Fluorescence imaging and DNA nanotechnology

02.06.2022

Viktorija Glembockyte
Philip Tinnefeld

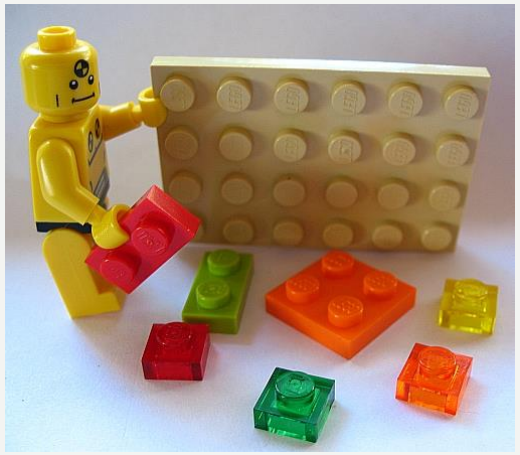
T1PP-M

Pw: DNArules

"see" molecules
on the nanoscale

Single molecule fluorescence imaging

+



place molecules
on the nanoscale

DNA nanotechnology

Reminder

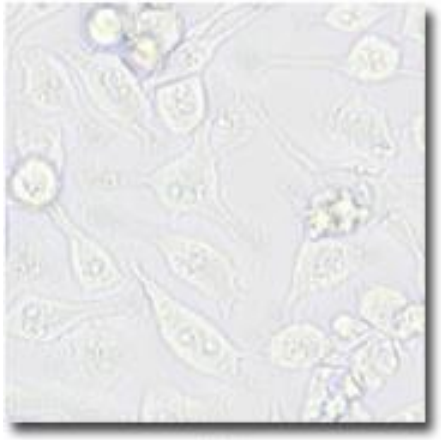
RECHTLICHER HINWEIS: Die Aufzeichnung und das Vervielfältigen einer Veranstaltung oder von Inhalten, z.B. von Folien oder Präsentationen, ist aus urheberrechtlichen und datenschutzrechtlichen Gründen untersagt.
Veranstaltungen und Inhalte hieraus dürfen weder ganz noch in Teilen unbefugt verbreitet werden, insbesondere nicht im Internet oder in sozialen Netzwerken. Soweit personenbezogene Daten verarbeitet werden, sind die maßgeblichen datenschutzrechtlichen Regelungen zu beachten, insbesondere der Datenschutzgrundverordnung und des Bayerischen Datenschutzgesetzes. Die Veranstaltungen und Inhalte sind ausschließlich für hochschulrechtliche Zwecke und zur Erfüllung von Hochschulaufgaben bestimmt. Zuwiderhandlungen jeglicher Art, insbesondere eine unbefugte Aufzeichnung von Veranstaltungen oder Inhalten, eine unbefugte Nutzung oder Verbreitung von Veranstaltungen oder Inhalten, Verstöße gegen Persönlichkeitsrechte, rechts- und zweckwidrige Datenverarbeitungen, Manipulationen von Veranstaltungen oder von Inhalten oder eine Weitergabe von Benutzerkennungen bzw. Passwörtern, können hochschulrechtlich, zivilrechtlich und strafrechtlich verfolgt und geahndet werden

Quick outline

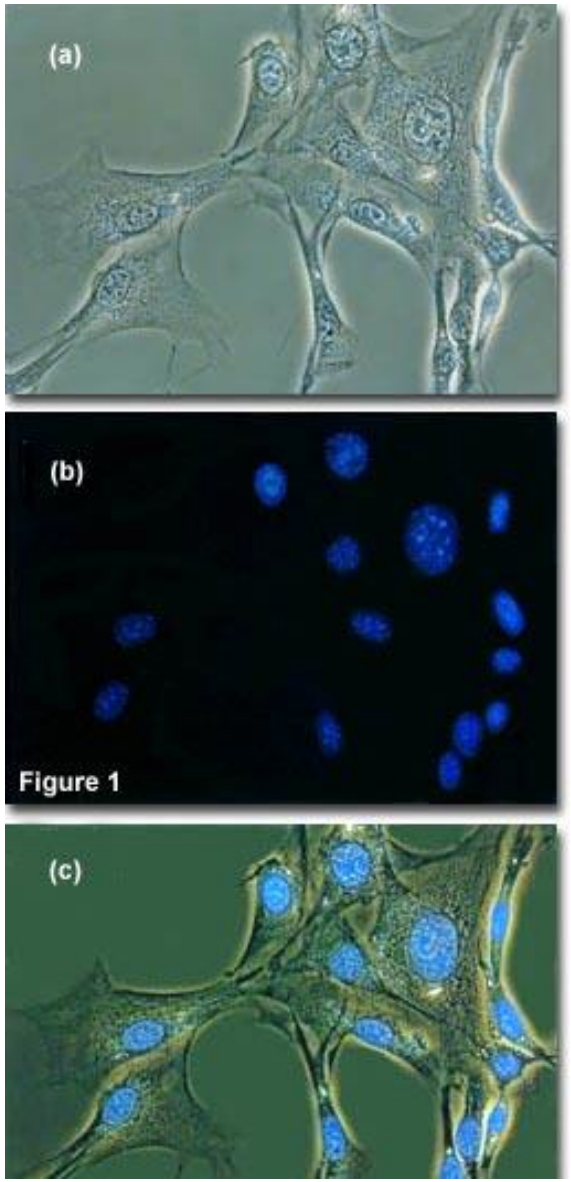
- Basics of fluorescence and fluorescence single-molecule imaging
- Fluorescence to study DNA and DNA nanostructures
- Intro to superresolution imaging
- DNA PAINT
- DNA PAINT to study DNA nanostructures
- DNA origami nanorulers
- DNA for barcoding and multiplexing

Why Fluorescence?

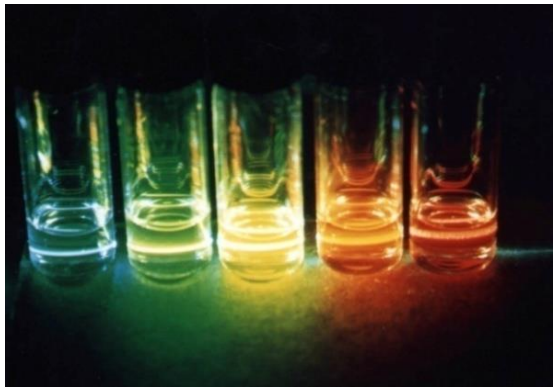
- selective
- sensitive
- almost non-invasive

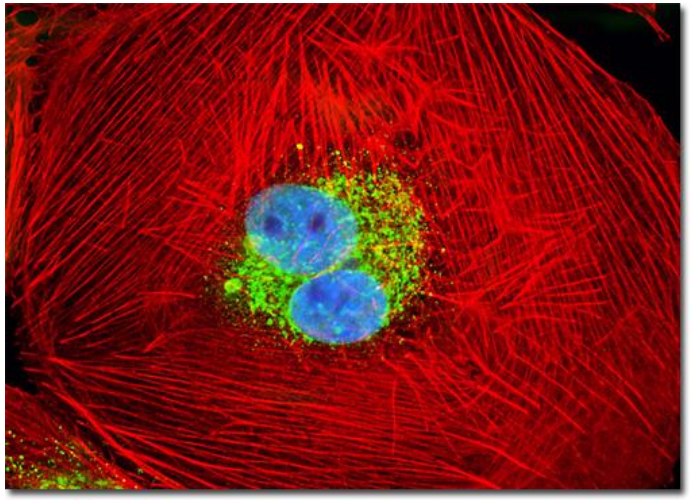


Living cells under the microscope



Staining with the respective dye-conjugates allows the visualization of distinct structures / features/ molecules

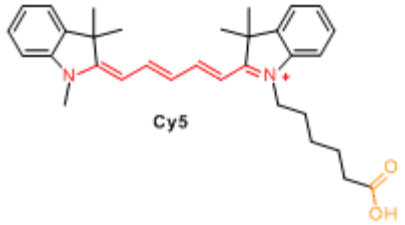


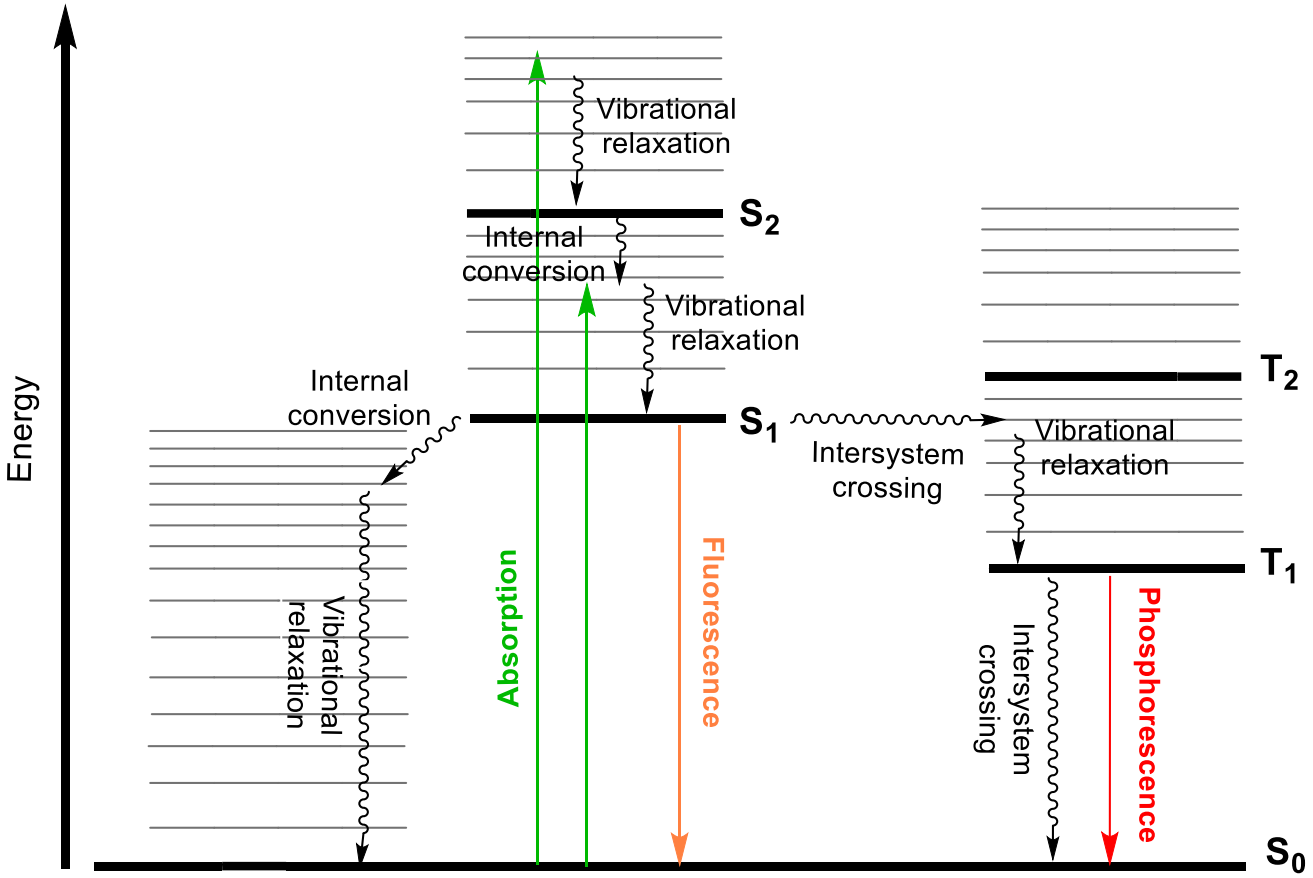


- Golgi apparatus: Oregon Green488- labelled Agglutinin
- Actin-cytosceleton: Alexa Fluor 568- labeled Phalloidin
- DNA in the nucleus: DAPI

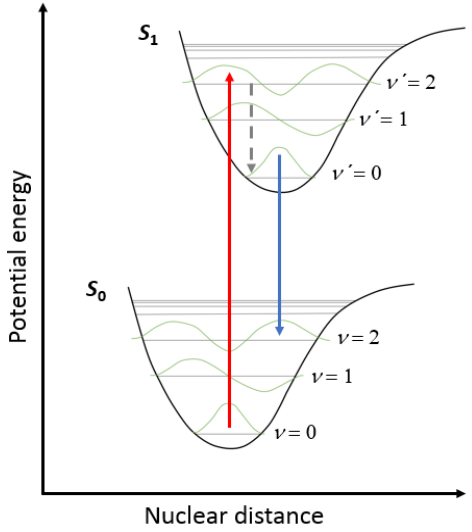
Fluorescence as a Physical Phenomenon

Fluorescence is caused by an electronic transition in a molecule

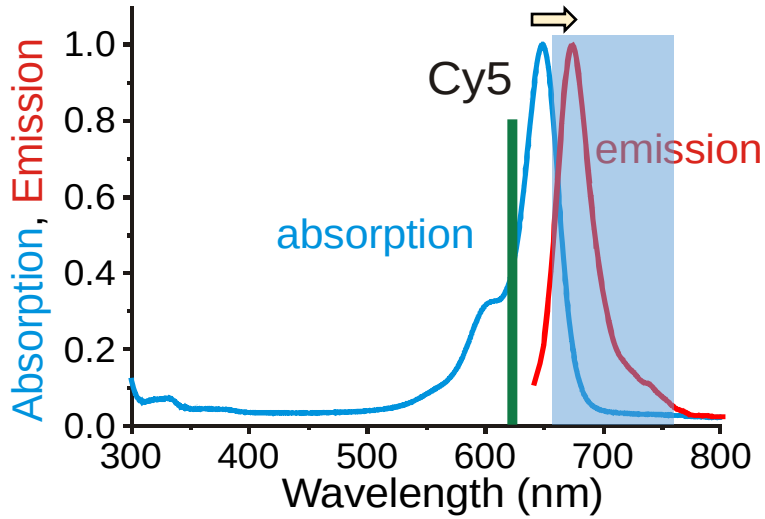




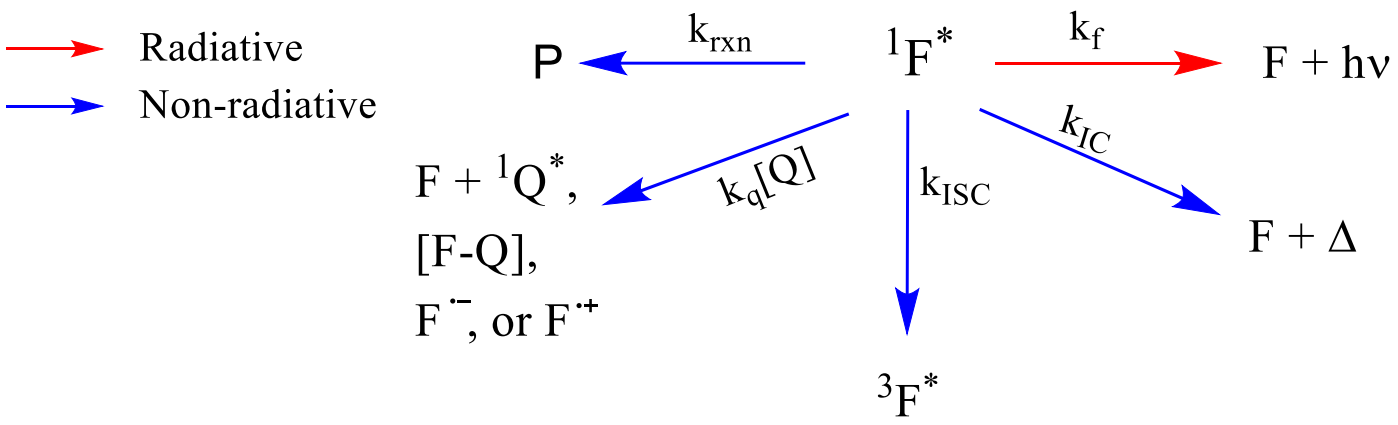
Frank-Condon principle



Stokes Shift



Important parameters of fluorophores



Fluorescence lifetime

Time molecule spends in the excited state

$$\tau_f = \frac{1}{k_{dec}} = \frac{1}{k_f + k_{IC} + k_{ISC} + k_q[Q] + k_{rx}}$$

Fluorescence Quantum Yield

Ratio of photons emitted vs photons absorbed

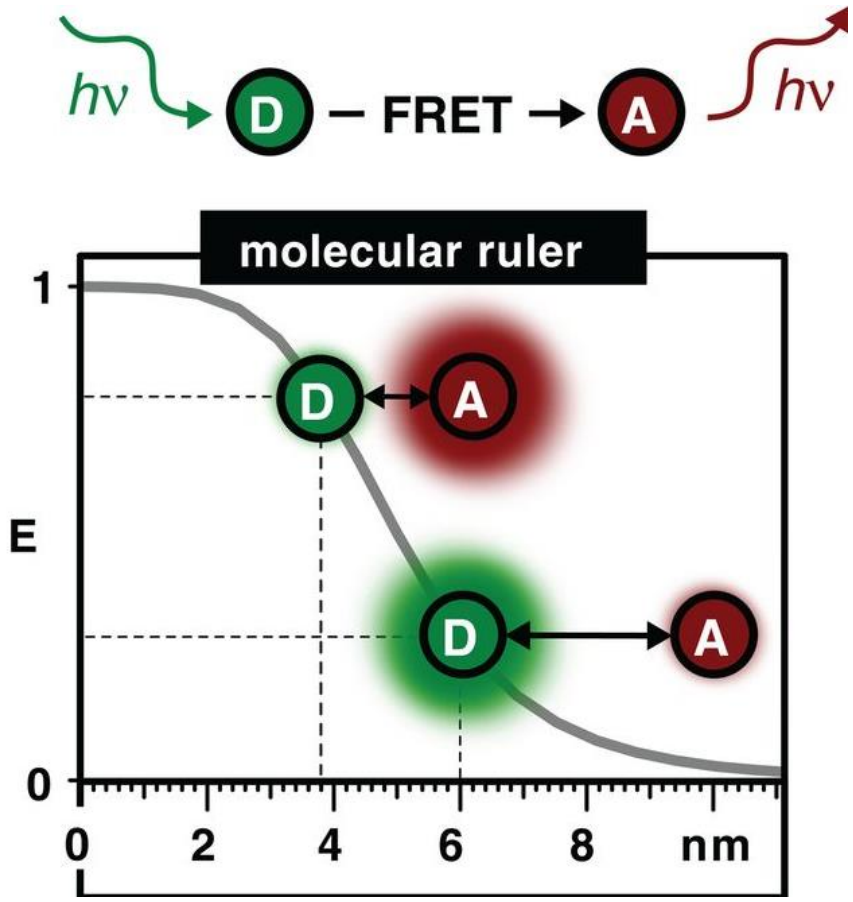
$$\Phi_f = \frac{k_f}{k_f + k_{nr}} = \frac{k_f}{k_f + k_{IC} + k_{ISC} + k_q[Q] + k_{rx}}$$

Brightness

$$\varepsilon \times \Phi_f$$

ε – extinction coefficient

Förster Resonance Energy Transfer (FRET) as a Molecular Ruler

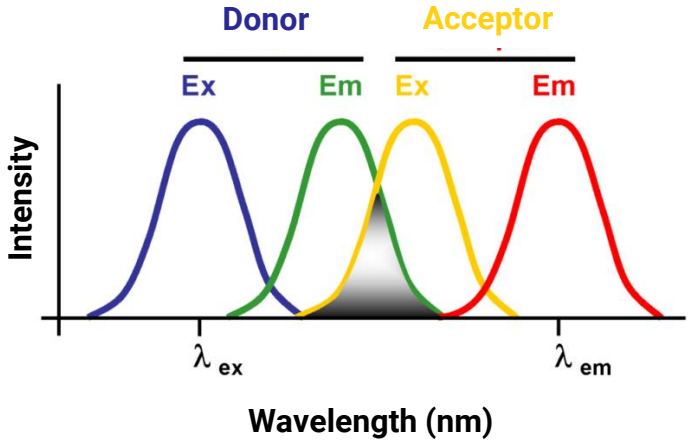


- Long range dipole-dipole interaction (not mediated by photons)
- Range ~ **2 – 9 nm**
- Depends on the spectral properties of and the orientation between **donor** and **acceptor** dyes:

Rate of energy transfer:

$$k_t(r) = \left(r^{-6} J(\nu) \kappa^2 n^{-4} \frac{\Phi_d}{\tau_d}\right) 8.71 \cdot 10^{23} s^{-1}$$

$J(\nu)$: spectral overlap integral
κ: orientation factor
r: distance between D-A
R_o: Förster distance (distance of 50% energy transfer)

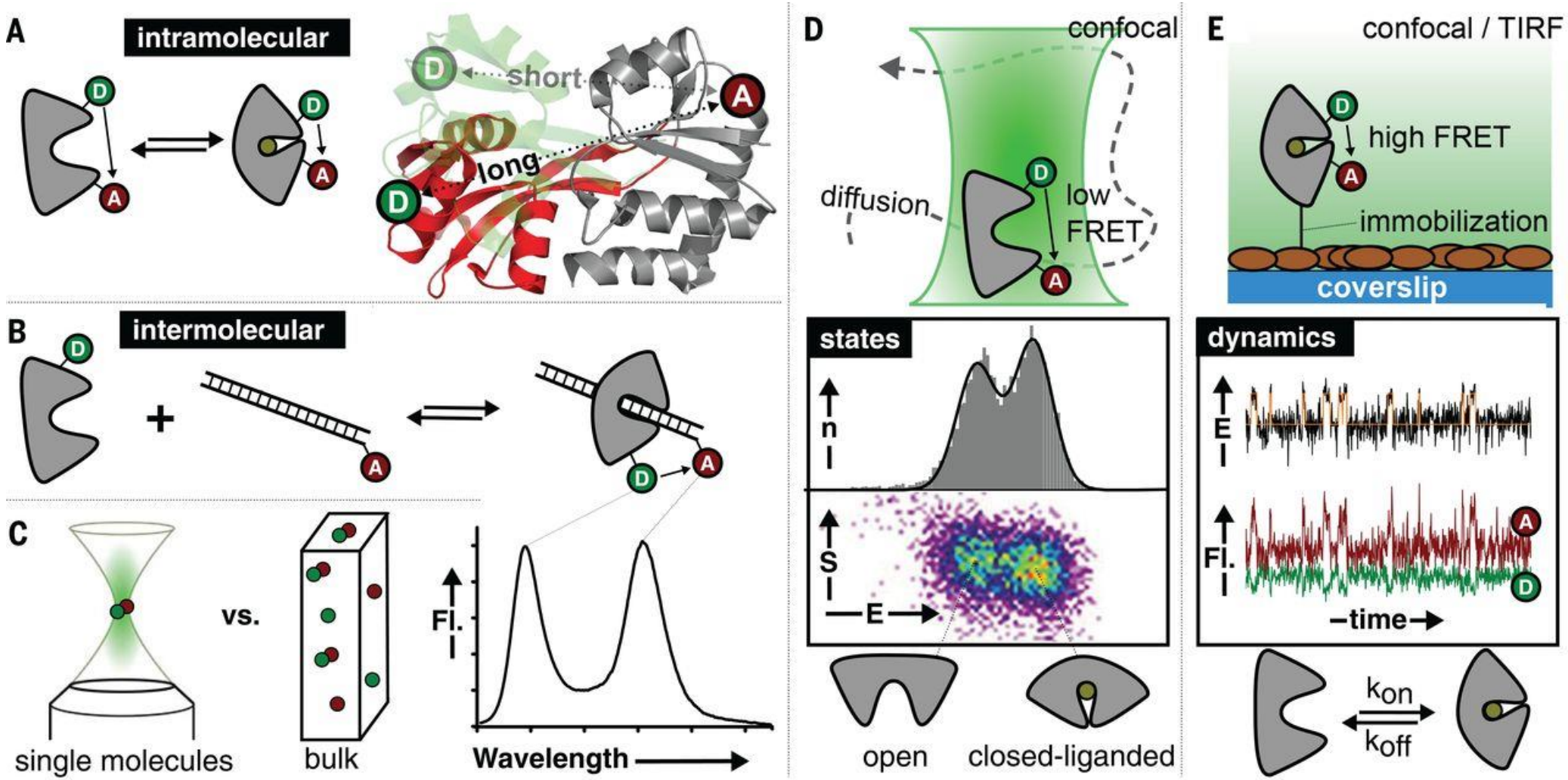


Energy transfer efficiency

$$E = \frac{k_t}{k_\Sigma + k_t} = \frac{R_o^6}{R_o^6 + r^6}$$

Lerner *et al. Science* **2018**, *359*, eaan1133

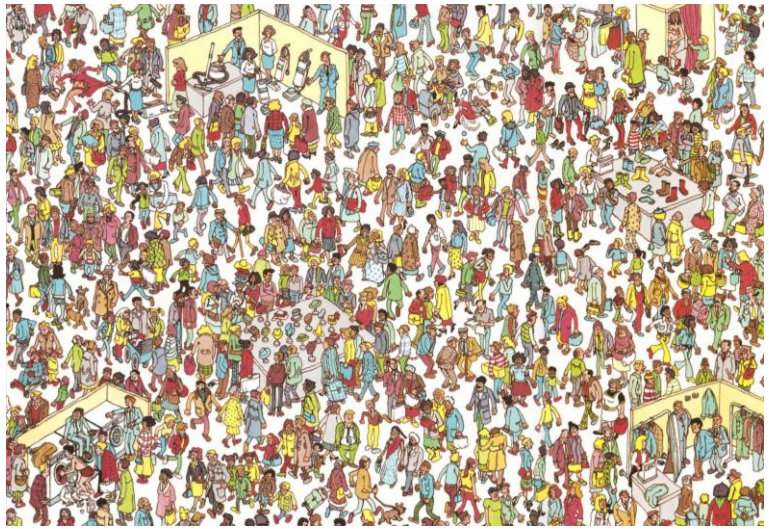
FRET to study molecular interactions



Lerner *et al. Science* **2018**, *359*, eaan1133

How to detect a single molecule?

‘Where is Waldo?’ problem

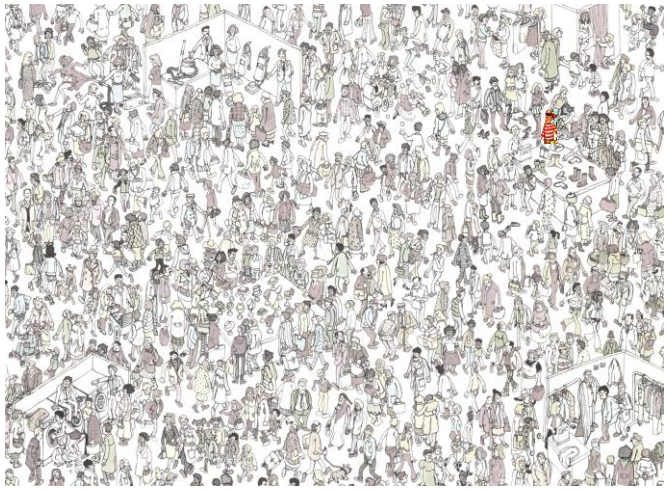


Issue: Signal to Noise Ratio (S/N) !!

