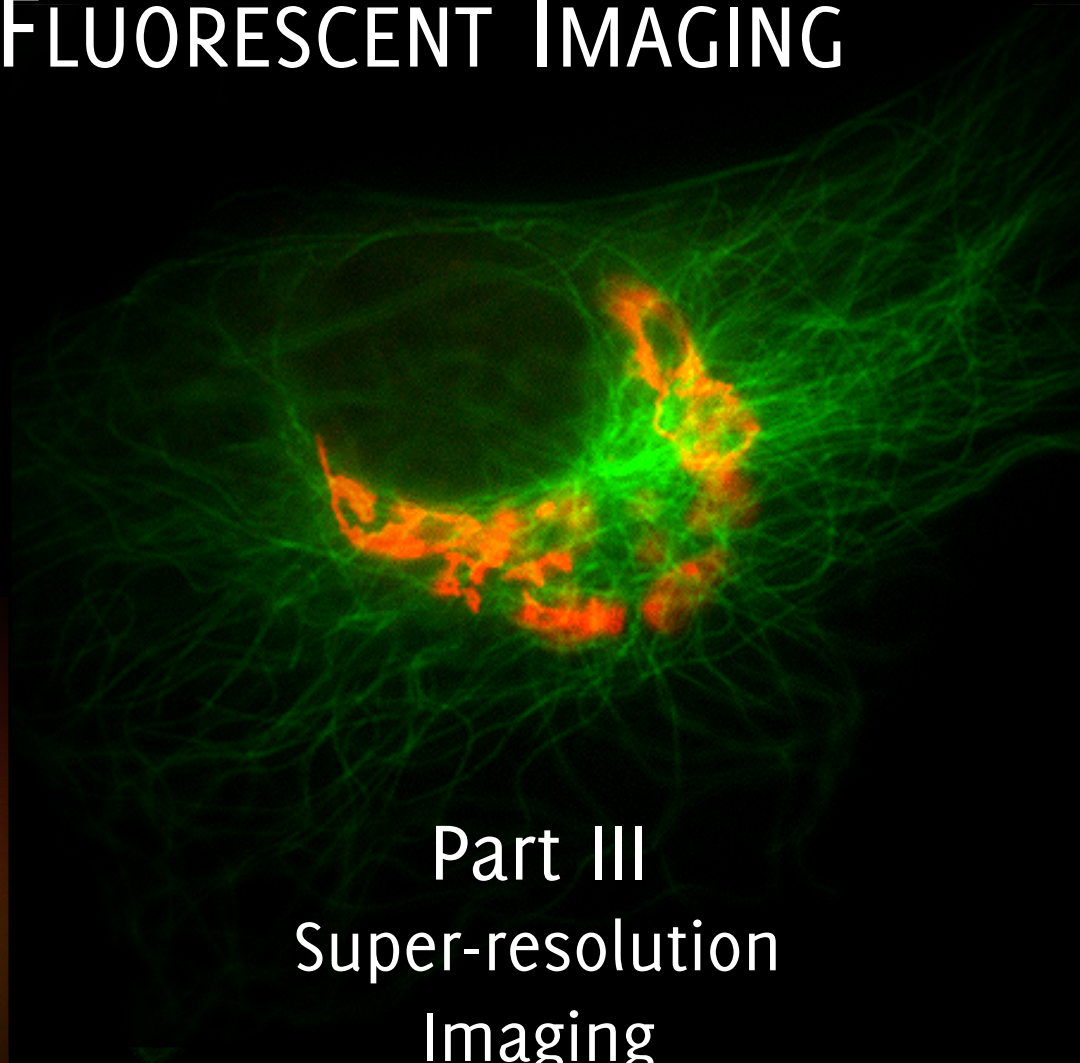
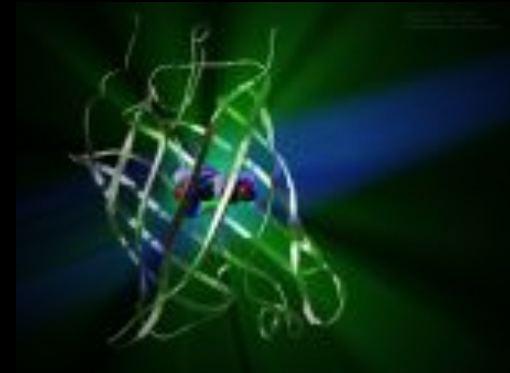
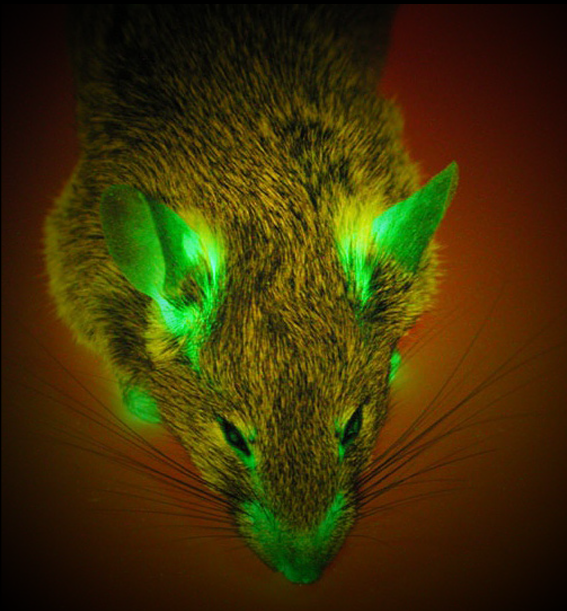


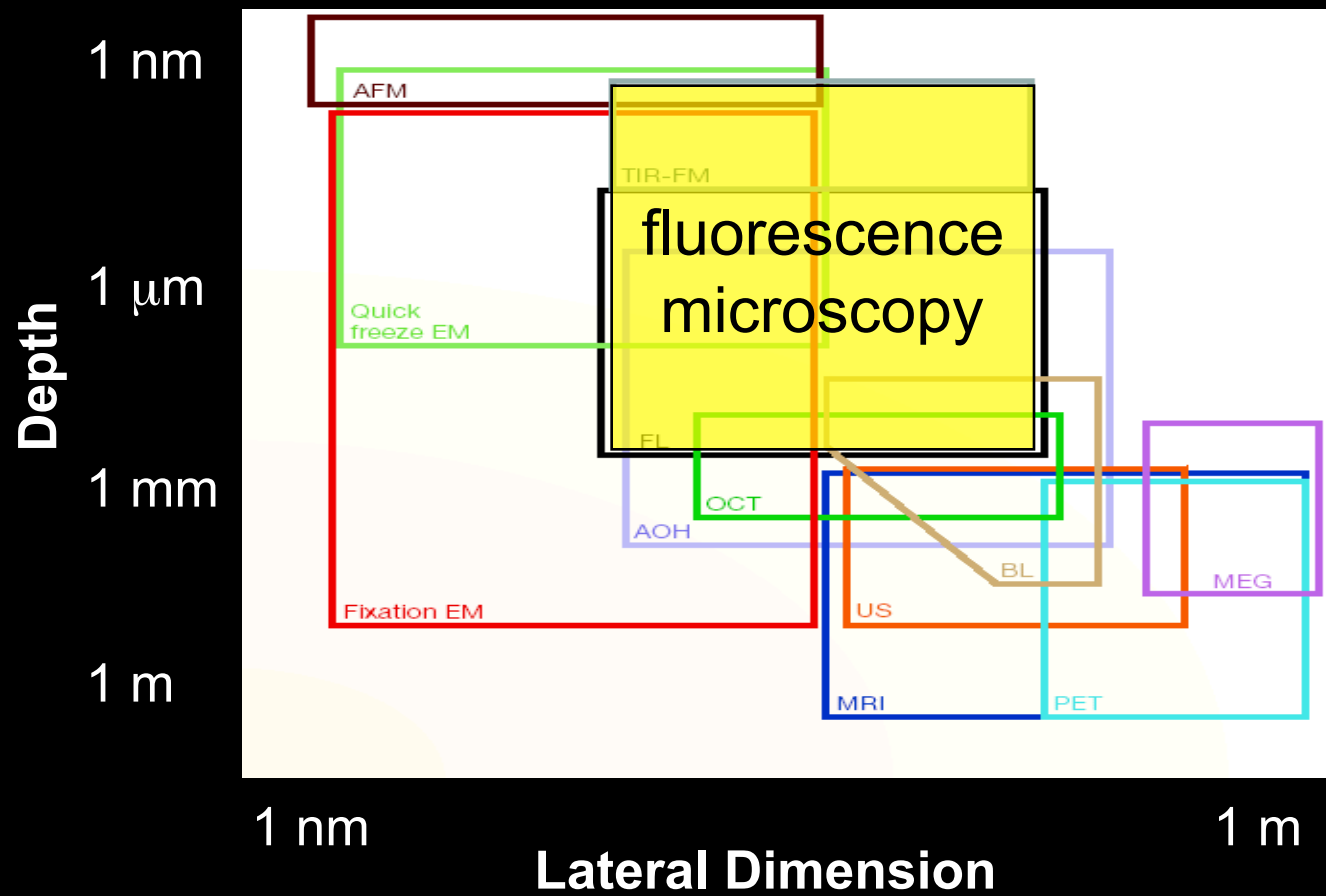
BREAKTHROUGHS IN INTRACELLULAR FLUORESCENT IMAGING



Part III
Super-resolution
Imaging

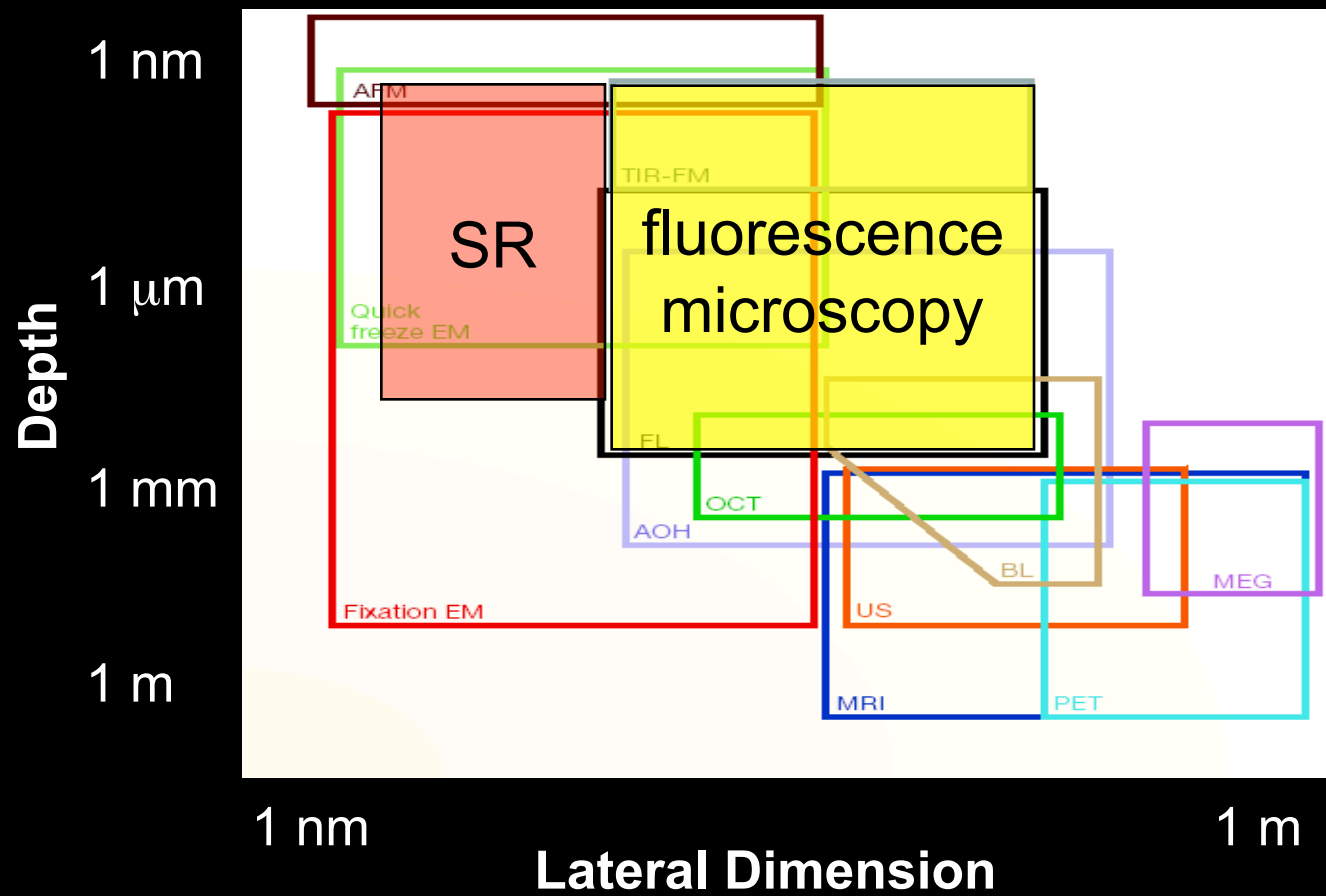


Imaging Methods



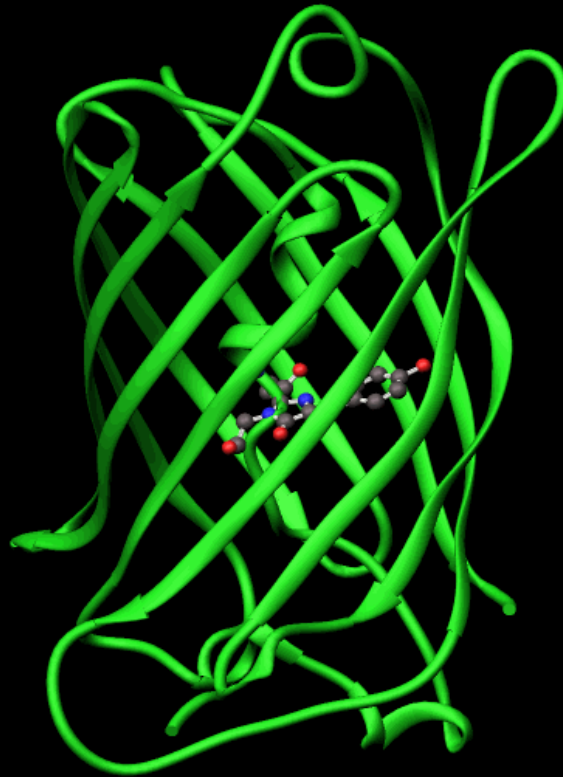
Tsien, Nat. Rev. (2003)

Imaging Methods



Tsien, Nat. Rev. (2003)

