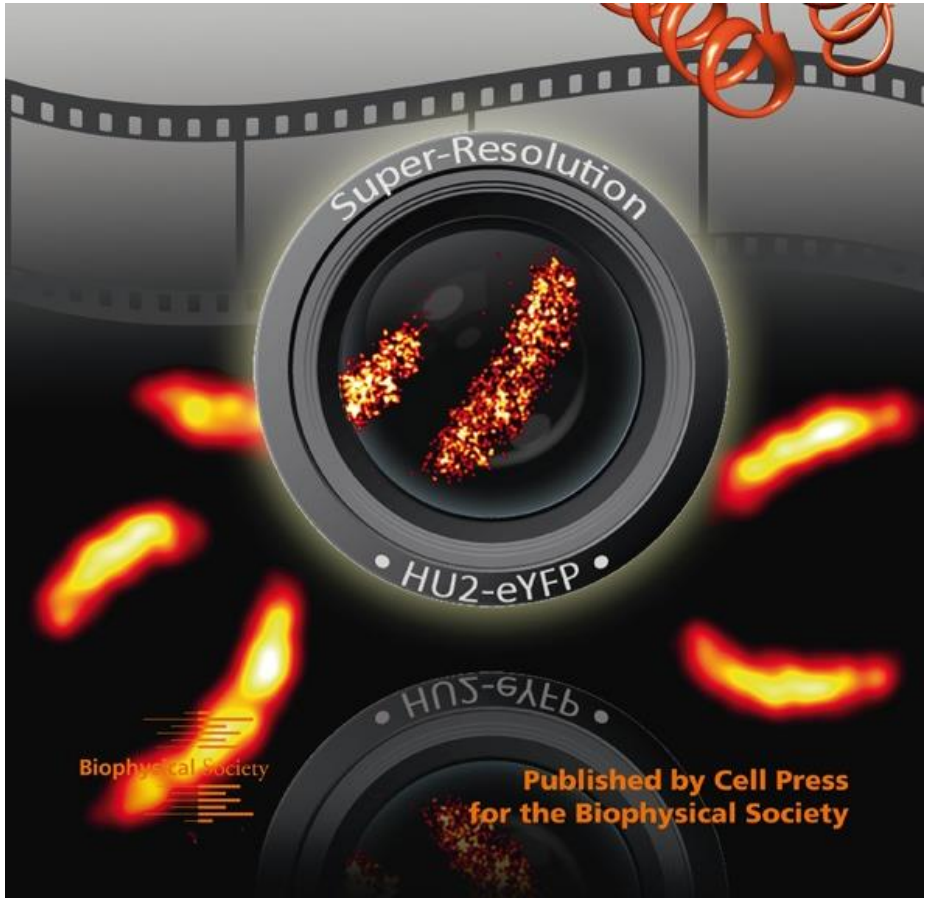
Advanced microscopy for microbiology



Steven Lee

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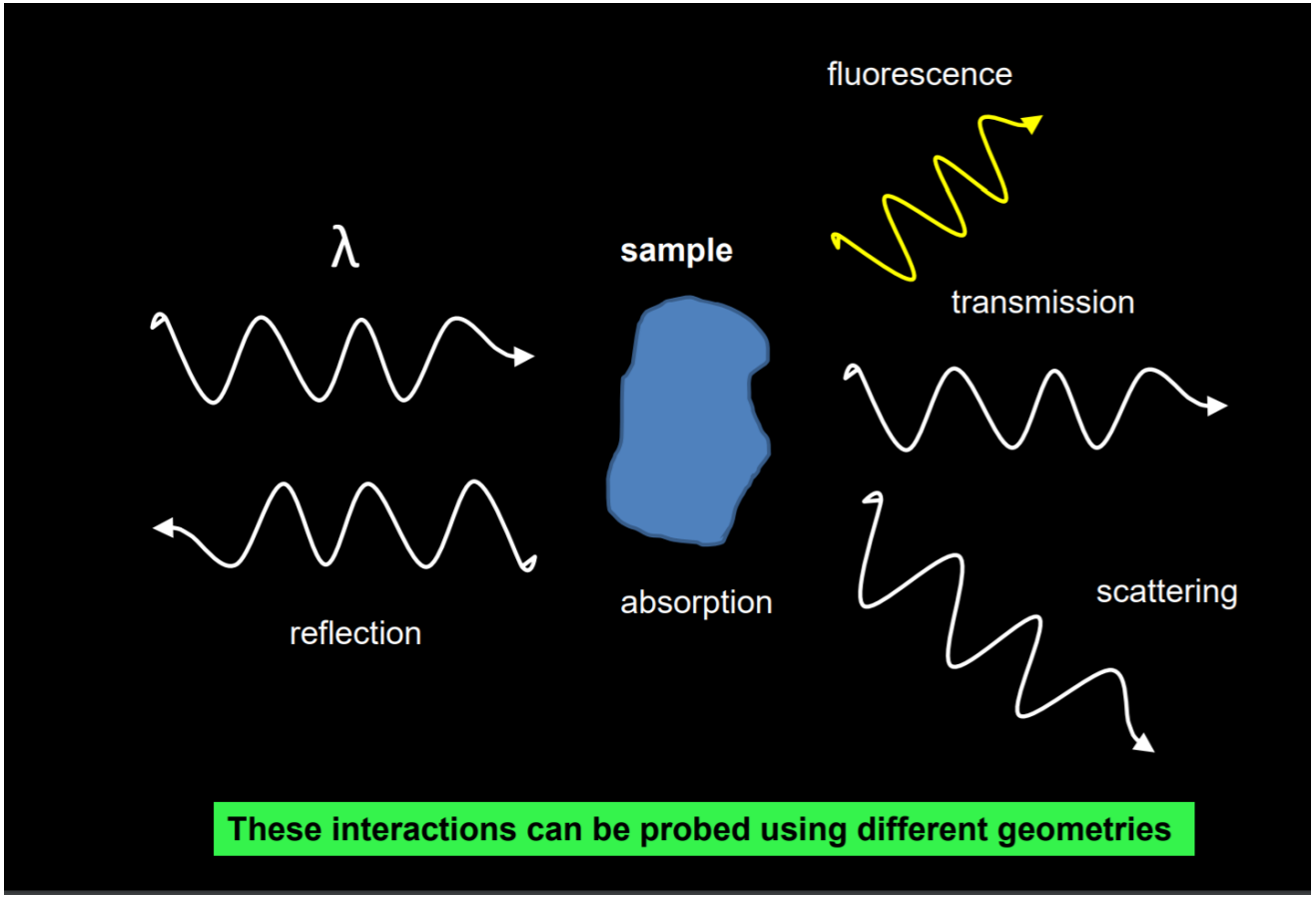
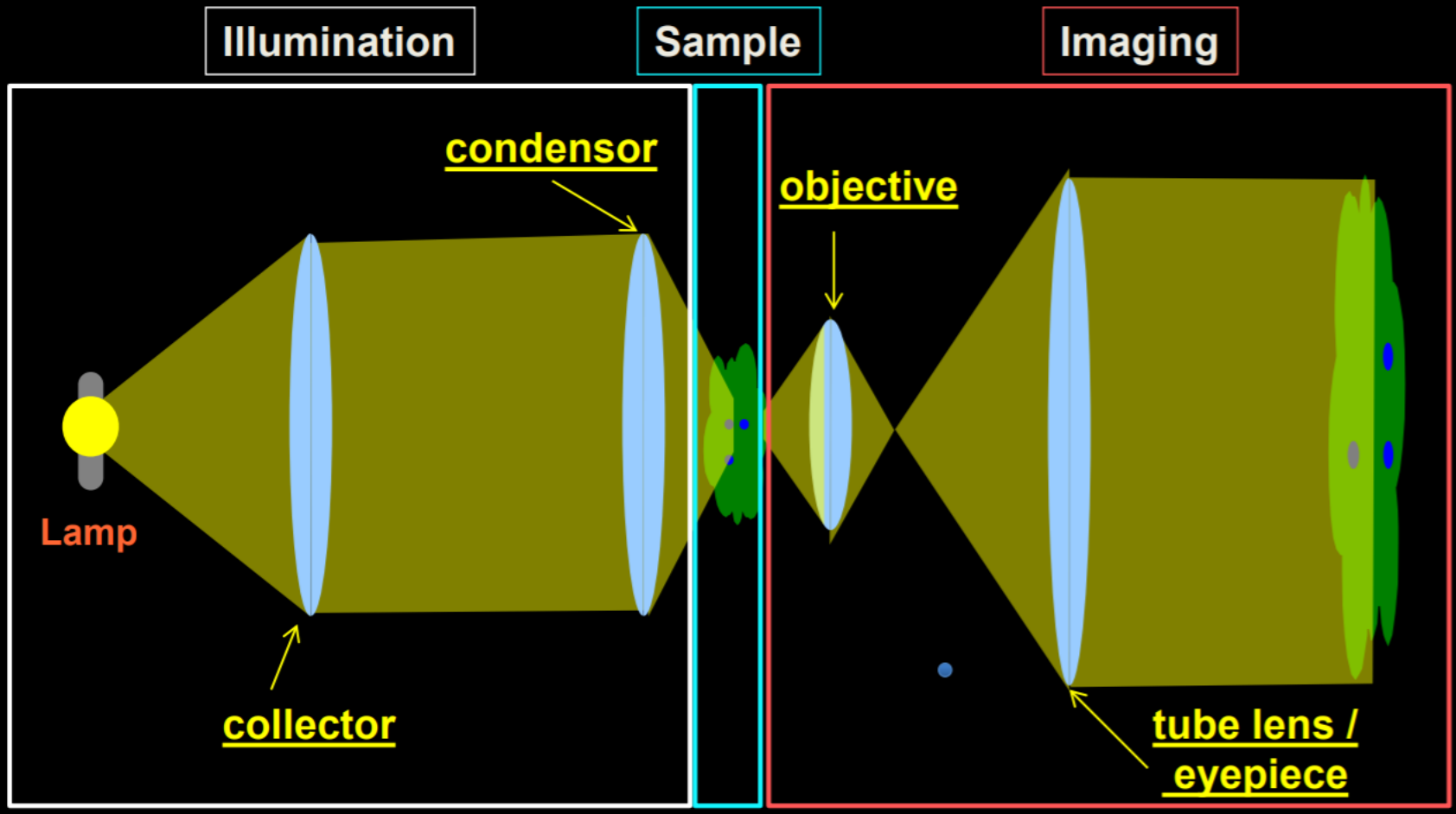
Light microscopy 101

Goal: Observe spatial and temporal organization of biology
Basic concept: use light to probe a sample



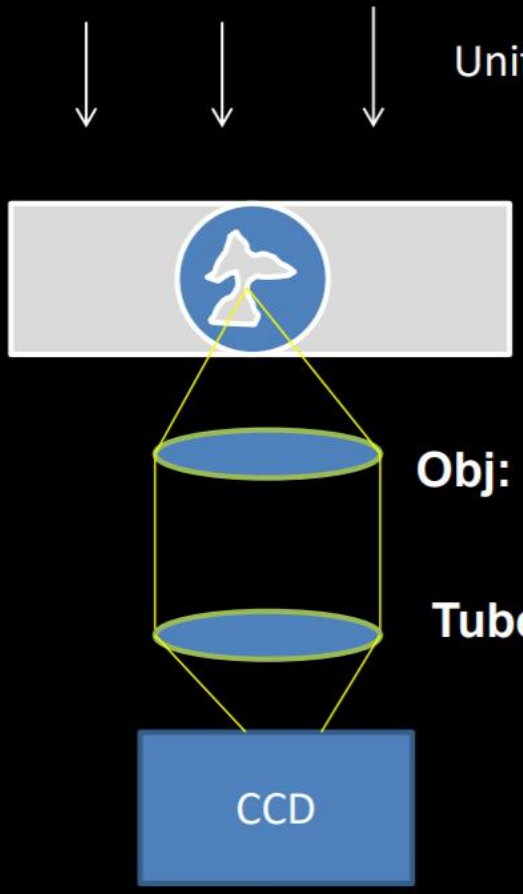
Anatomy of a microscope

(Lots of) More complex designs exist, but we'll first stick to this ☺

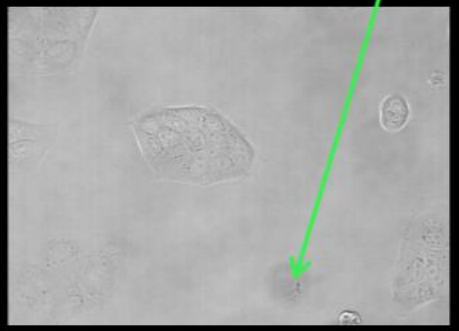


A microscope is ~ a light source, a detector, and some lenses in between

Brightfield microscopy (transmission)



Requires good illumination



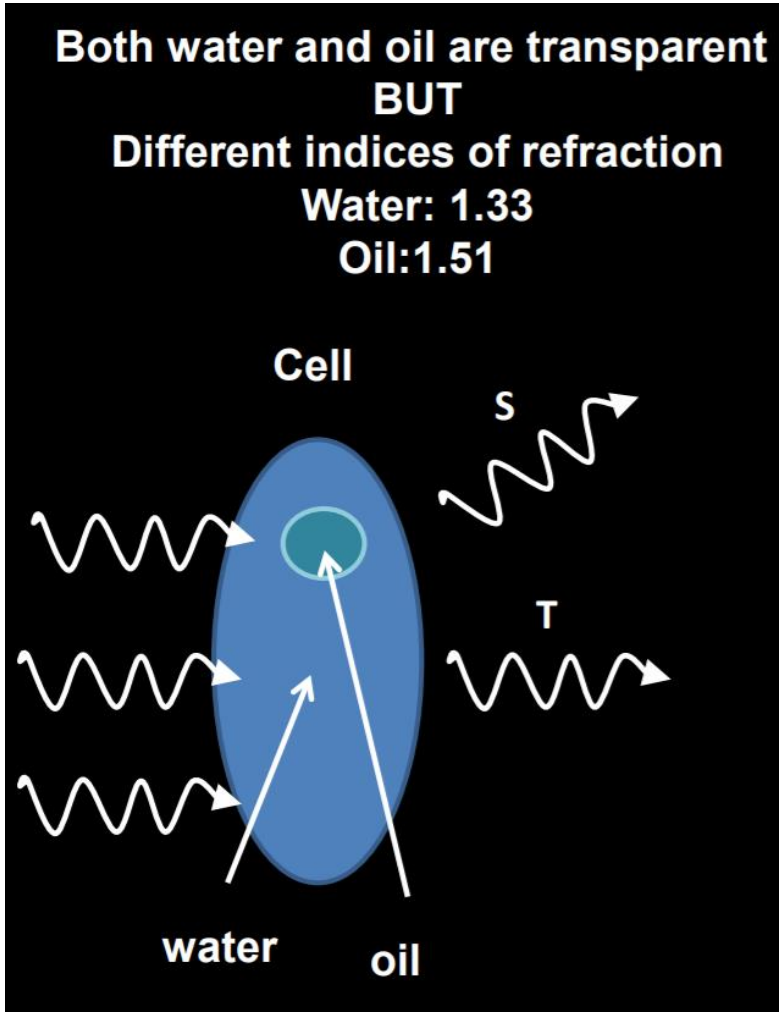
Low contrast on cells ☹

Widefield transmission microscopy:

sensitive to absorption, reflection and some scattering (absorbance)

Phase microscopy methods

- Methods such as Phase Contrast achieve much higher contrast transmitted light imaging
- By imaging changes in sample refractive index between cell and surrounding media.



