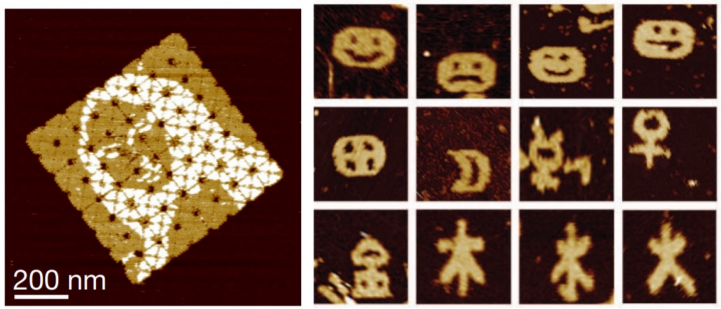
Fluorescence microscopy and super-resolution imaging

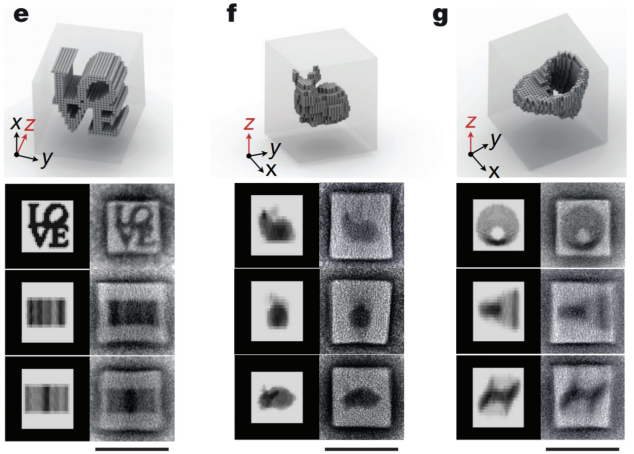
Shalin Shah & John Reif

Imaging sample using microscopy

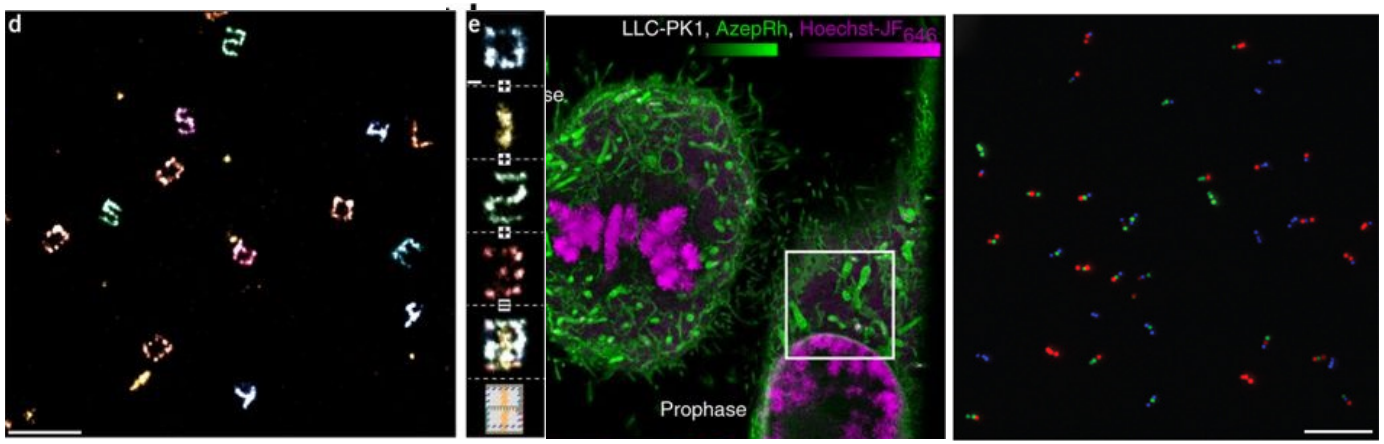
- **Goal:** To learn by visualizing mesoscale samples
  - Near field microscopy
  - Far field microscopy



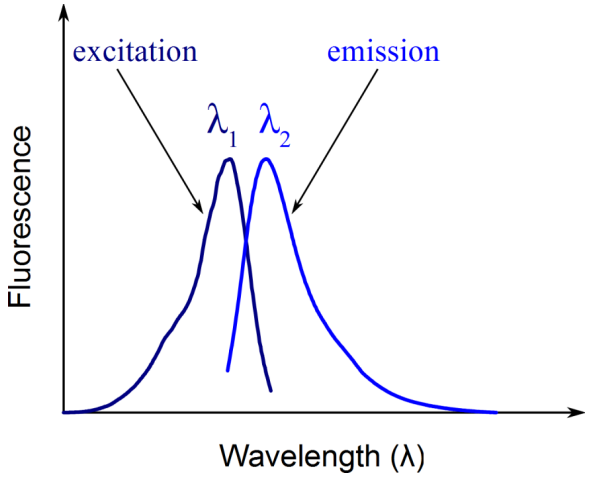
AFM imaging

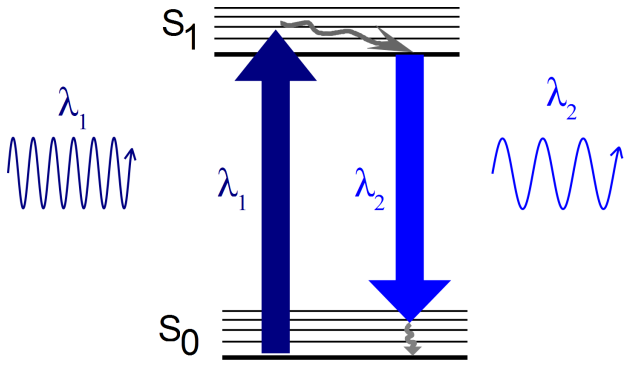


TEM imaging



Fluorescence microscopy





What is fluorescence?

- Emission of electromagnetic radiation by a substance that has absorbed light.
  - E.g. fluorophores, quantum dots, etc.
- The emitted light has a longer wavelength, and therefore lower energy, than the absorbed light.

Conventional fluorescence microscopy

- Performed by selectively staining the sample with fluorescent molecules, either:
  - linked to antibodies as in immunohistochemistry or
  - using fluorescent proteins genetically fused to the genes of interest.

- The more concentrated the fluorophores, the better the contrast of the fluorescence image.

- A single fluorophore can often be visualized under a microscope if the number of photons emitted is sufficiently high.

Limits of Conventional fluorescence microscopy

- The 2D image of a point source observed under a microscope is called the Airy disk (a section of the point spread function) of the imaging system.
- The ability to identify as two individual entities two closely spaced fluorophores is limited by the diffraction of light.
- Abbe's criterion [1830s] states that the minimal distance d that allows resolving two point sources is given by

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

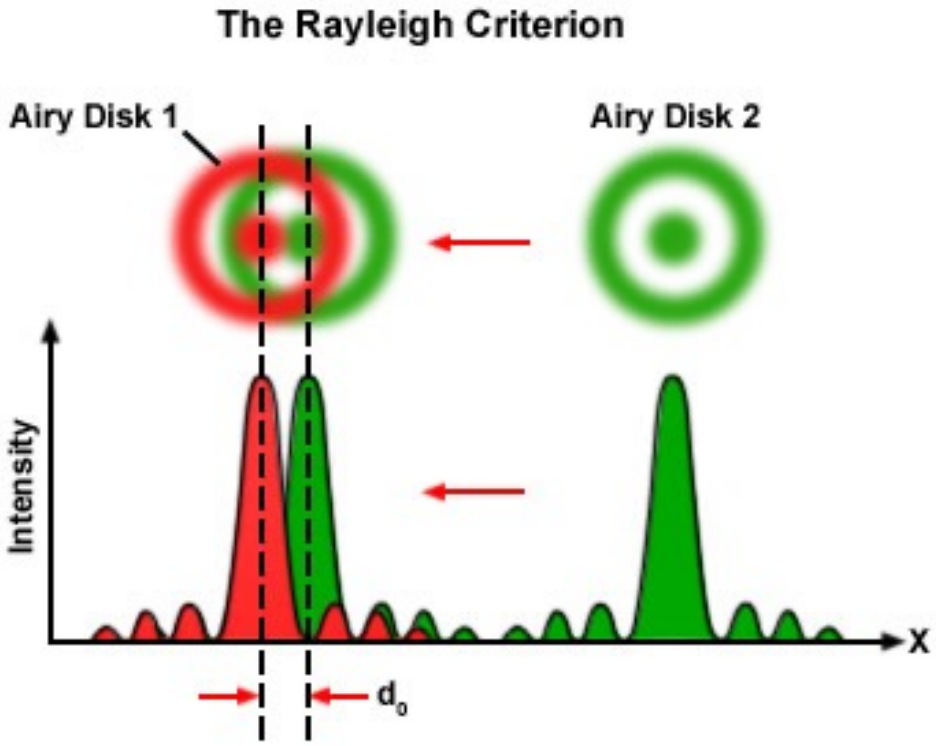
  - λ is the wavelength of the fluorescent emission and
  - NA is the numerical aperture of the microscope.