Optical Resolution vs. Molecular Dimensions



2.5 nm

The Diffraction Limit



The Diffraction Limit

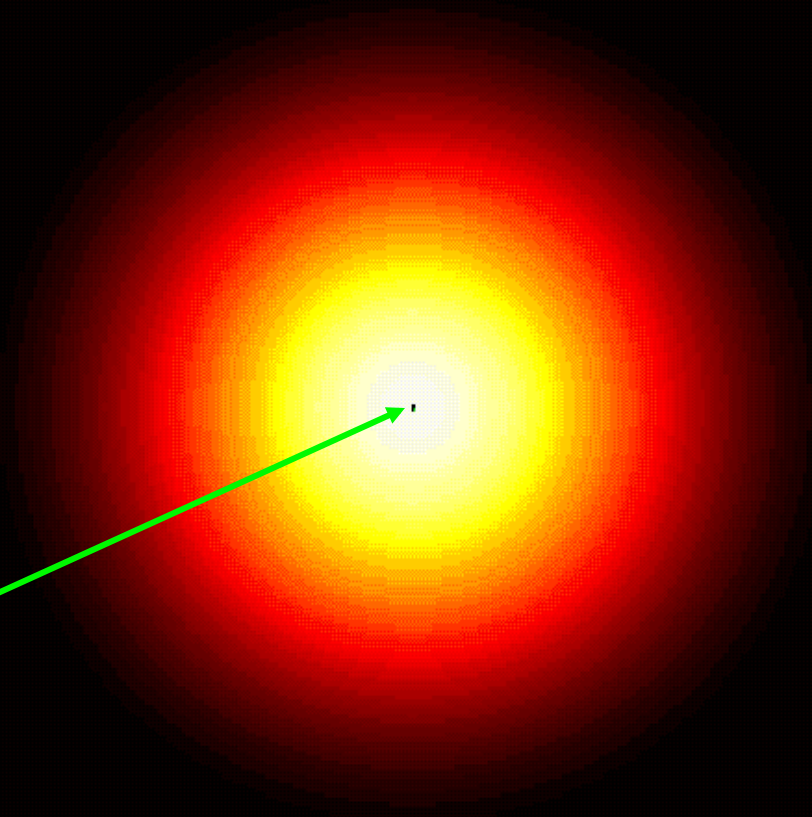
microscope point spread function

$NA=1.4$
 $\lambda_o=500\text{nm}$

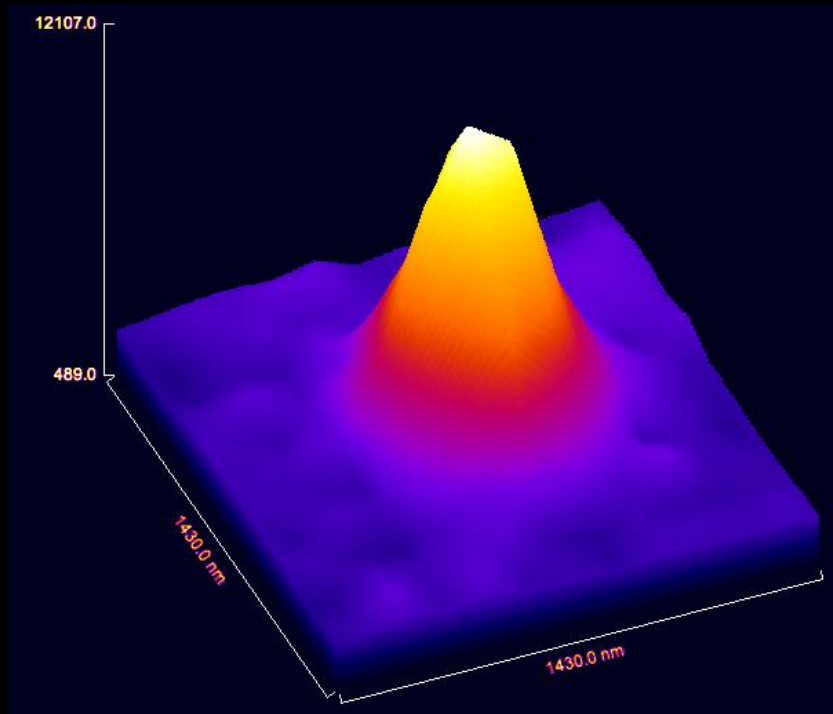
GFP

2.5 nm

100 nm



Localization of the molecule in x and y dimensions

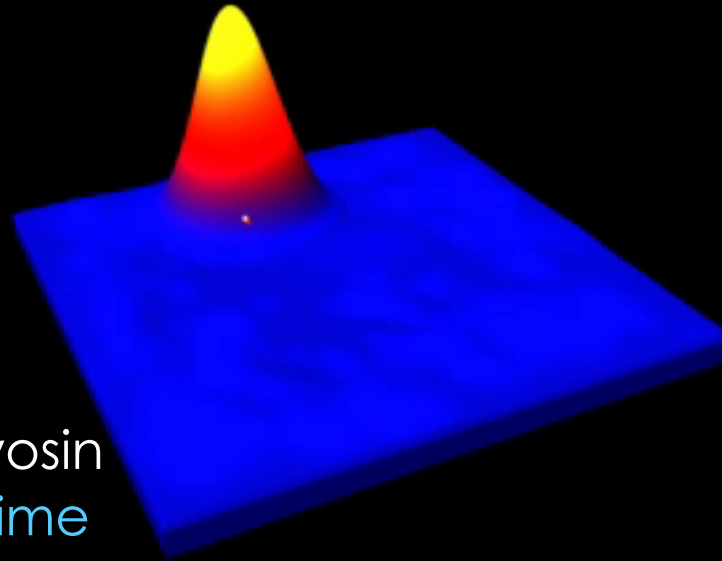
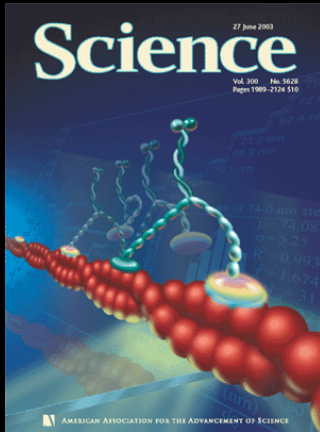


Apply 2D Gaussian least squares fit to find
center of centroid of point-spread-function

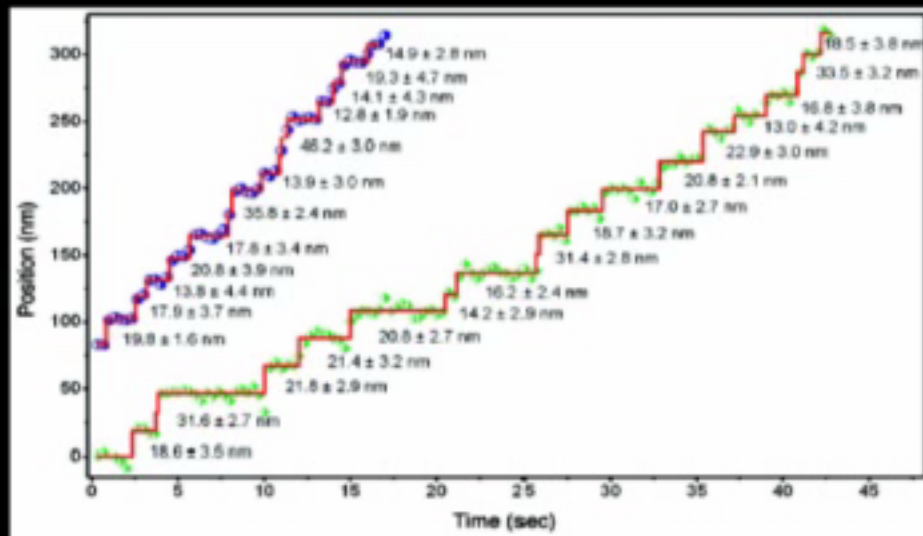
The greater the # of photons, the better the 2-D Gaussian fit

$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2} \right)}$$

Localizing Single Molecules

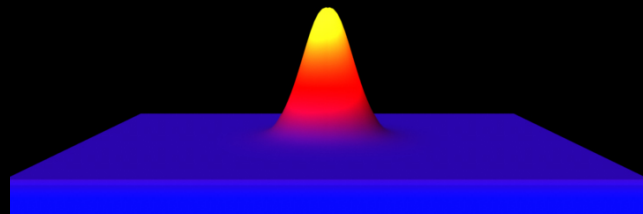
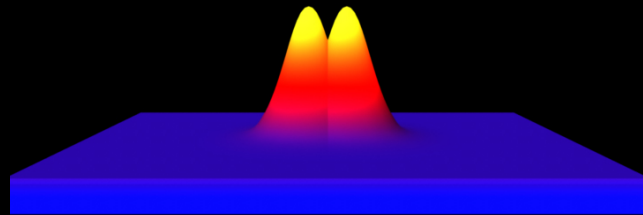
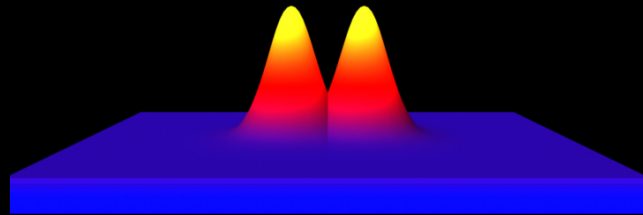
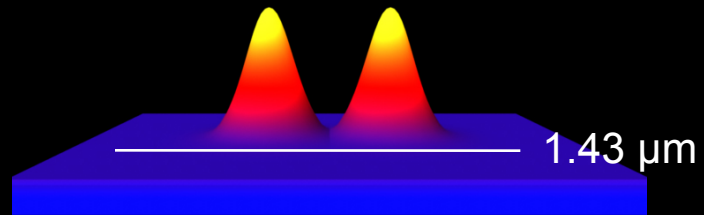


molecular motor: myosin
One molecule at a time



Probe-Based Super-Resolution

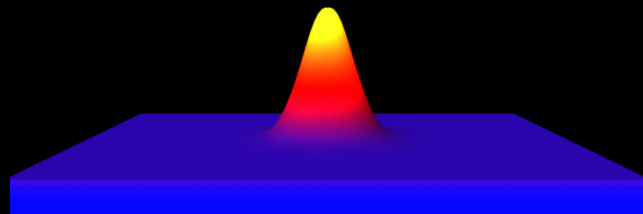
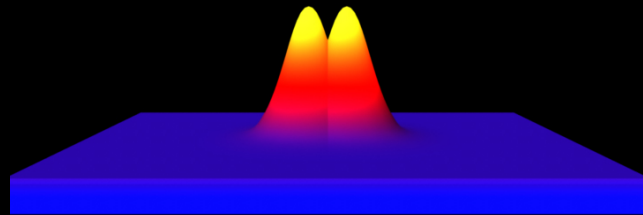
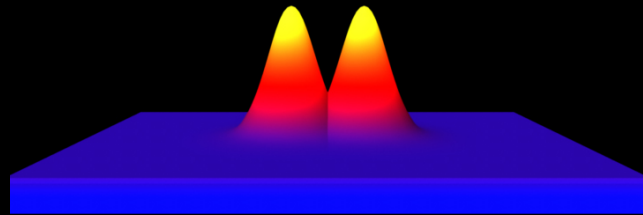
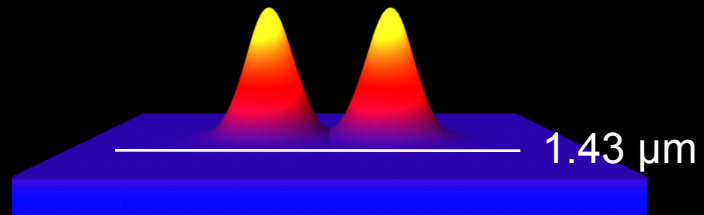
Two molecules



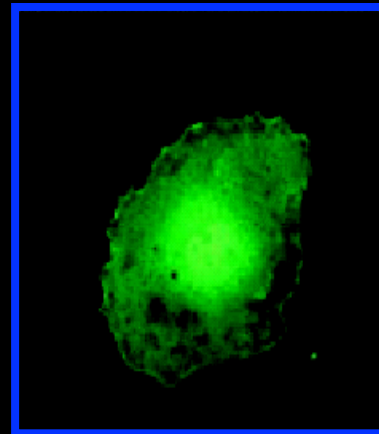
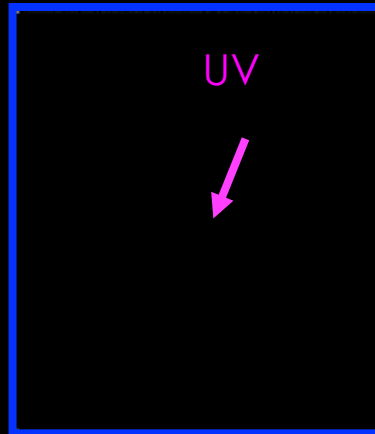
Localization

Probe-Based Super-Resolution

Two molecules



Photoactivatable FP

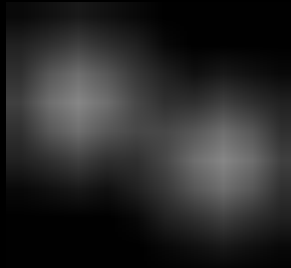


Photoactivated Localization Microscopy (PALM)

