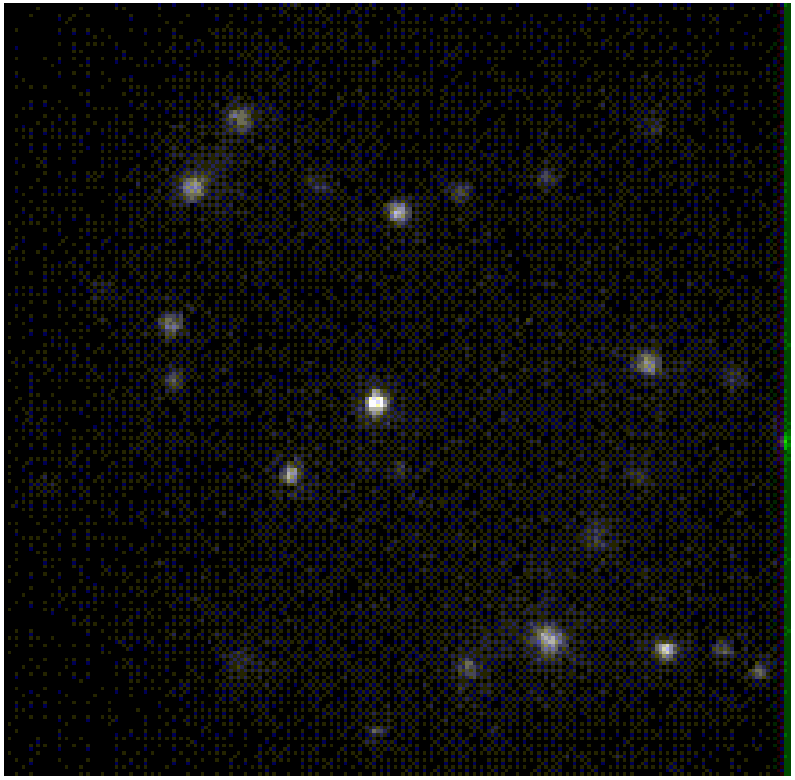
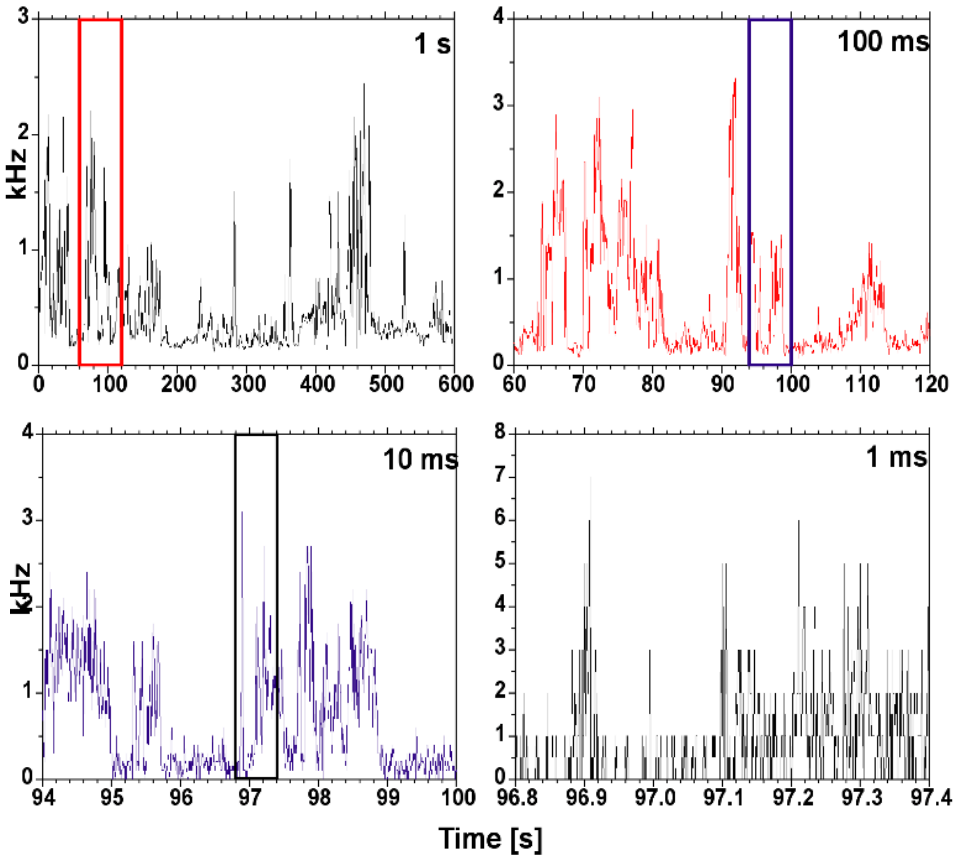
Fundamentals: Blinking of quantum dots

Blinking of semi-conductor nanoparticles (quantum dots) is very pronounced due to photo-ionisation



NK1 receptor (GPCR) labelled with Qdot 655 (Life technologies) on 293T cells



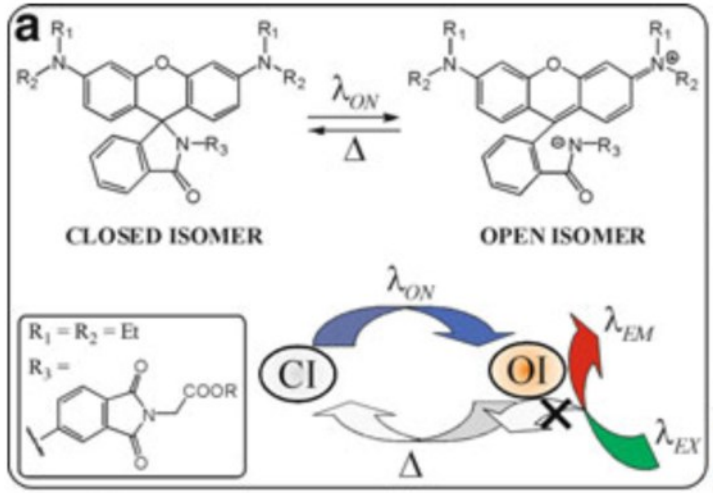
Blinking occurs at every timescale

Michalet, X, 2001 *Single Molecule, 2(4), 261–276.*

Blinking 1: Controlled intermittent emission of single molecule

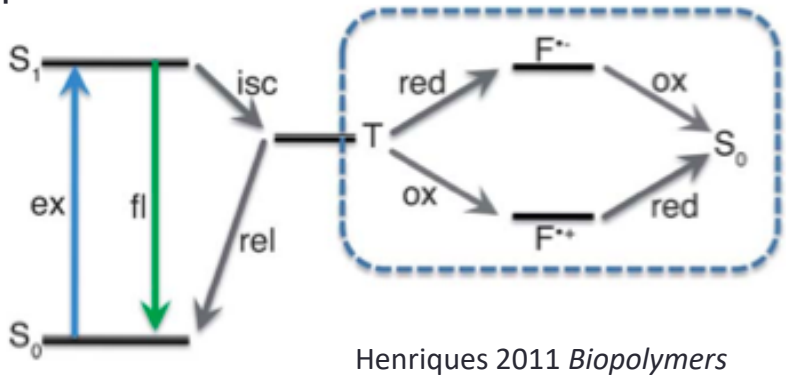
Organic fluorophore

Isomerization



P. Tinnefeld, *Far-Field Optical Nanoscopy*, Springer, 2015.

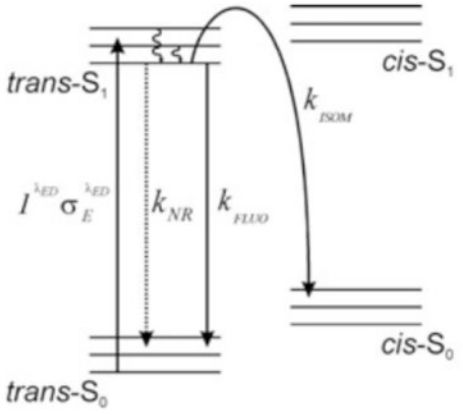
Triplet state



Henriques 2011 *Biopolymers*

Triplet state can be stabilized using reductant and oxidant solutions leading to a controlled blinking of the dye.

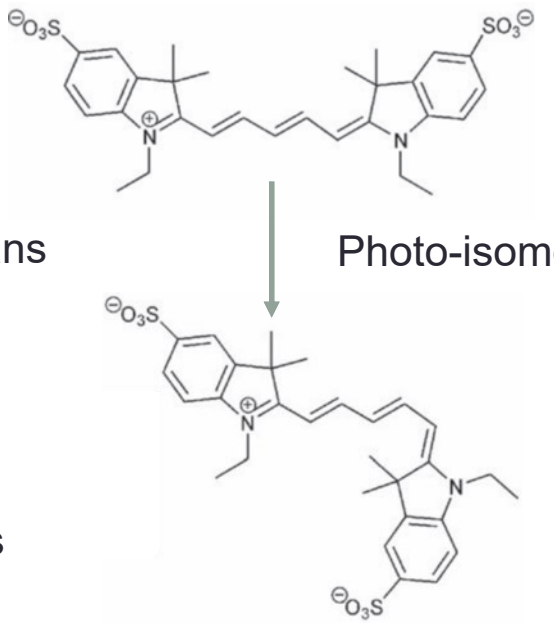
Cy5 cis-trans isomerization



$$\Phi_{FLUO} = \frac{k_{FLUO}}{k_{FLUO} + k_{ISOM} + k_{NR}}$$

$$\Phi_{ISOM} = \frac{k_{ISOM}}{k_{FLUO} + k_{ISOM} + k_{NR}}$$

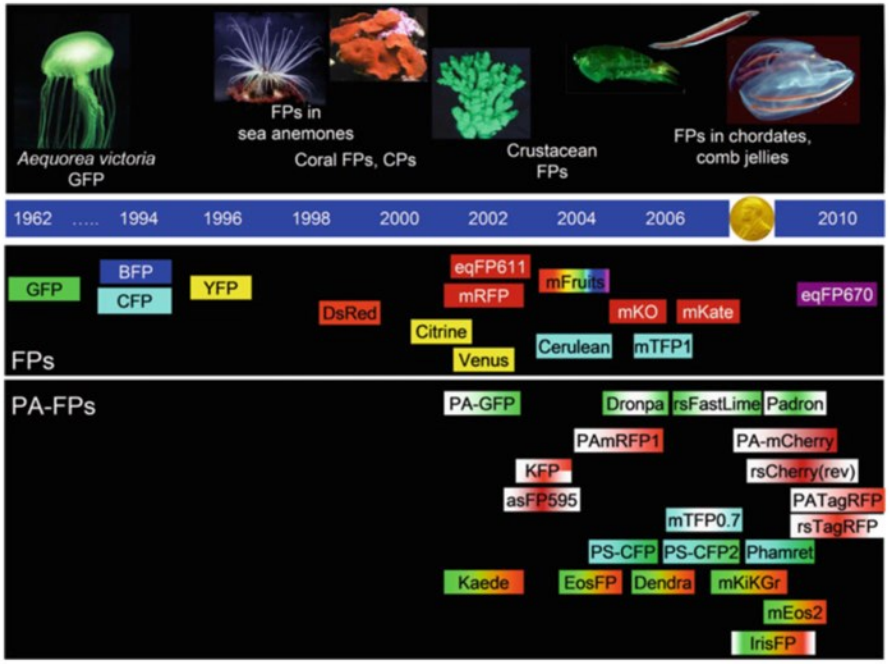
P. J. Walla, *Modern Biophysical Chemistry*. John Wiley & Sons, 2015.

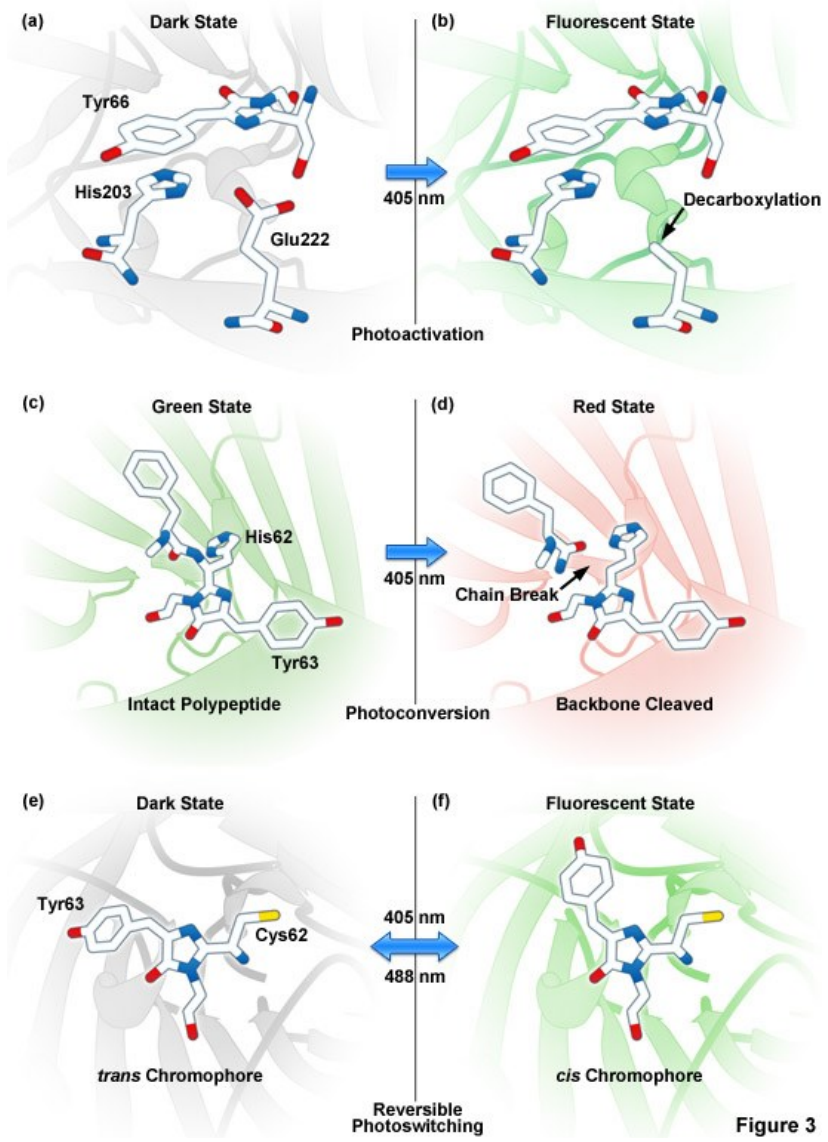


E. M. S. Stennett, et al, *Chem Soc Rev*, vol. 43, no. 4, pp. 1057–1075, 2014.

Blinking 2: Controlled intermittent emission of single molecule

Fluorescent proteins

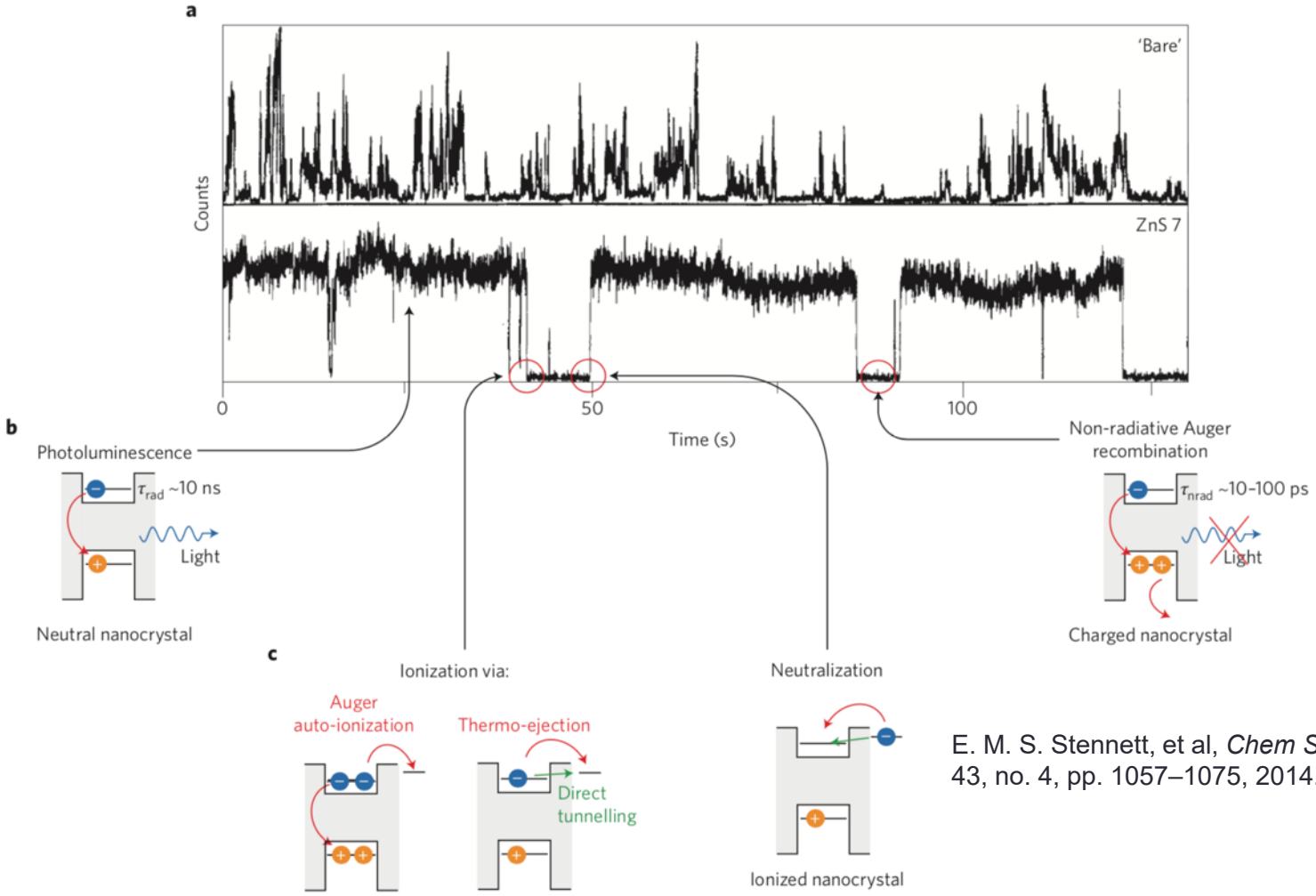




http://zeiss-campus.magnet.fsu.edu/articles/probes/highlighterfps.html

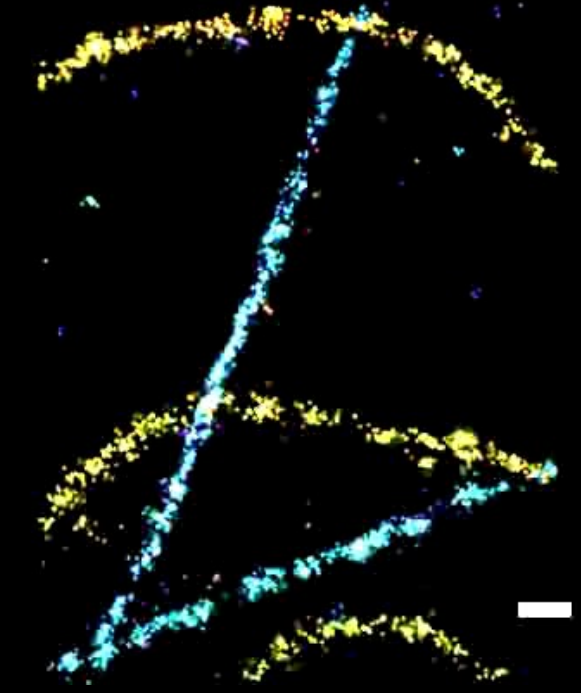
Blinking 3: Controlled intermittent emission of single molecule

Nanocrystals of semi conductors (quantum dots)

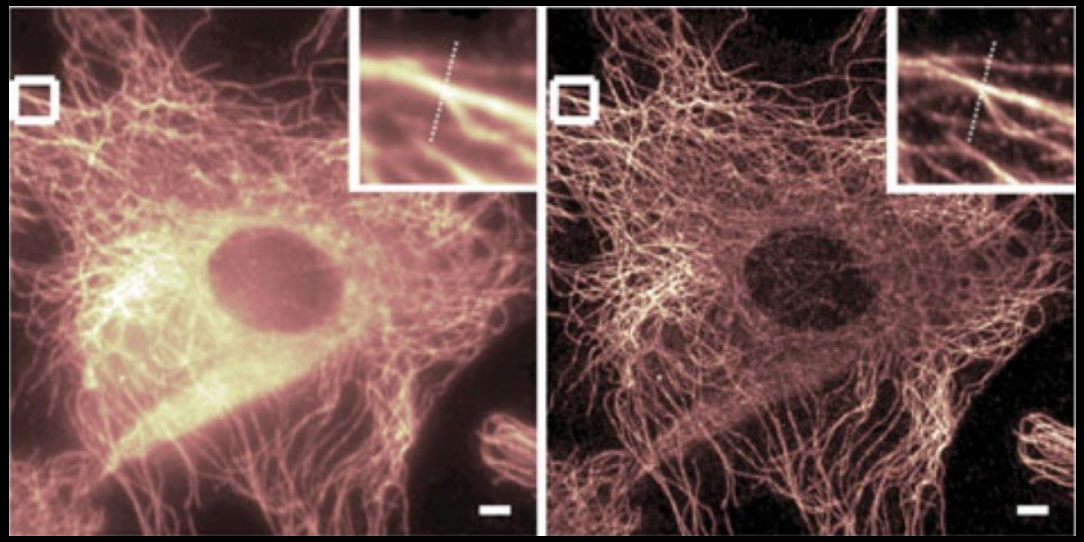


E. M. S. Stennett, et al, *Chem Soc Rev*, vol. 43, no. 4, pp. 1057–1075, 2014.

Quantum dots are naturally blinking molecules mainly due to photo-ionization at the surface of the particle. There is no easy method to control QD blinking.



