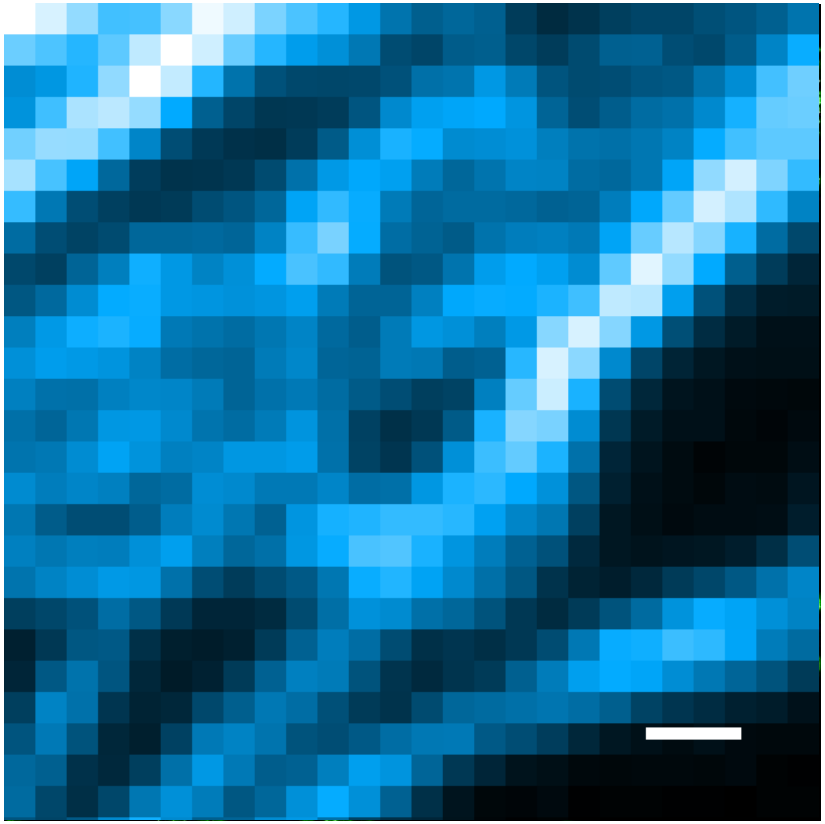
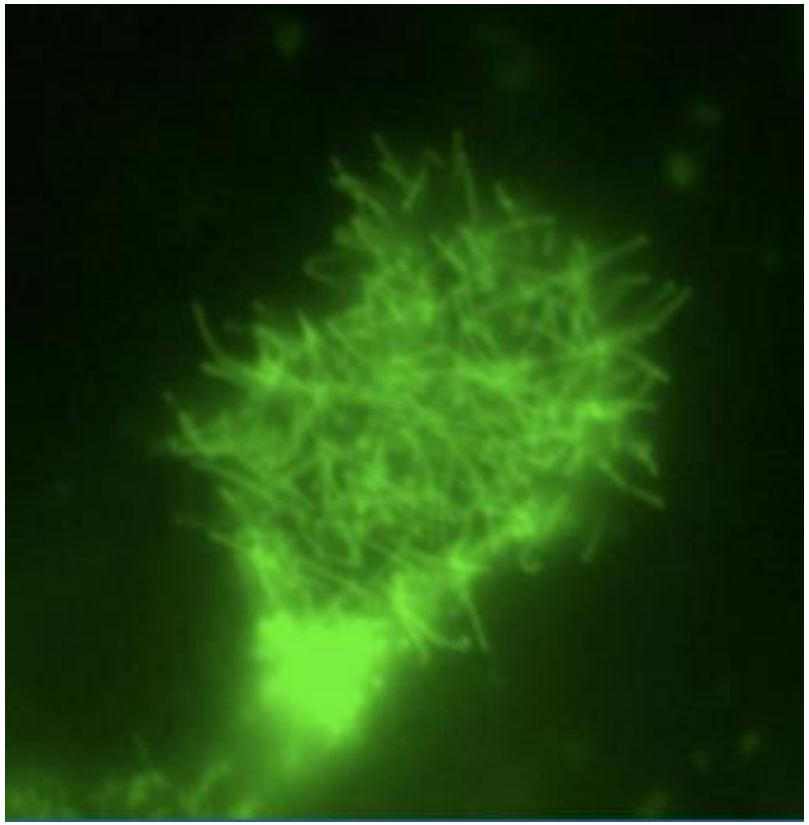
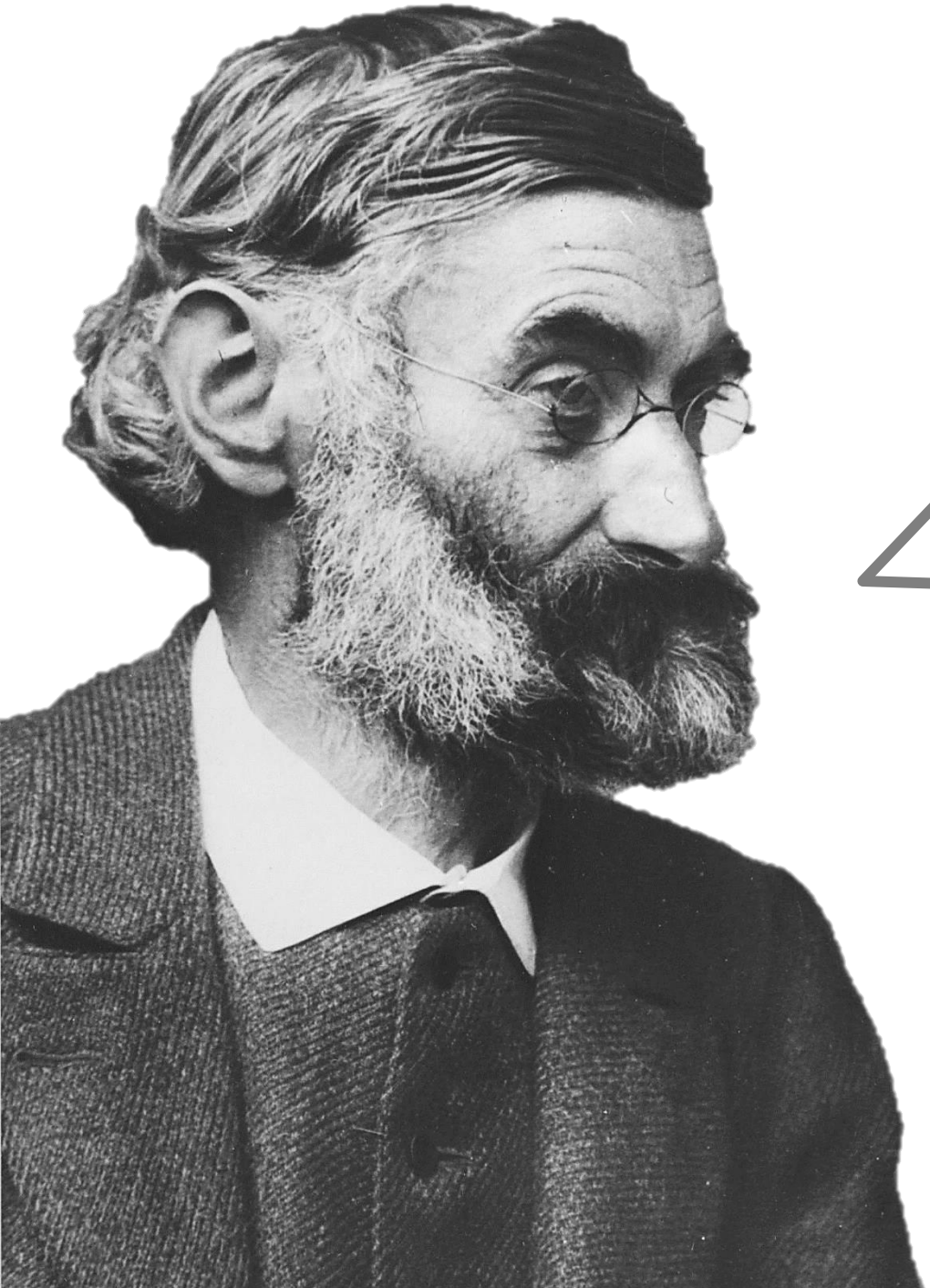
STORM TIRF





Mike Kerber
Bio-applications Manager
June 2015


N-STORM
Nikon - Stochastic Optical
Reconstruction Microscopy

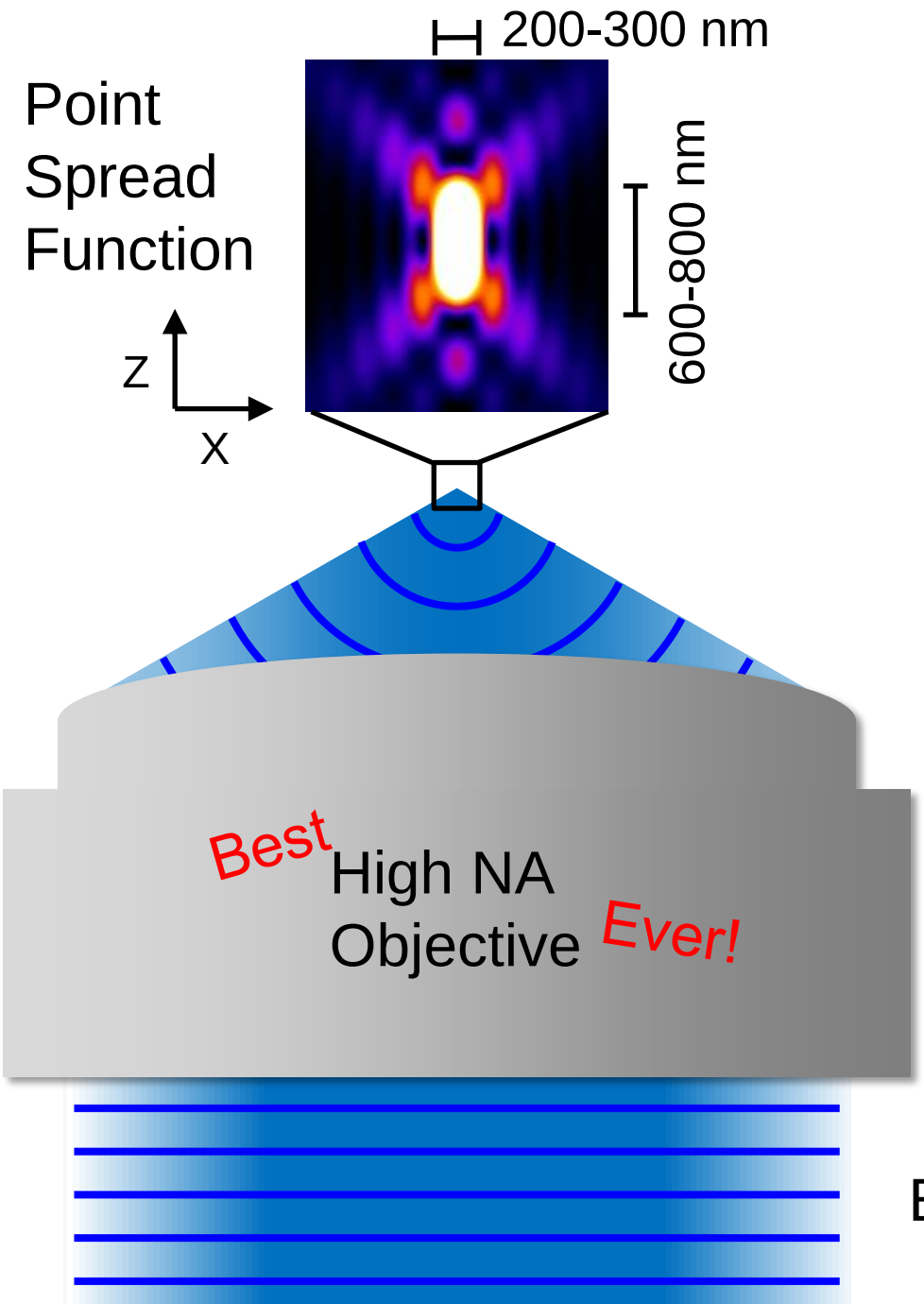


Resolution =

$$\frac{\text{Wavelength}}{2\ \text{NA}}$$

So…about **200 nm**

Ernst Abbe 1840-1905



Resolution =

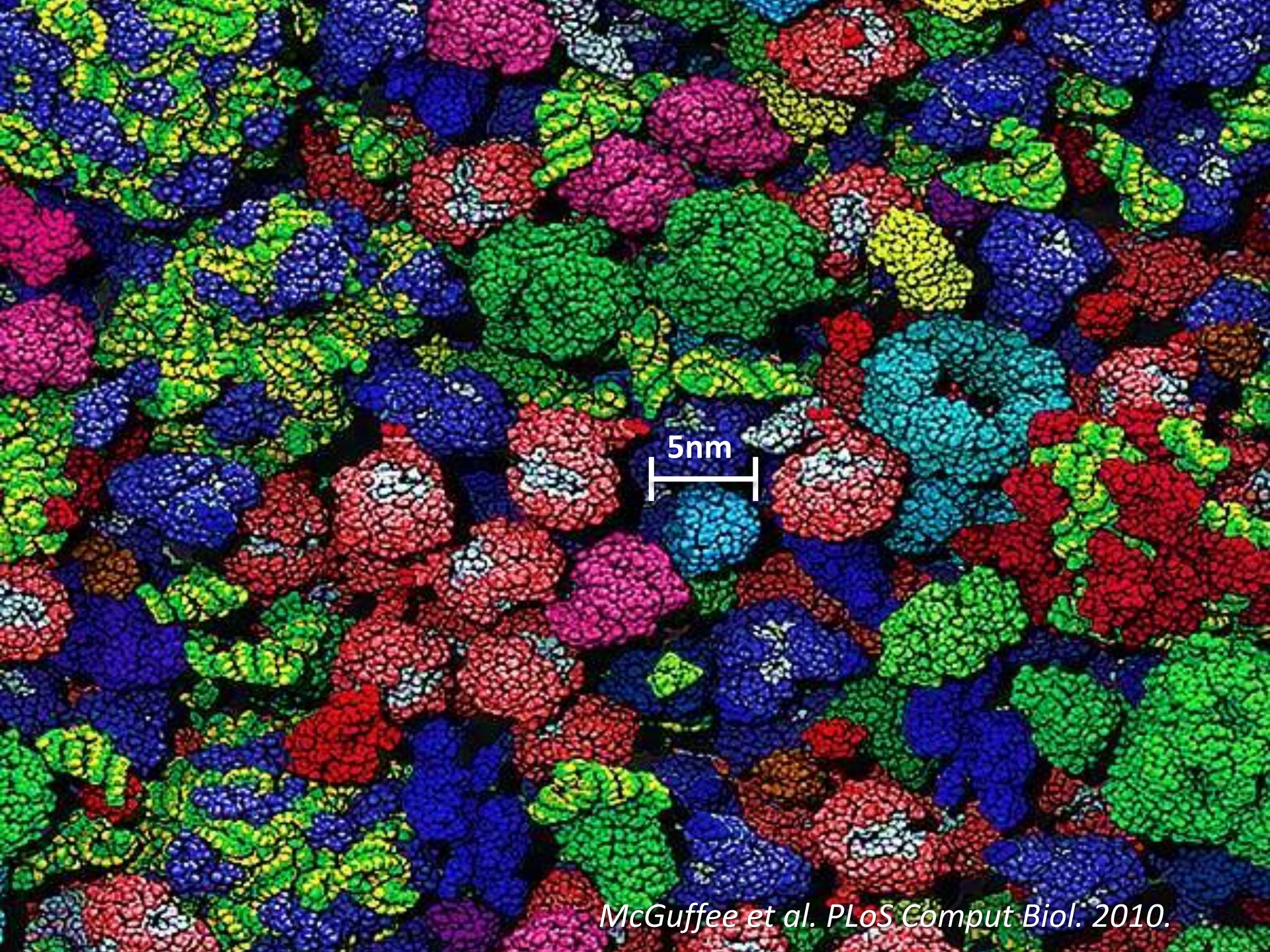
Wavelength

2 NA

So…about **200 nm**

How big is 200nm?

