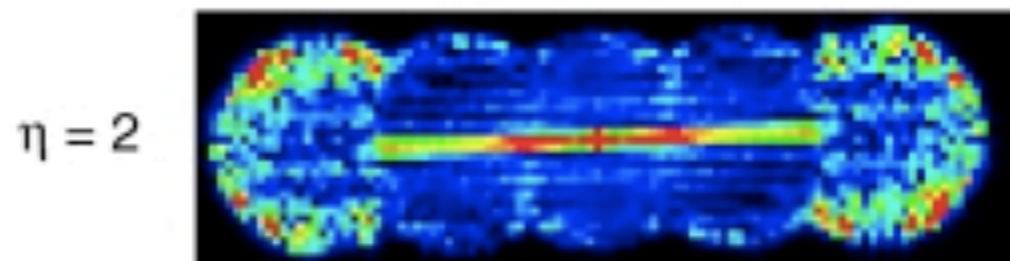
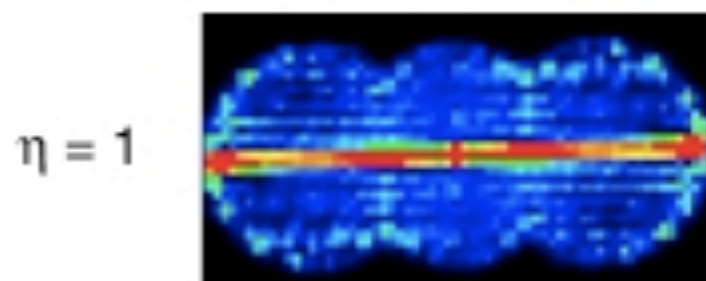
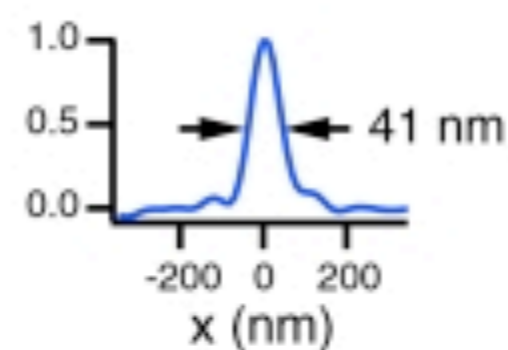
