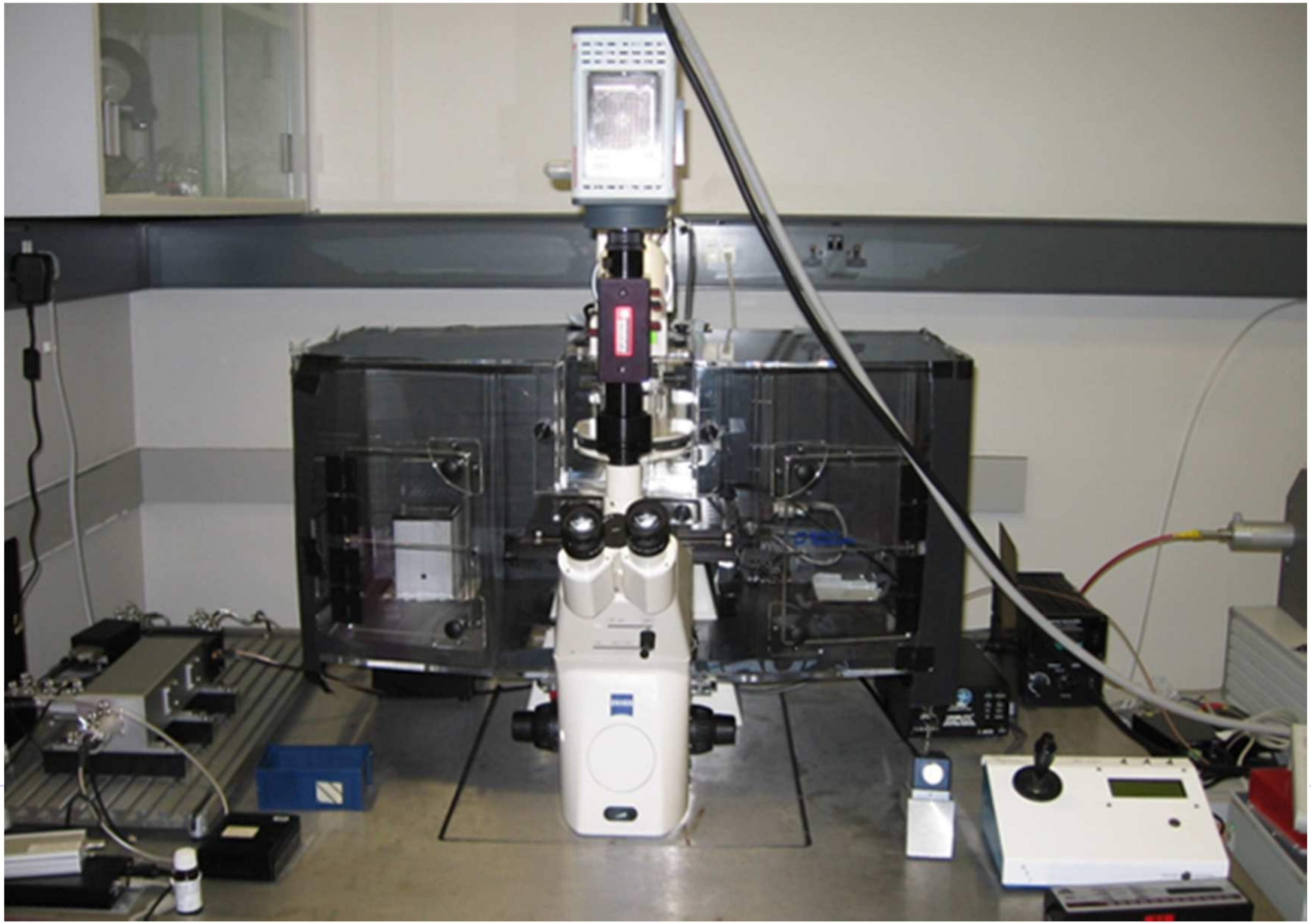


Special fluorescence techniques

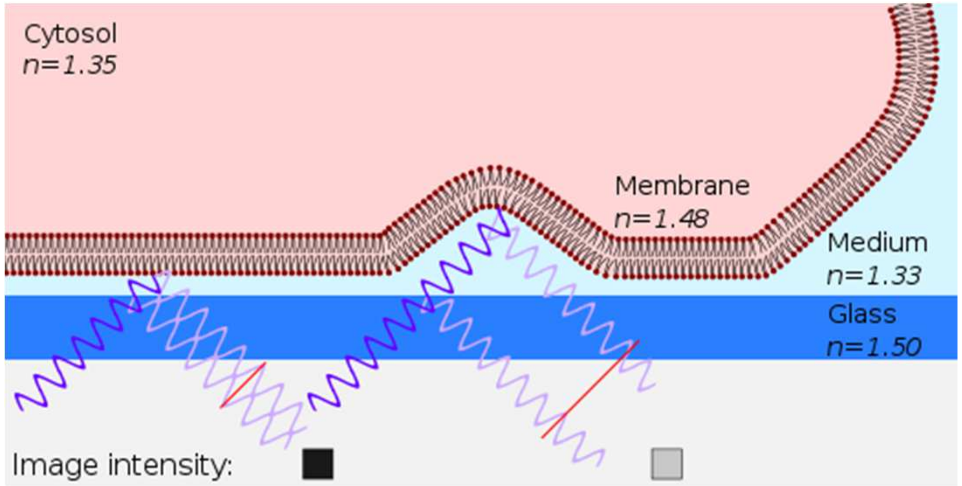
Imperial College London

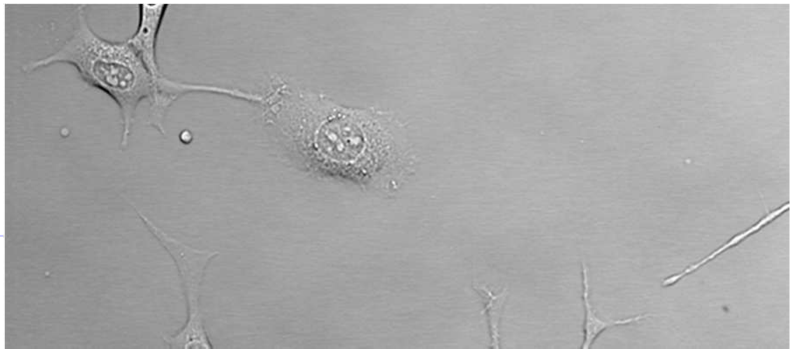
FILM

- IRM: Interference Reflection Microscopy
- STED: Stimulated Emission Depletion
- TIRF: Total Internal Reflection Fluorescence
- PALM: Photo-Activated Localization Microscopy
- STORM: Stochastic Optical Reconstruction Microscopy

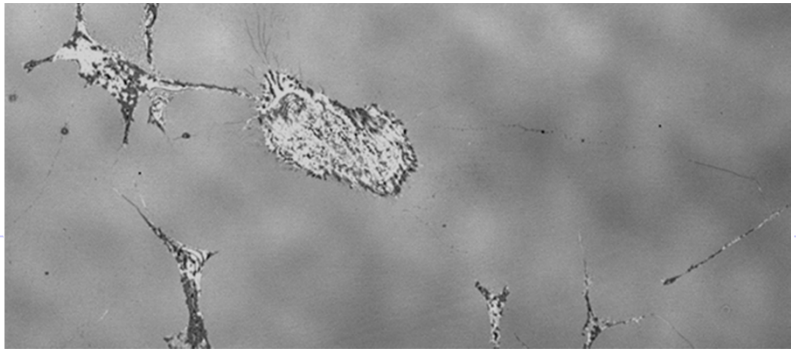
Interference Reflection Microscopy (IRM)

- Not really fluorescence technique, works on unlabelled samples
- Uses polarised laser light, best with confocal setup



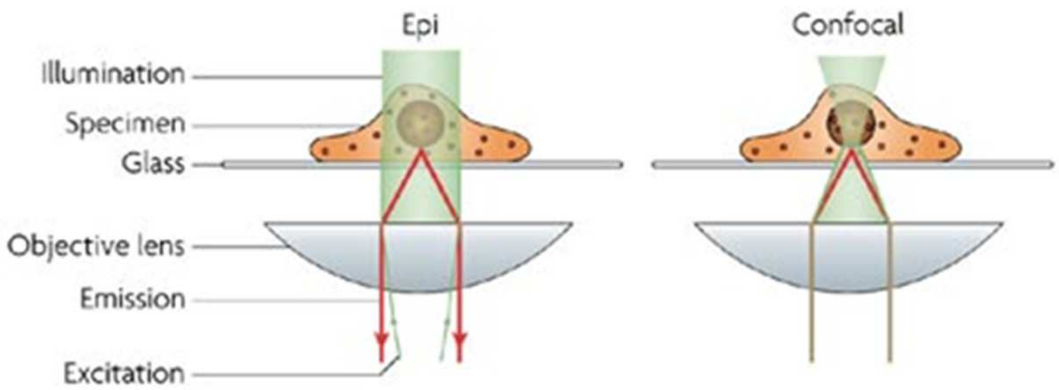


DIC

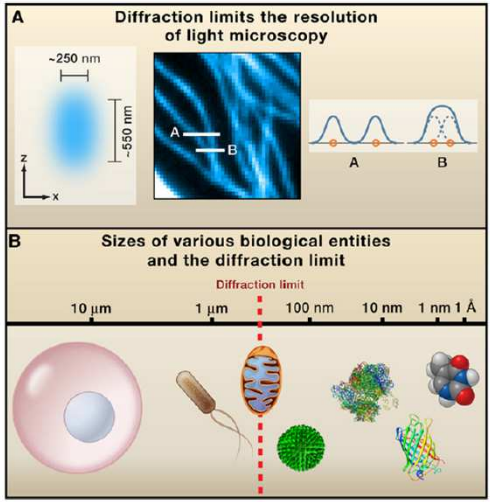


IRM

- Fluorescence microscopy - column of illumination and out-of-focus blurring
- Confocal microscopy employs a pair of pinhole apertures strategically placed in conjugate planes near the illumination source and detector to produce thin optical sections devoid of background fluorescence.
- Multiphoton excitation microscopy goes a step further by restricting the illuminated specimen area to an ellipsoid having micron or sub-micron dimensions.



