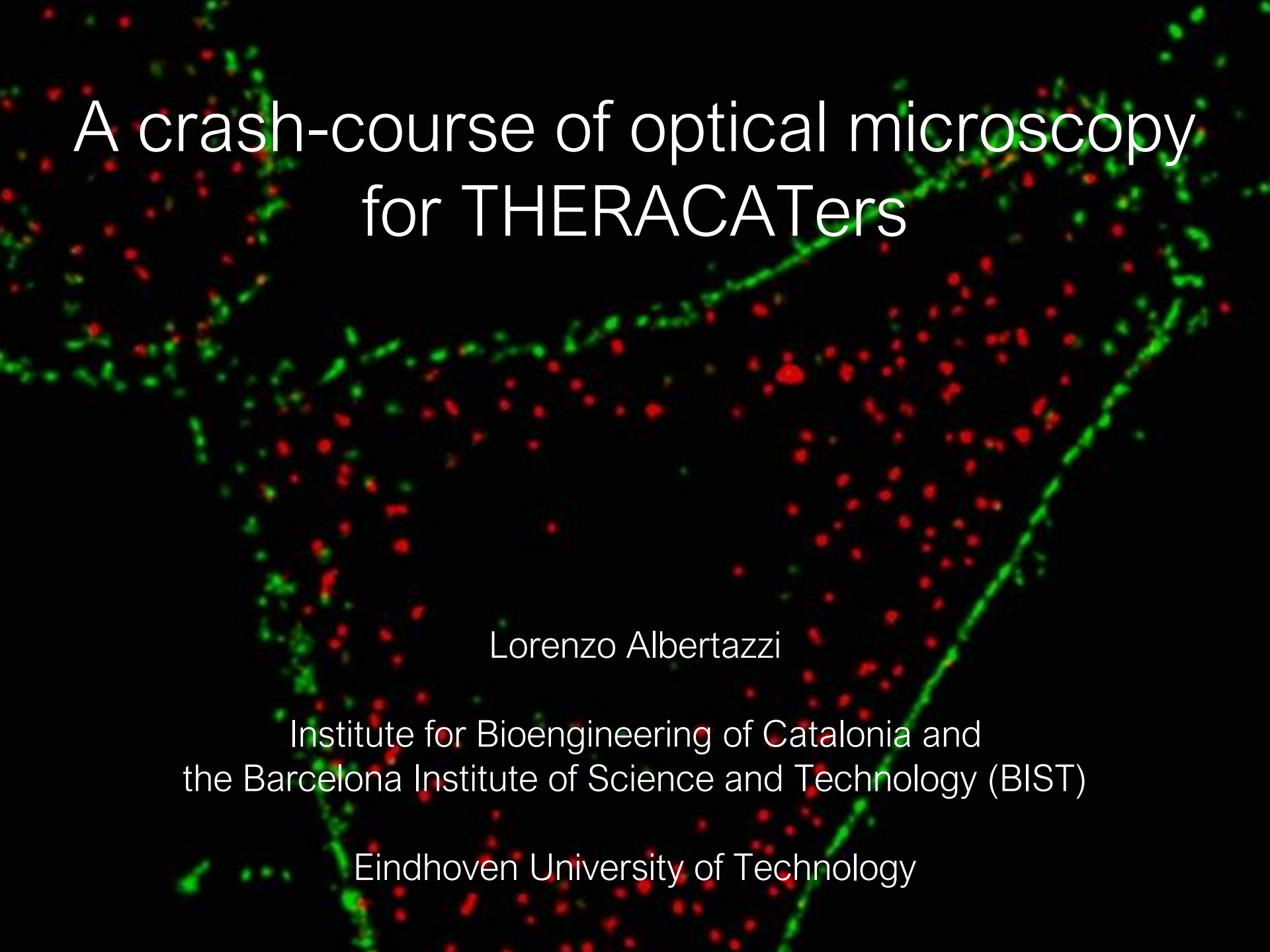
# A crash-course of optical microscopy for THERACATers

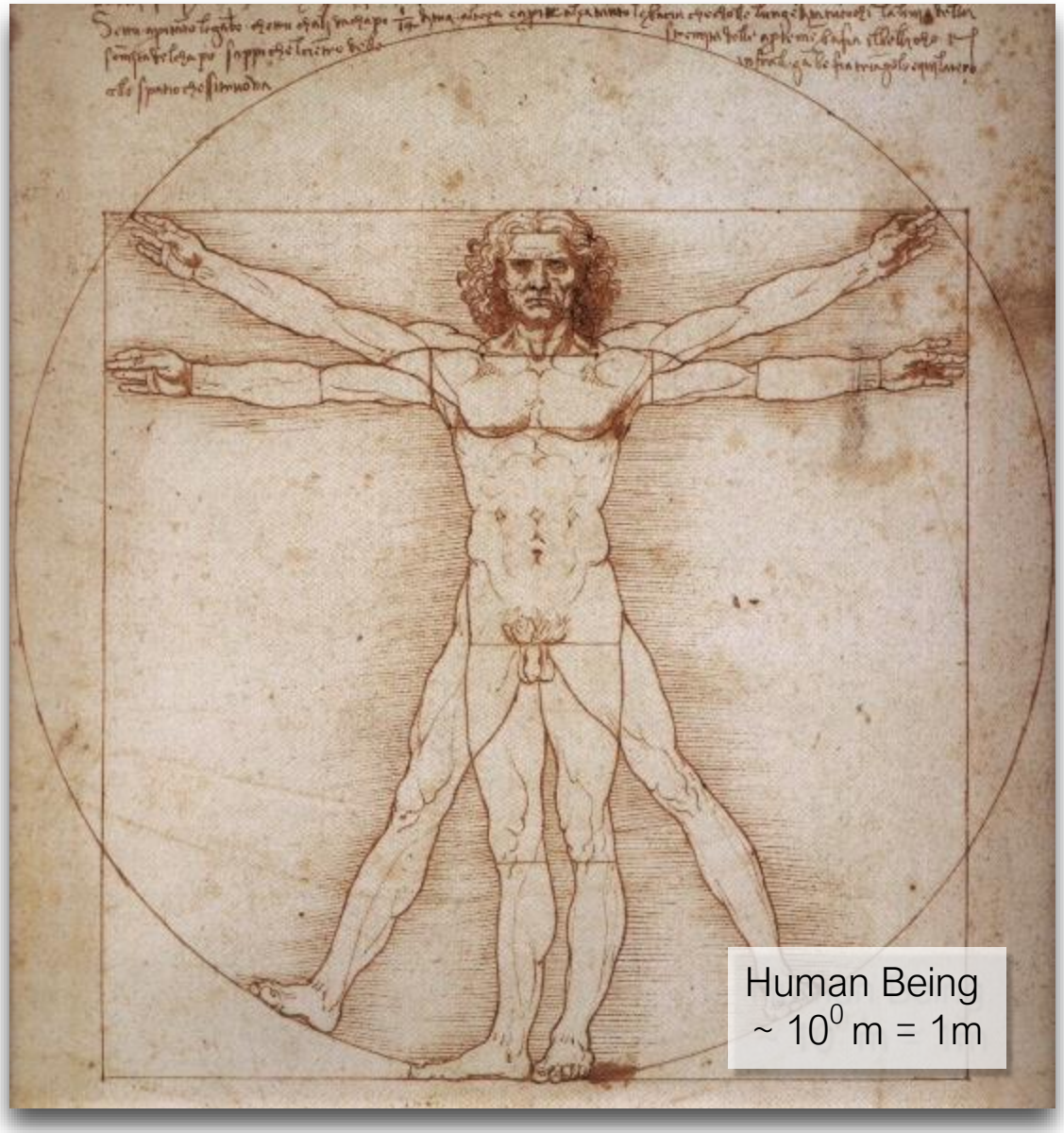


Lorenzo Albertazzi

Institute for Bioengineering of Catalonia and
the Barcelona Institute of Science and Technology (BIST)

Eindhoven University of Technology

# Why Resolution? A matter of size



# Why Resolution? A matter of size


https://www.smithsonianmag.com/

# Why Resolution? A matter of size

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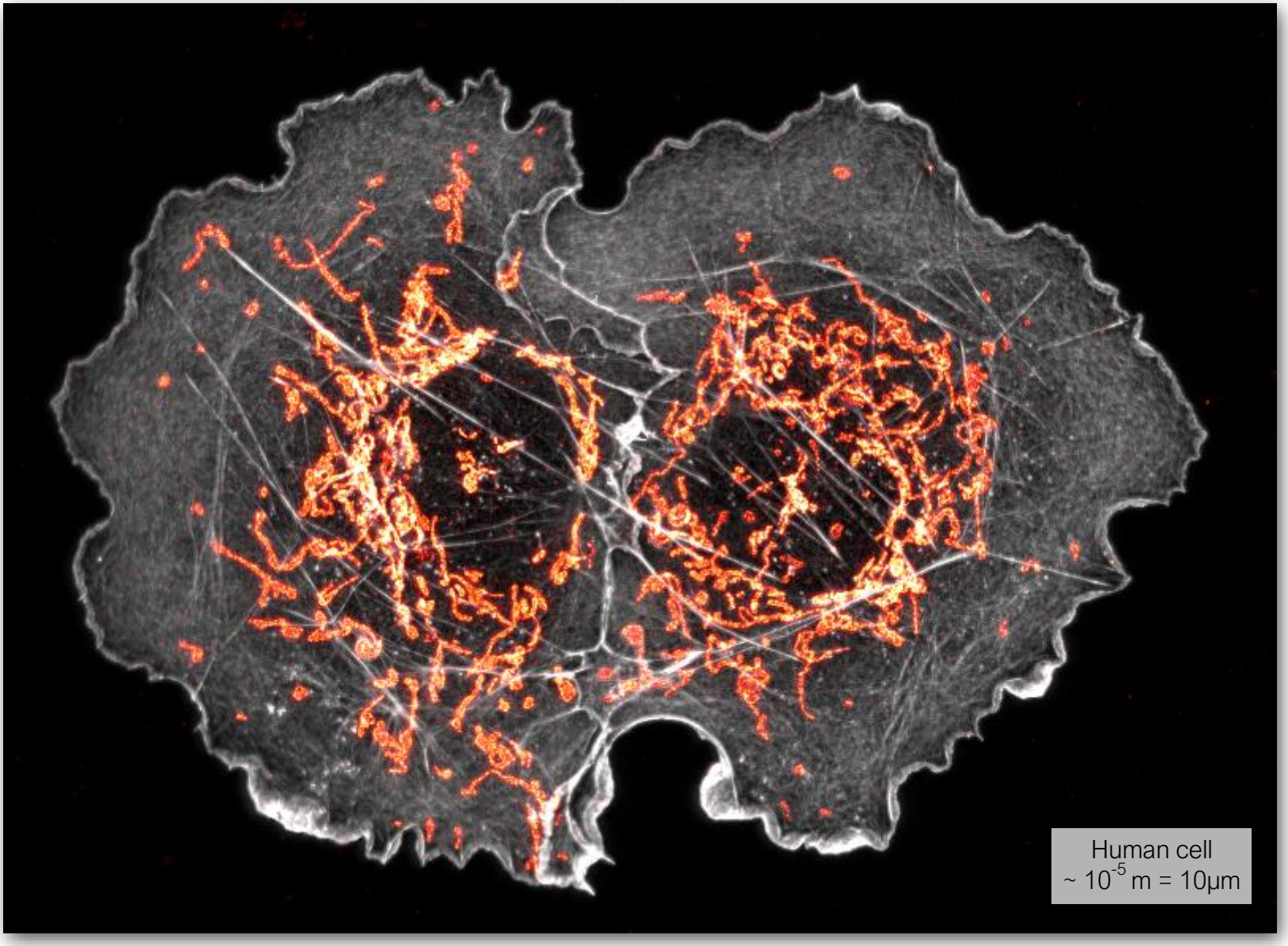
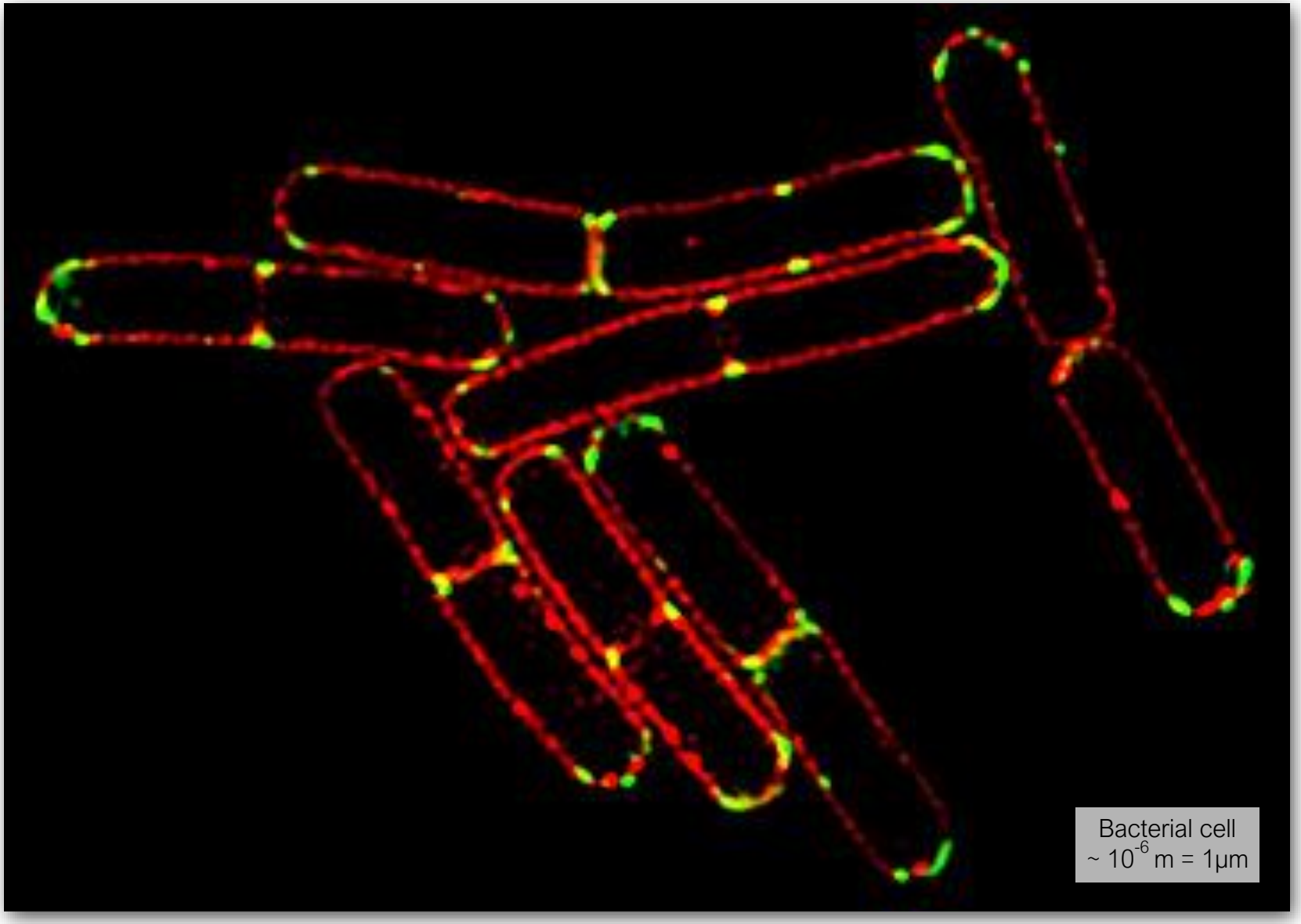


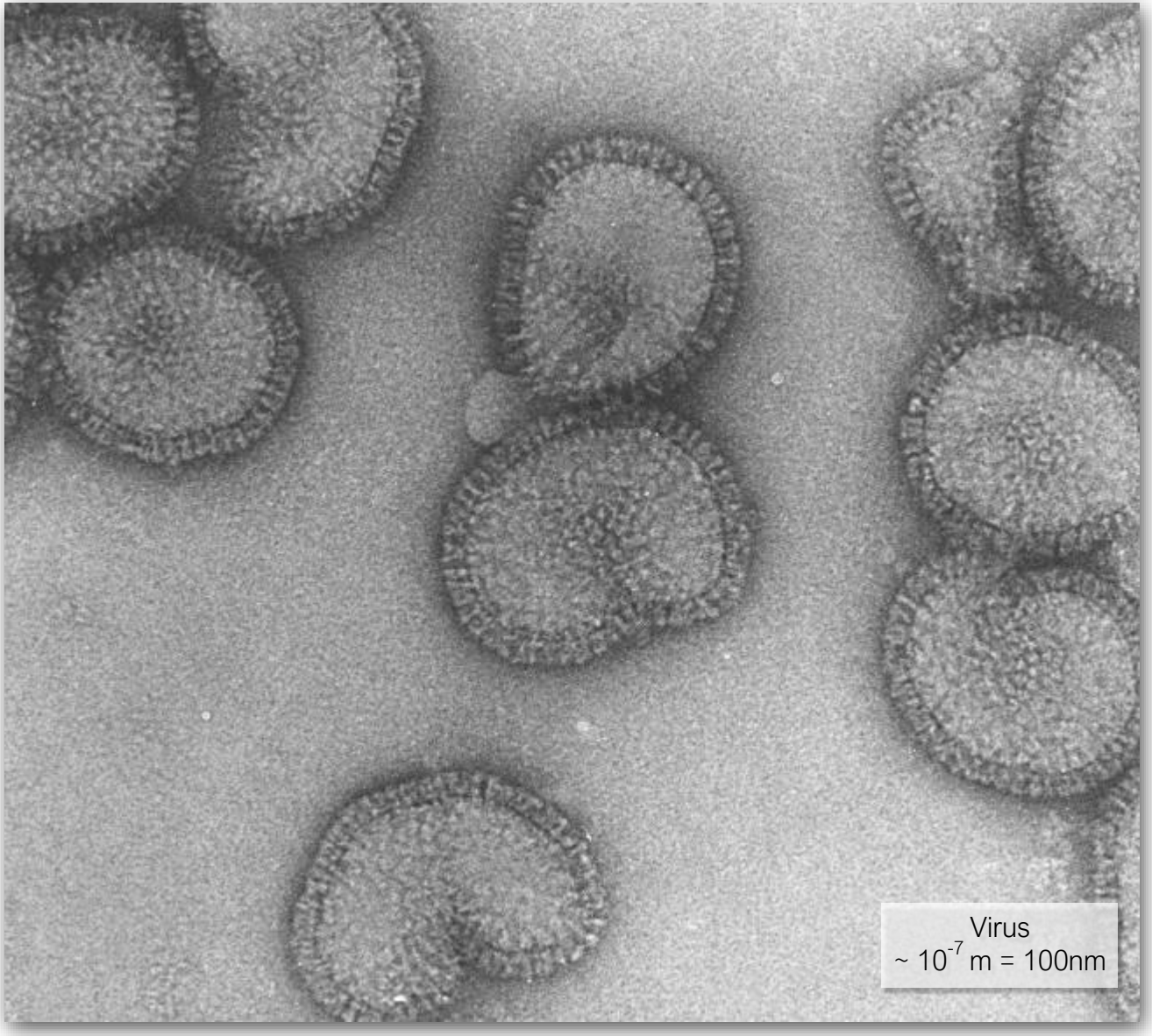

http://www.neurocytolab.org

# Why Resolution? A matter of size

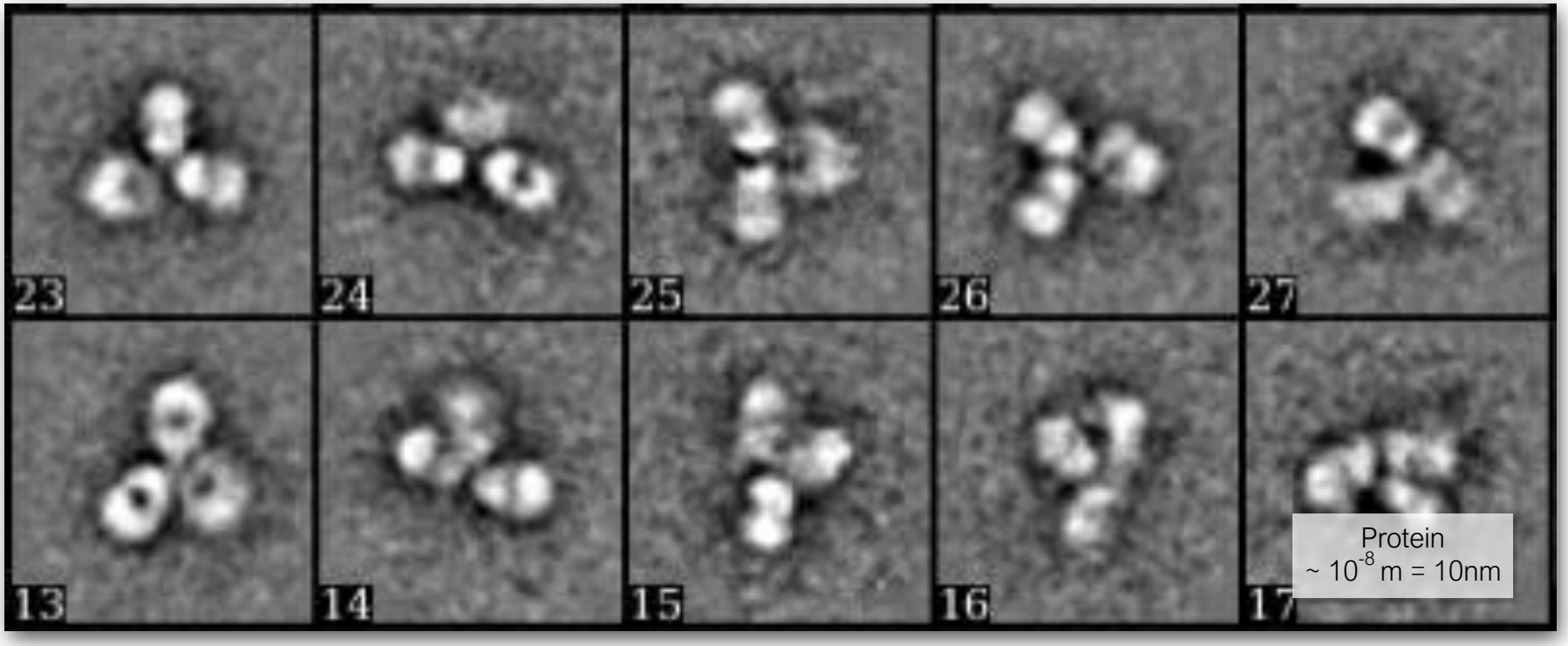



http://www.nikon.com/

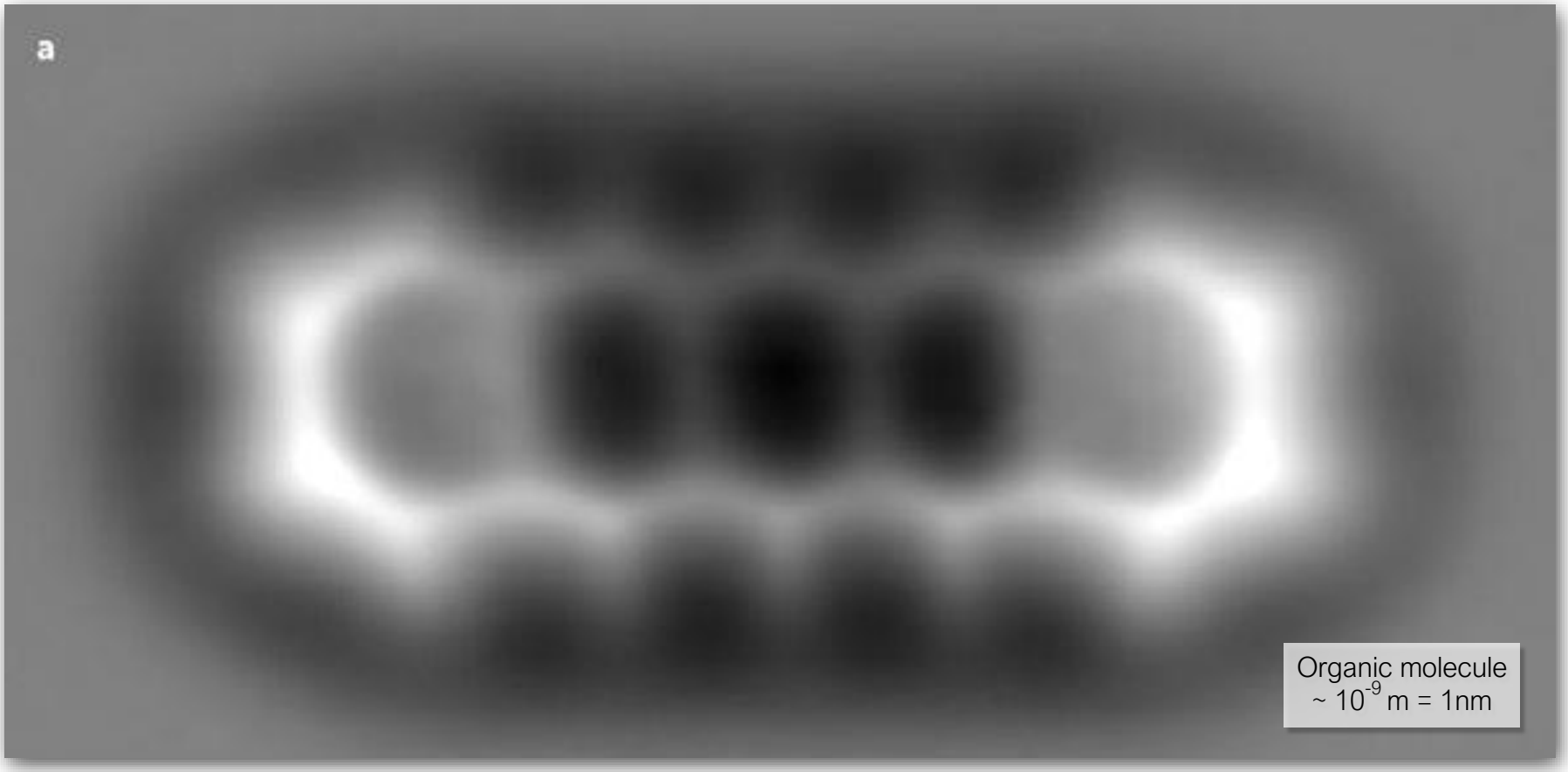
# Why Resolution? A matter of size



# Why Resolution? A matter of size

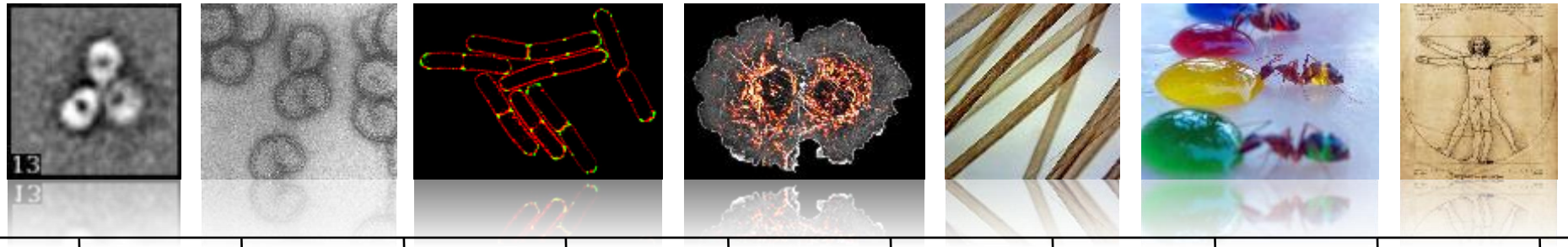


# Why Resolution? A matter of size




https://www.nature.com/articles/nchem.418

# Why Resolution? A matter of size



1nm 10nm 100nm 1µm 10µm 100µm 1mm 10mm 100mm 1m

Human eye

From microscopy…

MRI, PET

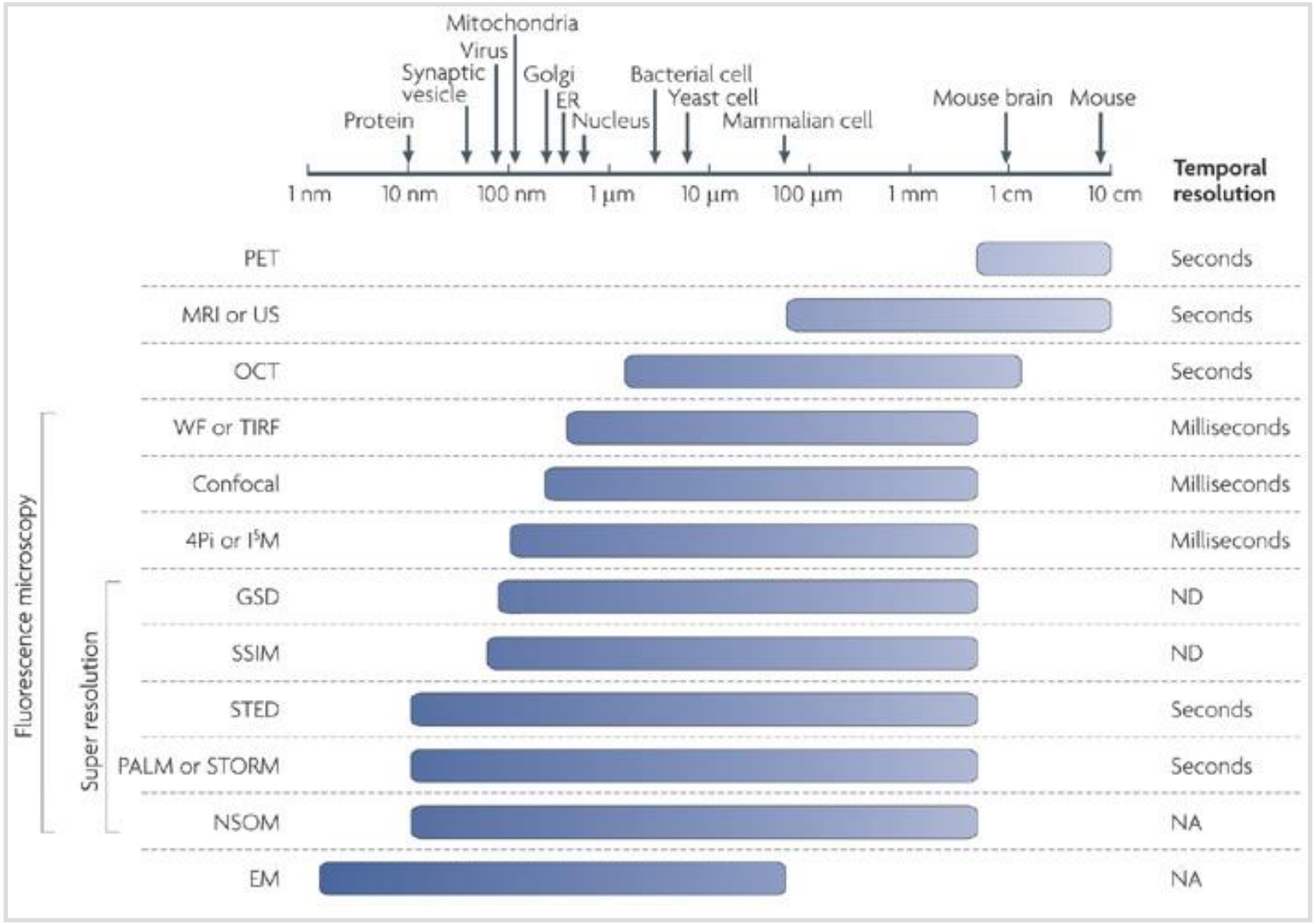
Light microscopy

Super resolution microscopy

…to nanoscopy

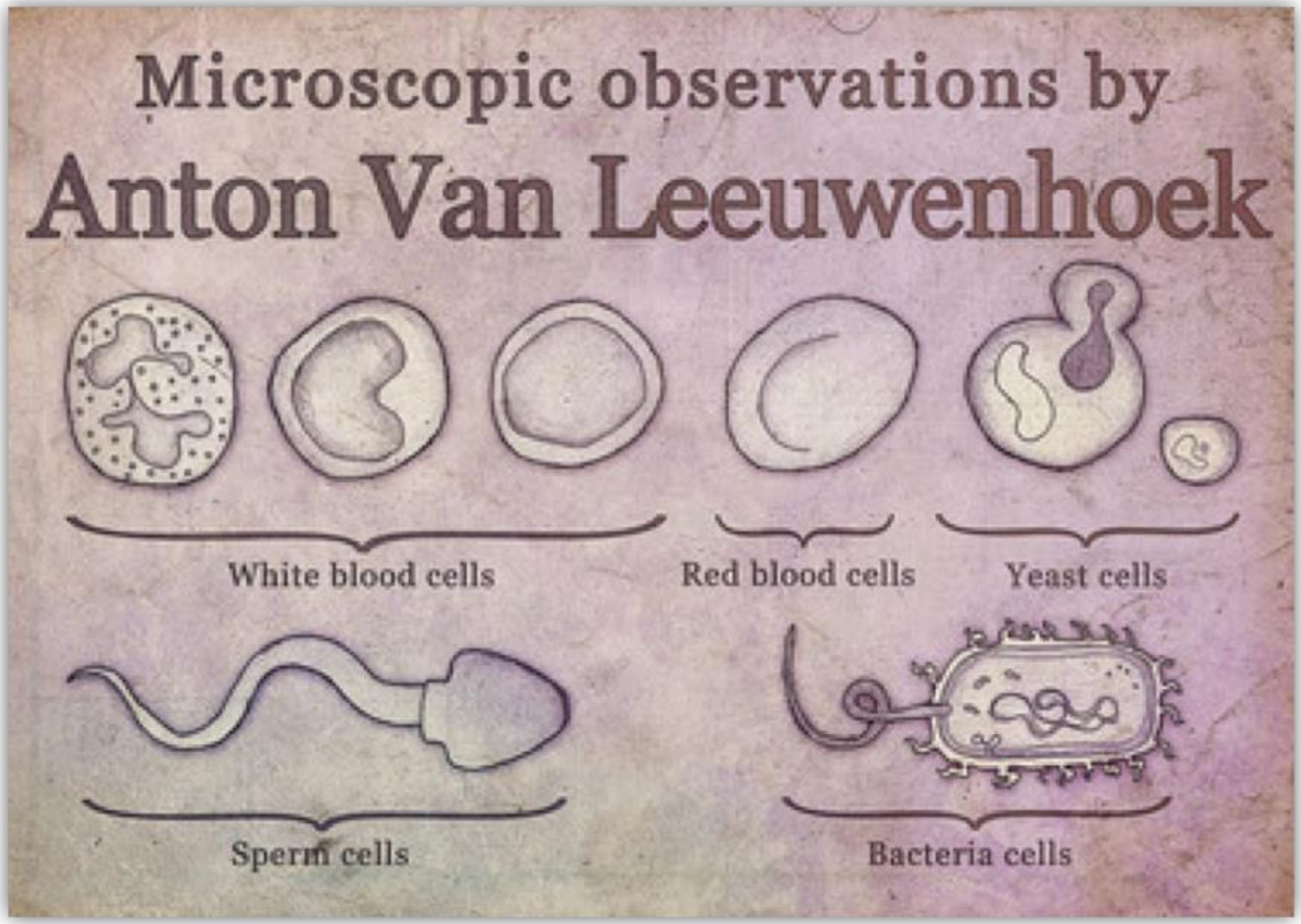
Electron microscopy

# Spatial + temporal




Not only resolution! (Penetration, biocompatibility, Ease, sensitivity)

# A bit of History: A. Van Leeuwenhoek (1632-1723)



# Galileo Galilei (1564-1642)



"Galileo's microscope was celebrated in the Accademia dei Lincei in 1624 and was the first such device to be given the name microscope"

# A bit of History: nowadays

Click to open expanded view

## My First Lab Duo-Scope Microscope - MFL-06

by My First Lab

996 customer reviews | 115 answered questions

List Price: ~~$79.99~~

Price: $63.99 & **FREE Shipping**. Details

You Save: $16.00 (20%)

In Stock.