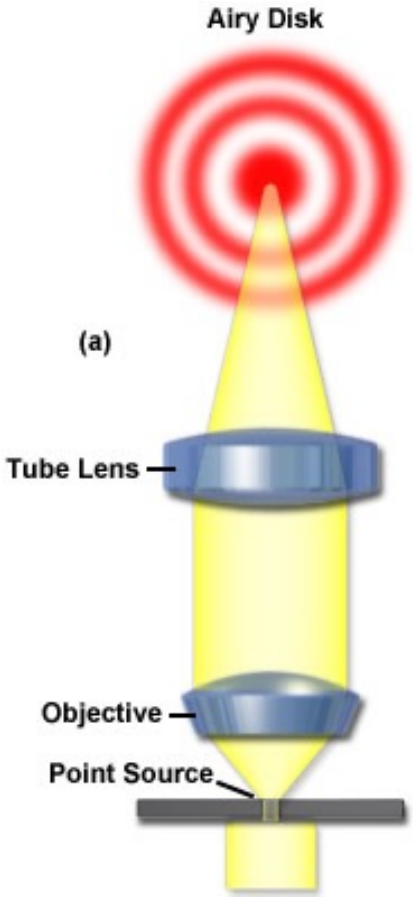
The center of this spot typically forms a 2D Gaussian function

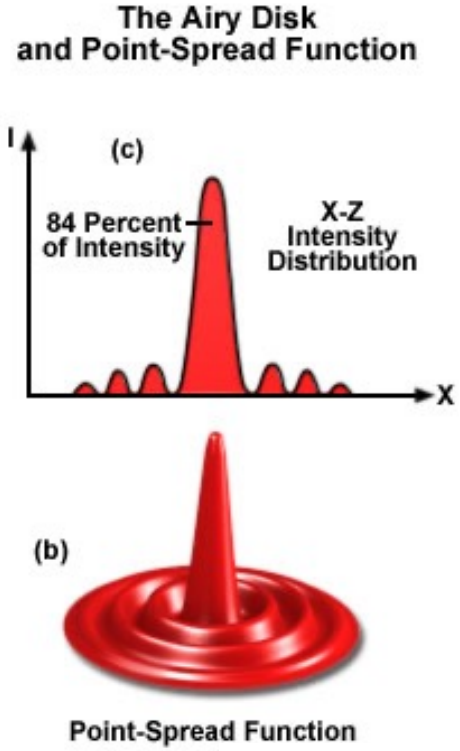
Limits of Conventional fluorescence microscopy

Diffraction limit of light

(Abbe 1830's) d = $\frac{\lambda}{2N\ sin\theta}$







Structured illumination microscopy (SIM)

- A technique that can create a high-resolution image by scanning two superimposed light sources.
- SIM works by taking multiple images with different illumination patterns and orientations.
- The illumination pattern is limited by light diffraction, so SIM can only double the spatial resolution.

Super Resolution Microscopy

Techniques that allow optical microscopy images to have higher resolutions than the diffraction limit

Super-resolution microscopy

Diffraction limit of light (Abbe 1830's) ~ 200 nm

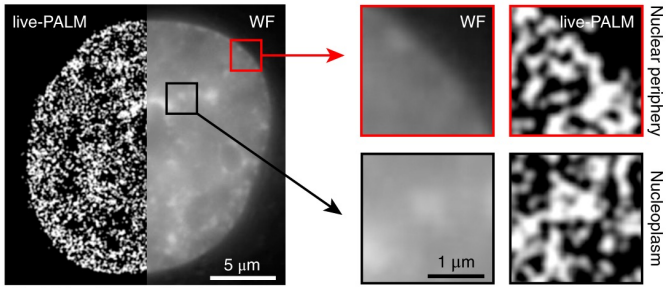
- $d = \frac{\lambda}{2N\ sin\theta}$

Major developments to overcome this barrier in last 2 decades:

Super Resolution Imaging Methods

- **Targeted Readout:**
  - **STED**
  - **SSIM/ SPEM**
- **Stochastic Readout:**
  - **PALM/ fPALM**
  - **STORM/ 3D-STORM**
  - **PAINT**
  - **GSDIM**
  - **DNA-PAINT/ Exchange-PAINT**

Super-resolution microscopy imaging techniques



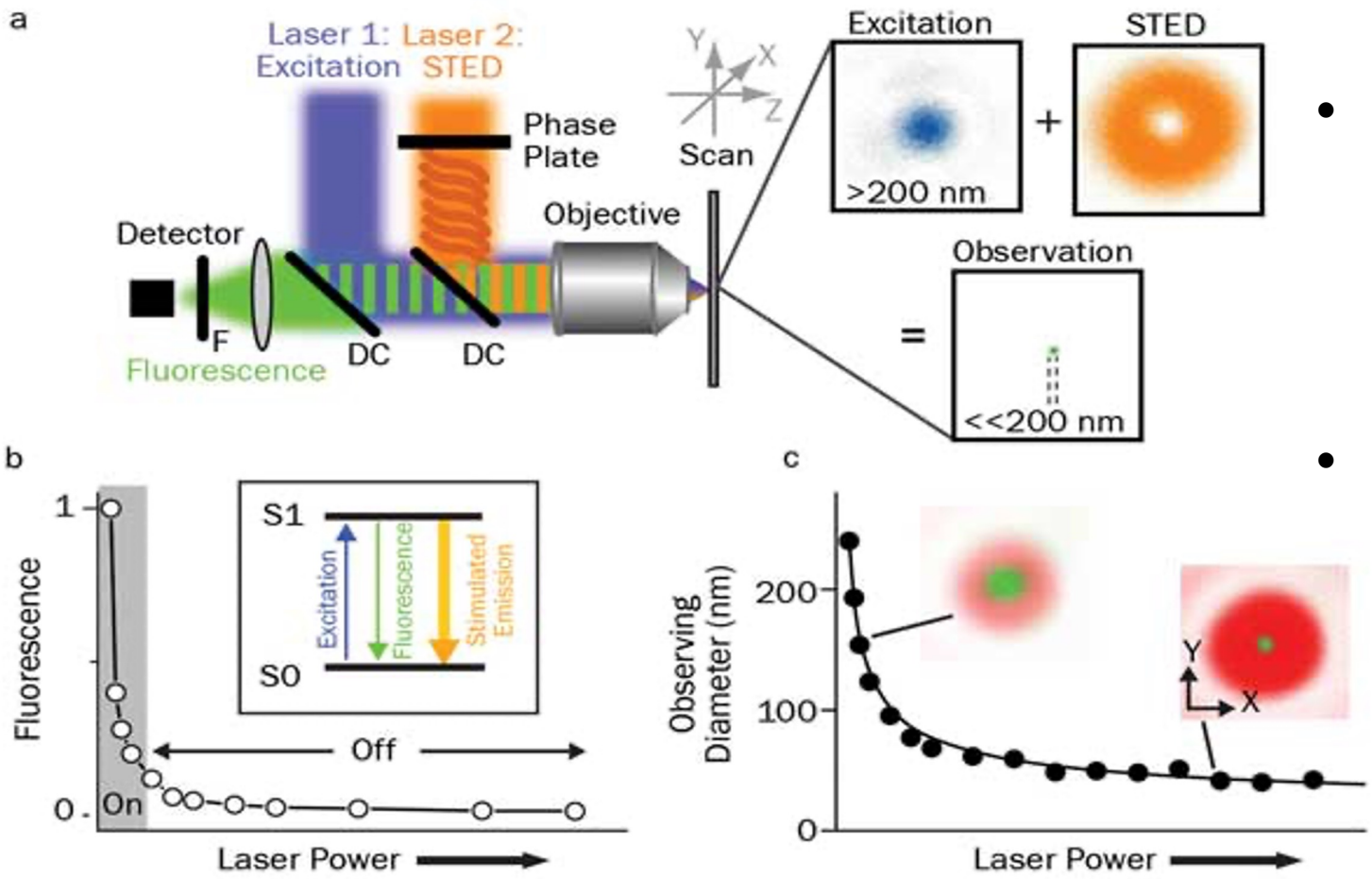
- **STED (1992)** - Stimulated relaxing of dyes using donut shaped beam.
- **STORM (2006)** - Triplet state switching depending on Cy3- Cy5 distance.
- **PALM (2006)** - Intensity based PA-FP separated temporally switch on/ off.
- **PAINT (2006)** - Targeting object with probes which fluoresce on binding.
- **Exchange-PAINT (2014)** - Multiplexing by washing strands.
- **Deep-STORM (2018)** – Uses deep learning for accelarated imaging.
- **Others:** qPAINT, dSTORM, iPALM, isoSTED, BALM, MINFLUX, etc.

STED (1992)

Stimulated Emission Depletion (STED) microscopy (1992):

- Creates super-resolution images by the selective deactivation of fluorophores, minimizing the area of illumination at the focal point, and thus enhancing the achievable resolution for a given system.
- A far-field optical nanoscopy technique that can image small objects with sub-diffraction-limit spatial resolution.
- Allows super-resolution imaging of living cells with low light intensity
- Can produce super-resolution images with resolutions as small as 50 nanometers (nm).

STED (1992)

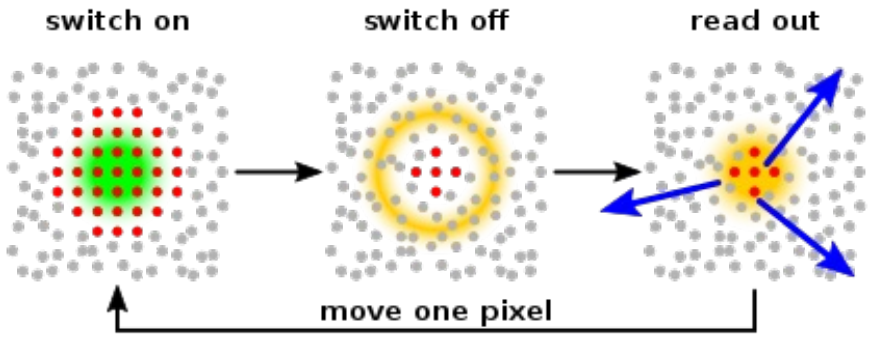


- There were a few other techniques named SSIM, SIM etc. which also were similar to STED with faster imaging speed.
- These techniques was generalized under the name RESLOT, later.