Breaking the Diffraction Limit
(Improving resolution)



The theoretical XY resolution of a light microscope is given by the wavelength of the light, which is limited by diffraction to be no less than approximately half the wavelength of the light. (approx 250nm). Minimum optical section thickness is approximately 500-600nm

- STED: Stimulated Emission Depletion
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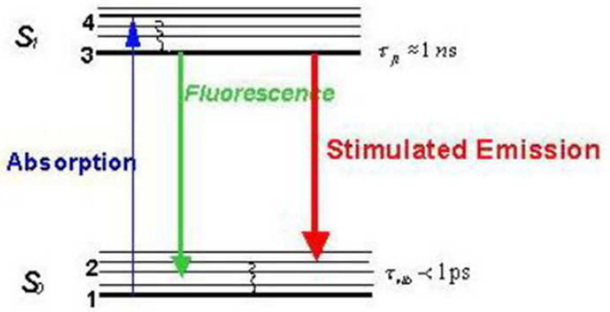
Stimulated Emission Depletion Microscopy

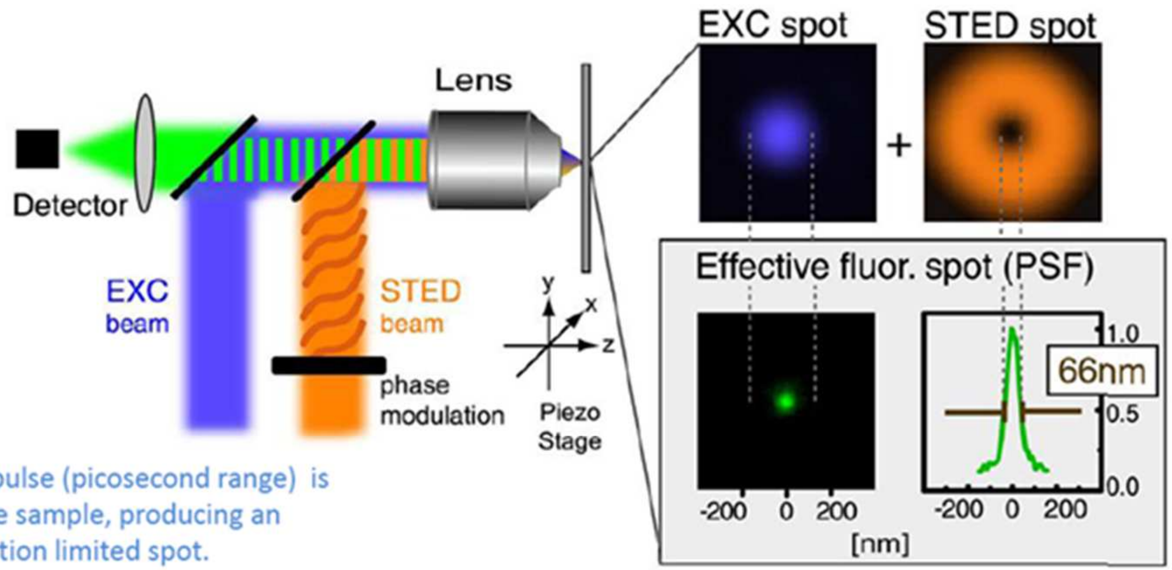
One way to sharpen the fluorescence focal spot is to selectively inhibit the fluorescence at its outer part.

A phenomenon that stops fluorescence is stimulated emission. If the nearby fluorescent objects is saturated by stimulated emission, one can break diffraction limit.

Scanning with a smaller fluorescent spot signifies increased spatial resolution



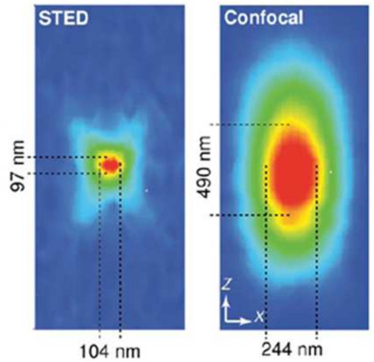
STED Setup



The excitation pulse (picosecond range) is focused into the sample, producing an ordinary diffraction limited spot.

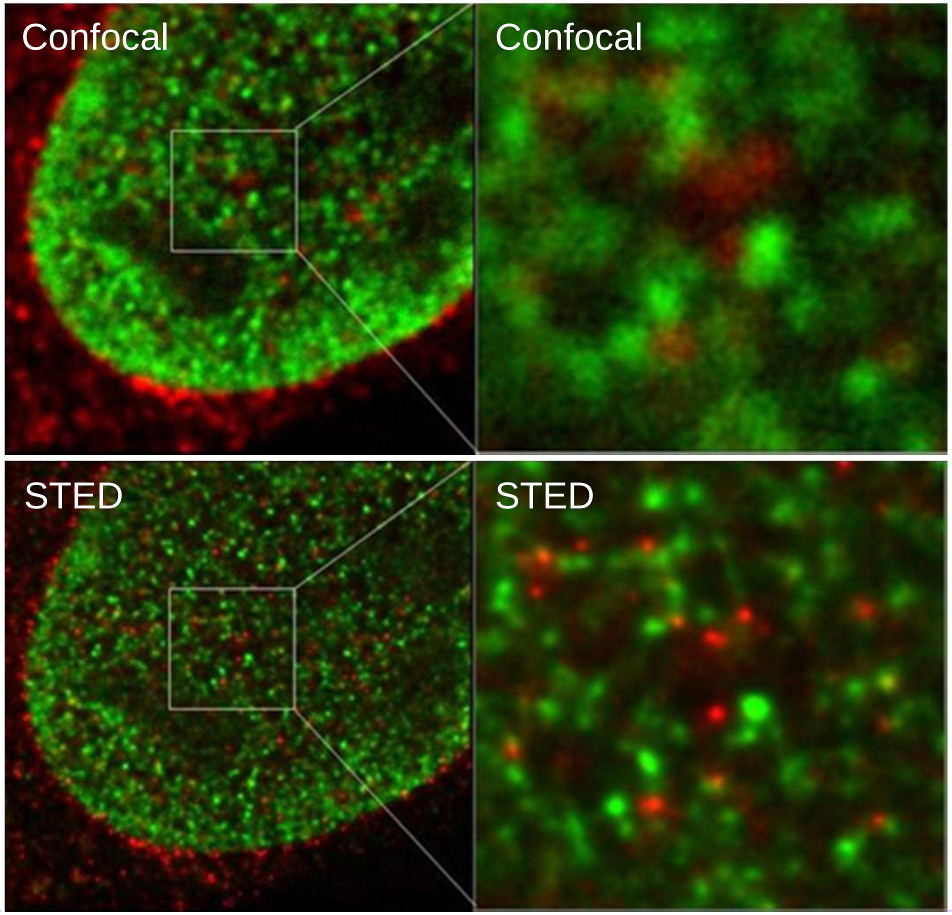
The excitation pulse is immediately followed by a depletion pulse which is red shifted.