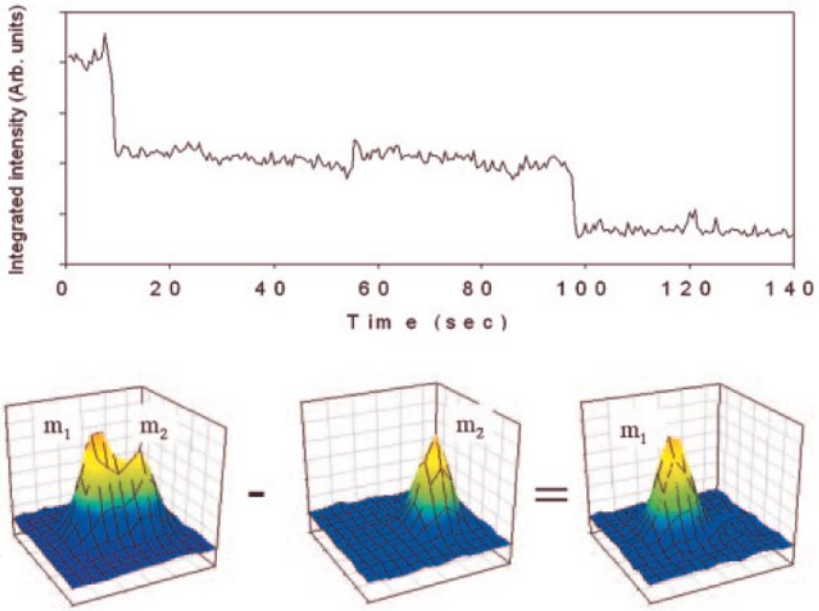
Fluorescence nanoscopy



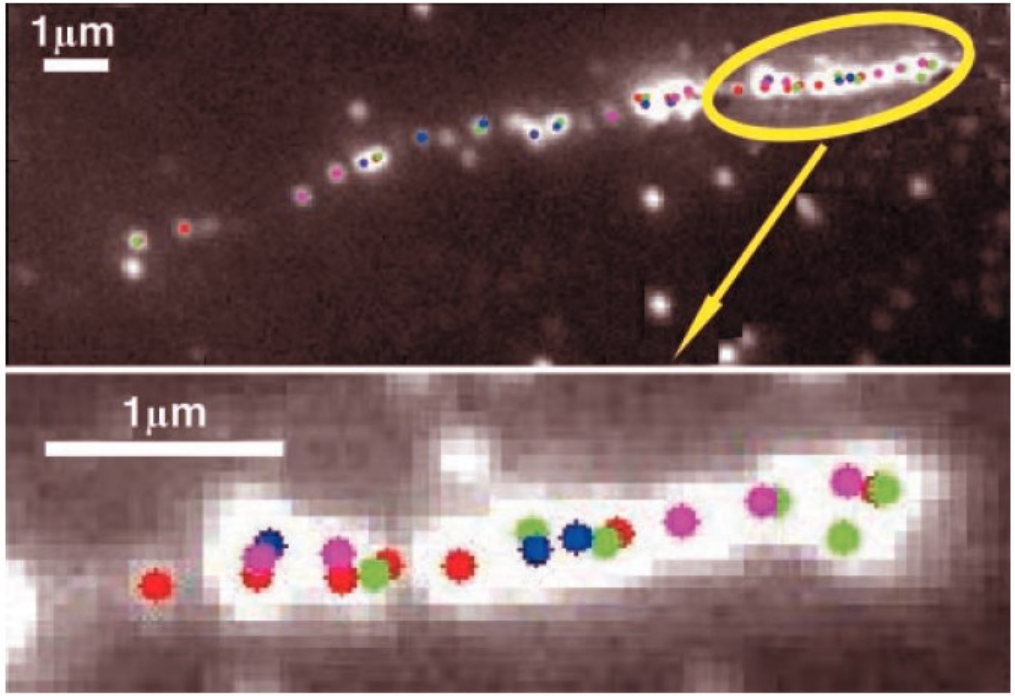
Photobleaching: Distance measurements in the nanometer range

Distance measurements have the same accuracy as position measurements (10-30 nm).
Principle: the last molecule to photobleach is fitted using a gaussian. This gaussian is then used to fit the two-molecule emission profile, and so on.

Example in a two-molecule case



DNA mapping

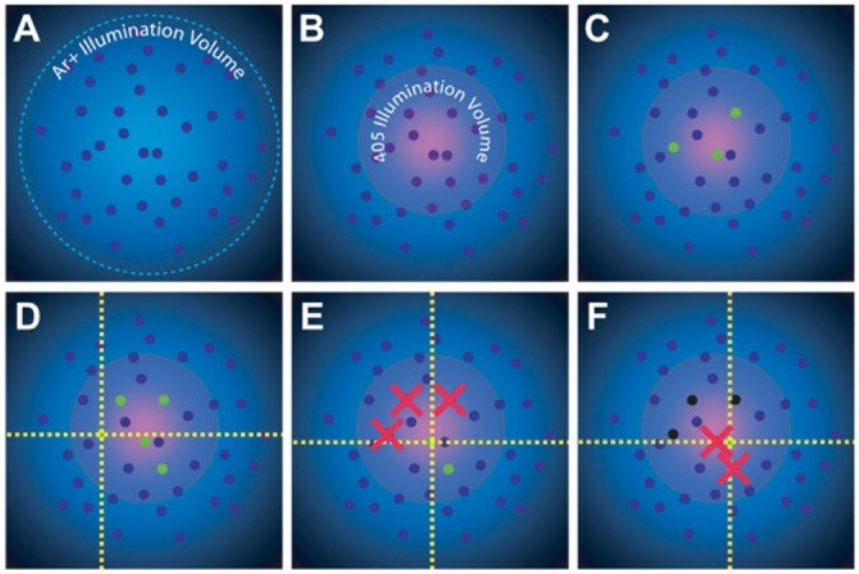


X. Qu, D. Wu, L. Mets, N.F. Scherer, *Proc. Natl. Acad. Sci. USA* **101**, 11298 (2004)

M.P. Gordon, T. Ha, P.R. Selvin, *Proc. Natl. Acad. Sci. USA* **101**, 6462 (2004)

Super-resolution: PALM

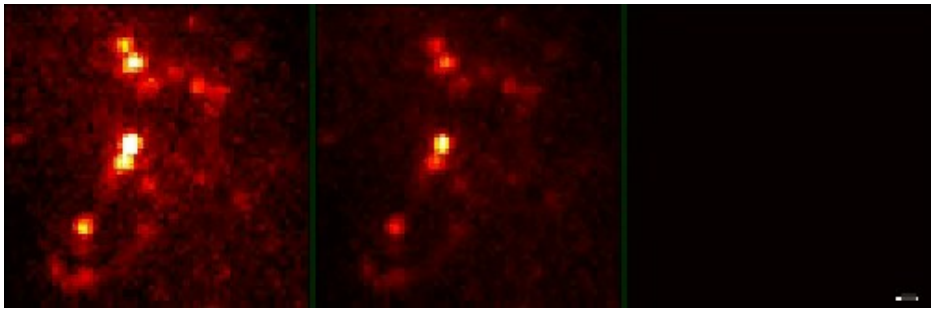
Photoactivatated localization microscopy (PALM)

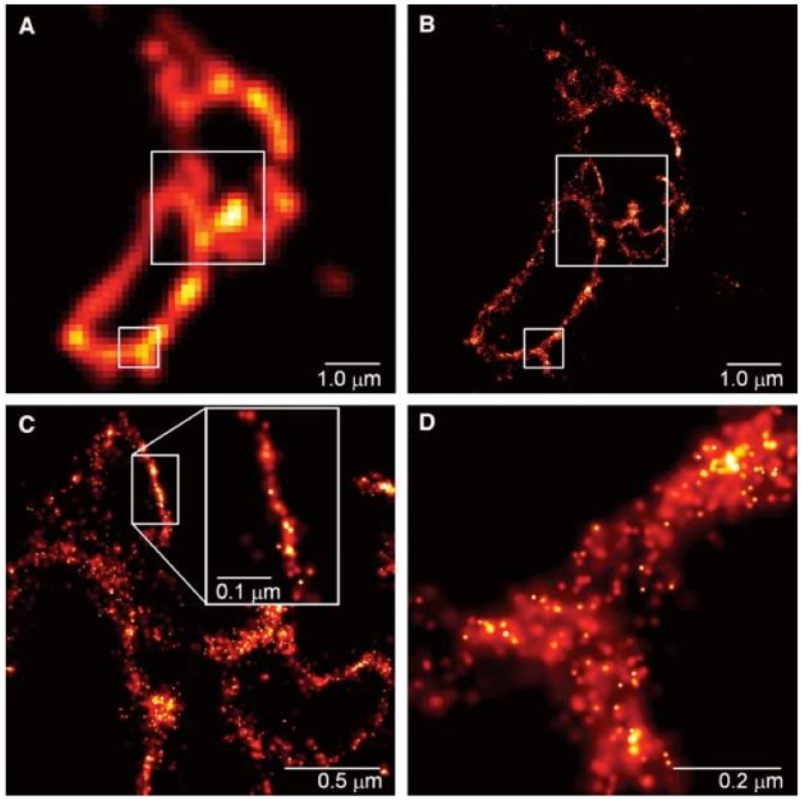


Hess, *Biophys J*, 2006

Fluorescent protein is activated or switched at low rate: molecules appear as single fluorescent spots.

Requirements:
-Photoactivatable or photoconvertible fluorescent protein (eg. mEOS2, Dronpa PA-GFP, PA-mCherry, EYFP)
-Relatively low protein expression



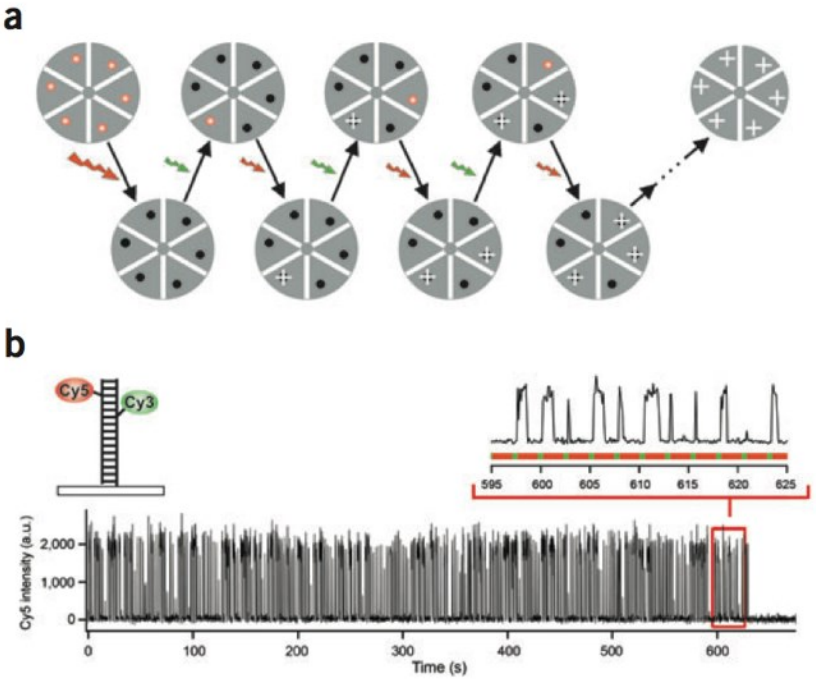


Betzig, *Science,* 2006

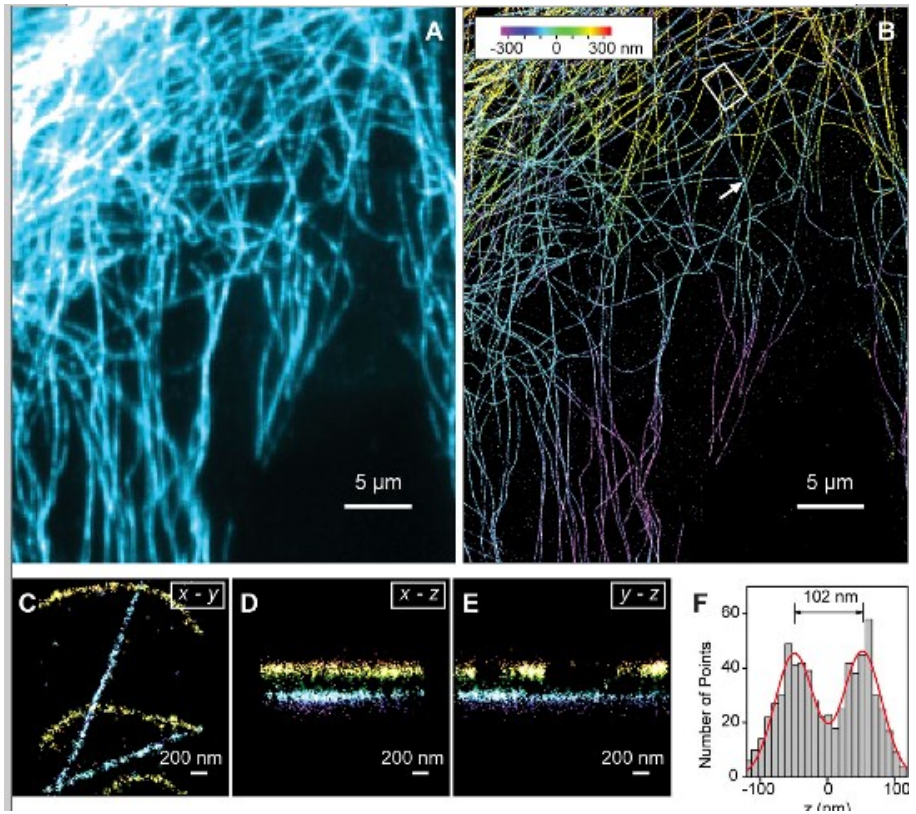
Super-resolution: STORM

Stochastic optical reconstruction microscopy (STORM)

Principle



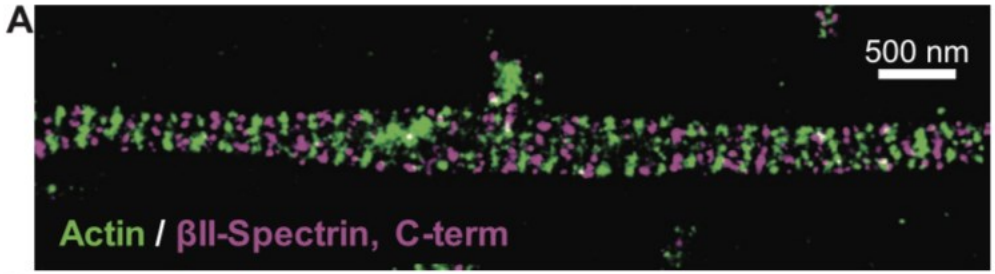
Rust, *Nat Meth*, 2006



Huang, *Science,* 2008

A dye is maintained in a dark state using strong excitation light. A fraction of the dye is reactivated using another wavelength. This can be mediated using another dye (STORM) or without (dSTORM)
Requirements:
-A pair of dye (eg Cy5 & Cy3) in close proximity (nanometer range) or a dye with stable triplet state
-An adapted buffer environnement (Reducing with thiol groups).
-Post-translational labelling (generally outside cells or *in vitro*)

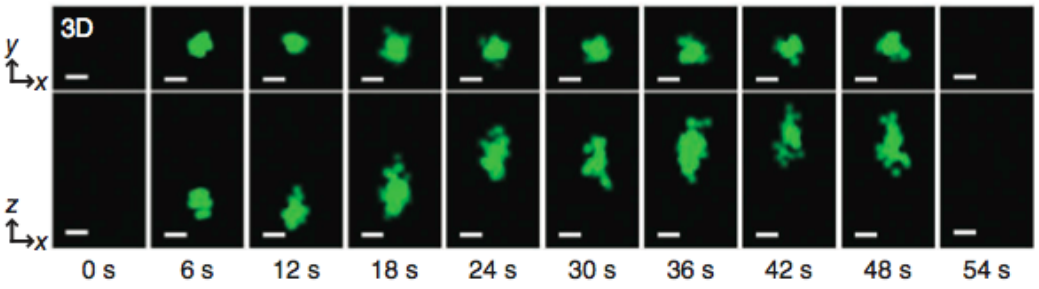
Super-resolution: STORM in neurons

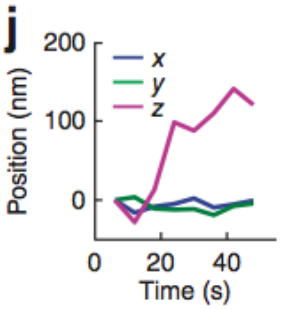


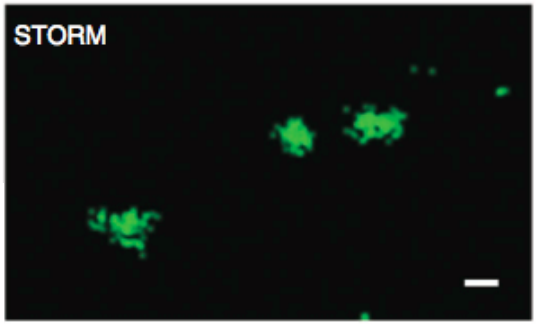
K. Xu, G. Zhong, and X. Zhuang, *Science*, vol. 339, no. 6118, pp. 452–456, Jan. 2013.

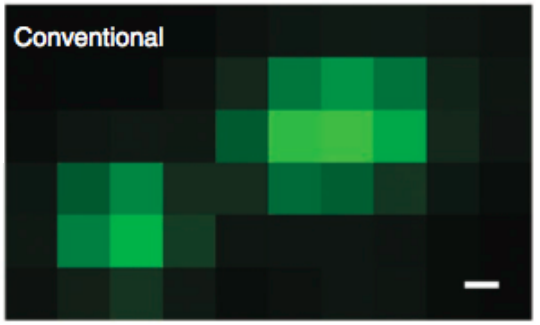
Super-resolution: Dynamic STORM in living cell

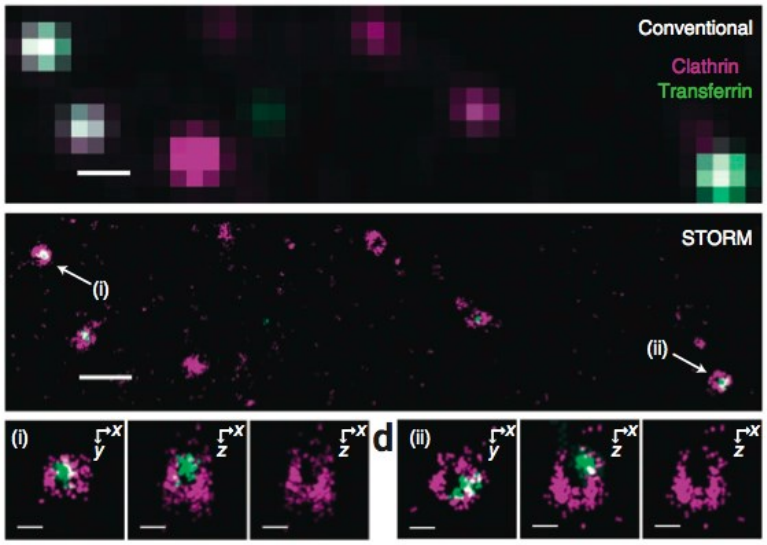
Dynamic STORM image of tranferrin in live cell









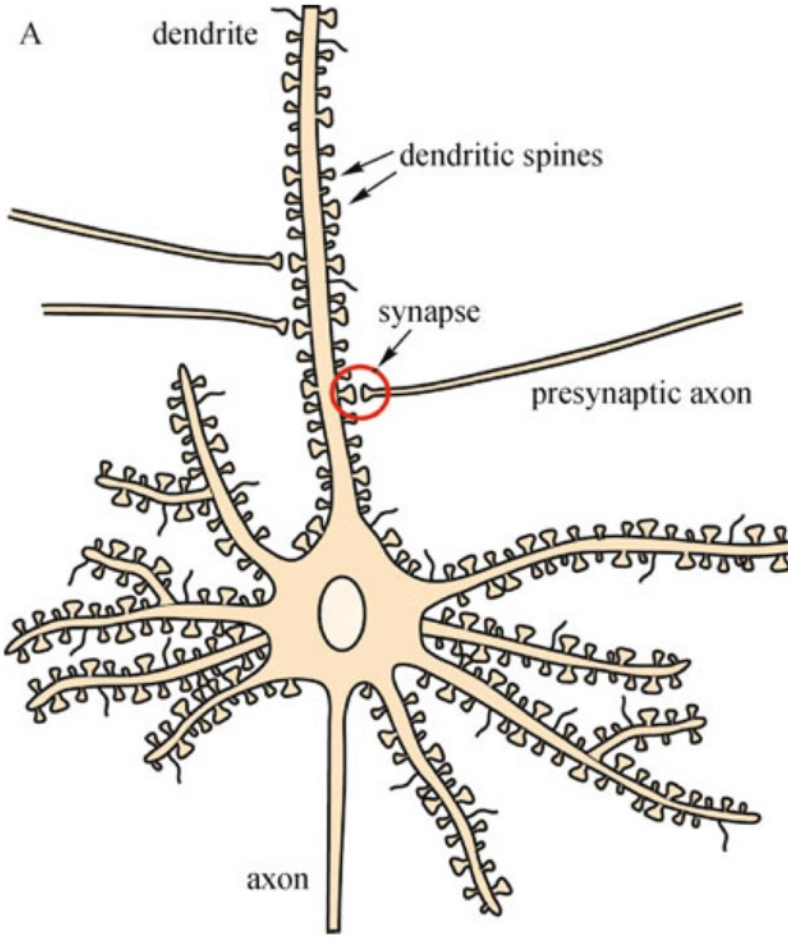


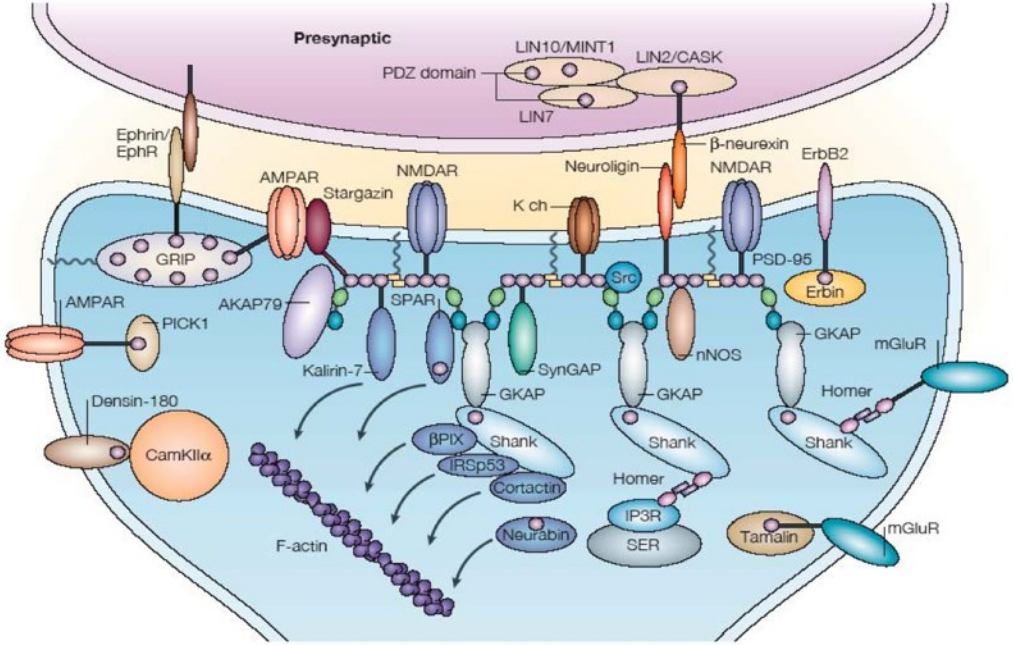
Dual color superresolution image of transferrin in clathrin-coated pits.