Sources of background:

- Detector dark counts
- Elastic and inelastic scattering
- Impurities

Solutions



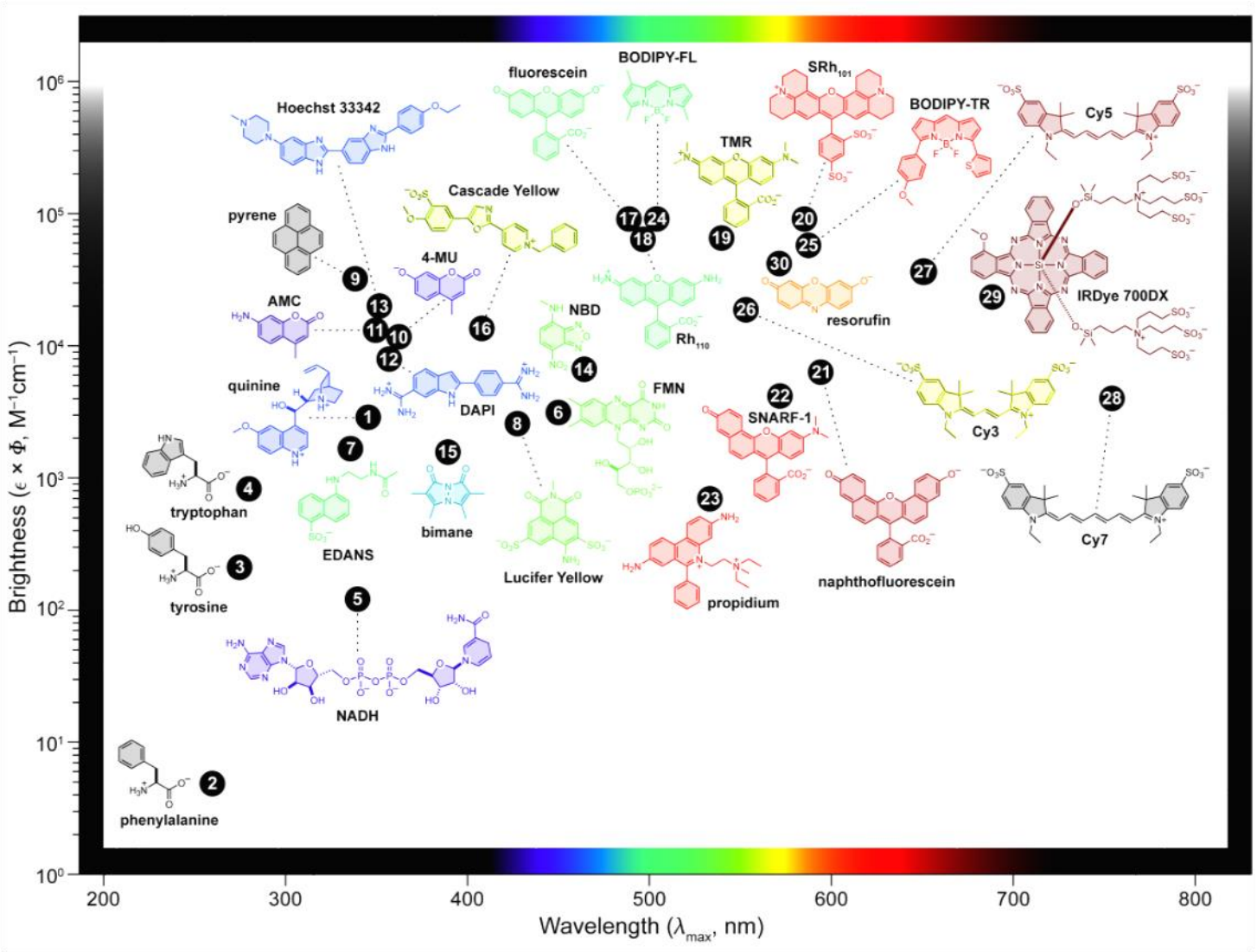
Maximize signal:

- High quantum yield
- High extinction coefficient
- High photostability
- Low fluorescence lifetime

Reduce the observation volume:

- TIRF microscopy
- Confocal microscopy
- Nanophotonic approaches/plasmonic hotspots

How to detect a single molecule: maximize the signal



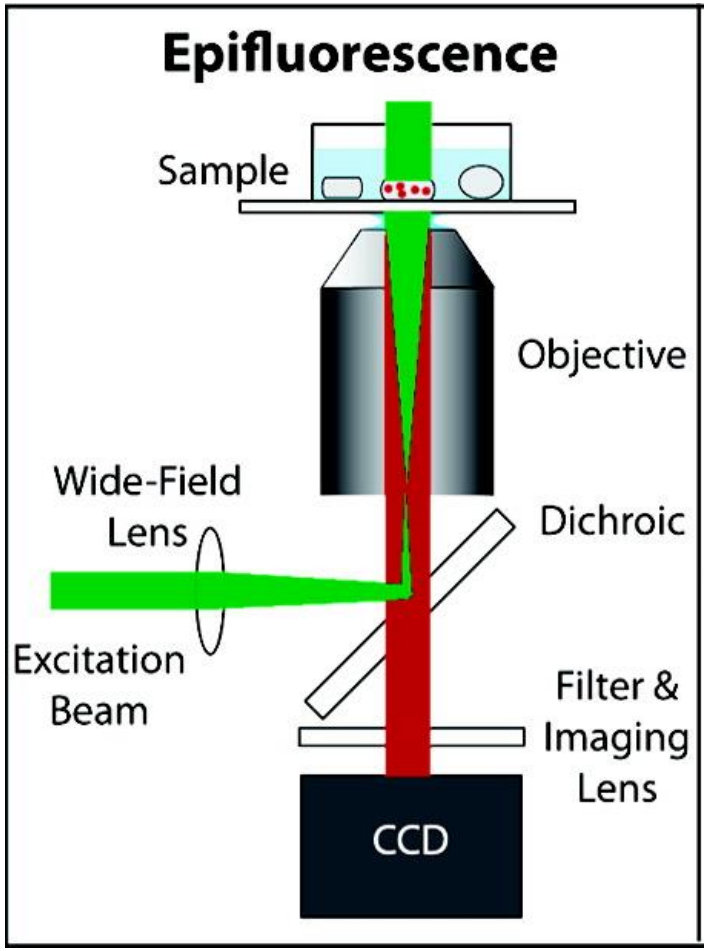
Many requirements for the fluorescent label:

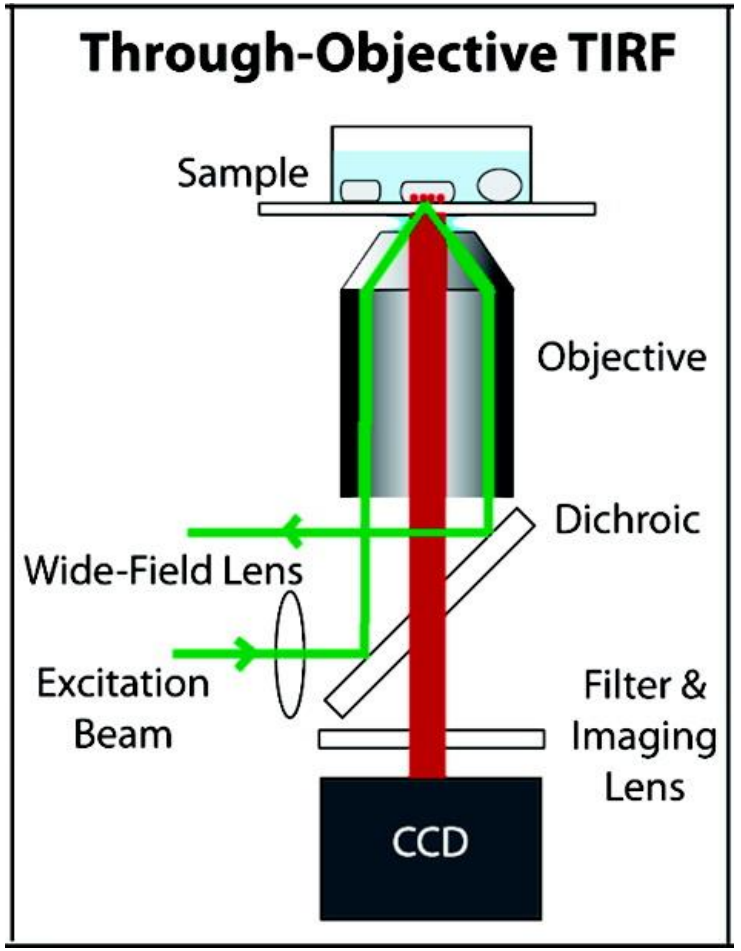
- High quantum yield
- High extinction coefficient
- High photostability
- Low fluorescence lifetime

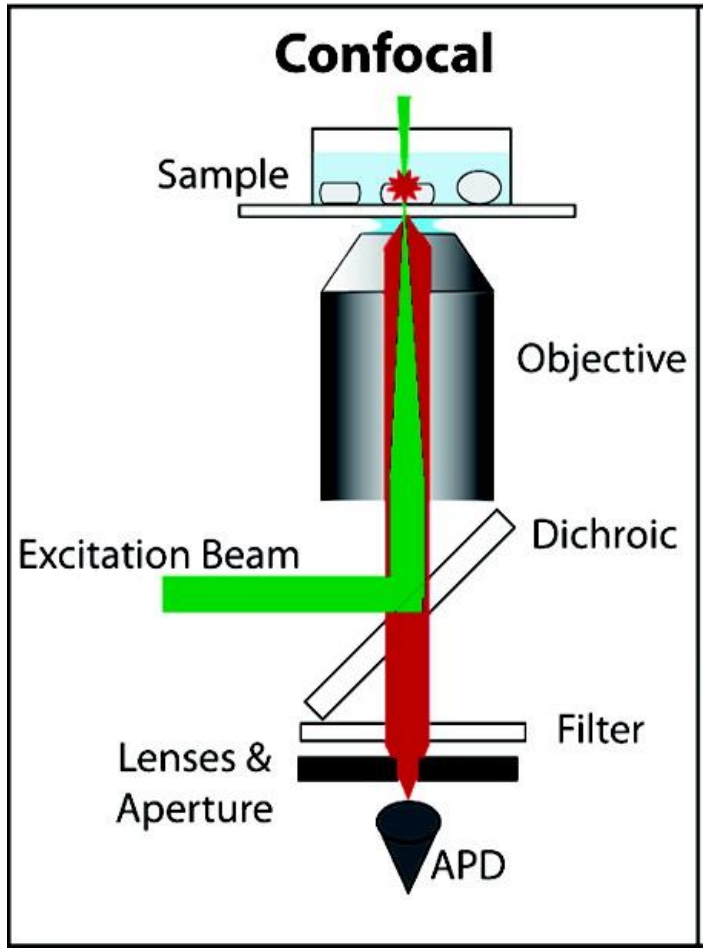
ACS Chem. Biol. **2008**, *3*, 142–155

How to detect a single molecule: reduce the observation volume

Reduced $V_{illumination}$ -> increased S/N!

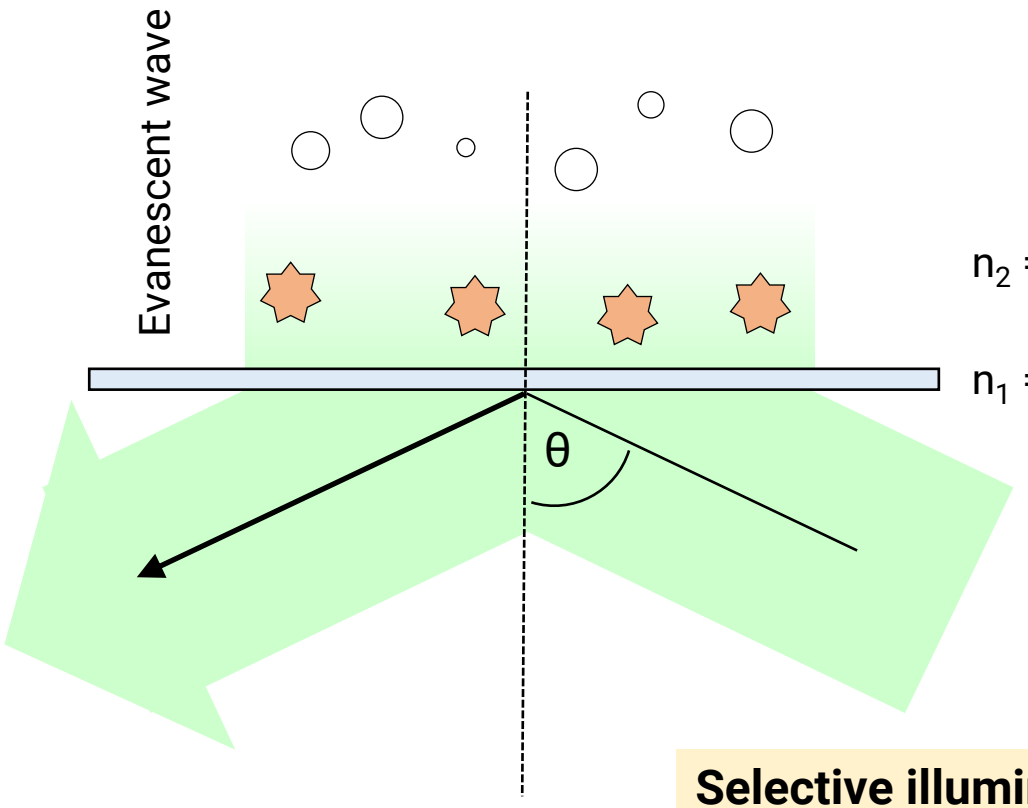






Anal. Chem. **2010**, *82*, 6, 2192-2203

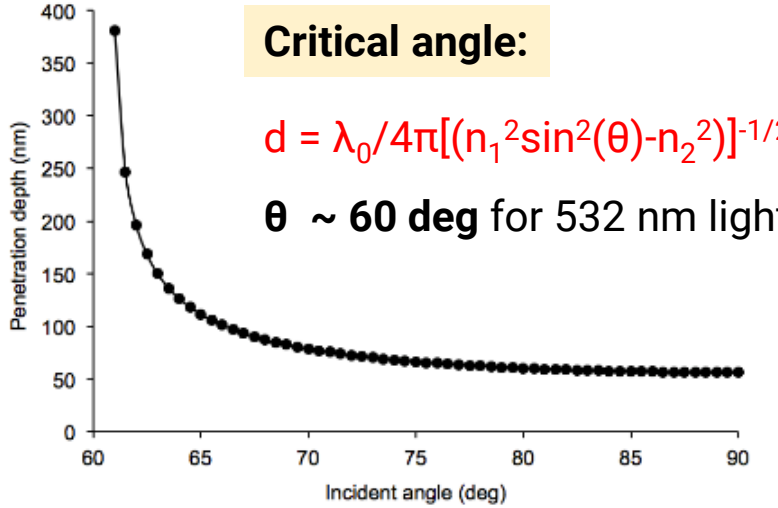
Total Internal Reflection (TIRF) Microscopy



n_2 = 1.33 (e.g., water)

n_1 = 1.52 (e.g., glass slide)

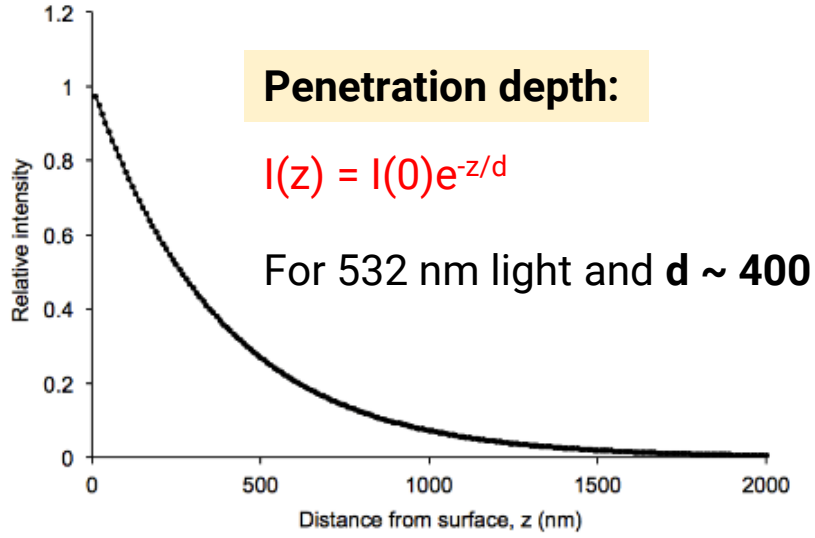
Selective illumination of molecules at the interface



Critical angle:

$d = \lambda_0/4\pi[(n_1^2\sin^2(\theta)-n_2^2)]^{-1/2}$

θ ~ 60 deg for 532 nm light



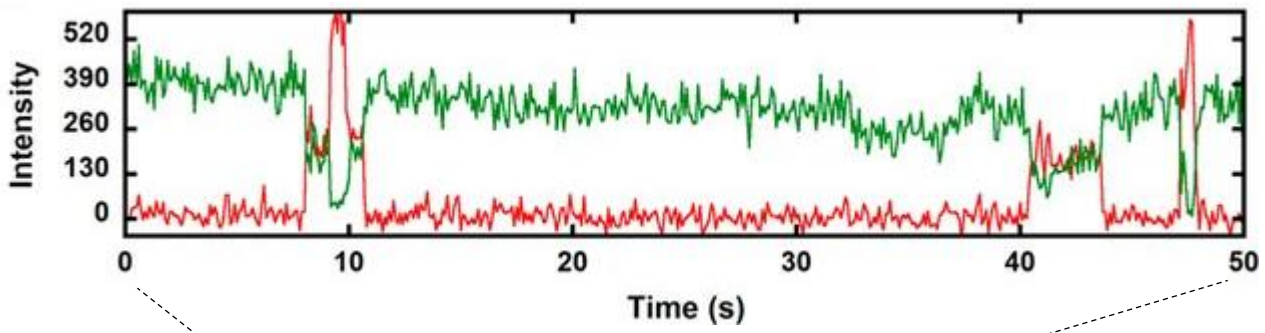
Penetration depth:

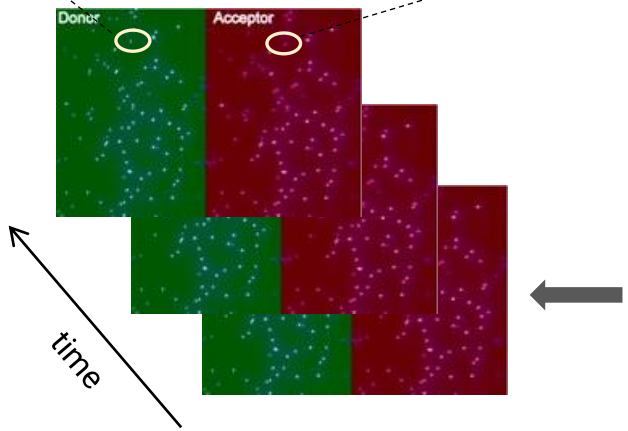
$I(z) = I(0)e^{-z/d}$

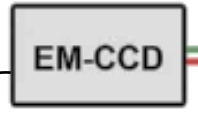
For 532 nm light and **d ~ 400 nm**

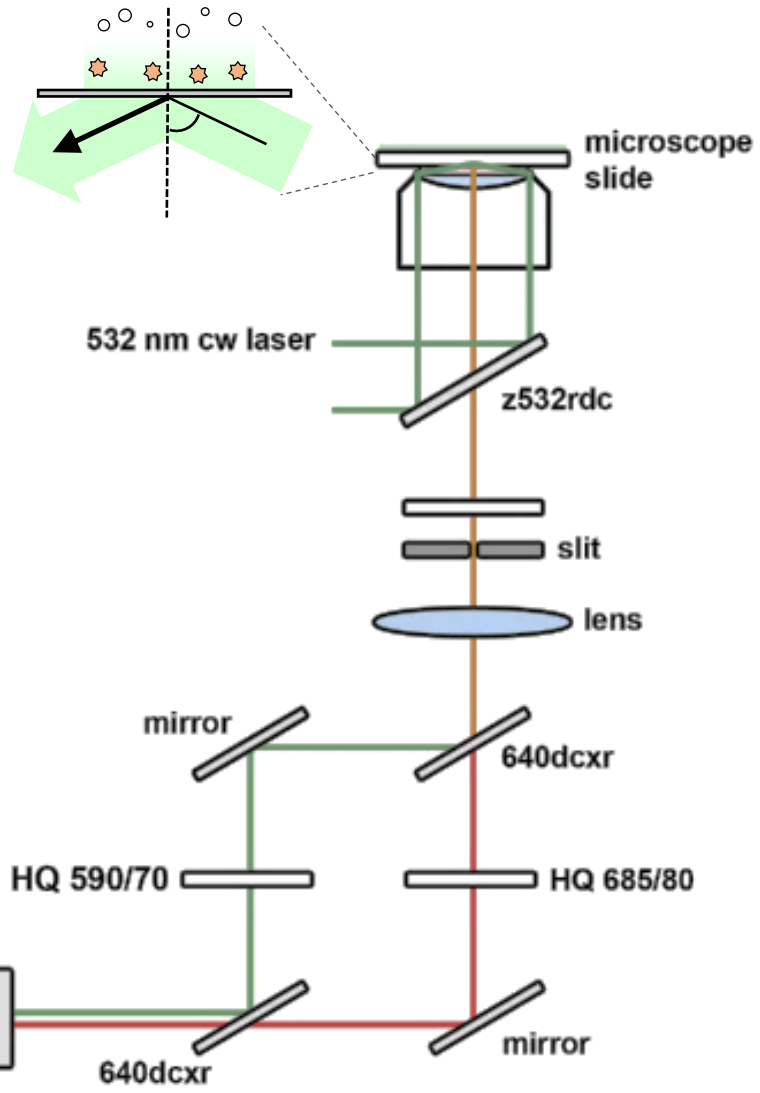
Total Internal Reflection (TIRF) Microscopy

Fluorescence intensity (dynamics) of hundreds of single molecules can be monitored over-time simultaneously

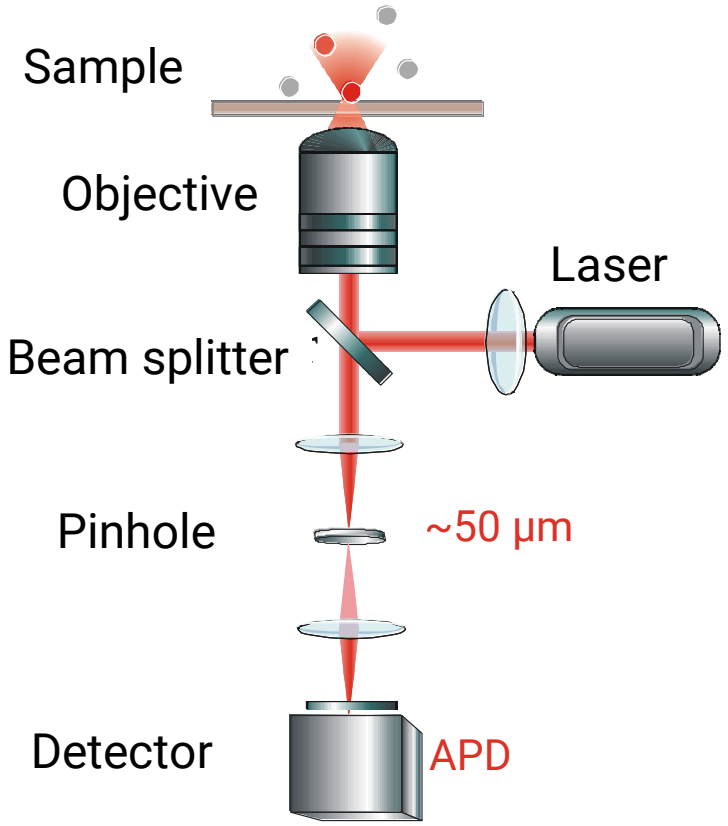








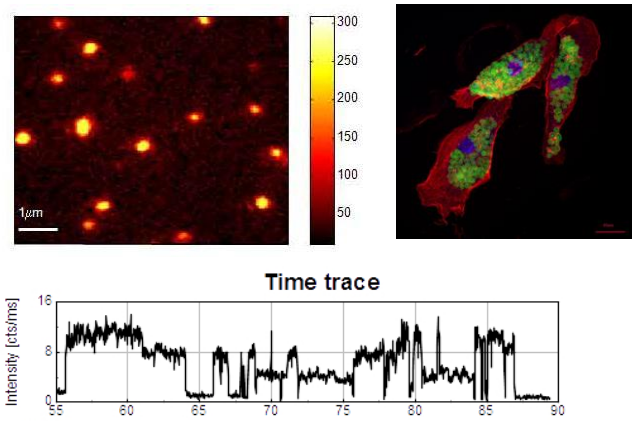
Confocal Microscopy



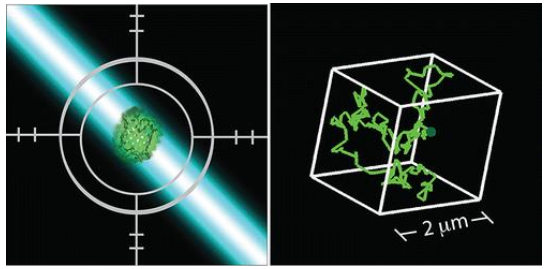
- Sample is excited by focused laser beam
- Filtering the out of focus light via pinhole
- Focal volume: ~ 250 x 750 nm

Different methods

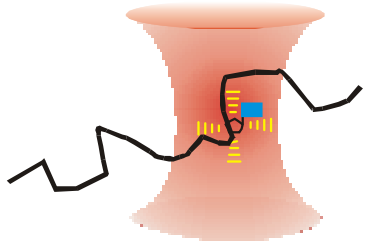
Scanning confocal microscopy:
produces and image of immobilized samples



Confocal microscopy in solution:
tracking of a molecule in solution over-time

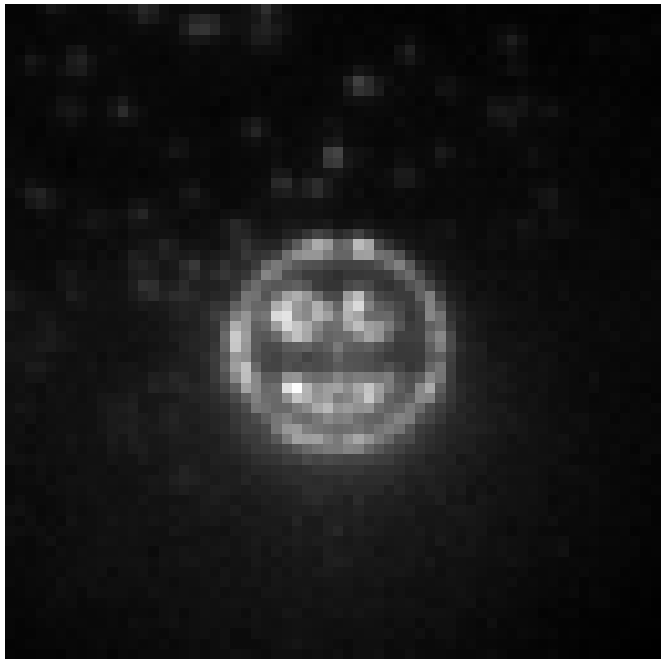


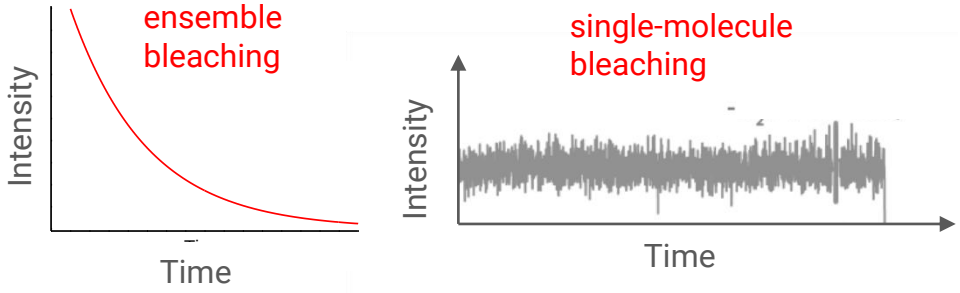
Fluorescence Correlation Spectroscopy (FCS):
monitoring the fluctuations caused by the diffusion of molecules through the focused laser beam