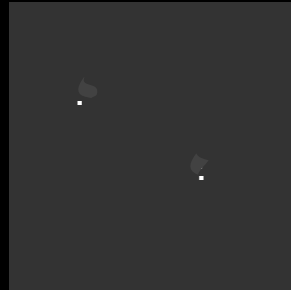
*Photoactivate 1st
subpopulation*



**Image & then
bleach
subpopulation**



Fit
2D
Gaussian

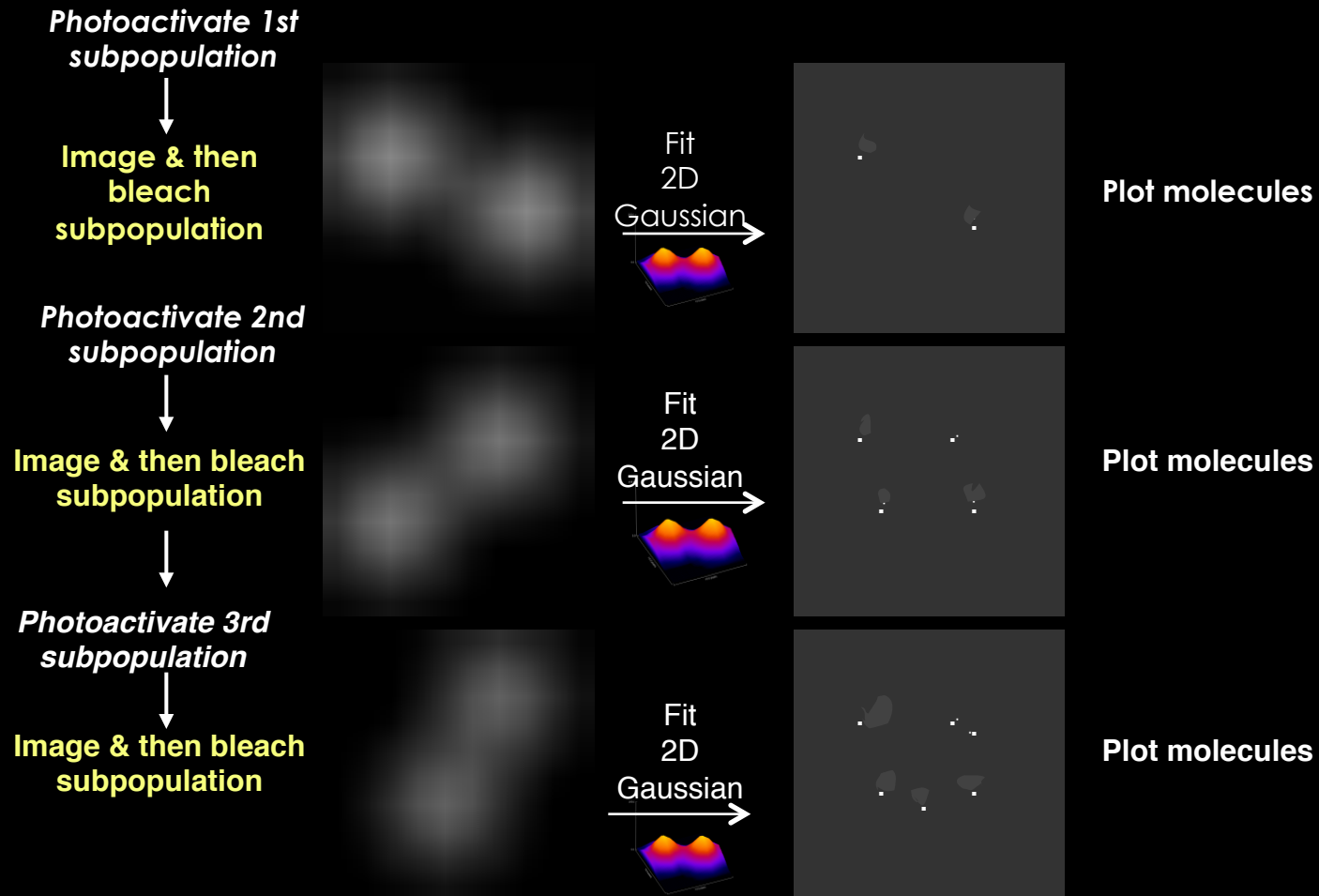


Plot molecules

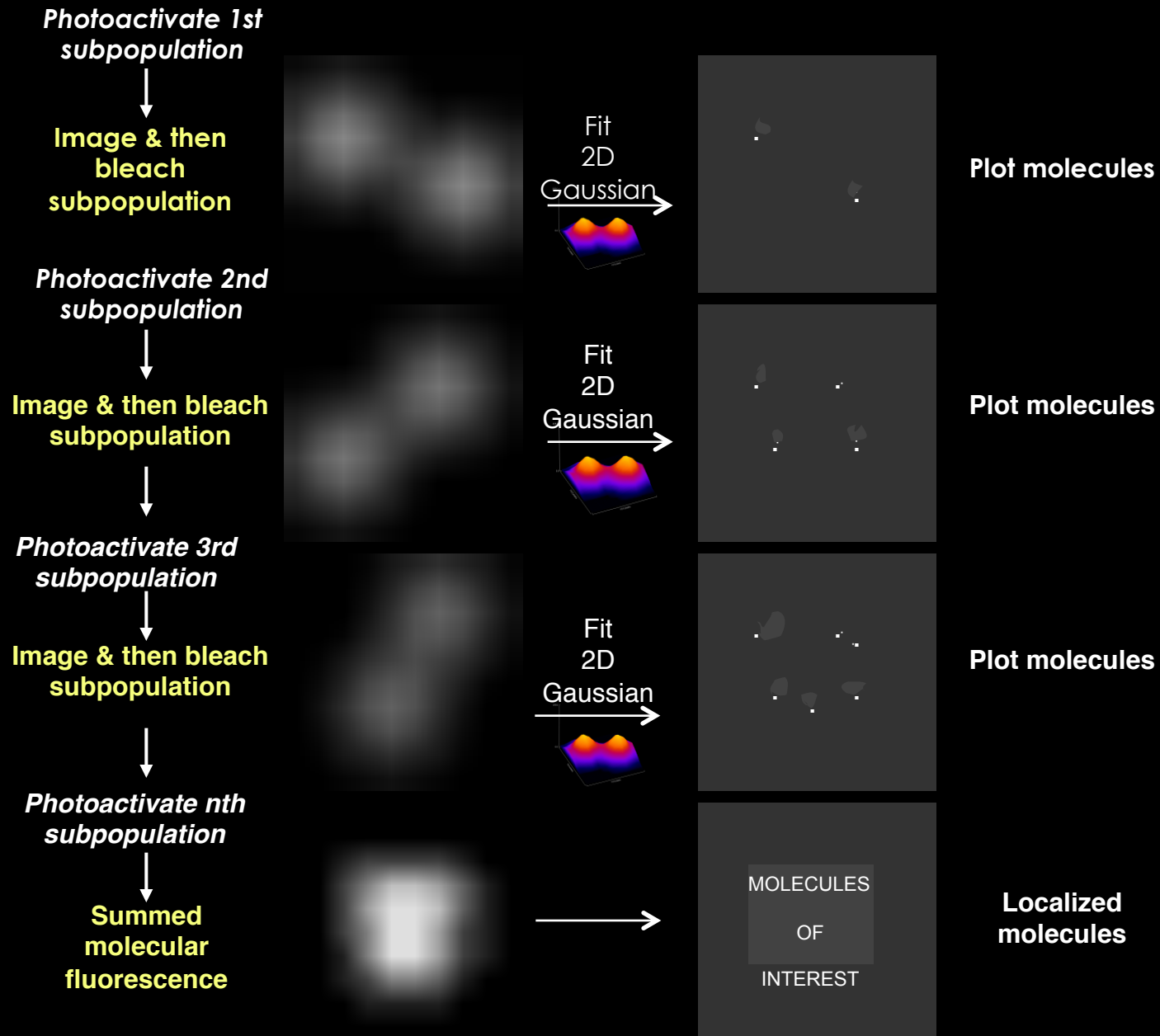
Photoactivated Localization Microscopy (PALM)



Photoactivated Localization Microscopy (PALM)



Photoactivated Localization Microscopy (PALM)

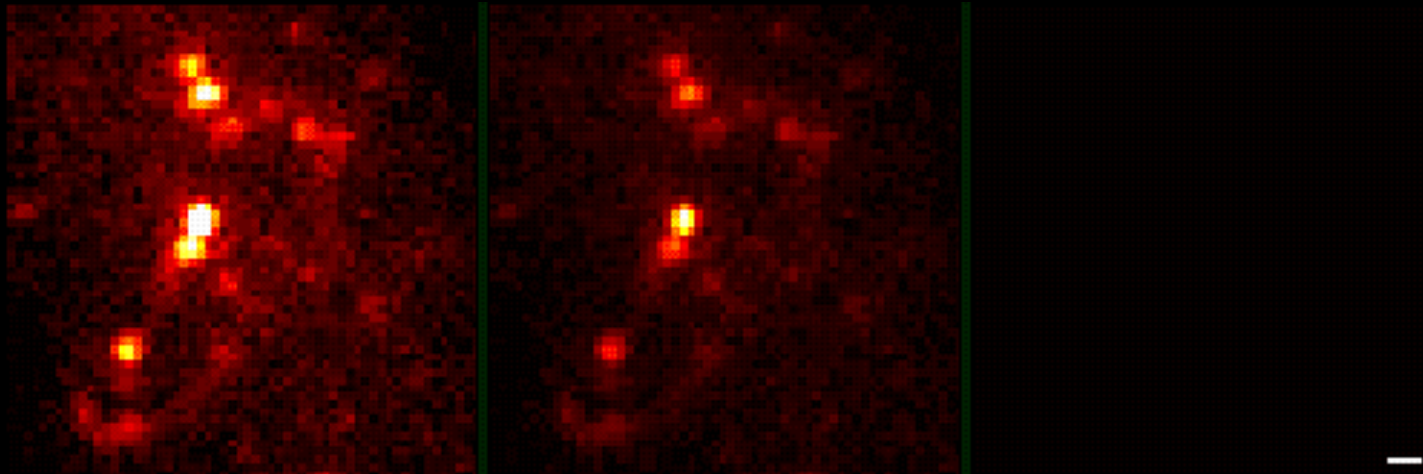


PALM DATA ACQUISITION

Raw

Sum

PALM



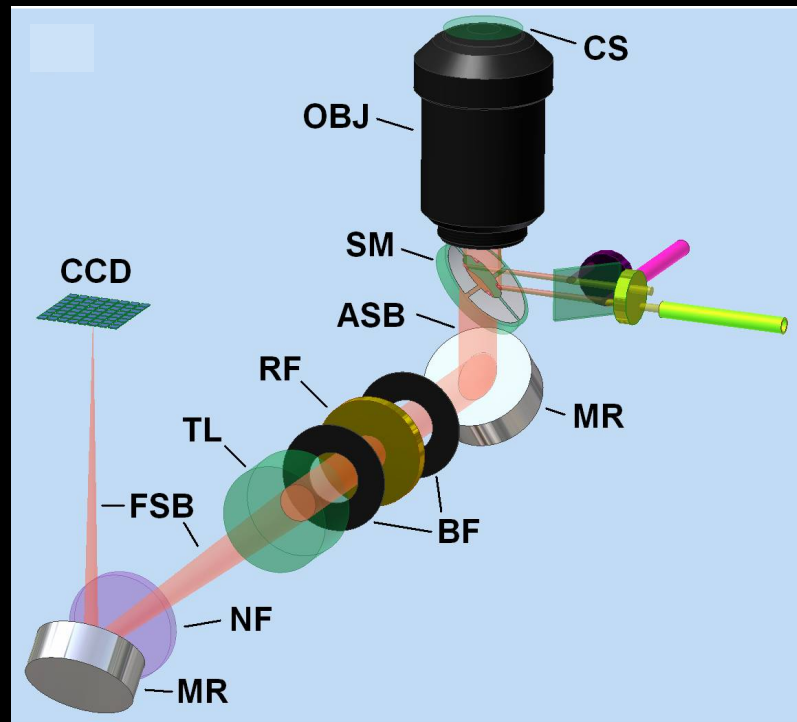
Thin section of PA-GFP labeled lysosomes

PALM INSTRUMENTATION

electron multiplying CCD

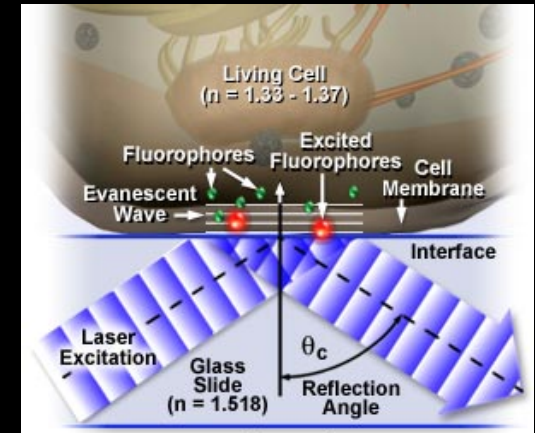


PALM optical path --
through objective TIRF excitation

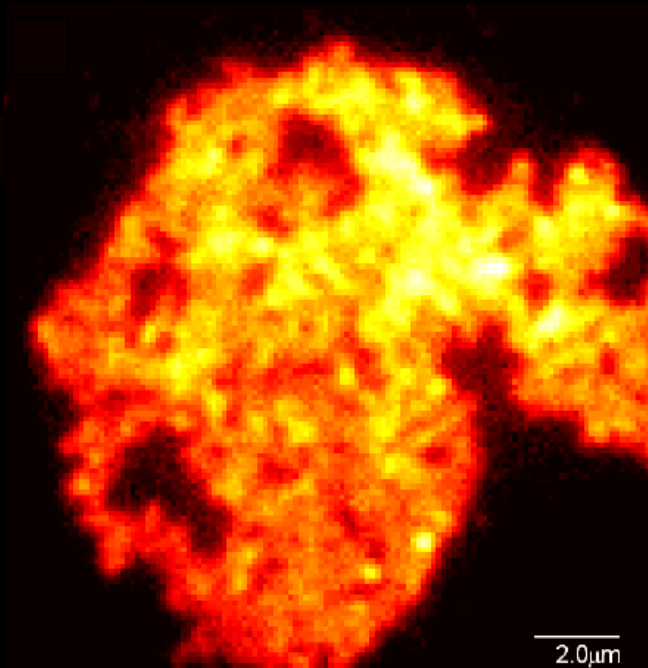


$\lambda = 405$ nm activation
 $\lambda = 491$ or 561 nm excitation

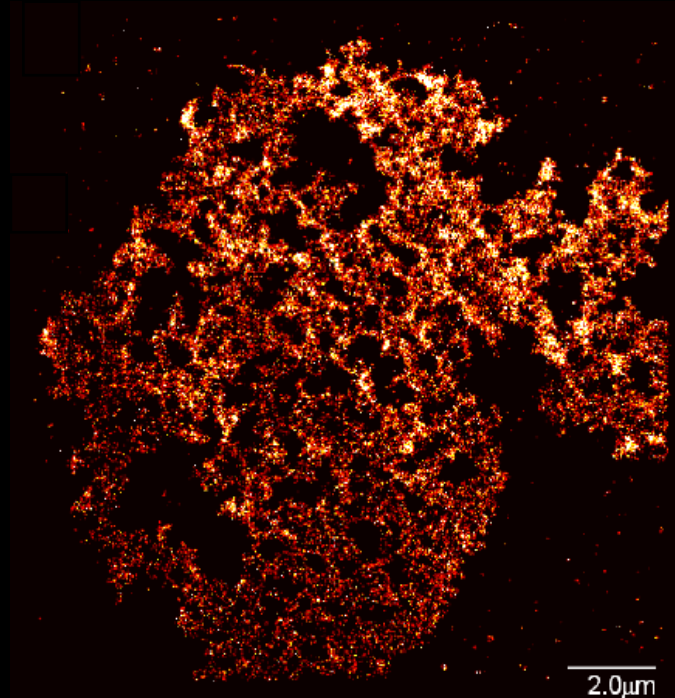
total internal reflection fluor. (TIRF)