RESLOFT (2004)

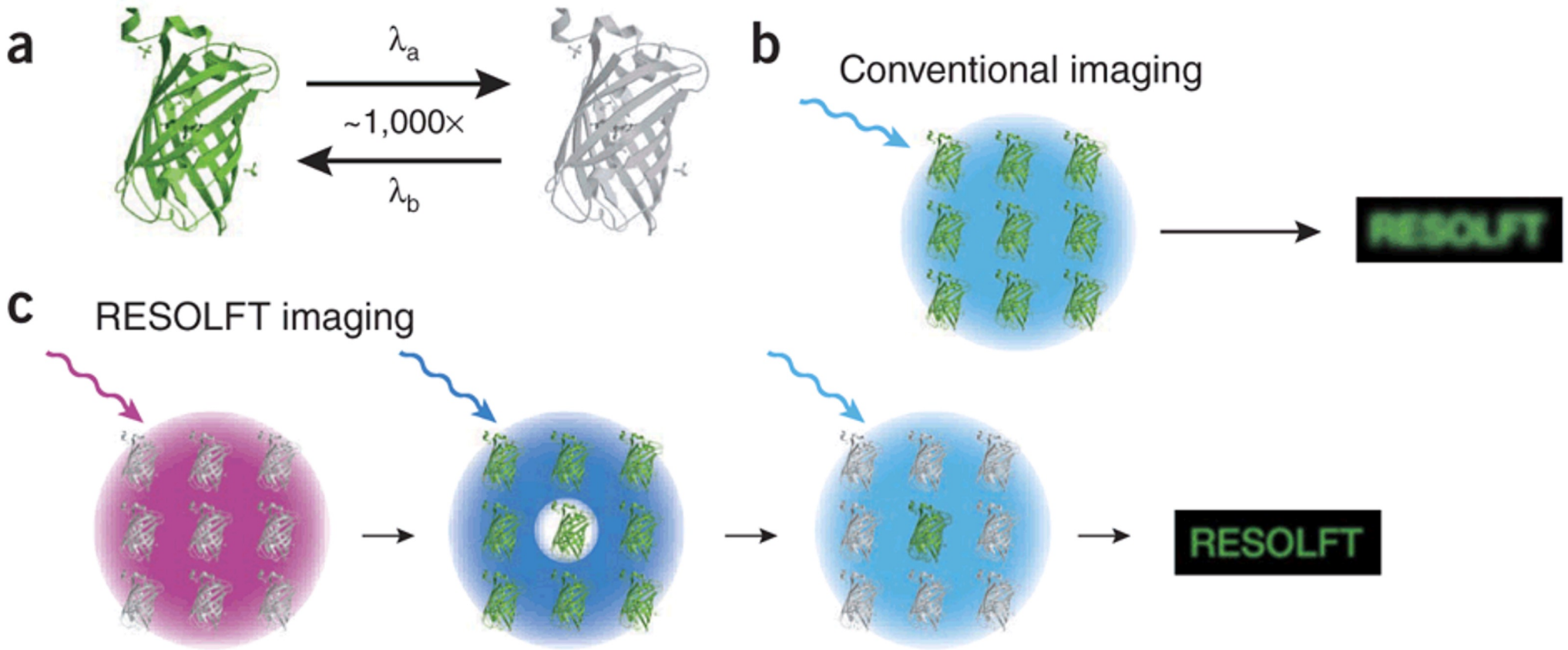
RESOLFT (REversible Saturable or switchable OpticaL Fluorescence Transitions) microscopy:

- Uses reversibly switchable fluorescent proteins (RSFPs) and a periodic light pattern for photoswitching.
- Works by scanning two superimposed light sources to create a super-resolved spot on a sample.
- It also selectively deactivates fluorophores, which minimizes the area of illumination at the focal point and improves the achievable resolution.
- Can achieve a lateral resolution of 20–70 nm and an axial resolution of 40–150 nm.



Switching fluorescence on and off in specific areas results in enabling fluorescence in areas smaller than the diffraction limit. Rasterisation of the whole sample results in a pixel image with extremely high resolution

RESLOFT (2004)



STORM/PALM (2006)

Photo-activated localization microscopy (PALM or FPALM):

- **Achieves a temporally resolved fluorescence of single molecules by putting the majority of the population into a dark state with the help of high power laser illumination**

Stochastic optical reconstruction microscopy (STORM):

- **Uses photo-activatable/convertible fluorescent proteins that can be "turned" on and off.**
- **After activation they are imaged until they bleach.**

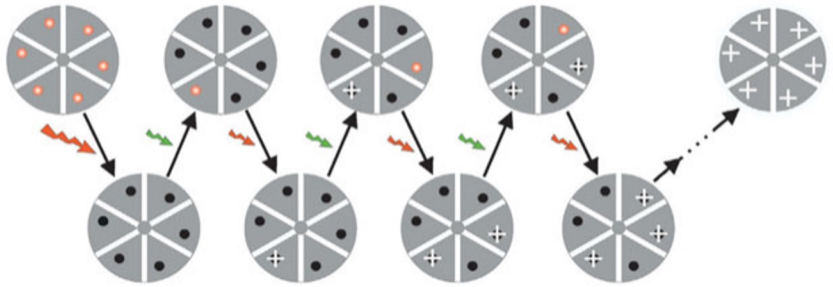
STORM/PALM (2006)

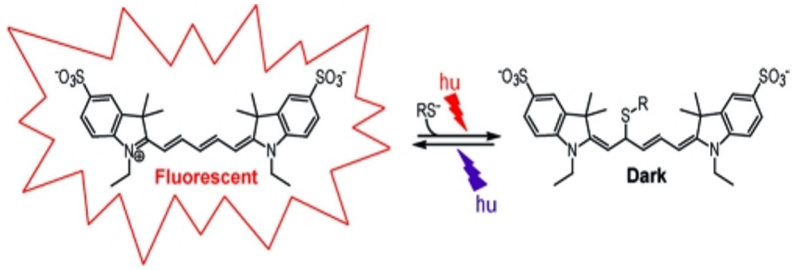
Photo-activated localization microscopy (PALM or FPALM) and stochastic optical reconstruction microscopy (STORM):

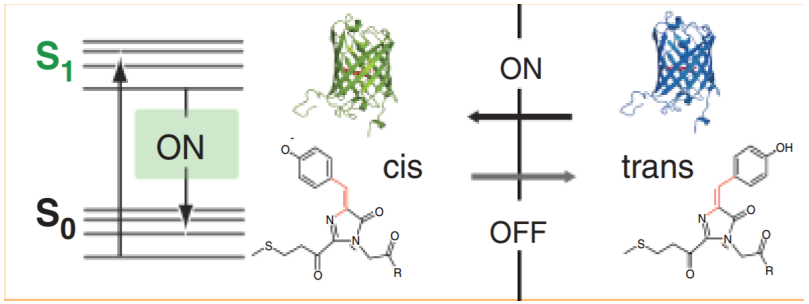
- **Widefield (as opposed to point scanning techniques such as laser scanning confocal microscopy) fluorescence microscopy imaging methods that allow obtaining images with a resolution beyond the diffraction limit.**
- **Use a fluorescent microscope to collect a large number of images each containing just a few active isolated fluorophores.**
- **The imaging sequence allows for the many emission cycles necessary to stochastically activate each fluorophore from a non-emissive (or less emissive) state to a bright state, and back to a non-emissive or bleached state.**
- **During each cycle, the density of activated molecules is kept low enough that the molecular images of individual fluorophores do not typically overlap.**
- **In each image of the sequence, the position of a fluorophore is calculated with a precision typically greater than the diffraction limit (typically a few to tens of nm).**
- **The resulting information of the position of the centers of all the localized molecules is used to build up the super-resolution image.**

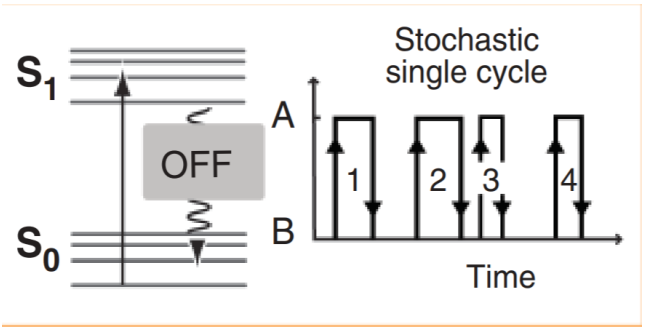
STORM/PALM (2006)

- **Wins**:
  - **Increased lifetime of dyes** because of longer dark state.
  - Non-invasive technique and can be operated at low laser power therefore there is no damage to sample.
- **Loses**:
  - Upper cap on the number of different available dye switches.
  - Multiple lasers are required for different switches. For example, green and red for Cy3 - Cy5 switch.
  - **Eventually, dye switches photobleach**.









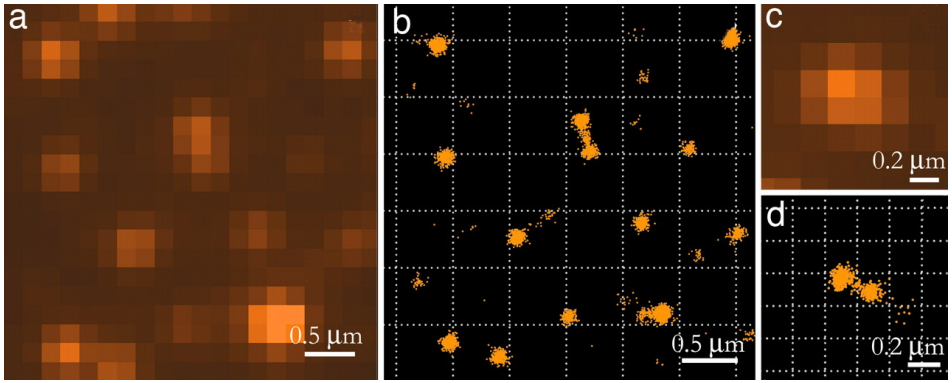
Universal-PAINT (2006)

uPAINT (universal Point Accumulation for Imaging in Nanoscale Topography):

- **Uses dynamic imaging of continuously labelled, arbitrary membrane biomolecules in living cells to reveal super-resolved images and single molecule trajectories at very high densities.**

- **Works by dynamically imaging single-molecule tracks on the cell surface under oblique illumination, hence resulting in a super resolved tracking of native biomolecule behavior at the surface of a living cell.**

Universal-PAINT (2006)



- **Wins**:
  - No photobleaching (excess imager strands).
  - No labelling errors because no labelling is required.
  - Control over probe hybridization kinetics (tuned length, frame rate of camera)
  - Imaging Efficiency calculation evaluated to 95%
  - Claimed strong multiplexing capabilities due to DNA.

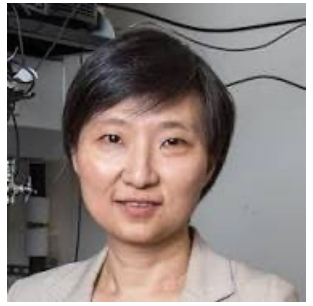
- **Loses**:
  - Restriction on the type of dyes (because imaging is done using electrostatic coupling/ hydrophobic interaction).
  - Universal-PAINT tried to overcome this but it is still not programmable.