Excitation Light Waves



McGuffee et al. PLoS Comput Biol. 2010.

10X Resolution Increase (X, Y, Z)

Modalities: 2D, TIRF, 3D, Multi-Color

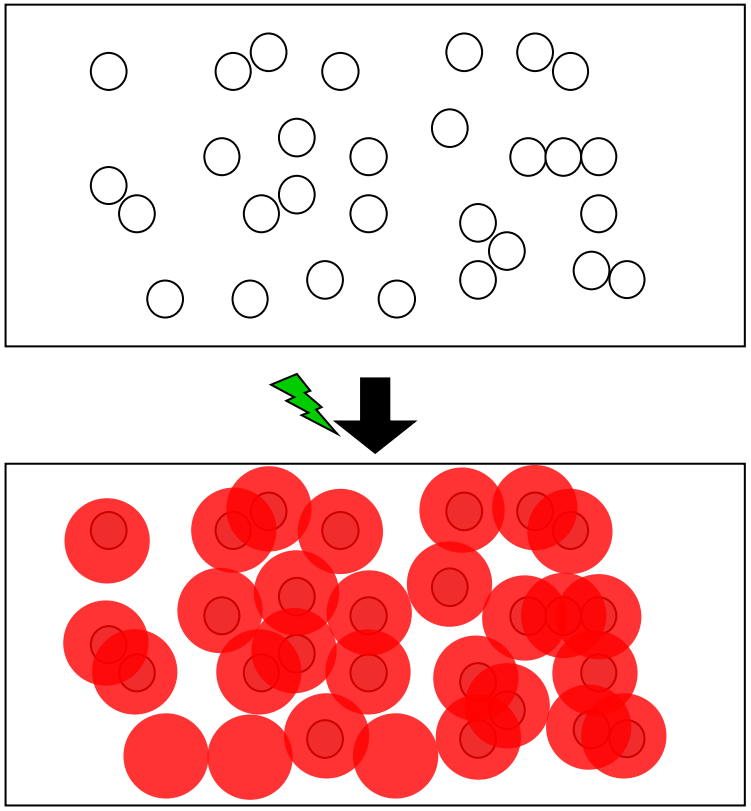
How does *STORM* work?

1. *Emitter isolation:* Only some lights are on at a time
2. *Localization:* Pinpoint centroids of visible lights
3. *Repeat!*

Requires *photoswitchable* fluorescent probes!

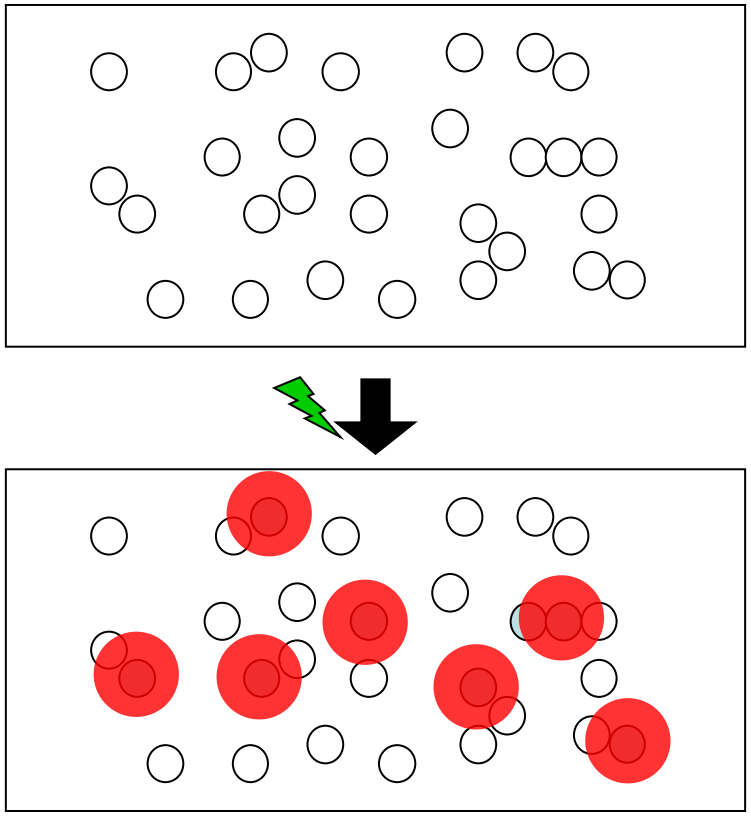
"Emitter Isolation" = Only a subpopulation of fluorophores is on at once

Conventional Fluorescence



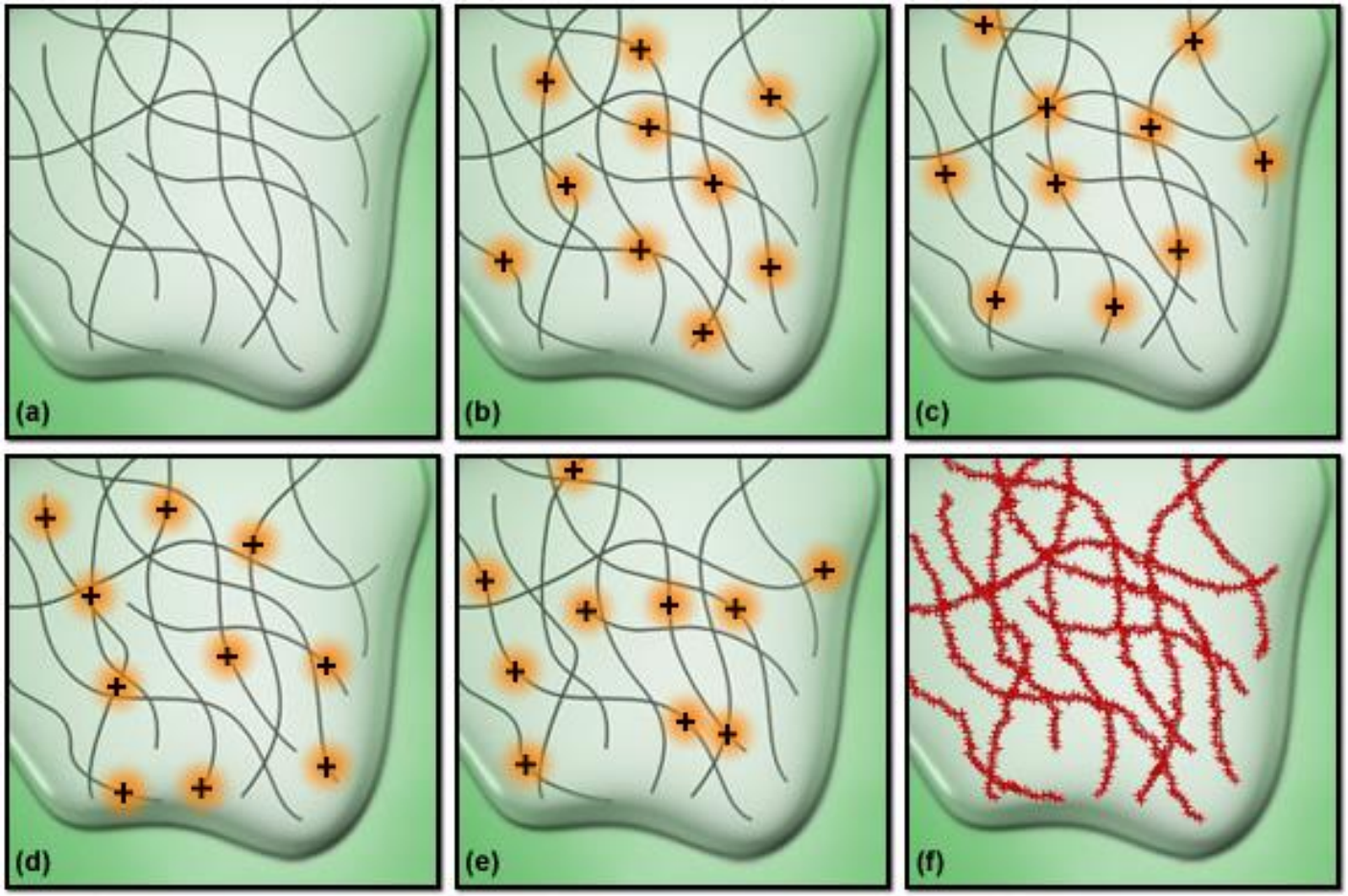
Can't see individuals in the crowd…

Emitter Isolation



Individuals distinguishable!

Continuous STORM

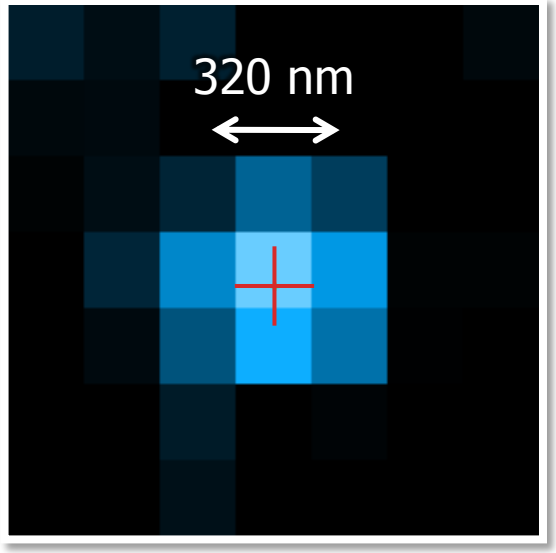


Common small molecule dyes will *"blink"* in the presence of *"imaging buffers"* and *high laser powers*.

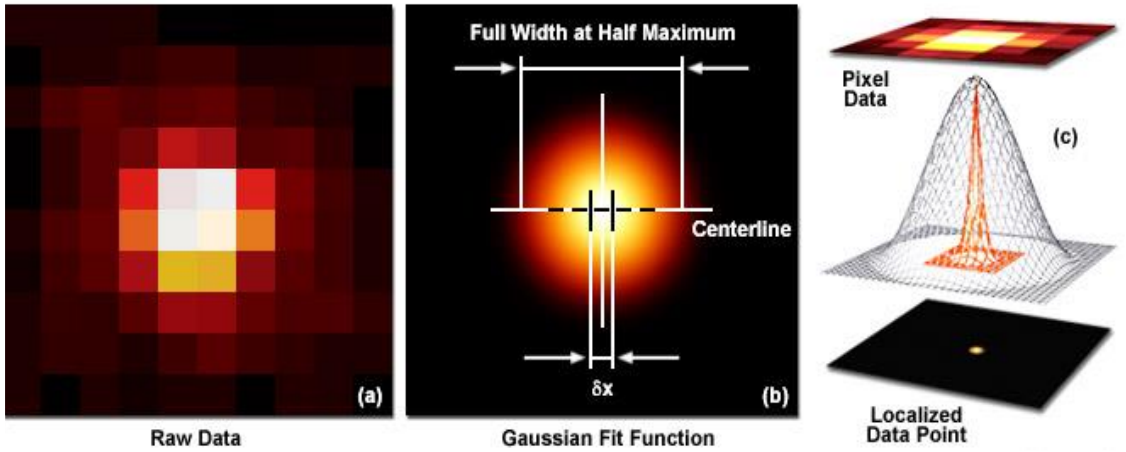
With some assumptions, we can accurately find the center of a diffuse spot

Single-Molecule *Localization*

Image of one fluorescent molecule



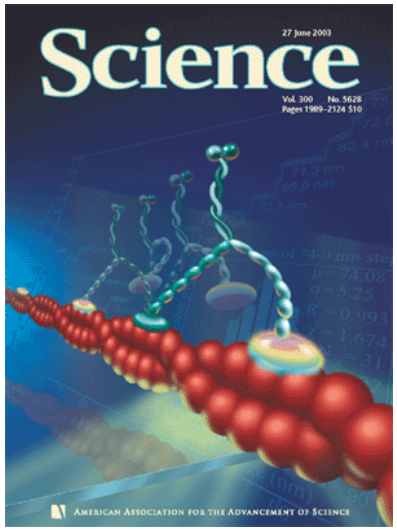
Fitting to a gaussian model



FIONA

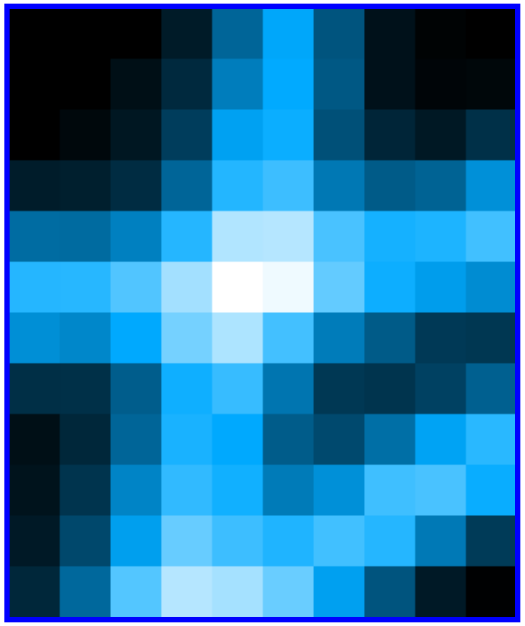
Fluorescence Imaging with One Nanometer Accuracy

Paul Selvin



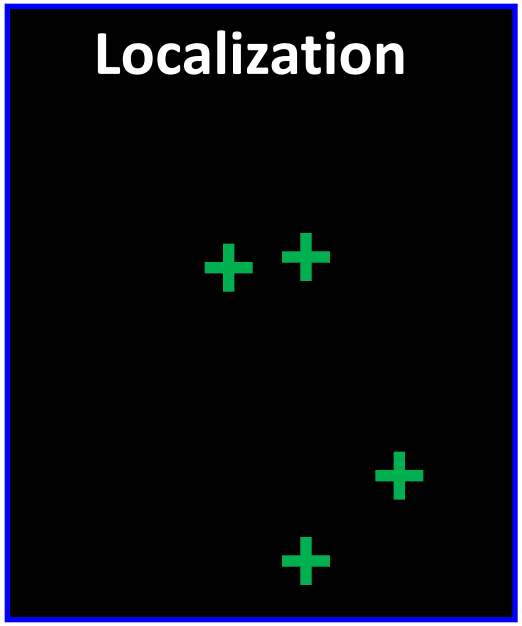
STORM = *Emitter Isolation* and *Localization*