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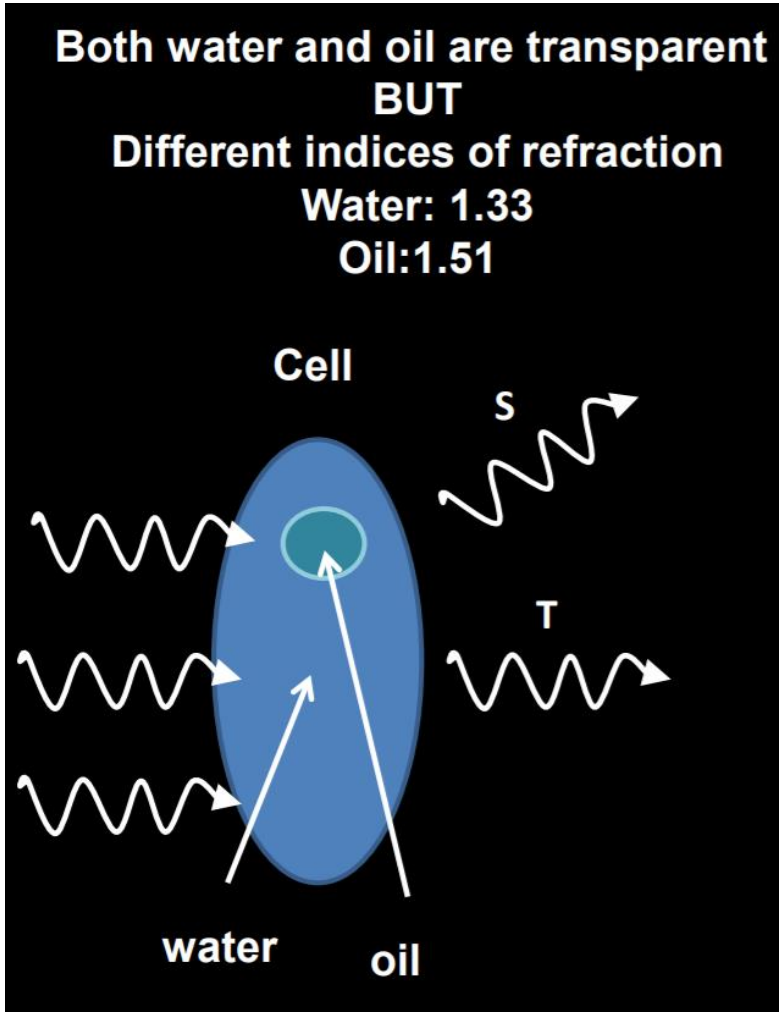
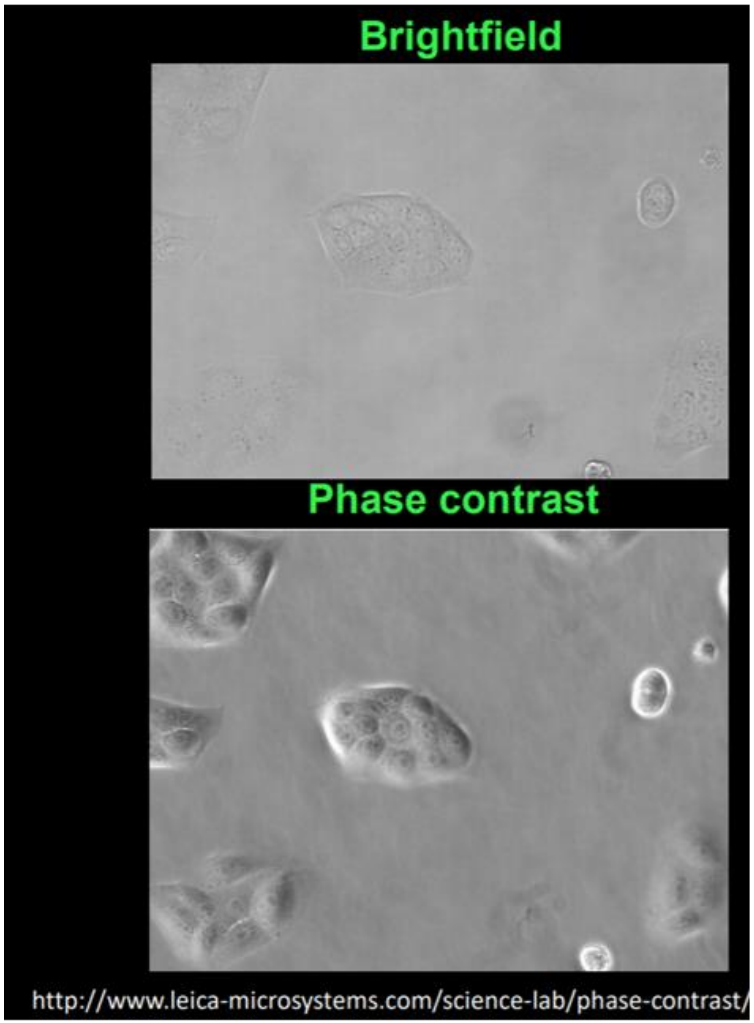
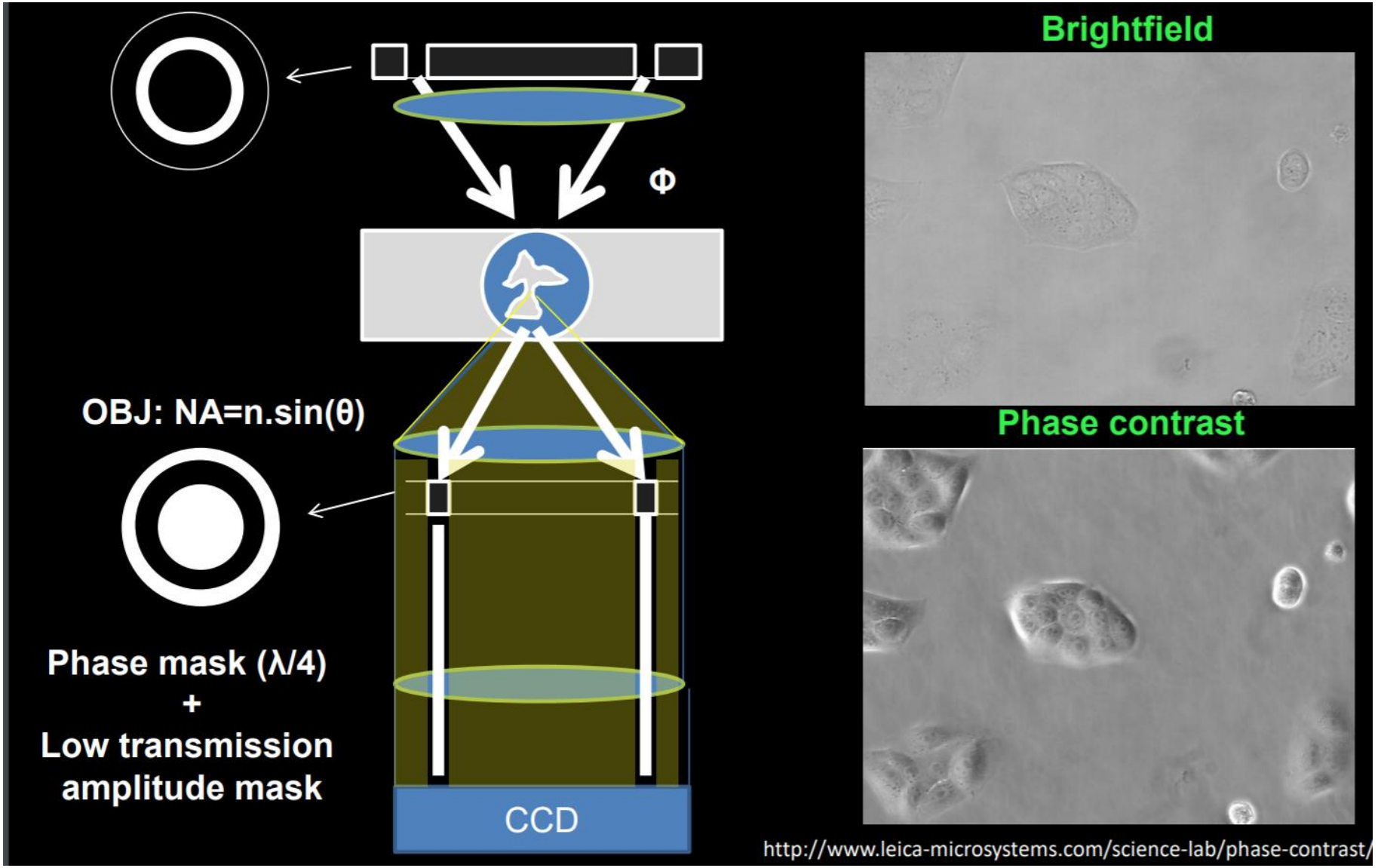




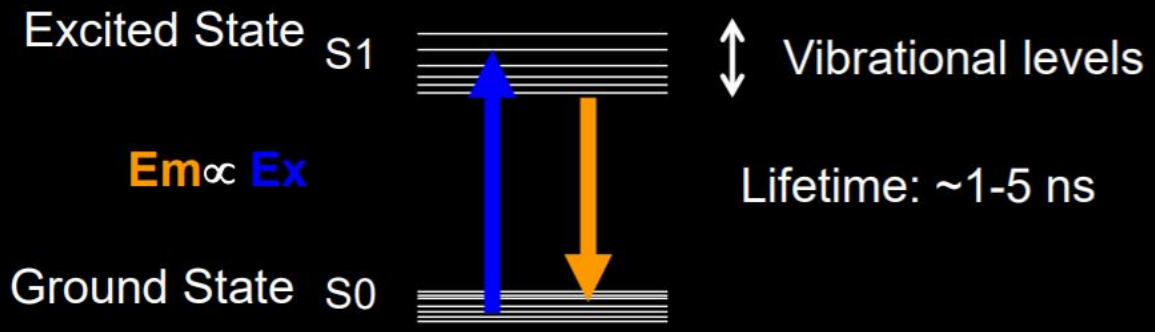
Phase microscopy methods

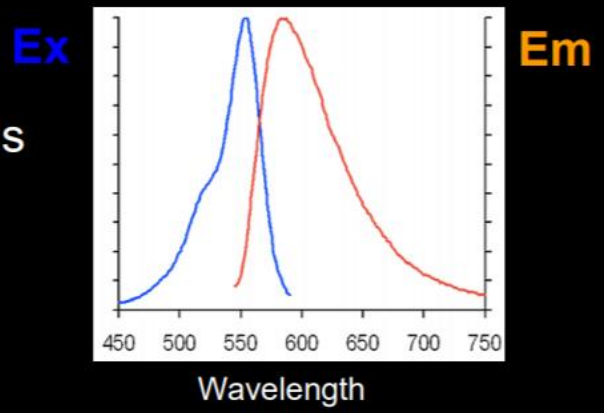


Fluorescence microscopy

Simplest and most powerful ☺

~ two level system

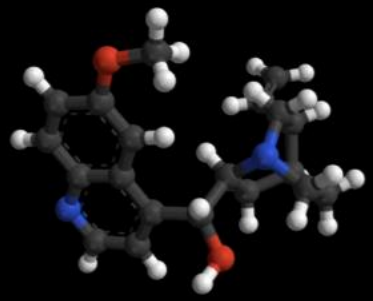




Organic dyes (XIX)

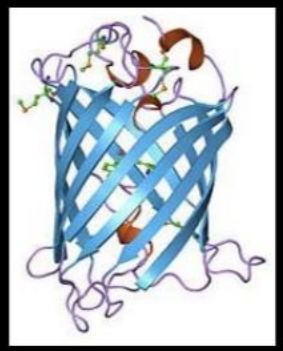
Ex: Rhodamine 6G

$C_{28}H_{31}N_2O_3Cl$



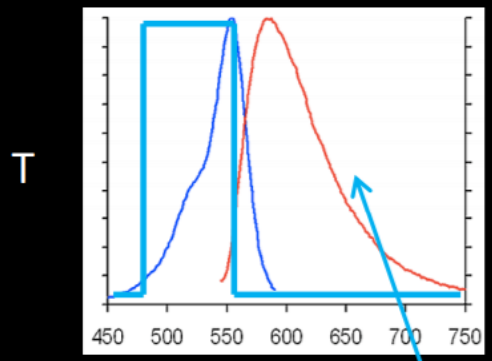
Proteins: GFP : 1994-1997

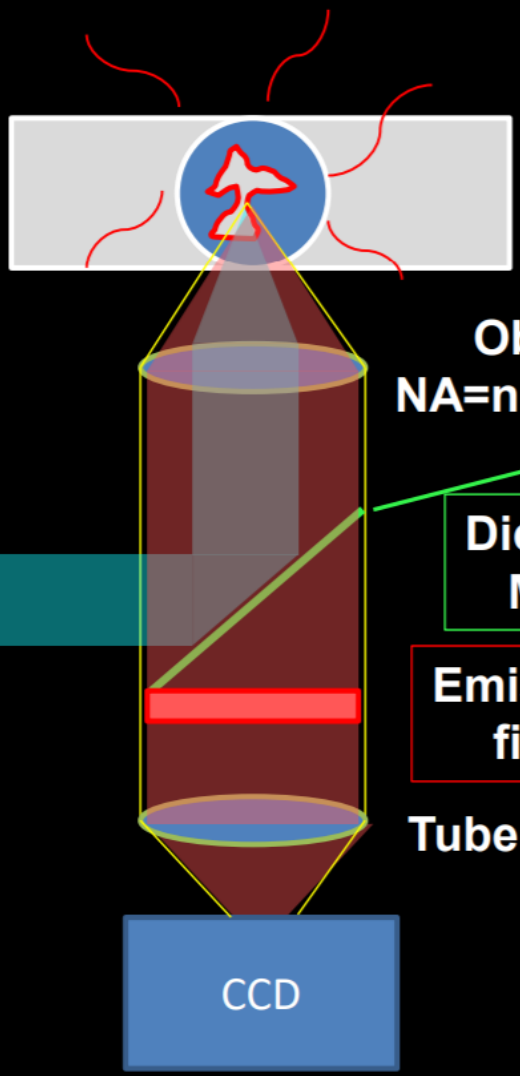
Nobel Prize Chemistry 2008
Tsien, Chalfie, Shimomura

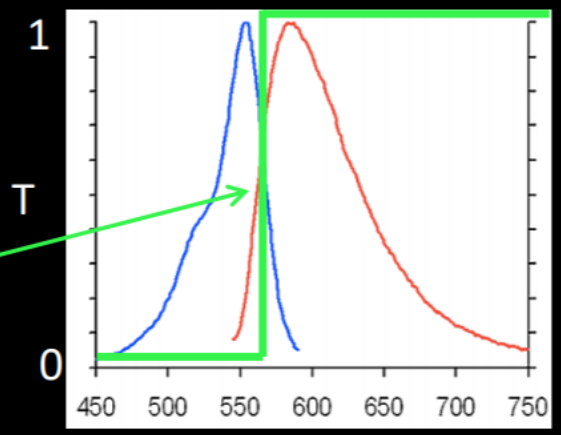


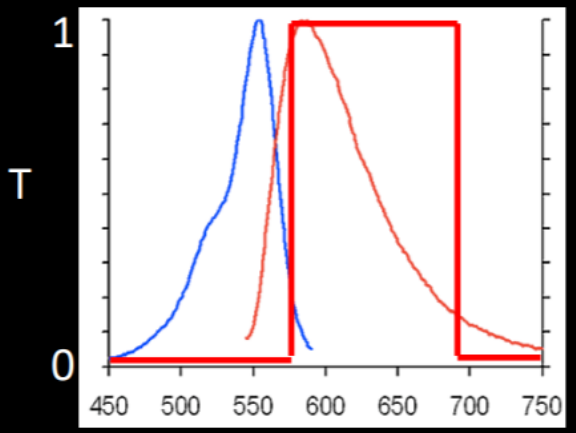
can target specific protein
in living cells