By spatially arranging the STED pulse in a doughnut mode, only the molecules at the periphery of the spot are ideally quenched

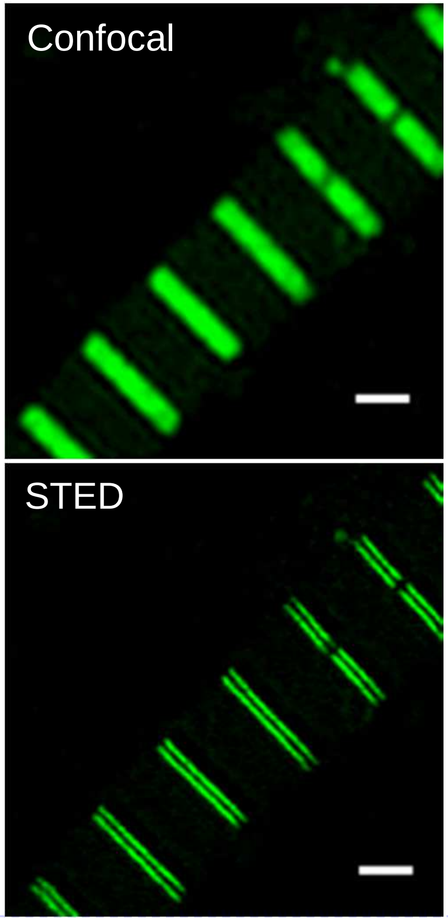


Imperial College London

Stimulated Emission Depletion Microscopy (STED)

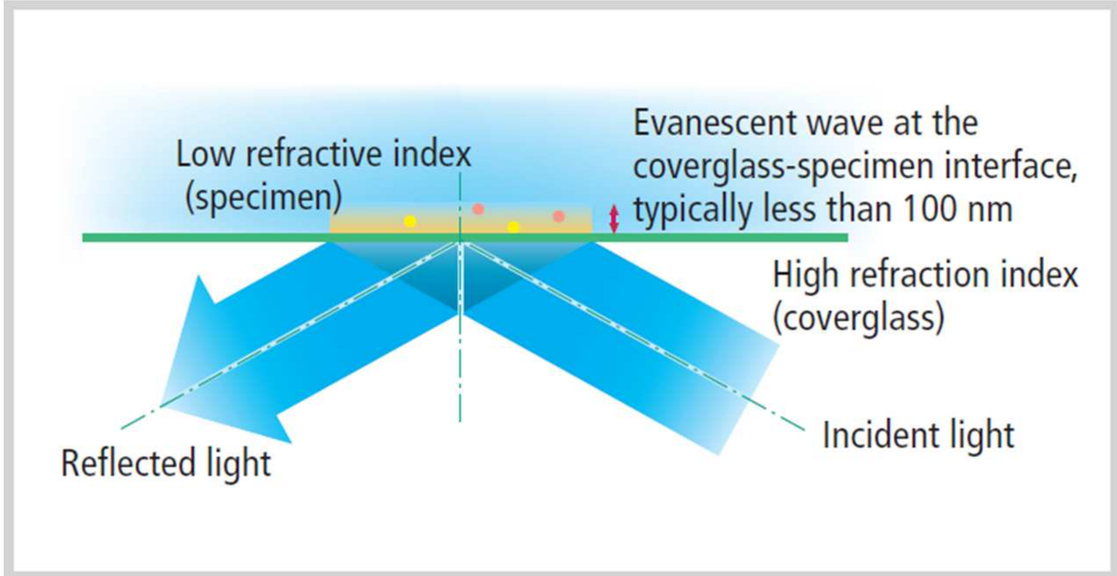


Nuclear structures visualized with Chromeo 494 (green) and Atto 647N (red)
Courtesy of Dr. L. Schermelleh, LMU Biozentrum, Munich



Rat myofibril sarcomeres stained with ATTO 647N
scalebar 1 µm.
Courtesy of Dr. E. Ehler, Kings College, London, UK

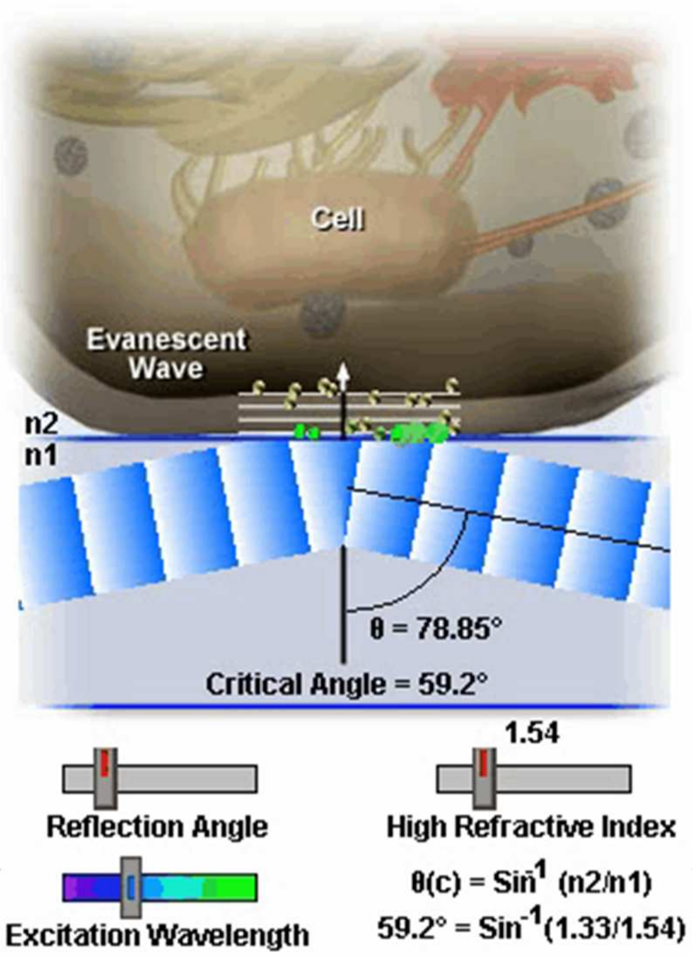
Total Internal Reflection Fluorescence Microscopy (TIRFM)

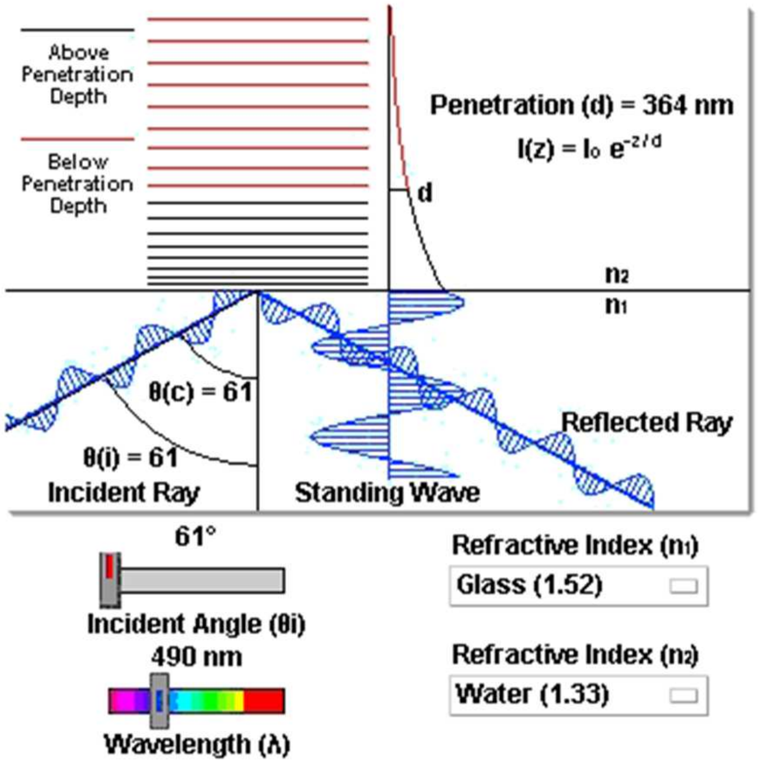


Principle

- Incident light angle greater than critical angle → light is reflected
- Generates a very thin electromagnetic field : "an evanescent wave "
- Penetration depth typically less than 100nm
- Intensity of wave decays exponentially with increasing distance from the surface

Total Internal Reflection Fluorescence Microscopy (TIRFM)

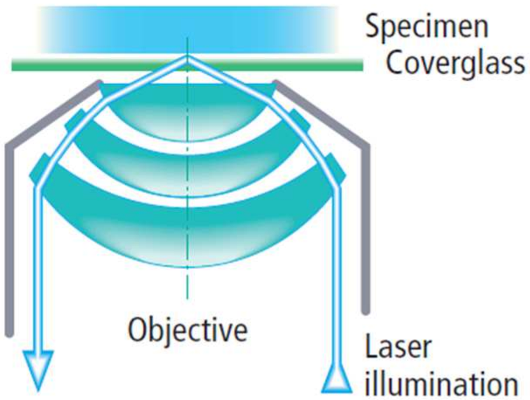




Total Internal Reflection Fluorescence Microscopy (TIRFM)

TIRFM Requirements

- High Na Objective lens (>1.4)
- Fast high sensitivity camera
- Different refractive indices (aqueous mounting media)