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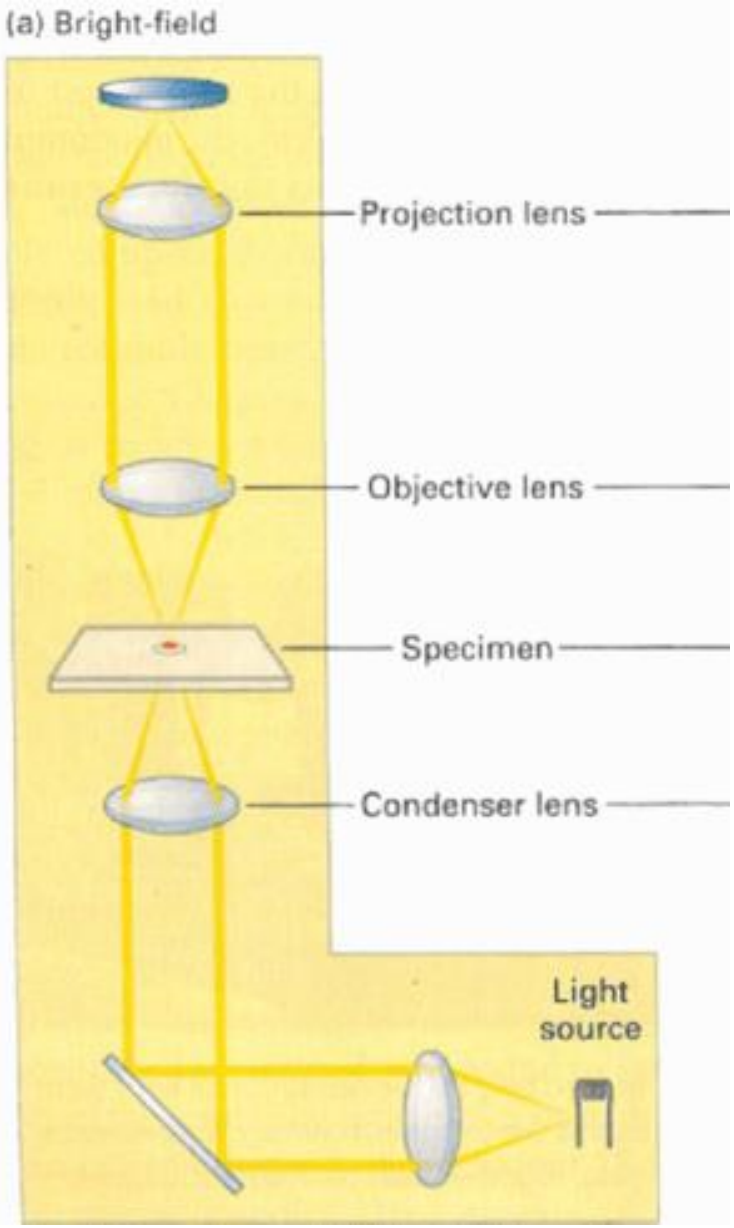
- Features 40x, 100x &400x magnifications
- Real Glass Optics
- 6 hole disk diaphragm
- Battery Operated
- Extensive, 50 pc accessory kit

8 new from $63.99 1 collectible from $55.06

# Brightfield microscopy



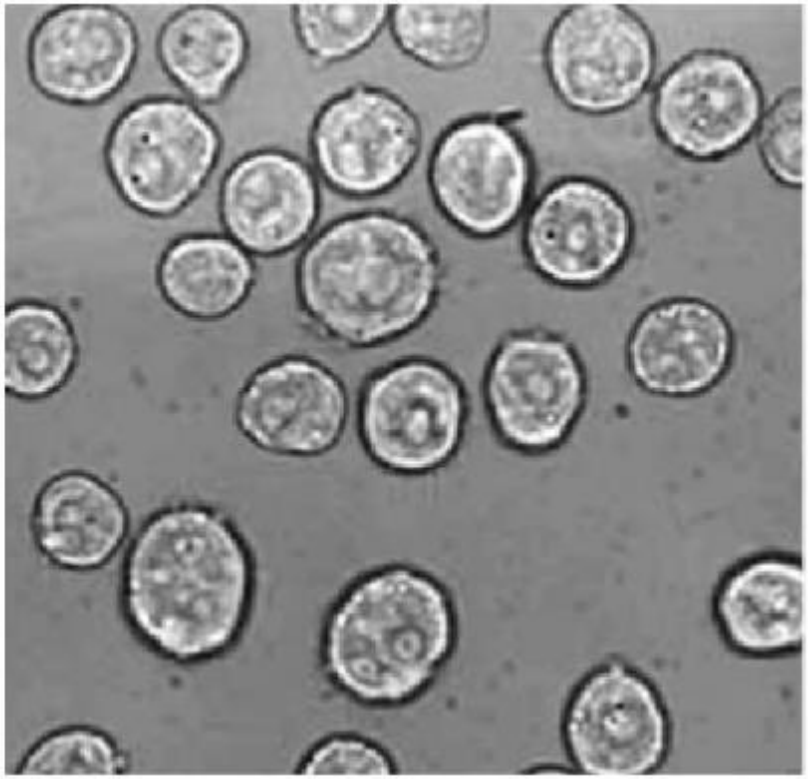

The light pass through the specimen.

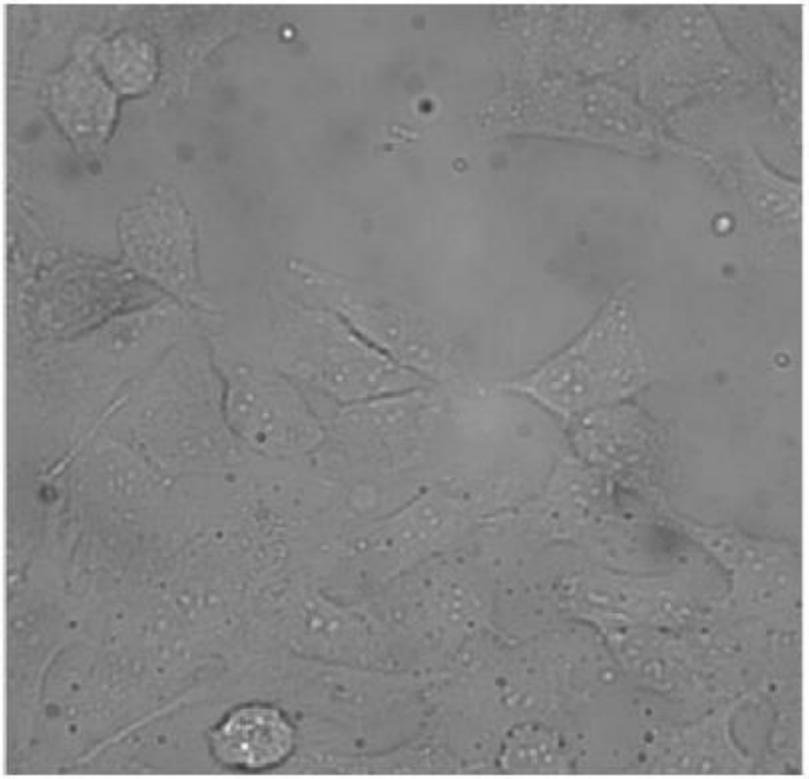
No object: light pass through —> Bright

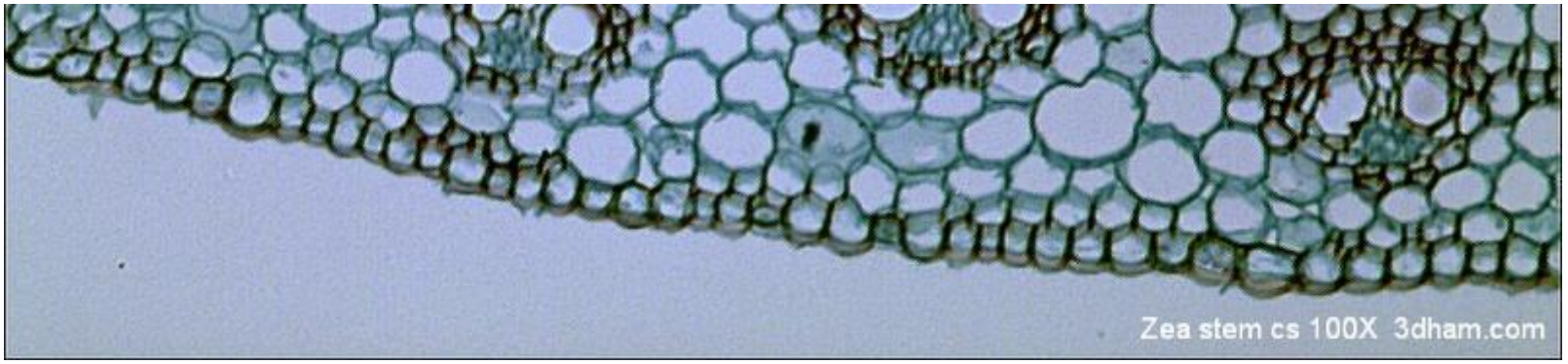
Object: light get scattered —> dark

DOI: 10.23889/Suthesis.50

# Brightfield microscopy








https://www.semanticscholar.org/paper/Automatic-segmentation-of-adherent-biological-cell-Ali-Gooding/c536a1cb523139d7dfc59a1f8570764c21555538

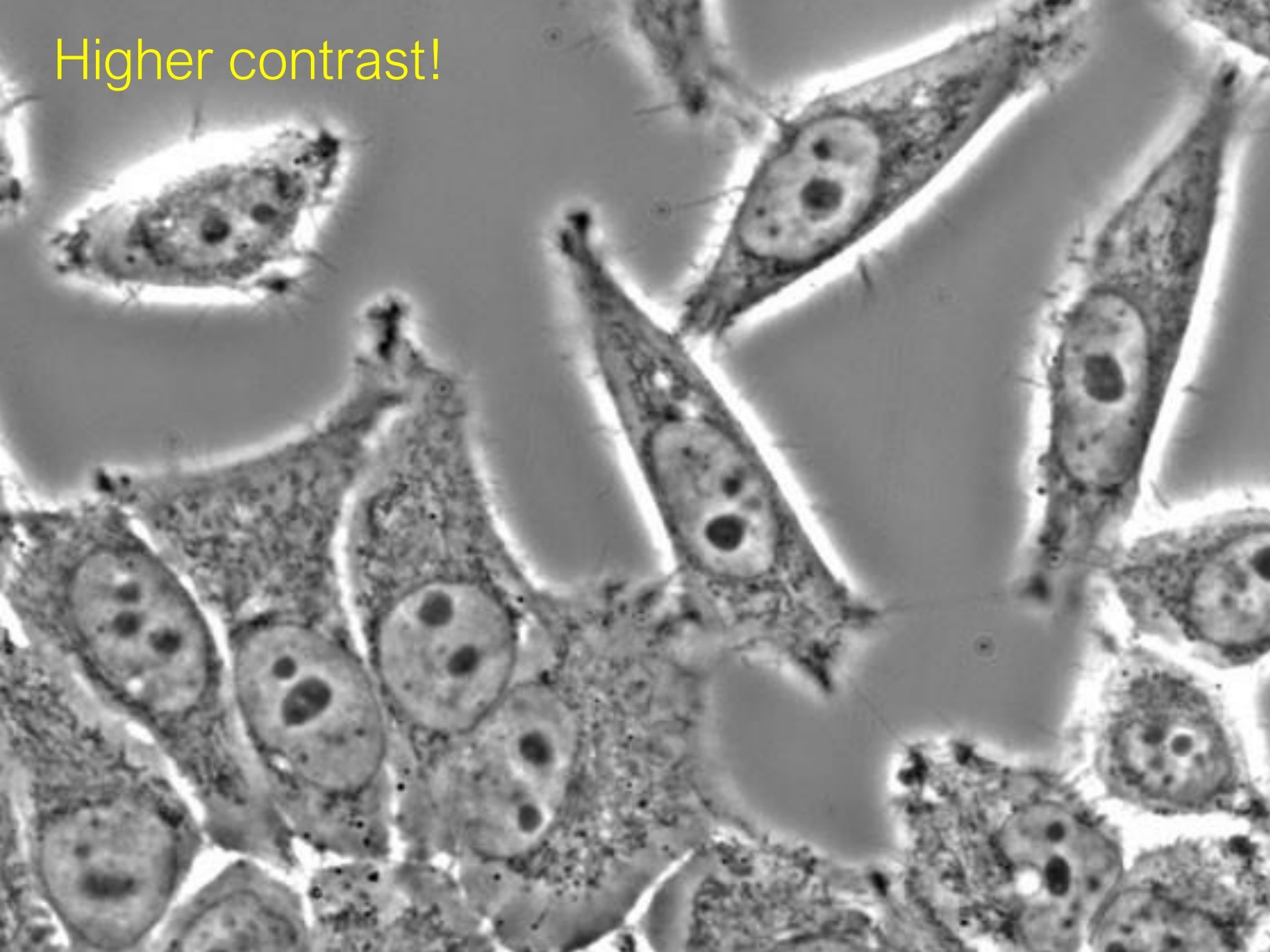
# Brightfield microscopy

+

Simple
Inexpensive
Fast

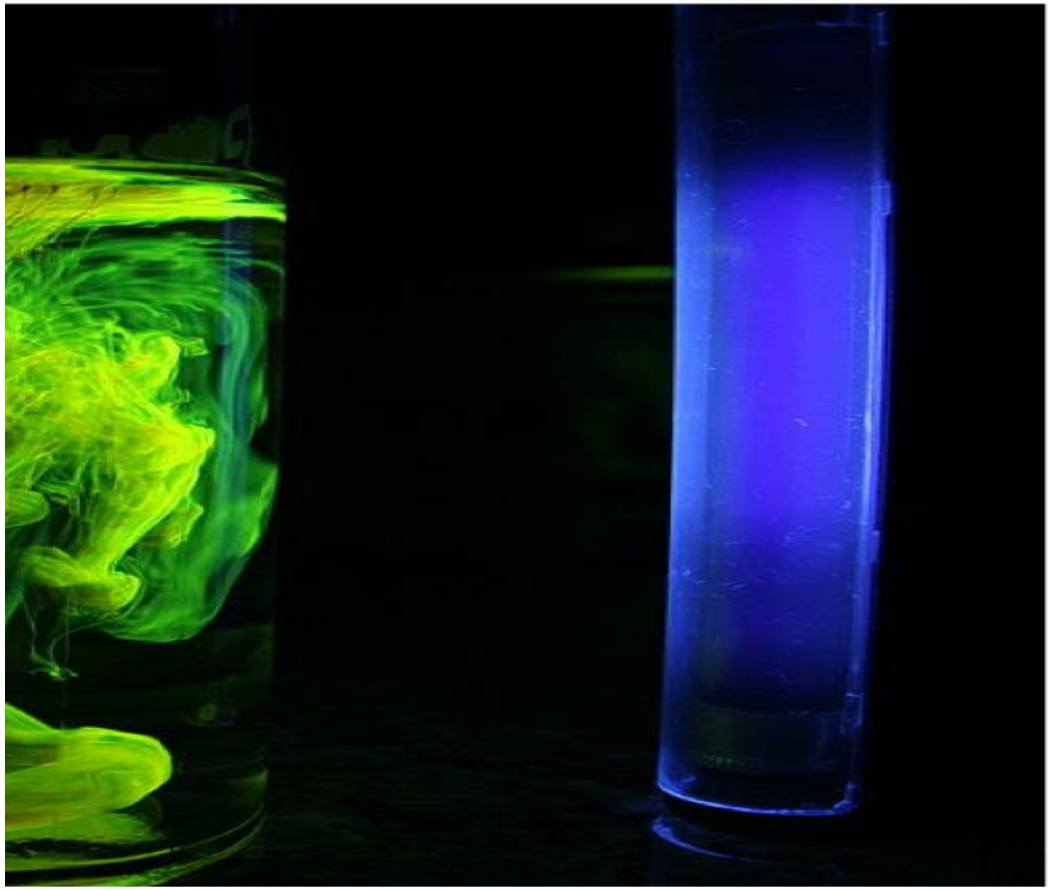
-

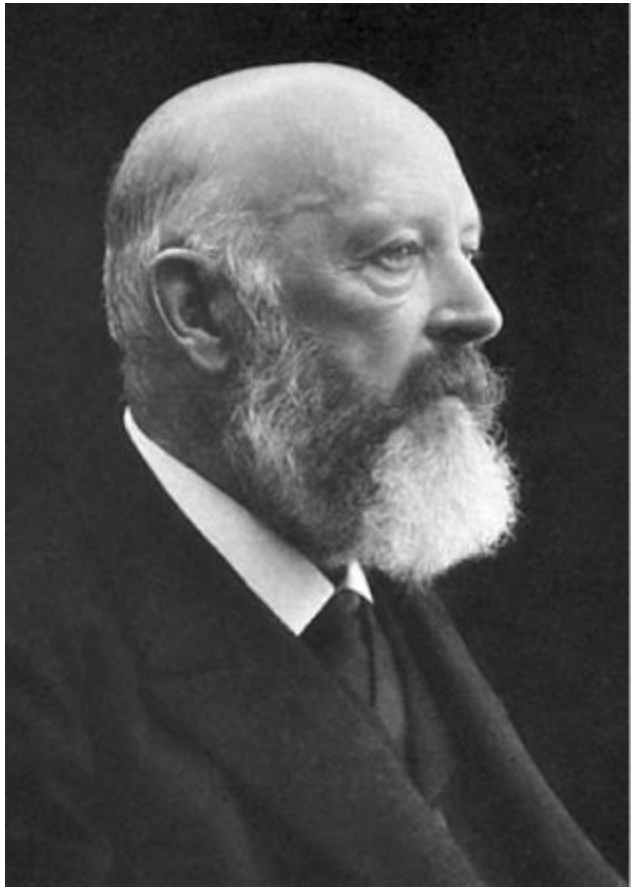
Low contrast
Blurry images due to out of focus objects
Requires thin sample


Higher contrast!


10 µm

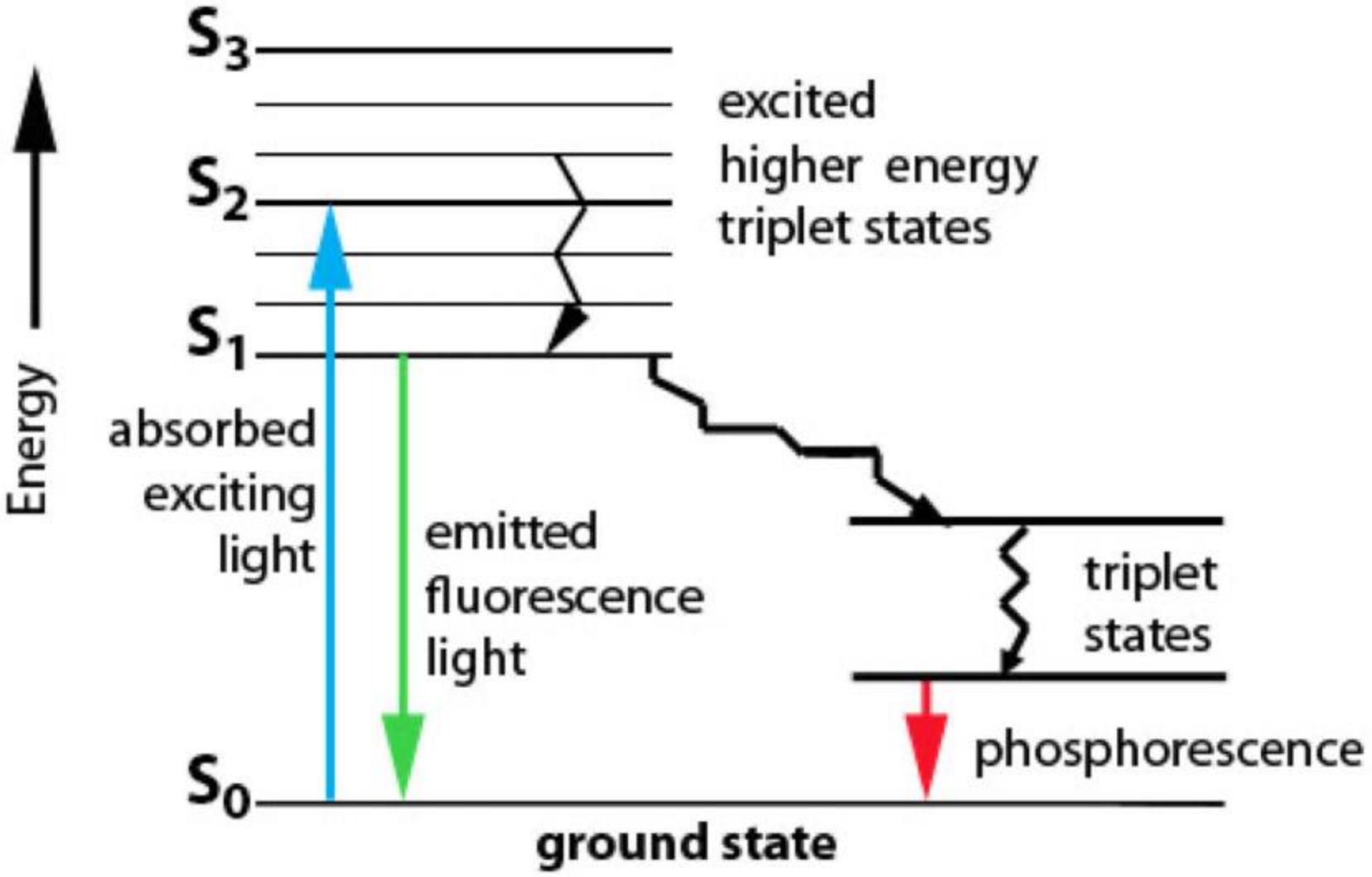
# Fluorescence microscopy



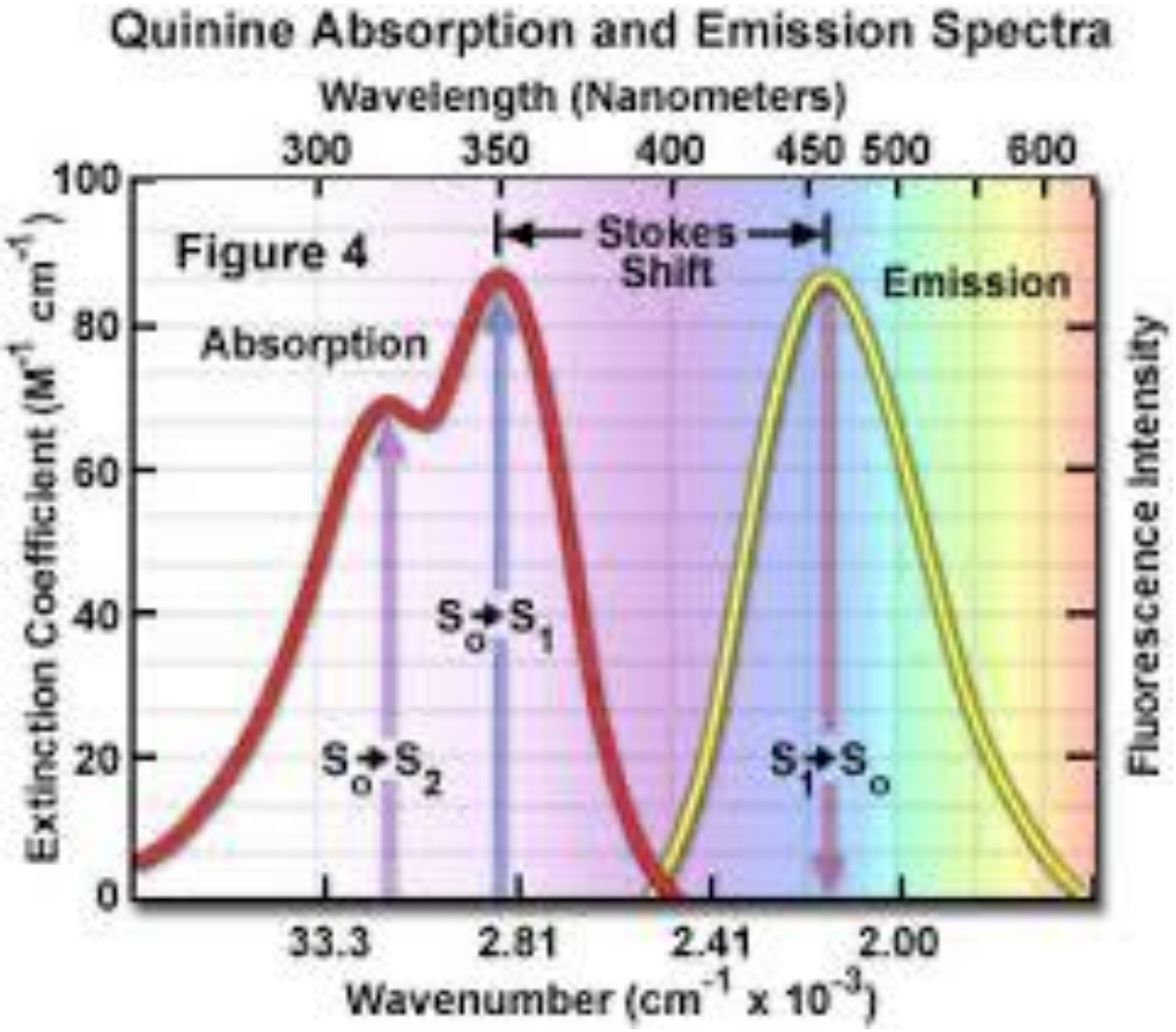


(1871) Synthesis of fluorescein - Adolf von Baeyer

# Fluorescence microscopy

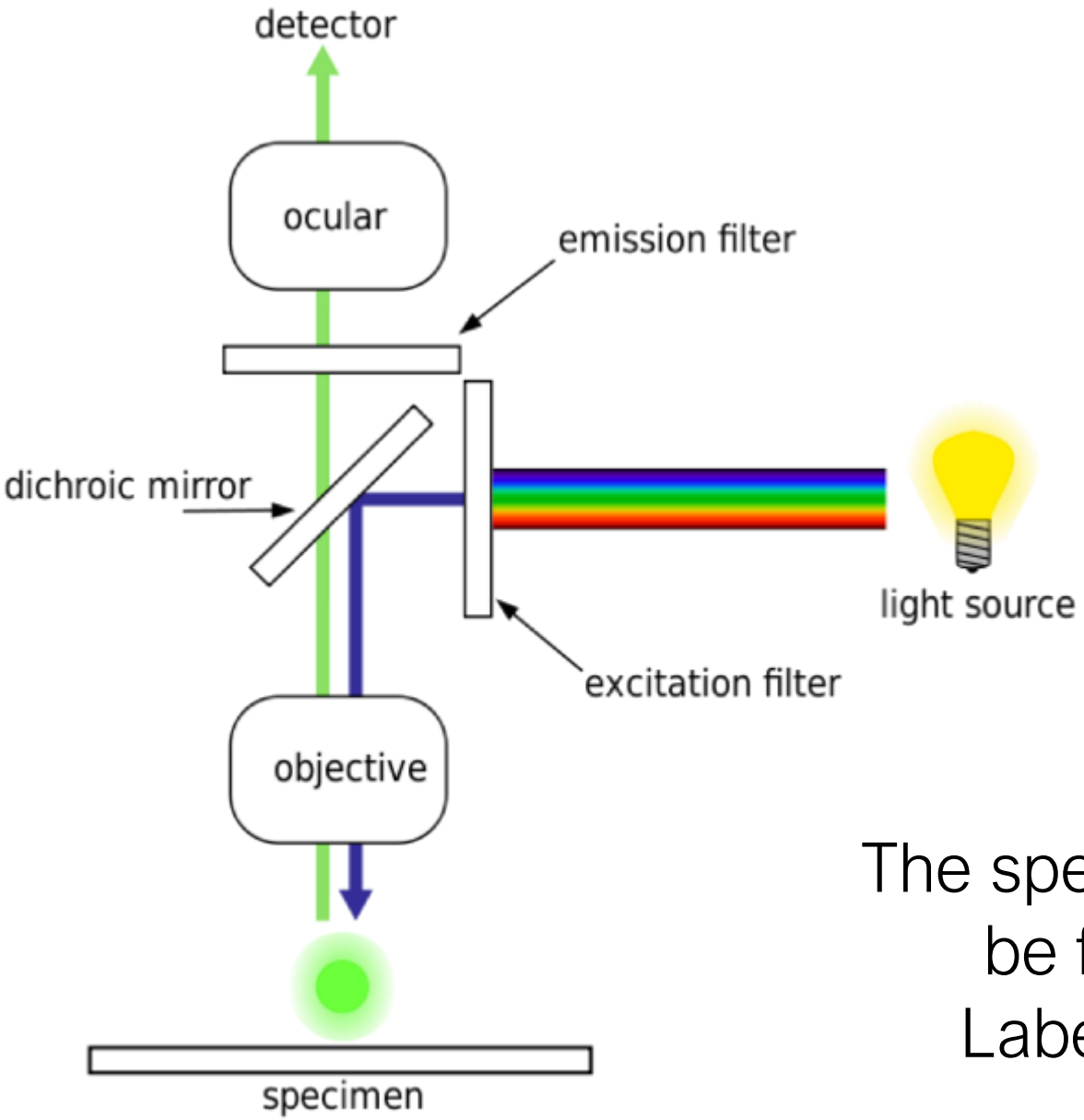


# Fluorescence microscopy



# From fluorescence to microscopy

# Fluorescence microscopy




The specimen needs to be fluorescent!
Labeling needed

# Fluorescence microscopy

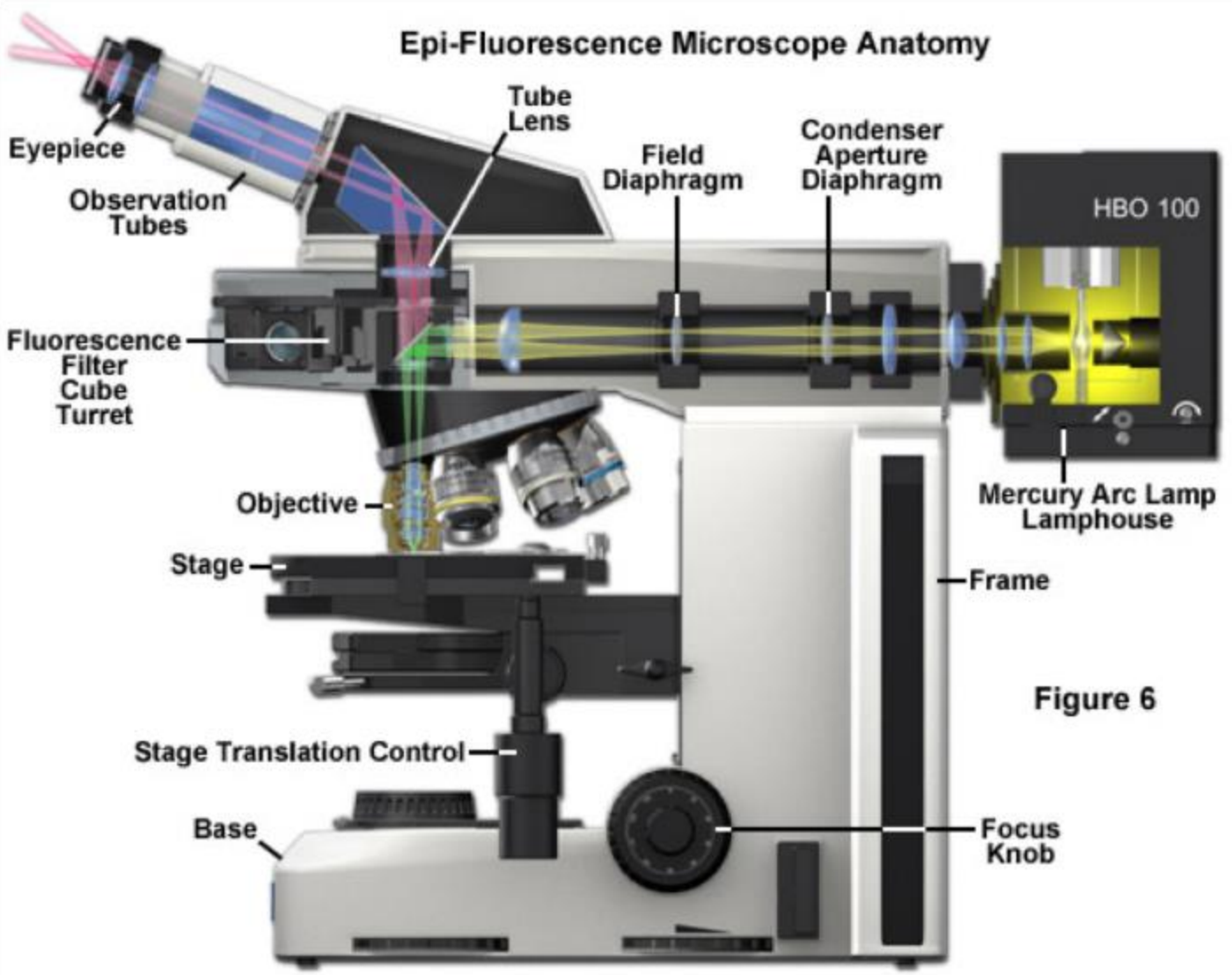
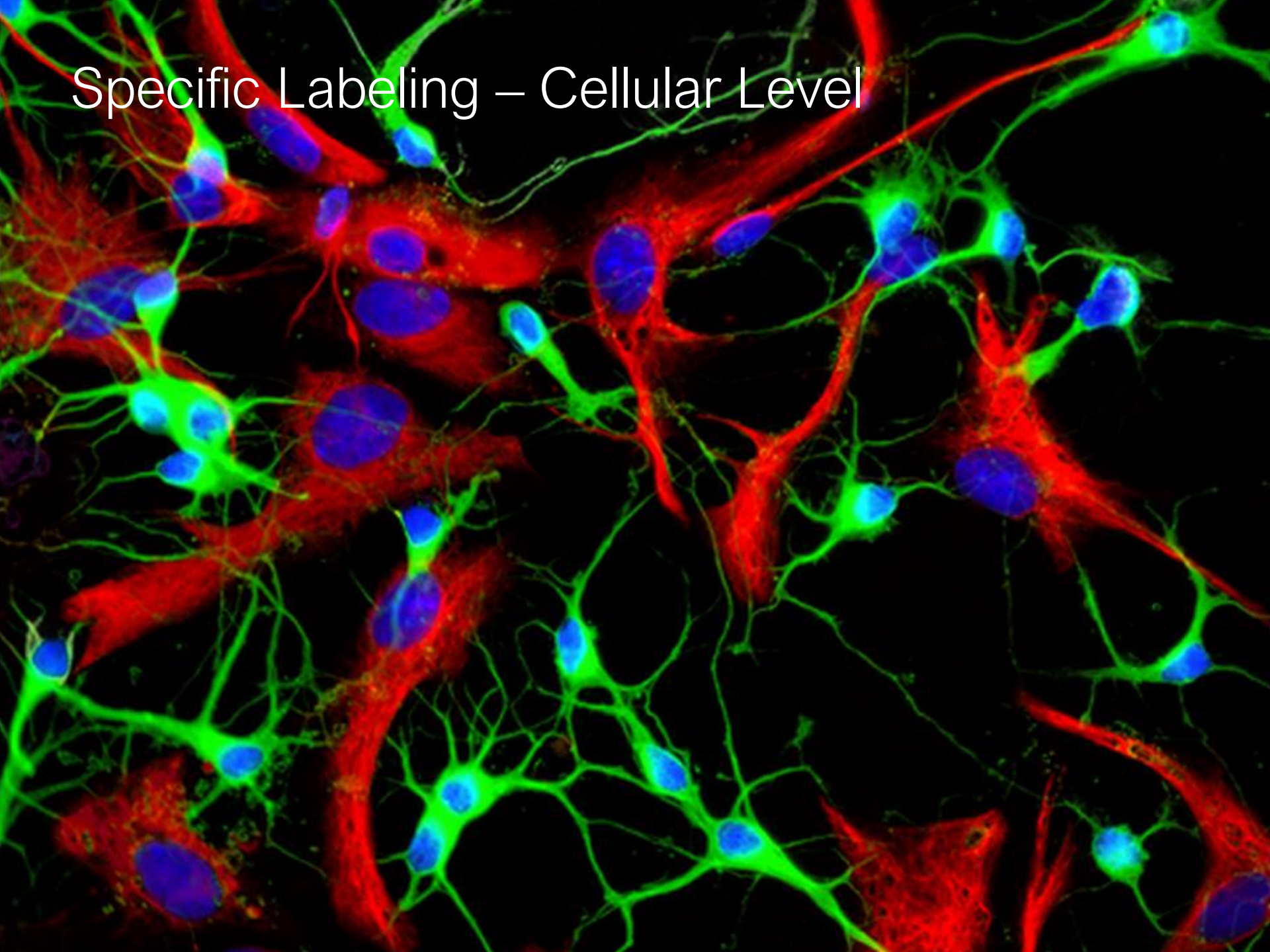