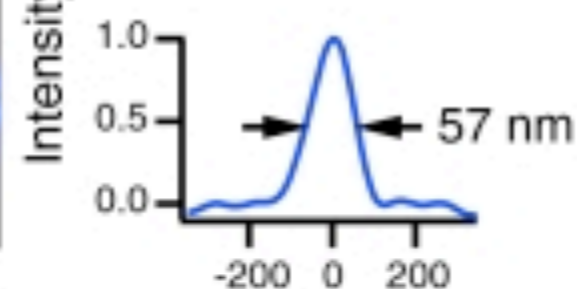
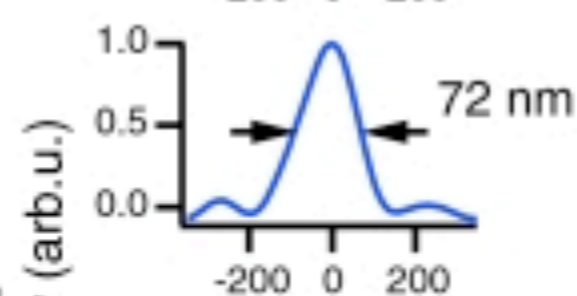
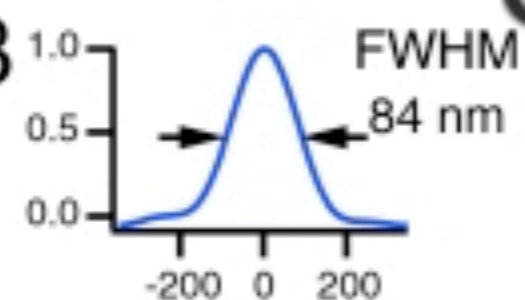