Advantages

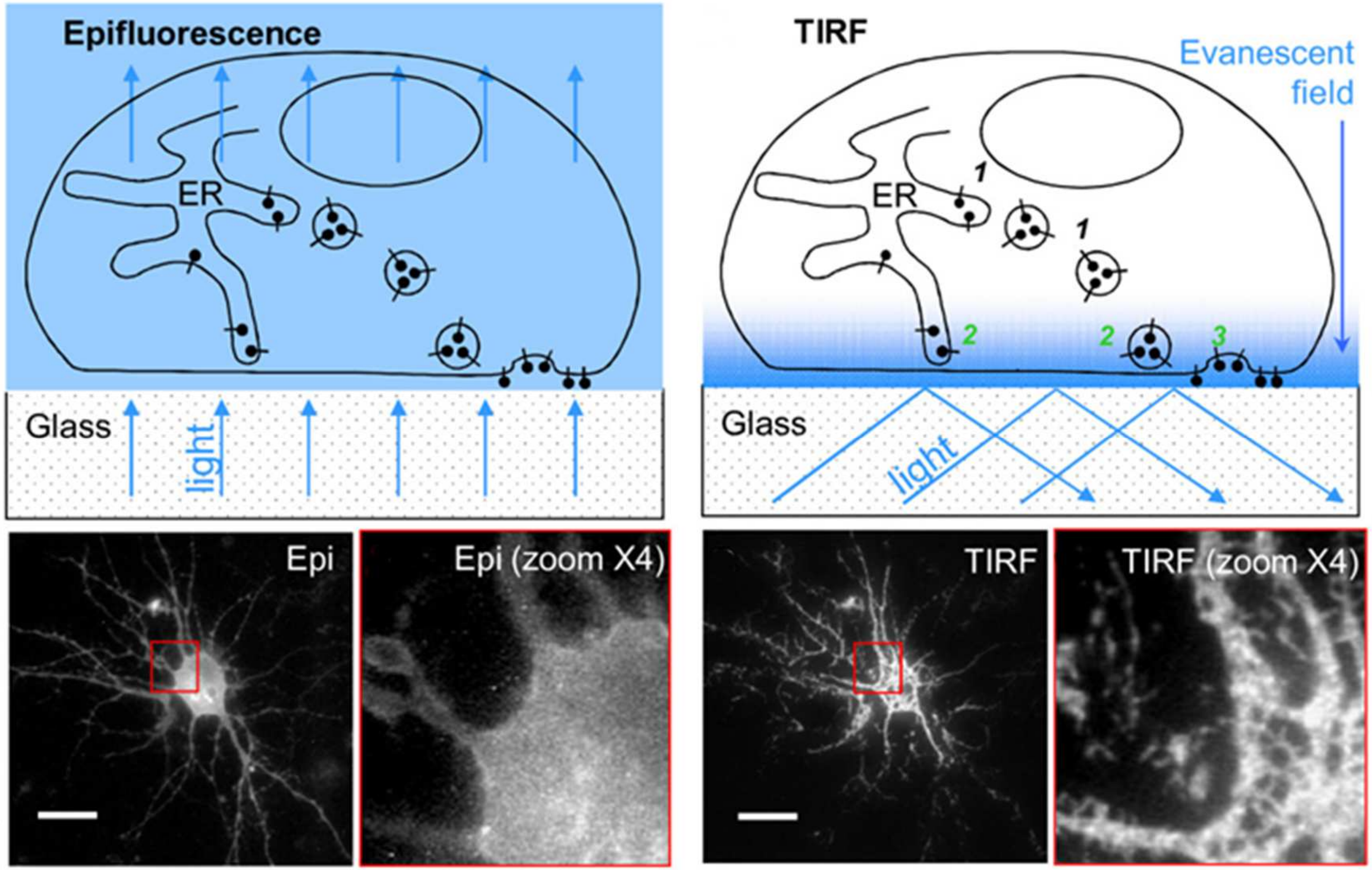
- Increased Z resolution (<100nm)
- Imaging of single fluorescent molecules
- Less phototoxicity - better for Live-cell imaging
- No out-of-focus light
- Excellent signal to noise ratio
- Images events at or near the membrane
- Fast camera based, not scanning
- Significant improvement to classical widefield techniques.

Applications

TIRFM is an ideal tool for the investigation of:

- Live cell imaging
- Protein interactions at the cell membrane surface: Cytoskeletal and membrane dynamics
- Membrane trafficking and fusion, (exocytosis, endocytosis), focal adhesions sites
- Study of reaction rates at surface.
- Single molecule interactions
- **Superesolution techniques**

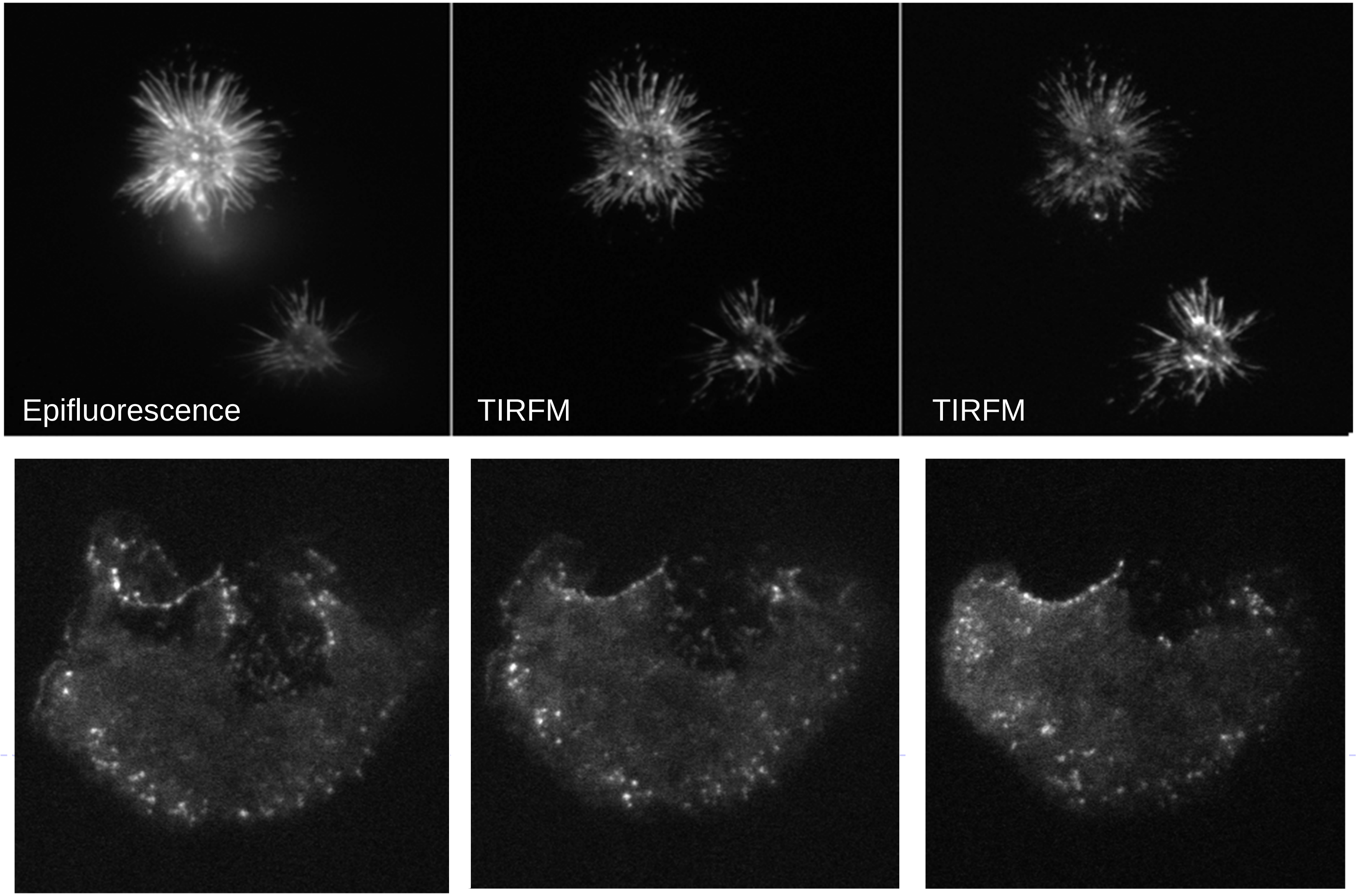
Total Internal Reflection Fluorescence Microscopy (TIRFM)



Epifluorescence and TIRF imaging of pHluorin-tagged molecules. (Scale bars: 10 μm.)
Khiroug *et al. BMC Neuroscience* 2009 **10**:141

Special fluorescence techniques

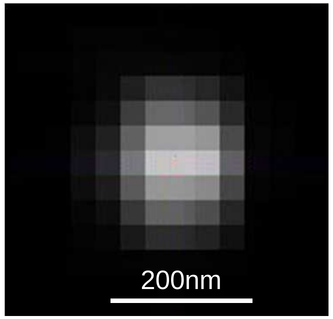
Total Internal Reflection Fluorescence Microscopy (TIRFM)



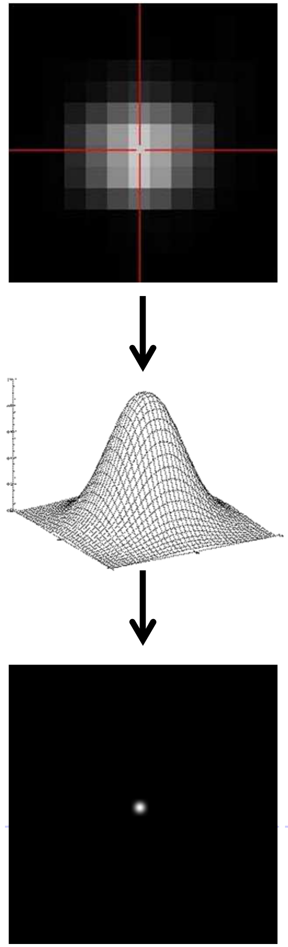
- <u>for a typical microscopy image in cell biology:</u>
  - λ = ~520-650 nm
  - objective 63x NA 1.4
  - diffraction limit of resolution: **~180-230 nm**