Jones, S. A., Shim, S.-H., He, J., & Zhuang, X. (2011) *Nature methods, 8(6), 499–505*

Super-resolution: Dynamic STORM in neuron

How do receptors diffuse in post-synaptic membrane ?

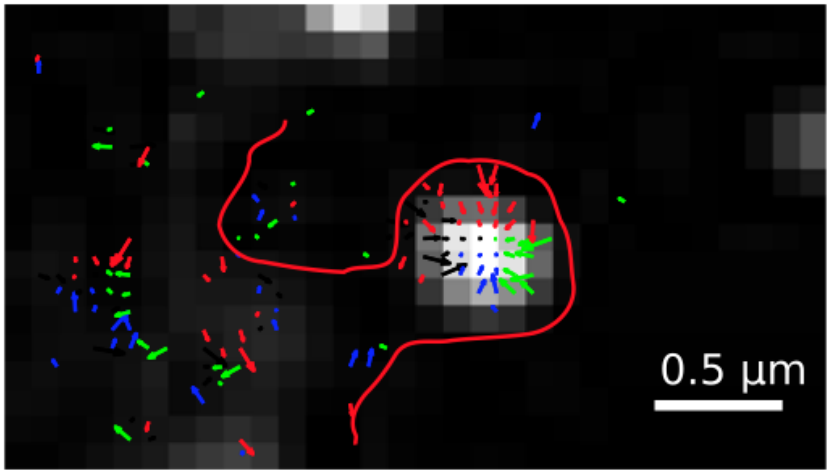




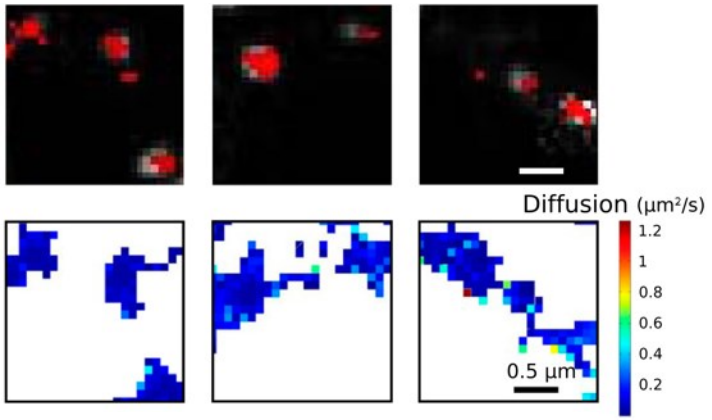
Smrt RD, Zhao X (2010) Frontiers in Biology 5:304–323.

Super-resolution: Dynamic STORM in neuron

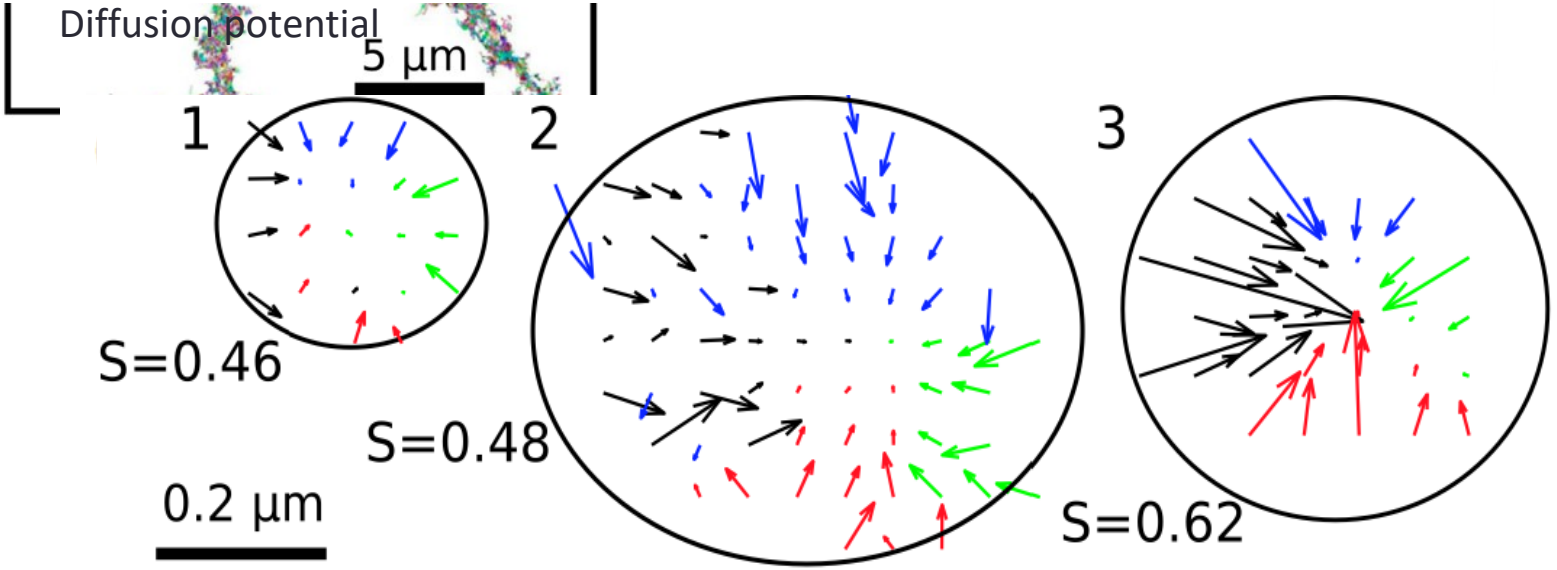
How do receptors diffuse in post-synaptic membrane ?



Diffusion mapping



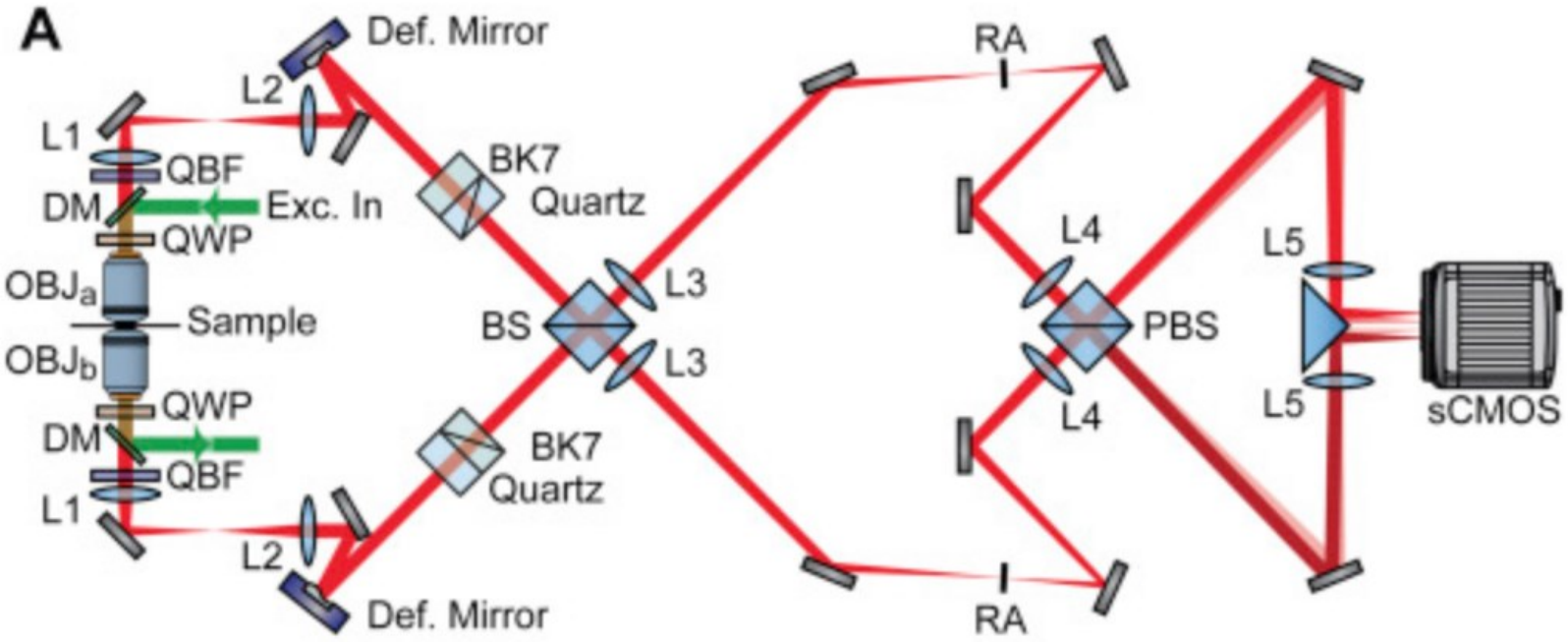
Diffusion potential



Hoze N et al. (2012) Proc Natl Acad Sci USA 109:17052-17057

Super-resolution 6: 3D image of whole cells

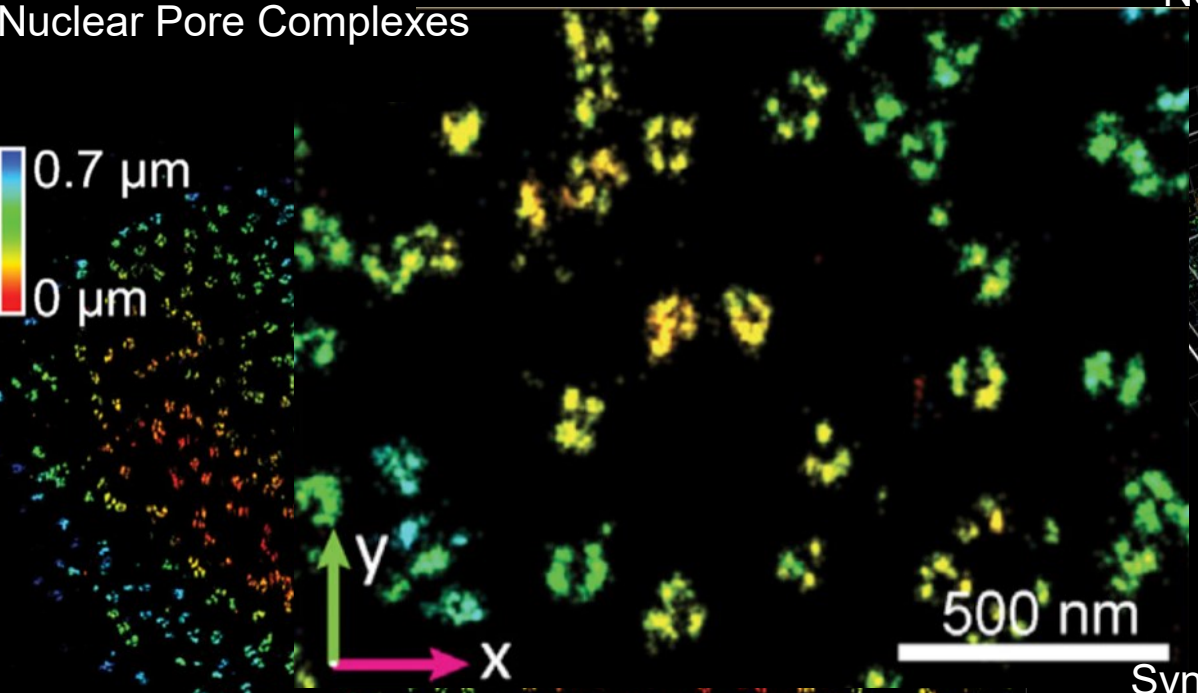
Whole-cell 4Pi single-molecule switching nanoscopy (W-4PiSMSN): Combination of PALM, 4Pi-microscopy and iPALM

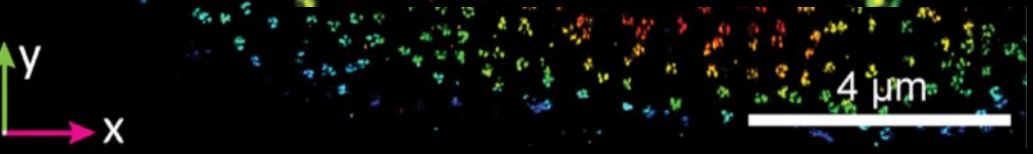


- The lateral resolution is increased by a factor 1.7 thanks to the 2 objectives
- The 4Pi configuration of the objective allows to reach a 10 nm axial resolution

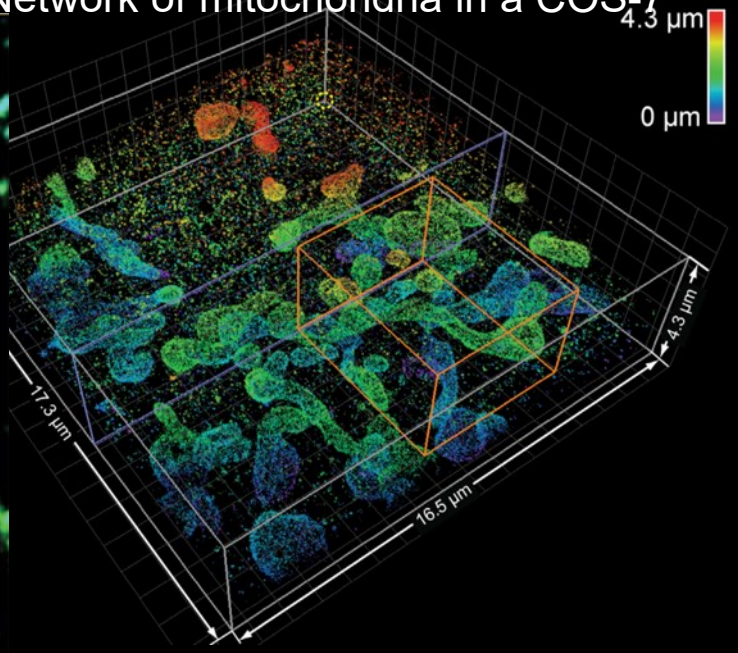
F. Huang, et al., *Cell*, vol. 166, no. 4, pp. 1028–1040, Aug. 2016.

