Super-resolution fluorescence microscopy systems compared.

First of all, thank you to the Committee for giving me the opportunity to come here and learn so much from you.

A personal view, concentrating on limitations related to too few photons and specimen refractive index heterogeneity

It is illustrated by dozens of stolen images.

My thanks to all of their owners, in particular to Stephan Hell and Jennifer Lippincott-Schwartz.

Jim Pawley

JBPawley@wisc.edu

The past decade has seen the introduction of a number of fluorescence light microscopy techniques that permit one to surpass the Abbe Resolution limit.

Although the resulting images are quite similar, differences in the operational details of these techniques can make them more or less suitable for specific types on biological studies.

It is my intent to highlight and explain these differences at a fairly basic level...

Assumptions:

- that the importance and utility of fluorescence microscopy is generally recognized and accepted.

- that what we really want is to be able to do living cell fluorescence microscopy better.

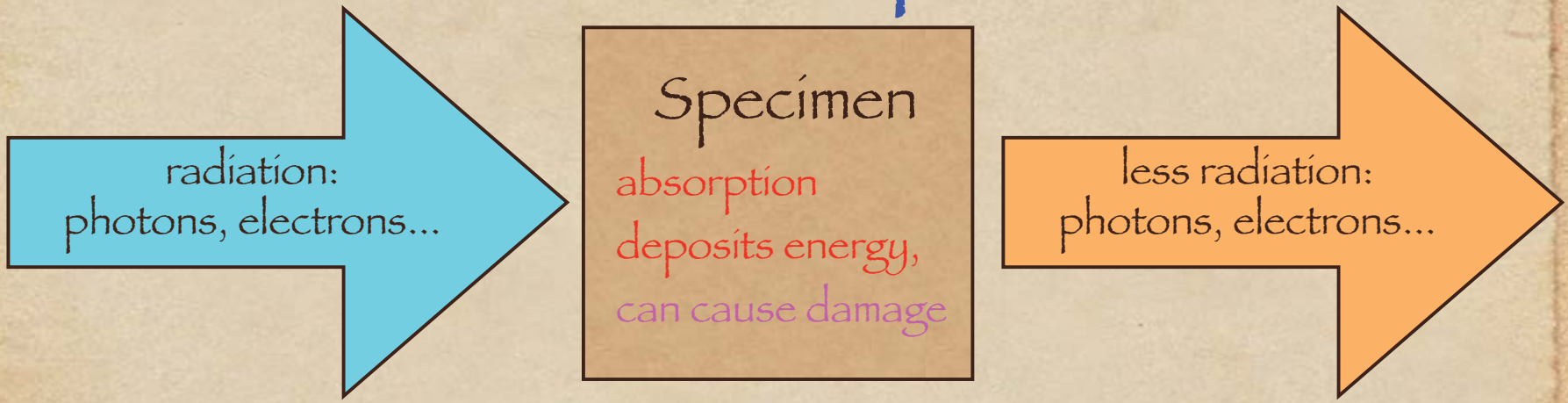
In Languedoc, I understand that you all search for the Holy Grail.

Perhaps the Cathars have it?

"The holy grail is to see dynamically imaged living cells noninvasively, to see at the level of an individual protein molecule, and to see how the molecules interact in a cell," Betzig says. "It's many years away, but you can dream about it.*"

Is this reasonable?

What is a microscope?



- Microscopy is about making images of small things.
- To form an image, a flow of radiation must strike the specimen, where it is modulated (differentially scattered?) by the structural features, producing contrast (and perhaps damage?).
- These scattered/emitted particles are then detected so as to form an image of the specimen.

- BETTER LIVE-CELL MICROSCOPY, involves getting more useful information w/o producing excessive damage.

What do we mean by better?

- Less damage to the specimen.
- More useful information/detected photon.

What's new?

- There are a lot of new contrast modes:
  - SHG, polscope, FLIM, FRET, CARS etc.
- New spectrometries:
- New lasers, scanning systems, photodetectors.
- Adaptive optics (!)

What makes information useful?

- Better spatial resolution?
- Better time resolution?
- More informative contrast mechanisms
  - FCS, pol-scope, SHG, THG, 3DFSR...
  - Silver cluster dyes,
  - Switchable fluorophors: BIPS, Merocyanine
- Being able to follow more cells (field of view).
  - HCS: computer aided image analysis
  - SPIM etc.

Today, I will concentrate on methods that provide higher spatial resolution when imaging living cells.

We have many such methods:

To overcome Abbe, one must saturate something:

- Stimulated Emission Depletion Microscopy
  (STED:Hell et al. MPG, Goettingen)
- Photoactivated Localization Microscopy
  (PALM:Betzig & Hess, HHMI, Janelia Farms)
- Stochastic Optical Reconstruction Microscopy
  (STORM:Rust, Bates & Zhuang, HHMI, Harvard)
- Incoherent Interference Illumination, Incoherent Illumination detection
  (I5M: Gustafsson, UCSF)
- Saturated structured Illumination microscopy (SSIM:Heintzmann, ICL).

I will now give brief descriptions of these...

They all share certain characteristics:

- They use fluorescence as the basic contrast mechanism ⇒ deposits energy ⇒damage.
- Higher spatial resolution implies both more and smaller voxels.
- i.e., More photons must be detected from the same number of dye molecules:

⇒ Stable dyes needed.

But there are also important differences.

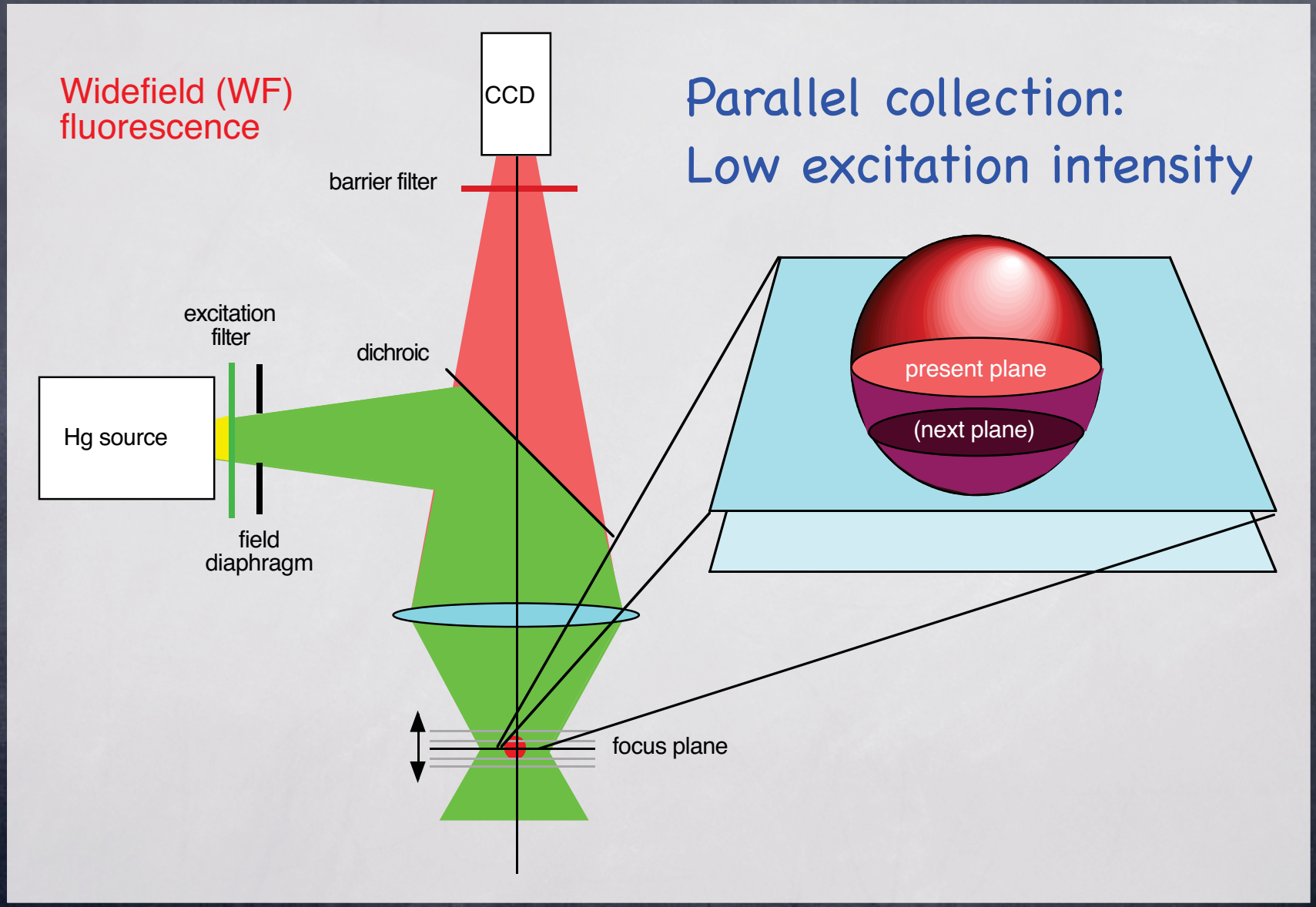
As the optics of these methods have been much discussed, I will concentrate my discussion to limitations related to:

- the detectors used
- rate at which data is accumulated
- the signal levels needed
- the effect of specimen RI.

As I do not own all these systems, most of my comments are inferences from published data.

Widefield HRLM techniques*

REVIEW: Widefield (WF) Deconvolution



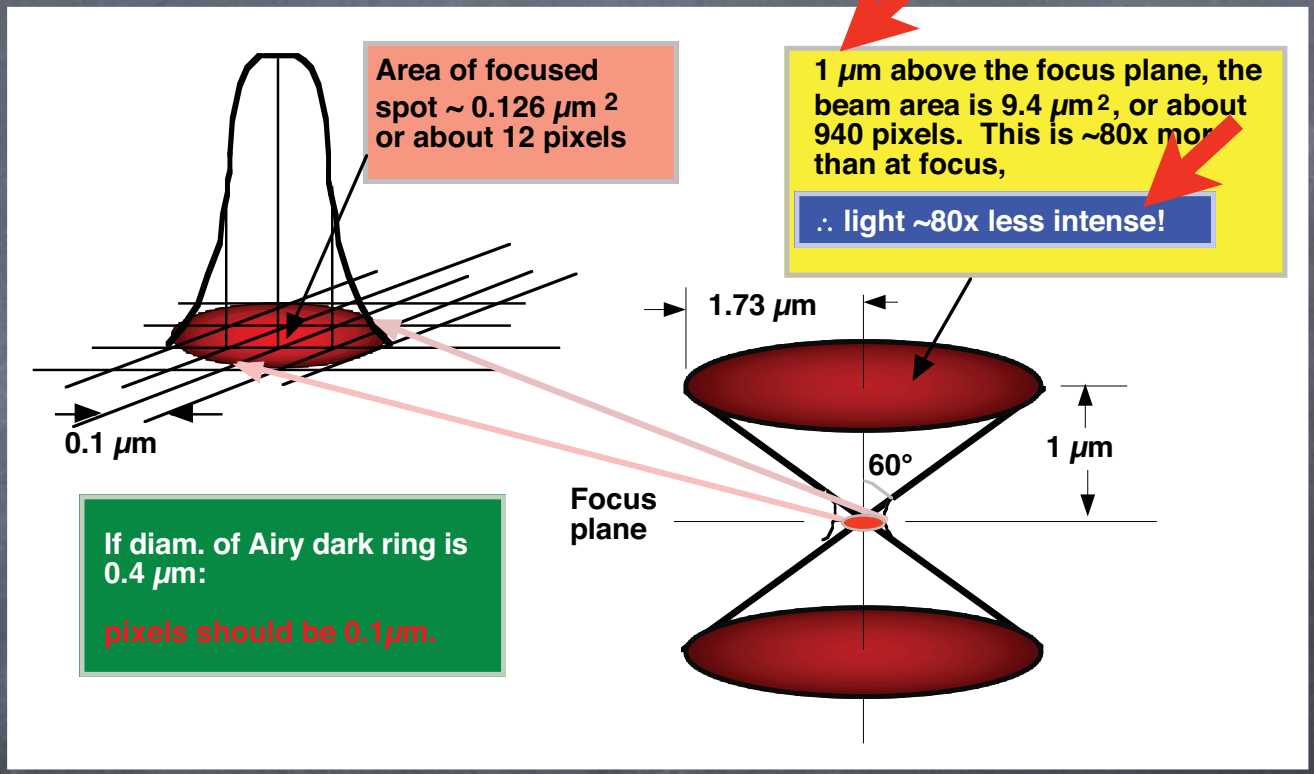
In WF fluorescence, the entire thickness of the specimen is exposed to the full intensity of the exciting light. This situation is ameliorated by the high QE of the CCD.

Q: Can one "Put the photons back where they come from?"

Suppose that, under WF illumination conditions, a CCD with Nyquist-sized pixels (0.1 μm at sample) records a total of 10k photons from a point feature in each image of the 3D stack.

In-focus: 10,000 photons over ~12 pixels => 830p/pix

Assuming a large NA lens (α = 60°) and simple geometrical optics,* by the time the point is 1 μm out of focus, these photons will be spread over a circle having an area of ~10 μm^2 or ~1,000 pixels.



If the intensity is spread evenly over this circle, then the detected signal level from the now-out-of-focus feature drops to ~10 photons/pixel. As this is only slightly more than the RMS read noise of the CCD, it is hard to see how the detection of this out-of-focus light can provide much useful structural information.

*an admitted oversimplification

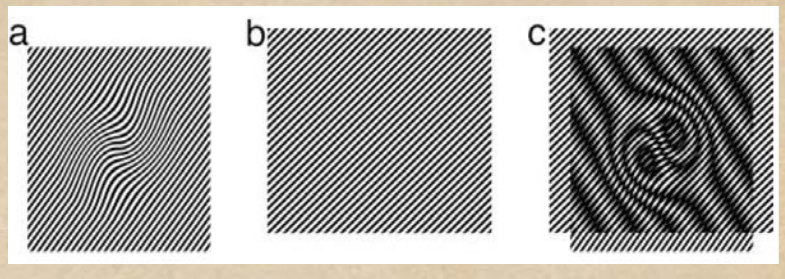
A: Only some of them can be "put back."

Structured Illumination Microscopy (SIM).

Start with ordinary WF 3D Iterative deconvolution and add structures illumination patterned at the λ/2.

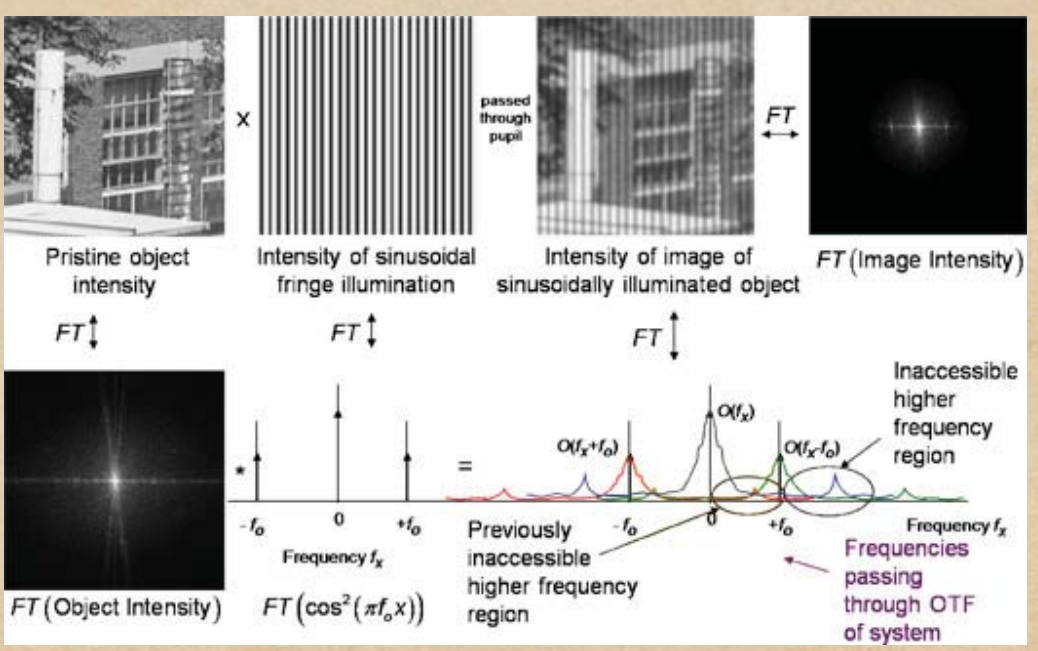
Mats Gustafsson

The resulting image represents the specimen structure multiplied by the sinusoidal illumination structure, rather like a Moiré pattern.



Such an image contains spatial frequencies up to 2x higher than those present with uniform illumination.

But to obtain a uniform response, ~9 images must be recorded: 3 phase positions and 3 angles.



The results, enlarged so that you can see them...

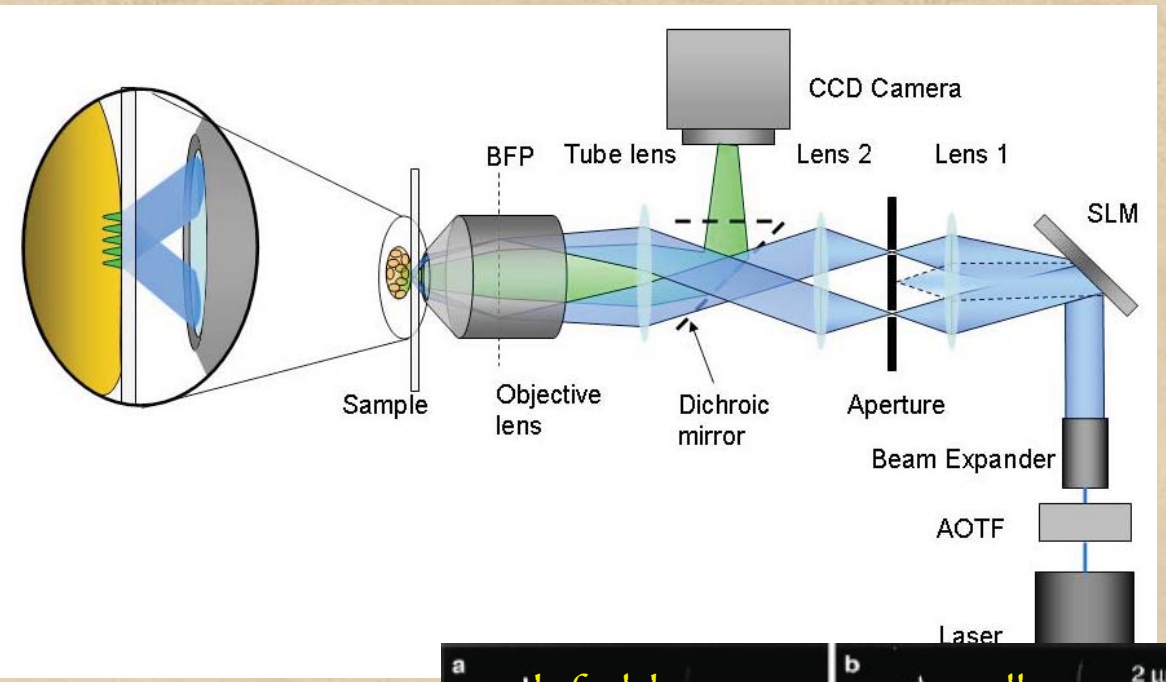
(a) Pristine object intensity

(b) Image with uniform illumination

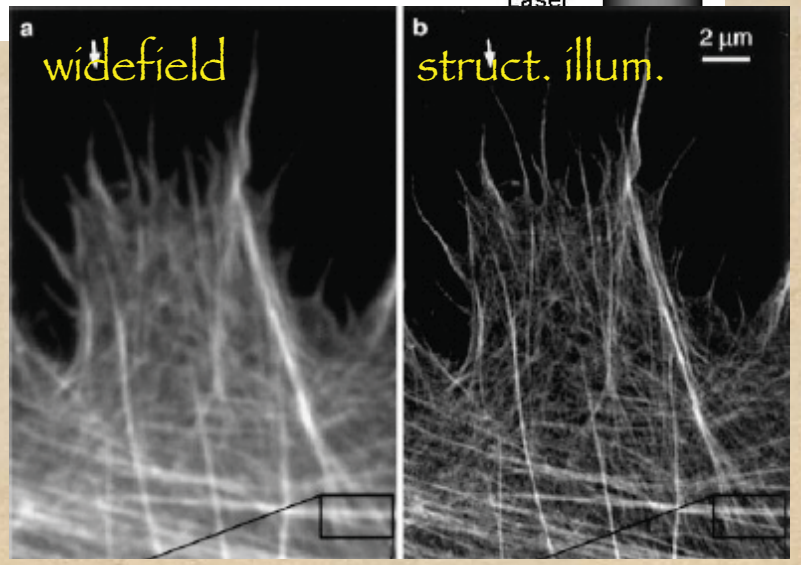
(c) Structured illumination recovered image with 75% superresolution

(d) Image with uniform illumination through 75% larger pupil

Making structured illumination by interference (for TIRF).

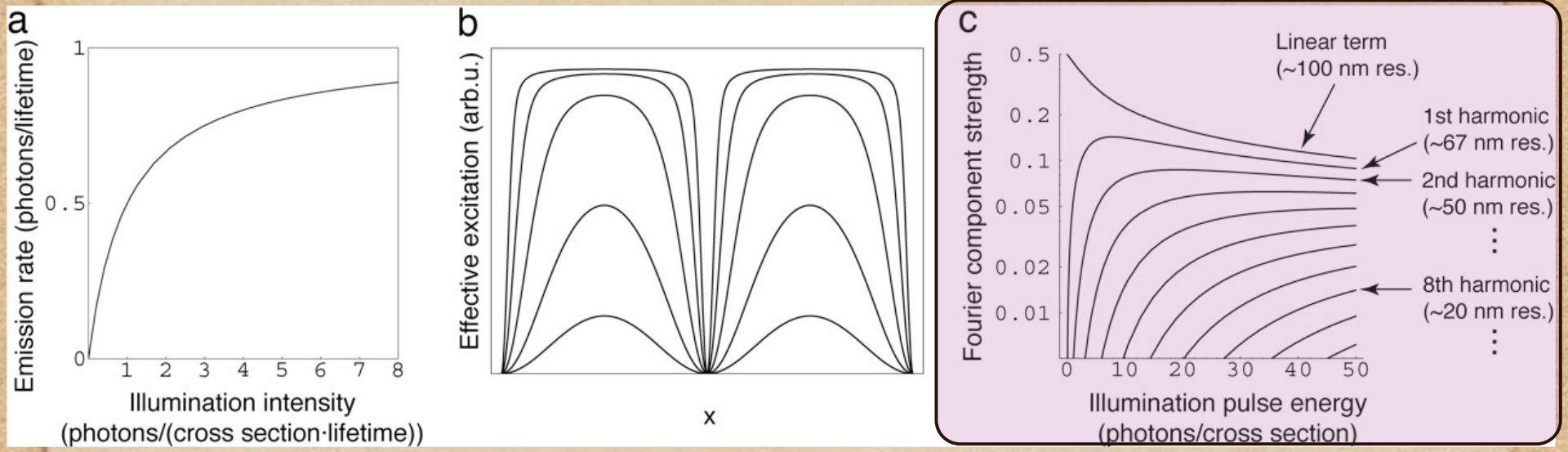


Although extracting this information requires recording and processing at least 9 images/plane (3-5 positions of the pattern in 3 orientations, it produces a significant resolution gain.



M. Gustafsson, *J. Microsc.*, **198**, 82 (2000)
Actin in fixed 3T3 fibroblast cell

Even greater improvement can be obtained by changing the sine-wave excitation to square-wave excitation, as can be approximated by using brighter illumination to saturate the singlet-state fluorescence process.



This produces yet higher harmonics in the resulting images.
(It can also be thought of as only NOT exciting near zeros.)

However, because these harmonics are only present at low intensity (contrast), one needs very good S/N to detect them.

In addition, this technique requires images to be recorded with a much finer separation of the phase of the illumination pattern.

And, saturating the singlet state requires very bright illumination.

For instance, common dyes approach singlet-state saturation when about 1mW of laser power is focused into a diffraction-limited spot by a high-NA objective.

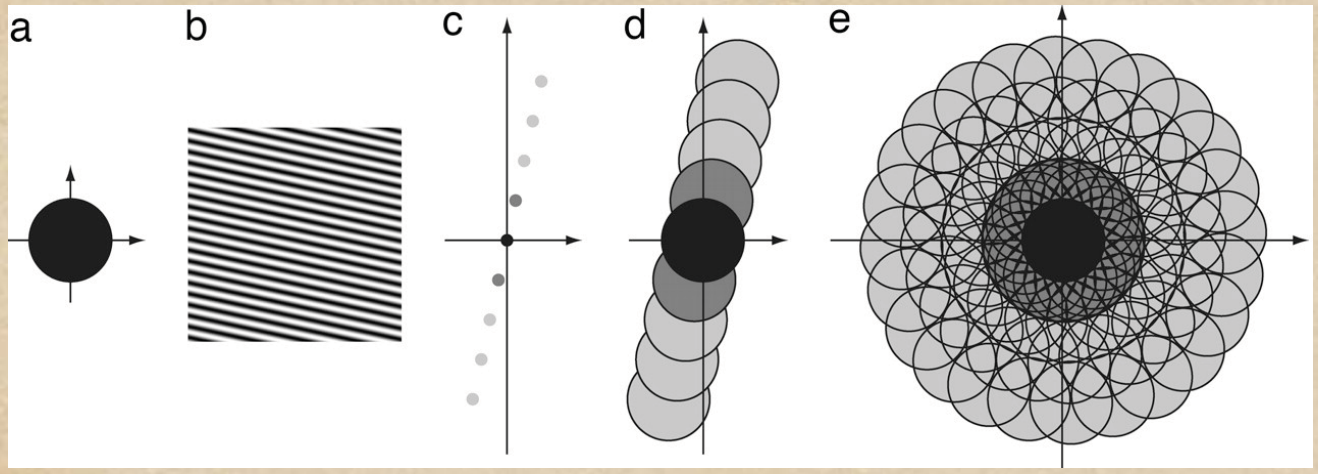
Saturating the singlet-state over the entire field of view with structured illumination requires either very high excitation power levels (watts or kW)

Such power levels can only be applied without vaporizing the specimen using a pulsed laser, a practice that significantly slows the rate at which data can be obtained.

Alternatively, one might use special dyes that remain in the excited state for a long time. (i.e., very long fluorescence decay times).

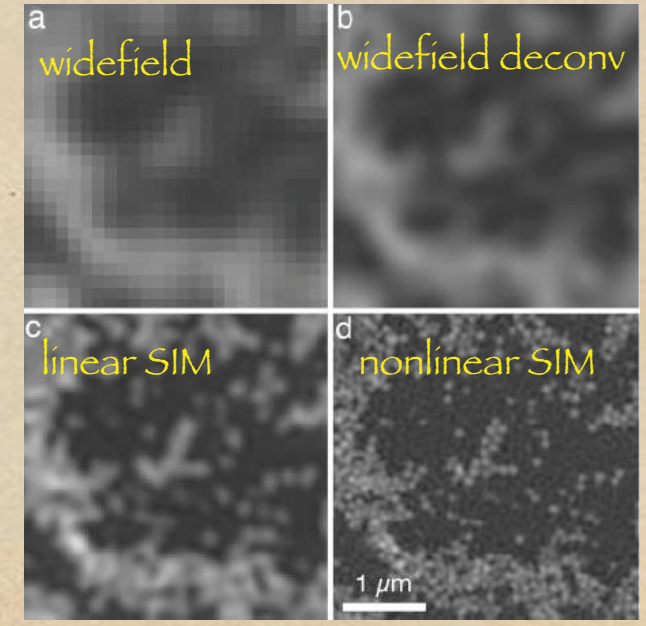
However, such dyes are usually especially prone to photodamage.

So, many pictures, bright light....



(In this case, 108 images/ plane)

but the resolution improvement is substantial!