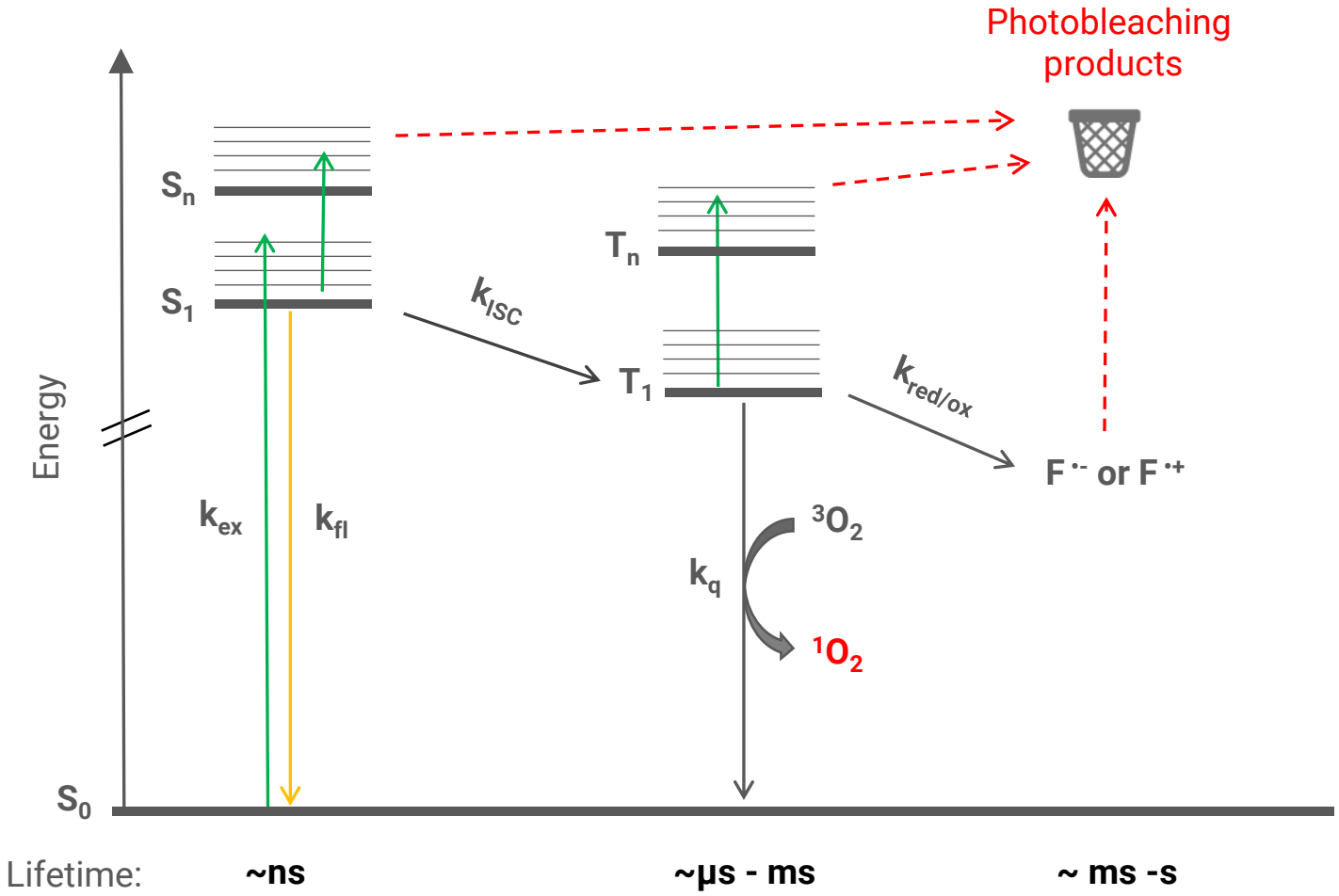


Single-molecule bleaching and fluorophore instabilities

# of photons from one molecule is limited!

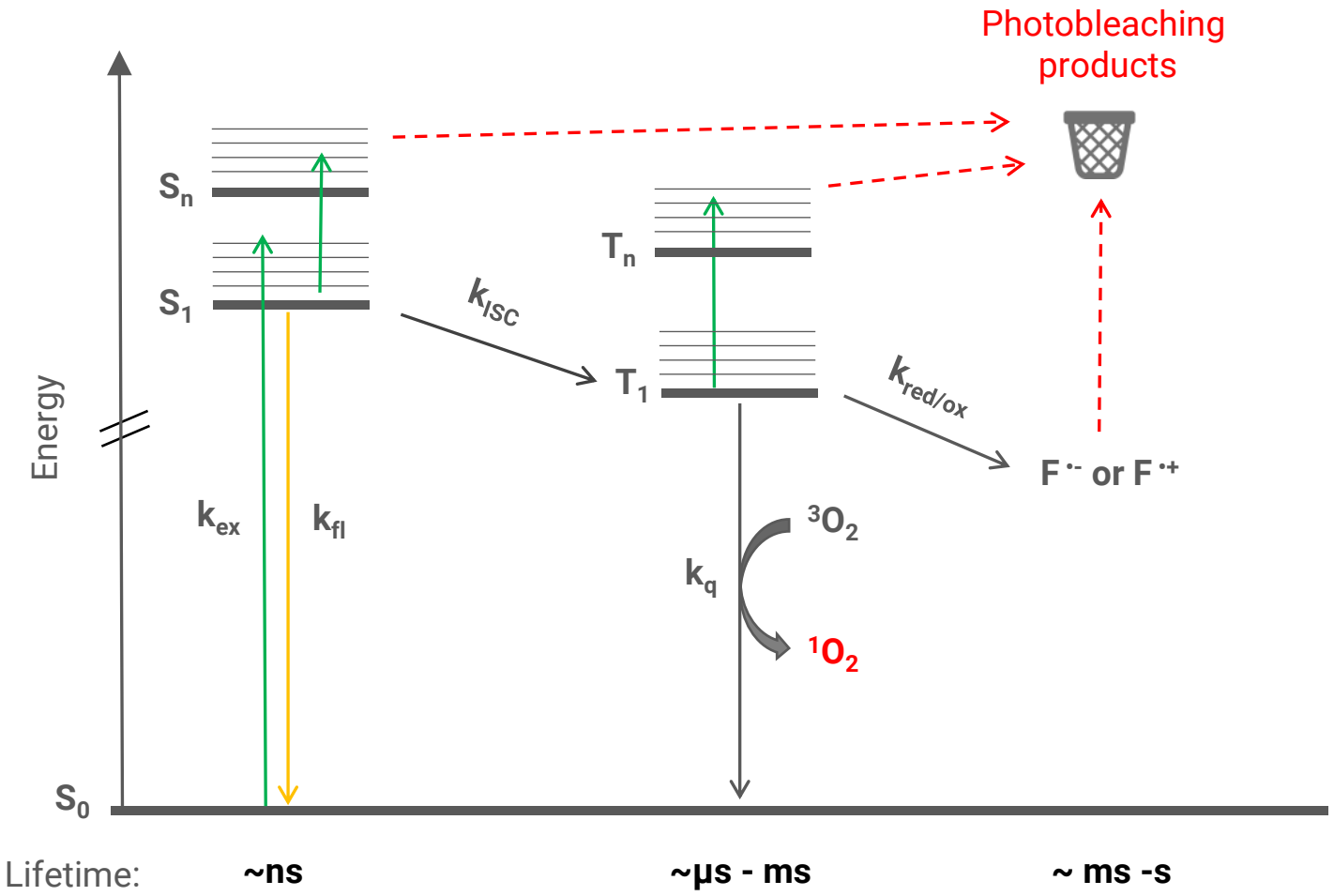


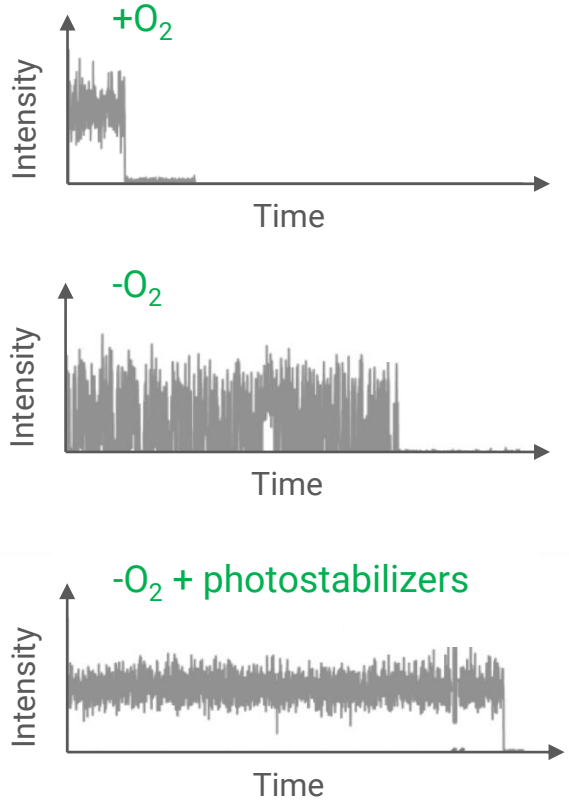




Molecules **2014**, 19, 15824 , Annu. Rev. Phys. Chem. **2012**, 63, 595

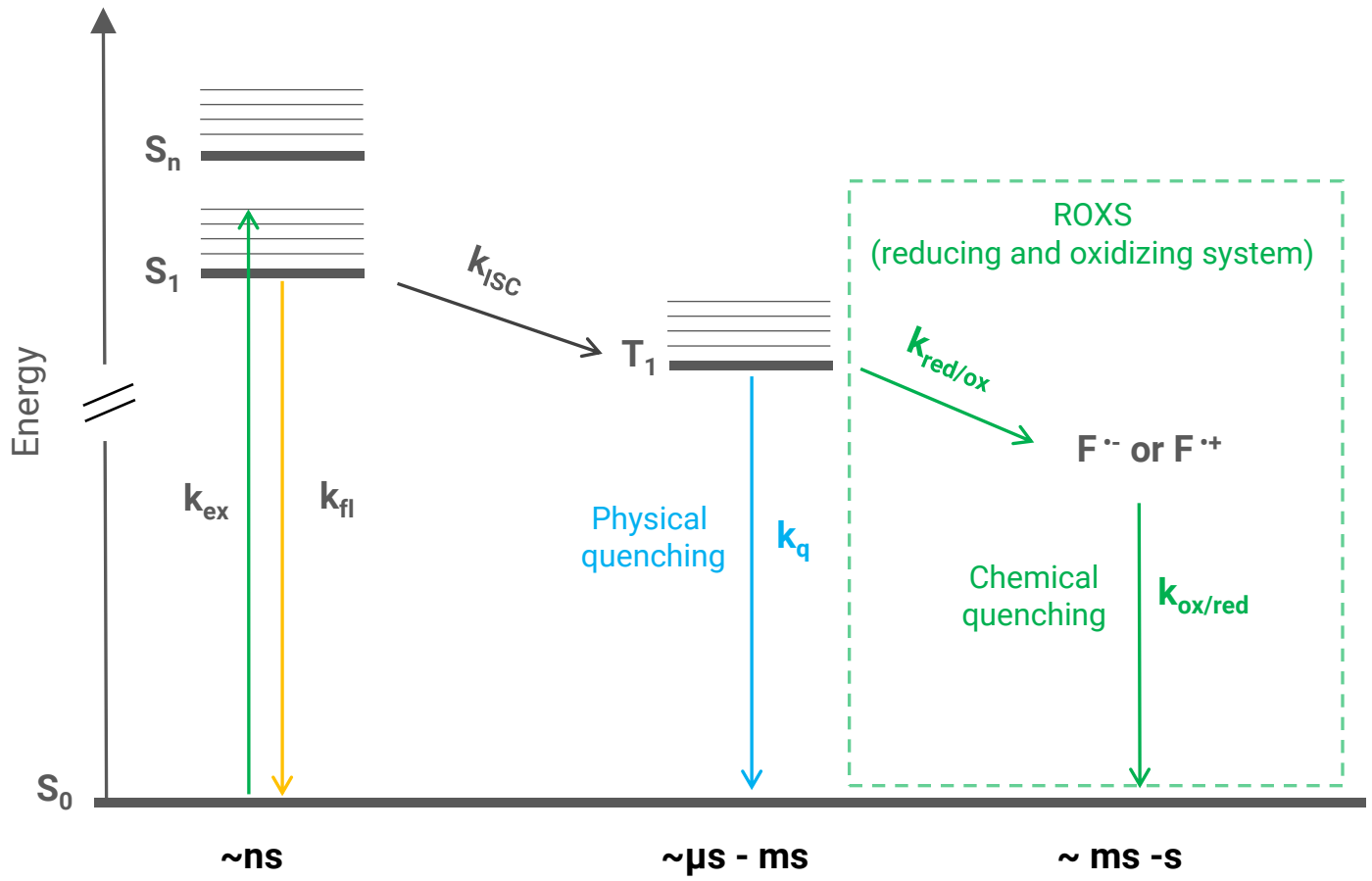
Single-molecule bleaching and fluorophore instabilities





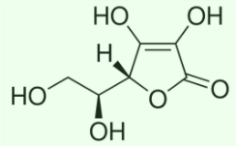
Molecules **2014**, 19, 15824 , Annu. Rev. Phys. Chem. **2012**, 63, 595

How to improve the photostability of fluorescence labels?

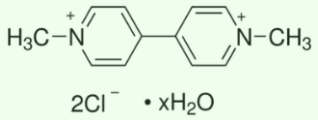


$-O_2$ +

Chemical (redox)
triplet quenchers



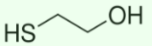
Ascorbic acid



Methyl viologen

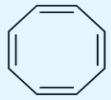
Trolox/Trolox quinone

β-mercaptoethanol



or

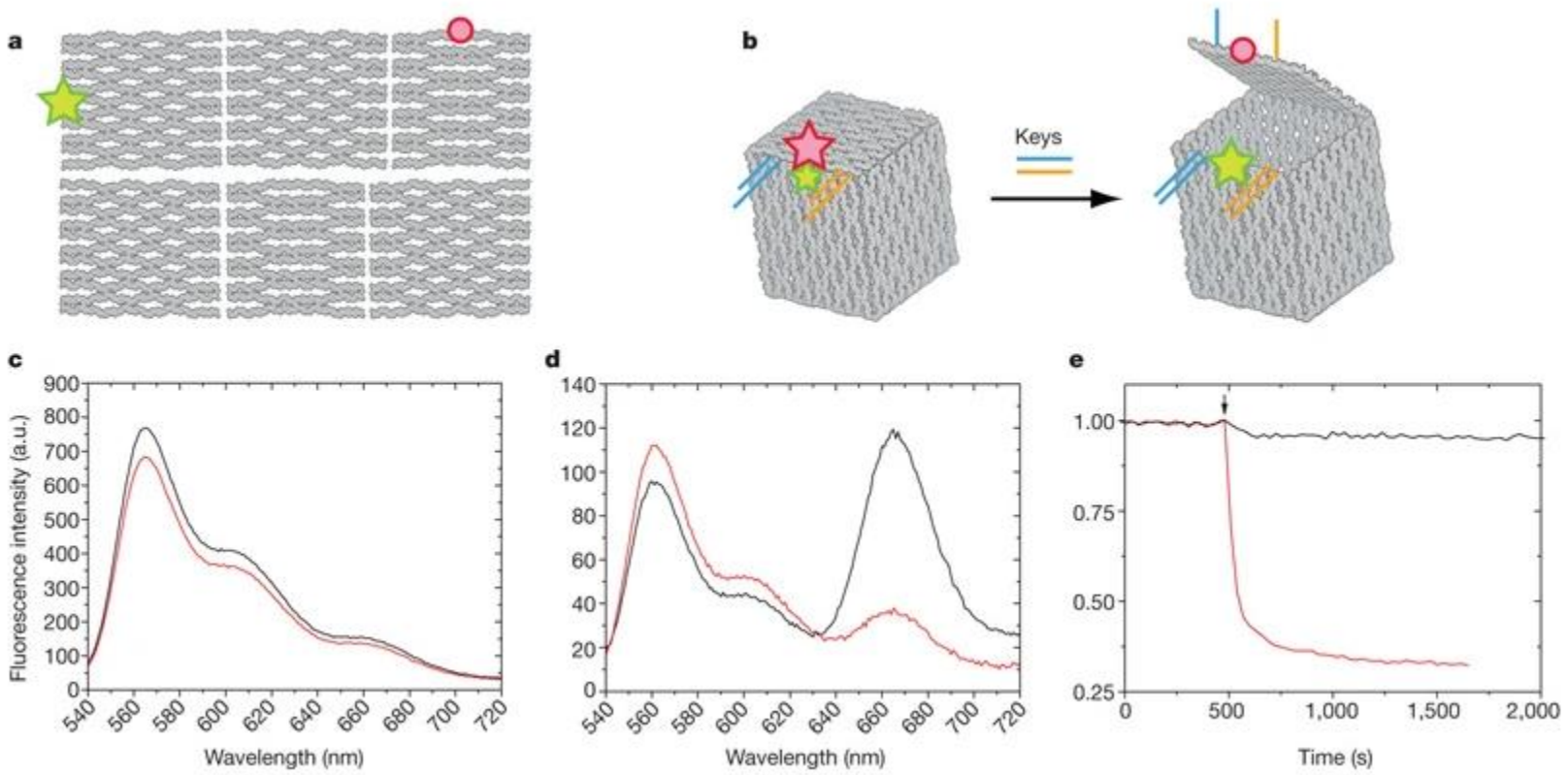
Physical triplet quenchers



Cyclooctatetraene (COT)
Ni^{2+} ions

Ang. Chem. Int. Ed. **2008**, 47, 5465l, Nat. Meth. **2006**, 3, 891, Annu. Rev. Phys. Chem. **2012**, 63, 595

Using fluorescence to study the opening of nanoscale DNA box



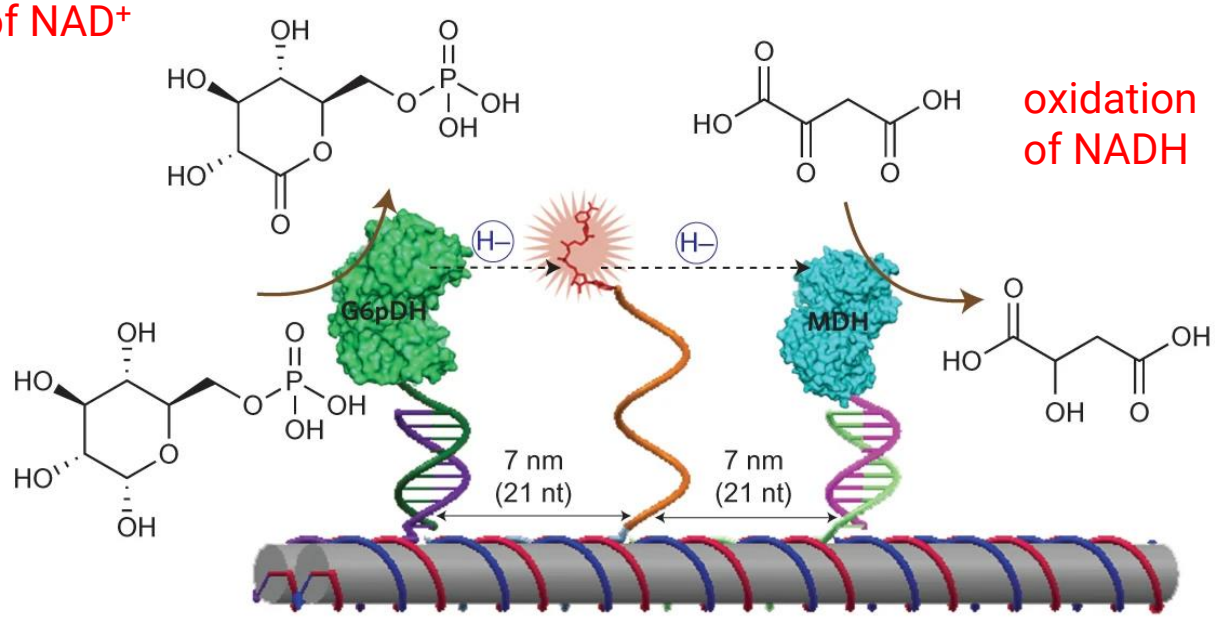
loss of FRET (Cy5 signal) upon opening of the box

Andersen *et al*., *Nature* **2009**, *459*, 73

Single-molecule FRET to study DNA-based swinging arm

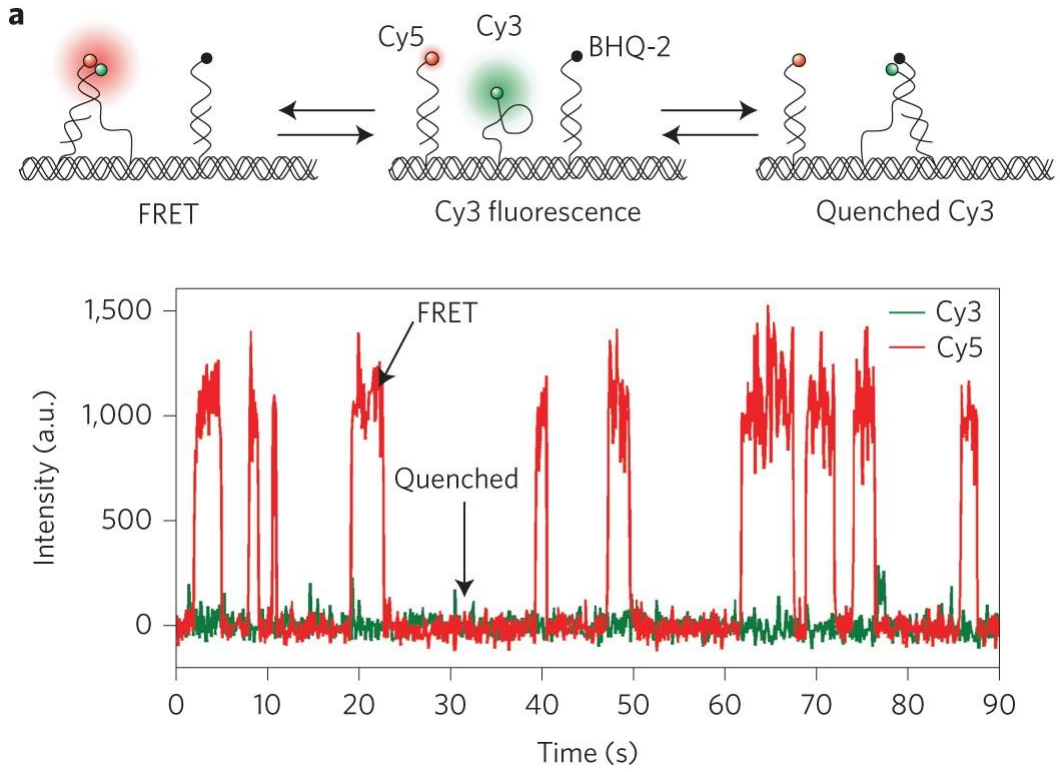
DNA-based swinging arm functionalized with NAD^+ capable of shuffling NAD^+ /NADH between two hydrogenases

reduction of NAD^+



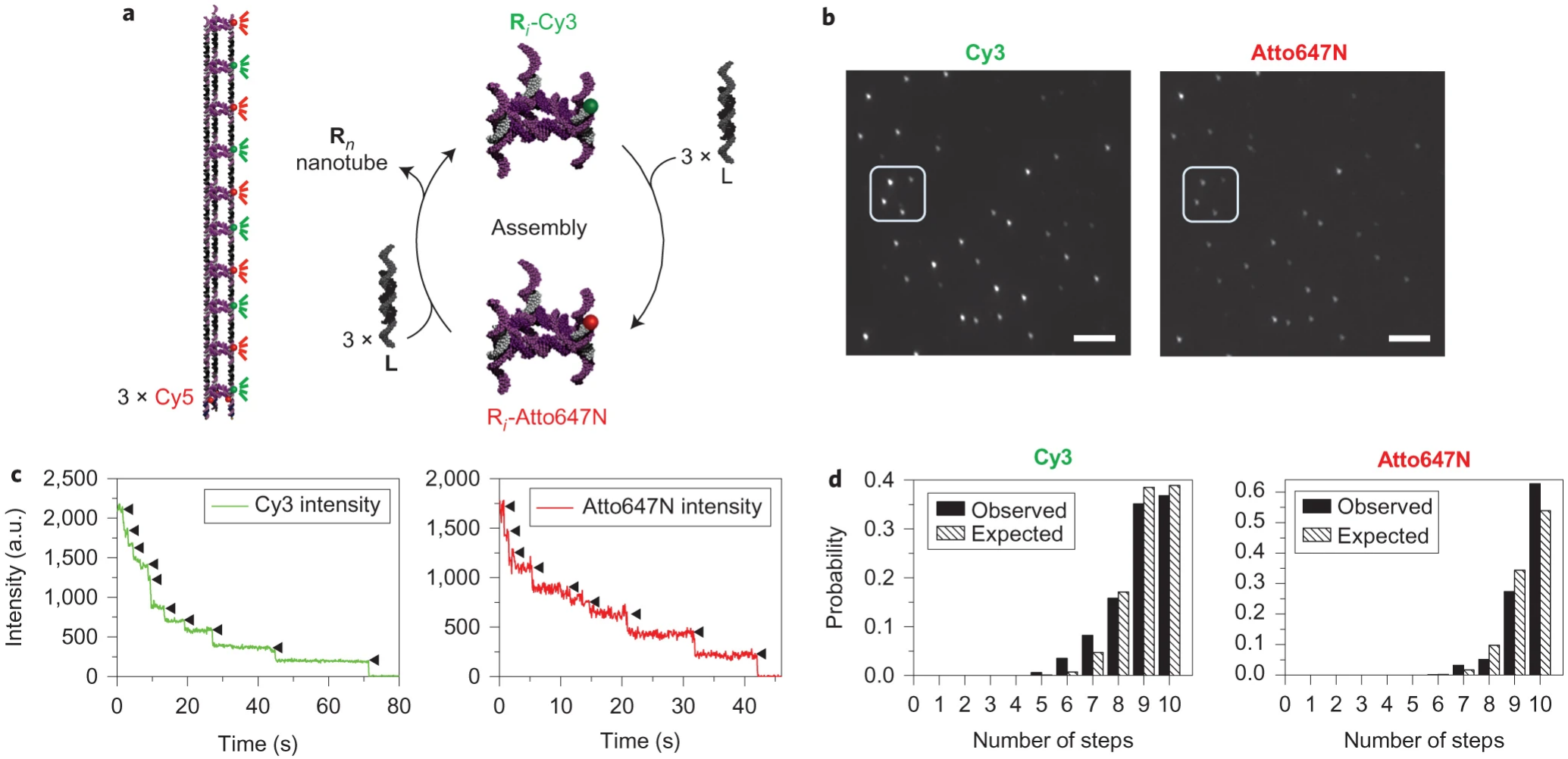
oxidation of NADH

Single-molecule FRET study to monitor the dynamics of the swinging arm:



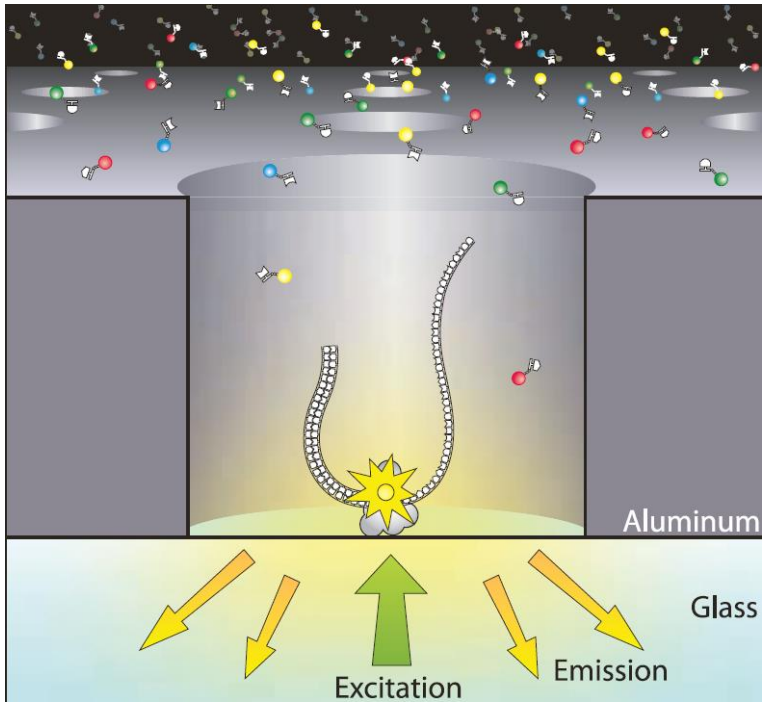
Fu *et al.*, *Nature Nanotechnology* **2014**, *9*, 531

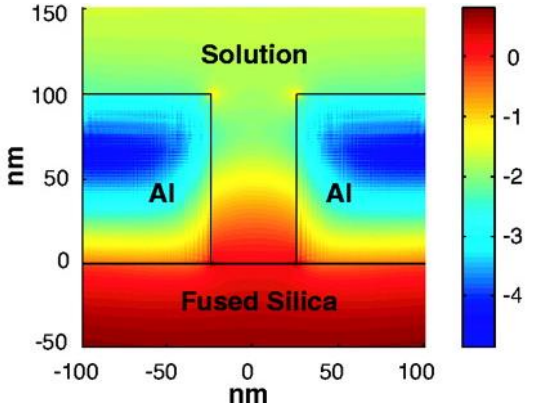
Viaualizing the stepwise-growth of DNA nanotubes at a single molecule level



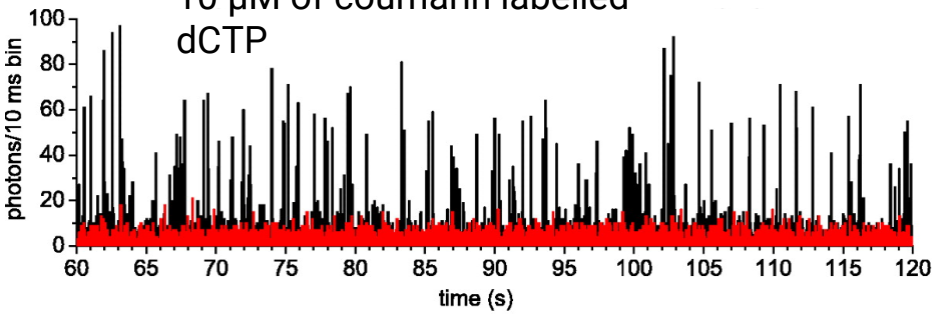
Hariri *et al.*, *Nature Chemistry* **2015**, *7*, 295

Single-molecule real-time DNA sequencing





Simulated intensity distribution (log scale)

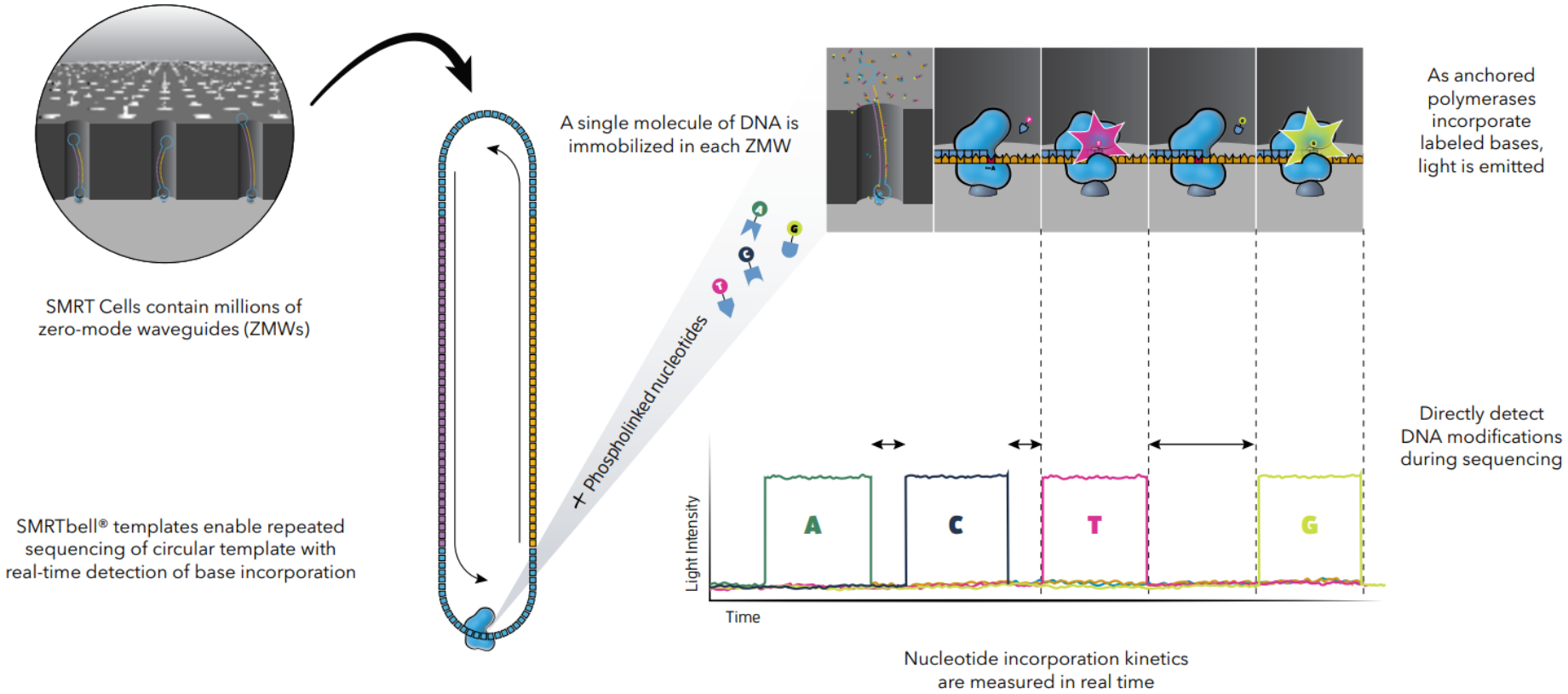


Zero Mode Waveguides:

- Subwalength holes in a metal film
- Reduced observation volume in zL range (compare to ~fL in confocal microscopy)
- Allow observation of fluorescent molecules in μM concentration range

Levene *et al.*, Science **2003**, *299*, 682
Eid *et al.*, *Science* **2008**, *323*, 133

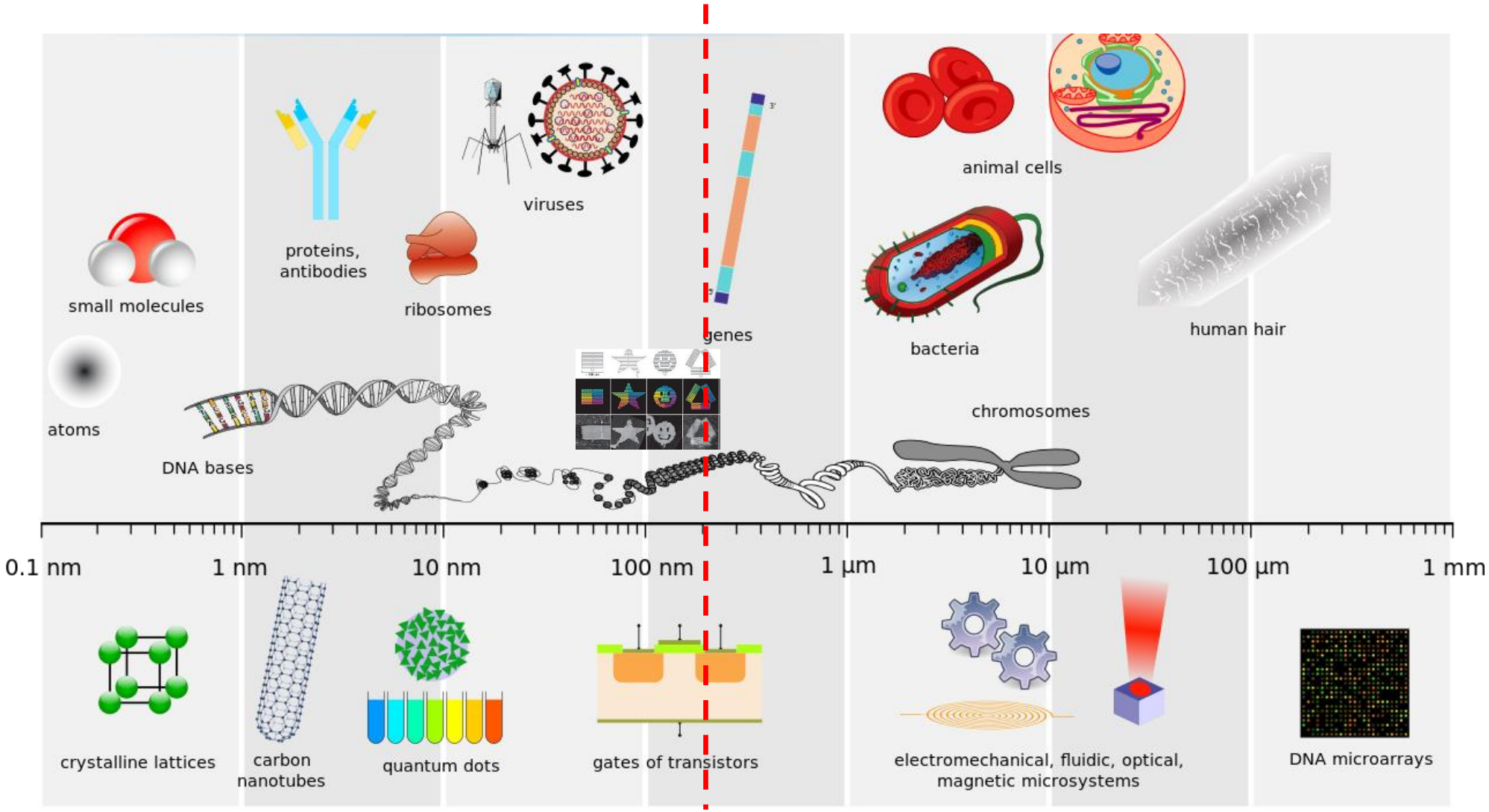
Single-molecule real-time DNA sequencing:



Video on single-molecule sequencing by PacBio:
https://www.pacb.com/smrt-science/smrt-sequencing/

Eid *et al.*, *Science* **2008**, *323*, 133

Abbey Diffraction Limit



https://commons.wikimedia.org/w/index.php?curid=4326462

Abbey Diffraction Limit

- Determines the size of the focal spot when light of the certain size passes through an optical lens
- Limits the distance at which two fluorescent molecules can be resolved:

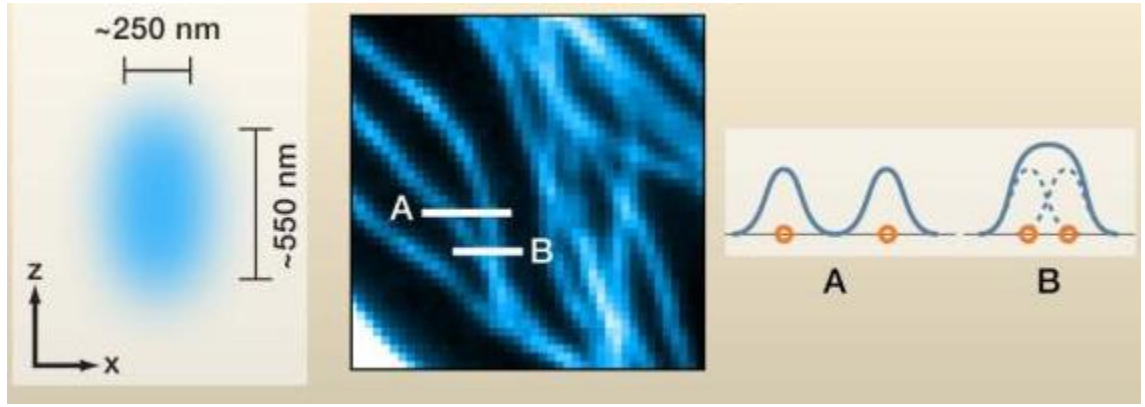
$$d = \frac{\lambda}{2nsin\theta}$$

λ – wavelength of light
$sin\theta$ – numerical aperture of the objective (NA)
n – refractive index

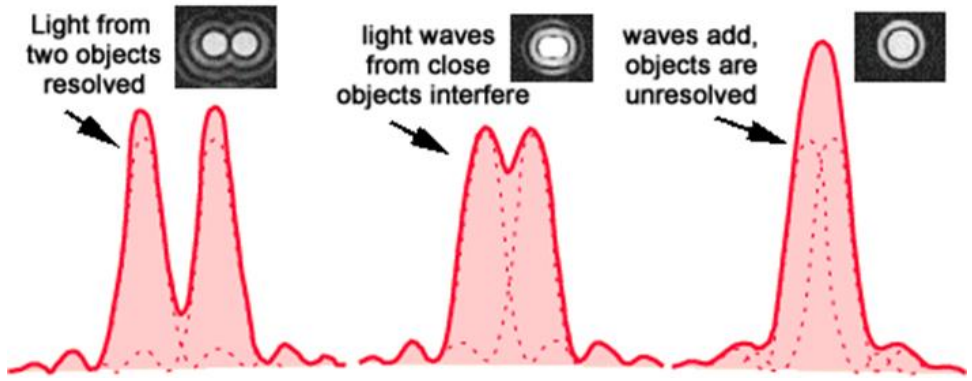
For typical fluorescence microscope:

λ ~ 400 – 700 nm
NA ~ 1.4

Resolution (d) is limited to **~ 200 nm**



Huang *et al*., *Cell* **2010**, *143*, 1047



Abbey Diffraction Limit: carved in stone?

$$d = \frac{\lambda}{2nsin\theta}$$

"similar objects closer than about half the wavelength should not be distinguishable in a light microscope"

Ernst Abbe 1873

Nobel Prize in Chemistry 2014: breaking the diffraction limit

© Nobel Media AB. Photo: A. Mahmoud

Eric Betzig

Prize share: 1/3

© Nobel Media AB. Photo: A. Mahmoud

Stefan W. Hell

Prize share: 1/3

© Nobel Media AB. Photo: A. Mahmoud

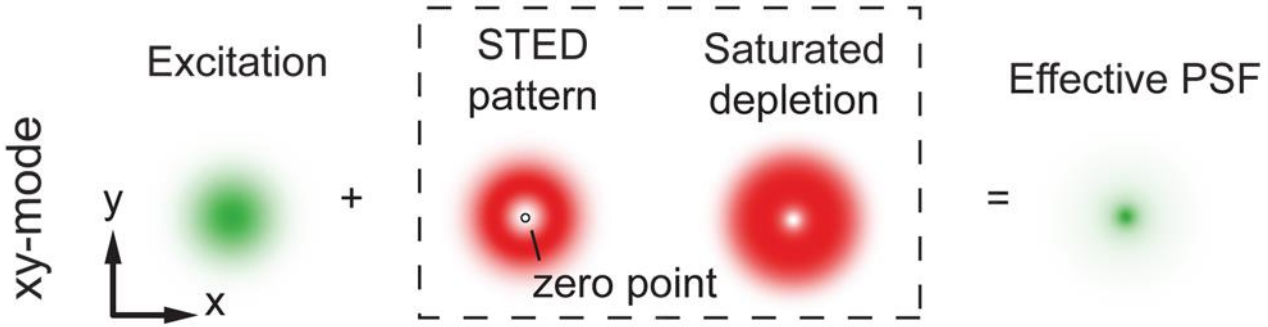
William E. Moerner

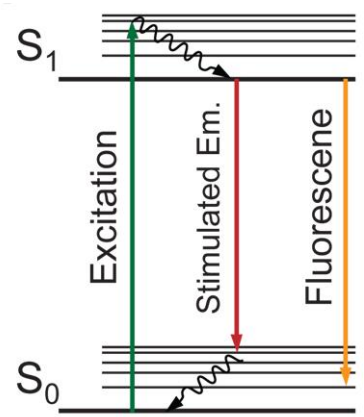
Prize share: 1/3

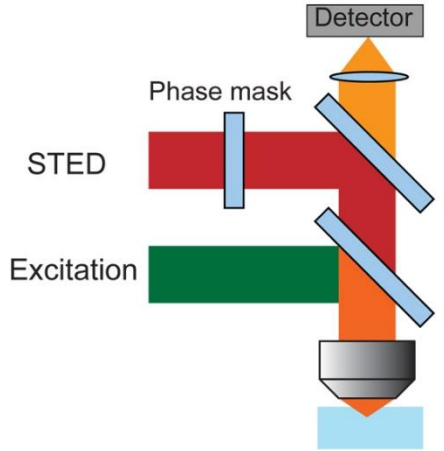
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner "for the development of super-resolved fluorescence microscopy."

https://www.nobelprize.org/prizes/chemistry/2014/summary/

Stimulated Emission Depletion (STED) Microscopy





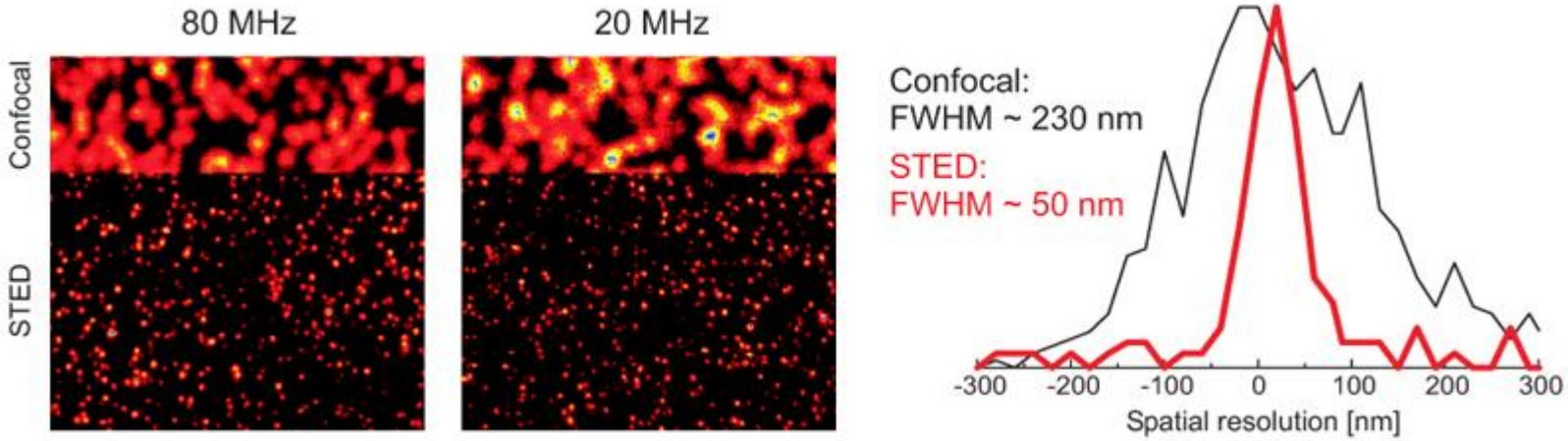


Huang et al., *Annu. Rev. Biochem.* **2009**, *78*, 993

The new resolution is defined by:

$$d = \frac{\lambda}{2n\,sin\alpha\sqrt{1+\frac{I}{I_{sat}}}}$$

I – intensity of the depletion beam
I_{sat} – saturation intensity of the fluorophore

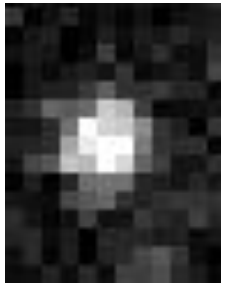


STED and confocal images of the Chrimson beads (www.picoquant.com/applications/category/life-science)

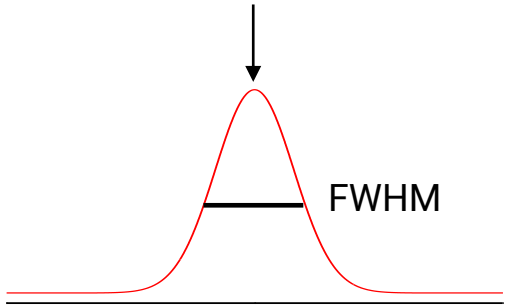
Hell and Wichmann, *Opt. Lett.* **1994**, *19,* 780; Klar, Engel and Hell Phys. Rev. E **2001**, *64*, 066613; Klar and Hell, *Opt. Lett*. **1999**, *24*, 954 ; Klar et al., *PNAS* **2000**, *97*, 8206.

Localization-based super resolution imaging

Point-Spread-Function of a single emitter



Precise localization of a single molecule

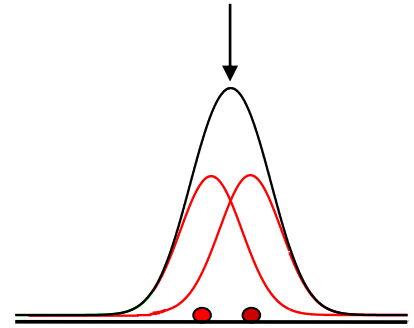


Single fluorescent molecules can be localized with an accuracy of:

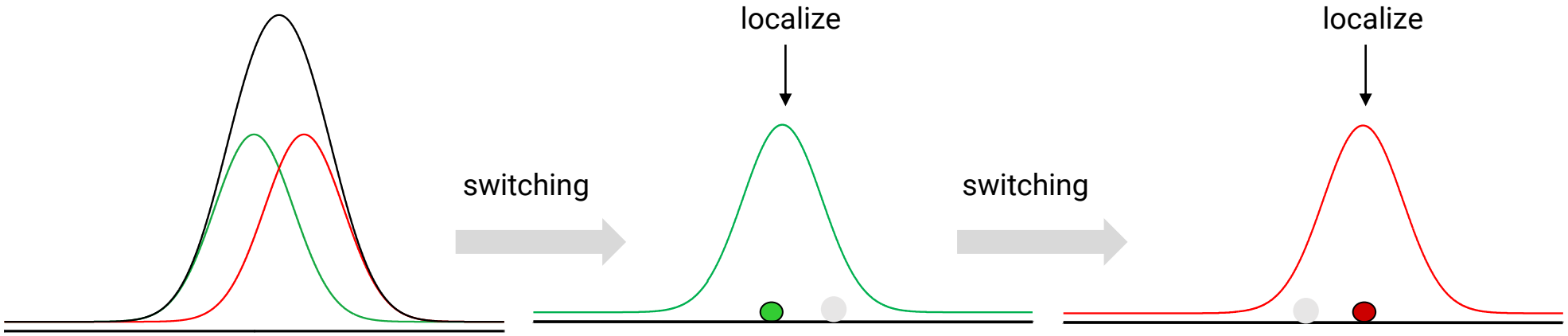
$$\sim \frac{FWHM}{\sqrt{N}}$$

Thompson et al., Biophys. J., 2002

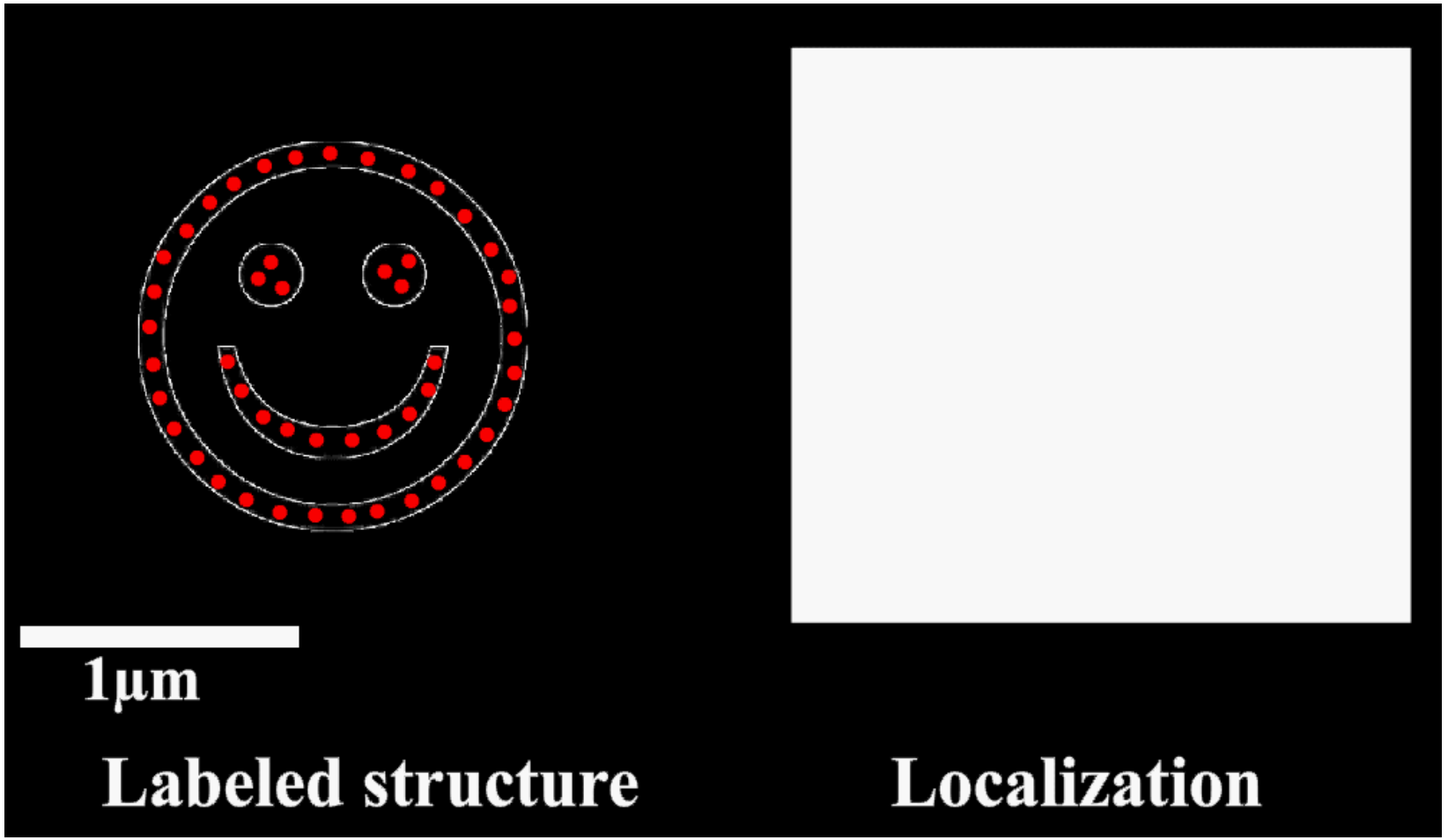
No localization possible



MAIN PRINCIPLE: separate the fluorescence from different emitters in time so that they can be localized one-by-one