Conventional fluorescence

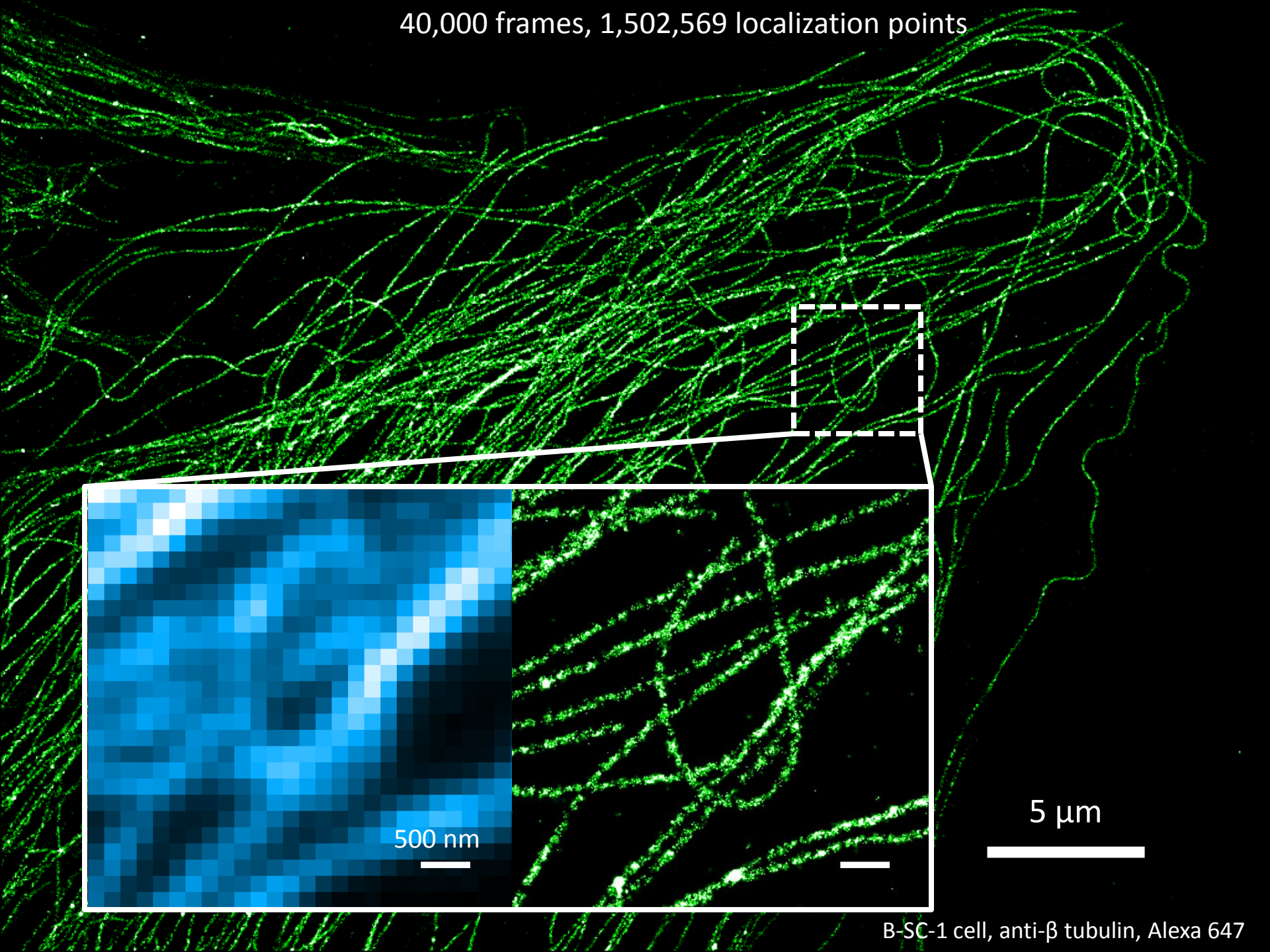


Raw images

STORM Image

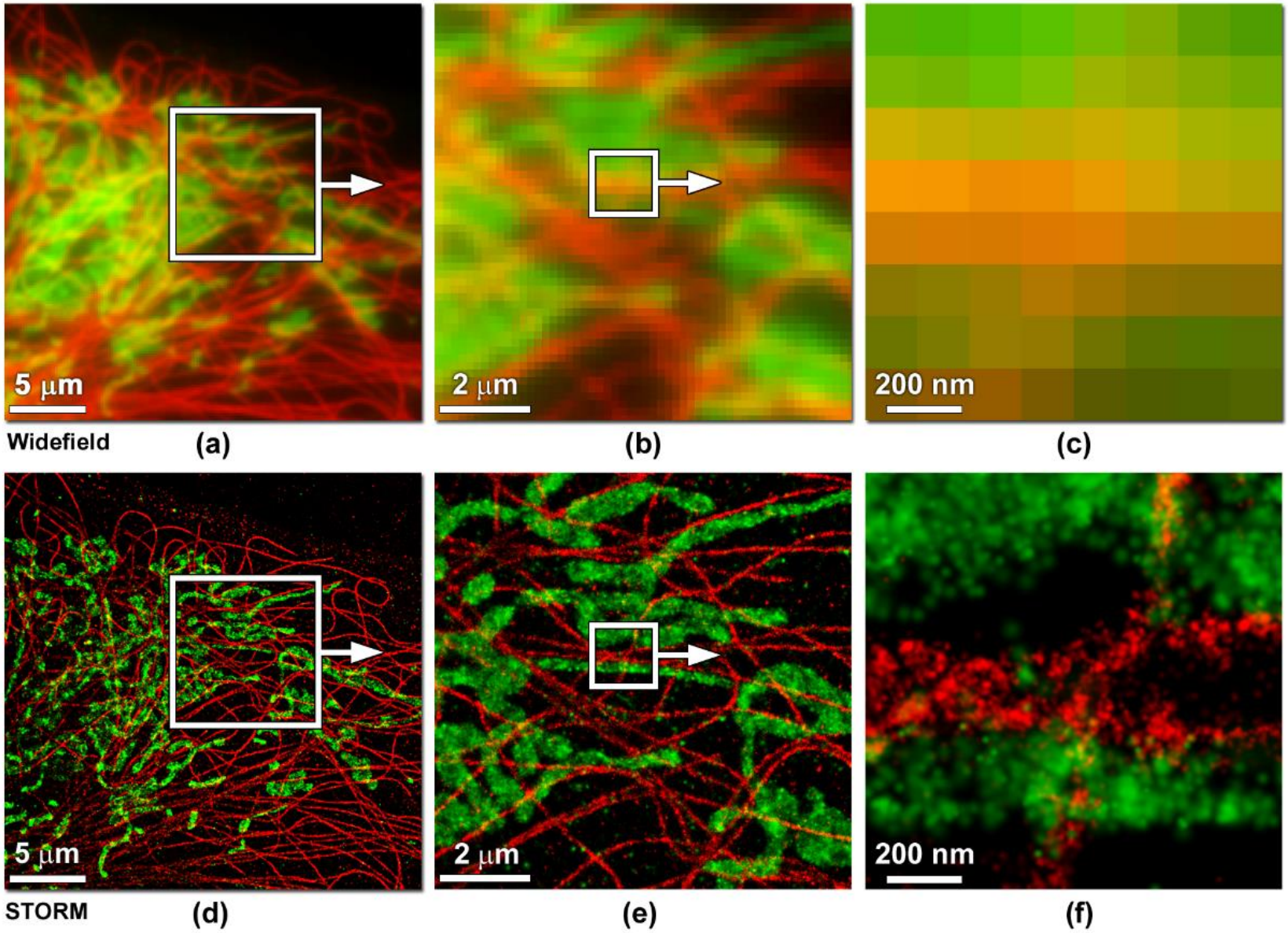


Rust, Bates & Zhuang, Nat. Methods, 2006
Bates, Huang, Dempsey & Zhuang, Science, 2007

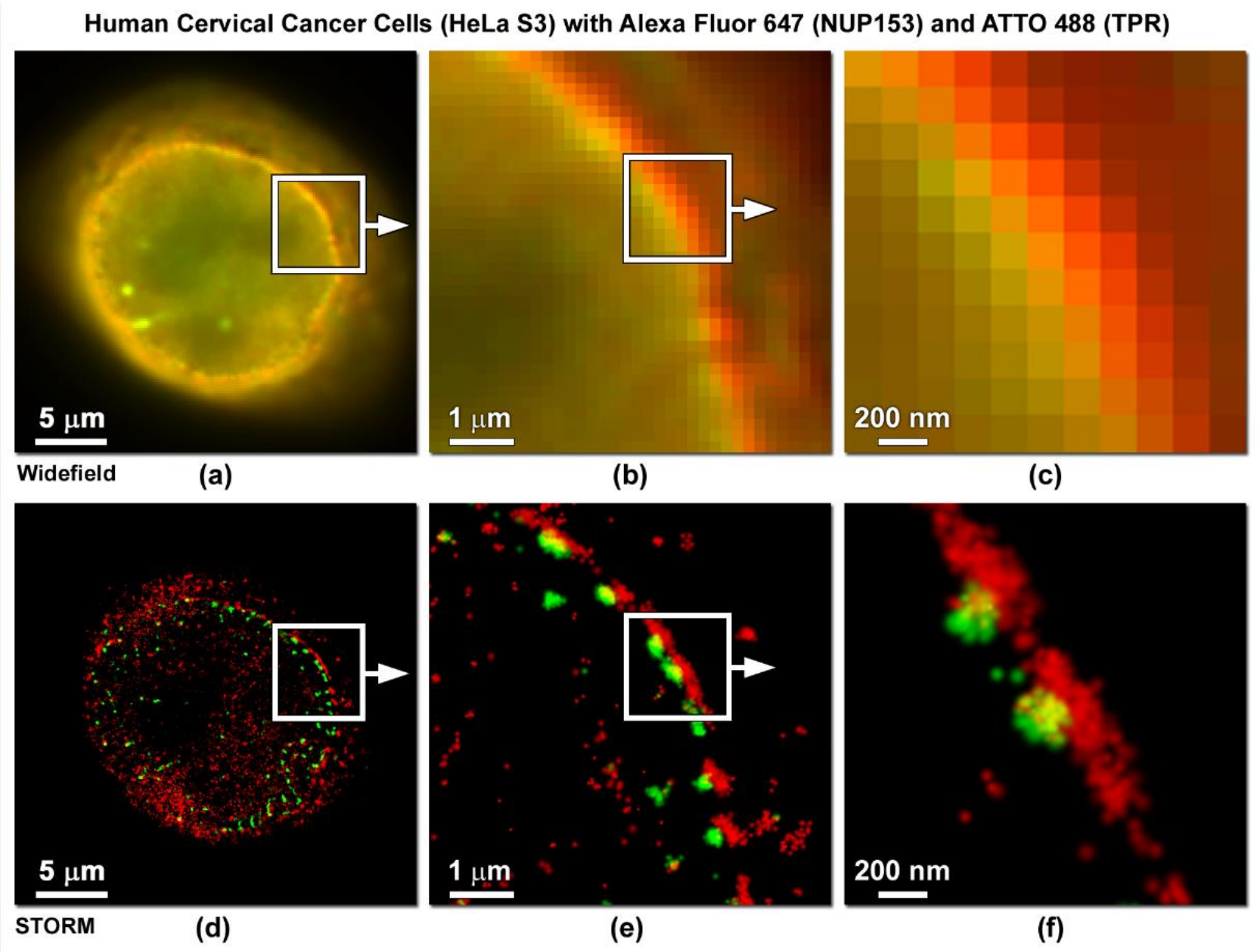

40,000 frames, 1,502,569 localization points
500 nm
5 µm
B-SC-1 cell, anti-β tubulin, Alexa 647

Dual Color STORM

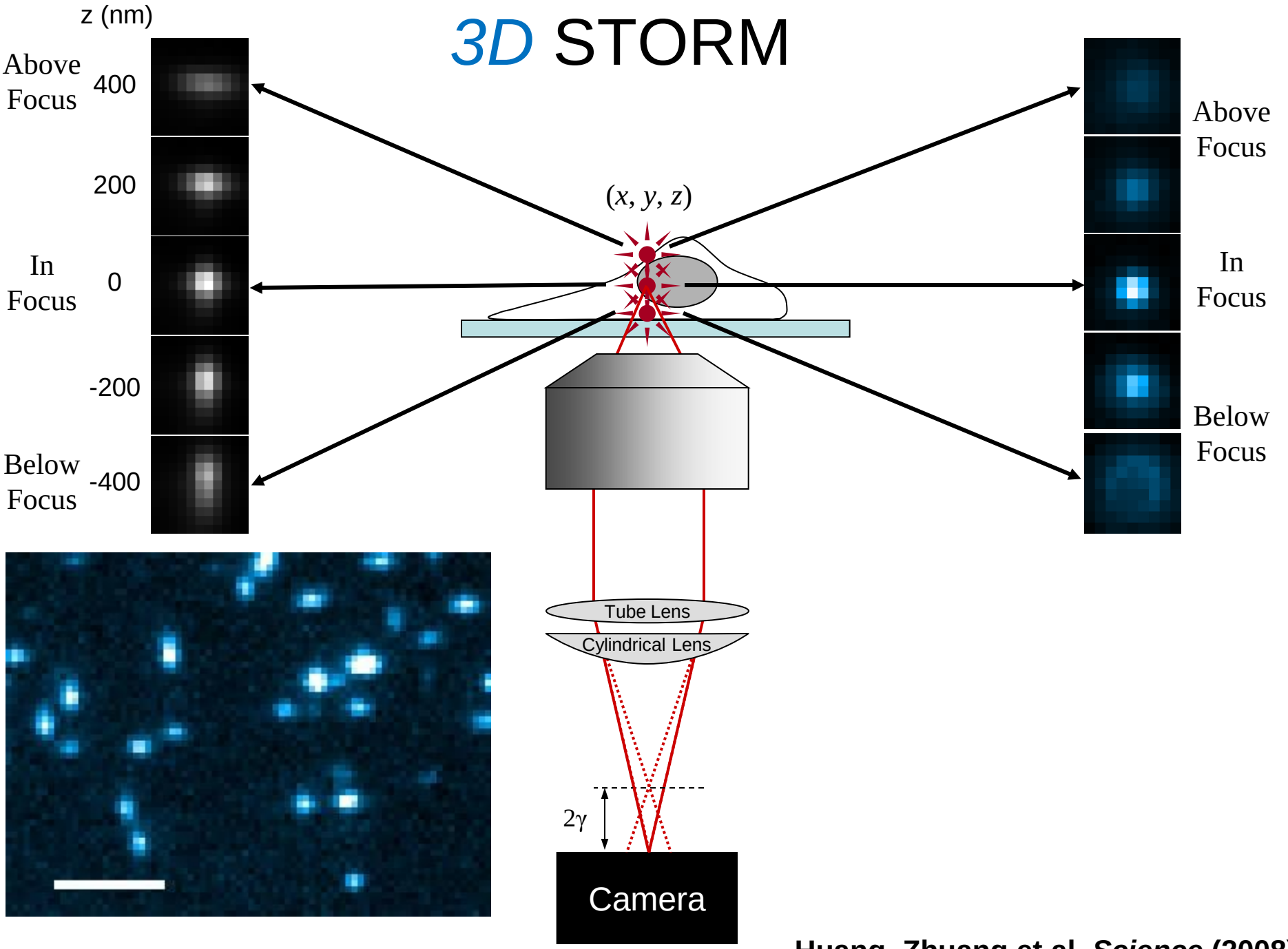
African Green Monkey Kidney (CV-1) Cells with Alexa Fluor 647 (Tubulin) and ATTO 488 (TOMM20)