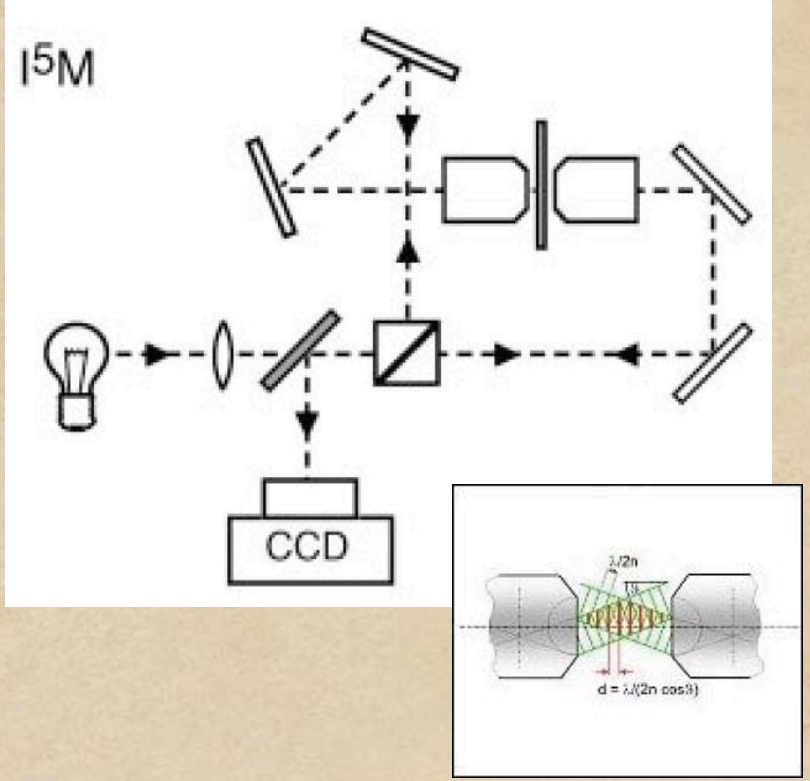


M. Gustafsson, *PNAS*, **102**, 13081 (2005)

To optimize axial resolution, add axial interfence: I5M

Incoherent Interference Illumination

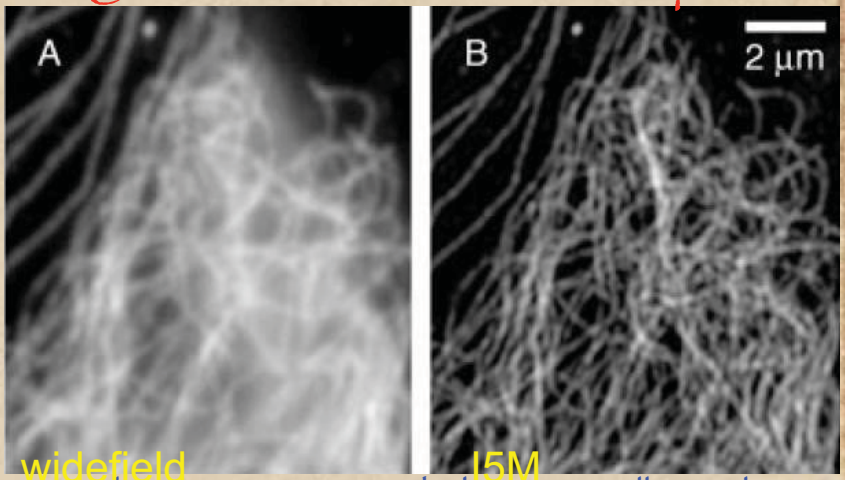
Incoherent Interference detection



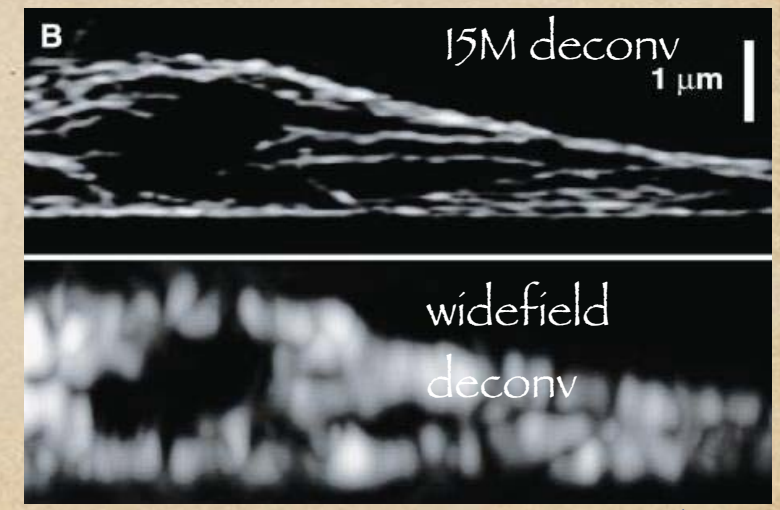
opposed objectives -> optimal axial resolution

M.G.L. Gustafsson, D.A. Agard, J.W. Sedat *J. Microsc.* **195**, 10 (1999)

But this requres a separate set of images at each illumination phase

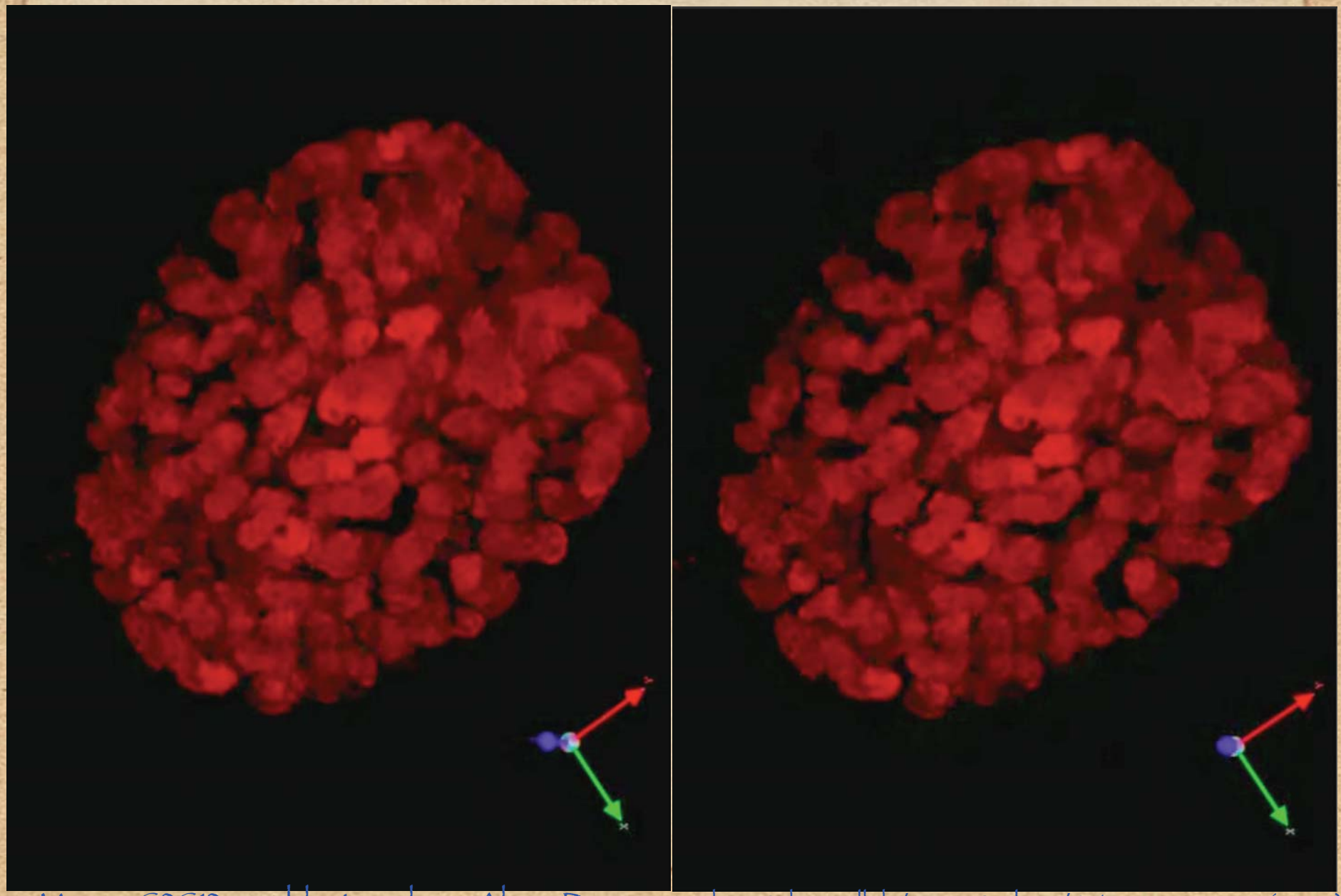


optical sectioning, microtubules,PtK2 cell xy plane

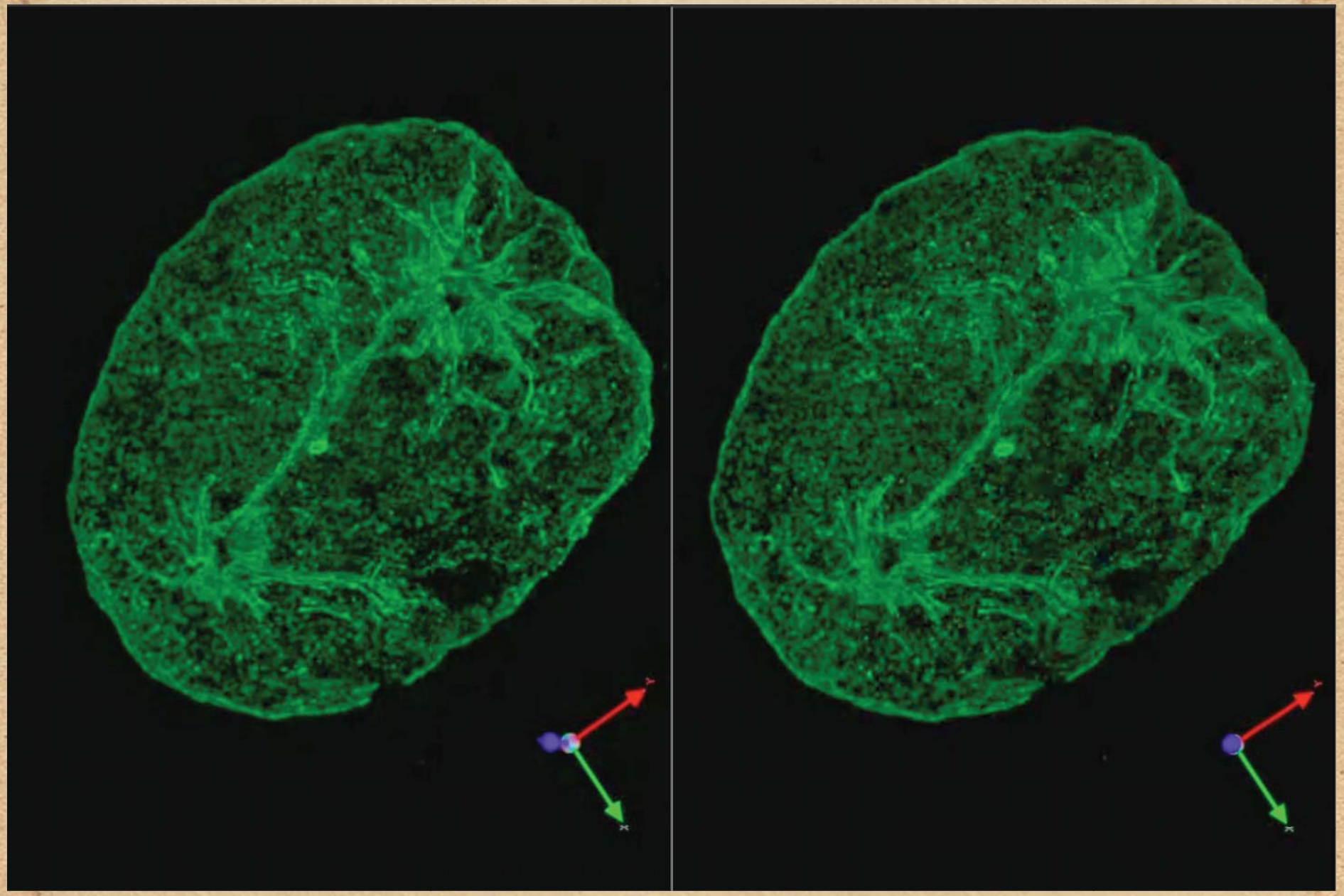


xz plane

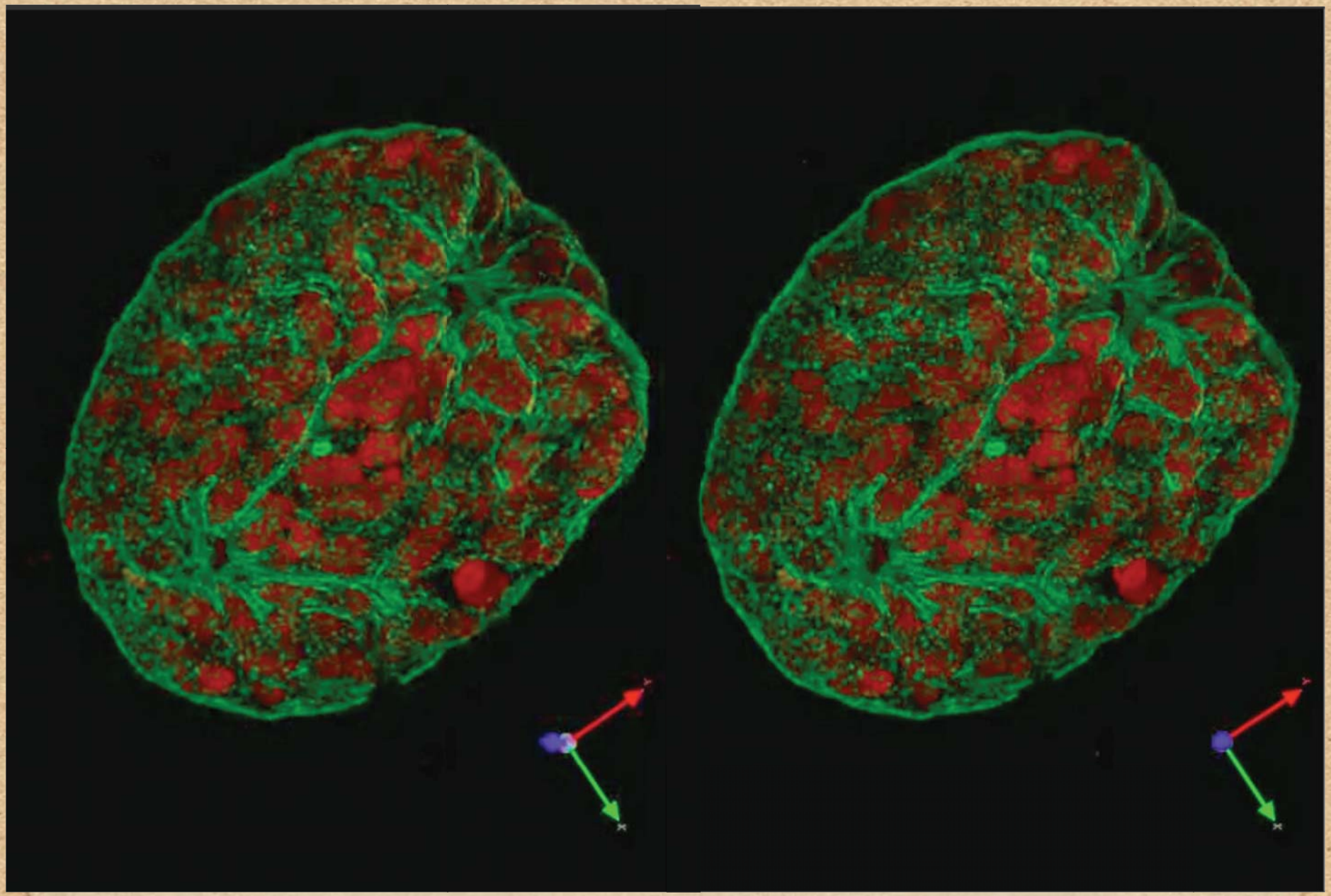
Advantages: High-resolution 3D , 2-color, widefield image



Mouse C2C12 myoblast nucleus. Alexa Dyes: Lothar Schermelleh/Peter Carlton/Science: 320;1332 (2008)



Mouse C2C12 myoblast nucleus. Alexa Dyes: Lothar Schermelleh/Peter Carlton/Science: 320;1332 (2008)



3 orientations, 5 phases, 2 colors, planes 0.125 µm apart = ~1,200 images @ 300 ms each (+100ms deadtime) on iXON 87 EMCCD camera, for a total of ~480s = ~8 minutes

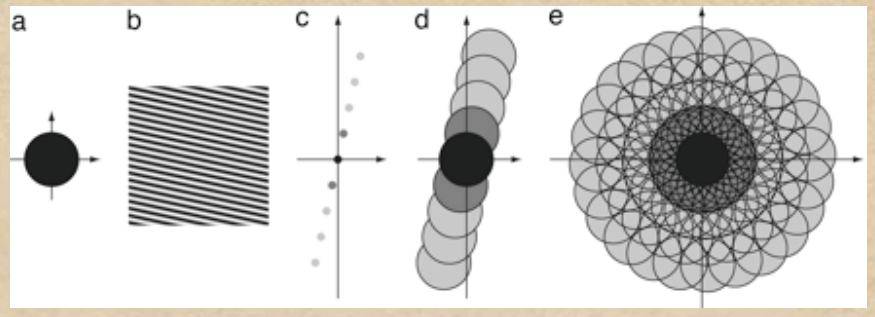
25 Lothar Schermelleh/Peter Carlton/Science: 320;1332 (2008)

The bad news:

- many images:
  bleaching
  slow: (dead?)
- sensitive to motion
  of specimen.
- "thinnish" samples
- difficult alignment
- laser wavelengths

Applied Precision OMX V2 with DeltaVision

Other, Linear I5M concerns include:



- Requires 4pi setup: 2 opposing objectives, aligned to λ/10 in xyz.
- Specimens need to have a uniform Refractive Index (RI).
- The need to record >100 frames/focus plane limits data rate.
  - May cause bleaching but is sensitive to bleaching.
  - Needs high frame-rate CCDs: greater noise.
  - Significant computational requirements.
  - Poisson noise created by bright, out-of-focus features may obscure data.

In Saturable Structured Illumination Microscopy (SSIM) or saturable I5M, concerns include:

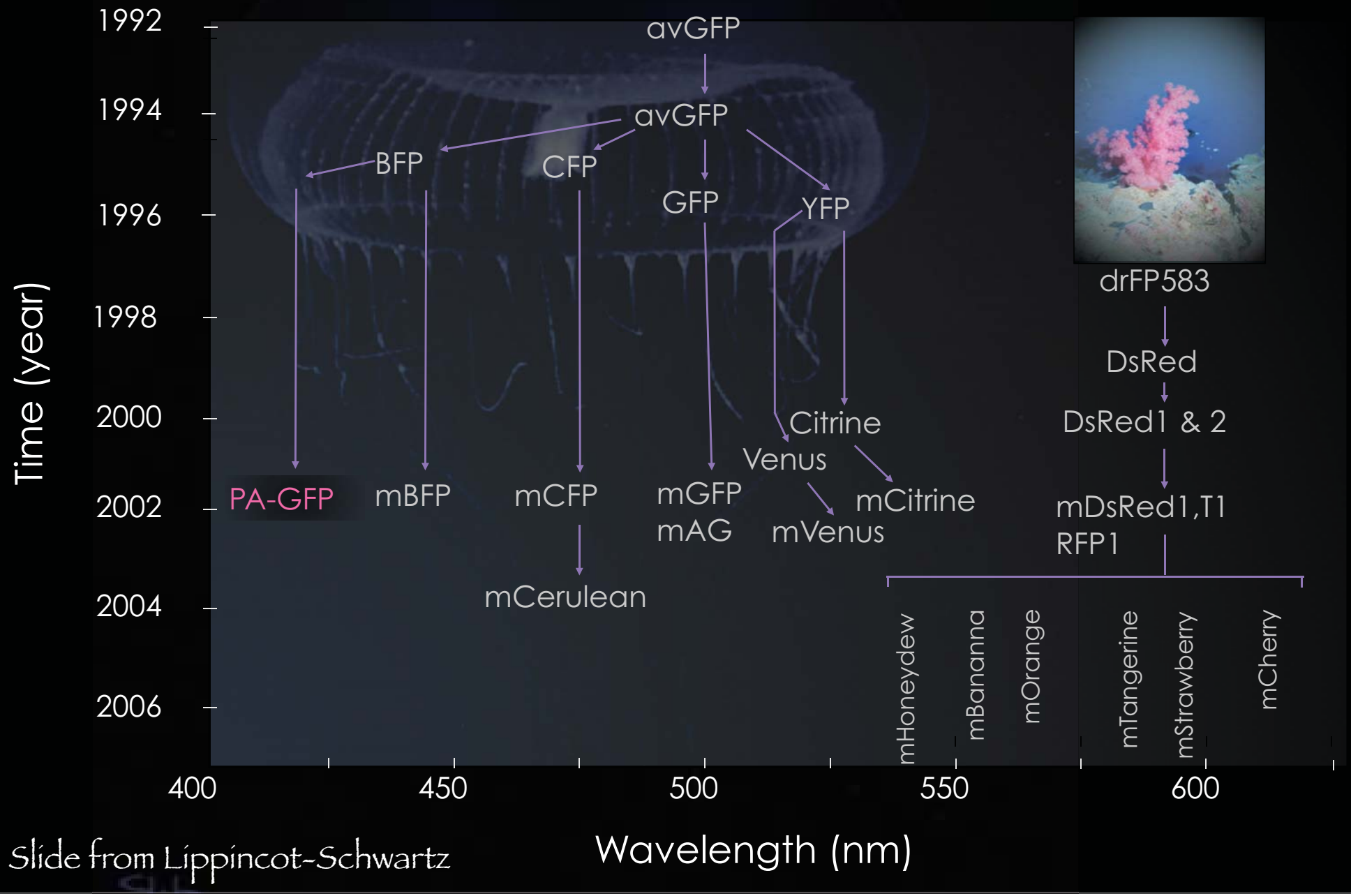
- Same as linear I5M plus...
- Although it requires saturation, and saturation increases the bleach rate, the technique is sensitive to bleaching.
- Full field, singlet saturation involves a lot of power.
  - ⇒ slow laser pulse rate ⇒
    - very low duty cycle.
      - Live-cell work unlikely

- Verdict
  - As always, there is a trade-off.
  - You can use this equipment to image moving cells in 3D, but only at close to "normal" (Abbe) resolution.
  - Highest resolution will require fixed cells to reduce specimen movement and to increase fading resistance.

Photoactivation location microscopy (PALM)

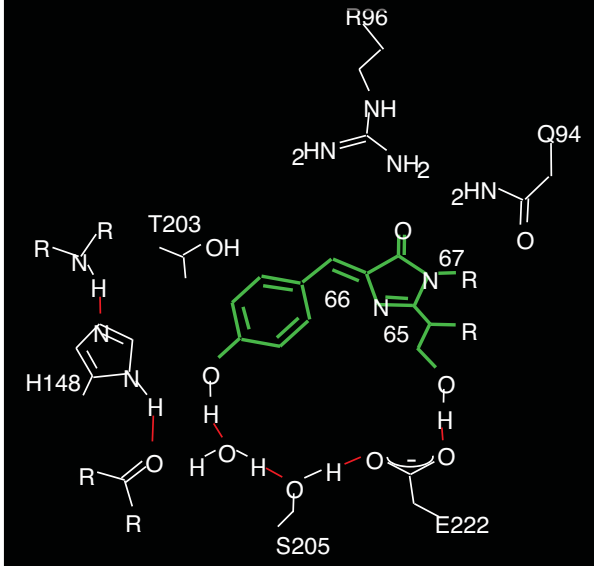
Jennifer Lippincott-Schwartz

PALM and STORM: Start with PhotoActivatable (PA) dyes

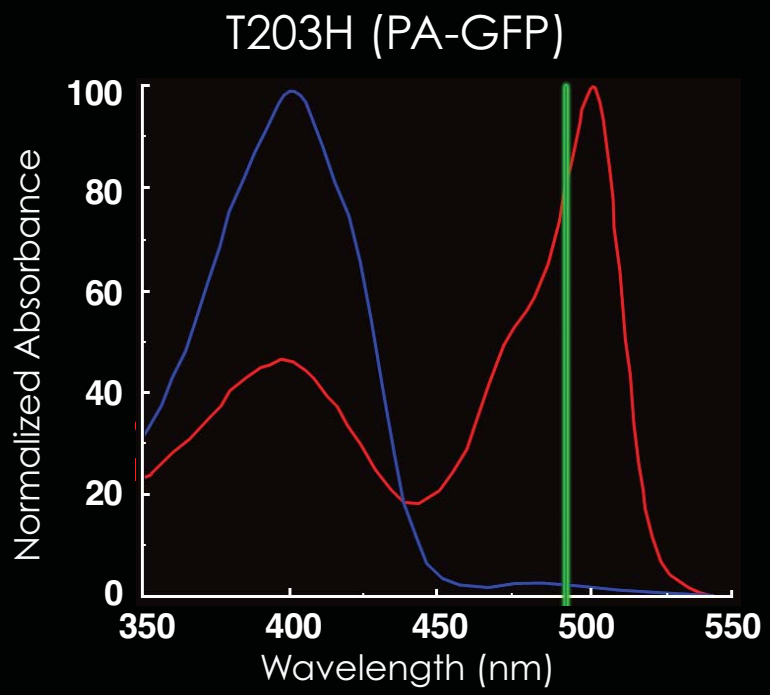


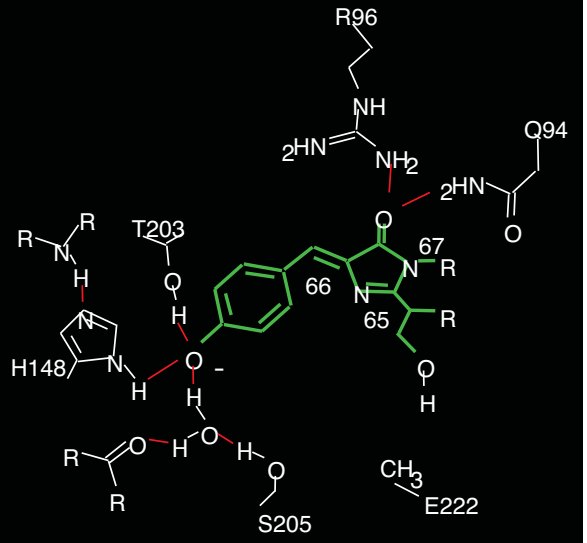
Slide from Lippincot-Schwartz

Photoactivatable Fluorescent Proteins



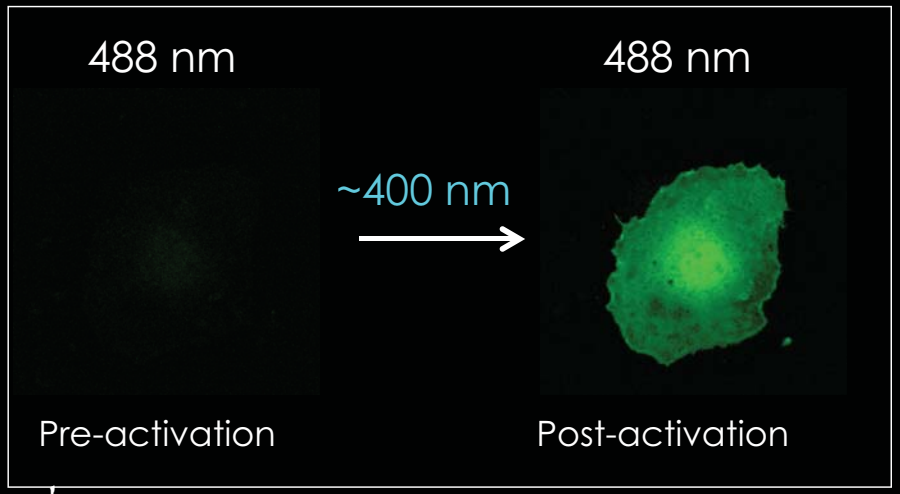
Native





Photoconverted
(photoactivated)

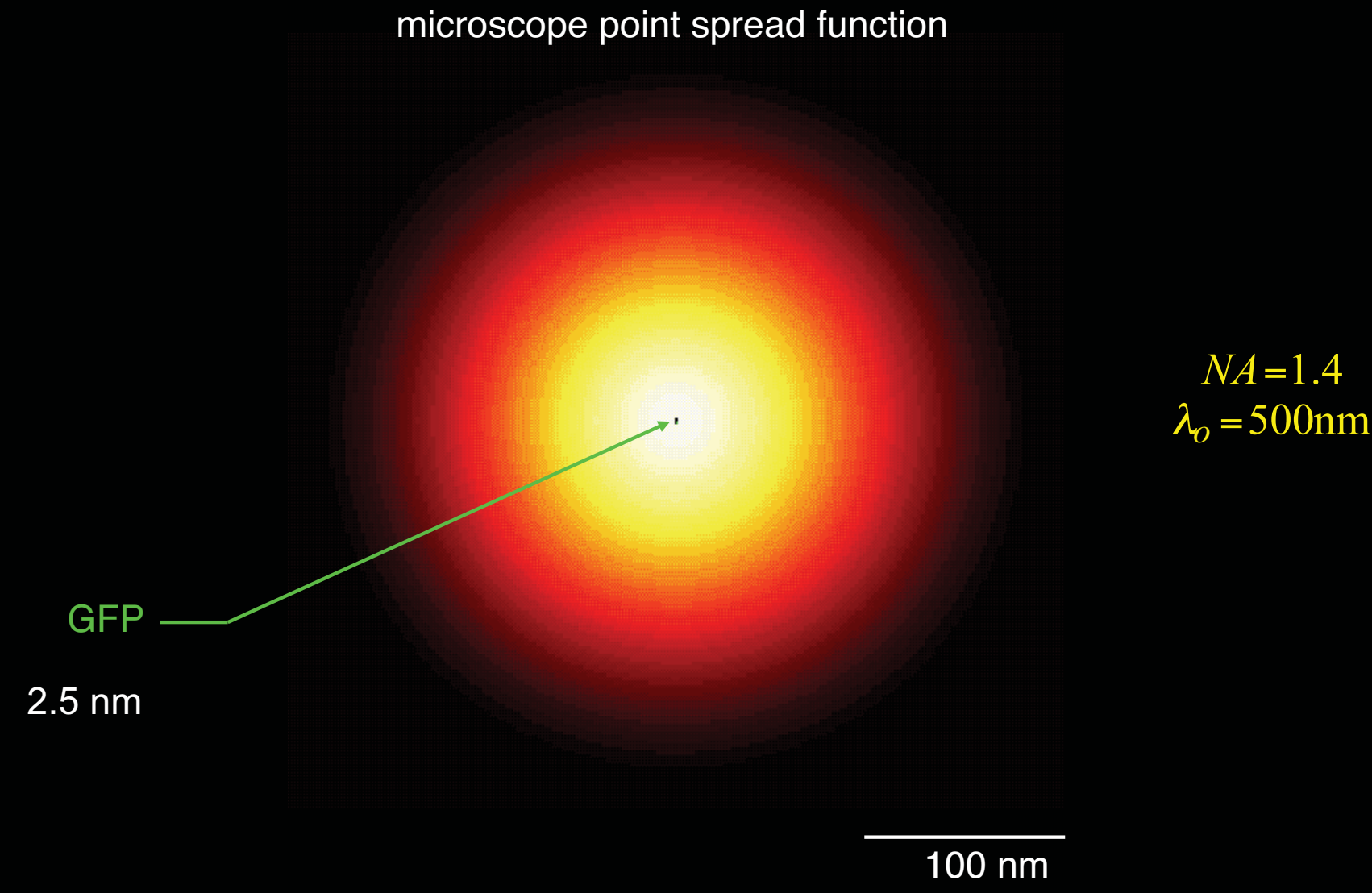
Patterson & Lippincott-Schwartz
Science 2001