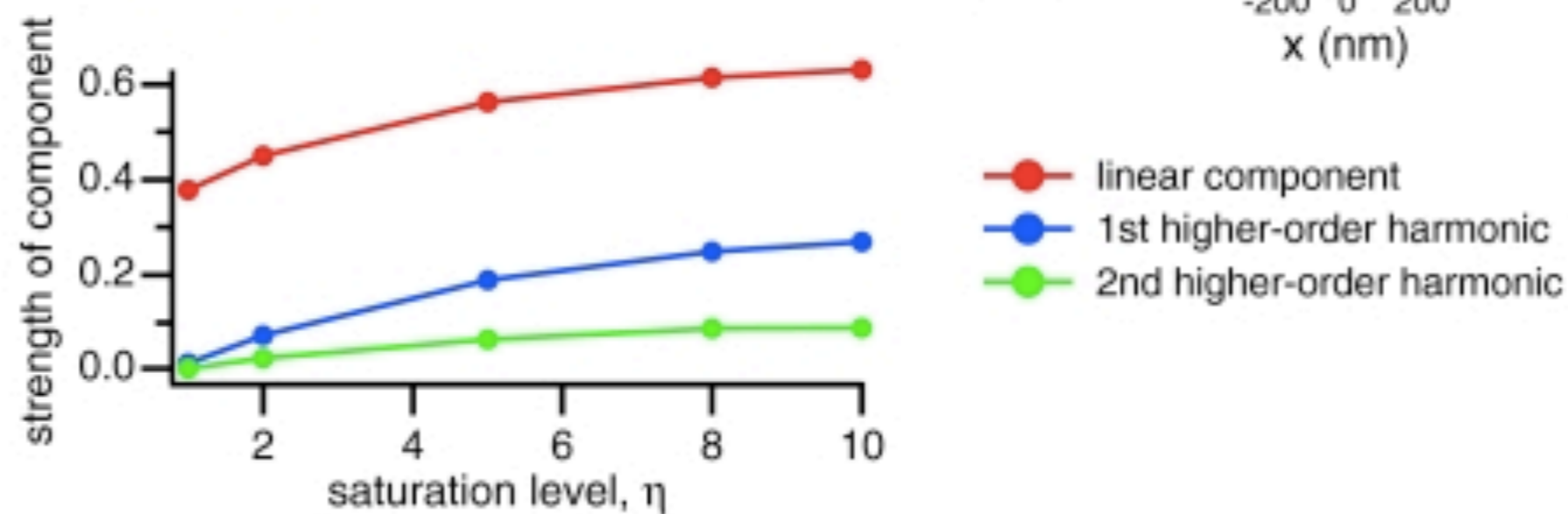
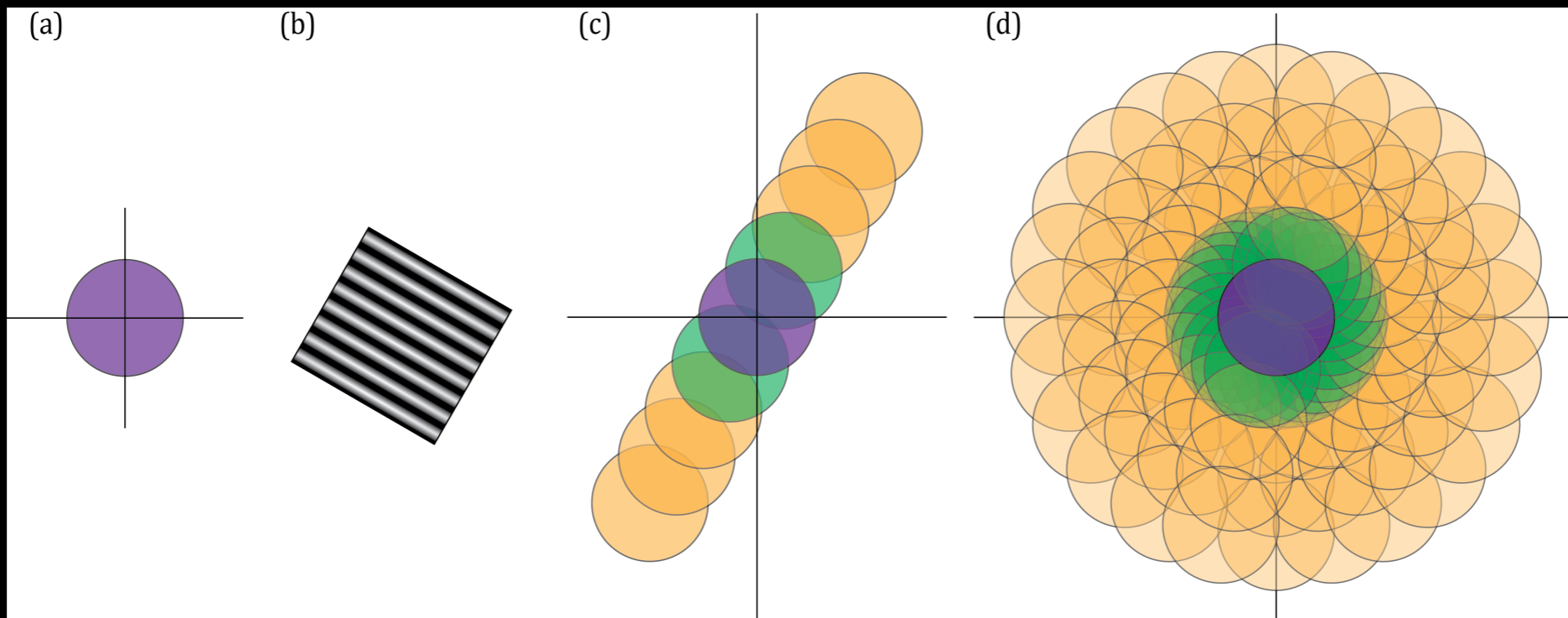
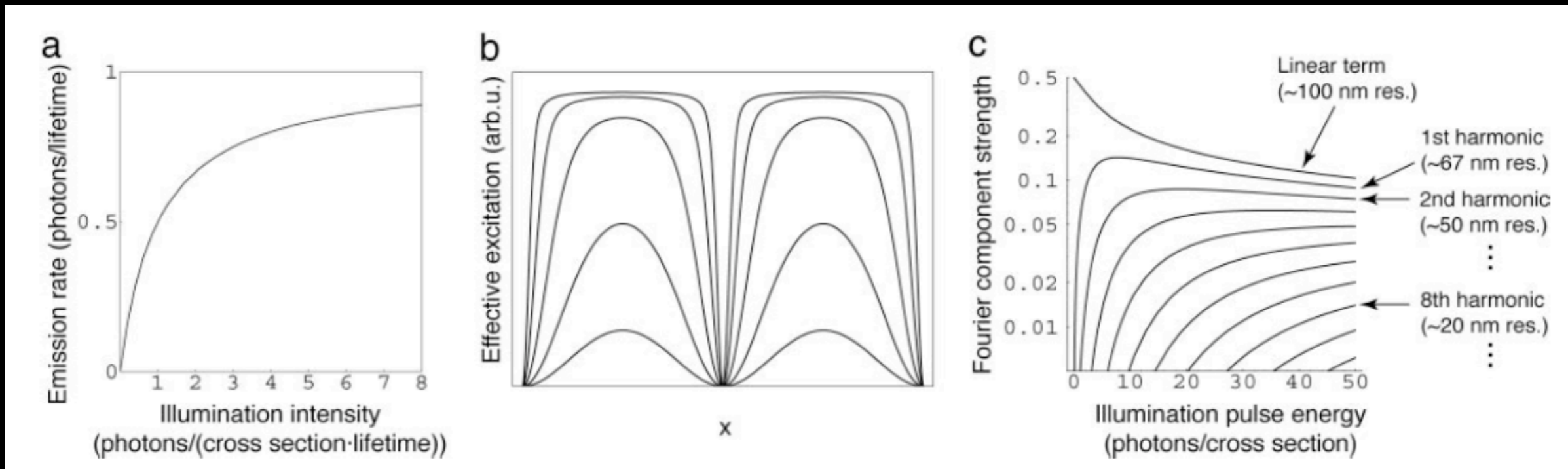