Dual Color STORM

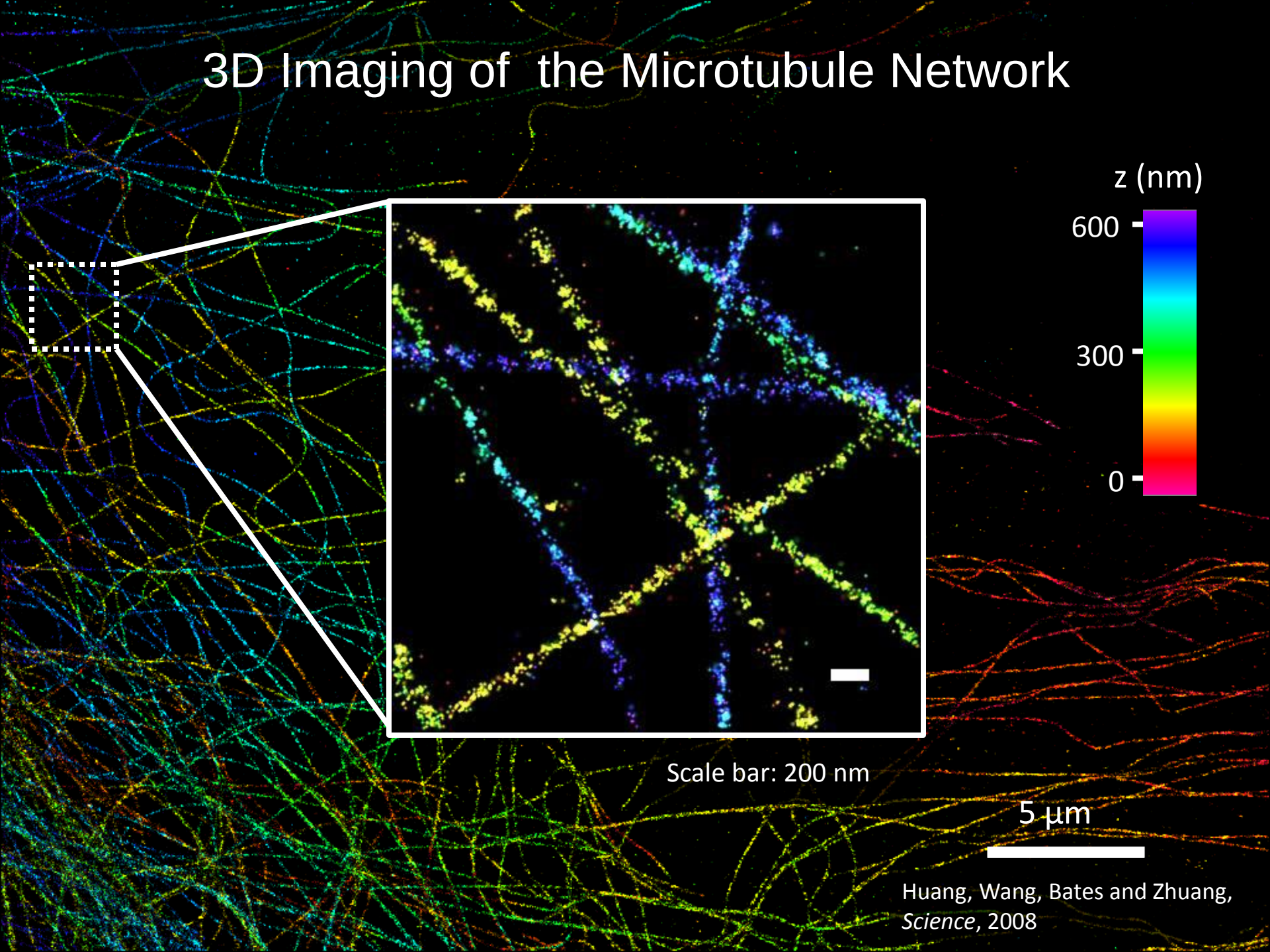


3D STORM



Huang, Zhuang et al, *Science* (2008)

3D Imaging of the Microtubule Network



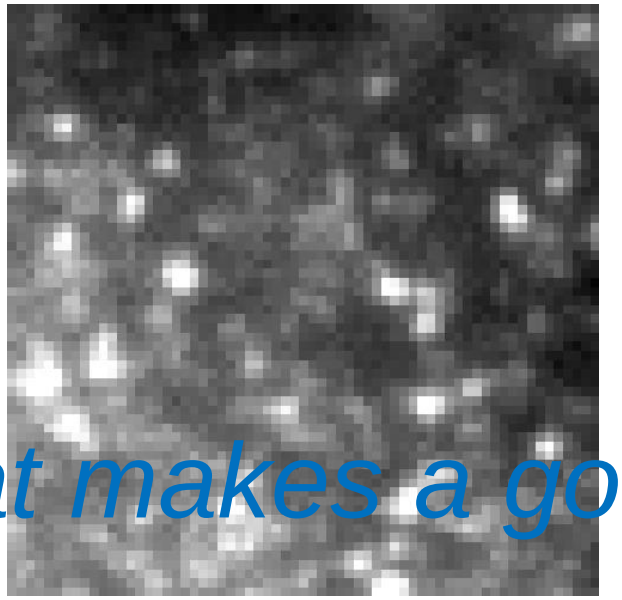
Huang, Wang, Bates and Zhuang, *Science*, 2008

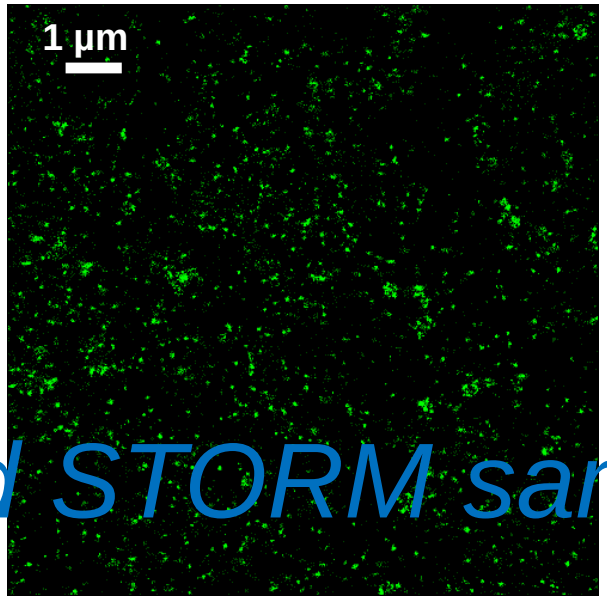
Bad *Sample* = Bad *STORM* Image

Widefield Clathrin

Clathrin-STORM

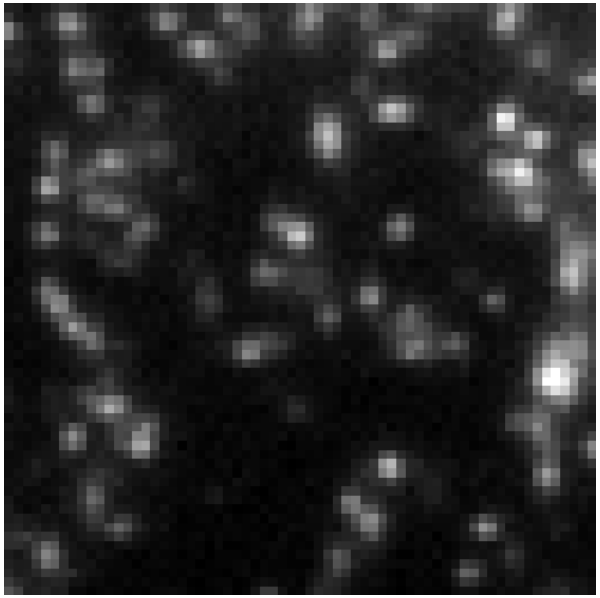
BAD

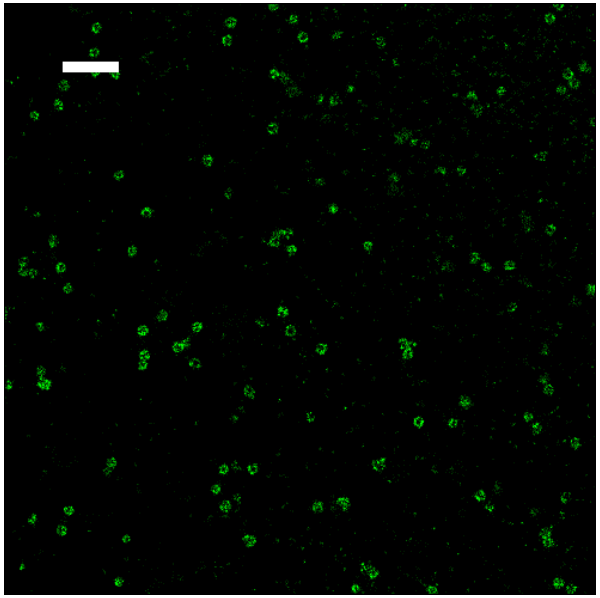




What makes a good STORM sample?

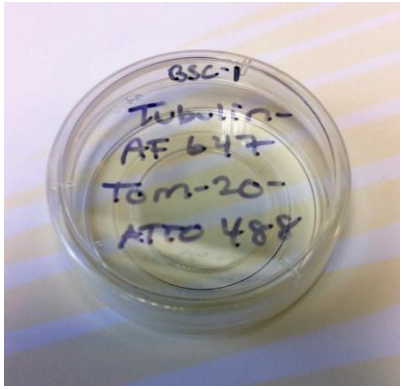
Good





Determinants of Sample Quality

1. *Probe Choice*
   - Dyes that work for N-STORM
2. *Labeling Strategies*
   - Fixation
   - Immunostaining



* Don't forget to use glass-bottom (#1.5) dishes to hold STORM imaging buffer!

Few Dyes Work for N-STORM

(Despite what is published)

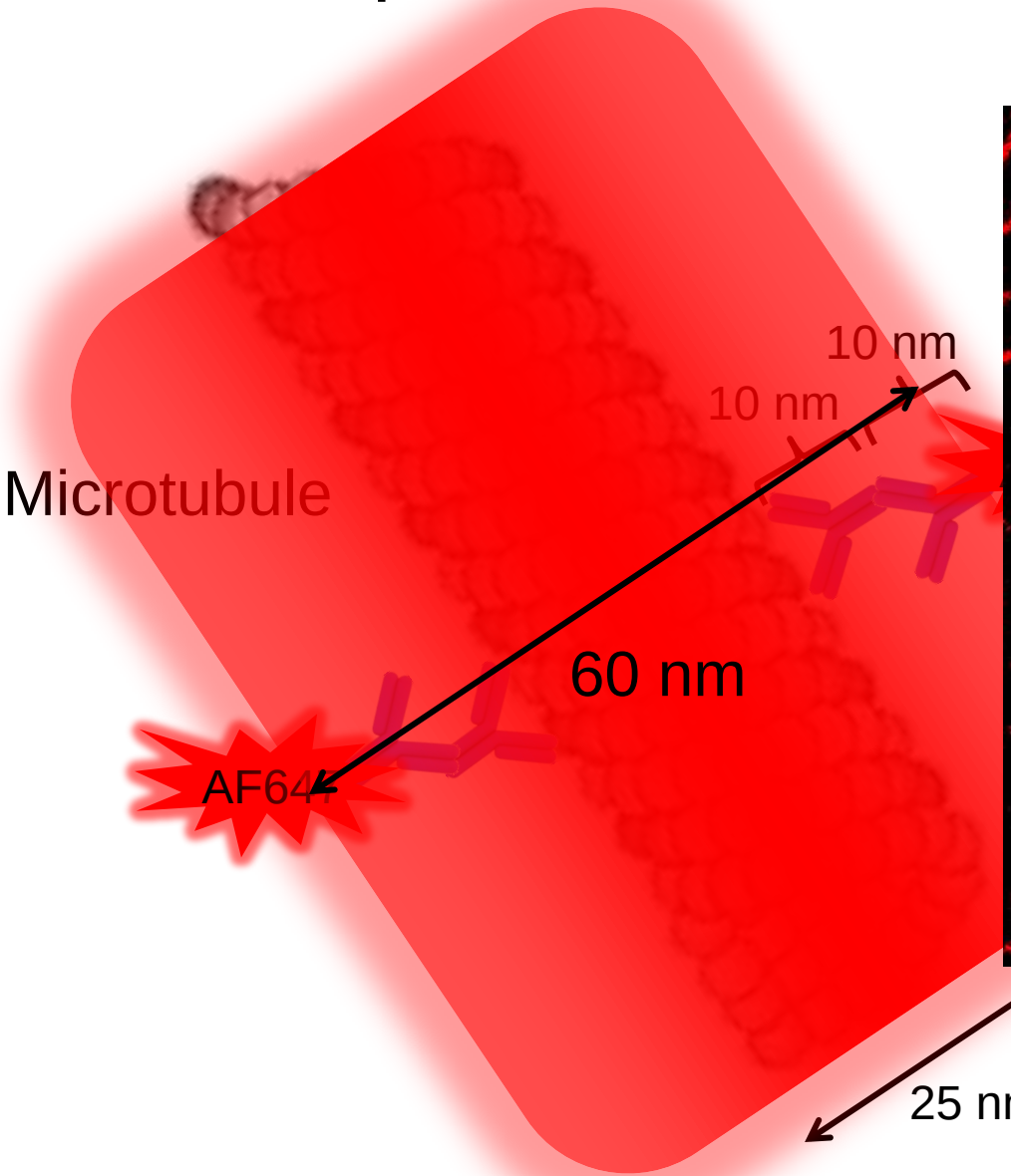
Dye	Excitation maximum (nm)[a]	Emission maximum (nm)[a]	Extinction (M^{-1} cm^{-1})[b]	Quantum yield[c]	Detected photons per switching event		Equilibrium on-off duty cycle (400–600 s)		Survival fraction after illumination for 400 s		Number of switching cycles (mean)	
					MEA	βME	MEA	βME	MEA	βME	MEA	βME
Blue-absorbing												
Atto 488	501	523	90,000	0.8	1,341	1,110	0.00065	0.0022	0.98	0.99	11	49
Alexa Fluor 488	495	519	71,000	0.92	1,193	427	0.00055	0.0017	0.94	1	16	139
Atto 520	516	538	110,000	0.9	1,231	868	0.0015	0.00061	0.92	0.86	9	17
Fluorescein	494	518	70,000	0.79	1,493	776	0.00032	0.00034	0.51	0.83	4	15
FITC	494	518	70,000	0.8	639	1,086	0.00041	0.00031	0.75	0.9	17	16
Cy2	489	506	150,000	0.12	6,241	4,583	0.00012	0.00045	0.12	0.19	0.4	0.7
Yellow-absorbing												
Cy3B	559	570	130,000	0.67	1,365	2,057	0.0003	0.0004	1	0.89	8	5
Alexa Fluor 568	578	603	91,300	0.69	2,826	1,686	0.00058	0.0027	0.58	0.99	7	52
TAMRA	546	575	90,430	0.2	4,884	2,025	0.0017	0.0049	0.85	0.99	10	59
Cy3	550	570	150,000	0.15	11,022	8,158	0.0001	0.0003	0.17	0.55	0.5	1.6
Cy3.5	581	596	150,000	0.15	4,968	8,028	0.0017	0.0005	0.89	0.61	5.7	3.3
Atto 565	563	592	120,000	0.9	19,714	13,294	0.00058	0.00037	0.17	0.26	4	5
Red-absorbing												
Alexa Fluor 647	650	665	239,000	0.33	3,823	5,202	0.0005	0.0012	0.83	0.73	14	26
Cy5	649	670	250,000	0.28	4,254	5,873	0.0004	0.0007	0.75	0.83	10	17
Atto 647	645	669	120,000	0.2	1,526	944	0.0021	0.0016	0.46	0.84	10	24
Atto 647N	644	669	150,000	0.65	3,254	4,433	0.0012	0.0035	0.24	0.65	9	39
Dyomics 654	654	675	220,000	–	3,653	3,014	0.0011	0.0018	0.79	0.64	20	19
Atto 655	663	684	125,000	0.3	1,105	657	0.0006	0.0011	0.65	0.78	17	22
Atto 680	680	700	125,000	0.3	1,656	987	0.0019	0.0024	0.65	0.91	8	27
Cy5.5	675	694	250,000	0.28	5,831	6,337	0.0069	0.0073	0.87	0.85	16	25
NIR-absorbing												
DyLight 750	752	778	220,000	–	712	749	0.0006	0.0002	0.55	0.58	5	6
Cy7	747	776	200,000	0.28	852	997	0.0003	0.0004	0.48	0.49	5	2.6
Alexa Fluor 750	749	775	240,000	0.12	437	703	0.00006	0.0001	0.36	0.68	1.5	6
Atto 740	740	764	120,000	0.1	779	463	0.00047	0.0014	0.31	0.96	3	14
Alexa Fluor 790	785	810	260,000	–	591	740	0.00049	0.0014	0.54	0.62	5	2.7
IRDye 800 CW	778	794	240,000	–	2,753	2,540	0.0018	0.038	0.6	1	3	127