George Patterson

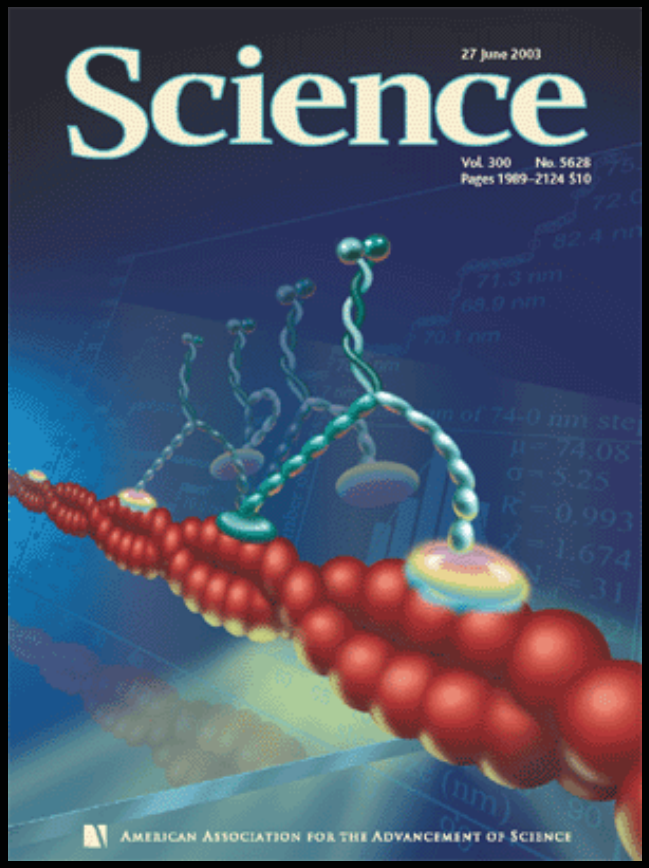
Slide from Lippincot-Schwartz

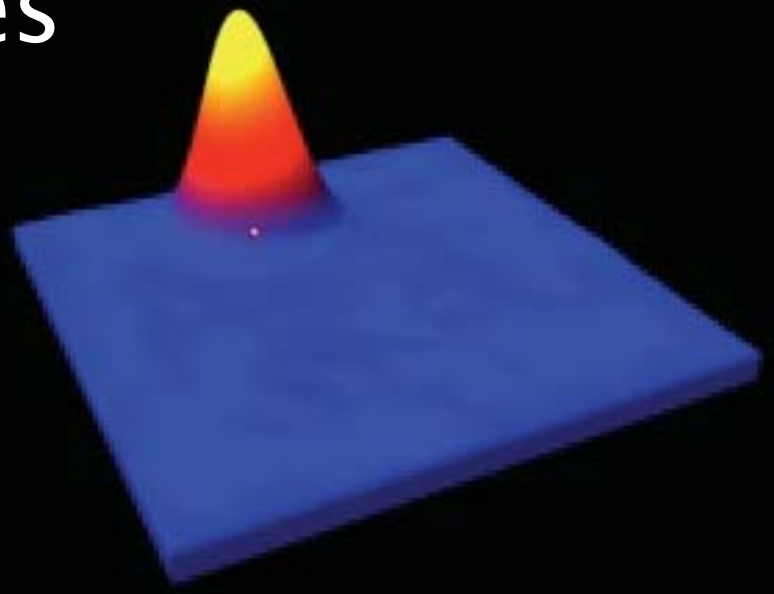
Although the Diffraction Limit is large,

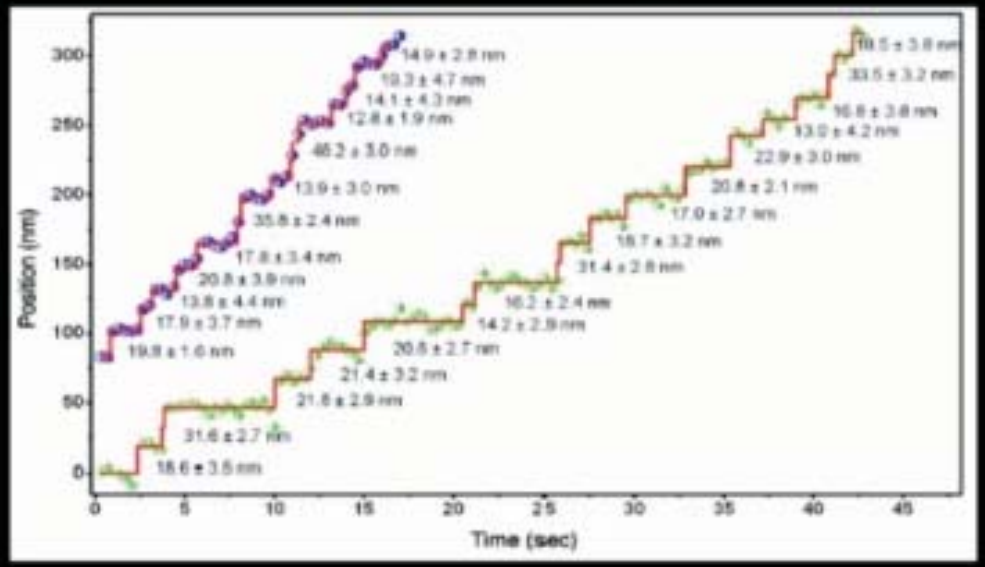


Slide from Lippincot-Schwartz

we can still localize single fluorescent molecules







molecular motor: myosin

One molecule at a time

Slide from Lippincot-Schwartz

Photoactivated Localization Microscopy

DATA ACQUISTION

Photoactivate 1st subpopulation

↓

Image & then bleach subpopulation

↓

Photoactivate 2nd subpopulation

↓

Image & then bleach subpopulation

↓

Photoactivate 3rd subpopulation

↓

Image & then bleach subpopulation

↓

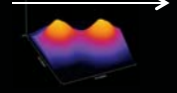
Photoactivate nth subpopulation

↓

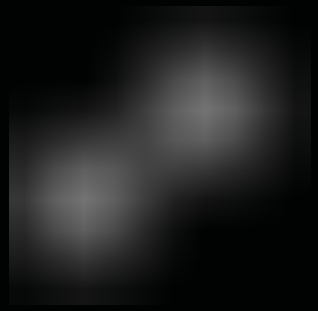
Summed molecular fluorescence

Eric Betzig

Fit 2D Gaussian →

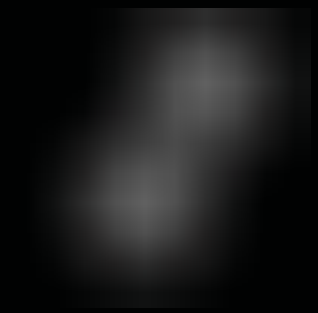


Plot molecules



Fit 2D Gaussian →

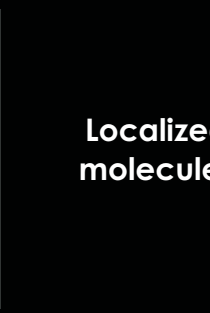
Plot molecules



Fit 2D Gaussian →

Plot molecules

→



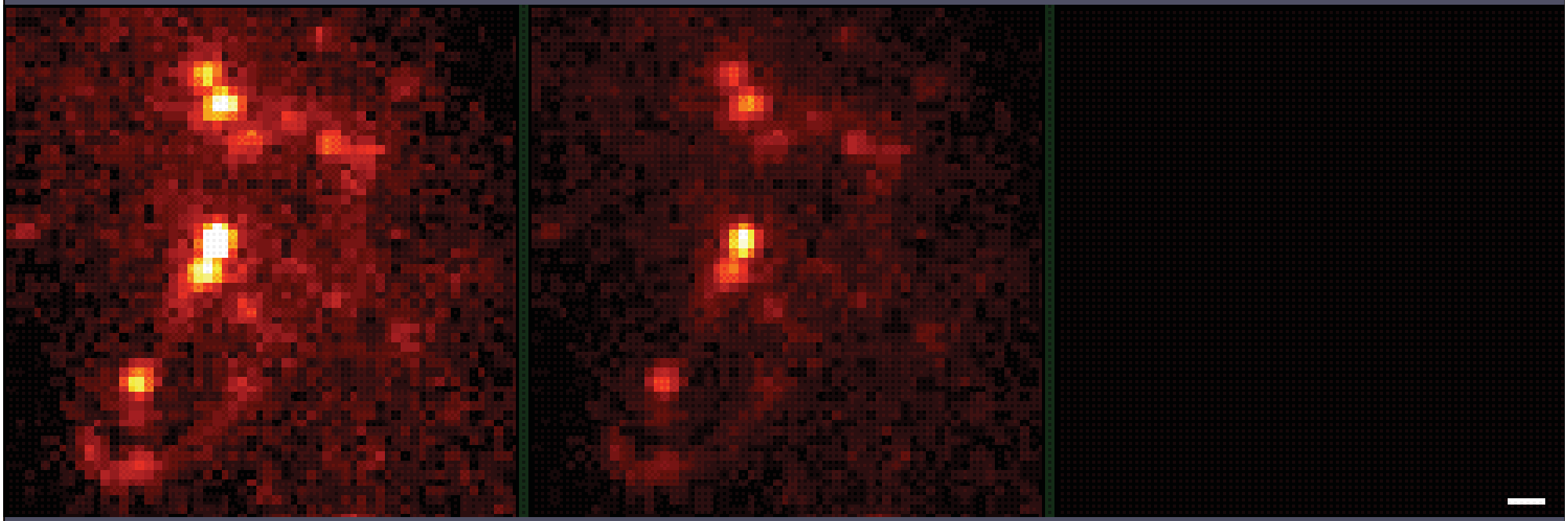
Localized molecules

Betzig et al., *Science* 2006

Slide from Lippincot-Schwartz

PALM in action

Current image | Sum of Current images | Sum of molecule centroids



- Molecules in each image must be far enough apart to localize.
- Use continuous activation and excitation to build composite.
- Apply the results to look at organization + single-molecule dynamics

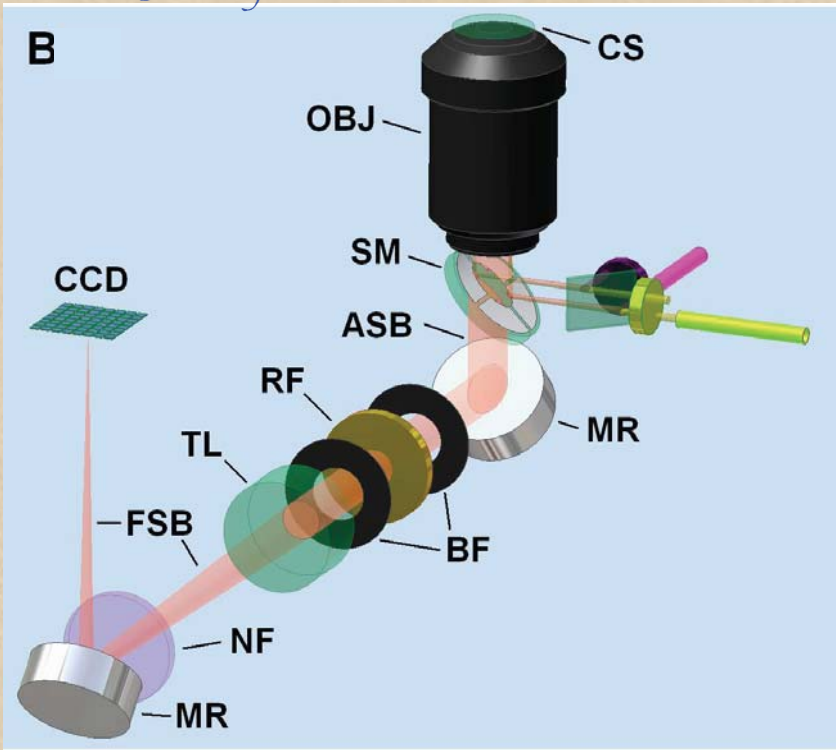
Interestingly, over thirty years ago, a similar system was used to improve the spatial resolution of a video-rate image intensifier system used in the TEM.
(Herrmann, Krahl Rust and Ulrichs, Optik, 44,(1976)-4, pp 393-412: see figure 7)

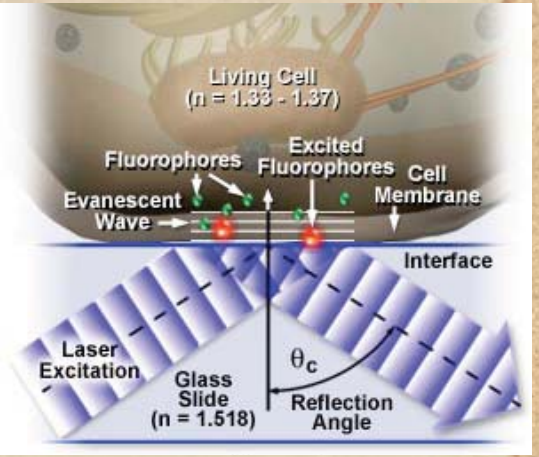
Slide from Lippincot-Schwartz

PALM-TIRF Instrumentation

electron multiplying CCD camera

PALM optical path --
through objective TIRF excitation





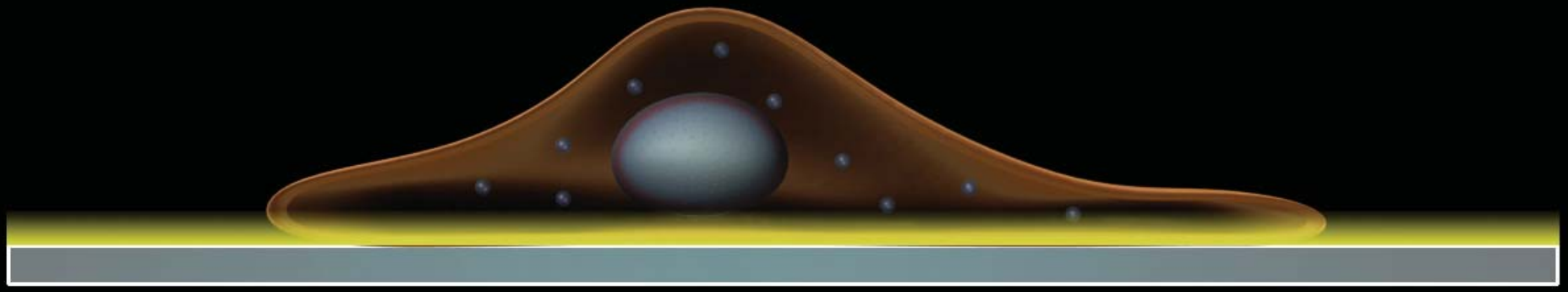
Total Internal Reflection Fluorescence (TIRF): reduces background photons so single molecules can be imaged.

l = 405 nm activation
l = 491 or 561 nm excitation

And a really fast, optimized computer

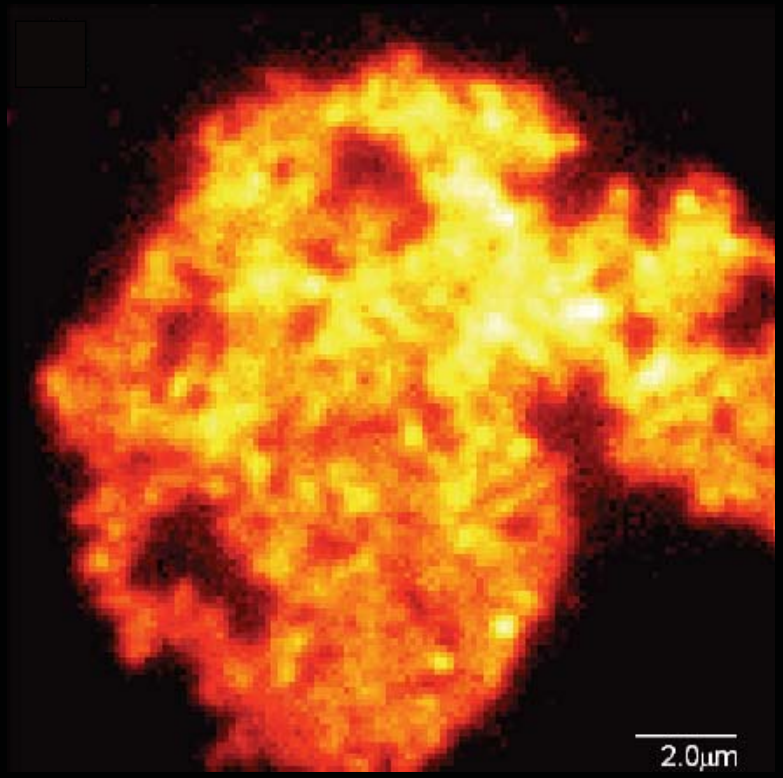
Slide from Lippincot-Schwartz 37

PALM Imaging of Fixed Cells Expressing PA-FPS

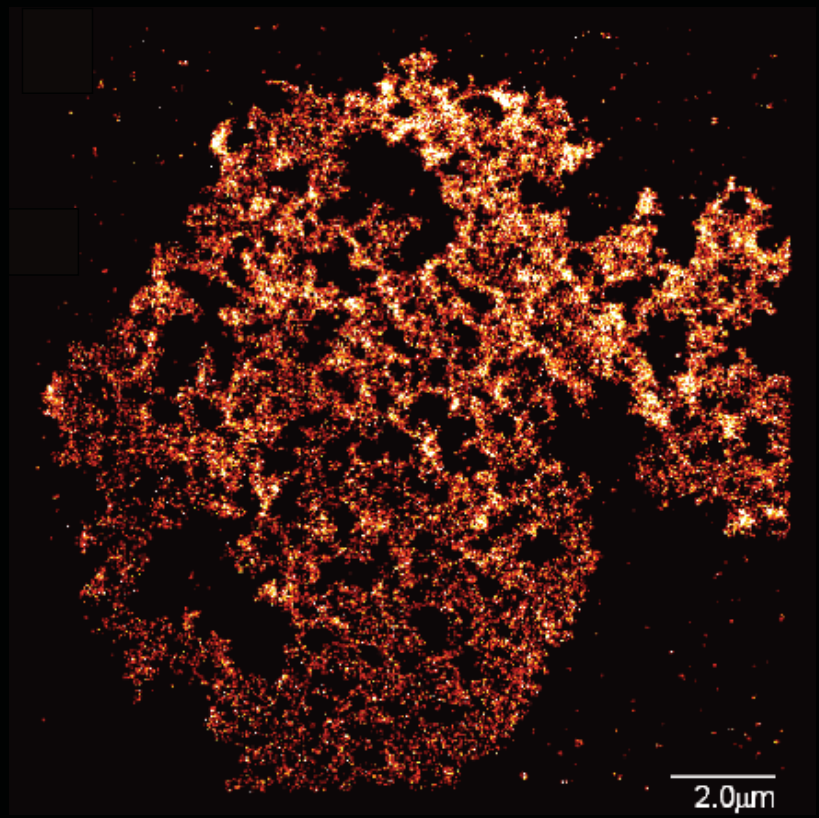


Total Internal Reflection microscopy limits imaging to range ≤100nm distance from the coverslip

PALM Super-Resolution Organization



Conventional TIRF

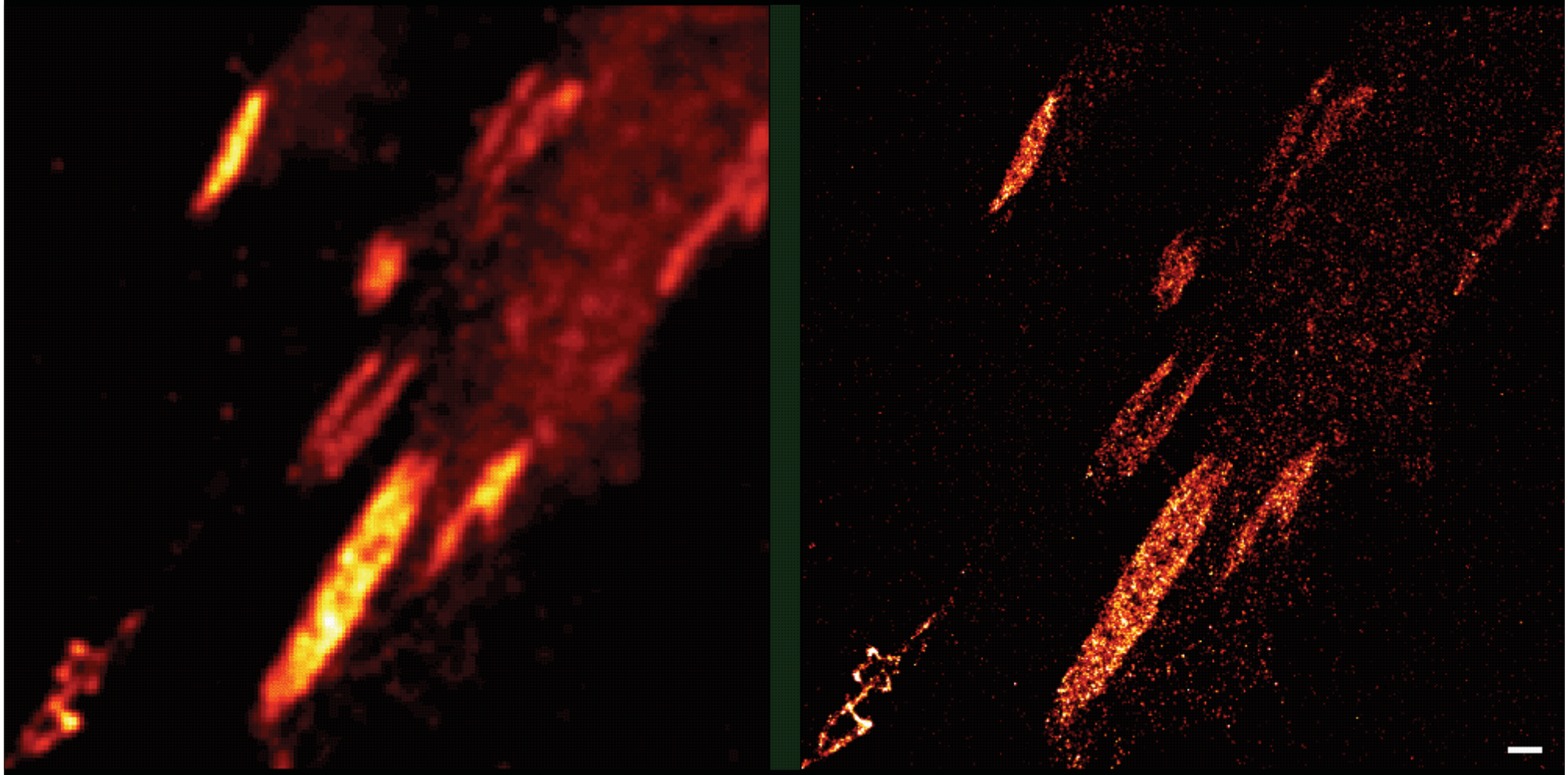


PALM Image

Constructed by summing position probability gaussians determined for all localized molecules in data stack

Aggregate of 50 nm polystyrene beads coated with PA-FP Kaede

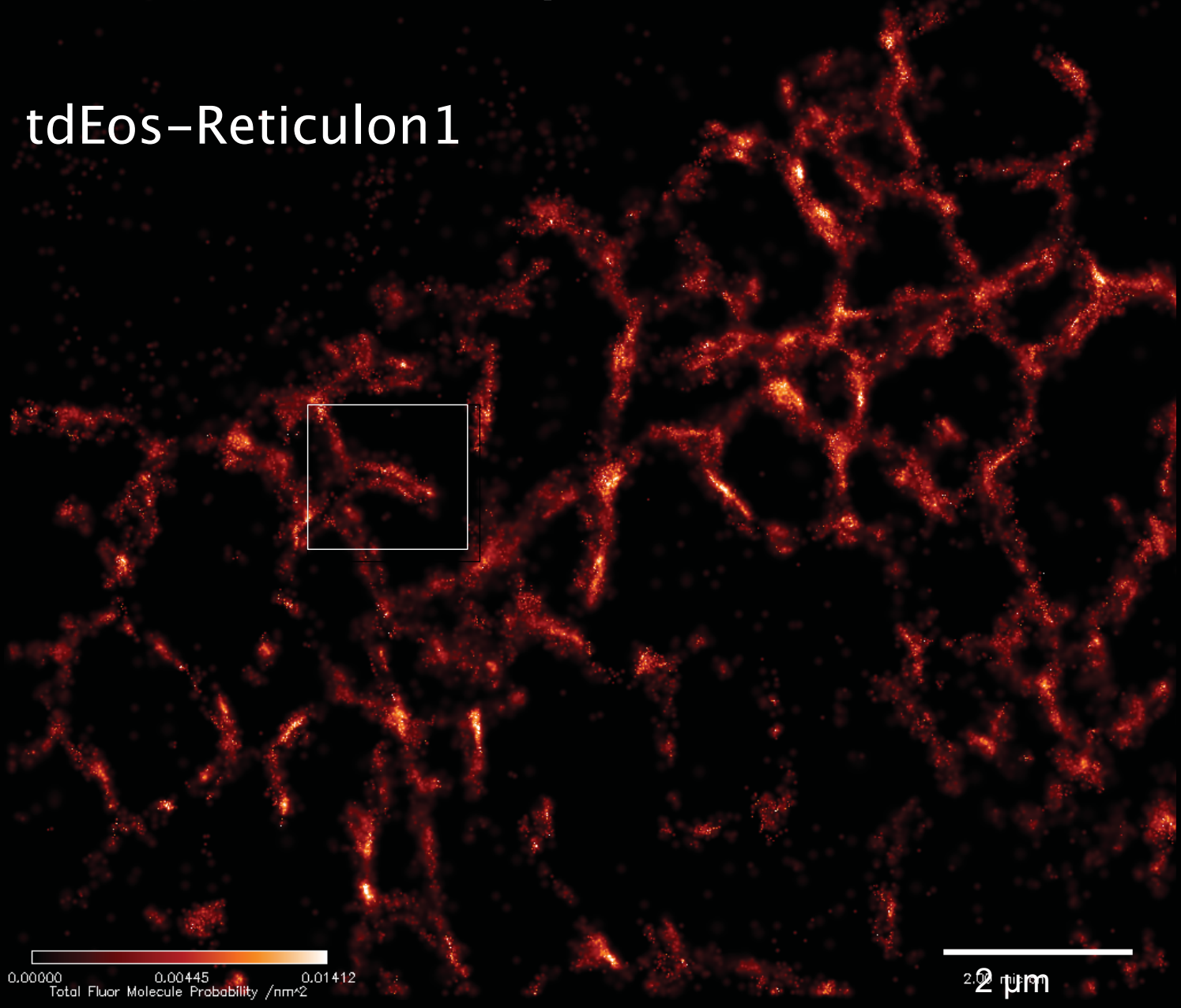
PALM Imaging of fixed cells expressing PA-FPs



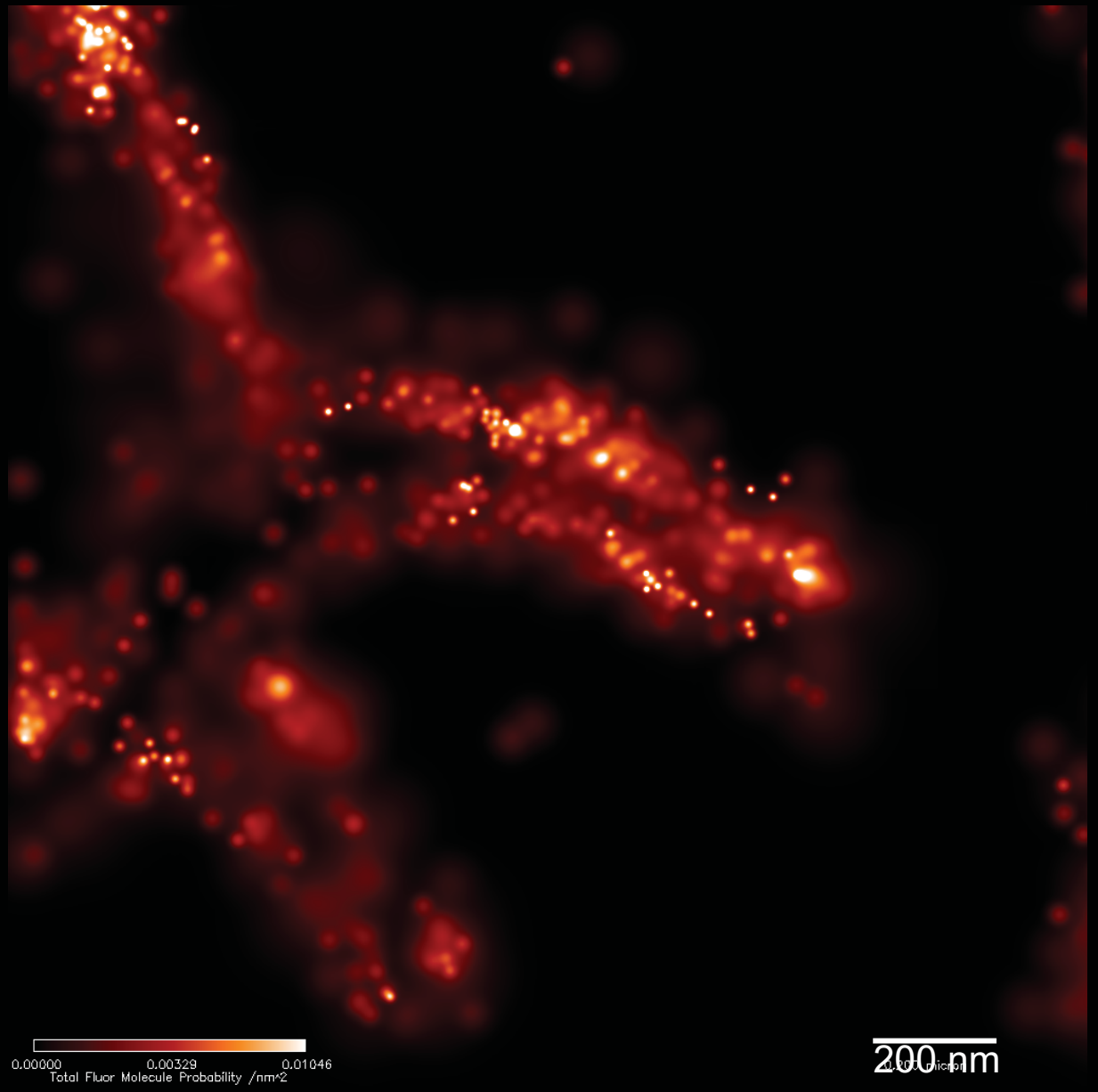
Focal adhesions, FoLu cell, dEosFP-tagged vinculin

PALM of Endoplasmic Reticulum

tdEos-Reticulon1



Zoom of PALM of ER

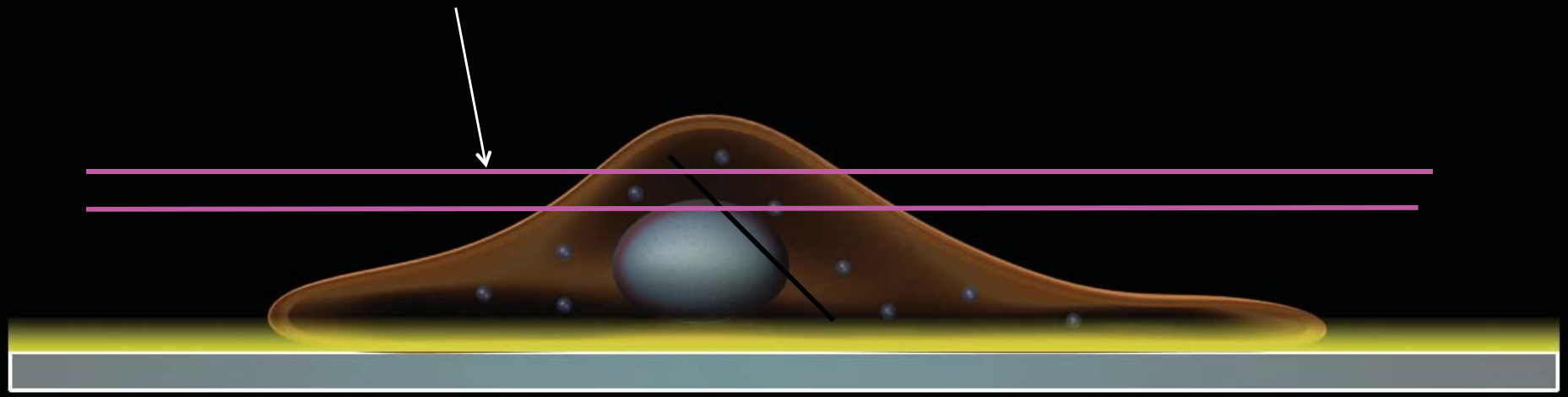


tdEos-Reticulon1

To see Intracellular Components.

