Image analysis and data processing in superresolution microscopy 2021

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The fundamental limits in light microscopy

- Resolution
  - ability to resolve objects in close proximity
  - ability to observe fine details
  - governed by diffraction (photon is a wave) and labeling density

- Signal to noise ratio
  - ability to observe small/faint features
  - strongly depends on contrast (e.g. fluorescence)
  - governed by photon statistics (photon is a particle)

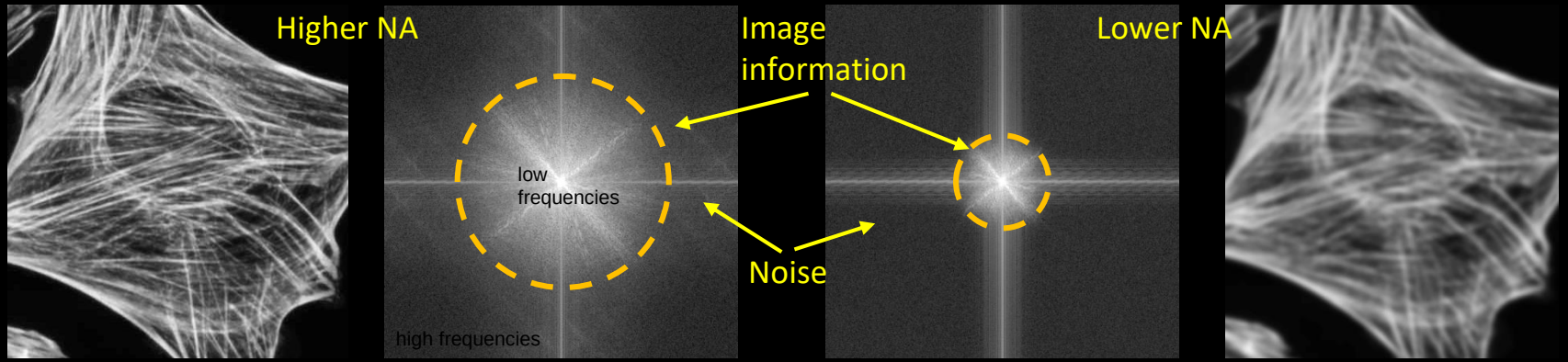
"If it isn't diffraction, it's statistics!"
James B. Pawley

Photon statistics

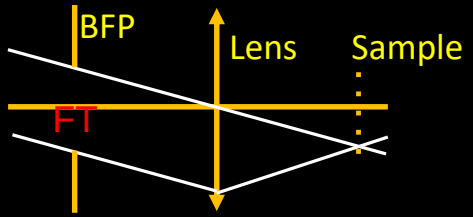
- Photons are not coming in regular intervals, but absolutely randomly!
- Poisson statistics ->
  when *N* photons are collected, the expected error is $\sqrt{N}$

Resolution in light microscopy is

- Can be understood in ”real” space or in “spatial frequency” domain using FT (Fourier transformation) (best in both :-)



- given, limited by numerical aperture
- cannot be arbitrarily large
- *NA < n (refractive index)*

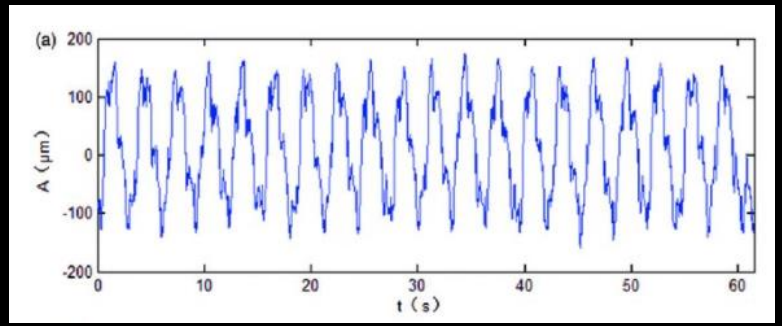


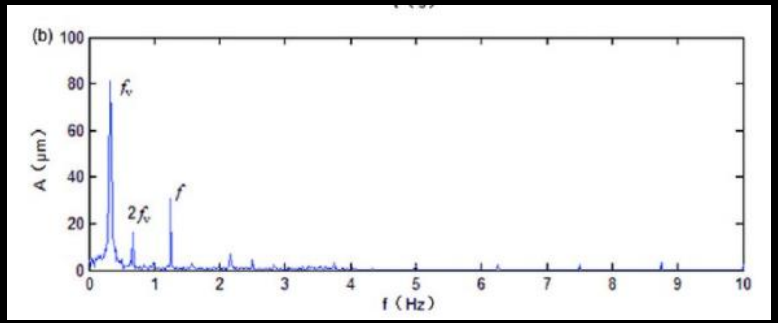
A word about Fourier Transform

$$\mathcal{F}\{g(t)\} = G(f) = \int_{-\infty}^{\infty} g(t)e^{-i2\pi ft}\,dt$$

$$\mathcal{F}^{-1}\{G(f)\} = g(t) = \int_{-\infty}^{\infty} G(f)e^{i2\pi ft}\,df$$

- Linear, complex integral transform
- $e^{ix} = \cos x + i \sin x$
- Link between intensities (e.g. acoustic pressure) and frequency content (spectrum)





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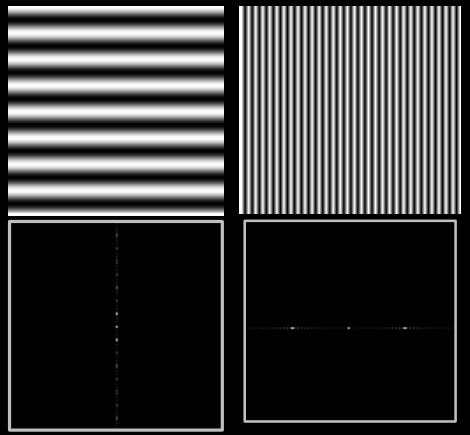
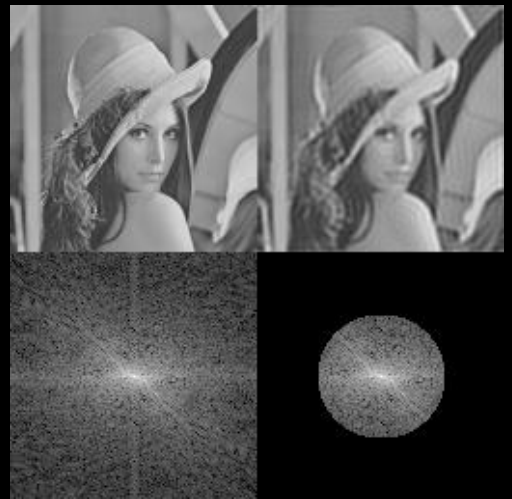
- Works in multiple dimensions, too

$$\mathcal{F}\{g(x,y)\} = G(k_x,k_y) = \iint g(x,y)e^{-i2\pi(xk_x+yk_y)}\,dx\,dy$$



vlabs.ac.in



Resolution in light microscopy

- Can be understood in ”real” space or in “spatial frequency” domain (best in both :-)

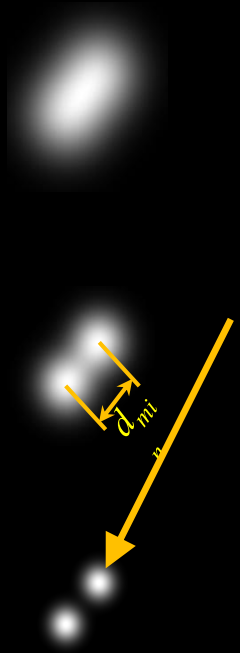
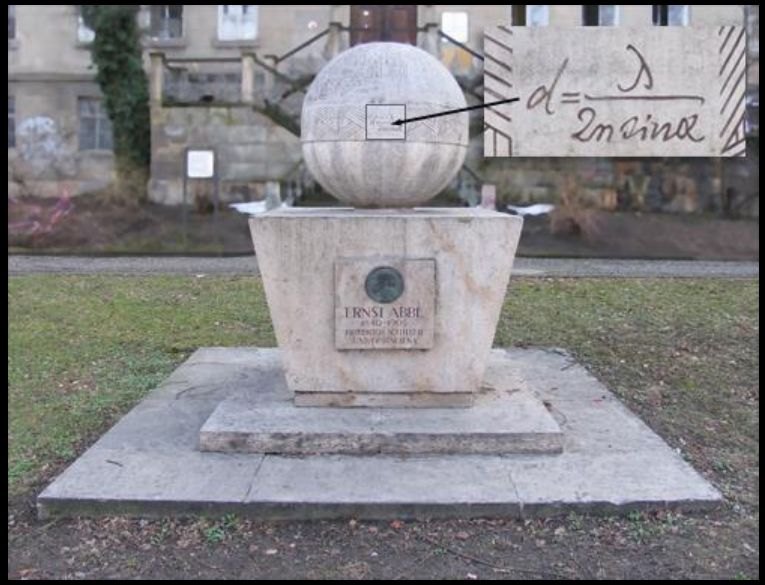


