

The use of imaging technology in cell biology

- 1. Introduction: what it is & how did it all start**
- 2. Applications & techniques, including some examples from our research**
- 3. Super-resolution microscopy**
- 4. Facilitates at SUSTC & how to start using them**
- 5. What does the future hold...**

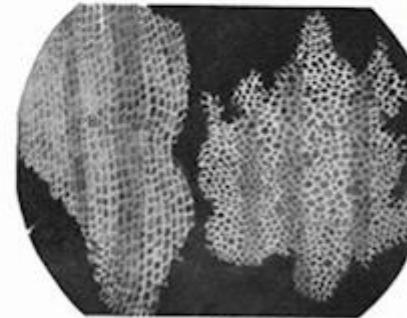
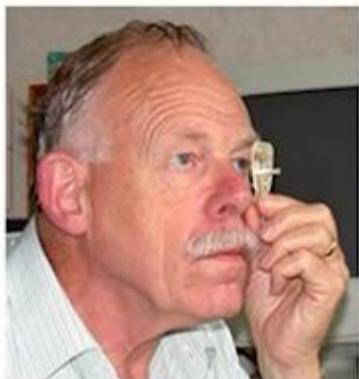
The advent of microscopy in biology



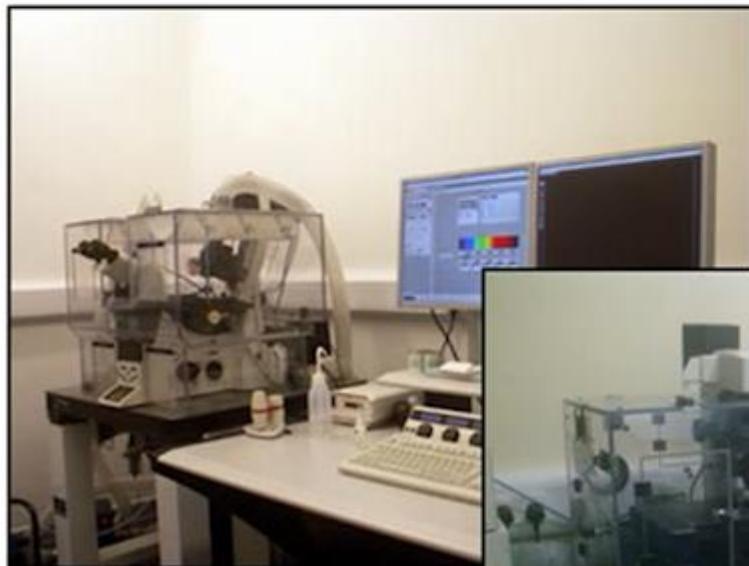
Antonie van Leeuwenhoek
1632-1723



Robert Hooke
1635-1703



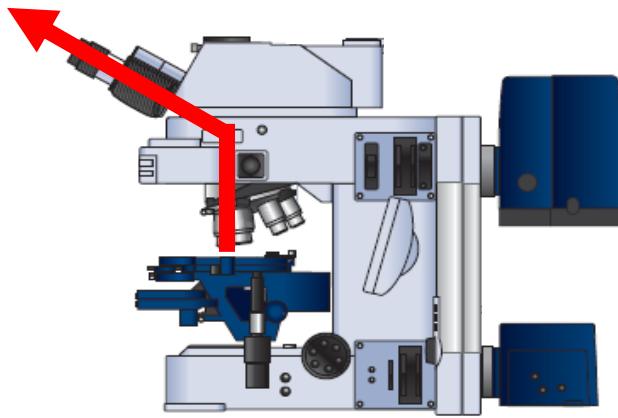
To present day.....



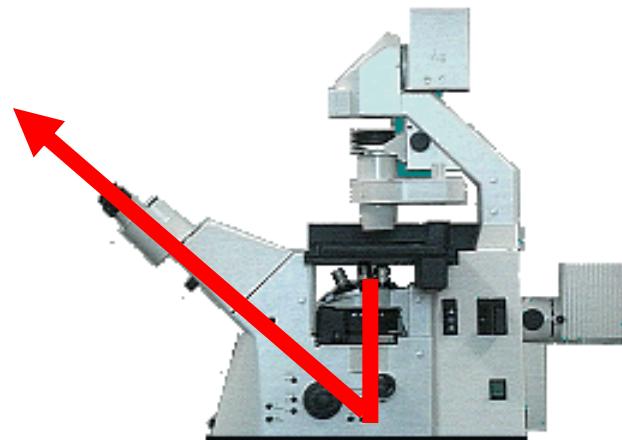
Optical Microscope



The basic light microscope types

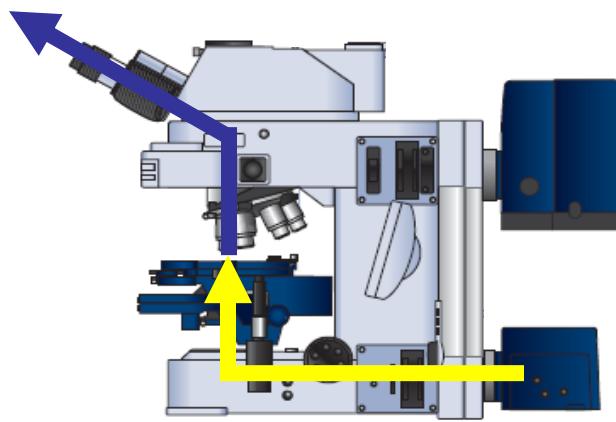


Upright microscope

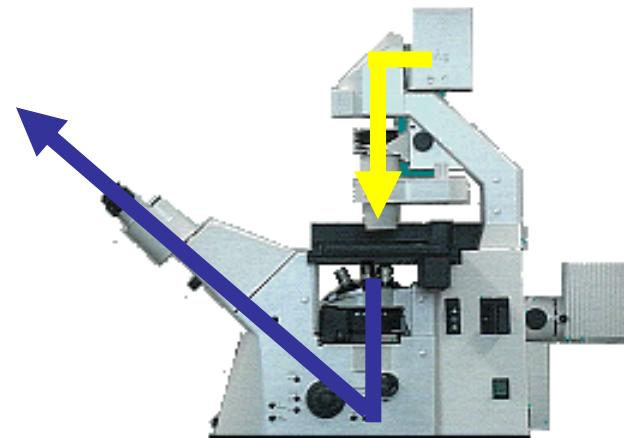


Inverted microscope

Illumination via Transmitted Light

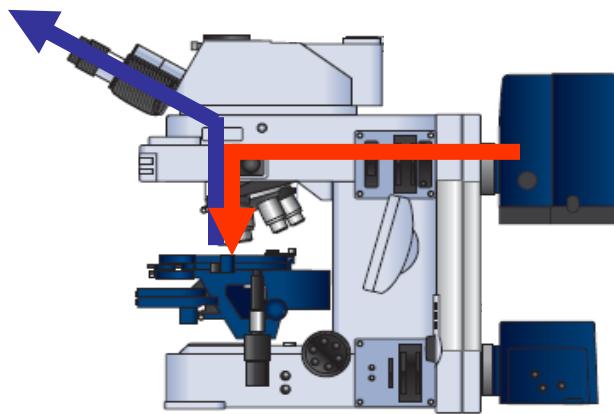


Upright microscope

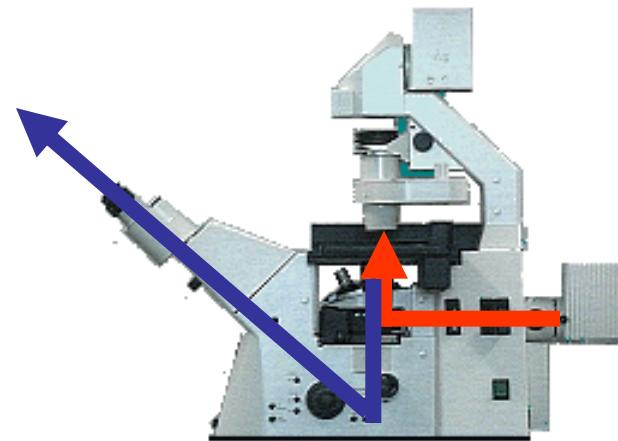


Inverted microscope

Illumination via “Reflected” (Incident) Light

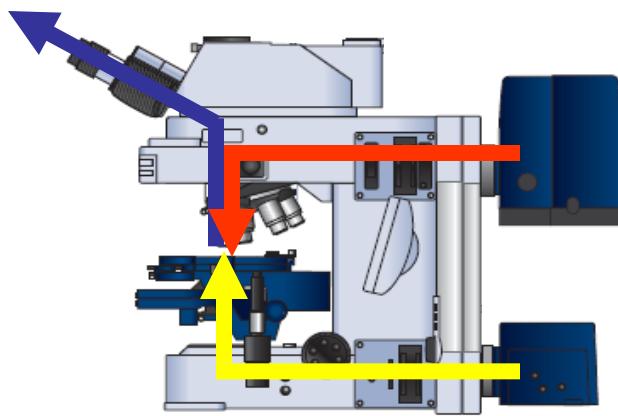


Upright microscope

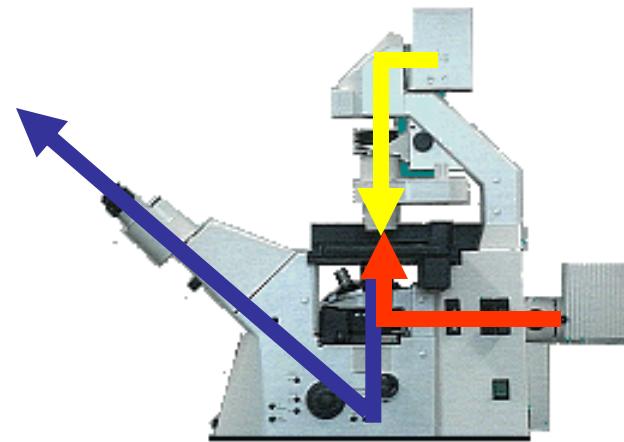


Inverted microscope

Mixed Illumination

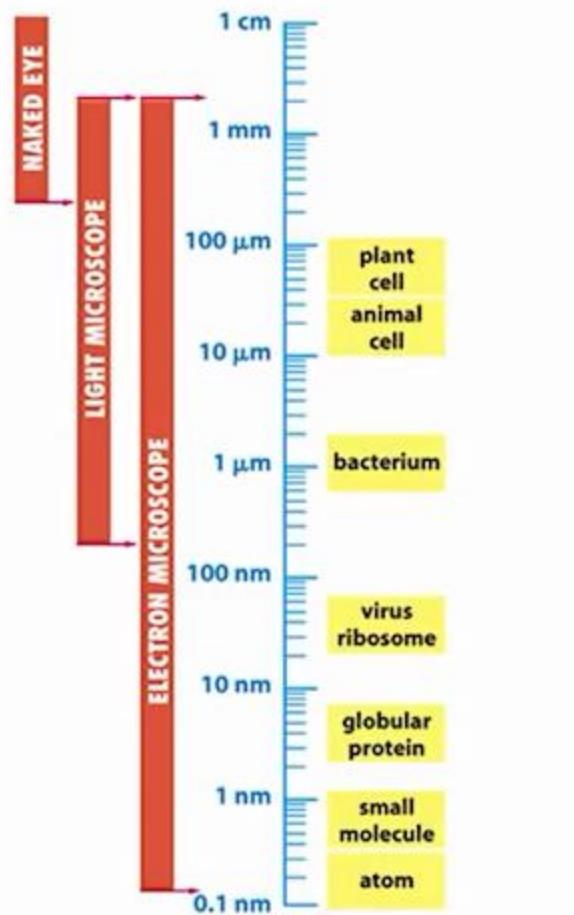
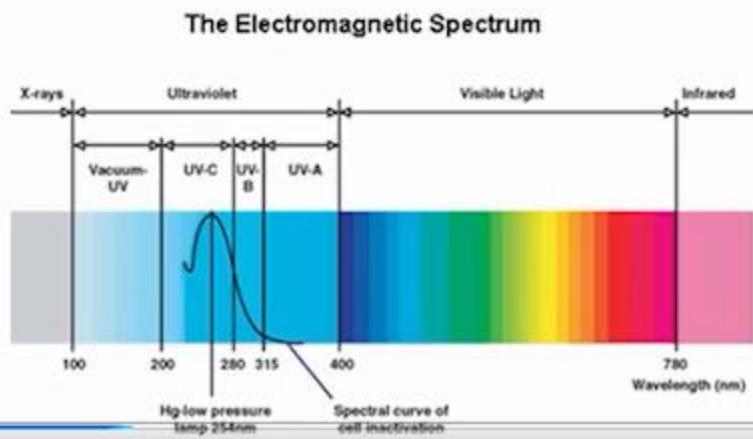
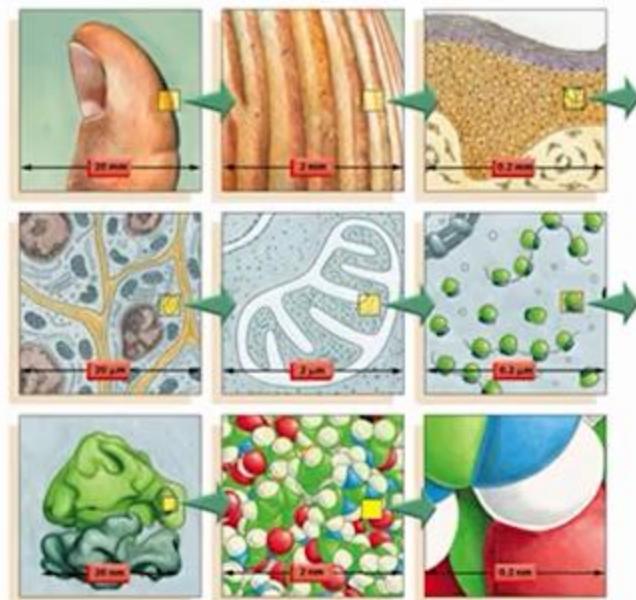


Upright microscope



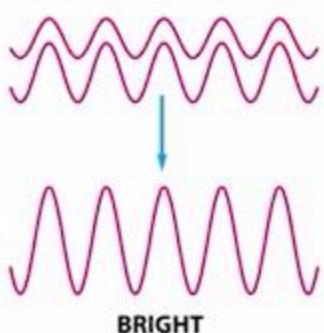
Inverted microscope

The resolution of light microscopes

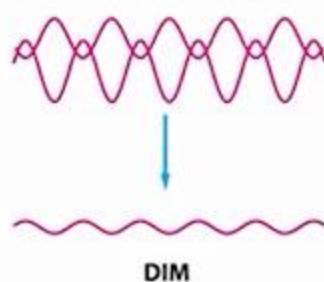


The principle of light microscopy

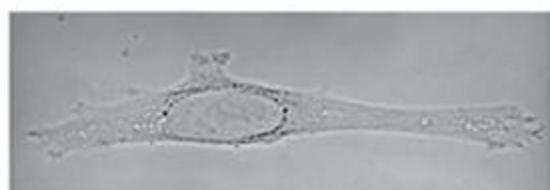
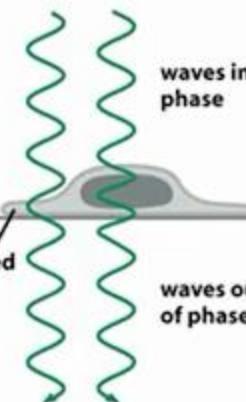
TWO WAVES IN PHASE



TWO WAVES OUT OF PHASE



incident light
(green)



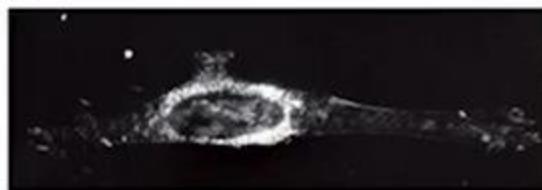
(A)



(B)



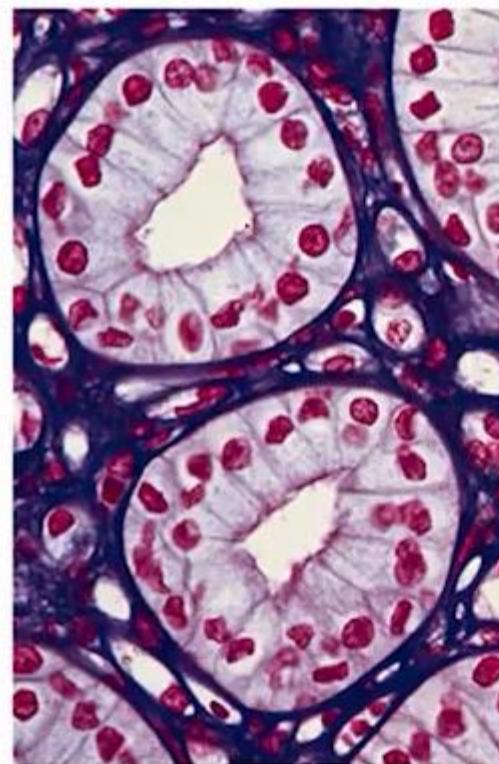
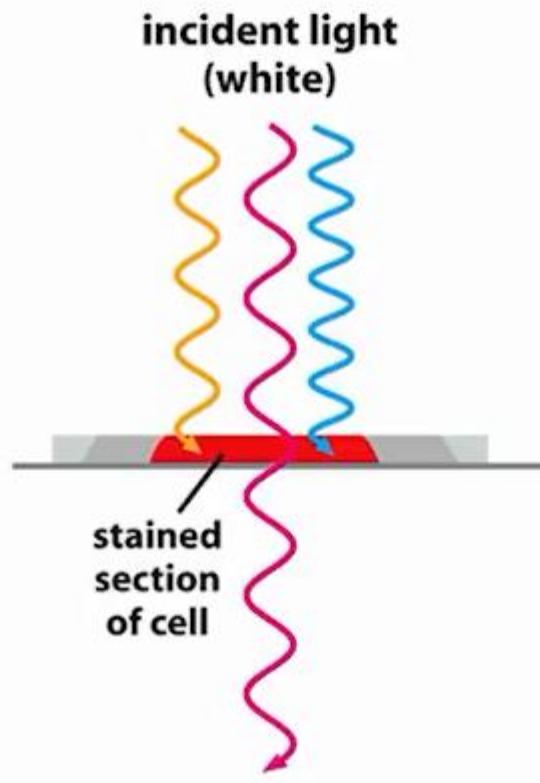
(C)



(D)

50 μm

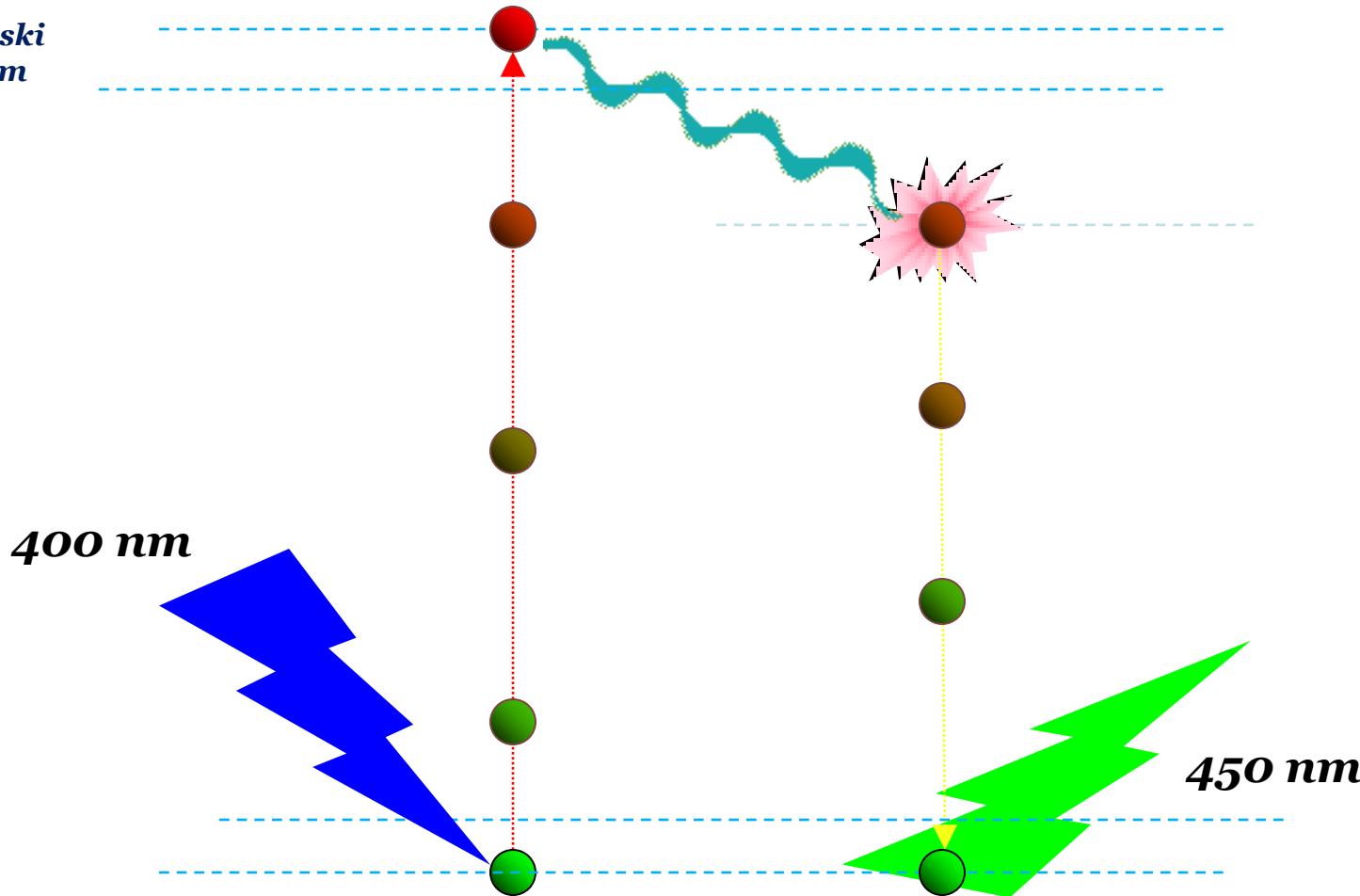
The development of chemical stains



50 μm

What is Fluorescence?

Jablonski diagram



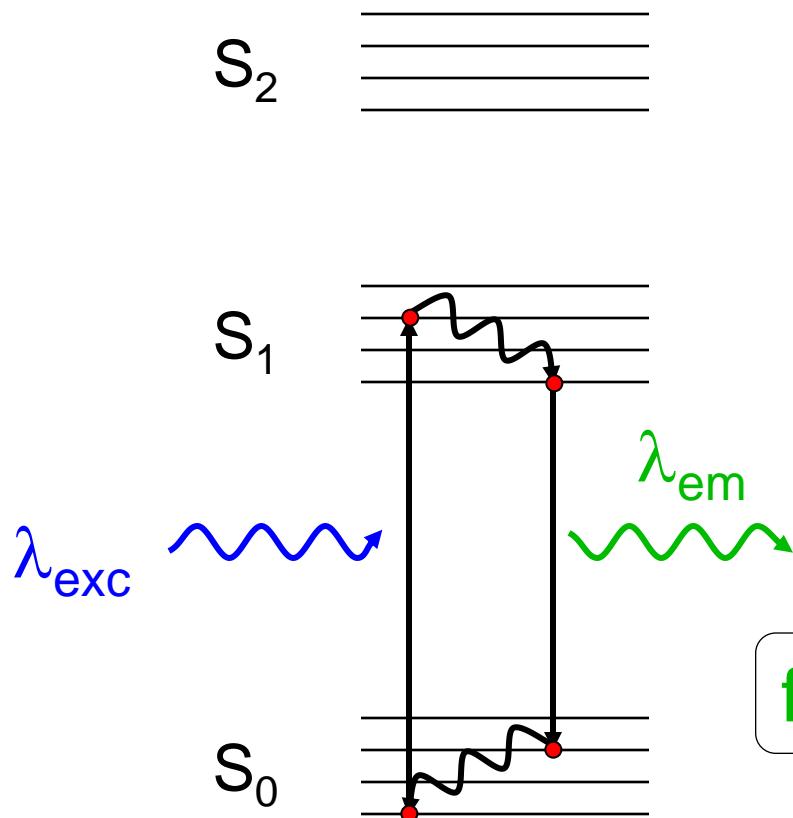
Quantum yield = no. of fluorescent photons emitted/no. of photons absorbed
e.g. EGFP QY=0.6 For every 10 photons absorbed, 6 are emitted.
(at optimal temp, pH etc.)

Photo bleaching Important: Dye emits $10^5 \rightarrow 10^7$ photons, then dies!

Jabłonski diagram

(Molecular energy diagram)

Singlet states
Spin S=0

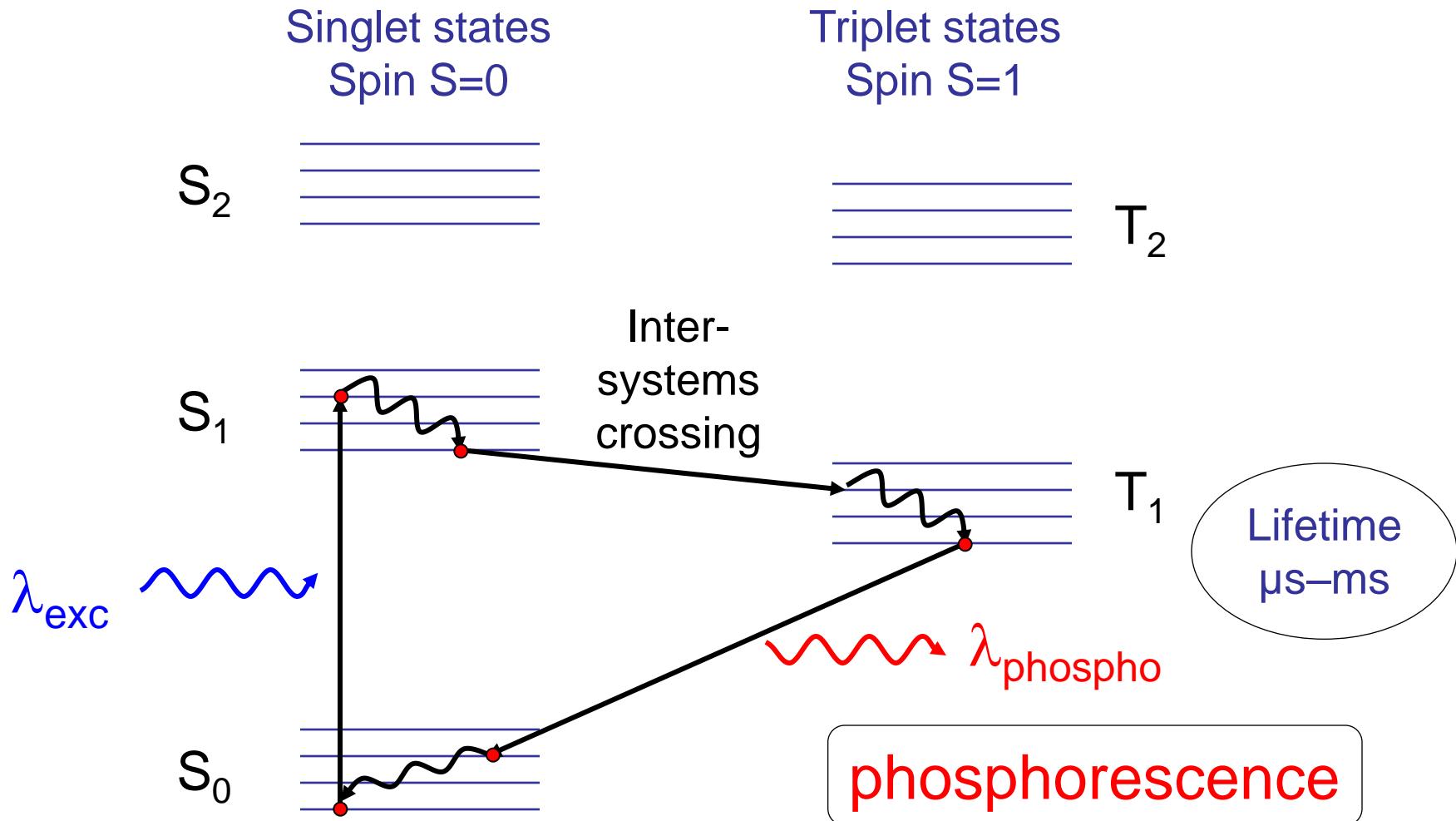


fluorescence

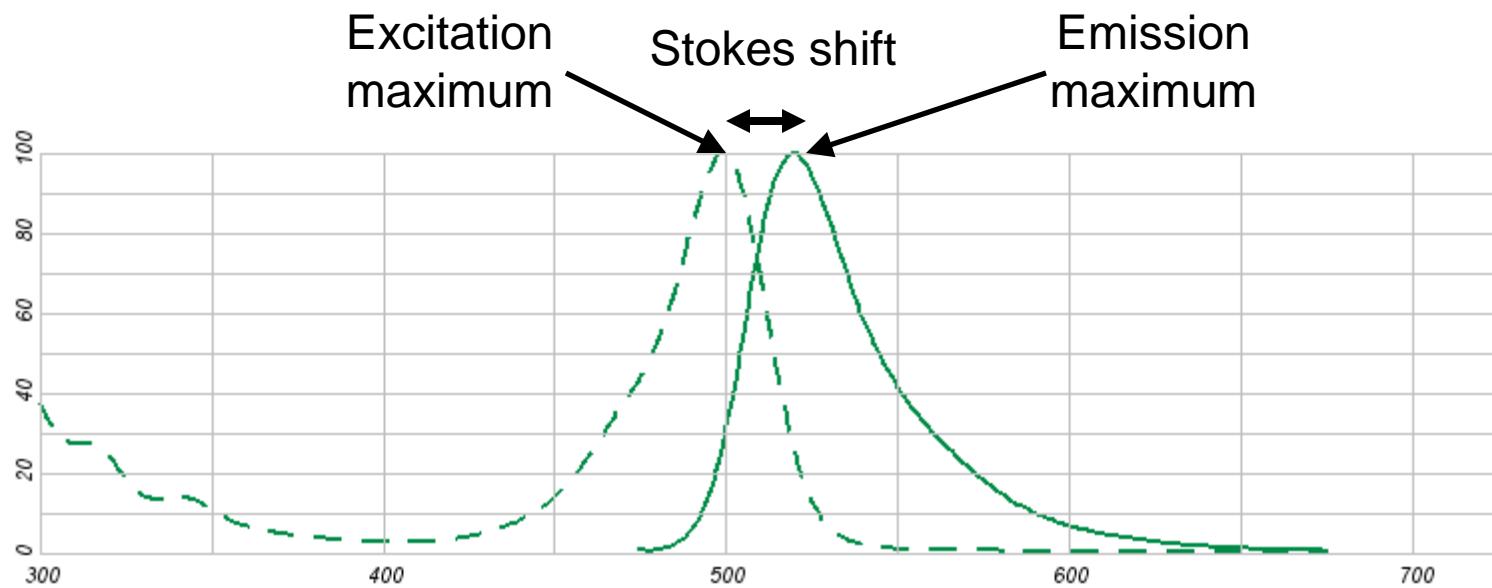
Lifetime
 $\tau \sim 1\text{--}4 \text{ ns}$