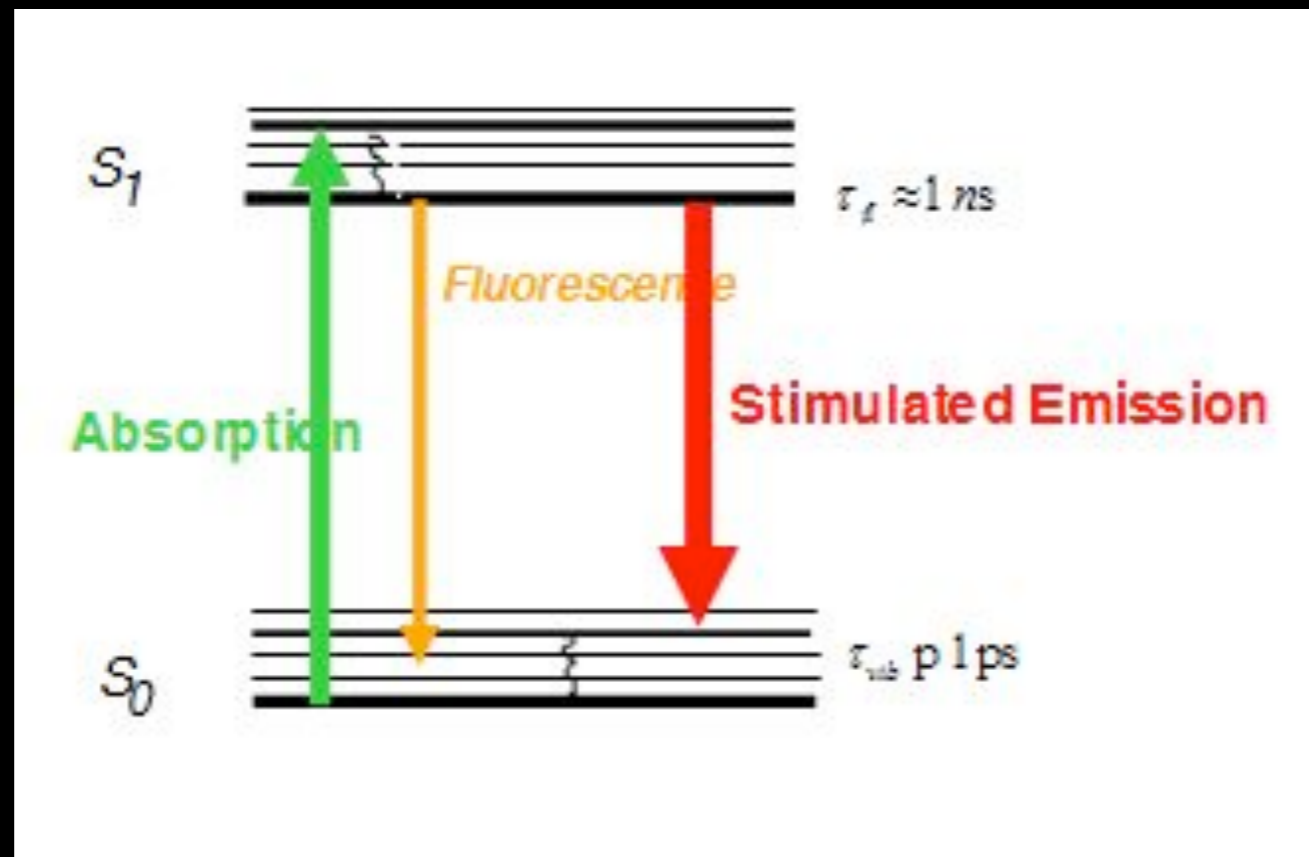
Fig. 6. Dronpa-Lifeact in a mammalian CHO cell imaged with NL-SIM. (A) Because SIM is a wide-field technique, high-resolution data over a large field of view is possible. On the left, a portion of the entire image is shown with conventional microscopy. The image is displayed using a nonlinear intensity scale ($\gamma = 0.65$) to highlight the small filaments in the background over the thick and bright stress fibers. A subset of the data is enlarged and shown with (B) conventional TIRF microscopy, (C) linear SIM-TIRF, and (D) nonlinear SIM-TIRF. Lifeact marks the actin network, the structure of which is most clearly resolved with nonlinear SIM-TIRF as demonstrated by the normalized intensity profiles (*Inset*) taken between the white triangles in B–D). Two filaments are clearly resolved with NL-SIM (solid), but are not resolved with either conventional microscopy (dot dashed) or linear SIM (dashed). Scale bars, 5 μm (A) and 1 μm (B–D).