Nobel prize for Super Resolution Microscopy

Eric Betzig
PALM

Stefan Hell
STED

Stefan Hell
Localization microscopy

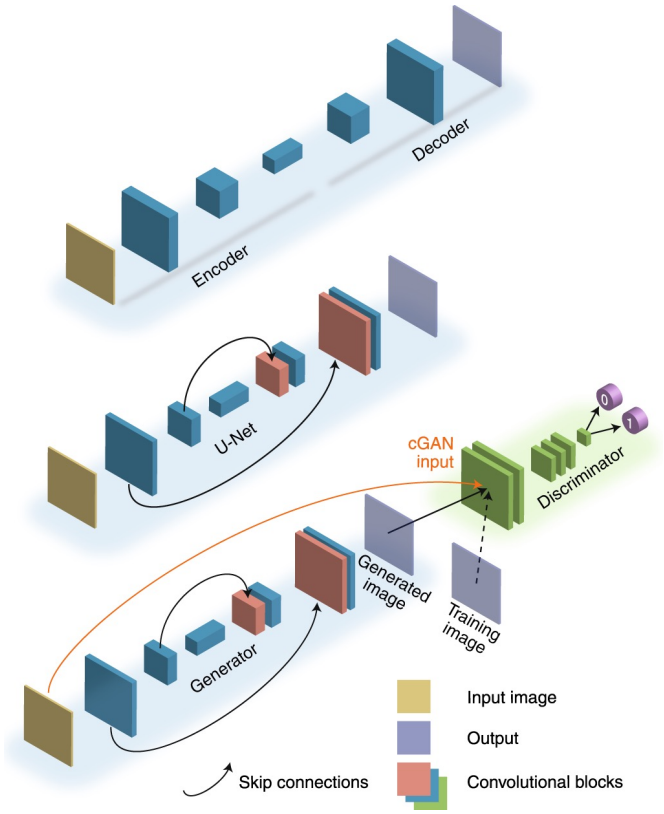


Xiaowei Zhuang
Localization microscopy

Super resolution imaging using AI

- **Various methods using also AI:**
  - **ANNA-PALM**
  - **Deep-STORM**
  - **Etc.**

- Review paper: *Belthangady, Chinmay, and Loic A. Royer. "Applications, promises, and pitfalls of deep learning for fluorescence image reconstruction." Nature methods (2019): 1-11.*

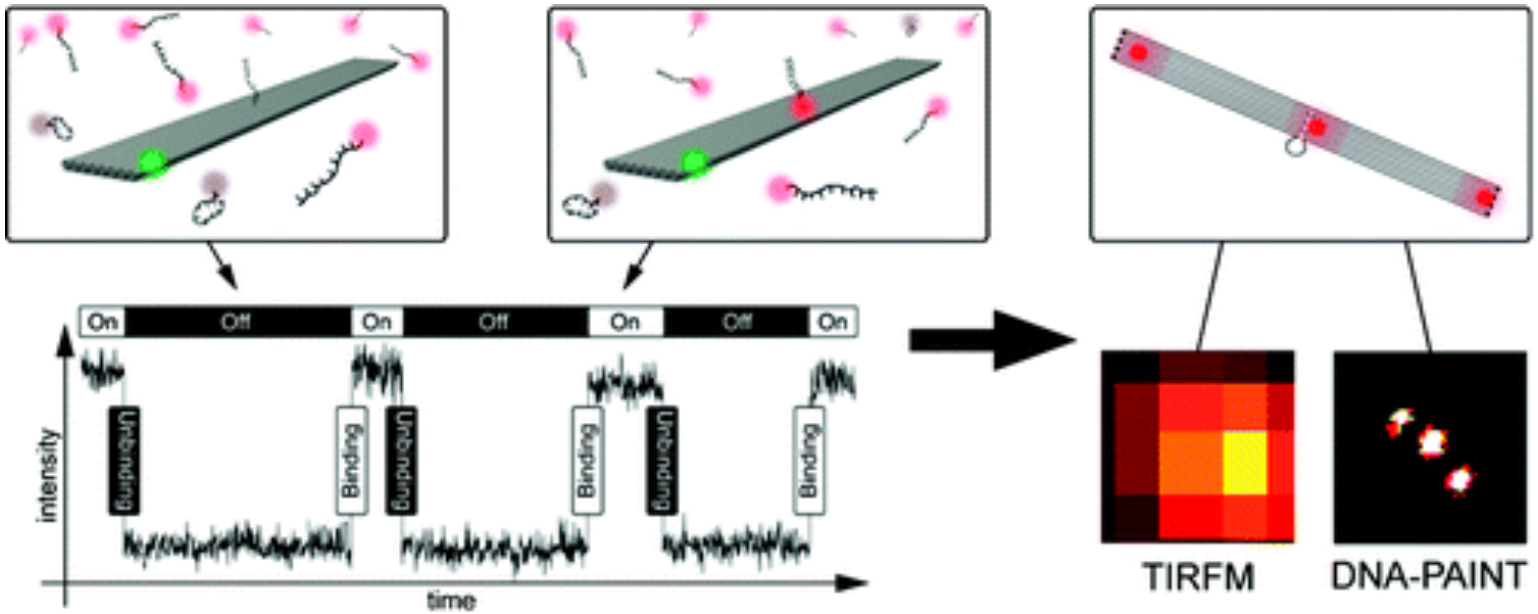


- **DNA-PAINT (2010)**

  In DNA-PAINT, transient binding of short dye-labeled ('imager') oligonucleotides to their complementary target ('docking') strands creates the necessary 'blinking' to enable stochastic super-resolution microscopy.

  Using the programmability and specificity of DNA molecules as imaging and labeling probes allows it to decouple blinking from dye photophysics, making them compatible with virtually any single-molecule-compatible dye.

DNA-PAINT: A Super-Resolution Imaging Technique



- **Short, fluorescent labeled DNA imager strands are used to bind transiently to complementary docking strands attached to a target.**
- **The spontaneous binding and unbinding causes the fluorescence at a given point to switch between the on and off state, thus allowing individual target sites to be imaged with sub-10-nm resolution using total internal reflection microscopy.**
- **The reversible nature of DNA-PAINT means that it is not limited by the number of fluorophores, and sequential labelling allows the reuse of fluorescent dyes.**

R. Jungmann, M.S. Avendano J.B. Woehrstein M. Dai, W.M. Shih and P. Yin, Multiplexed 3D Cellular Super-Resolution Imaging with DNA- PAINT and Exchange-PAINT, Nat Methods. 2014 March ; 11(3): 313–318. doi:10.1038/nmeth.2835

Jungmann, R. et al. Single-molecule kinetics and super-resolution microscopy by fluorescence imaging of transient binding on DNA origami. Nano Le7. 10, 4756–4761 (2010).

Jungmann, R., Scheible, M. & Simmel, F.C. Nanoscale imaging in DNA nanotechnology.Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 4, 66–81 (2012).

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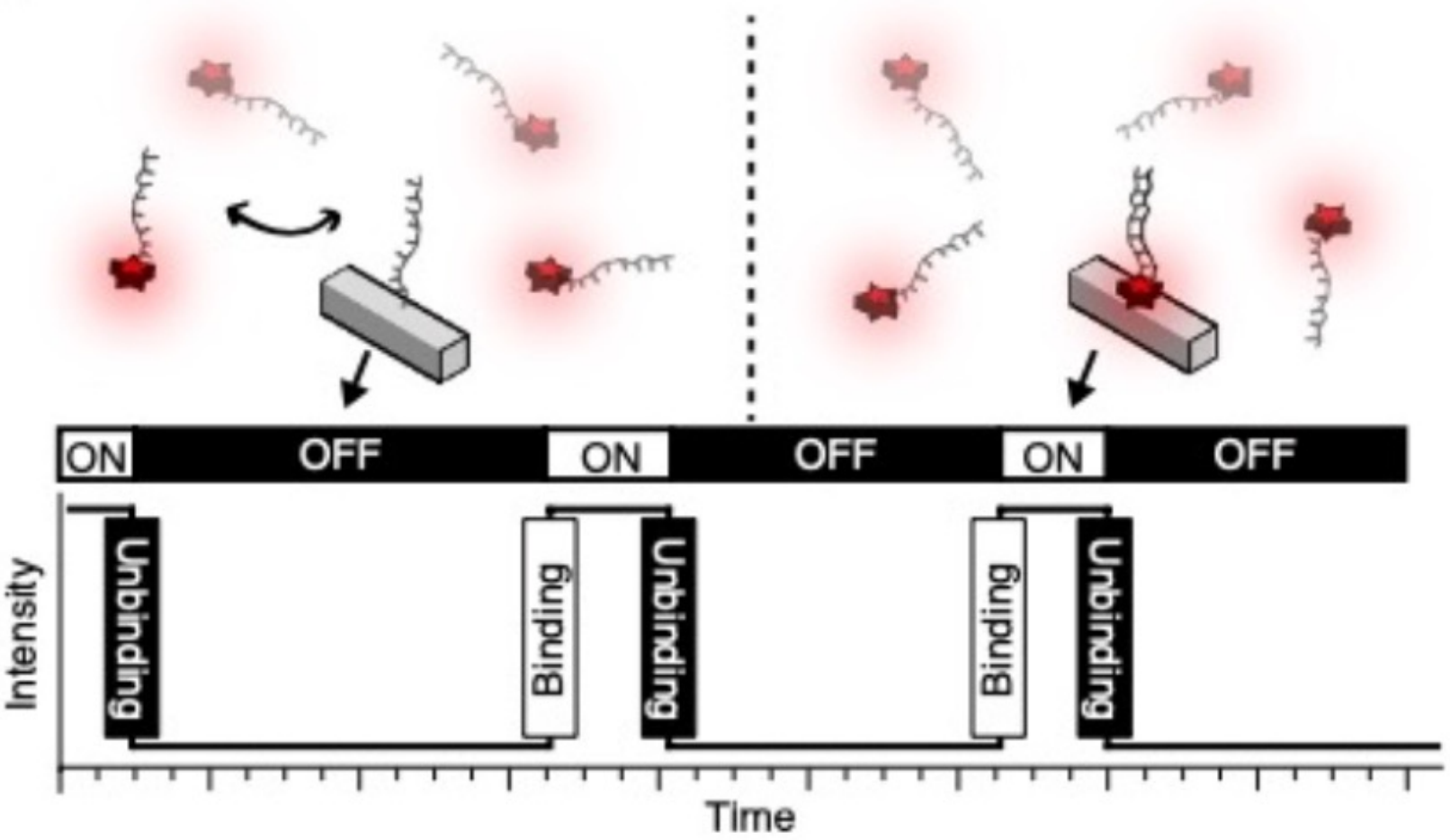
Joerg Schnitzbauer, Maximilian T Strauss, Thomas Schlichthaerle, Florian Schueder & Ralf Jungmann Super-resolution microscopy with DNA-PAINT, Nature Protocols volume 12, pages1198–1228 (2017)