Nuclear Pore Complexes

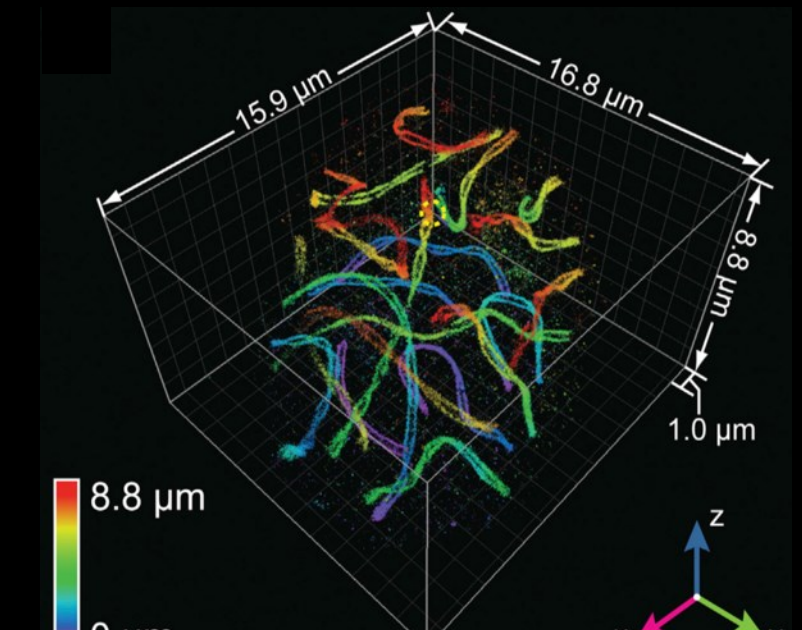




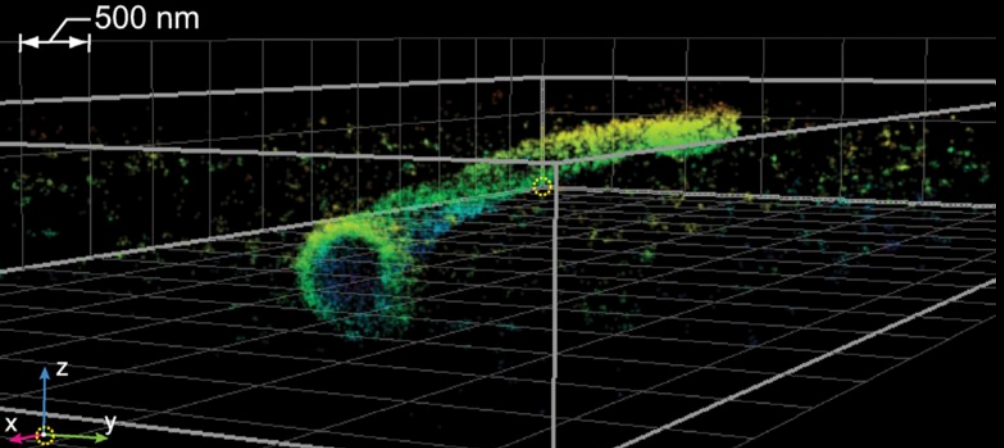
Network of mitochondria in a COS-7



Synaptonemal Complexes in a Whole-Mouse Spermatocyte

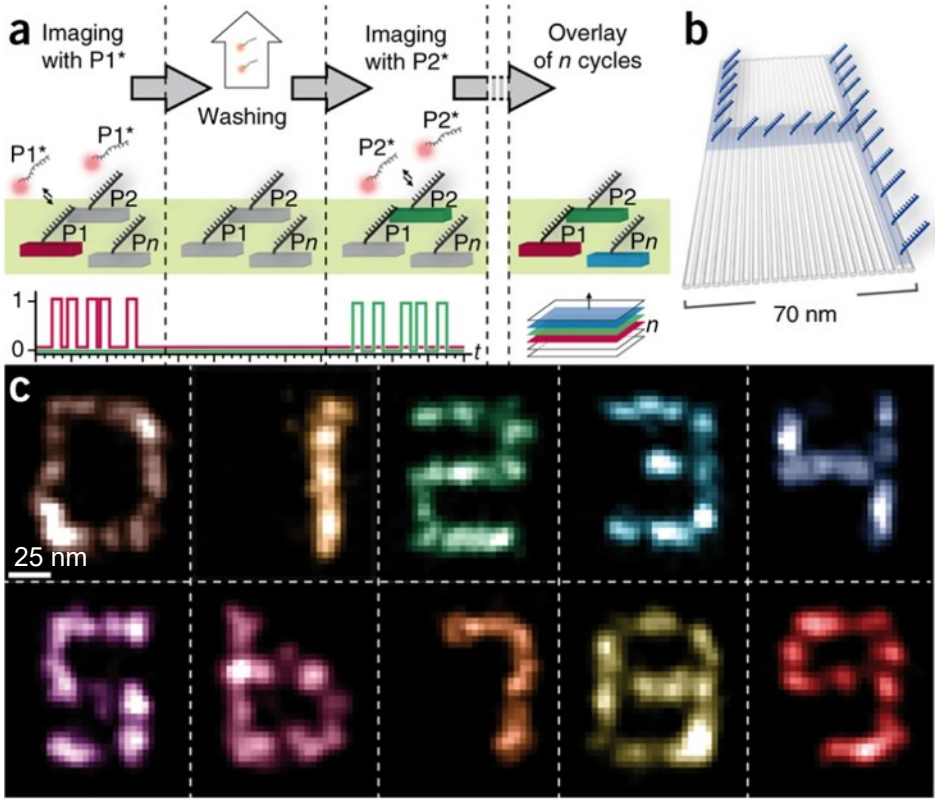


pH- SMO (a GPCR) on the primary cilium of an hTERT-RPE1 cell



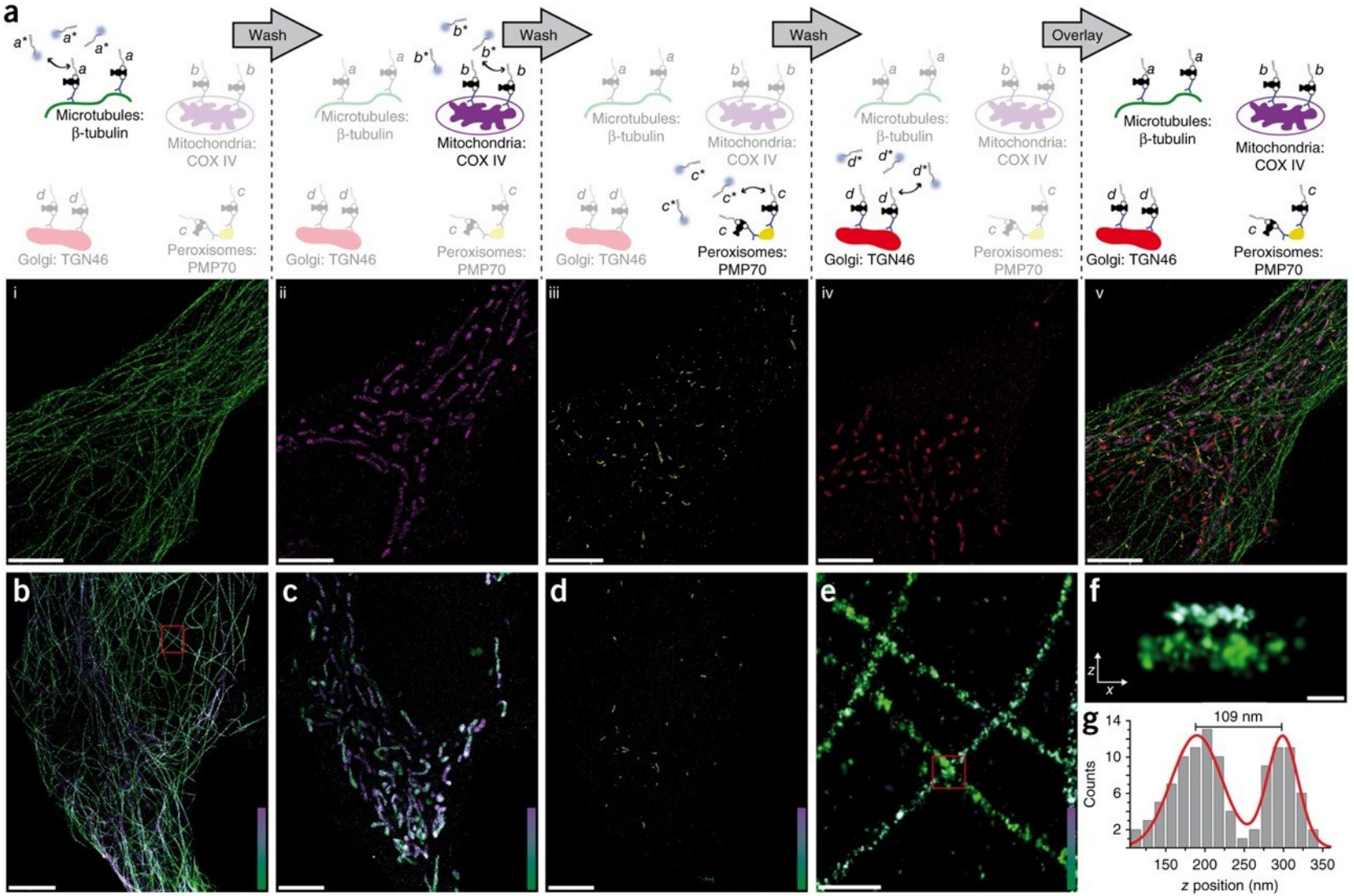
Super-resolution : (DNA)-PAINT

Sequential imaging of multiple targets using DNA-hybridization



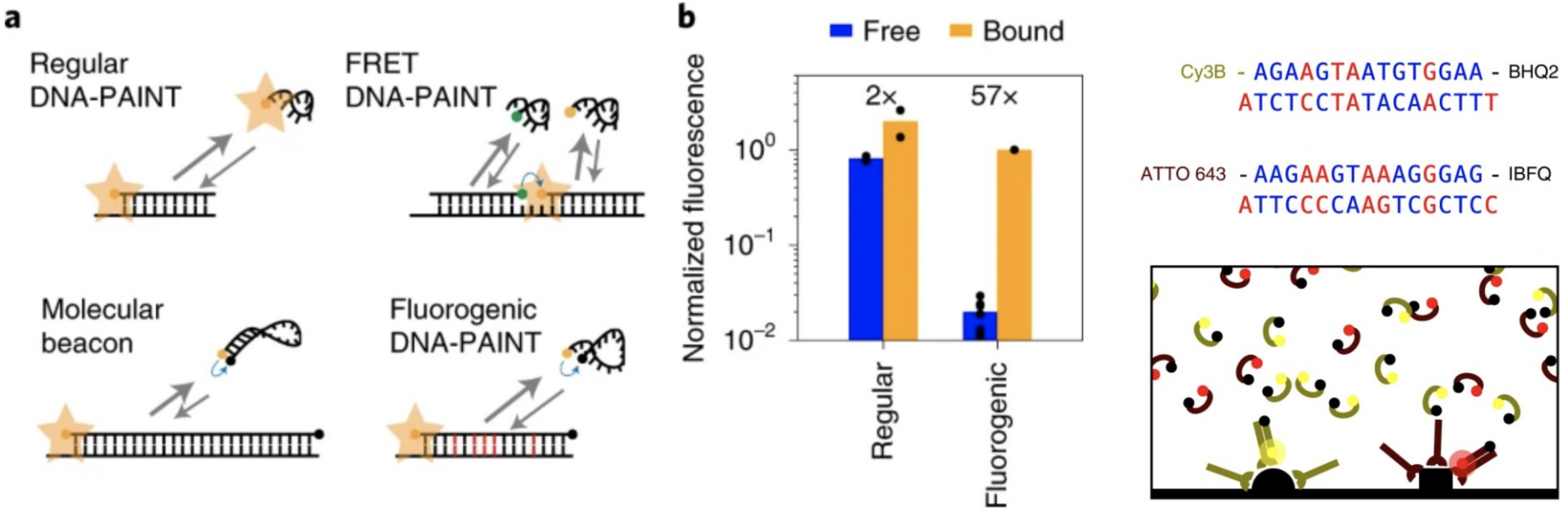
Jungmann, R., Avendaño, M., Woehrstein, J. *et al. Nat Methods* **11**, 313–318 (2014). https://doi.org/10.1038/nmeth.2835

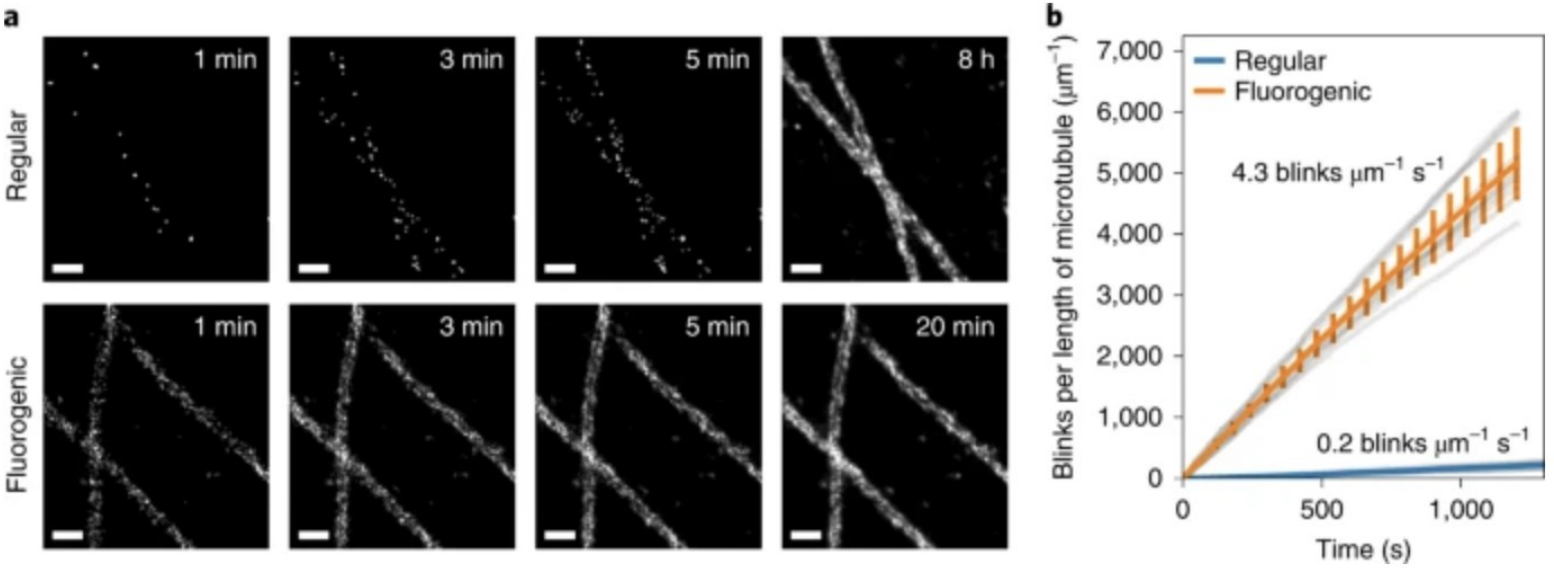
Super-resolution : (DNA)-PAINT

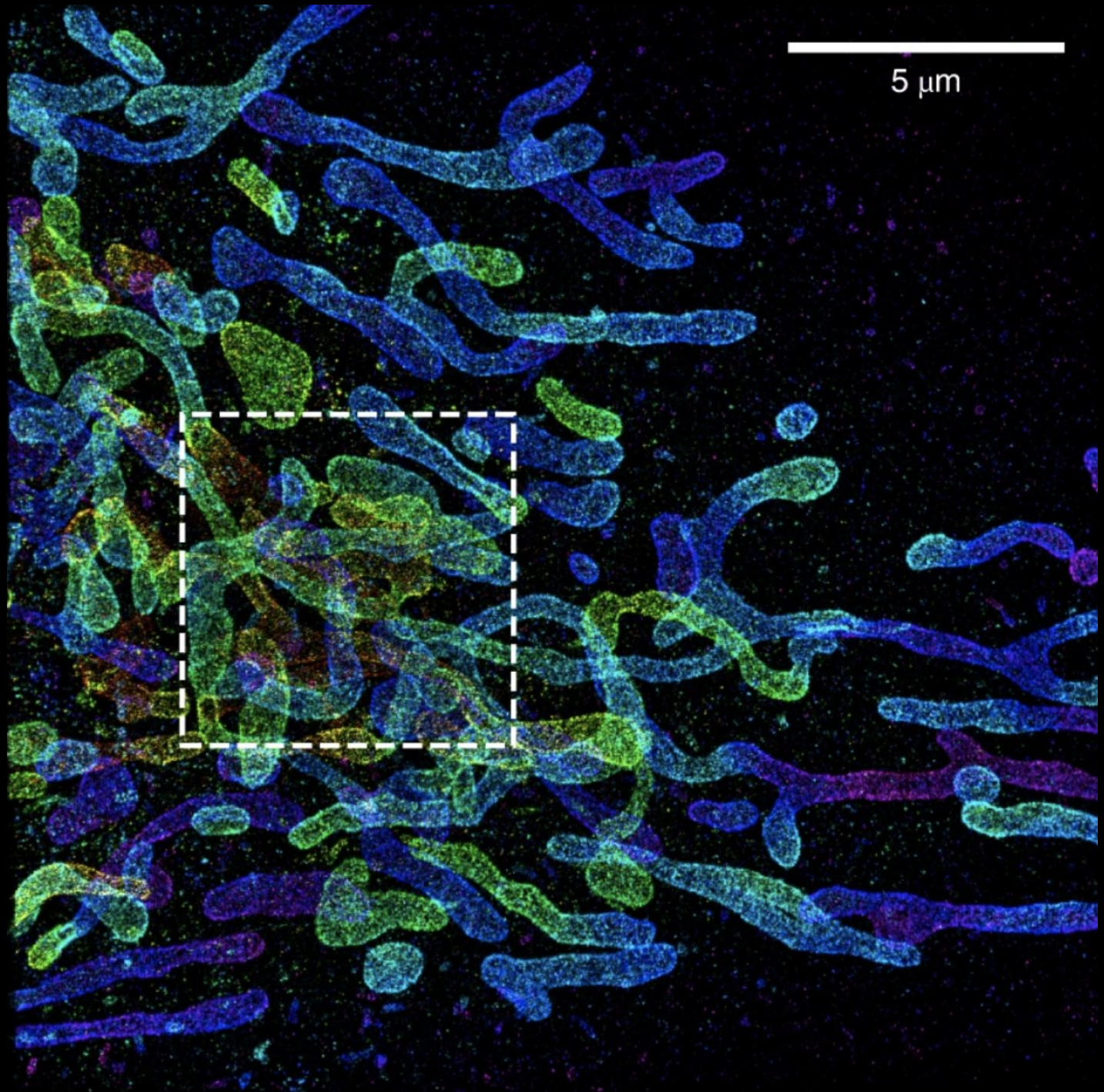


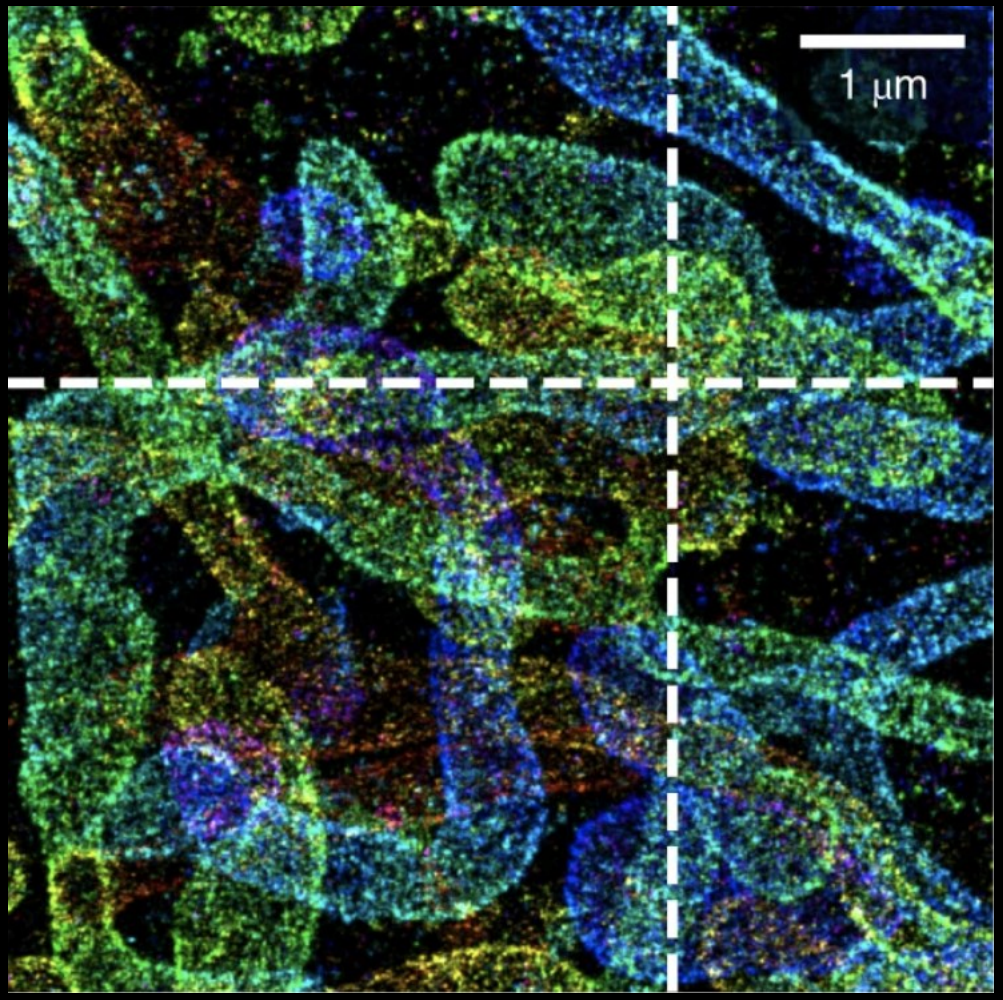
Jungmann, R., Avendaño, M., Woehrstein, J. *et al. Nat Methods* **11**, 313–318 (2014). https://doi.org/10.1038/nmeth.2835

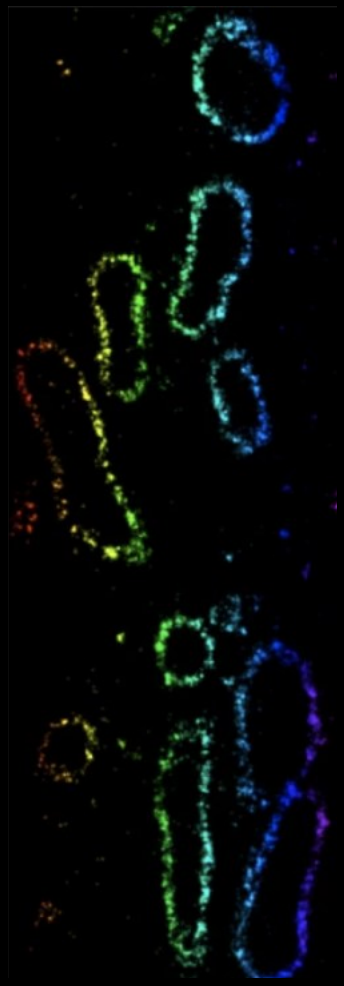
Super-resolution : Fluorogenic DNA-PAINT

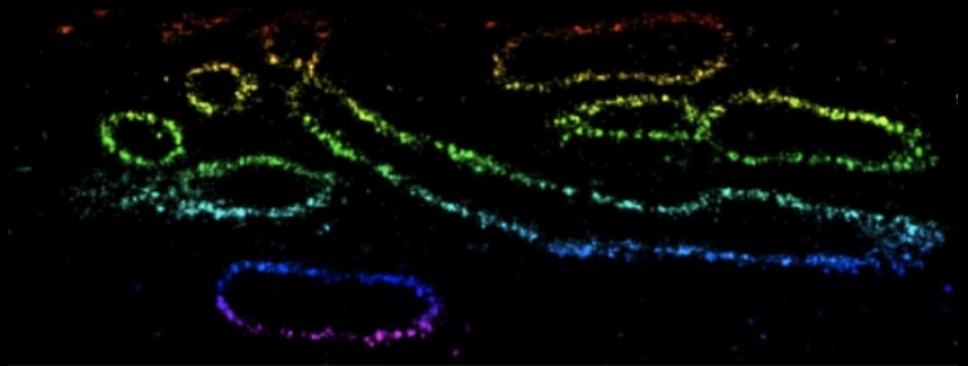


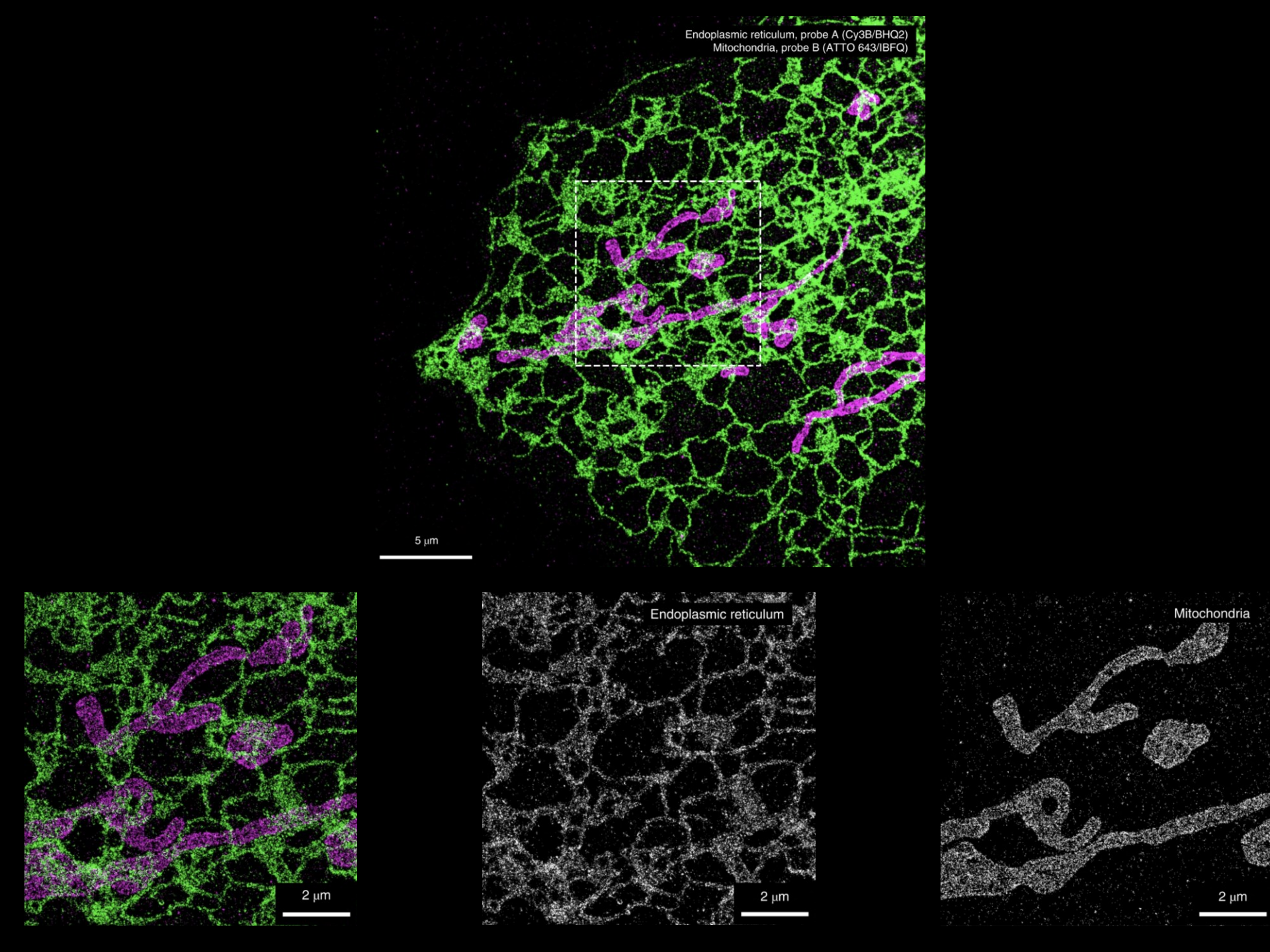



5 µm

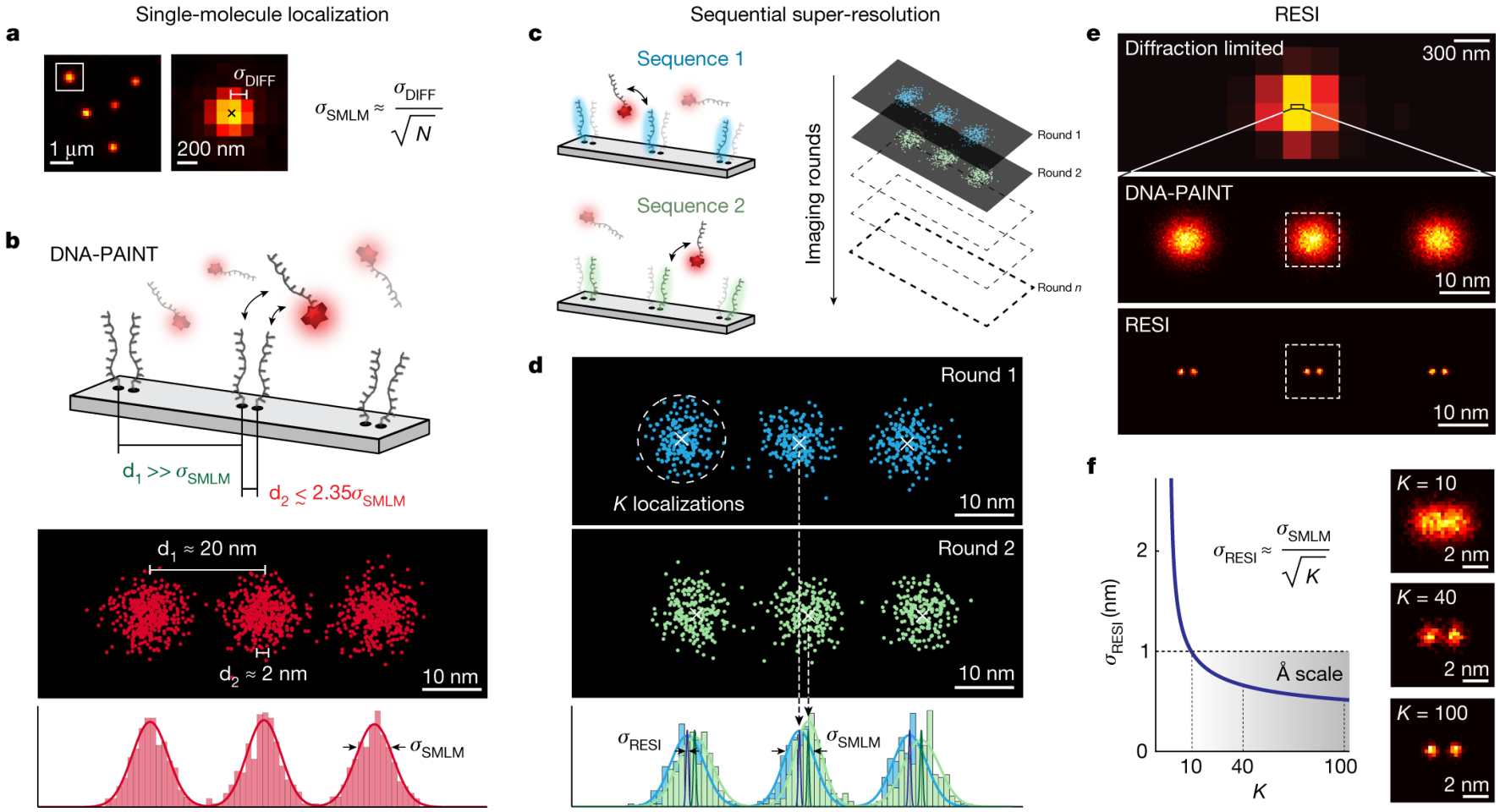

1 µm





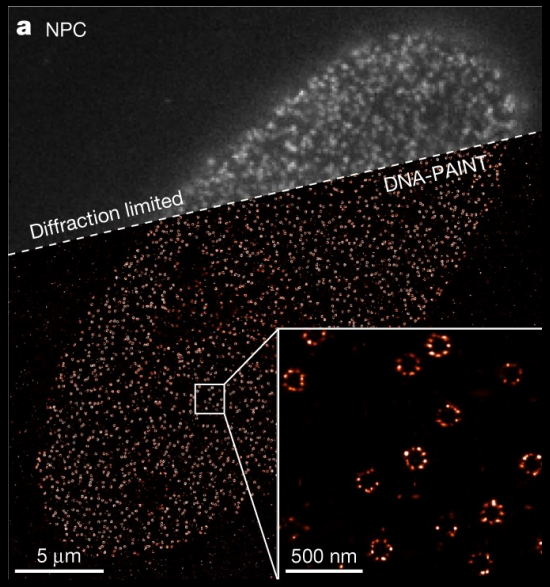

Endoplasmic reticulum, probe A (Cy3B/BHQ2)
Mitochondria, probe B (ATTO 643/IBFQ)
5 µm
2 µm
Endoplasmic reticulum
2 µm
Mitochondria
2 µm