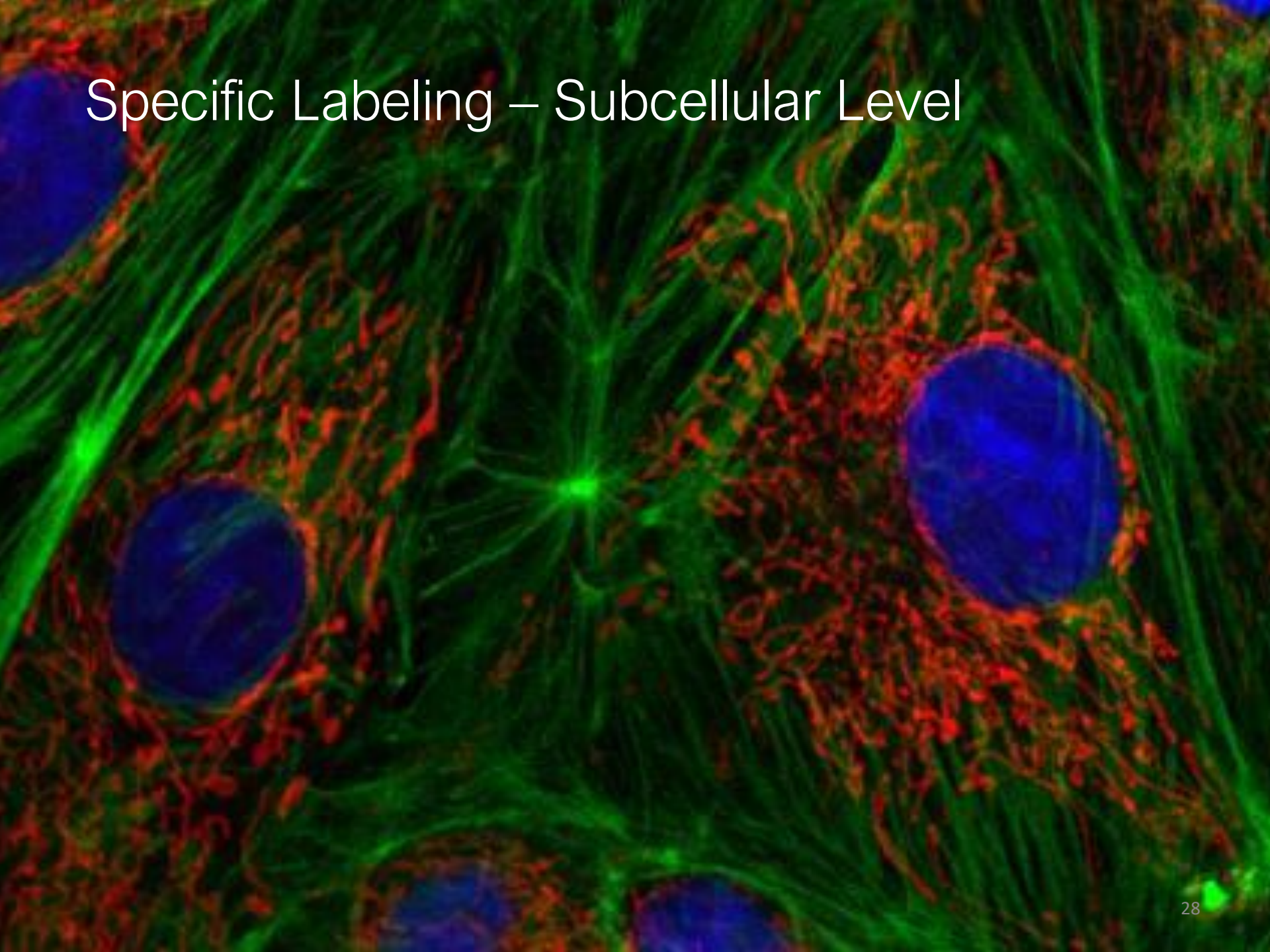

Figure 6

# Why Fluorescence?

# Specific Labeling – Cellular Level

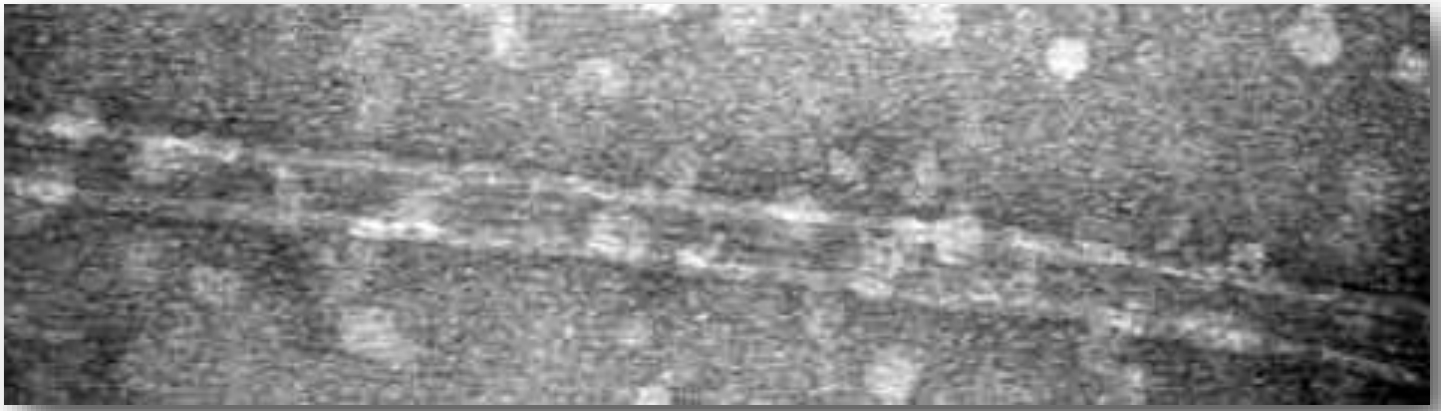


# Specific Labeling – Subcellular Level

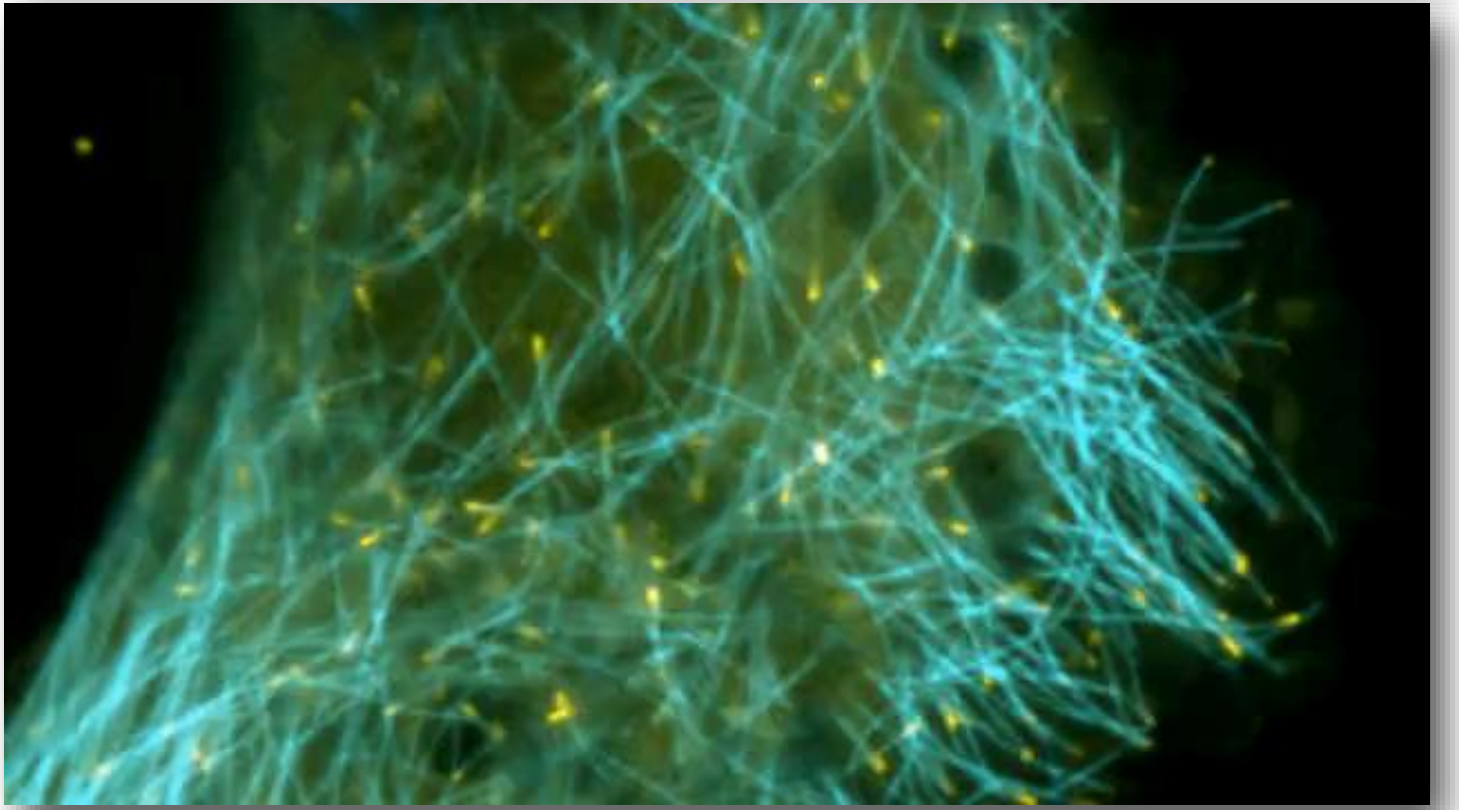


# Fluorescence microscopy

EM

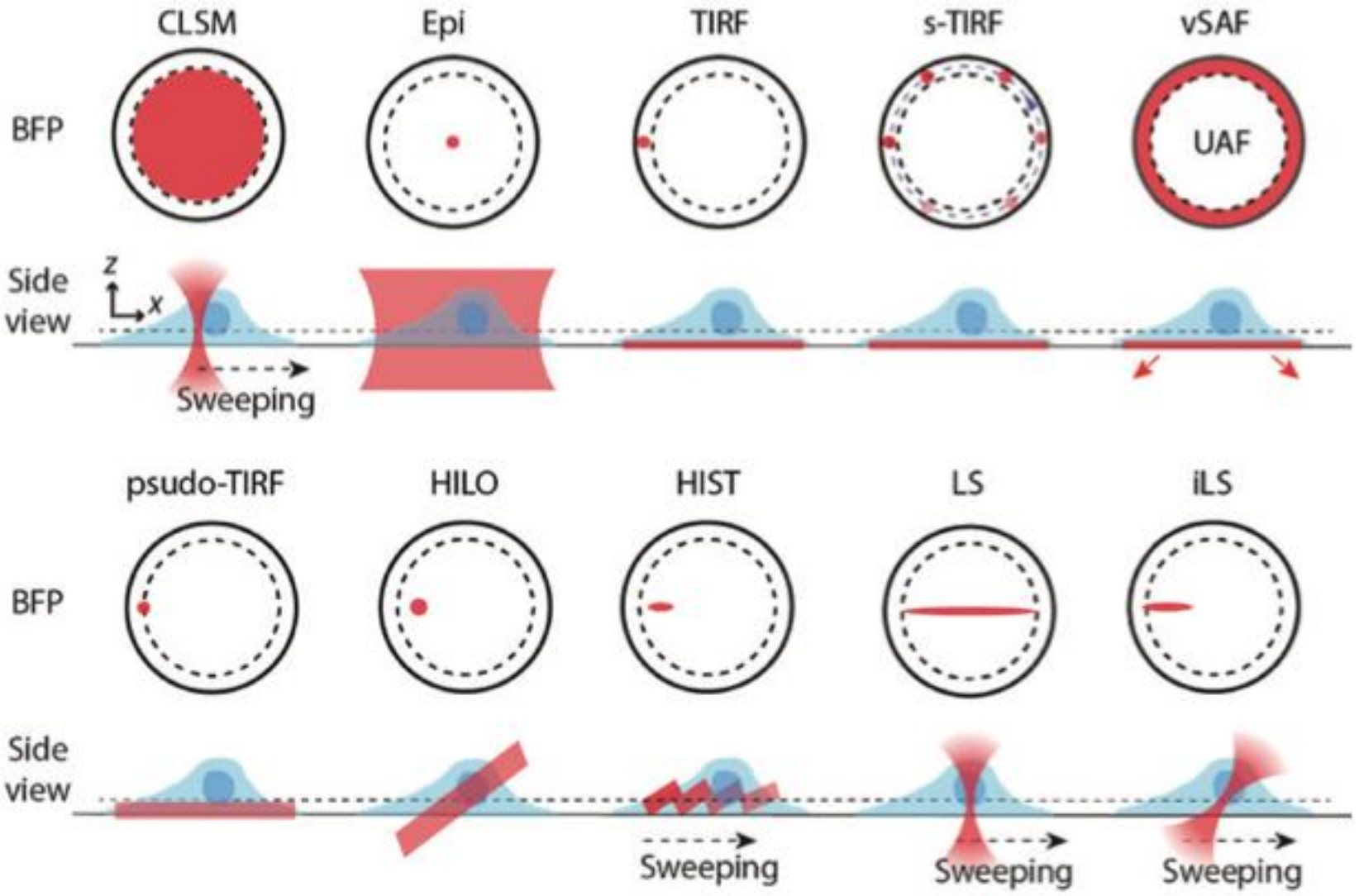


LSCM



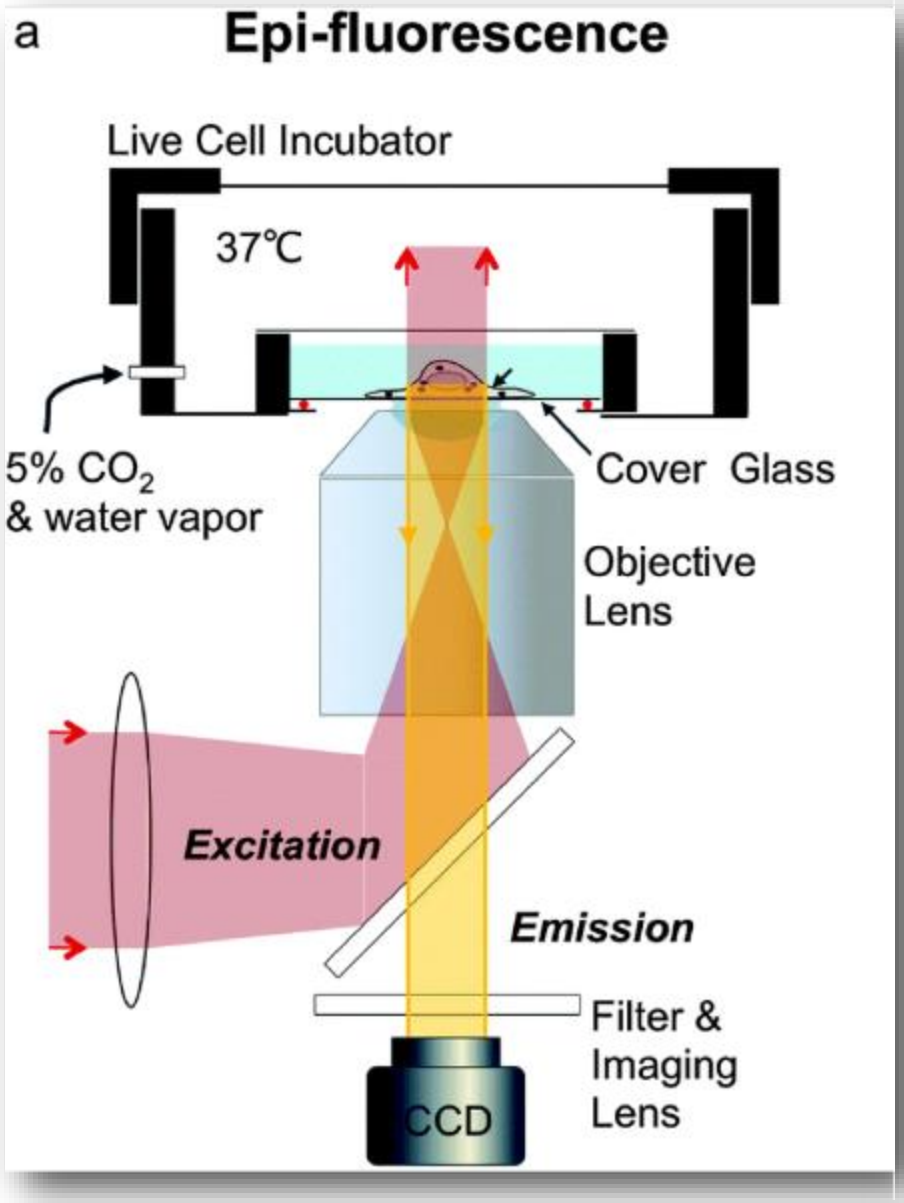
# Fluorescence microscopy

# Different illumination different microscope




J. Tang et al.: Fluorescence imaging with tailored light

# Widefield or Epifluorescence



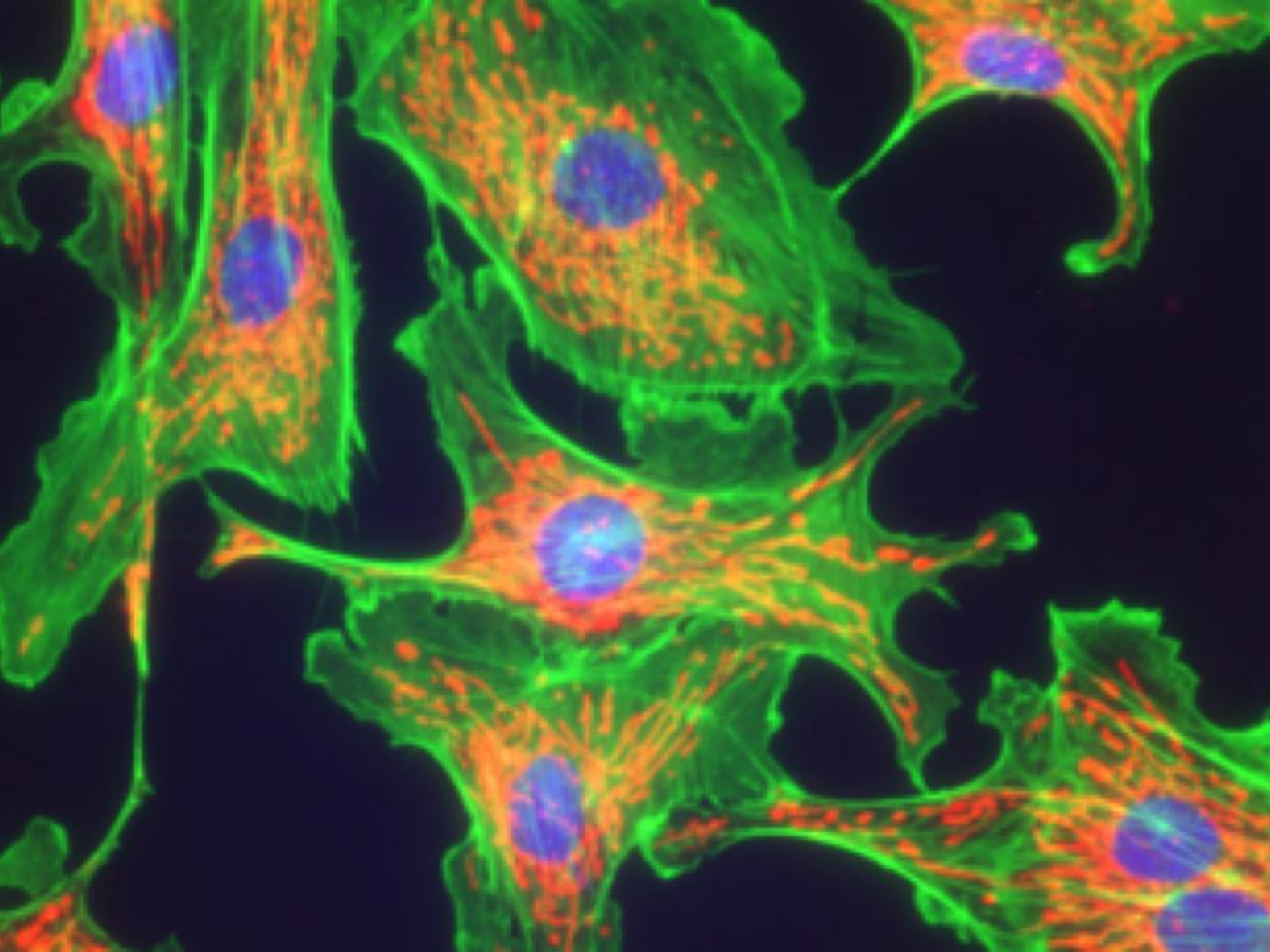

\+

Simple
Inexpensive
Fast

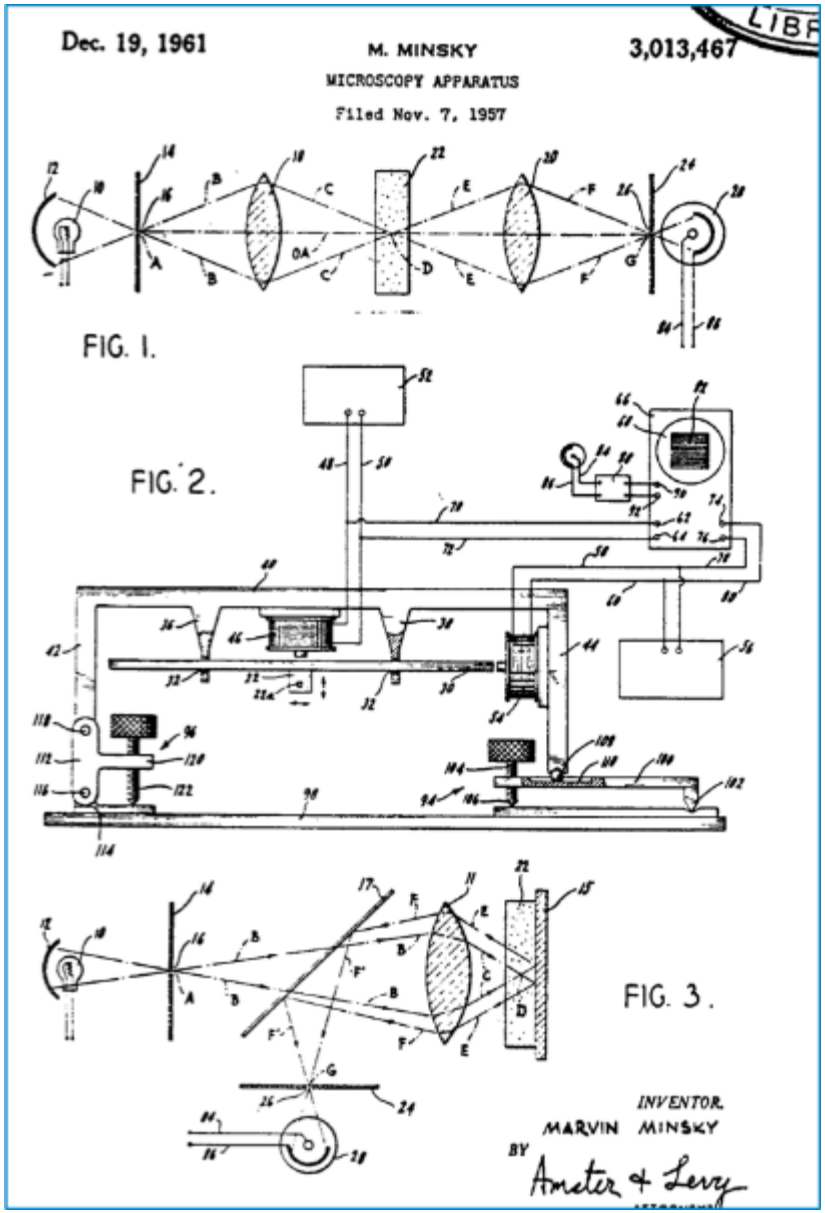
\-

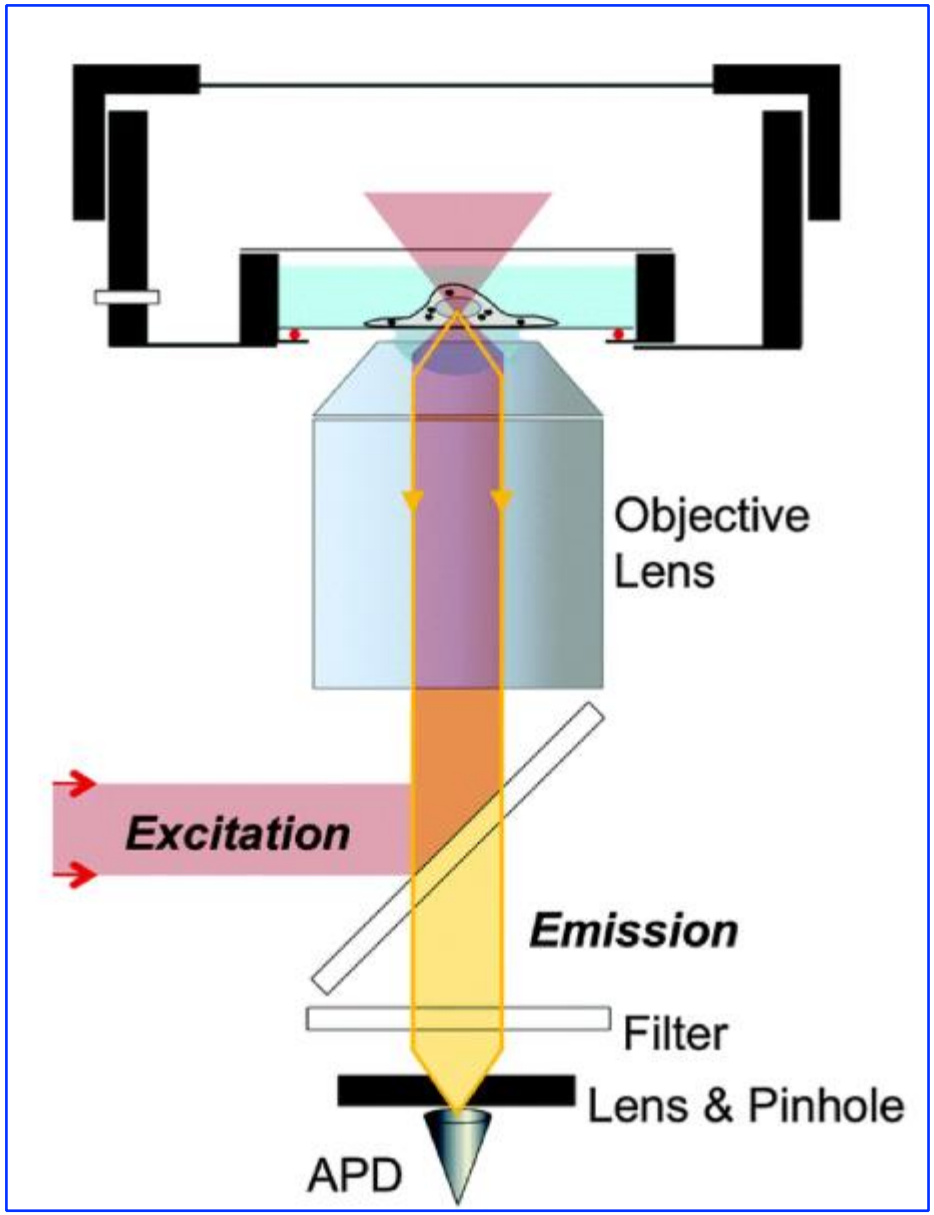
Low resolution
Out of focus light (blurred)

Widefield or Epi… NOT THE NORMAL MICROSCOPE

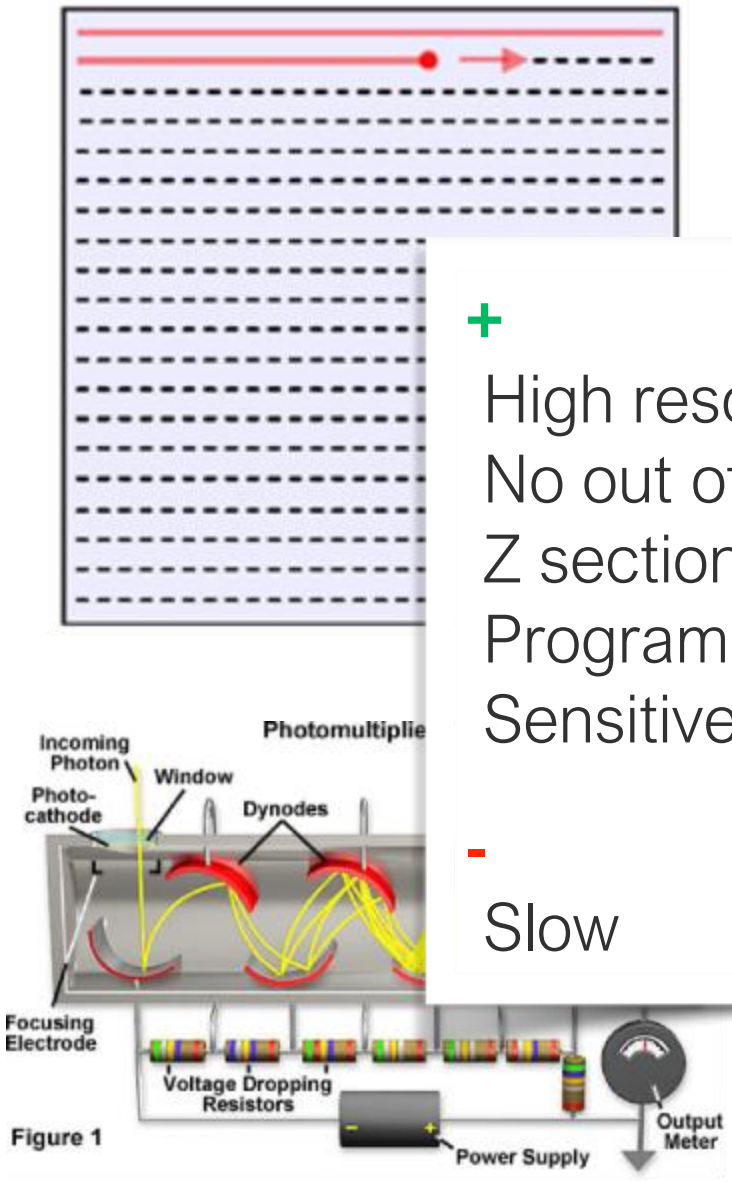


# Confocal microscopy



# Confocal microscopy

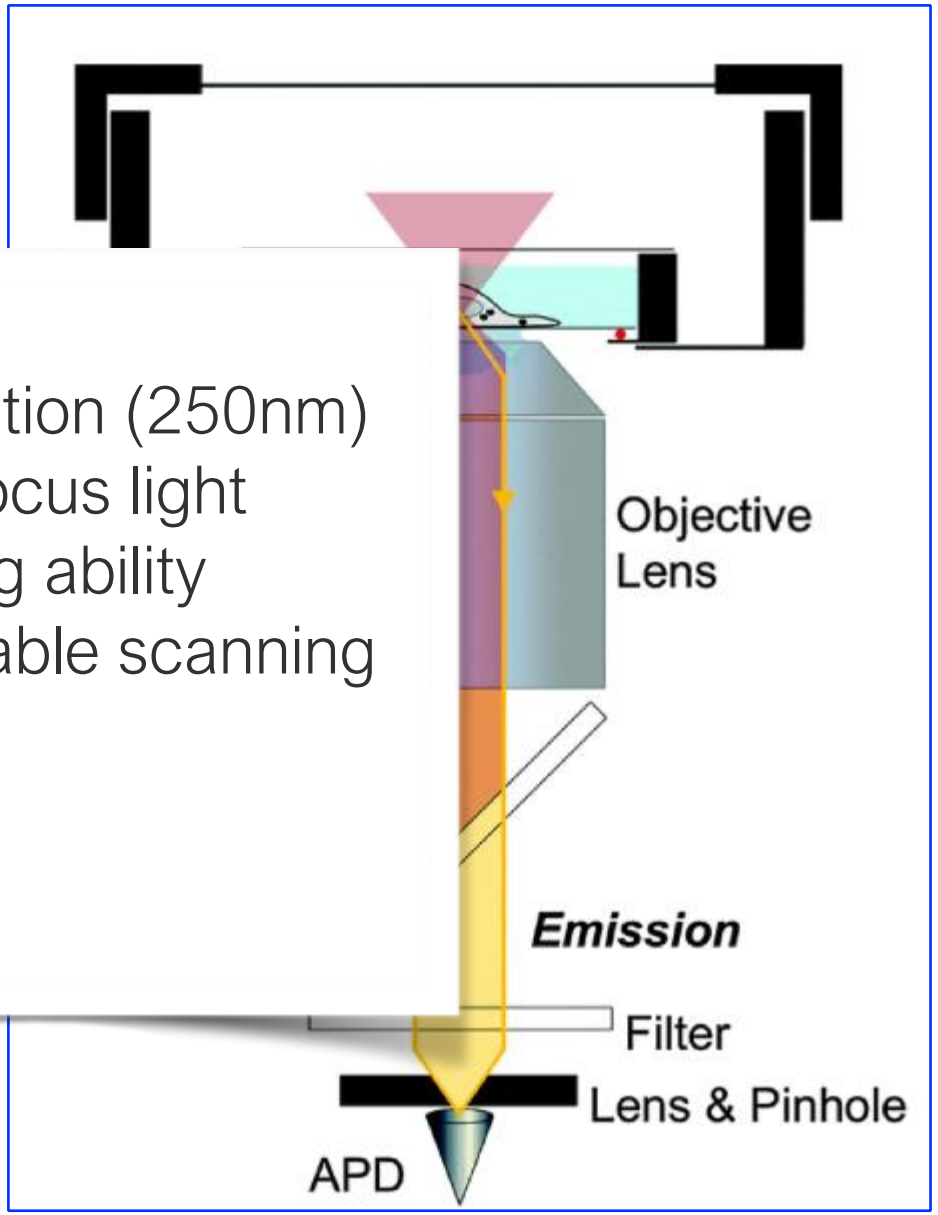



\+

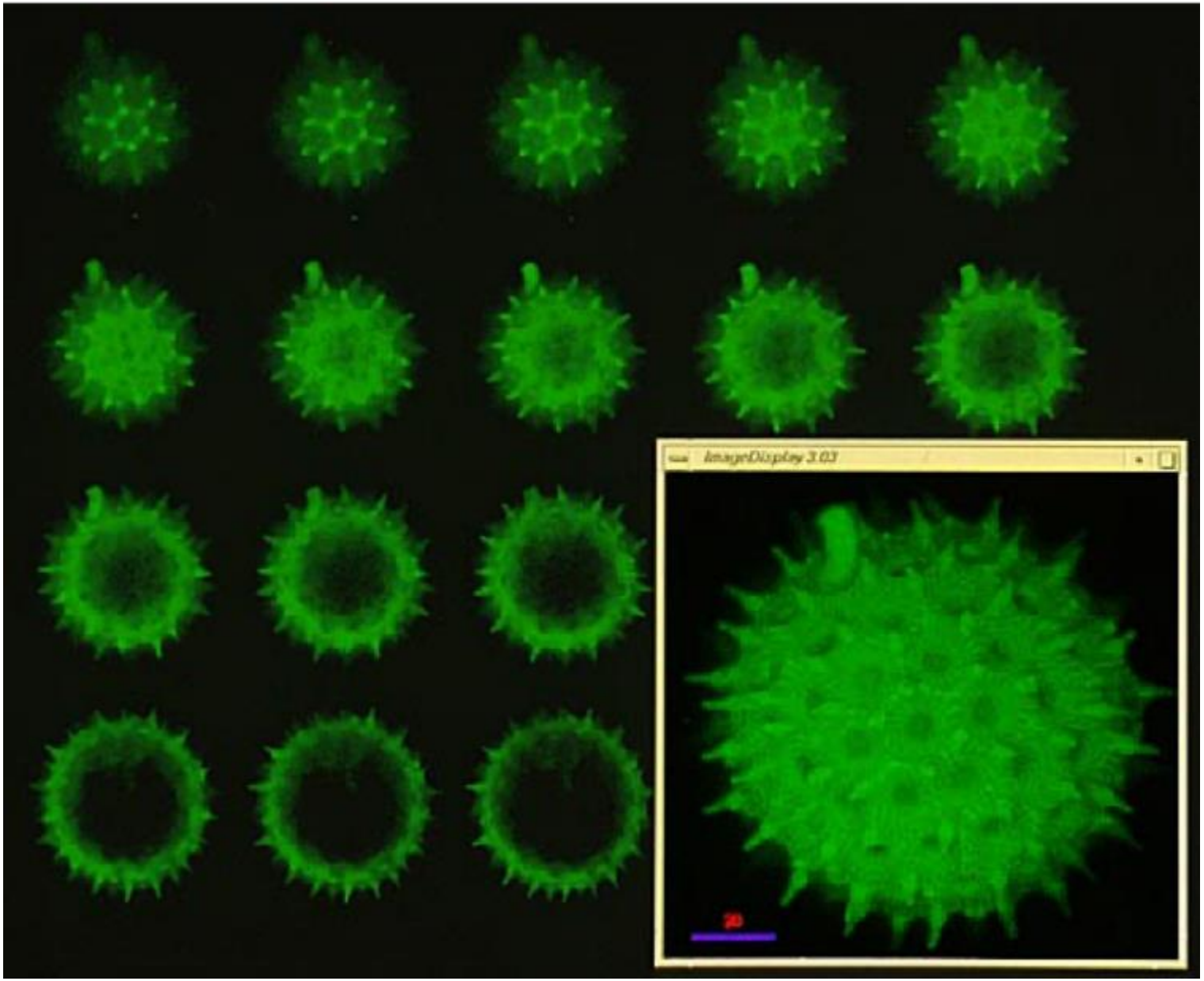
High resolution (250nm)
No out of focus light
Z sectioning ability
Programmable scanning
Sensitive