Jablonski diagram

(Molecular energy diagram)

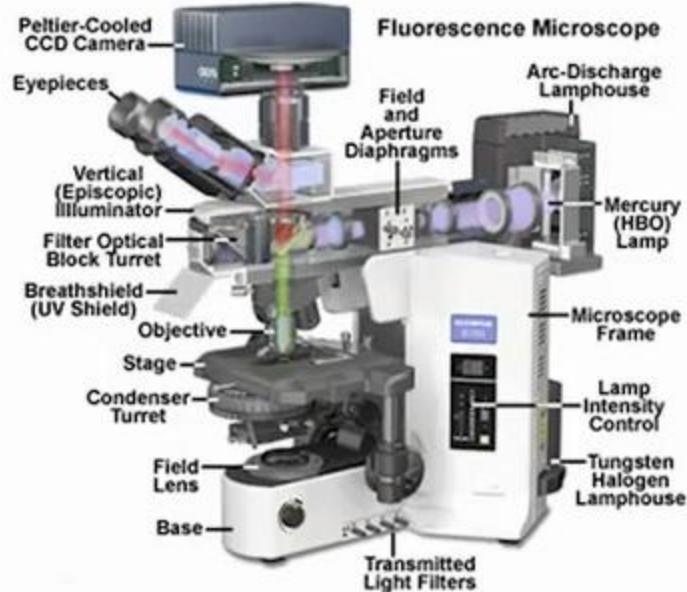
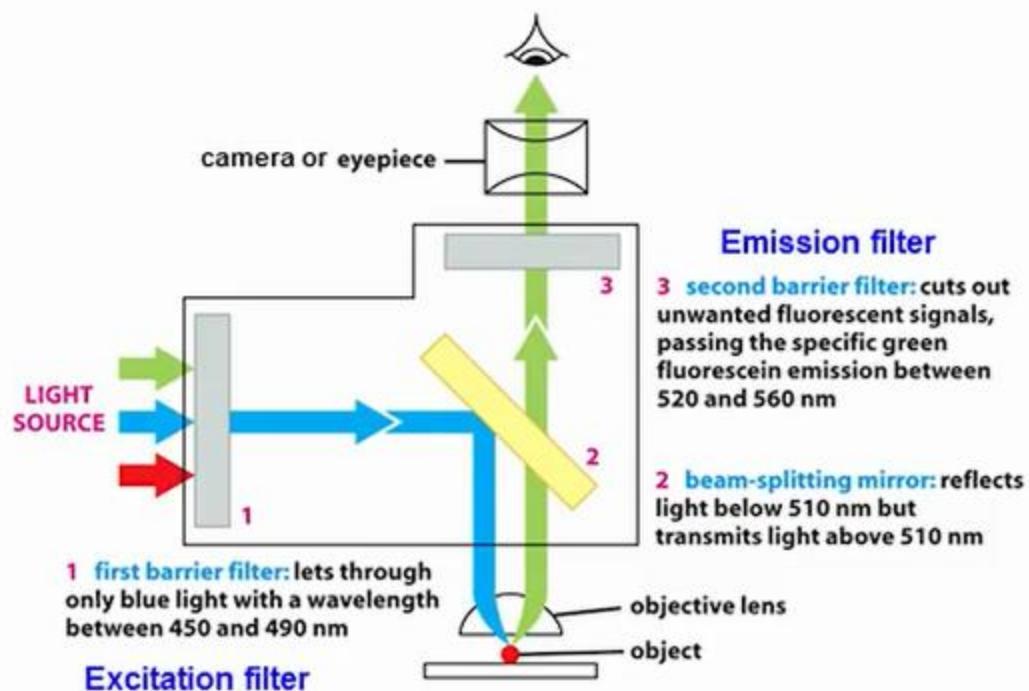


Fluorescence Spectra

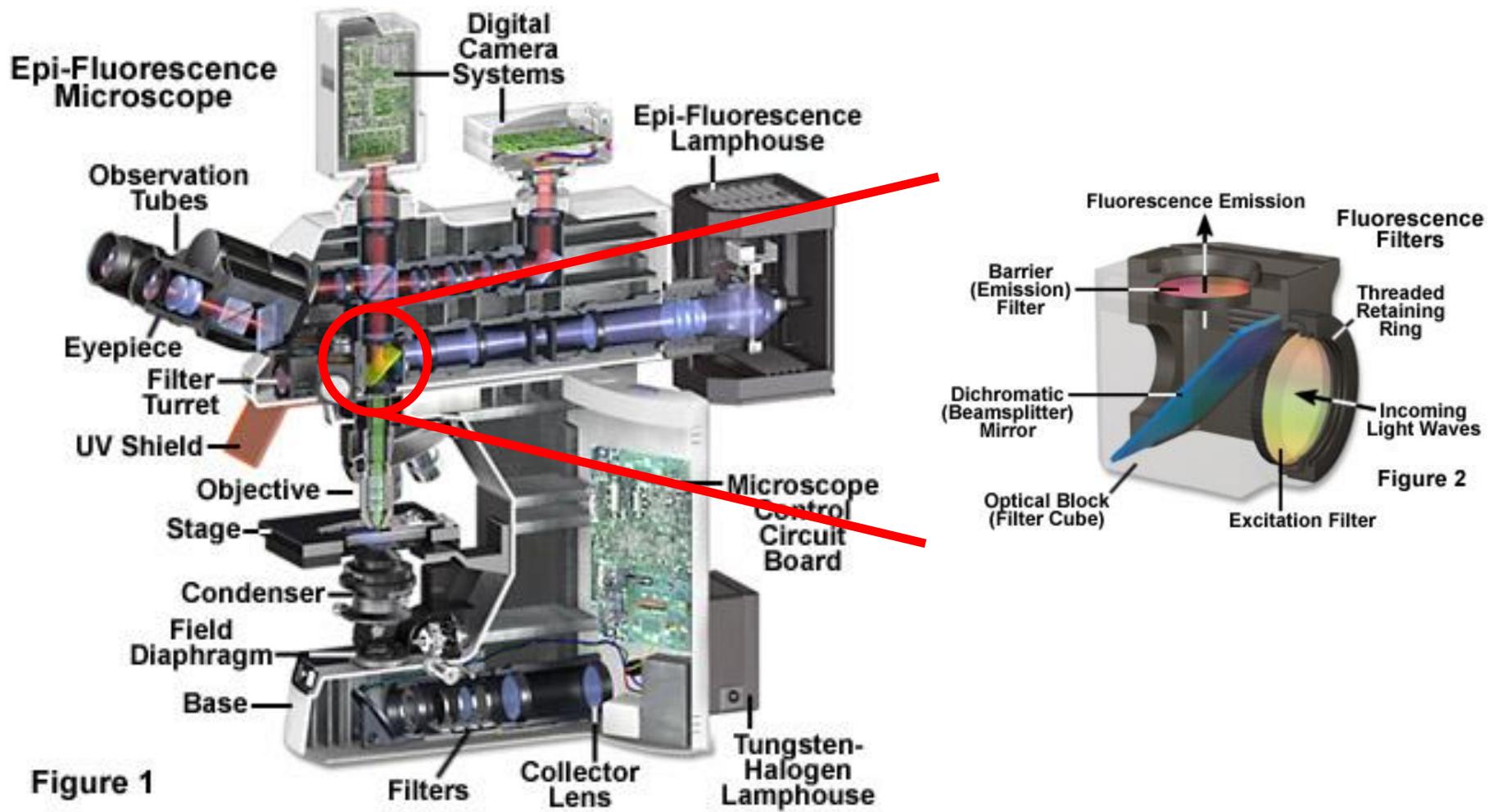


Alexa 488

The basic components of a fluorescence microscope



The Epifluorescence Microscope



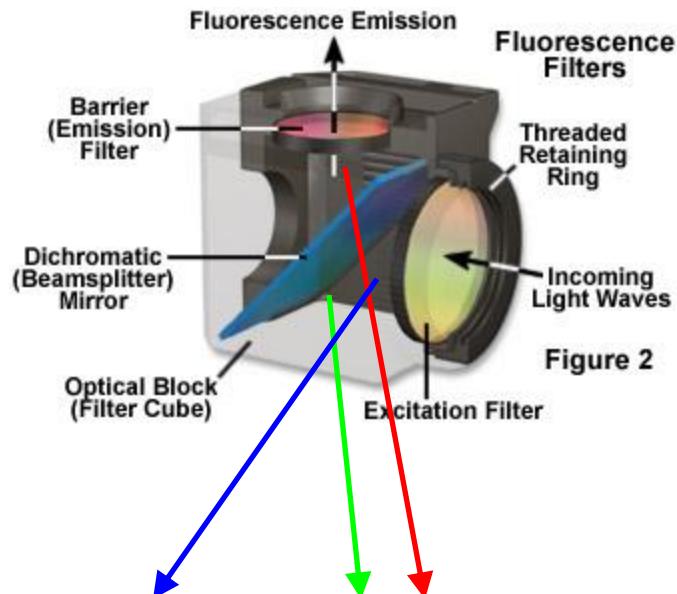
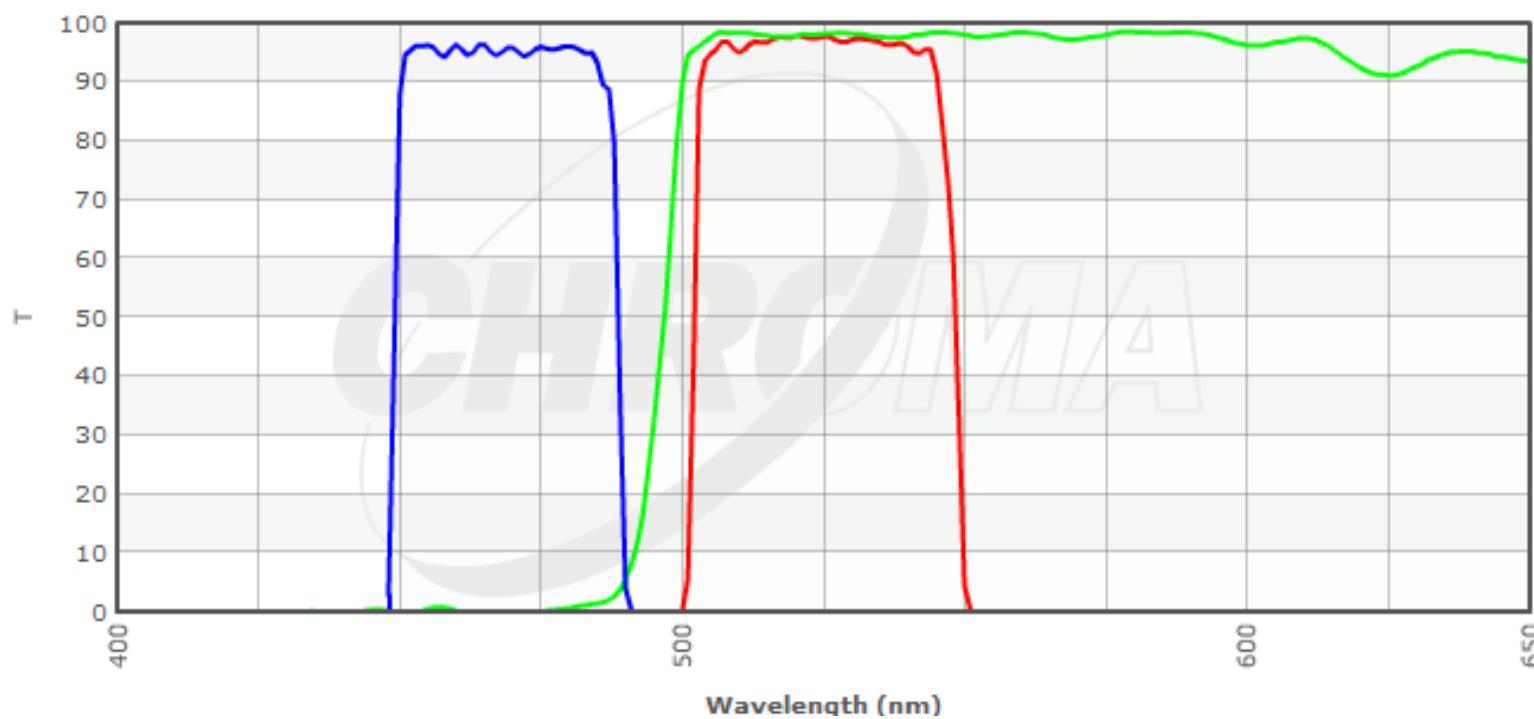


Figure 2



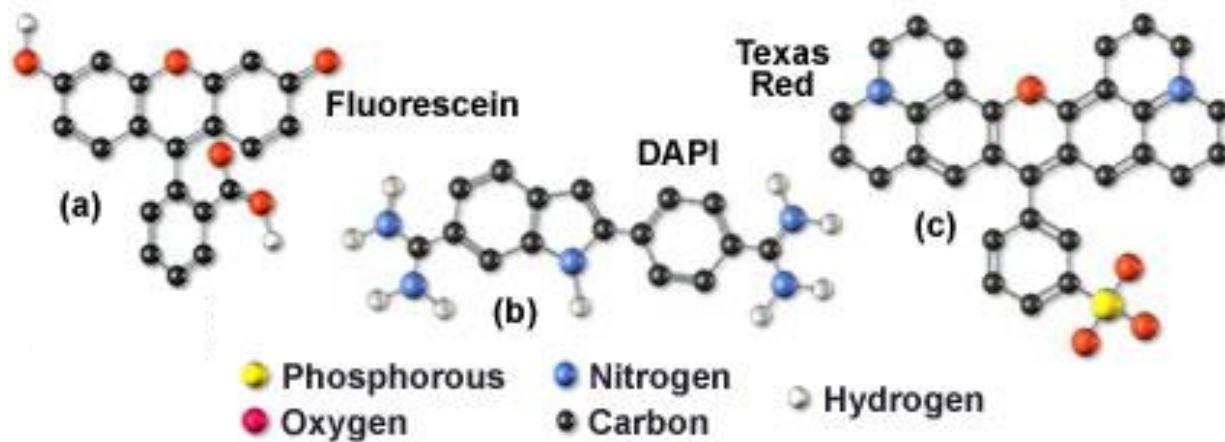
Filter cube nomenclature

- Chroma labels filters as center wavelength / passband (e.g. D350/50x)
- Dichroics are labeled by cut-on wavelength (e.g. 505DCLP)
- Nikon filters use a letter to specify illumination wavelength – e.g. UV, B, G, R
- Letters afterward specify emission profile – e.g. UV-2A vs UV-2E/C

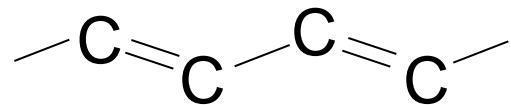
Light Sources

- Arc Lamps
 - Hg and Xe
 - Metal Halide
- LEDs
- Lasers
 - Generally only for collimated illumination
(Confocal, TIRF)

Fluorescent molecules



Systems of conjugated bonds
that share electrons



Larger system → longer wavelength

Parameters of fluorescent molecules

- Excitation & emission maxima

- Extinction coefficient ε

\propto absorption cross section

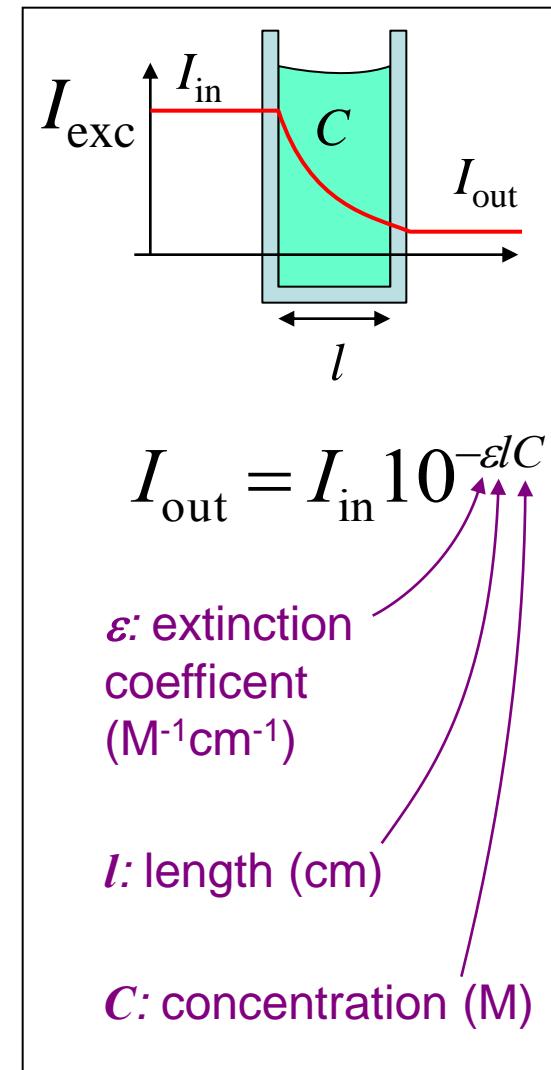
$$\varepsilon \approx 50,000\text{--}100,000 \text{ M}^{-1}\text{cm}^{-1}$$

- Fluorescence quantum yield Q_f

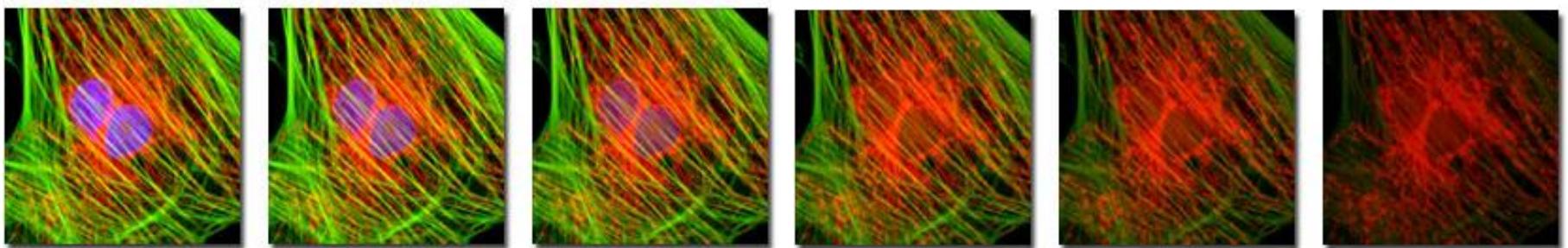
= # Photons emitted / # photons absorbed

$$Q_f \approx 25\text{--}90\%$$

Brightness $\propto \varepsilon Q_f$



The Enemy: Photo-bleaching



Decrease in emission intensity after exposure

Exciting a molecule once has a probability Q_b of killing it

Each molecule will emit only a finite number of photons

What Sort of Molecules are Fluorescent?

Organic fluorophores

especially

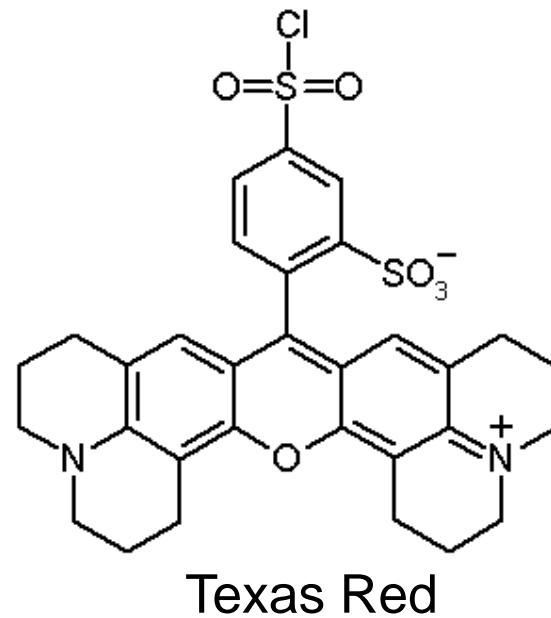
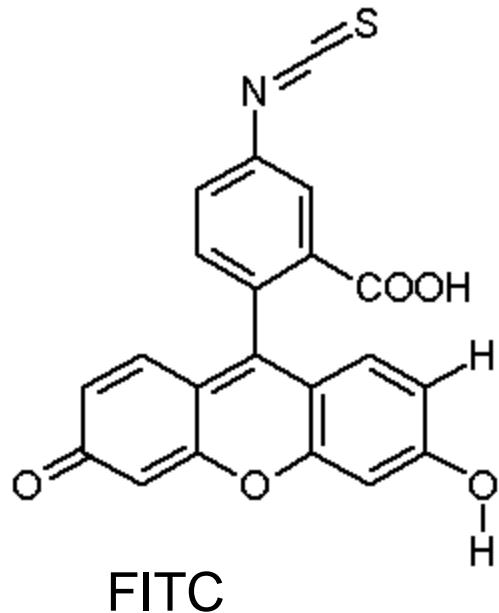
1. Intrinsic fluorophores (source of auto fluorescence)
2. Dyes: FITC, TRITC, etc..
3. Biological fluorophores - Fluorescent proteins: GFP, Phycoerythrin (PE)

Inorganic fluorophores

especially

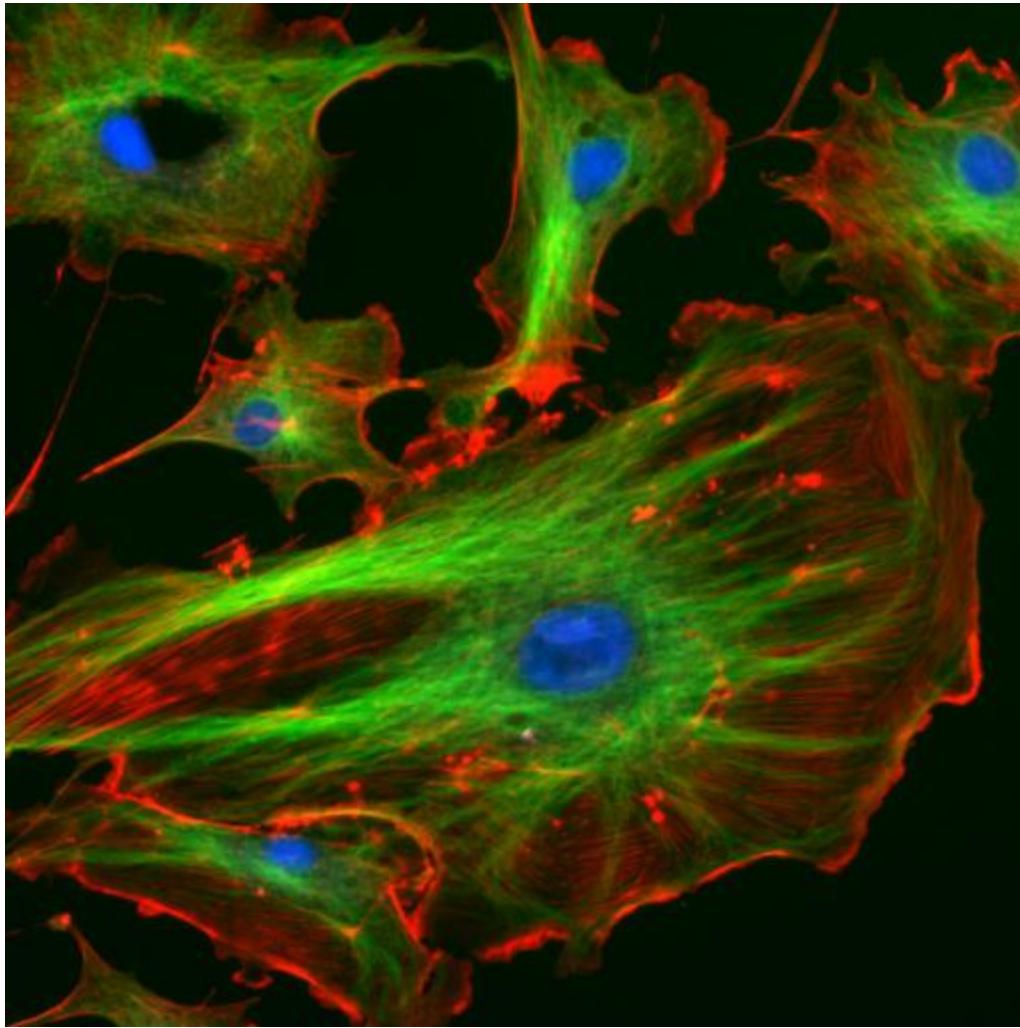
1. Quantum dots

Fluorescent dyes in Biology



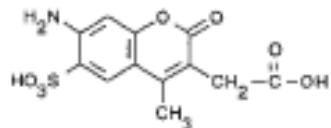
- Protein labeling: couple to amino- or sulfhydryl groups
- Direct and indirect (immuno-) fluorescence

Bind to: free cysteines ($--\text{SH}$) (often only one or a few in proteins)
free lysines ($--\text{NH}_2$) (many per protein)

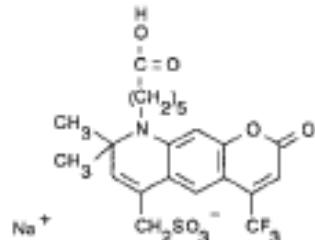


Endothelial cells under the microscope. Nuclei are stained blue with DAPI, microtubules are marked green by an antibody bound to **FITC** and actin filaments are labeled red with phalloidin bound to **TRITC**. Bovine pulmonary artery endothelial (BPAE) cells

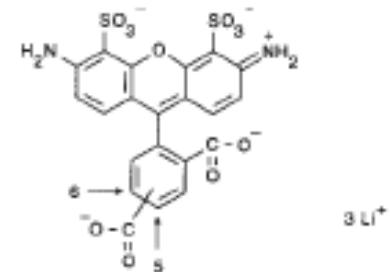
Traditional small molecule dyes



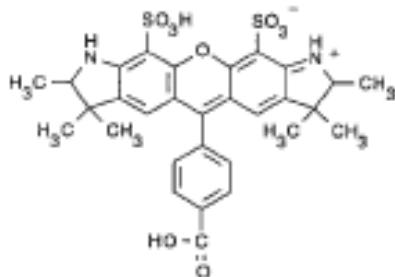
Alexa 350



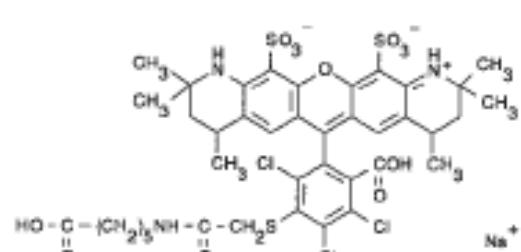
Alexa 430



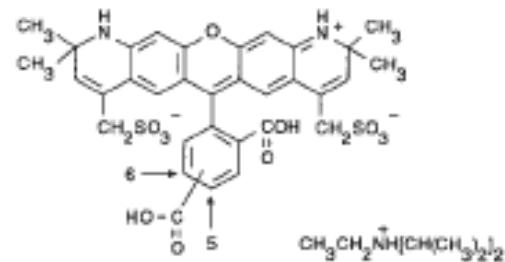
Alexa 488



Alexa 532



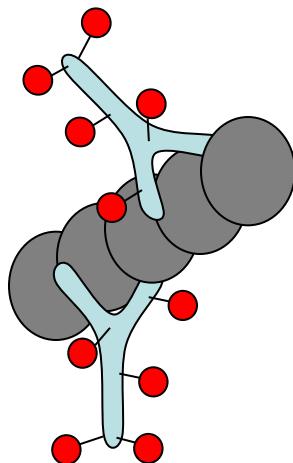
Alexa 546



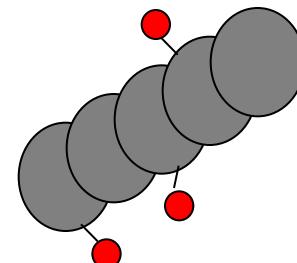
Alexa 568

Fluorescent labeling

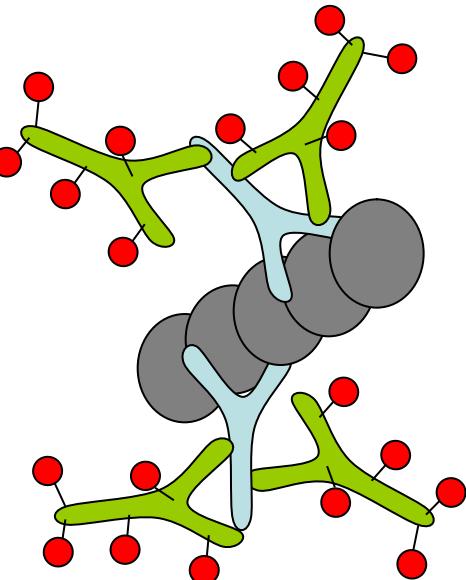
Direct immunofluorescence:
labeled antibodies against target



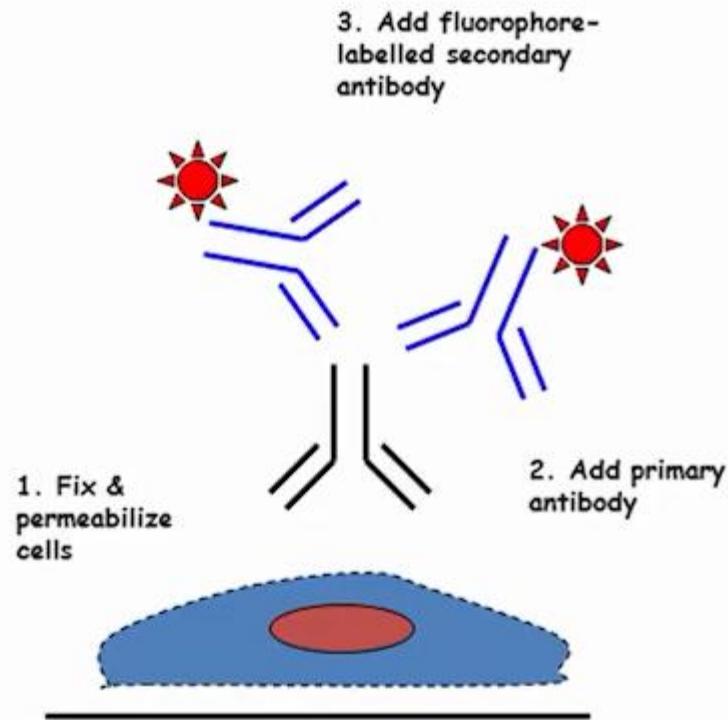
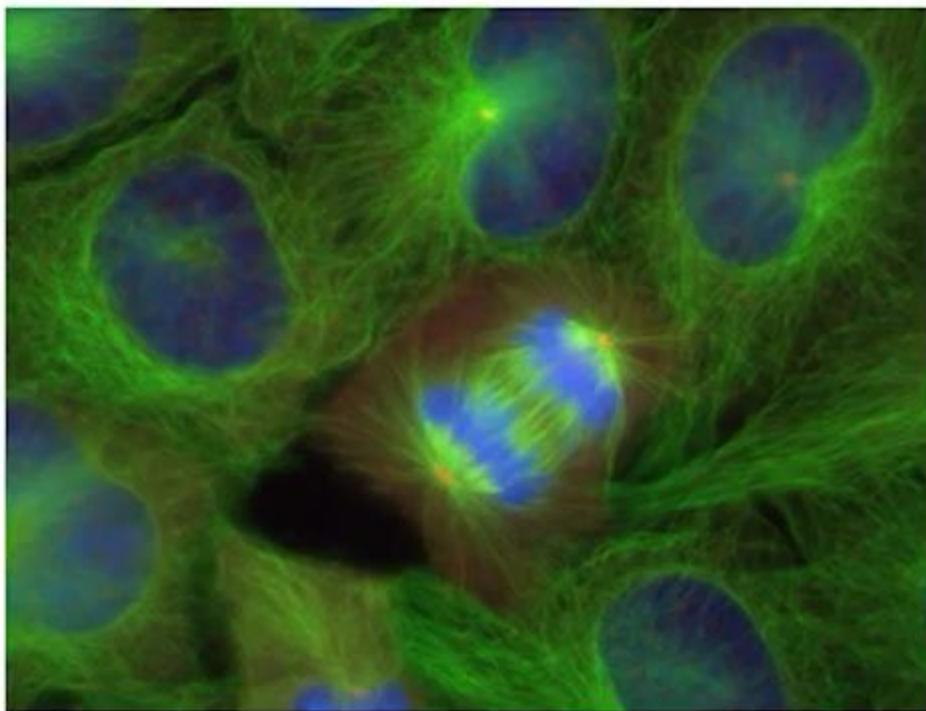
Direct labeling (& microinjection)
of target molecules