Image formation:
replace each point of the object
with PSF (point spread function)
of the microscope
(typically an Airy pattern)

Ernst Abbe (1873):

$$d_{min} = \frac{\lambda}{2NA}$$



Superresolution light microscopy - methods

Various attempts to overcome the Abbe diffraction limit

- Linear methods – “structured” illumination (Intensity (I) of emission is linear to I of excitation)
  - Confocal -> OPRA/ISM/ReScan -> Airyscan
  - InstantSIM, MSIM, SoRa
  - SIM, Lattice SIM
  - max. increase of resolution 2x
- Nonlinear methods (unlimited increase of resolution)
  - STED, SSIM
  - higher resolution information can be extracted (more phases needed)
- Stochastic methods (based on random blinking of molecules)
  - SMLM (PALM, STORM, …)
  - BALM (DNA-PAINT, IRIS)
  - SOFI, SRRF

Superresolution light microscopy - history

STED

Einstein 1917
stimulated emission

LASER 1960

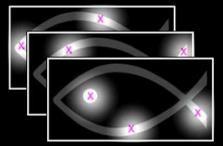
Hell 1994 (concept)

Hell 2000

Leica 2007

Abberior ~2013

SMLM



Moerner 1989 (@ 4 K)

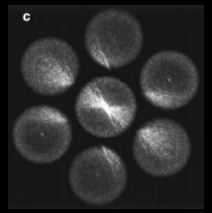
PALM Betzig 2006

STORM Zhuang 2006

Nikon, Zeiss, Vurata, … 2010

SIM

theory ???? related to confocal, axially modulated excitation



Cremer 1998

Gustaffson 2000 (†2011)

Nikon, Zeiss, GE… 2010

confocal / Airyscan

Minsky 1955

Petran 1966

Sheppard 1988 (concept)

Bertero 1987 – 1992

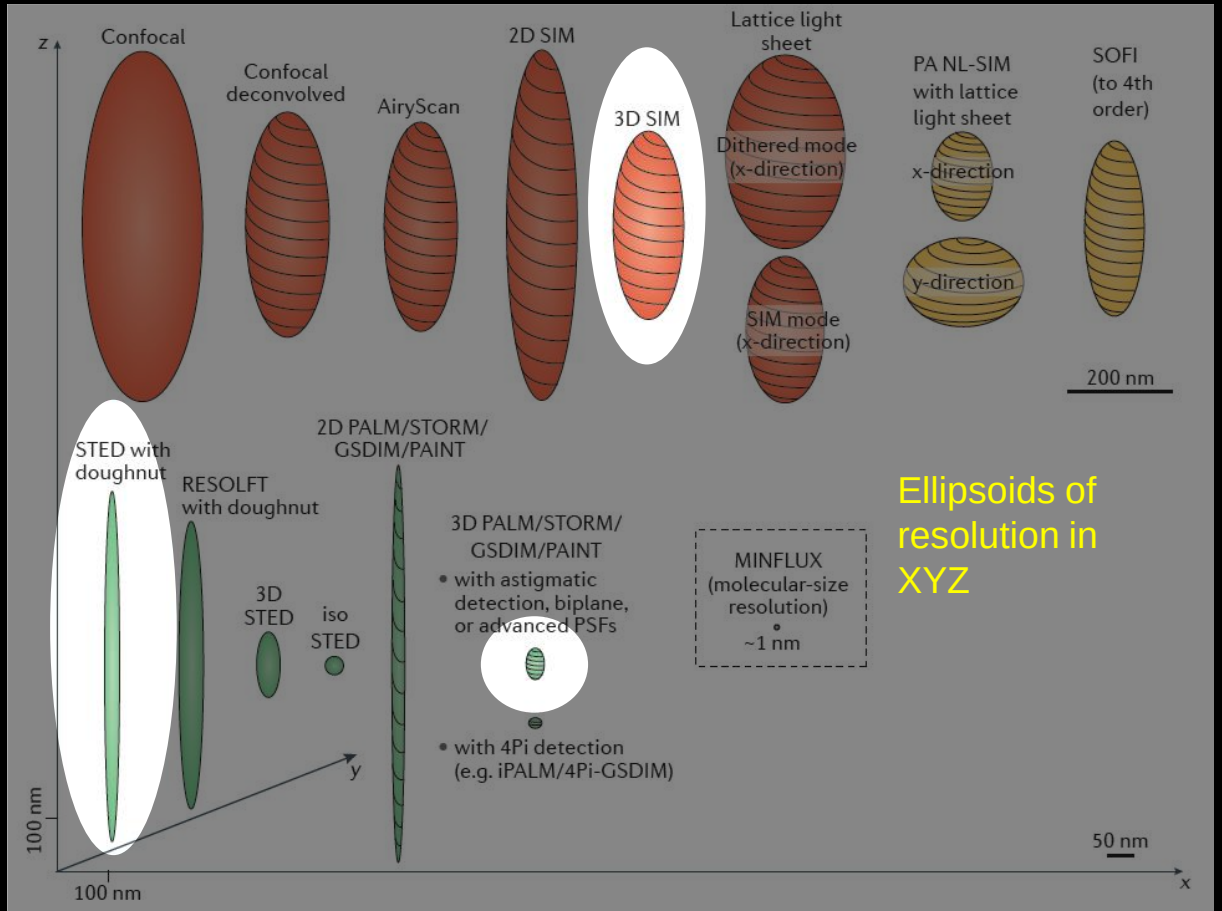
Enderlein 2010

Sheppard 2013

Zeiss Airyscan 2015

SoRa 2018

Superresolution light microscopy – comparison of resolution



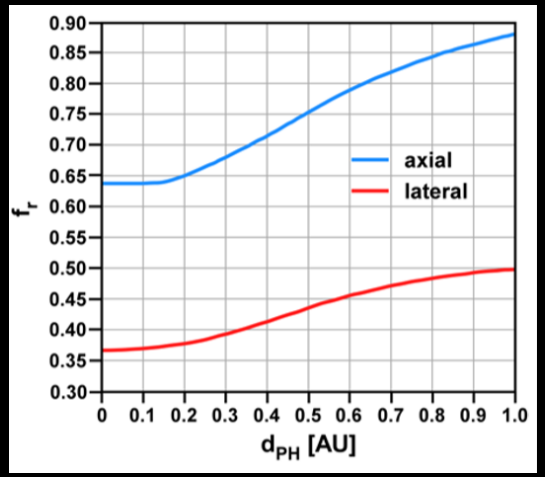
Nobel prize 2014:
Eric Betzig,
Stefan W. Hell,
William E. Moerner

from Steffen, Stefan and Stefan, *Nat Rev. Mol. Cel. Biol.* **18** (2017) 685

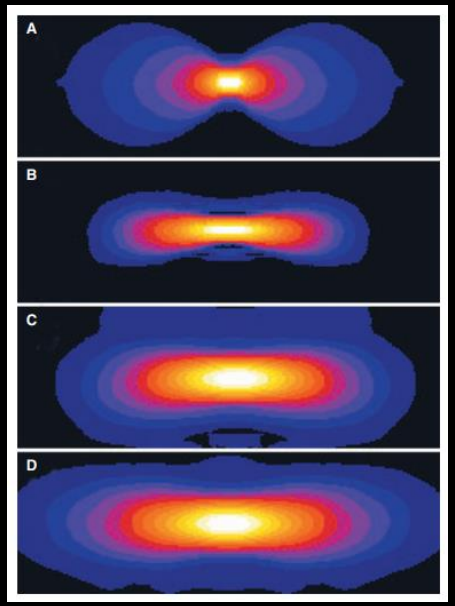
Confocal – is it superresolution technique?

OTF (Optical Transfer Function) = Fourier Transform of the PSF (Point Spread Function)

- Theoretically … yes!



Axial and lateral resolution of a confocal microscope improves with smaller pinhole (below 1 AU)...
… but the signal decreases quickly!



Widefield

Confocal, 4.3 AU

Confocal, 2.5 AU

Confocal, 0.7 AU

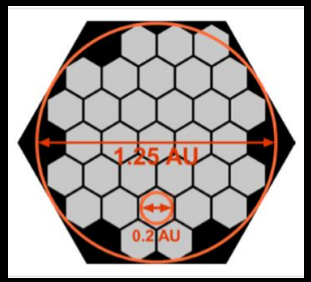
Peter Shaw, Pawley's Handbook, chap. 23

Ideal microscope: resolution of a small pinhole & light collection of a large pinhole!