\-

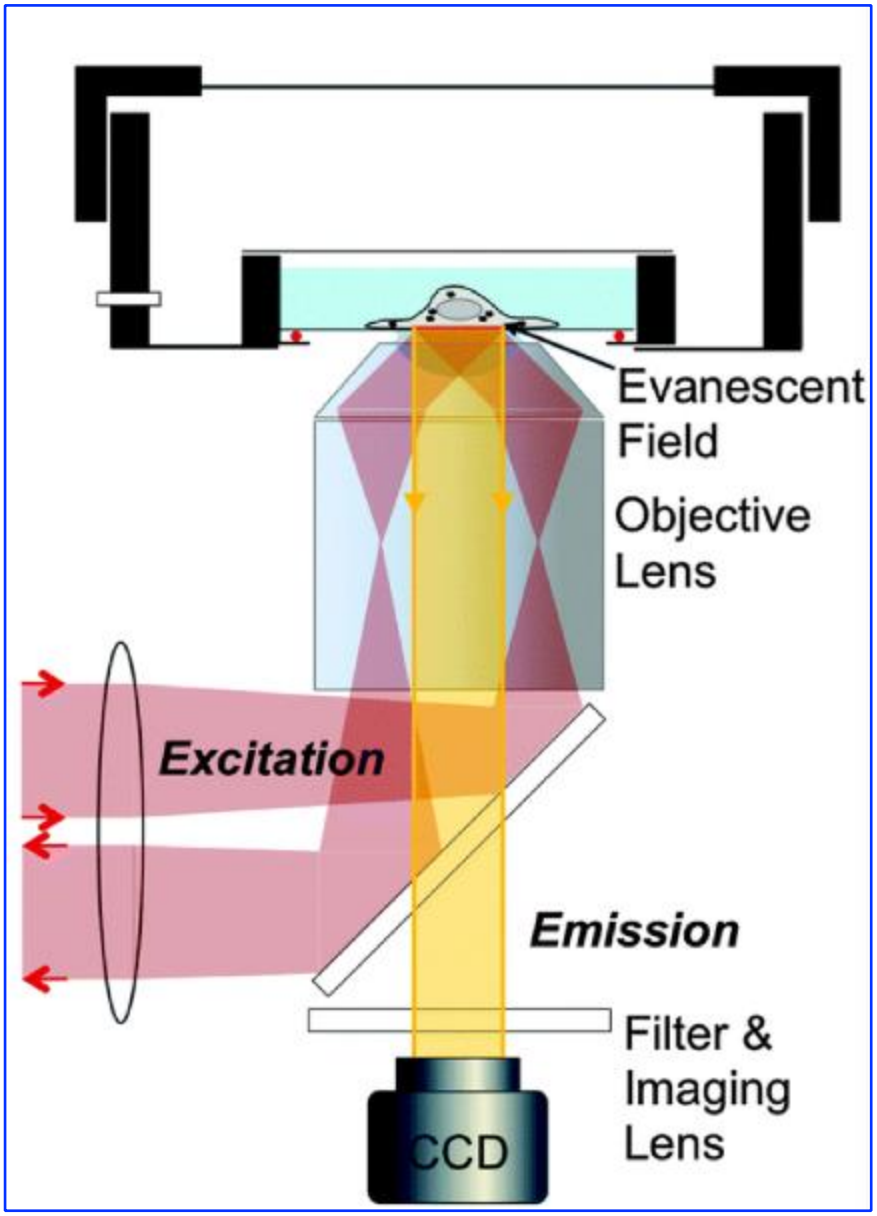
Slow



# Confocal microscopy - 3D microscopy



# TIRF




Total Internal Reflection Fluorescence

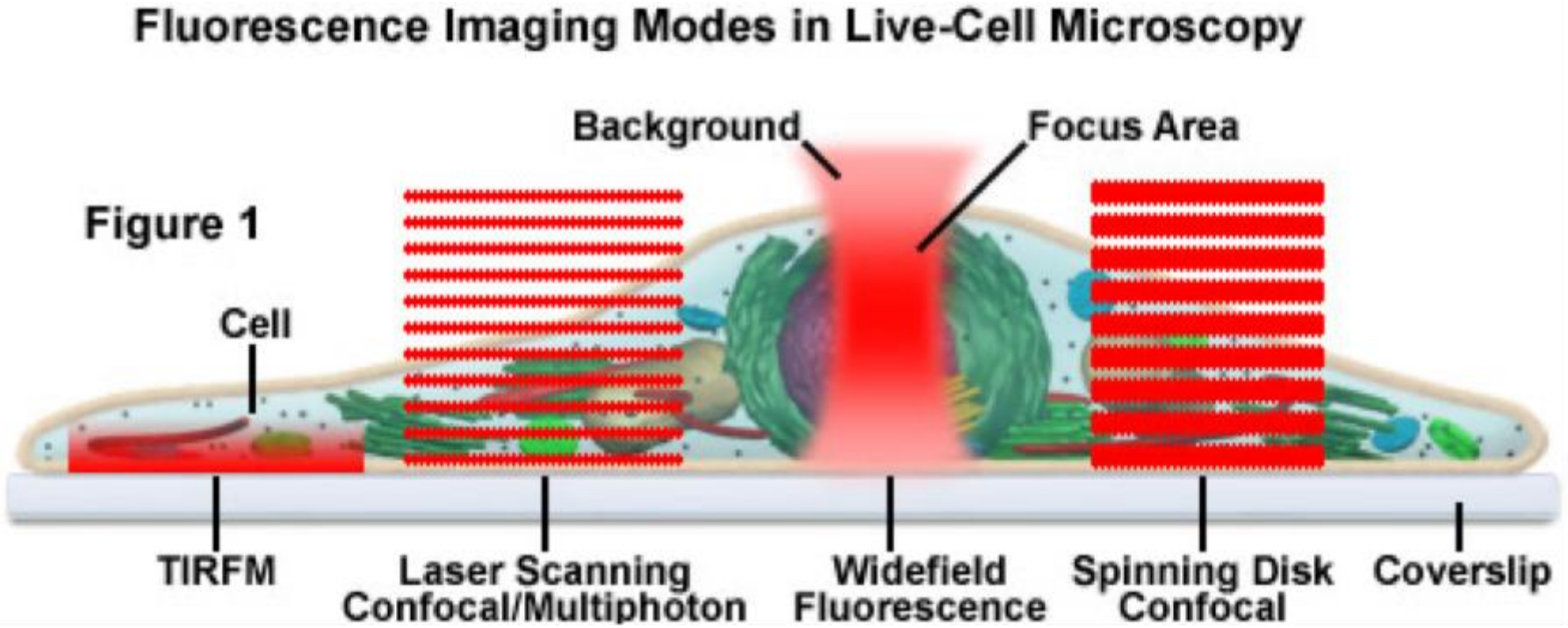
\+

Unique S/N
Fast
Single molecule sensitivity

\-

Limited to surface
Expensive

# No one is perfect



# The need for the dye

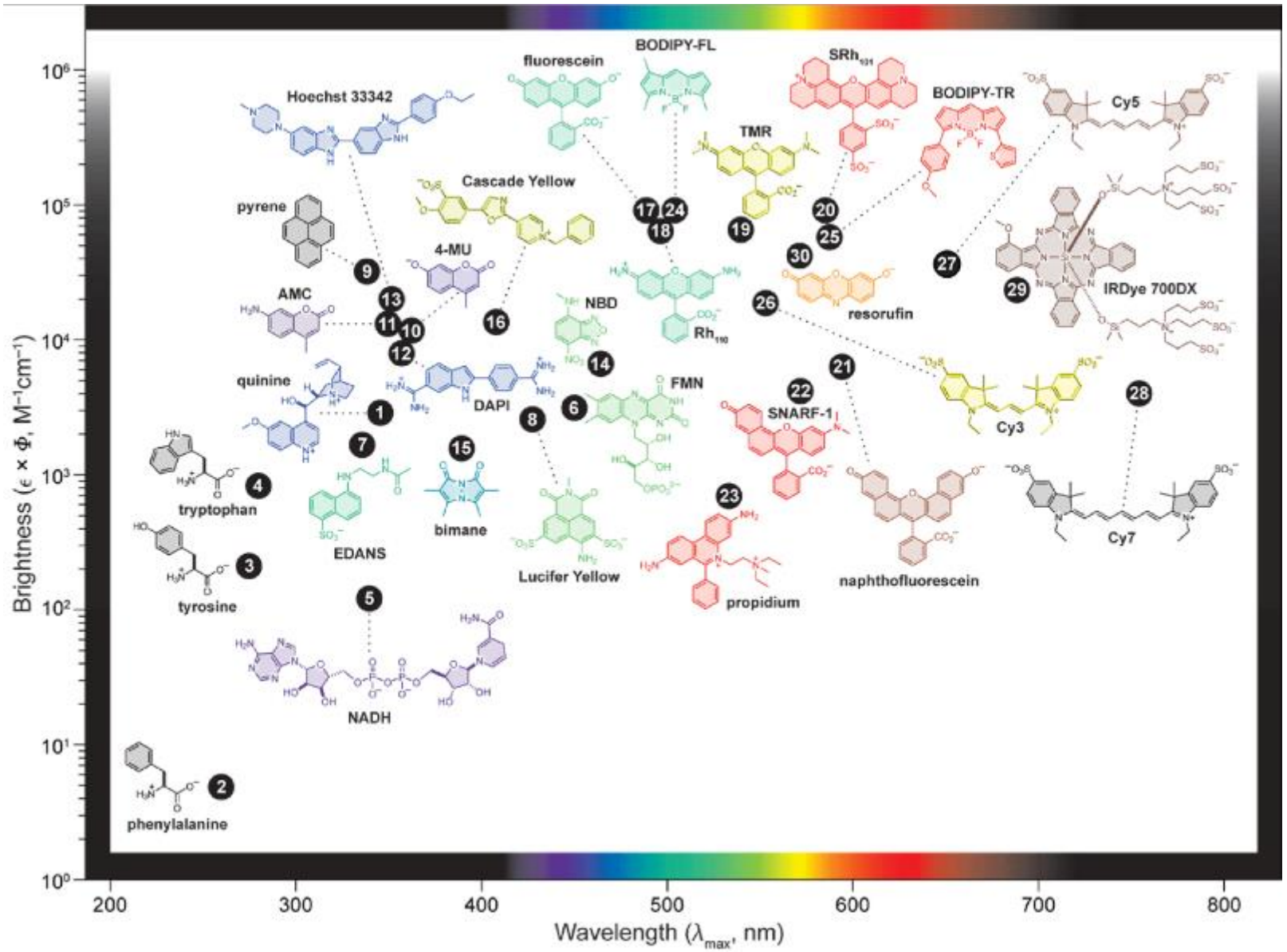
Generally specimen are NOT fluorescent.

Need for labeling = attach a fluorescent marker to the object of interest

# What are the properties of an ideal dye?

- Super bright (Abs * QY)
- Small
- Never Bleaching
- Significant Stoke shift
- Easy to conjugate
- Not-perturbing

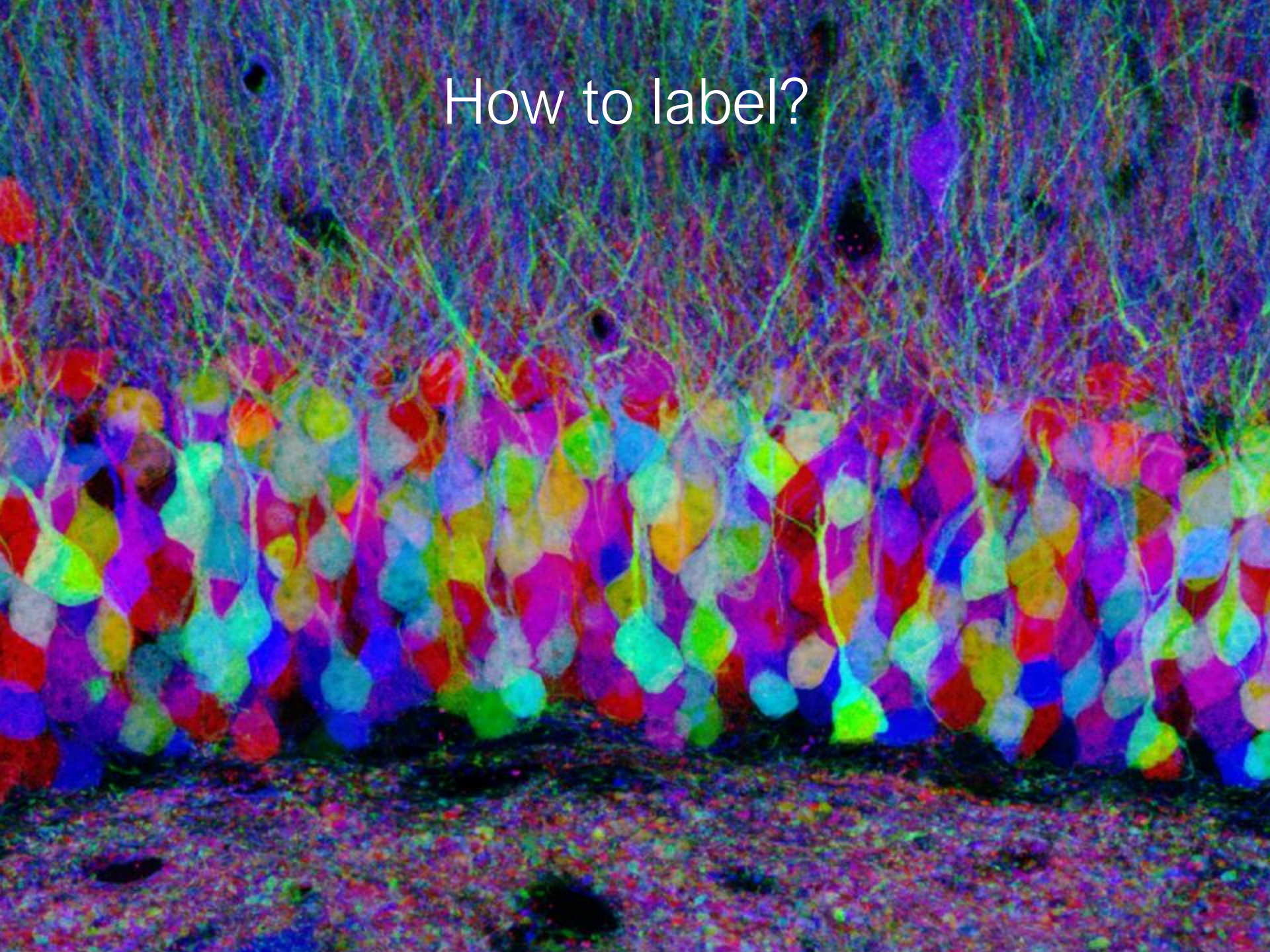
# Organic flurophores



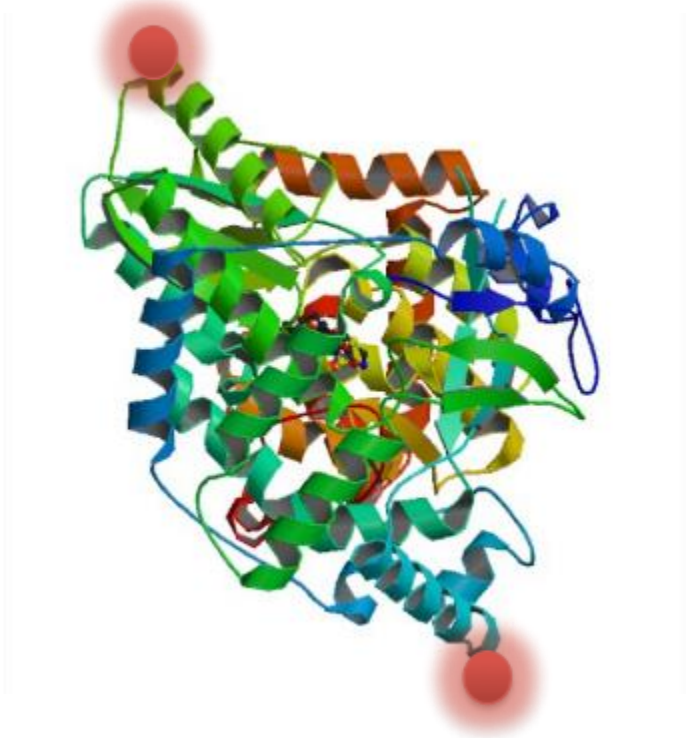
# Quantum dots

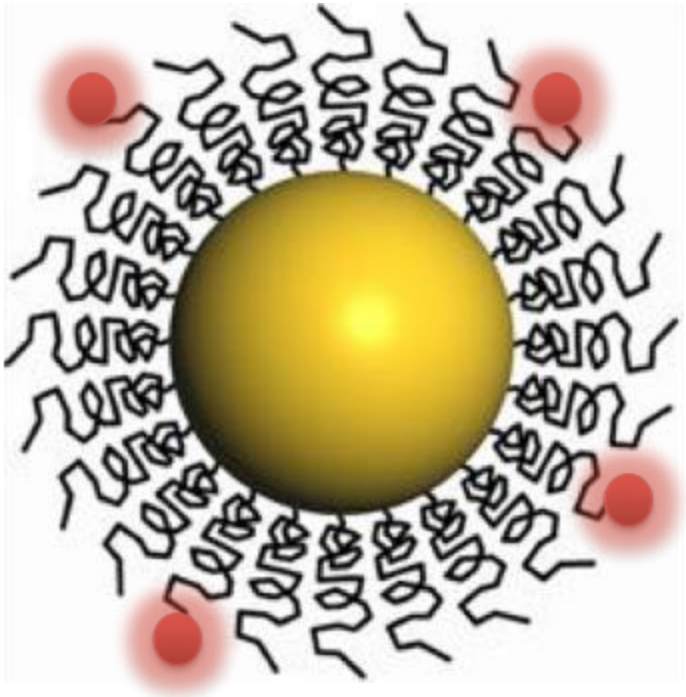
/S

Se


How to label?

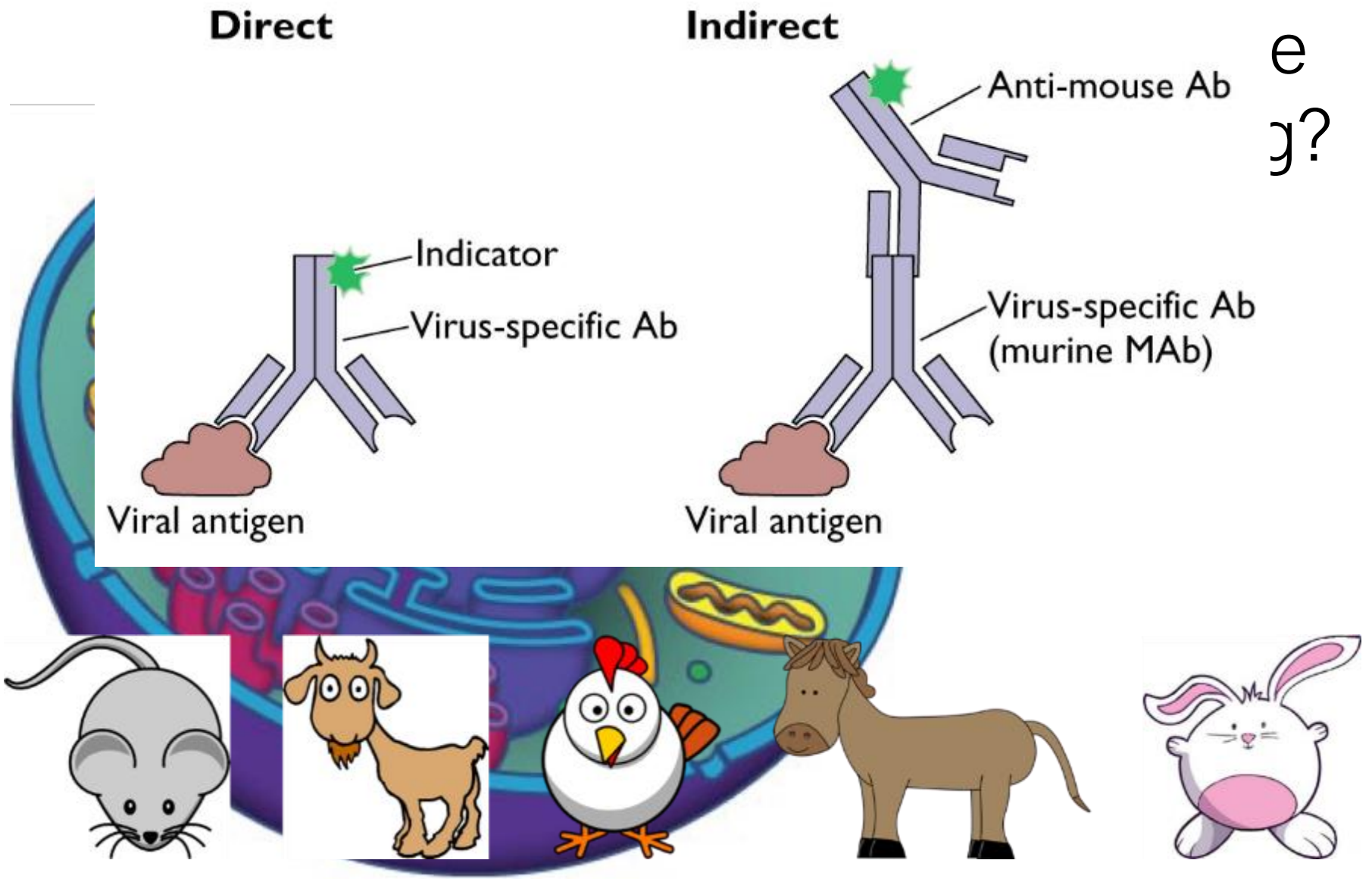
# Covalent labeling





EDC/NHS - Maleimide-Thiol - Click-Chemistry

# Cell labeling



# Cell labeling

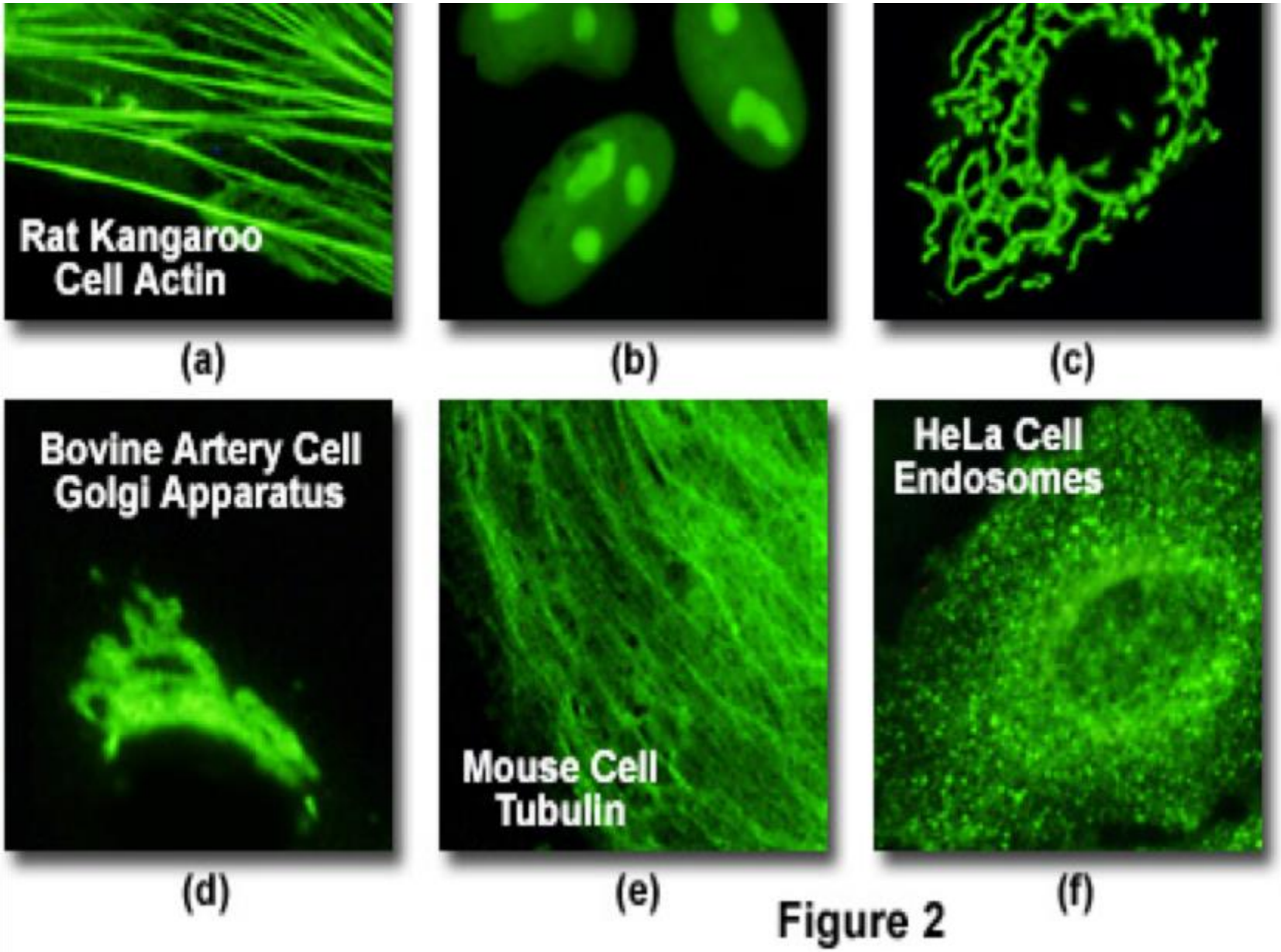
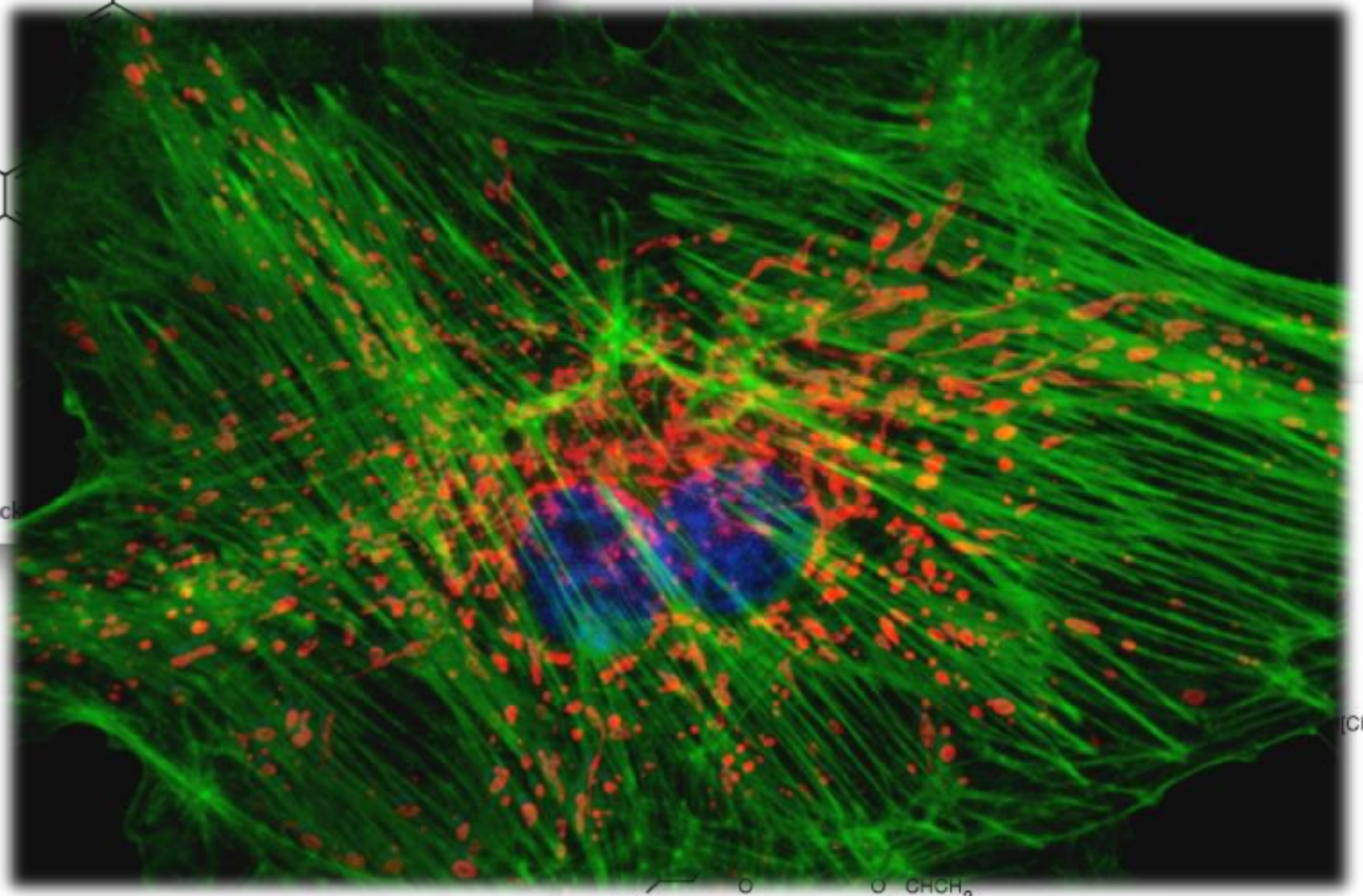
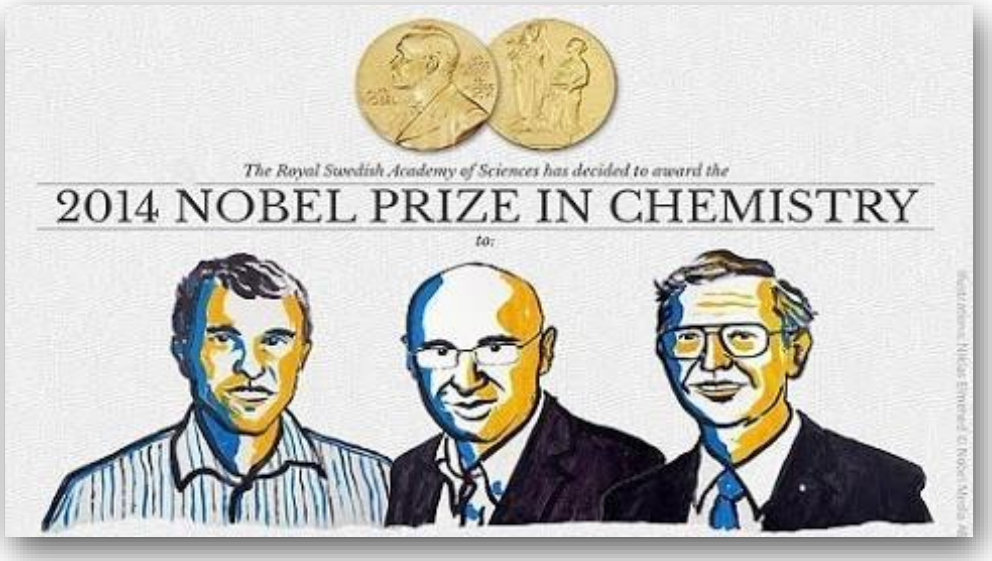



Figure 2

# Cell labeling



# Super-resolution microscopy



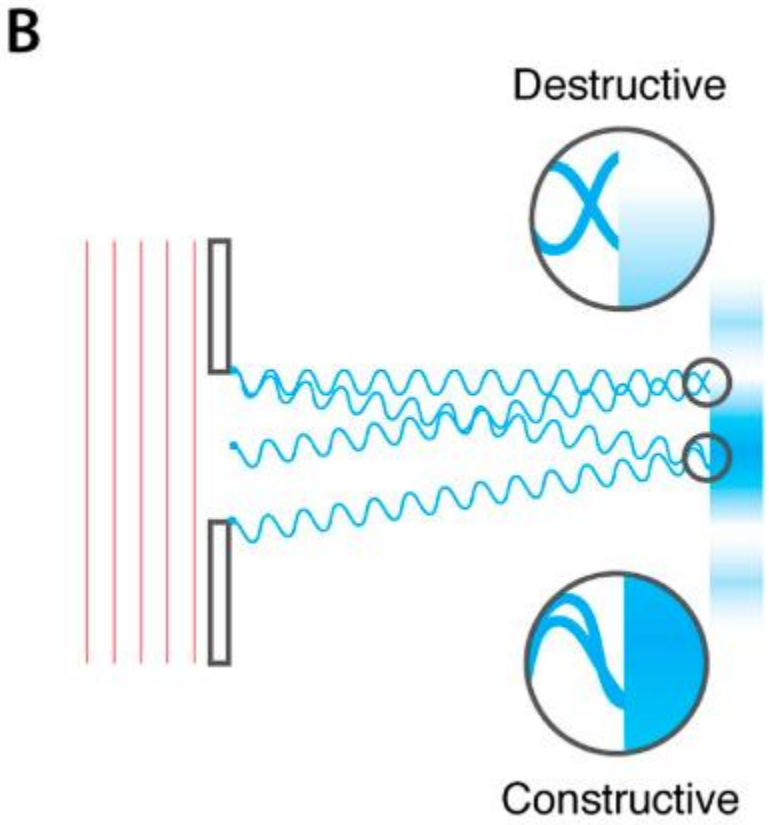

Eric Betzig, Stefan W. Hell and William E. Moerner

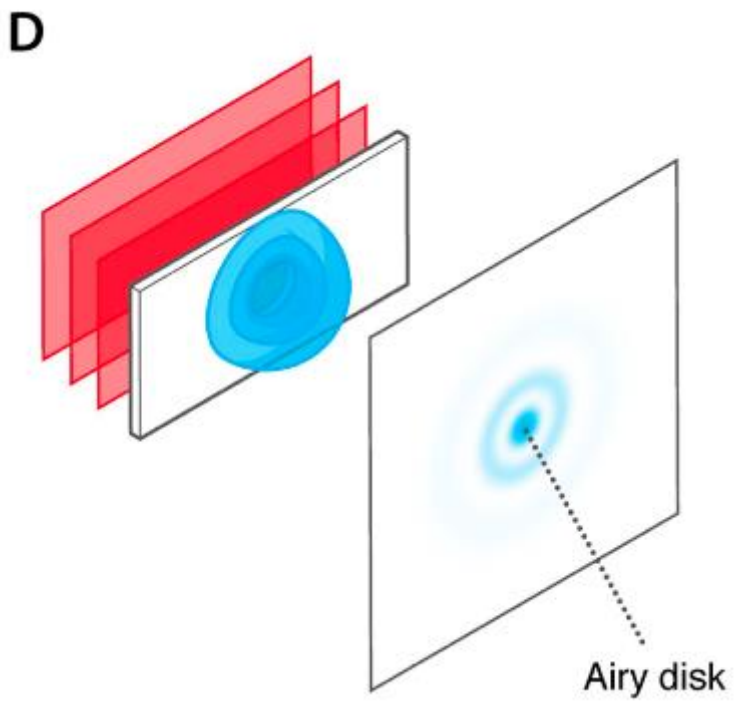
For the development of super-resolved fluorescence microscopy

aims to keep some of the advantages of fluorescent microscopy but wi


Abbe's study influenced the field so greatly that very few attempts were made to overcome the diffraction limit...

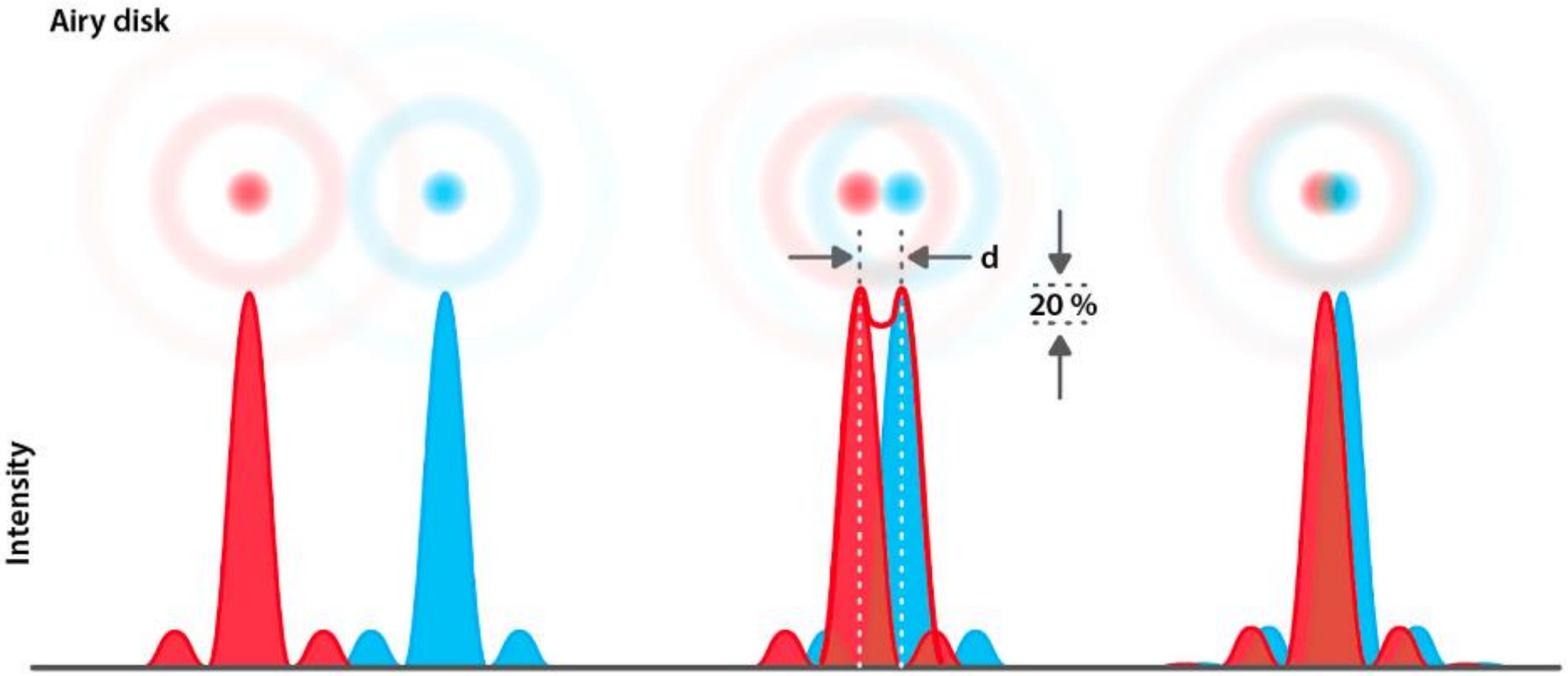
# Diffraction limit




Methods Appl. Fluoresc. 6 (2018) 022003

# Diffraction limit




$$d = \frac{\lambda}{2NA}$$

**Abbe Criteria**

$$d = \frac{0.61\lambda}{NA}$$

**Rayleigh Criteria**

# Super-resolution microscopy

*STED*

*GSD*

*STORM*

*PALMIRA*

*RESOLFT*

*dSTORM*

*PALM*

*SIM*

*PAINT*

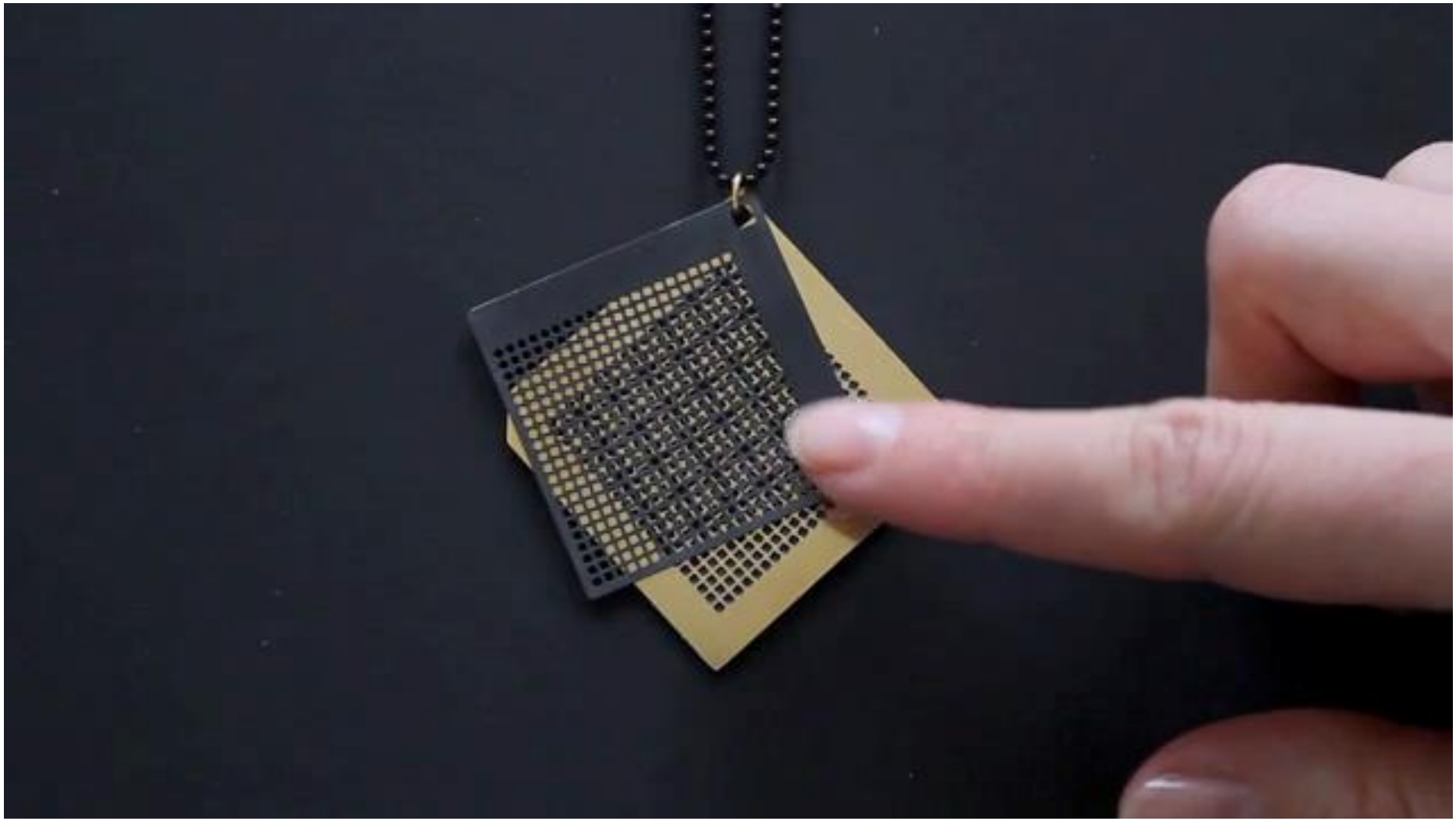
*SMACM*

They all based on three main principles to overcome the diffraction limit.
SIM-like - STED-like - SMLM-like