Indirect immunofluorescence:
Unlabeled antibodies against target
Labeled antibodies *against those antibodies*

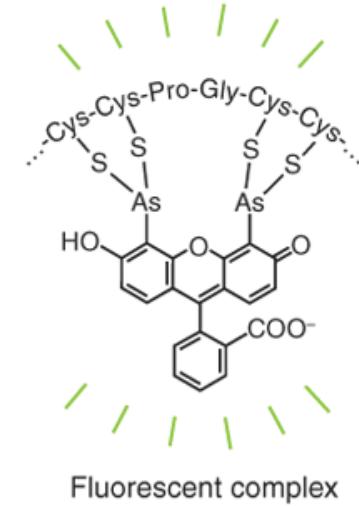
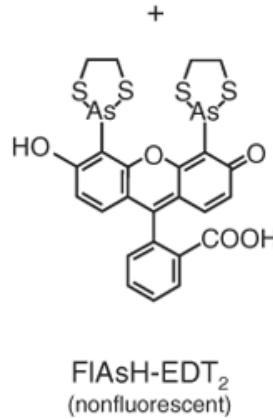


Antibody-based “immunofluorescence” microscopy of fixed cells

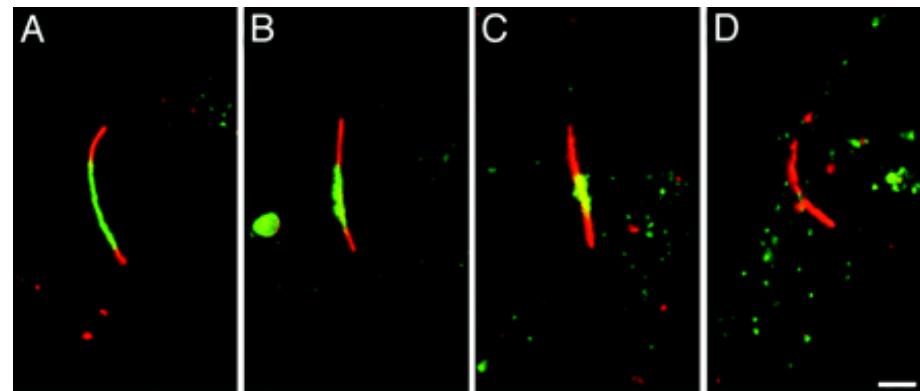


FLAsH/ReAsH

...-Cys-Cys-Pro-Gly-Cys-Cys-
(genetically encoded FLAsH
recognition sequence)



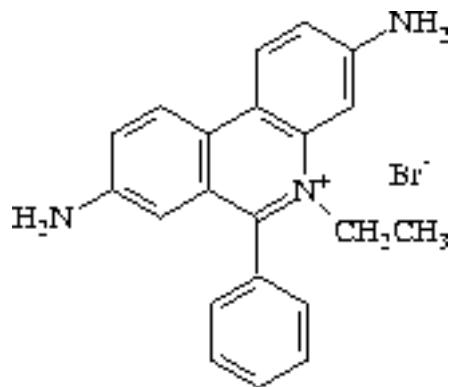
Bi-Arsenic FLAsH (green), ReAsH (red)



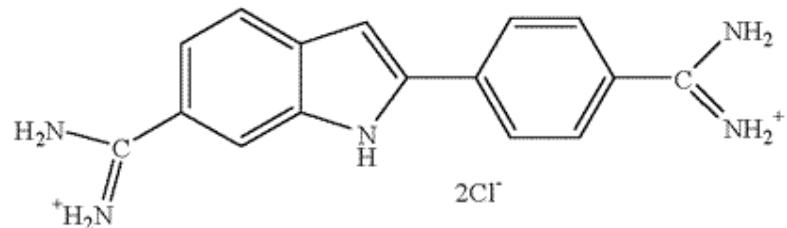
Bi-arsenic fluorescein binds to tetracysteine motif
Griffin, B. A. et al., *Science*, 1998

Gaietta G. et al., *Science*, 2002

DNA Probes



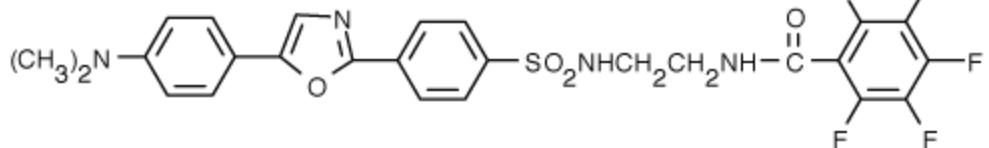
Ethidium Bromide
~30 fold enhancement



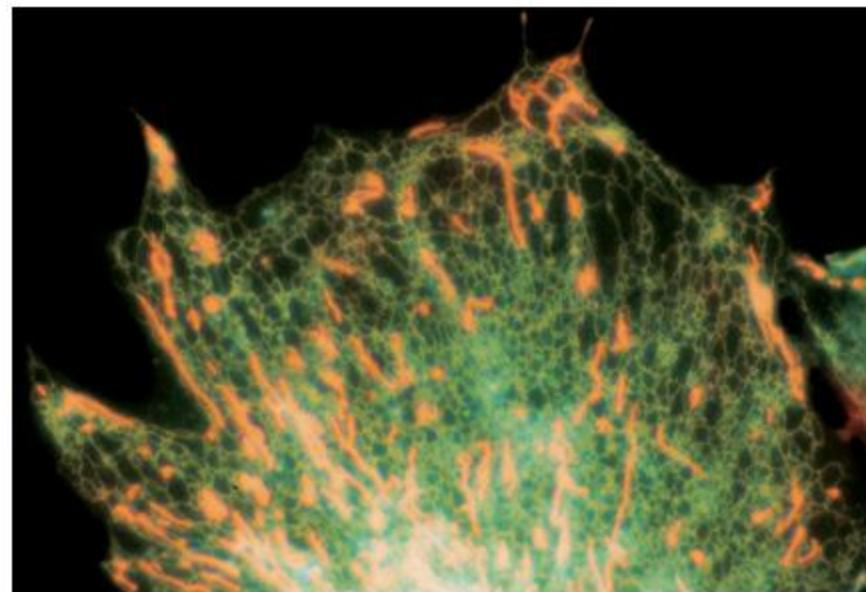
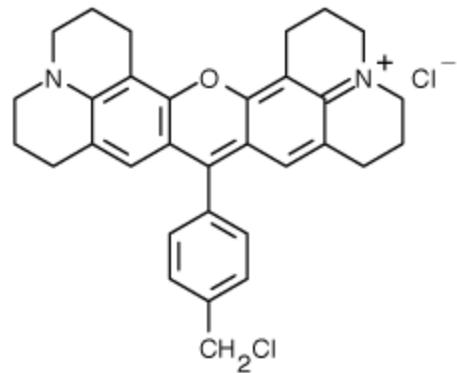
DAPI
Hoechst 33258
Hoechst 33342
~20 fold enhancement

Other probes

ER-Tracker™ Blue-White DPX

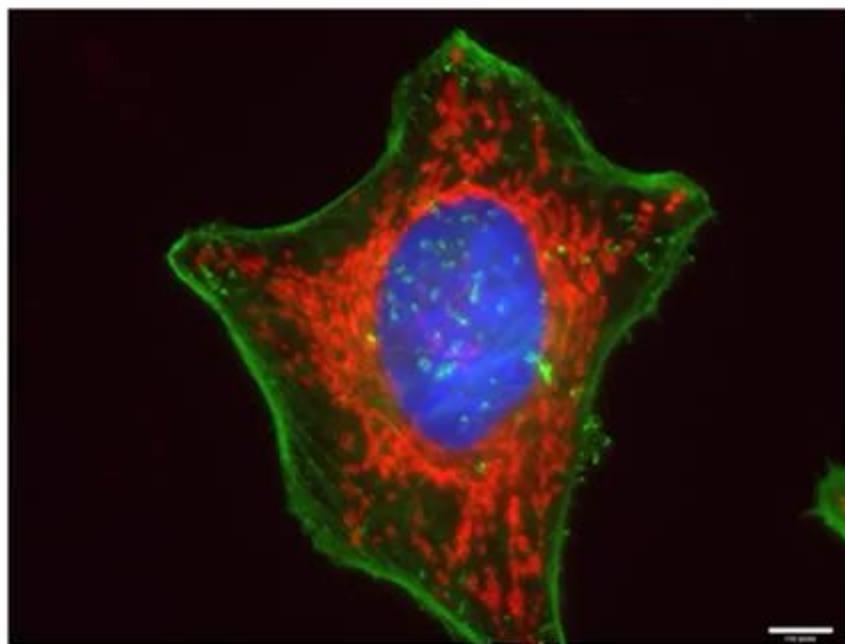


MitoTracker Red CMXRos

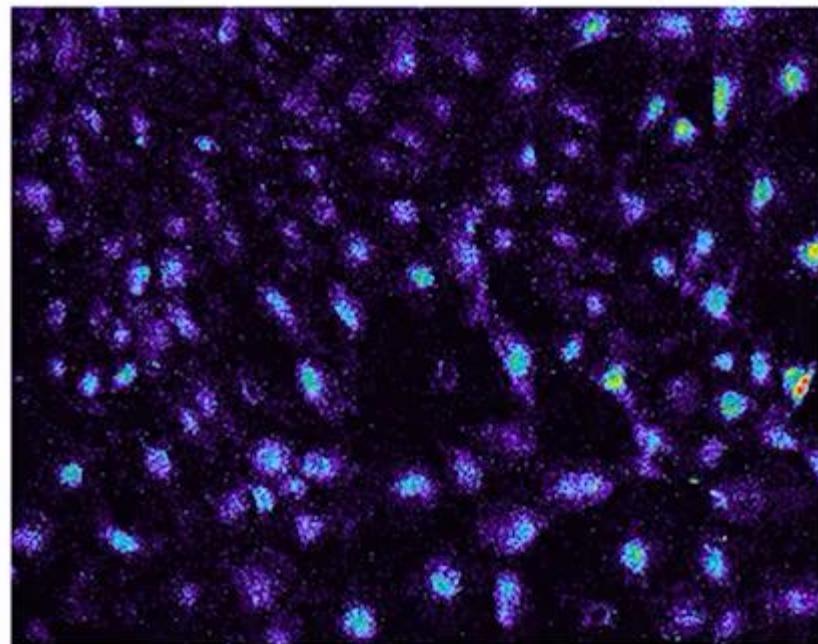


Probes for Golgi, lysosomes, and peroxisomes are also available

Organelle and chemical specific fluorescently-labelled dyes



Mitotracker labelling mitochondria
Phalloidin labelling actin
Hoechst 33258 labelling DNA



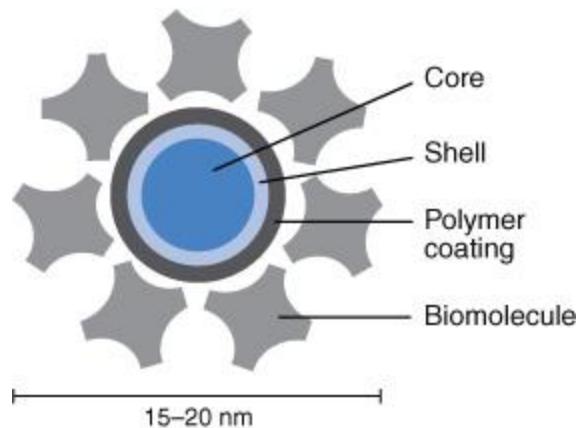
Fluo-3 imaging of intracellular calcium.
Leiper et al. *BMC Biology* 2006, 4:27.

Small molecules – pros / cons

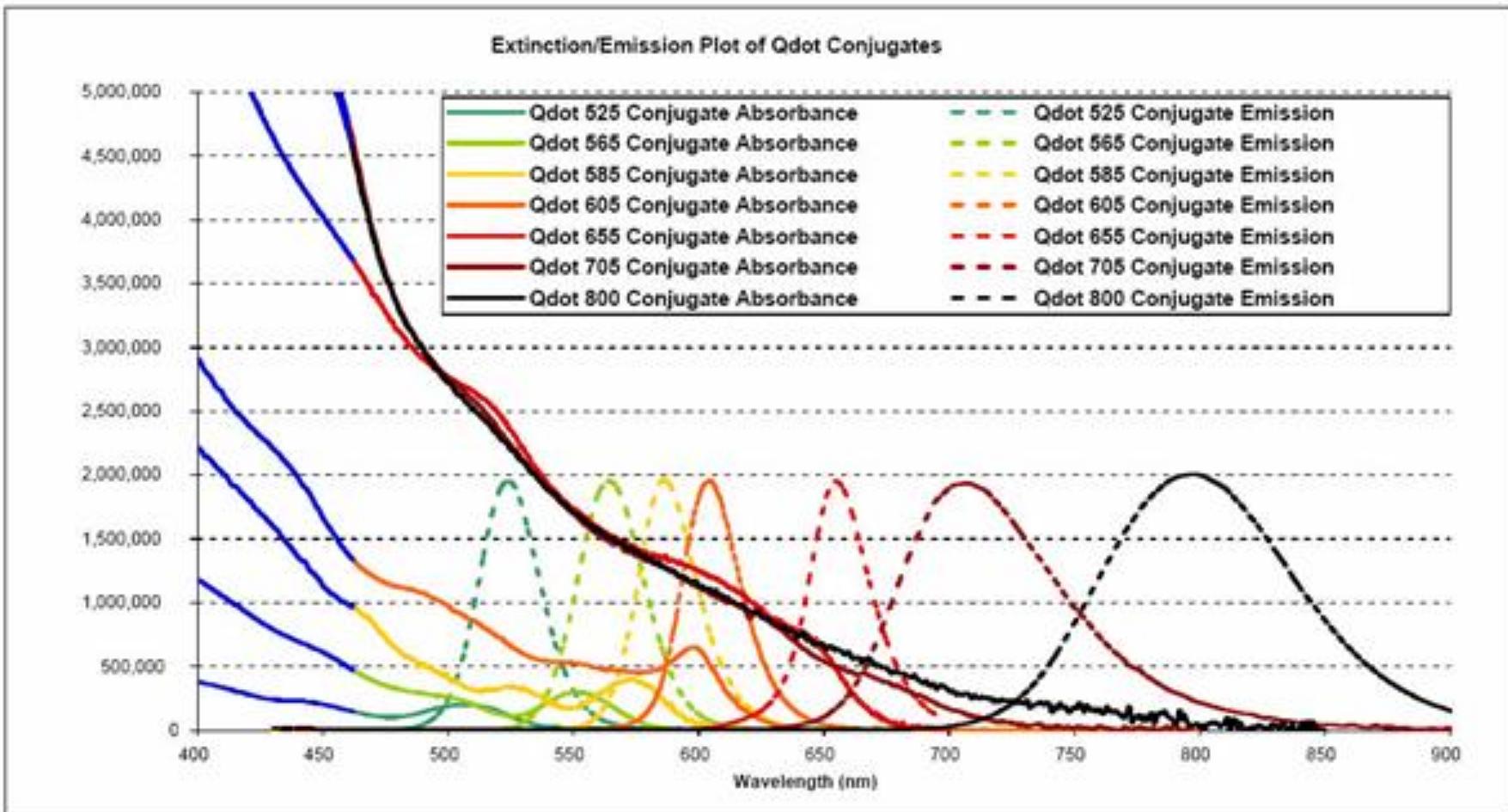
- 1000s available – huge spectral range
- Easy to acquire
- Precisely tailored properties, including environmental sensitivity
- Require fixing and staining, which can lead to artifacts
- Potential self-quenching and environmental sensitivity

Quantum dots

- “Artificial atoms” composed of small semiconductor nanocrystals



Quantum dots - spectra

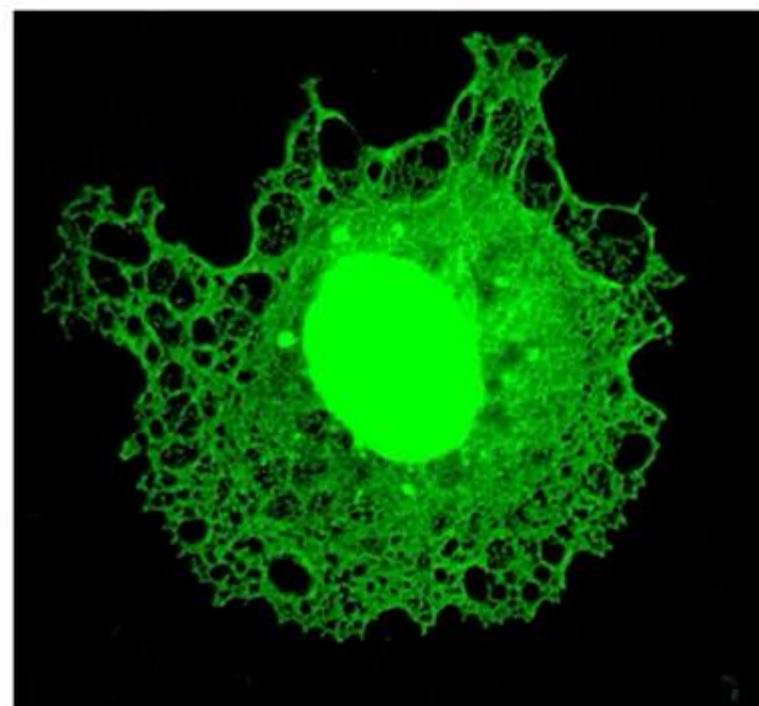
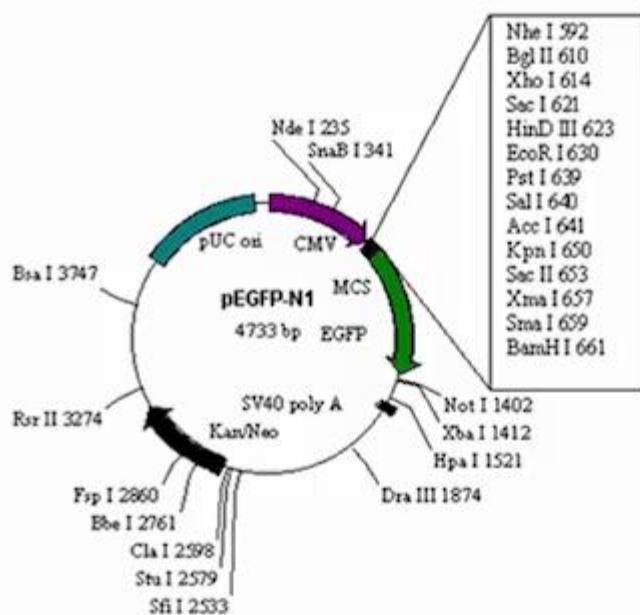


Quantum dots – pros / cons

- Little to no photobleaching
- Very bright
- Narrow emission spectra
- Can use single excitation wavelength for multiple dyes

- Large compared to small molecule dyes
- Problems with non-specific binding

GFP-based fluorescence microscopy

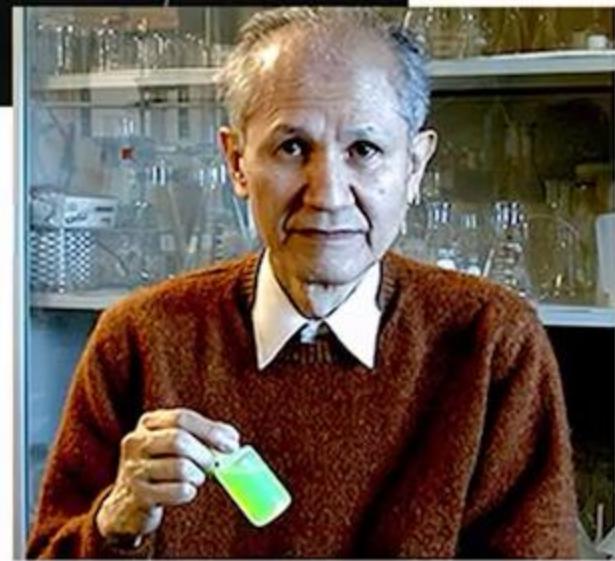
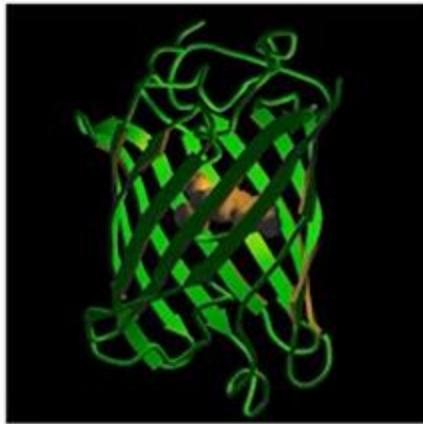


N Protein X GFP C

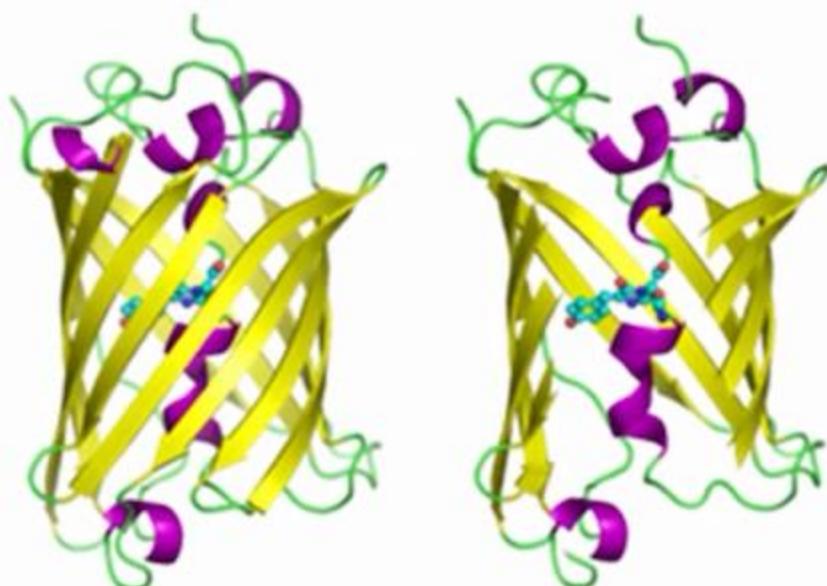
The GFP revolution (part I)

Green fluorescent protein (GFP)
from the jellyfish *Aequoria victoria*.

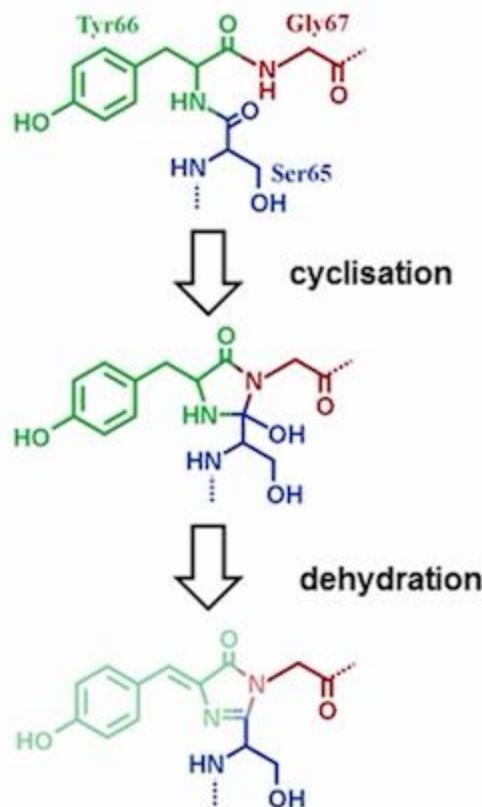
1962 Discovery in jellyfish
Osamu Shimomura



How GFP works



3 amino-acid based chromophore
embedded in β -barrel

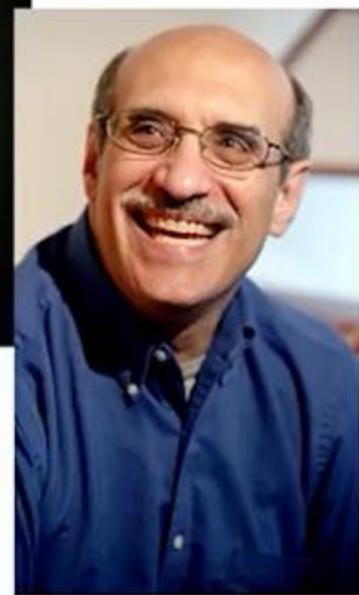
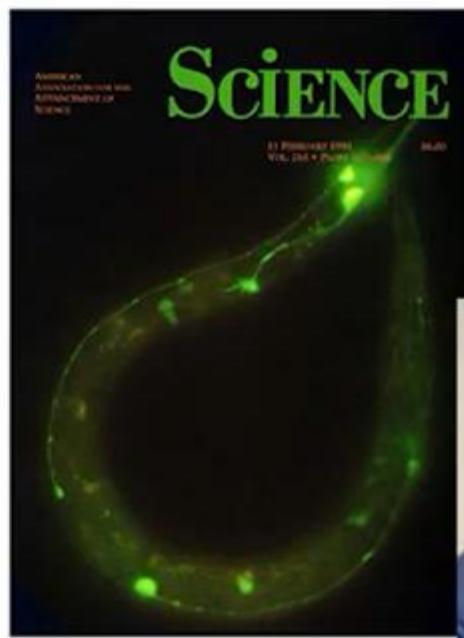


The GFP revolution (part II)

Green fluorescent protein (GFP)
from the jellyfish *Aequorea victoria*.

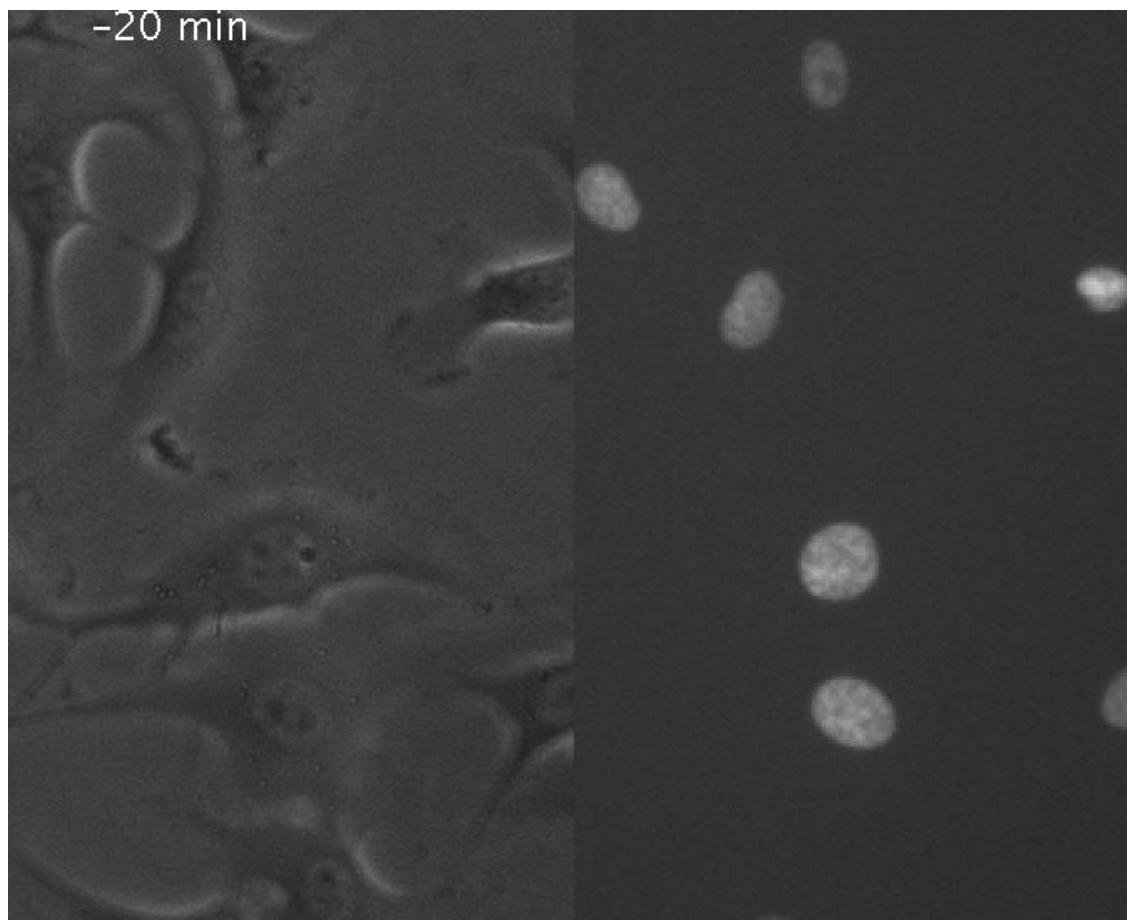
1962 Discovery in jellyfish
Osamu Shimomura

1994 Used to tag a *C. elegans* protein
Martin Chalfie



**Fluorescent proteins allow live cell
time-lapse imaging**

Fluorescent proteins allow live cell time-lapse imaging



The GFP revolution (part III)

Green fluorescent protein (GFP) from the jellyfish *Aequoria victoria*.