- <u>size of biological structures:</u>
  - cells: ~10-20 µm
  - nucleus: ~5-10 µm
  - intracellular vesicles: **~50-200 nm**
  - membranes: **~7-9 nm**
  - proteins: **~1-10 nm**

Diffraction limited image of a point source on a charge-coupled device

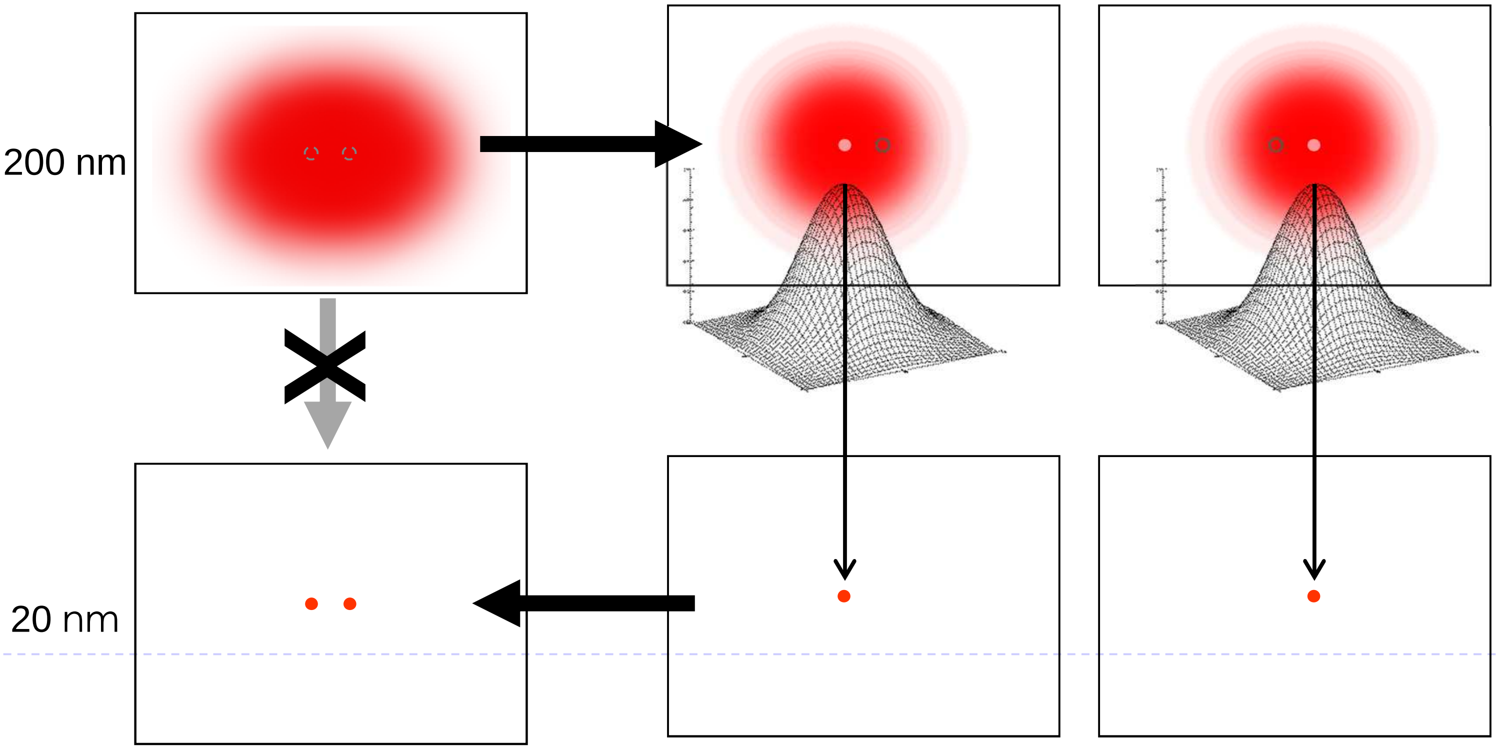


Fitting a Gaussian



Taking TIRF a step further

Fitting the point-spread function (PSF).



PALM

(Photoactivated Localisation Microscopy)
Eric Betzig

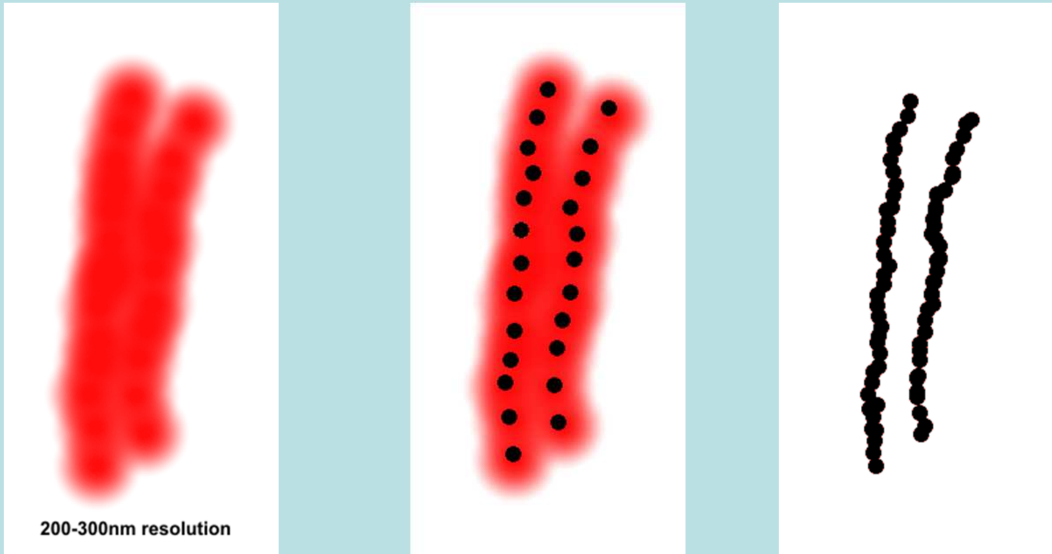
STORM

(Stochastic Optical Reconstruction Microscopy)
Xiaowei Zhuang at Harvard
Sam Hess at University of Maine

- Exploits the photoswitchable nature of certain fluorophores
- Photoactivation is stochastic:- only a few well-separated molecules "turn on."
- Gaussians are fit to their PSFs to high precision and centres calculated with sub-resolution accuracy
- Large number of images required

Super-resolution image reconstructed from localisations

TIRFM Image | PALM/STORM | Reconstruction



Fluorophores too close to resolve

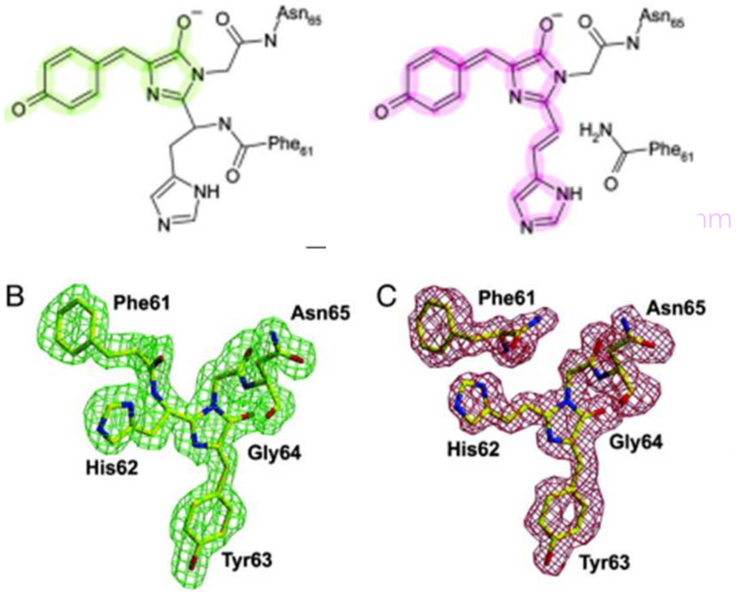
Stochastic activation and localisation of individual molecules

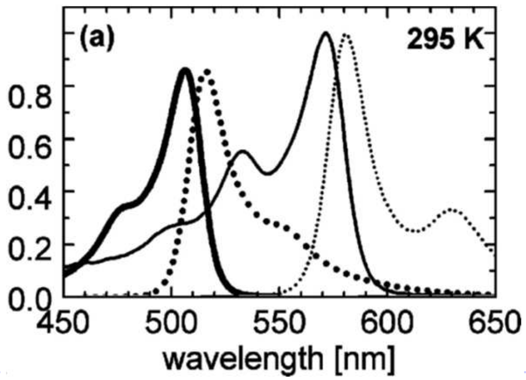
Photoconvertible fluorescent proteins

- EosFP
- pDendra2
- PA-GFP
- PS-CFP
- KFP-Red

EosFP

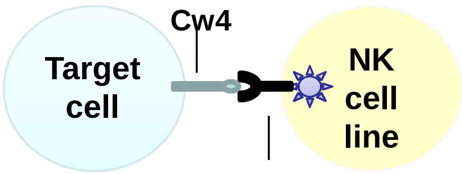
- monomeric
- conversion wavelength separate from excitation wavelength (no bleaching during conversion!)
- stable photoconversion (irreversible: cleavage)





Example: Human Natural Killer cells - Courtesy Sophie V. Pageon (Imperial)