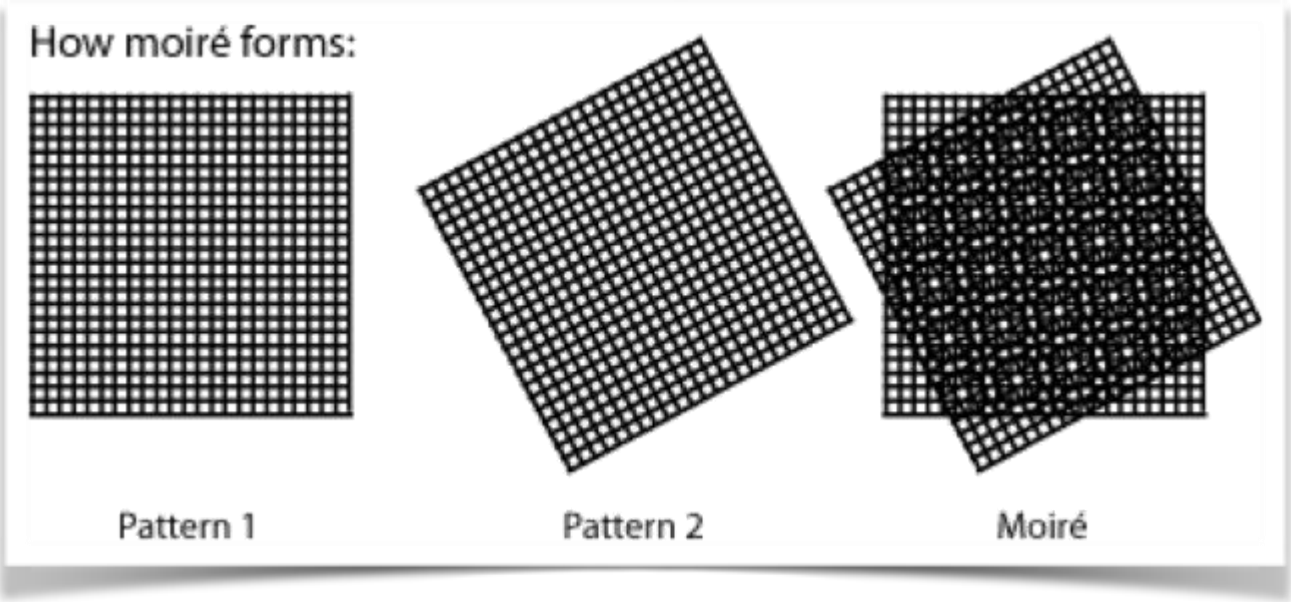
# Mats Gustaffson & SIM

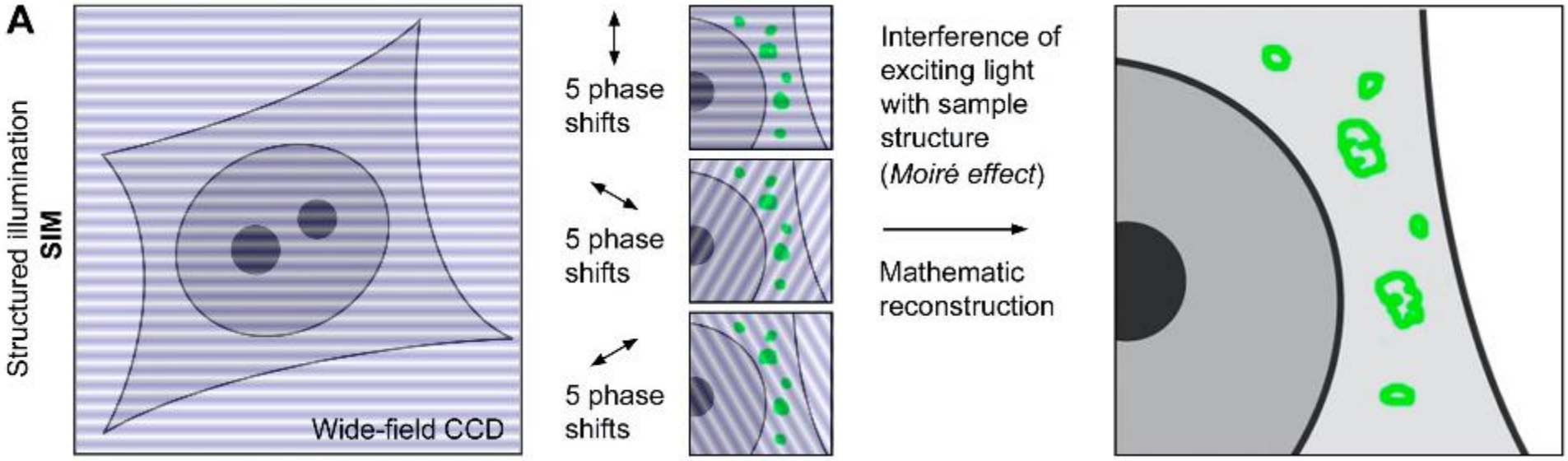
Even though the classical resolution limits are imposed by physical law, they can, in fact, be exceeded. There are loopholes in the law or, more precisely, the limitations are true only under certain assumptions.
Mats Gustaffson

# SIM: the Moiré effect

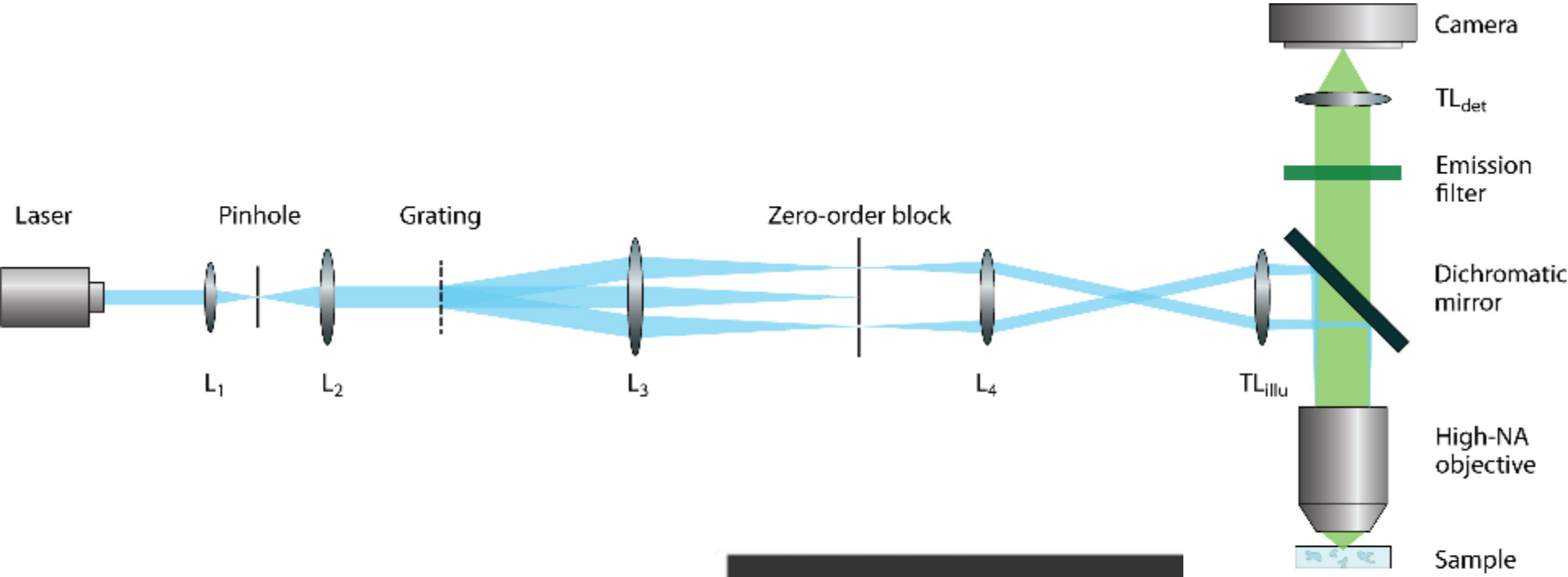


# Moiree fringes inside the microscope

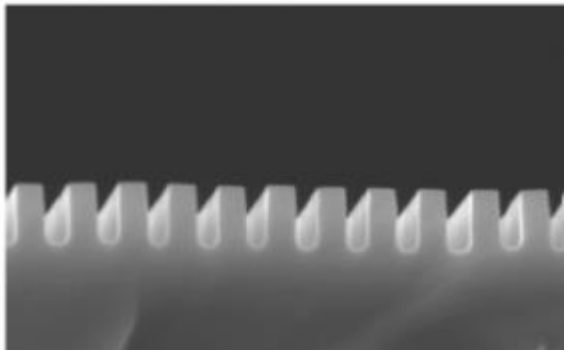


# The SIM microscope - Grating



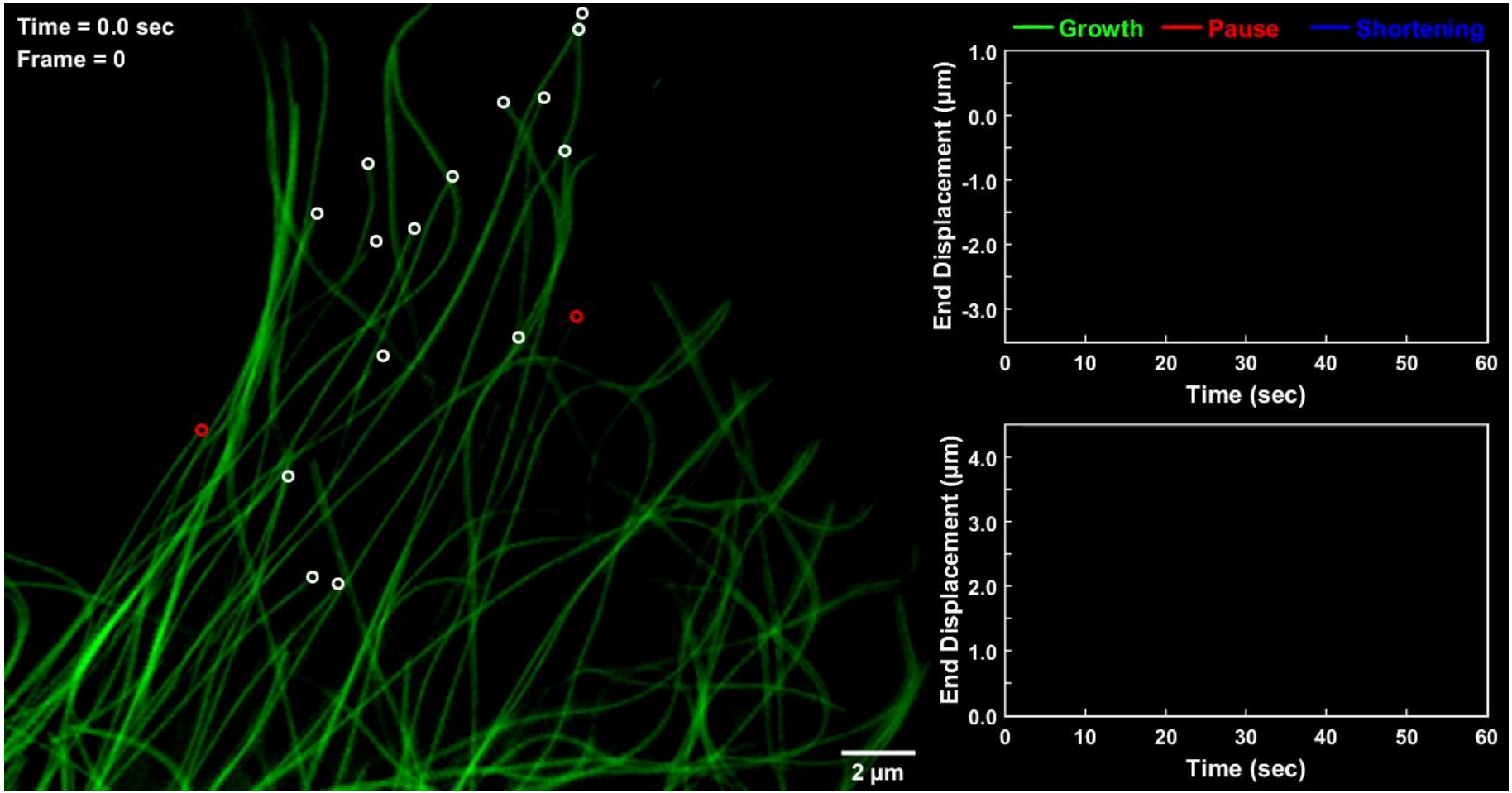

Jost A, Heintzmann R. 2013.
Annu. Rev. Mater. Res. 43:261–82



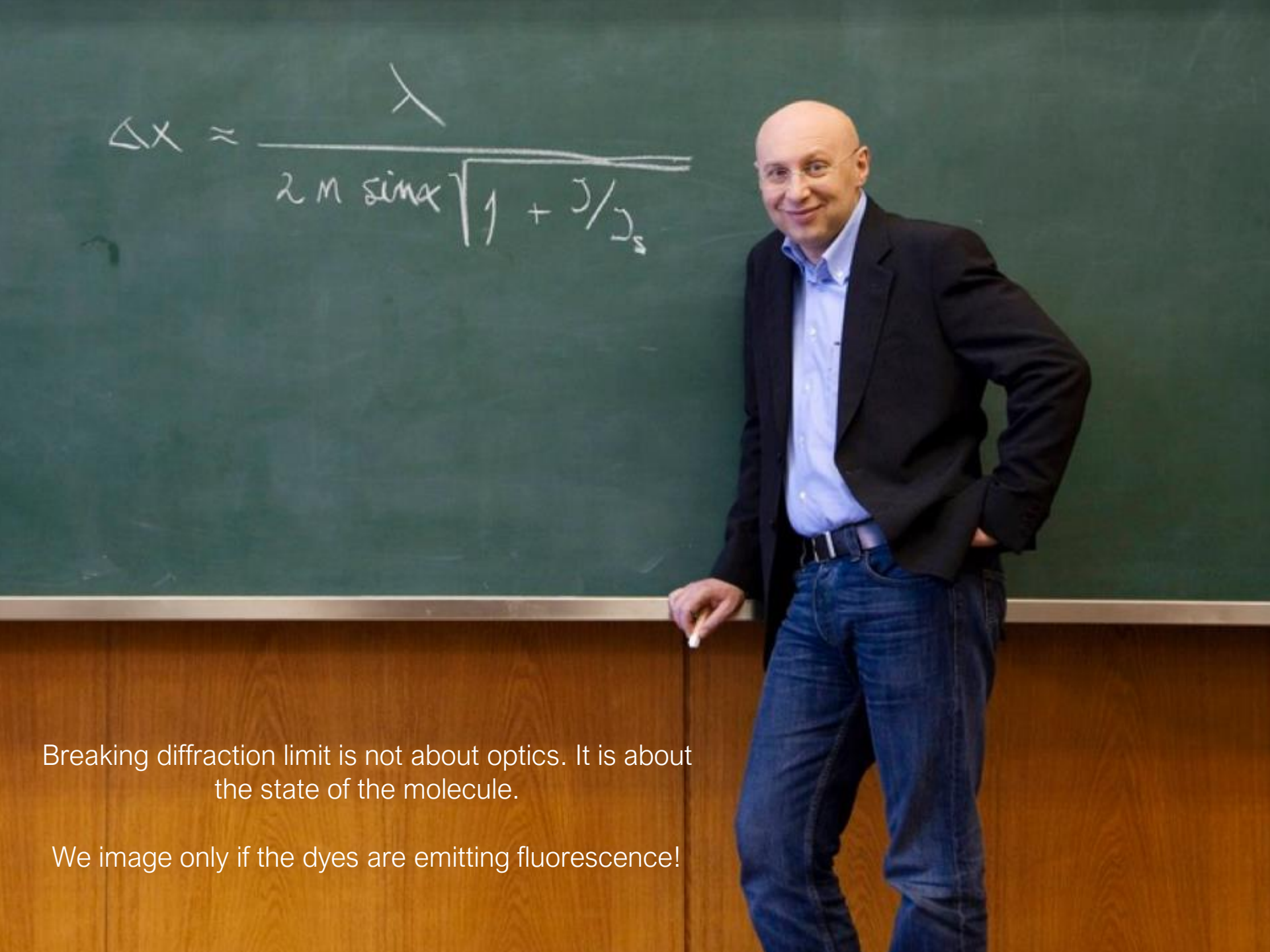
# SIM

| + | - |
| --- | --- |
| Simple | Limited Resolution (2x) |
| No special dyes required | Limited sensitivity |
| Fast (ms-s) | |
| Low light dose | |

# Low perturbation —> Live cell measurements



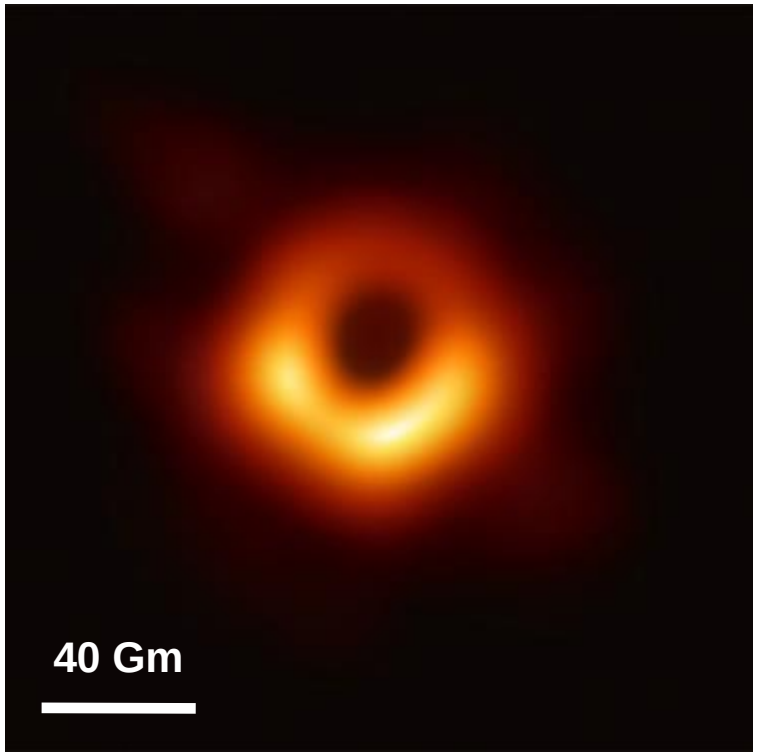

Cell. 2018 Nov 15;175(5):1430-1442.e17



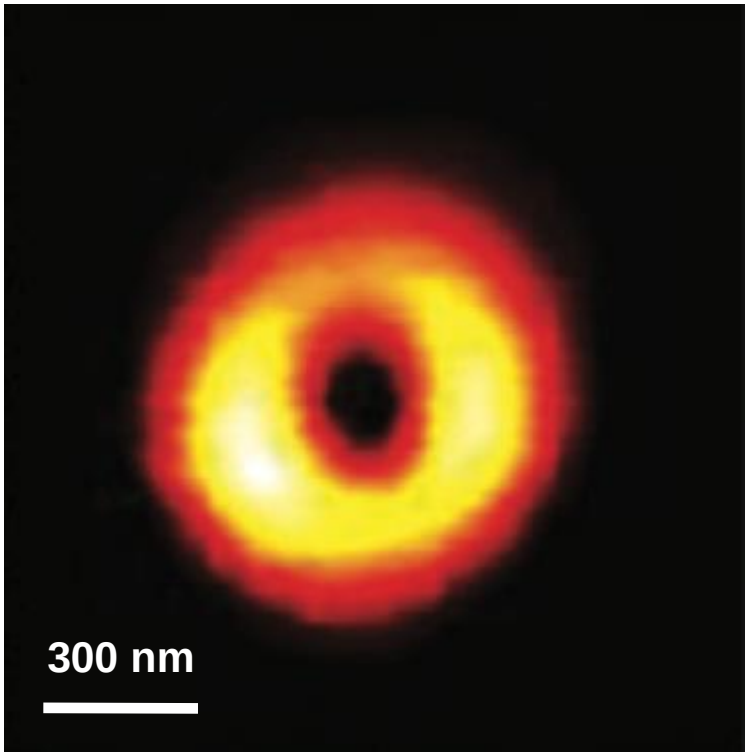

Breaking diffraction limit is not about optics. It is about the state of the molecule.

We image only if the dyes are emitting fluorescence!

# The depletion donut




Supermassive black hole at the centre of the galaxy M87

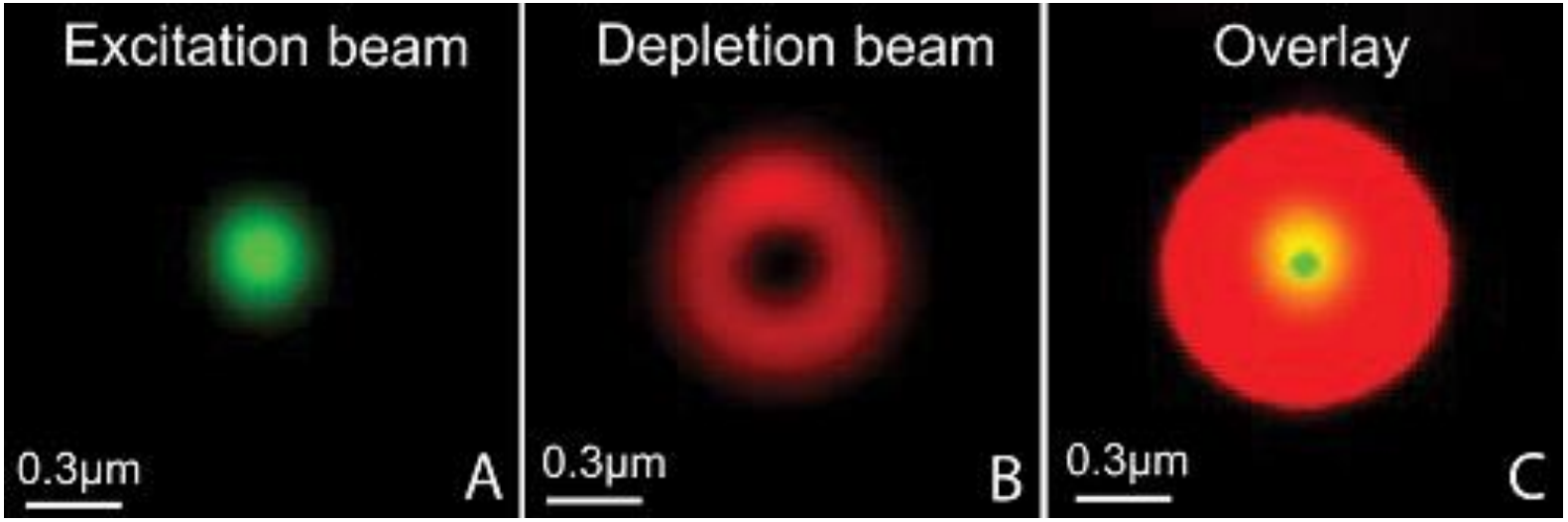



Depletion beam of a STED microscope

https://www.nature.com/articles/d41586-019-01155-0

Nanophotonics 2018; 7(12): 1971–1980

# The depletion donut

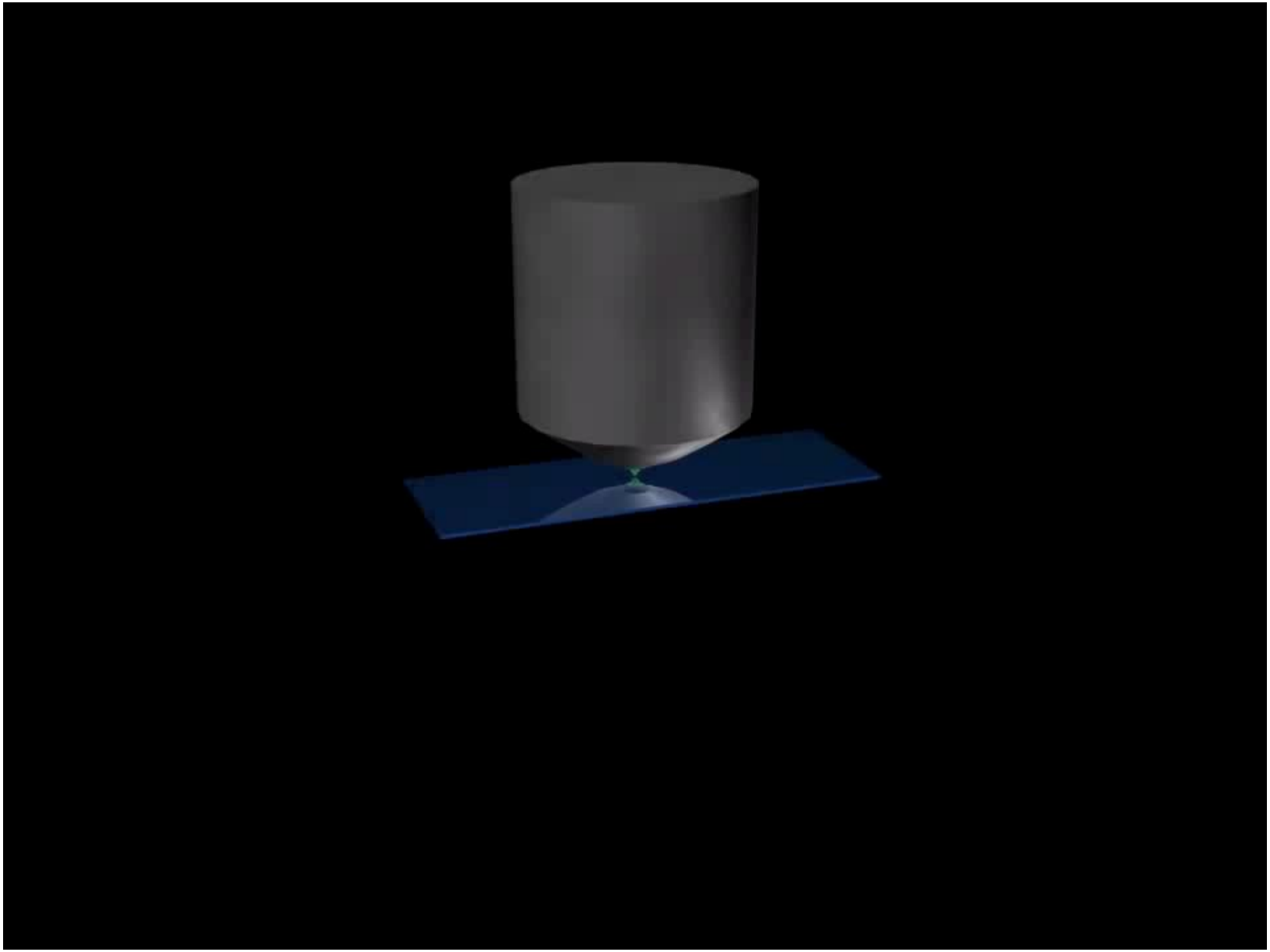



Only the fluorescence inside the donut will remain on.
The rest is depleted by stimulated emission

We see fluorescence only from the center.
The effective PSF gets smaller!

https://www.ntnu.edu/mh/cmic/equipment-and-services-s

# STED enhanced resolution



# STED

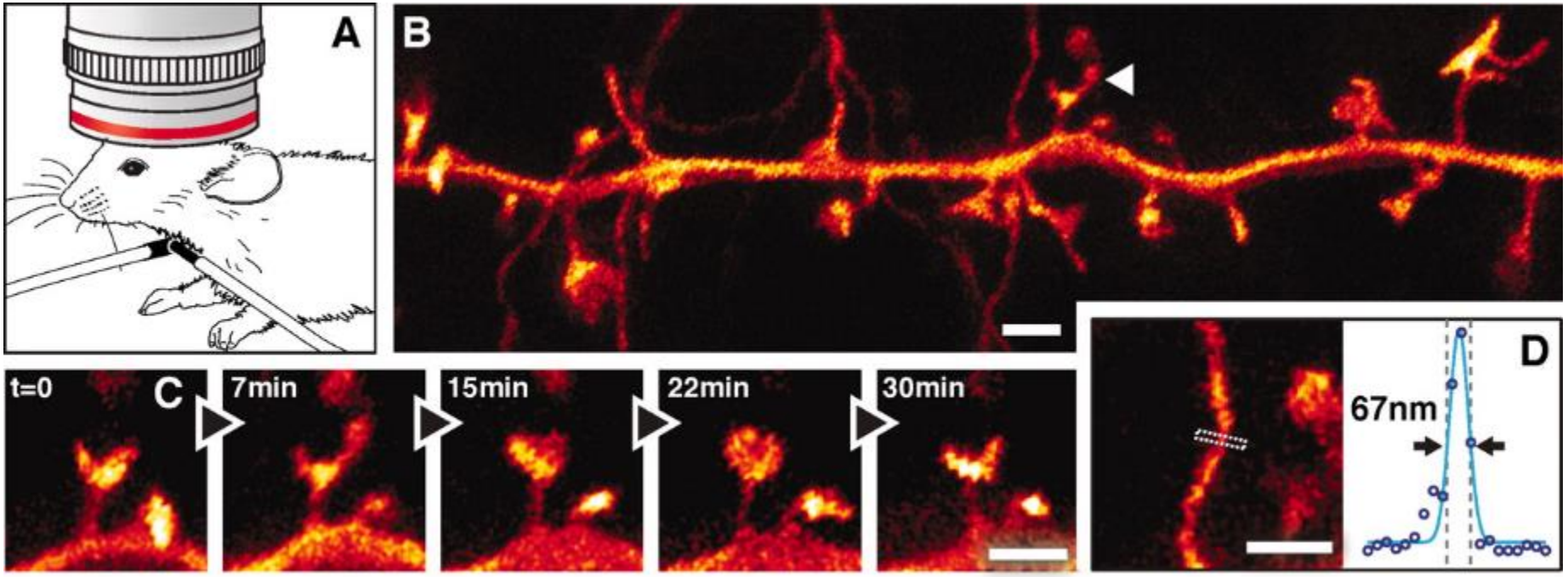
| + | - |
| --- | --- |
| Good resolution (30-50nm) | Difficult to build/calibrate |
| Simple to use | High power needed |
| In vivo capability | Good but not great resolution |
| Moderately fast | Special dyes needed |

# Biological examples: in vivo STED

## Nanoscopy in a Living Mouse Brain

Sebastian Berning[1], Katrin I. Willig[1,*], Heinz Steffens[1], Payam Dibaj[2], Stefan W. Hell[1,*]
+ See all authors and affiliations

*Science* 03 Feb 2012:
Vol. 335, Issue 6068, pp. 551
DOI: 10.1126/science.1215369



# Example in nanotech: nanofibers

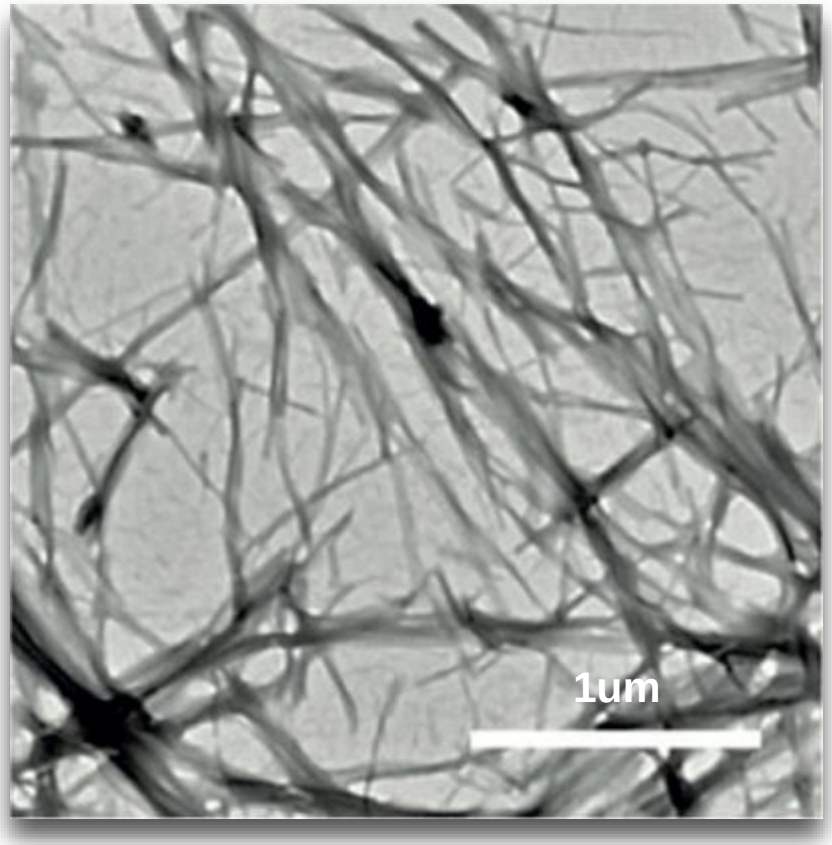
nature chemistry

Article | Published: 30 May 2016

*In situ* real-time imaging of self-sorted supramolecular nanofibres

Shoji Onogi, Hajime Shigemitsu, Tatsuyuki Yoshii, Tatsuya Tanida, Masato Ikeda, Ryou Kubota & Itaru Hamachi

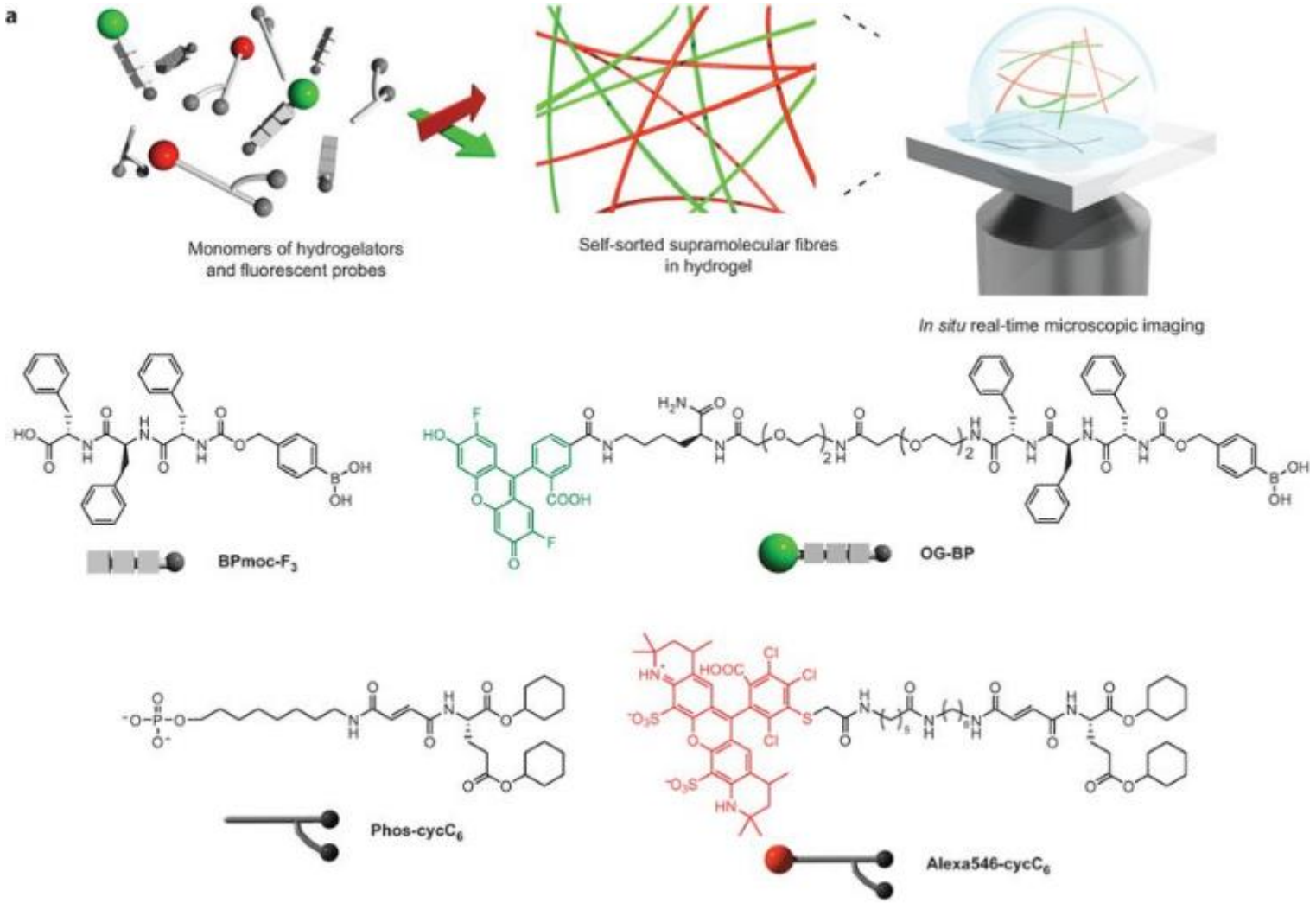
*Nature Chemistry* **8**, 743–752 (2016) | Download Citation



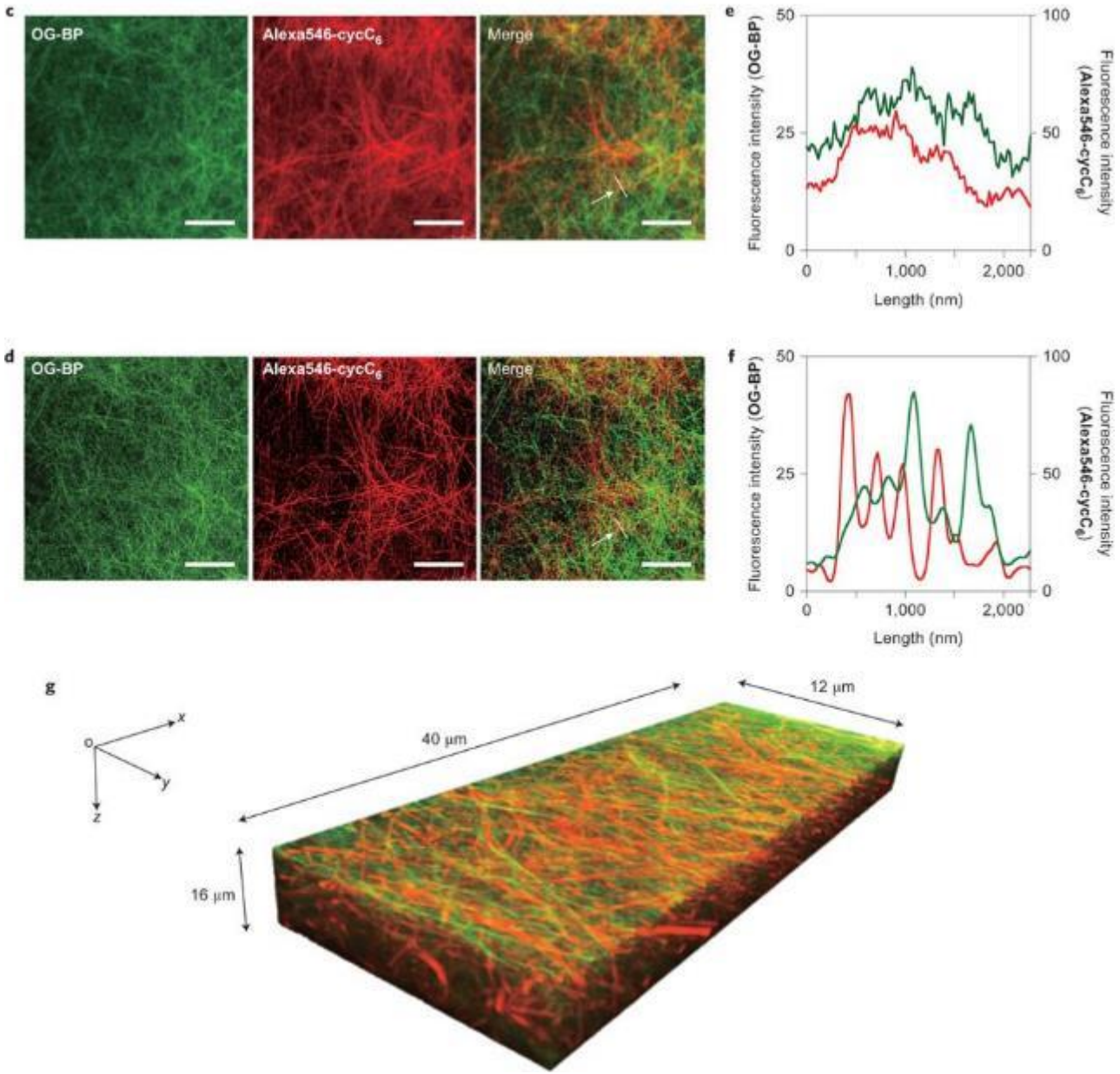

Double network of supramolecular nanofibers.