Fluorescence microscopy

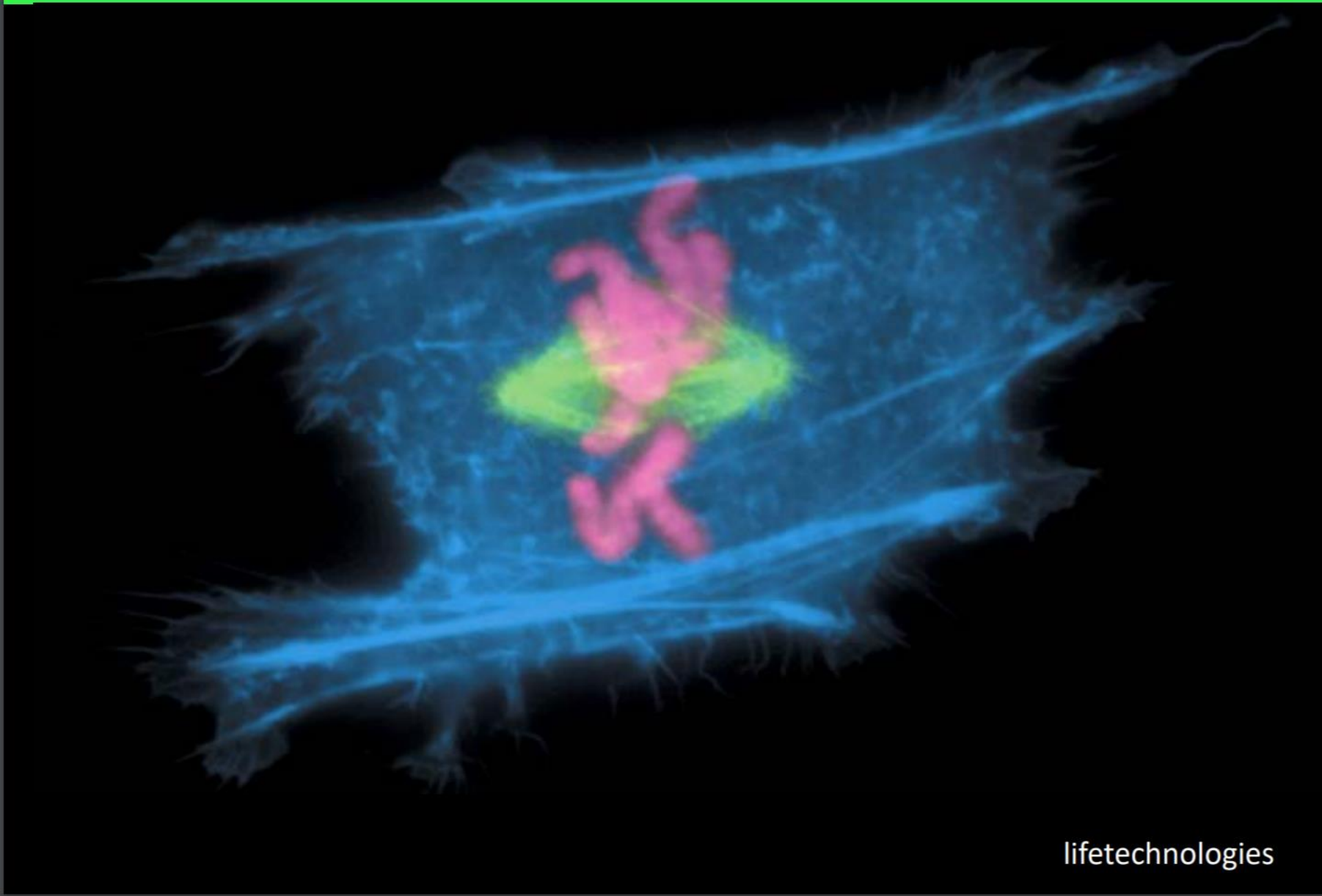




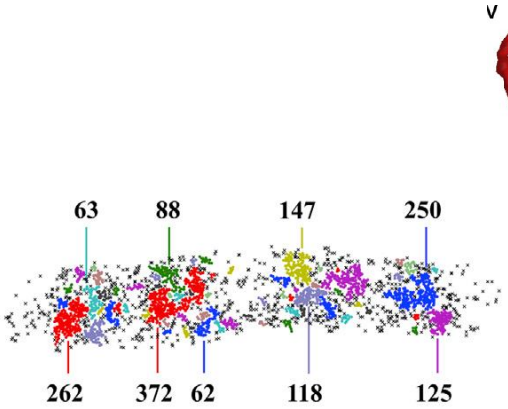


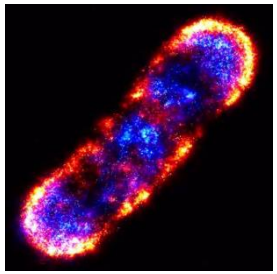


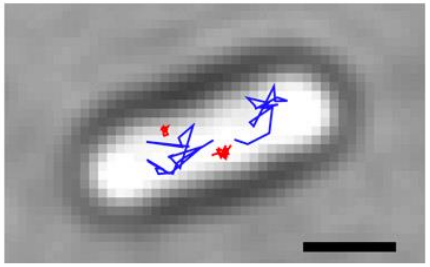
Fluorescence microscopy



Super-resolution and single molecule imaging for microbiology



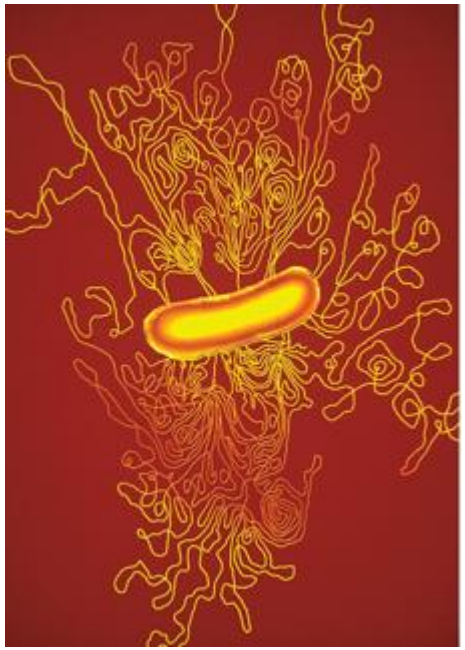




- These techniques dramatically increase resolution and allow us to probe the behaviour of single proteins in live cells
- Revolutionary throughout biology
- But particularly useful in bacteria due to their small size and their relative simplicity

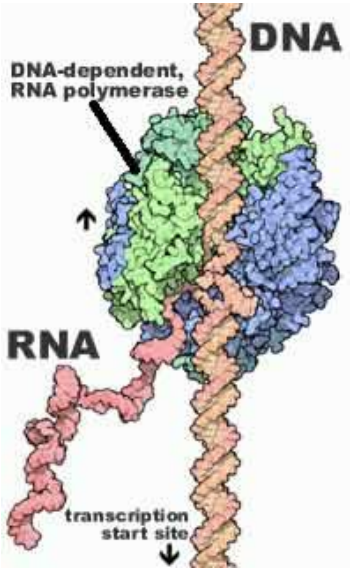
Single molecule microbiology

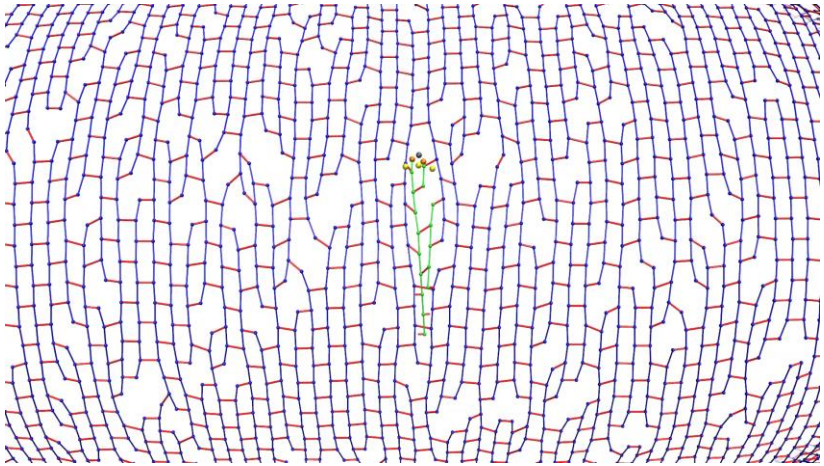
Biology works at the single molecule level!



Examples:

- Chromosome is a single molecule!
- Gene expression is performed by a single molecule nanomachine - RNA polymerase
- Cell wall remodelling is performed by single multi-enzyme complexes

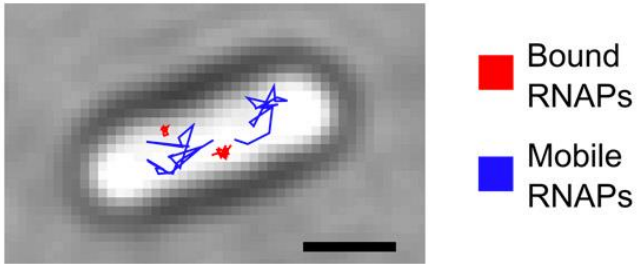




Nguyen et al PNAS 2015

Single molecule microbiology

Different copies of a protein will be in multiple different states in the cell
Eg, RNAP bound/ unbound to DNA:

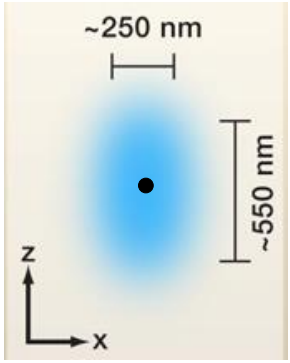


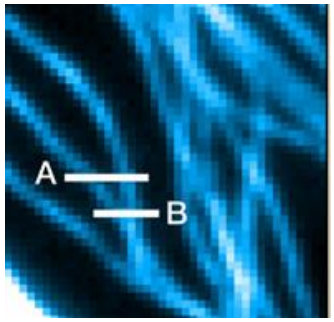
Stracy et al PNAS 2015

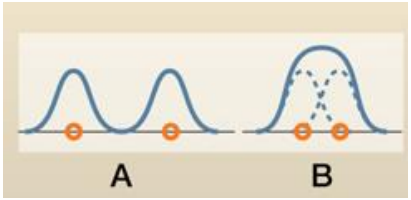
“Ensemble” methods average over these different states -
To get accurate information we need to measure one molecule at a time

Diffraction poses serious problems in bacteria…

Diffraction limits the resolution of light microscopy:

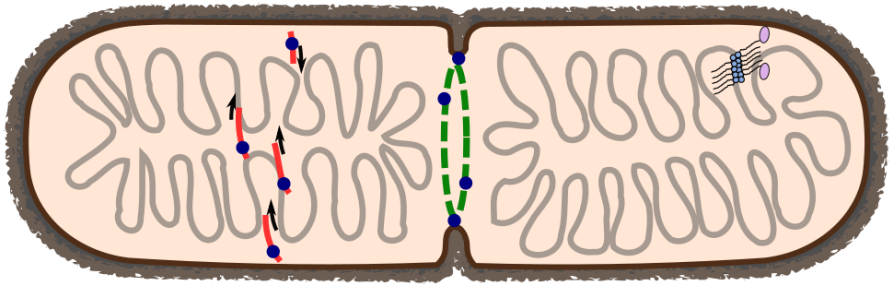






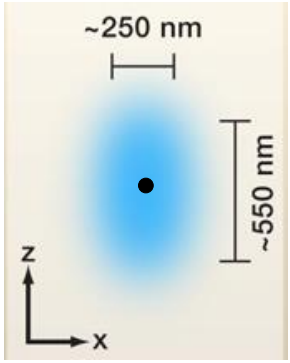
Huang et al, *Cell* (2010)

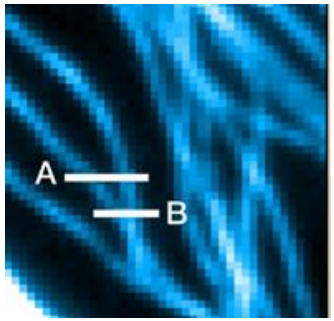
In practice this is a serious limitation!

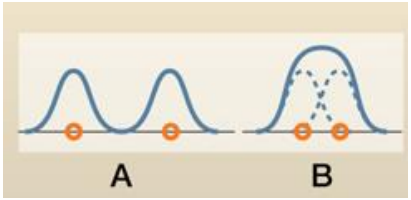


Super-resolution microscopy resolves this problem

Diffraction limits the resolution of light microscopy:







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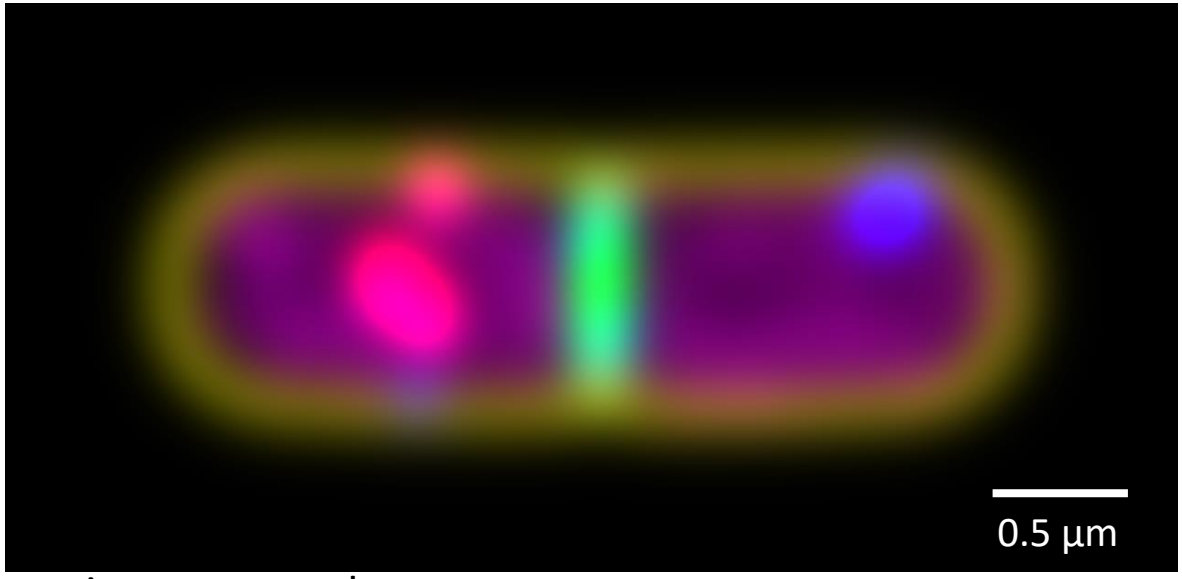
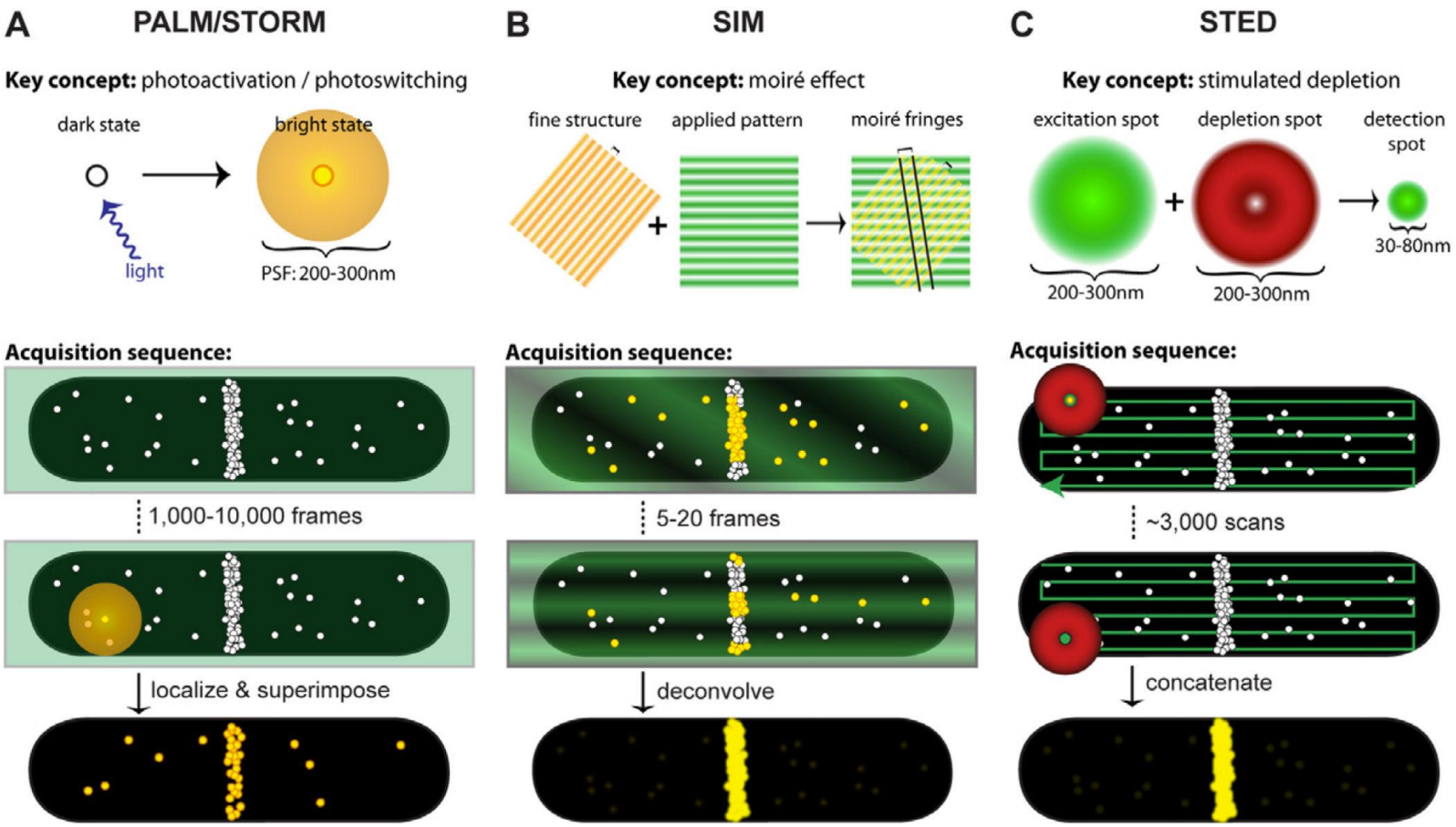