Cell system



tdEosFP = photo-switchable fluorescent protein

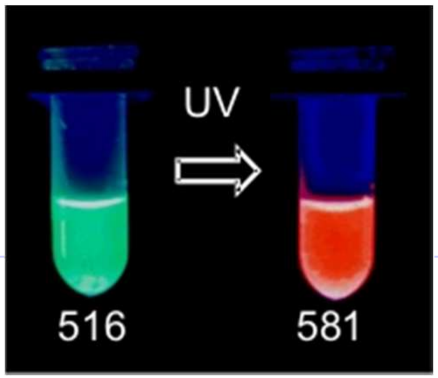
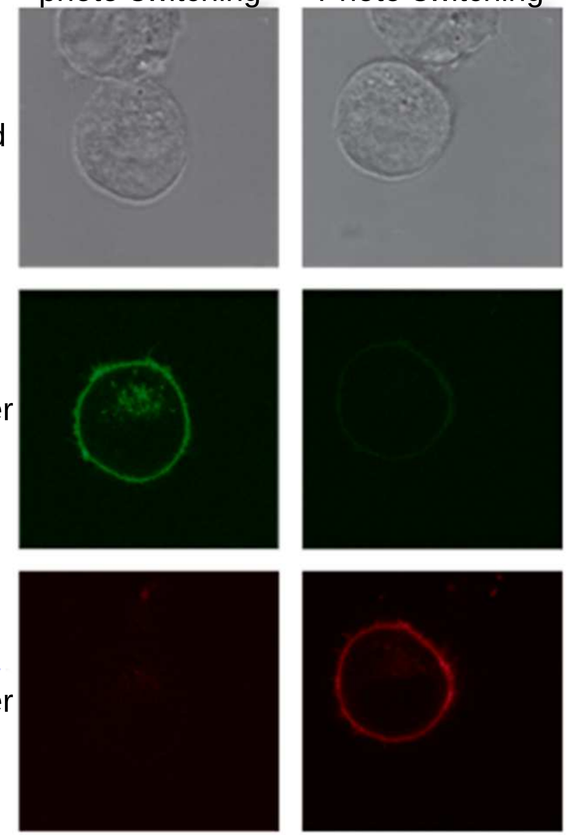
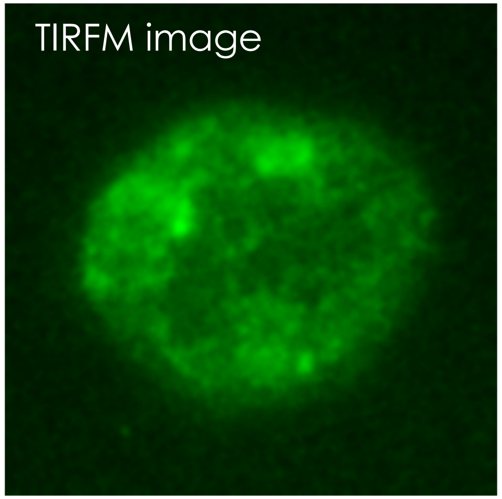


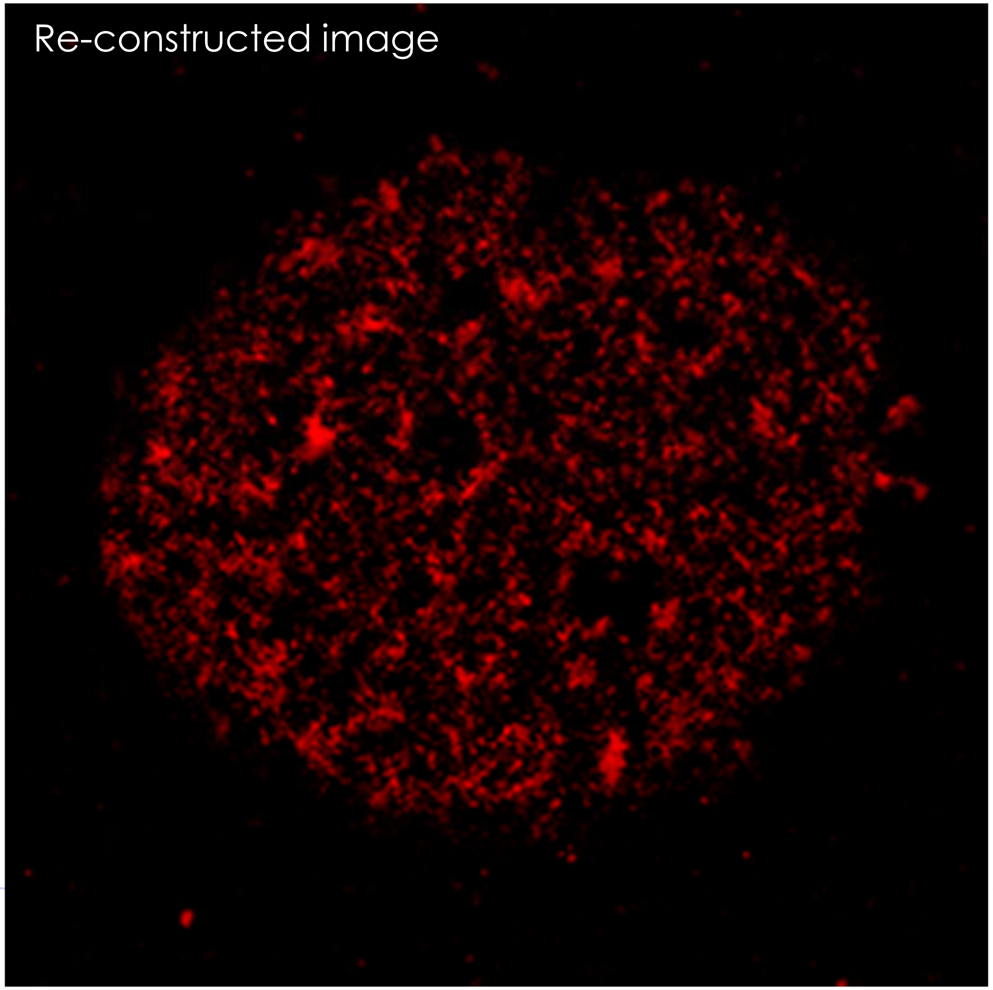
Photo-switching with UV light (390)



High-resolution microscopy: PALM/STORM



Raw Palm data



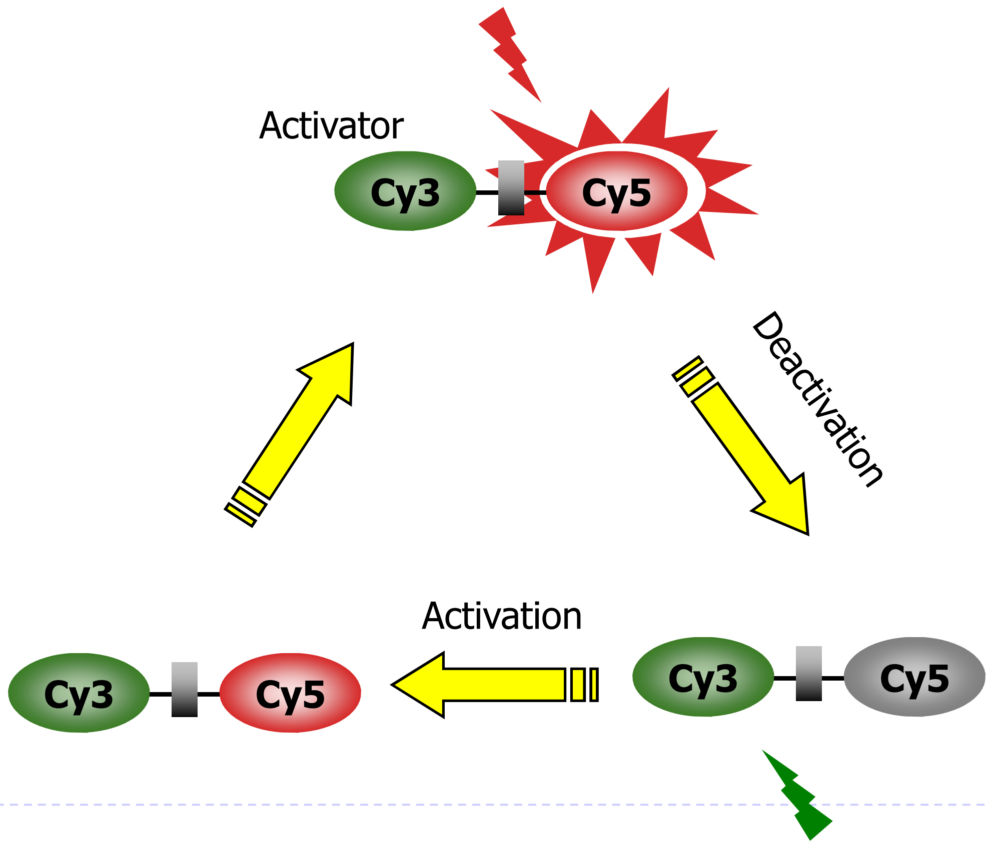
STORM

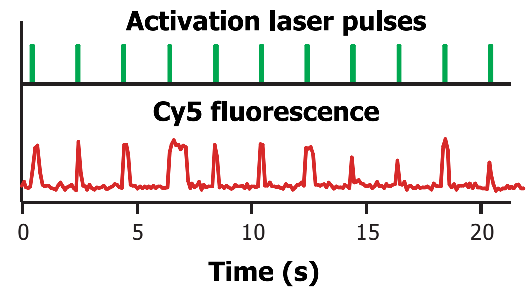
Standard organic fluorophores such as Carbocyanine, Alexa Fluor and ATTO-dyes - Wide range covering the whole visible spectrum