Confocal – is it superresolution technique?

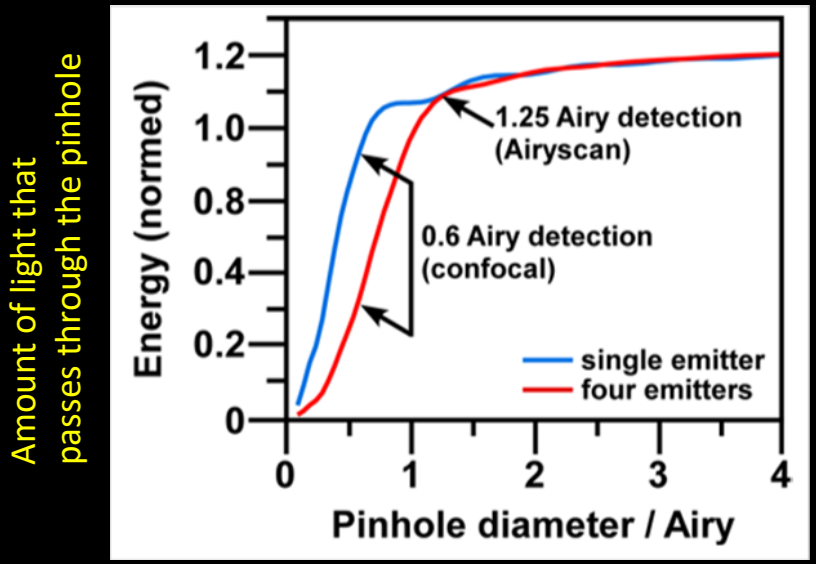
- Practically… not so much!

… solution: Airyscan …



32 channel area detector collects a pinhole-plane image at every scan position. Each detector element functions as (0,2 AU) pinhole.

The 32 detectors combine to a total pinhole size of 1.25 AU



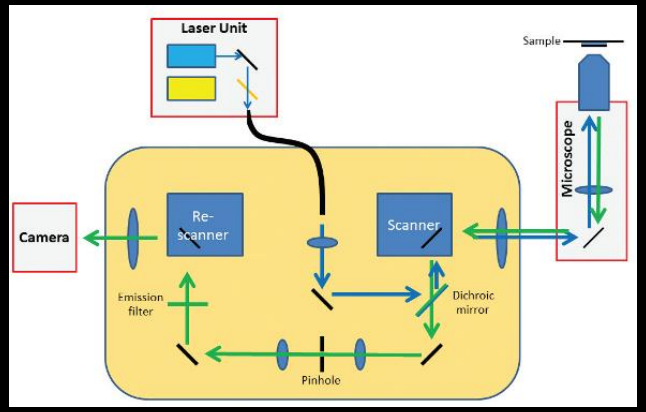
ZEISS application note

Airyscan: resolution of a small pinhole (0.2 AU) & light collection of a large pinhole (1.25 AU)!

“Photon Reassignment” methods

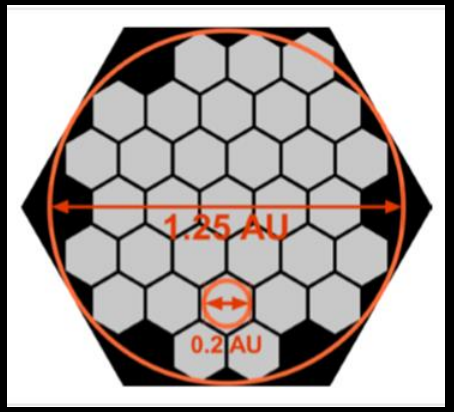
Linear superresolution methods based on the same “Photon Reassignment” principle:

- ReScan confocal



- Cheap and easy to operate
- Basic 4-color confcal
- Relatively slow (0.25 fps)
- point scanning confocal with second set of XY galvos that re-scans the signal onto CMOS camera

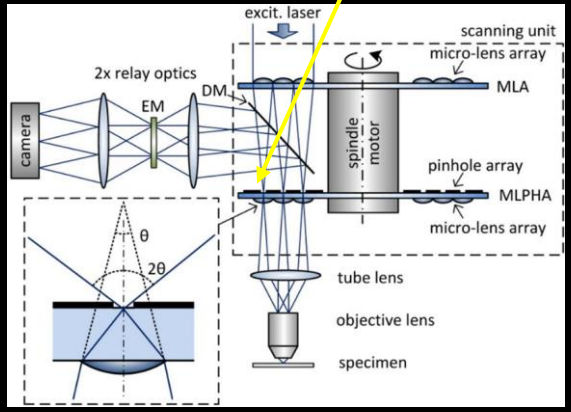
- AiryScan



- Easy to operate
- Flexible confocal microscope
- Advanced image processing
- based on computer processing

- SoRa

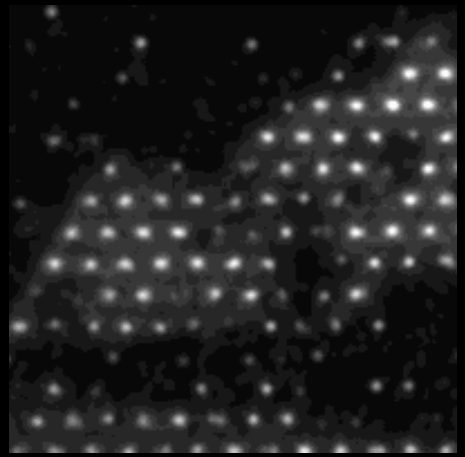
Spinning disk with extra microlenses



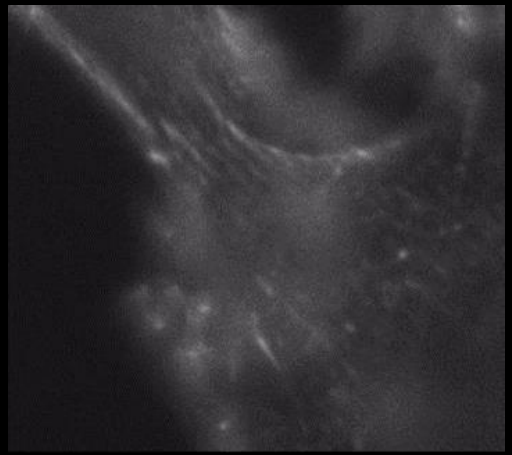
- Cheap and easy to operate
- Fast (it’s spinning disk)
- Less superres
- spinning disk with extra microlenses

SIM

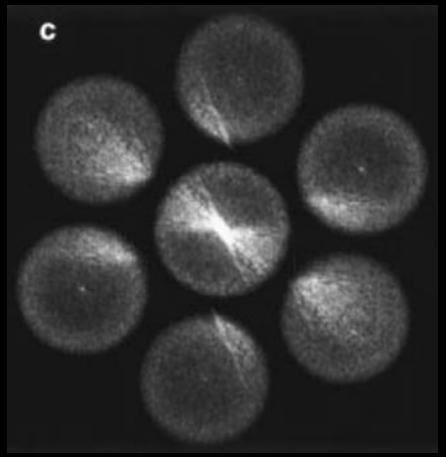
- Resolution improvement dictated by the spatial frequency of the illumination features
- SIM projects line patterns with different angles and phases (or thousands of dots) and processes the camera images by FT