PALM SUPER-RESOLUTION ORGANIZATION



Conventional TIRF

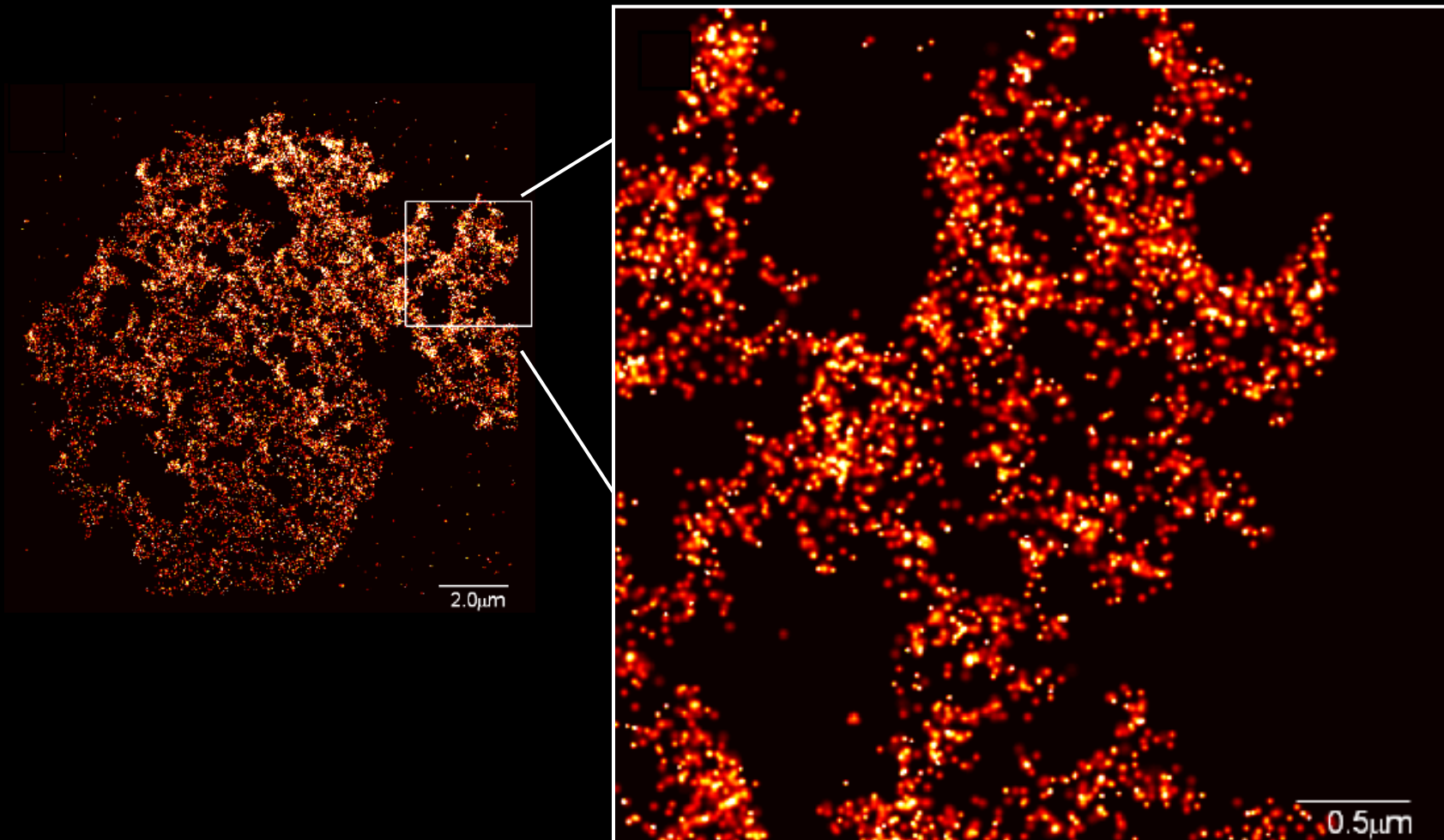


PALM Image

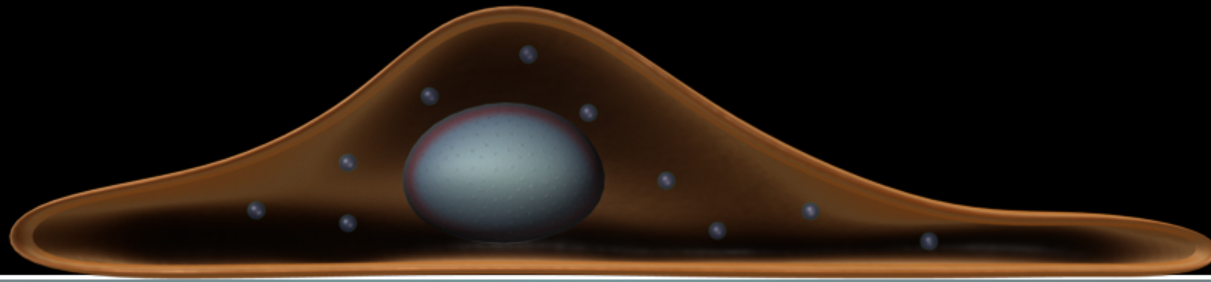
Constructed by summing position
probability gaussians determined for all
localized
molecules in data stack

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

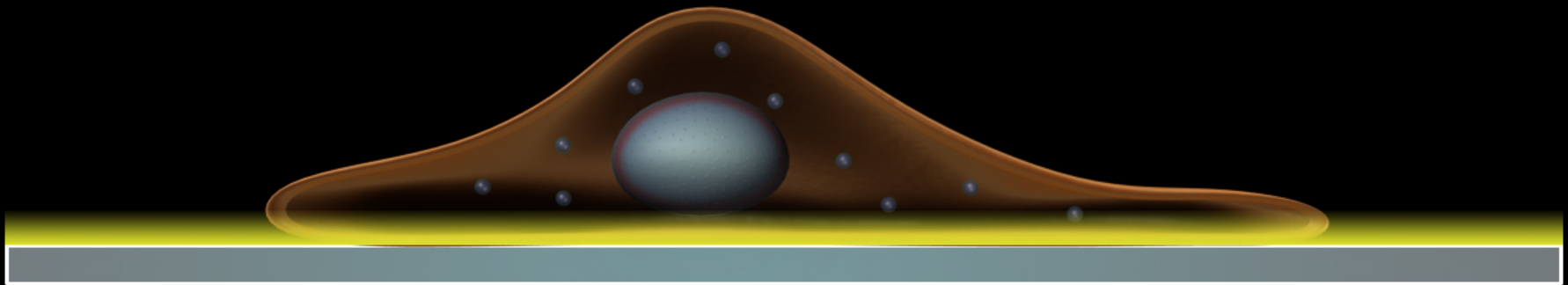
Zoom of PALM Image



PALM IMAGING OF FIXED CELLS EXPRESSING PA-FPS

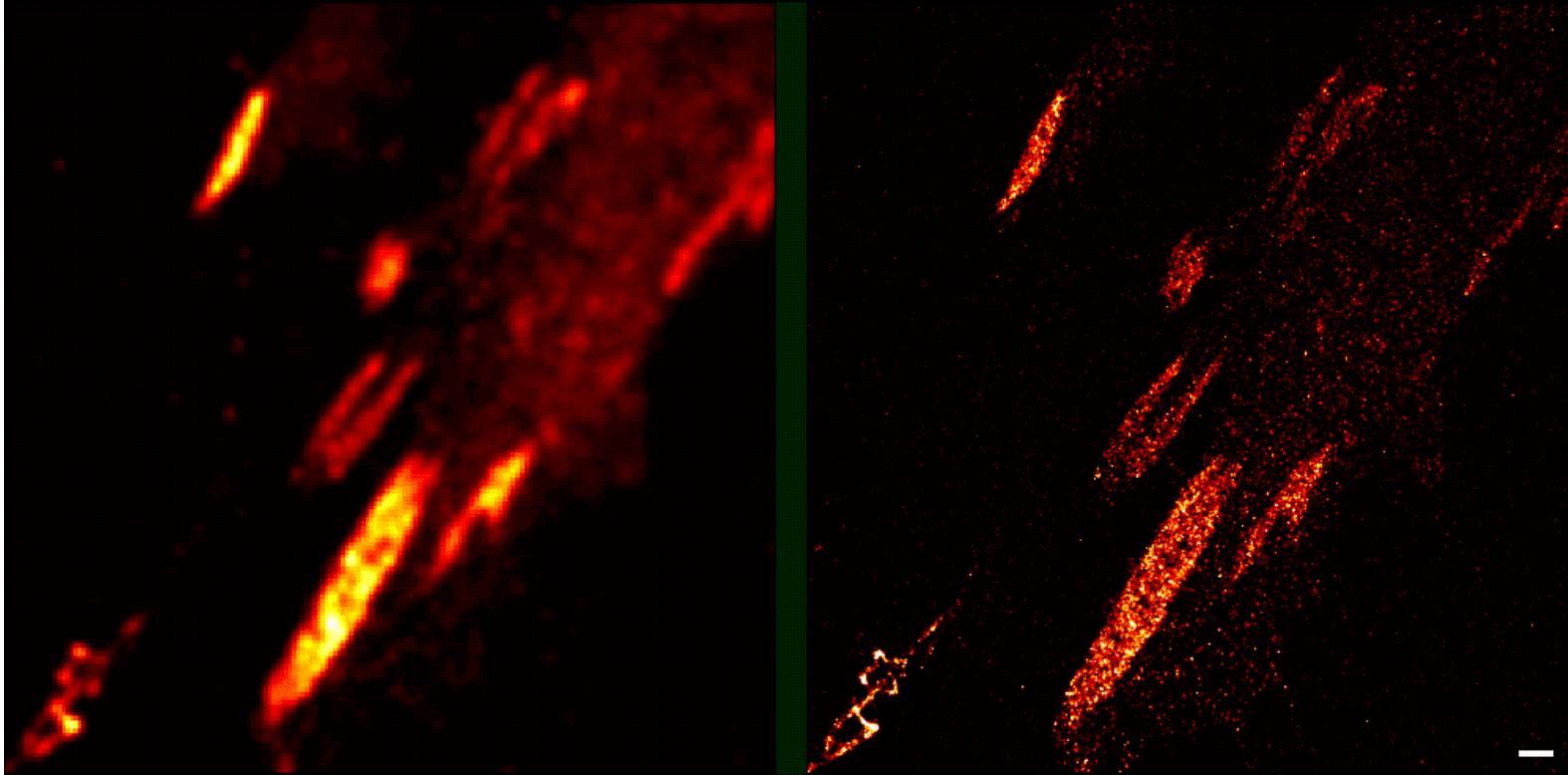


PALM IMAGING OF FIXED CELLS EXPRESSING PA-FPS



Total Internal Reflection microscopy
limits imaging to range $\leq 100\text{nm}$
distance from the coverslip

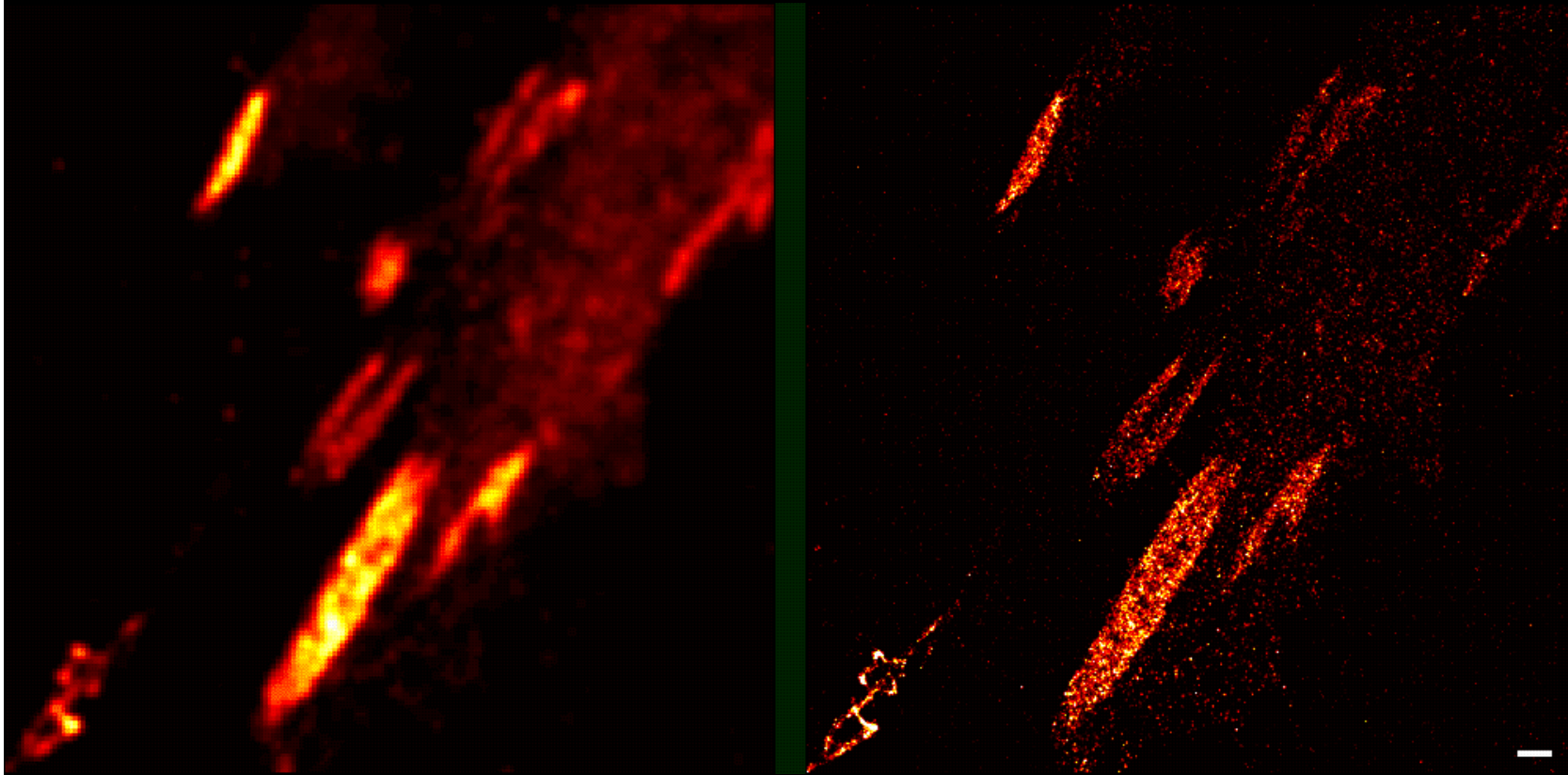
PALM Imaging of fixed cells expressing PA-FPs