Super-resolution : Resolution enhancement by sequential imaging (RESI)



Reinhardt, S.C.M., Masullo, L.A., Baudrexel, I. *et al. Nature* **617**, 711–716 (2023). https://doi.org/10.1038/s41586-023-05925-9

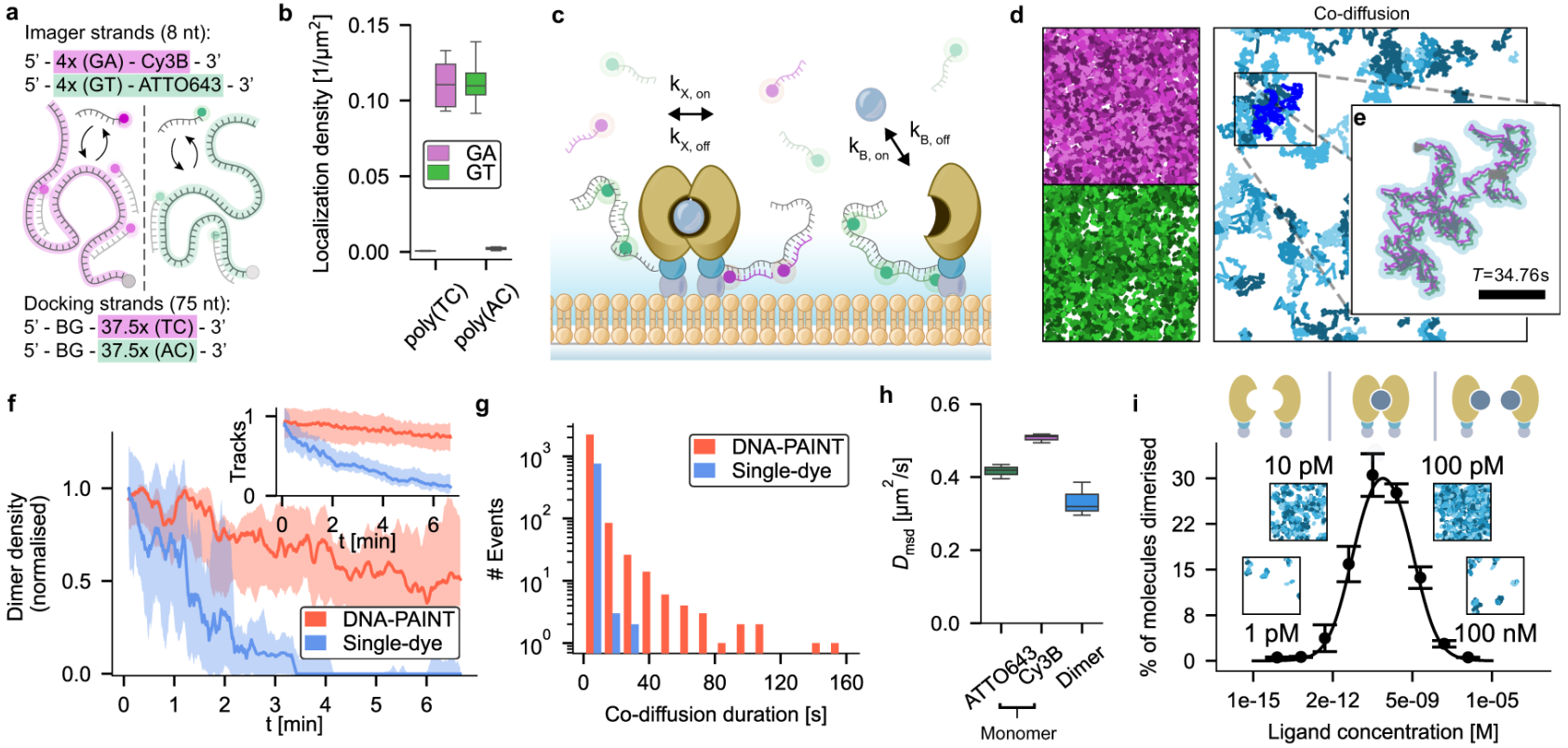
Super-resolution : Resolution enhancement by sequential imaging (RESI)



Reinhardt, S.C.M., Masullo, L.A., Baudrexel, I. *et al. Nature* **617**, 711–716 (2023). https://doi.org/10.1038/s41586-023-05925-9

Super-resolution : Dual-color DNA-PAINT single-particle tracking

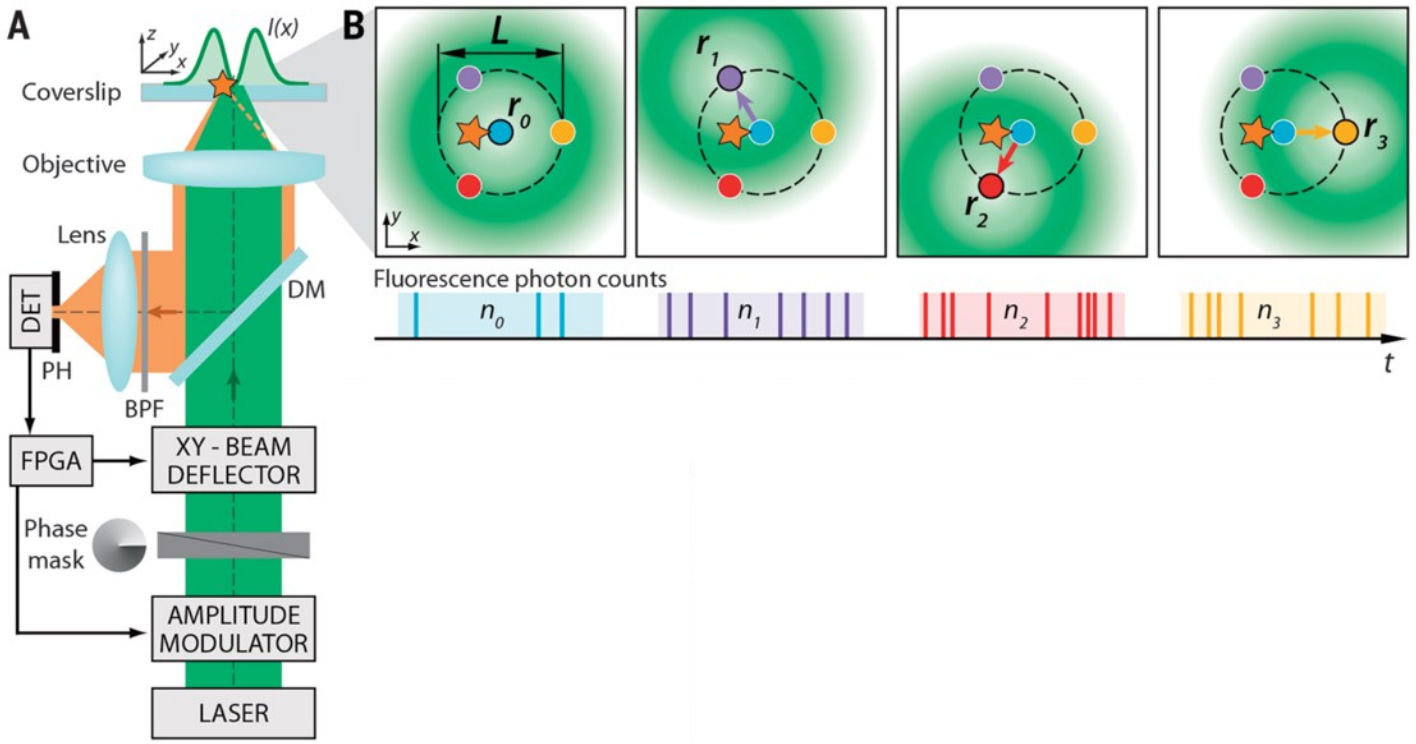
Several imager strands can bind the docking strand:
- No “Photobleaching”
- Actual interaction time accessible
=> **Quantitative** study of FKBP homodimerization



Niederauer, C., Nguyen, C., Wang-Henderson, M. et al. Nat Commun 14, 4345 (2023). https://doi.org/10.1038/s41467-023-40065-8

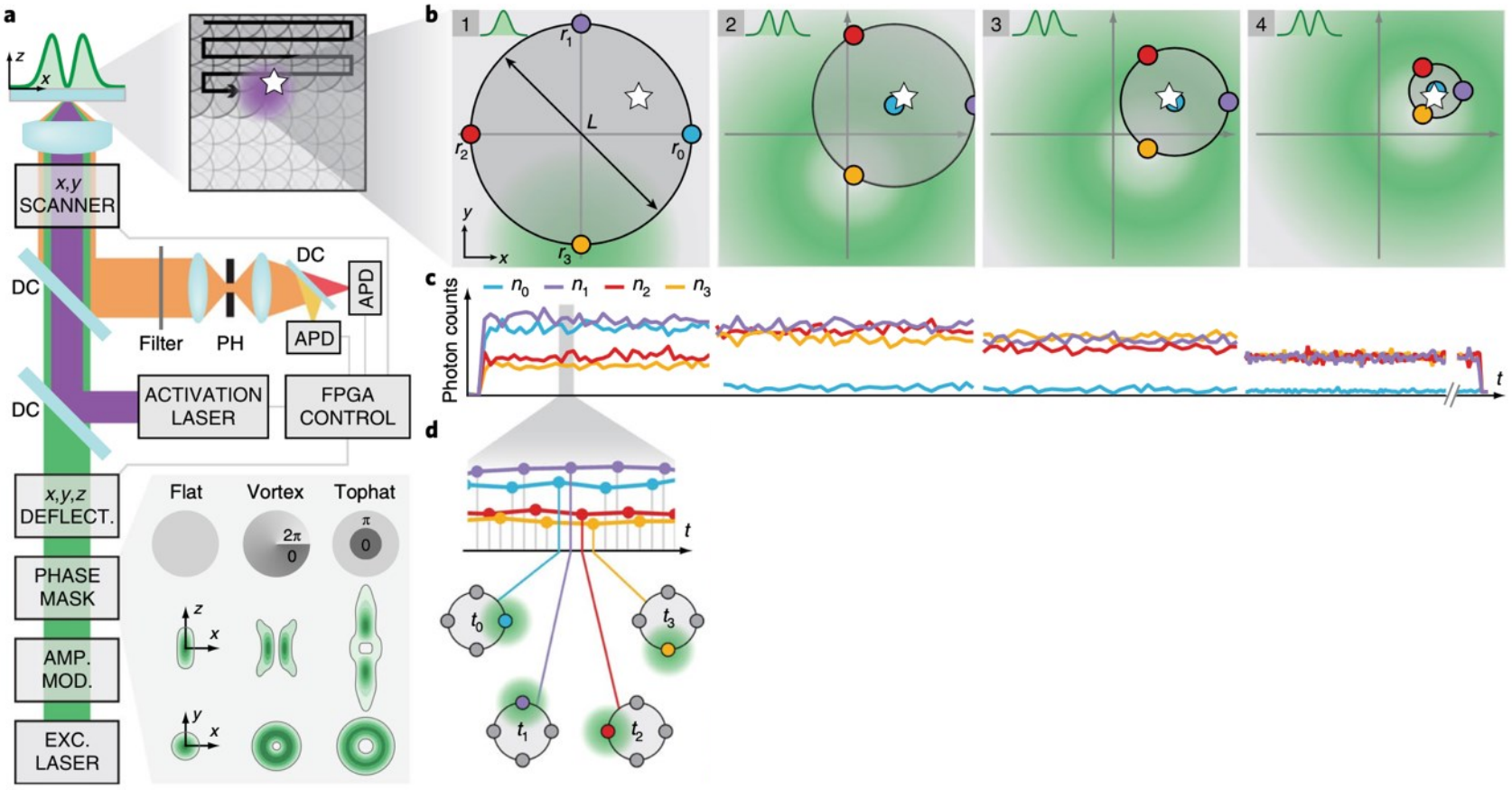
Super-resolution: Miniflux

Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes



Balzarotti, F. *et al. Science* **355,** 606–612 (2017).

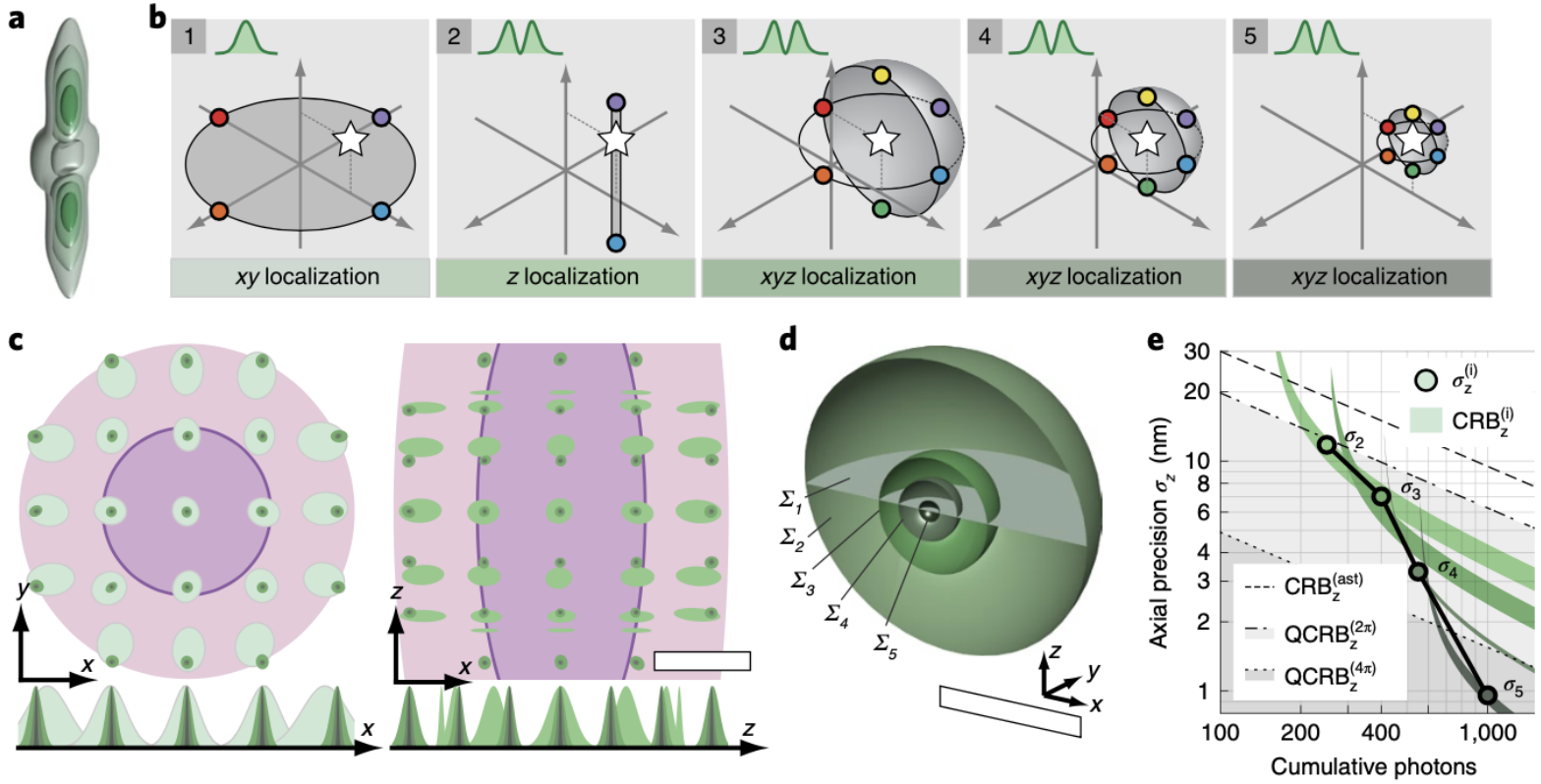
Super-resolution: Iterative 3D-Miniflux



Multiple localization steps using gaussian and doughnuts profiles

Super-resolution: Iterative 3D-Miniflux

K. C. Gwosch et al., *Nat Methods*, **17**, 2, 217–224, 2020

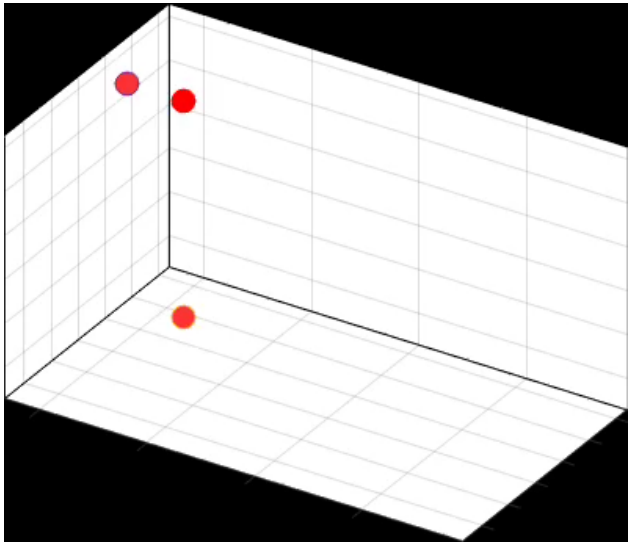


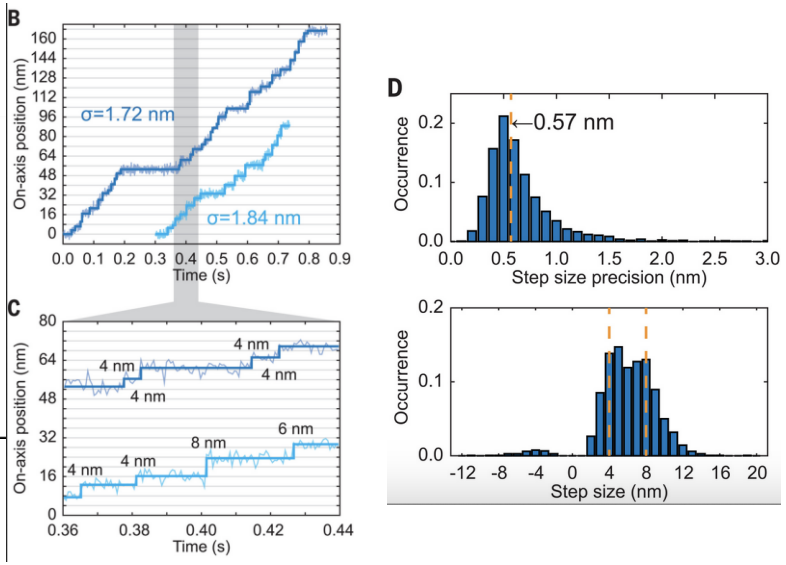
Super-resolution: MINFLUX Kinesin Again!!