2-colors and nanometric resolution needed

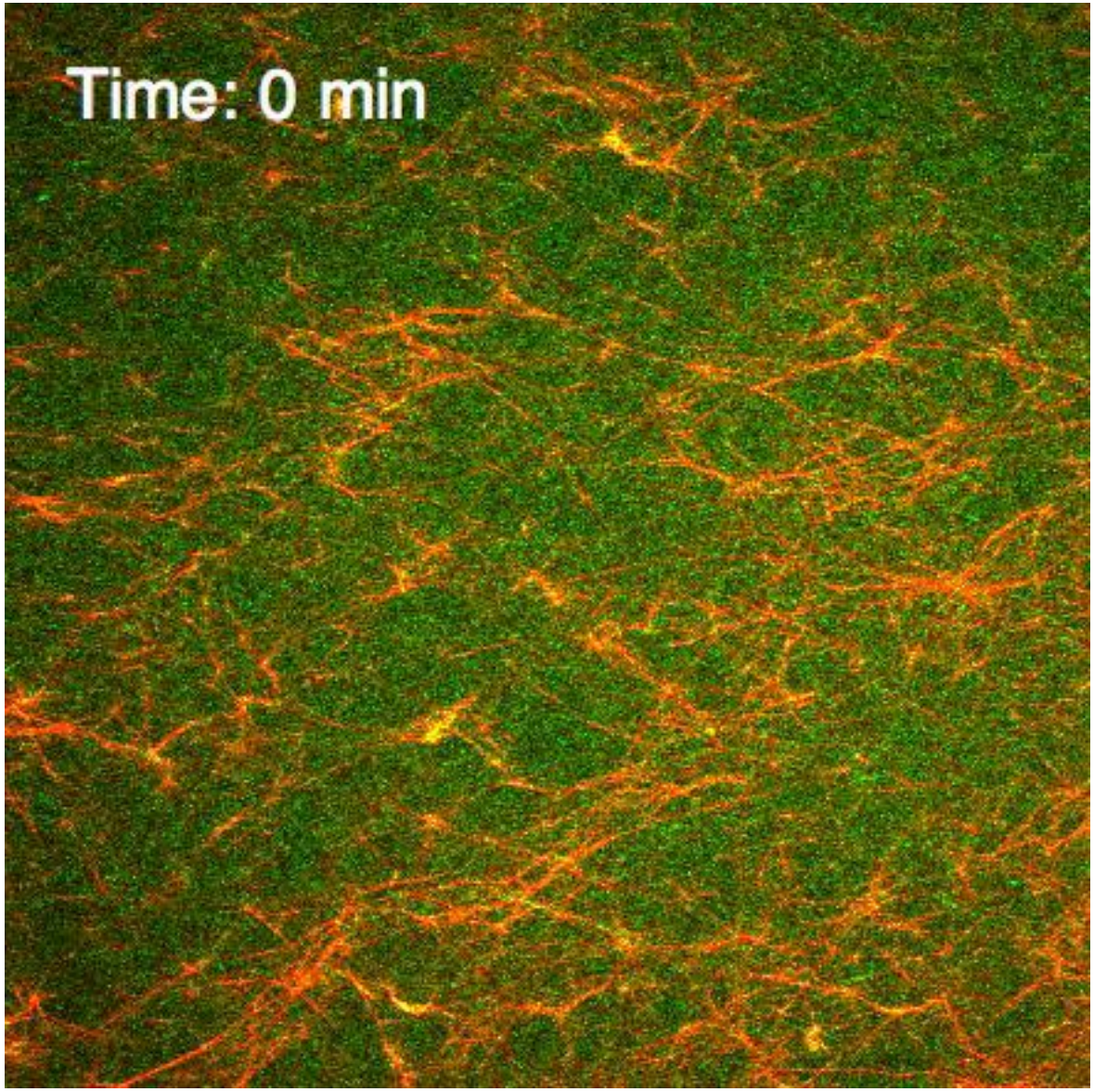
# Example in nanotech: nanofibers



# Example in nanotech: nanofibers



# Example in nanotech: nanofibers



# 2006 the SMLM year

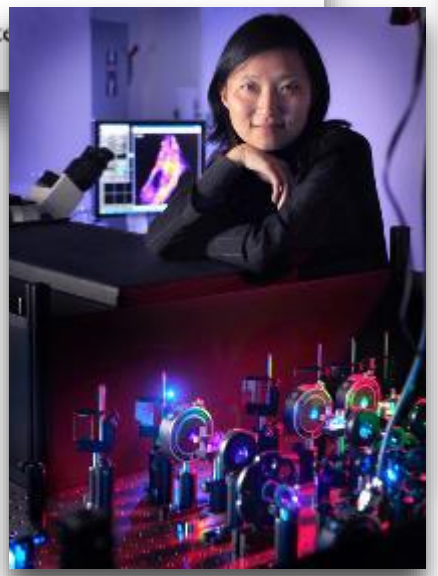
4258 Biophysical Journal Volume 91 December 2006 4258–427

## Ultra-High Resolution Imaging by Fluorescence Photoactivation Localization Microscopy

Samuel T. Hess,*† Thanu P. K. Girirajan,†‡ and Michael D. Mason†
*Department of Physics and Astronomy, †Institute for Molecular Biophysics, and ‡Department of Chemical and Biological Engineering, University of Maine, Orono, Maine

## Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)

Michael J Rust[1,5], Mark Bate



## Imaging Intracellular Fluorescent Proteins at Nanometer Resolution

Eric Betzig,[1,2]*† George H. Patterson,[3] Rachid Sougrat,[3] O. Wolf Lindwasser,[3] Scott Olenych,[4] Juan S. Bonifacino,[3] Michael W. Davidson,[4] Jennifer Lippincott-Schwartz,[3] Harald F. Hess[5]*

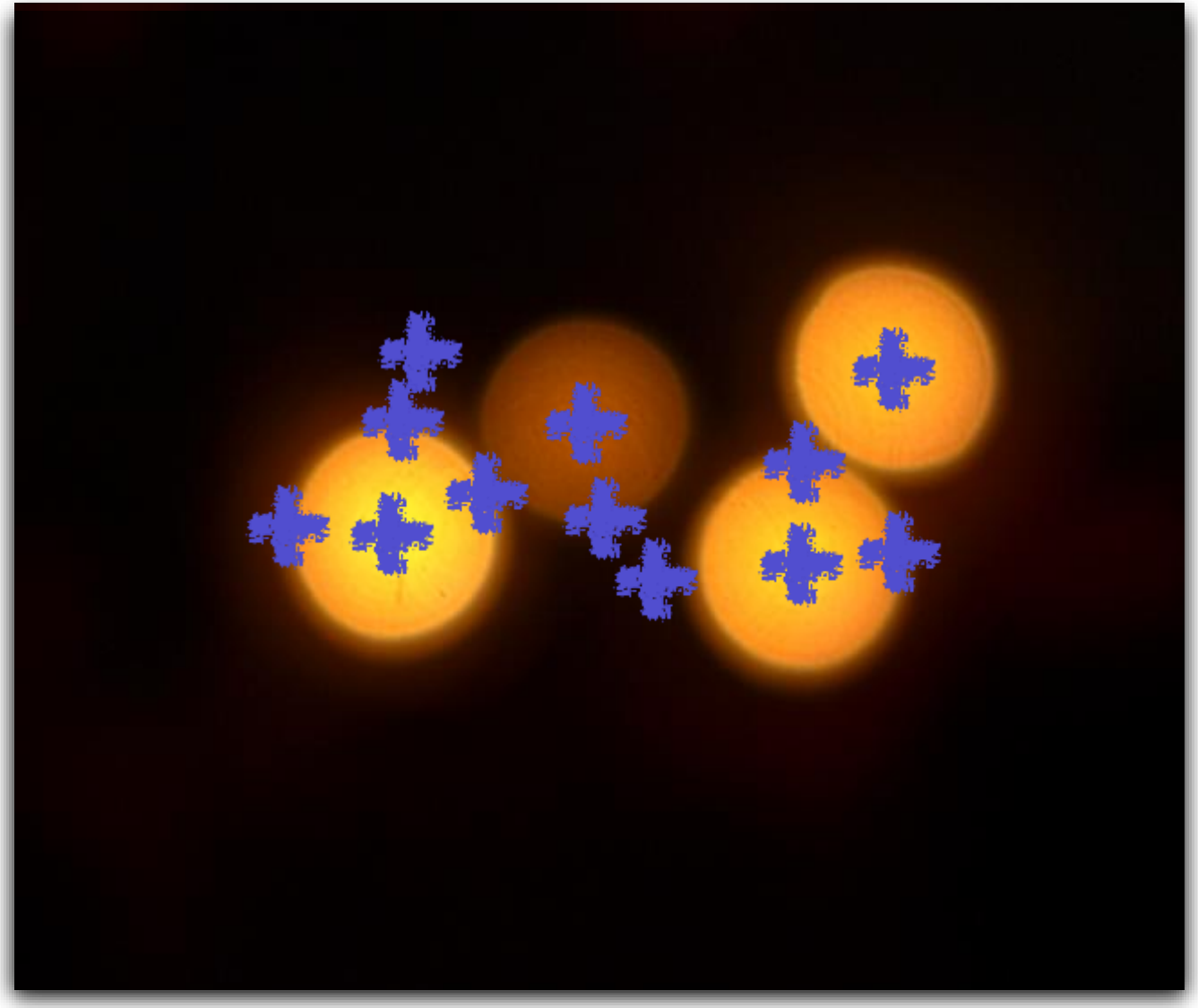
# SMLM: how does it work?

A macroscopic example:

Candles = dyes

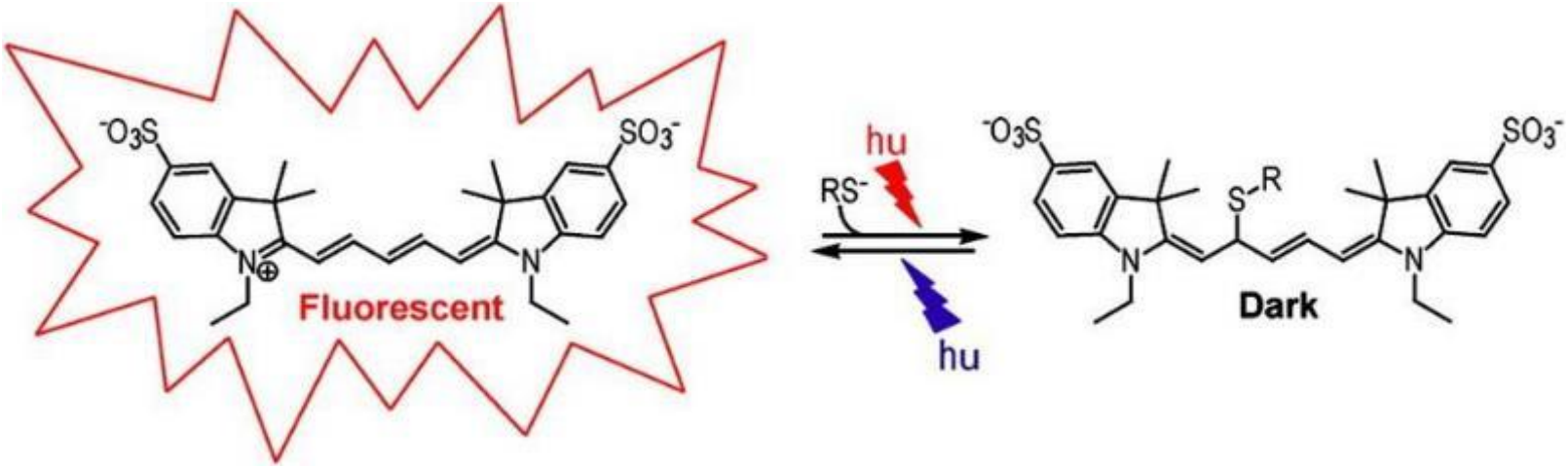
Phone = EMCCD camera

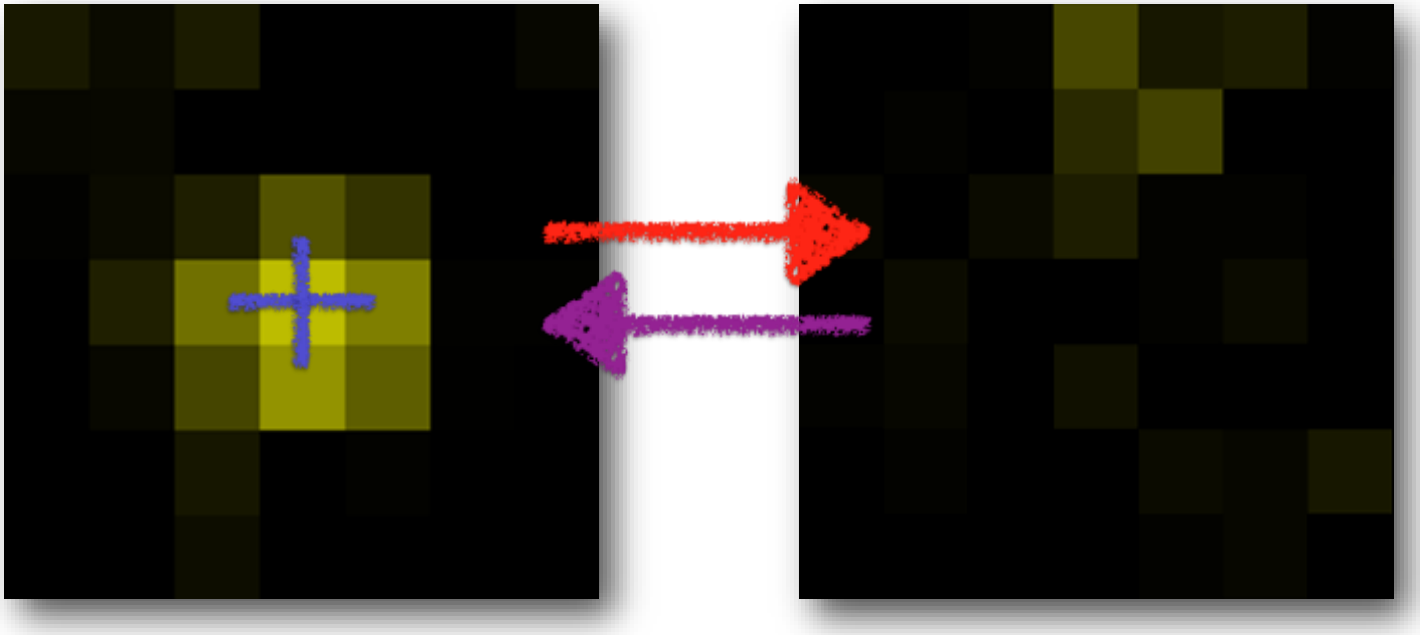
# SMLM: how does it work?



# SMLM: how does it work?

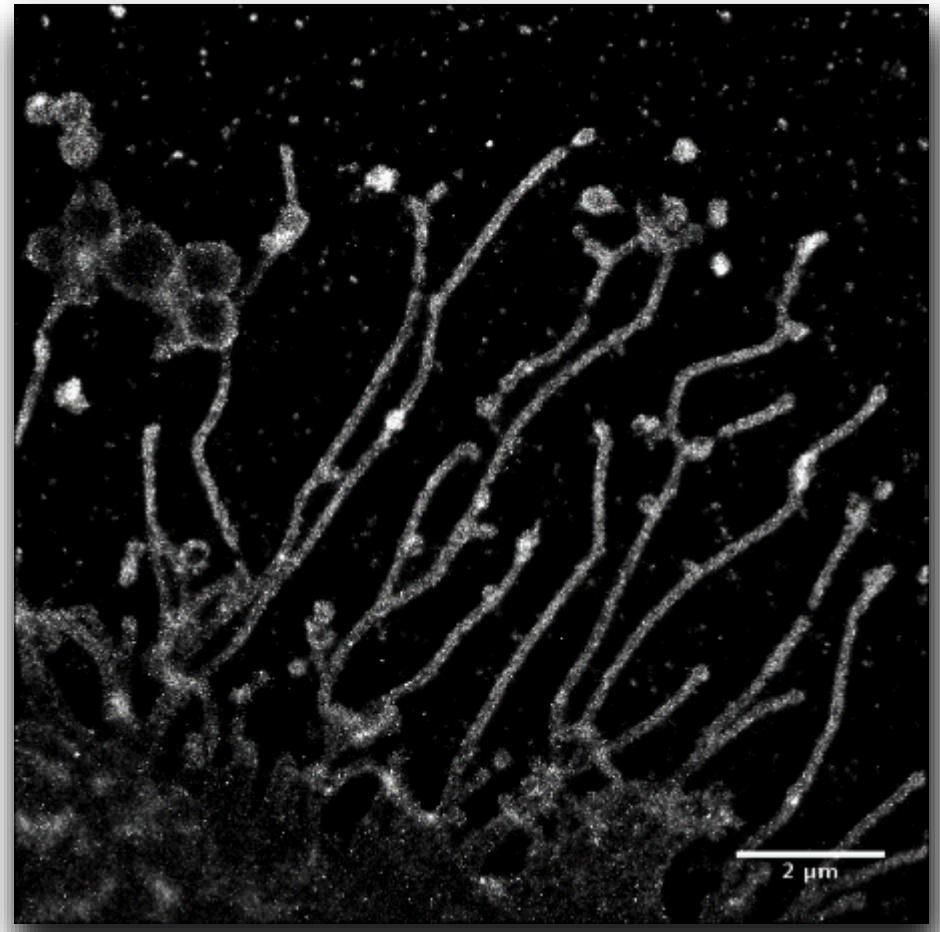
# Photoswitching



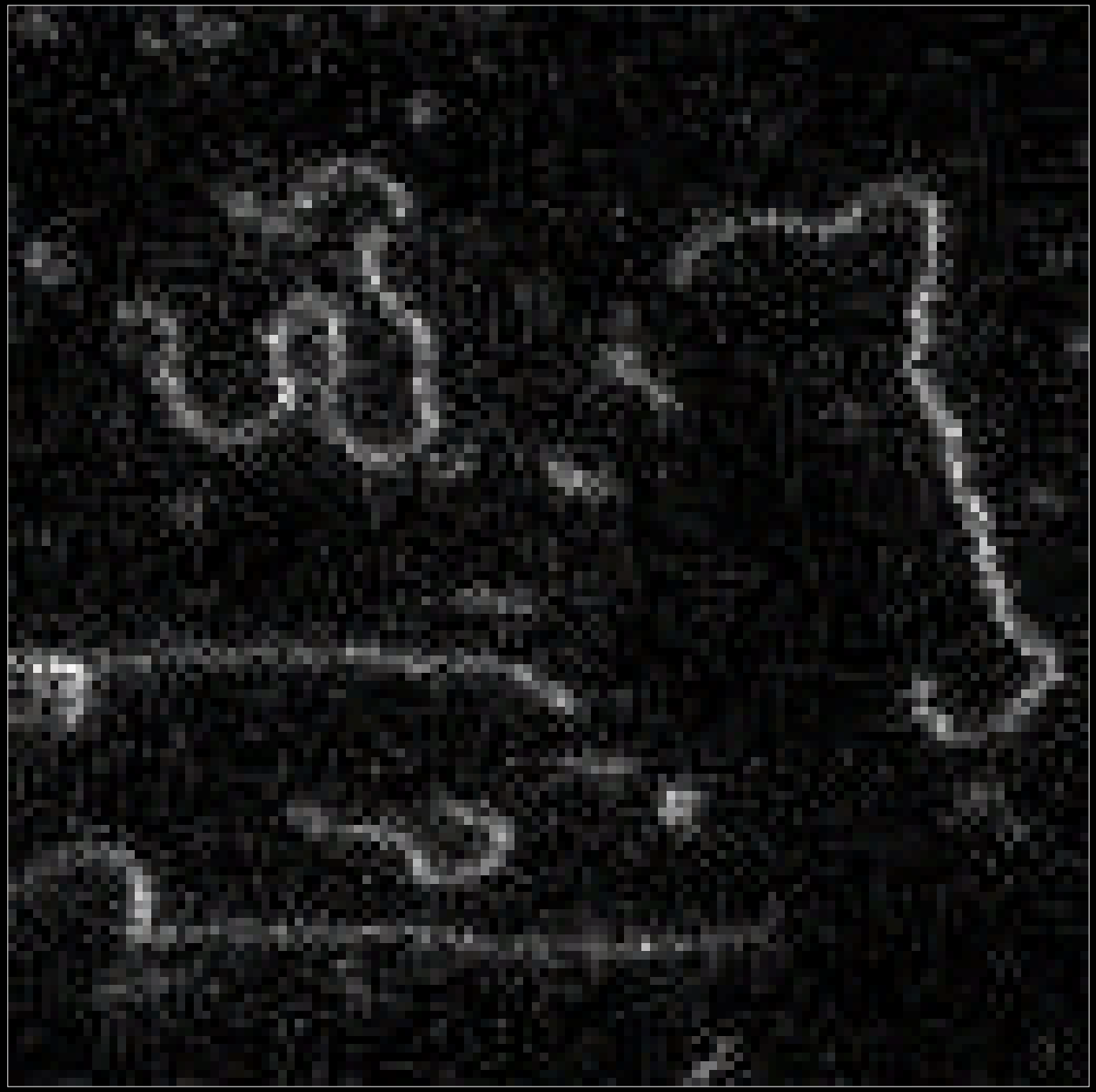
*Dempsey et al, JACS (2009)*

# Super-resolution imaging (SMLM)




Spatial Resolution xy: 20 nm
Spatial Resolution z: 50-80nm
Temporal resolution: min
Laser power: kW $cm^{-2}$

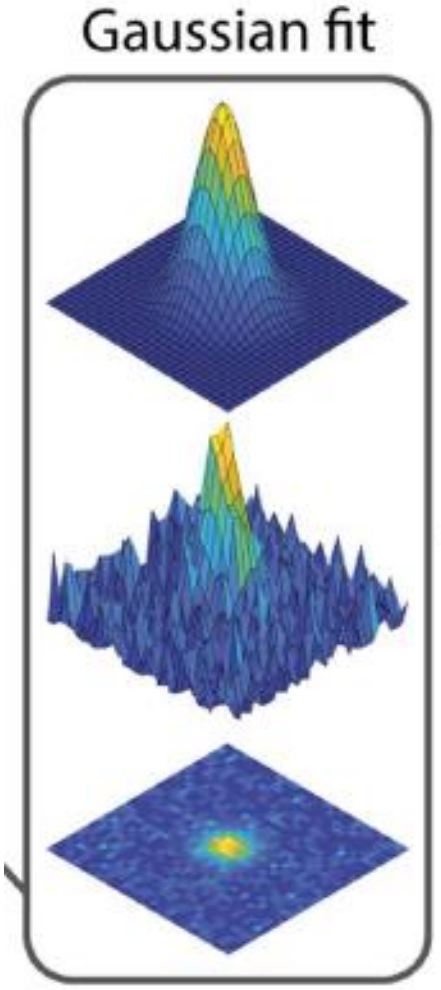
Scientific Reports (2019)



# SMLM: how does it work?

The “super-resolution” comes from the high localisation accuracy of individual events. How accurate?

$$\sigma = \frac{s}{\sqrt{N}}$$

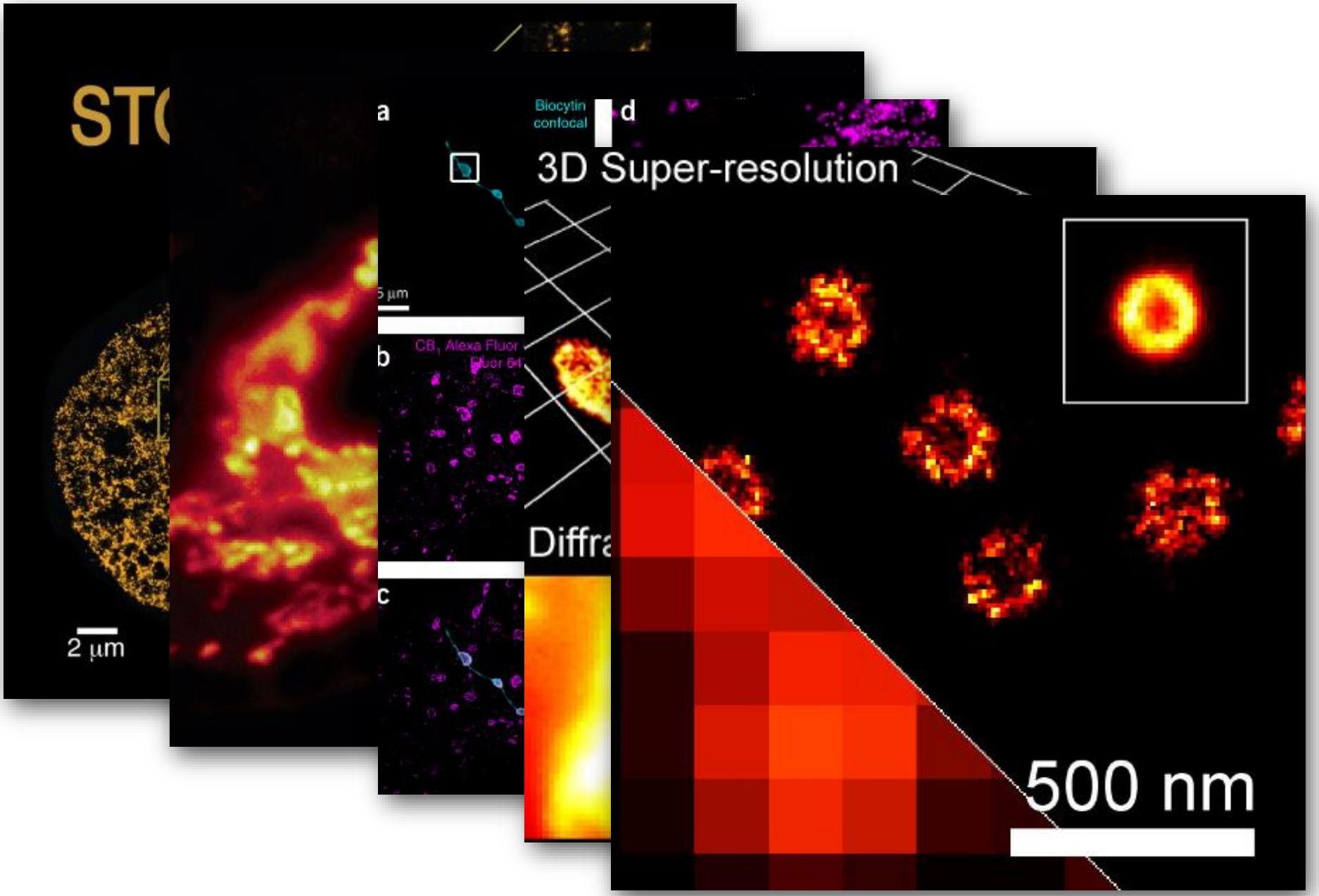



It is possible to achieve resolution in the nm range.
Resolution is not localization accuracy!

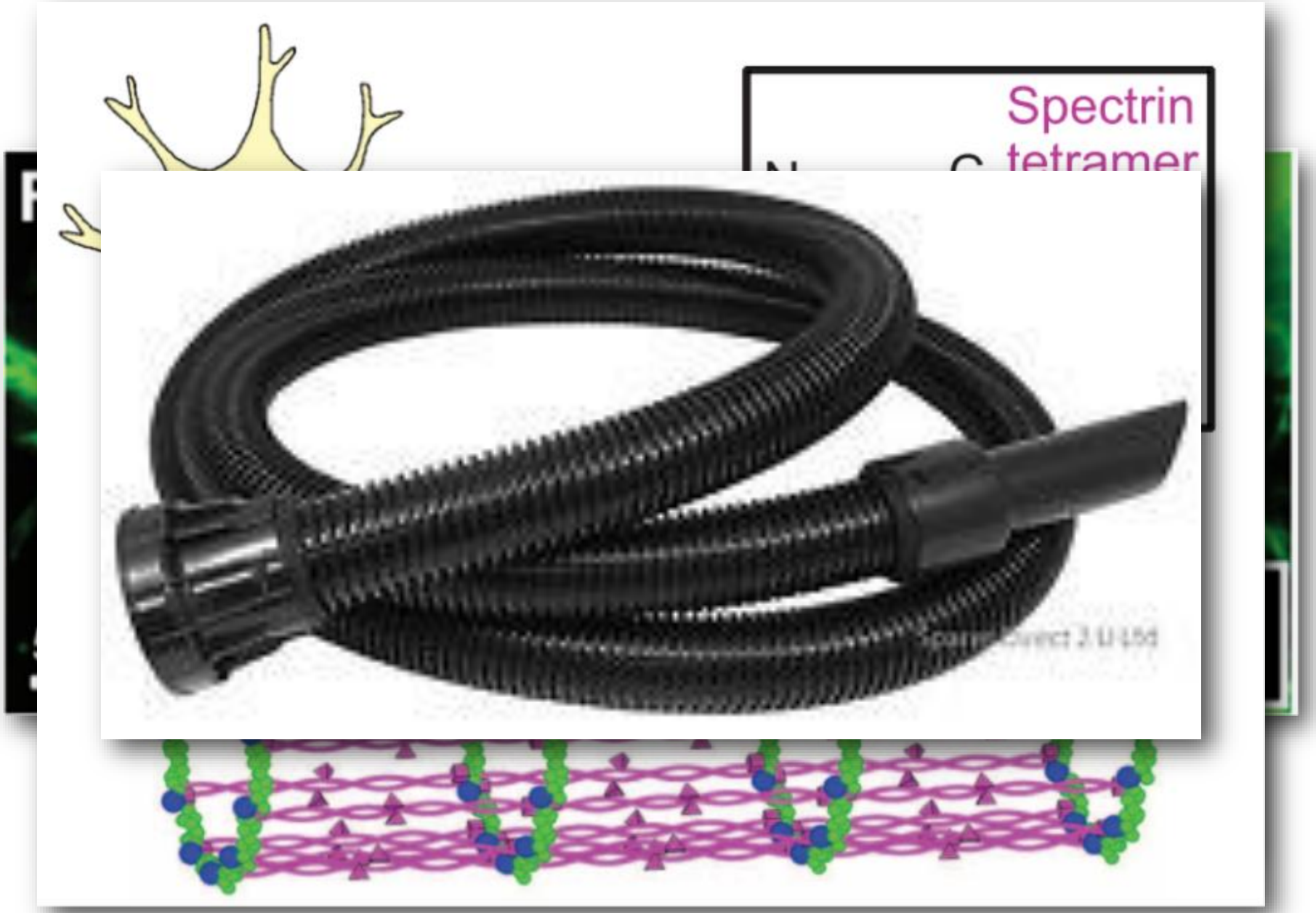
# SMLM

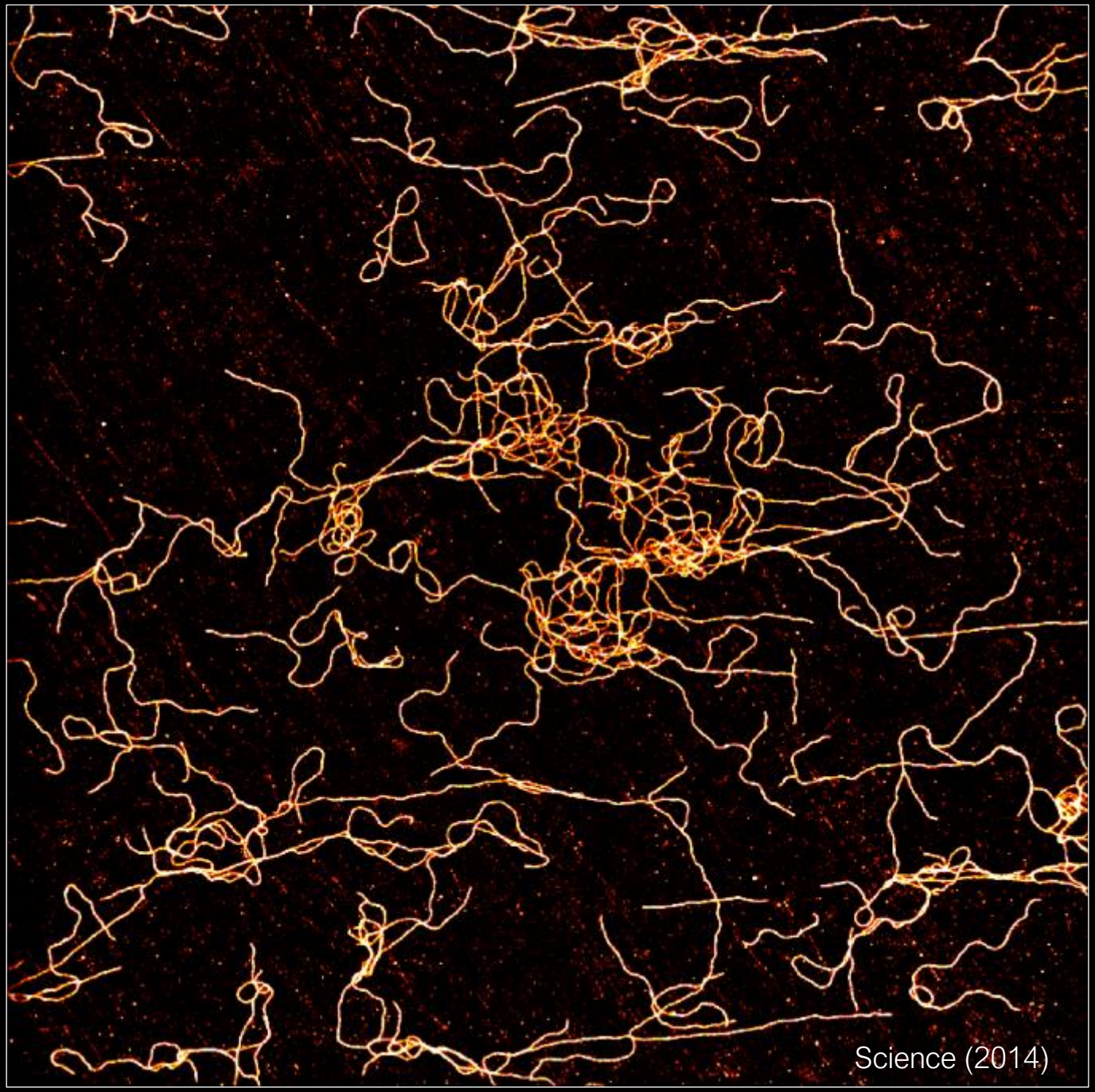
| + | - |
| --- | --- |
| Best resolution (15-20nm) | Slow |
| Quantitative (molecular counting) | Special dyes needed |