Dempsey et al., 2011

Where to get secondary antibodies for STORM

cSTORM 2º Antibodies	Sources
Alexa647	Life Technologies, Jackson
Cy5	Jackson
Alexa568	Life Technologies
Cy3B	DIY (Dye from GE)
Atto488	Rockland, Sigma

Linkage Error: Antibodies separate the fluorophore from the structure of interest



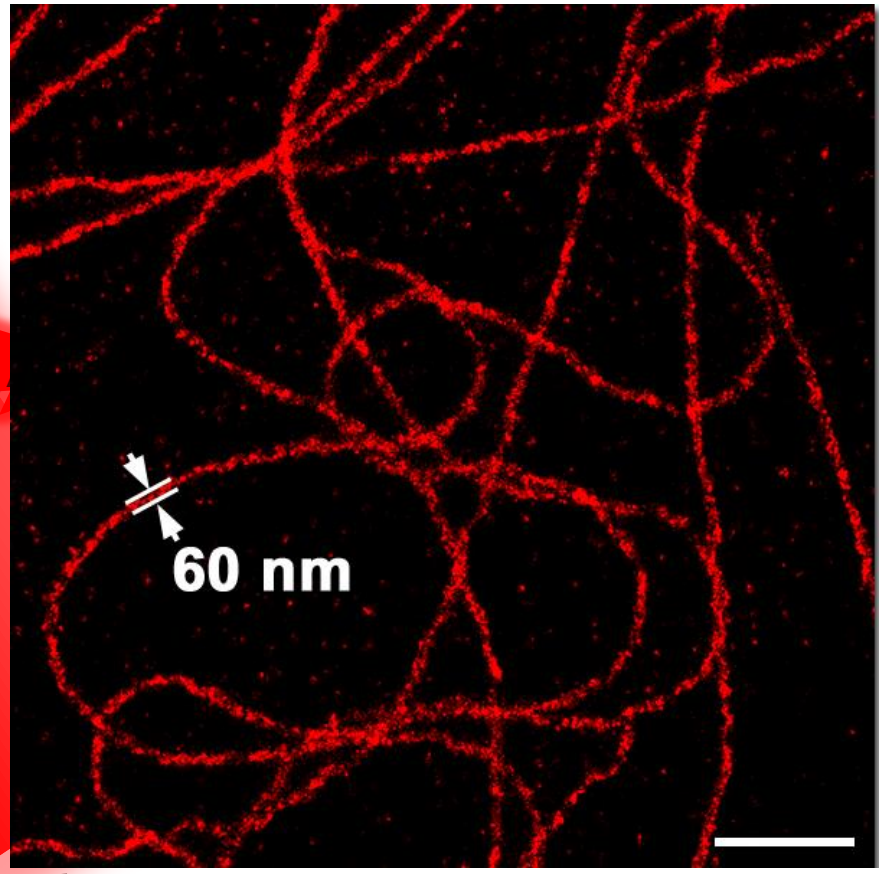


Image courtesy of Michael Davidson, FSU

Our sample prep protocol: *a good starting point*

The following *optimizations* are also necessary…

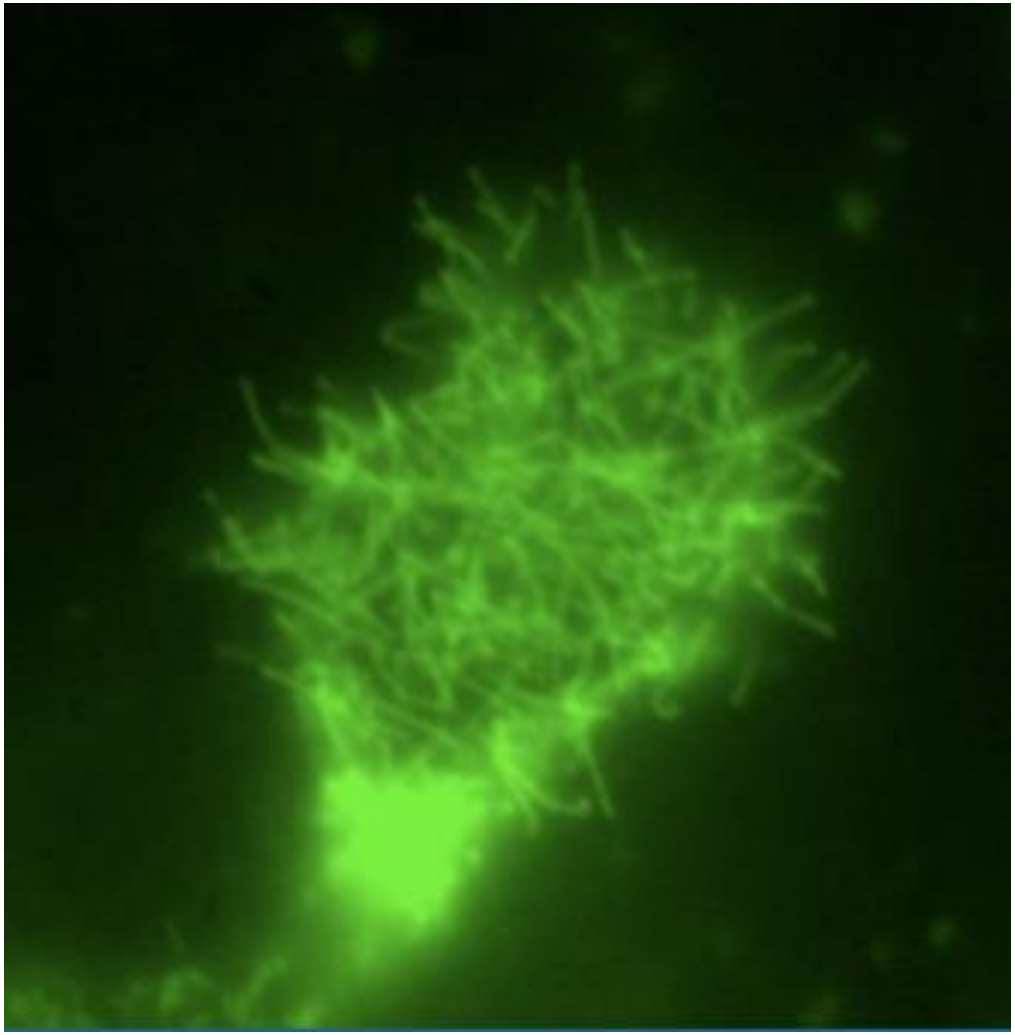
Tips for STORM Sample Preparation

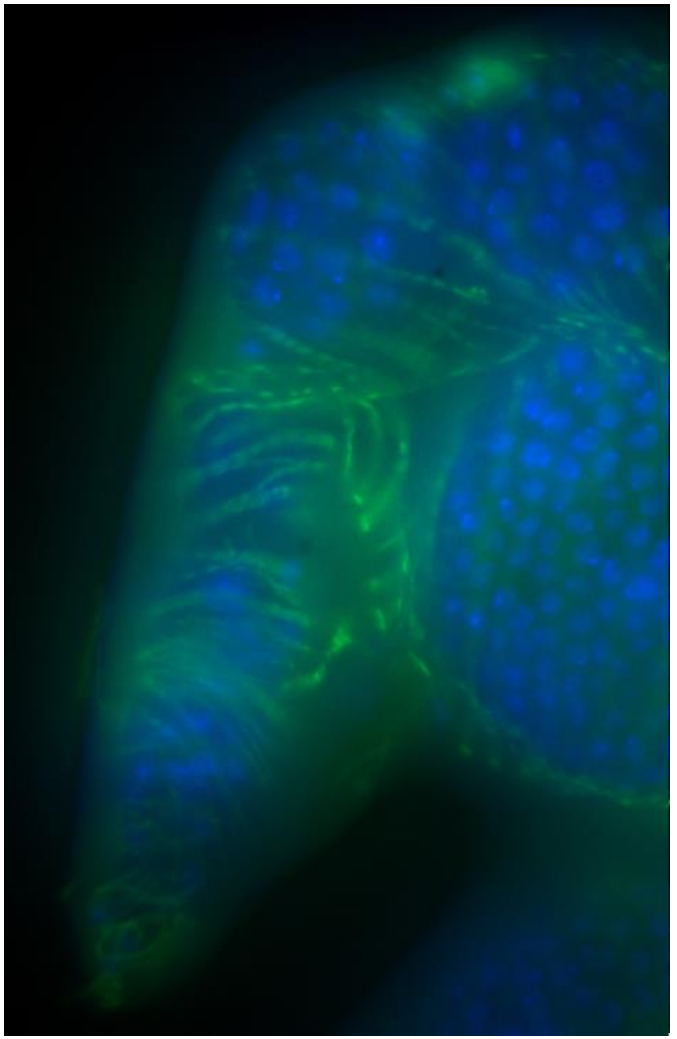
- *Compare antibodies* from multiple sources.
- *Optimize fixation* (fixative concentration, permeabilization, etc.) to maximize structural preservation and antibody binding.
- *Minimize background* signal levels by titrating primary antibody.
- *Block* with heat-treated sterile filtered blocking serum.
- Don’t skip on the *washing steps* and use 1% blocking serum to remove antibodies AT EVERY STEP.
- Lock secondary antibodies in place with *post-staining fixation*.
- Remove residues with *Tween 80 wash*.

STORM Sample Prep Summary

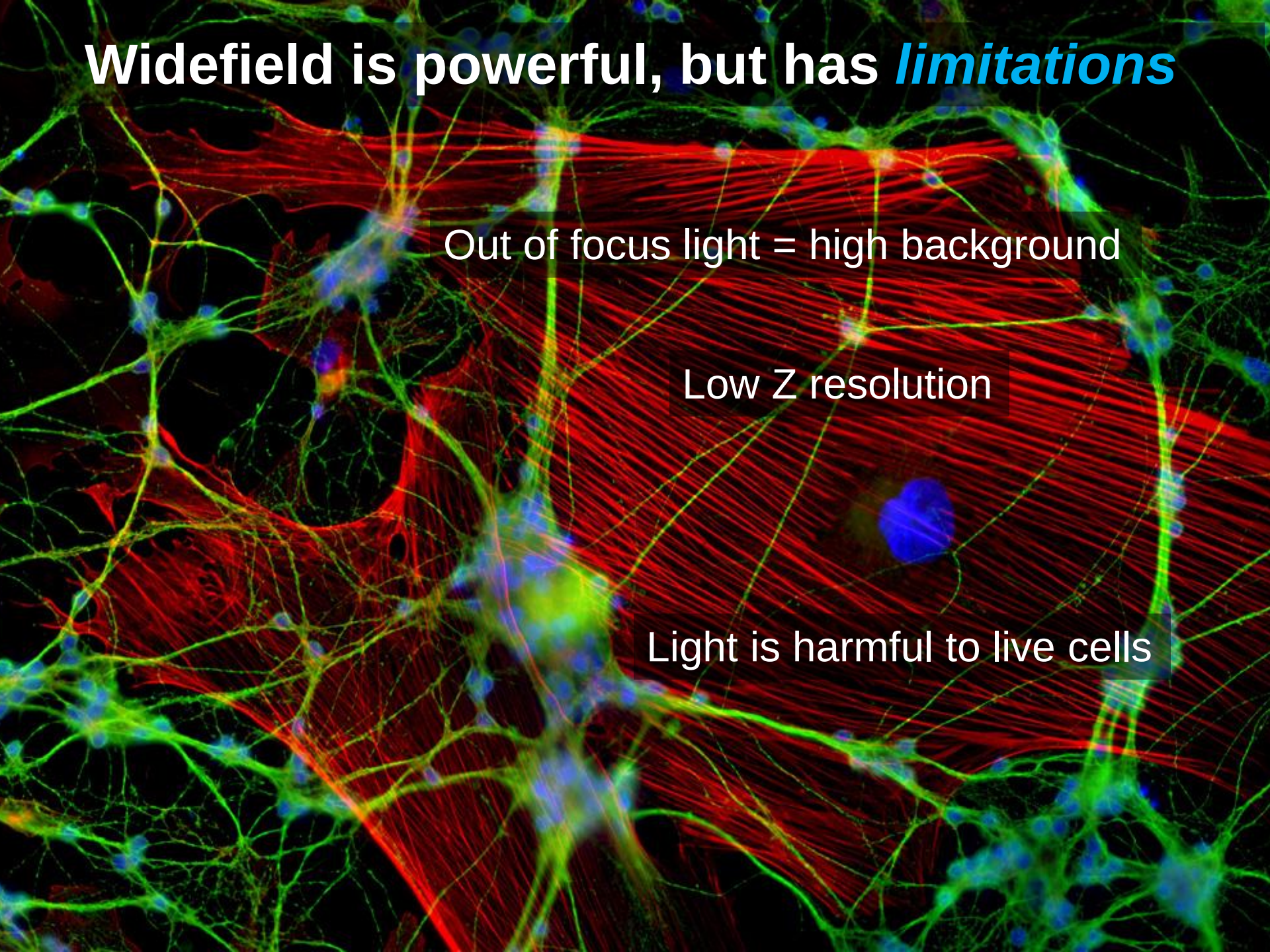
- Use glass-bottom (#1.5) dishes
- Label with Alexa 647 and Atto 488
- Block and wash samples thoroughly
- Use MEA-containing imaging buffer and keep it fresh
- *Optimization is usually necessary for the best results!*