Cut a physical section.

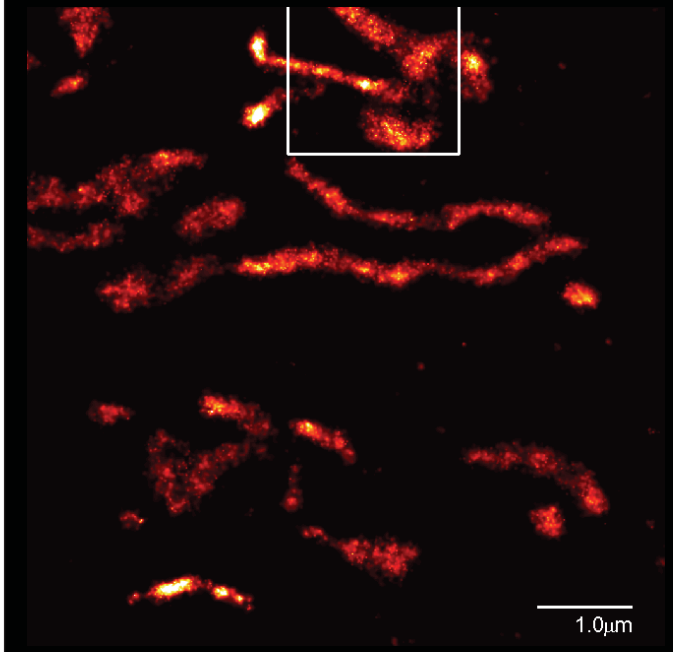
Place it at the coverslip surface.



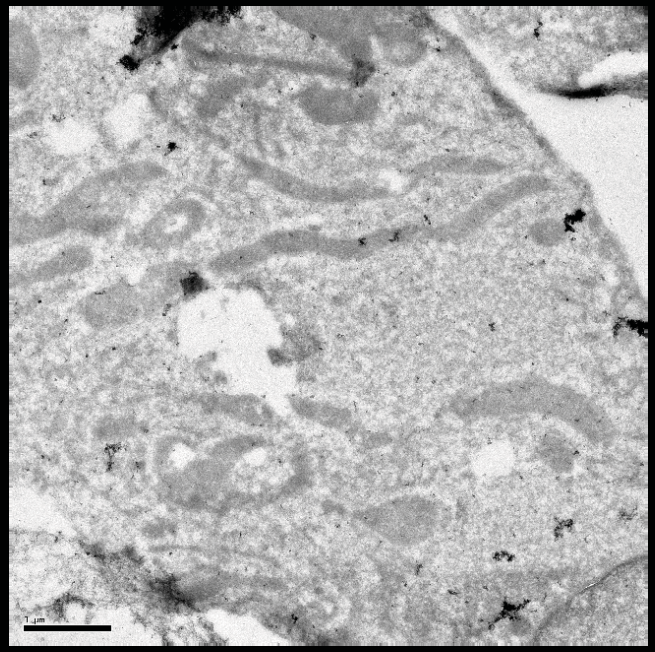
Rachid Sougrat

This also facilitates Correlative PALM/TEM.

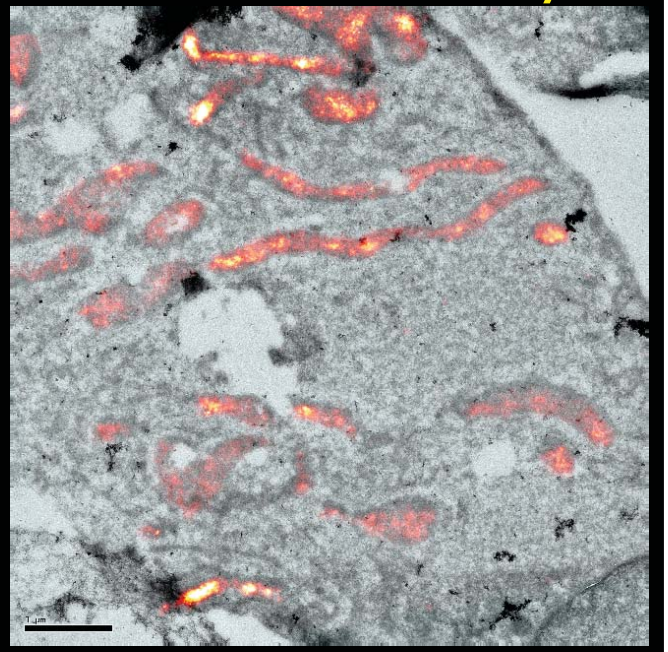
PALM



TEM



PALM/TEM overlay



Mitochondrial matrix, COS-7 cell

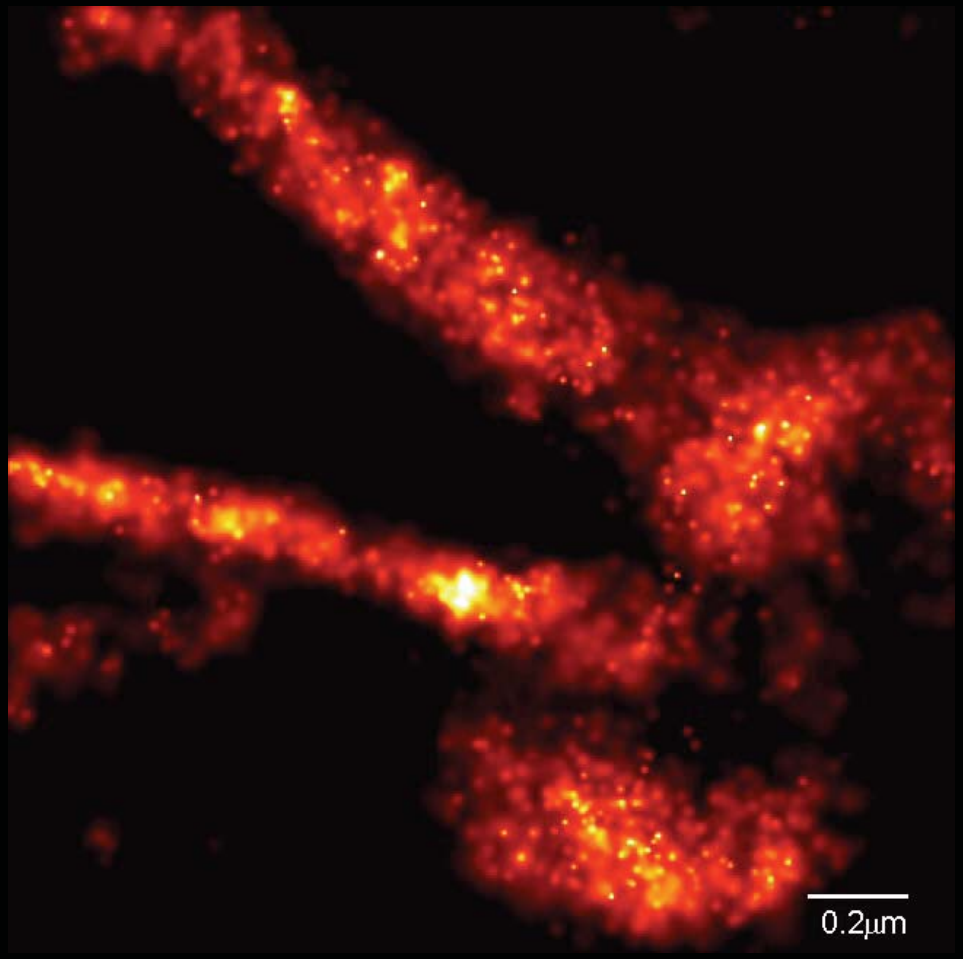
dEosFP-tagged cytochrome-c oxidase targeting sequence

Slide from Lippincot-Schwartz

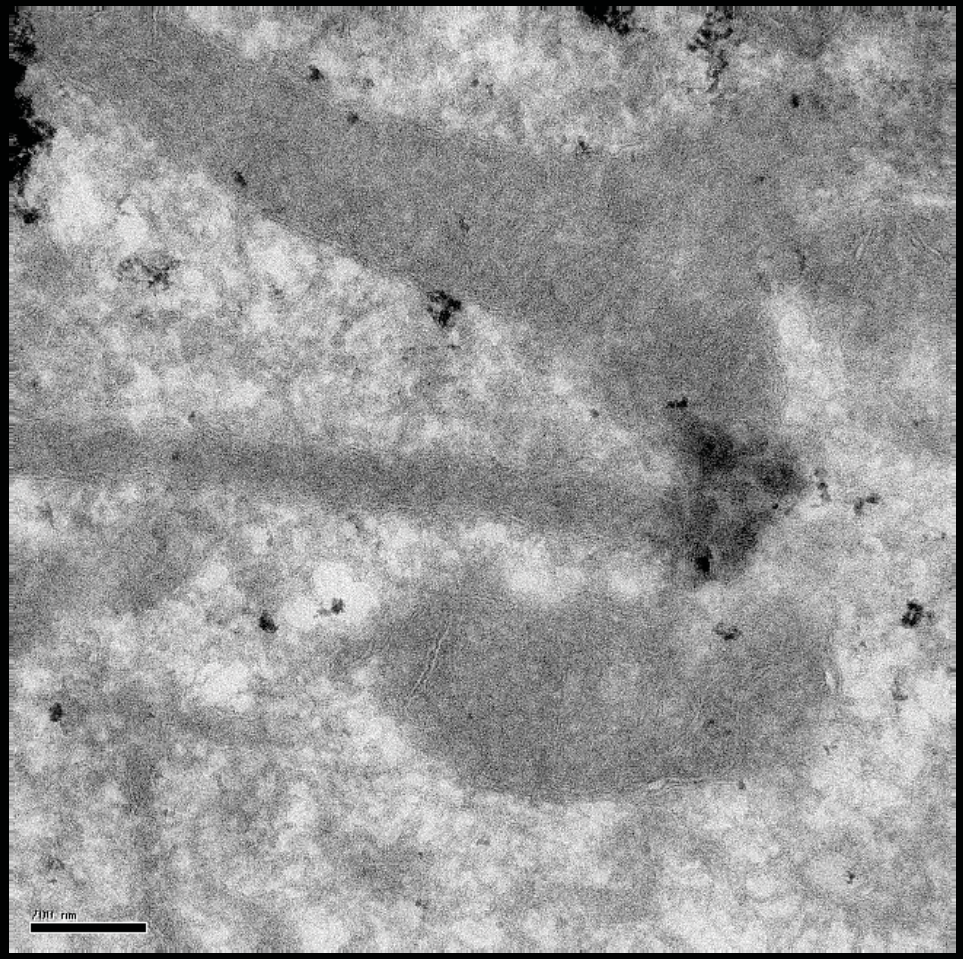
E. Betzig, *et al.*, *Science* **313**, 1642 (2006)

Higher mag of inset. Compare the number of labels.

PALM

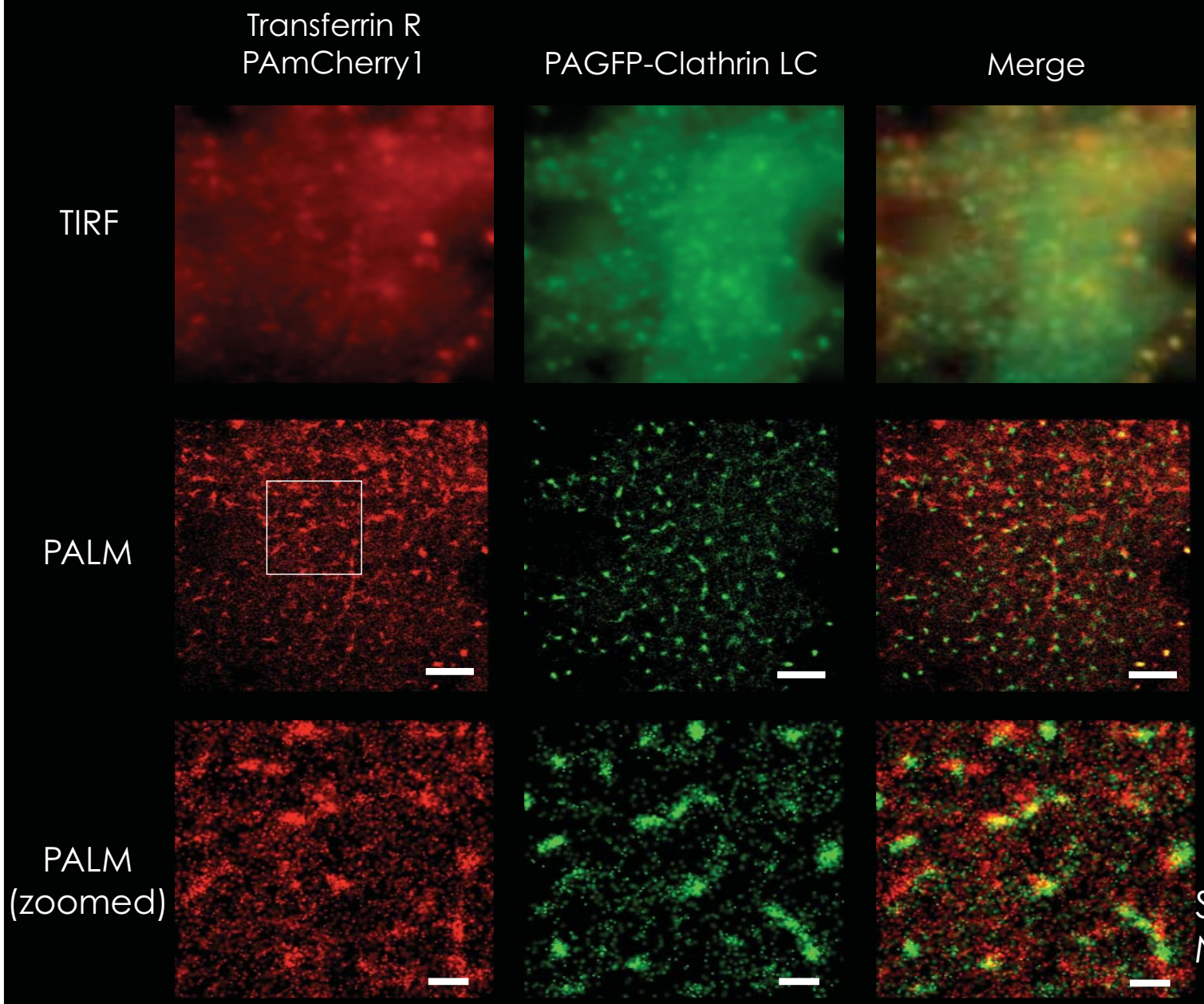


TEM



Mitochondrial matrix, COS-7 cell
dEosFP-tagged cytochrome-c oxidase targeting sequence

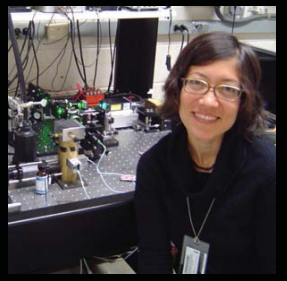
Two-Color (Simultaneous) PALM



Subach et al., 2009
Nature Methods

Dynamics: Single Particle Tracking

Su Manley

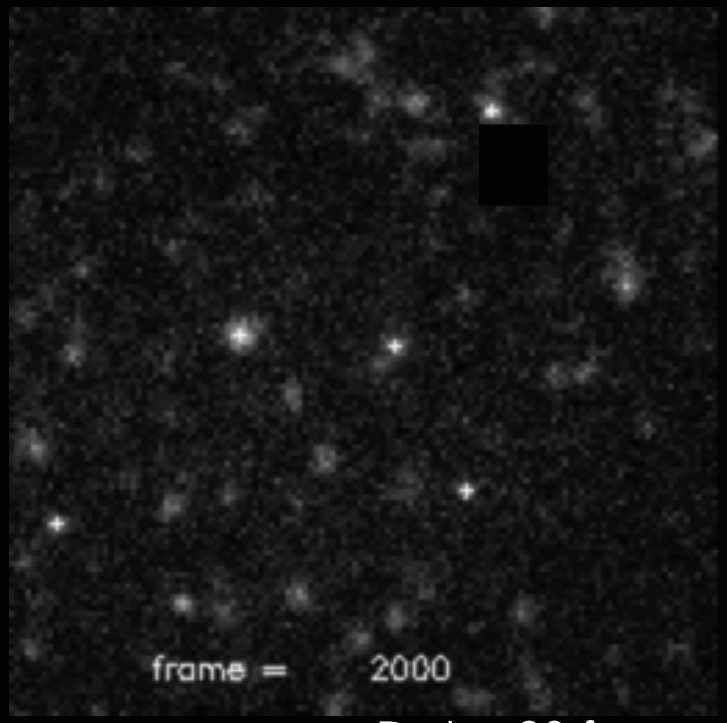


Jen Gillette

Single Particle Tracking + PALM:

- TIRF microscopy (1.65 NA)
- Eos-FP
- 10,000 images
- 20 frames/sec
- require molecules move < 300 nm

Manley et al, Nature Methods (2008)



Data: 20 frames/sec, 100 images

VSVG-Eos

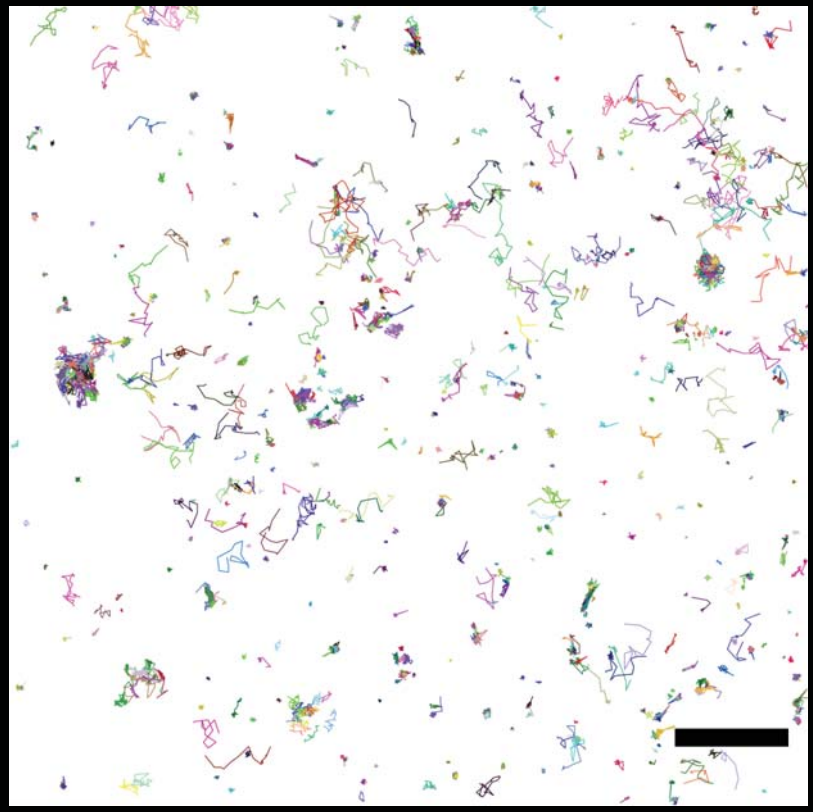
Create spatial maps of SPT: sptPALM

Dynamics: Single Particle Tracking

SINGLE PARTICLE TRACKING WITH PALM

VSVG-EosFP

Gag-EosFP



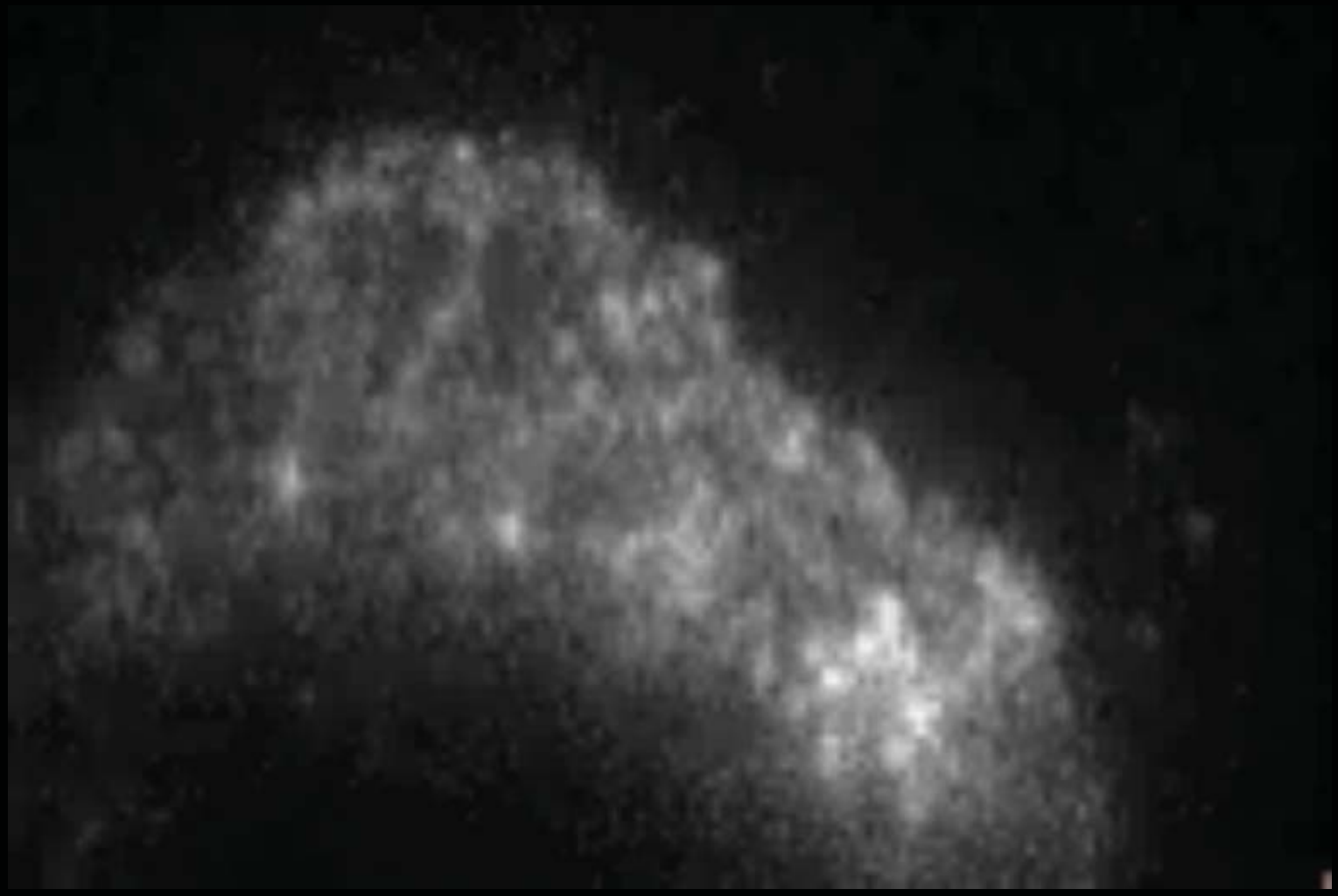
Single molecule tracks

High-density information on dynamics

http://lippincottschwartzlab.nichd.nih.gov/pdf/Manley_NatMeth2008.pdf

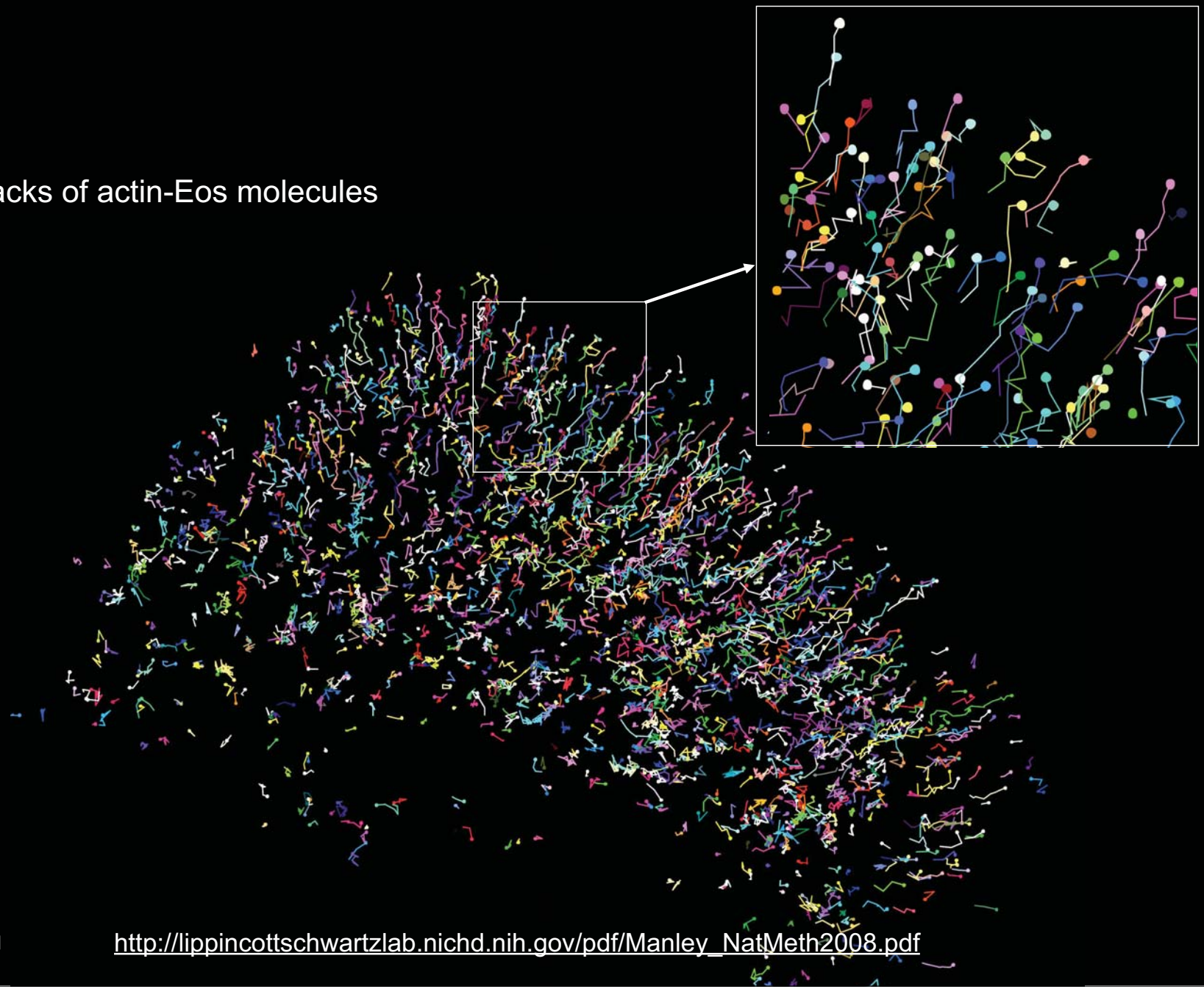
Probing the dynamics of single actin filaments with sptPALM