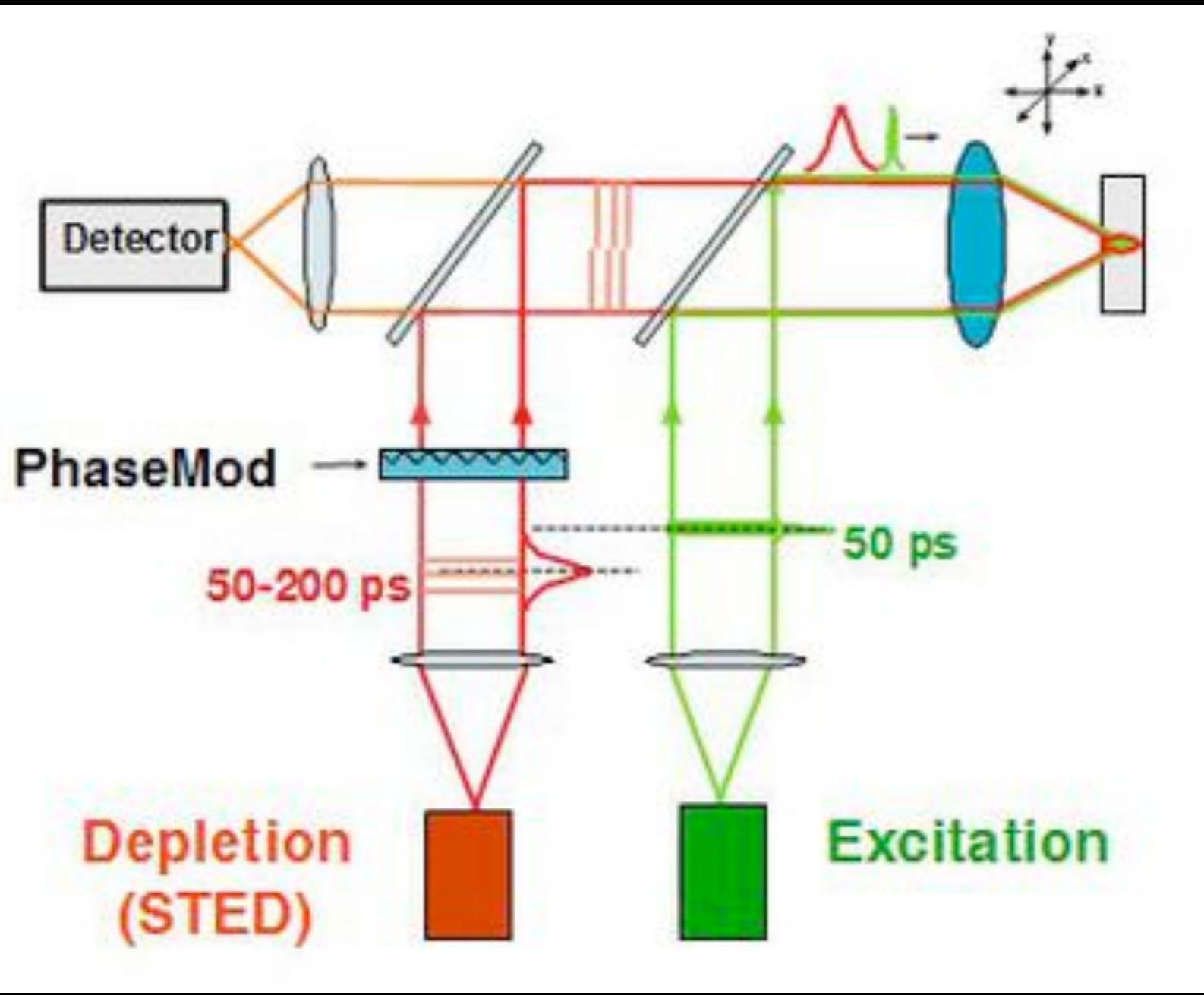
A**B****C**



STED: Scanning super-resolution

- Stimulated emission depletion

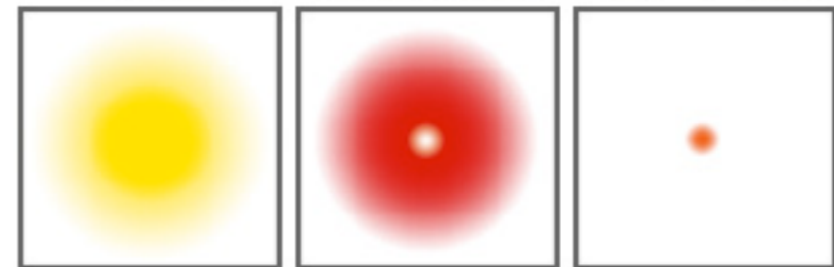




detector



exc. donut STED



dichroic mirror



laser head LDH Series

dichroic mirror