TIRF





Widefield is powerful, but has *limitations*



Out of focus light = high background

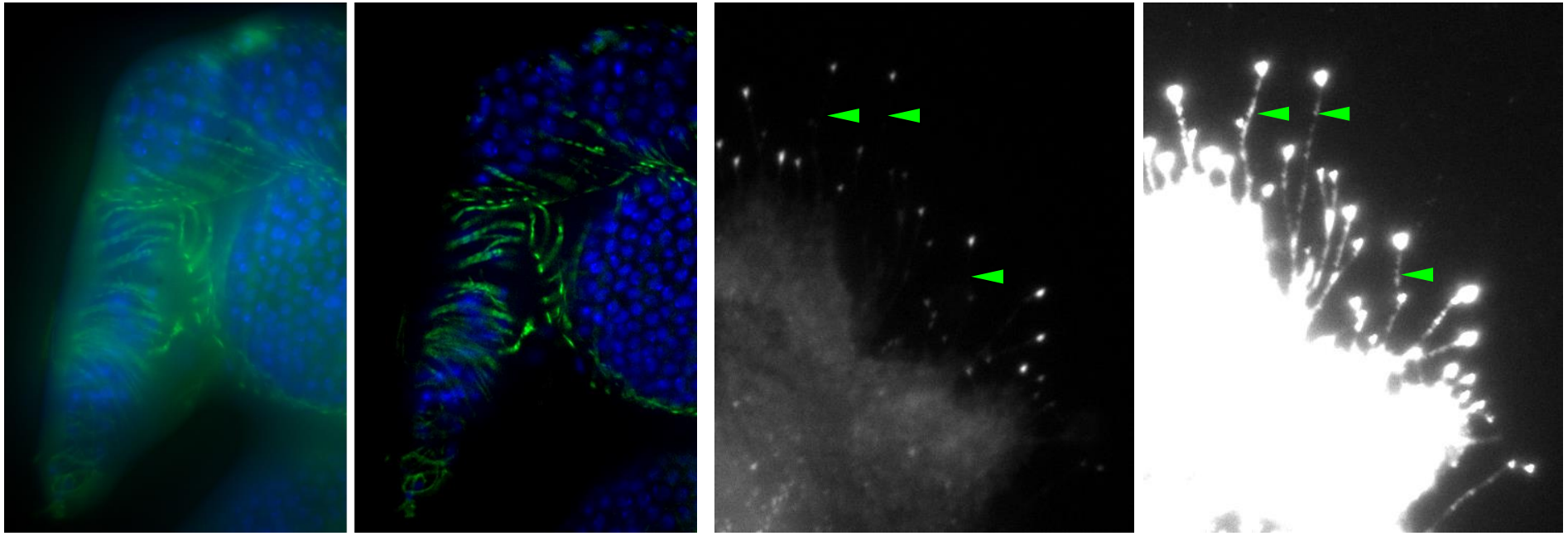
Low Z resolution

Light is harmful to live cells

TIRF = Total Internal Reflection Fluorescence

No out of focus fluorescence

Single-molecule sensitivity



Plus:

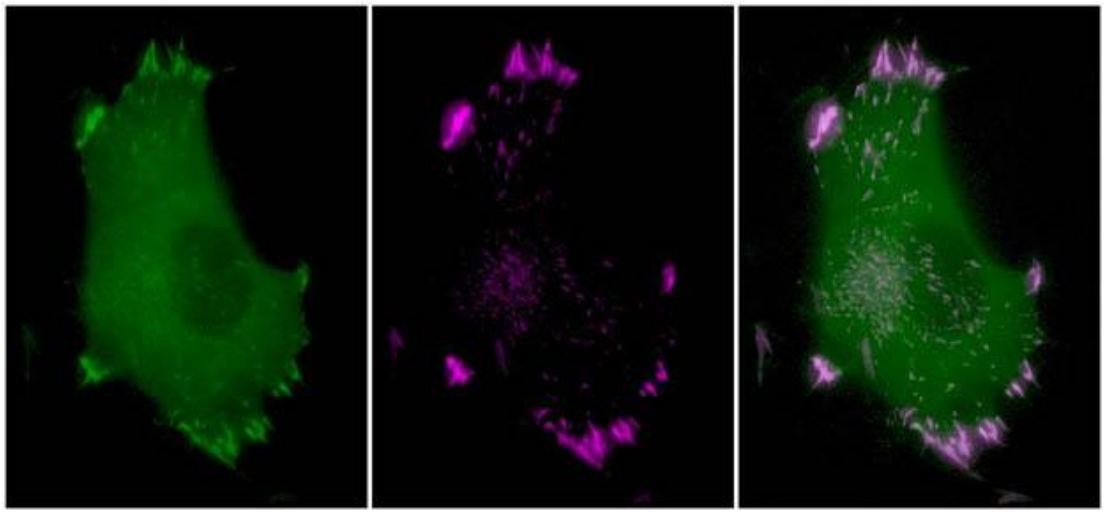
- 150nm Z-resolution
- Reduced phototoxicity to live cells
- Very high speeds possible

So…What's the catch?

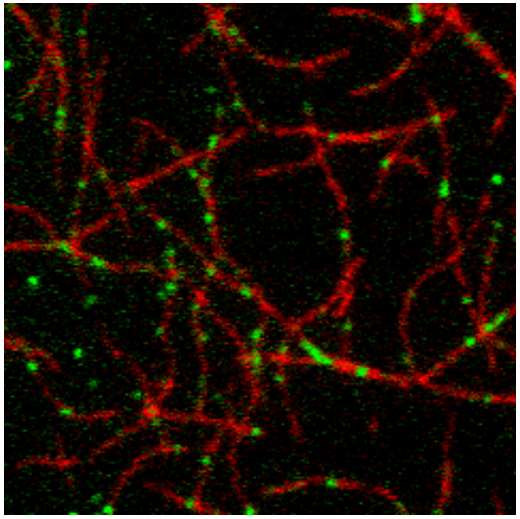
Can only image the 150nm next to the coverslip!

What near-coverslip applications require

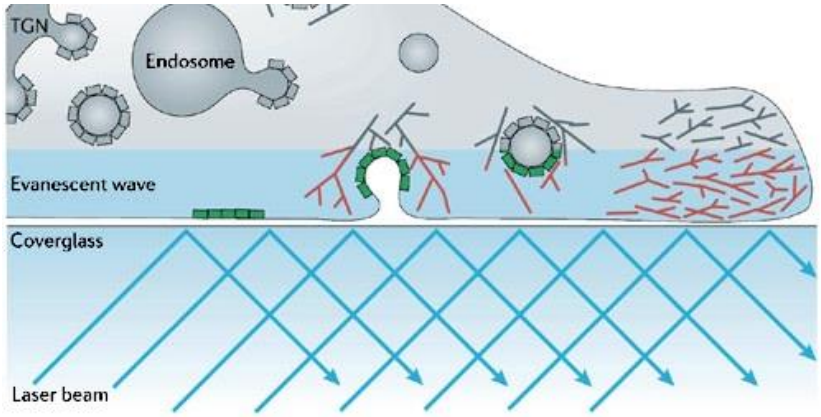
high sensitivity, high speed, and low background?

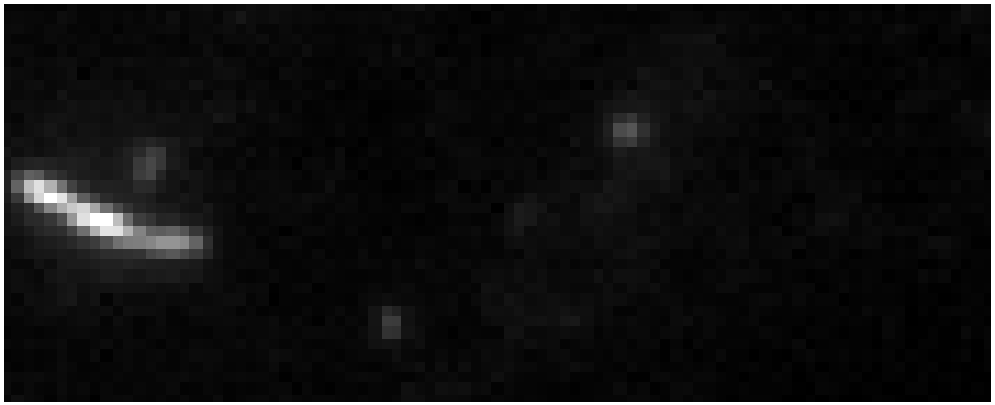


Focal adhesions



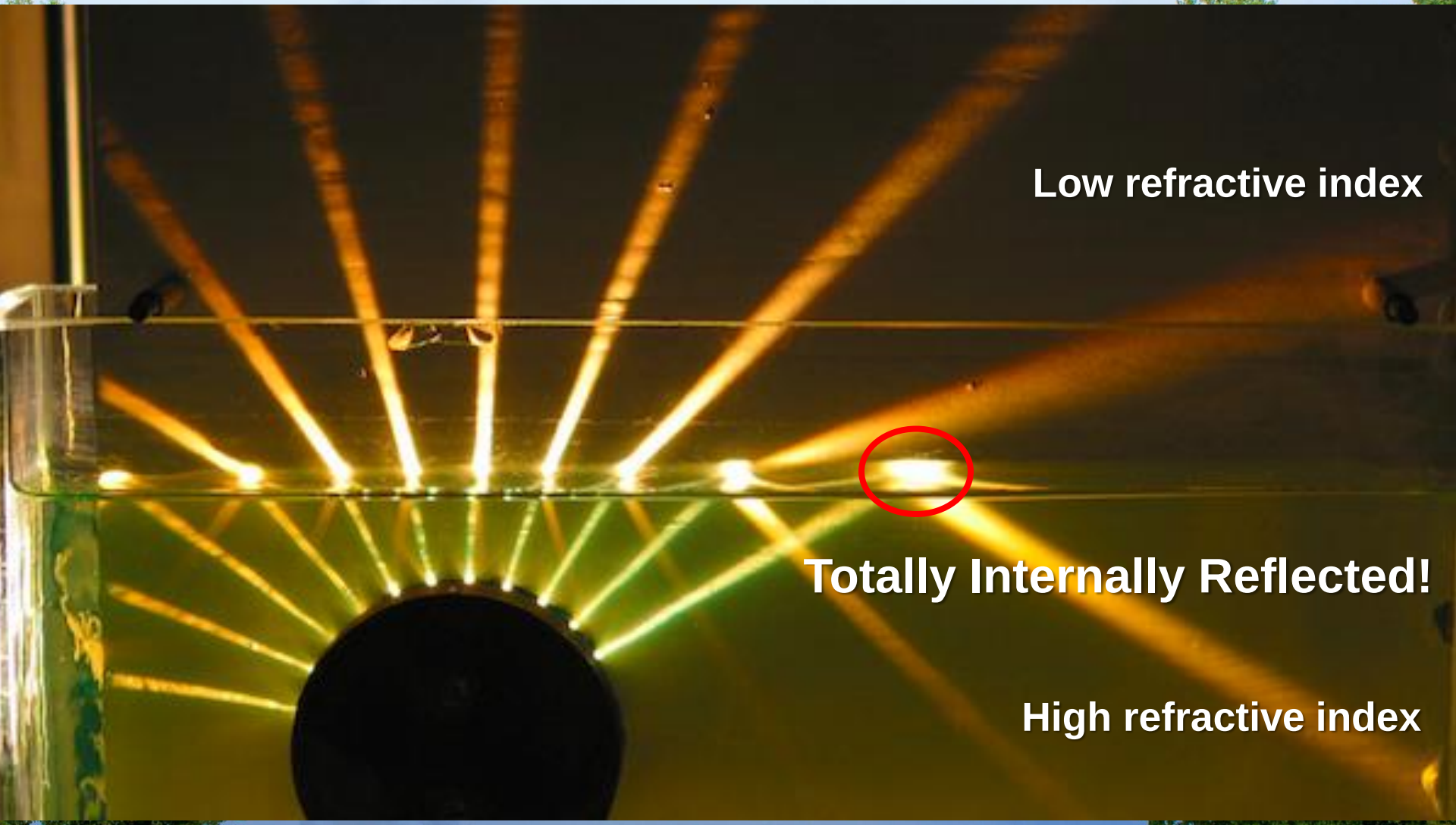
Single molecules



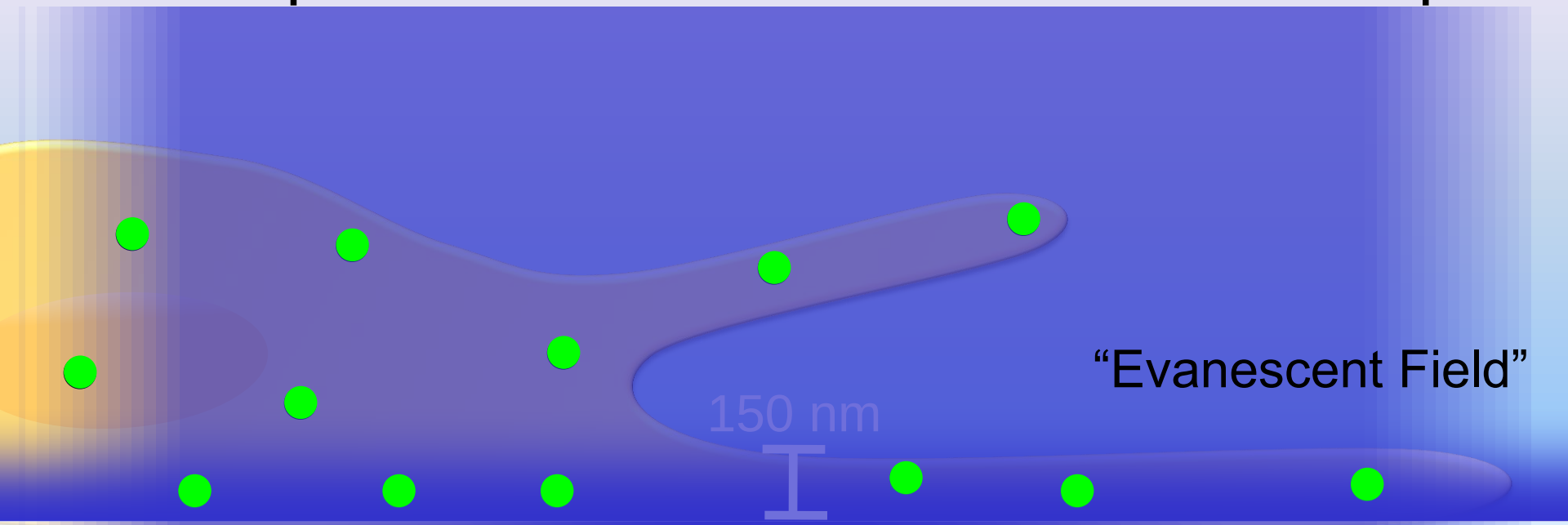


Membrane transport

How does TIRF work?
High angle light reflects off of interfaces



TIRF creates an *"Evanescent Field"* that excites fluorophores within *~150nm* of the coverslip