Single actin-Eos molecules imaged with TIRF
Images acquired at 1 sec intervals
Video displayed 30x real time



Probing the dynamics of single actin filaments with sptPALM

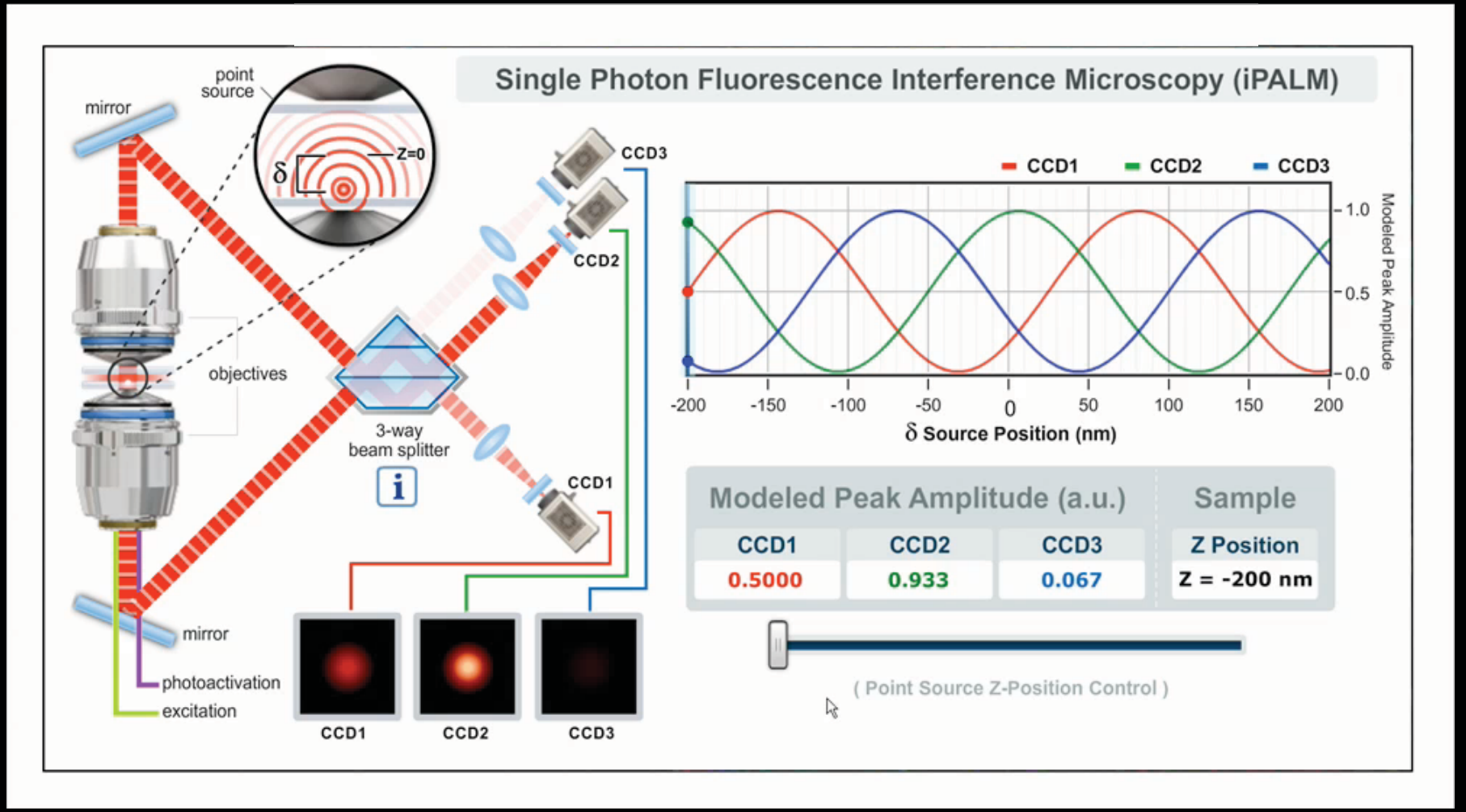
sptPALM tracks of actin-Eos molecules



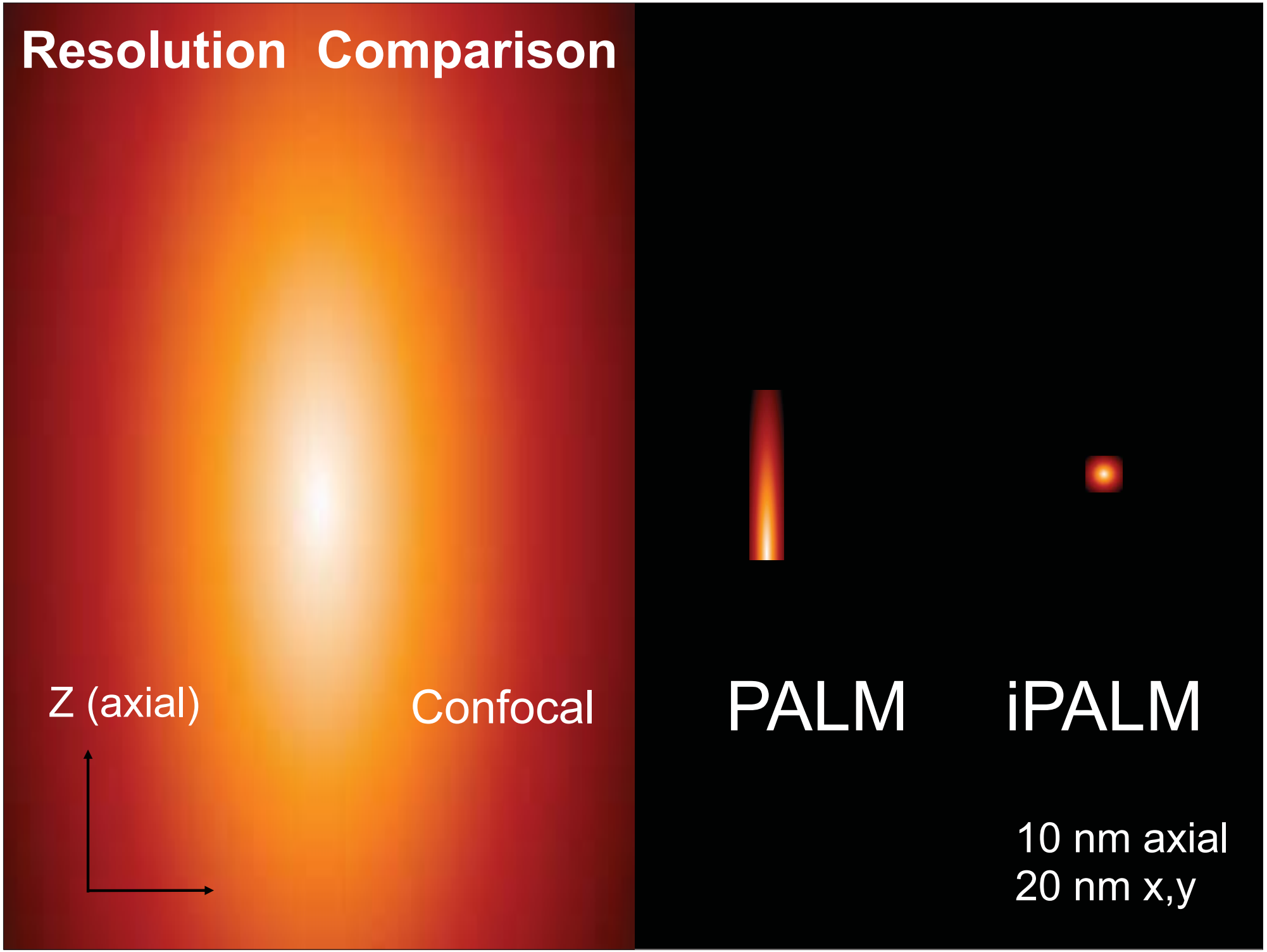
5 μm

http://lippincottschwartzlab.nichd.nih.gov/pdf/Manley_NatMeth2008.pdf

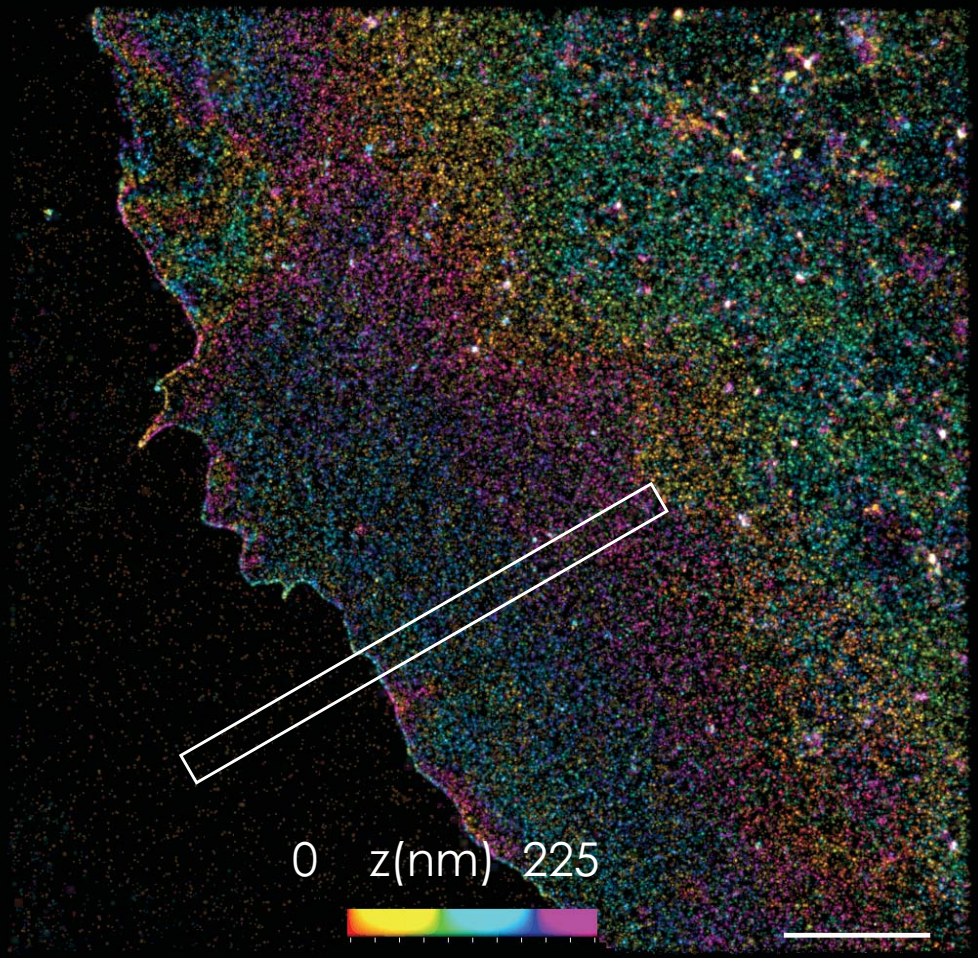
The Principle of iPALM



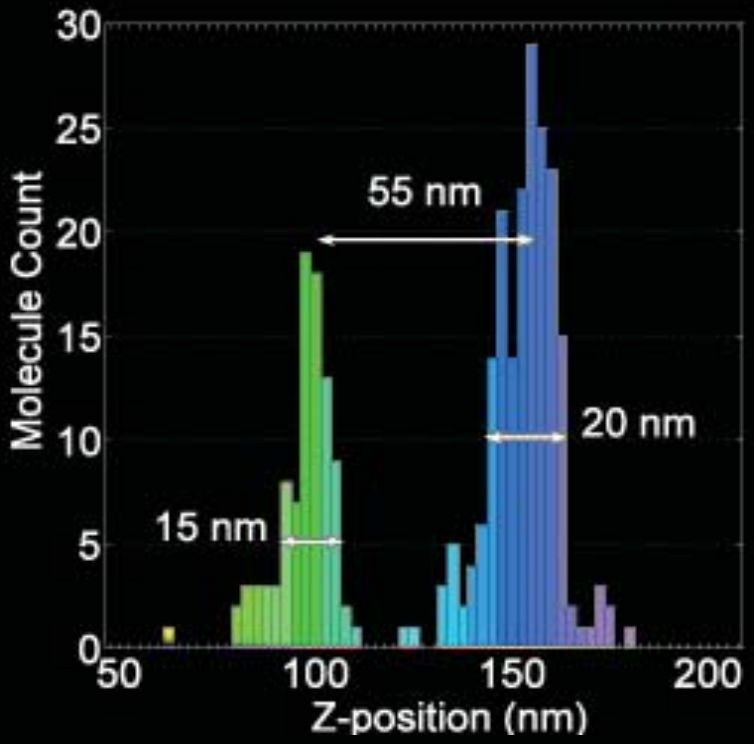
Harald Hess + Gleb Shtengel

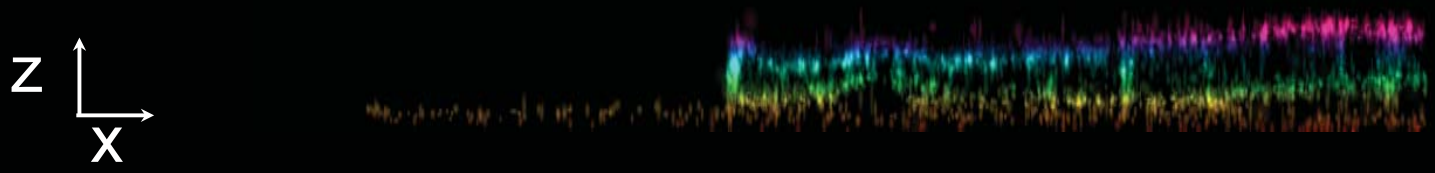

Resolution Comparison
Z (axial)
Confocal
PALM
iPALM
10 nm axial
20 nm x,y

Imaging with iPALM



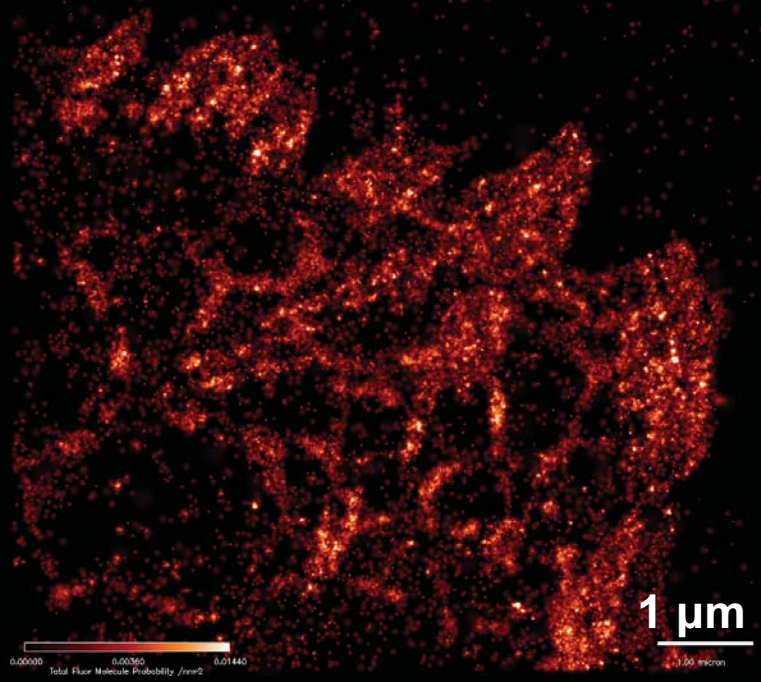
VSVG-tdEos on PM

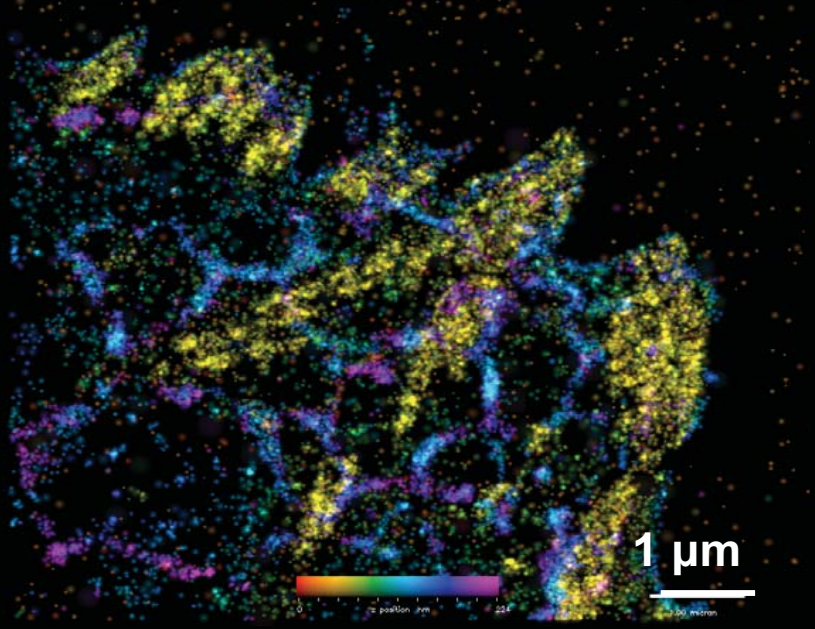




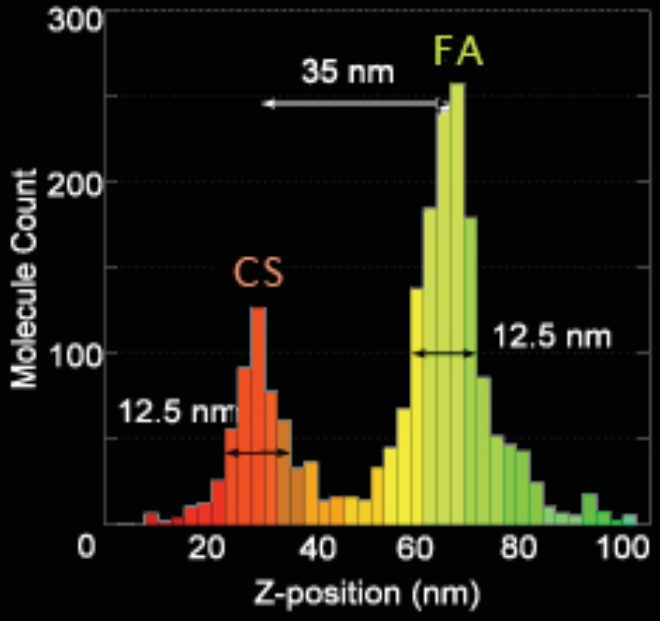
Shtengel et al., *PNAS* 2009

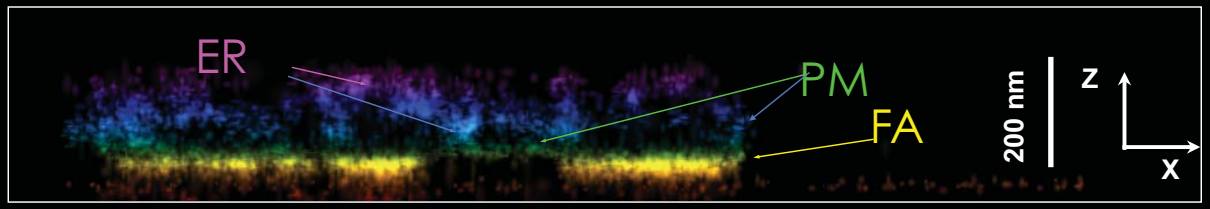
iPALM OF Eos-α_v-integrin





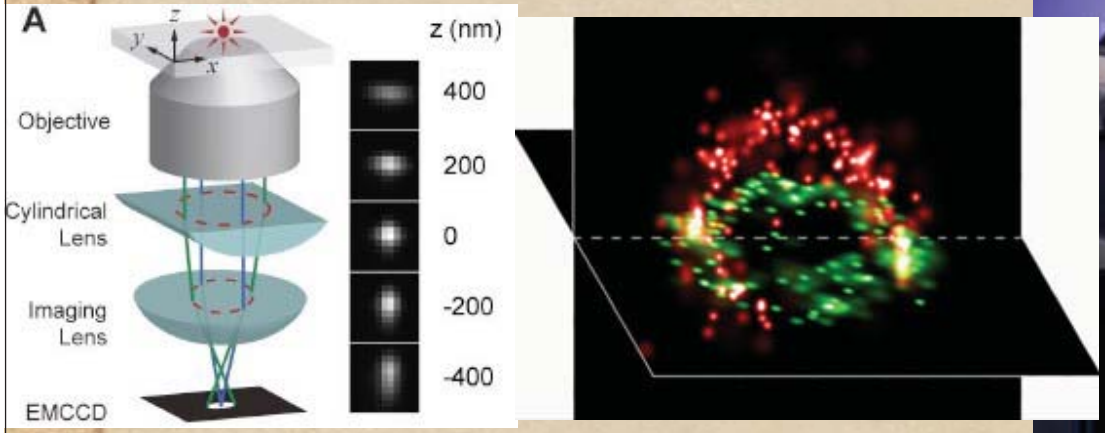
Top view

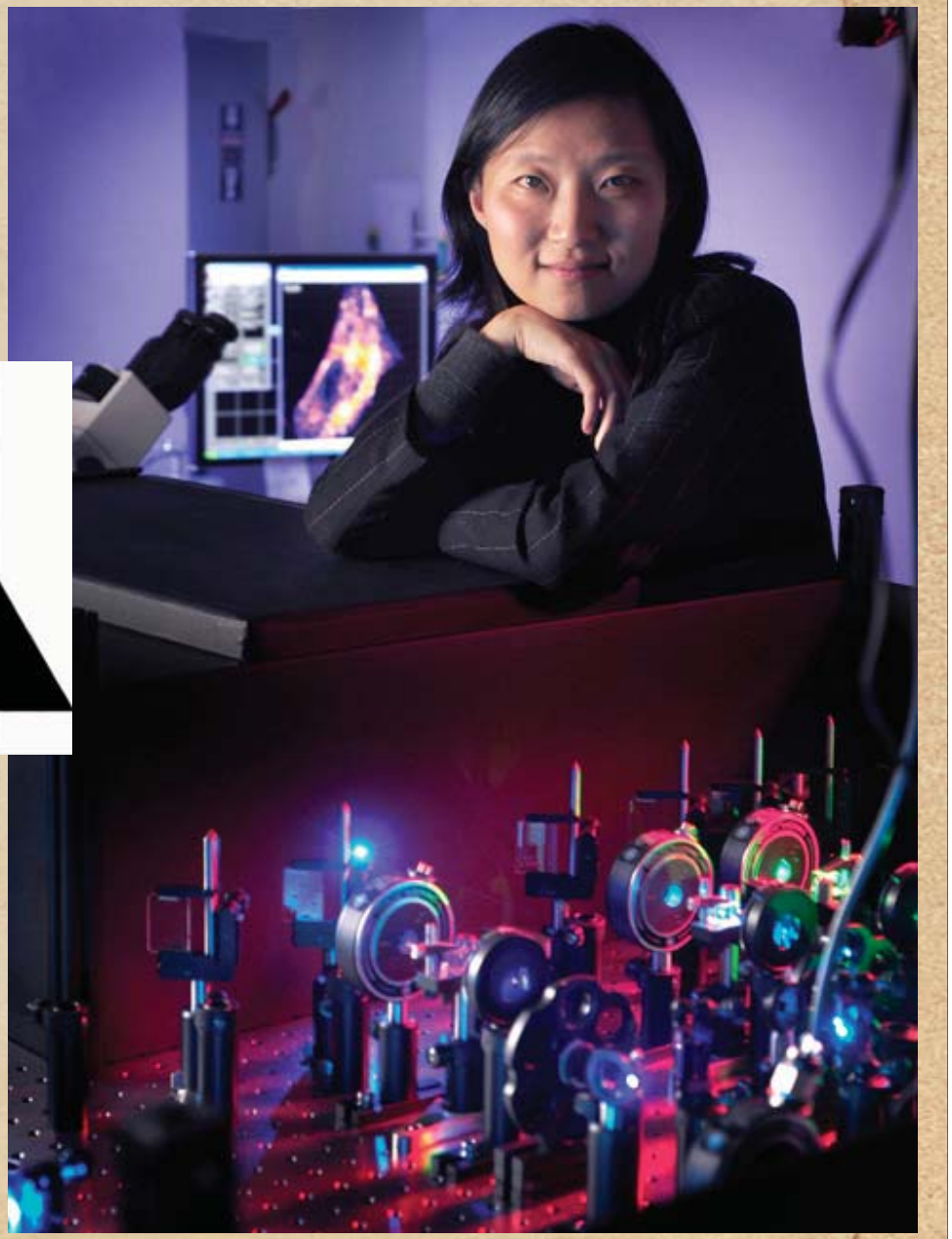




Side view

STORM is like PALM but uses probes that are photoswitchable rather than photoactivatable.





And the third dimension is obtained using intentional astigmatism, rather than interference.

Xiaowei Zhuang, HHMI and Harvard

In PALM/STORM, concerns include:

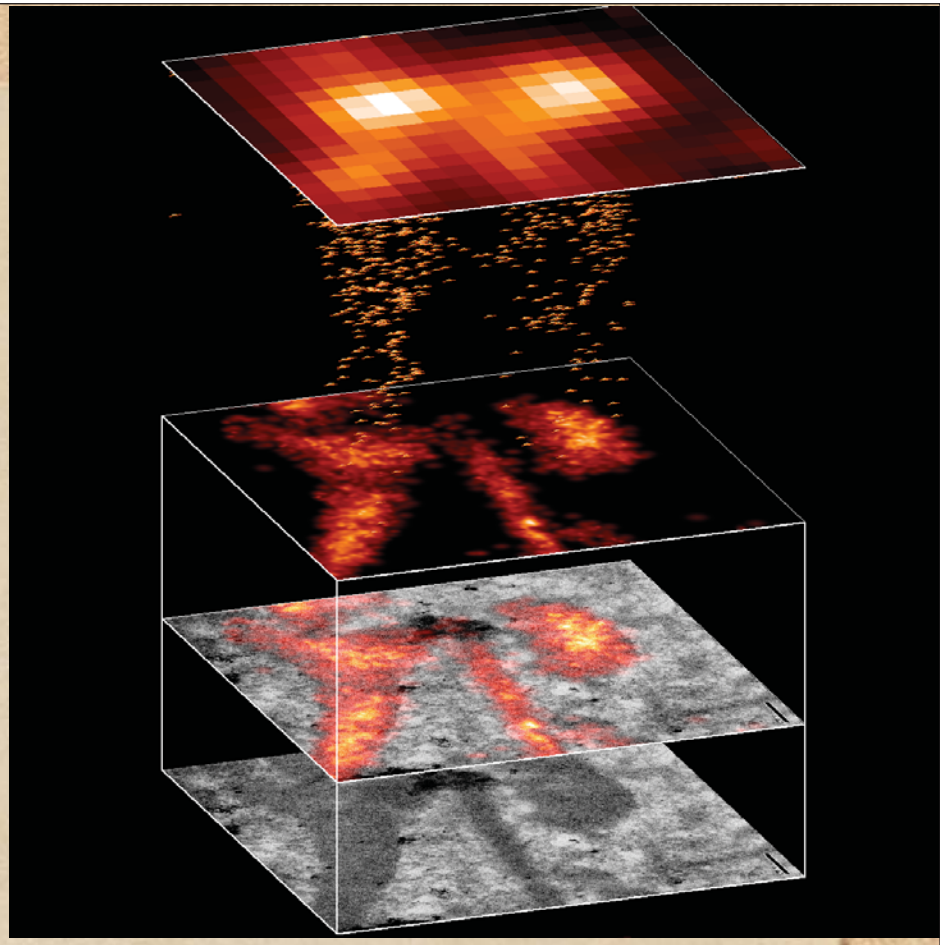
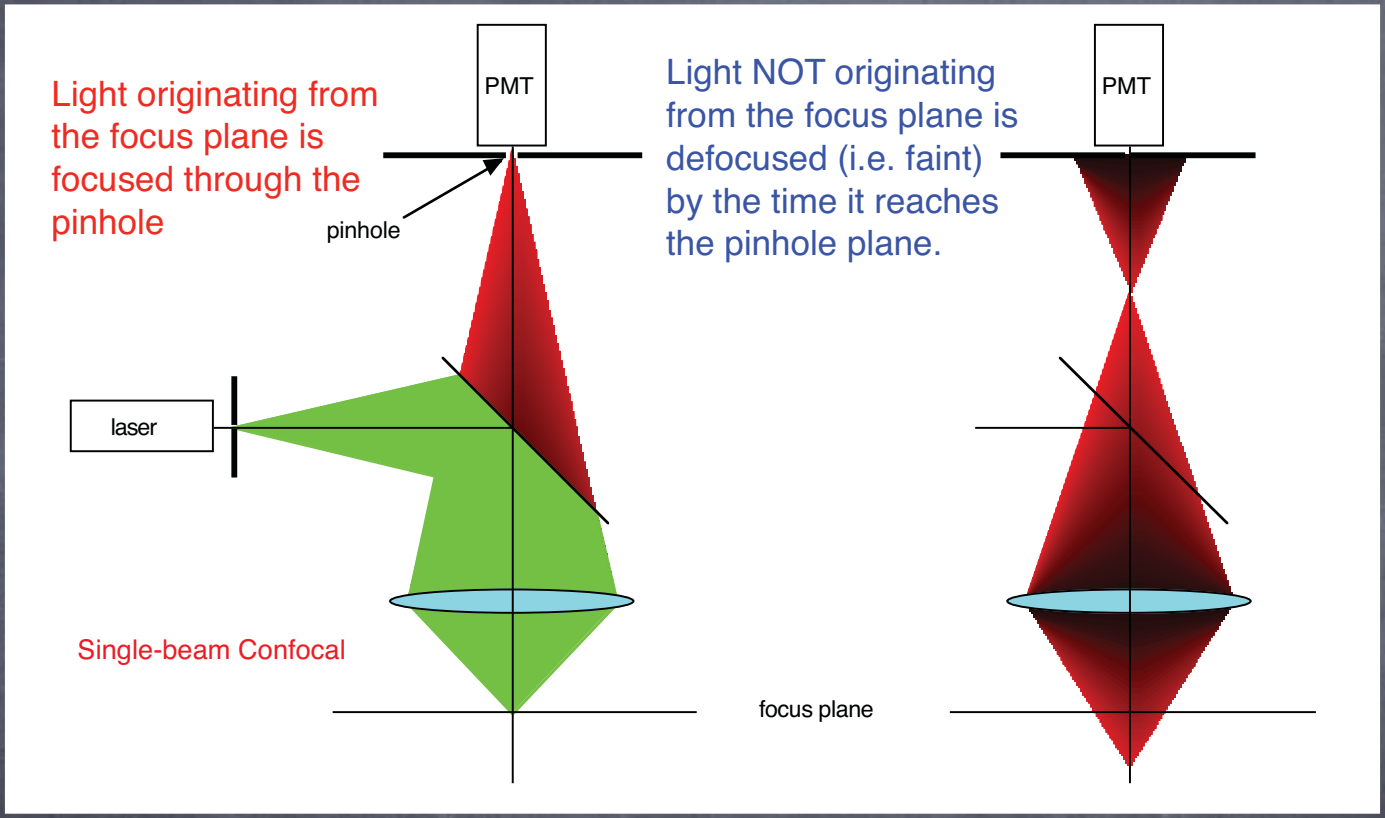


Image from Lippincot-Schwartz

- Works only with un-cageable or photoswitchable dyes.
- As low background is essential, it works better in TIRF/2D.
- Data rate limited by need to prevent spots overlapping.
  - Higher total data rate for larger field of view but small CCDs readout faster.
  - Needs high frame-rate EM-CCDs: greater CIC noise.
  - Significant computational requirements
- Although PALM requires bleaching: ⇒ photoproducts ⇒ phototoxicity, at least it bleaches only a single plane and each molecule is imaged only once.