Super-resolution microscopy to the rescue…

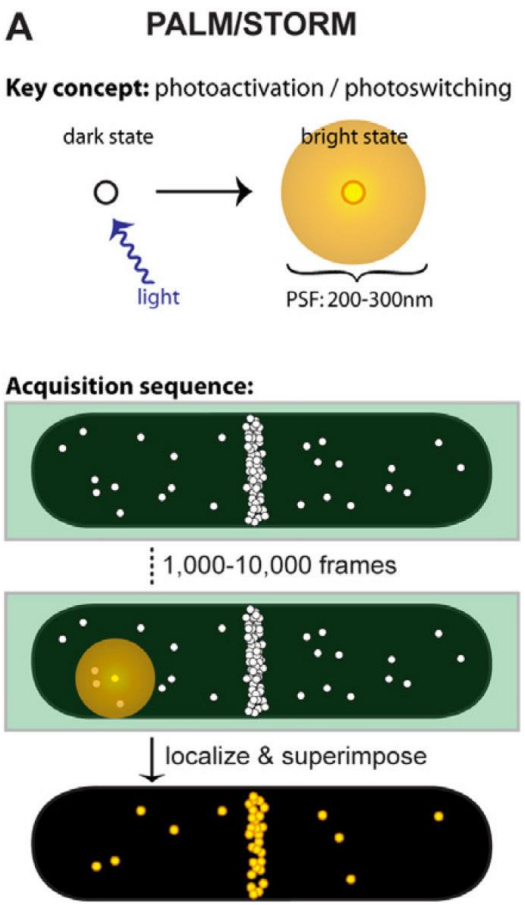
Super-resolution methods



Resolution: 20 nm 100 nm ~50 nm

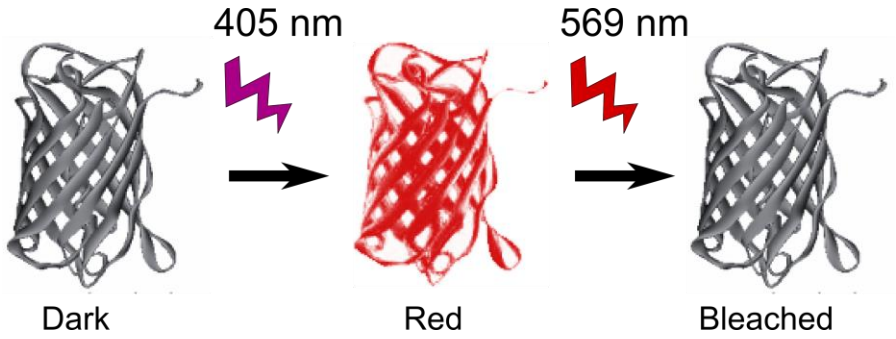
Coltharp & Xiao, Cell Microbiol 2012

Localization microscopy: principle

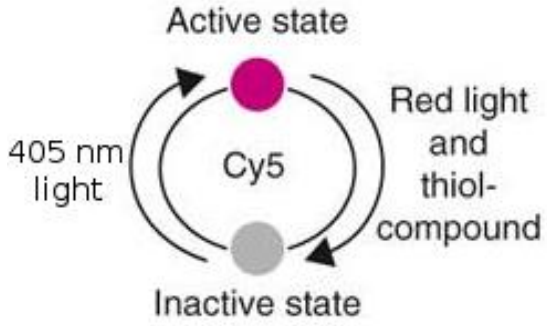


It’s all about making fluorophores blink!

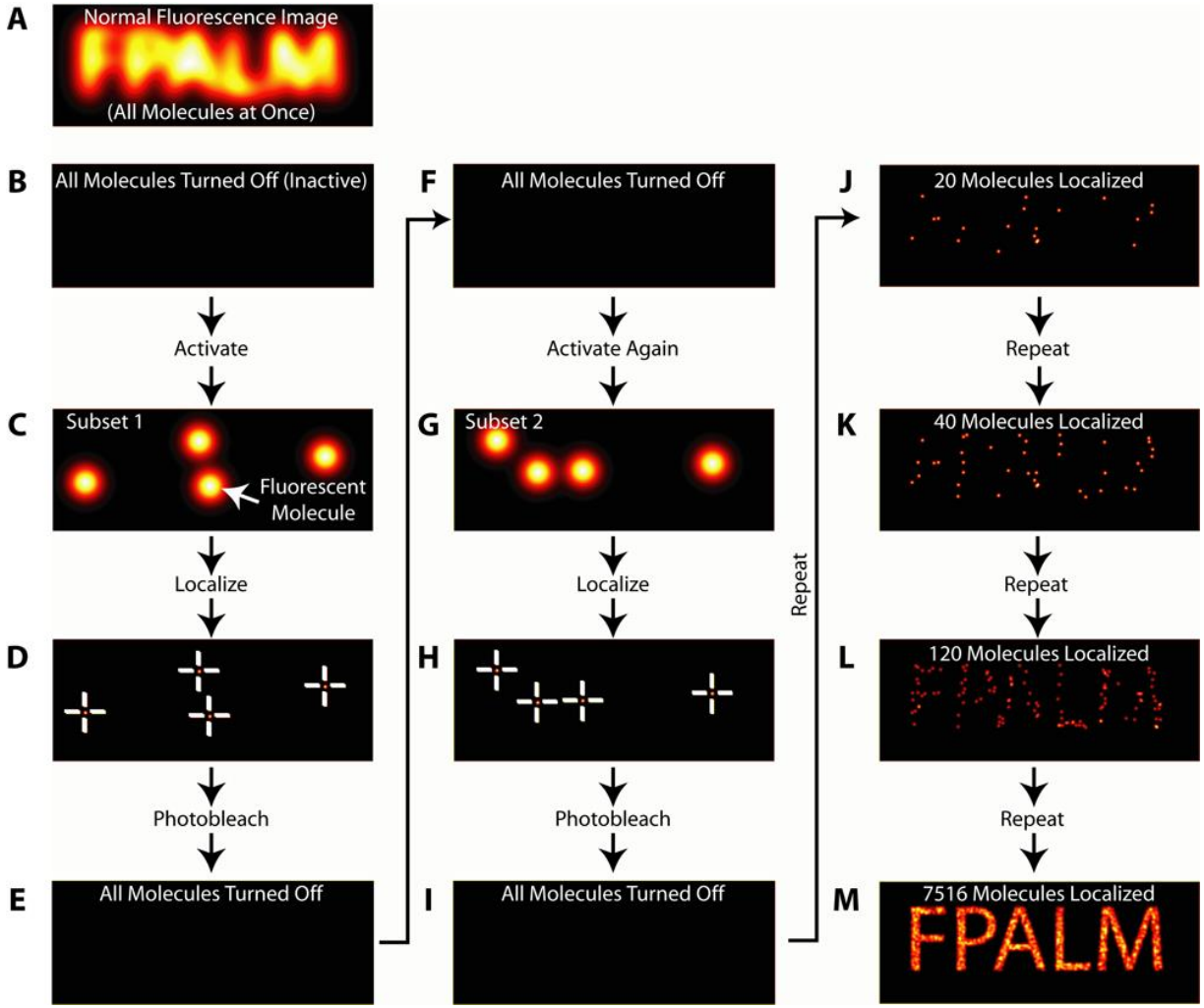
Photoactivatable fluorescent proteins:



Photoswitchable organic dyes



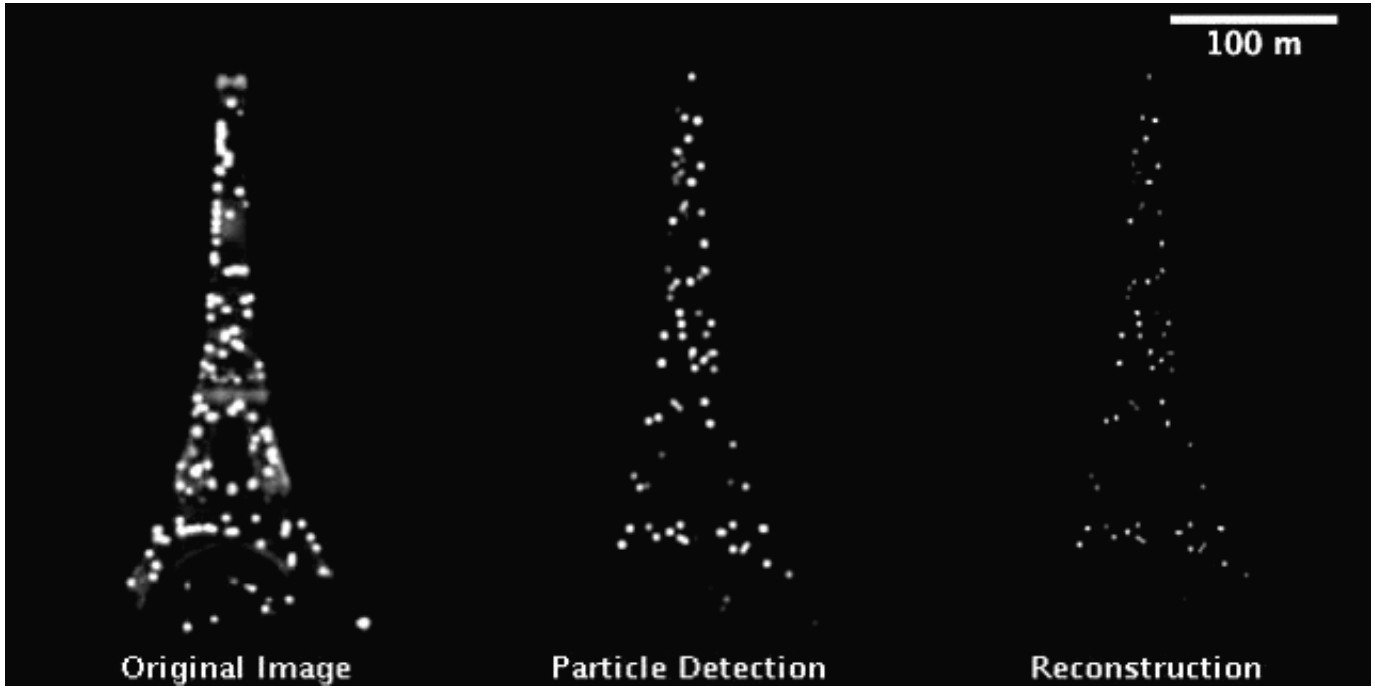
… and then finding their centres



Stochastic Optical Reconstruction Microscopy (STORM)/
Photoactivation Localization Microscopy (PALM)

Betzig et al., *Science (*2006)
Rust et al, *Nat. Methods* (2006)
Hess et al., *Biophys. J.* (2006)

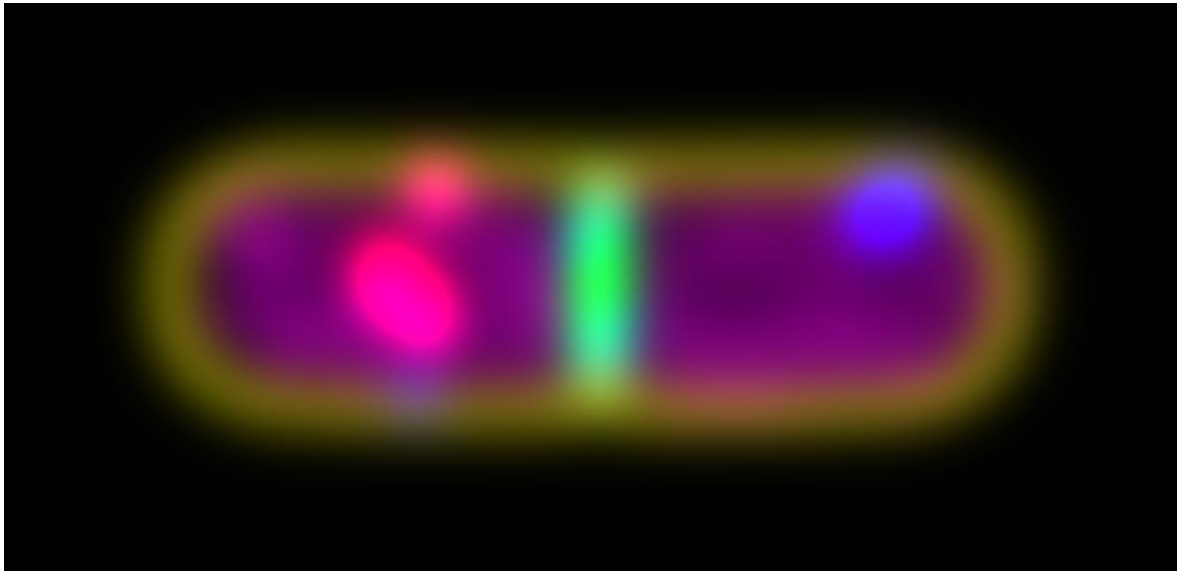
STORM over the Eiffel Tower



Henriques et al., *Nat. Methods* (2010)

Bacteria’s small size is a big problem

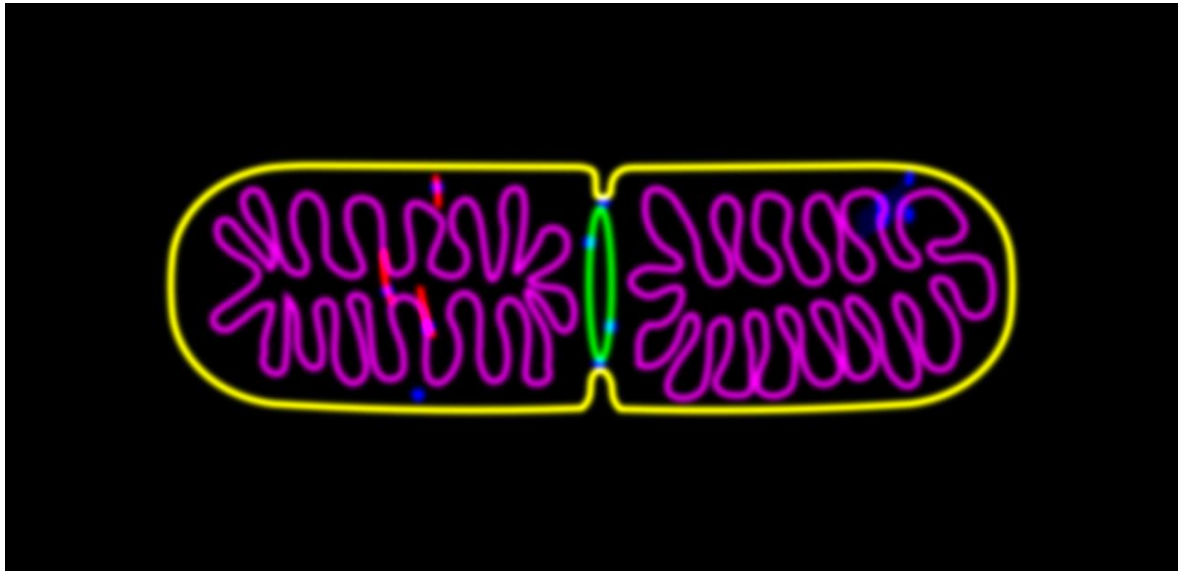
Spatial resolution
XY: 25 nm
Z: 100 nm



0.5 µm

Super-resolution microscopy allows *in vivo* imaging of bacterial ultrastructure

Spatial resolution
XY: 25 nm
Z: 100 nm



Time (typ.): 3 – 5 mins
Time (best): 2 s (FPs), 30 ms (dyes)