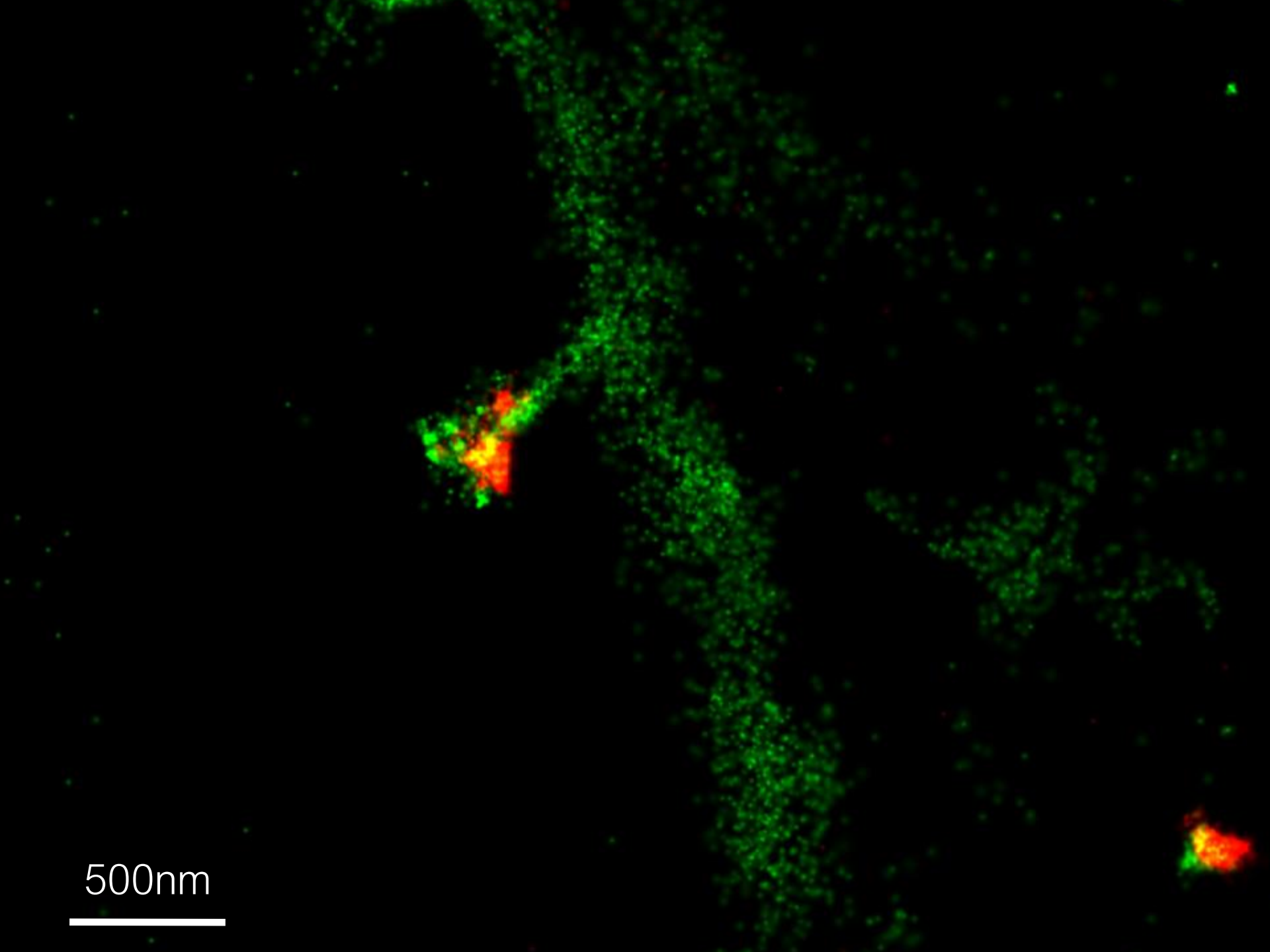
# Biological examples



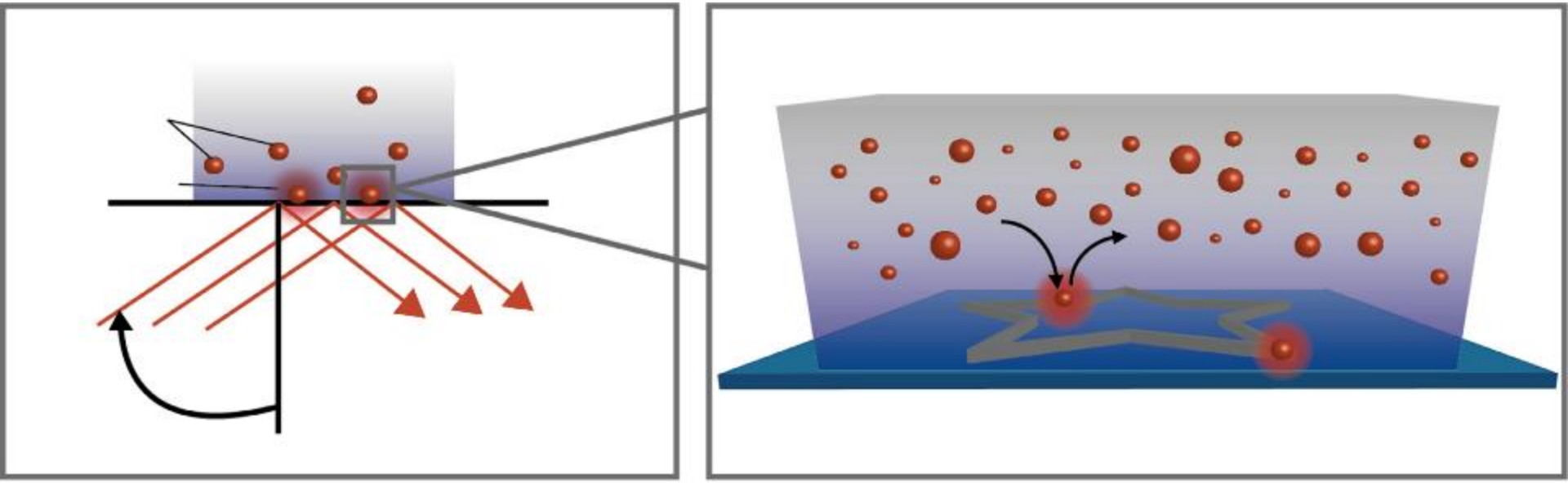
# Discovering new structures

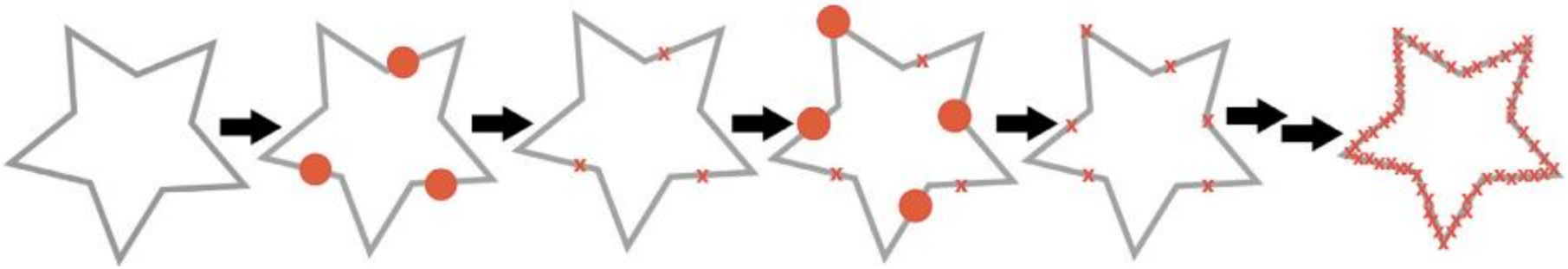



Science (2014)


500nm

# Not only photoswitching: PAINT

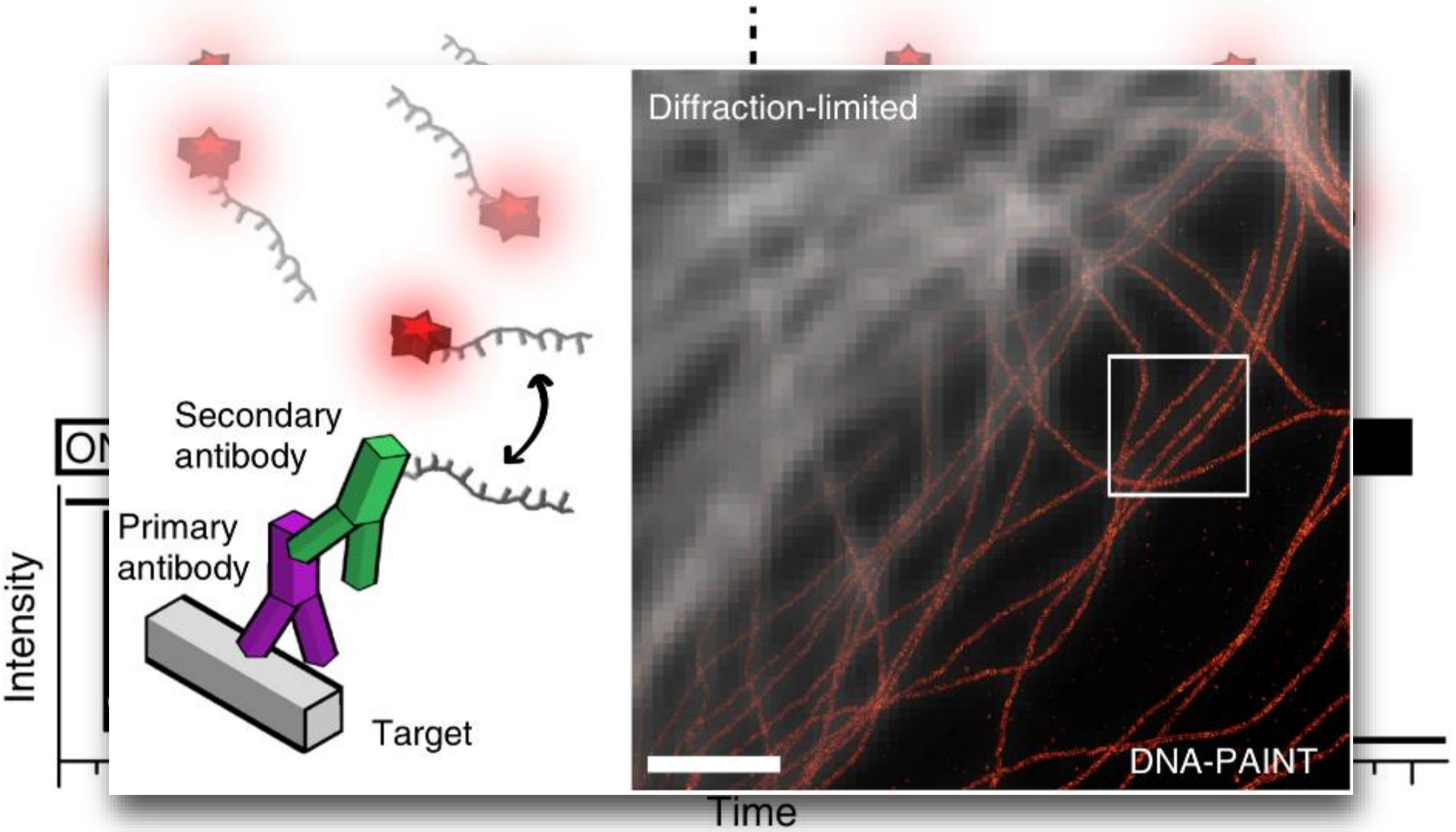




No bleaching - high accuracy - multicolor

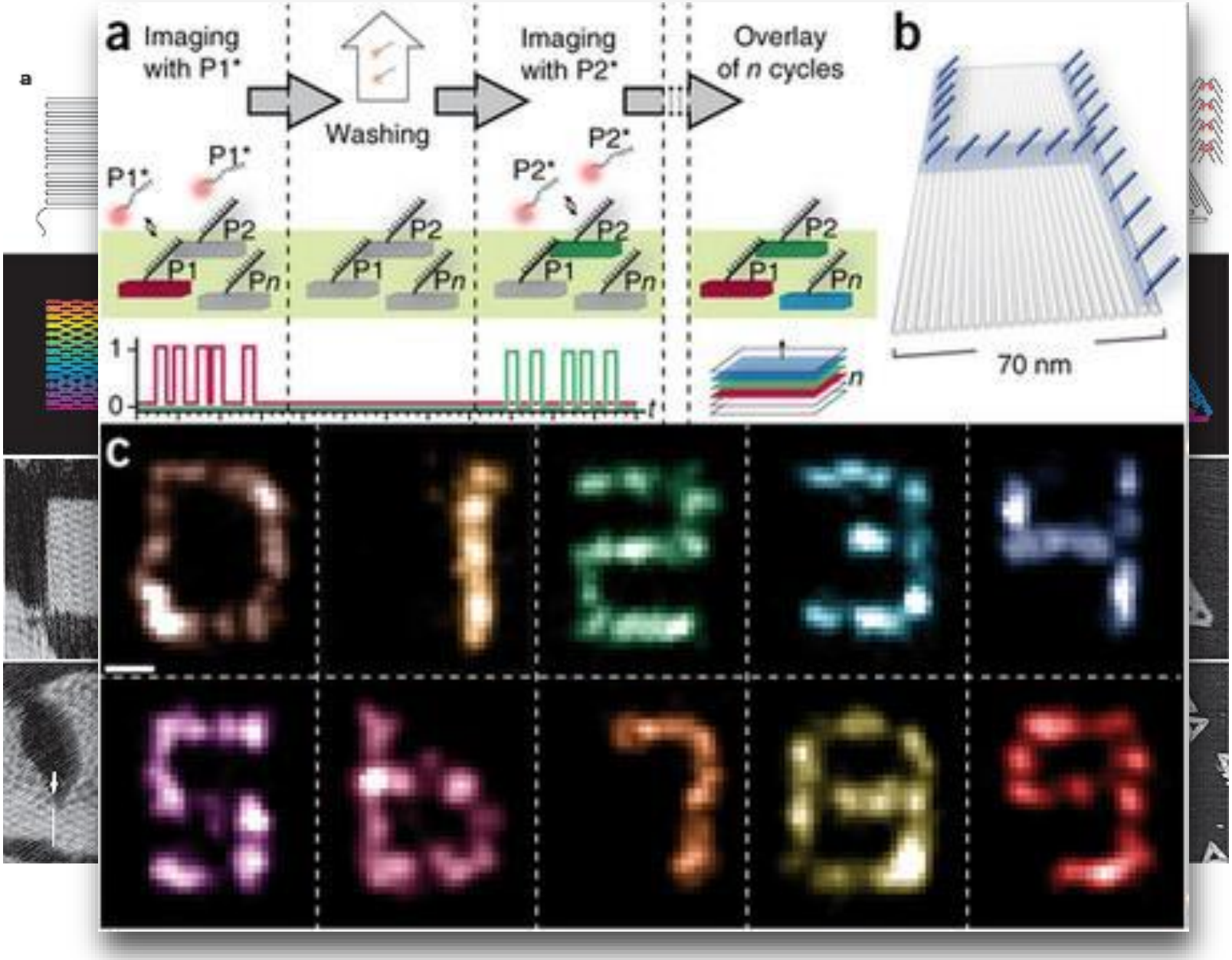
Current Opinion in Biotechnology 2016, 39:8–16

# How does it work in reality? (PAINT)

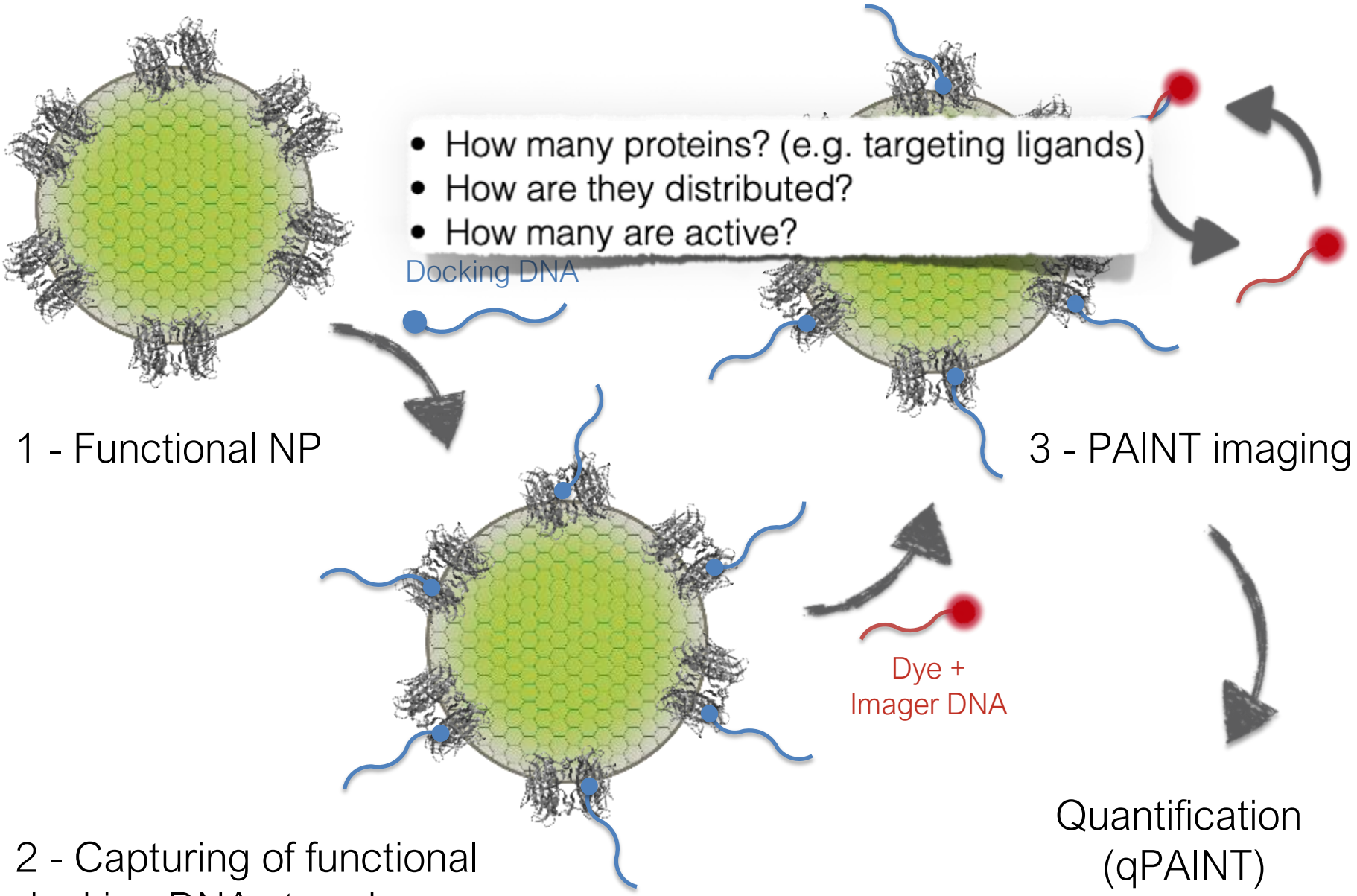


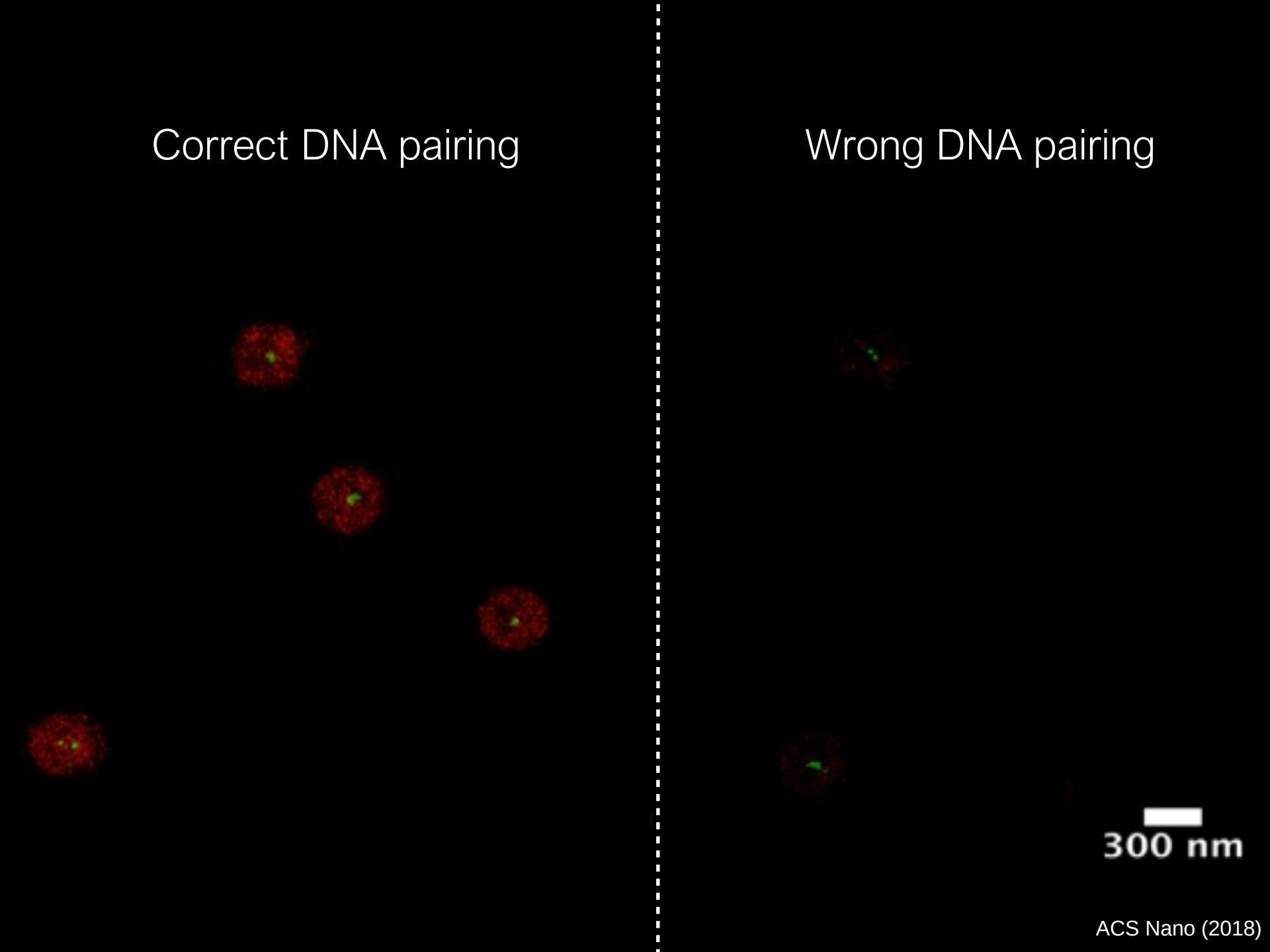

Jungmann et al, Nat. Protocols (2017)

# PAINT + DNA Origami



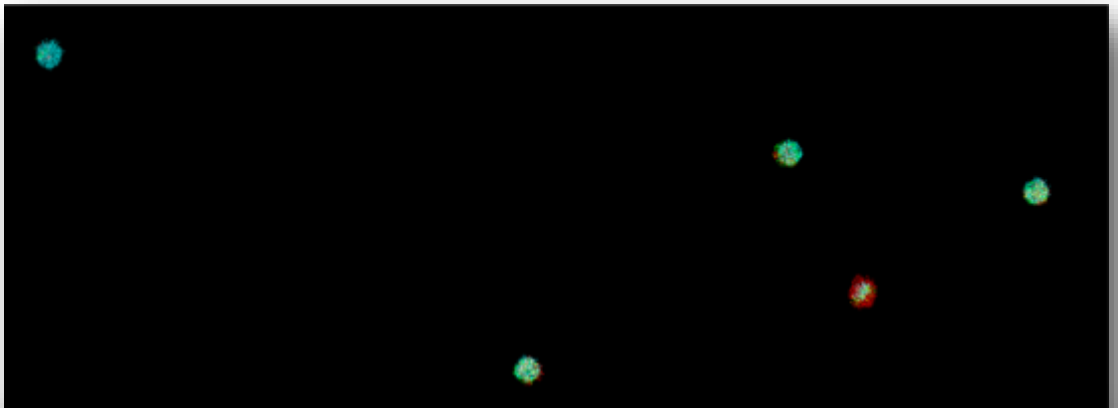
# Mapping functionalities on NP surface

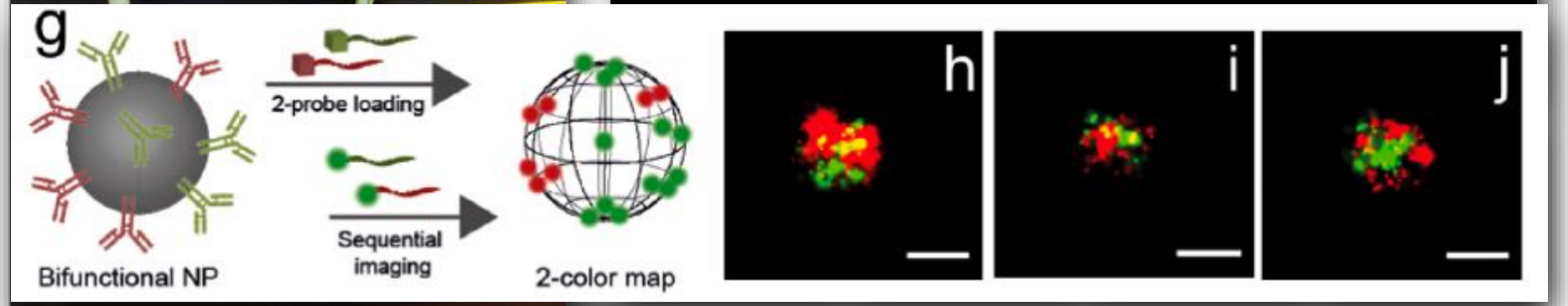


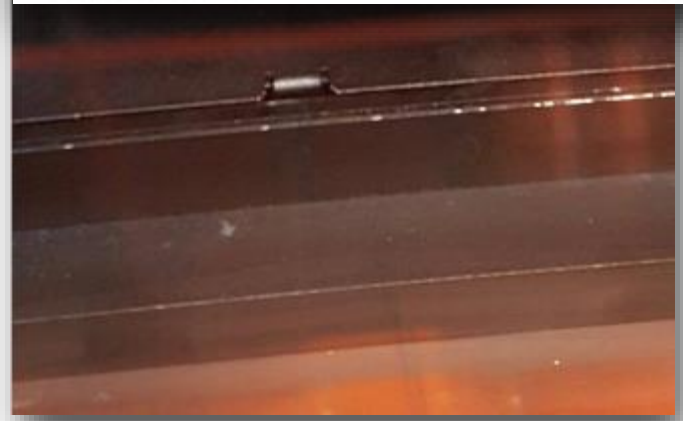



ACS Nano (2018)

# Multicolor qPAINT

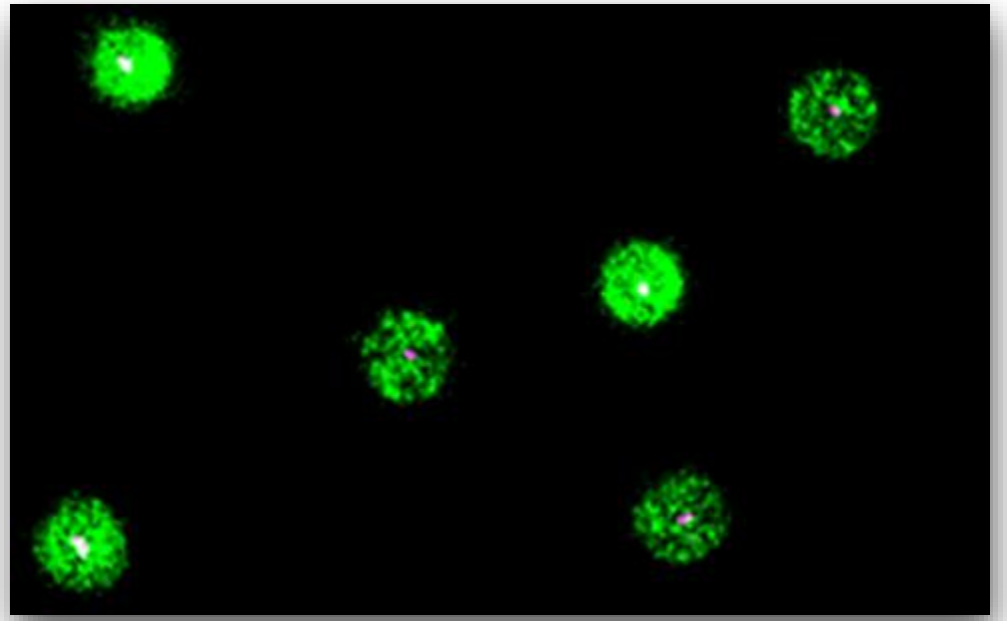


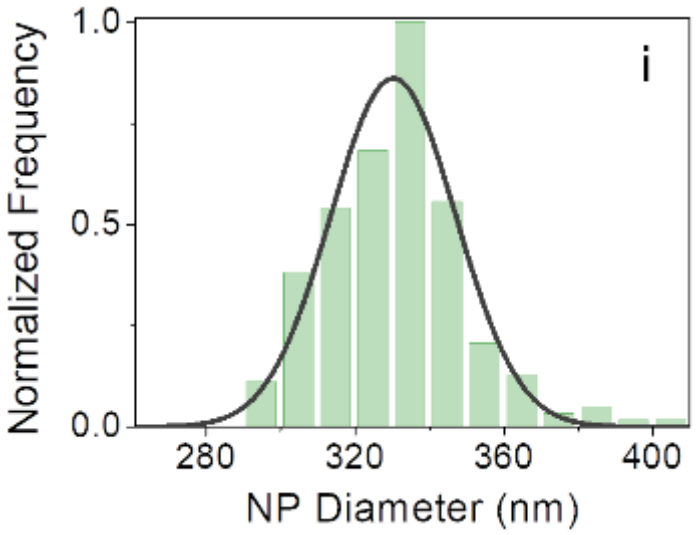



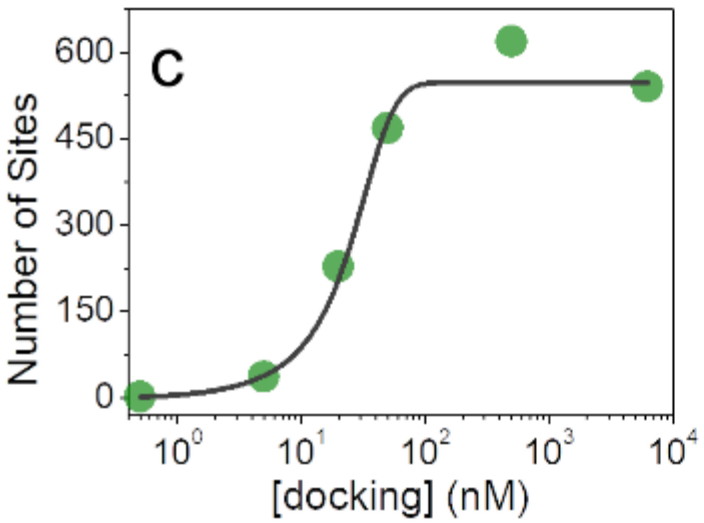
It is always Atto647N:
"Multicolor" imaging with only one color!
Theoretically unlimited colors

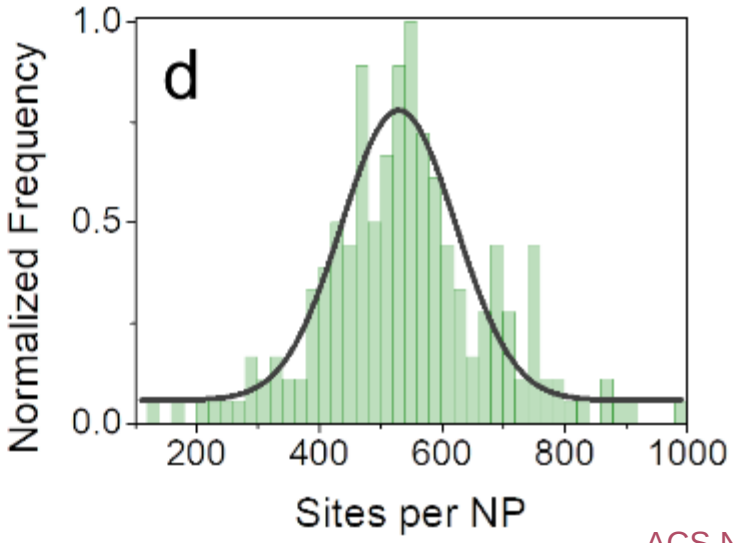
ACS Nano (2018)

# Inter-particle heterogeneity



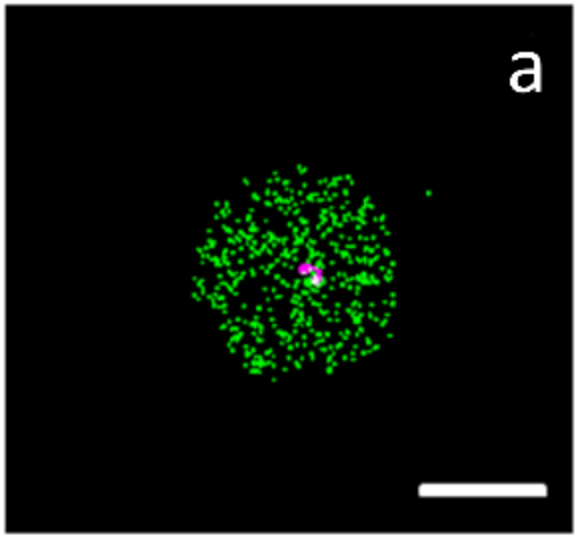


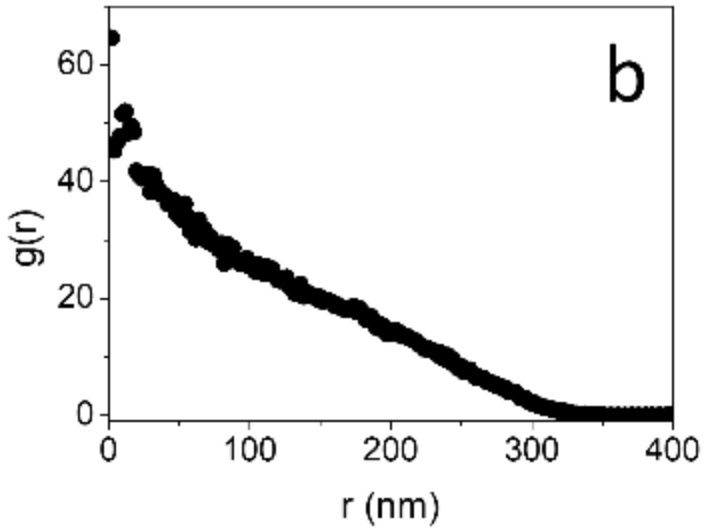



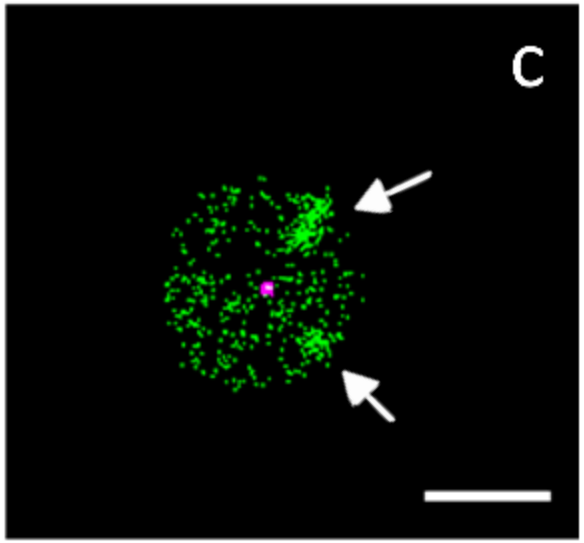



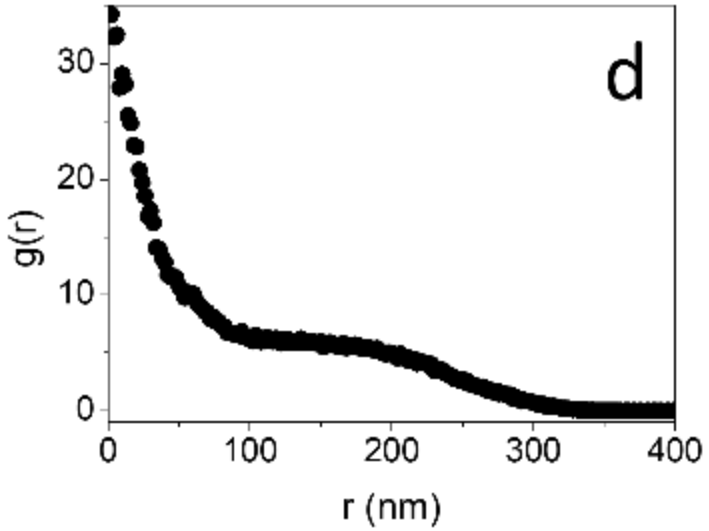

ACS Nano (2018)