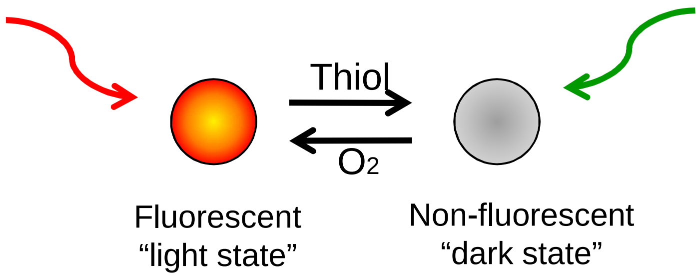
Requirement:

- Reversible between a fluorescent and non-fluorescent state
- Bright “on” state – high photon yield
- Long lifetime of the “dark” state
- Controllable cycling for a large number of cycles

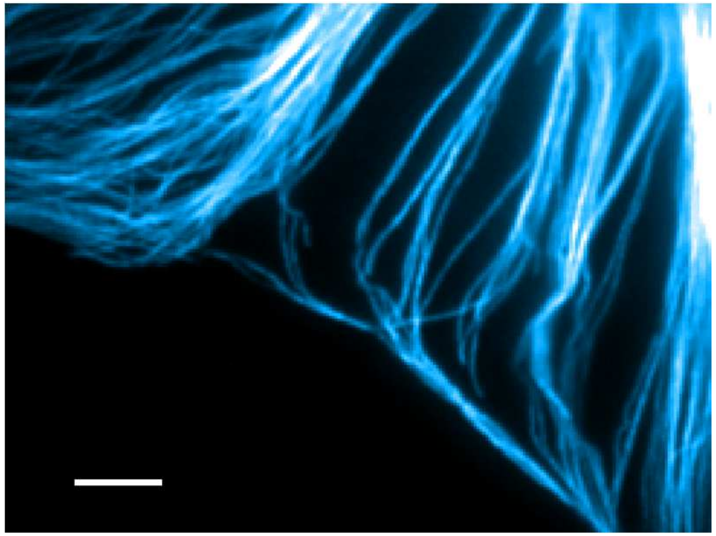
A Photo-switchable Probe

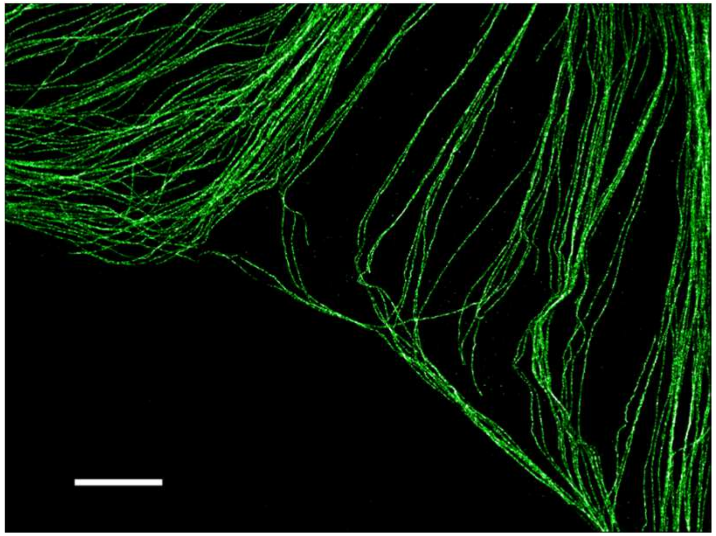


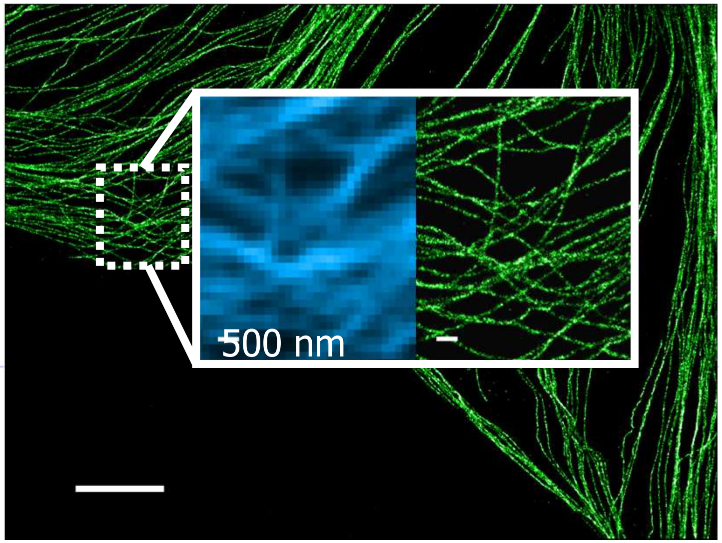


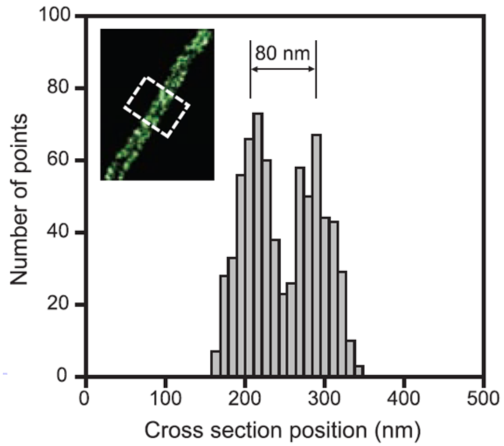


High-resolution microscopy: PALM/STORM





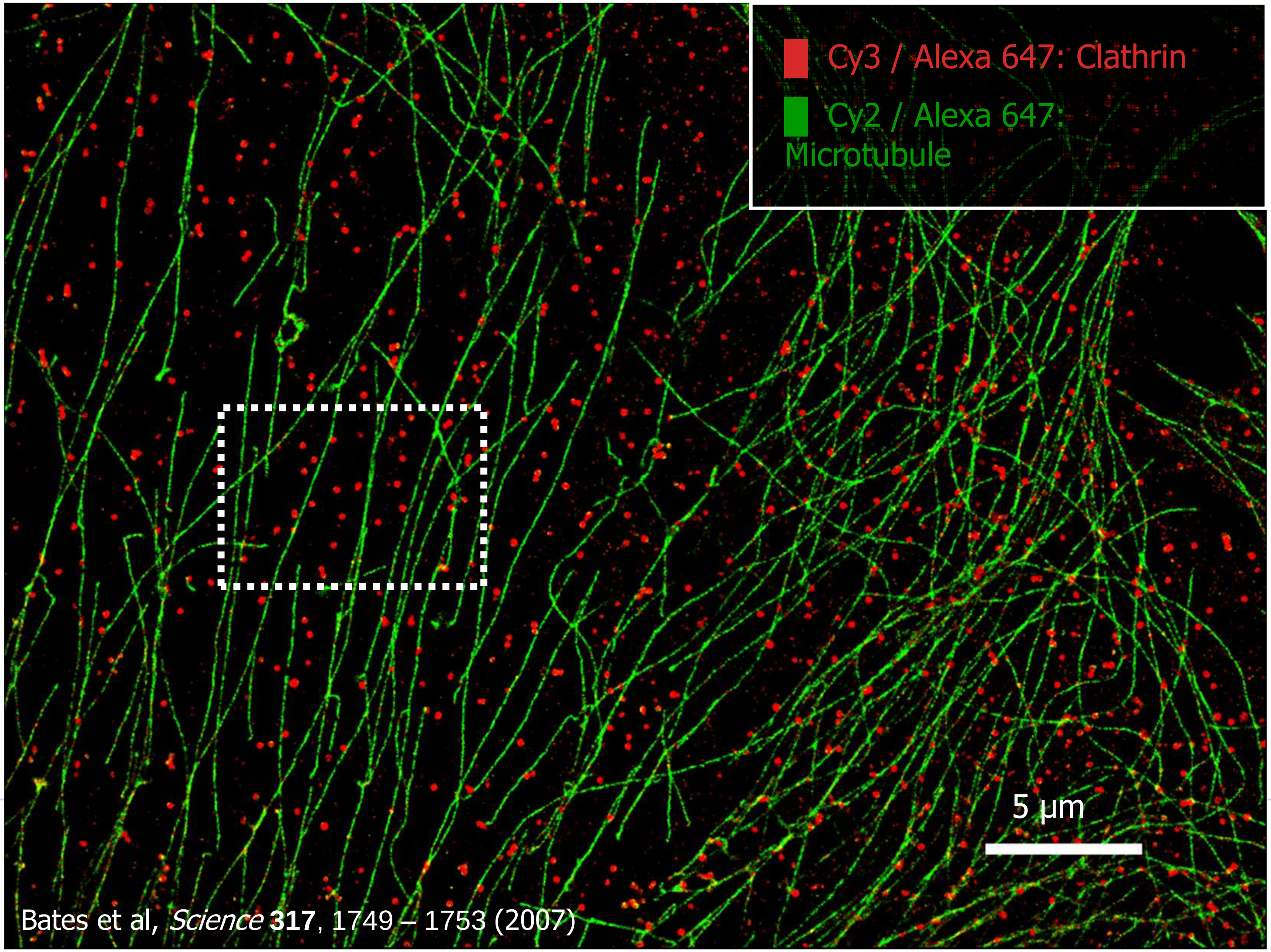




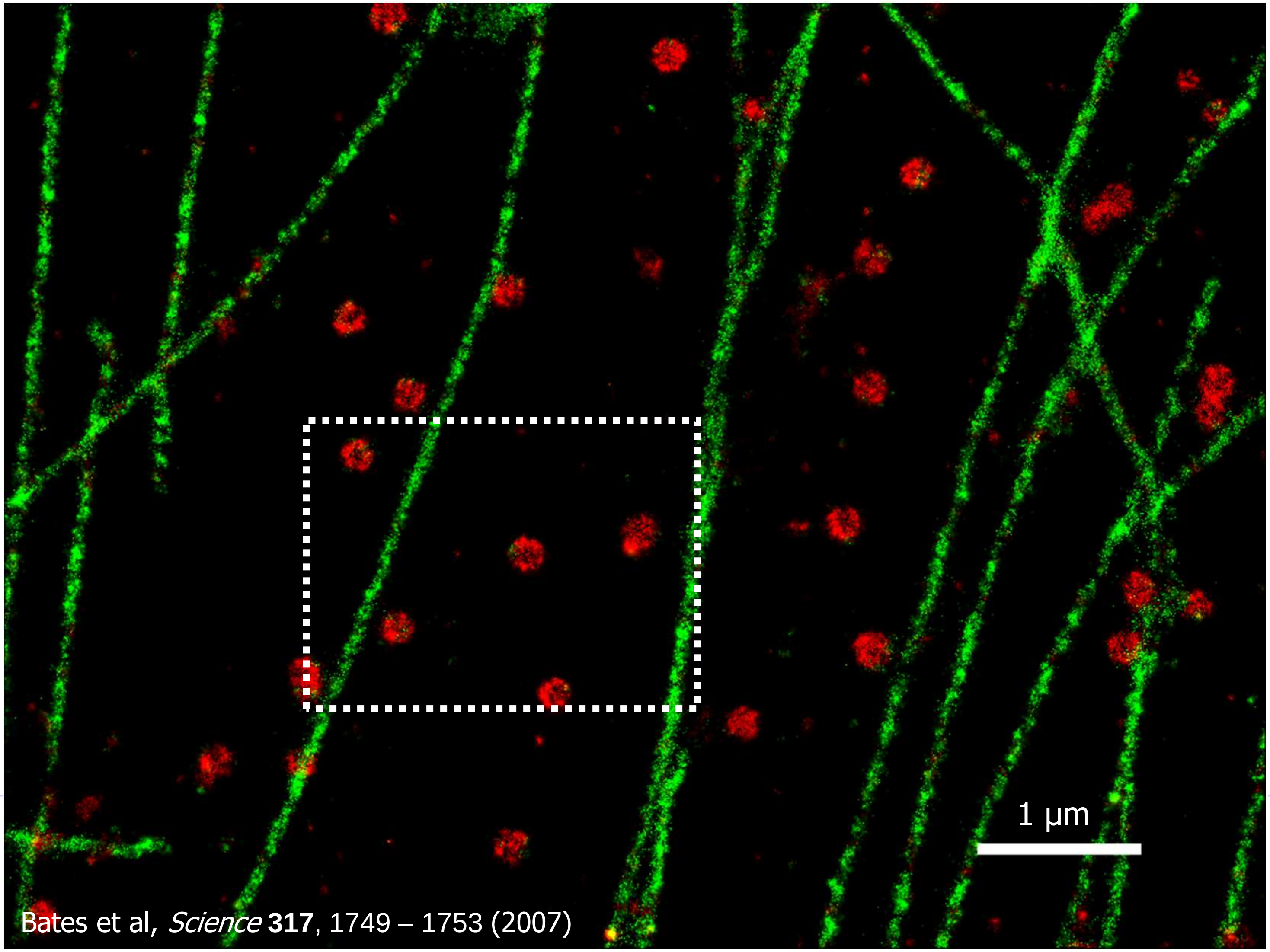
B-SC-1 cell,
Microtubules stained with
anti-β tubulin
Cy3 / Alexa 647
secondary antibody

Bates et al, *Science* **317**, 1749 – 1753 (2007)