1962 Discovery in jellyfish

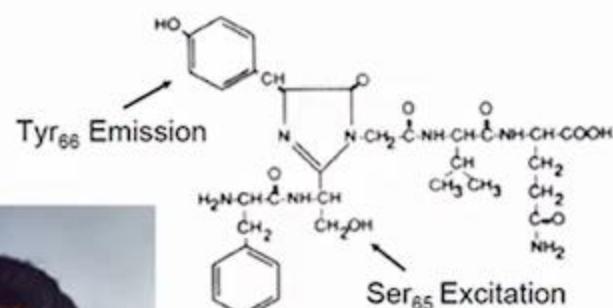
Osamu Shimomura

1994 Used to tag a *C. elegans* protein

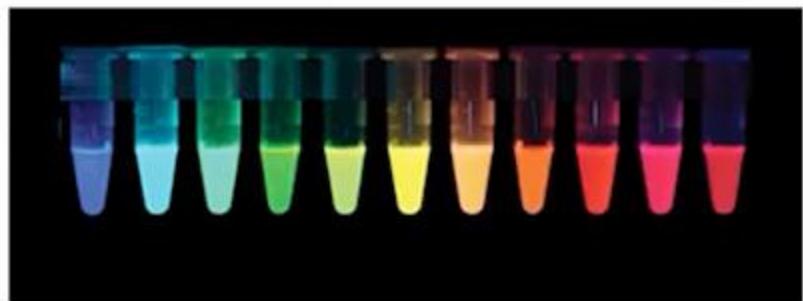
Martin Chalfie

1992 GFP colour variants developed

Roger Tsien



>100 variants now
available



GFP in vivo

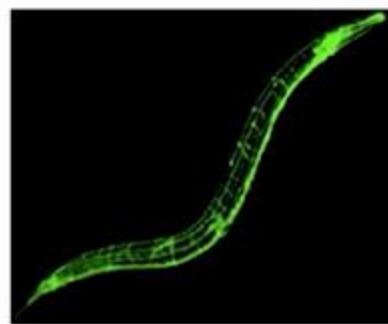
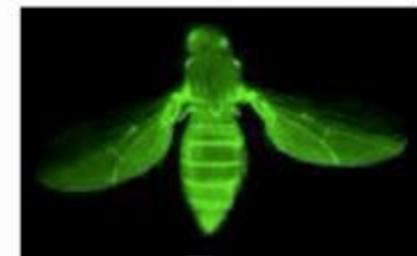
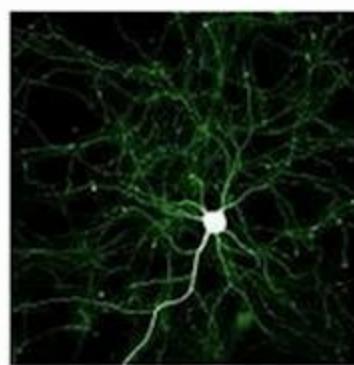
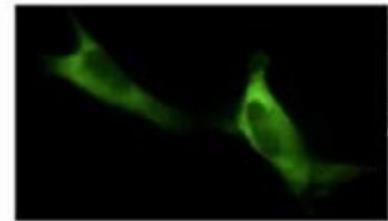
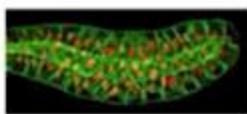


Photo-active GFP

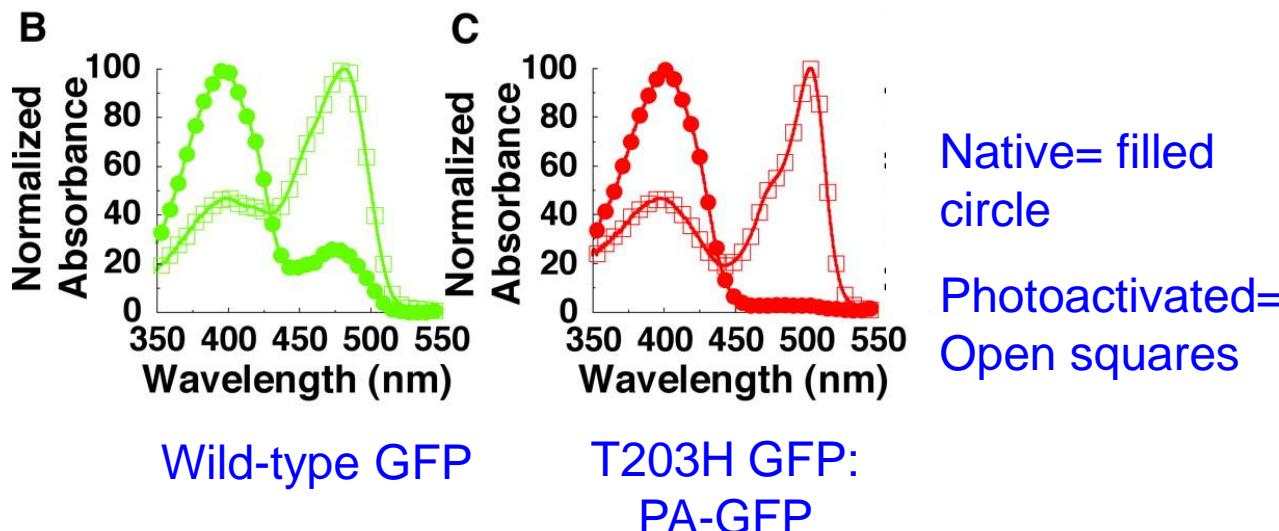
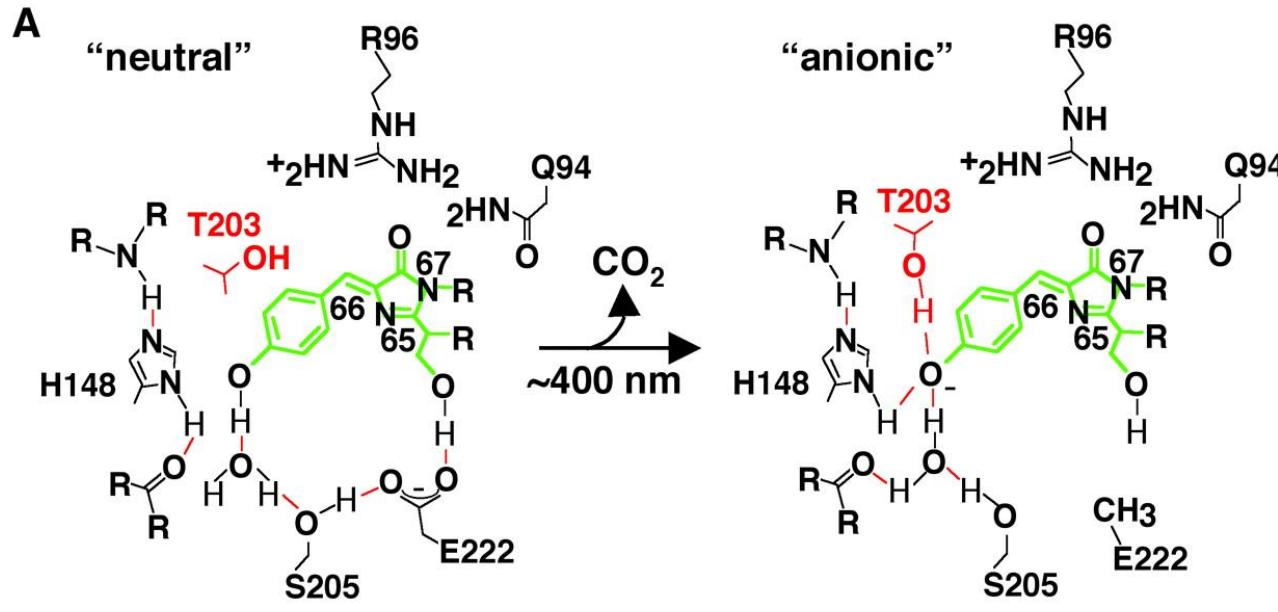
G. H. Patterson et al., *Science* 297, 1873. (2002)

Photo-activation and Imaging *In Vitro*

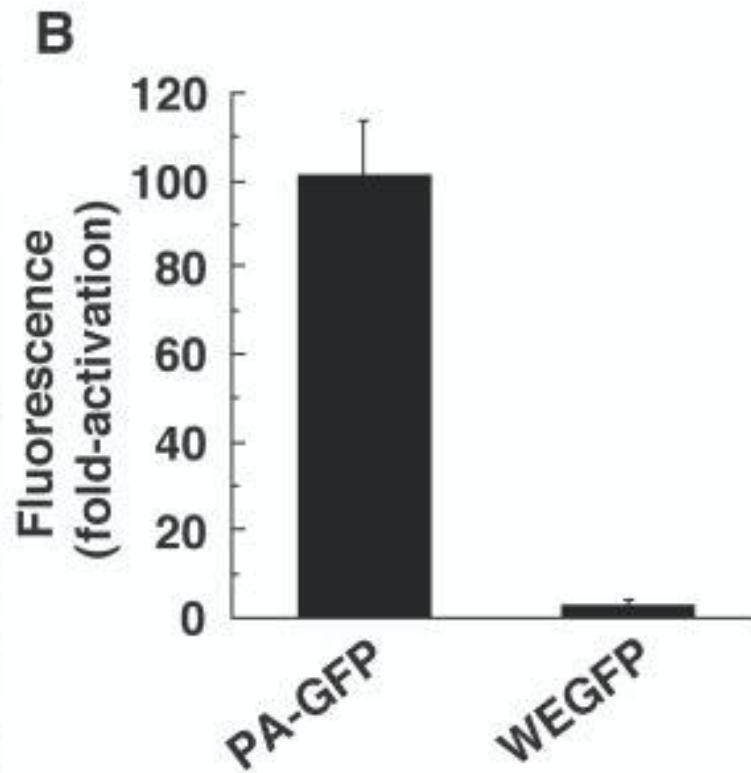
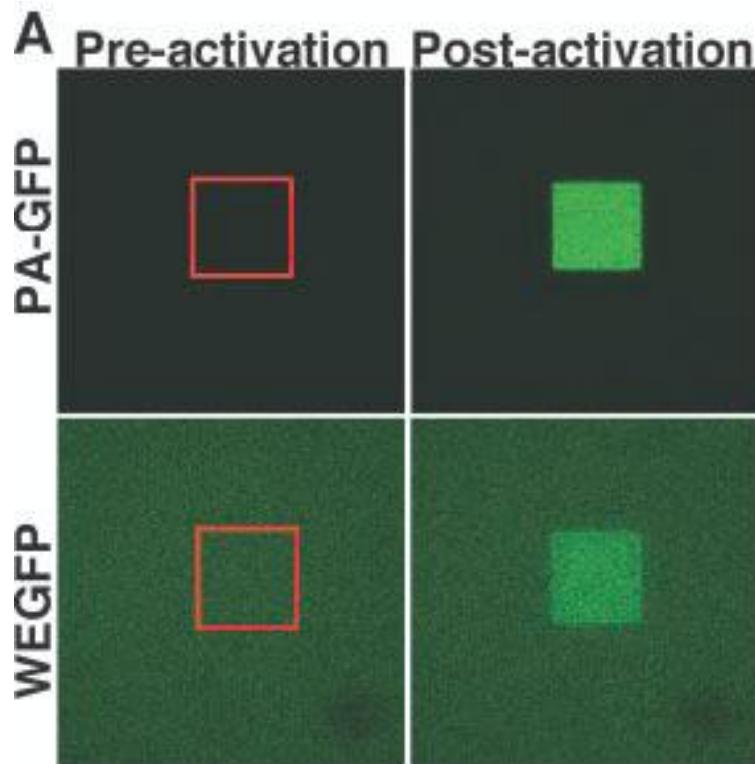
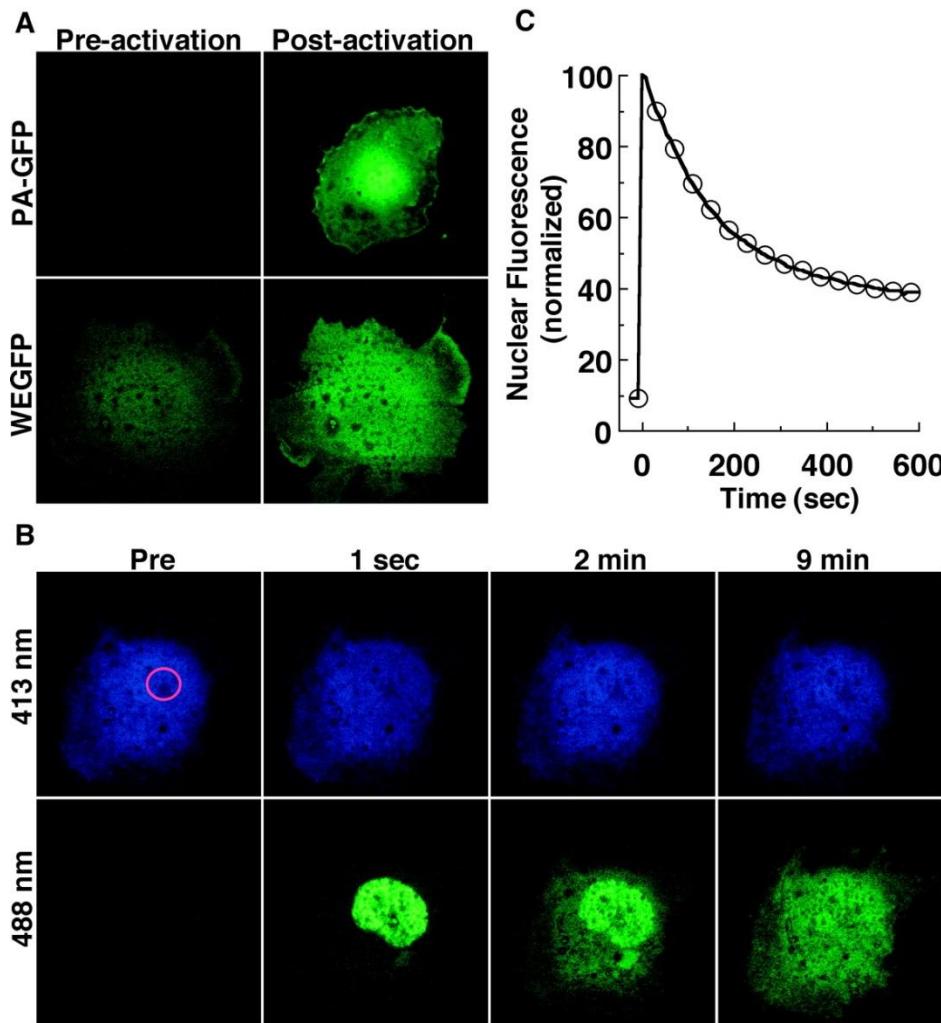
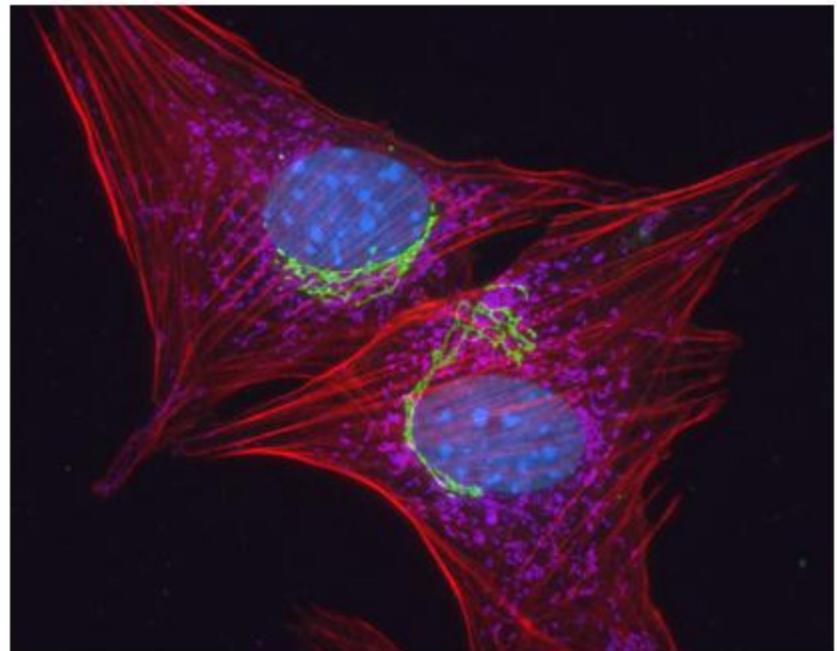
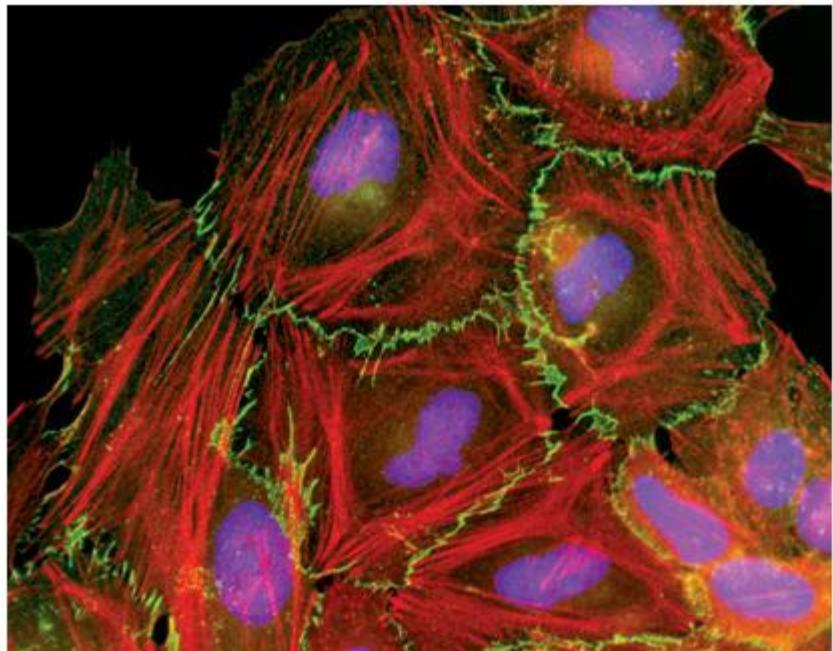


Photo-activation and Imaging *In Vivo*



You can get beautiful pictures



The use of imaging technology in cell biology

- 1. Introduction: what it is & how did it all start**
- 2. Applications & techniques, including some examples from our research**
- 3. Super-resolution microscopy**
- 4. Facilitates at SUSTC & how to start using them**
- 5. What does the future hold...**

Fluorescence imaging is the principle tool of cell biology

1. Individual proteins / DNAs / RNAs

Subcellular localization

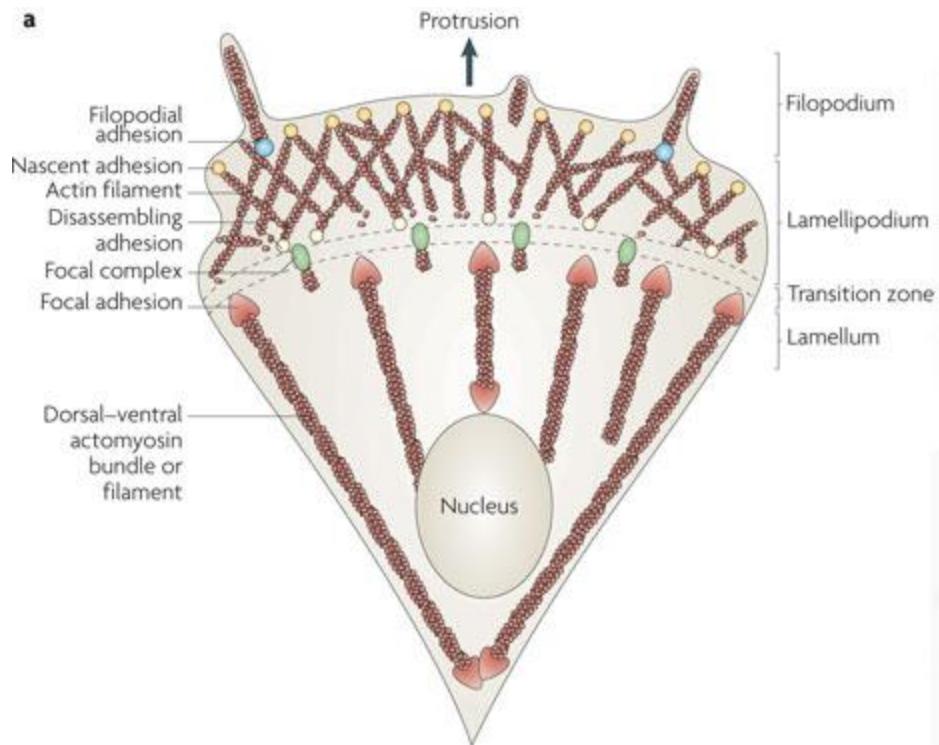
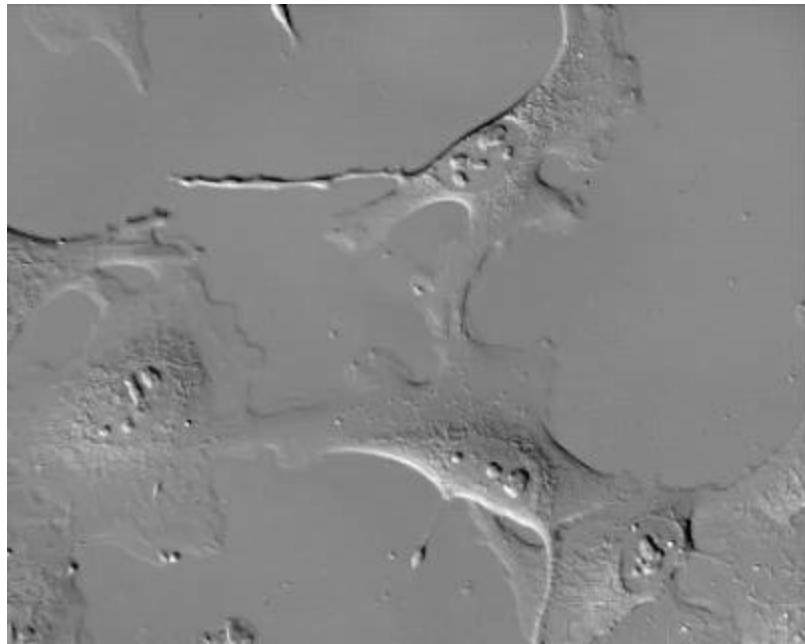
Colocalization

Dynamics

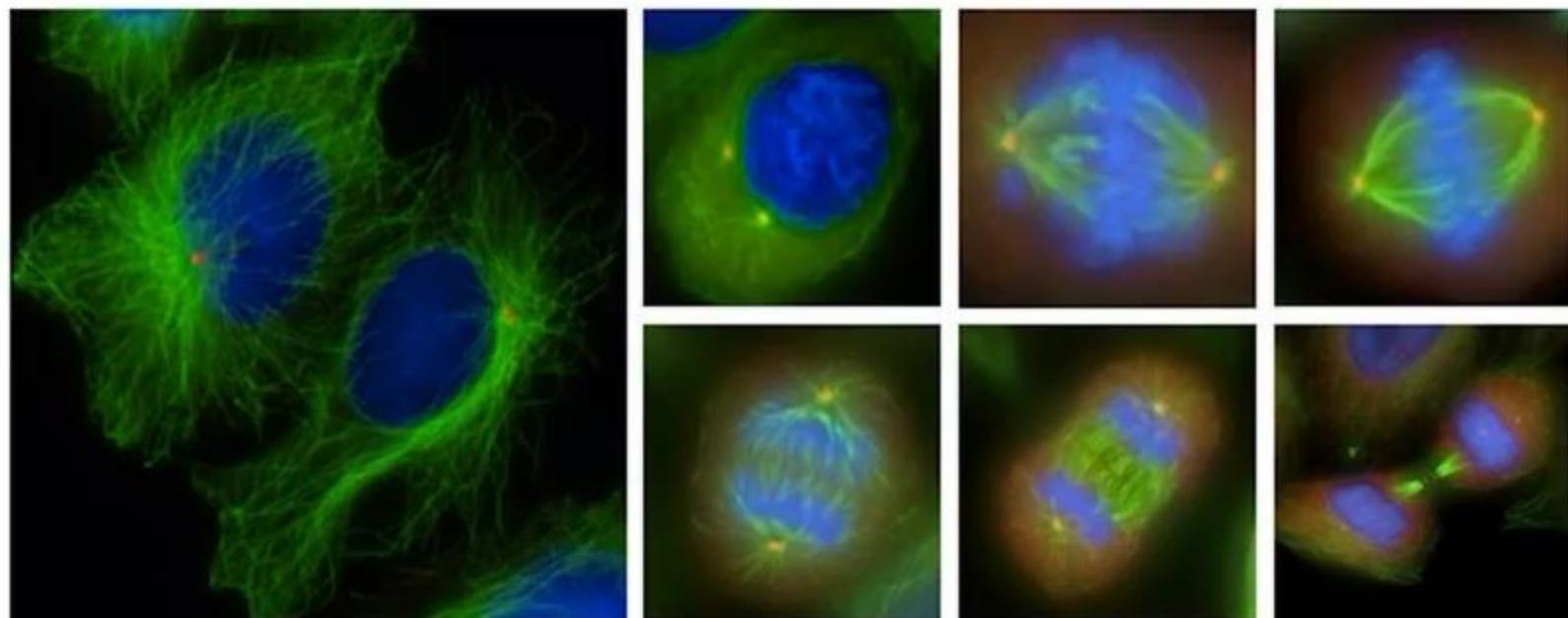
2. Biological processes

Functional phenotypes through analysis of cell structures or behaviour

What we study: The molecular control of cell movement

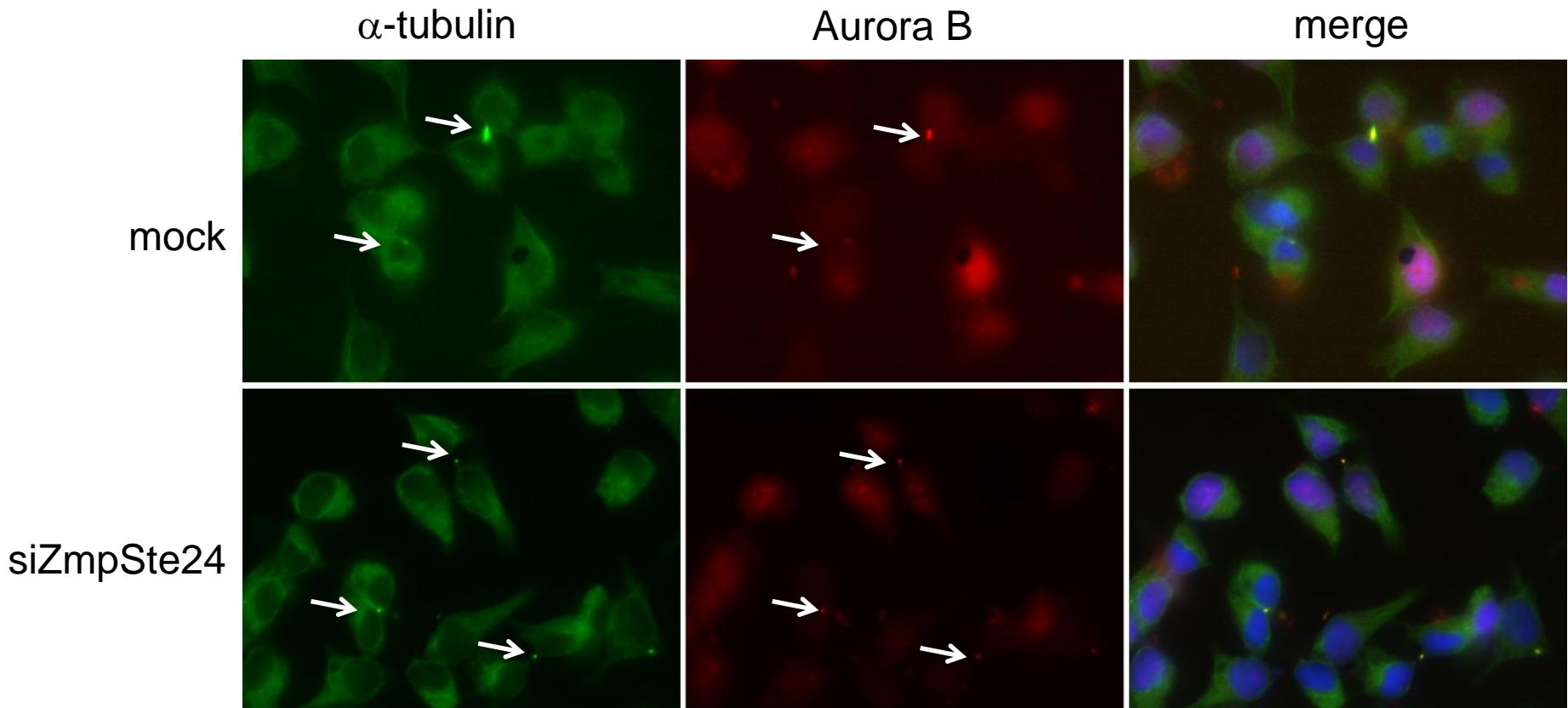


Immunofluorescence microscopy of fixed cells: observing subcellular localization

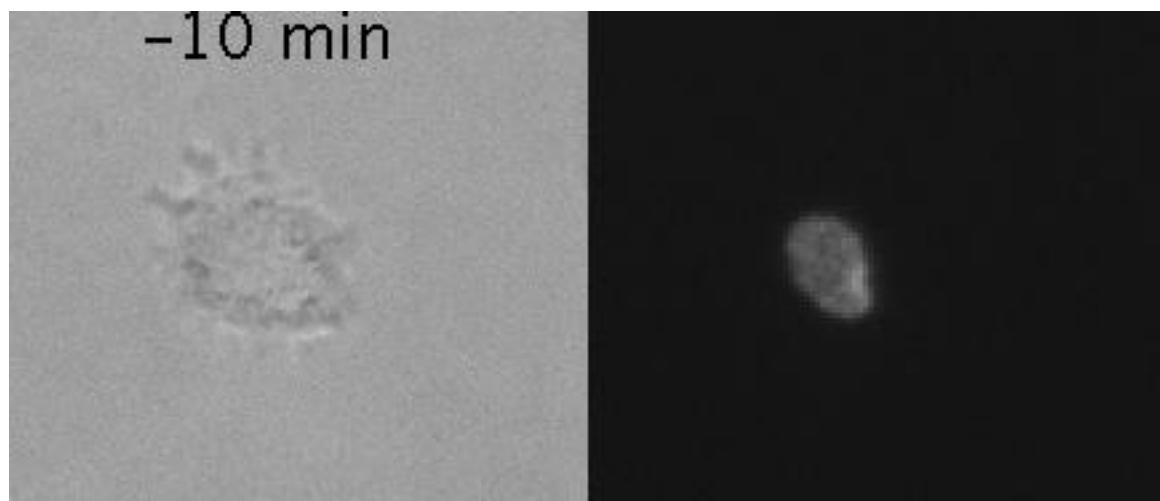


Nuclei / Microtubules / Centrosomes

Immunofluorescence microscopy of fixed cells: colocalization

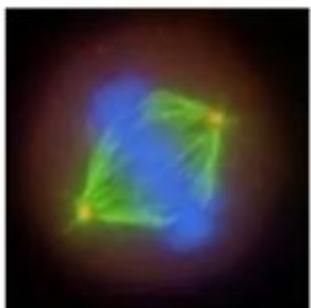


Time-lapse imaging of cells expressing GFP-tagged proteins

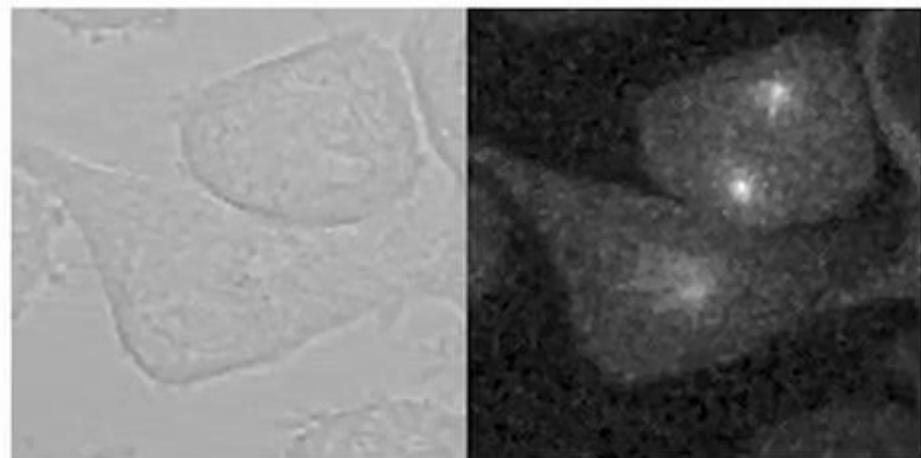
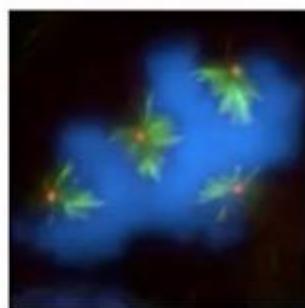
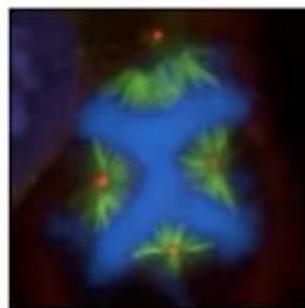