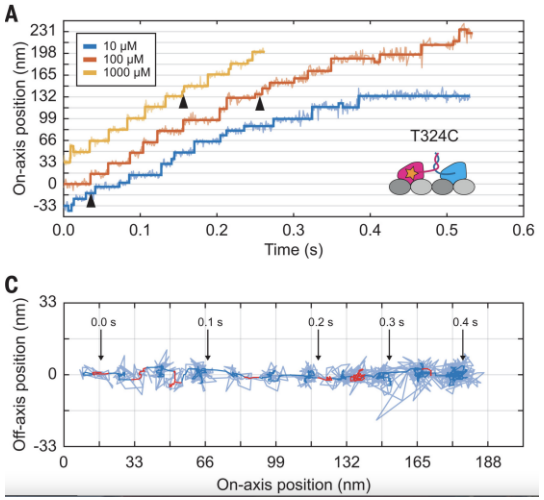
MINFLUX allow a new perspective on motor movement

Movement in living cells

3D motor-PAINT in permeabilized cells





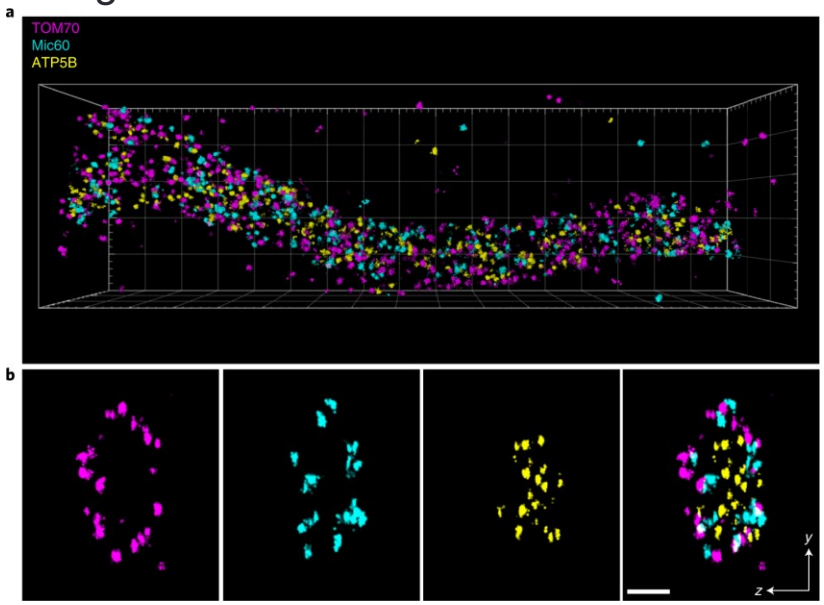


Science 9 Mar 2023 Vol 379, Issue 6636 pp. 1010-1015 DOI: 10.1126/science.ade2676

Science 9 Mar 2023 Vol 379, Issue 6636 pp. 1004-1010 DOI: 10.1126/science.ade2650

Super-resolution: MINFLUX DNA-PAINT

Multiplexing

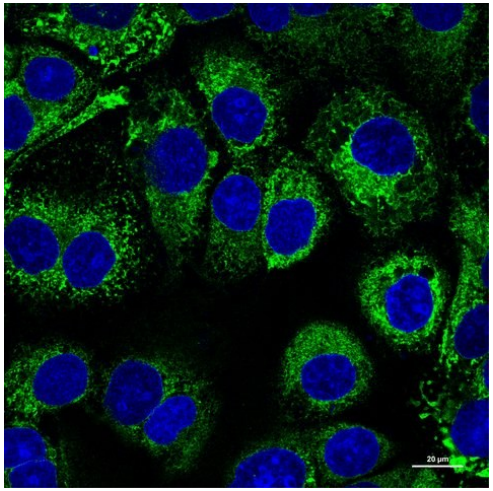


Sequential labelling and imaging using DNA-PAINT

Ostersehlt, L.M., Jans, D.C., Wittek, A. et al. Nat Methods 19, 1072–1075 (2022). https://doi.org/10.1038/s41592-022-01577-1

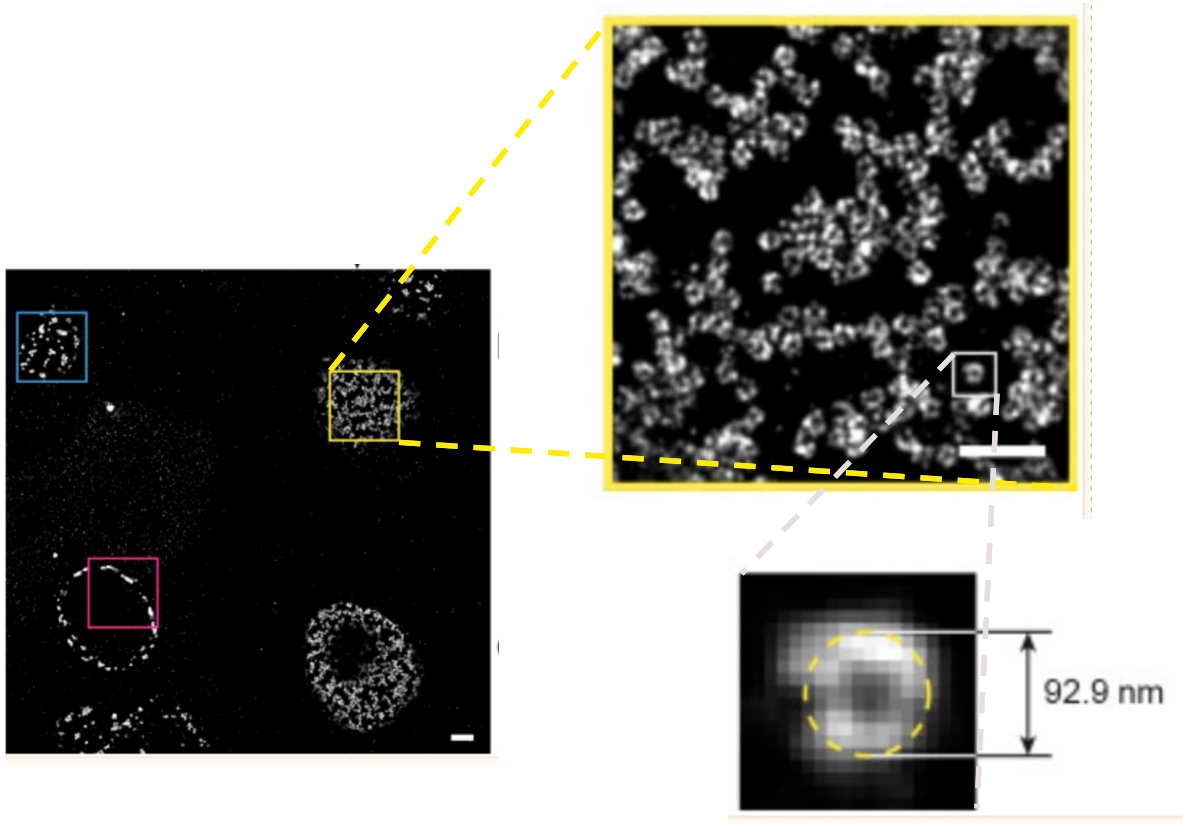
Super resolution: a scale issue for mechanisms understanding

Confocal microscopy: whole cell
~300 nm resolution



ER structure can be well defined using DAPI staining of the nucleus

STORM nuclear pores ~30 nm resolution

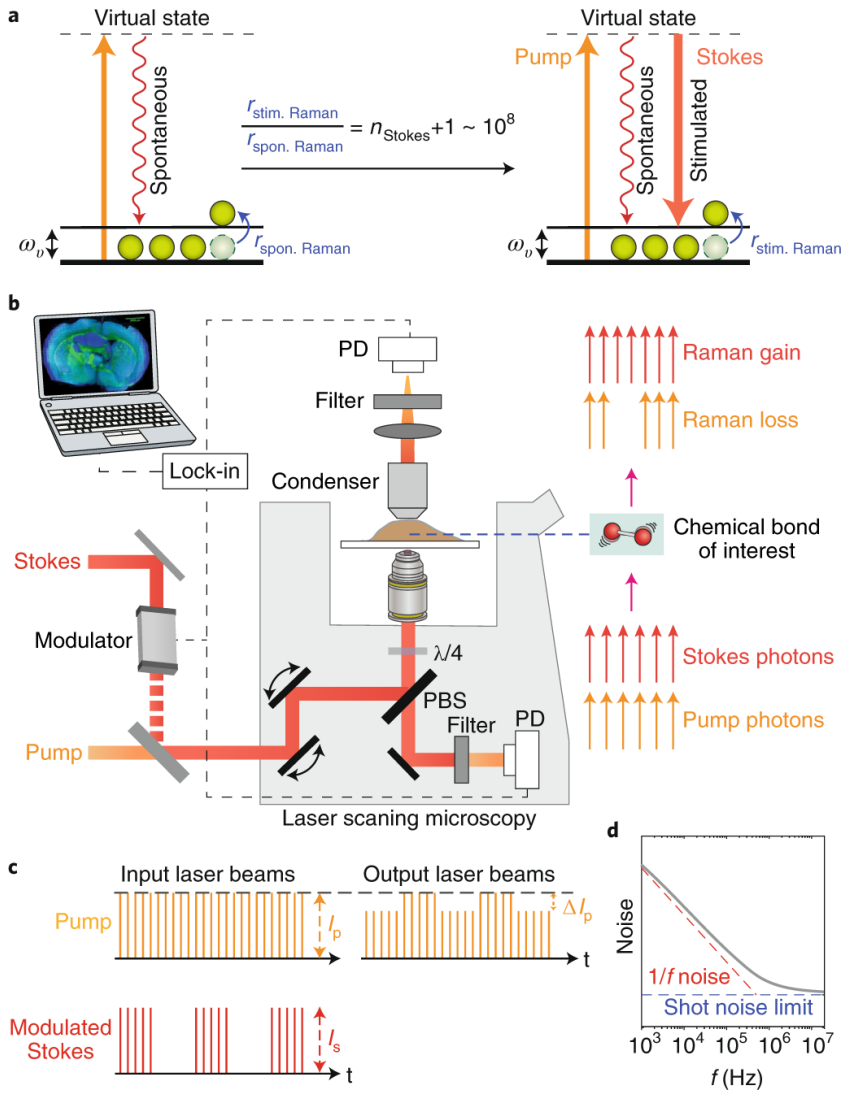


The region of interest is a ROI in a ROI taken in the nucleus

How is it possible to get background information for MINFLUX at 3 nm resolution?

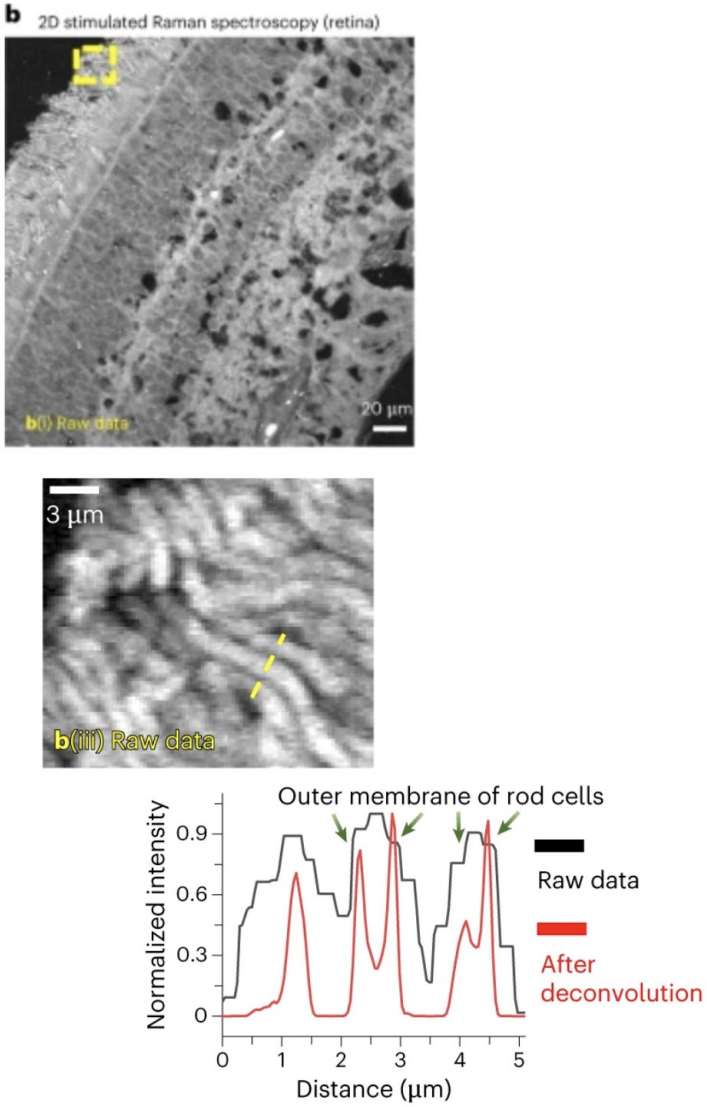
Super-resolution: towards **label-free** high resolution SRS?

Stimulated Raman Spectroscopy



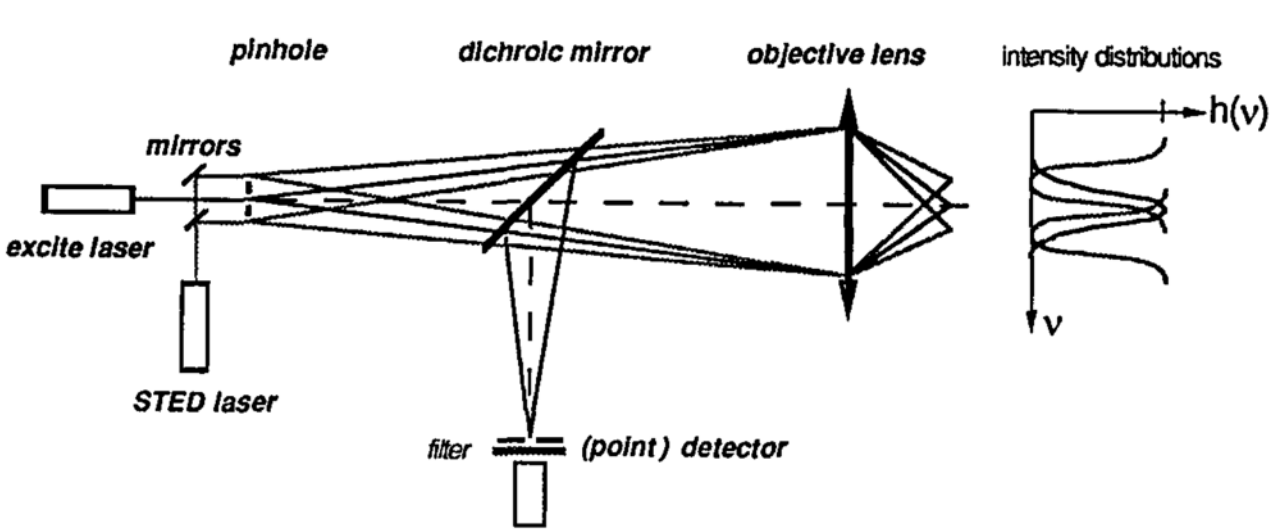
Hu, F., Shi, L. & Min, W. *Nat Methods* **16**, 830–842 (2019). https://doi.org/10.1038/s41592-019-0538-0

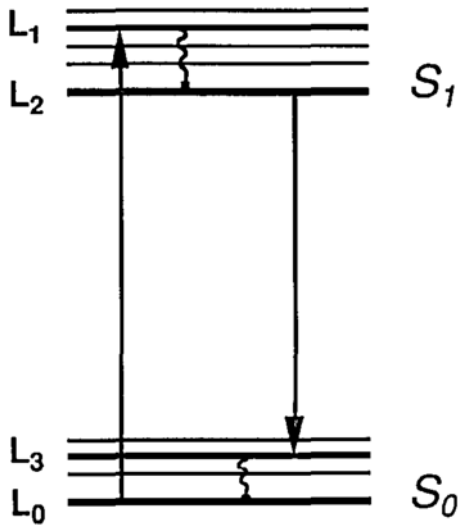
Standard SRS



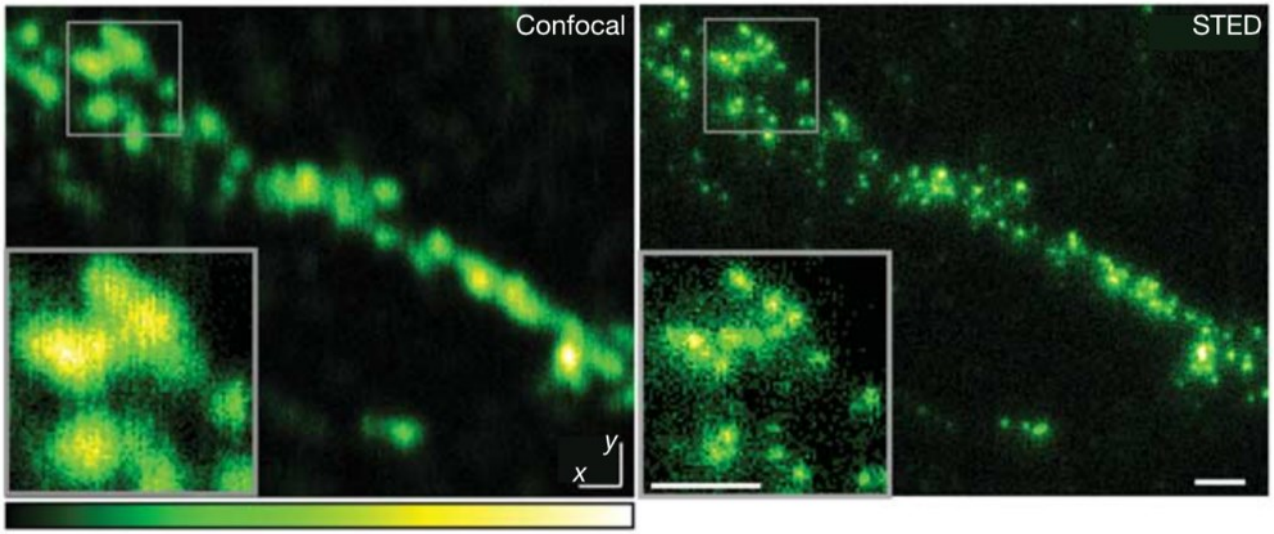
Jang, H., Li, Y., Fung, A.A. *et al. Nat Methods* **20**, 448–458 (2023). https://doi.org/10.1038/s41592-023-01779-1

Super-resolution : Confocal STED microscopy



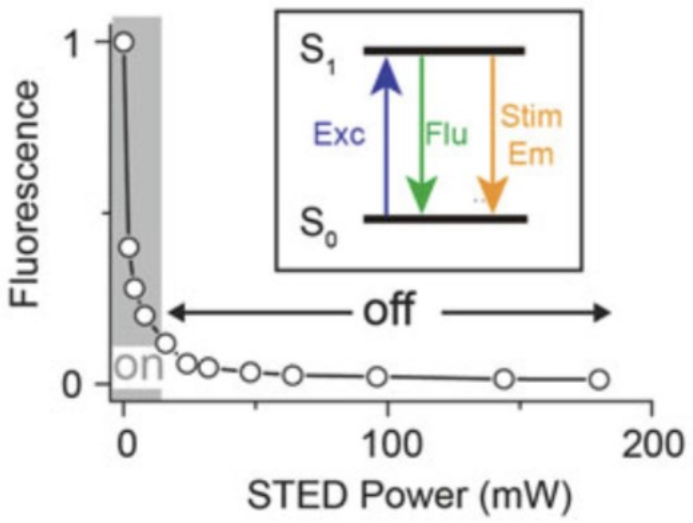


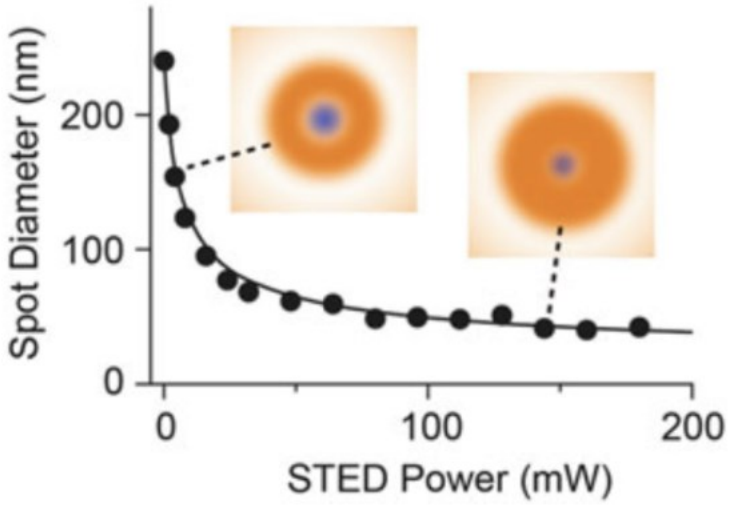
Hell, S. W., & Wichmann, J. (1994). *Optics Letters*, *19*(11), 780–782.

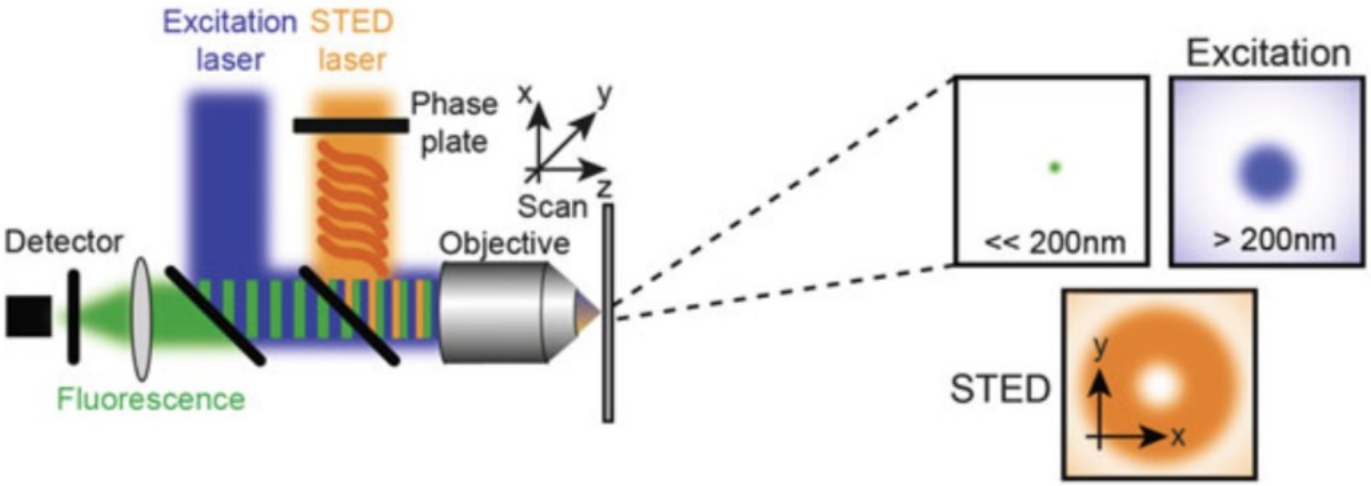


Willig, K. I., Rizzoli, S. O., Westphal, V., Jahn, R., & Hell, S. W. (2006). *Nature Cell Biology*, *440*(7086), 935–939. http://doi.org/10.1038/nature04592

Super-resolution: Confocal STED microscopy







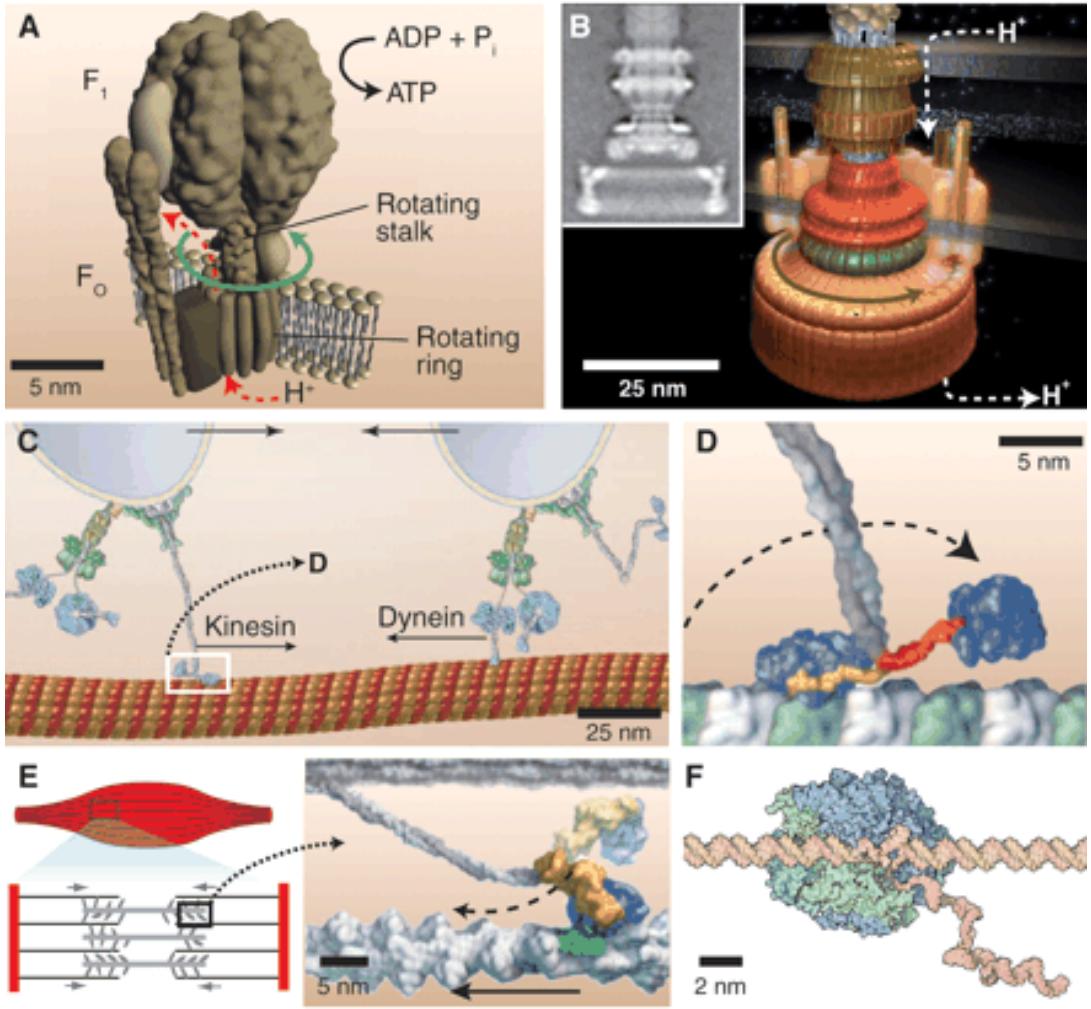
Blom, H., & Widengren, J. (2014). *Current Opinion in Chemical Biology*, *20*, 127–133. http://doi.org/10.1016/j.cbpa.2014.06.004

Functional devices based on single-molecule

Outlook: How can one integrate nano-machines into functional devices?