"Lattice" style
-honeycomb structure projected across the sample

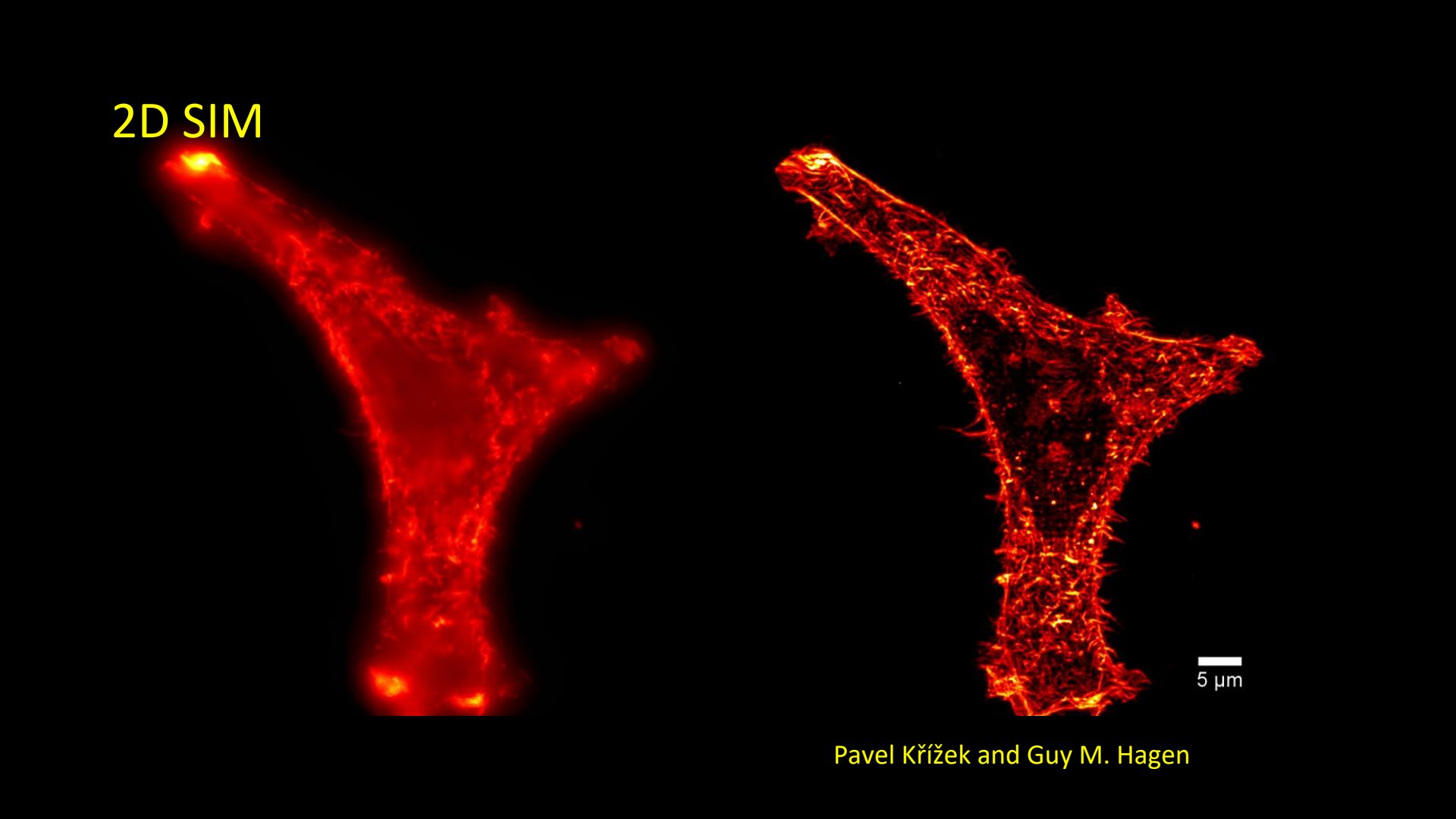


"Traditional" style
- lines projected across the sample



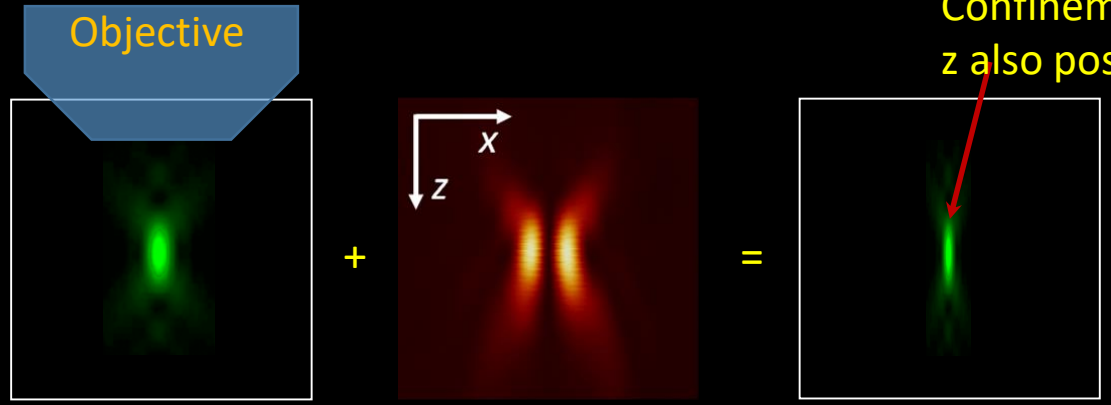
Fourier domain processing

2D SIM



Pavel Křížek and Guy M. Hagen

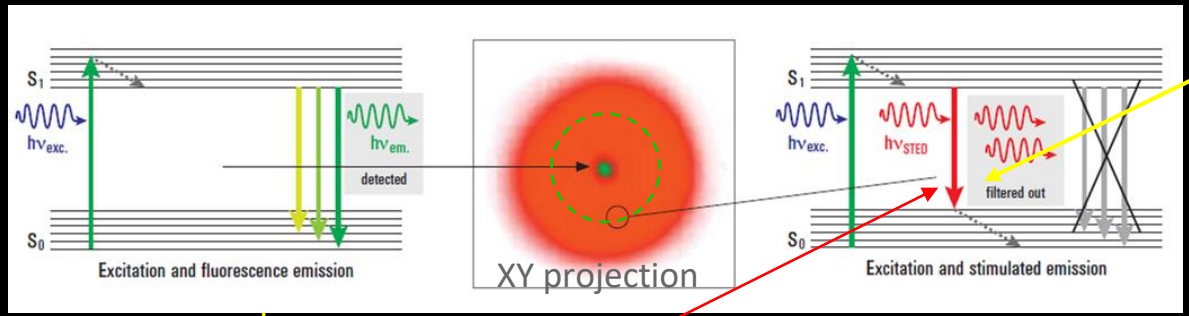
STED – CLSM with extra depletion beam



confocal excitation PSF

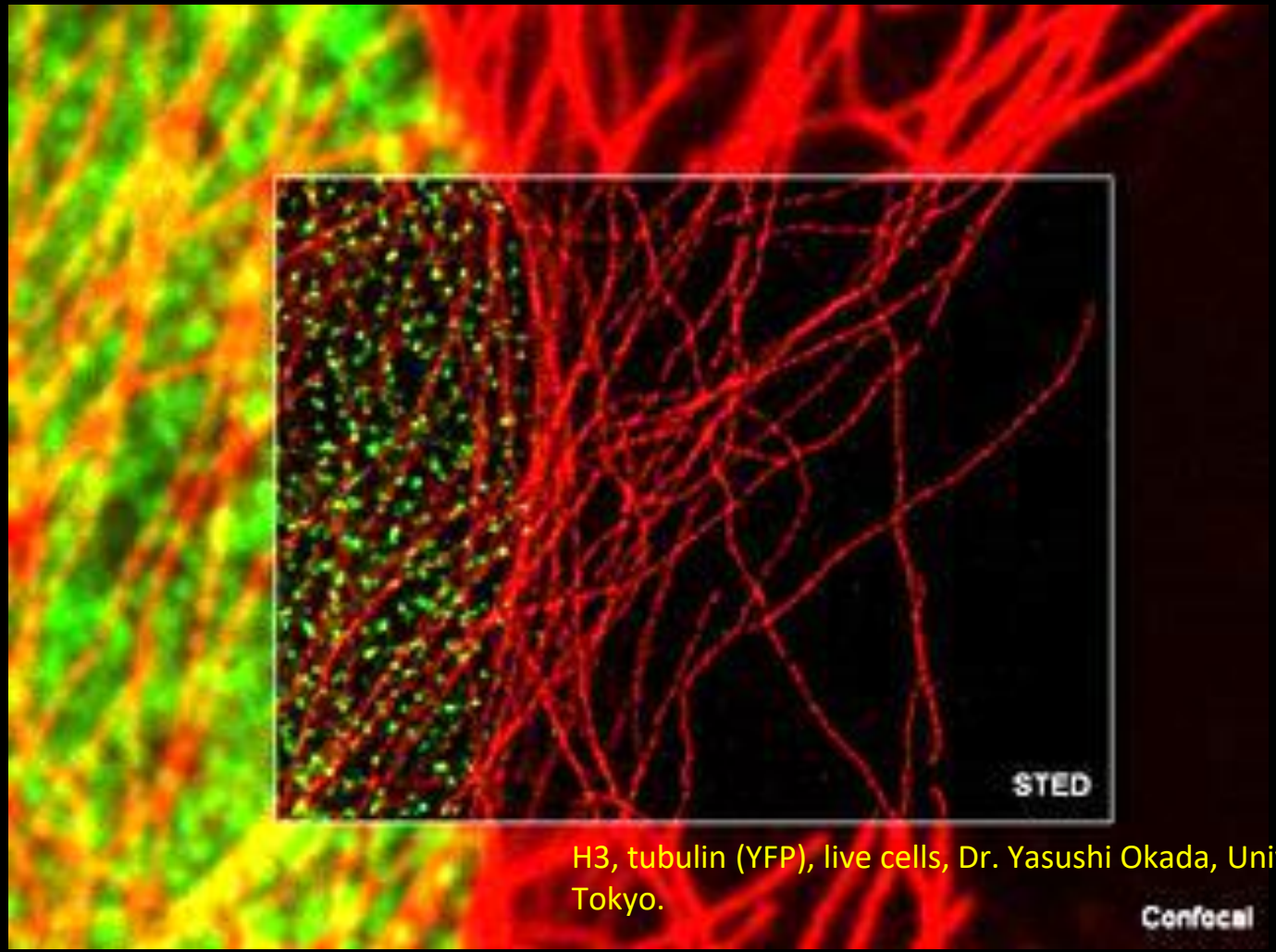
STED beam (doughnut) PSF (no light in the middle)

effective excit ation PSF



Molecule is de-excited by the STED beam, and does not contribute to the fluorescence signal

592 nm fiber laser, 600 mW



H3, tubulin (YFP), live cells, Dr. Yasushi Okada, University of Tokyo.