Abnormal cell division in cancer cells

Bipolar spindles



Multipolar spindles

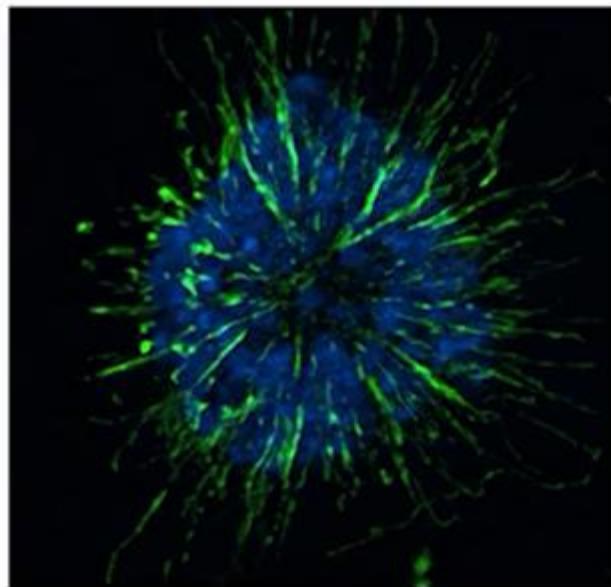
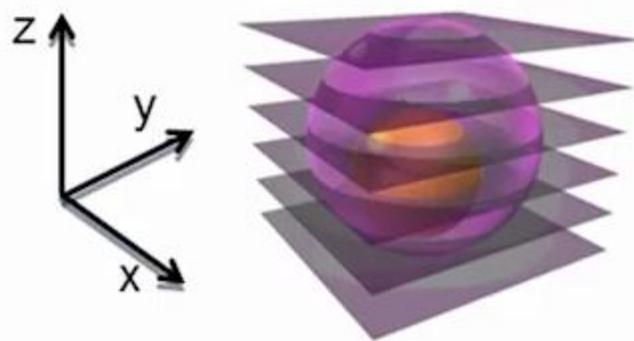


Brightfield

GFP-tubulin

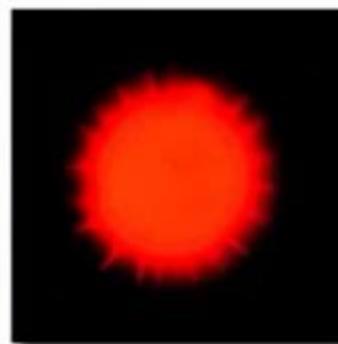
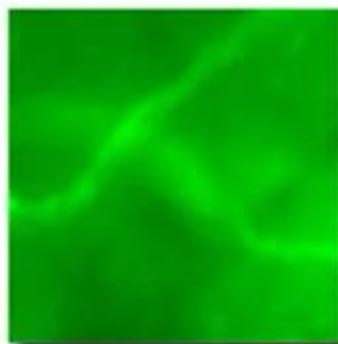
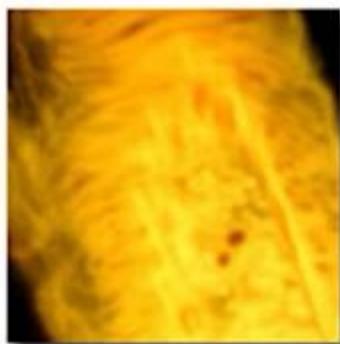
Nuclei / Microtubules /
Centrosomes

Obtaining sharper images I: deconvolution microscopy

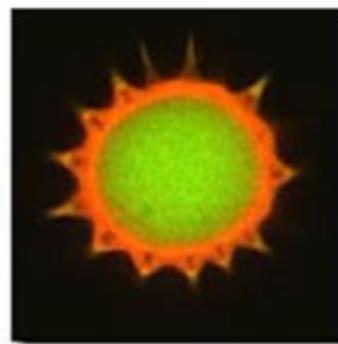
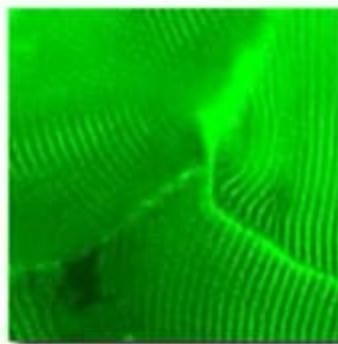
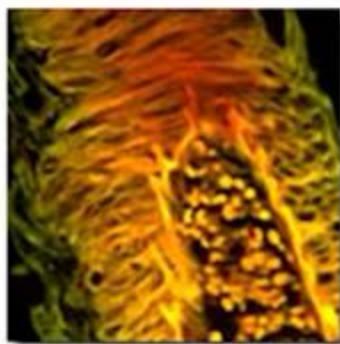


- Capture multiple z-sections using widefield microscopy (piezo device)
- Use computer-based algorithms to remove out of focus light

Obtaining sharper images II: confocal microscopy

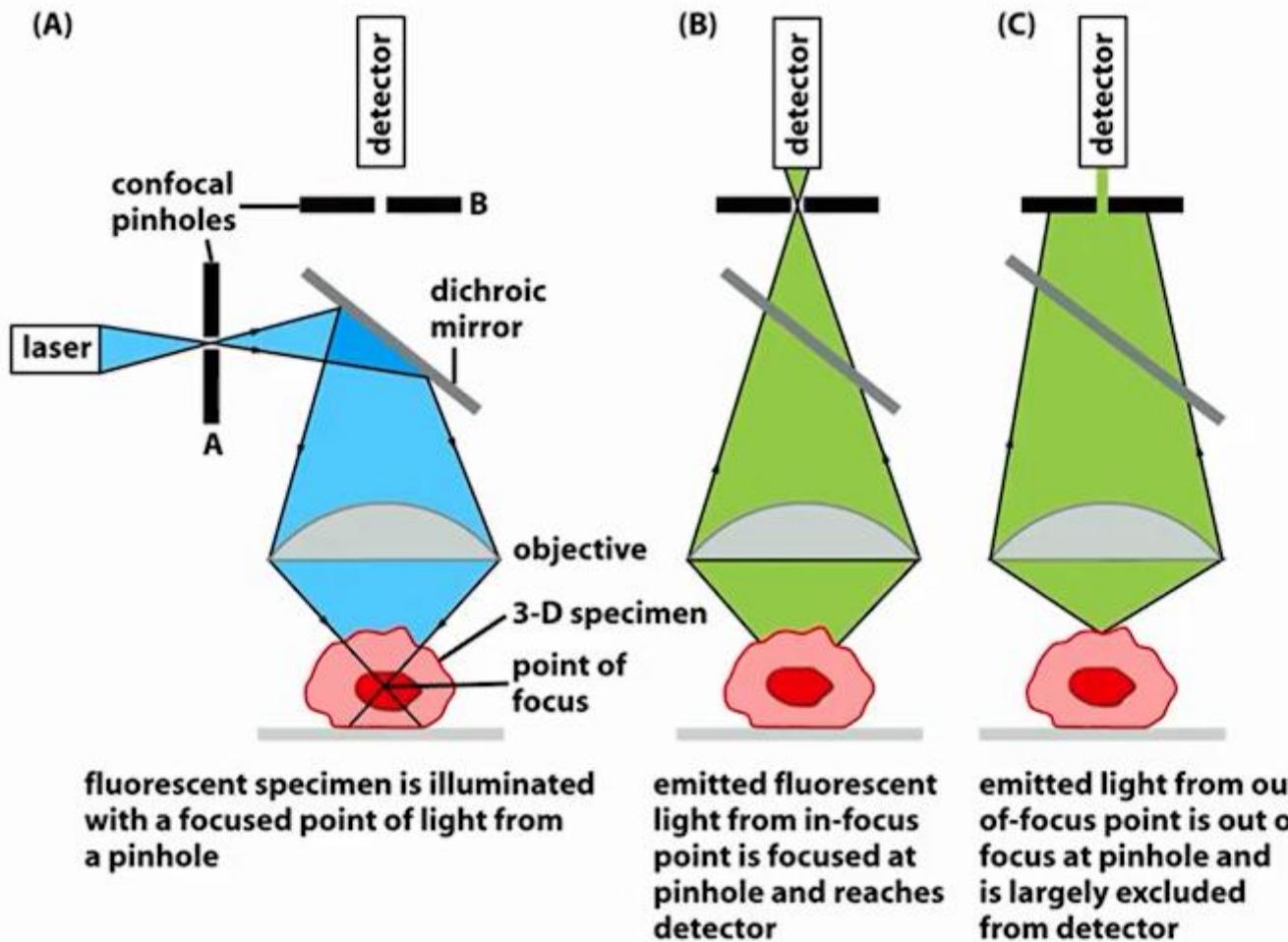


Nonconfocal images



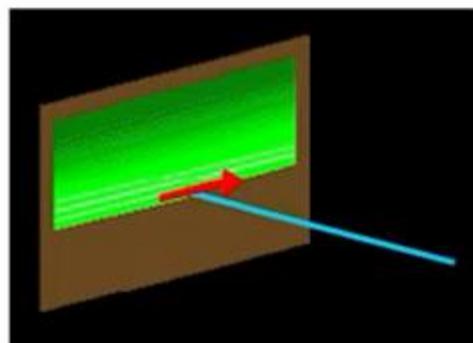
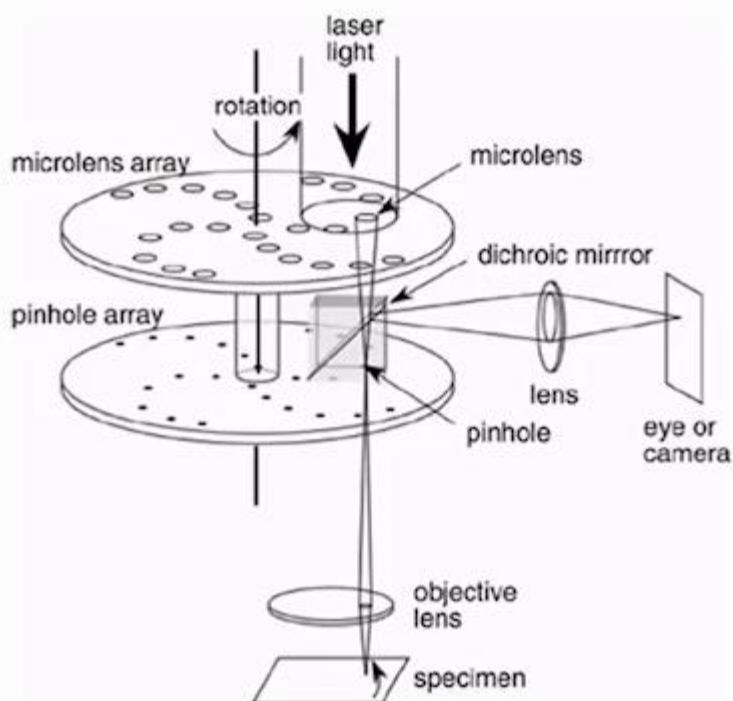
Confocal images

The principle of confocal microscopy

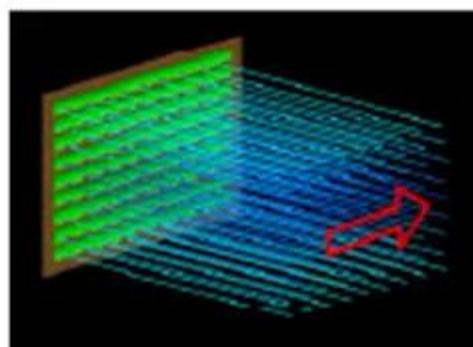


Spinning disk microscopy

A. Nakano



CLSM

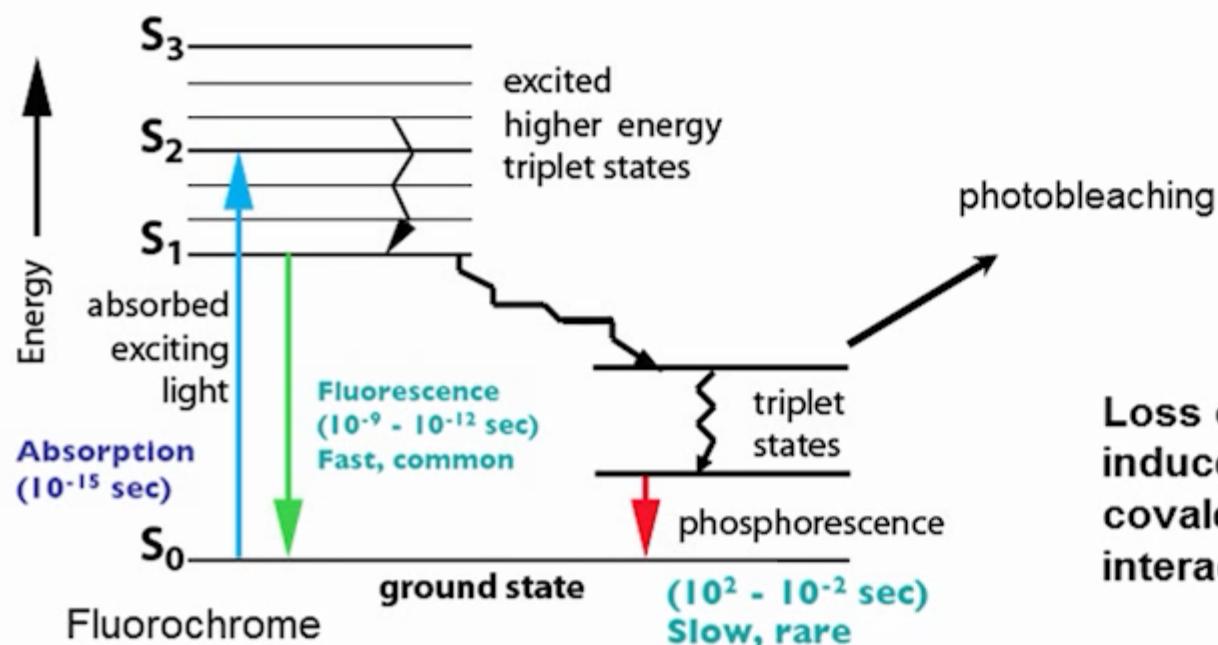


Spinning
disk

Advantages over CLSM: Much faster acquisition rate & reduced photodamage

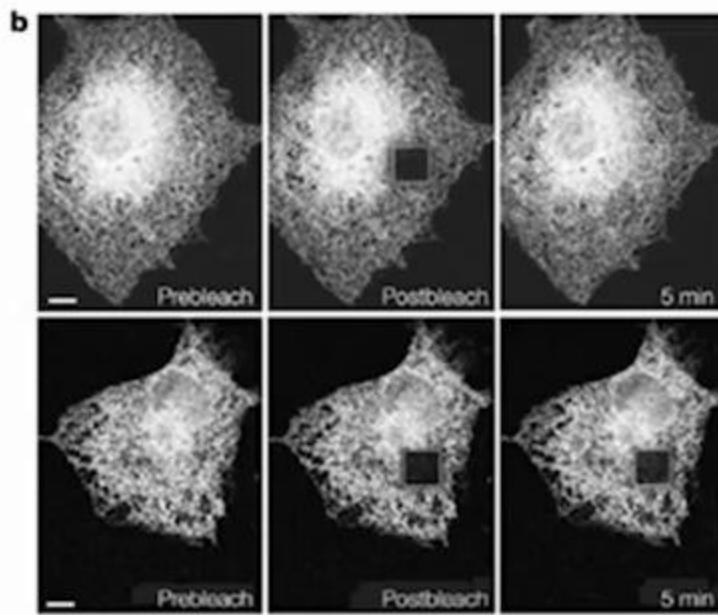
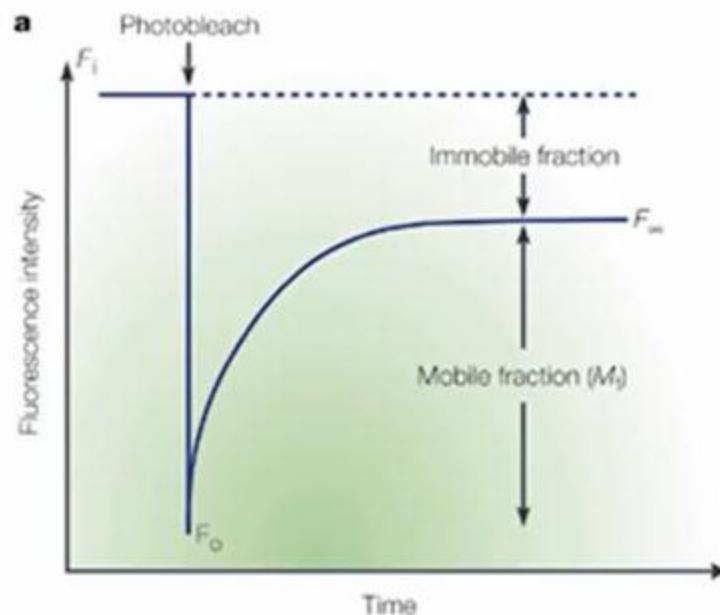
Disadvantages compared to CLSM: generally not as flexible, e.g. fixed resolution

Photobleaching techniques



Loss of fluorescence due to photon-induced chemical damage and covalent modification as a result of interaction with other molecules

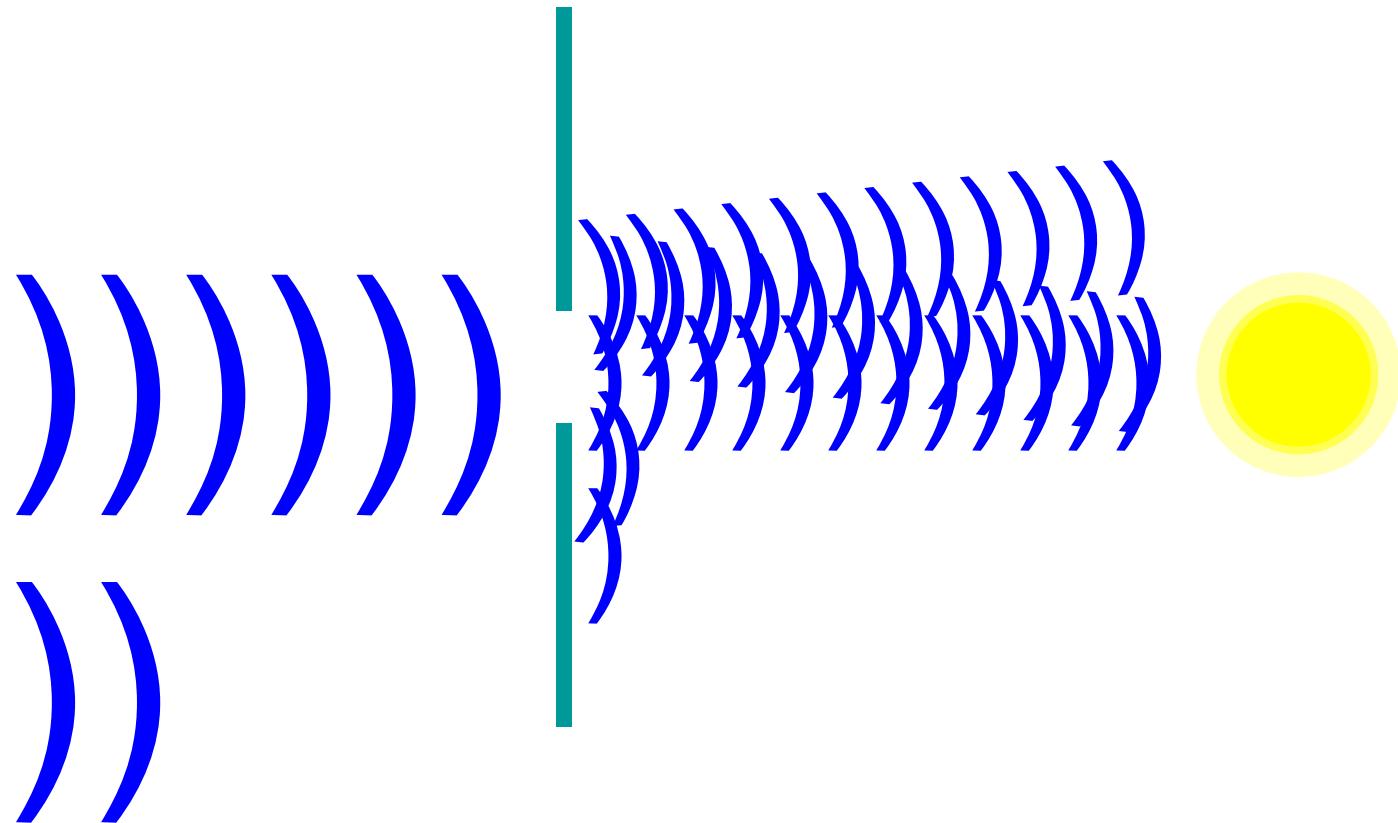
Fluorescence Recovery After Photobleaching (FRAP)



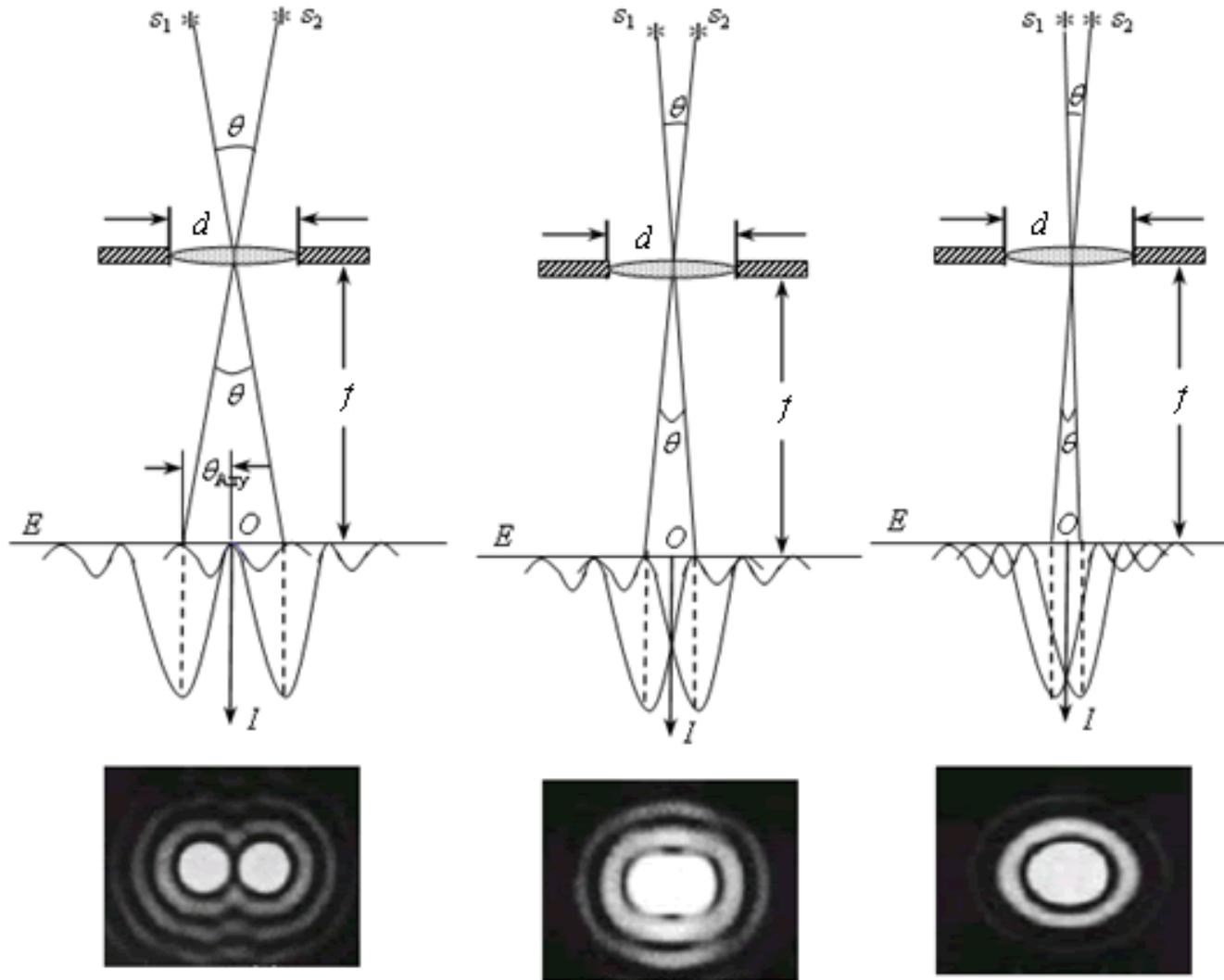
The use of imaging technology in cell biology

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- 3. Super-resolution microscopy**
- 4. Facilitates at SUSTC & how to start using them**
- 5. What does the future hold...**

Diffraction



Limits of Resolution



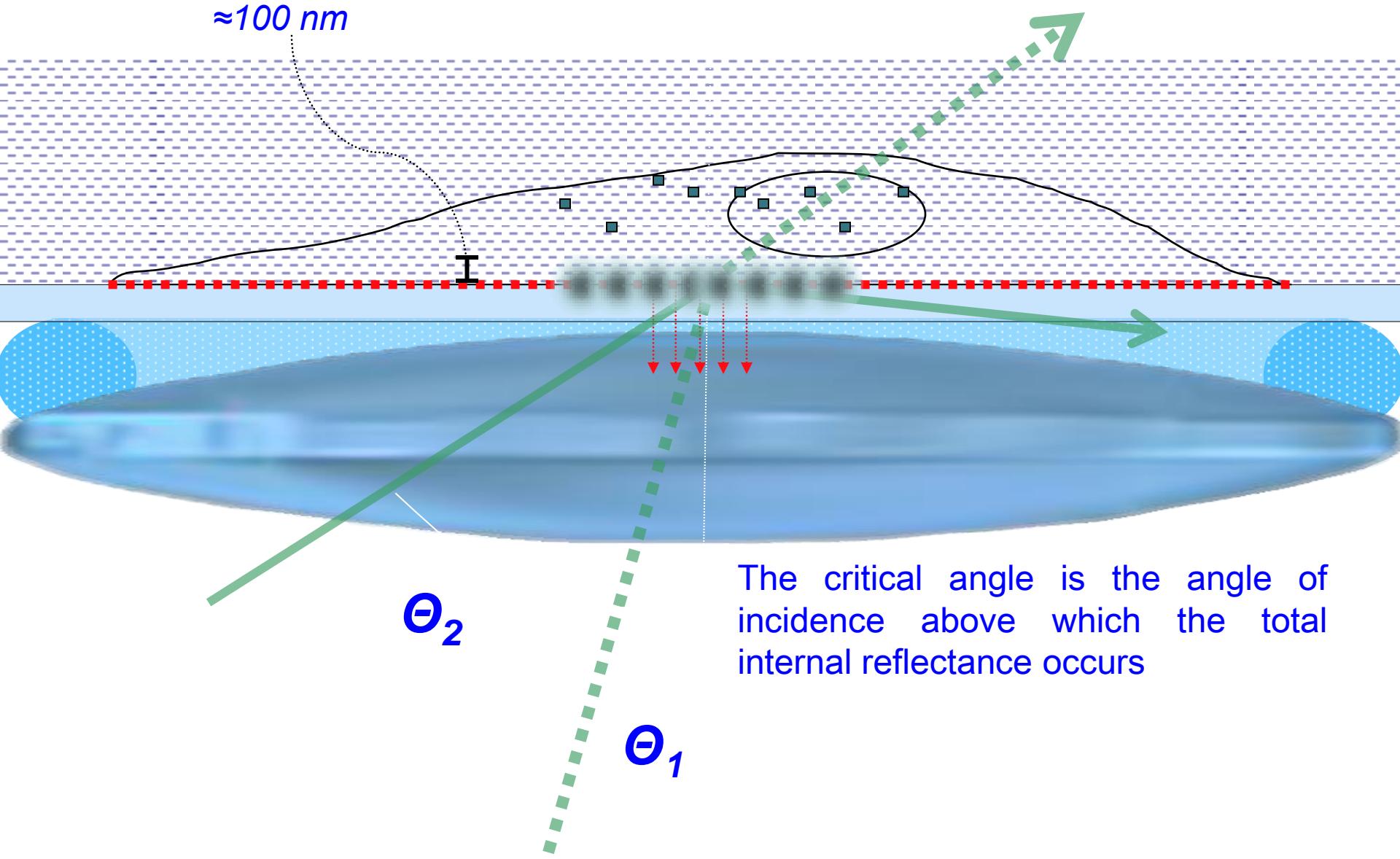
Diffraction Limit - The Bane of Imaging

Abbe diffraction limit

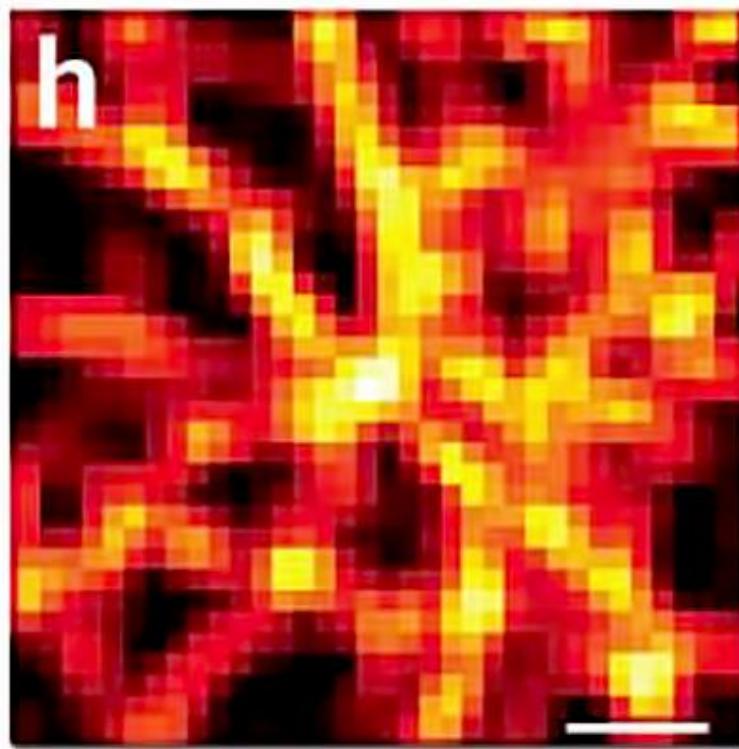
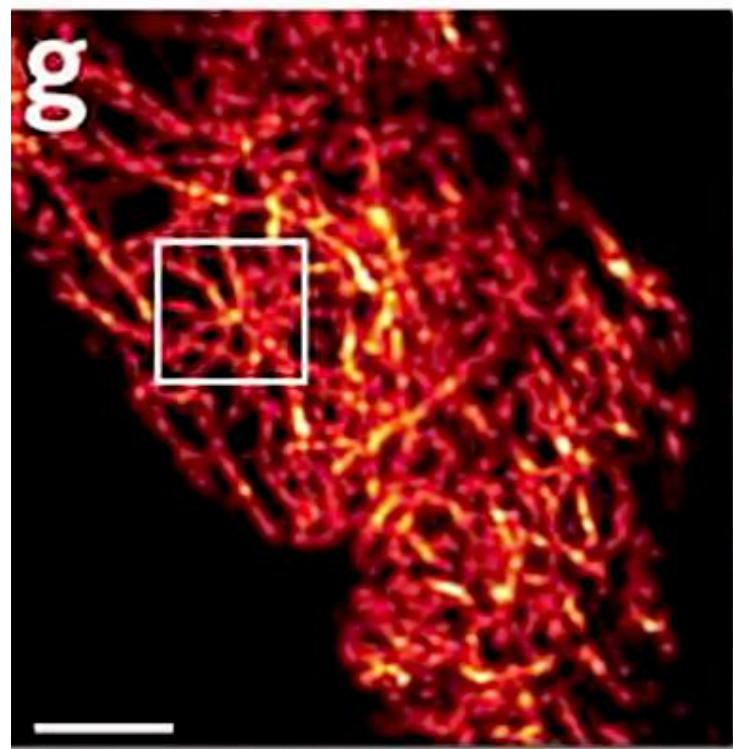