1.0 μm

Focal adhesions
dEosFP-tagged vinculin

PALM Imaging of fixed cells expressing PA-FPs

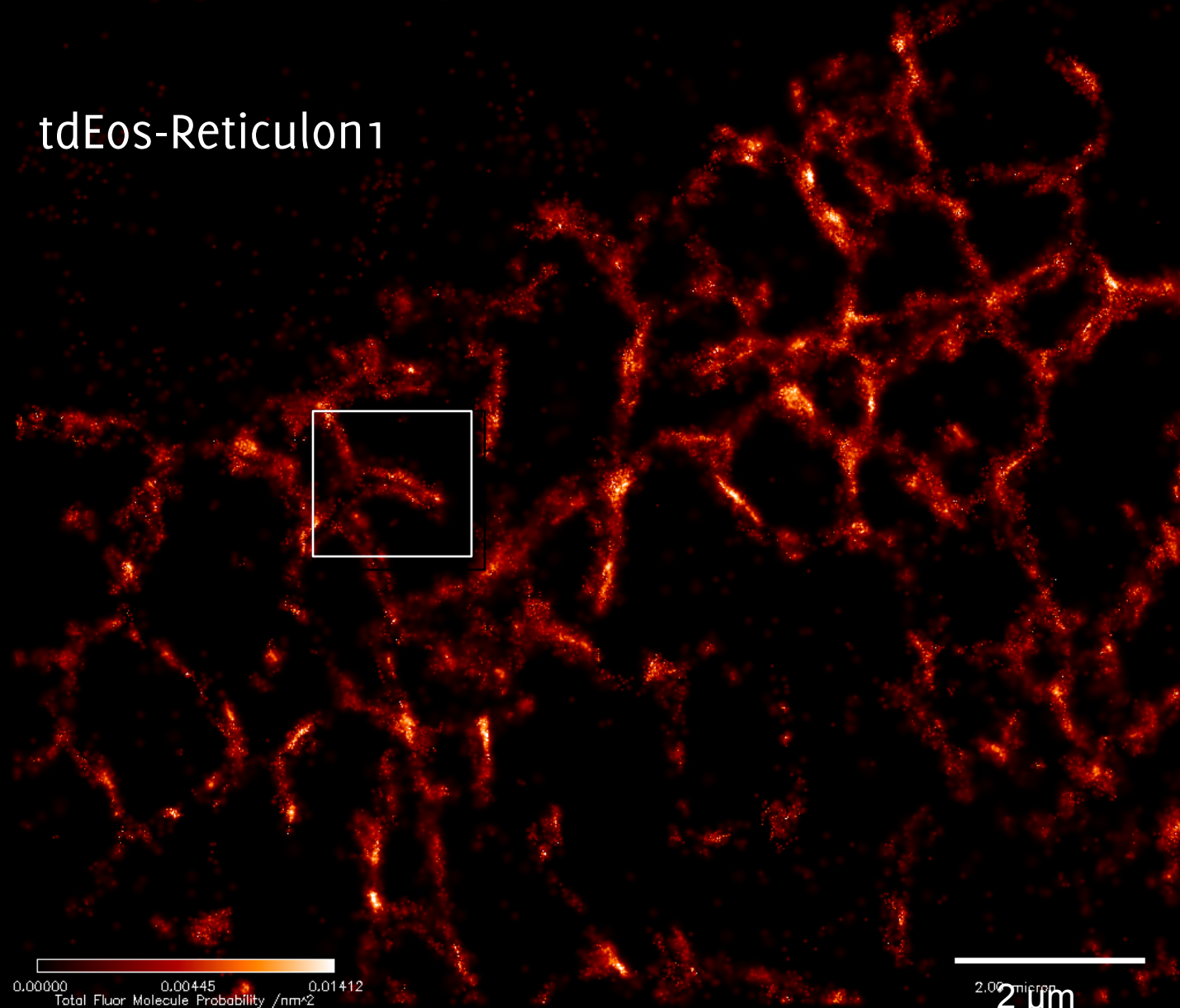


1.0 μm

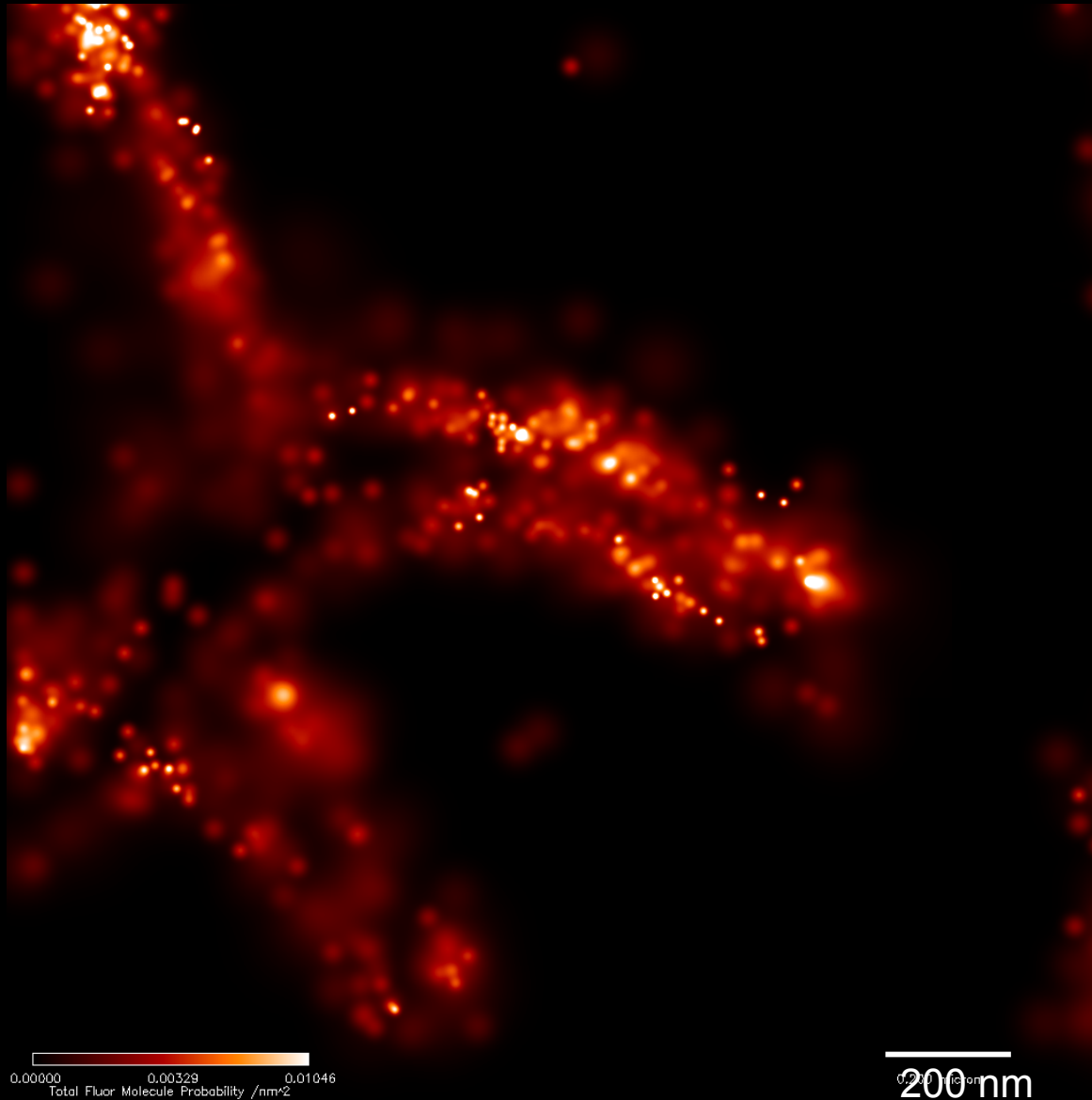
Focal adhesions, FoLu cell, dEosFP-tagged vinculin