# Intra-particle heterogeneity



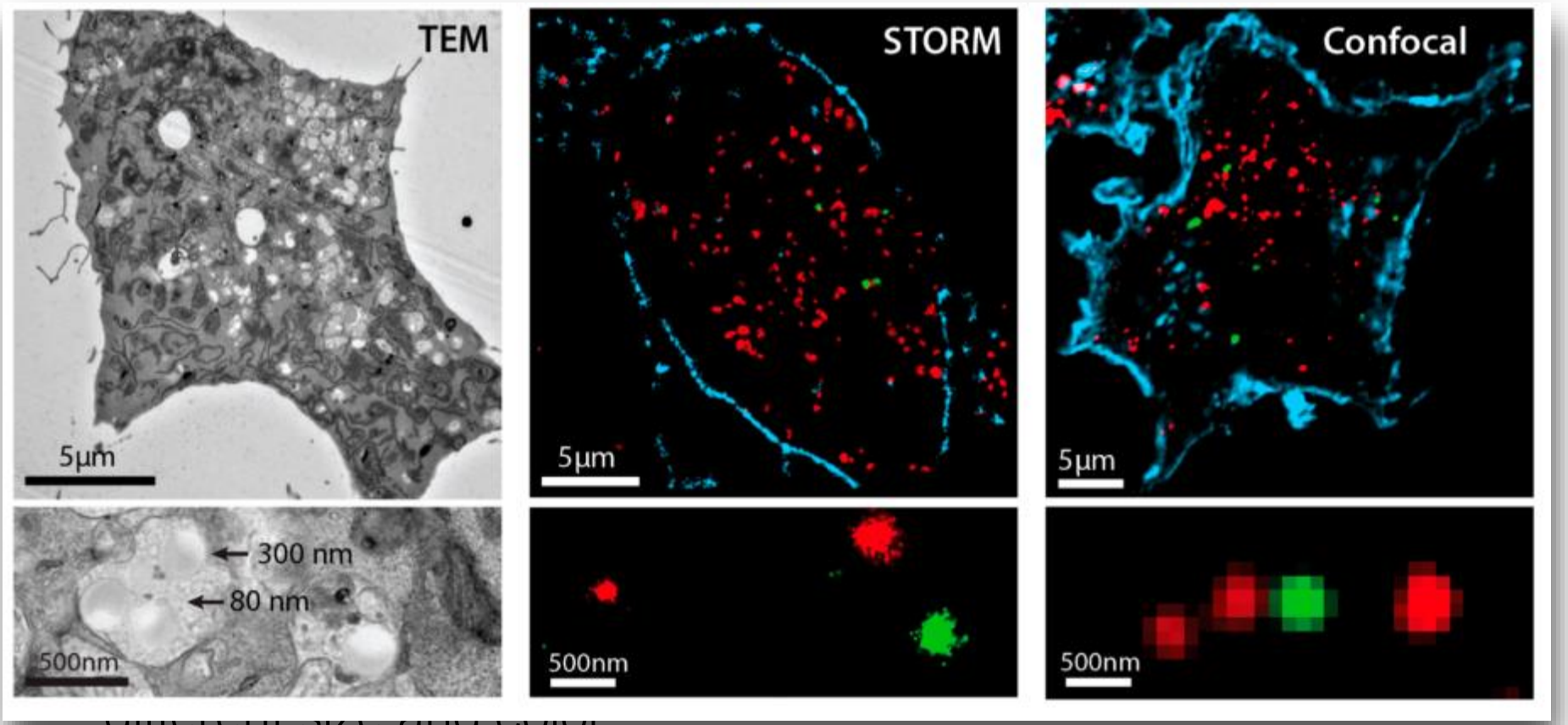



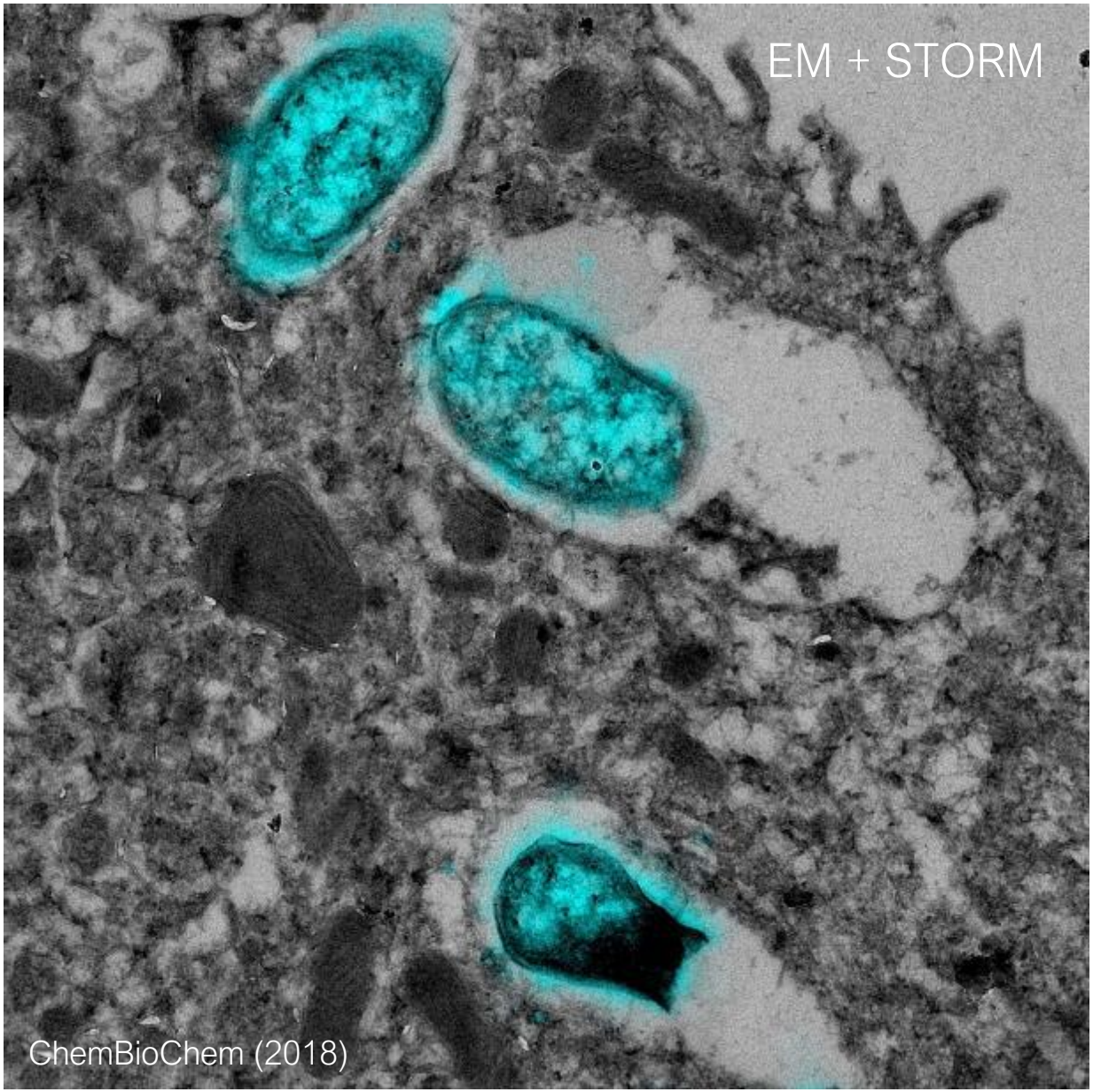

ACS Nano (2018)

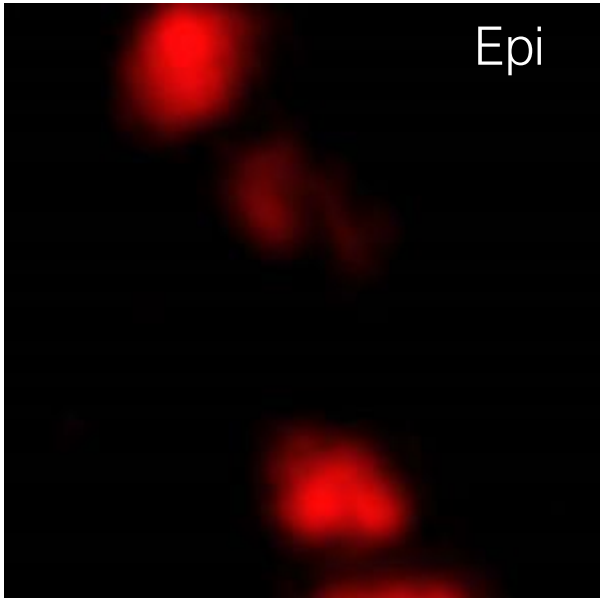
# Benchmarking

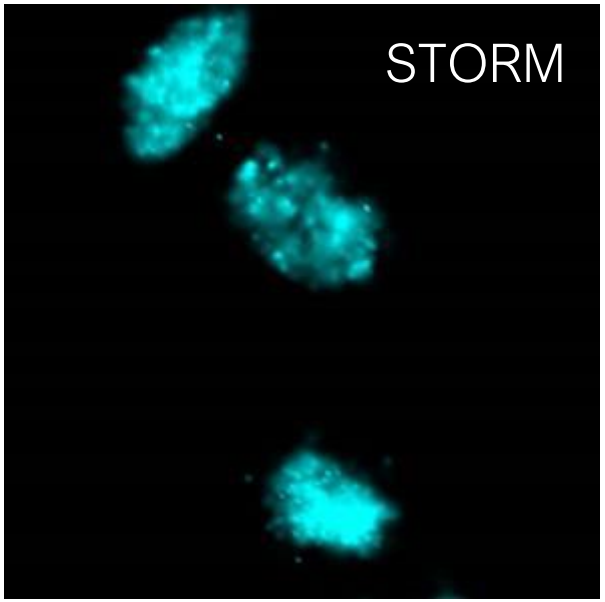



Find the best technique for your question! Or…

# Correlative: the best of both worlds



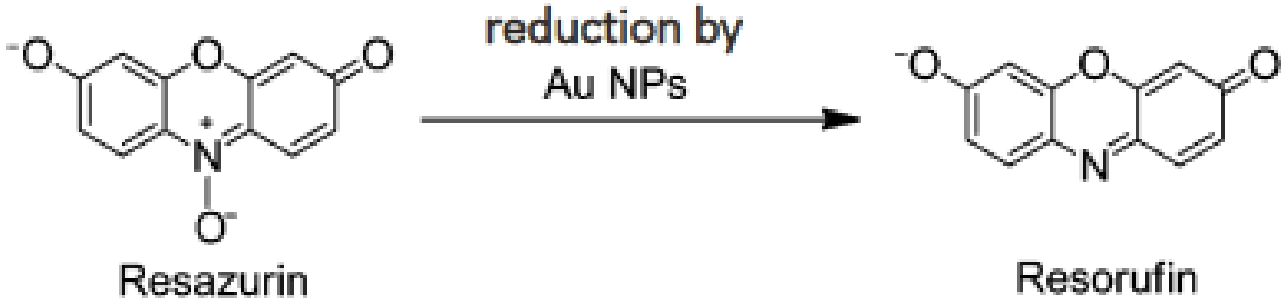



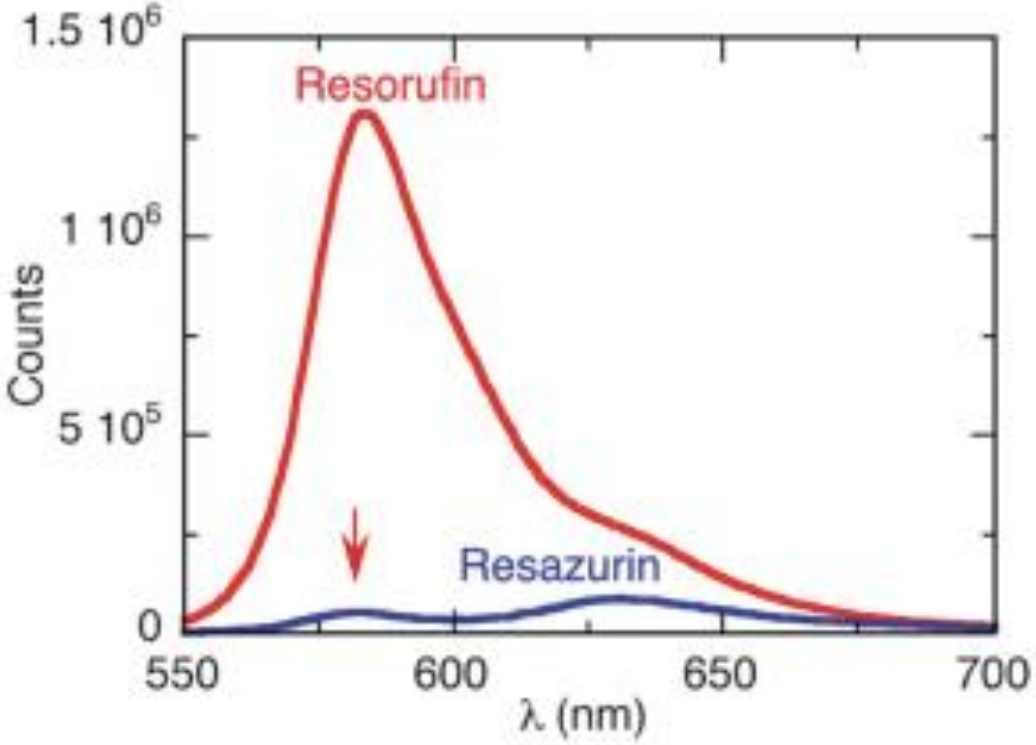
# Fluo for THERACATers

Why fluorescence microscopy for THERACAT?

- Where is you catalyst?
- How does the cell react to it?
- Is it catalyzing (pro-dyes)
- Characterization of catalytic activity

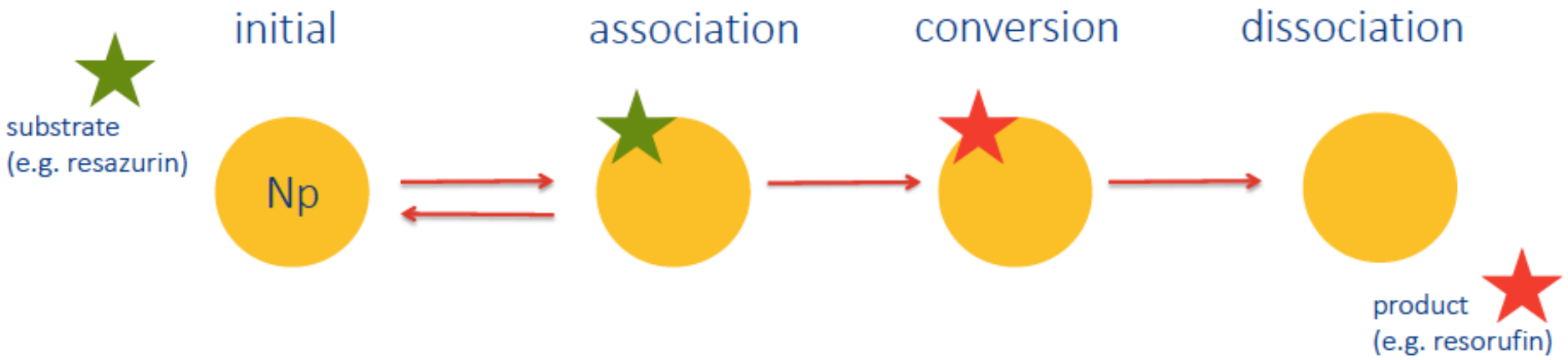
# Pro-dyes




Fluorescent enhancement is proportional to catalytic activity

# A bit of kinetics




$$Np + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} NpS \xrightarrow{k_{cat}} Np + P$$

$$\frac{d[Np]}{dt} = -k_1[Np][S] + (k_{-1} + k_{cat})[NpS]$$

$$\frac{d[NpS]}{dt} = k_1[Np][S] - (k_{-1} + k_{cat})[NpS]$$

$$\frac{d[P]}{dt} = k_{cat}[NpS]$$

# A bit of kinetics

Assume $\frac{d[NpS]}{dt} = 0$ (steady-state approximation) (use $[Np] = [Np_0] - [NpS]$)

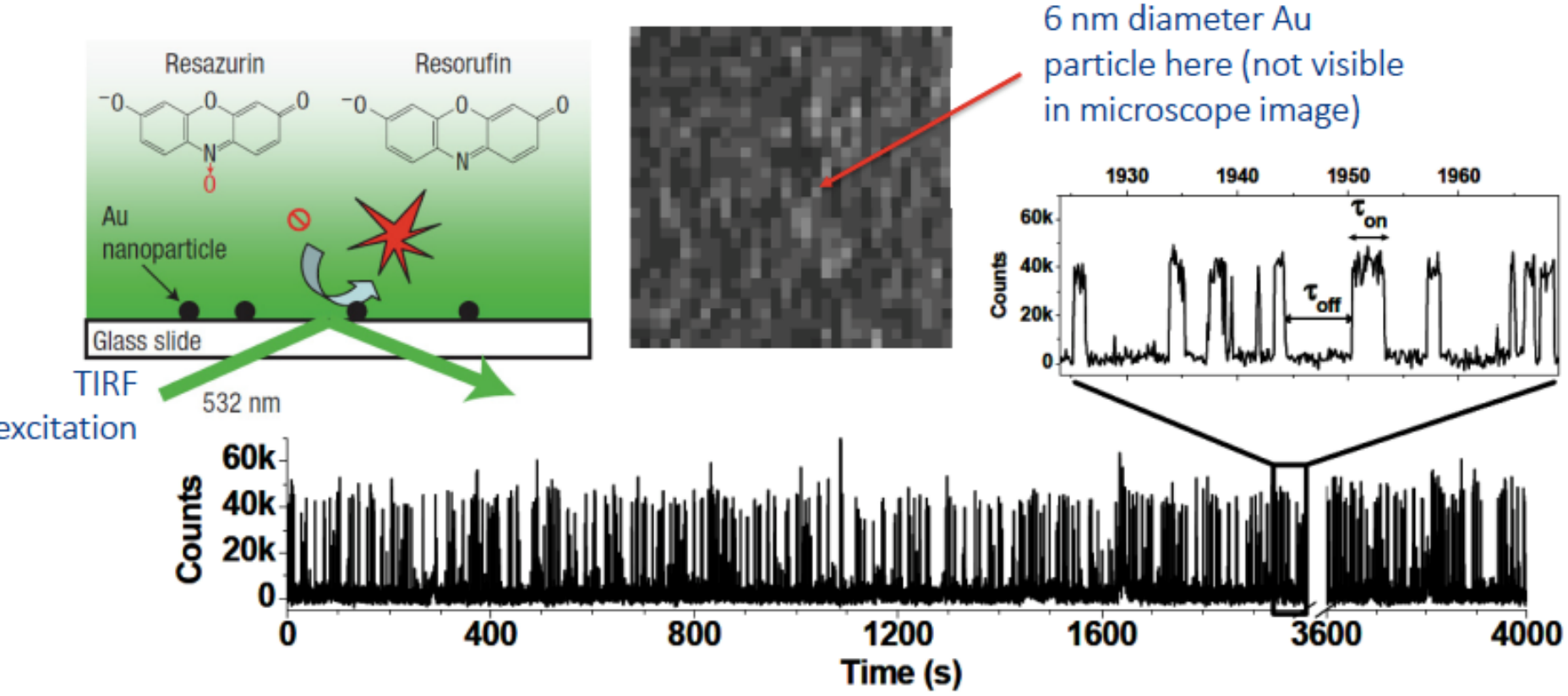
$$v_0 = \frac{d[P]}{dt} = \frac{k_{cat}[Np_0][S]}{K_m + [S]}$$

Michaelis-Menten equation

with $K_m = \frac{k_{-1} + k_{cat}}{k_1}$

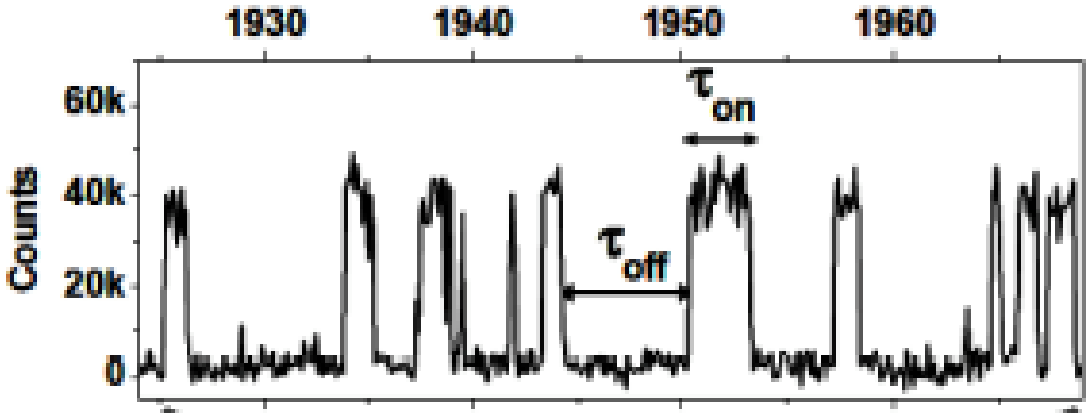
(equilibrium dissociation constant of NpS complex)

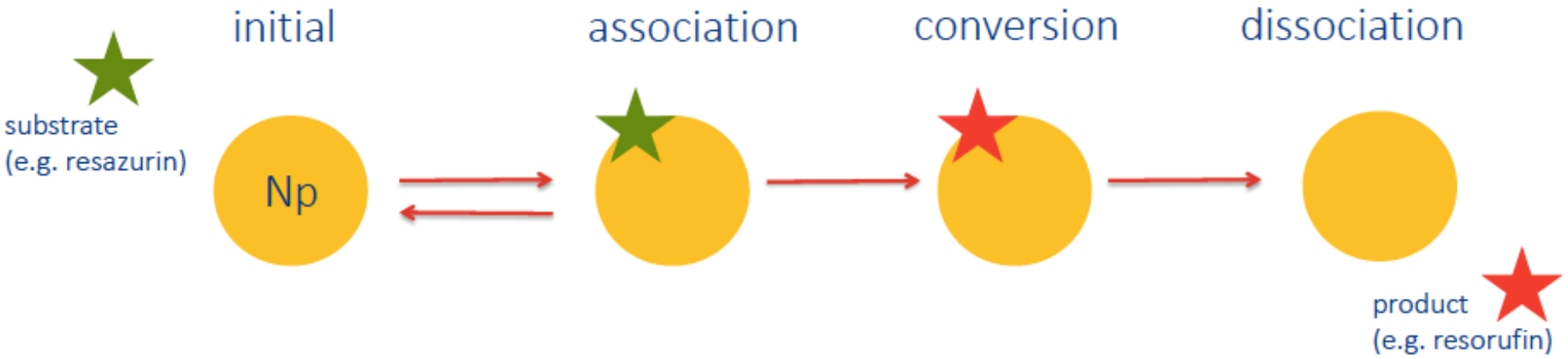
# Fluorescence measurements of kinetics



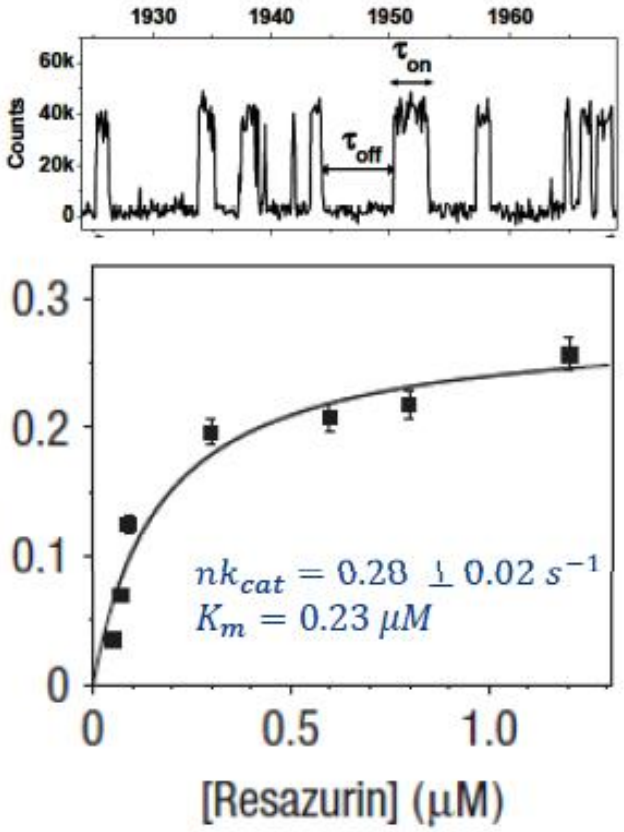

Xu et al., Nat. Mater. 7, 992-996 (2008)

# Now single-catalyst and single molecule level!



# Now single-catalyst and single molecule level!

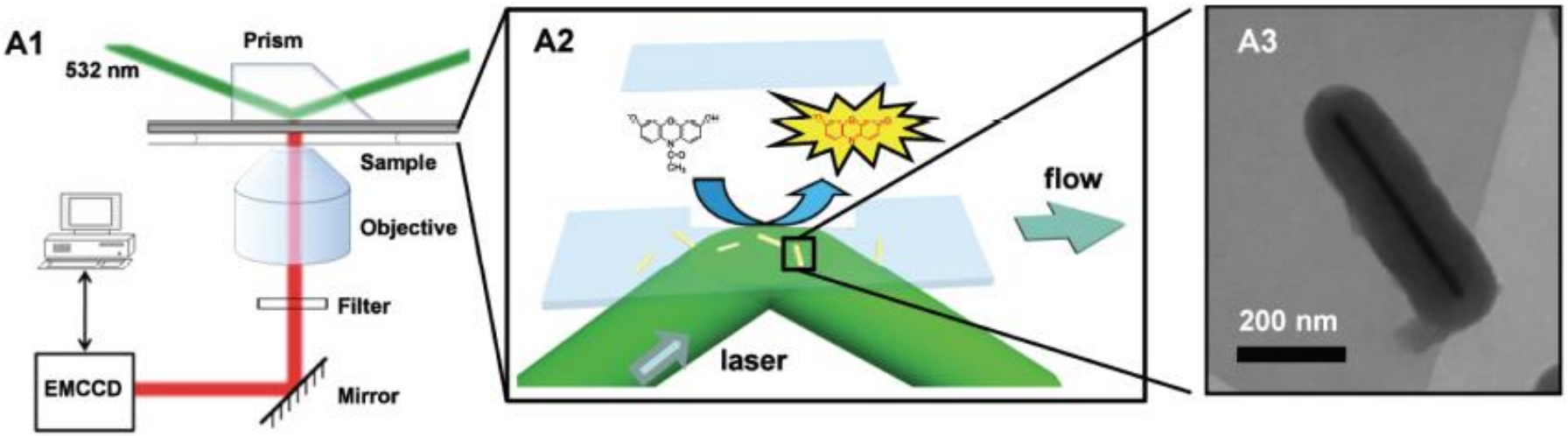



Waiting time between events indeed follows single-molecule Michaelis Menten reaction kinetics:

$$\frac{1}{\langle \tau_{off} \rangle} = \frac{nk_{cat}[S]}{K_m + [S]}$$

( $n$ = number of sites per particle)

# Super-res + catalysis

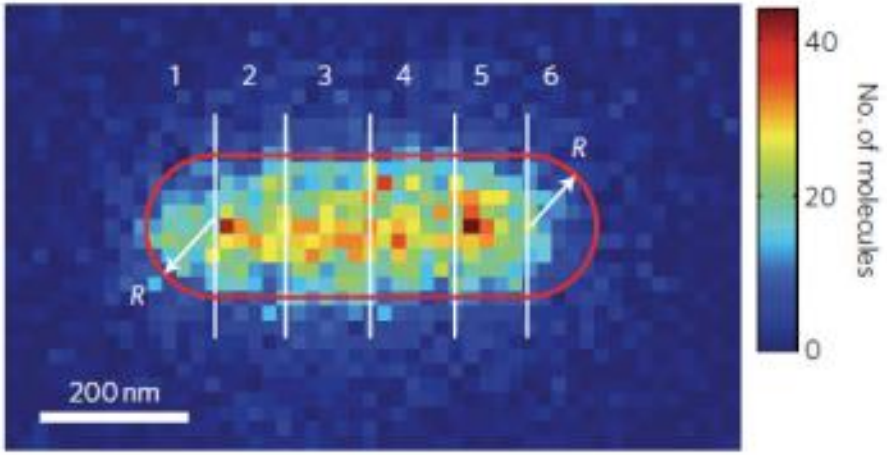


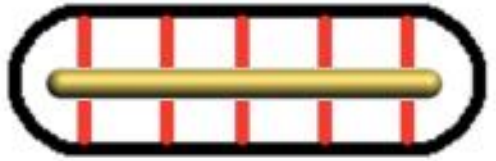

Amplex red to resorufin conversion on single gold nanorods in $SiO_2$ shell.

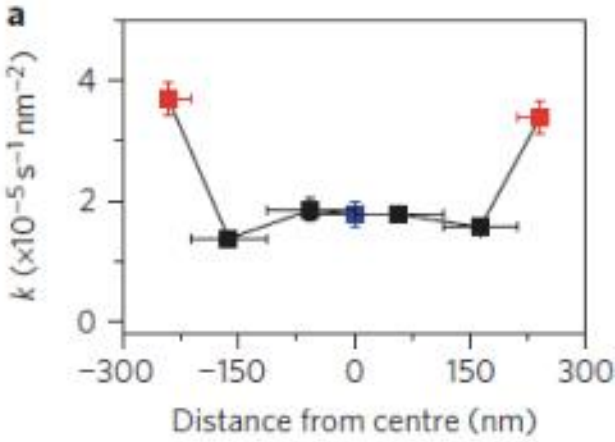
Each event gives a "burst" of fluorescence signal, which can be localized.

Zhou et al. Nature Nanotechn. **7**, 237 – 241 (2012)

# Super-res + catalysis

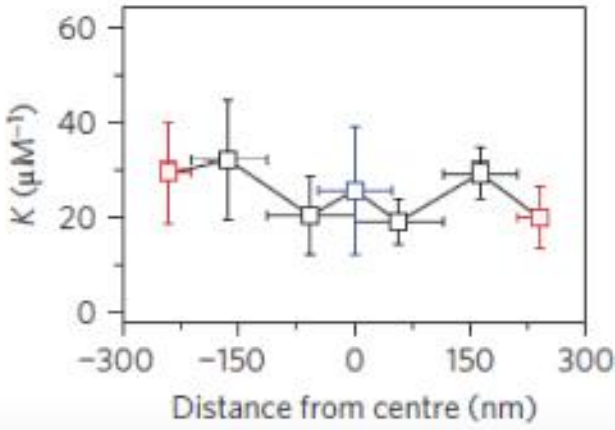


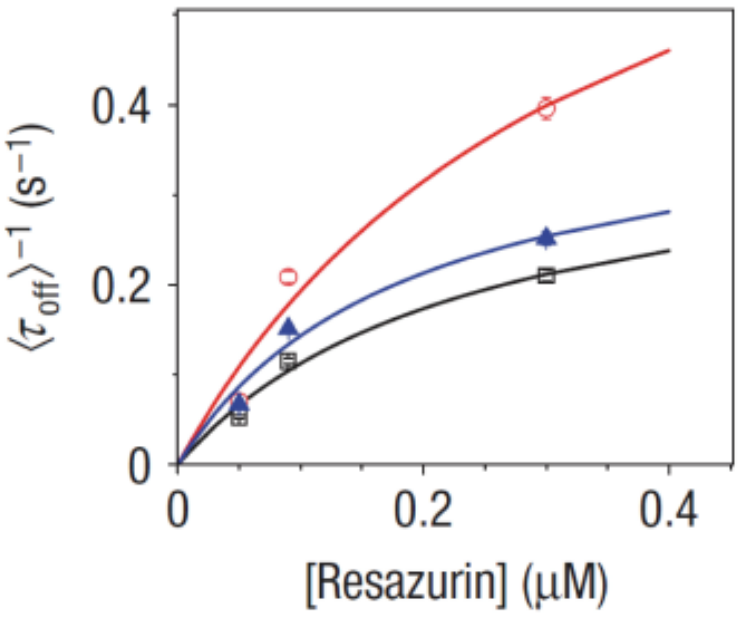


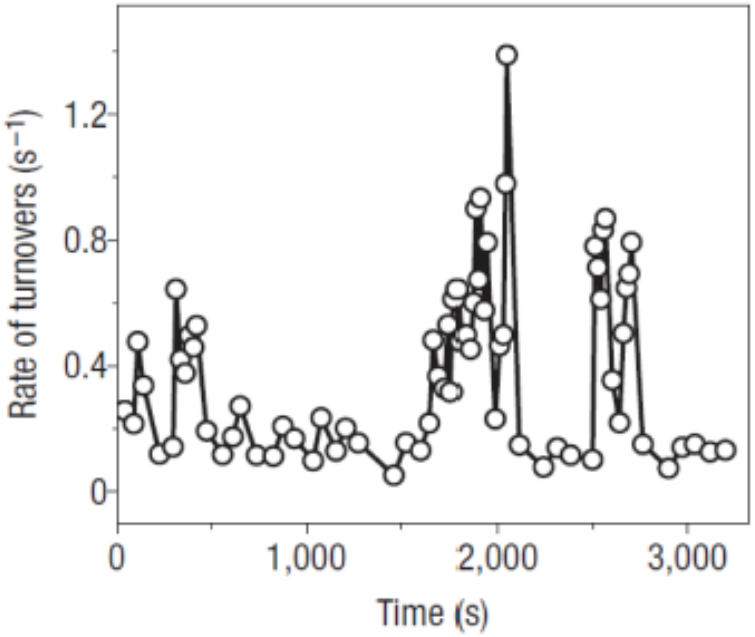

For this particle, activity per unit surface area is higher at tips.

$$\frac{1}{\langle \tau_{\text{off}} \rangle} = \frac{nk_{cat}[S]}{K_m + [S]}$$

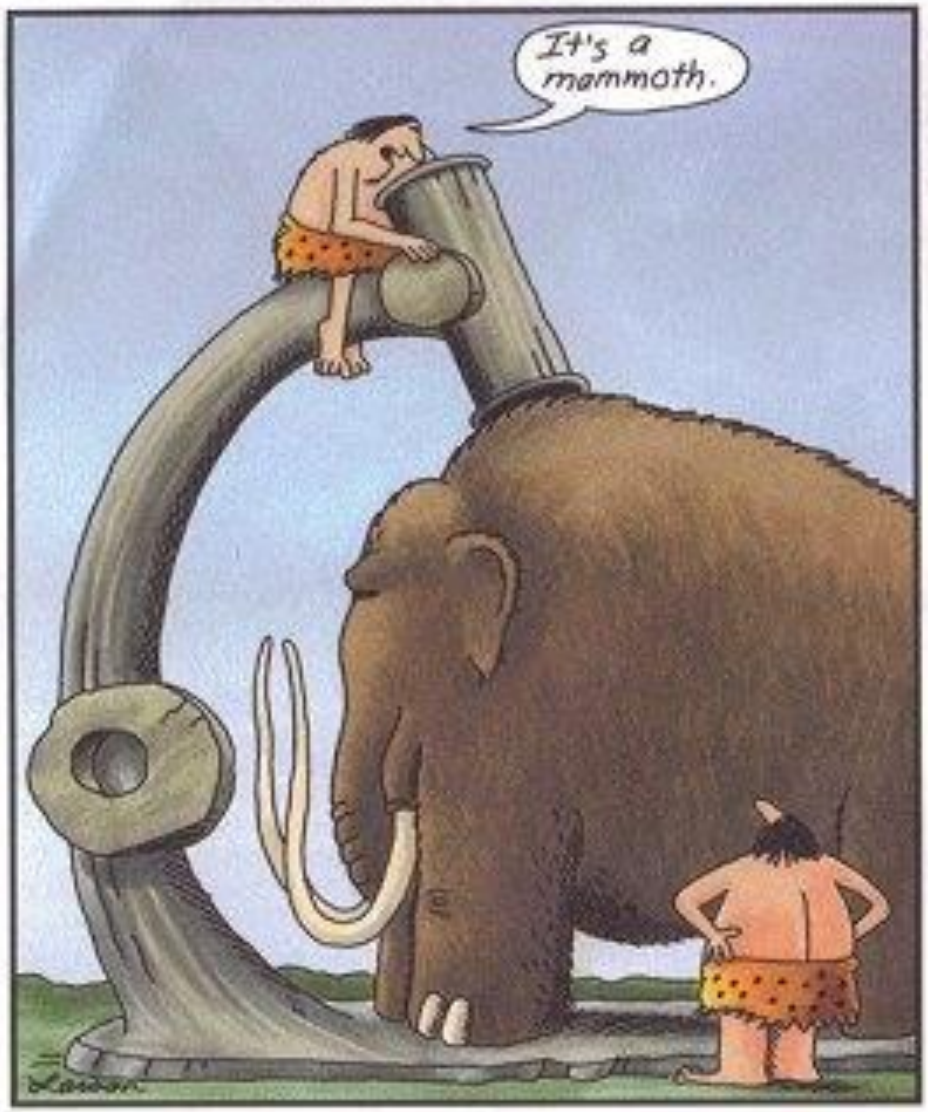


# Super-res + catalysis




- Kinetics of three different particles are not the same!
- Turnover rate fluctuates over time!




Early microscope

There is no “better” technique. Pick the best technique for your sample!