Total internal reflection

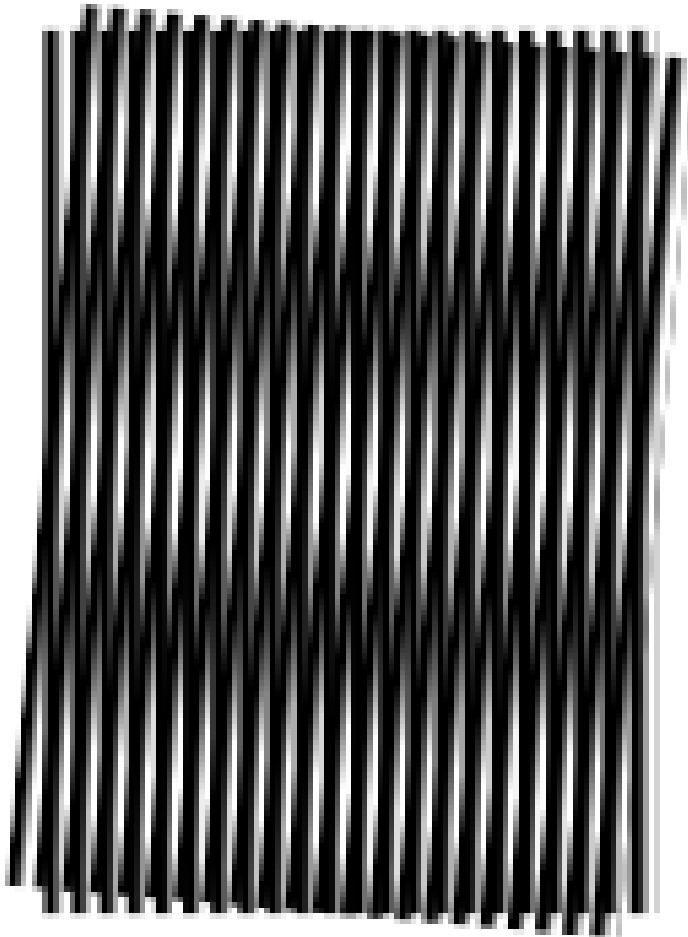
Evanescence wave



Total Internal Reflection Fluorescence Microscopy (TIRF)

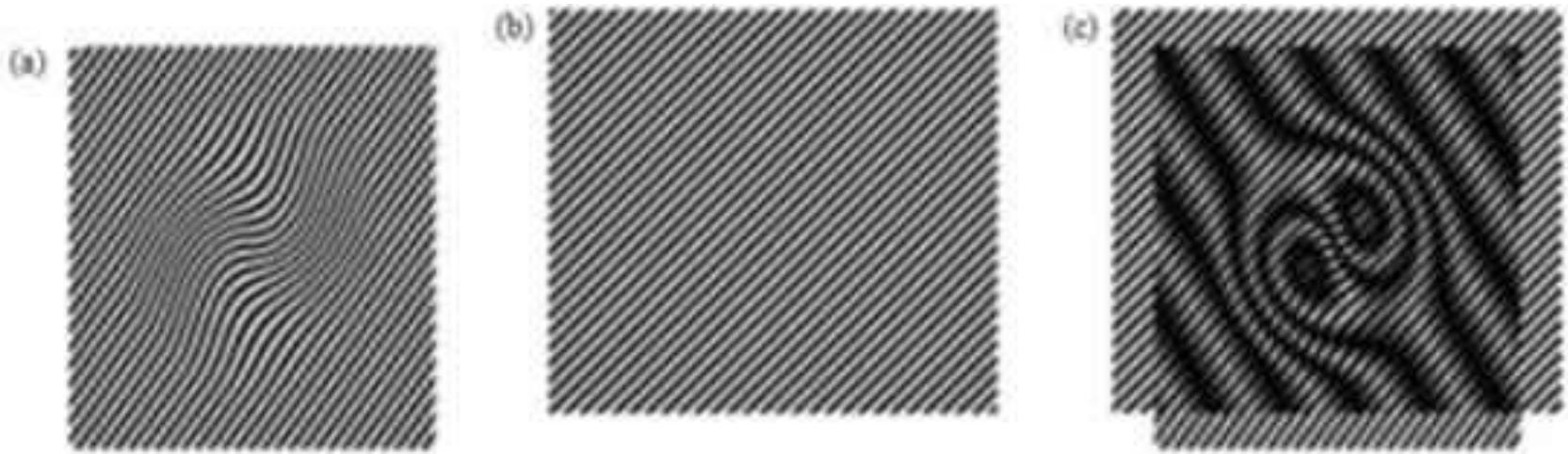


Moiré Pattern (莫尔条纹)

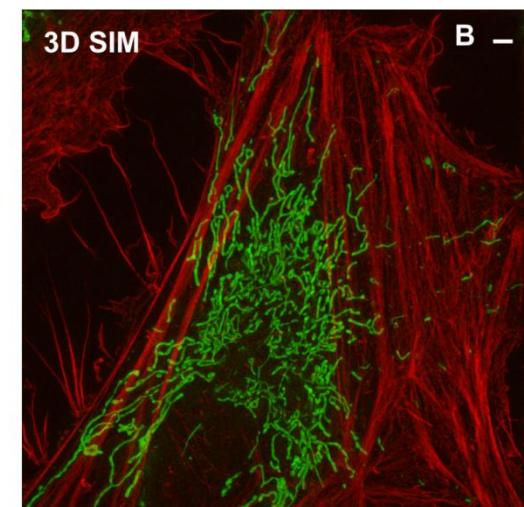
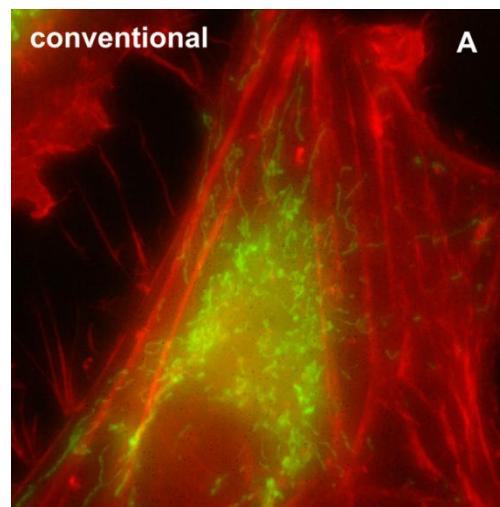
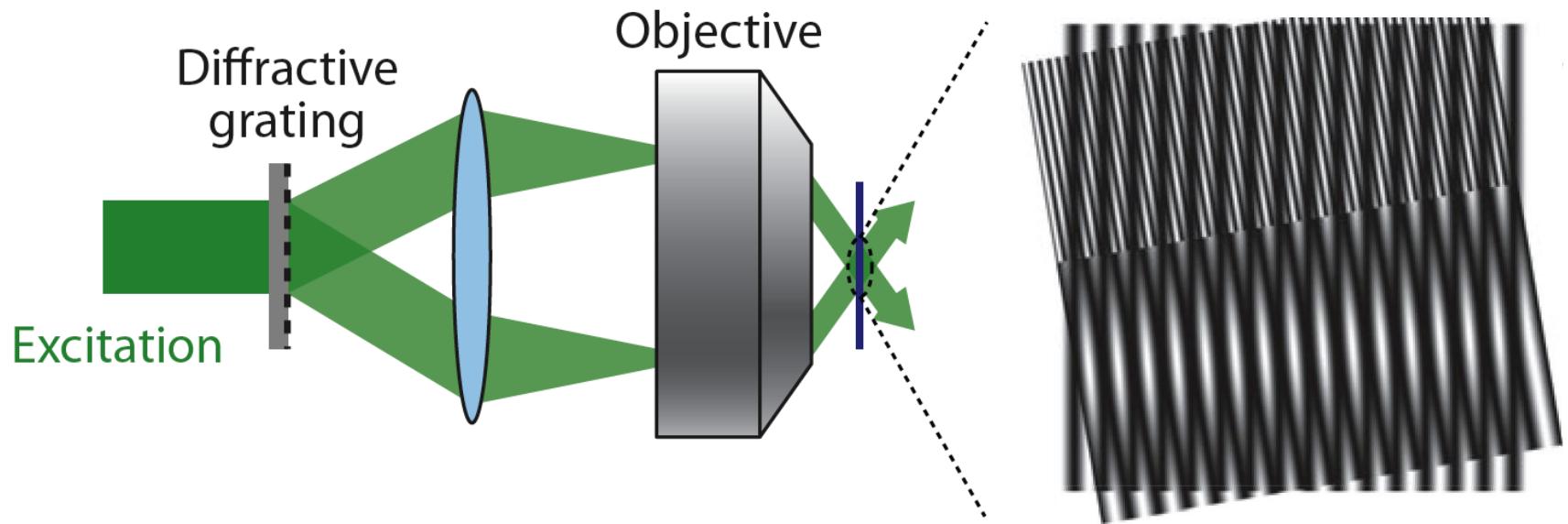


Structured Illumination Microscopy (SIM)

The Moiré effect



Structured-Illumination Micropscopy (SIM)



SIM (pros & cons)

- + up to 4 colors, standard dyes (e.g., Alexa, GFP...)
- + 3D with 2x resolution in XY and Z (8x volumetric)
- + Optical sectioning over larger volumes (10 µm in z)
- + Sensitive (EMCCD/sCMOS) and fast (OMX Blaze)
 - live cell imaging
 - o Only moderate xy-resolution improvement
 - Mathematical reconstruction → artifact proneness
 - High requirements on sample quality and system calibration

Super-Resolution Fluorescence Microscopy

Goal: obtain sub-100 nm resolution

Two methods:

(i) Spatially Patterned Excitation
STED, SIM,

(ii) Localization Methods
STORM, PALM, FPALM



The Nobel Prize in Chemistry 2014
Eric Betzig, Stefan W. Hell, William E. Moerner

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The Nobel Prize in Chemistry 2014



Photo: Matt Staley/HHMI
Eric Betzig
Prize share: 1/3



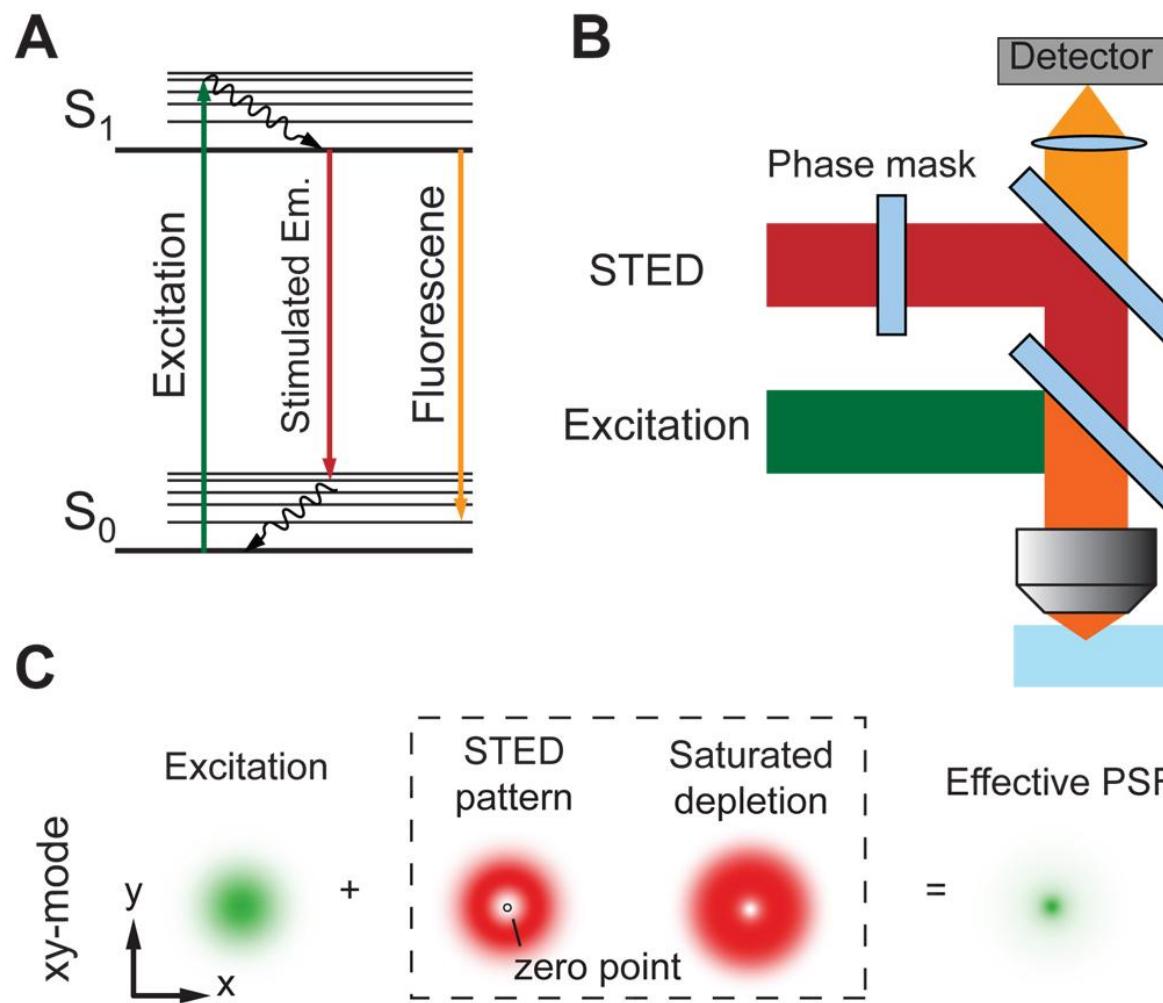
© Bernd Schuller,
Max-Planck-Institut
Stefan W. Hell
Prize share: 1/3



Photo: K. Lowder via
Wikimedia Commons,
CC-BY-SA-3.0
William E. Moerner
Prize share: 1/3

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner "for the development of super-resolved fluorescence microscopy".

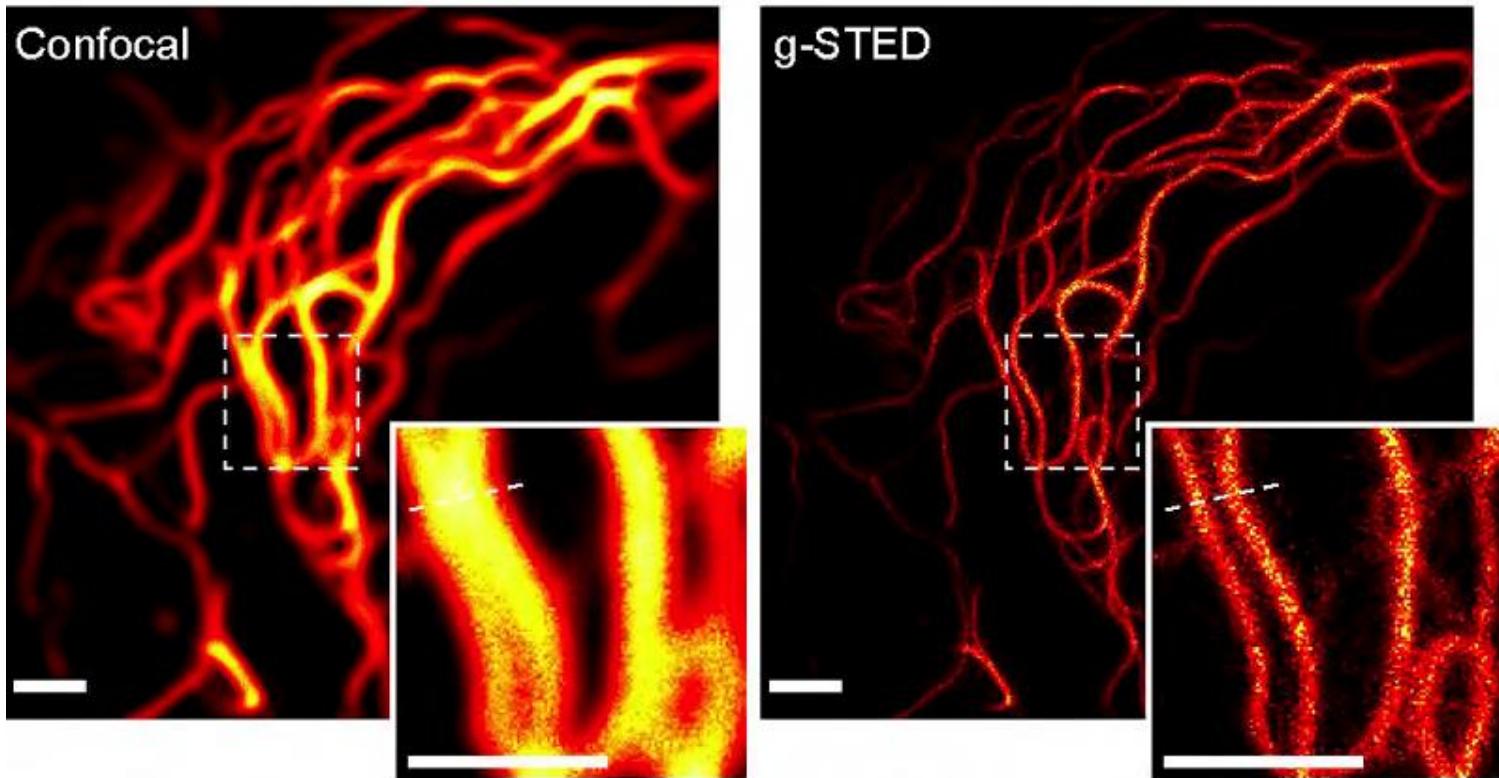
STimulated Emission Depletion (STED)



Net result is a smaller Point Spread Function

Example of STED

Up to 50 nm of lateral resolution and 500 nm of axial resolution

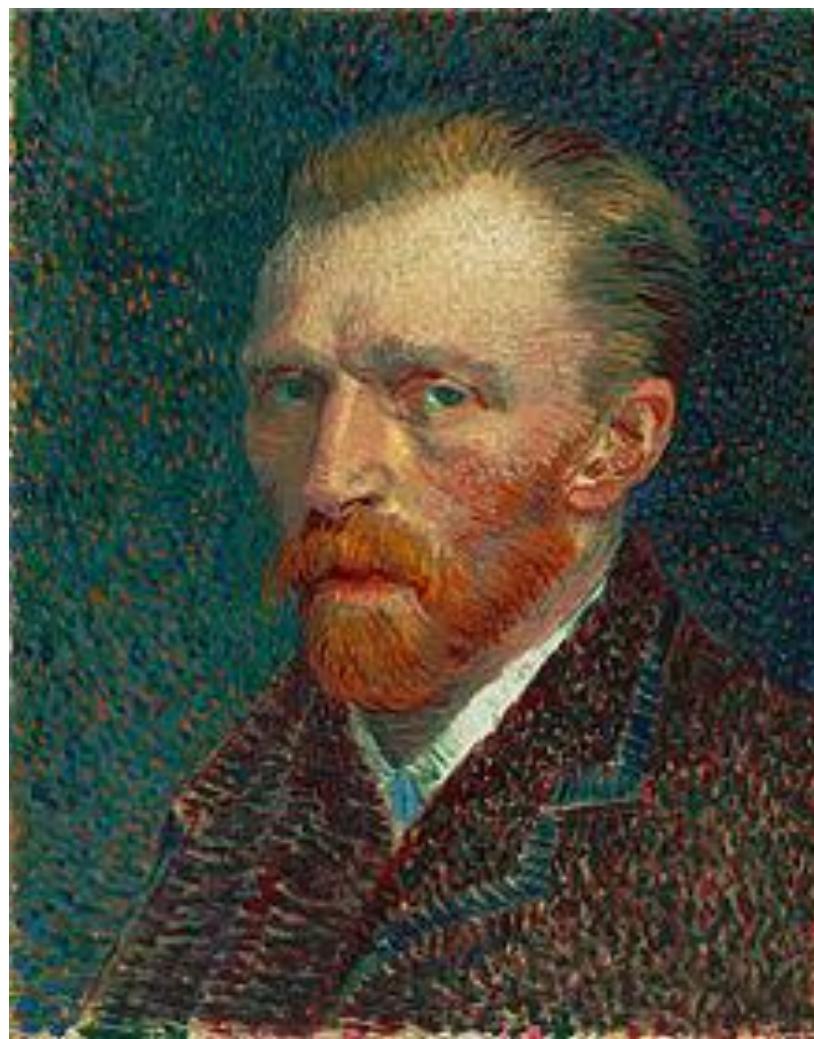


STED (pros & cons)

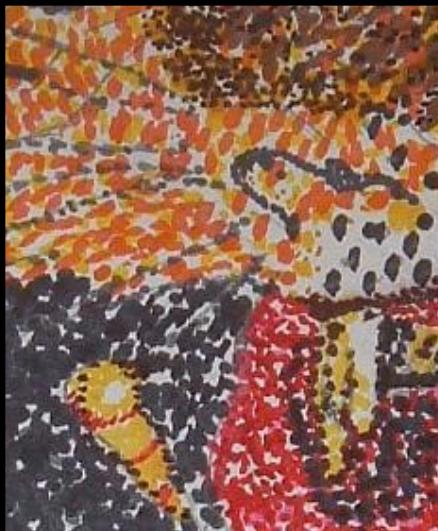
- + What you see is what you get → no math required!
- + High xy-resolution (50-70 nm, Leica TCS STED)
- + > 20 µm depth
 - o Maximum 2 colors (no UV!)
 - o Special dyes required for optimal performance
 - o Speed scales with size → full frame rather slow!
 - o PMT/APD detectors less sensitive
- Relative high energy load → photodamage
- Not ideal for 3D and live cell imaging
- Complex instrumentation, price tag

Super-resolution: Localization Microscopy
PALM, STORM

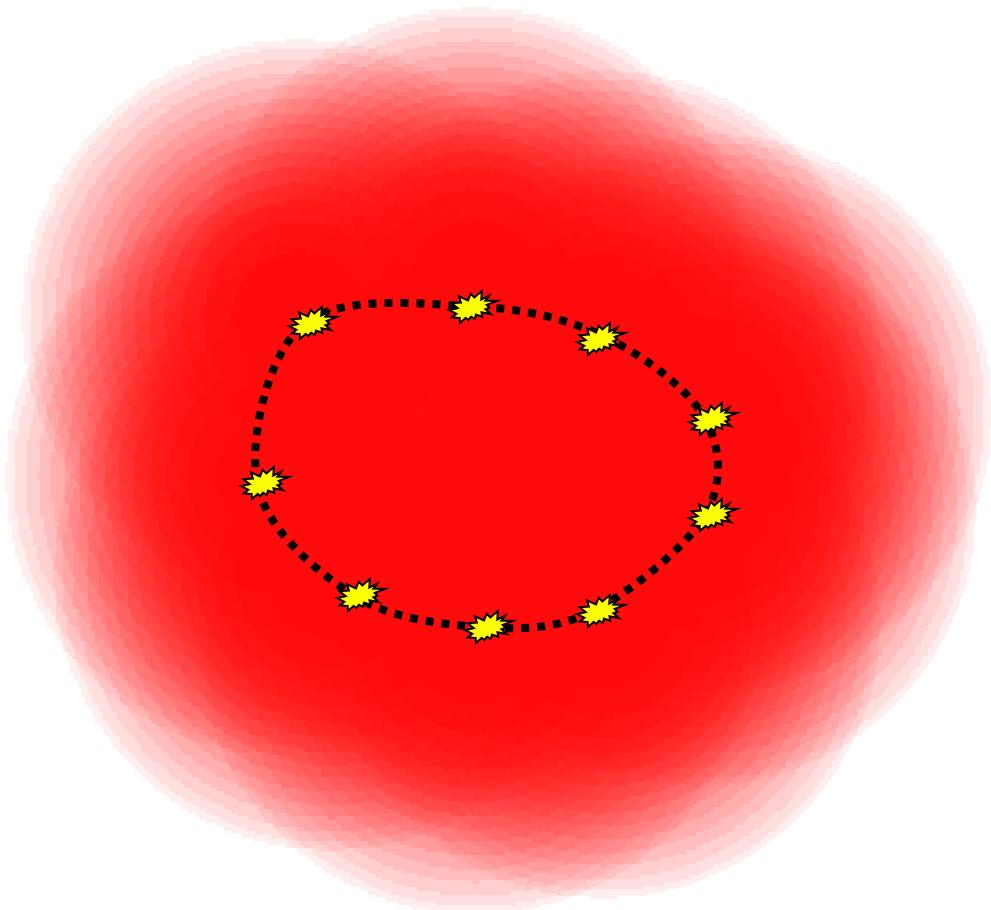
Side Point: Pointillism



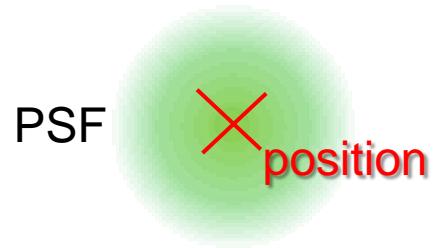
If positions are known you can
paint a picture!



Many Molecules



Localization, not resolution



Localization, not resolution

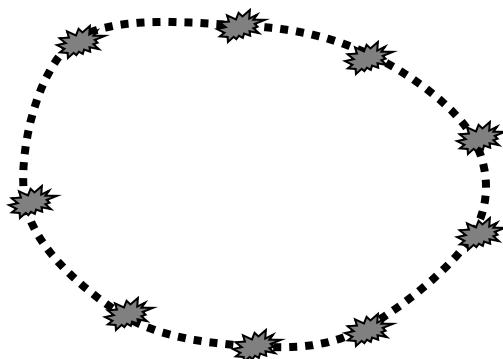


How to separate particles?