PALM of Endoplasmic Reticulum

tdEos-Reticulon1

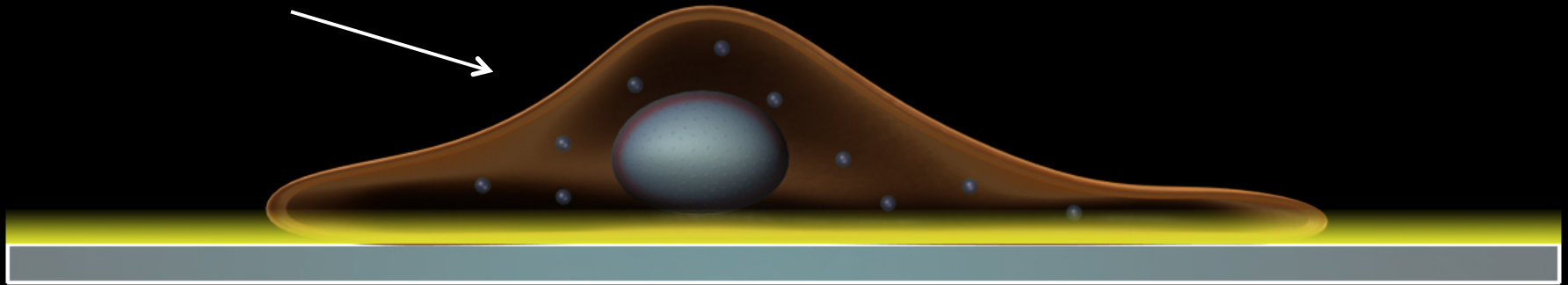


Zoom of PALM of ER

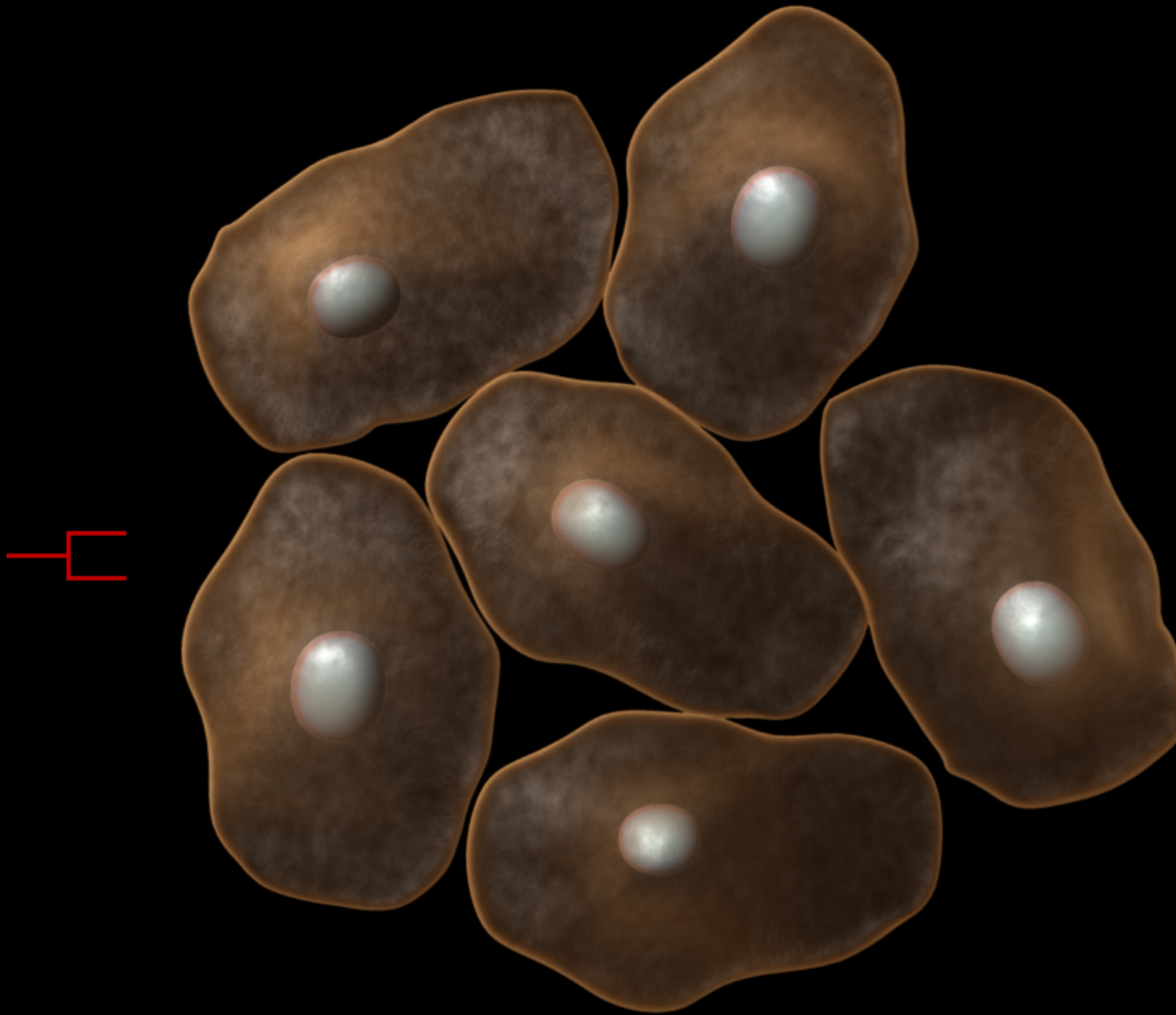


tdEos-Reticulon1

PALM of Intracellular Components



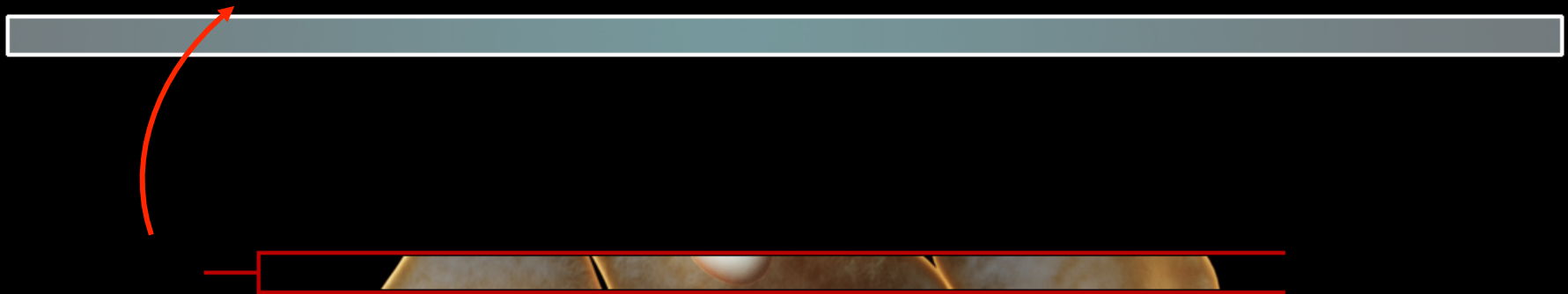
PALM of Intracellular Components



PALM of Intracellular Components



PALM of Intracellular Components

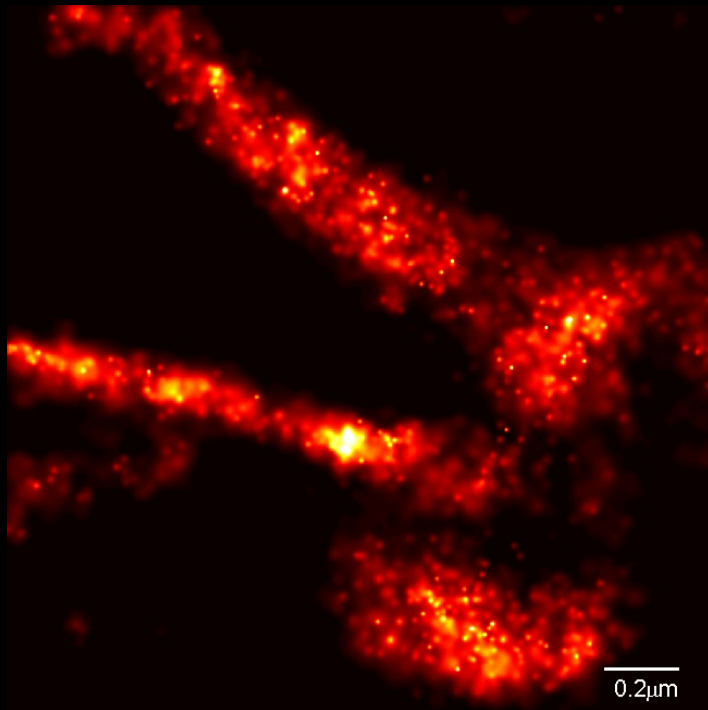


PALM of Intracellular Components

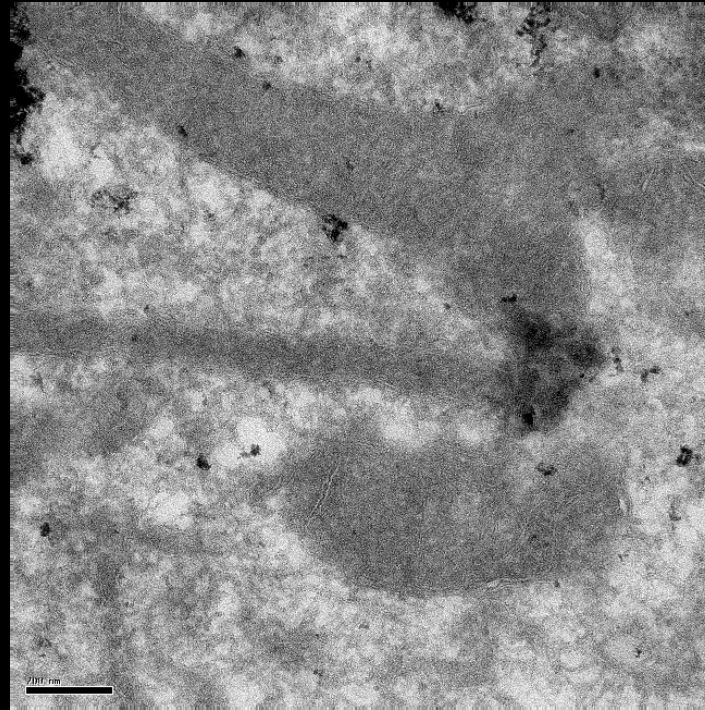


Correlative PALM / TEM

PALM



TEM

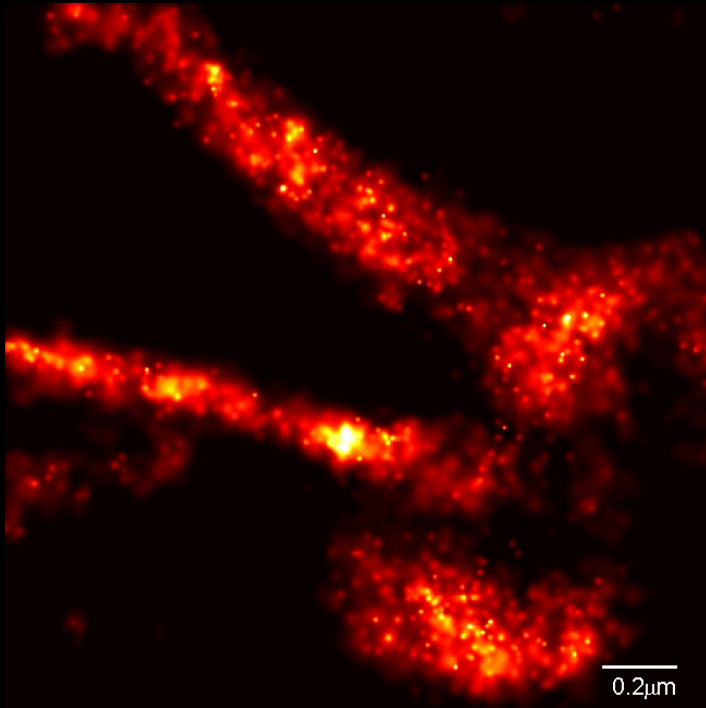


Mitochondrial matrix

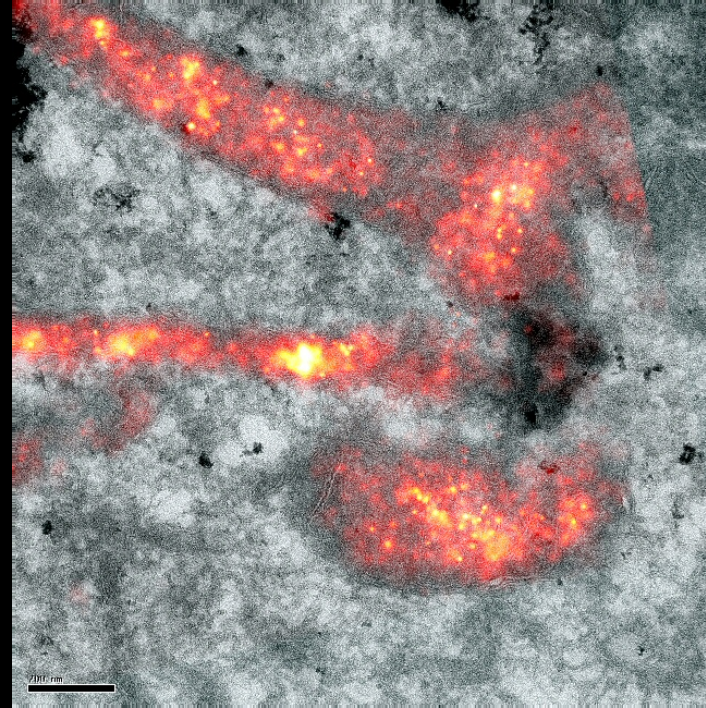
*dEosFP-tagged cytochrome-c oxidase
targeting sequence*

Correlative PALM / TEM

PALM



TEM



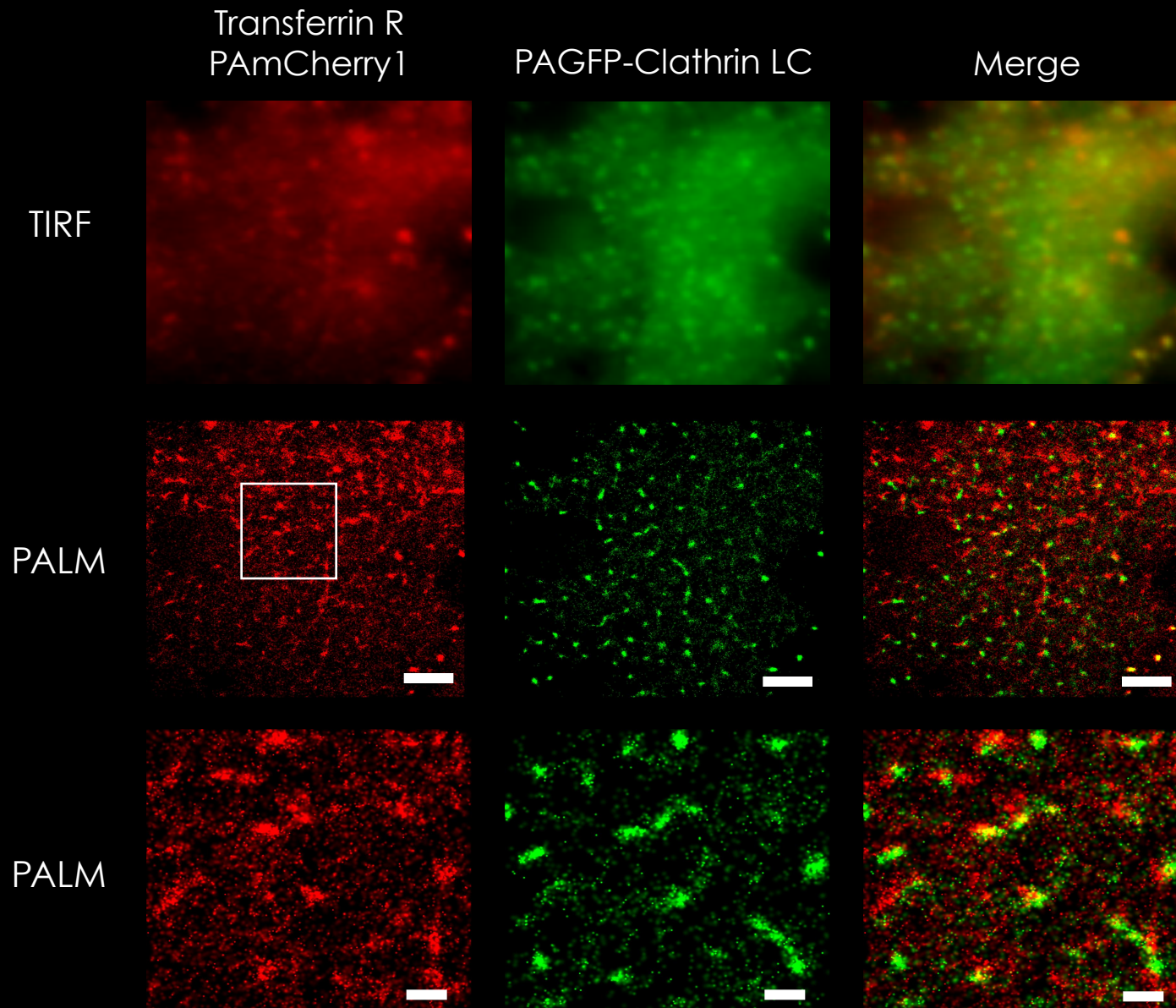
5500 molecules

A high density alternative to immunogold labeling!

PA-FP classes

PA-FP classes	λ_{ex} (nm)	λ_{em} (nm)	Brightness ^a	Oligomeric state	Contrast
Irreversible, off to on					
PAGFP	504	517	13750	monomer	70
PAmCherry1	564	595	8280	monomer	4000
Photoconversion, wavelength shift					
PS-CFP2	490	511	10810	monomer	>2000 ^b
Kaede	572	582	19900	tetramer	2000 ^c
KikGR	583	593	21200	tetramer	>2000 ^c
mKikGR	580	591	17650	tetramer	NR
Dendra2	553	573	19250	monomer	300
EosFP	571	581	22600	tetramer	NR
mEos2	573	584	30300	monomer	NR
Reversible photoactivation					
Dronpa	503	518	80800	monomer	NR
rsFastlime	496	518	30100	monomer	70 ^d
Padron	396	522	27500	monomer	140 ^d
KFP1	580	600	4100	tetramer	>30
rsCherry	572	610	1600	monomer	7 ^d
rsCherryRev	572	608	420	monomer	20 ^d

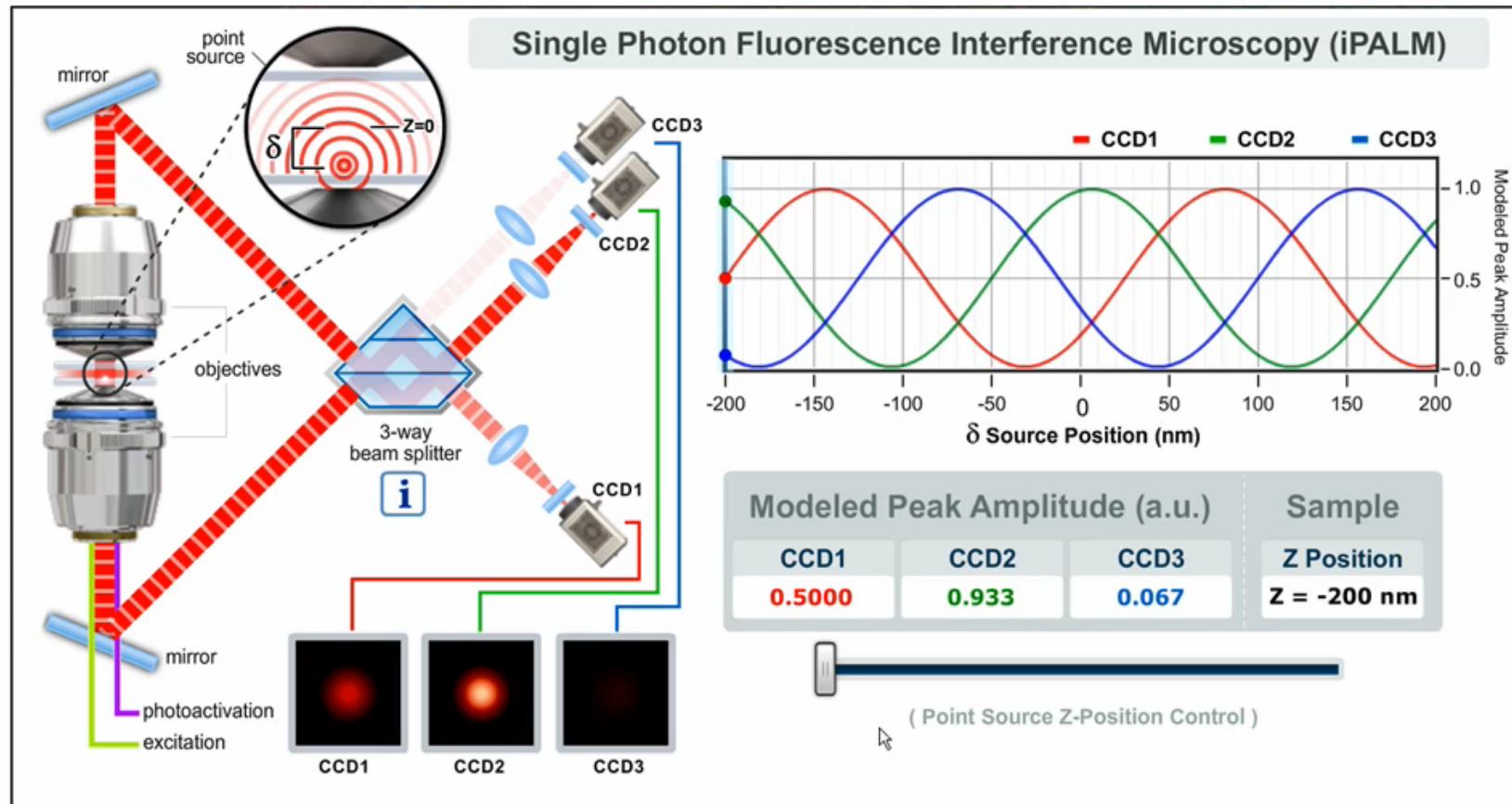
TWO-COLOR (SIMULTANEOUS) PALM



3D Interferometric PALM (iPALM)

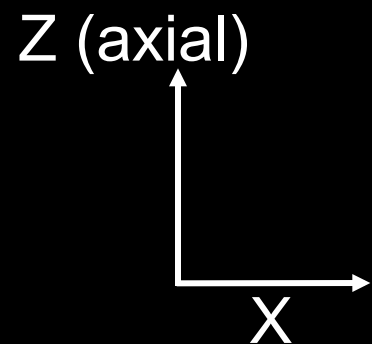
- Full 3D imaging
- 2- nm horizontal PALM & 10-nm vertical resolution interferometry size scale of a protein complex.
- Interferometry + PALM = **iPALM**
- Works with genetically expressed labels.
- No sample compromise of immuno-label.

The Principle of iPALM

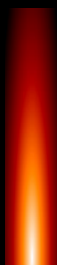


Resolution Comparison

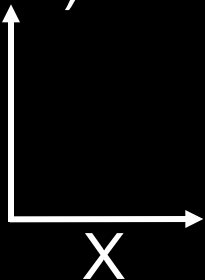
Confocal



PALM



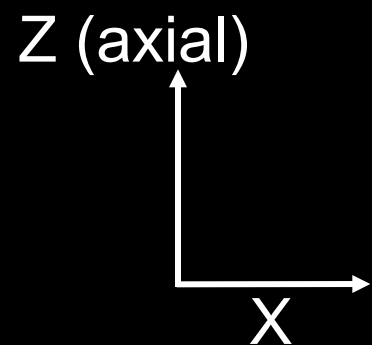
Z (axial)