DNA-PAINT: A Super-Resolution Imaging Technique

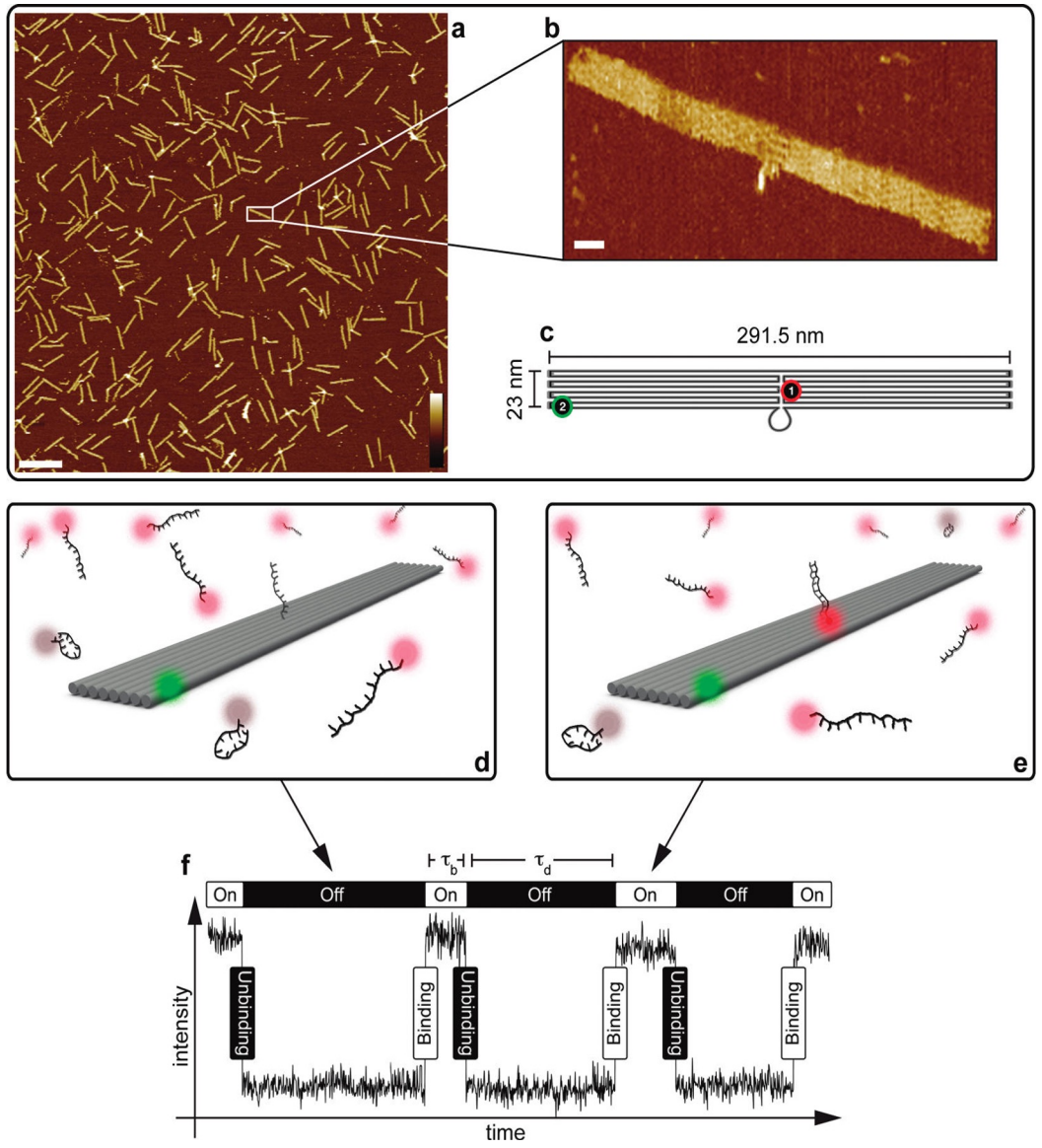
DNA-PAINT concept:

- Transient binding of dye-labeled DNA strands (imagers) to their complementary target sequence (docking site) attached to a molecule of interest.

- The transient binding of imager strands is detecte as 'blinking', illustrated by the intensity versus time trace.



DNA-PAINT (2010):



DNA-PAINT (2010): References:

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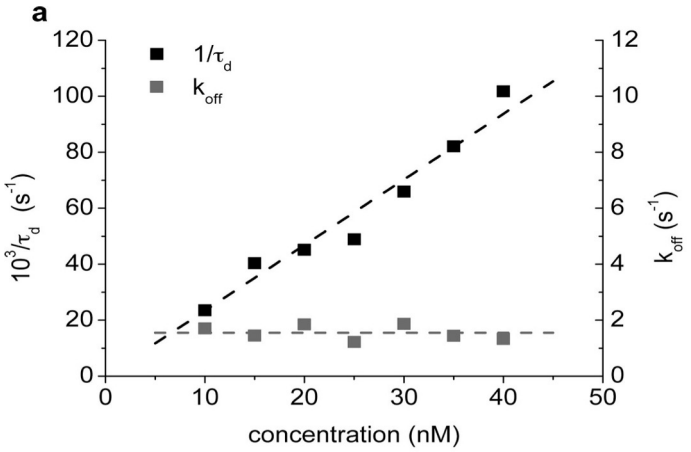
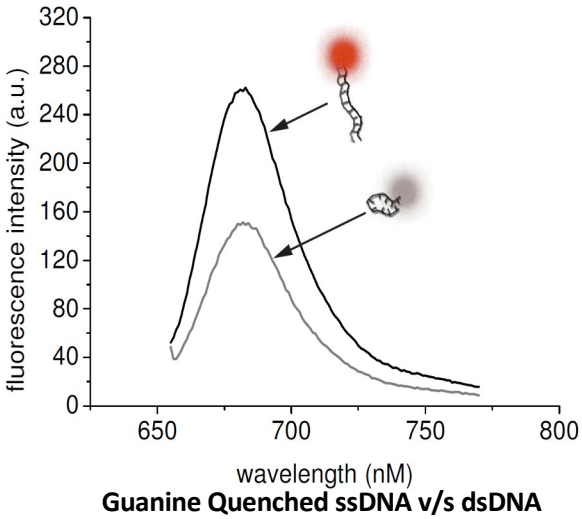
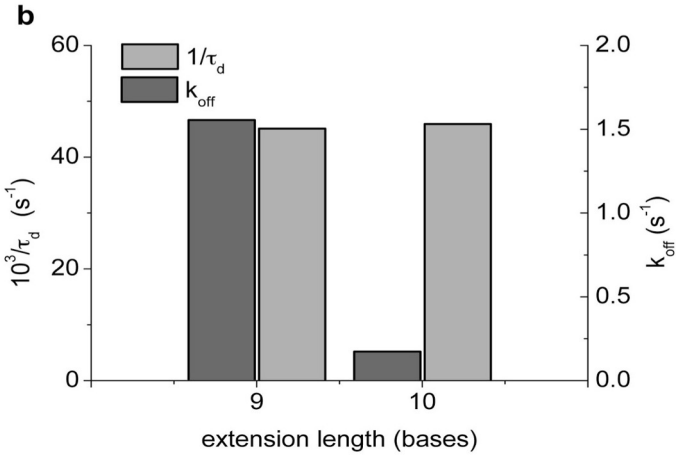
DNA-PAINT (2010)

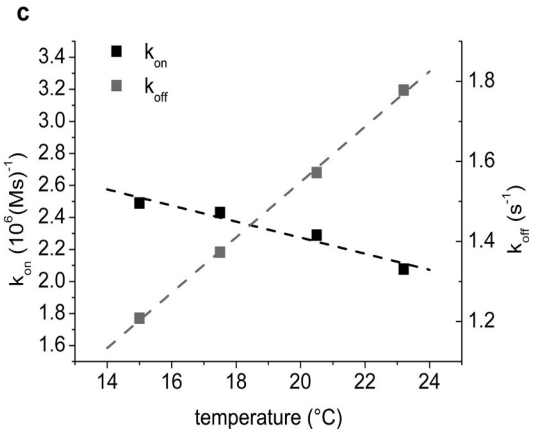
Bind/Un-Bind Rates for imager strand concentration and length:





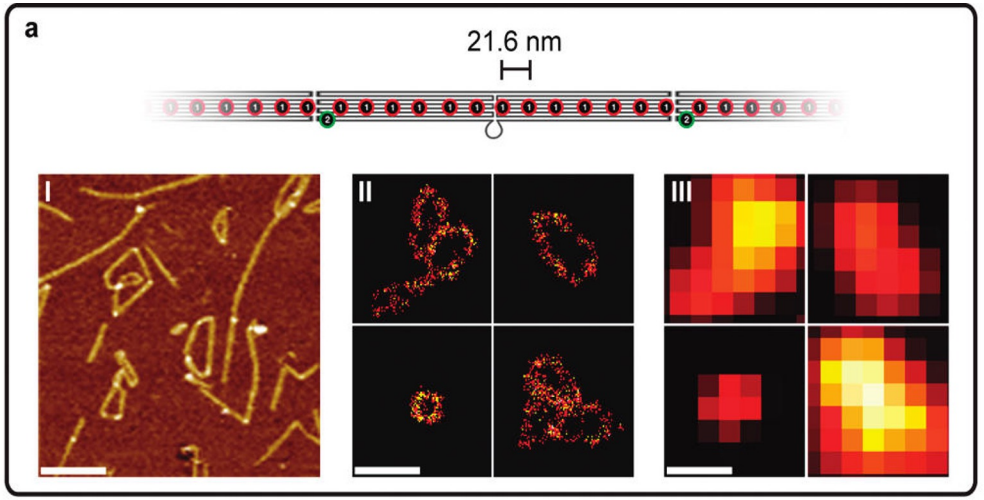
Guanine Quenched ssDNA v/s dsDNA

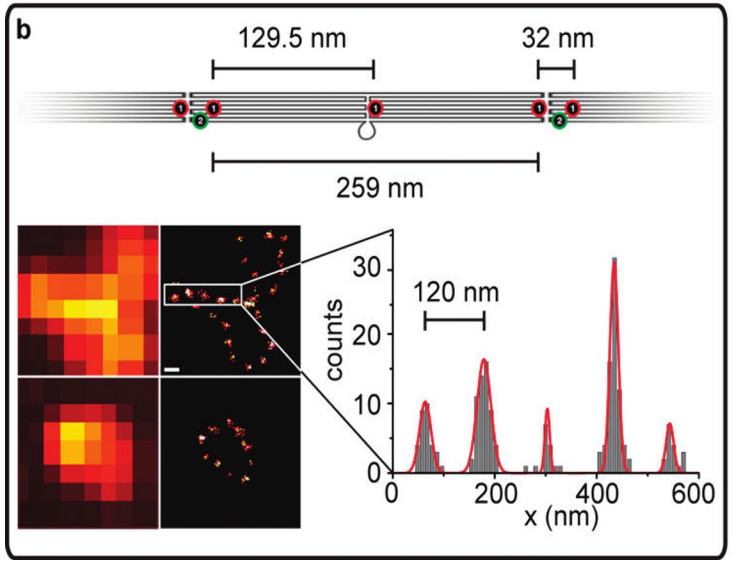


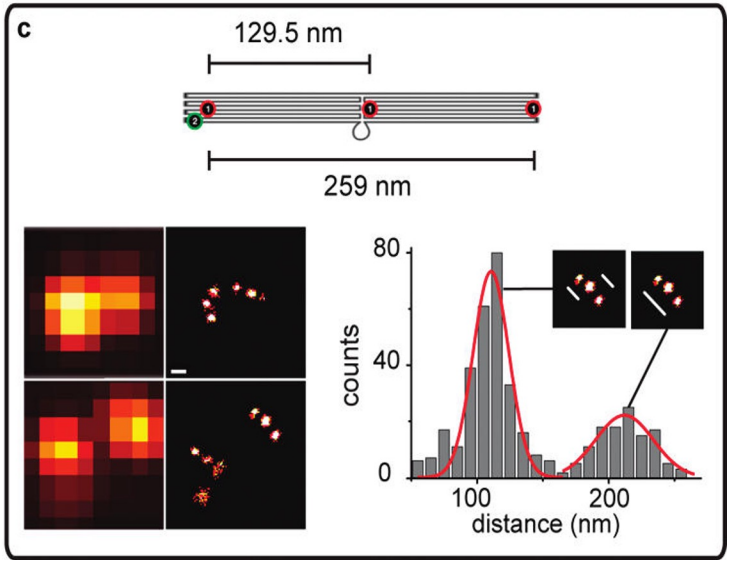


DNA-PAINT: A Super-Resolution Imaging Technique

- On left, diffraction-limited image (approx. 2 µm scale) and
- On right, a super-resolved DNA-PAINT image (approx. 500 nm scale).



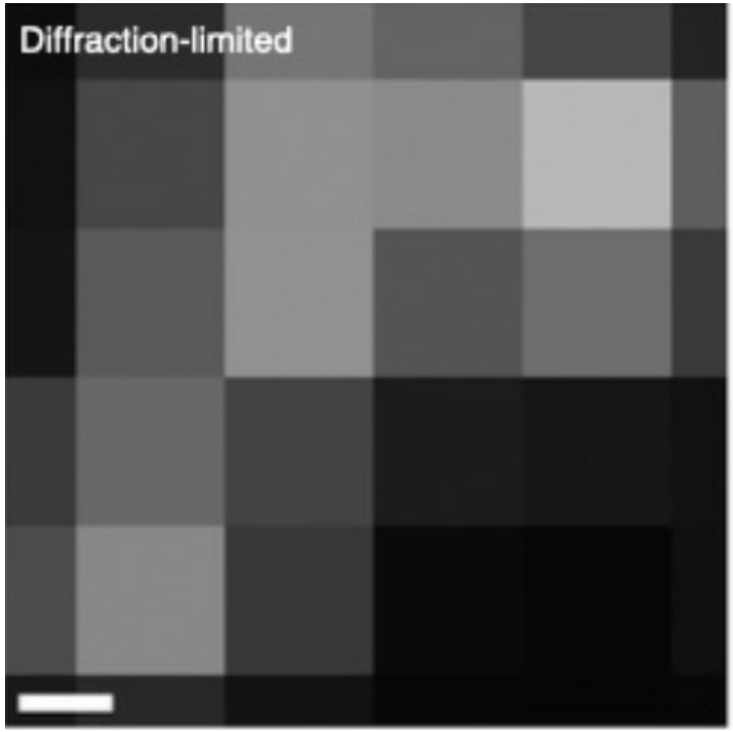




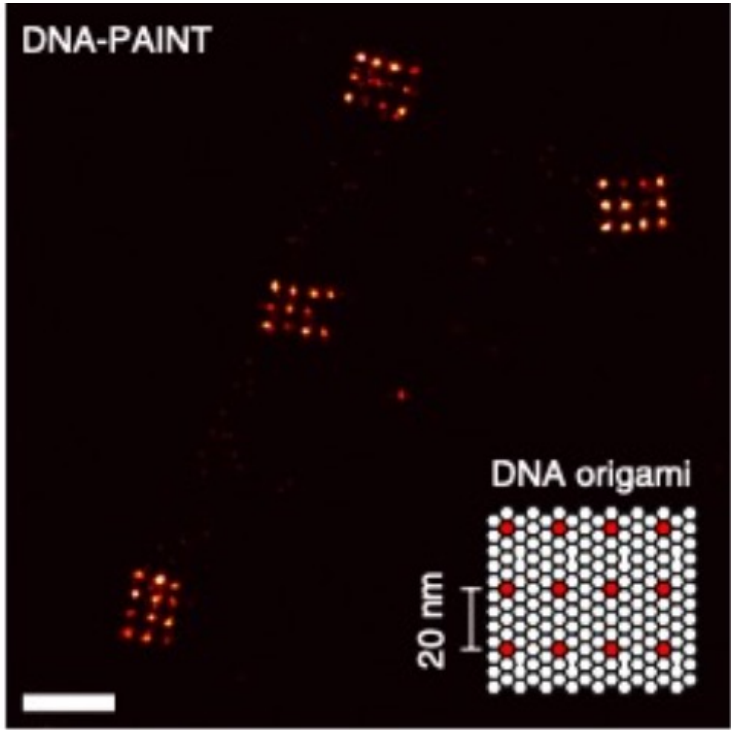
DNA-PAINT: A Super-Resolution Imaging Technique

Diffraction-limited Image (left) and super-resolved DNA-PAINT images (right) of DNA origami nanostructures:

Each structure consists of 12 docking strands that are arranged in a 20-nm grid (scheme in lower right corner).



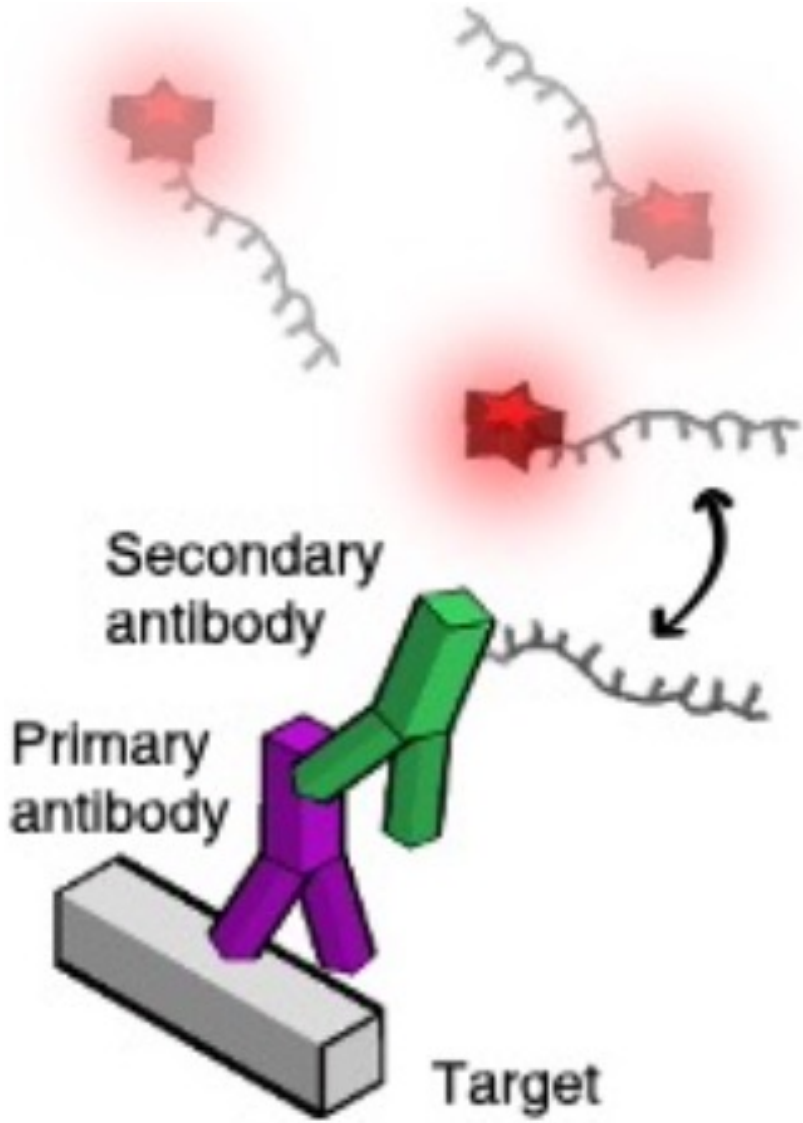
Diffraction-limited Image of DNA origami nanostructures