Single Molecule Localization

✓**STORM, PALM, …**

✓ based on blinking of individual molecules

"photonic" background

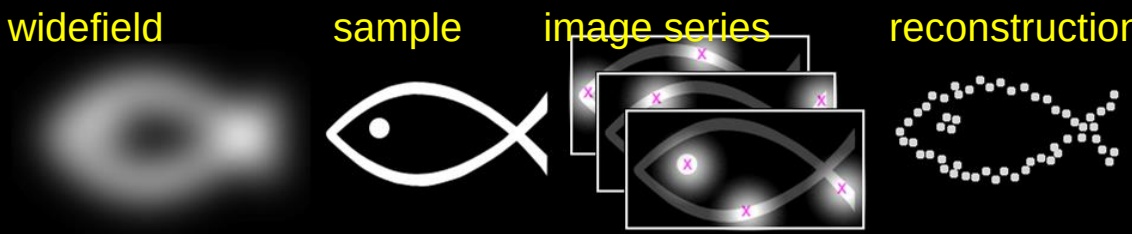
widefield (Abbe) "resolution"

pixel size

$$\sigma_{xy} = \frac{1}{\sqrt{N}}\sqrt{\sigma_{PSF}^2 + a^2/12 + 25\frac{\sigma_{PSF}^4 b^2}{Na^2}} \approx \frac{\sigma_{PSF}}{\sqrt{N}}$$

number of captured photons

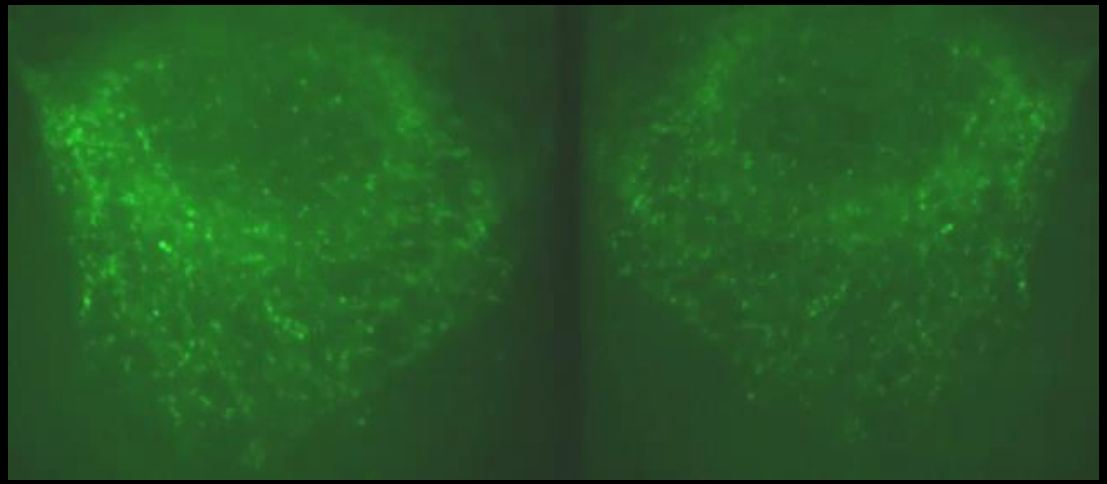
number of "captured" photons

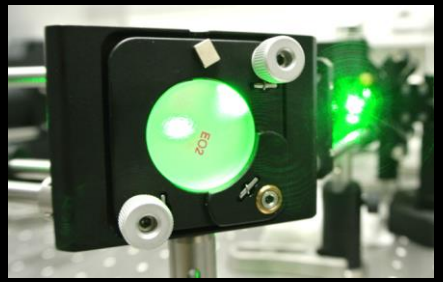


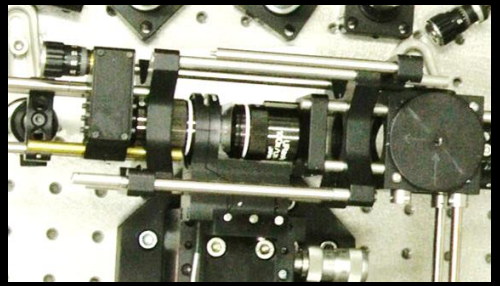
Single Molecule Localization

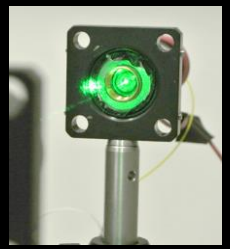
Key features:

- Powerful illumination
- Fast, sensitive camera
- Isolated blinking
- Low background
- High label density
- Long acquisition
- 3D is a bit tricky



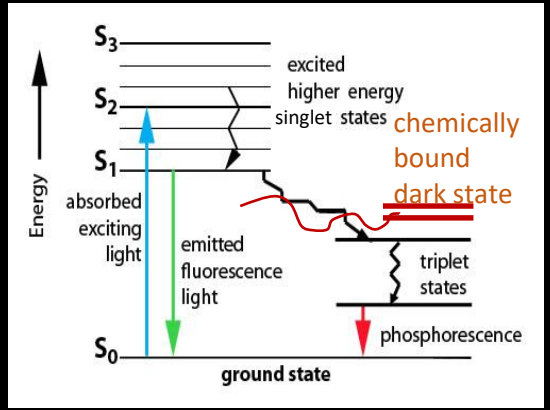


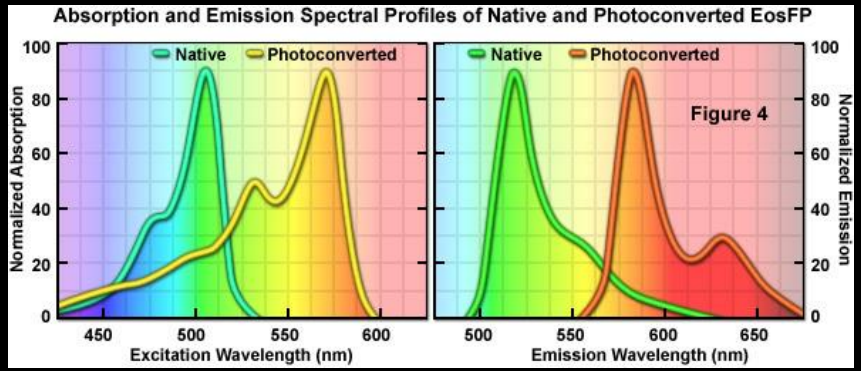




Stochastic fluorophore blinking

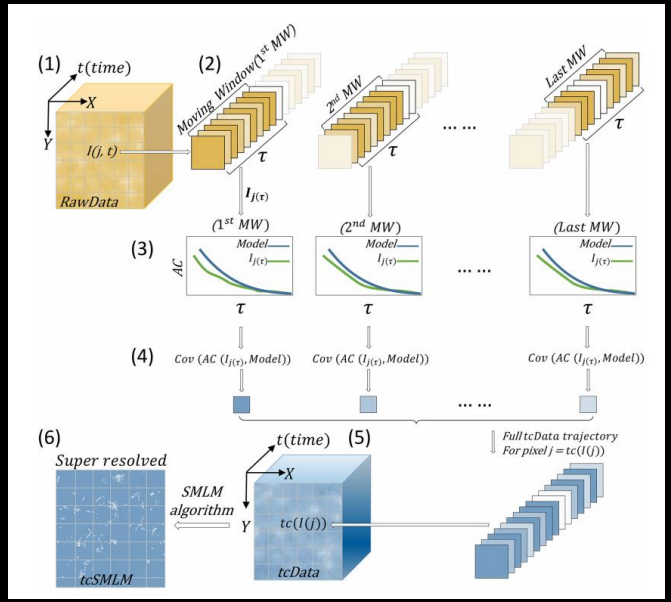
- Common Cyanine, Rhodamine dyes
  - special ‘blinking buffer’ stabilizes dark state
  - typically thiols + oxygen scavengers
  - require very intense illumination
  - ≈10,000 photons per burst
- Photo-switchable proteins
  - mEOS, CFP, …
  - no special buffers needed
  - moderate light intensities
  - live cell imaging demonstrated
- Transient binding of fluorophores = BALM