- Molecular motors
- Nano-bioanalytics, nano-containers: Reducing the sample volumes
- Nano-sensors: Combination with electrophysiology
- Nano-sequencer: DNA, RNA and peptide sequencing

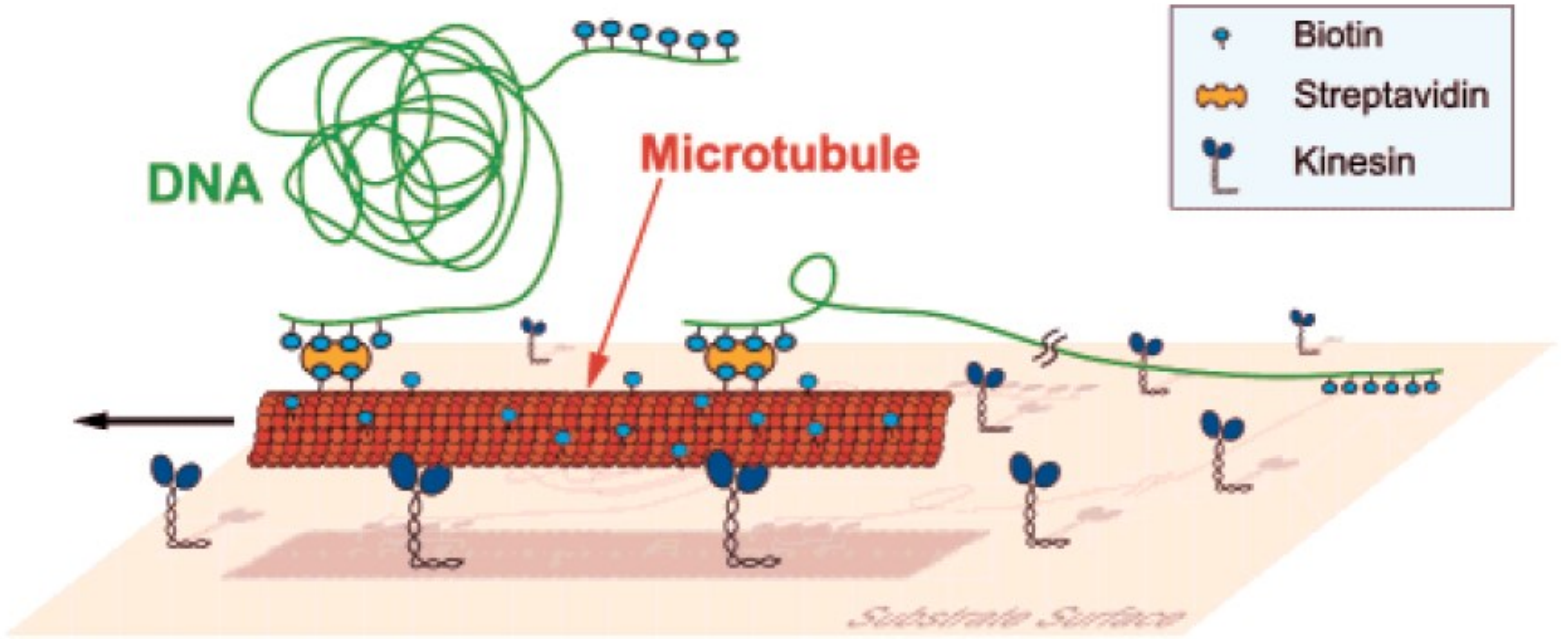
Molecular Motors



M. G. L. van den Heuvel, C. Dekker, *Science* **317,** 5836, (2007) 333 – 336

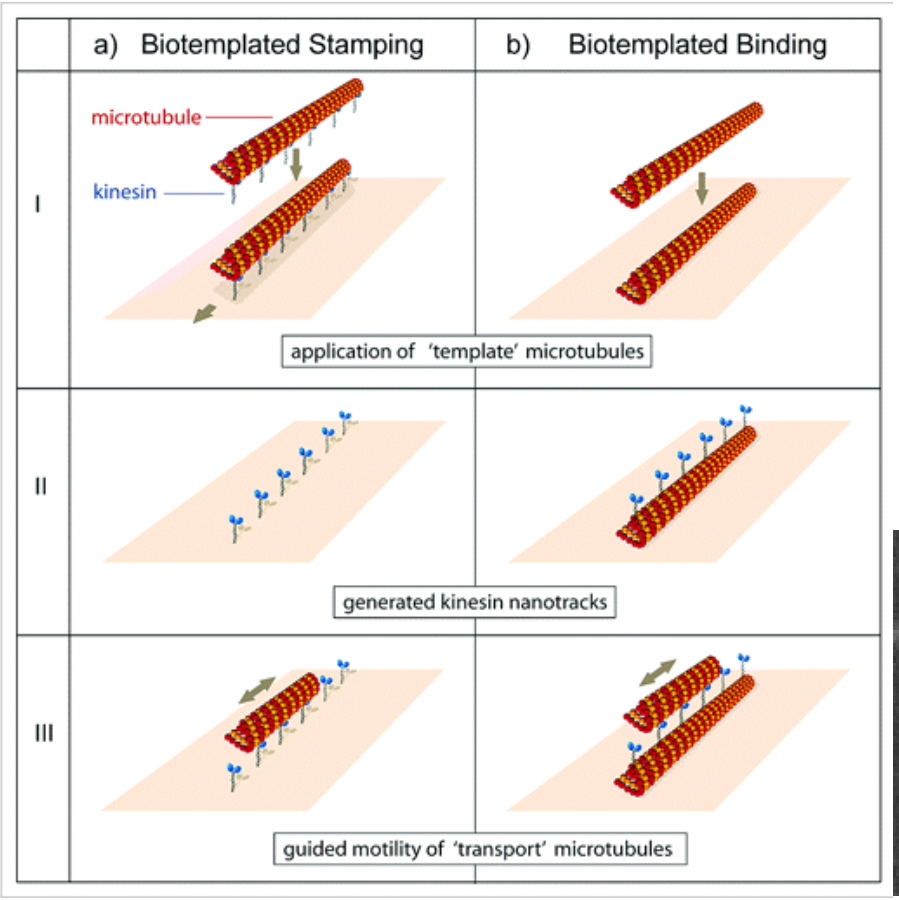
Processing motors: Moving cargos

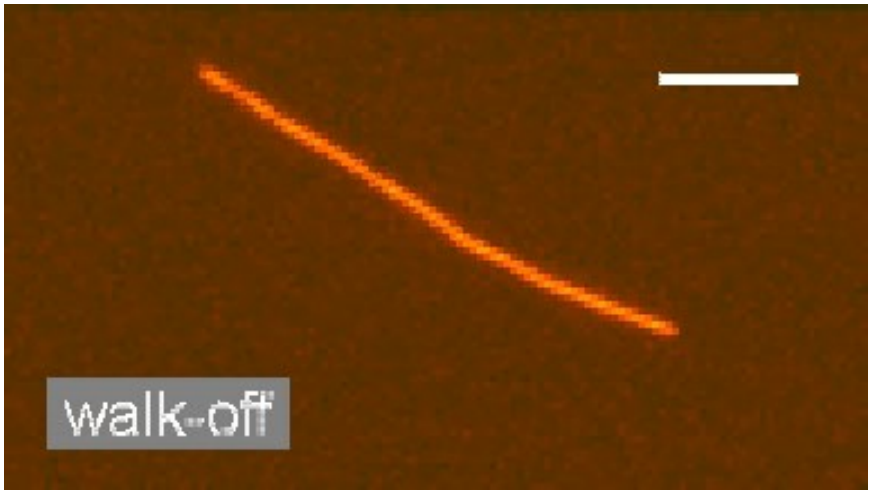
Motor: kinesin. Cargos: DNA attached to microtubules

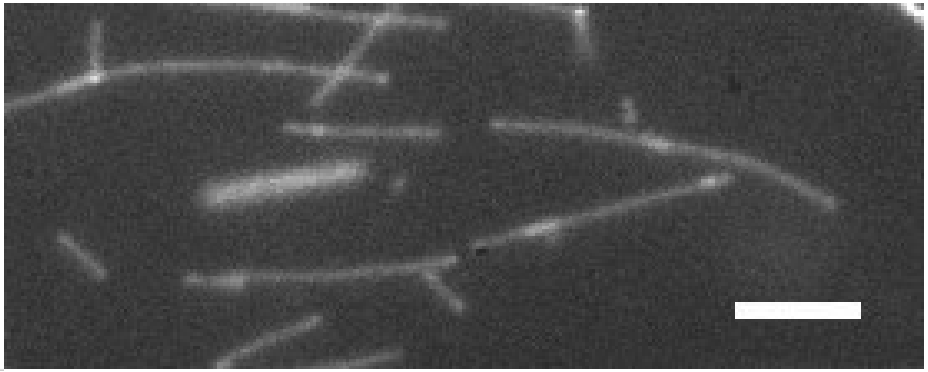


S. Diez, C. Reuther, C. Dinu, R. Seidel, M. Mertig, W. Pompe, J. Howard, *Nano Lett.* **3**, 1251 (2003)

Kinesin nanotracks



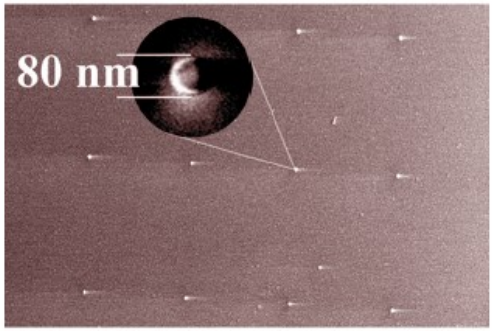


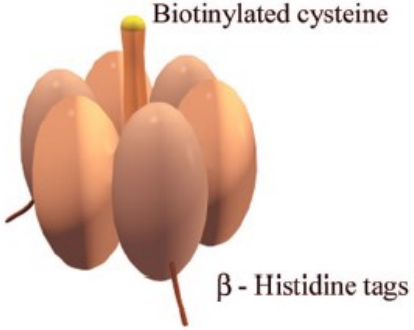


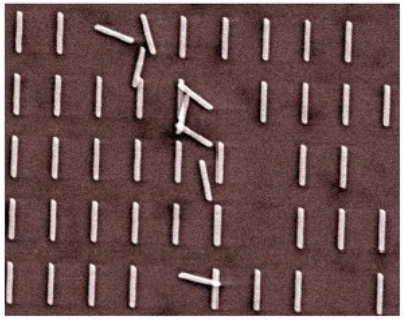
C. Reuther,[†] L. Hajdo,[‡] R. Tucker,[§] A. A. Kasprzak,[‡] S. Diez*[†] *Nano Lett.*, 2006, 6 (10), 2177–2183

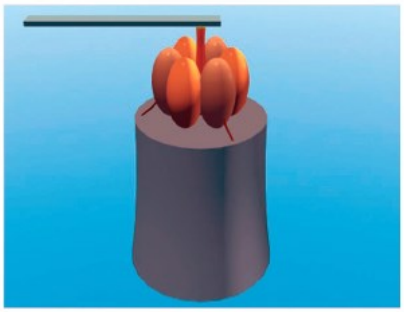
Rotary motors: F1 ATPase

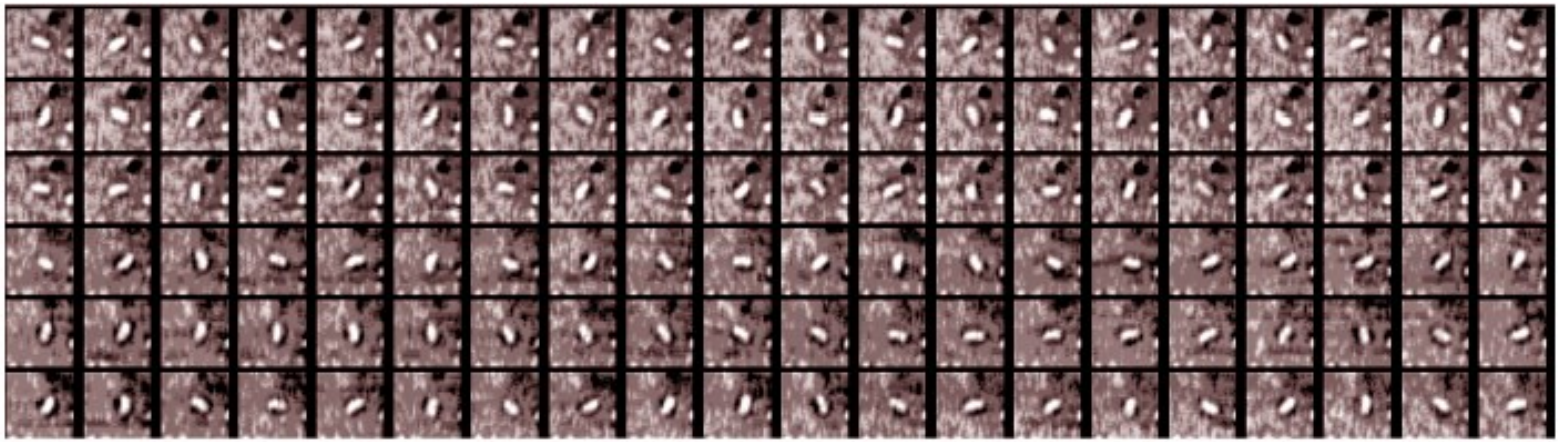
F1 ATPase attached to Ni posts by His Tags is used to rotate Ni arms





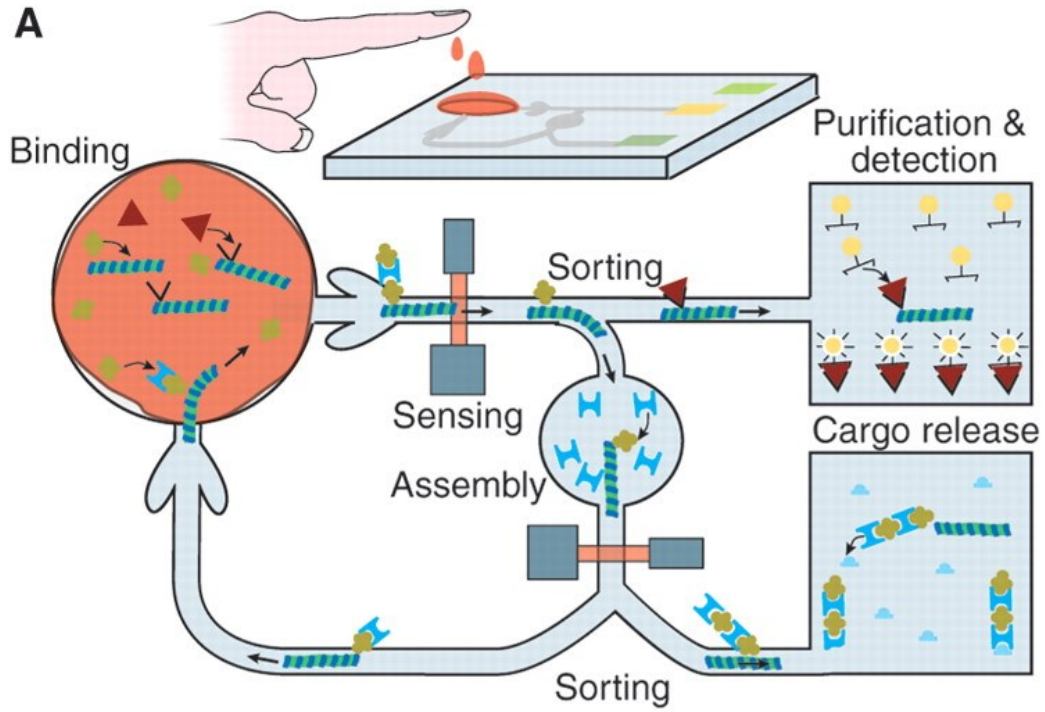


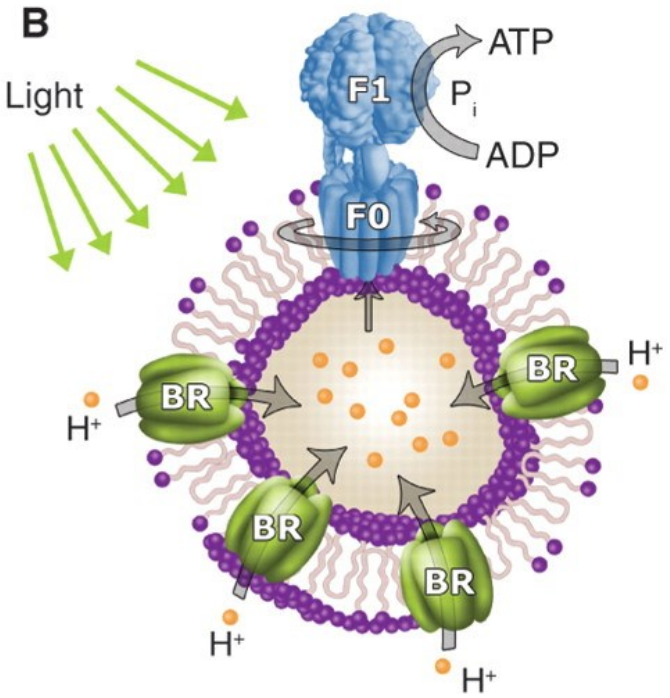


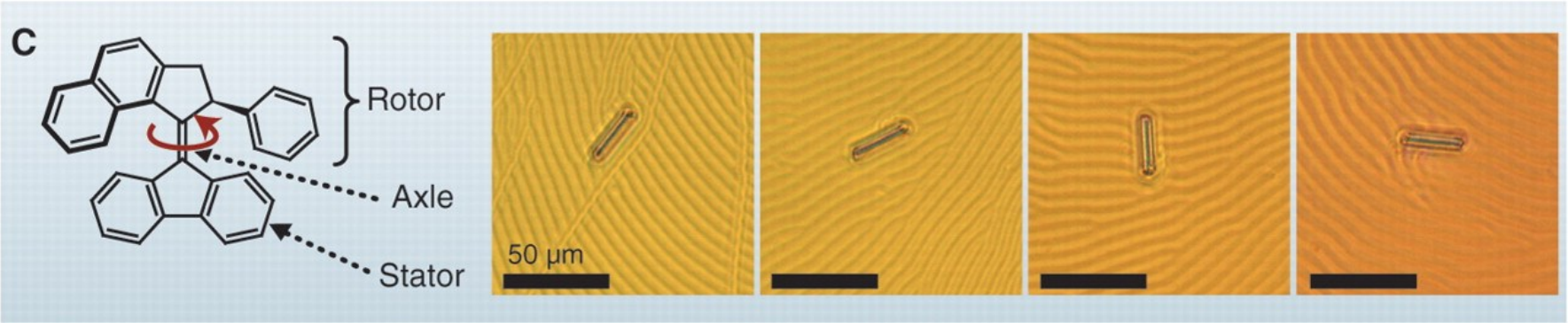


R.K. Soong, G.D. Bachand, H.P. Neves, A.G. Olkhovets, H.G. Craighead, C.D. Montemagno, *Science* **290**, 1555 (2000)