High-resolution microscopy: PALM/STORM



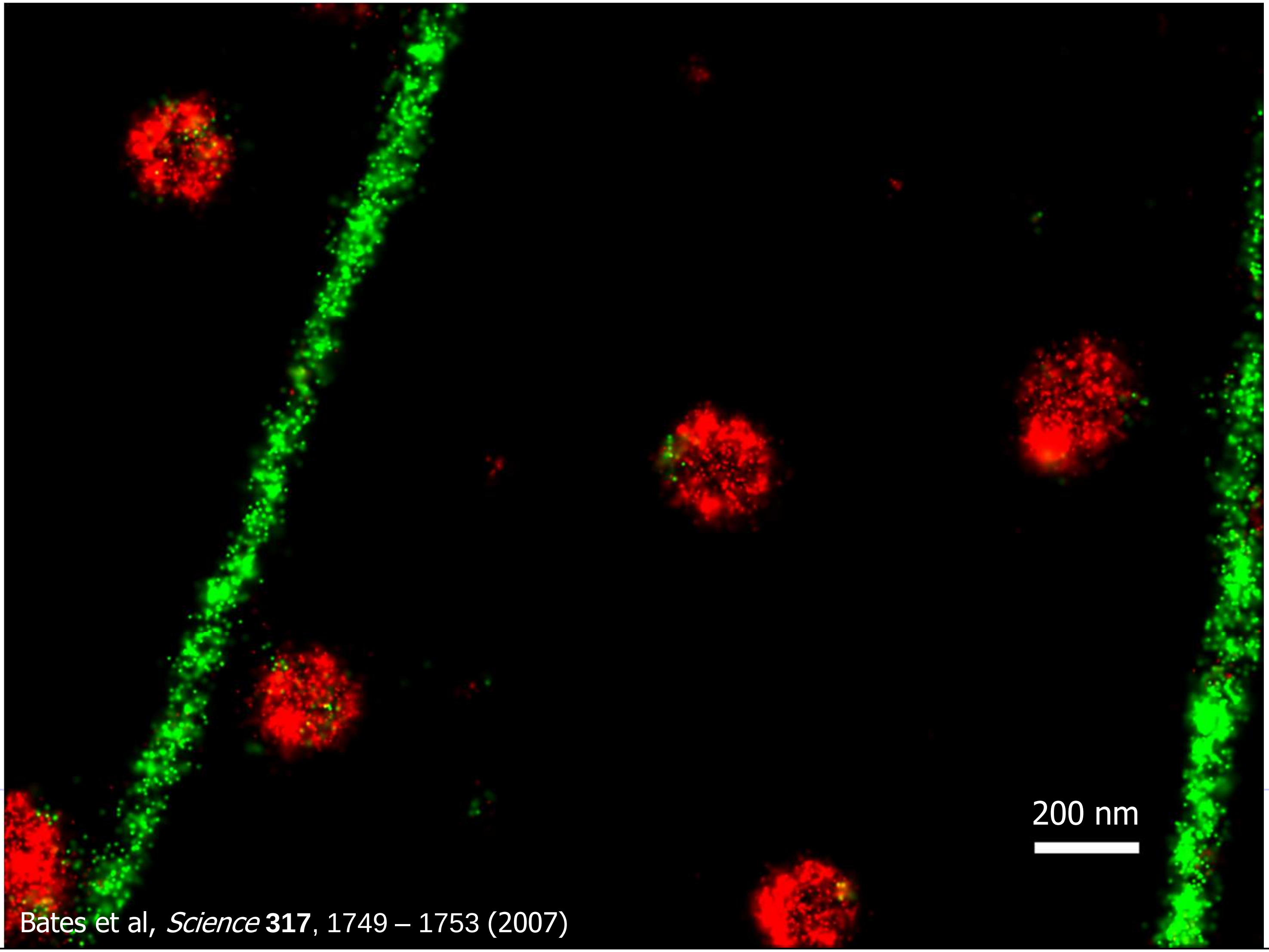
High-resolution microscopy: PALM/STORM



Bates et al, *Science* **317**, 1749 – 1753 (2007)

Special fluorescence techniques

High-resolution microscopy: PALM/STORM



Avg = 172 nm

Special fluorescence techniques

Summary

- STORM and PALM can achieve very high (20-60 nm) spatial resolution.
- Use TIRF microscopy
- Image formation require very large number of raw images.
- Time resolution is on the order of minutes/hours, not ideal to study dynamics
- PALM one image only per sample
- STORM possible to record several final images per sample before permanently photobleaches.