LDH-FA

phase plate



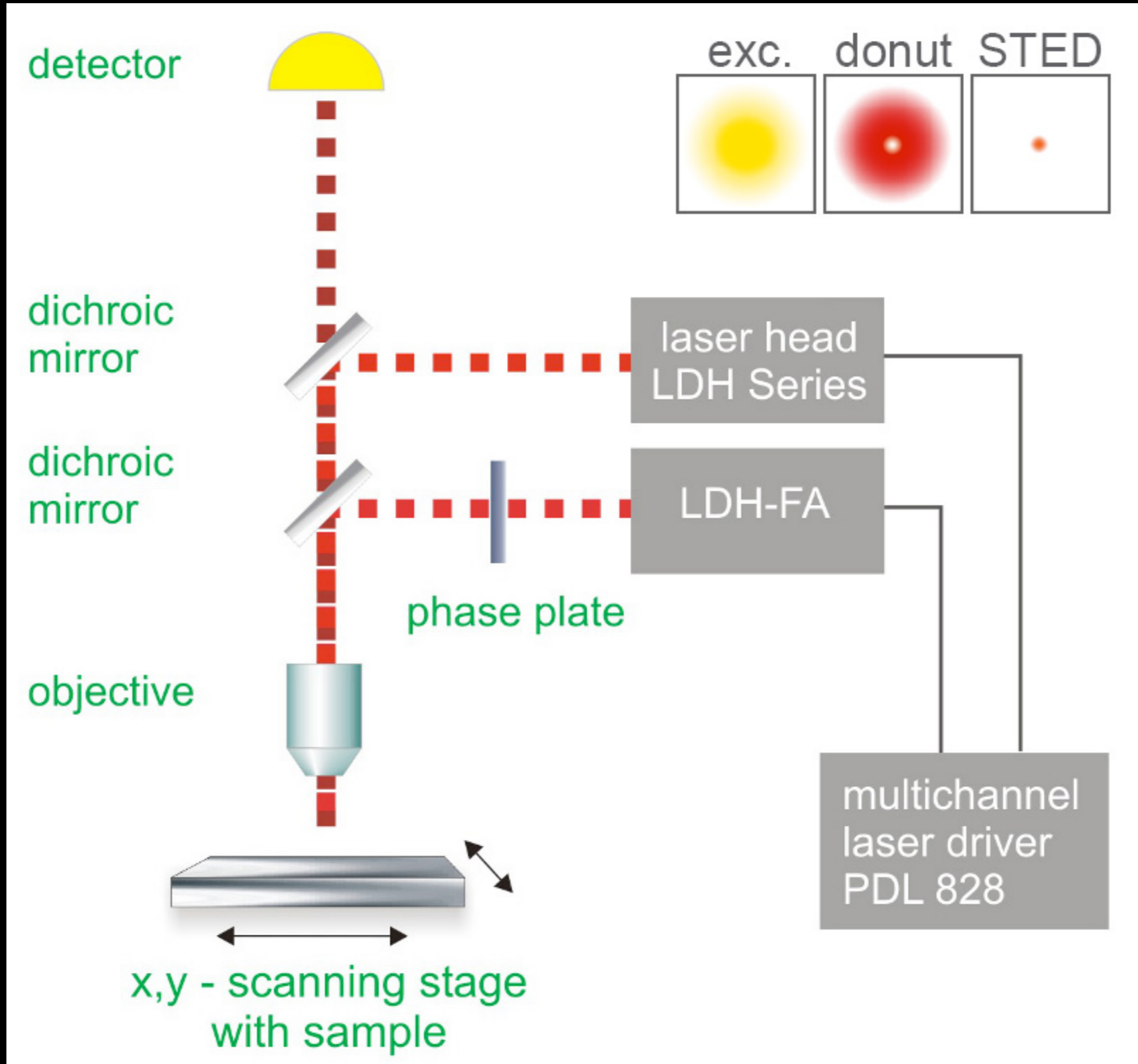
objective



multichannel laser driver PDL 828



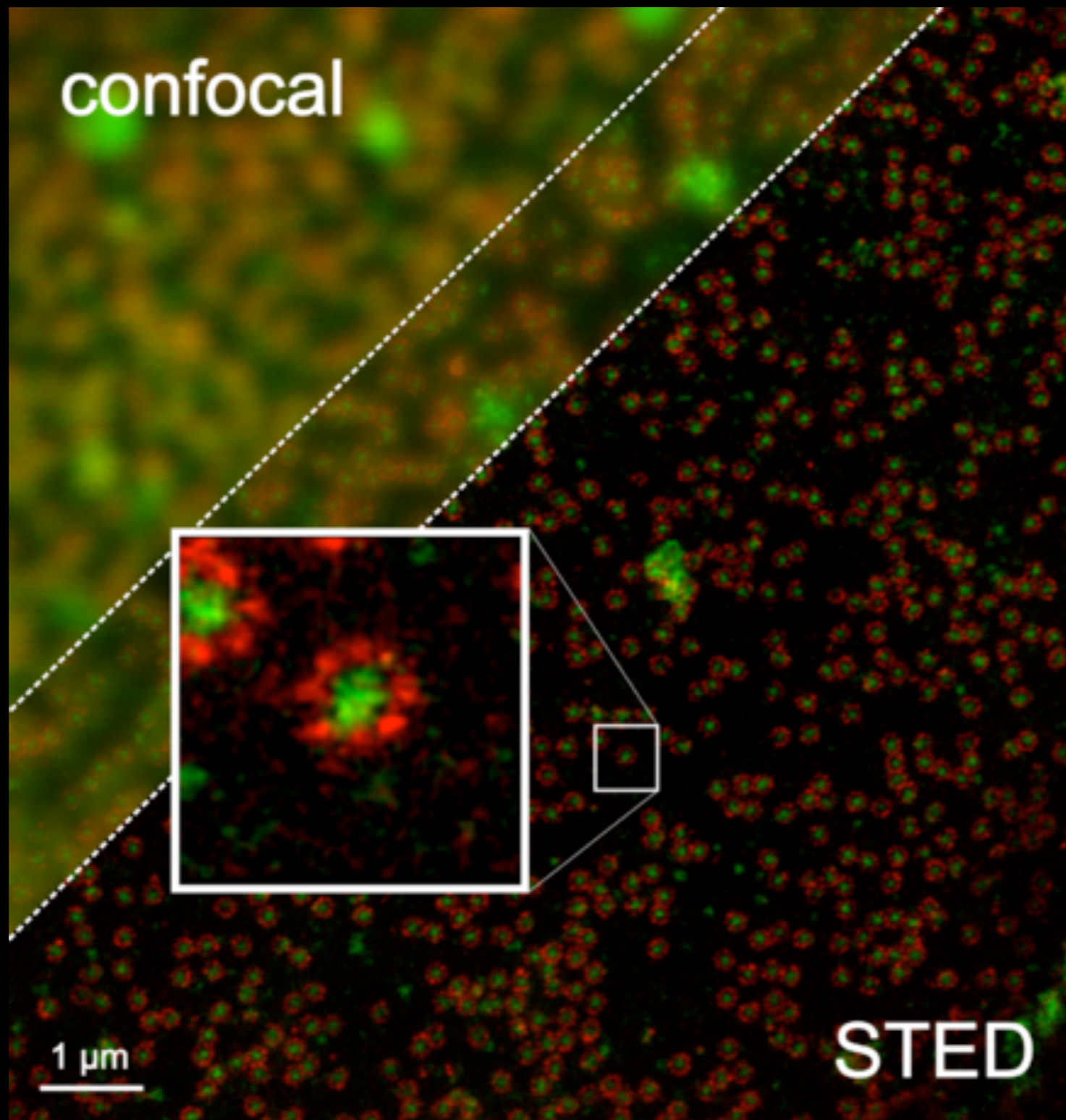
x,y - scanning stage with sample



Nonlinear effect

- Alternative way (Stefan's way) to describe it:
“It's all about the switching”

Insane resolution



Excitation light in the MW-GW/cm² range



Trying to illuminate a spot, but effectively we illuminate an hourglass volume. The unwanted light is blocked out. Image is “scanned” and “de-scanned”