A significant improvement: tcSMLM

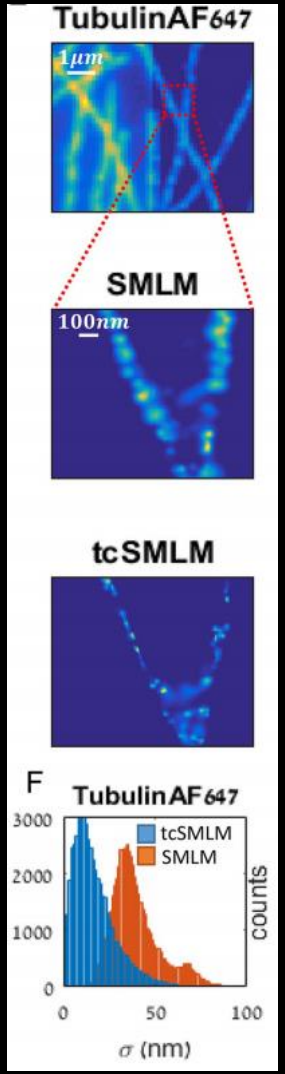
- “time-correlated Single Molecule Localization Microscopy”
- Harnessing the temporal properties of the blinking fluorophores
- Frames are not processed separately, but in groups (< 50 frames)
- The autocorrelation of the temporal profile of each pixel is compared to a model
- The last step is ThunderSTORM...



Hermon *et al.* Time-correlated single molecule localization microscopy enhances resolution and fidelity. *Sci Rep* **10,** 16212 (2020).

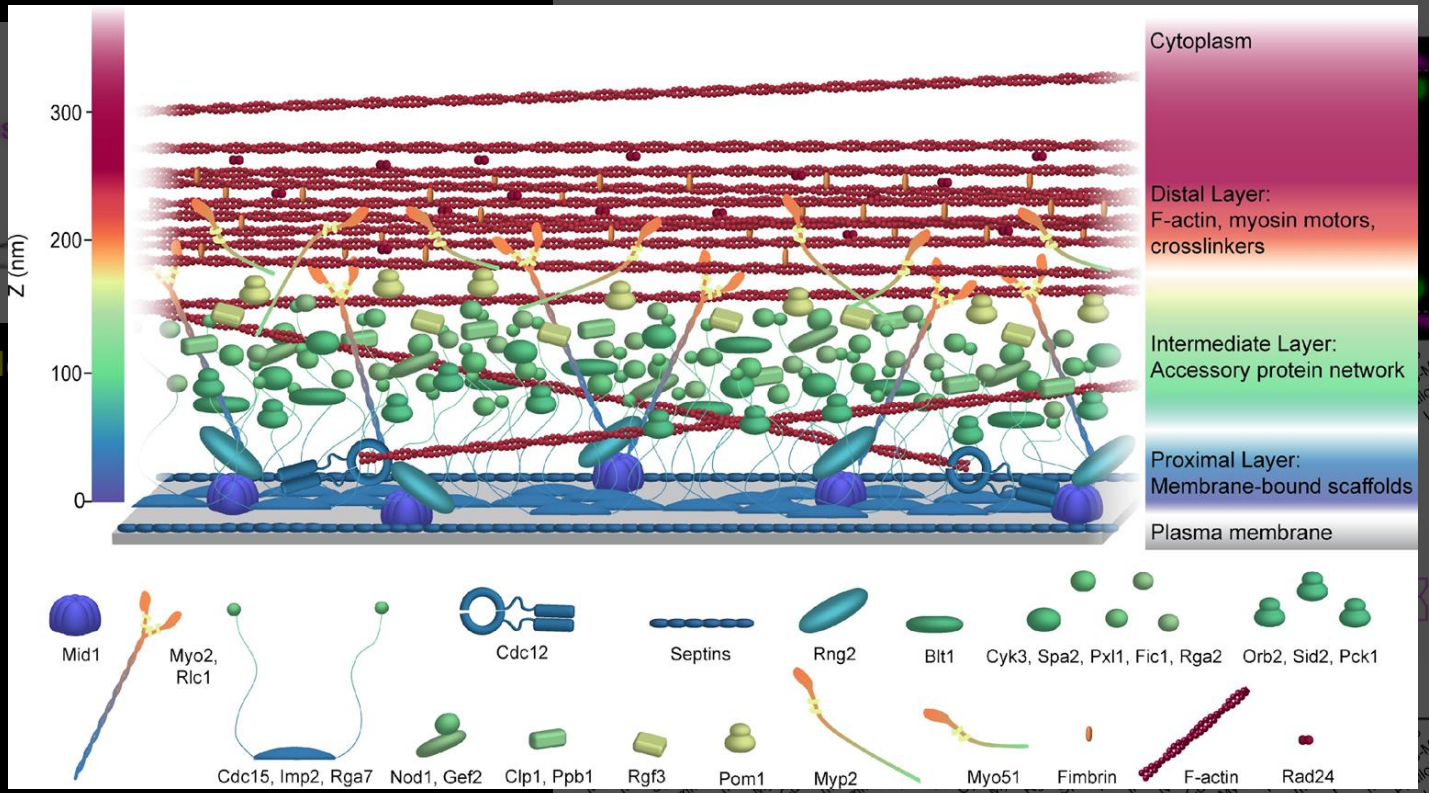
A significant improvement: tcSMLM

- Especially useful with fast frame rates (> 100 fps)
- Typical resolution improvement: 50 nm -> 30 nm
- Matlab code available https://github.com/ShermanLab/tcSMLM
- Processing is slow, but can be parallelized...

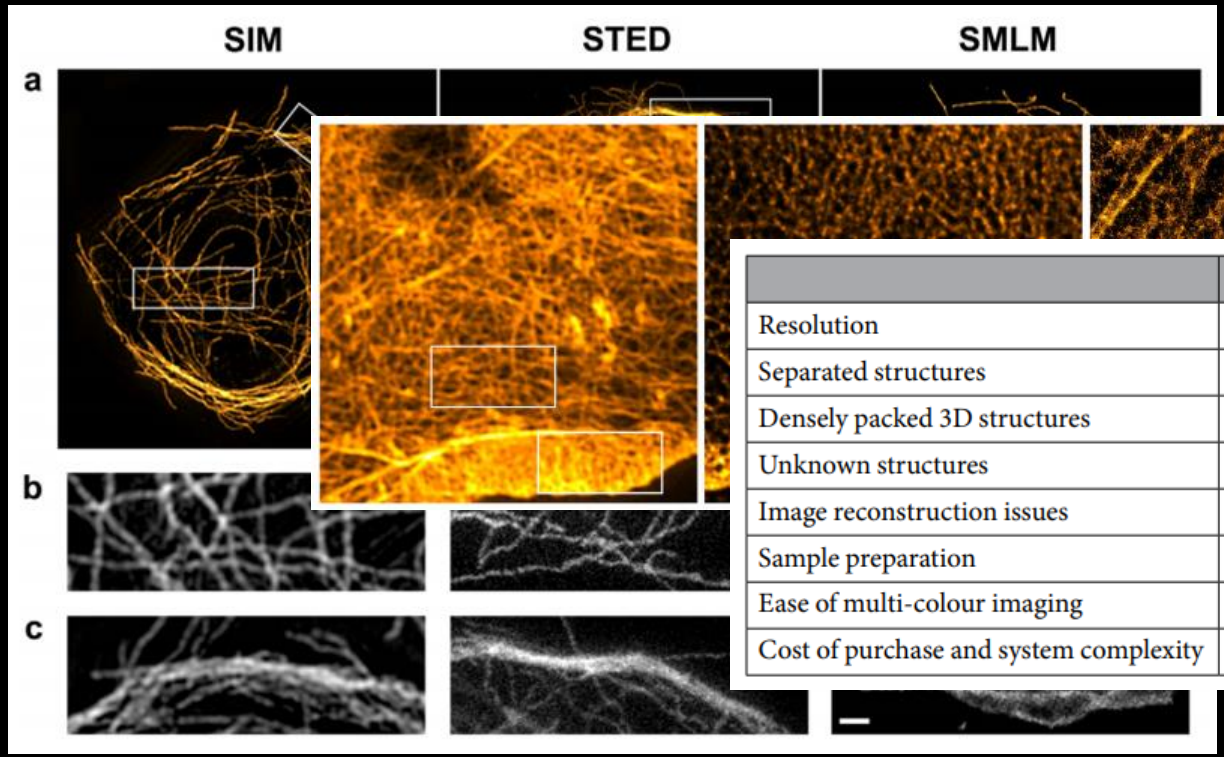


Hermon *et al.* Time-correlated single molecule localization microscopy enhances resolution and fidelity. *Sci Rep* **10,** 16212 (2020).