REVIEW: Confocal microscopy:

Produces optical sections (and eventually 3D STACKS).



However, there is a snag! This setup gives an image of only one point.

To get a useful 2D/3D image, one must scan the beam/detector system.

AND to get a comparable image in the same time,
the light intensity in the spot must be ~100,000x more than WF.

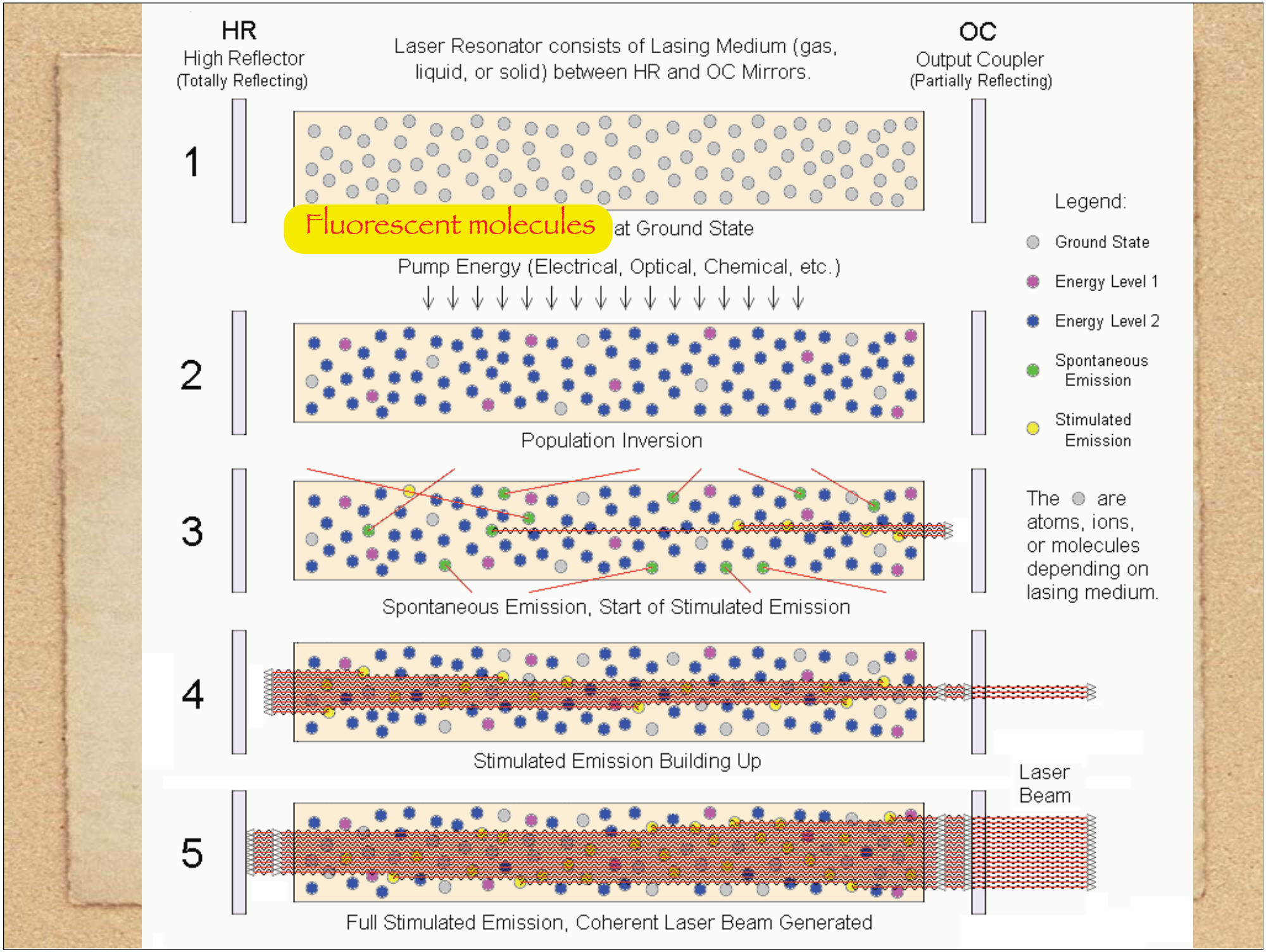
Scanned HRLM techniques

Stephan Hell

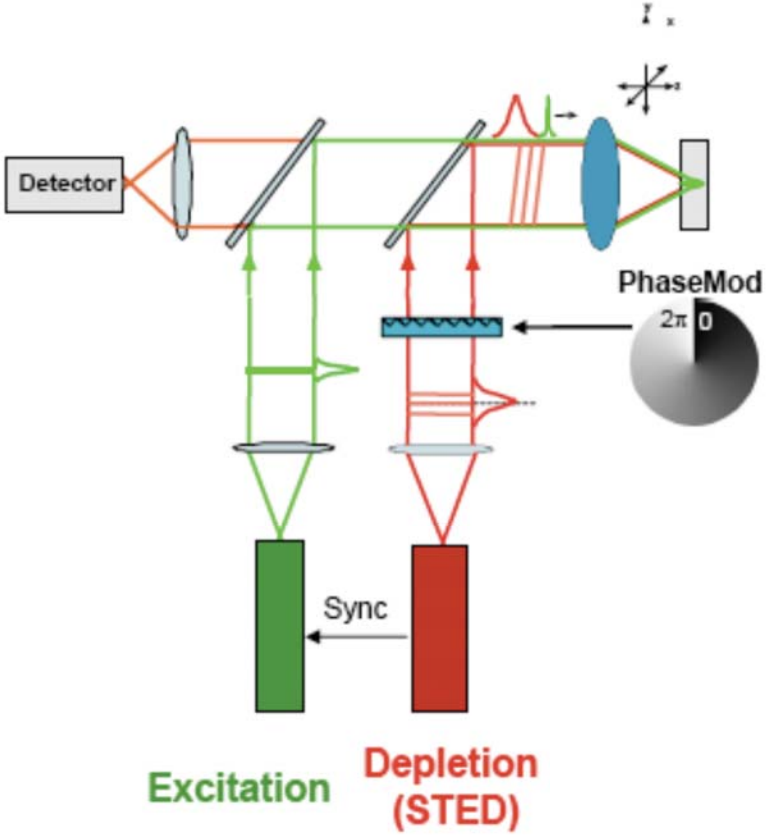
Stimulated emission depletion microscopy (STED)

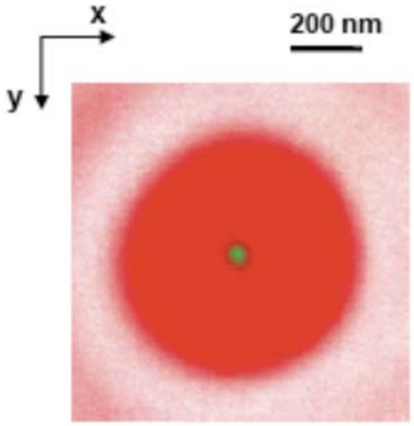
$$d = \frac{\lambda}{2n\sin\alpha\sqrt{1+I/I_s}}$$

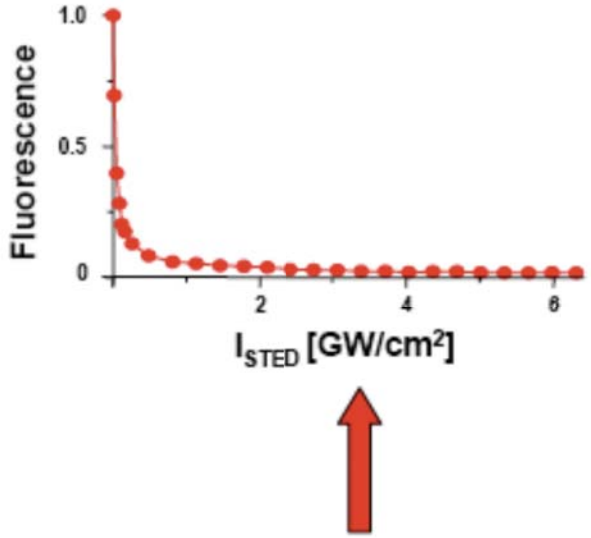
First, about how lasers work...


HR
High Reflector
(Totally Reflecting)
Laser Resonator consists of Lasing Medium (gas, liquid, or solid) between HR and OC Mirrors.
OC
Output Coupler
(Partially Reflecting)
1
Fluorescent molecules
at Ground State
Pump Energy (Electrical, Optical, Chemical, etc.)
2
Population Inversion
3
Spontaneous Emission, Start of Stimulated Emission
4
Stimulated Emission Building Up
5
Laser Beam
Full Stimulated Emission, Coherent Laser Beam Generated
Legend:
Ground State
Energy Level 1
Energy Level 2
Spontaneous Emission
Stimulated Emission
The are atoms, ions, or molecules depending on lasing medium.

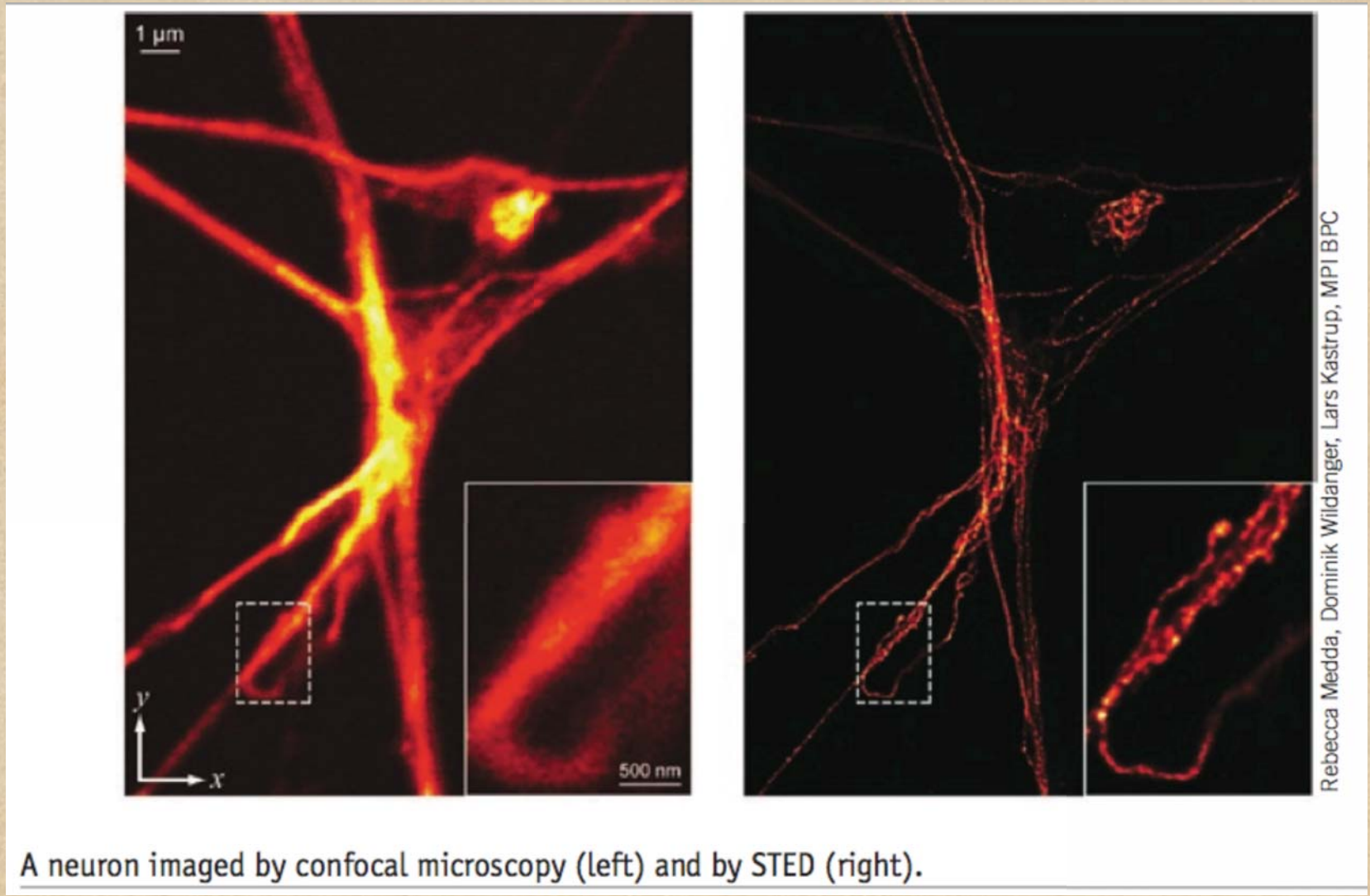
STED microscope



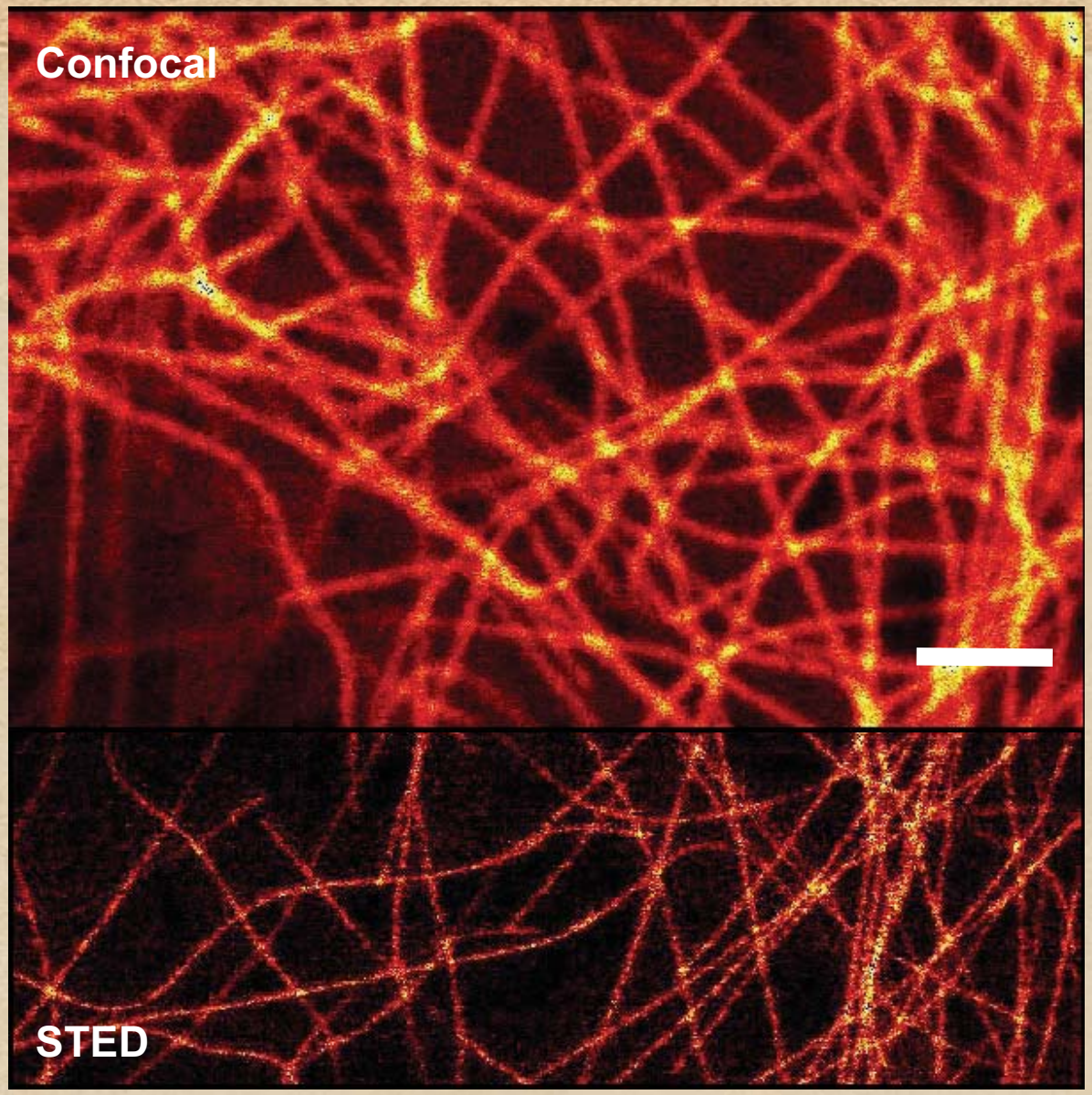




and it works at near-normal scan speeds



A neuron imaged by confocal microscopy (left) and by STED (right).



What is more, you can get accurate photon counts.

Confocal intensity, 0-50 photons/15µs

STED intensity, 0-15 photons/15µs

Hein, Willig and Hell
PNAS, 2008

Subdiffraction resolution fluorescence imaging of Citrine-labeled microtubules inside a living PtK2 cell. In both the confocal reference and the STED counterpart, excitation was performed in the pulsed mode with 7.6μW of time-averaged focal power. In the STED mode, a STED beam of 31mW average power was added, which improves the lateral resolution inside the living cell from 180 to 60 nm. (Scale bar, 2μm.)

2-color STED

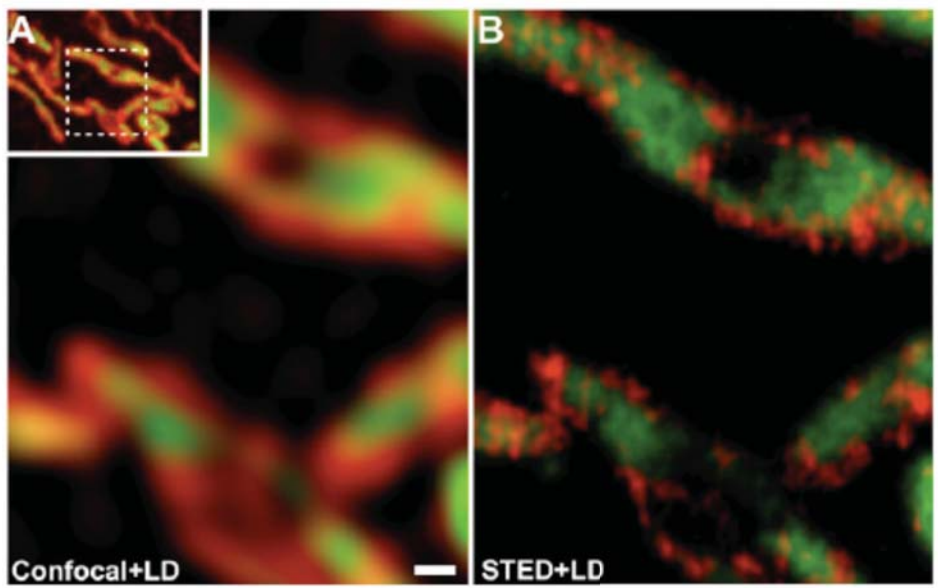


FIGURE 4 Mitochondria with labeled F_1F_0ATP synthase (*green*) and TOM complex (*red*); reverted antibody labeling as compared to Fig. 3. Again, the TOM complex appears as clusters; unlike the confocal (*left*), the STED recording (*right*) resolves the TOM complex clusters. In both recordings, the raw data were subject to a linear deconvolution (*LD*) with the respective PSF. (*Inset*) Low magnification image. Scale bar, 200 nm.

Note scale bar size.

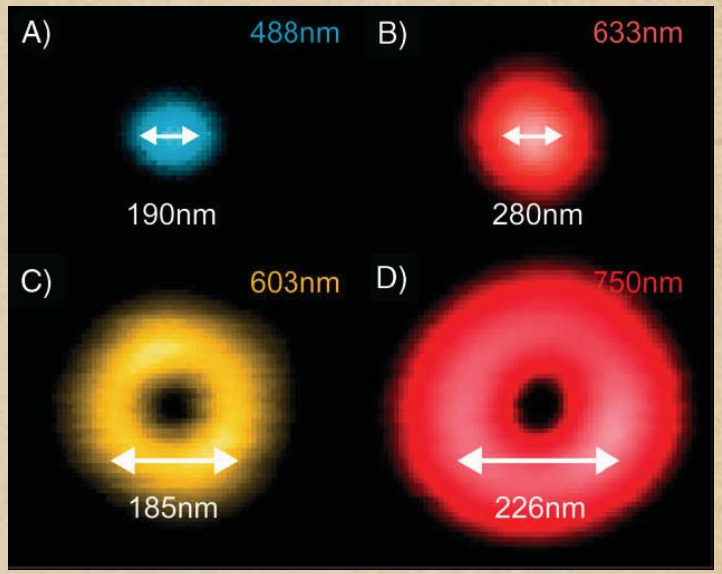
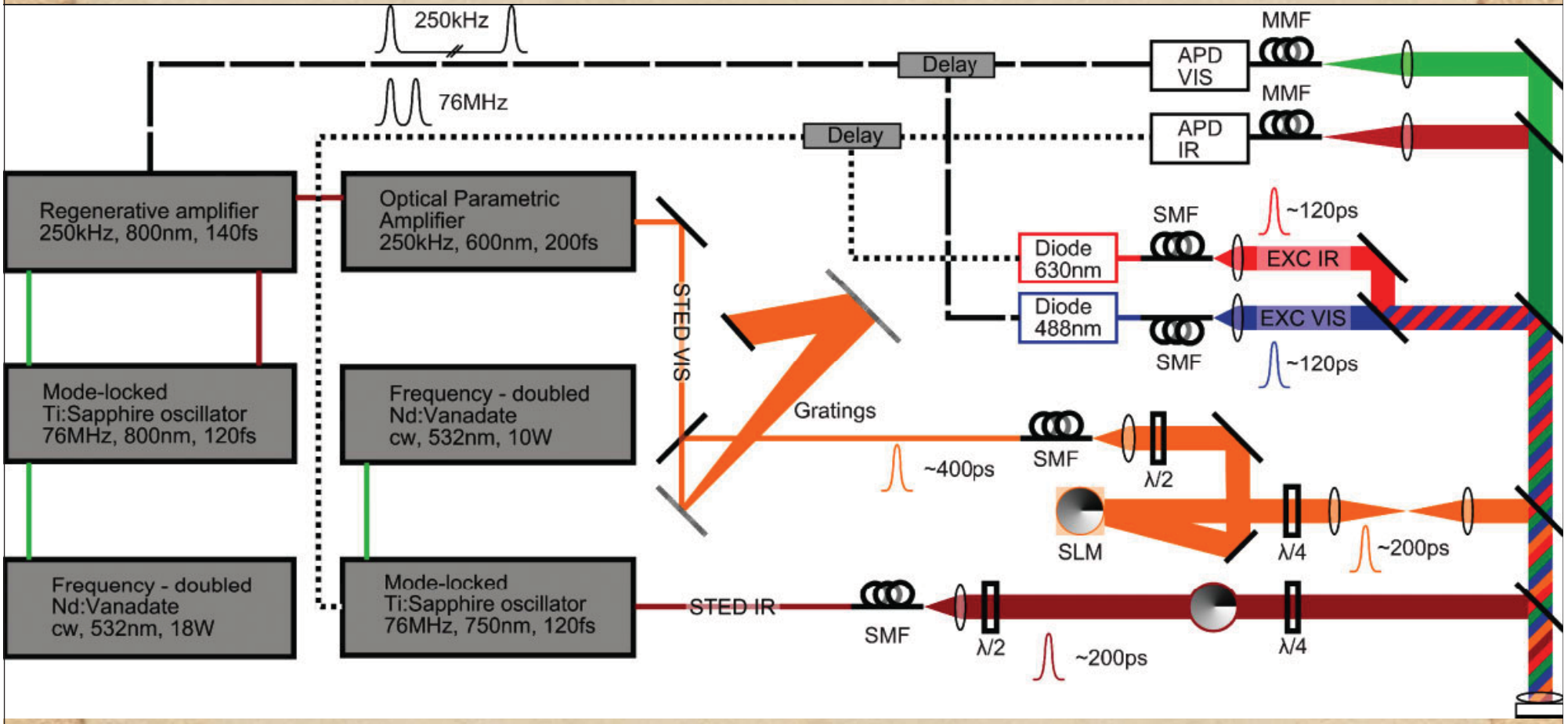


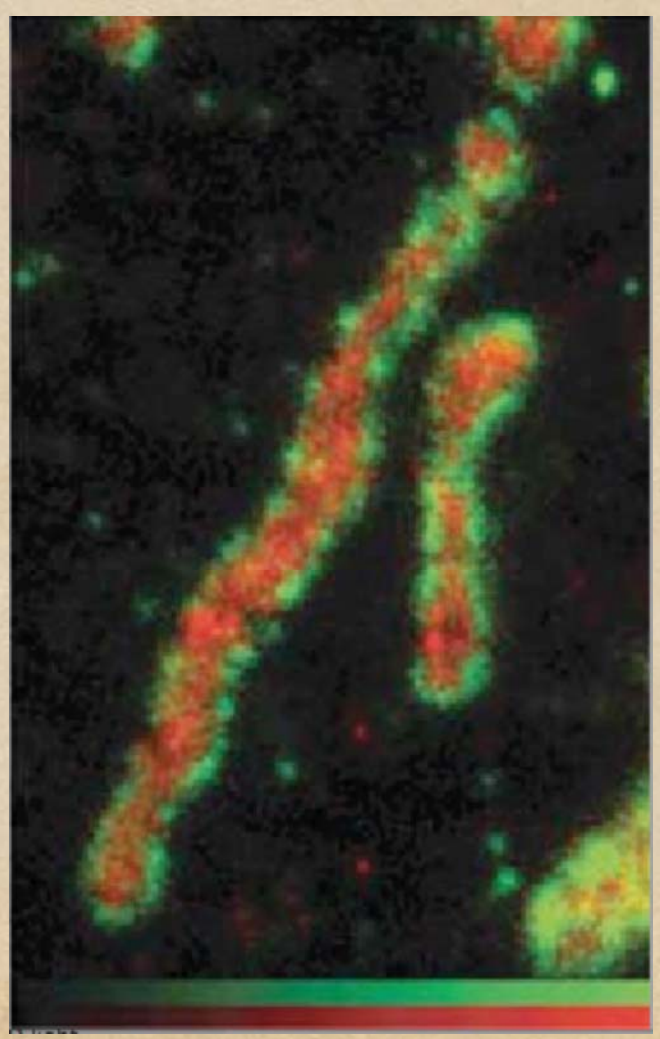
Figure 7. Focal spots (PSF) of the two-color STED microscope, measured by recording the scattered light from a subdiffraction-sized bead. Excitation PSF for the A) green and B) red channel are overlapped with the corresponding doughnut-shaped STED-PSF shown in (C) and (D). The numbers at the arrows indicate the FWHM of the diffraction maximum (A, B) or the diffraction doughnut minimum (C, D).