Localization-based super resolution imaging

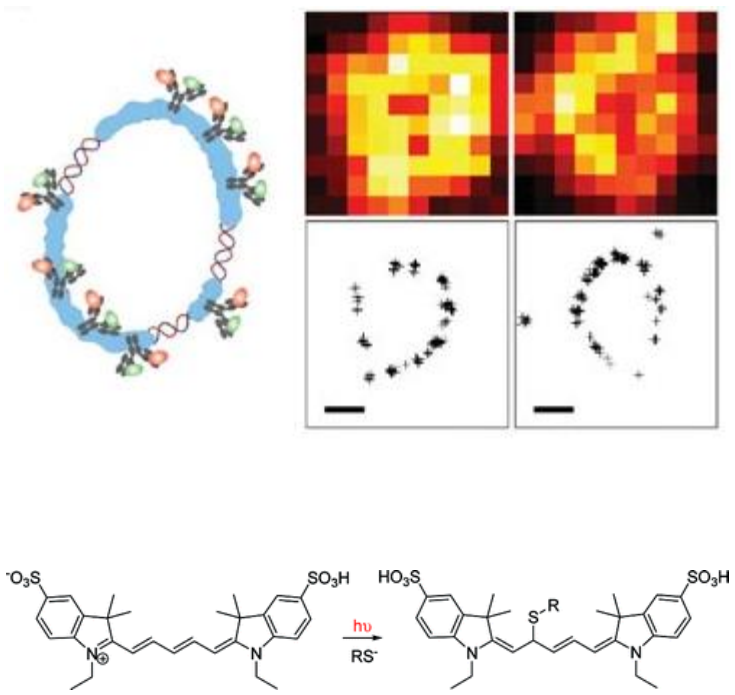


Requirements for dyes in localization-based superresolution

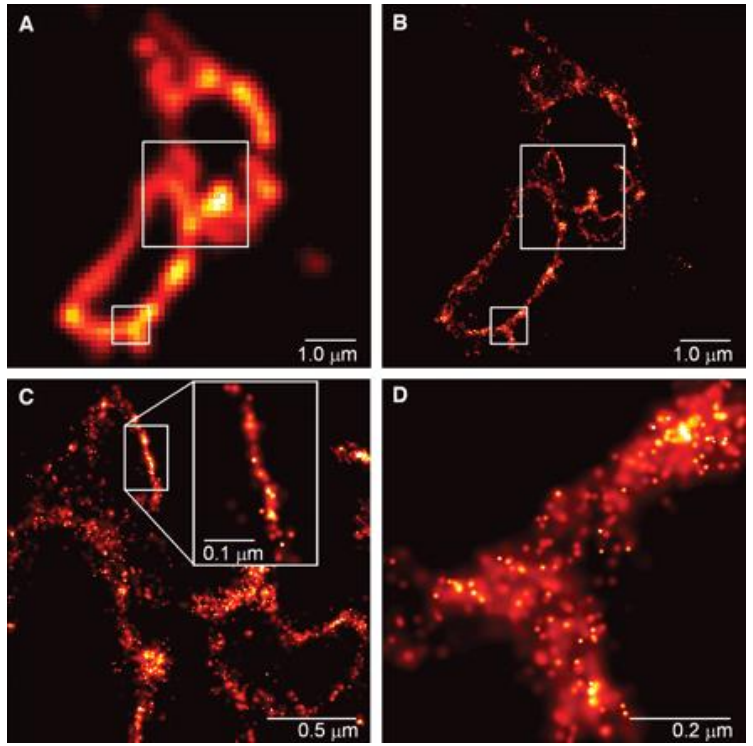
- **Bright** (high extinction coefficient, high quantum yield)
- **Photostable** (emit many photons before photobleaching)
- **Switchable** (or photoactivatable or color switching; or reversible binding)

Betzig *et al*., *Science* **2006**: PALM (photoactivated localization microscopy)
Rust *et al*., *Nat. Meth.* **2006**: STORM (stochastic optical localization microscopy)
Hess *et al. Biophys. J*. **2006**: FPALM (fluorescence photoactivated localization microscopy)

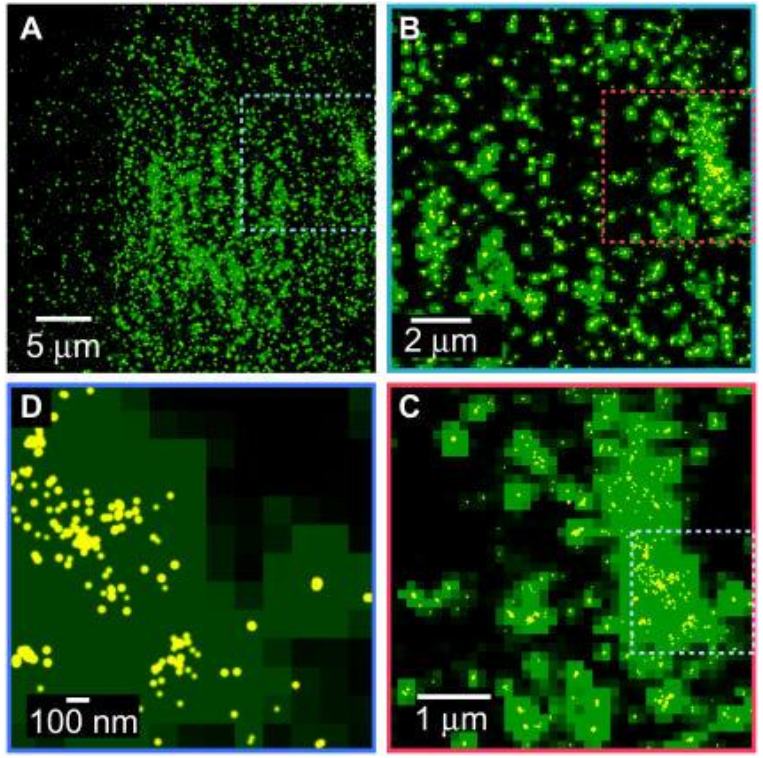
Localization-based super resolution imaging



Using thiol-induced blinking of cyanines
Rust *et al.*, *Nat. Meth.* **2006**: **STORM**
(stochastic optical localization microscopy)



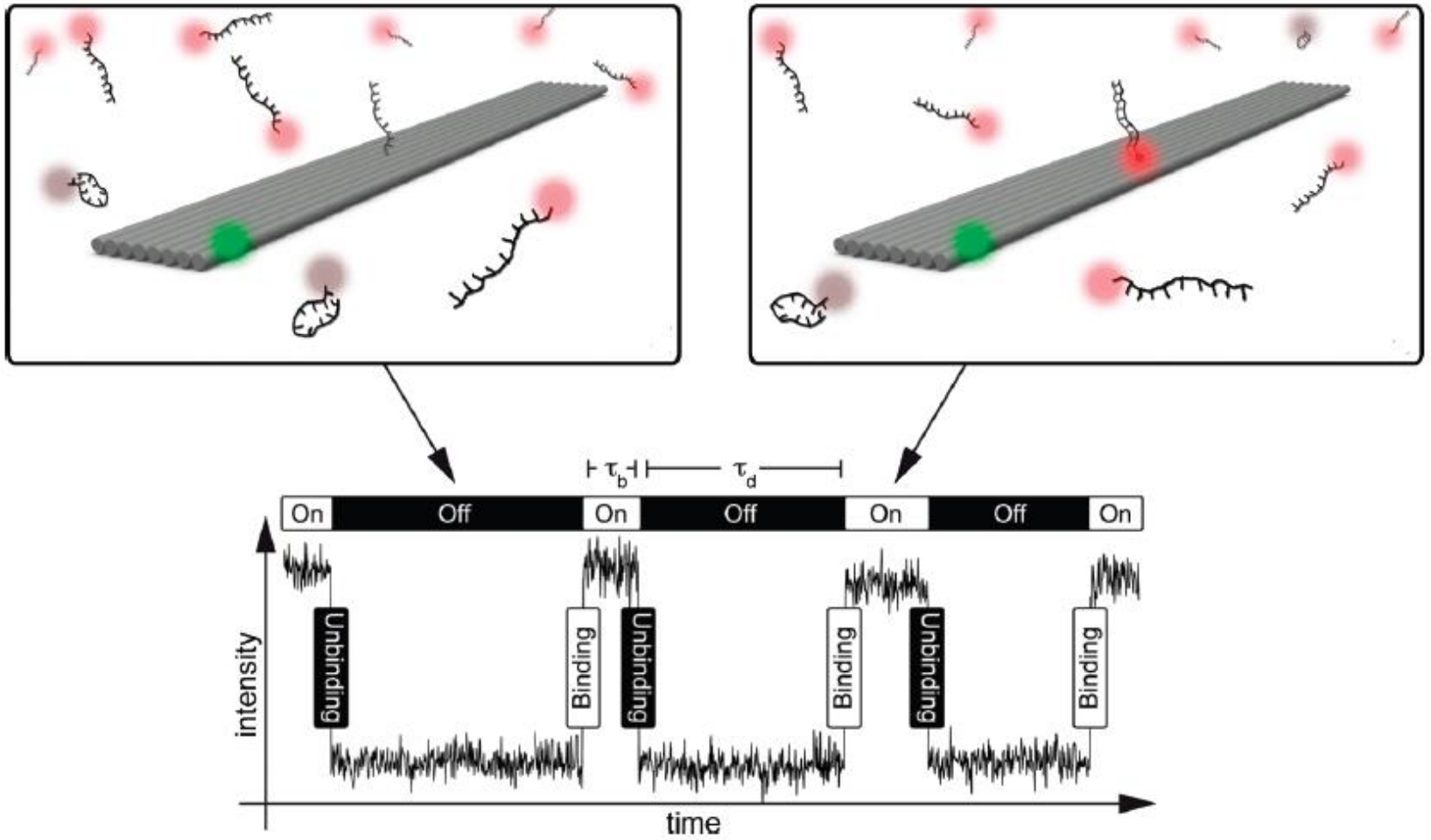
Using photoactivatable proteins
Betzig *et al.*, *Science* **2006**: **PALM**
(photoactivated localization microscopy)



Using photoactivatable GFP protein
Hess *et al. Biophys. J.* **2006**: **FPALM** (fluorescence photoactivated localization microscopy)

Stochastic blinking via DNA-PAINT

DNA PAINT: DNA Point Accumulation for Imaging in Nanoscale Topography

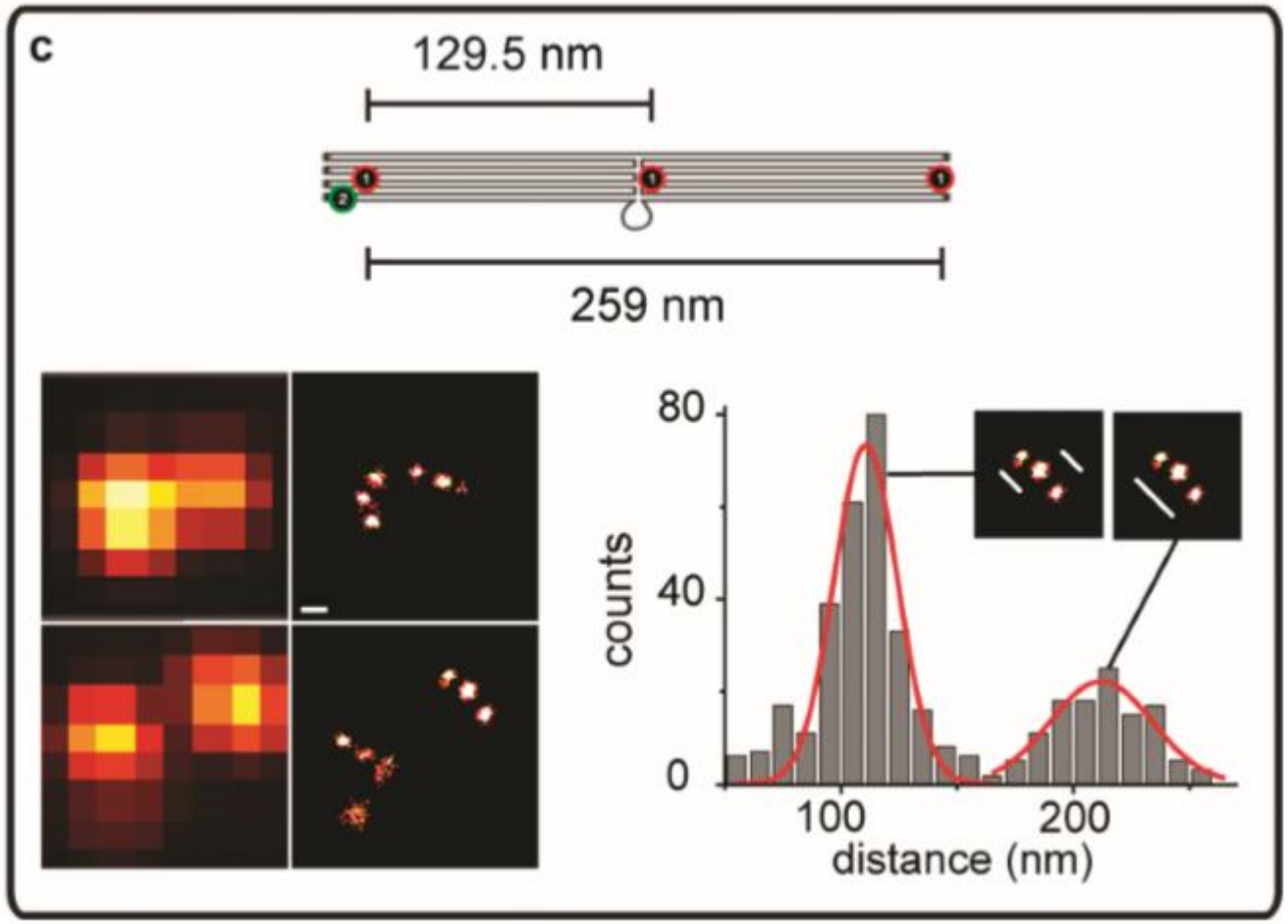


- Short, dye labeled oligos are binding transiently to the target
- Full control over blinking kinetics
- No photobleaching issues
- Full photon budget of the best dyes

Ralf Jungmann, Christian Steinhauer, Max Scheible, Anton Kuzyk, Philip Tinnefeld and Friedrich C. Simmel, *Nano Letters* **2010**, *11*, 2475-2490

Stochastic blinking via DNA-PAINT

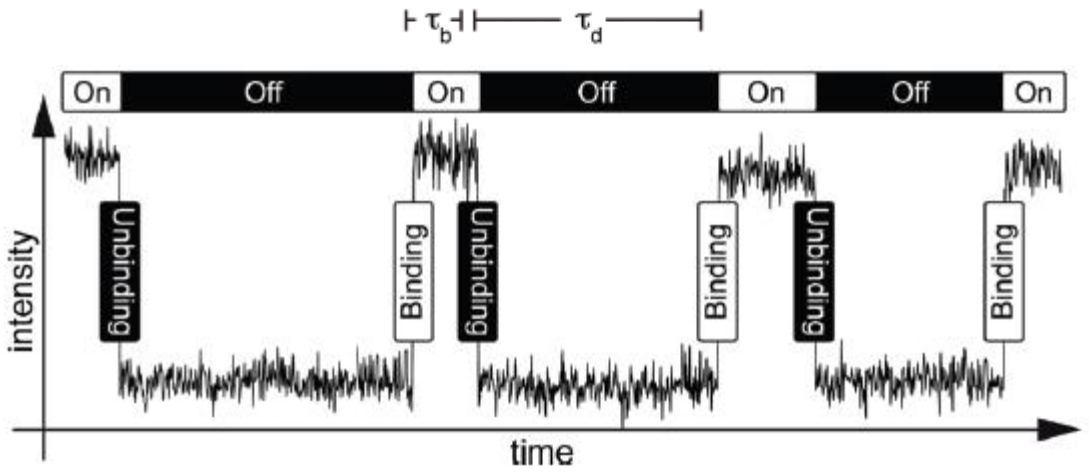
DNA PAINT: DNA Point Accumulation for Imaging in Nanoscale Topography

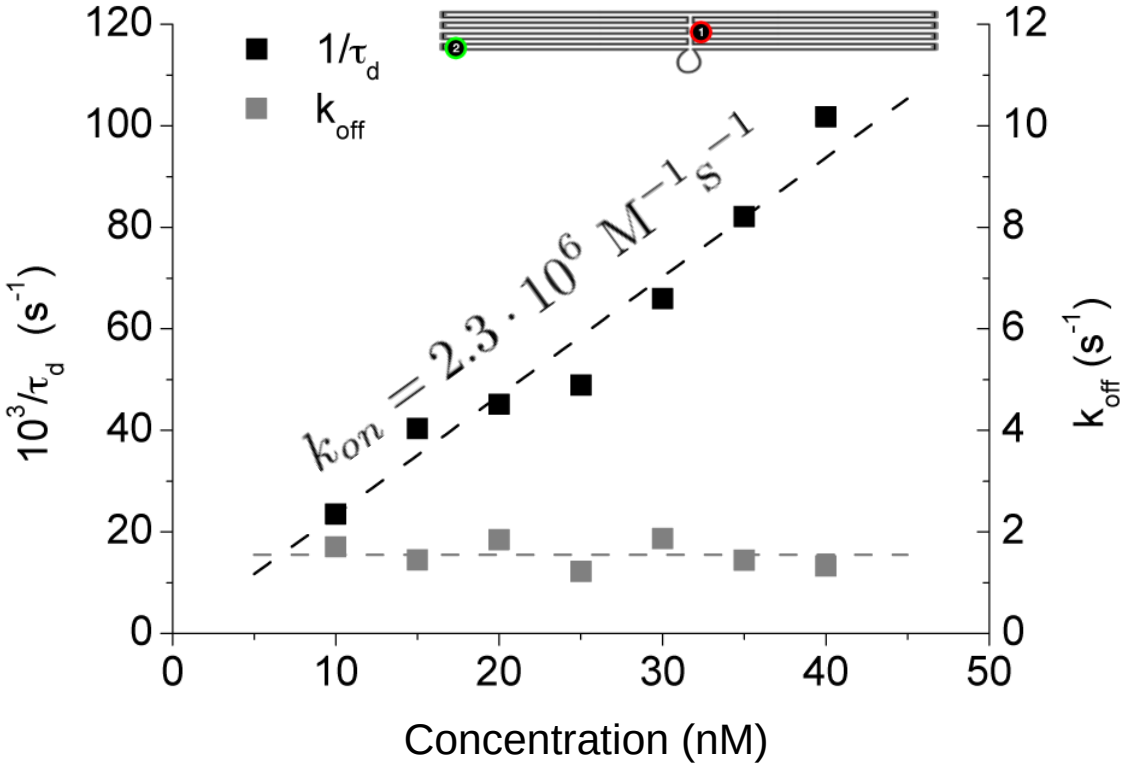


- Short, dye labeled oligos are binding transiently to the target
- Full control over blinking kinetics
- No photobleaching issues
- Full photon budget of the best dyes

Ralf Jungmann, Christian Steinhauer, Max Scheible, Anton Kuzyk, Philip Tinnefeld and Friedrich C. Simmel, *Nano Letters* **2010**, *11*, 2475-2490.

Studying Binding and Unbinding Dynamics on DNA Origami





Association time:
concentration dependent

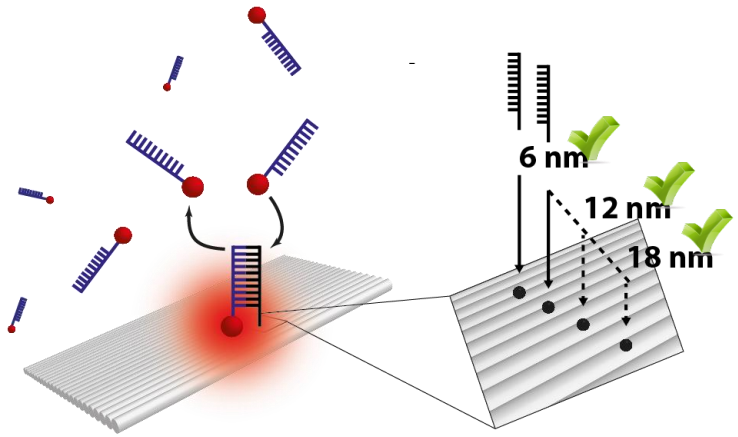
$$\frac{1}{\tau_d} = c \cdot k_{on}$$

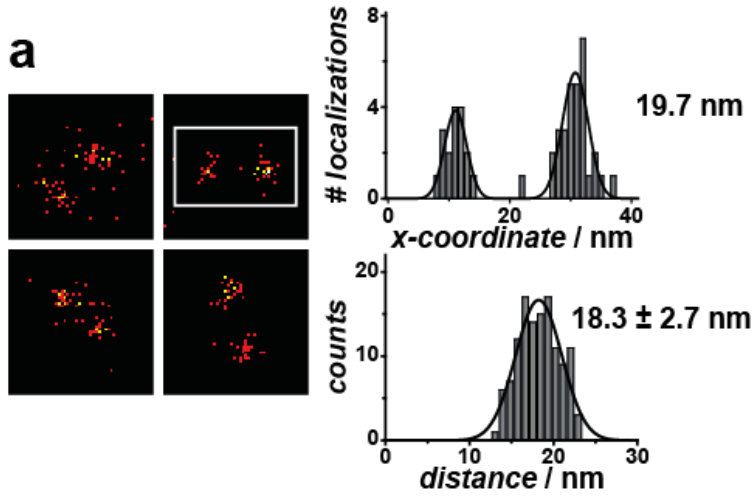
Dissociation time:
DNA sequence dependent

$$\frac{1}{\tau_b} = k_{off}$$

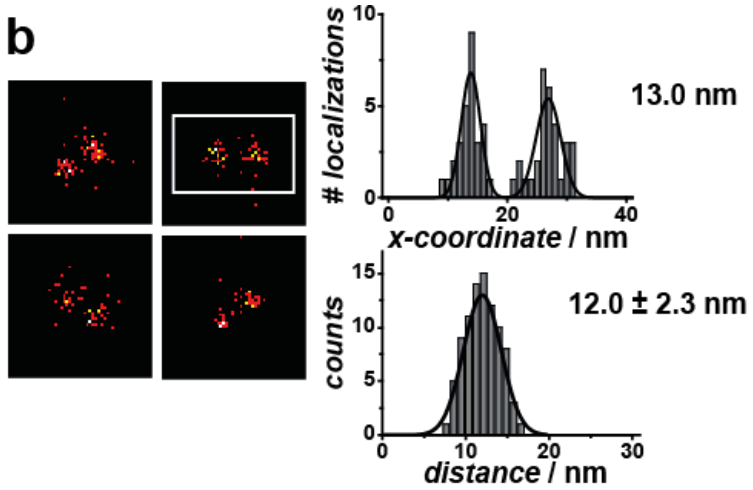
Ralf Jungmann, Christian Steinhauer, Max Scheible, Anton Kuzyk, Philip Tinnefeld and Friedrich C. Simmel, *Nano Letters* **2010**, *11*, 2475-2490.