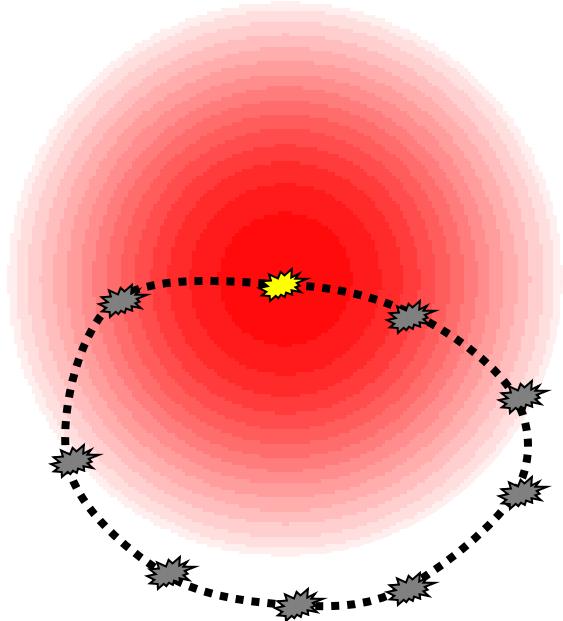
Avoid overlap entirely by temporally separating the particles

E. Betzig, "Proposed method for molecular optical imaging", *Opt. Lett.* **20**, 237 (1995)

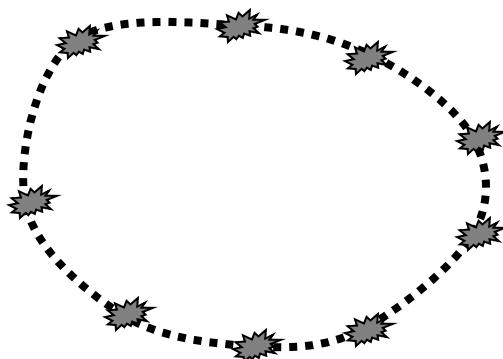
The principle of PALM/STORM



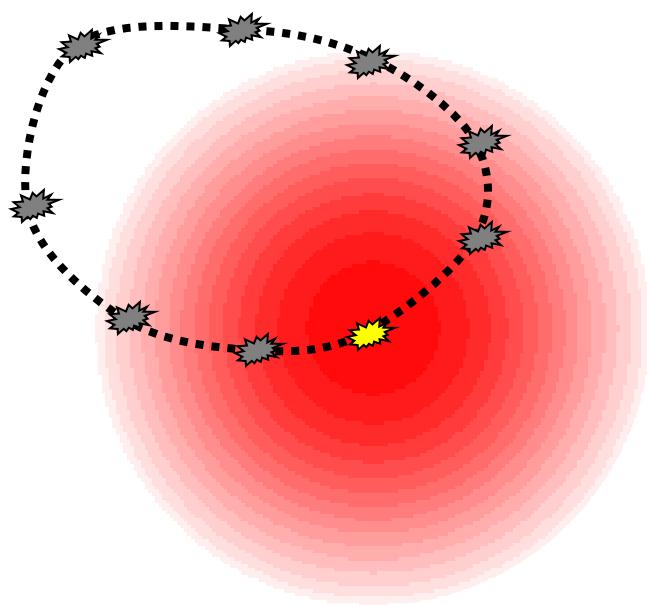
The principle of PALM/STORM



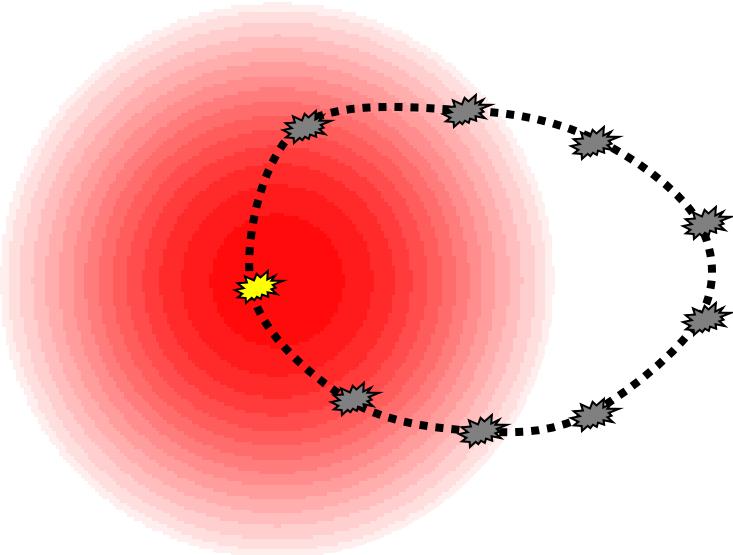
The principle of PALM/STORM



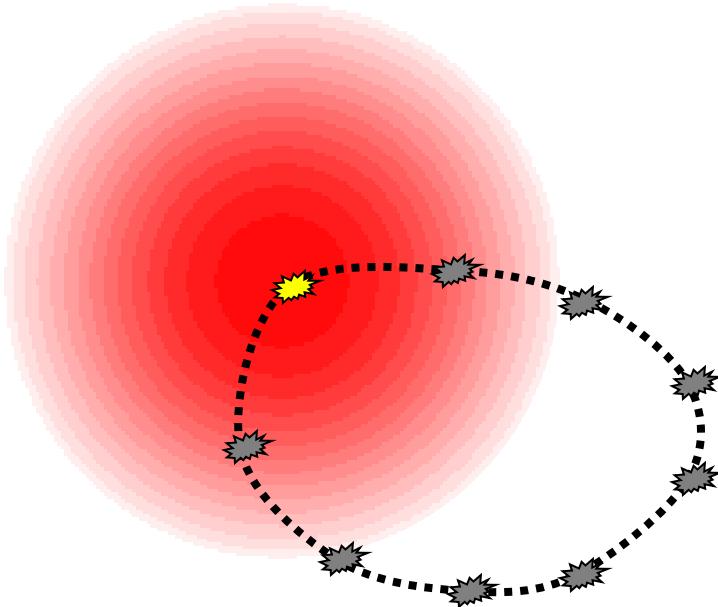
The principle of PALM/STORM



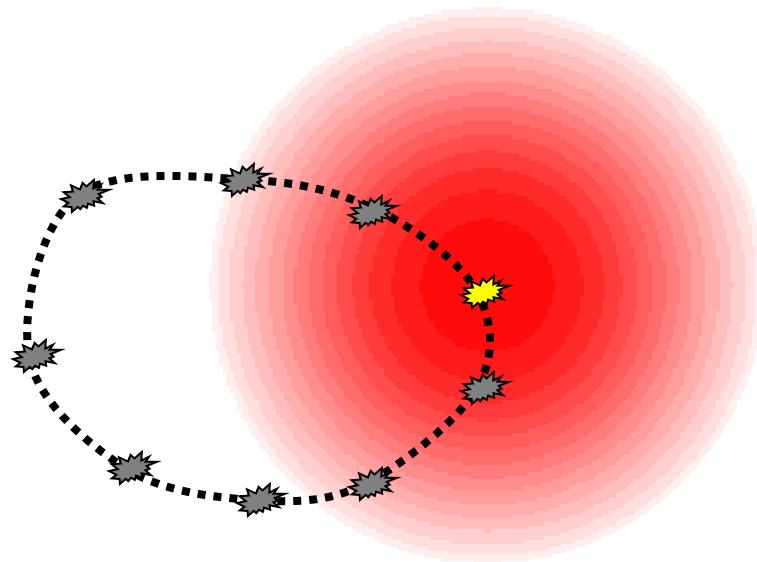
The principle of PALM/STORM



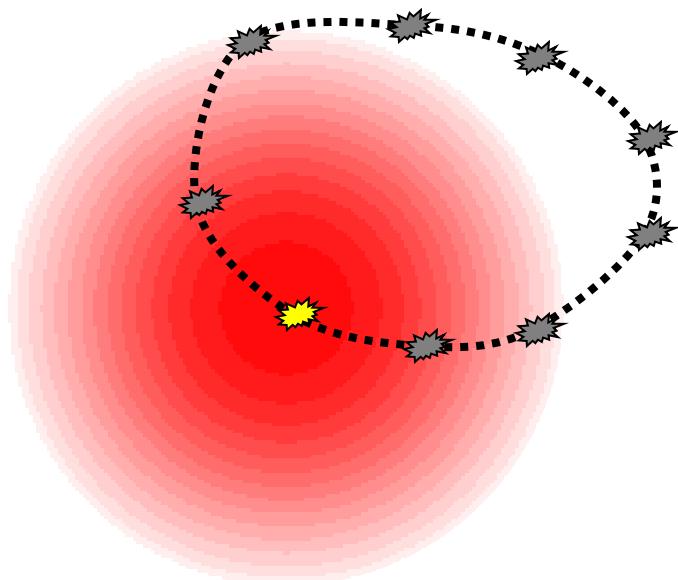
The principle of PALM/STORM



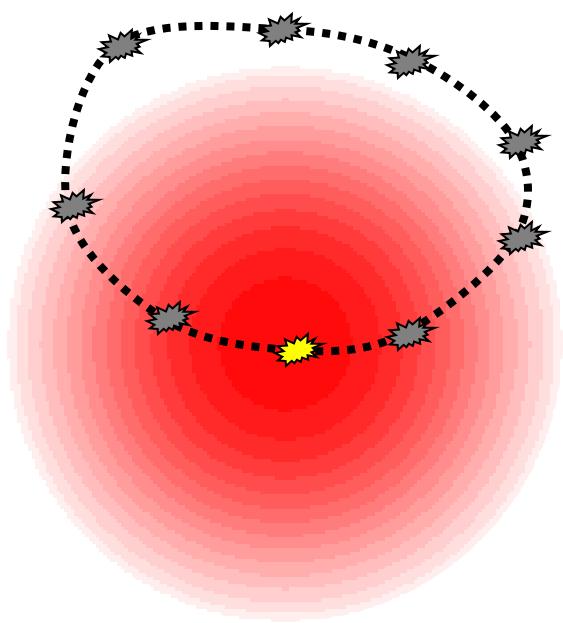
The principle of PALM/STORM



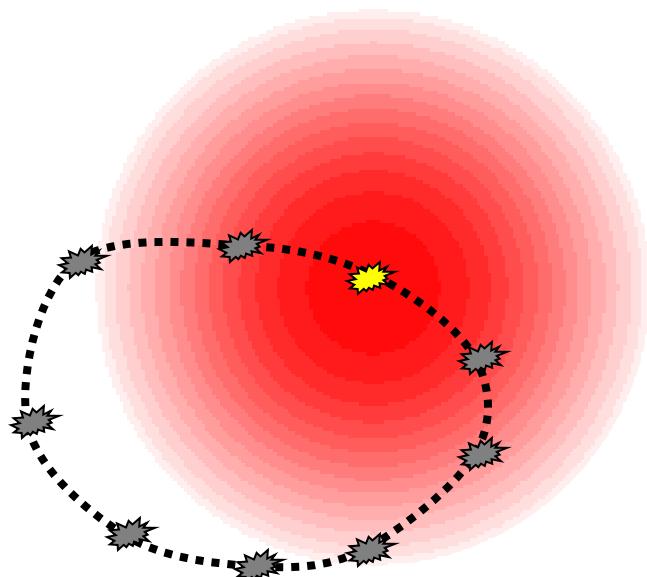
The principle of PALM/STORM



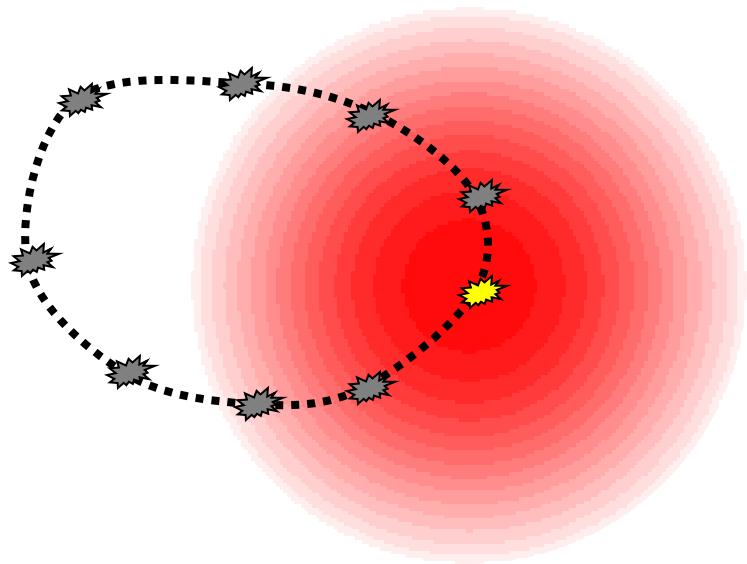
The principle of PALM/STORM



The principle of PALM/STORM



The principle of PALM/STORM



Jena at night

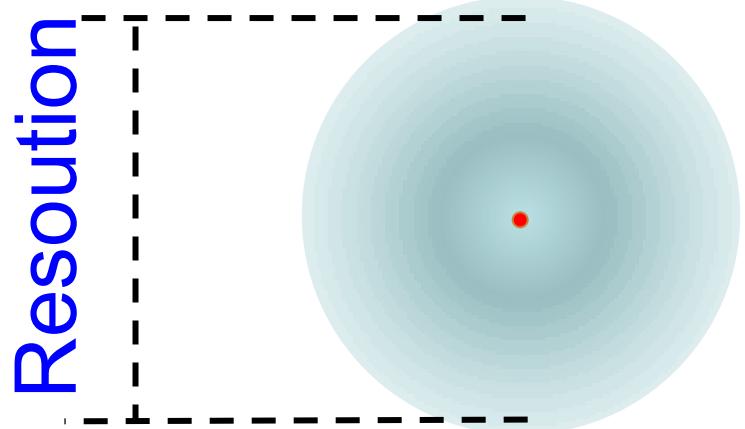
...Google

Jena at night

Task: Localization of the university buildings

How: Each Professor has to turn on the light for one minute

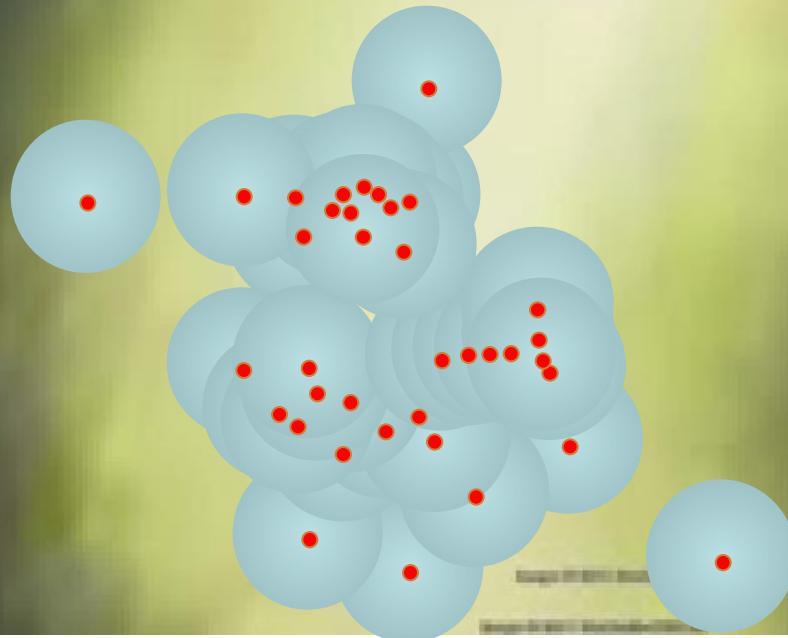
Localizing is much more precise than resolution



Separation over time



Separation over time



Without labelling:
everything is bright

Labelling the university buildings
widefield: bad resolution

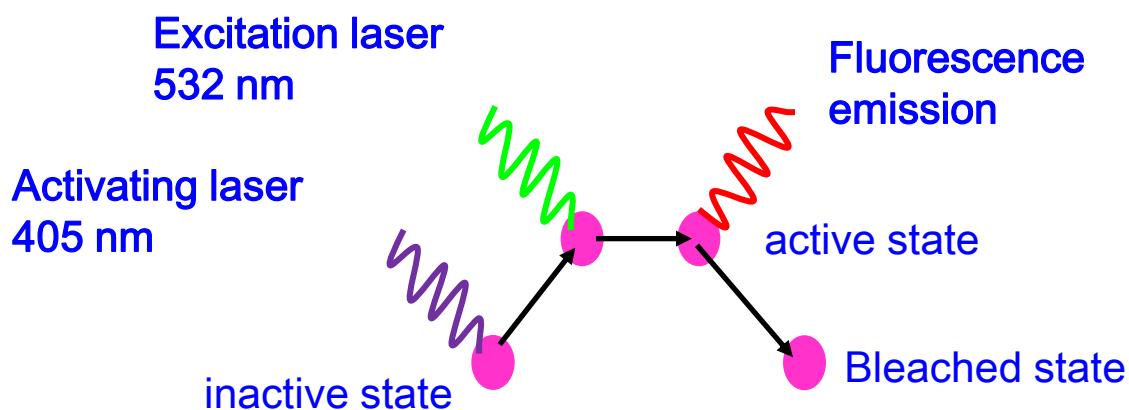
Pointillistic: accurate map

Super-Resolution Single Molecule Localization Methods

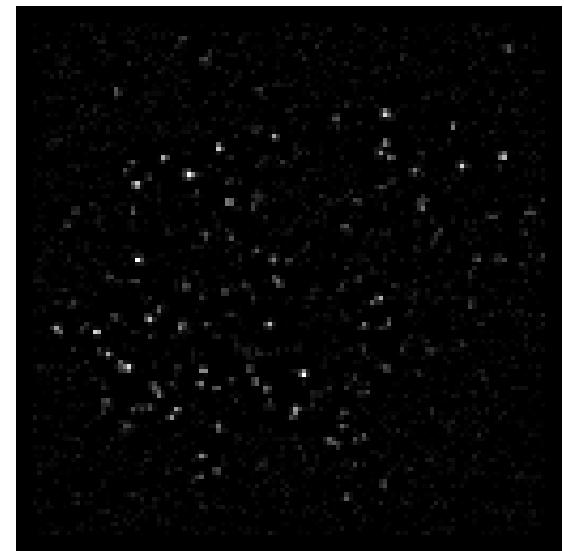
Photo-Activated Localization Microscopy (PALM)

Eric Betzig et al Imaging intracellular fluorescent proteins at nanometer resolution
Science 313, 1642 (2006).

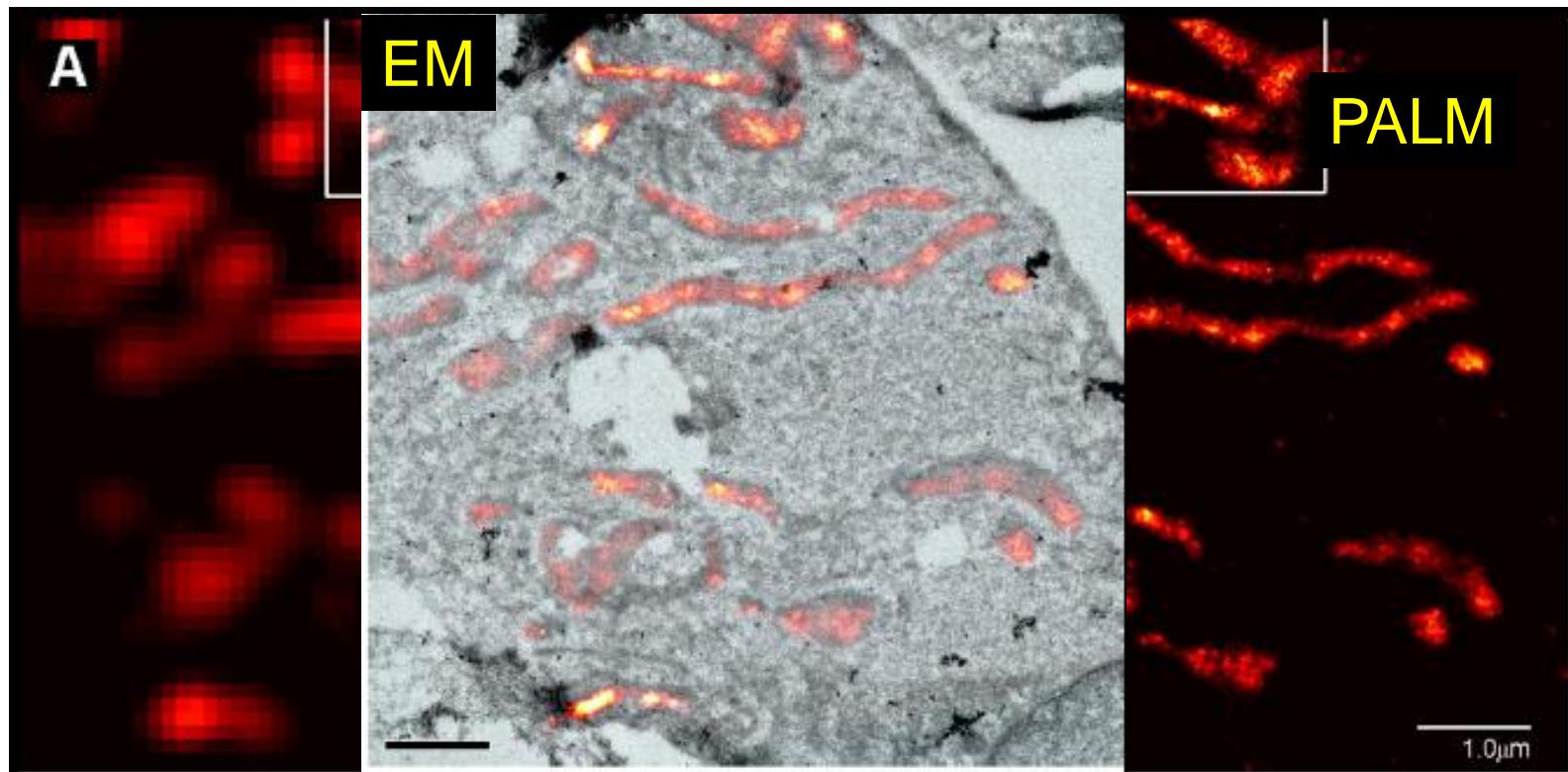
- Express protein of interest tagged with a photo-activatable fluorescent protein in cell
- Stochastically photo-activate a low density of molecules per frame and localize using Gaussian function



Repeat thousands of times



Example of PALM



E. Betzig *et al.*, *Science*, DOI: 10.1126/science.1127344, Aug. 2006

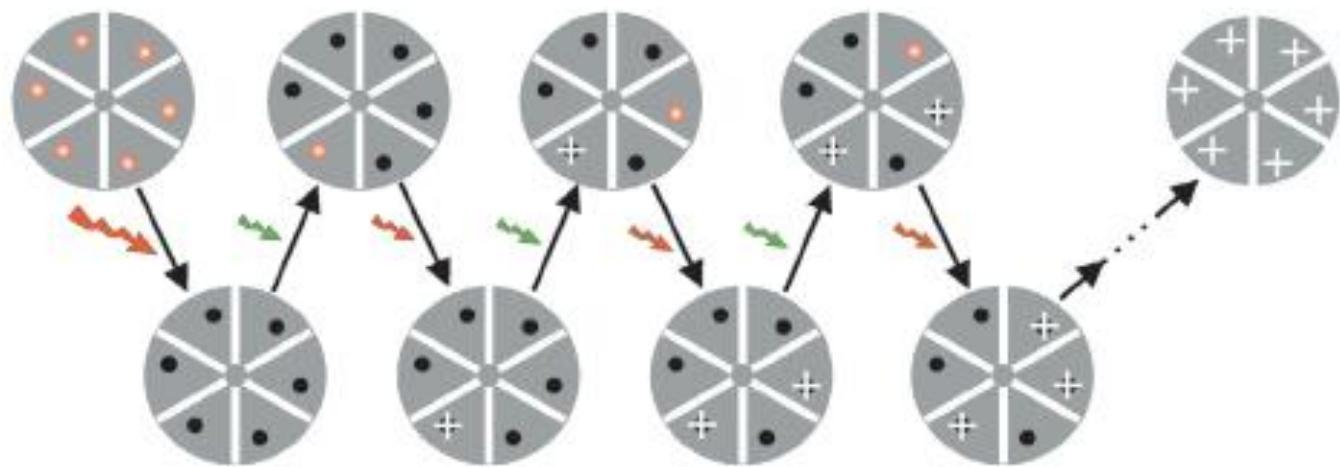
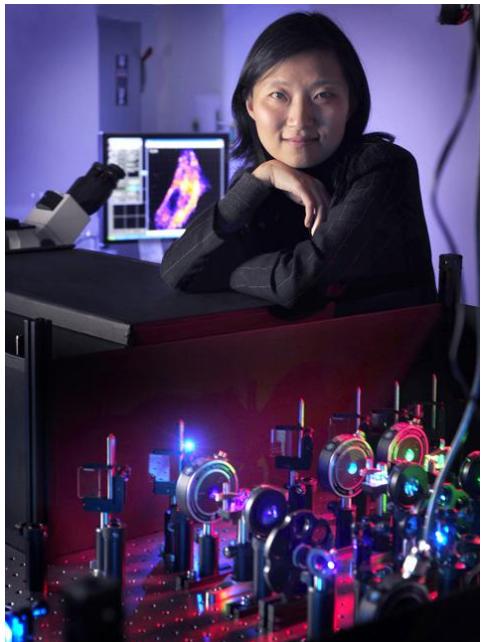
Mitochondria

COS-7 Zellen

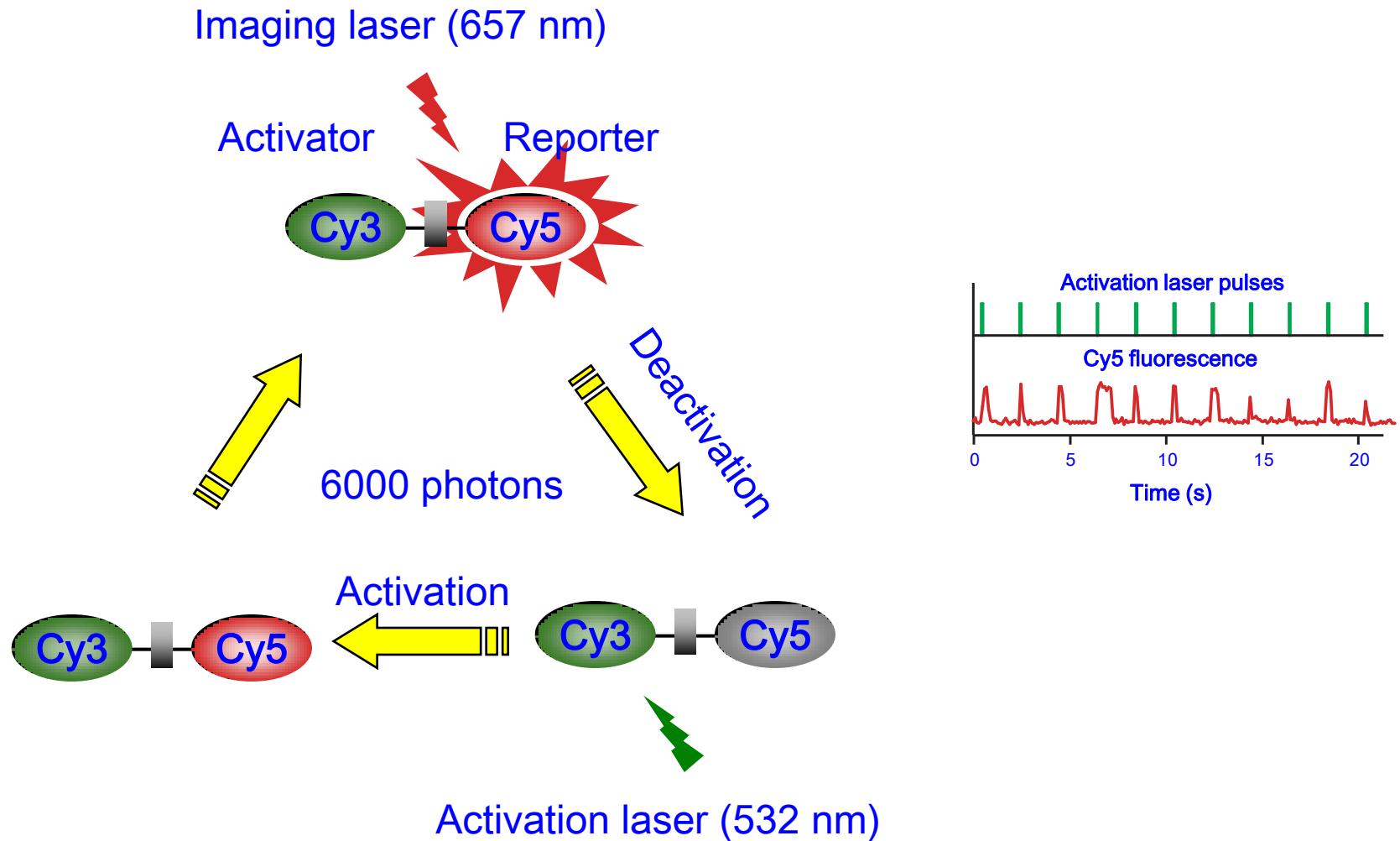
Cryo-Schnitte

Cytochrome C Oxidase import Sequenz - dEosFP

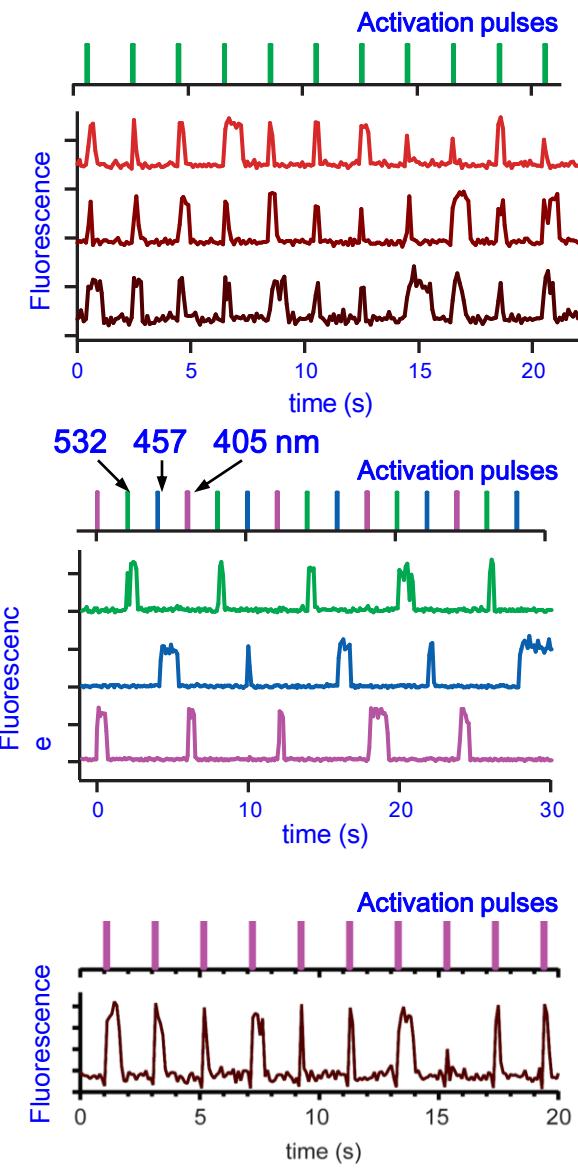
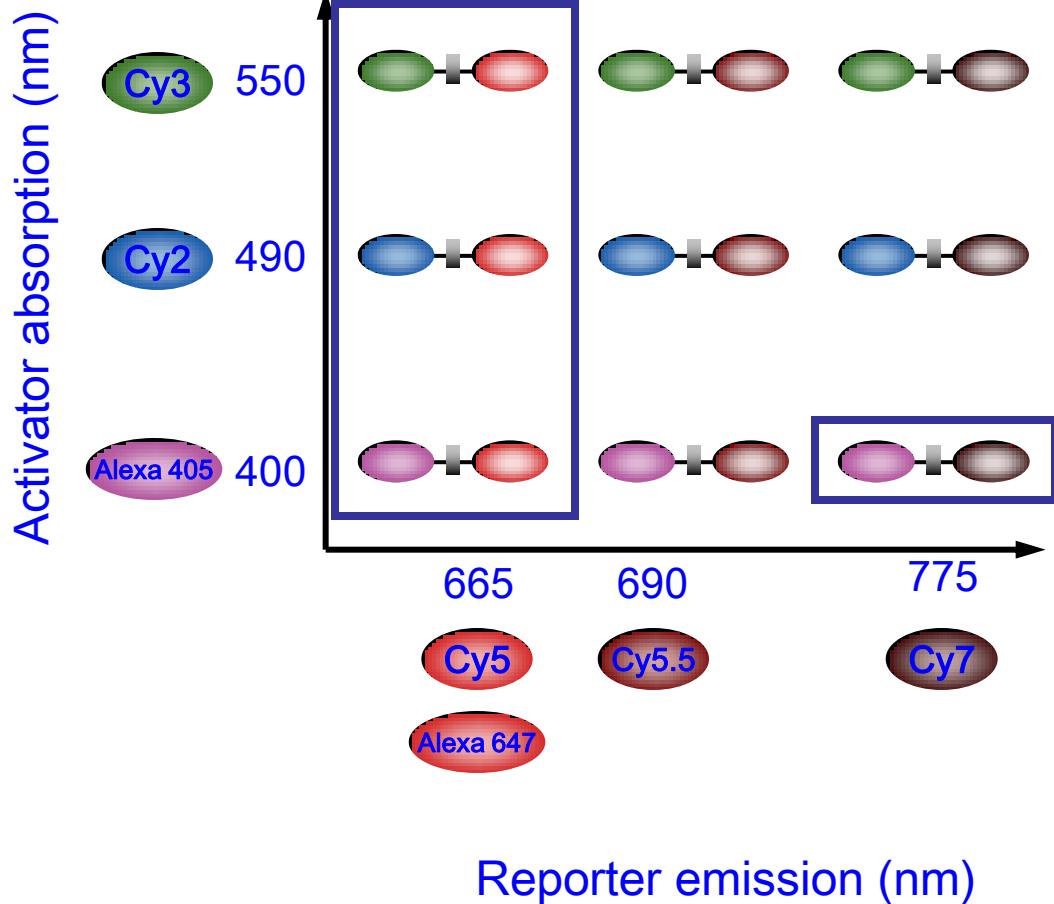
Stochastic Optical Reconstruction Microscopy (STORM)