DNA-PAINT images of DNA origami nanostructures

DNA-PAINT:
A Super-Resolution Imaging Technique

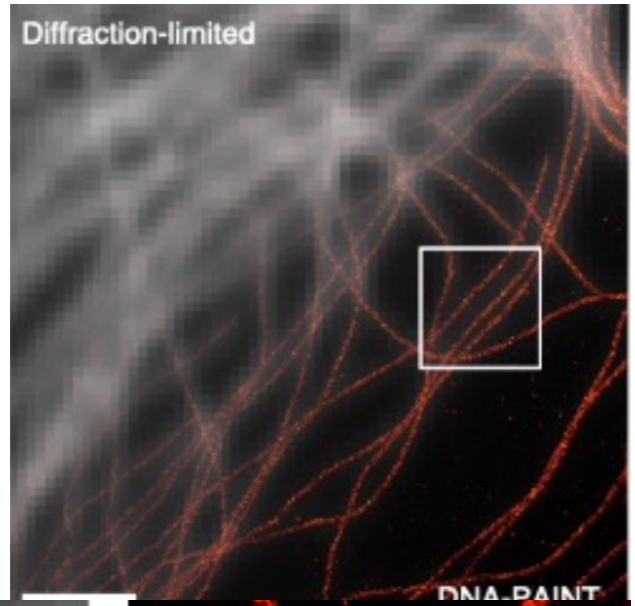
In situ protein-labeling strategy for DNA-PAINT using primary and DNA-conjugated secondary antibodies:



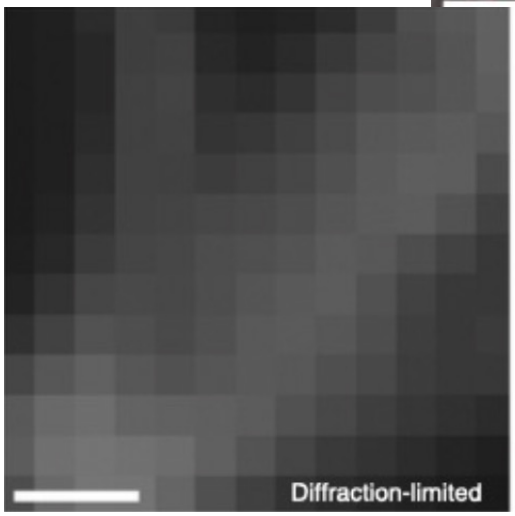
DNA-PAINT:
A Super-Resolution Imaging Technique

Overlay of:

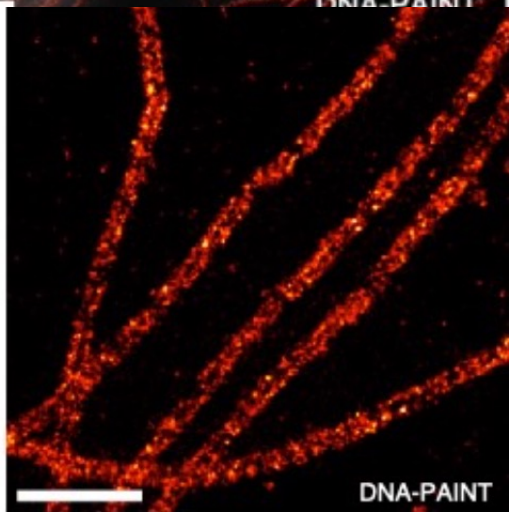
- **a diffraction-limited α-tubulin image (2 µm scale) and**
- **a super-resolved DNA-PAINT image (500 nm scale).**



Close-ups of the highlighted area above, comparing diffraction-limited image (left) with DNA-PAINT super-resolved image (right).



Diffraction-limited image



DNA-PAINT super-resolved image

DNA-PAINT (2010)

- **Wins:**
  - No photobleaching (excess imager strands).
  - No labelling errors (or inactive fluorophores).
  - Control over probe hybridization kinetics (tuned length, frame rate of camera).
  - Imaging Efficiency calculation evaluated to 95%.
  - Claimed strong multiplexing capabilities due to DNA.

- **Loses:**
  - Mostly restricted to DNA based structures.
  - Didn't demonstrate any multiplexing using DNA sequences/ length.
  - Very few dyes spaced at 32 nm were resolved.
  - Used green and red dye for imaging.

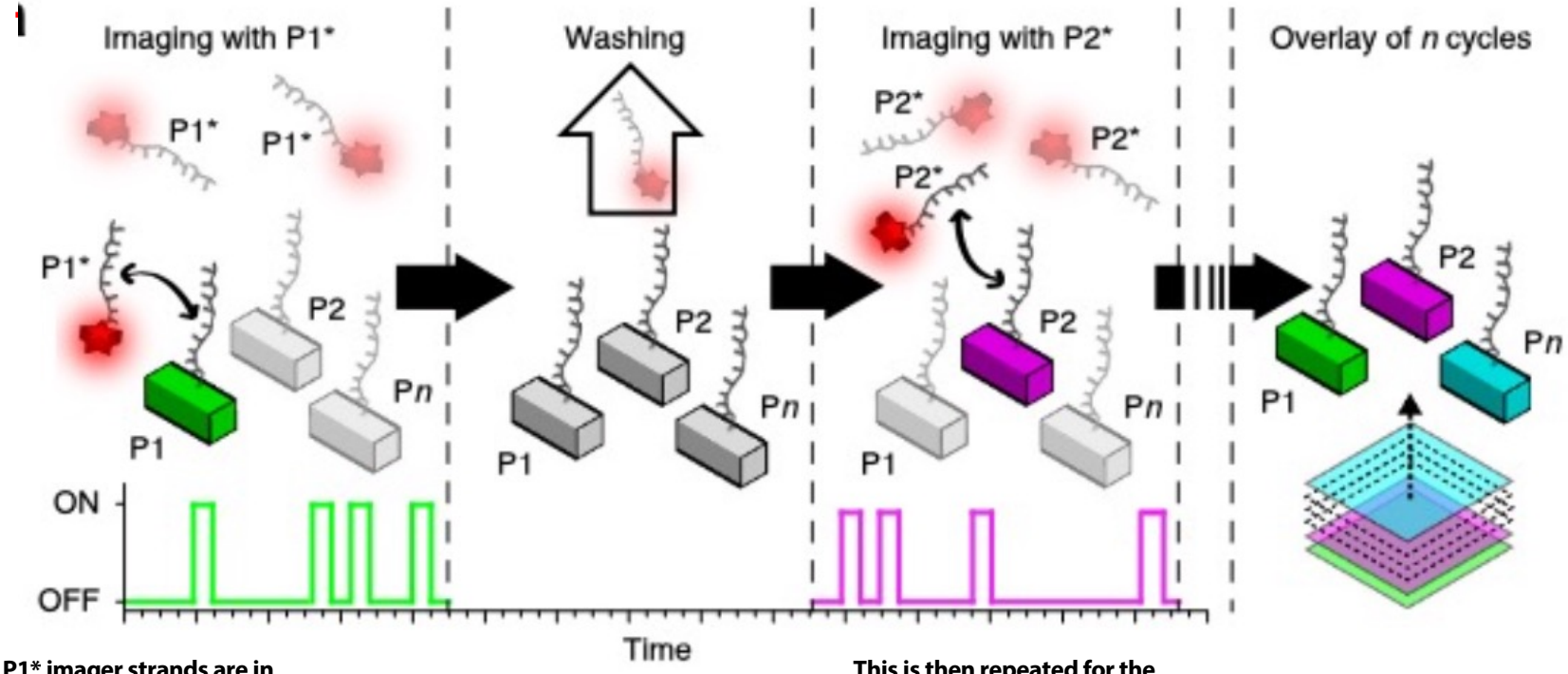
Exchange-PAINT (2014):

- **Another DNA-Based Super-Resolution Imaging Technique**

- **A DNA-based technique that uses reversible binding between complementary DNA sequences to create an effect similar to the "flicker" of fluorescent molecules.**

Exchange-PAINT (2014): Another DNA-Based Super-Resolution Imaging Technique

Schematic representation of sequential Exchange-PAINT imaging of multiple targets with orthogonal sequences using the same



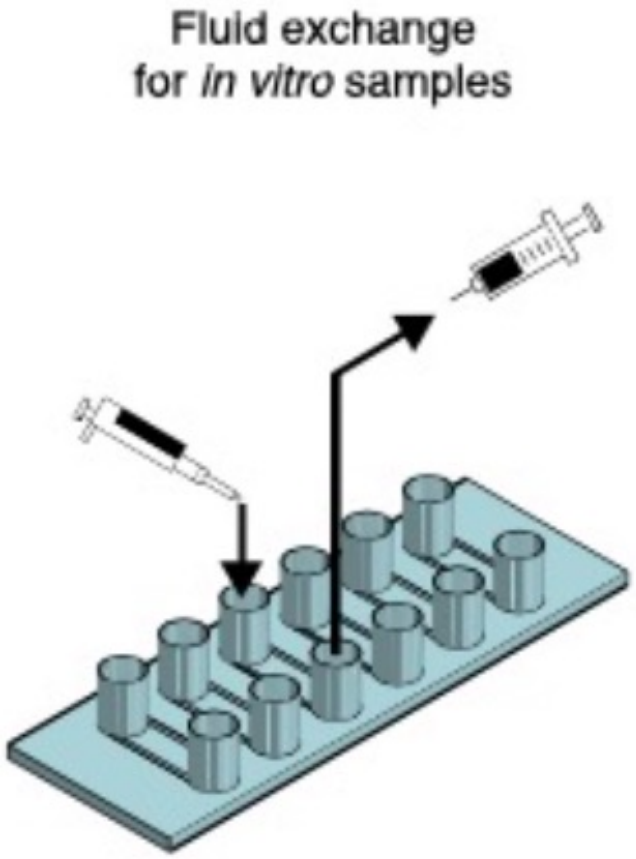
P1* imager strands are in solution and interact with their complementary target sequence, P1.