iPALM

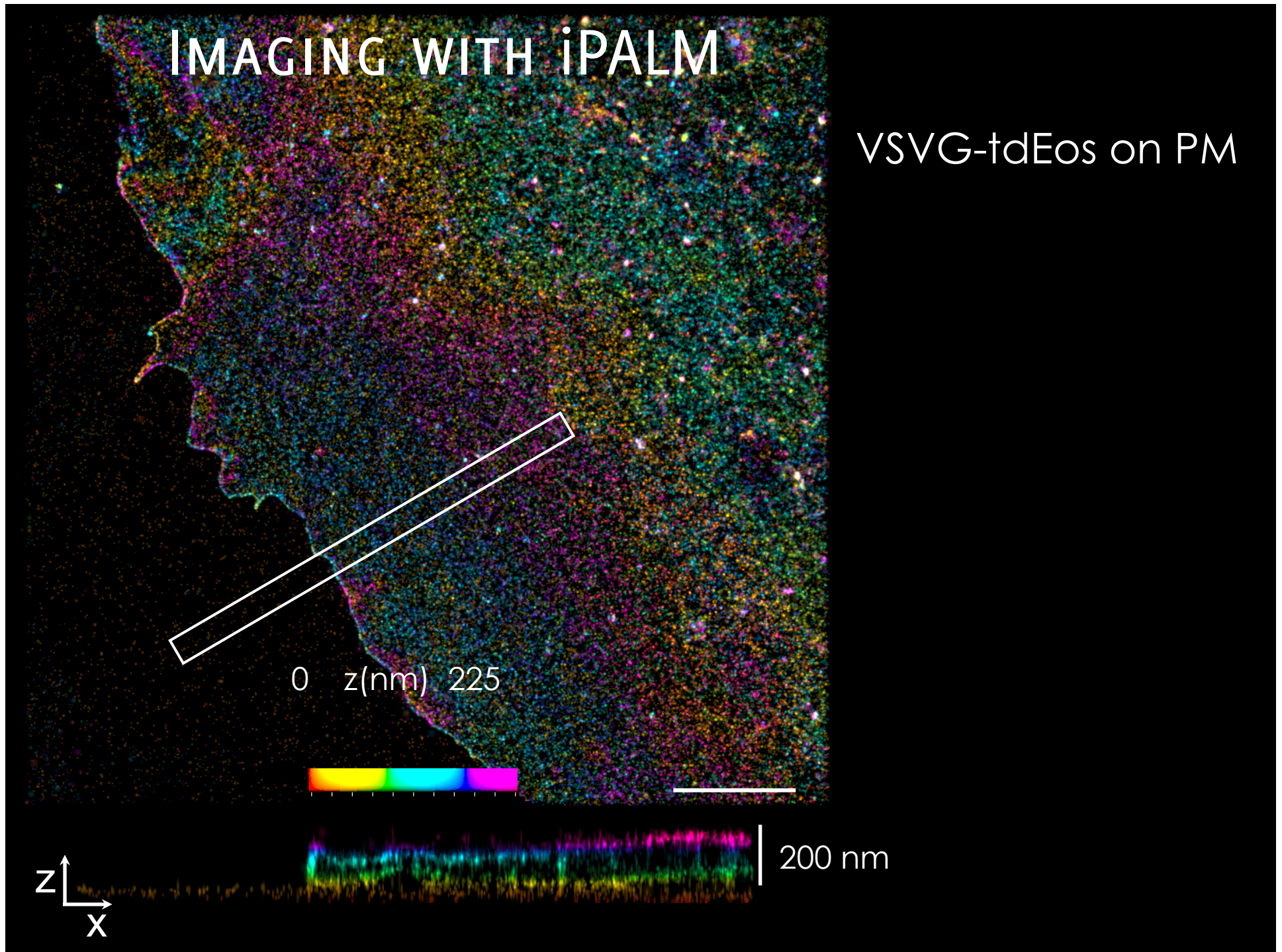
10 nm axial

20 nm x,y



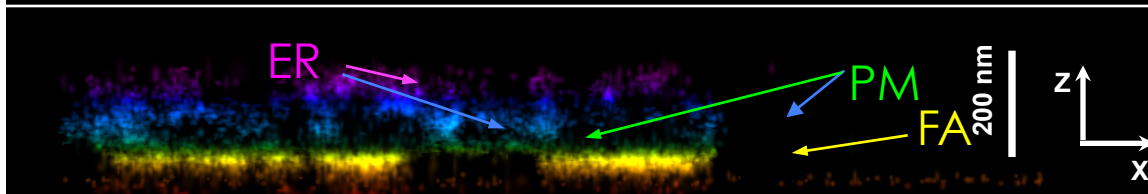
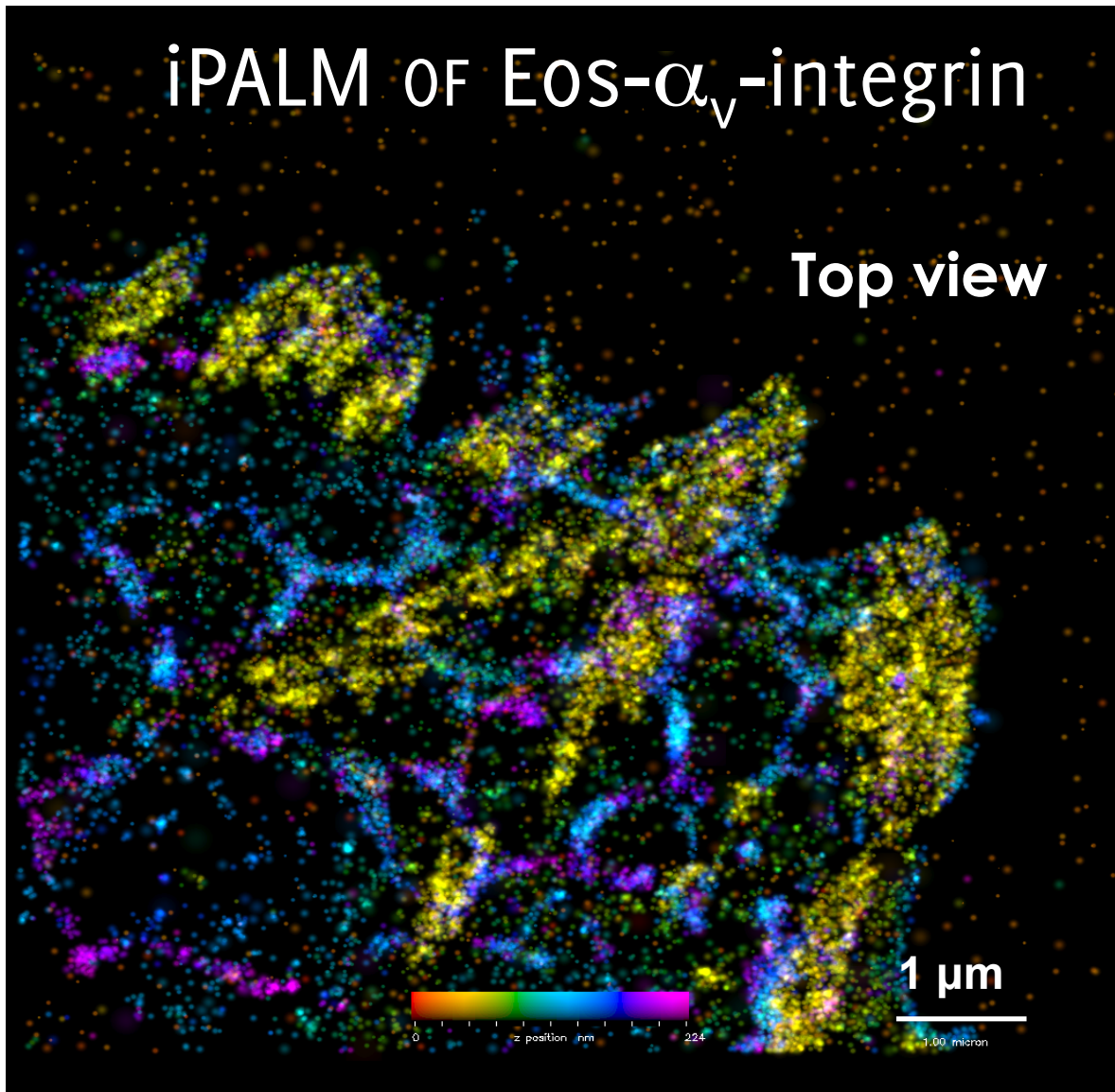
IMAGING WITH iPALM