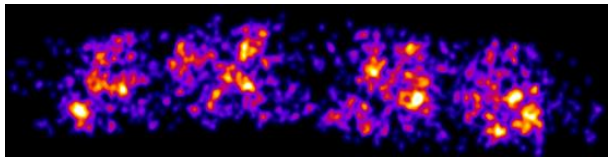
0.5 µm

Advantages:
- Highest resolution of SR microscopies
- Single molecule information

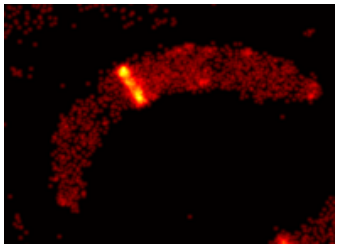
Disadvantages:
- high laser powers

→ phototoxicity
→ Best for fixed cells

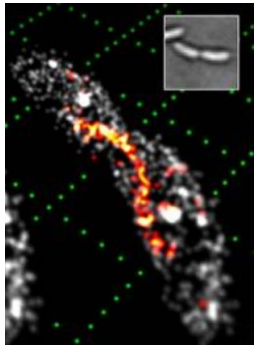
Localization microscopy: applications



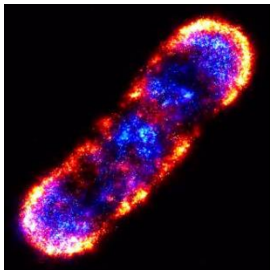
RNA polymerase



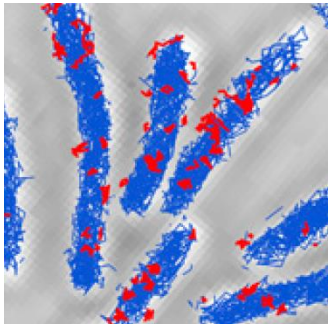
FtsZ



Crescentin



CheY

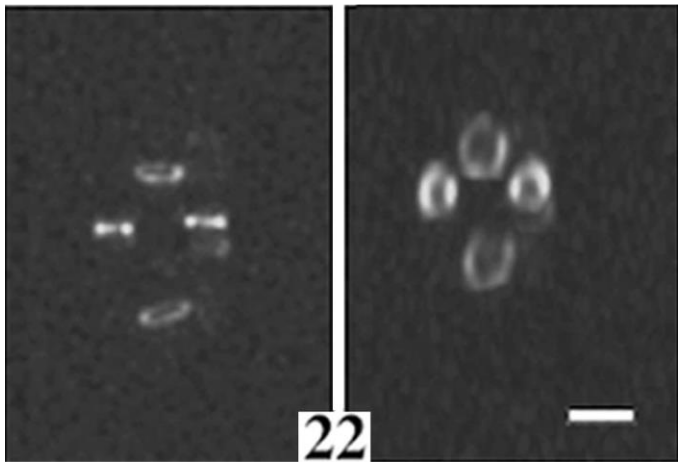


DNA polymerase

Endesfelder, Finan, Holden et al., *Biophys J.* (2013)
Holden et al, *PNAS* (2014)
Greenfield et al, *Plos Biol* (2009)
Lew et al, PNAS (2011)
Uphoff et al, *PNAS* (2013)

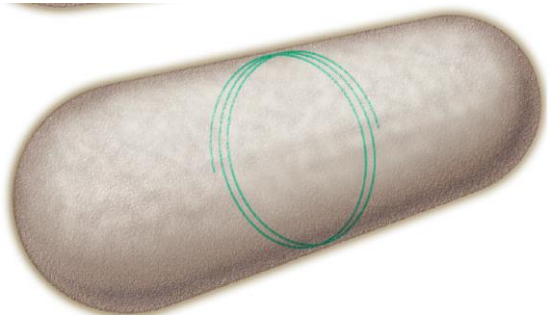
FtsZ ultrastructure

Diffraction limited imaging of the cell division cytoskeletal “Z-ring” look continuous:



Sun & Margolin J. Bac 1998

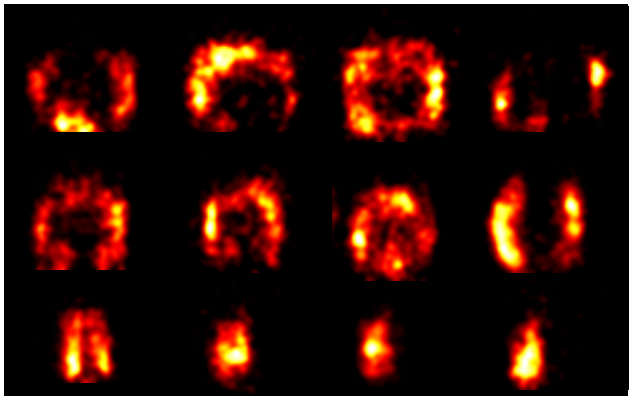
Consistent with the idea of a force generating constrictive ring:



Erickson et al. Mirco & Mol Bio Rev 2010

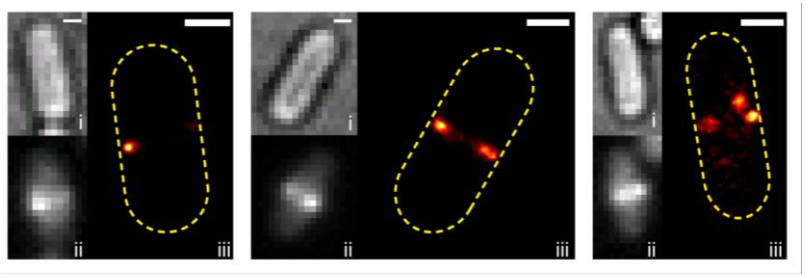
Super-resolution suggests a patchy Z-ring

C. crescentus 3D PALM



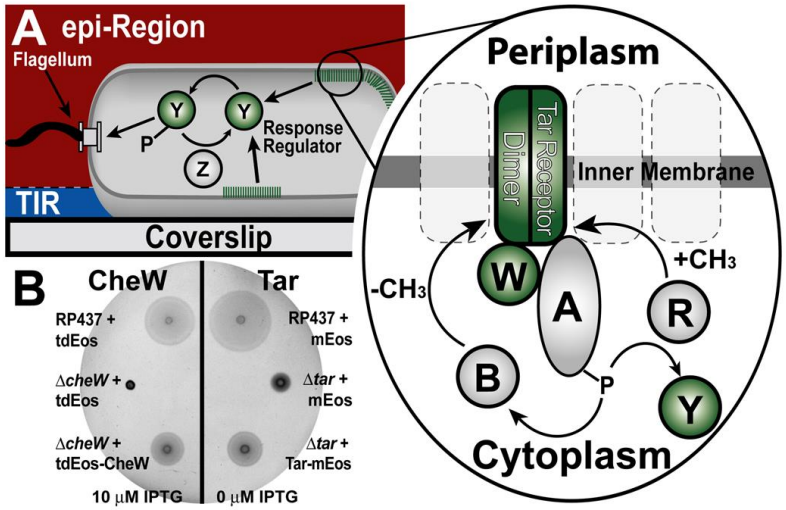
Holden et al PNAS 2014

E. coli 2D PALM



Buss et al PLoS Genetics 2015

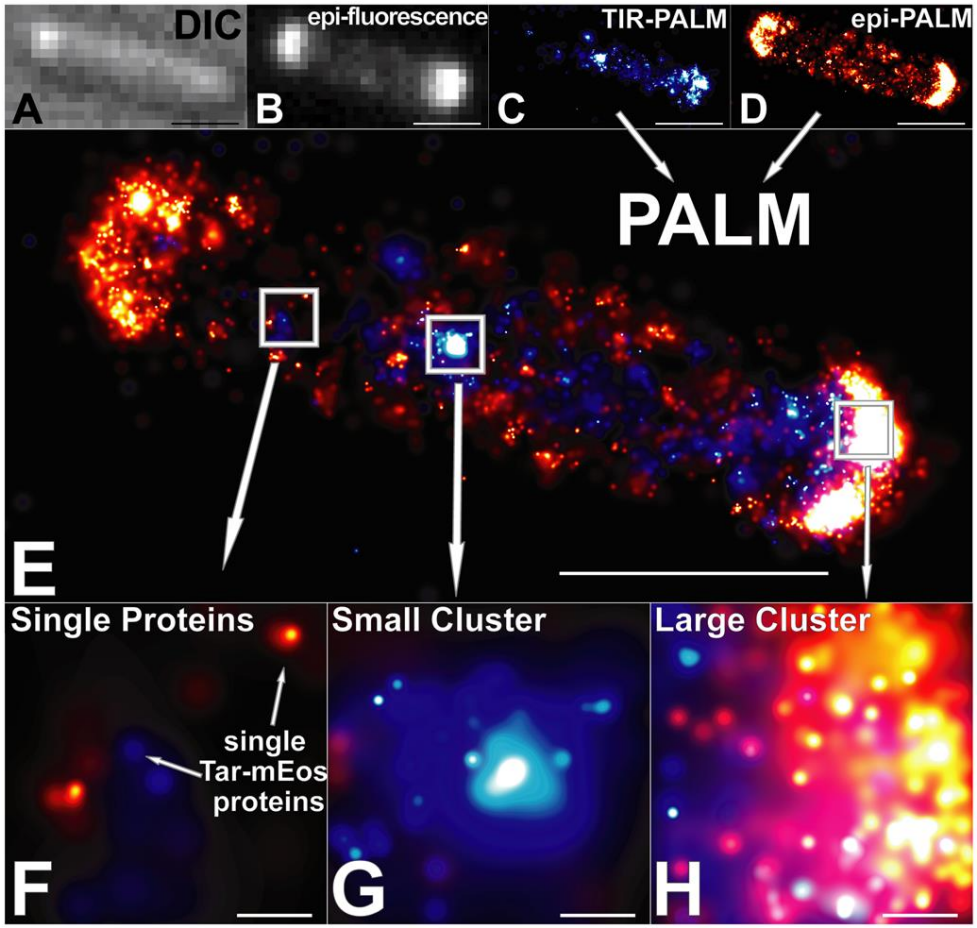
Chemotaxis sensors



Tar proteins senses chemicals outside of cell
Large clusters of Tar act cooperatively to amplify signals
How are clusters organised?

Greenfield et al PLoS Biol 2009

Chemotaxis sensors



Continuously varying distribution of cluster sizes

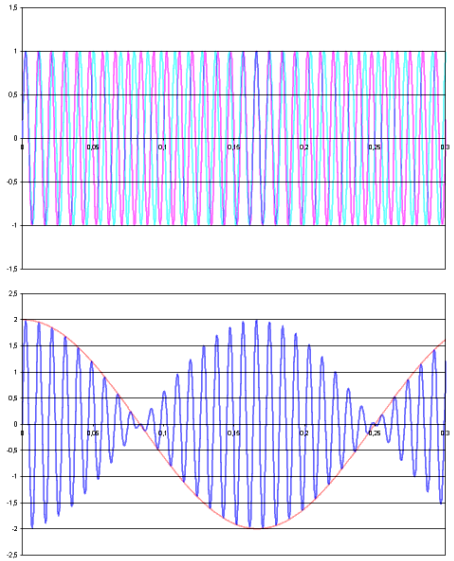
→ Suggests stochastic nucleation (ie no defined cluster size)

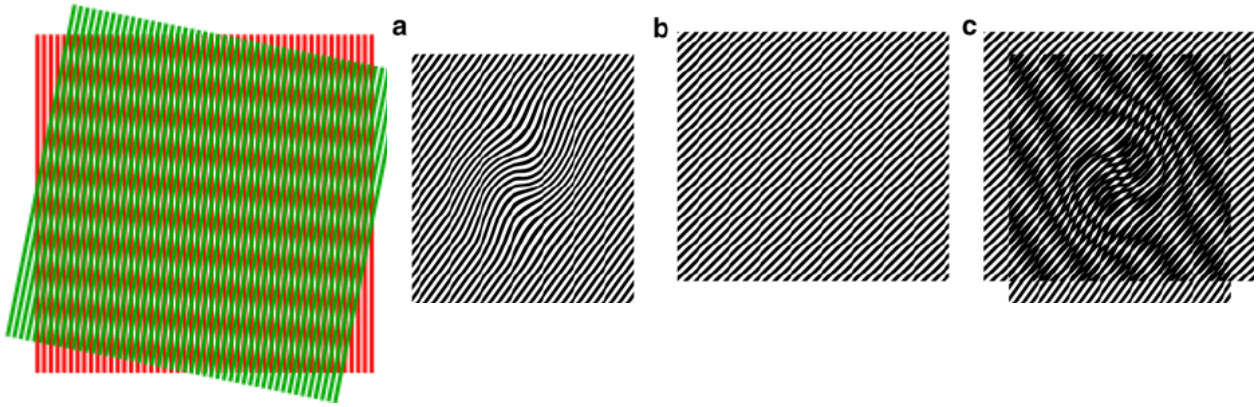
→ Potential explanation for spontaneous polar clusters

Greenfield et al PLoS Biol 2009

Structured illumination microscopy: principle

Moire fringes project high frequency information (invisible) to lower frequency





Example in practice:
http://zeiss-campus.magnet.fsu.edu/tutorials/superresolution/hrsim/indexflash.html