SMLM Application: yeast contractile ring - search for proteins localisation



So, which one is the best?

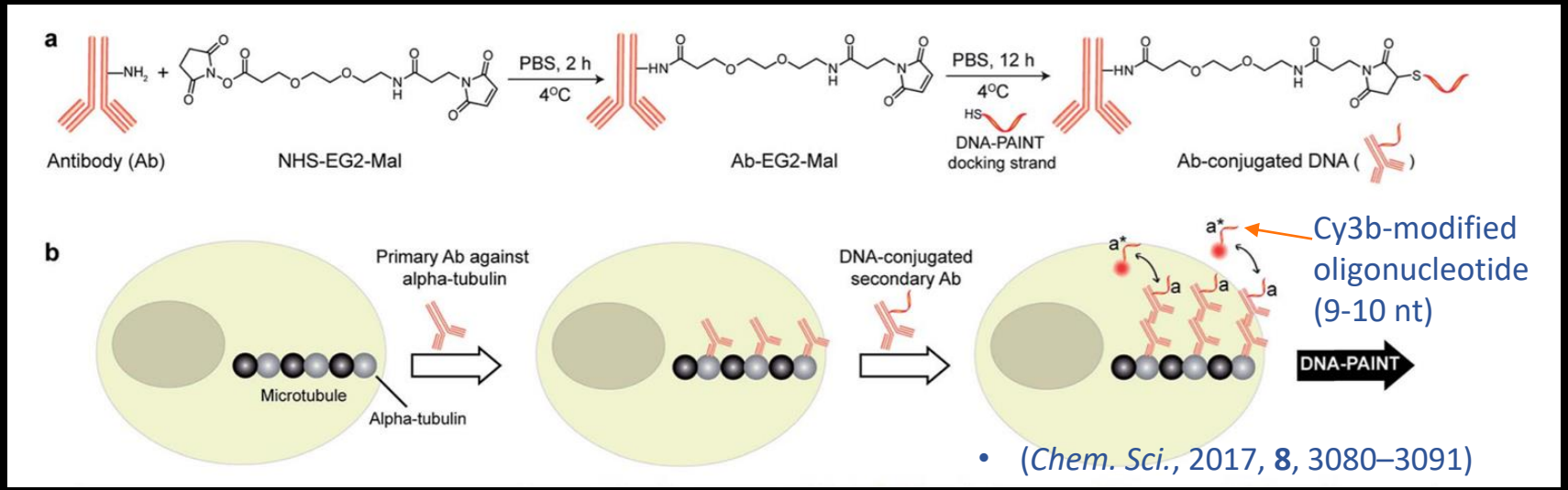


	SIM	STED	SMLM
Resolution	●	●	●
Separated structures	●	●	●
Densely packed 3D structures	●	●	●
Unknown structures	●	●	●
Image reconstruction issues	●	●*	●
Sample preparation	●	●	●
Ease of multi-colour imaging	●	●	●
Cost of purchase and system complexity	●	●	●

Wegel, …, Dobbie, S.Rep. (2016) **6**:27290

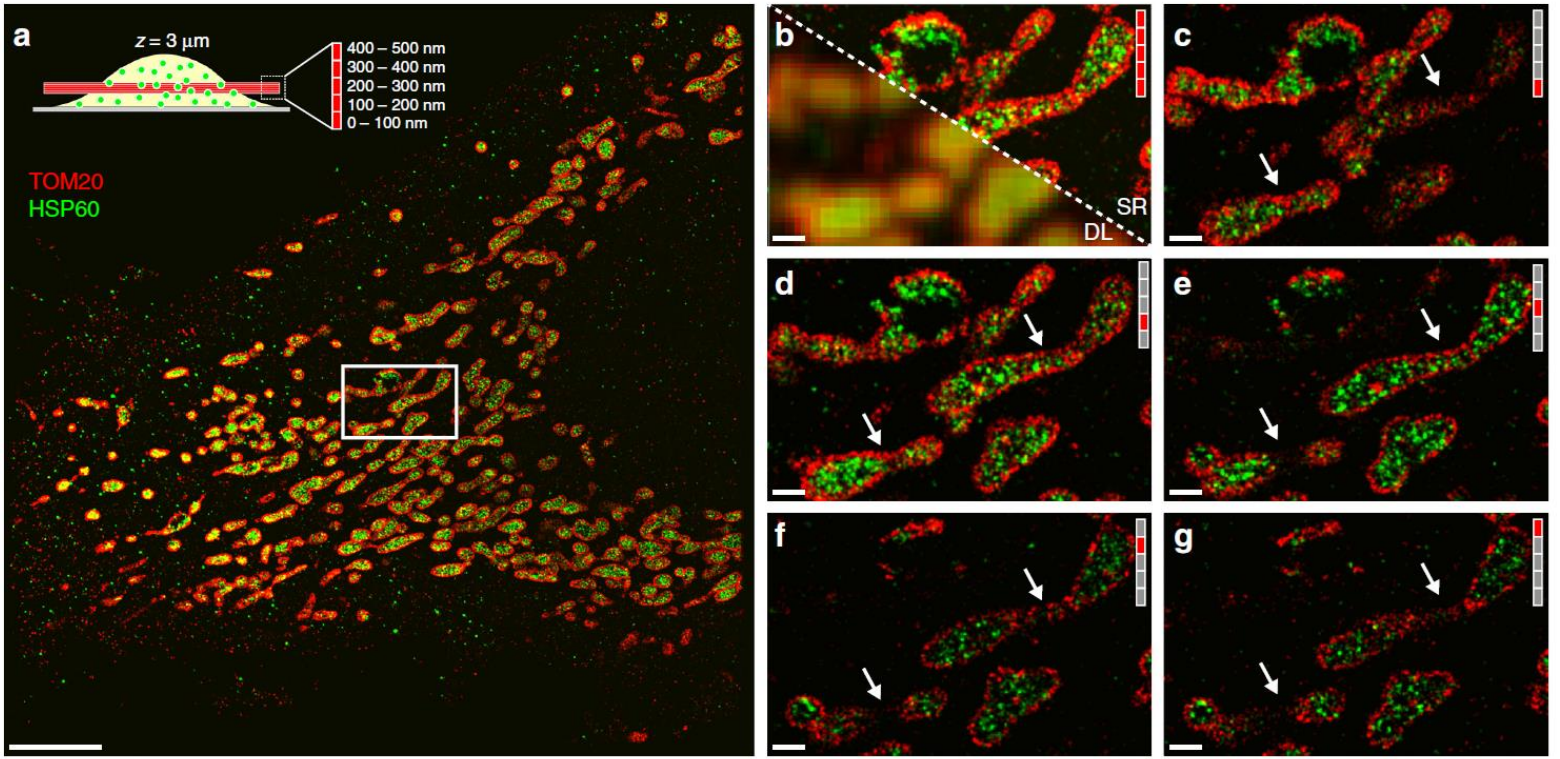
New strategy: DNA-PAINT

Transient binding of fluorescent probes
Based on DNA-DNA hybridization (good tunability, multicolor-compatible)
Various conjugation methods (biotin-streptavidin-biotin, NHS-xxx-Mal)
Problems with high background -> fluorogenic probes / confocal microscopy



- (*Chem. Sci.*, 2017, **8**, 3080–3091)

DNA-PAINT + spinning disk confocal combination



Acquisition ~ 1 h per plane !