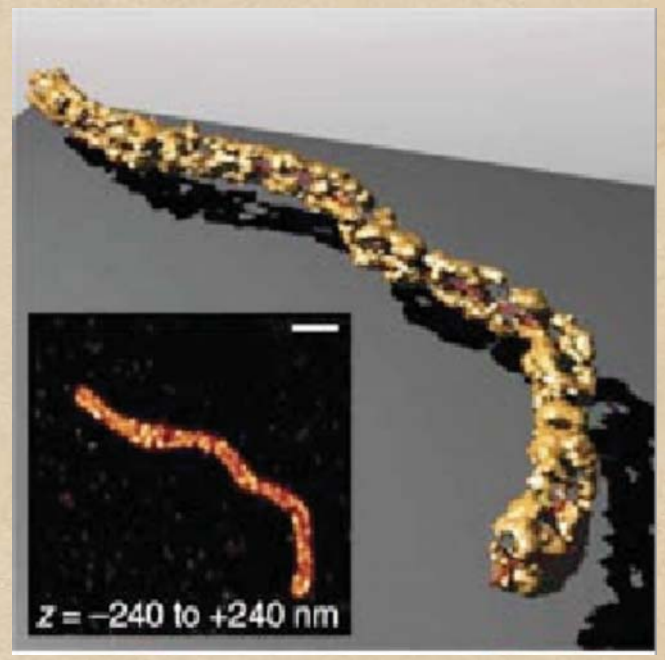
But 2-color STED does require some complexity…



Although most STED has been done using optics optimized for 2D,
it is also possible to do

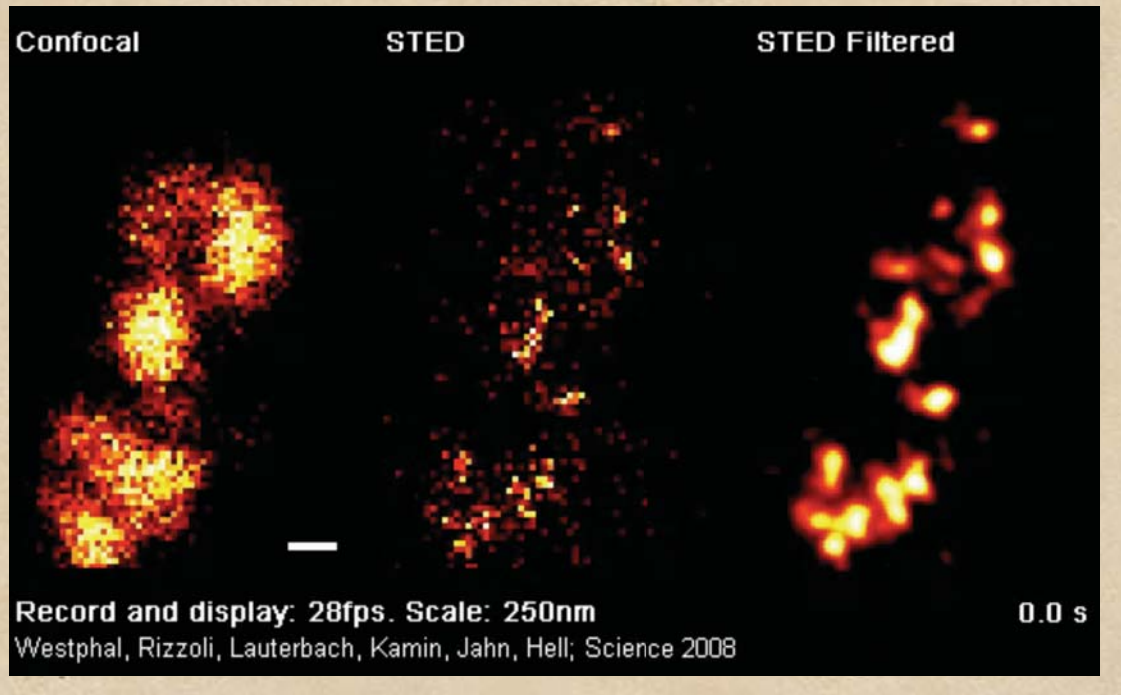
3D, 2-color STED





Cellular powerhouse: These images of a cellular organelle called the mitochondrion were taken with the highest-resolution 3-D light microscope yet developed. Researchers at the Max Planck Institute, in Germany, have used the microscope to image individual, fluorescently labeled protein clusters in the mitochondria of live cells (below) and to combine them to form 3-D images (above). Credit: Nature Methods/Stefan Hell

As there is no requirement to avoid imaging overlapping PSFs,



you can do STED fast.

Real time, 28 fps,
total of 140 frames

Movie from Stephan Hell,
Science WWW

Fig. 1. Real-time STED microscopy resolves single synaptic vesicles in living neurons. Images of a short fragment of axon labeled with mouse anti-synaptotagmin and Atto647N-labeled anti-mouse Fab fragments, in confocal mode (left) and in STED mode (center and right). The increased resolution in the STED image and the reappearance of vesicles in subsequent frames can be seen (three arrowheads indicate relatively stable vesicles; also, many other spots can be recognized in both frames). Smoothing helps to identify super-resolved vesicles in the STED images (right).

STED puts considerable stress on the dye molecules and consequently the most successful studies have been carried out using specific dyes...

The following dyes have been successfully used in STED microscopy:

Dye name (Manufacturer / Distributor)	Exc. Wavelength	Exc. Pulse Length	STED Wavelength	STED Pulse Length	Repetition Rate	Avg. STED Power
ATTO 532 (ATTO-TEC GmbH)	470 nm	100 ps	615 nm	200 ps	80 MHz	14 – 18 mW
ATTO 532 (ATTO-TEC GmbH)	470 nm	80 ps	603 nm	280 ps	250 kHz	0.5 mW
ATTO 532 (ATTO-TEC GmbH)	488 nm	100 ps	615 nm	200 ps	80 MHz	16 mW
Chromeo 488 (Actif Motif)	488 nm	140 ps	602 nm	~ 160 ps	250 kHz	0.6 mW
Chromeo 488 (Actif Motif)	488 nm	< 100 ps	590 nm	200 – 300 ps	80 MHz	40 mW
DY-485XL (Dyomics GmbH)	488 nm	< 100 ps	647 nm	~ 200 ps	72 MHz	(20 + 3) mW
GFP	490 nm	100 ps	575 nm	200 ps	80 MHz	7.2 mW
ATTO 565 (ATTO-TEC GmbH)	532 nm	~ 90 ps	640 – 660 nm	~ 90 ps	1 – 2 MHz	
ATTO 565 (ATTO-TEC GmbH)	532 nm	cw	647 nm	cw	cw	114 mW
MR 121 SE (Roche Diagnostics)	532 nm	10 ps	793 nm	107 ps	76 MHz	10.4 mW
NK51 (ATTO-TEC GmbH)	532 nm	< 100 ps	647 nm	~ 200 ps	72 MHz	(20 + 3) mW
Sulfonated & rigidized rhodamine derivatives (V. Boyarskiy, NanoBiophotonics, MPI Göttingen)	532 nm	100 ps	640 nm	~ 300 ps	80 MHz	
Pyridine 2 / LDS 722 (Exciton, Radiant Dyes GmbH)	554 nm	250 fs	760 nm	13 ps	76 MHz	
Pyridine 2 / LDS 722 (Exciton, Radiant Dyes GmbH)	554 nm	250 fs	745 nm	50 – 200 ps		12.2 mW
RH 414 (Invitrogen Corp.)	554 nm	250 fs	745 nm	13 ps	76 MHz	8.78 mW
ATTO 590 (ATTO-TEC GmbH)	570 nm	~ 90 ps	690 – 710 nm	~ 90 ps	1 – 2 MHz	
ATTO 633 (ATTO-TEC GmbH)	630 nm	~ 90 ps	735 – 755 nm	~ 90 ps	1 – 2 MHz	
ATTO 633 (ATTO-TEC GmbH)	635 nm	100 ps	750 nm	~ 200 ps	76 MHz	
ATTO 647N (ATTO-TEC GmbH)	635 nm	cw	750 nm	cw	cw	423 mW
ATTO 647N (ATTO-TEC GmbH)	635 nm	100 ps	780 nm	300 ps	250 kHz	
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	781 nm	303 ps	40 MHz	10.1 mW
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	775 nm	300 ps	76 MHz	
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	780 nm	300 ps	80 MHz	90 – 100 mW
JA 26 (K.H. Drexhage, Siegen University)	637 nm	54 ps	778 – 785 nm	303 ps	40 MHz	

Good features of STED:

- Almost unlimited improvement in xy-resolution possible.
- Can work in 2 colors.
- Illumination need only come from one side.
- Phase plate can be optimized to improve z-resolution.
- 3D imaging possible.
- Data rate sufficient to permit "video-rate" imaging.
- Speed can be further increased by using many STED spots but this will also increase total light input/damage.
- Hi-QE Avalanche photo-diode photodetector used .
- Relatively immune to RI inhomogeneity.

In STED, the matters of concern include:

- Optimal dyes are needed: work in near-IR and must have low crossing to triplet state, and significant Stokes shift.
- Will the very small effective PSF contain enough dye?
- The high intensity of the STED beam (use red, near-IR).
- What is the importance of damage/signal far way from the focus plane, where the STED beam is weak?
- Pulsed illumination implies that the excitation duty cycle is low. This limits the data rate: small rasters or few frames/s.
- Considerable technical complexity

STED, PALM/STORM, I5M and SSIM have different "sensitivities" to Poisson Noise.

	Detector, (QE_{eff}, noise)	Sensitivity to out-of-focus light	Inherent contrast, $\Delta I/I$	Recorded electrons/ pixel	Data rate limited by
STED	APD, pulsed signal	Little	high	10s	scanned image, pulsed ill.
PALM	EM-CCD	none	high	100s	few molecules/ frame, 100s of frames/image. EM-CCD frame rate limiting
STORM	EM-CCD	none	high	100s	
I5M	EM-CCD	Serious	Much lower	1,000s to 10,000s	Many frames/ plane: Limited by CCD camera frame rate
SSIM	EM-CCD	Serious	Much lower	1,000s to 10,000s	

STED, PALM, STORM, I5M etc. use a variety of different photodetectors.

Method/Det	CCD	EM-CCD	APD	PMT
STED			x	x
PALM/STORM		x		
I5M	x	x		
QE_{EFF}, %	80-95	40-45 multiplicative noise	40-45	2-10
NOISE, e^-/p	3 - 10	0.003	pulse counting	0.003
Other factors	slow read	Frame transfer Vertical smearing	Signal loss to pulse pile-up	multiplicative noise

These detectors all involve tradeoffs.

STED, PALM, STORM, I5M etc. subject the specimen to different excitation intensities.

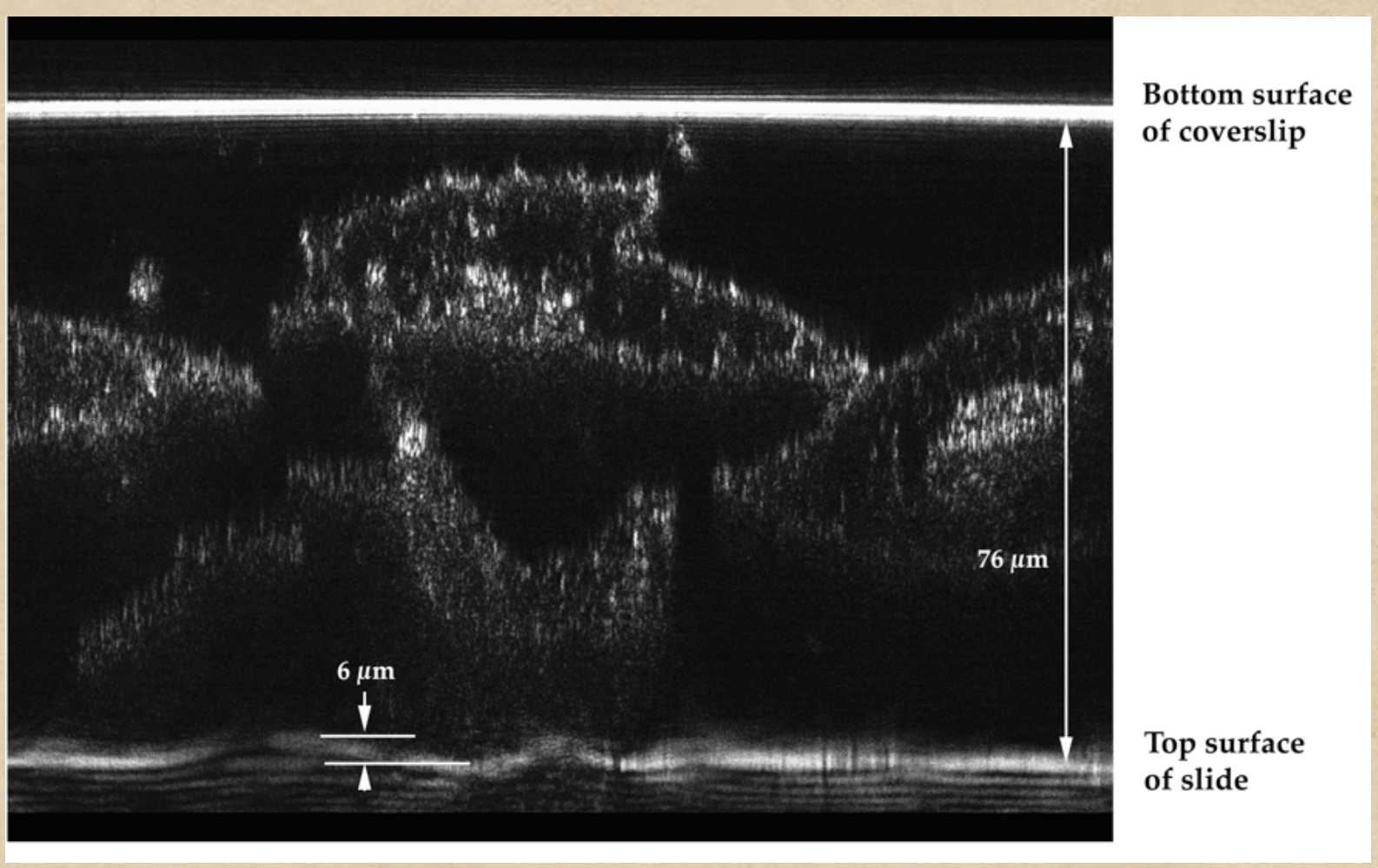
Method/ excitation	Spot/ Area	Input power	Input power density	Mitigating factors	Exacerbating factors
STED	spot	mW	Very high	Most excitations depleted. Pulsed illumination	Near-IR/red dyes
PALM/STORM	area	mW	Very low	Evanescent illumination	Slow recording
I5M	area	mW	Low	Modest equipment	Slow recording, Immotile spec.
I5M saturated	Area, pattern, saturated	>100s of mW	high	Pulsed illumination	Dyes with long singlet lifetime Slow recording Immotile spec.

What about the specimen?
Do the optical characteristics of the cell limit microscope performance?

About 8 years ago, I took a test image of a cheek-cell using backscattered light (BSL).

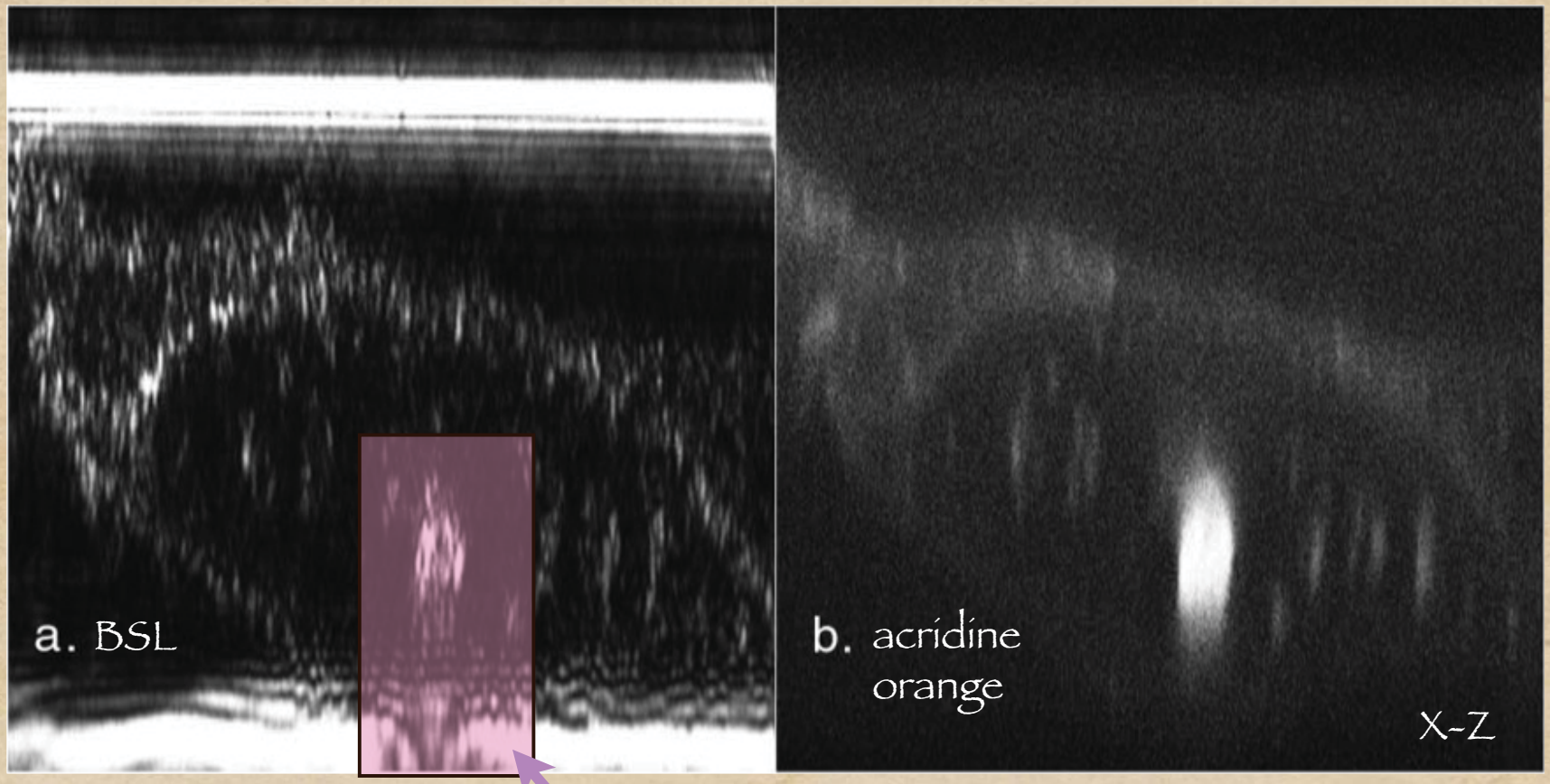
It turned out to be more interesting than I expected...

This is an xz image of a cheek cell made using backscattered light.



Something was wrong with this image.

Some cheek!



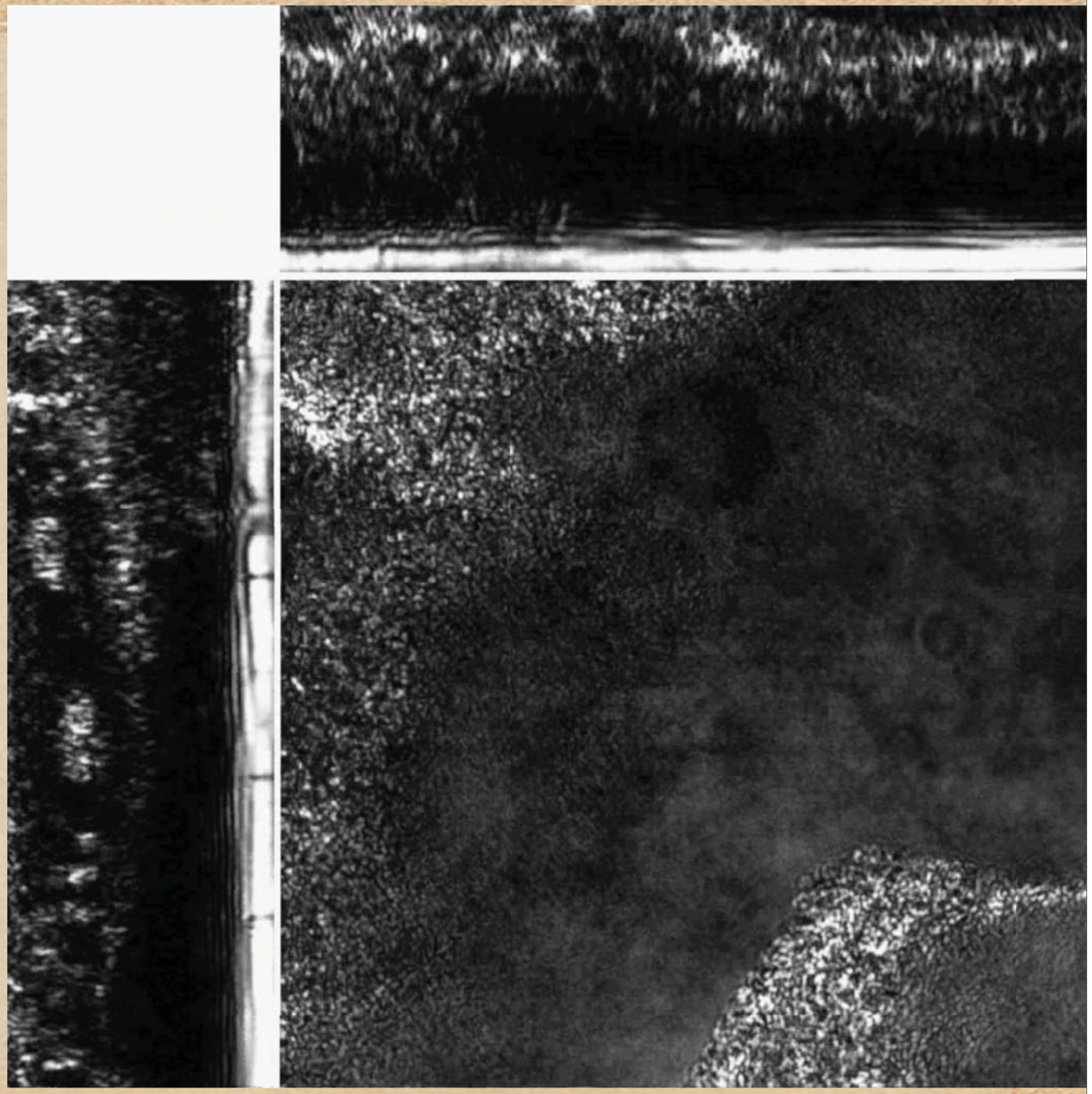
What is going on here?

Clearly, there is a lot of spherical aberration.

But why the apparent dip in the slide surface?

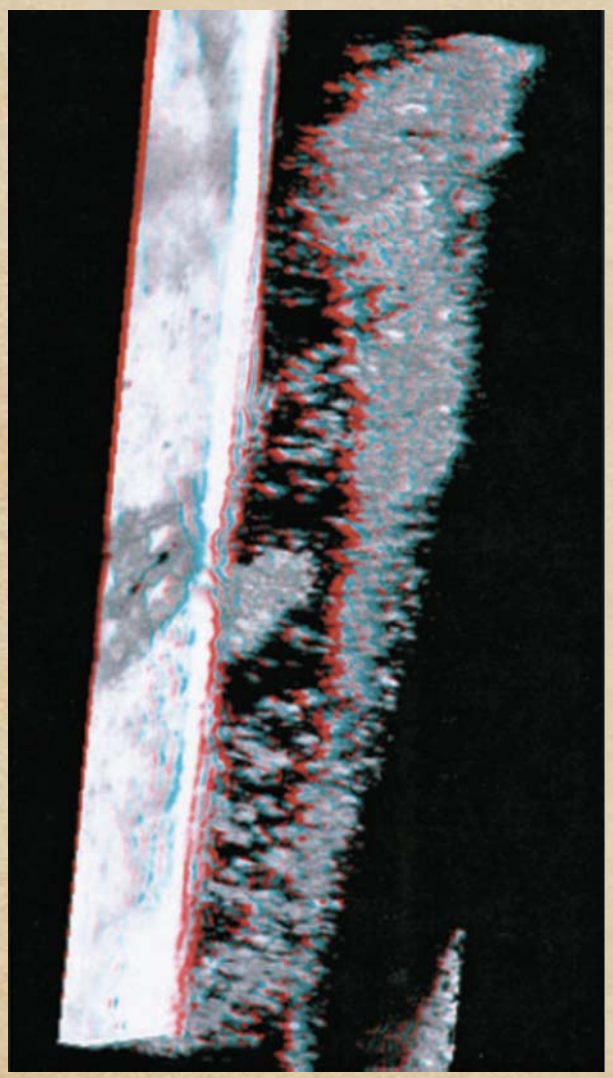
So let's try with a water lens...

Same problem...

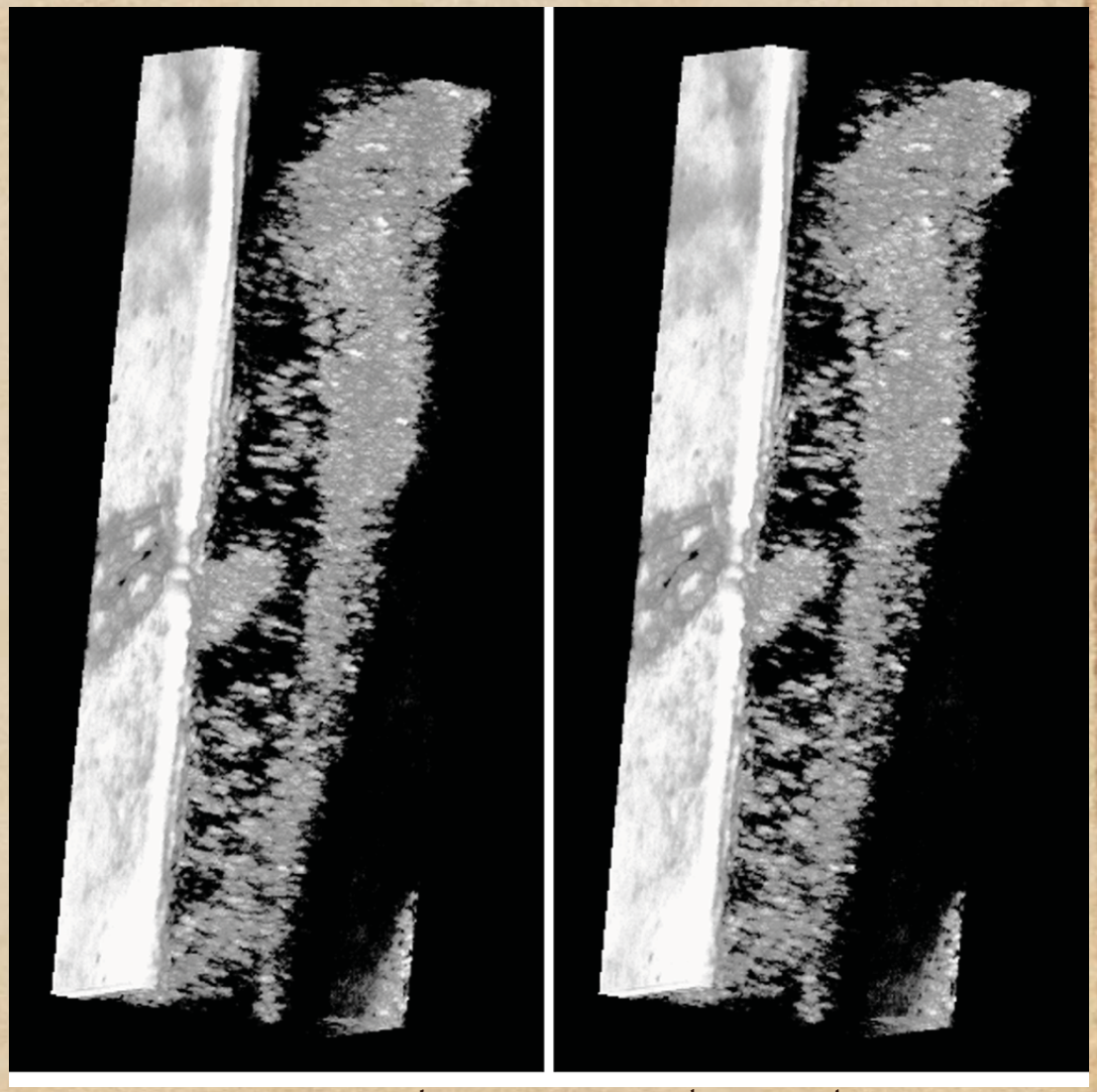


Thanks to Glen MacDonald for making this movie from my data.

a stereo view of the same data...