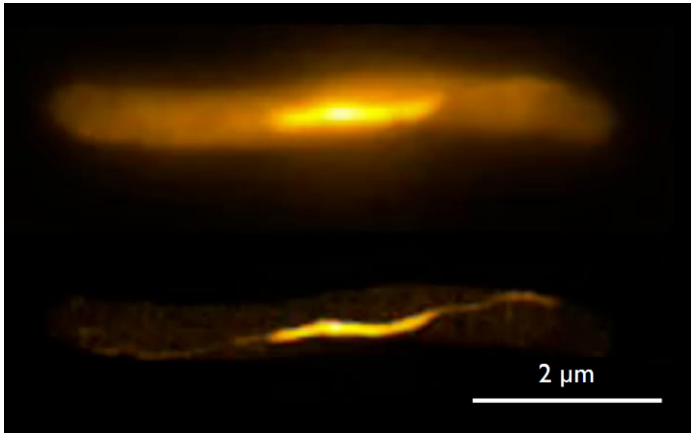
Related techniques: iSIM, Airyscanning

Moiré fringes Wikipedia
Rego, Shao, Methods Mol Biol 2015

Resolution

Spatial resolution
XY: 115 nm
Z: 350 nm

Time (typ.): 0.6- 1 s



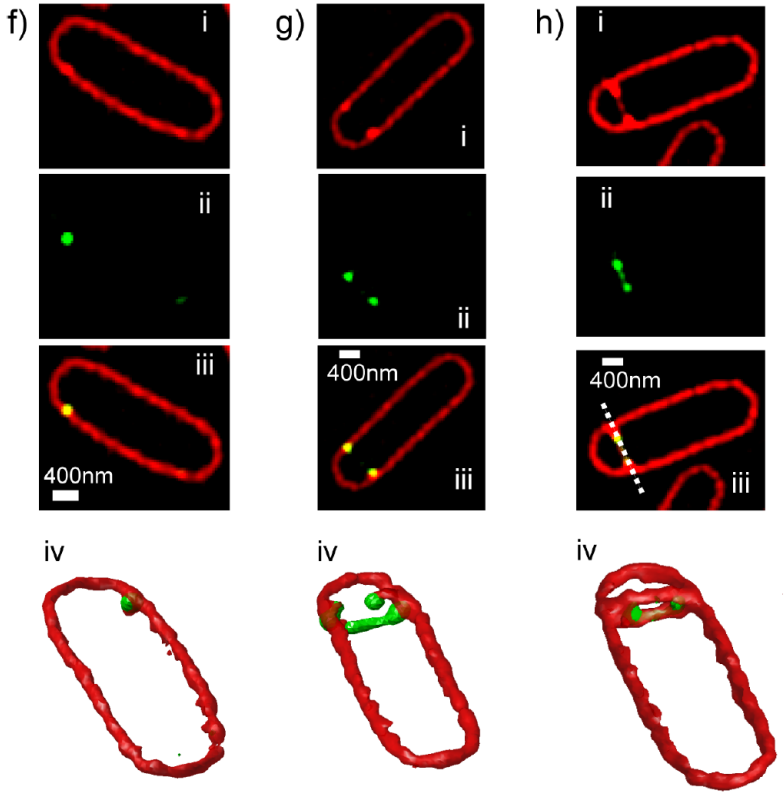
E. coli RecA

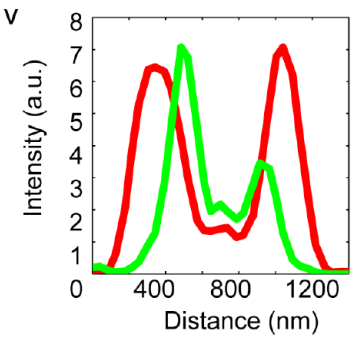
Lesterlin et al Nature 2014

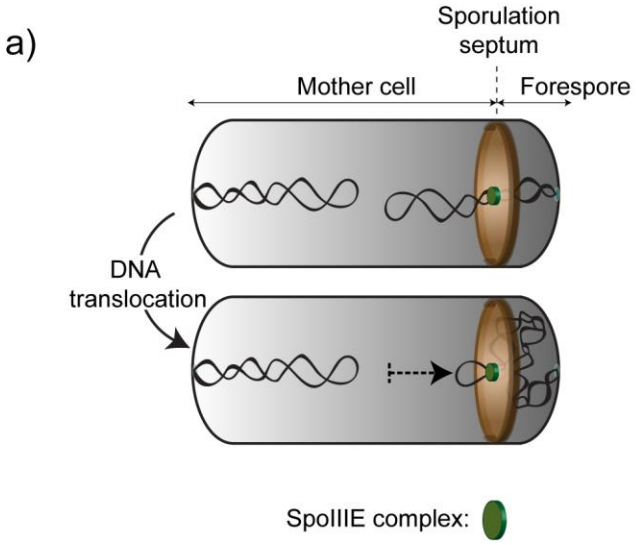
Advantages:
+ FAST!
+ Low-ish laser power
→ Low phototoxicity
→ Extended time lapse
+ Really good at multicolour

Disadvantages:
- "Only" doubles diffraction limited resolution

SpoIIIE DNA pump recruitment to *B. subtilis* septation sites



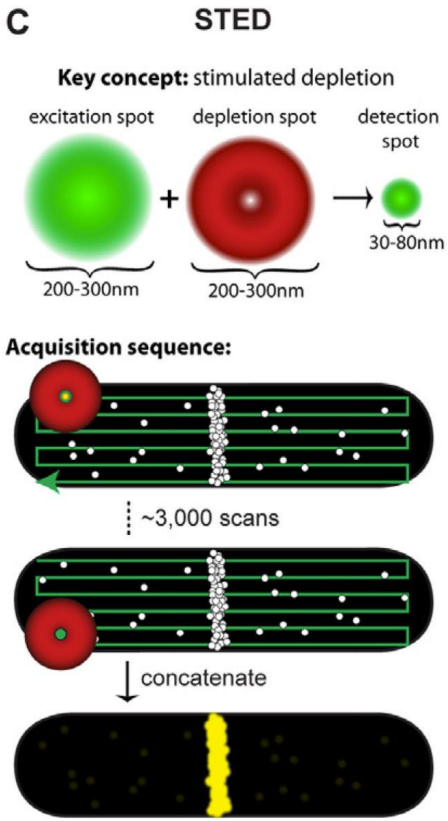




SpoIIIE is a translocase – pumps chromosome into forespore
Directly visualized localization to leading edge of closing septum

Fiche et al PLoS Biol 2013

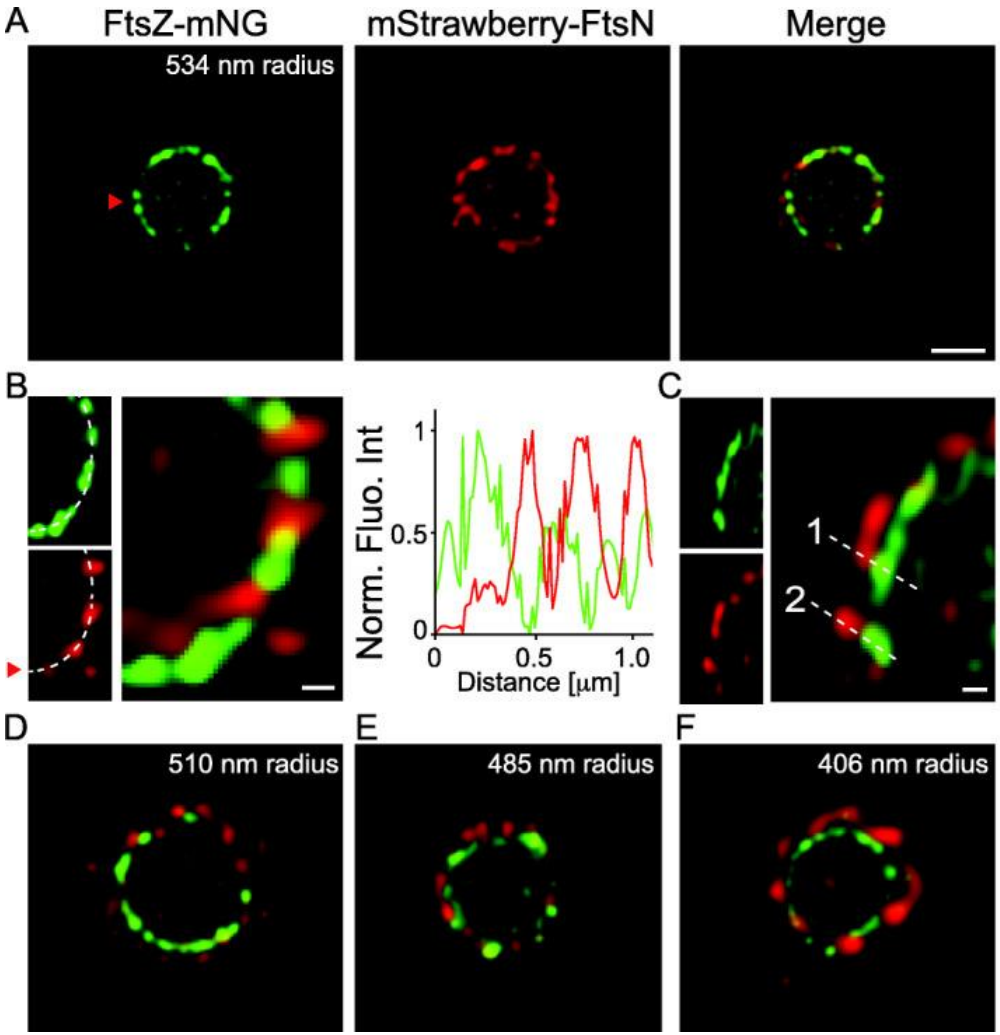
Stimulated Emission Depletion microscopy



Resolution: 50 nm. Time resolution: 1-2s

- Works for ordinary dyes & fluorescent proteins
- 2 colour is not too complex.
- Similar workflow to confocal -> reasonably straightforward (with help of a good technician)
- Bleaching is a big issue → only works for very bright samples

STED of cell division proteins



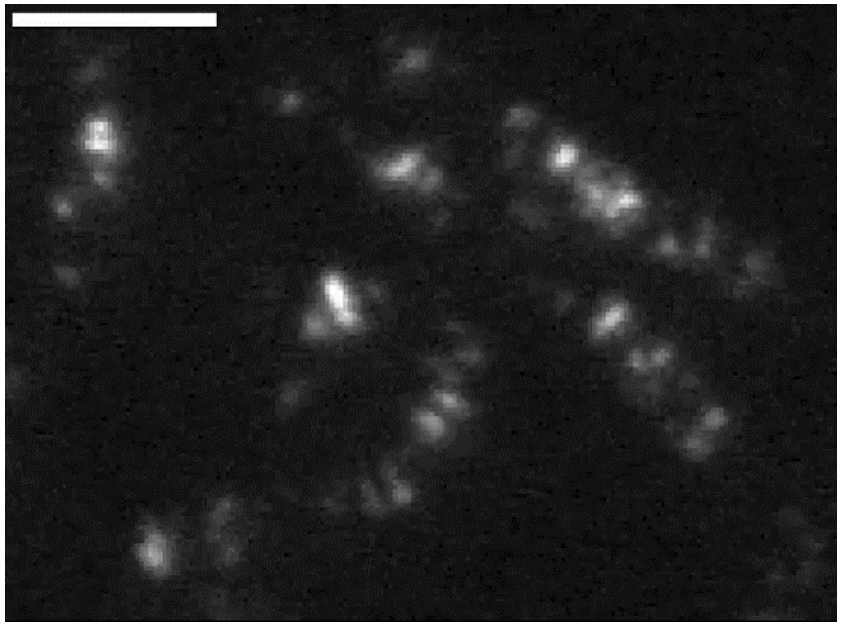
- STED shows that E. coli FtsZ and FtsN do not colocalize. (Rather interface at the membrane?)
- Patchiness: imaging artefact vs real is always a concern → best image by multiple techniques.

Soderstrom et al Mol Micro (2018)

Single molecule imaging

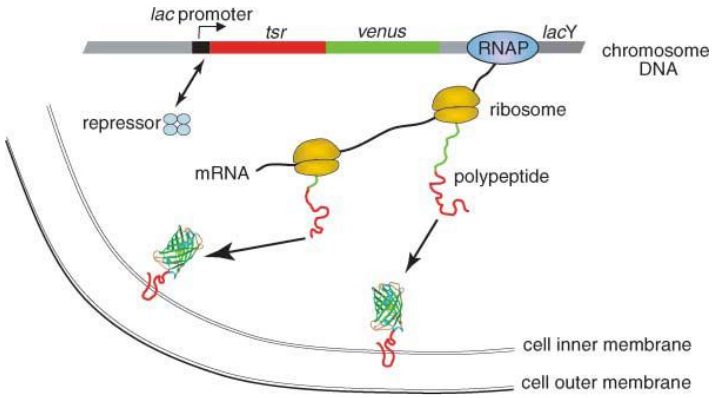
- Closely related to localization microscopy

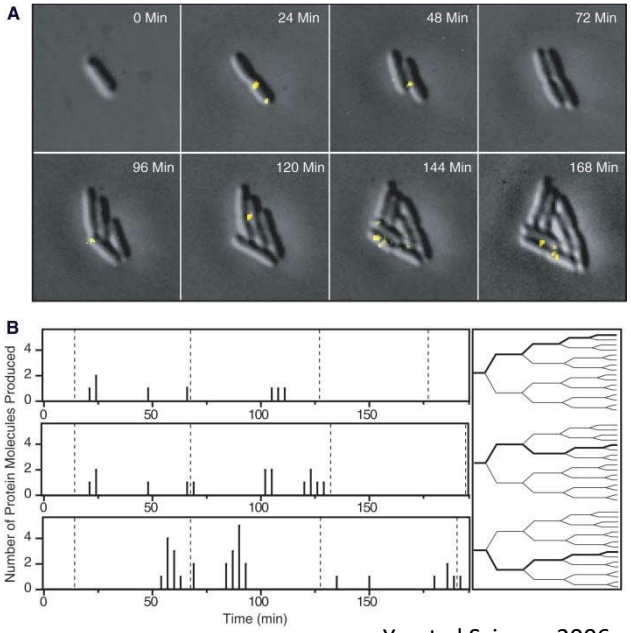
- Key techniques
  - Single molecule tracking
  - Molecule counting



Hussain et al eLife 2018

Single molecule gene expression

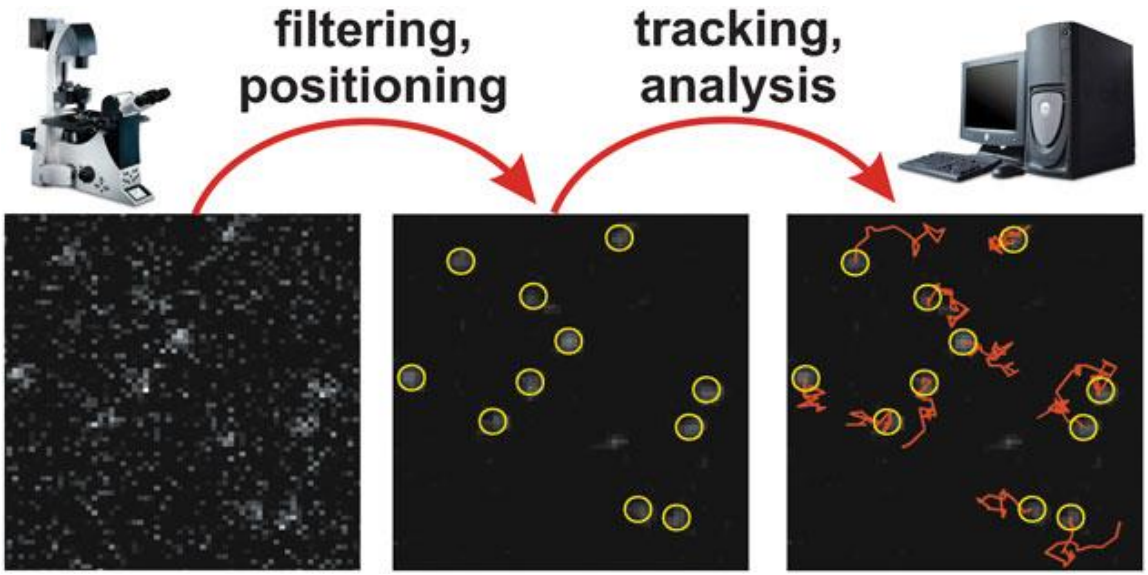




Yu et al Science 2006

One of the earliest really powerful applications of single molecule imaging
Proteins are expressed and observed in real time
Direct observation of "bursty" expression
– ie. multiple protein expressed rapidly after transcription of a single mRNA

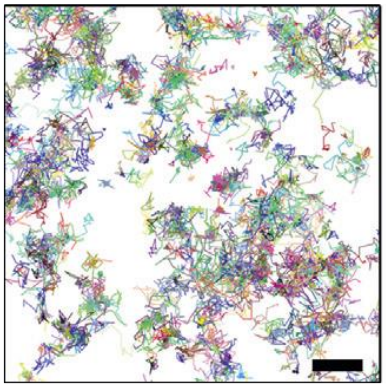
Single molecule tracking: principle



Woll et al Phy Chem Chem Phys 2013

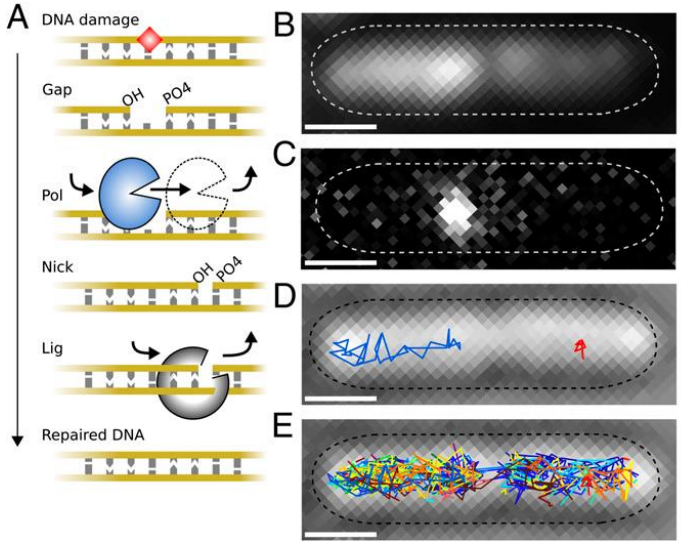
These days often combined with photoactivation to obtain 1000s of tracks
→ Single particle tracking PALM (sptPALM) – extremely powerful