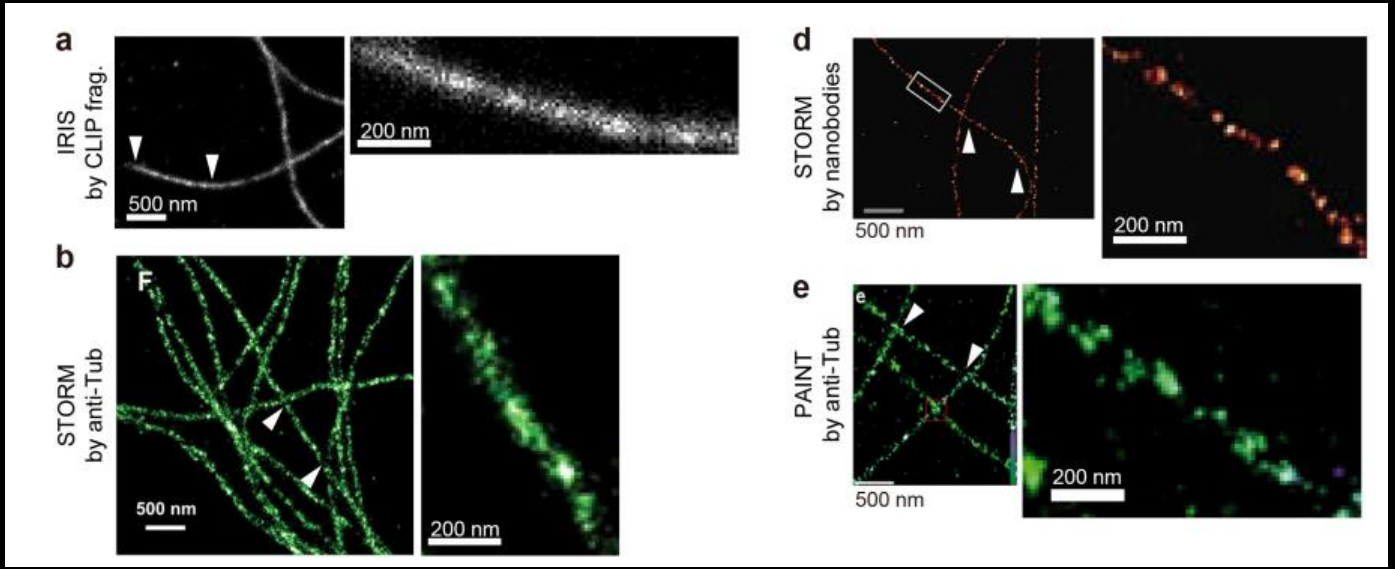
Schueder et al, *Nat. Comm.*, **8** (2017) 2090

New strategy: IRIS

Transient binding of fluorescent probes
Based on weak protein-protein interactions

- LifeAct, protein fragments

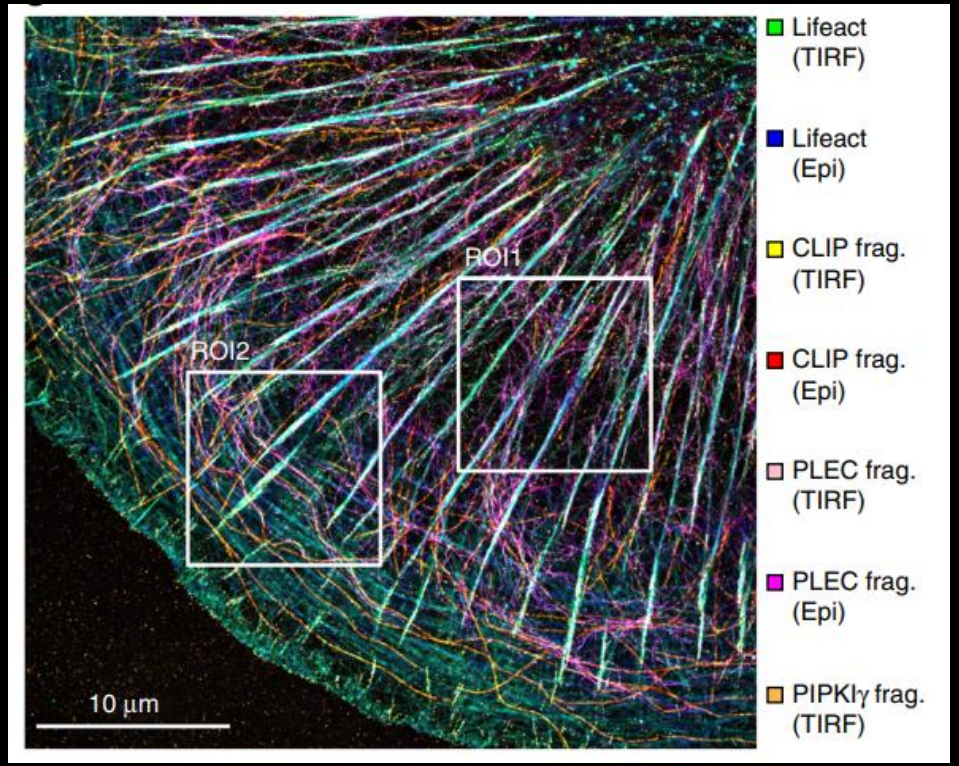
- Solves the labeling density problem



Kikuchi et al, *Nat. Meth.*, **12** (2015) 743

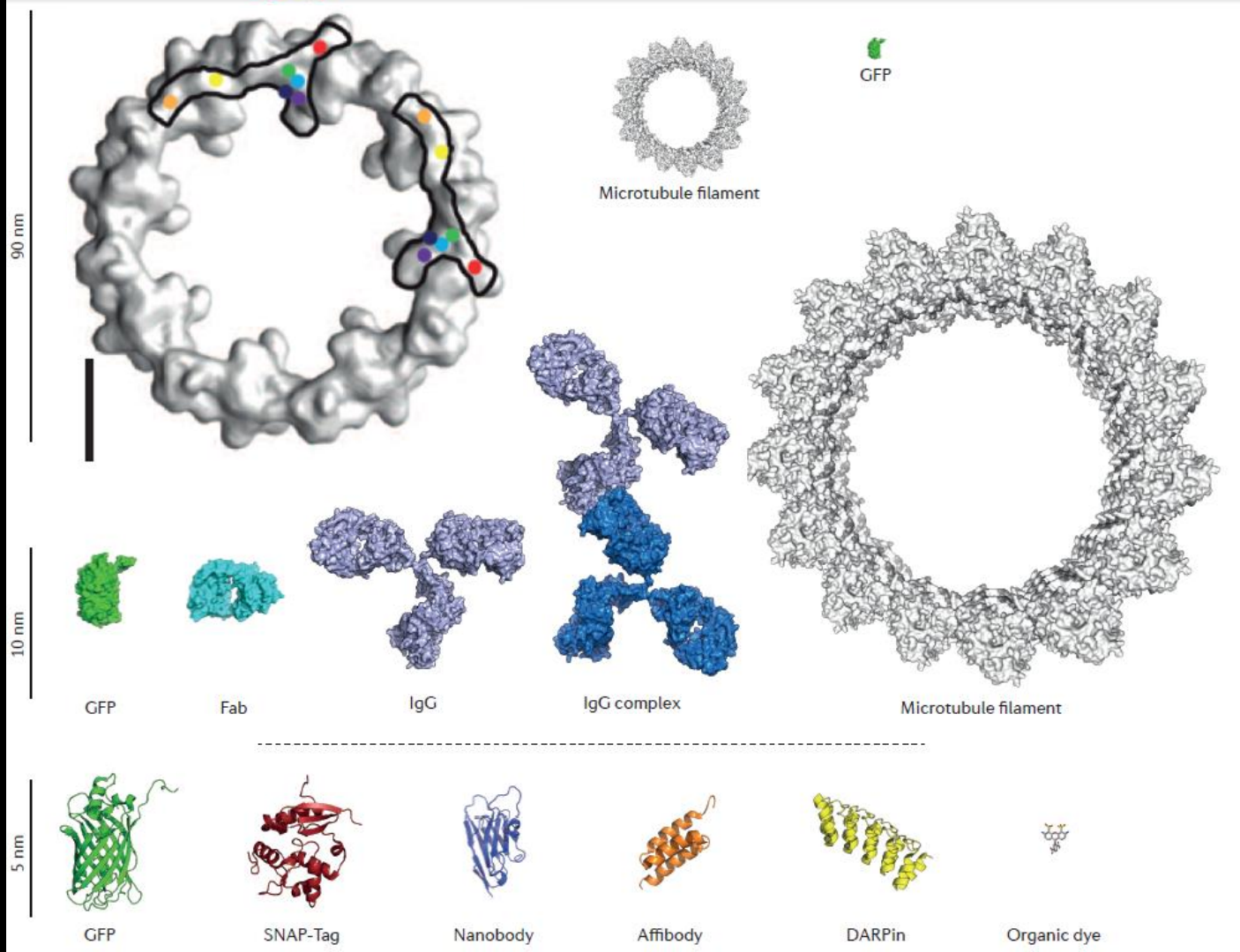
IRIS Application: Focal Adhesions

- Multiple exchangeable probes in fixed cells
- Oxygen scavenging buffer
- 20 – 400 min per channel
- TIRF, widefield



Kikuchi et al, *Nat. Meth.*, **12** (2015) 743

Fluorescent labeling



from Sahl et al, *Nat Rev. Mol. Cel. Biol.* **18** (2017) 685

Summary and outlook

- The hardware is more or less mature
  - Airyscan may see some upgrades
  - Superresolution with spinning disk confocal - optimization
- Software keeps evolving
  - Image processing algorithms (AI is coming!)
- Rapid development of new fluorescent probes
  - new labeling strategies, delivery into cells
  - fluorogenic probes, photoswitching, STED probes
  - live cell compatibility
  - faster imaging

Thank you

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