Coverslip

Oil

LASER

Conventional Widefield:

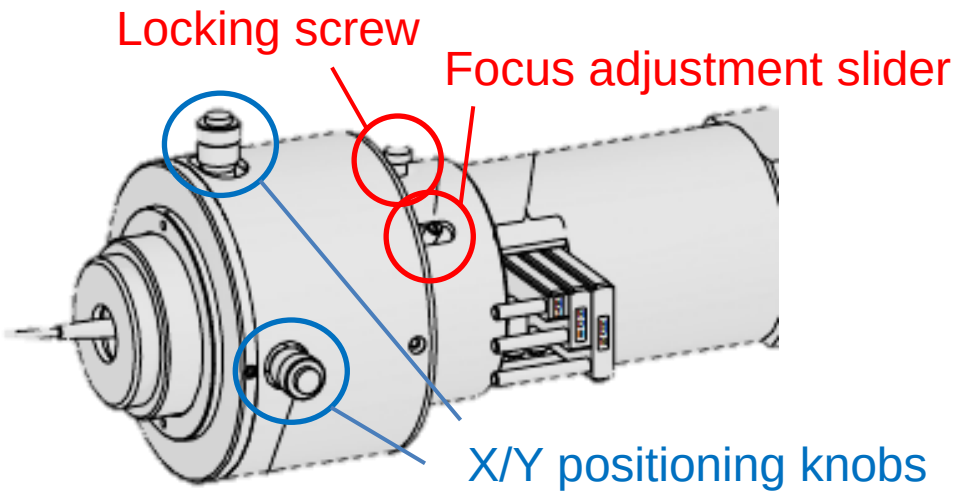
- All fluorophores in the volume are excited

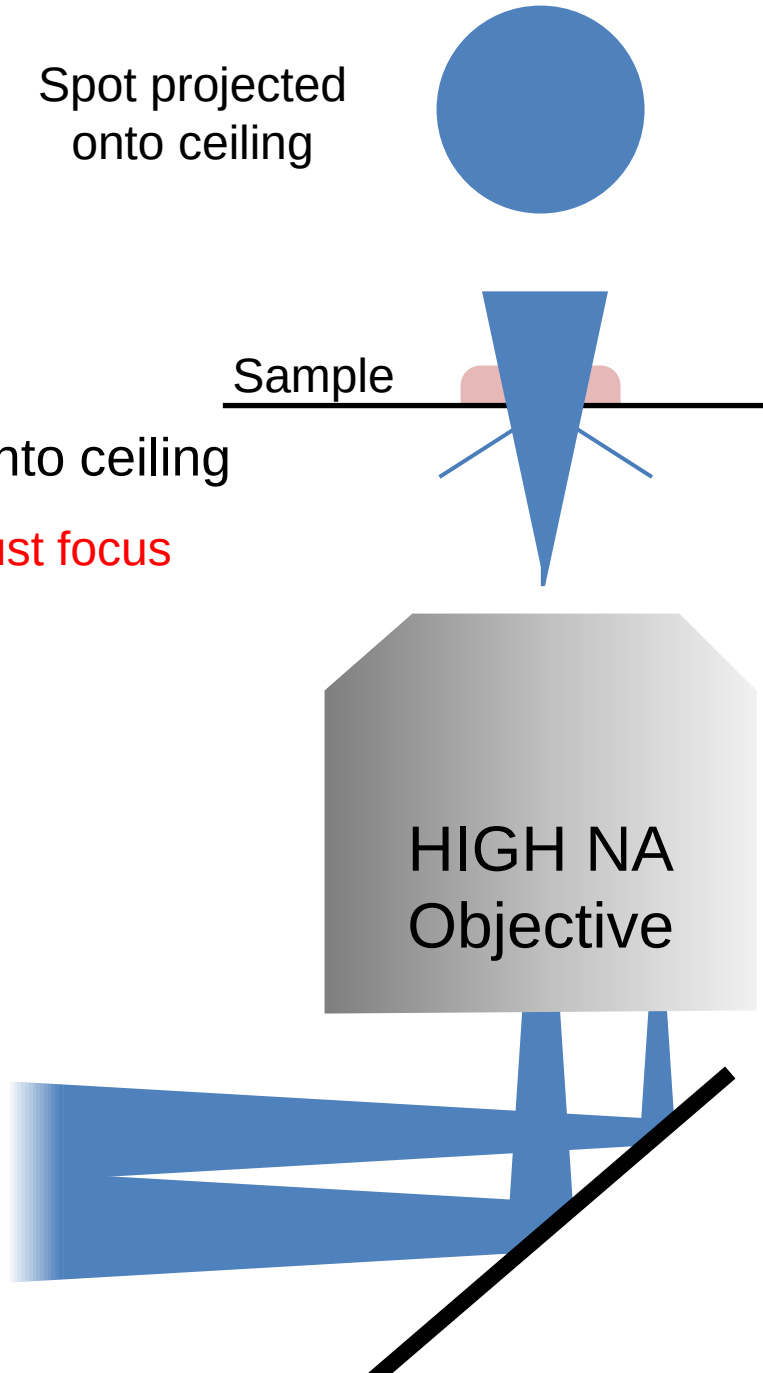
Higher signal:noise!

How to do TIRF:

1. Focus on your sample interface
2. Aim beam directly through sample and onto ceiling
3. Focus beam
4. Adjust beam angle until TIR is achieved

This is why you must focus the objective first!



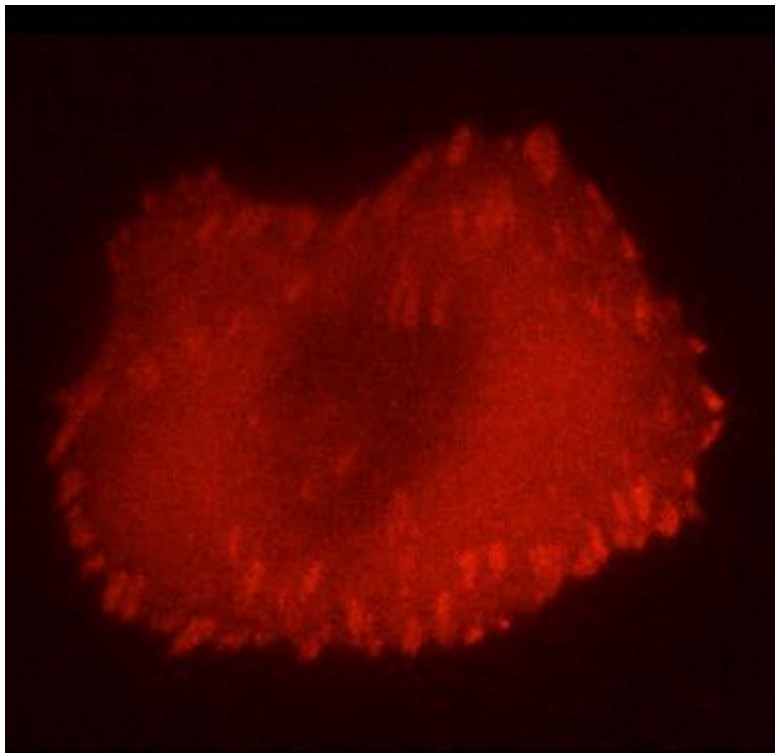


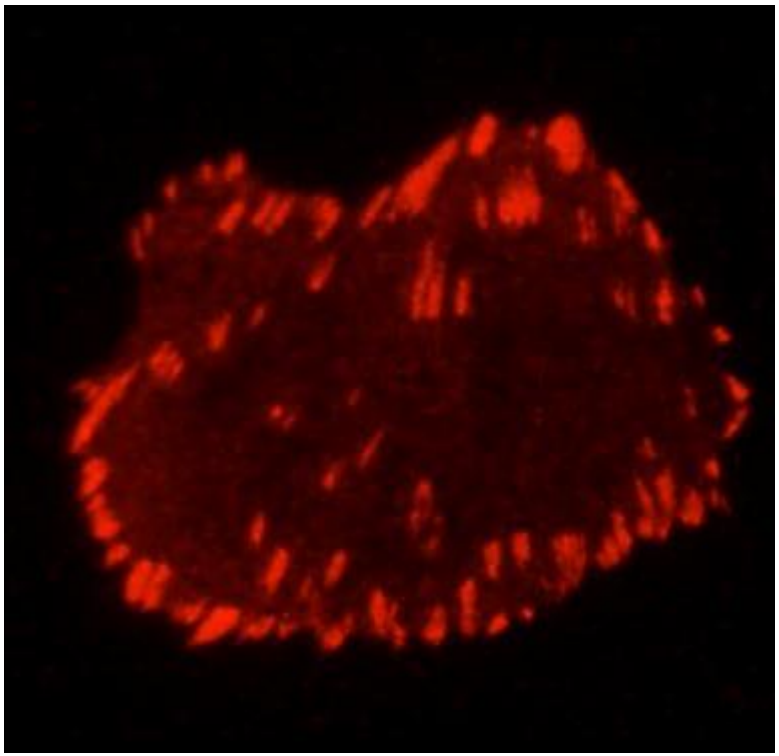
How do I know if I'm *"in TIRF?"*

Widefield

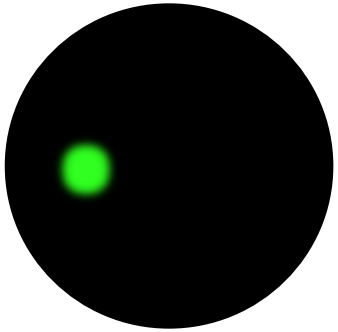
TIRF

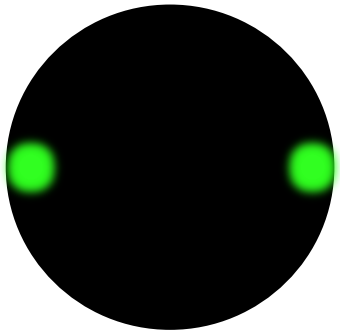
Image Plane



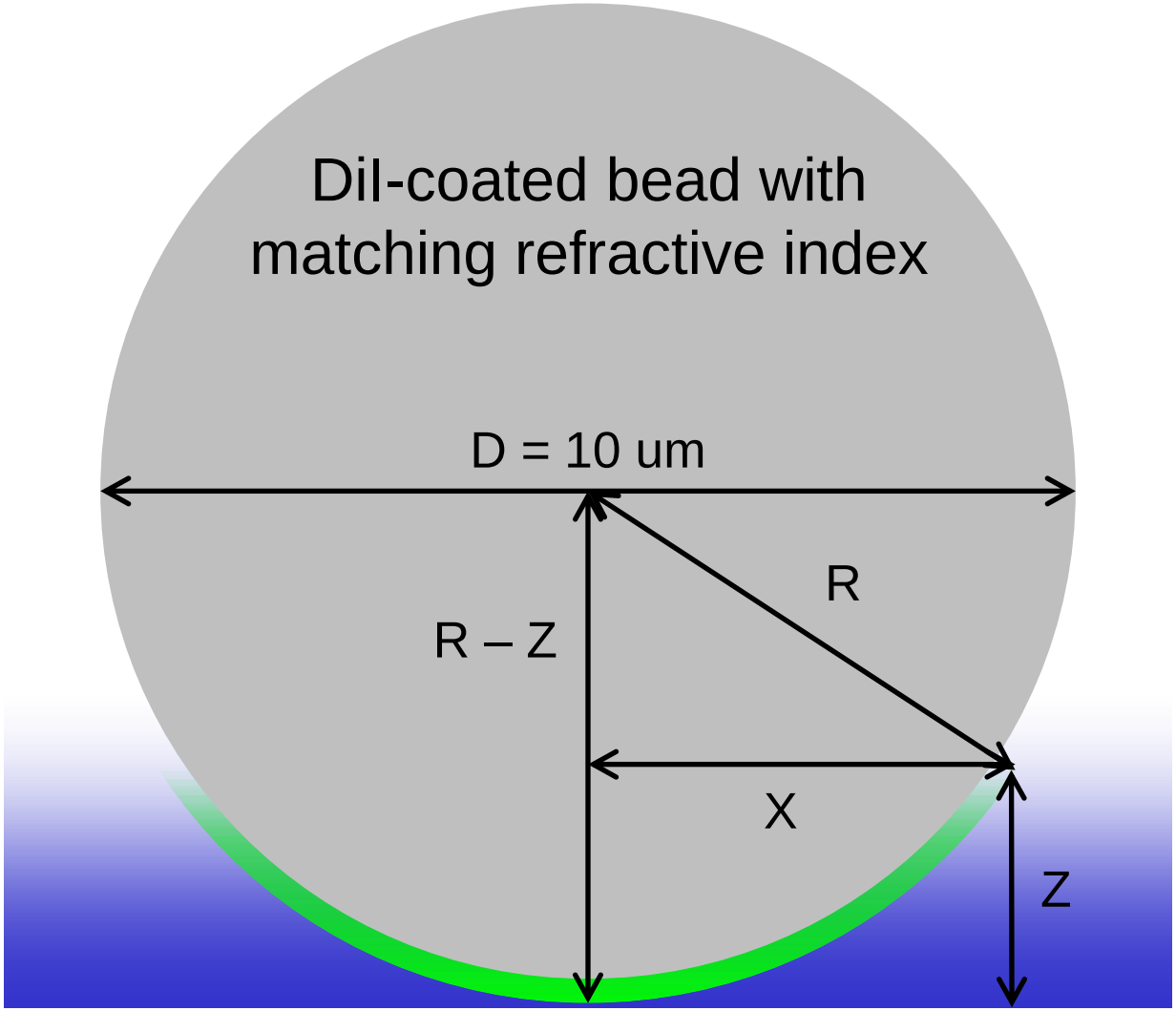


Aperture Plane



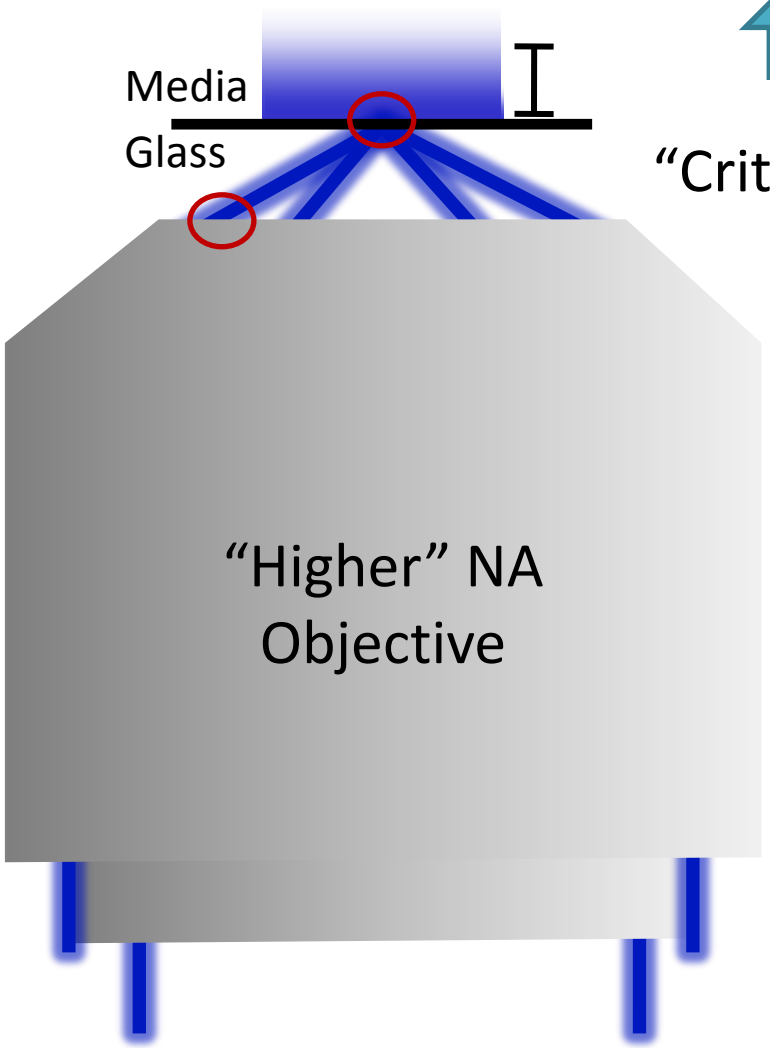


What if you want to actually *measure* the penetration depth of the evanescent field?