Can study the binding/ diffusion of **all** the copies of a labelled protein in a cell



Manley et al Nat Methods 2008

Single molecule tracking of E. coli DNA polymerase I



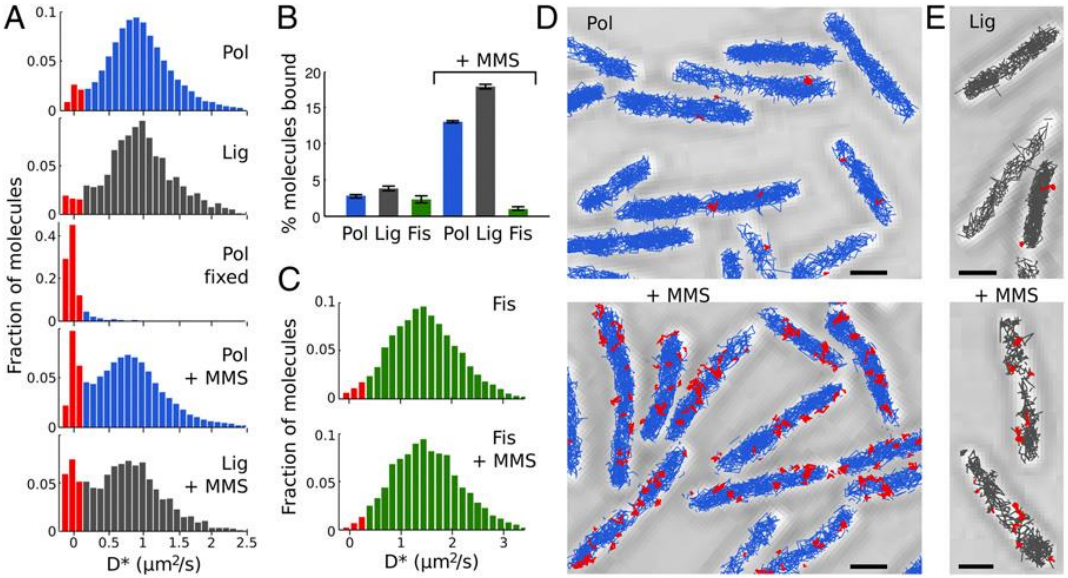
DNAP I is a repair polymerase

Track its motion:

- Fast diffusion – DNA unbound
- Slow diffusion – DNA bound

Uphoff et al PNAS 2013

Single molecule tracking of E. coli DNA polymerase I

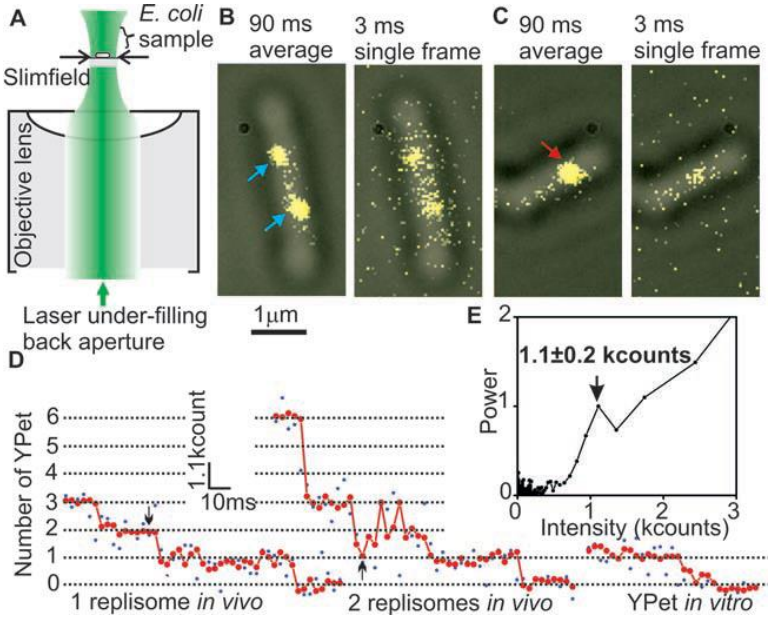


Direct observation of DNAPs actively repairing DNA gaps & nicks

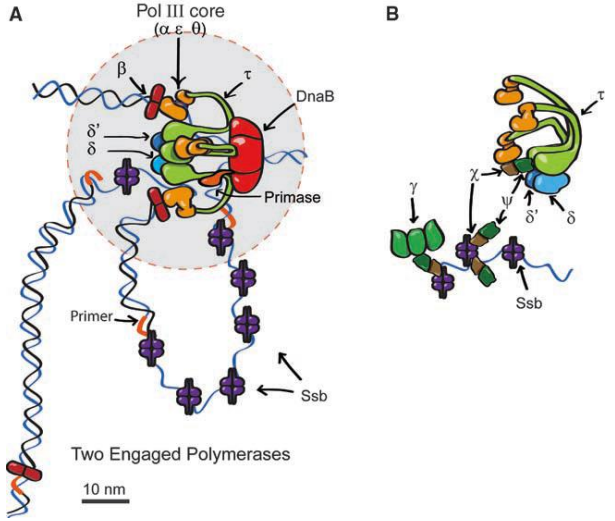
- Repair times
- Search times

Uphoff et al PNAS 2013

Single molecule counting by photobleaching



Watch foci bleach step-by-step
→ Tells you how many proteins are in the focus



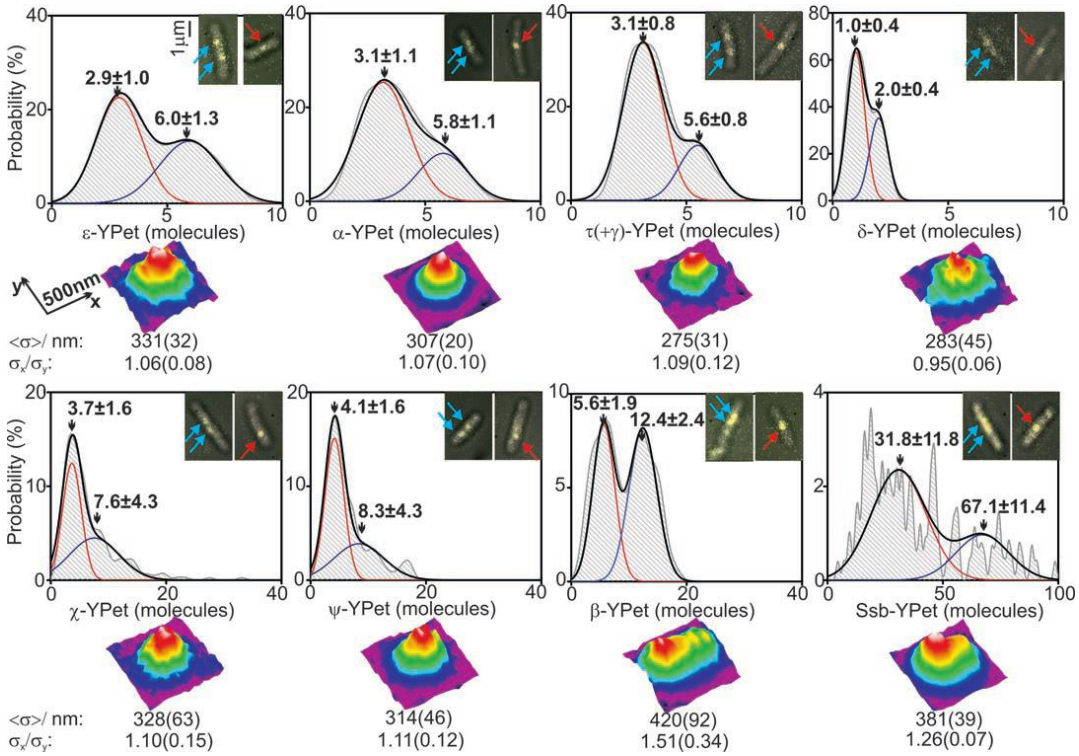
Reyes-Lamothe et al, Science 2010

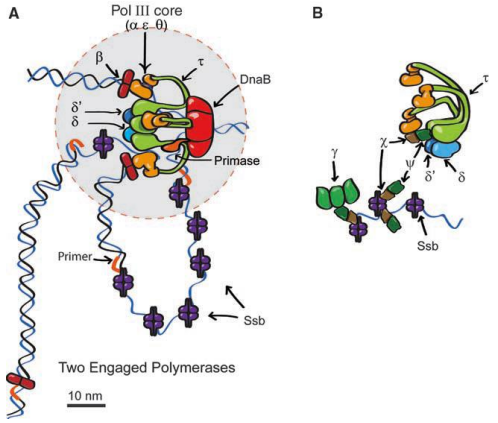
Single molecule counting by photobleaching

Very cool paper

By measuring numbers of all the key replisome proteins, determined in vivo stoichiometry of replisome

They found an extra polymerase!

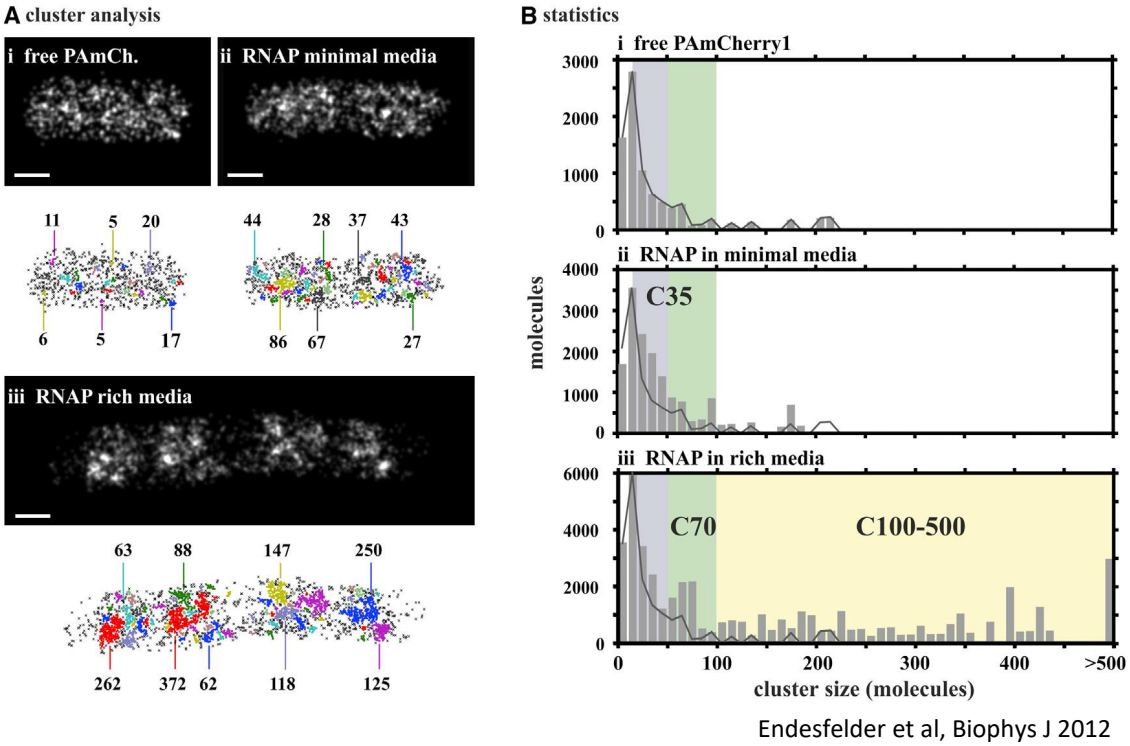




Reyes-Lamothe et al, Science 2010

Single molecule counting by localization microscopy

Since you localize the molecules one-by-one, why not count them?



Endesfelder et al, Biophys J 2012

Potentially very powerful for large complexes where photobleaching would not work
BUT - determining absolute numbers (rather than relative stoichiometry) is an ongoing challenge - mainly due to difficulty establishing 'dark' fraction of FPs
Need good "counting standards"

Quantitative image analysis for microbiology

Images are not just pretty pictures!

Image analysis lets us analyse cell shape and protein localization in space and time

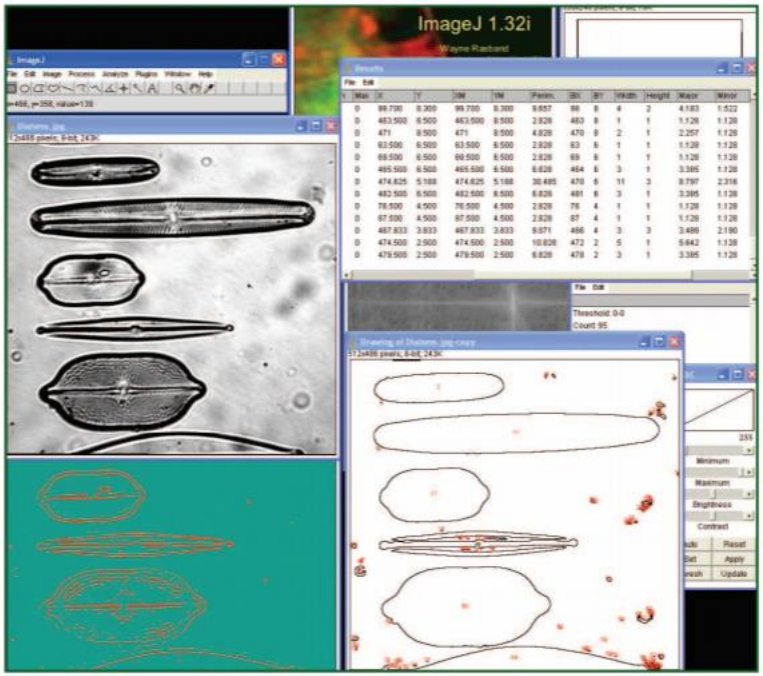
Extensive user-friendly tools allow us to quantify:

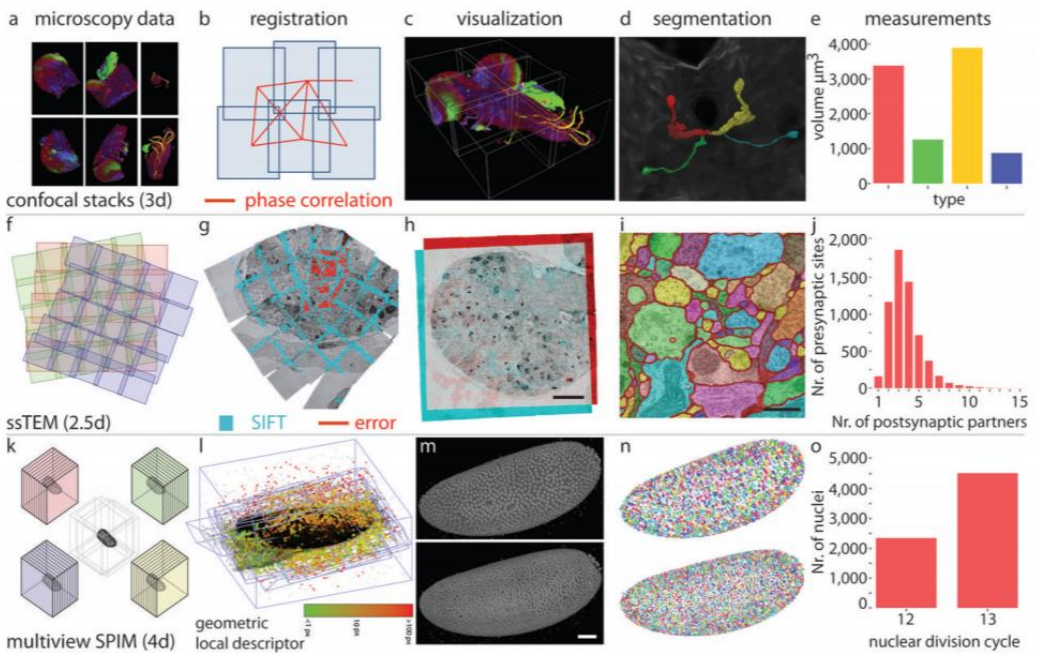
- Intensity
- Cell number
- Cell morphology
- Subcellular localization of proteins
- 3D rendering
- And more…

If you use a microscope *at all* in your research, this is probably useful to you.