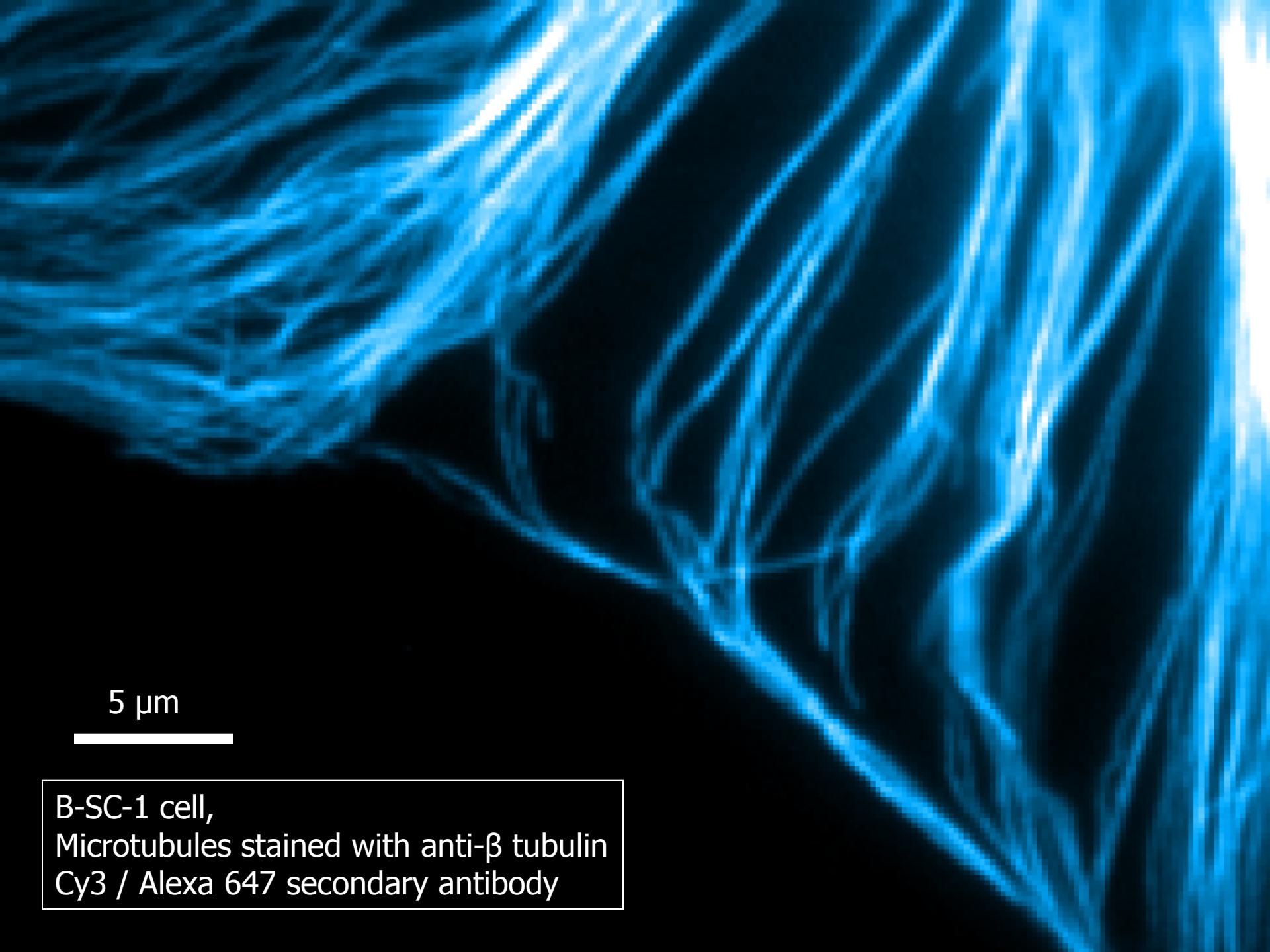


A Photo-switchable Probe



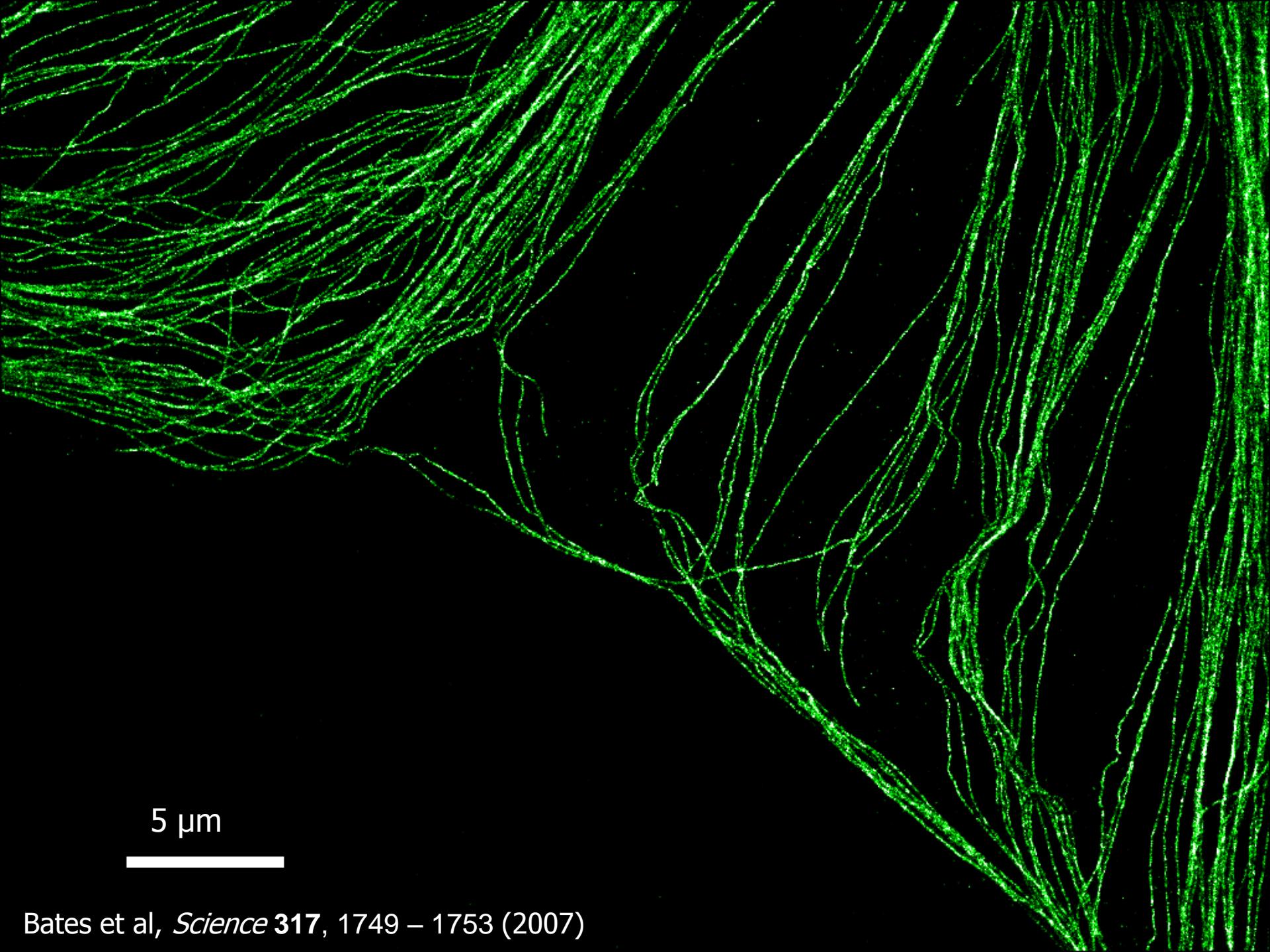
More Colors



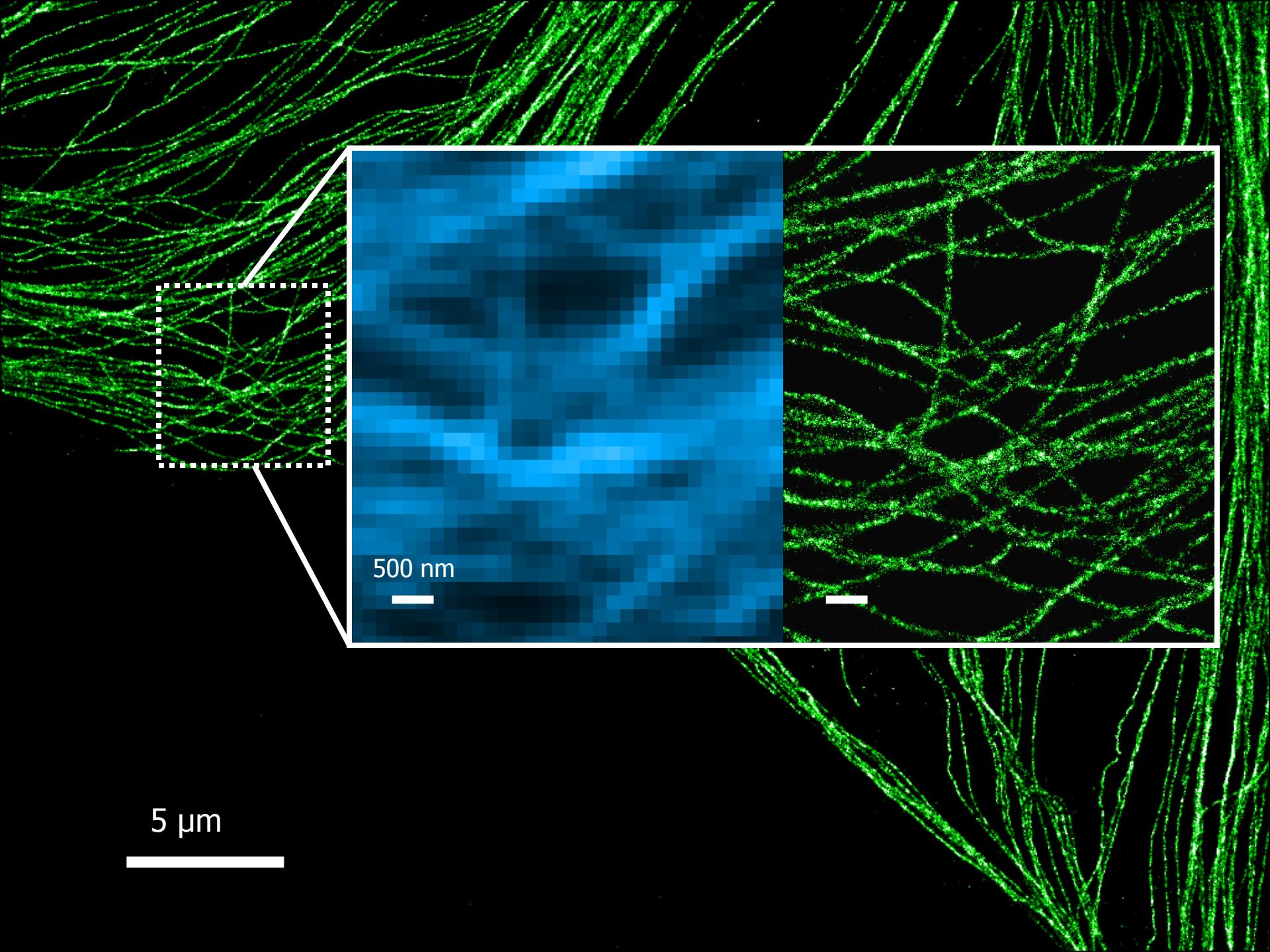
A fluorescence micrograph showing a dense network of microtubules in a B-SC-1 cell. The microtubules are stained with anti-β tubulin antibody and visualized using Cy3 or Alexa 647 secondary antibody, appearing as bright blue filaments against a dark background.

5 μ m

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

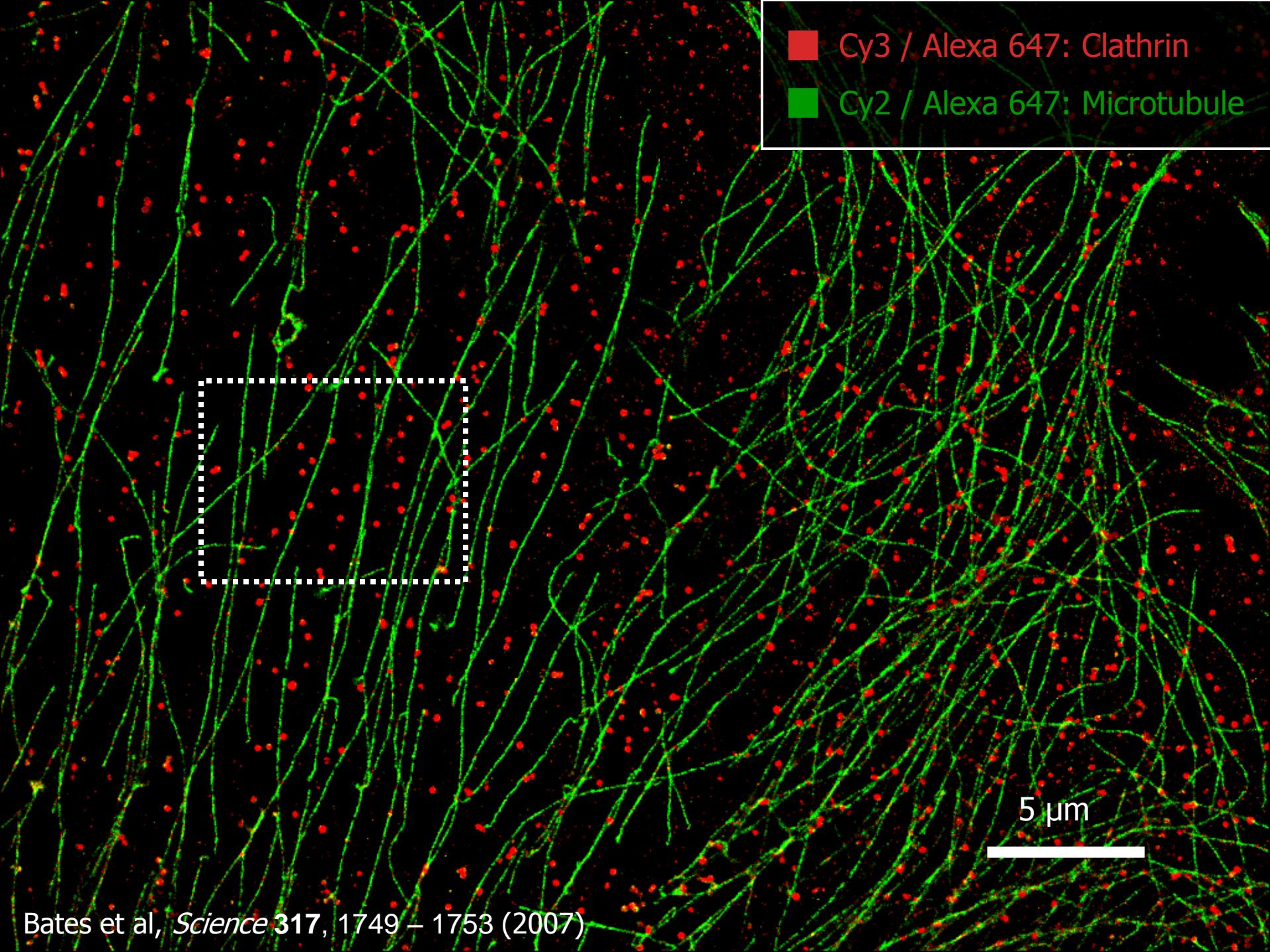


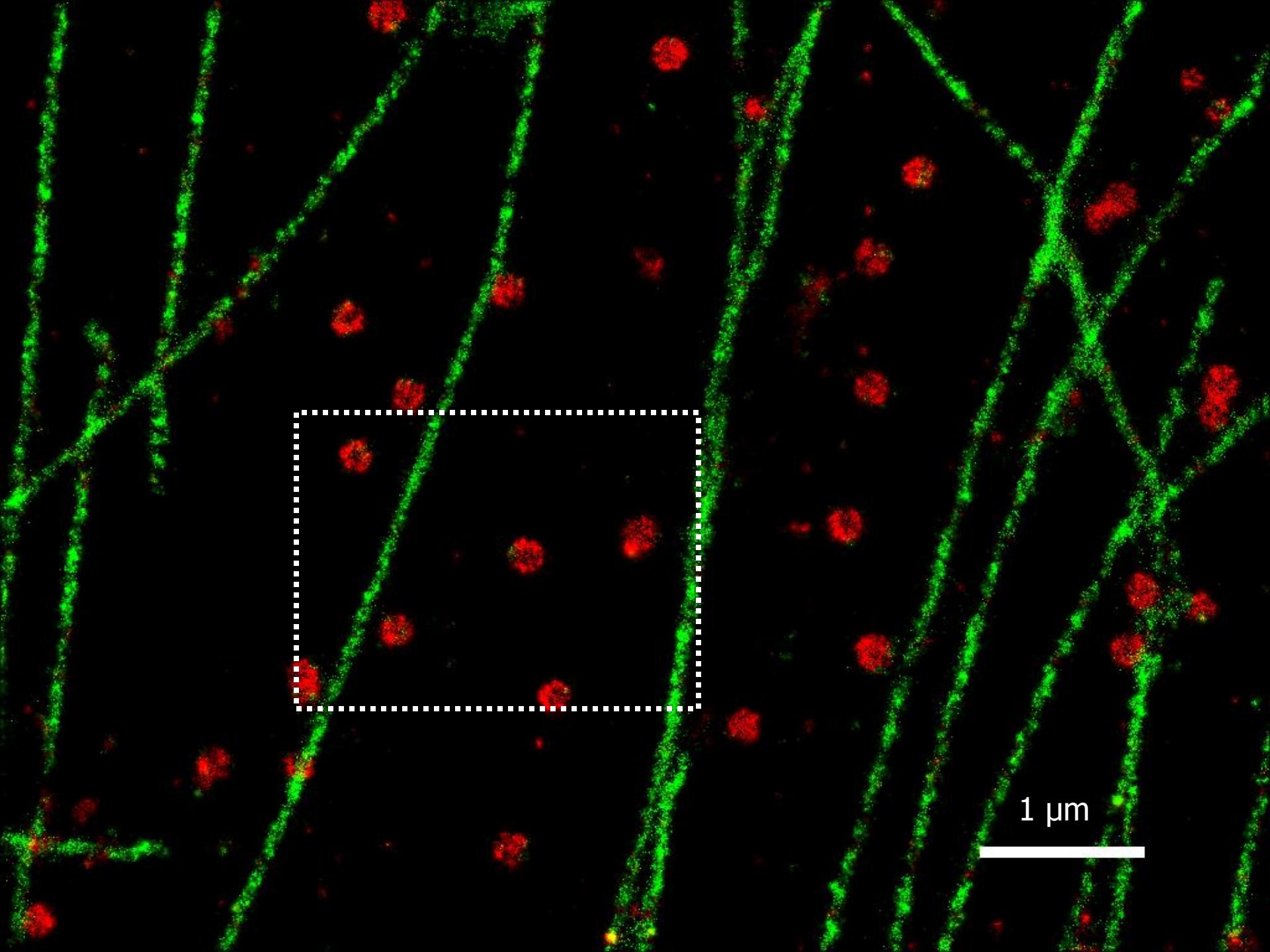
5 μm



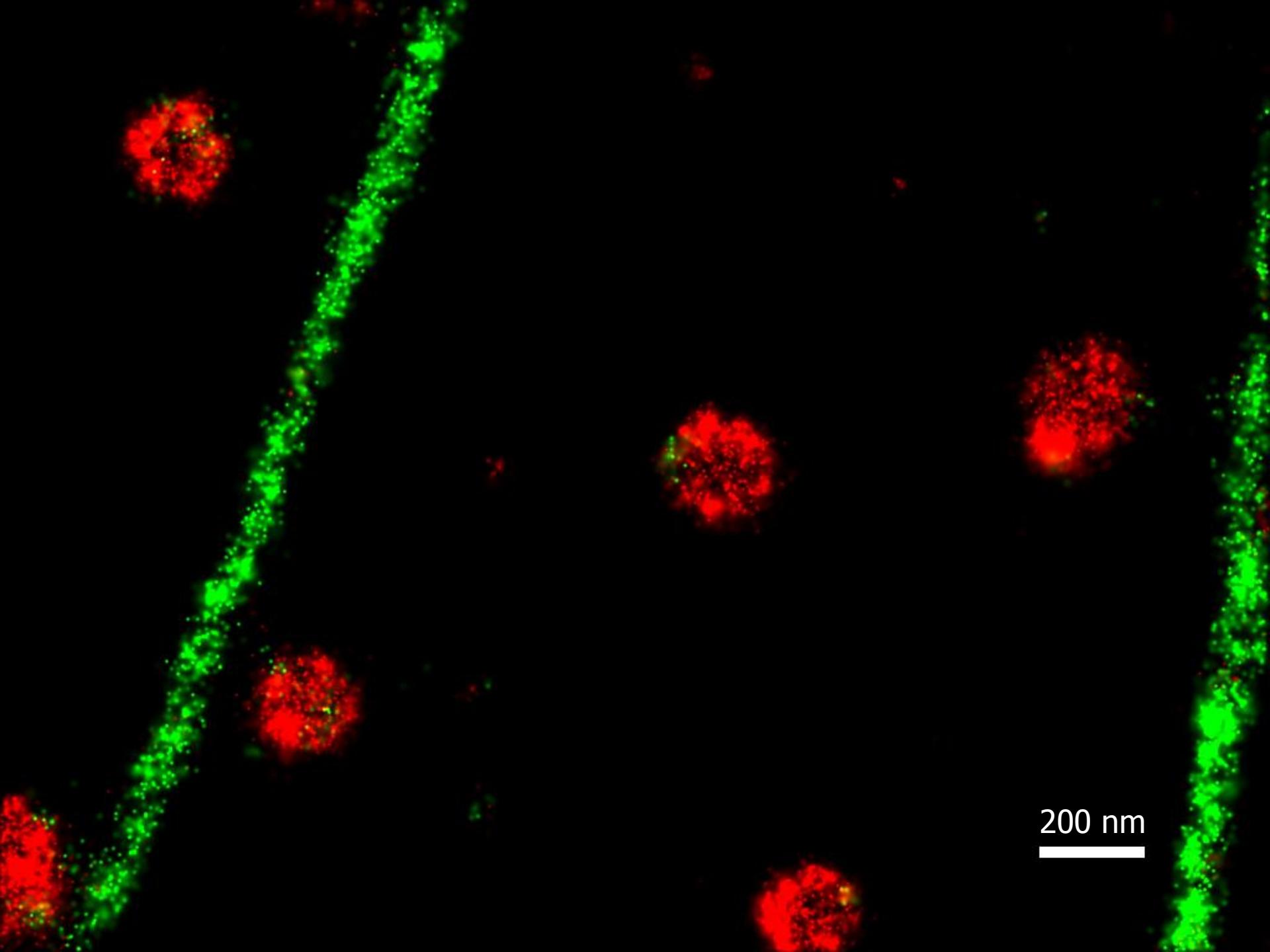
5 μm

500 nm





1 μm



200 nm

Examples of PALM

Two-Color PALM Imaging of Actin and Paxillin in Focal Adhesions

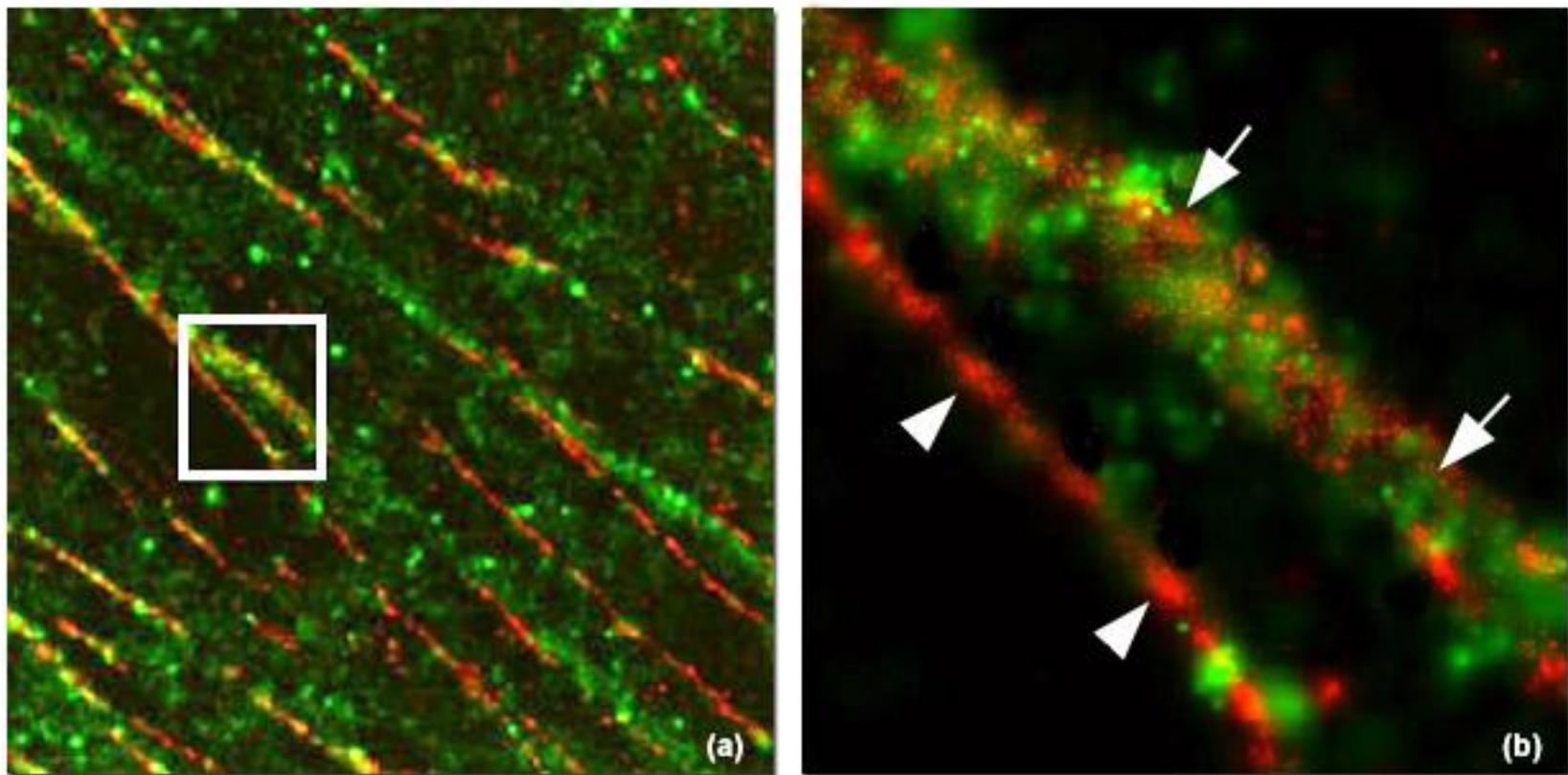


Figure 8

Problem: sparseness of labelling

Localization Resolution and Molecular Density in PALM

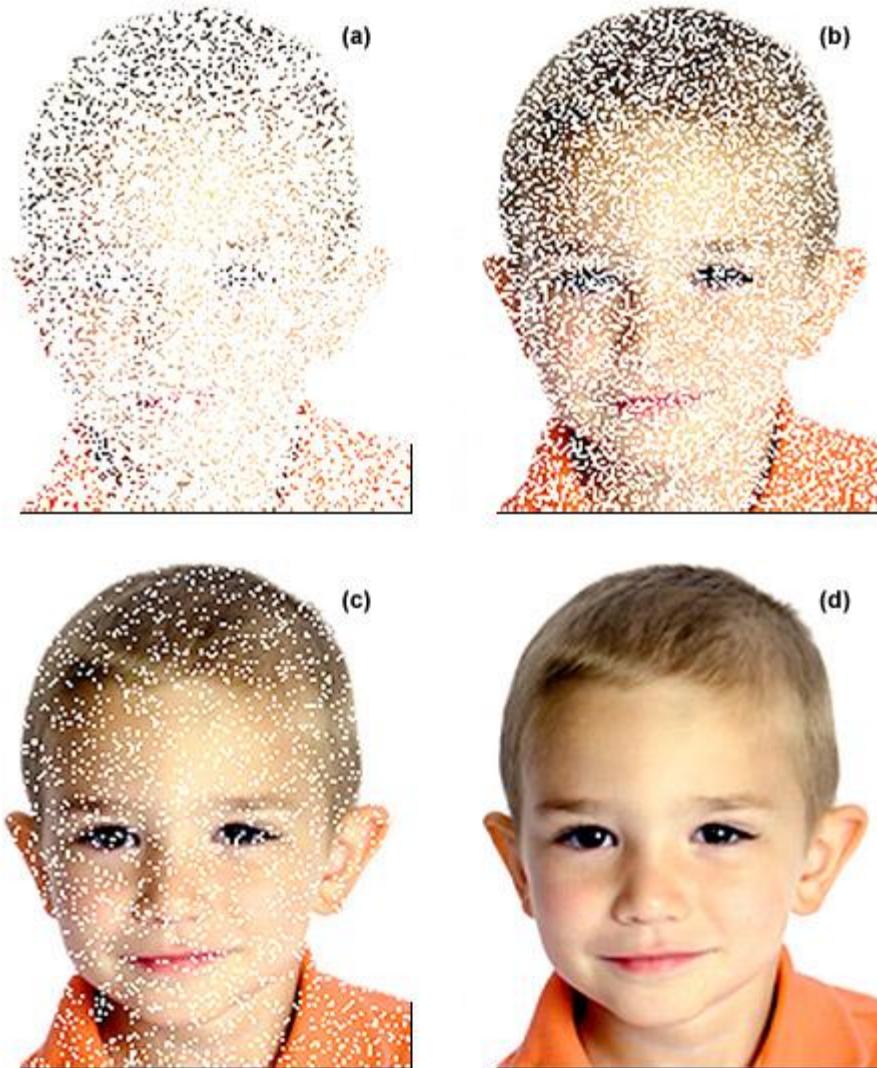


Figure 5

Dyes for Localization Microscopy

- have on and off state
- easily able to switch from on/off state
- on/off can be non-fluorescent or have a change in *either* excitation or emission wavelengths
- best if reversible but not necessary

Localization Microscopy (pros & cons)

- + High localization precision (± 20 nm)
- + Quantification of single molecules
 - (e.g., cluster analysis, single particle tracking)
- + Instrumentation relatively simple
 - o Localization precision \neq structural resolution
(± 50 nm, dependent on labeling density)
 - o Only single plane, best with TIRF, fixed samples
 - o Dye/embedding restrictions (photophysics)
- Slow
- Not suited for z-extended 3D structures

Conclusions

still very young technology and best dyes have yet to be discovered

impact is big since imaging is such a popular tool

a lot of opportunity for innovation/development

The use of imaging technology in cell biology

- 1. Introduction: what it is & how did it all start**
- 2. Applications & techniques, including some examples from our research**
- 3. Super-resolution microscopy**
- 4. Facilitates at SUSTC & how to start using them**
- 5. What does the future hold...**

The Facilities at SUSTC

- CLSM (Leica SP8, Nikon A1-R)
- Spinning-disk Confocal
- Super-resolution (SIM & STORM)
- Single molecule imaging
- Fluorescence Microscopes

Where do we want to go in the future?

- High speed
- Super-resolution
- Single molecule imaging
 - Fluorescence correlation spectroscopy (FCS)
 - Total internal reflectance microscopy (TIRF)

Resources

www.microscopyu.com

micro.magnet.fsu.edu

www.chroma.com (esp. their handbook on filter design)

www.probes.com (esp. their handbook/catalog)

Douglas B. Murphy “Fundamentals of Light Microscopy and Electronic Imaging”

James Pawley, Ed. “Handbook of Biological Confocal Microscopy, 3rd ed.”

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