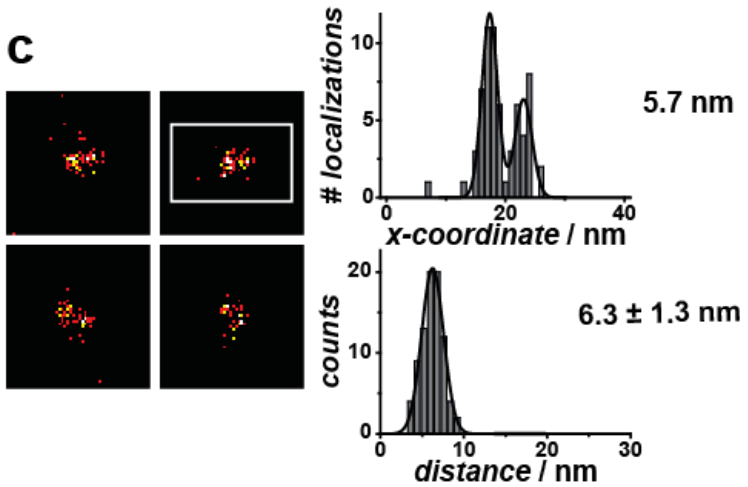
6 nm resolution with DNA-PAINT





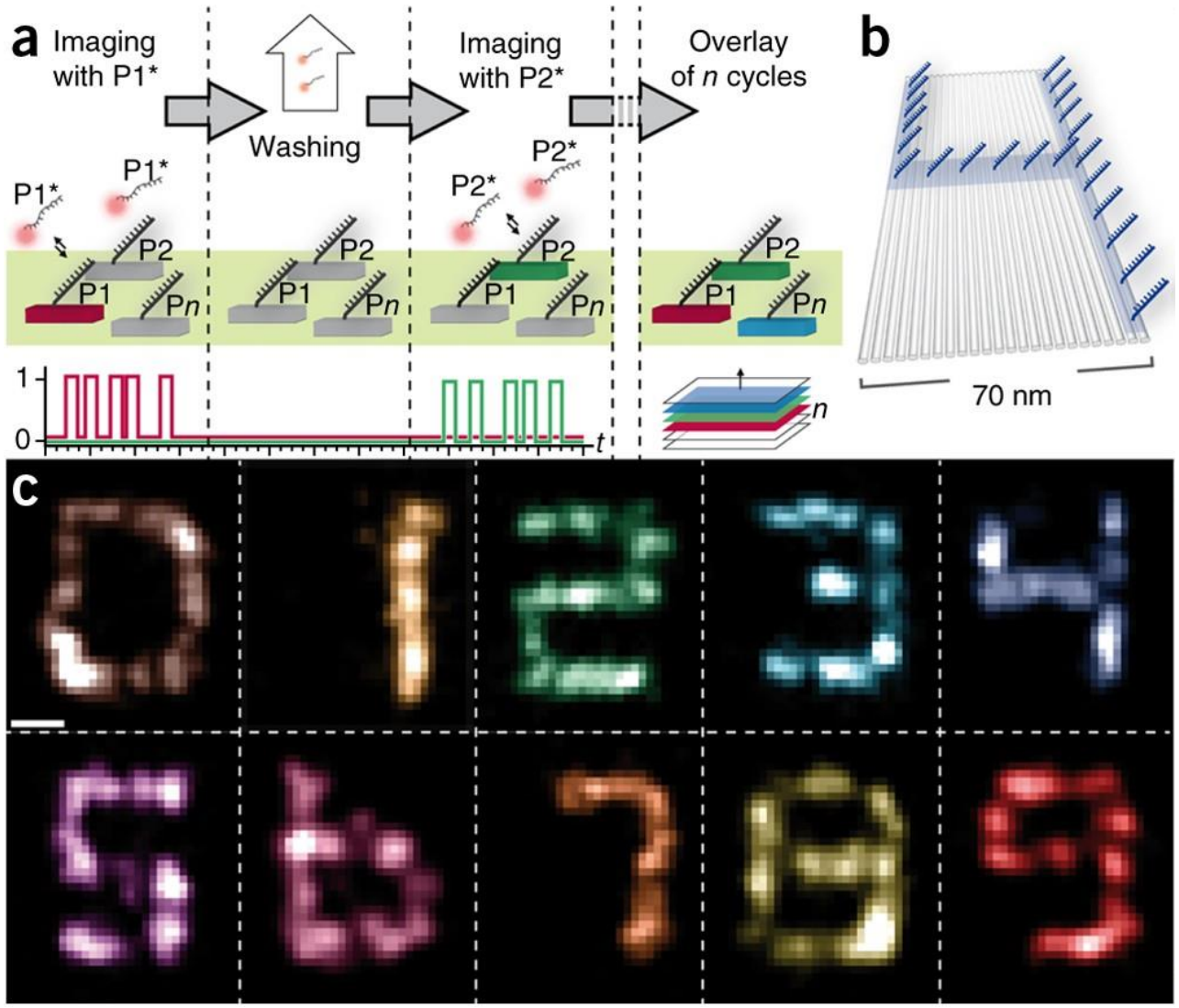
Average number of photons = 36600!





Raab *et al.*, *Chem Phys Chem* **2014**, *15*, 2431

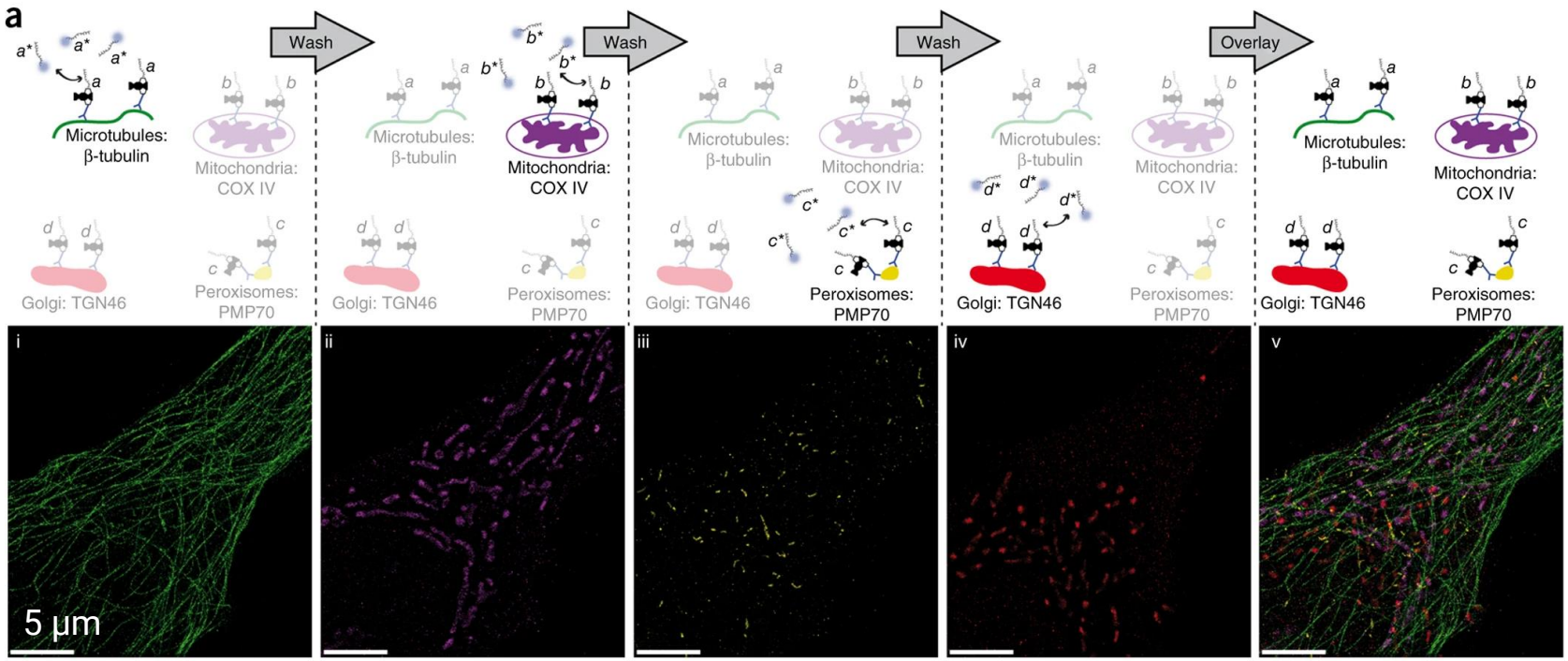
Exchange-PAINT

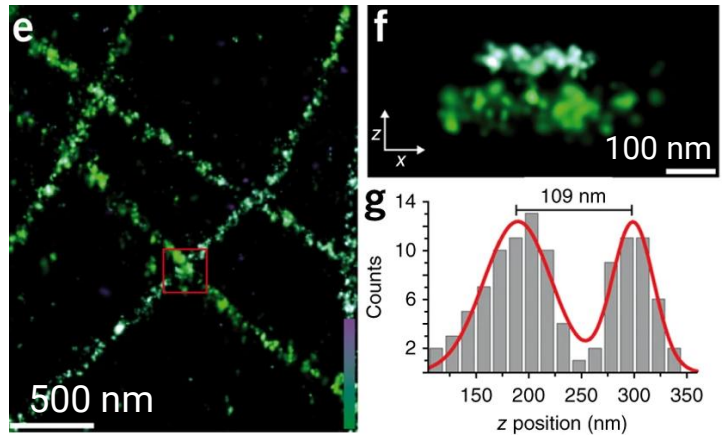


- Sequential exchange of PAINT imager strands
- Each strand target different structure/ compartment
- Only one fluorophore is employed
- Sub-10 nm spatial resolution

Jungmann *et al.*, *Nature Methods* **2014**, *11*, 313-318

2D and 3D Exchange-PAINT in fixed cells

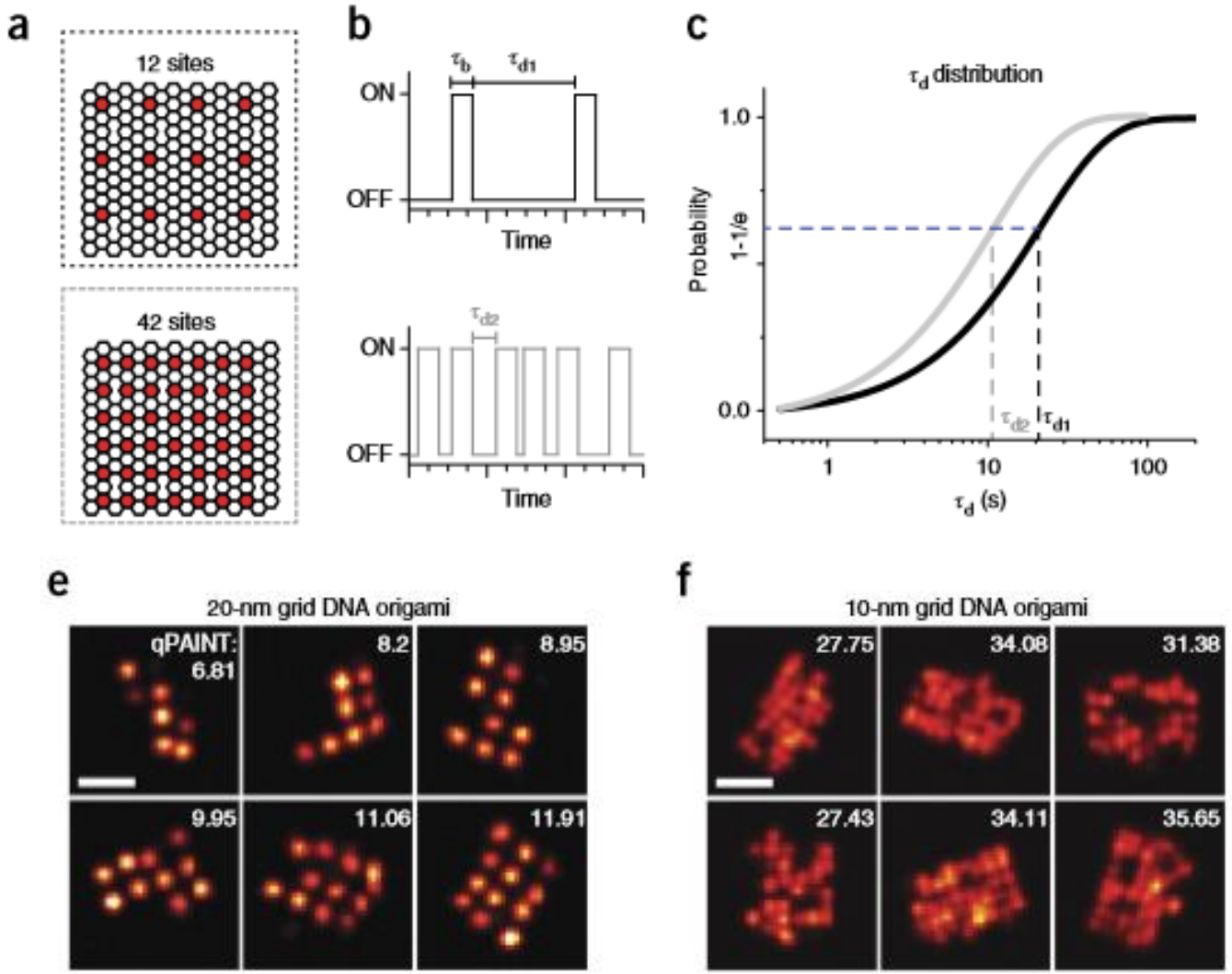




- each target is labelled with an antibody carrying a unique DNA-PAINT docking sequence
- 2D and 3D imaging on fixed cells

Jungmann *et al.*, *Nature Methods* **2014**, *11*, 313-318

Quantitative DNA PAINT (qPAINT)

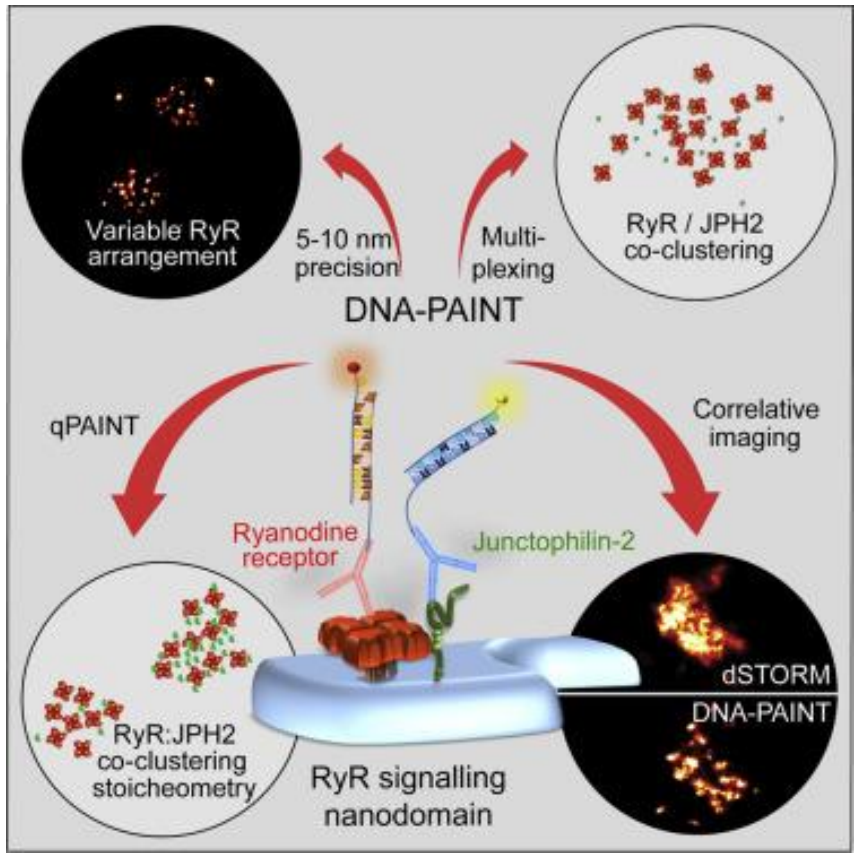


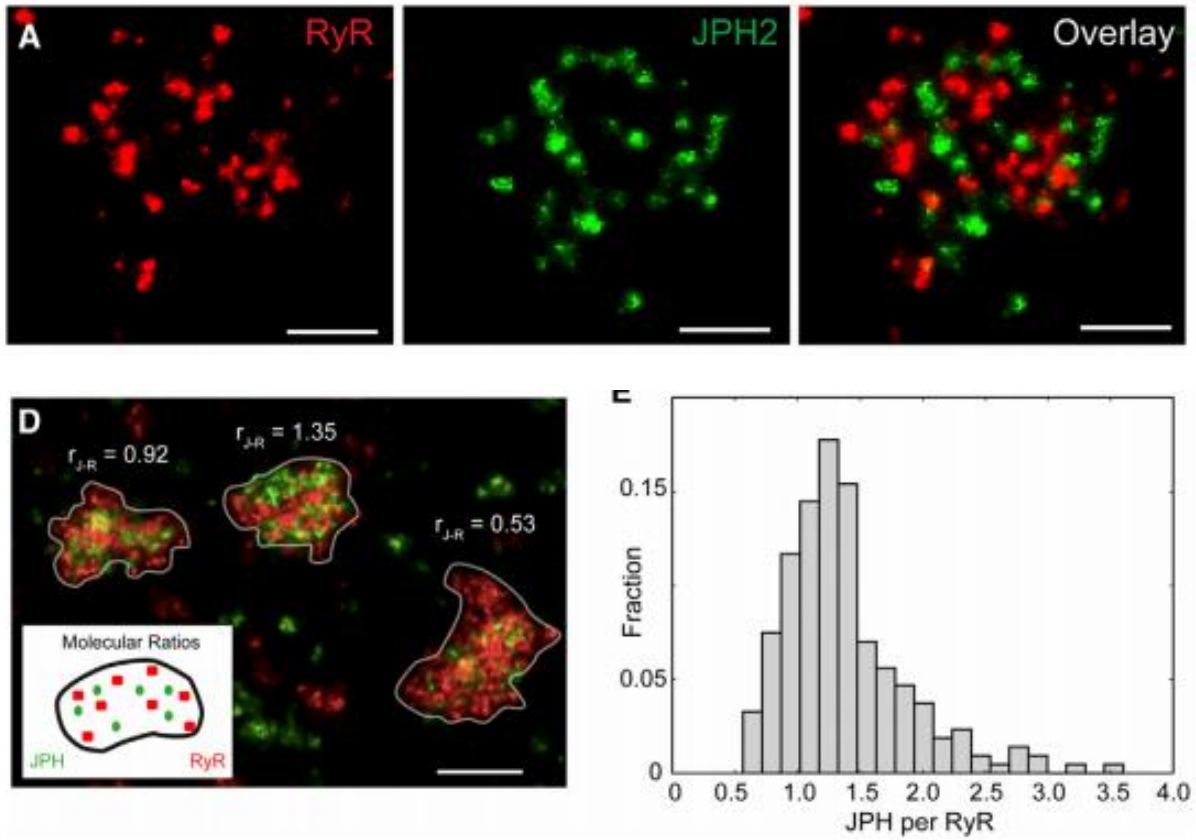
Main principle

- Frequency of binding (blinking) events ∝ # of binding sites
- Use DNA origami structures with a known number of binding sites for calibration
- Determine # of binding sites in the unknown sample

Jungmann *et al.*, *Nature Methods* **2016**, *13*, 439-442; Schnitzbauer et al., *Nature Protocols* **2017**, *12*, 1198

Using qPAINT to study clustering of receptors





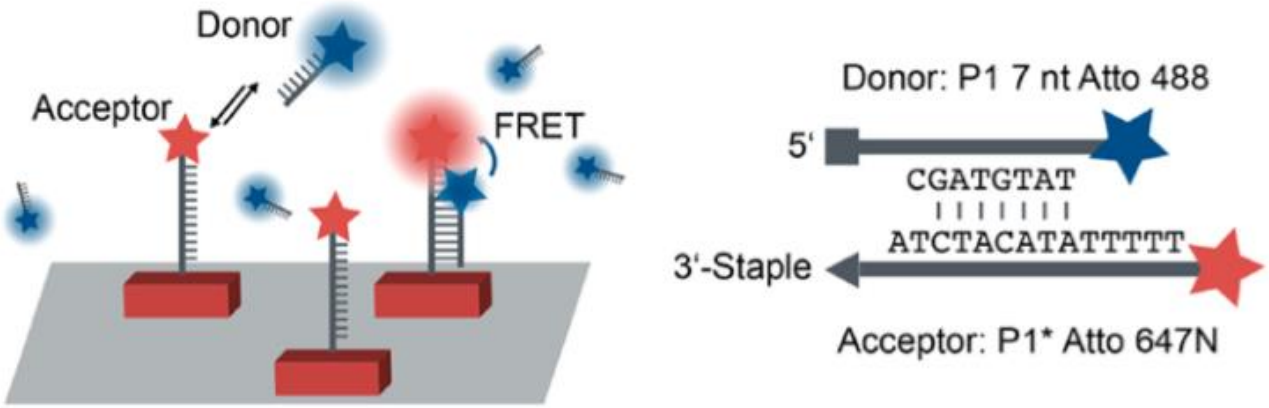
- Different PAINT binding sites are designed for each receptor
- Reveals heterogeneous clustering of two receptors in cells

Jayasinghe et al., *Cell Reports* **2018**, *22*, 557-567

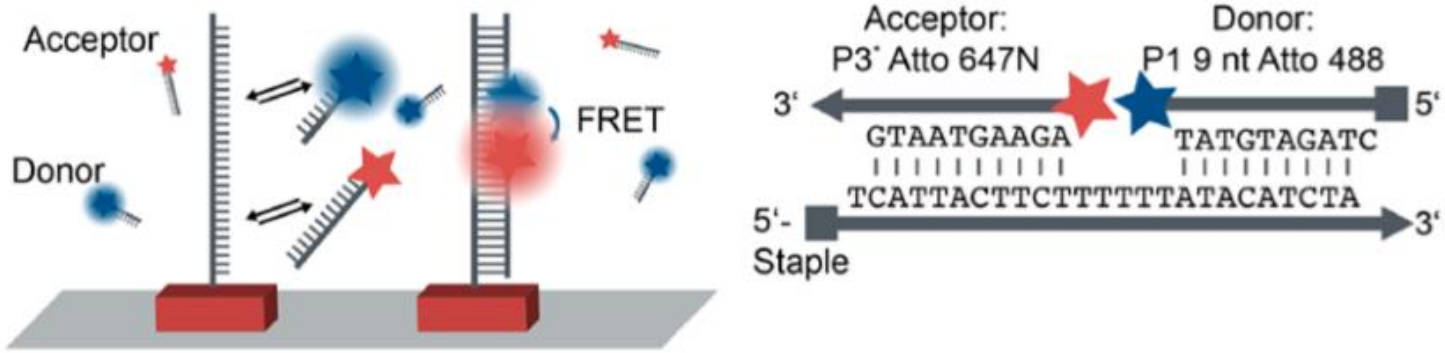
FRET PAINT: reducing the background

- Background from diffusing dye labelled imager strands can be an issue
- [Imager] is limited to 1-10 nM
- FRET-PAINT approaches provide a way to overcome the background problems

FRET-PAINT

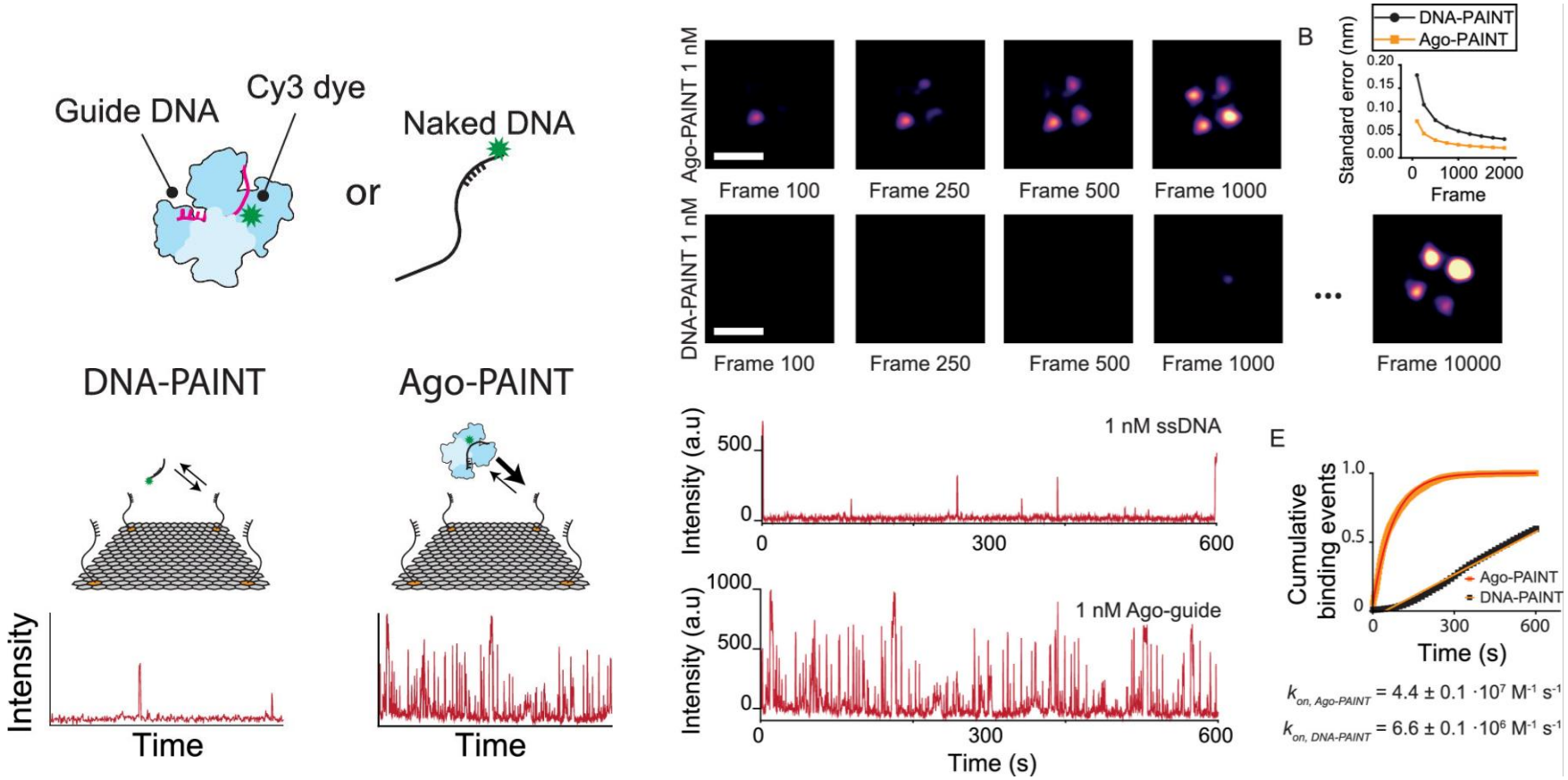


“Dynamic” FRET-PAINT



Auer *et al*., *Nano Lett*. **2017**, *17*, 6428; Nieves *et al*., *Genes* **2018**, *9*, 621

Protein-assisted high-speed DNA PAINT



In **regular DNA PAINT** # of binding events/time is limited by:

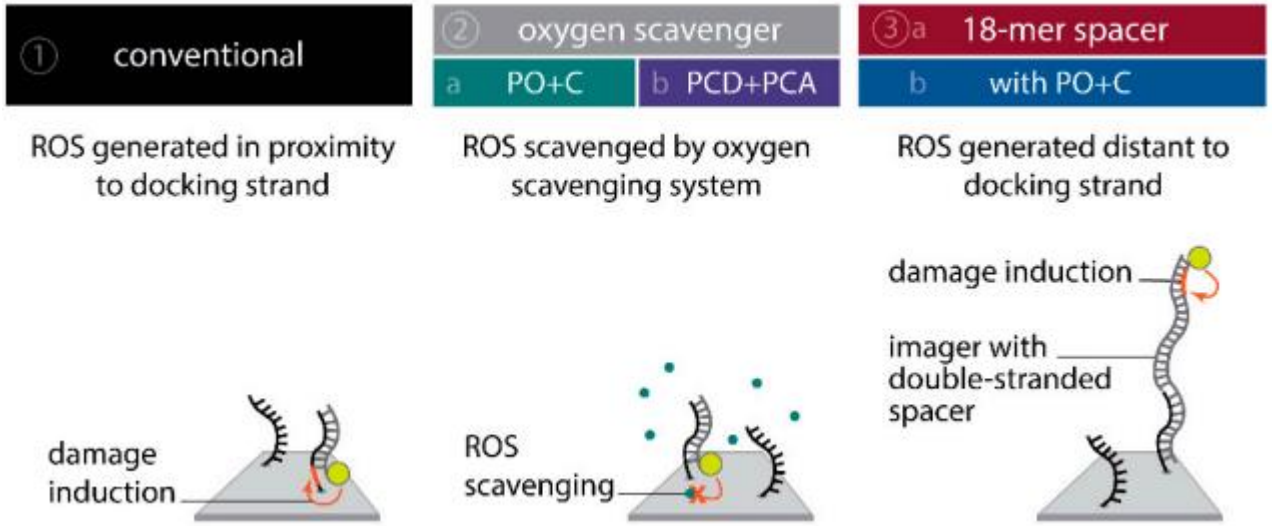
k_{on} ~10^6 M^{-1} s^{-1}
[Imager] ~ 1-10 nM