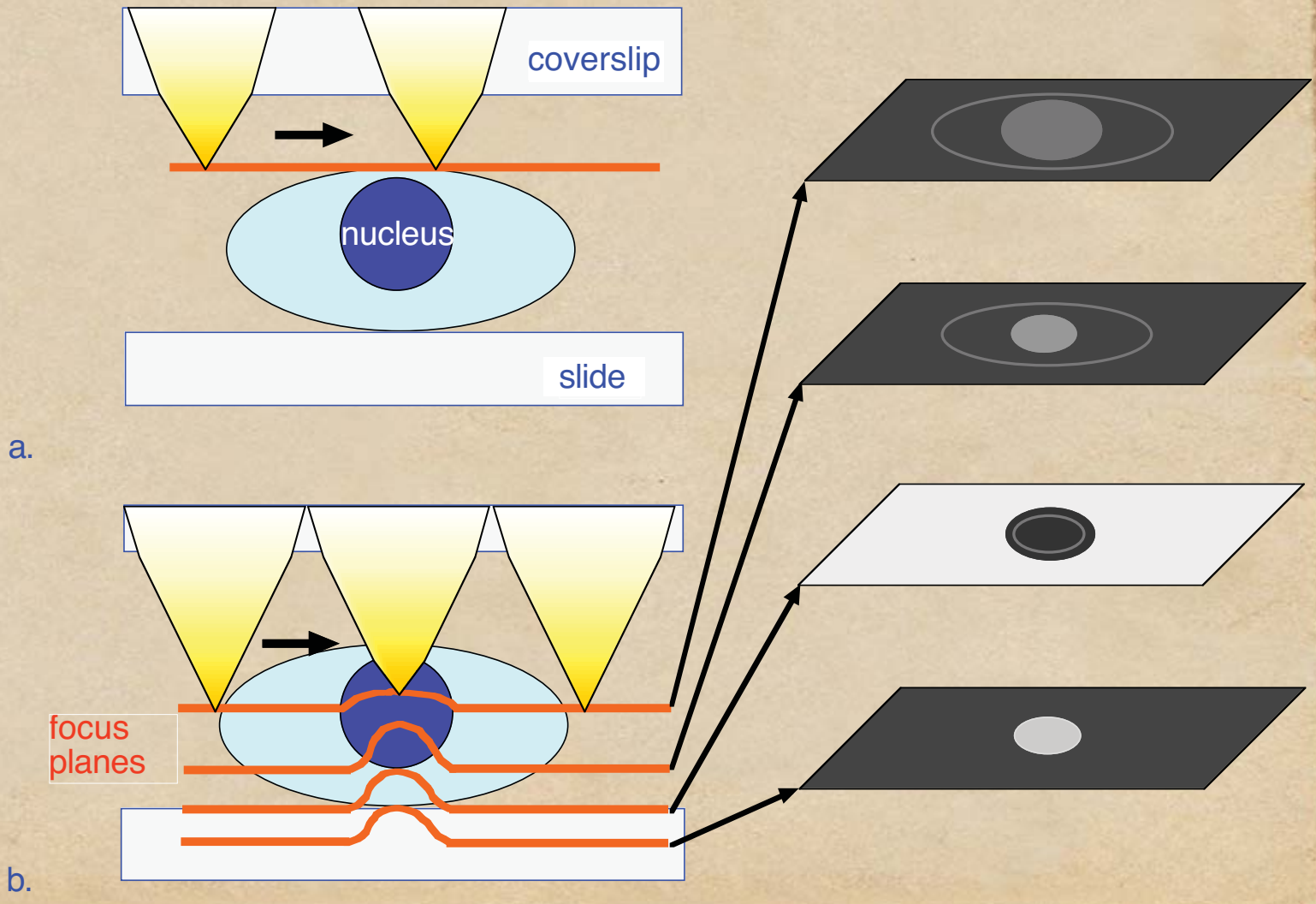


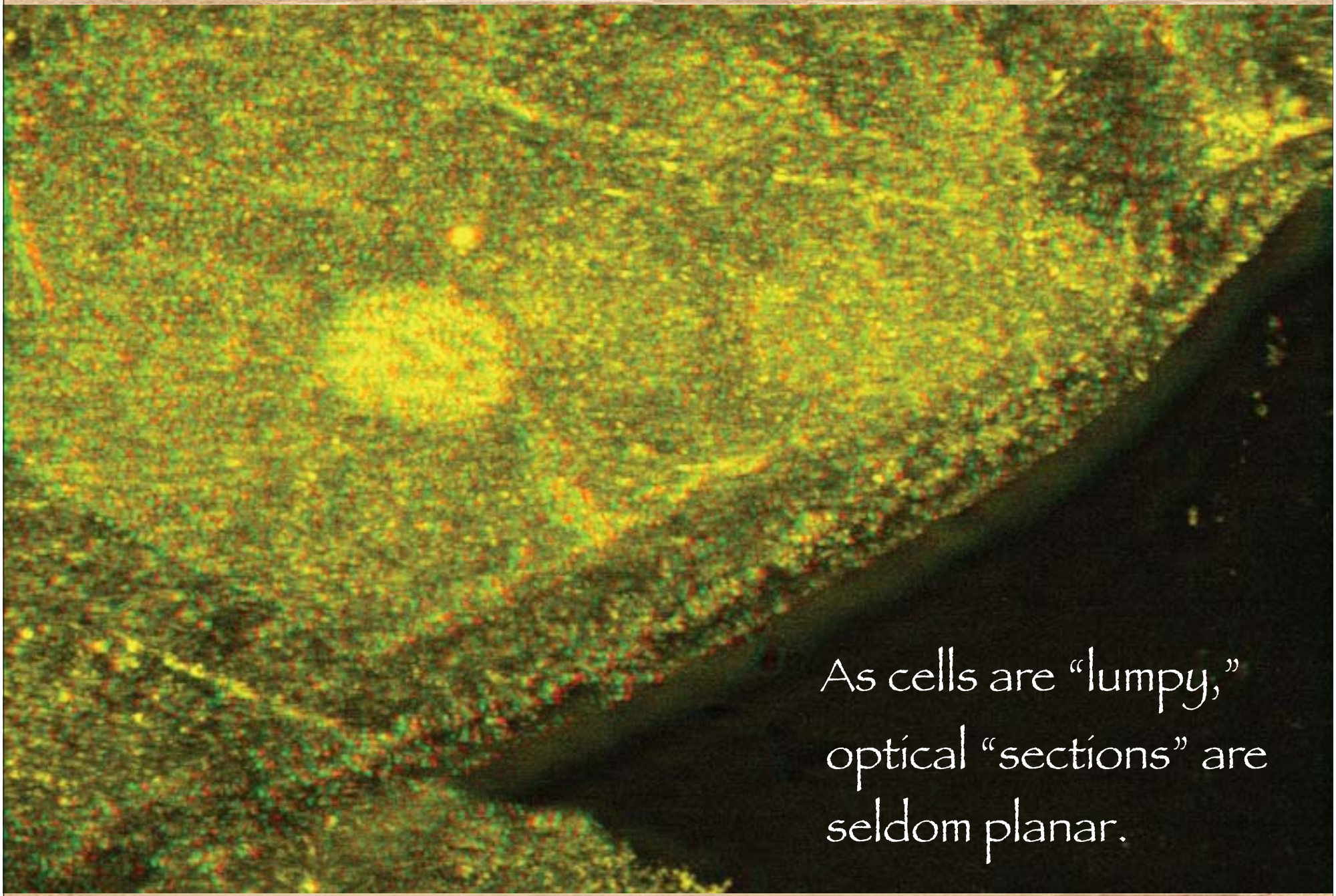
Use your red and green glasses.



Cross your eyes and concentrate on the central image.

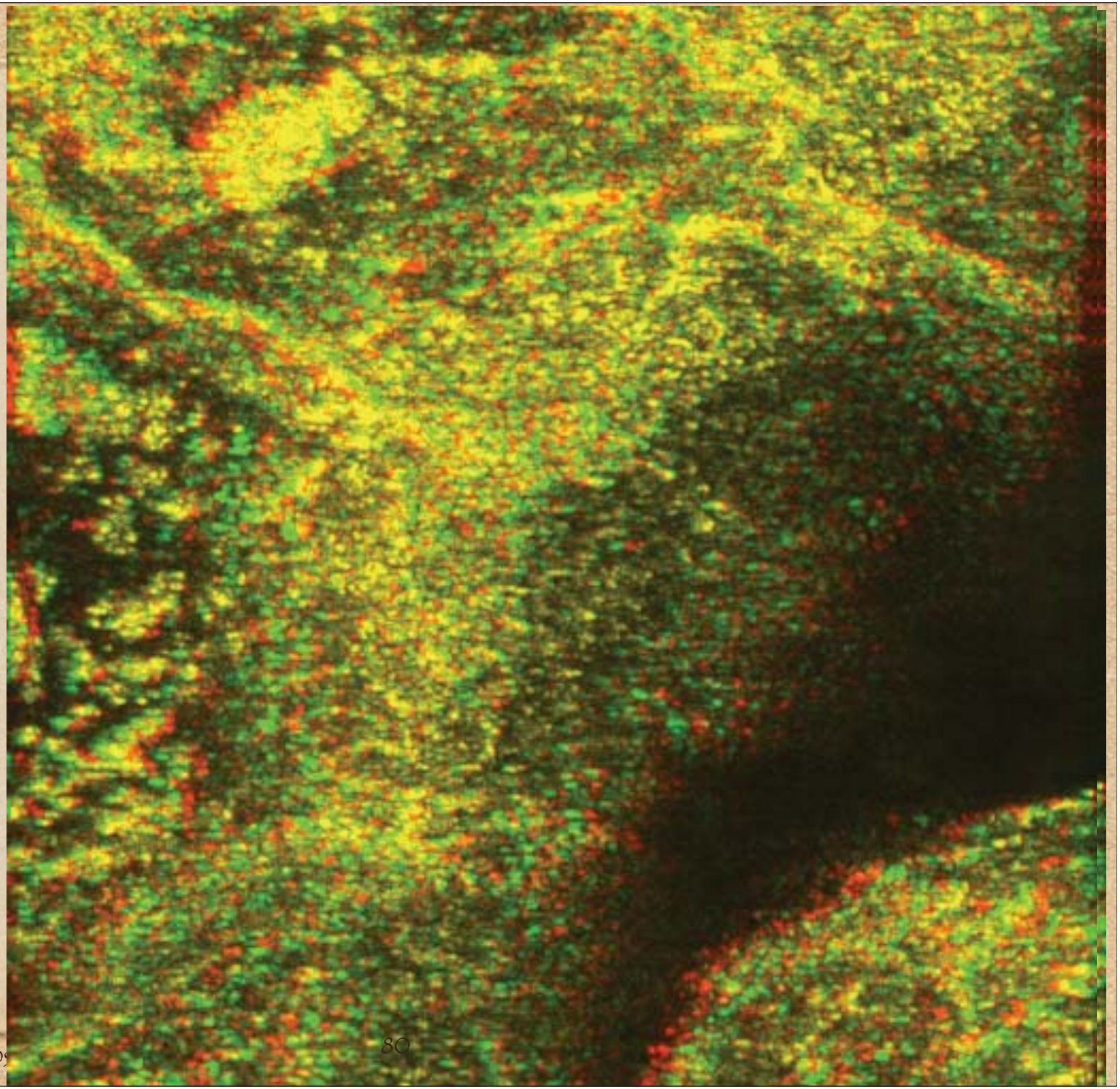
What appears to be happening...





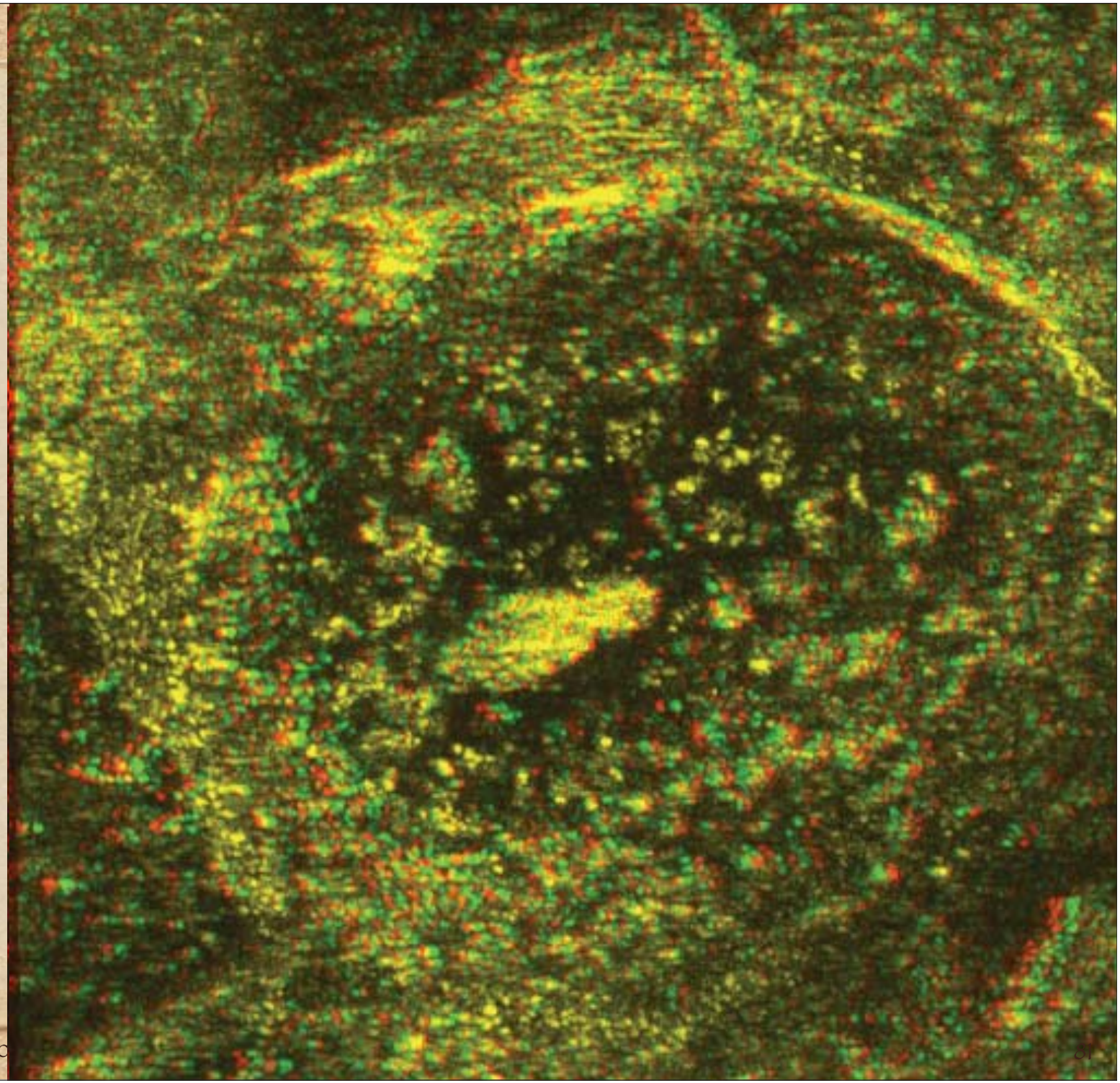
Use your red and green glasses.

BSL image of cheek cell, 40x NA 1.2 cApo-water, BioRad BSL



BSL stereo-pair, ±7°, from 16 optical sections, 1-6 µm past the coverslip surface.
40x/1.2 cAPO water

Use your red and green glasses.



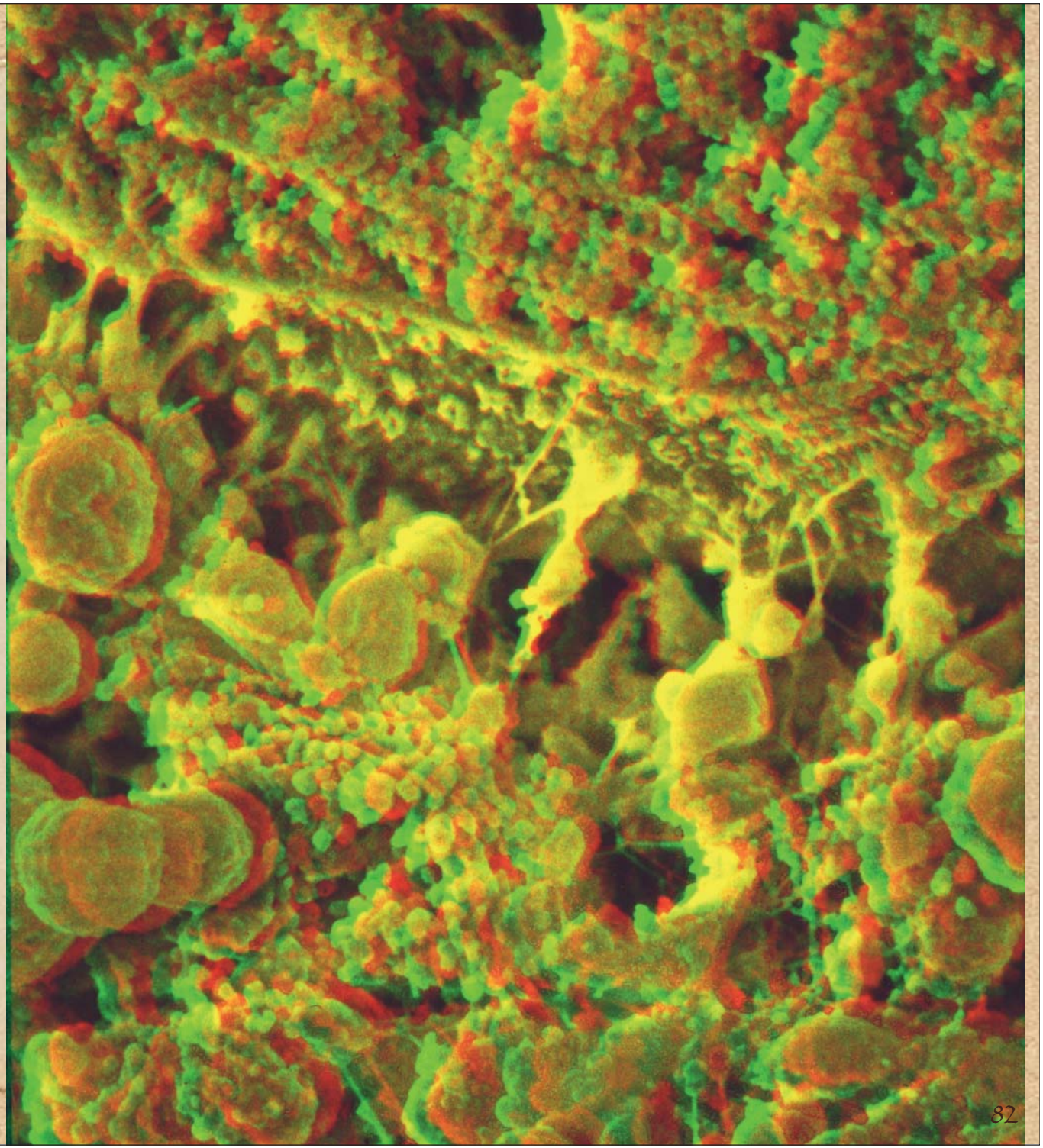
BSL stereo-pair, ±7°,
from 60 optical
sections, 4-24 µm
past the coverslip
surface.
40x/1.2 cAPO water

Use your red and
green glasses.

INRA:Sophia-Antipolis, 12/200

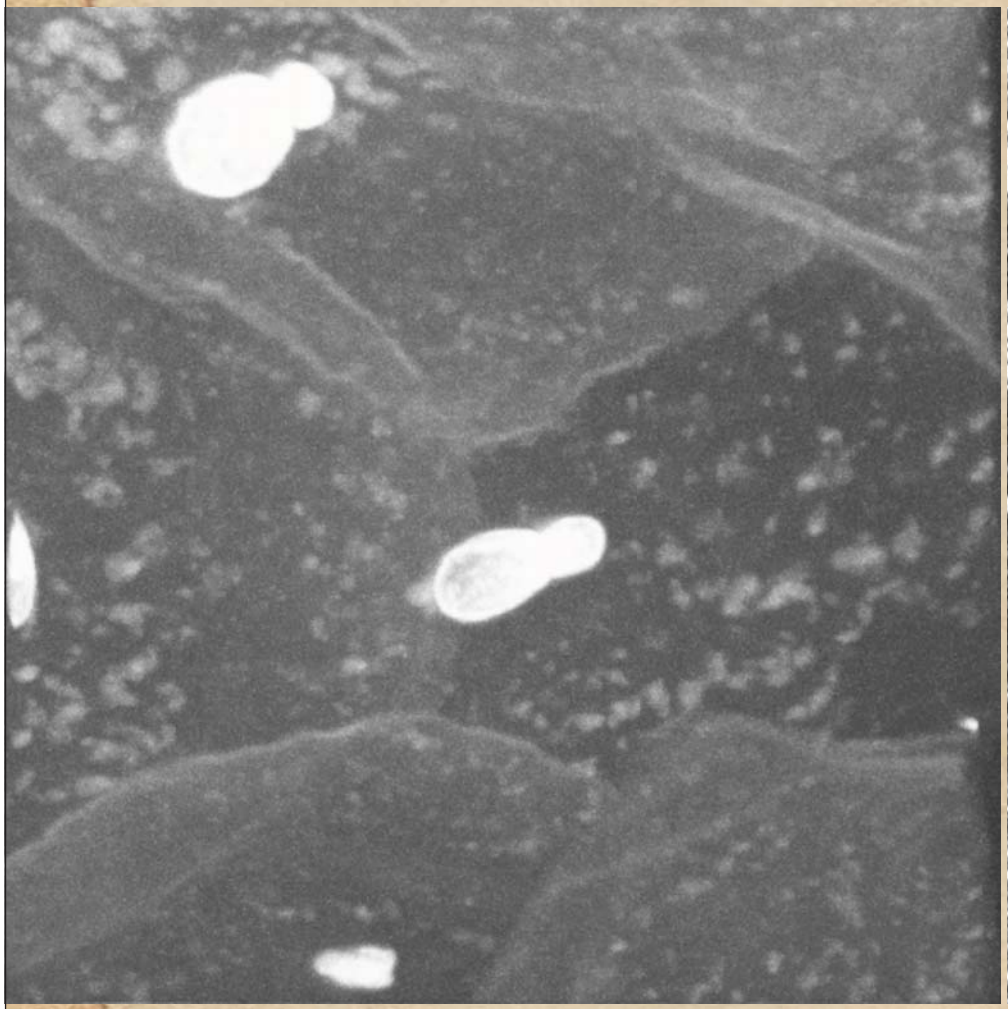
Cells are full of stuff.
It can be interesting to see what some of those "refractile bodies" look like.

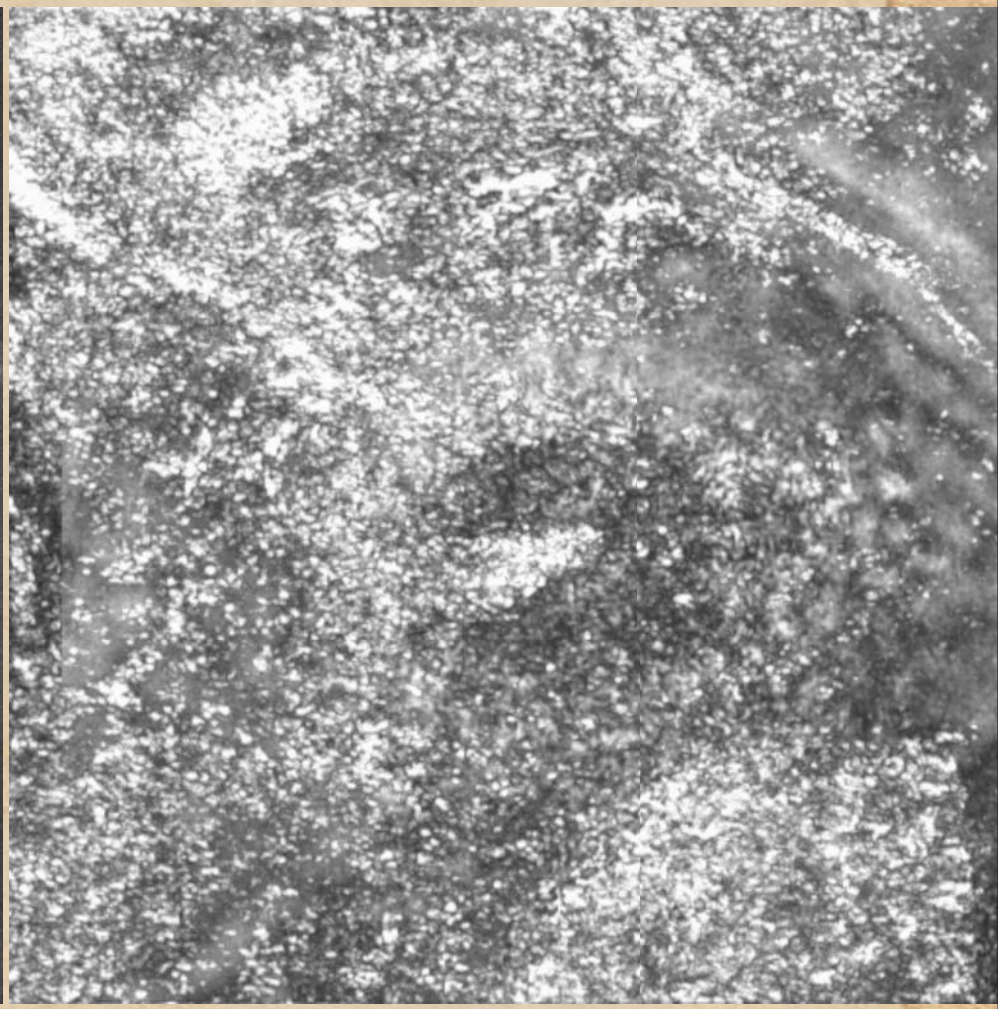
Use your red and green glasses.



Don't let a nice Acridine-orange fluorescence image ...

Acridine-orange stained cheek cell, 40x, NA 1.2 cApo-water, BioRad BSL





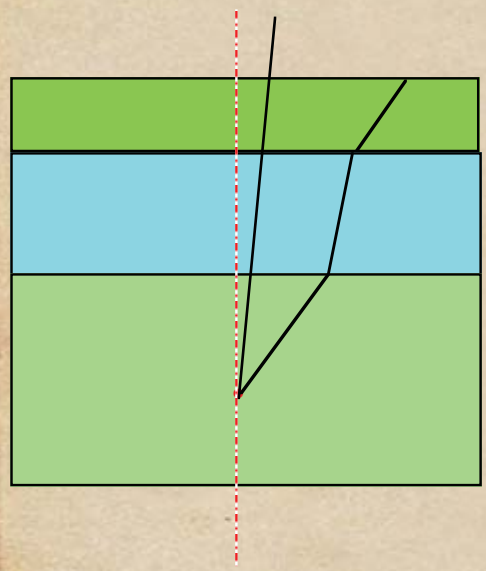
fool you into thinking that nothing else is going on.

30

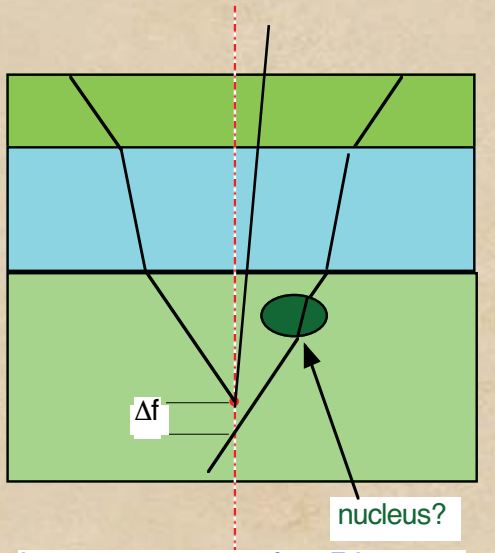
Biological specimens are optically inhomogenious:

(They produce phase-constrast and DIC images.)

This inhomogeneity severely limits the optical performance that can be obtained on biological specimens, a limitation that is particularly important when viewing living specimens because the “embedding medium” cannot be changed.



An axially-symmetric, spherical aberration corrector can correct for discrepancies in RI **that occur only along the optical axis.**

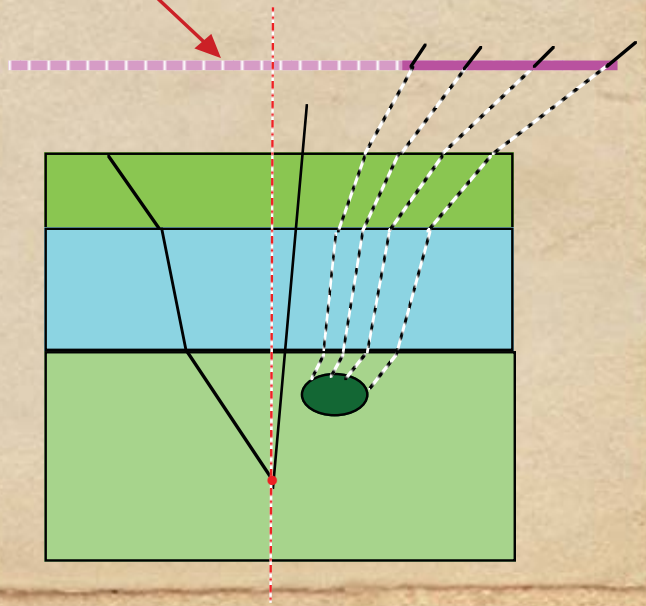


It cannot correct for RI changes occurring in the x-y plane. Here, the only possibility is...

The only possibility suggested so far involves putting an LCD into the back focal plane of the objective to eliminate troublesome rays. (Wilson, et al. 2000)

This assumes that it is possible to determine which rays to eliminate.

an LCD array in BFP that



STED, PALM/STORM, I5M and SSIM place different conditions on the specimen.

Method/Det	STED	PALM	STORM	I5M	SSIM
RI uniformity	slight	NA	NA	serious	serious
Thin specimen	no	TIRF	TIRF	4Pi	4Pi
Dye:triplet	serious	bleaching		bleaching	bleaching
Dye:stability	serious, best in IR	photo-activatable	photo-switchable	serious	serious
Dye: other		requires bleaching	may double count		
Use of emitted photons	good	very good	very good	out-of-focus light	out-of-focus light

Always, there are tradeoffs.

The Holy Grail still becons

"The holy grail is to see dynamically imaged living cells noninvasively, to see at the level of an individual protein molecule, and to see how the molecules interact in a cell," Betzig says. "It's many years away, but you can dream about it.*"

...but we have to recognize that we probably cannot do everything at once.

*HHMI Bulletin, 19:1 February, 2006,

In a way, we have only scratched the surface.

Although we may be approaching practical limits in the optics and photodetectors, we have not discussed new dyes, such as some ps metal-cluster dyes, with less intersystem crossing, and hence less damage.

Surely, finer LM resolution is most important when imaging cells that are both alive and happy.

Dead cells can often be seen more easily in EM.

Clearly, we will need all the help we can get to proceed much farther! Let the discussion begin!

My thanks to all those who have
developed and perfected these
dazzling new techniques
And thank you even more for
allowing me to use your data.

Finally, thank you for your attention.