FIJI/ ImageJ

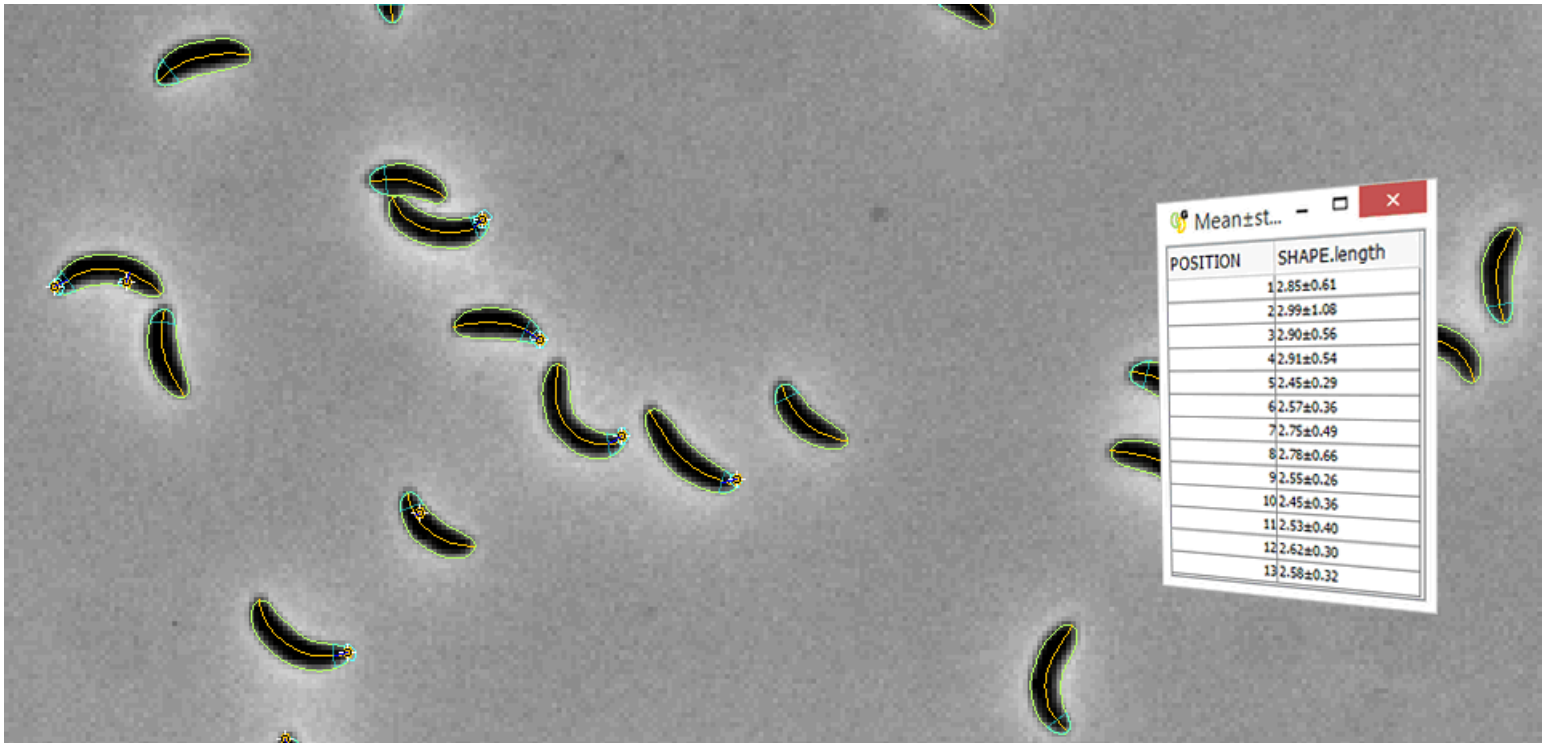
- The standard image processing tool in biology: https://imagej.net/Fiji
- Easy to use
- Immensely powerful due to enormous array of plugin for almost any image processing task
- Open source = open reproducible science





Adamoff et al Biophotonics 2004
Schindelin et al Nature Methods 2012

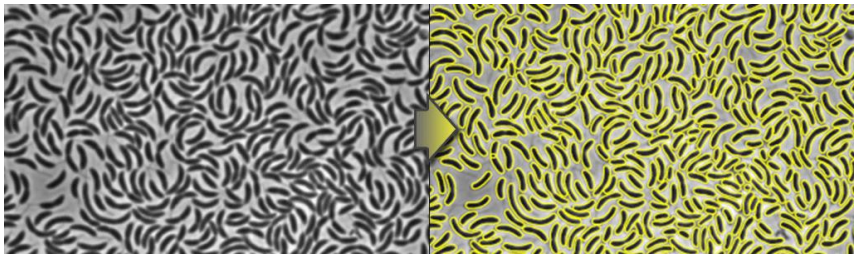
Quantitative image analysis for microbiology: examples



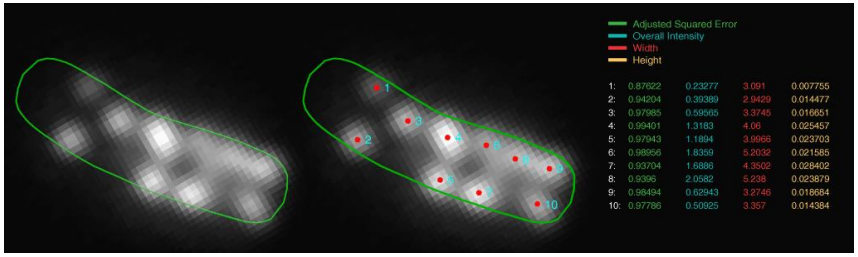
http://www.microbej.com

Quantitative image analysis for microbiology: examples

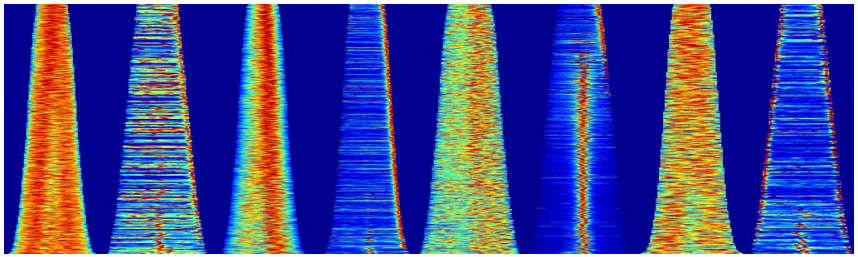
Cell shape



Protein localization



Cell cycle dependent protein localization (kymographs)



Fiji

Microbe J by the Brun Lab

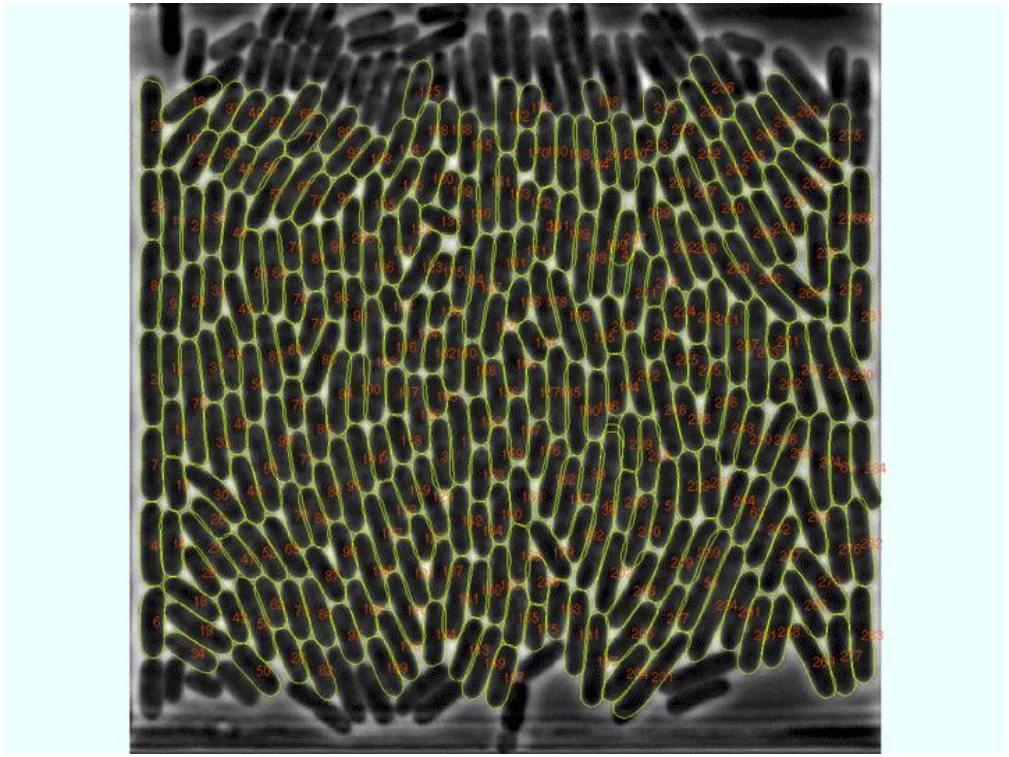
Oufti

The recent BactMAP paper has a nice summary of the microbiology packages :
van Raaphorst et al, Mol Micro, 2019

https://fiji.sc/
https://www.biodip.de
http://www.microbej.com
http://oufti.org/

Quantitative image analysis for microbiology: examples

Single cell growth rates



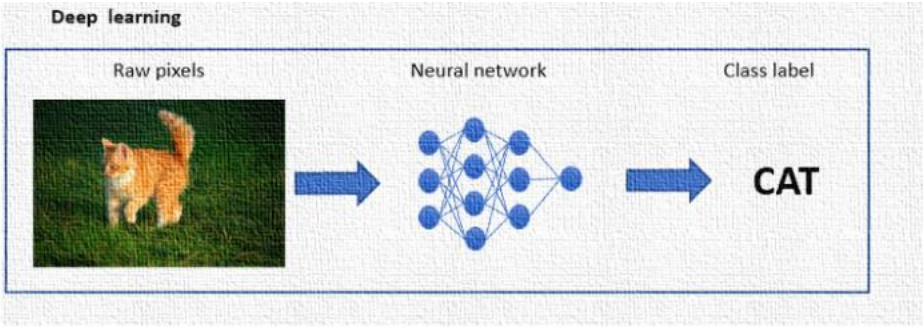
Fiji

Microbe J by the Brun Lab

Oufti

https://fiji.sc/
https://www.biodip.de
http://www.microbej.com
http://oufti.org/

A note about deep learning



1. Pre-train a computational neural network
2. Use that network as a classifier or image filter
3. Profit

https://towardsdatascience.com/convolutional-neural-network-a-step-by-step-guide-a8b4c88d6943

Deep convolutional neural nets have tremendous potential…

https://www.youtube.com/watch?v=_OqMkZNHWPo

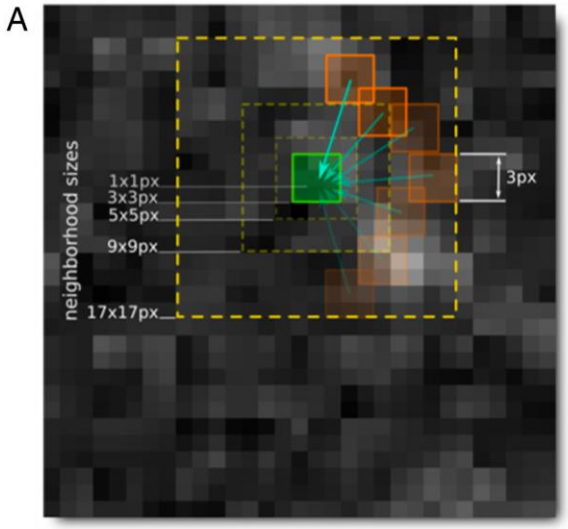
Also in the life sciences

Deep learning based denoising:
Dramatically improve image quality of noisy data with DL
→ More images with less light
→ Higher time resolution
→ Happier cells
→ Just beginning to be adopted but potentially game changing

Weigert et al 2018

“Conventional” denoising is already pretty awesome

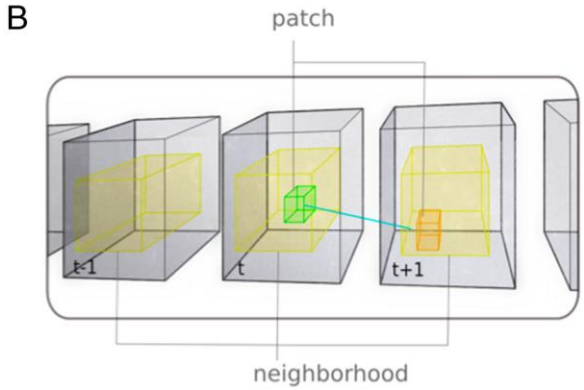
- Finds correlated patches in space & time
- Only assumption is Poisson + Gaussian noise
- No assumptions on sample structure
- Preserves edges and intensities



- Enhances SNR
- Reduces light dose
- “for free”

- Straightforwardly available for ImageJ

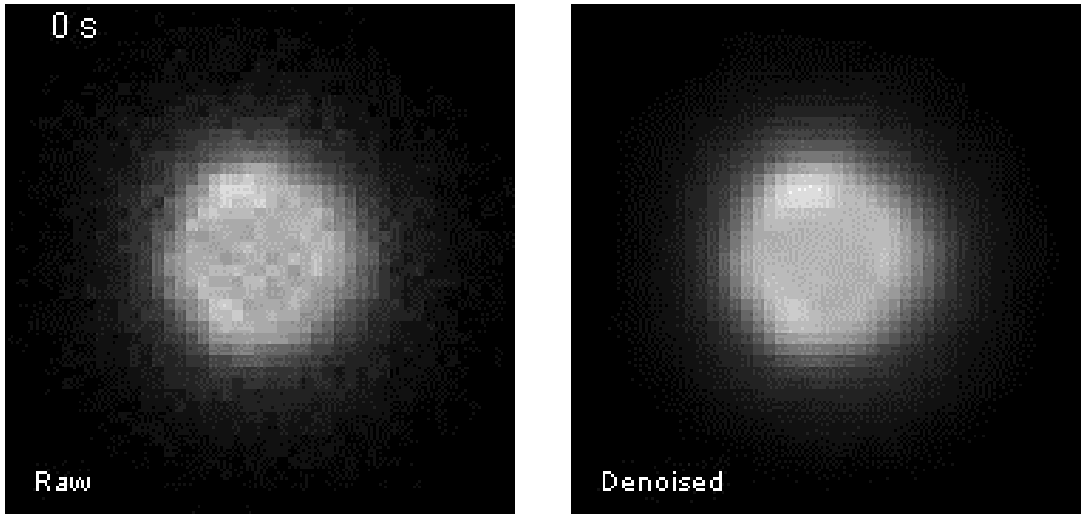
http://bigwww.epfl.ch/algorithms/denoise/



In my lab, we use this on almost every single image

Carlton et al PNAS 2010
Luisier et al, Signal Processing, 2010

Image denoising allows extended FtsZ-ring imaging at very low light levels



Whitley et al bioRxiv 2020

LIVE DEMO OF IMAGEJ & MICROBEJ

Resources:
FIJI - the standard image processing tool in biology:
https://imagej.net/Fiji
MicrobeJ
https://www.microbej.com/
MicrobeJ YouTube channel:
https://www.youtube.com/channel/UC_CxvjXezYXE9xgRIP7cK5Q

Test data Caulobacter.tif on Canvas

Lots of great online courses, especially for FIJI

Summary

- Advanced microscopy and image analysis are immensely powerful tools for microbiology

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