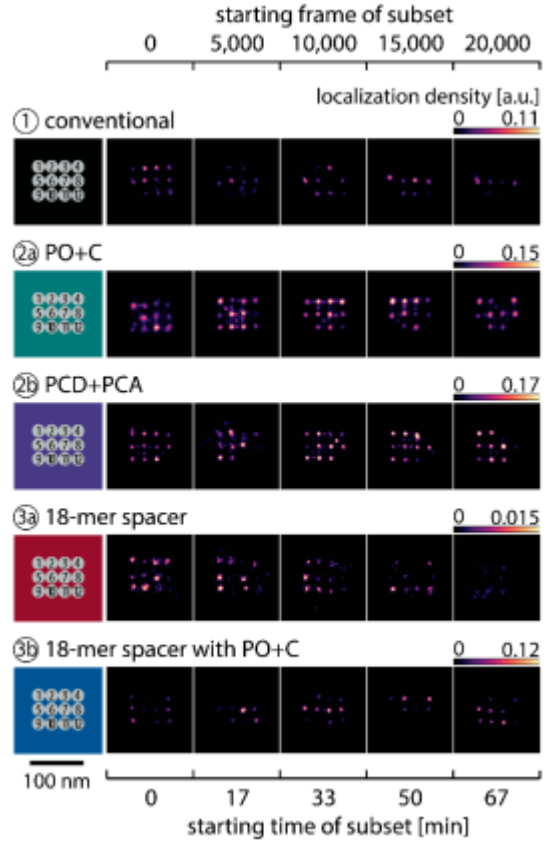
- **Argonaute proteins (Agos)** are a class of enzymes that utilize a DNA or RNA guide to find a complementary target
- Ago preorders the guide strand into helical conformation to bind the target strand -> **increase in association rate -> faster superresolution imaging**

Nano Lett. **2020**, *20*, 2264–2270

Challenge: photodamage of PAINT binding sites

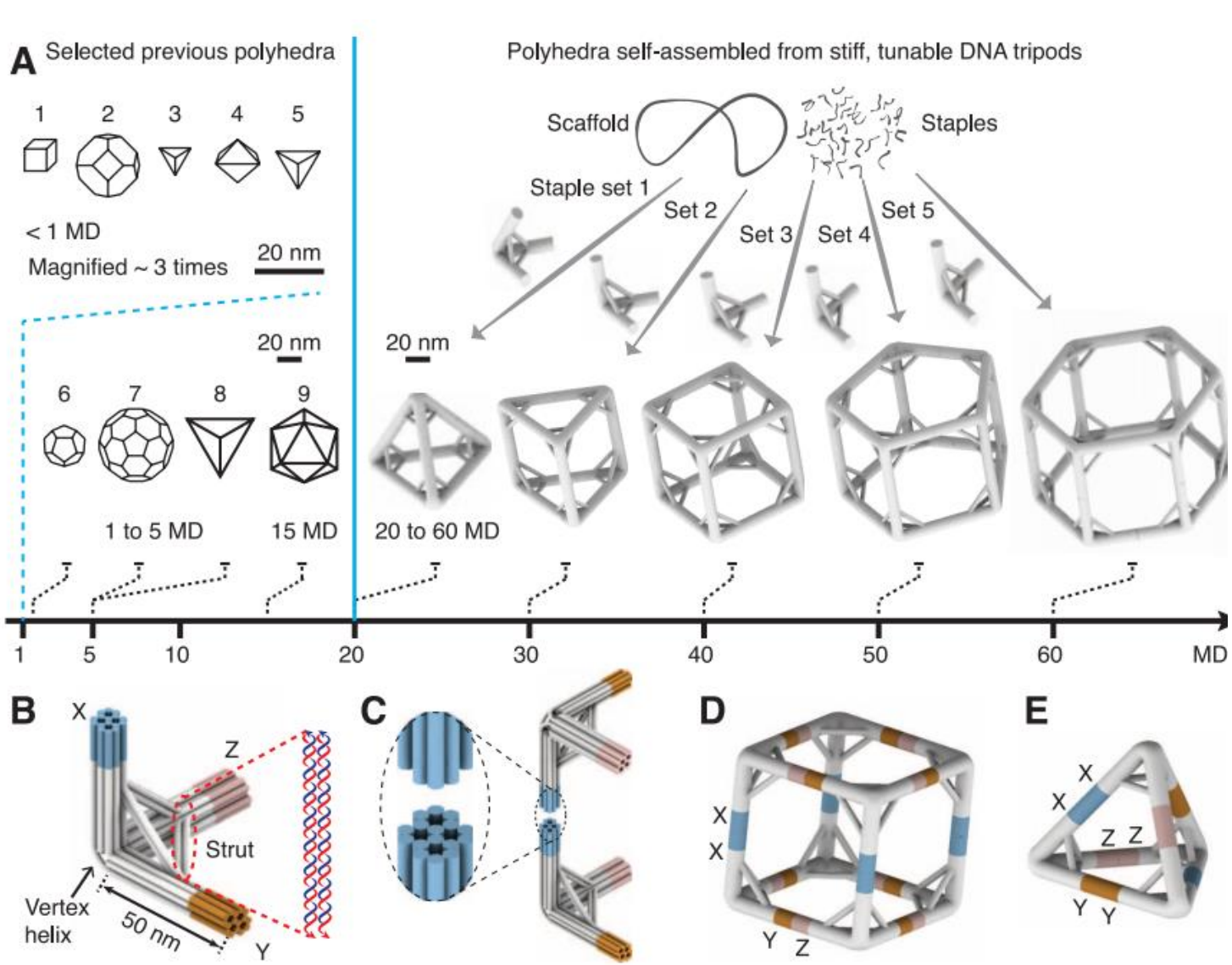
- over extended imaging times PAINT binding sites are depleted
- limits the quality of super-resolved images
- proposed damage to DNA docking site by continuous generation of reactive oxygen species (ROS)
- suppression of the damage by the use of oxygen scavengers and docking spacer

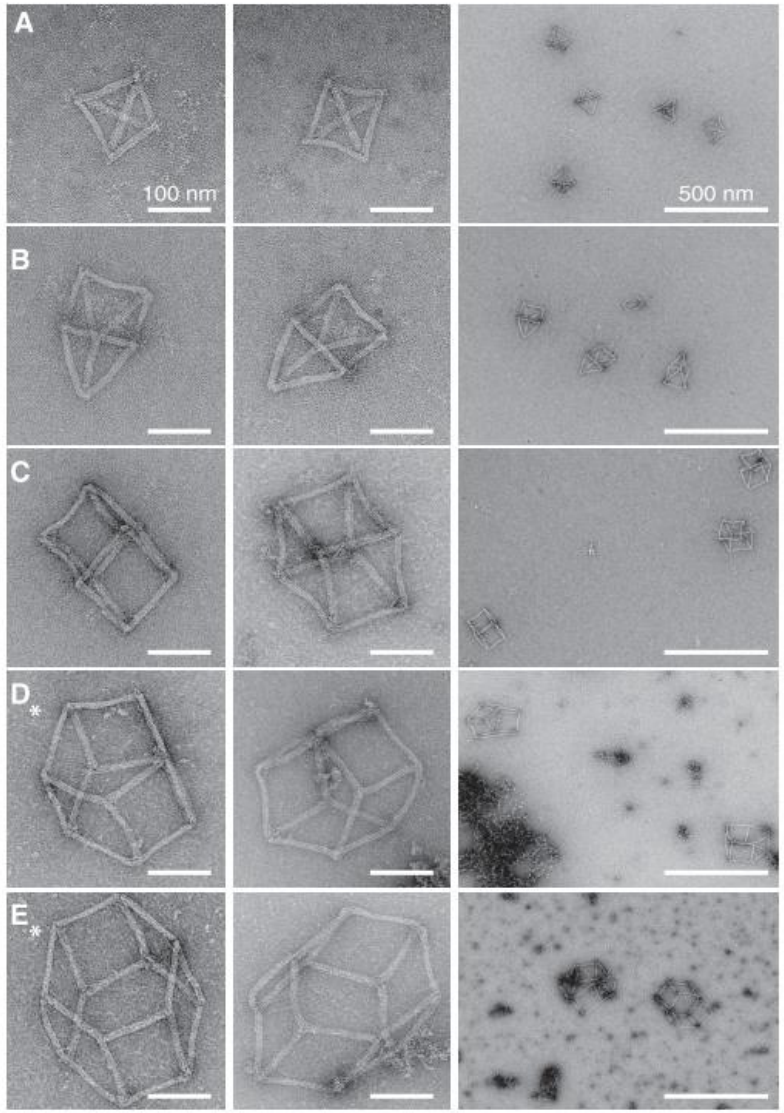




Blumhardt *et al. Molecules* **2018**, *23*, 3165

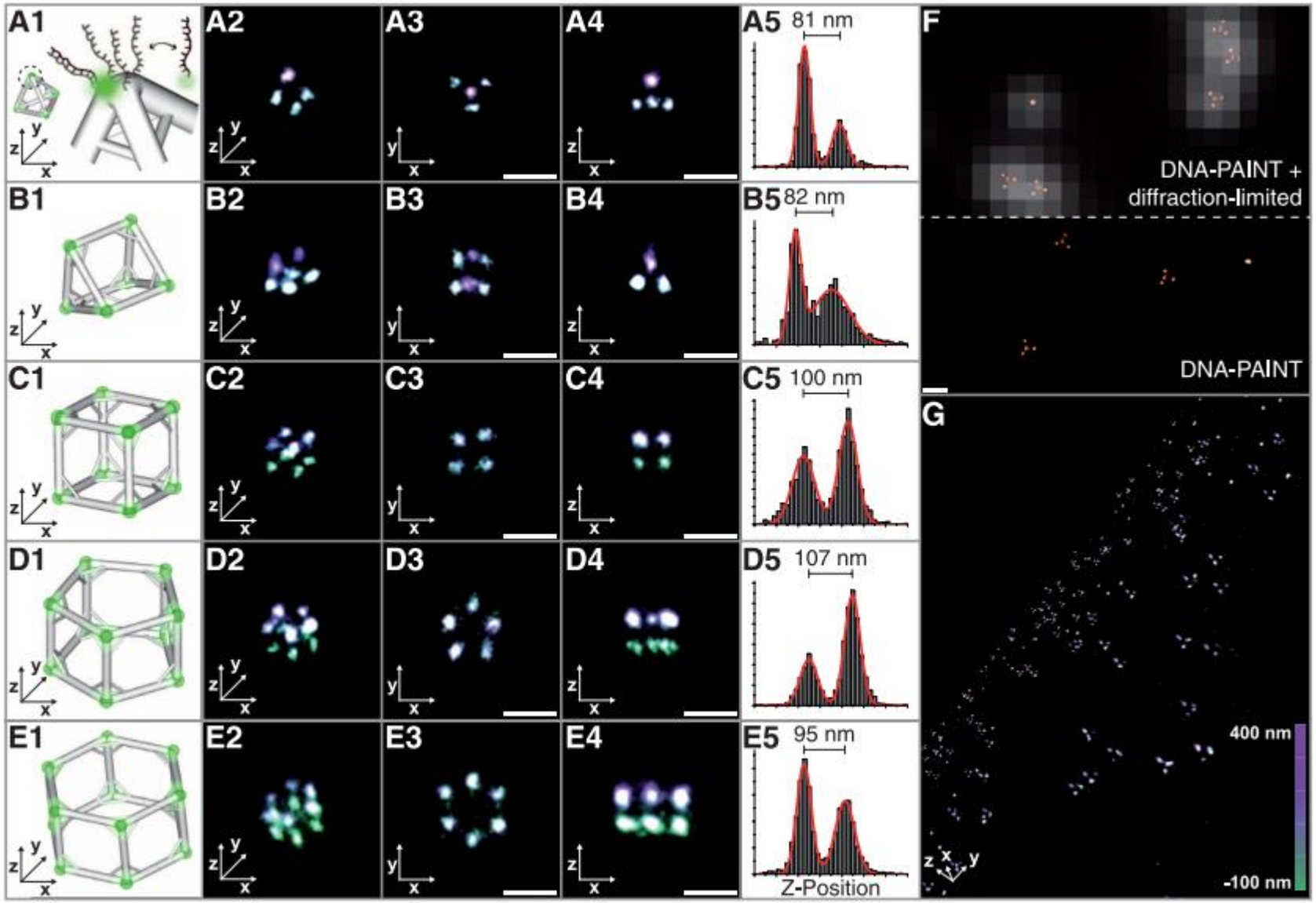
Megadalton DNA Polyhedra Characterized with 3D DNA PAINT



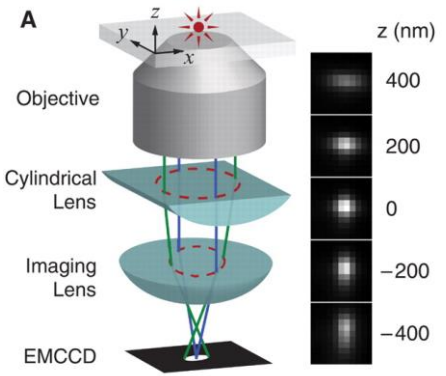


Iinuma, Ke, and Jungmann, *Science* **2014**, 344, 65

Megadalton DNA Polyhedra Characterized with 3D DNA PAINT



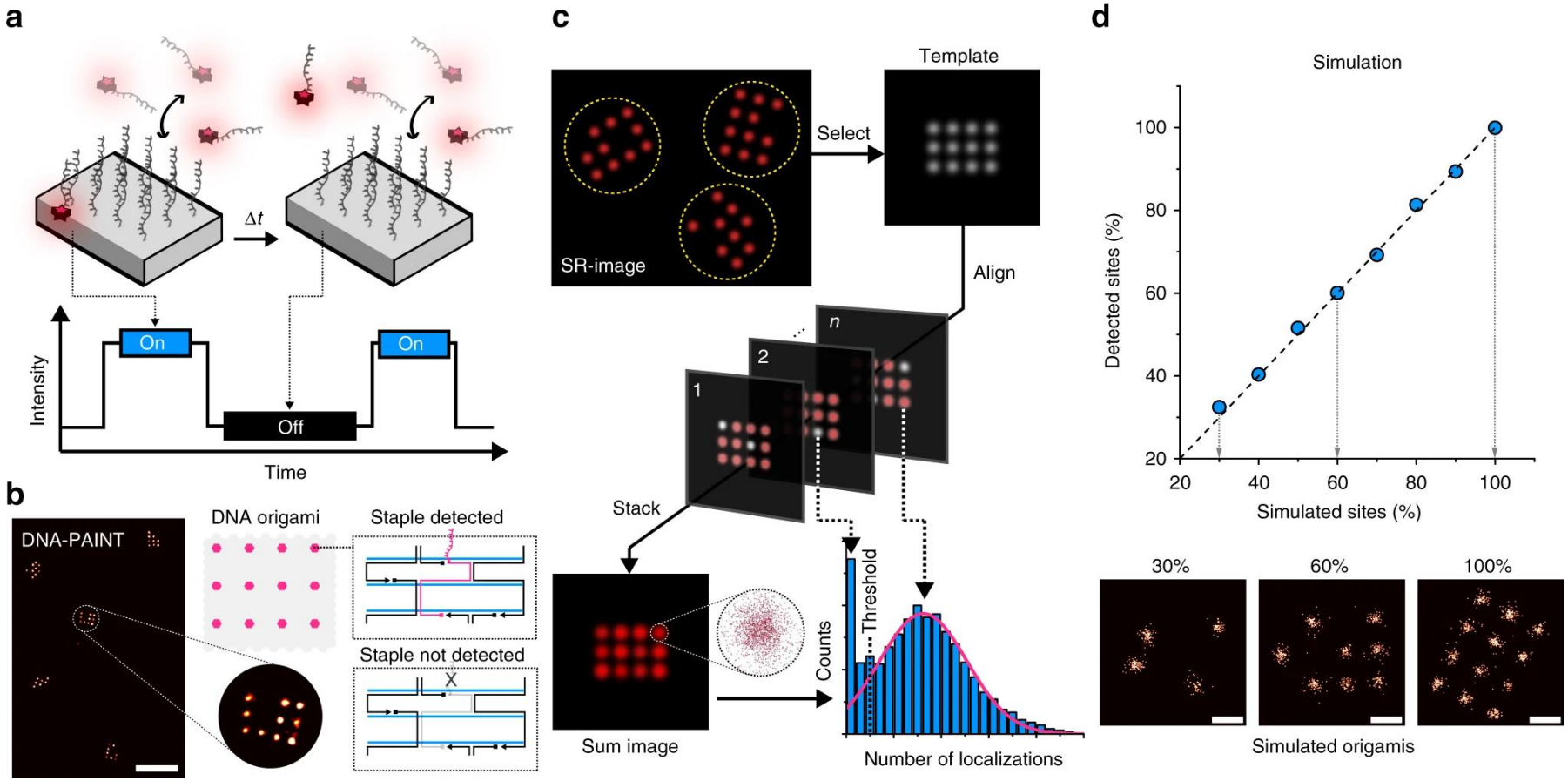
- imaging of the large 3D polyhedra structures in “native” hydrated environment
- each vertex modified with ~18 9-nt docking sites
- Localization precision:
  ~13 nm in x-y
  ~24 nm in z



Iinuma, Ke and Jungmann, *Science* **2014**, 344, 65

Quantifying addresability of DNA origami with DNA-PAINT

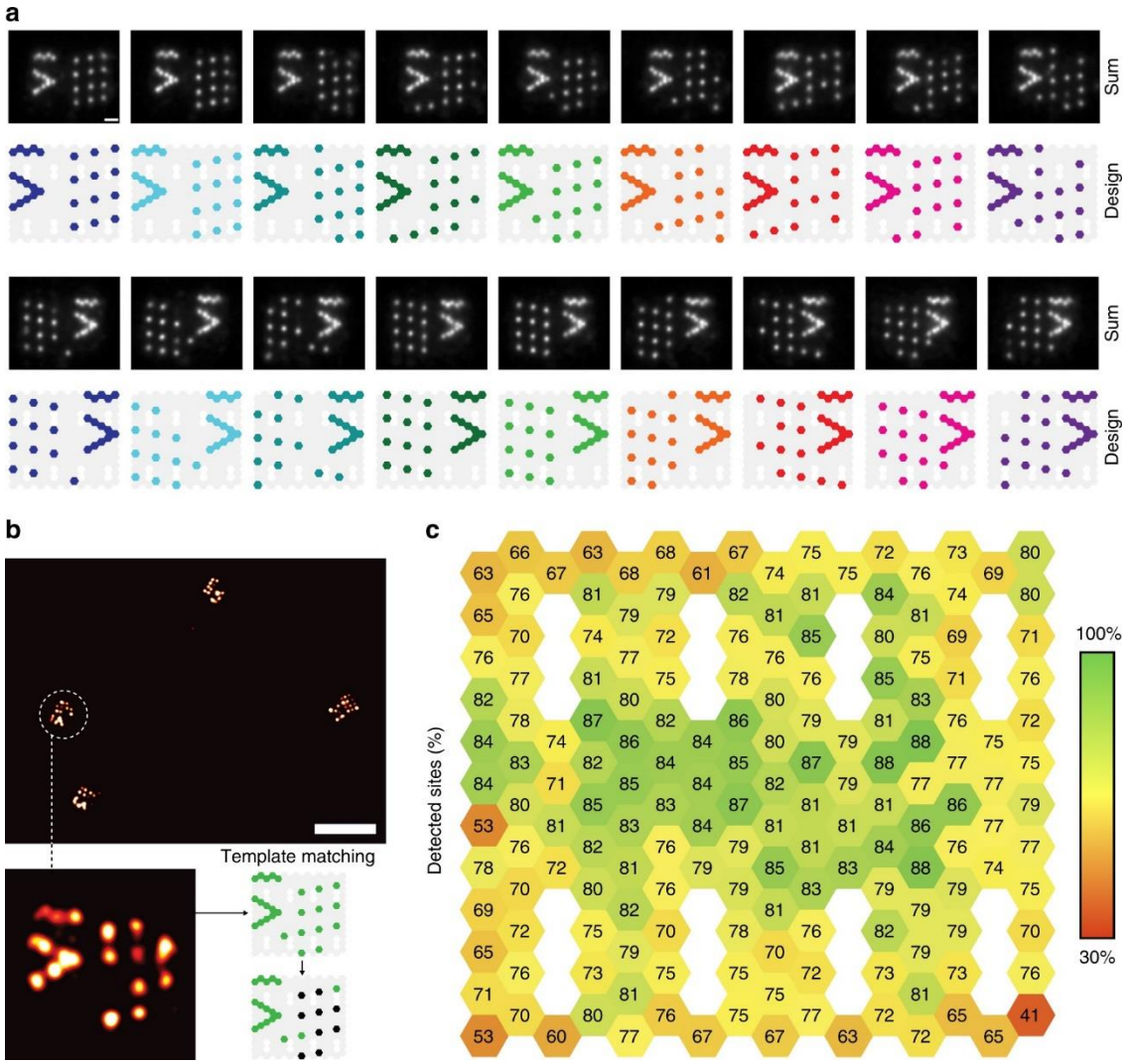
Are all the staples equally incorporated and accessible?



Strauss et al., *Nature Communications* **2018**, *9*, 1600

Quantifying addresability of DNA origami with DNA-PAINT

Are all the staples equally incorporated and accessible?

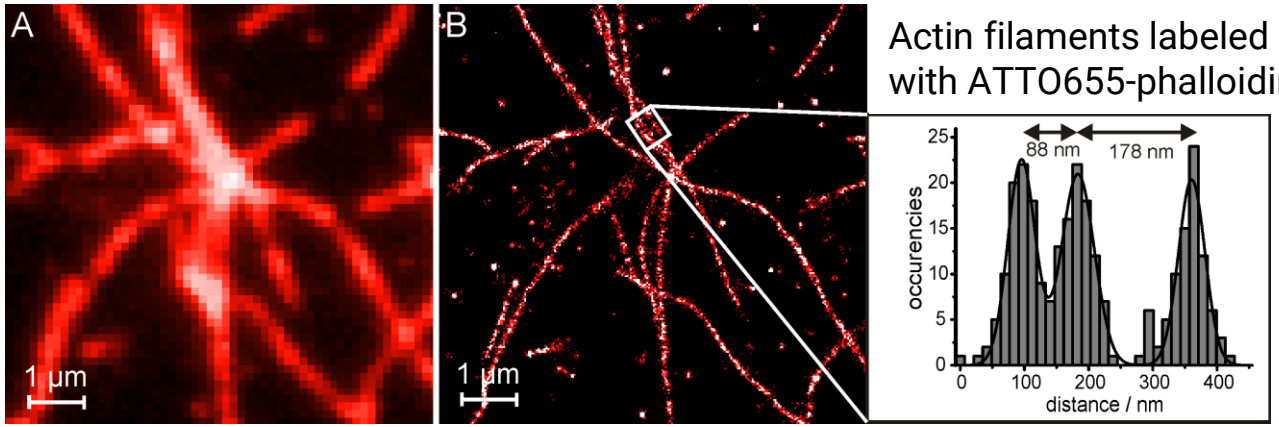


Conclusions:

- incorporation efficiency of the target strands is strongly influenced by the position on origami
- lowest incorporation at the periphery (as low as 40-50 %)
- highest incorporation efficiency in the middle of the tile (maximum 95 %)

Strauss et al., *Nature Communications* **2018**, *9*, 1600

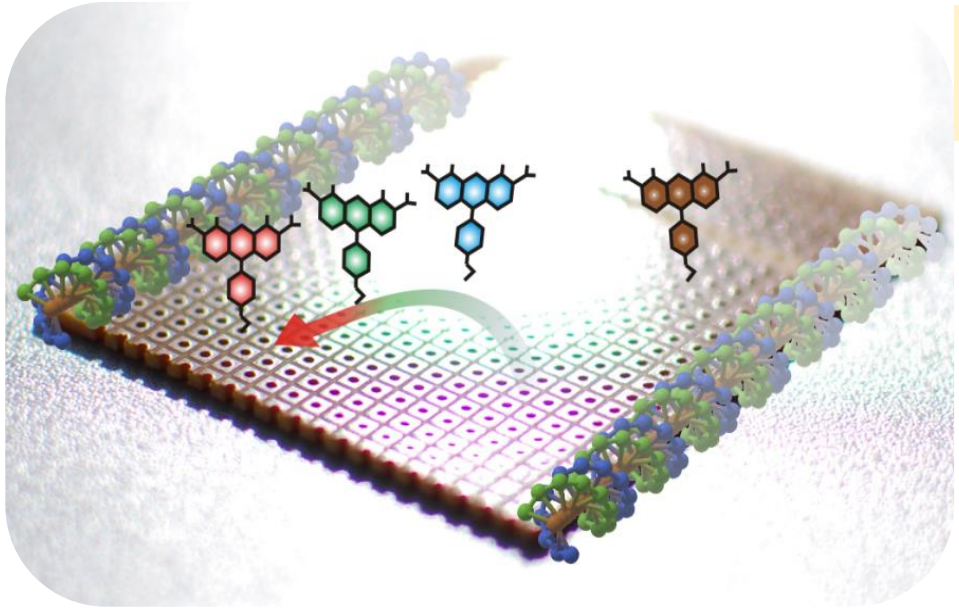
DNA Origami-based Superresolution Standards



Actin filaments labeled with ATTO655-phalloidin

A defined standard is required to:

- quantify and demonstrate the resolution
- study and calibrate dye blinking
- very optical magnification
- correct for aberrations



Can we place fluorophores with nm-accuracy?