New directions with nanomotors

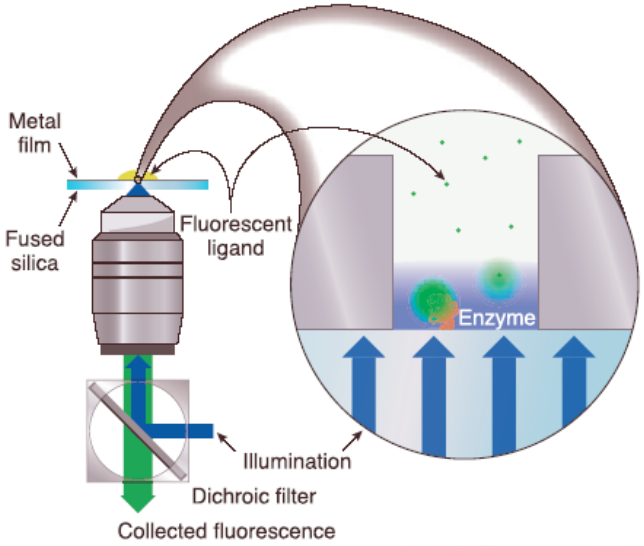


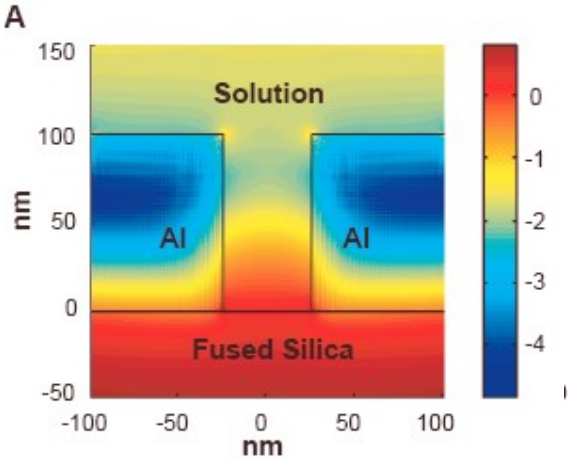


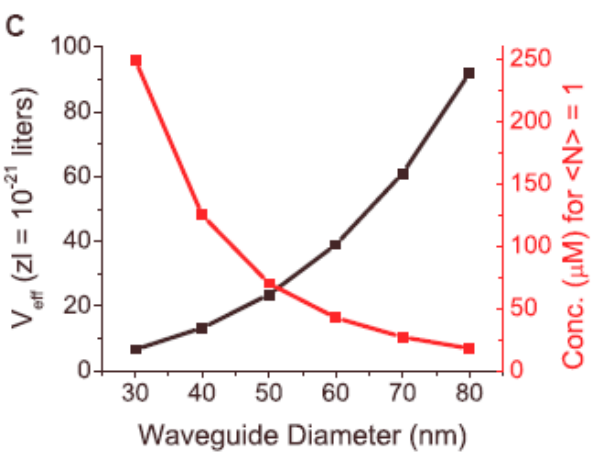


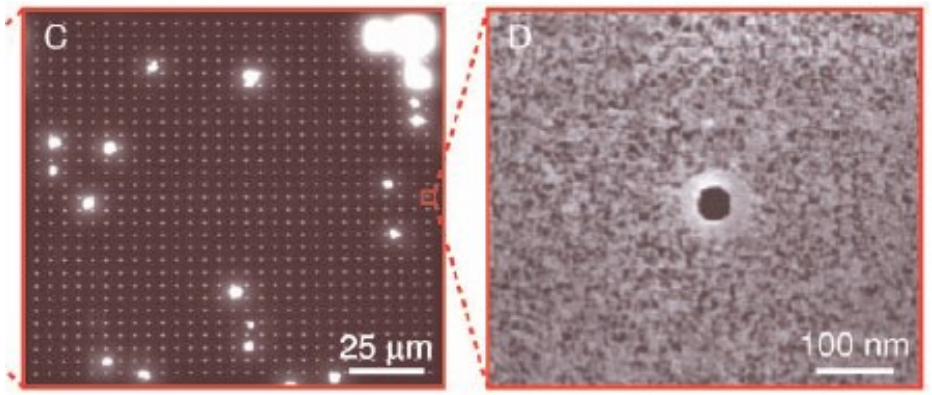
M. G. L. van den Heuvel, C. Dekker, *Science* **317,** 5836, (2007) 333 – 336

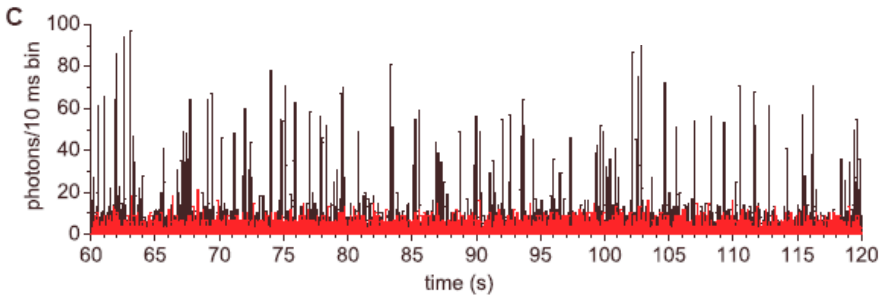
Reducing the volumes for nano-bioanalytics: Nanowells











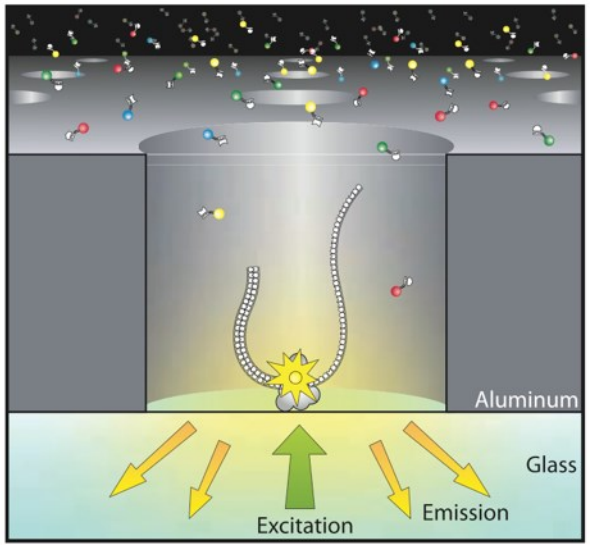
$$I(z) = \exp(-z/\Lambda)$$

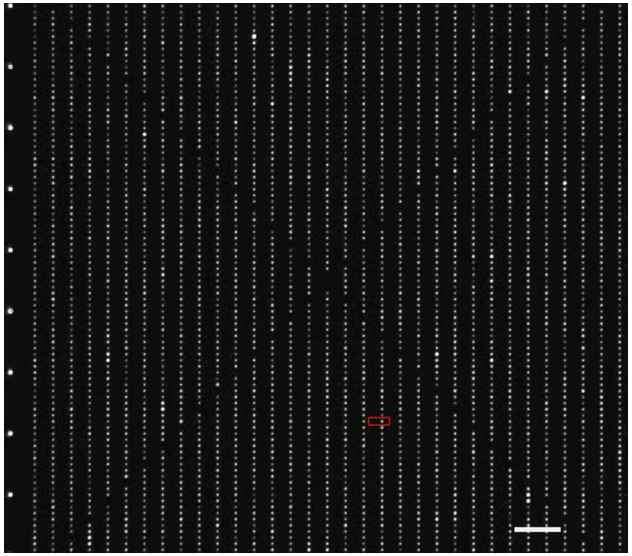
$$\frac{1}{\Lambda} = \sqrt{2\frac{1}{\lambda_c^{\,2}} - \frac{1}{\lambda_m^{2}}}$$

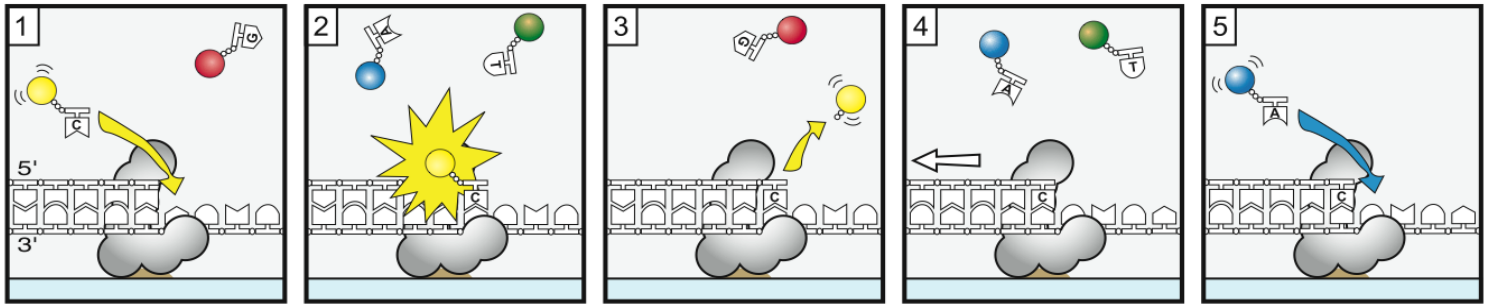
Principle: Small wells in Al with a diameter smaller than the wavelength of light. The light penetrates only up to a small depth in z.

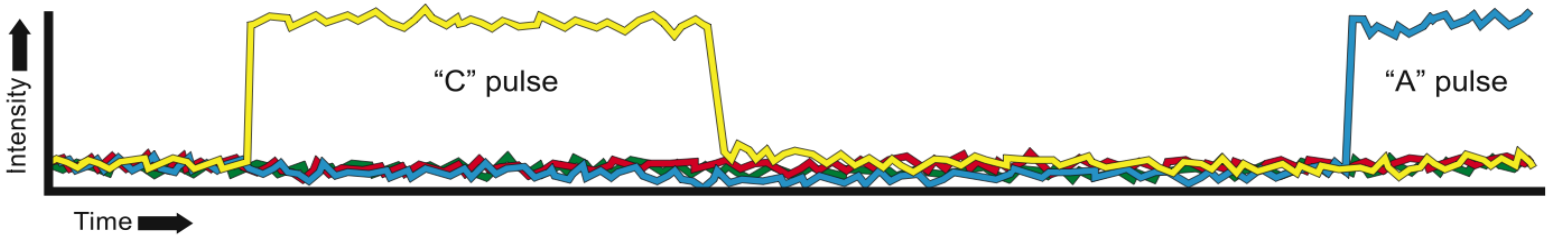
M.J. Levene, J. Korlach, S.W. Turner, M. Foquet, H.G. Craighead, W.W. Webb, *Science* **299**, 682 (2003)

Nanowells (Zeroth mode waveguide): DNA sequencing



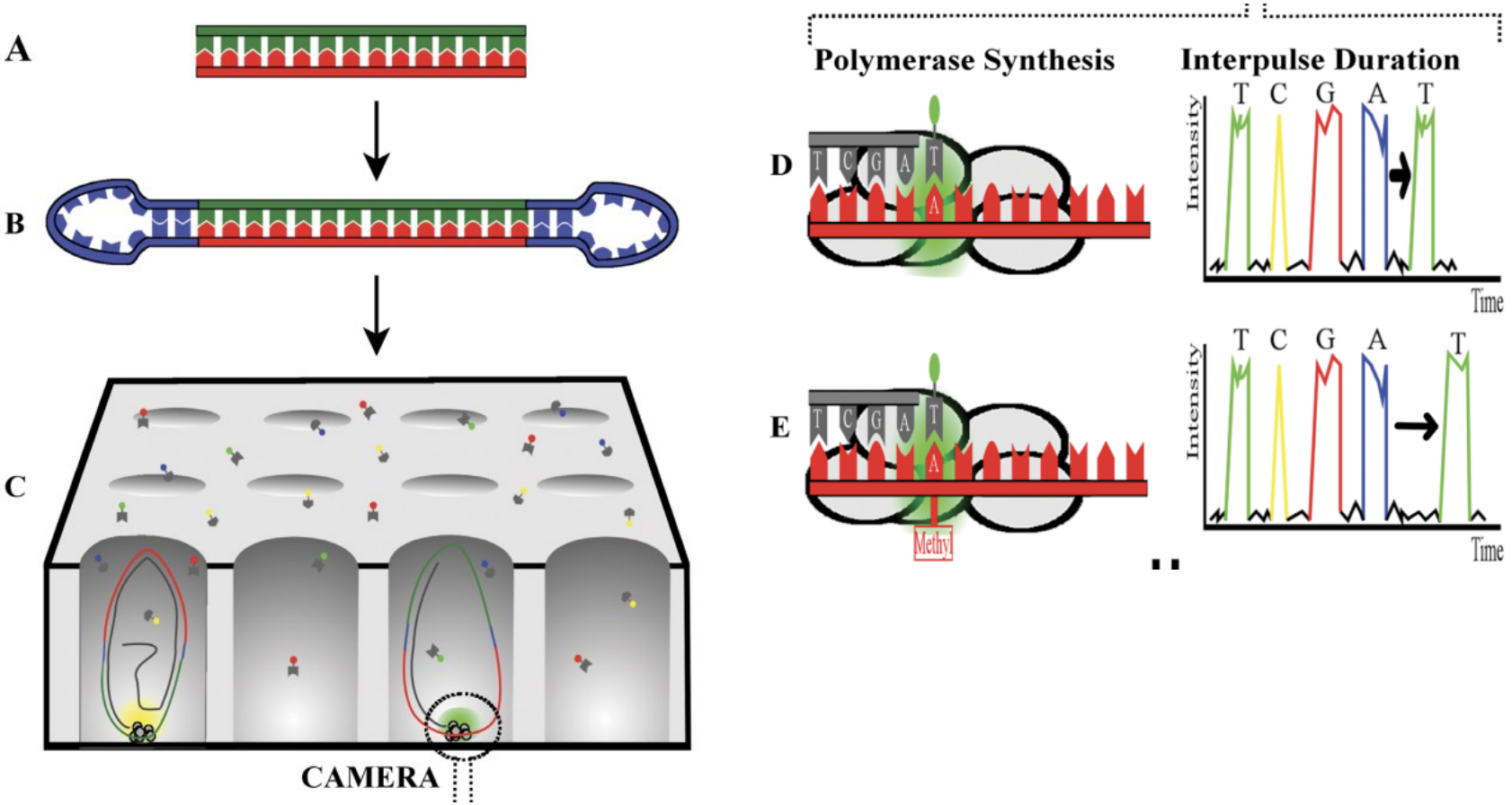






J.Eid, et al., *Science* **323** (2009) 133-138

Nanowells (ZMW): Single molecule real-time (SMRT) sequencing



S. Ardui, et al, *Nucleic Acids Res*, vol. 46, no. 5, pp. 2159–2168, 2018.

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P. J. Walla, *Modern Biophysical Chemistry*. John Wiley & Sons, 2015

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Single-Molecule

S. Weiss, "Fluorescence spectroscopy of single biomolecules", Science **283**, 1676

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P. Tinnefeld and M. Sauer, “Branching Out of Single-Molecule Fluorescence

Spectroscopy: Challenges for Chemistry and Influence on Biology”, Angew. Chem. Int. Ed. **44,** 2642 (2005)

X. Michalet, Sh. Weiss, and M. Jäger, “Single-Molecule Fluorescence Studies of

Protein Folding and Conformational Dynamics”, Chem. Rev. **106**, 1785 (2006)

C. Bräuchle, D. C. Lamb, and J. Michaelis, *Single Particle Tracking and Single Molecule Energy Transfer*. John Wiley & Sons, 2009.

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P. Tinnefeld, C. Eggeling & S. W. Hell, *Far-Field Optical Nanoscopy*. (Springer, 2015)

http://www.microscopyu.com/references/single-molecule-localization

MINFLUX

Francisco Balzarotti *et al.* Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes.*Science***355**,606-612(2017).DOI:10.1126/science.aak9913

Ostersehlt LM, Jans DC, Wittek A, Keller-Findeisen J, Inamdar K, Sahl SJ, Hell SW, Jakobs S. DNA-PAINT MINFLUX nanoscopy. Nat Methods. 2022 Sep;19(9):1072-1075. doi: 10.1038/s41592-022-01577-1

Typical fluorophores properties

Fluorophore	λ_{exc} (nm)	λ_{em}^{max} (nm)	QE (in H_2O)	τ_f (ns)	I_s (kW/cm^2)	k_∞ (kcnts/ms)	τ_{bl}^{∞} (ms)	N_{max} (10^6 photons)	ε ($M^{-1}cm^{-1}$)	Brightness Rel to EGFP
fluorescein	488	520	0.71	-	-	-	-	< 0.1	68000	146
TMR	514	580	0.28	2.1	5.6±1.6	4.0±1.	11.5±2.5	0.6±0.2	95000	81
Cy3	532	568	0.14	~1	-	-	-	-	150000	64
Cy5	630	670	0.18	~1	2.2±1.0	2.3±0.3	14.8±0.3	0.67±0.11	250000	136
Atto488	501	523	0.80	4	-	-	-	-	90000	218
Atto565	563	592	0.90	4	-	-	-	-	120000	327
STAR635	634	654	0.51	2.8	-	-	-	-	63000	97
QF640R	630	670	-	-	-	-	-	-	105000	295
Atto647N	644	669	0.65	3.5	-	-	-	-	150000	295
Alexa647	650	665	0.33	1	-	-	-	-	237000	237
ECFP	439	476	0.40	-	-	-	-	-	32500	39
mTurquoise	434	474	0.84	3.7	-	-	-	-	30000	76
EGFP	488	512	0.60	3.2	13±3	2.9±0.2	2.8±0.2	0.14±0.05	55000	100
EYFP	514	527	0.61	3.7	9±2	3.1±0.3	2.6±0.1	0.14±0.05	83400	154
mCitrine	516	529	0.76		-	-	-	-	77000	177
mCherry	587	610	0.22	1.46	-	-	-	-	72000	48
mPlum	590	649	0.10	~1	-	-	-	-	41000	12
PA-GFP d	400	515	0.13	-	-	-	-	-	20700	8
PA-GFP a	504	517	0.79	-	-	-	-	-	17400	42
PA-mCherry a	564	595	0.46	-	-	-	-	-	18000	25
Dronpa (G)	503	517	0.85	-	-	-	-	-	95000	245
mEOS2 (G)	506	519	0.74	-	-	-	-	-	56000	126
mEOS2 (R)	573	584	0.66	-	-	-	-	-	46000	92

Dyes for superresolution microscopy (nanoscopy)

TABLE 1. Spectral properties of photoswitchable fluorescent proteins and organic dyes

		Before activation		After activation		
Fluorophore	Activation (nm)	Absorption max (nm)	Emission max (nm)	Absorption max (nm)	Emission max (nm)	Reference(s)
Fluorescent proteins						
PA-GFP	405	400	515	504	517	Patterson and Lippincott-Schwartz (2002)
PS-CFP2	405	400	468	490	511	Chudakov et al. (2004)
Dendra-2	405	490	507	553	573	Gurskaya et al. (2006)
Kaede	405	508	518	572	582	Ando et al. (2002)
EosFP, mEos2	405	506	516	571	581	Wiedenmann et al. (2004), McKinney et al. (2009)
mKikGR	405	507	517	583	593	Tsutsui et al. (2005), Habuchi et al. (2008)
Dronpa	405	—	—	503	518	Habuchi et al. (2005)
Dronpa-2	405	—	—	486	513	Ando et al. (2007)
bs-Dronpa	405	—	—	460	504	Andresen et al. (2008)
rsFastLime	405	—	—	496	518	Stiel et al. (2007)
eYFP	405	—	—	514	529	Dickson et al. (1997), Biteen et al. (2008)
PA-mCherry	405	—	—	570	596	Subach et al. (2009)
Synthetic dyes						
Cy5	350–570[a]	—	—	649	670	Bates et al. (2005, 2007), Heilemann et al. (2005), Dempsey et al. (2009a)
Cy5.5	350–570[a]	—	—	675	694	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Cy7	350–570[a]	—	—	747	776	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Alexa Fluor 647	350–570[a]	—	—	650	665	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Photochromic rhodamine B	375	—	—	565	580	Folling et al. (2007)
Rhodamine spiroamides	375			537–591[b]	555–620[b]	Belov et al. (2009)
Azido-DCDHF		—	—	570	613	Lord et al. (2009)

PA, photoactivatable; GFP, green fluorescent protein; PS, photoswitchable; CFP, cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; azido-DCDHF, azido-2-dicyanomethylene-3-cyano-2,5-dihydrofuran.

[a]Dependent on the activator dye, if present.

[b]Dependent on the specific compound.

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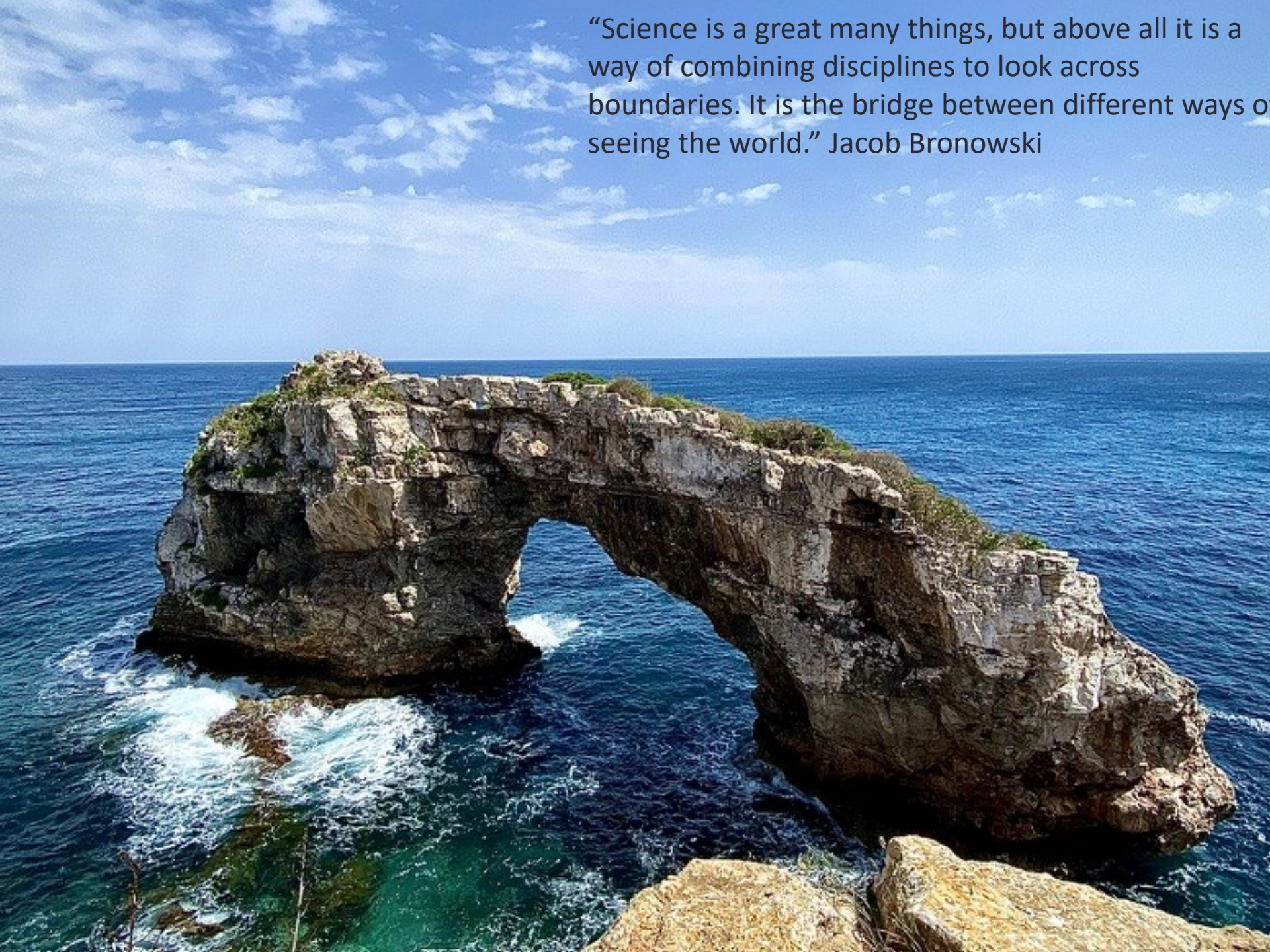
“Science is a great many things, but above all it is a way of combining disciplines to look across boundaries. It is the bridge between different ways o seeing the world.” Jacob Bronowski