VSVG-tdEos on PM



iPALM of Eos- α_v -integrin

Top view

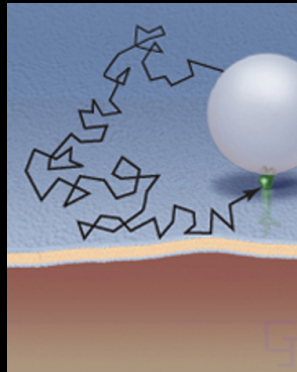


Side view

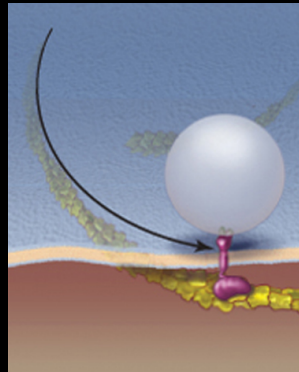
iPALM OF EOS- α_v -integrin



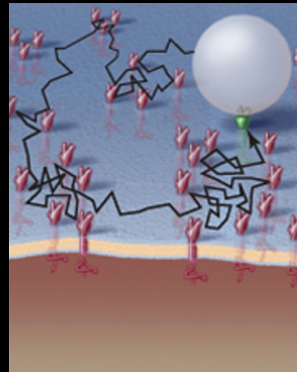
Tracking single molecules with PA-FP



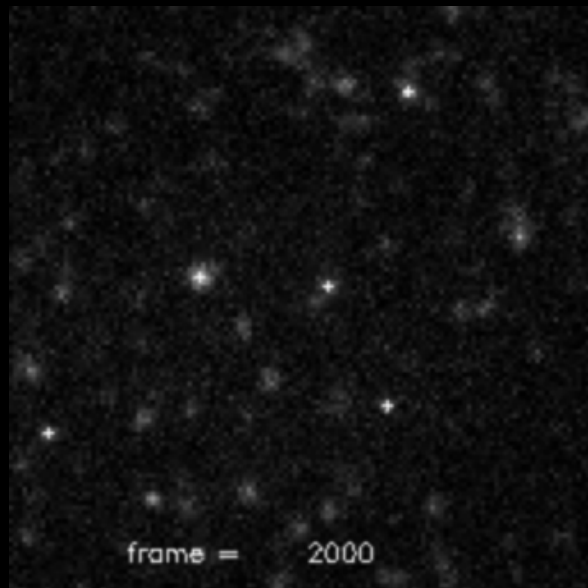
free diffusion



active transport



confinement

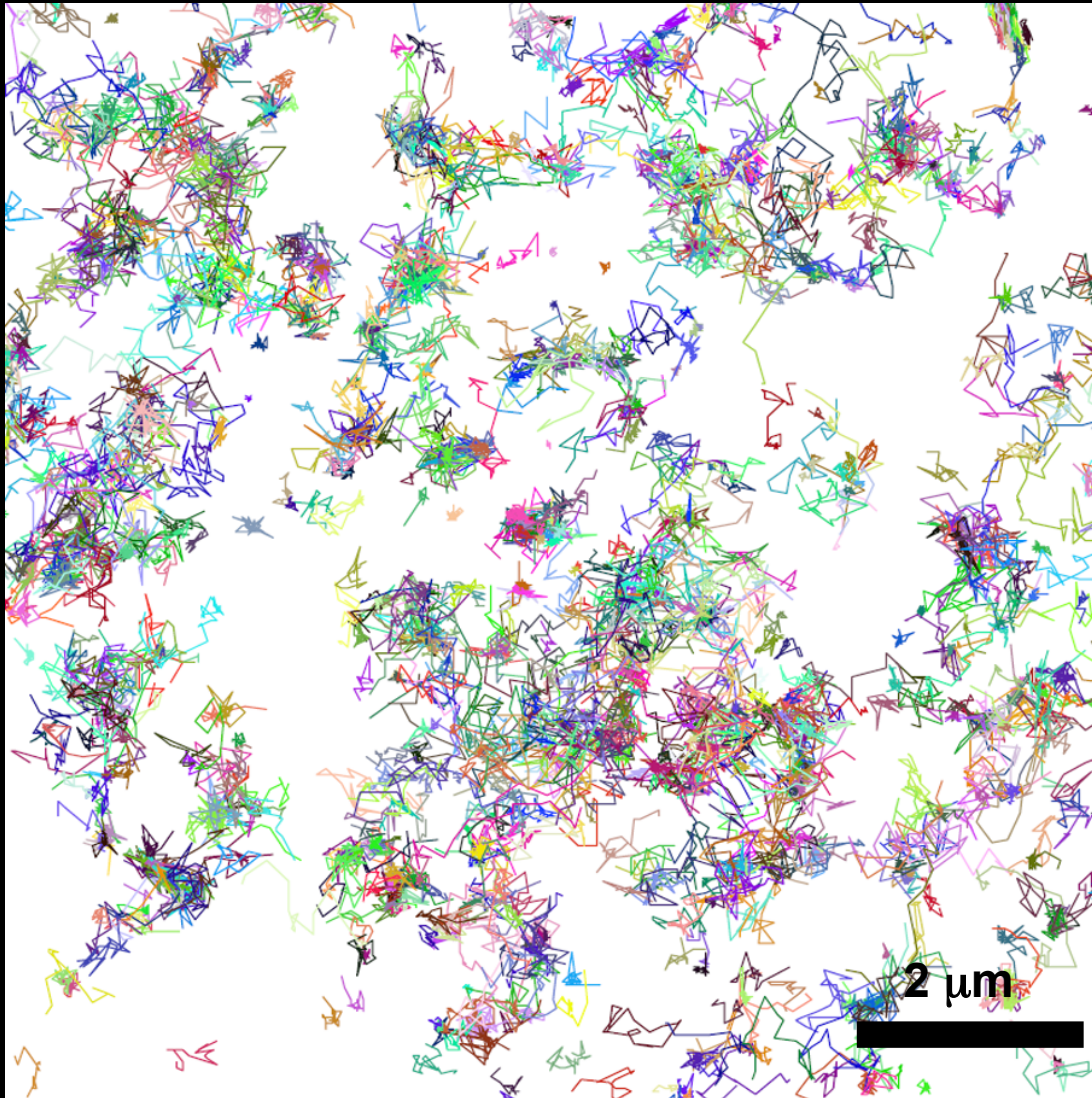


20 frames/sec, localizing to 25 nm

VSVG-EosFP

DYNAMICS: SINGLE PARTICLE TRACKING

SINGLE PARTICLE TRACKING WITH PALM

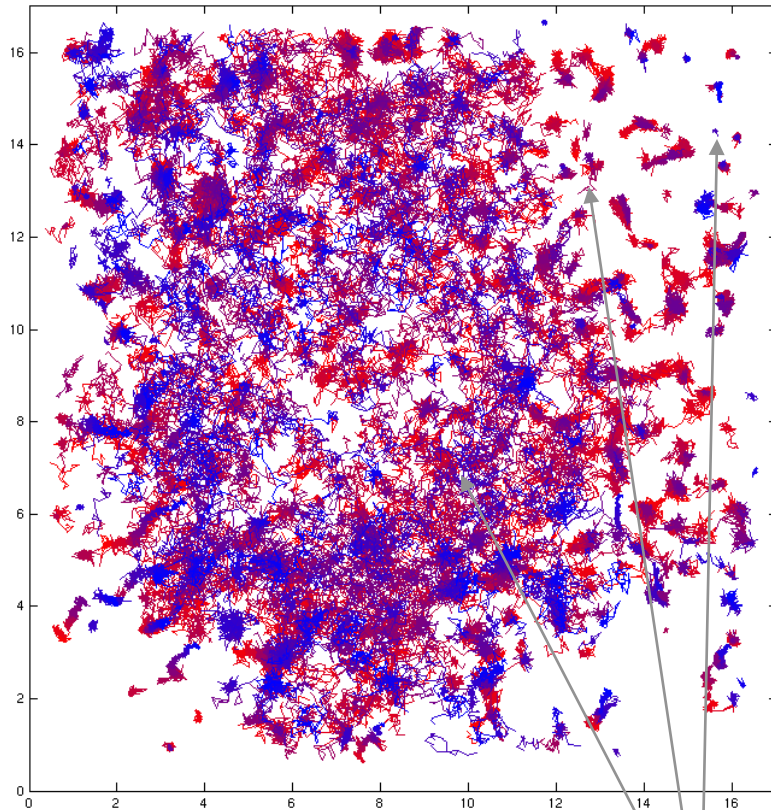


VSVG-EosFP
Single molecule tracks

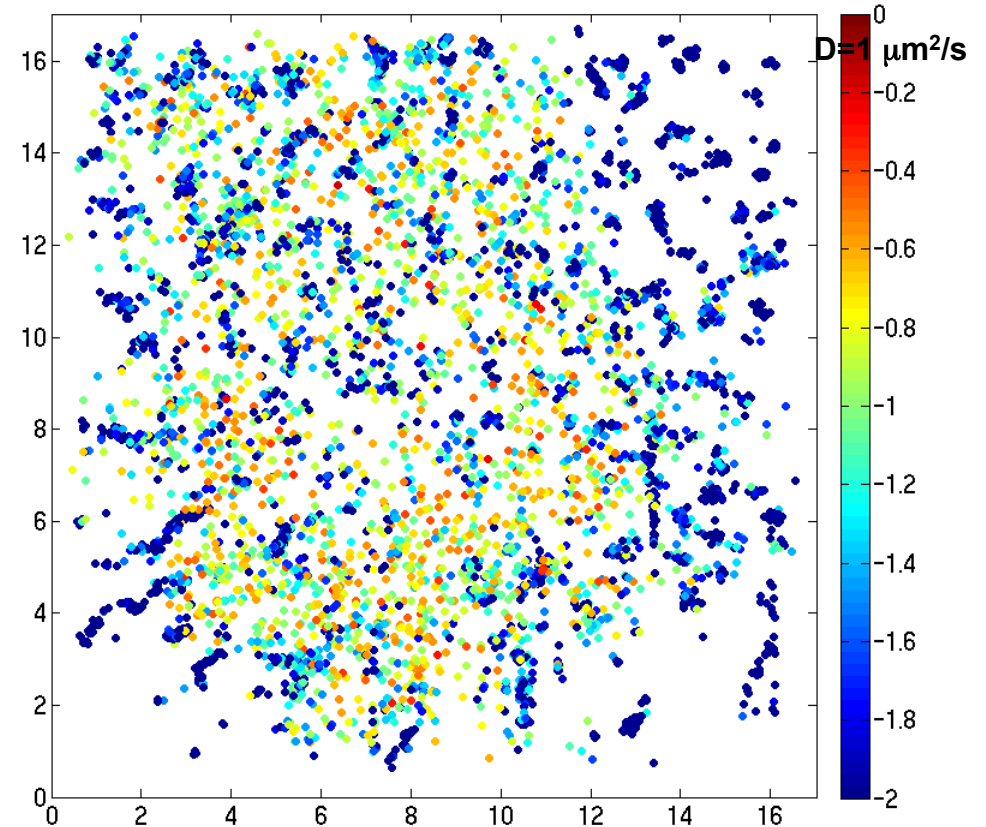
High-density information on dynamics

DIFFUSION MAPS FROM SINGLE MOLECULE TRACKING

SINGLE MOLECULE TRACKS



DIFFUSION COEFFICIENT MAPS



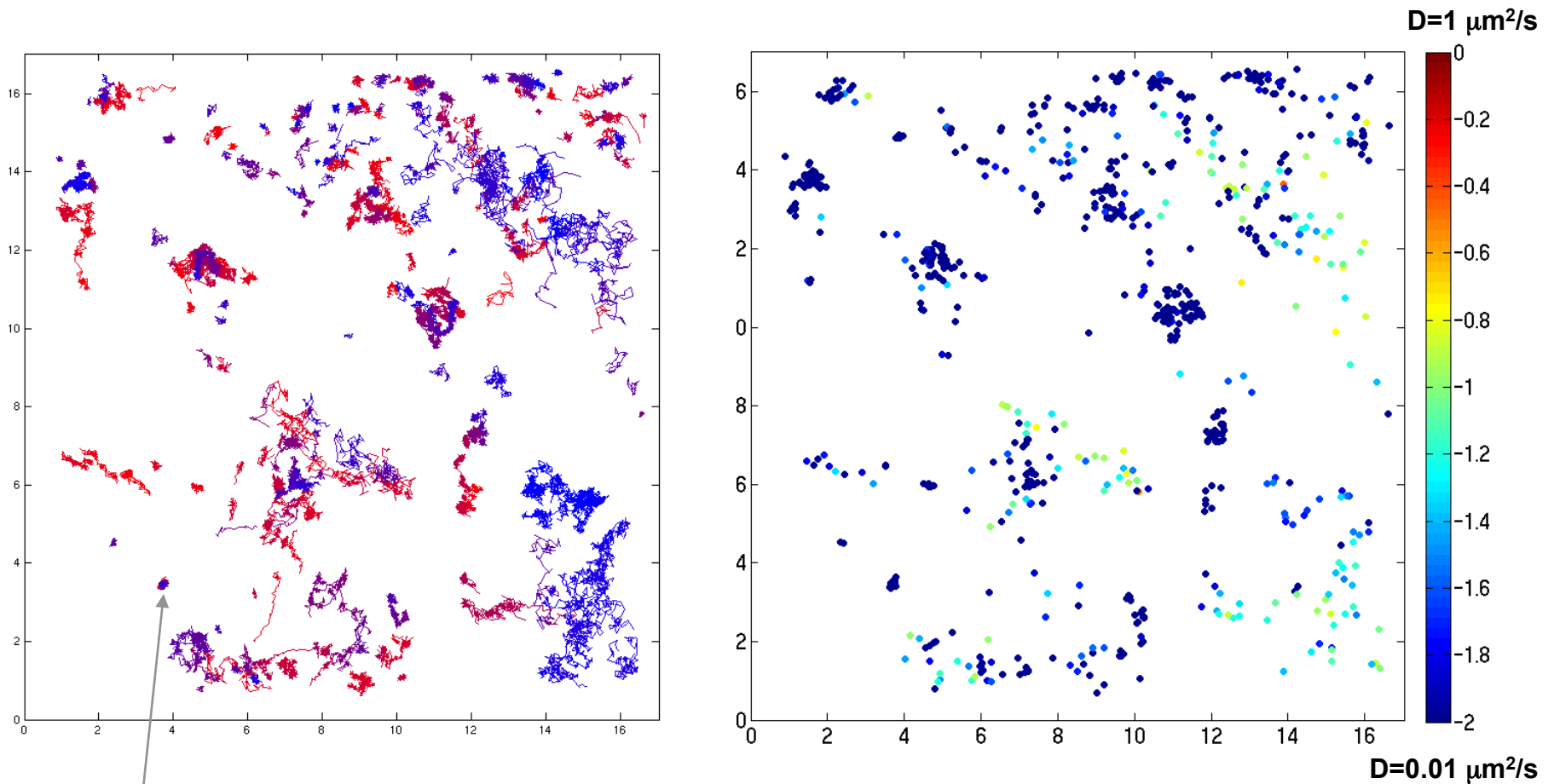
$D=0.01 \mu\text{m}^2/\text{s}$

Gold fiducial

VSVG-EosFP

Each track is analyzed to yield a diffusion coefficient
Color scale is log, immobile molecules binned

DIFFUSION MAPS FROM SINGLE MOLECULE TRACKING



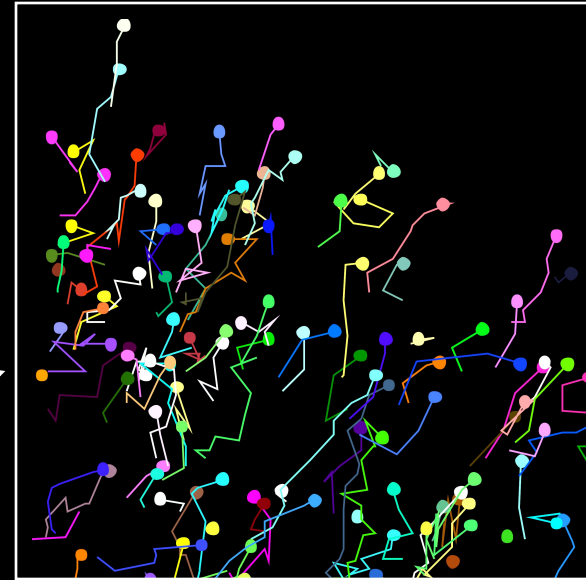
Gold fiducial

EosFP-Gag

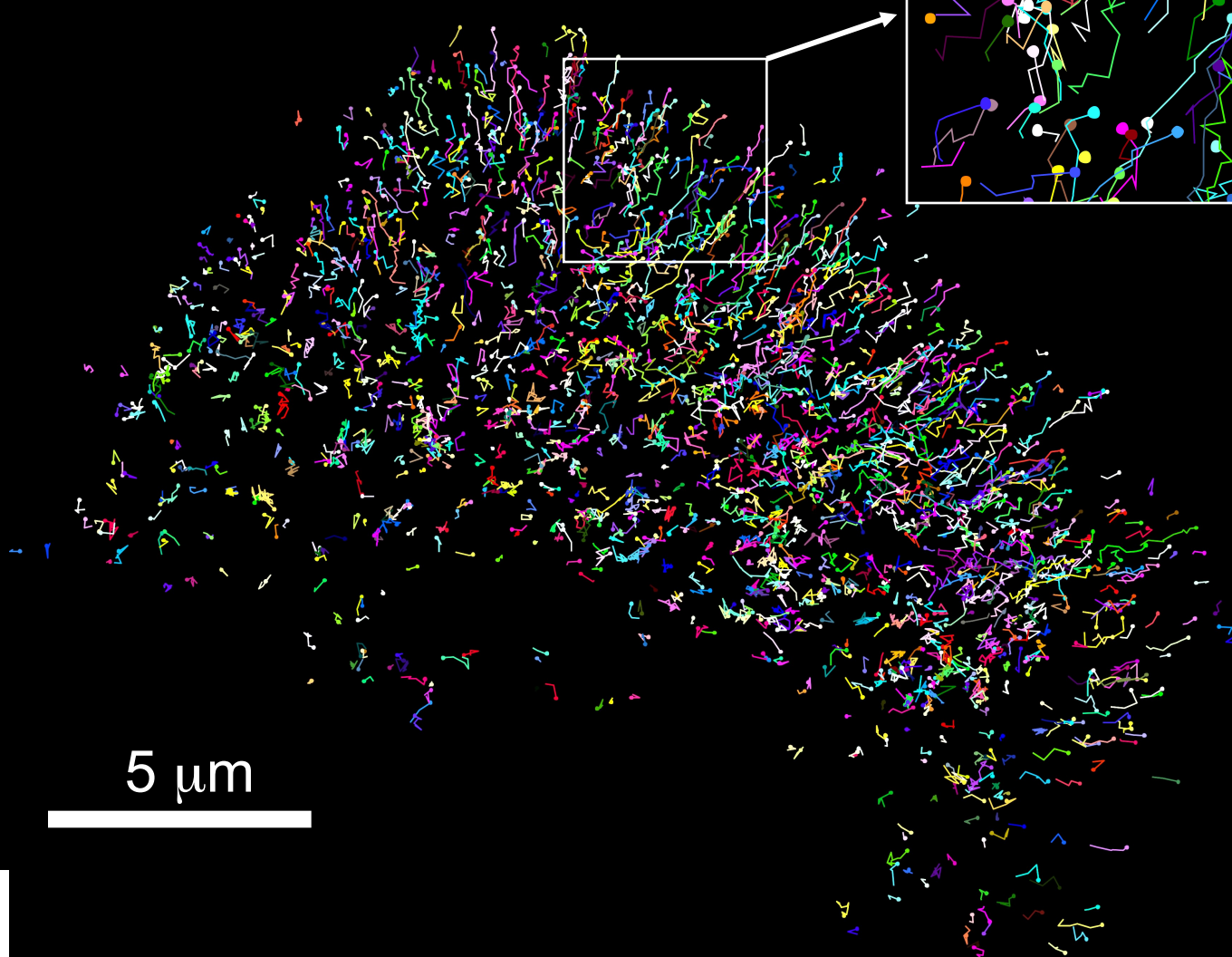
Each track is analyzed to yield a diffusion coefficient
Color scale is log, immobile molecules binned

Spt-Palm of Single Actin Filaments

sptPALM tracks
of actin-Eos molecules

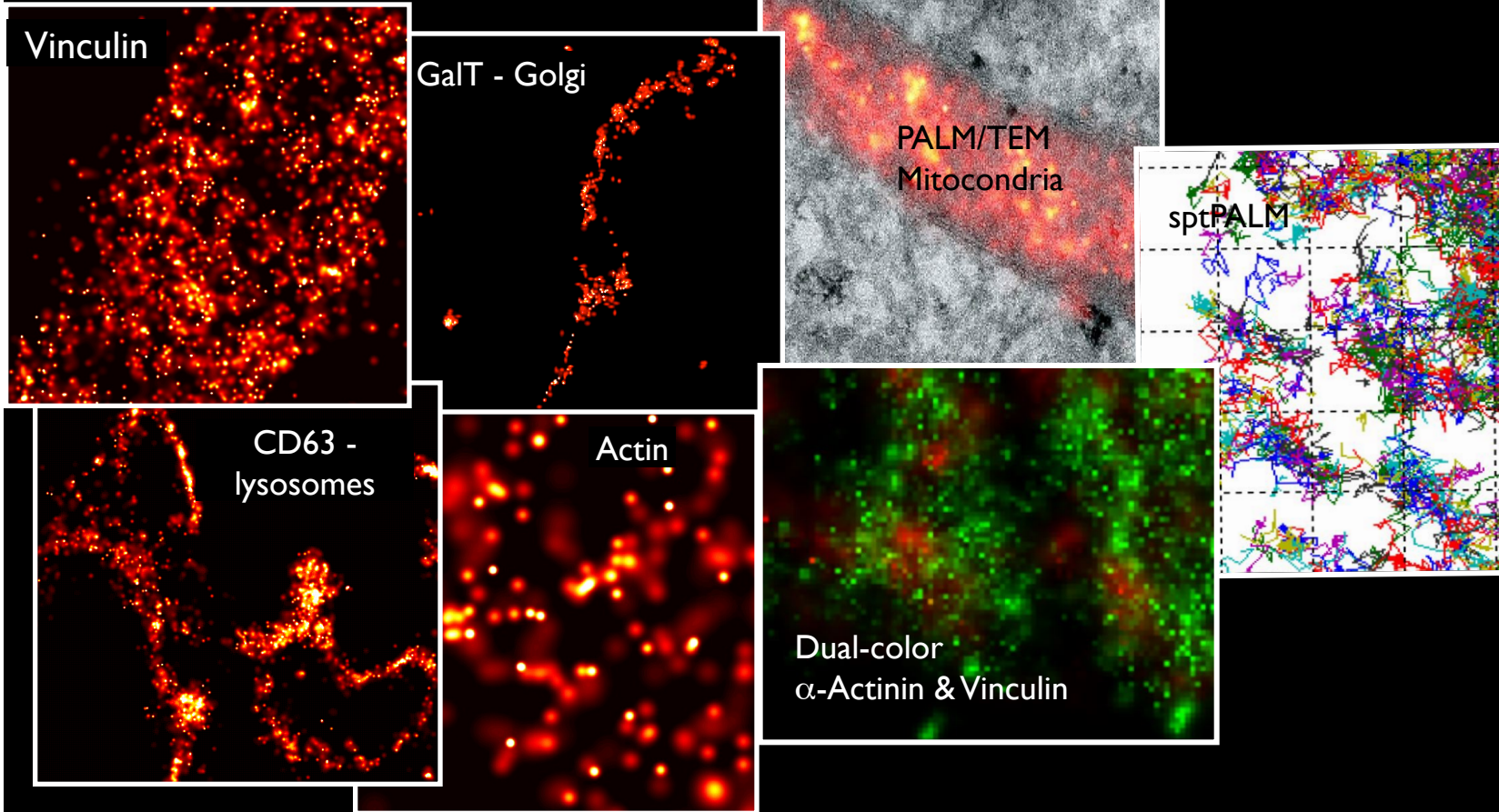


5 μm



SUPERRESOLUTION POSSIBILITIES

PALM



Protein-specific structural imaging at near-molecular resolution in fixed or live cells