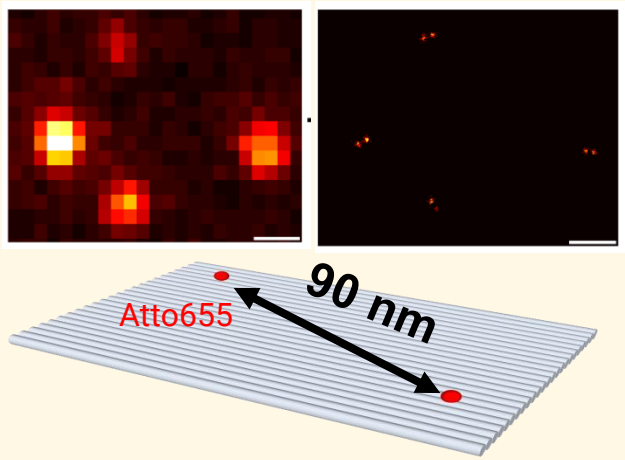
DNA-Origami as a nanoscopic ruler for super-resolution microscopy

GATTAquant DNA NANOTECHNOLOGIES

First commercial application of DNA origami nanotechnology

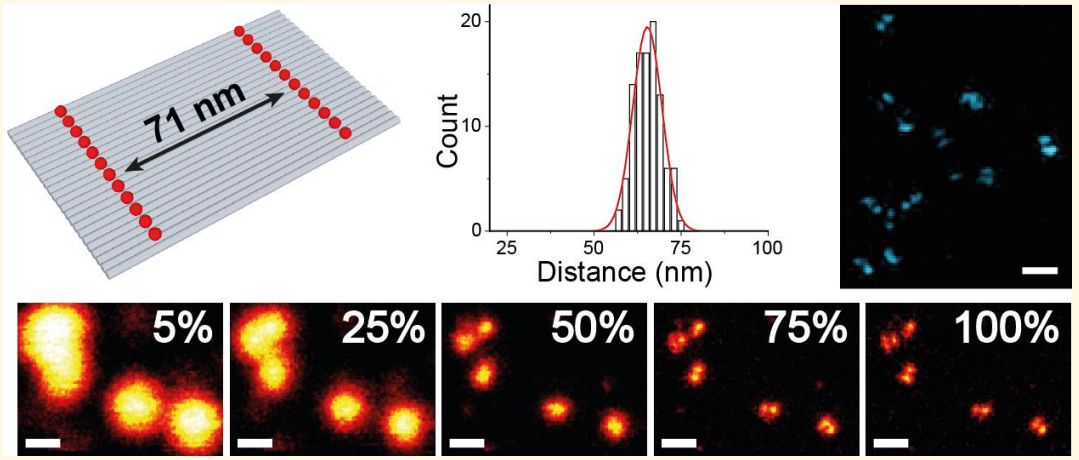
DNA Origami-based Superresolution Standards

Localization-based SR Ruler



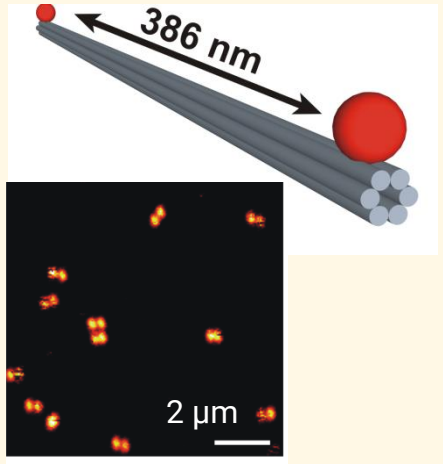
Steinhauer *et al.*, *Angew. Chem.* **2009**, *121*, 9030

STED Ruler

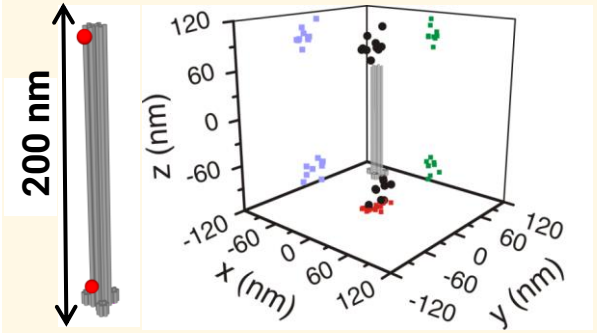


Schmied *et al.*, *Nat. Methods* **2012,** *9*, 1133

Confocal Ruler

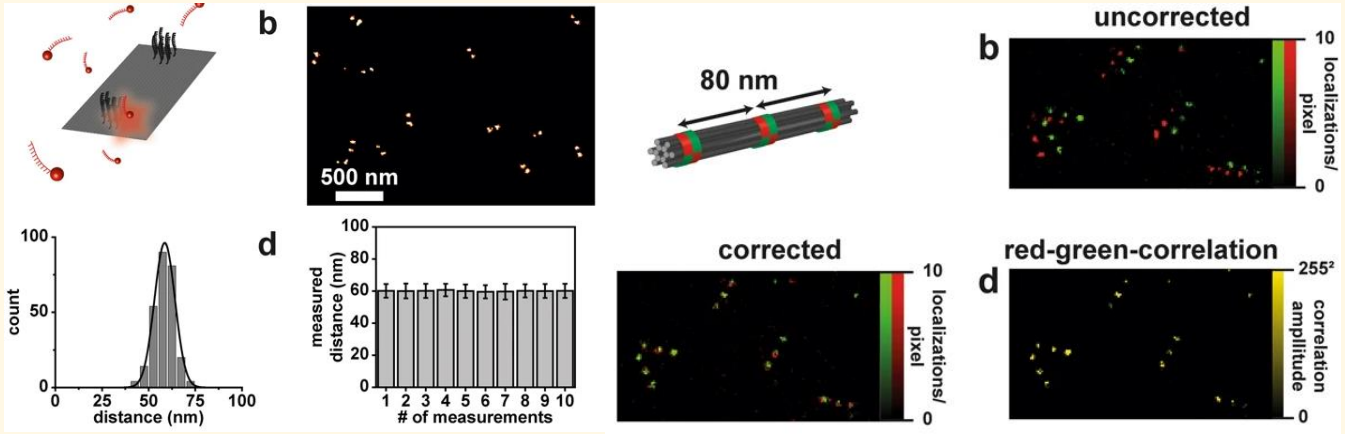


3D Superresolution Ruler



Schmied *et al.*, *Nano Lett.* **2013**, *13*, 781

Distance and chromatic shift rulers



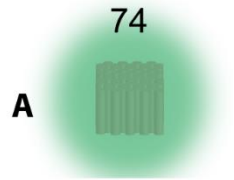
Raab *et al.*, *Scientific Reports* **2013**, *8*, 1780

DNA origami-based brightness standards

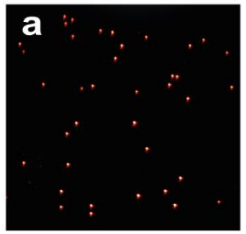
- Precise positioning of dyes
- Avoids quenching
- Precise # of dyes
- Brightness ∝ number of dyes

How many dyes can be detected with a smartphone camera?

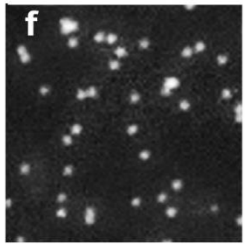
DNA origami with X fluorophores

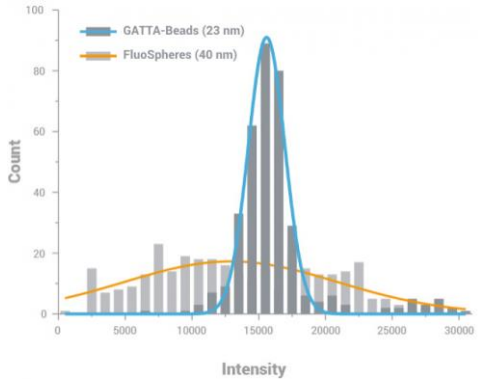


Commercial confocal microscope



Monochrome smartphone camera

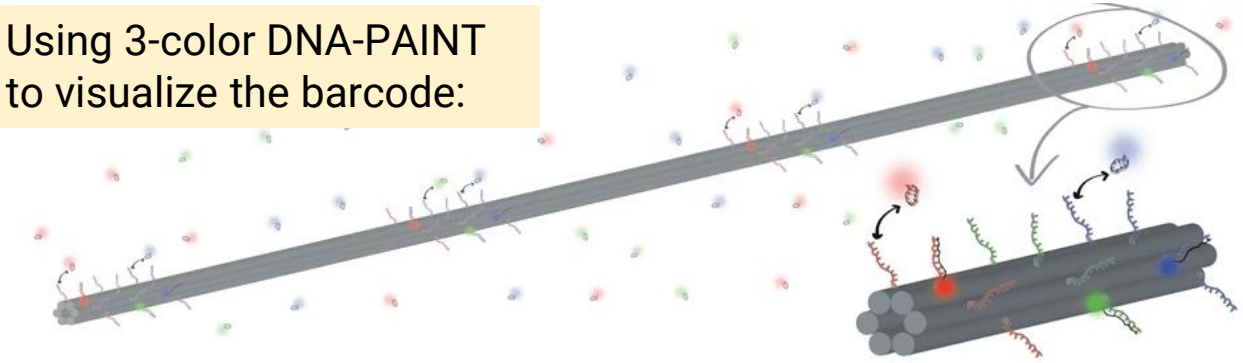


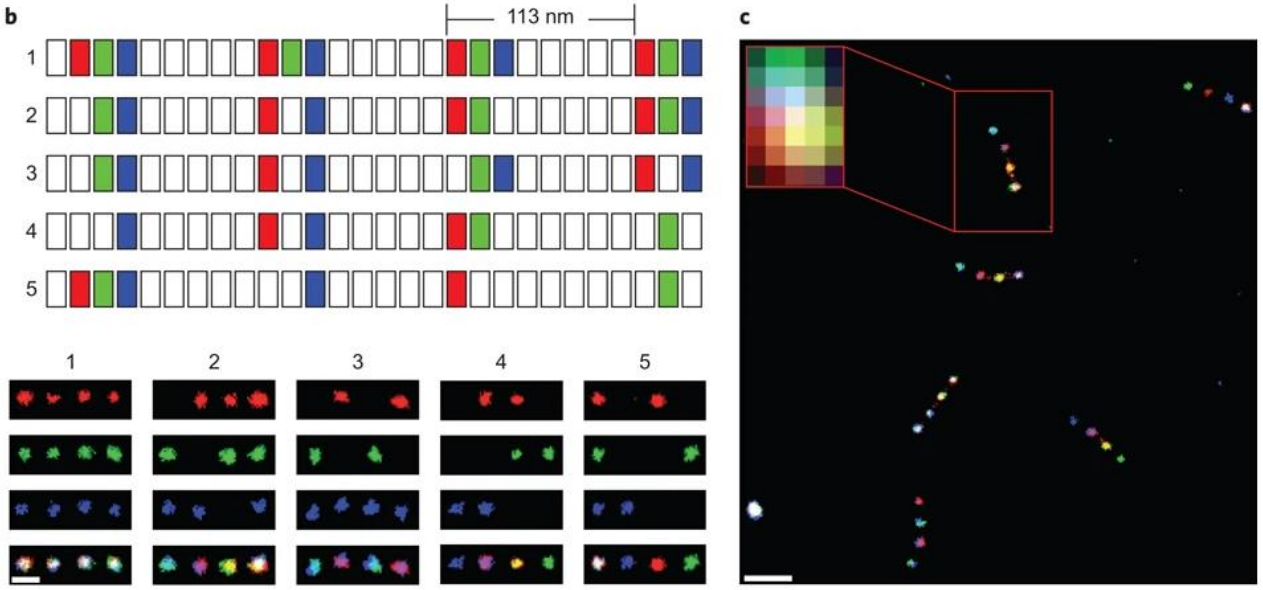


Schröder *et al. Nano Lett.* **2019**, *19*, 1275–1281; Vietz *et al.*, *ACS Omega* **2019**, *4*, 637

DNA Origami Barcodes

Using 3-color DNA-PAINT to visualize the barcode:



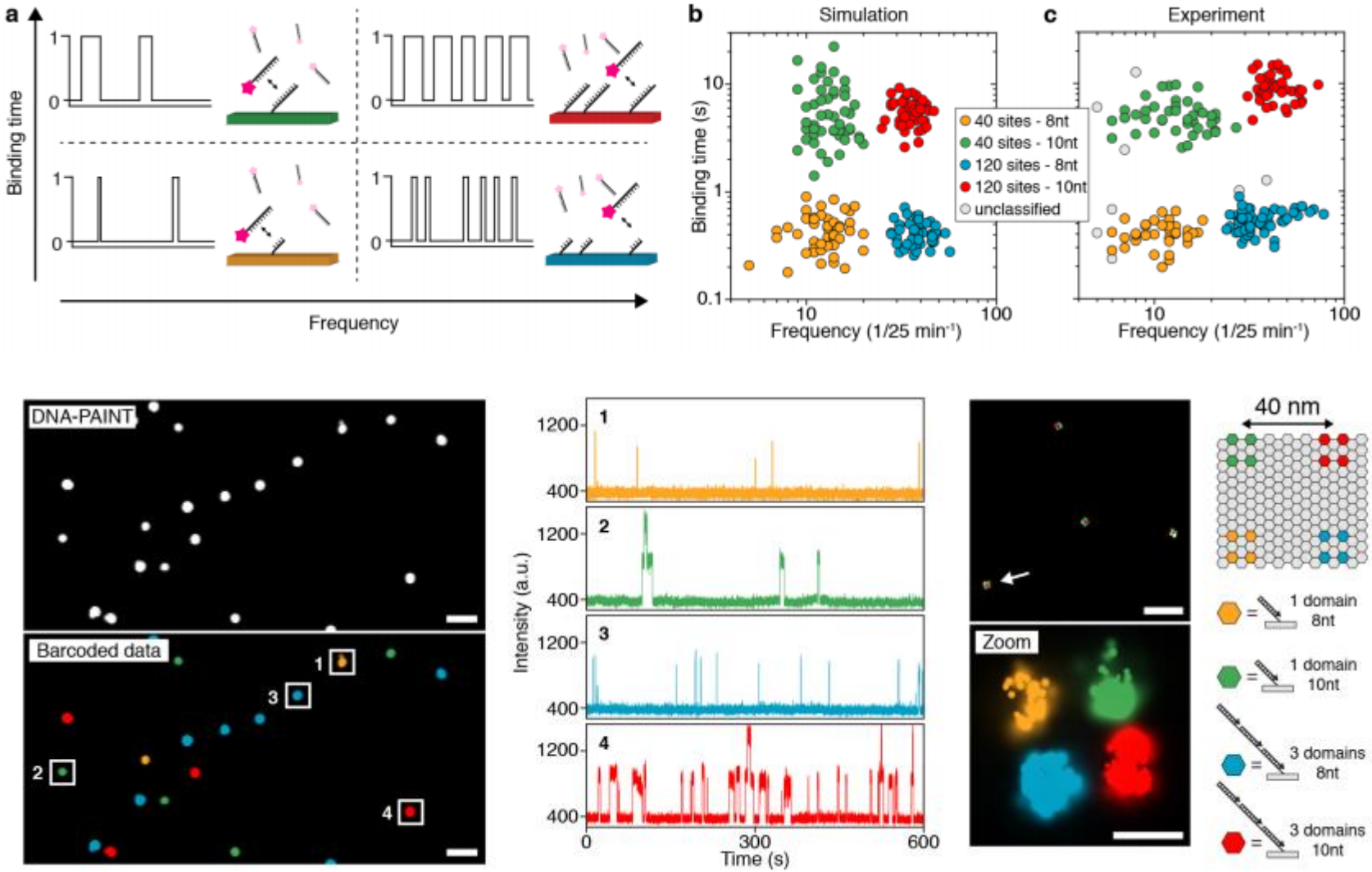


First spot						Second spot	Third spot
B	G	R	B+G	B+R	G+R		
						B	B
							G
							R
							G+B
							B+R
							G+R
						G	B
							G
							R
							G+B
							B+R
							G+R
						R	B
							G
							R
							G+B
							B+R
							G+R
						G+B	B
							G
							R
							G+B
							B+R
							G+R
						B+R	B
							G
							R
							G+B
							B+R
							G+R
						G+R	B
							G
							R
							G+R
							B+R
							G+R

- Six pseudo-colors: B, R, G, BG, BR, GR
- $6^3 = 216$ different barcodes

Nature Chemistry **2012**, *4*, 832

Barcoding based on DNA-PAINT blinking kinetics



- Multiplexing based on the PAINT blinking kinetics of

- Blinking kinetics are sorted based on:

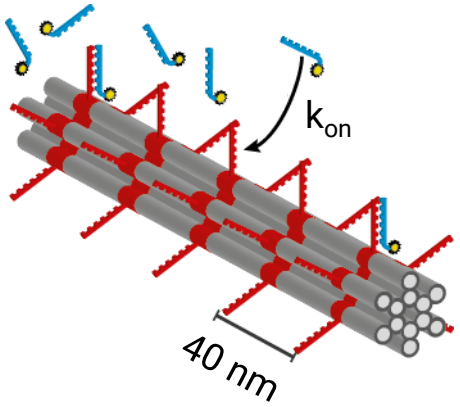
binding frequency (# of sites)
binding time (length of DNA sequence)

- 124 artificial colors can be created!

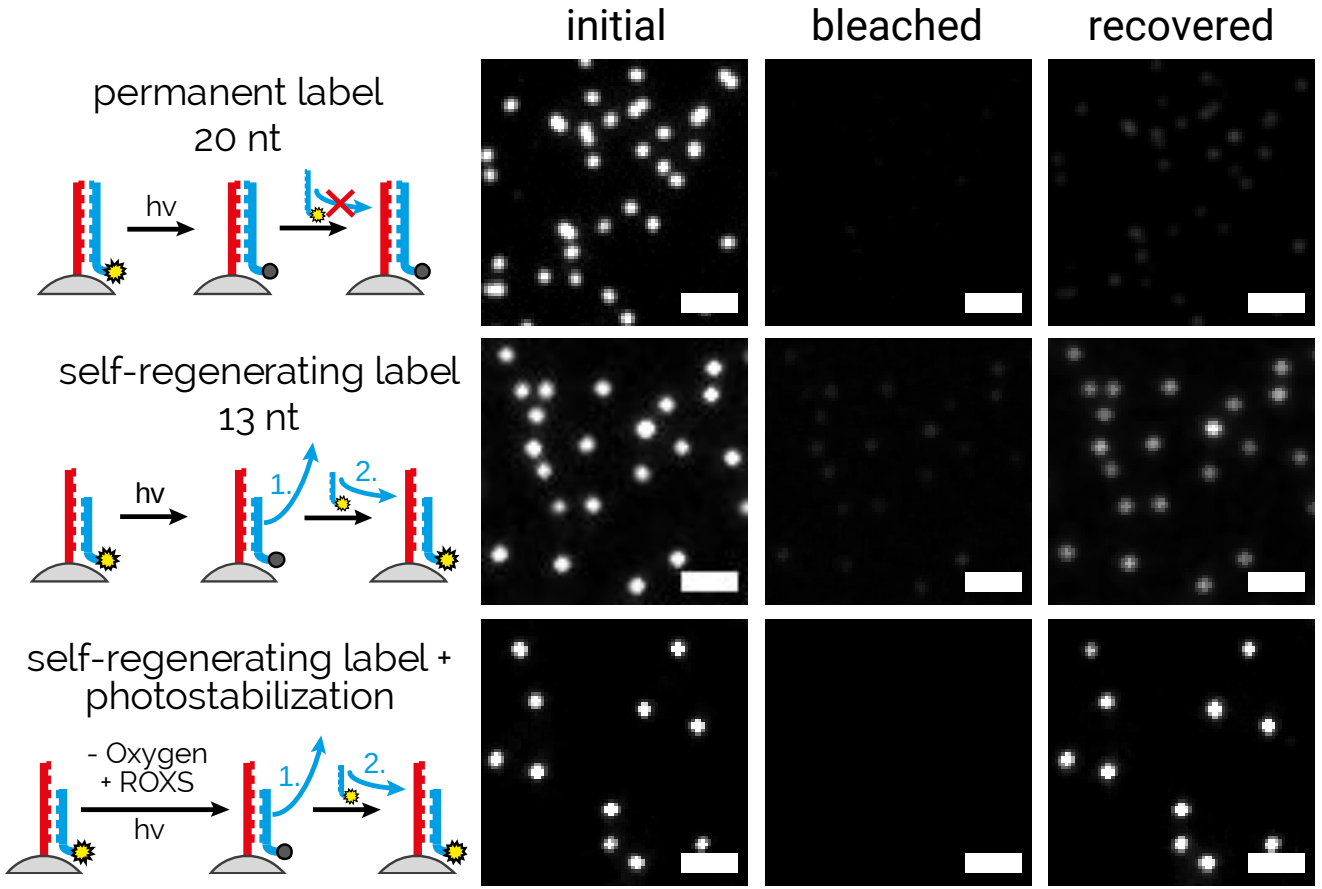
Nano Lett. **2019**, *19*, 2641−2646

Self-regenerating fluorescence labels

12 HB brightness ruler



5 x 20 nt docking sites to bind labelled imager strands



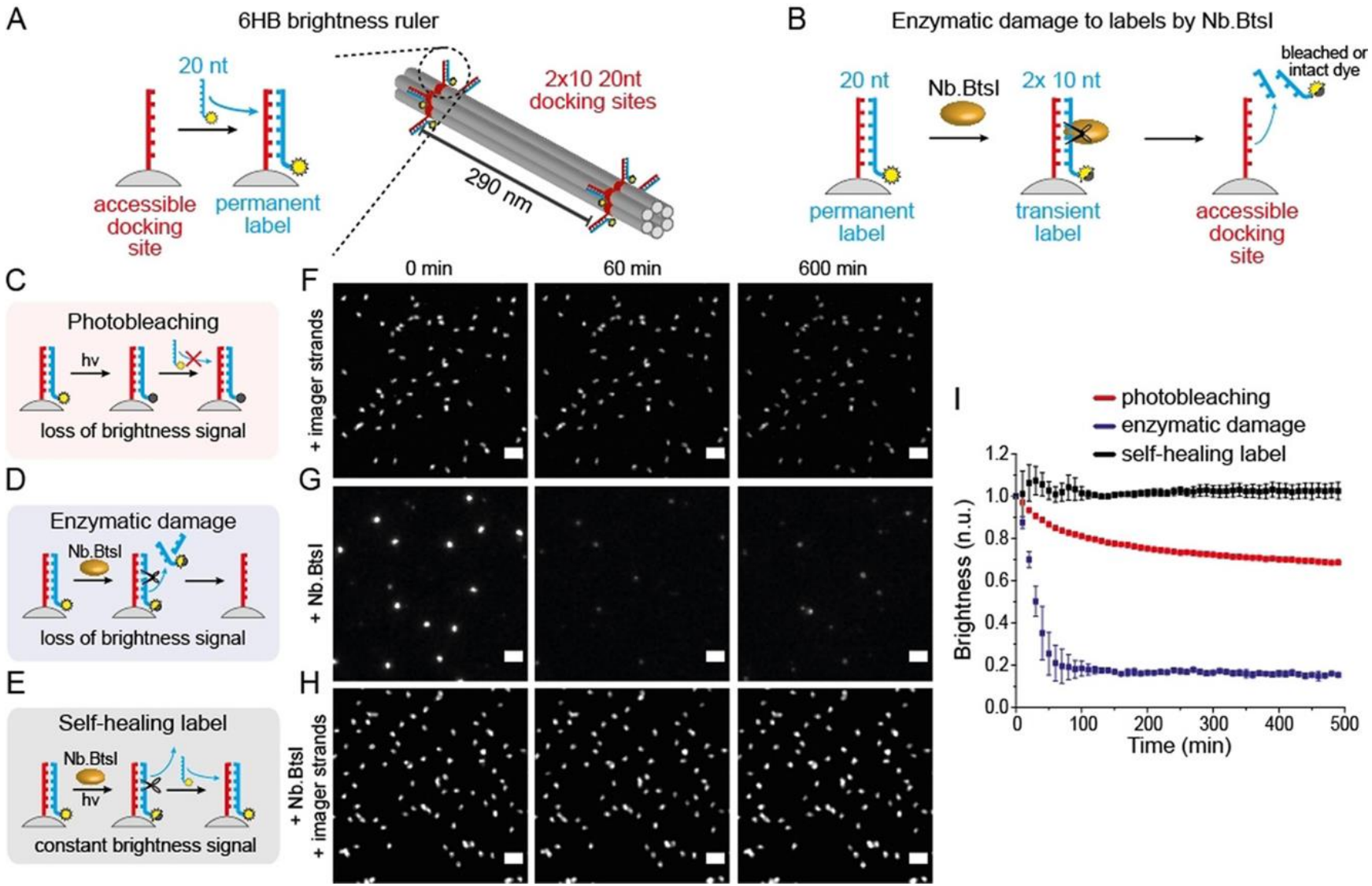
can only be imagined once

recovers to ~40 % in 30 min

quantitative recovery in 180 min

M. Scheckenbach et al. Angew. Chem. Int. Ed. **2021**, 60, 4931

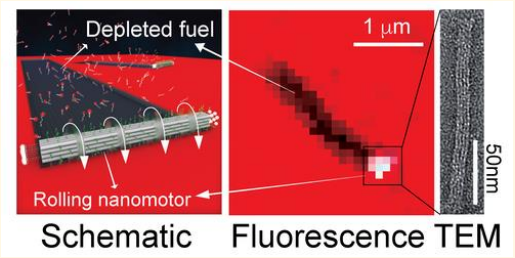
Self-regenerating fluorescence labels



- **self-healing with respect to enzymatic damage**: only the strands damaged by the enzyme are replaced
- **self-regenerating with respect to the fluorescent label** : dyes are exchanged irrespectively weather they are damaged or not

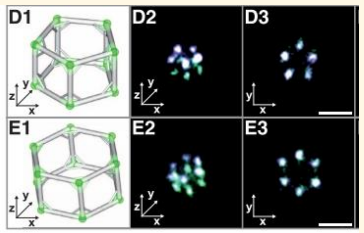
M. Scheckenbach et al. Angew. Chem. Int. Ed. **2021**, 60, 4931

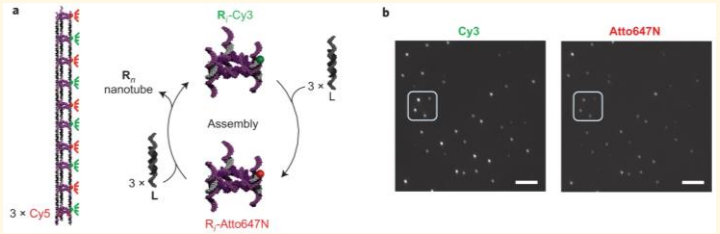
Fluorescence imaging in DNA nanotechnology



Mechanism and dynamics of DNA nanostructures

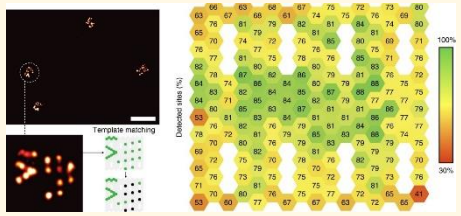
3D characterization of DNA nanostructures in the native environment





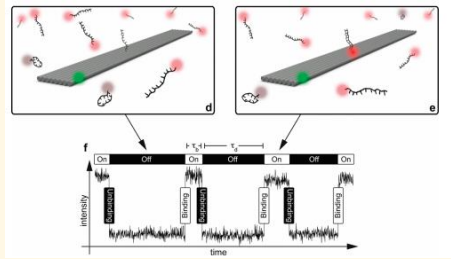
Stability and assembly of DNA nanostructures

Assessing the addressability of DNA nanotechnology



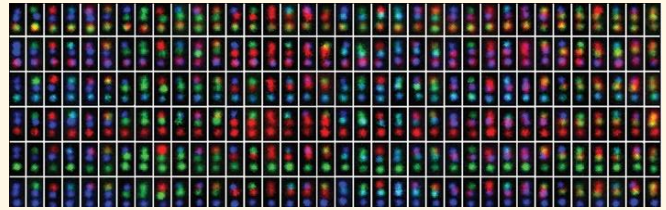
DNA nanotechnology in fluorescence imaging

DNA-aided super resolution imaging (DNA-PAINT)



DNA nanometrology (DNA nanorulers)

DNA barcodes for multiplexing



Bright and stable DNA-based labels