$$R^2 = X^2 + (R - Z)^2$$

Why *"High NA?"*



↑ ɵ → ↓ Penetration Depth

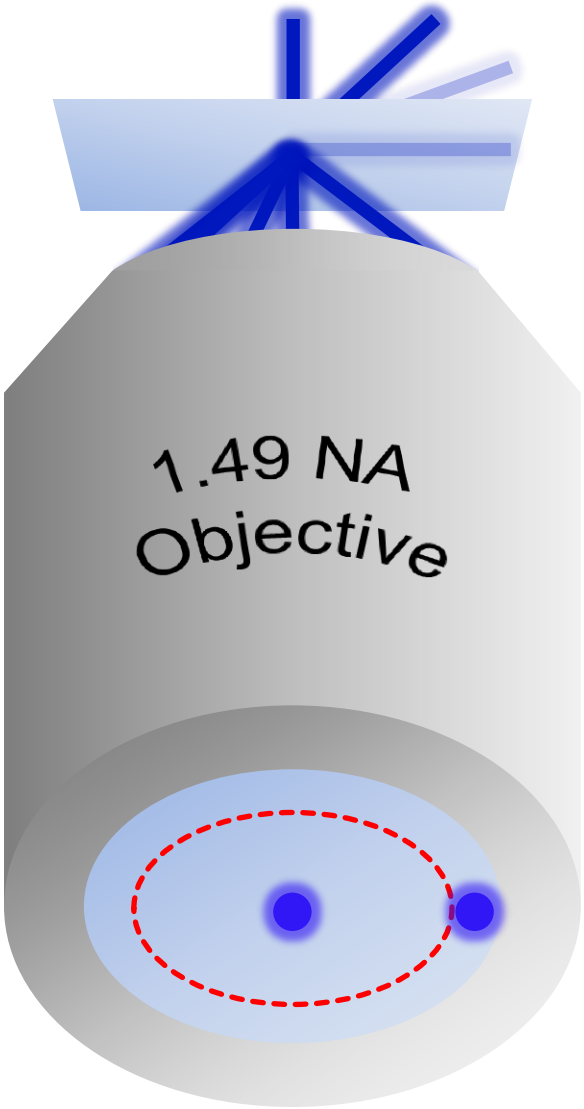
"Critical angle" = Angle necessary for reflection
= $\sin^{-1}(n2/n1)$ = **63°**

Maximum angle for an objective
NA = n sin **ɵ**

NA	ɵ
1.4	67°
1.45	72°
1.49	79°

So, higher NA lenses allow for thinner evanescent fields!

Also remember: Laser beams have a *width*

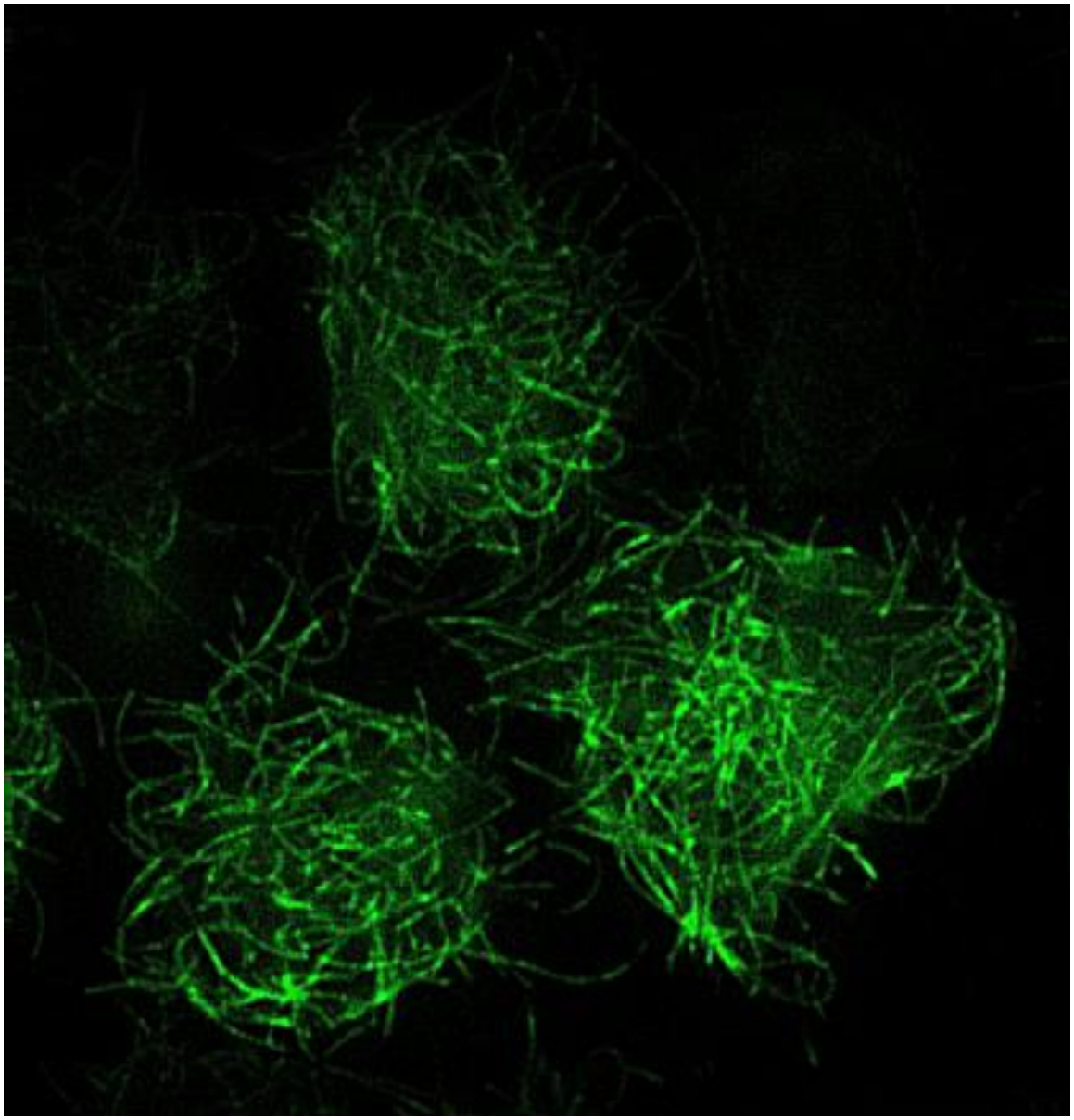


Some laser light at critical angle and some not
Total internal reflection!

This is the other reason to use "Higher NA" objectives

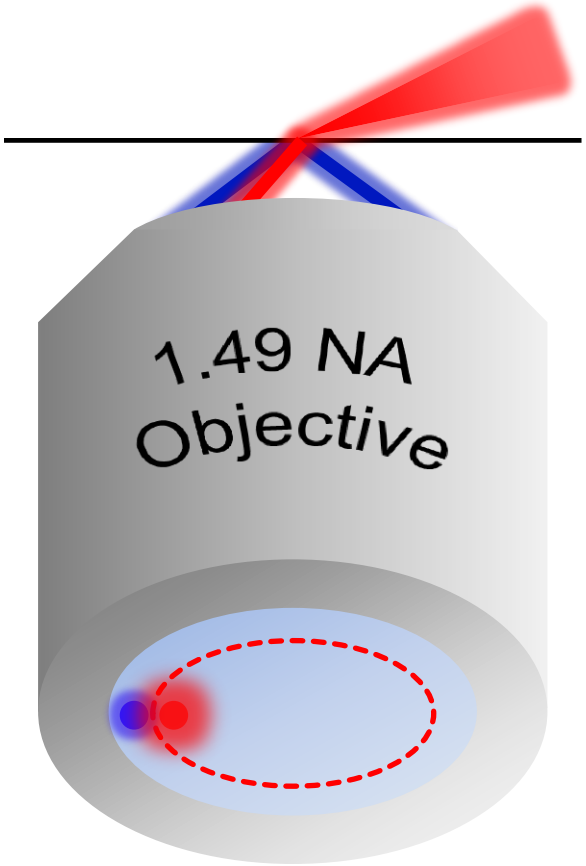
Critical angle threshold

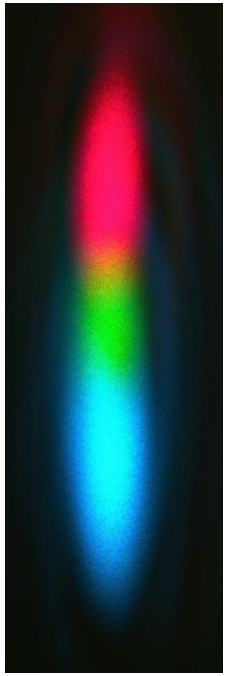
What if I want to do TIRF with multiple colors?



Multichannel TIRF considerations

Remember: shorter wavelengths of light are refracted more at interfaces.





Some options:

- Use *chromatically corrected* optics in your TIRF illuminator
- Use a *motorized* TIRF illuminator
- Use a *separate* TIRF illuminator for each wavelength

Different wavelengths will not strike the back aperture at the same *location* or in the same *focus*

What do you need to do TIRF?

- High NA objective
  - 60X 1.49 Apo
  - 100X 1.49 Apo
  - 100X 1.49 SR Apo
  - 100X 1.45 ʎ Plan Apo

- TIRF cubes

- Laser

- Samples
  - Need a glass-water interface!
  - Get as close to coverslip as possible, minimize ECM!

Extra hardware for *single-molecule live-cell* TIRF:

- Maintaining focus is a must!
  - Perfect Focus System!

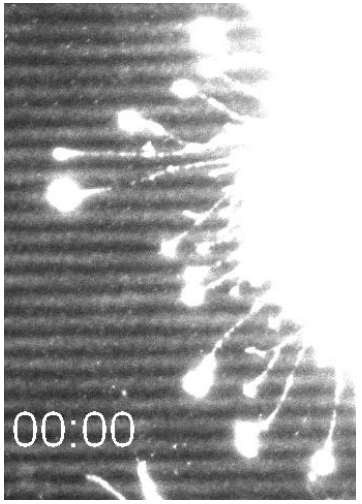
- Single molecules are dim
  - EMCCDs

- Molecular motors move fast
  - Triggering

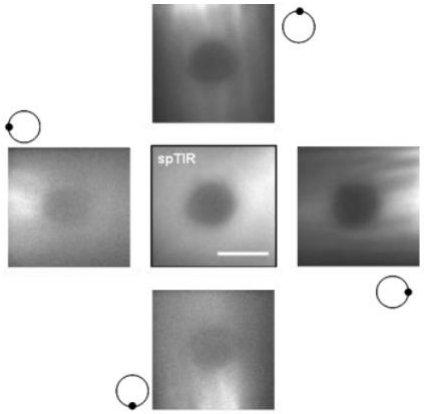
- Live-cell fluorophores, like GFP, bleach very fast
  - Minimize exposure to light when not imaging
  - Find focus using some other imaging modality (transmitted, SRIC)

Common Pitfalls of TIRF

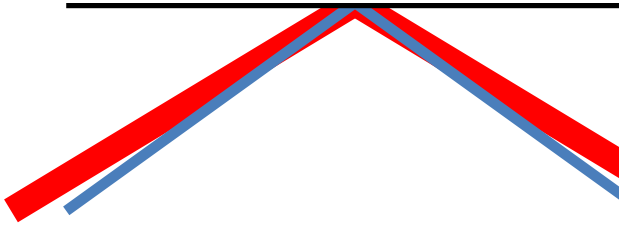
- Very small imperfections in your optics will cause an *interference fringe*
  - DIC prism, etc. can worsen interference patterns and cause ghosting



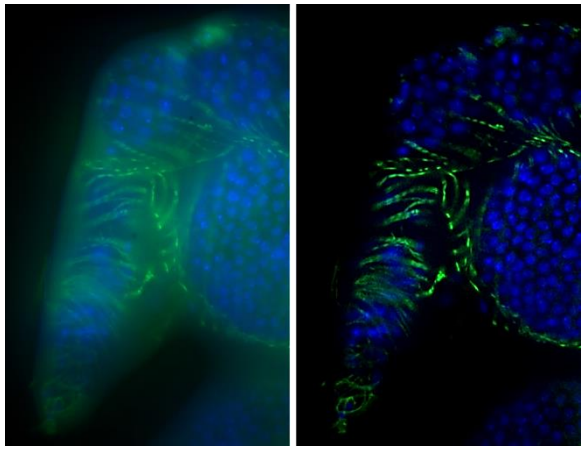
- Light scatter causes *directional smearing*
  - Scattering exacerbated by extra coatings
  - Thinner evanescent fields cause less scatter
  - Take images at multiple orientations



- *Different wavelengths* will not strike the specimen at the same incident angle or same focus
  - Ti TIRF arms have chromatic correction for focus
  - Use different angles for each channel (Motorized or multiple arms)



TIRF Summary



- *TIRF advantages*: high speed, high signal:noise, thin z-sectioning, and low phototoxicity
- *TIRF disadvantages*: can only see ~150nm adjacent to coverslip

- An evanescent field is created by reflecting laser light off of a *glass/water interface*
- *Penetration depth* depends on wavelength, angle, and refractive index
- Penetration depth can be calculated or *measured experimentally*
- Objective lenses with at least *1.45 NA* are recommended

Thank you!

Acknowledgements

- **Xiaowei Zhuang, Harvard University**
- **Mike Davidson, FSU**
- **microscopyu.com**

Your local Nikon representatives:

- **Edwin DeFeijter**
- **Jim Winkelmann**