After the first acquisition round, the P1* imager strands are washed away and P2* imagers are introduced to image the next target.

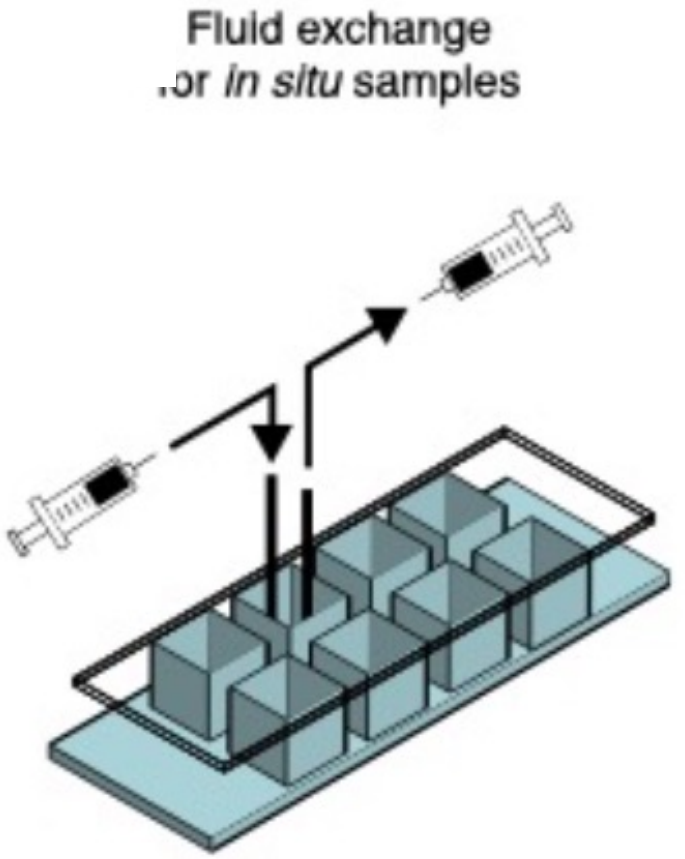
This is then repeated for the remaining target cycles, and pseudocolors are assigned for each respective imaging round.

- Last, all rounds are aligned and overlaid to form the final multiplexed Exchange-PAINT image of n targets.

Exchange-PAINT (2014): Another DNA-Based Super-Resolution Imaging Technique

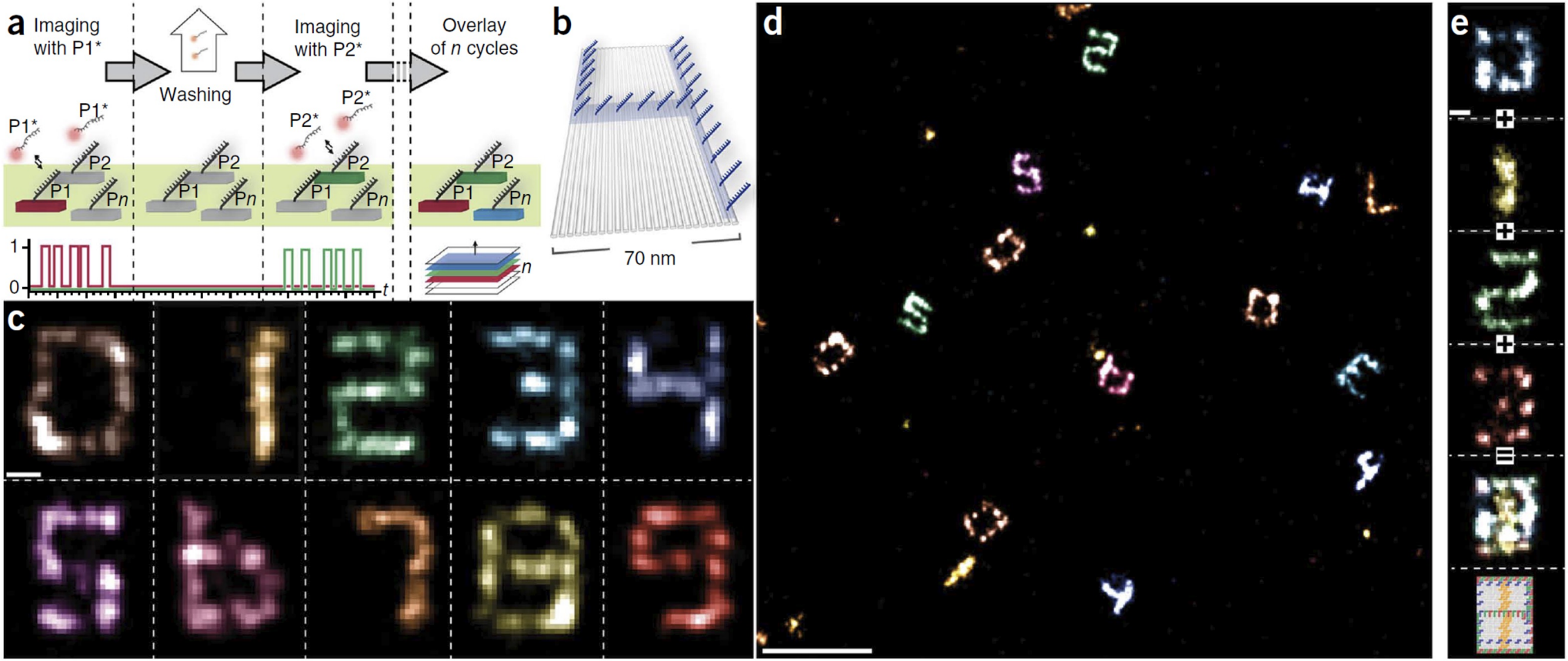


Fluid exchange chamber for in vitro samples (e.g., for DNA origami imaging).
Liquid is introduced by pipetting into the inlet. The outlet is attached to a syringe with a flexible tube to remove the liquid.



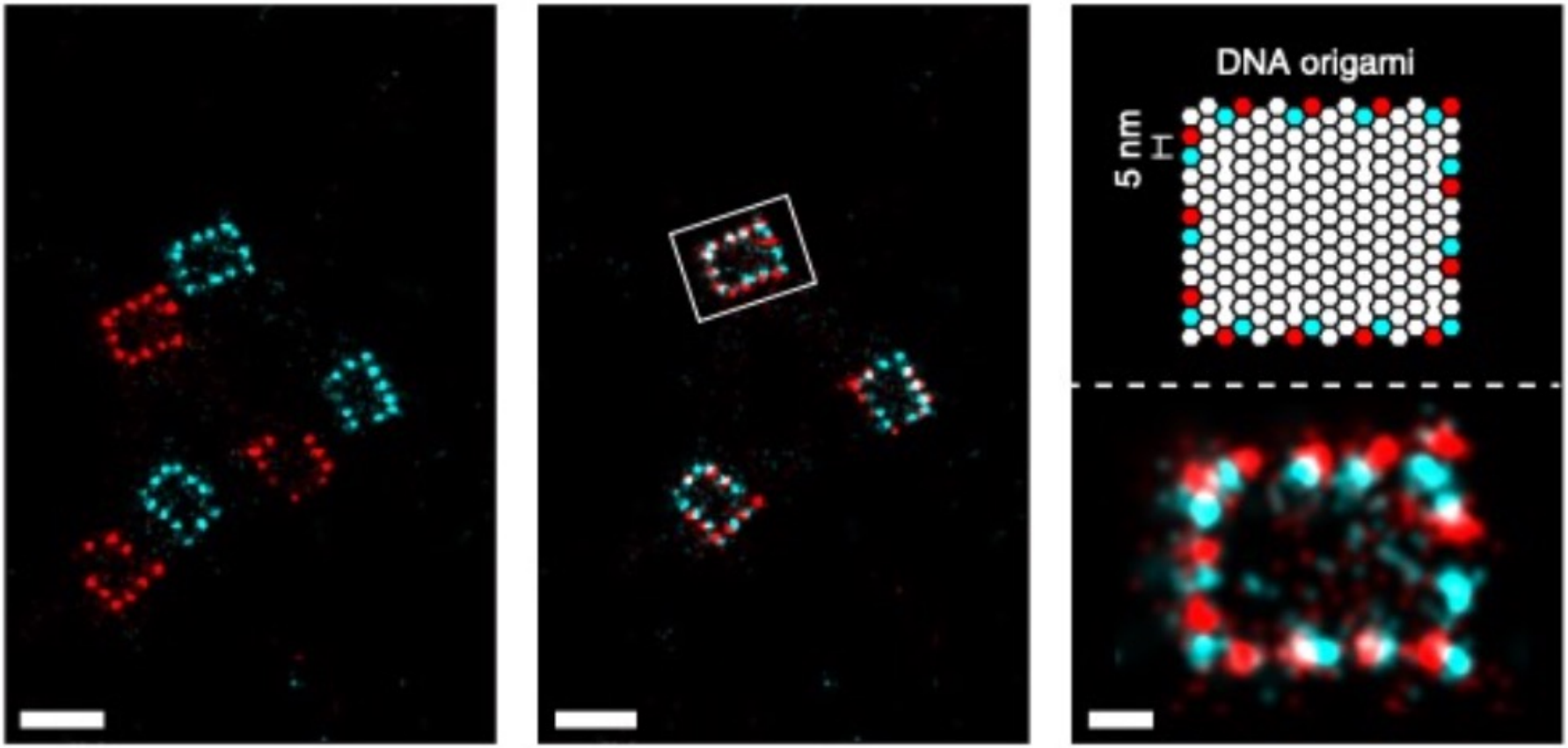
Fluid exchange chamber for in situ samples (i.e., used for in situ cellular imaging).
Two tubes with syringes are connected to an 8-well chambered cover glass to facilitate fluid exchange.

Exchange-PAINT (2014): Another DNA-Based Super-Resolution Imaging Technique



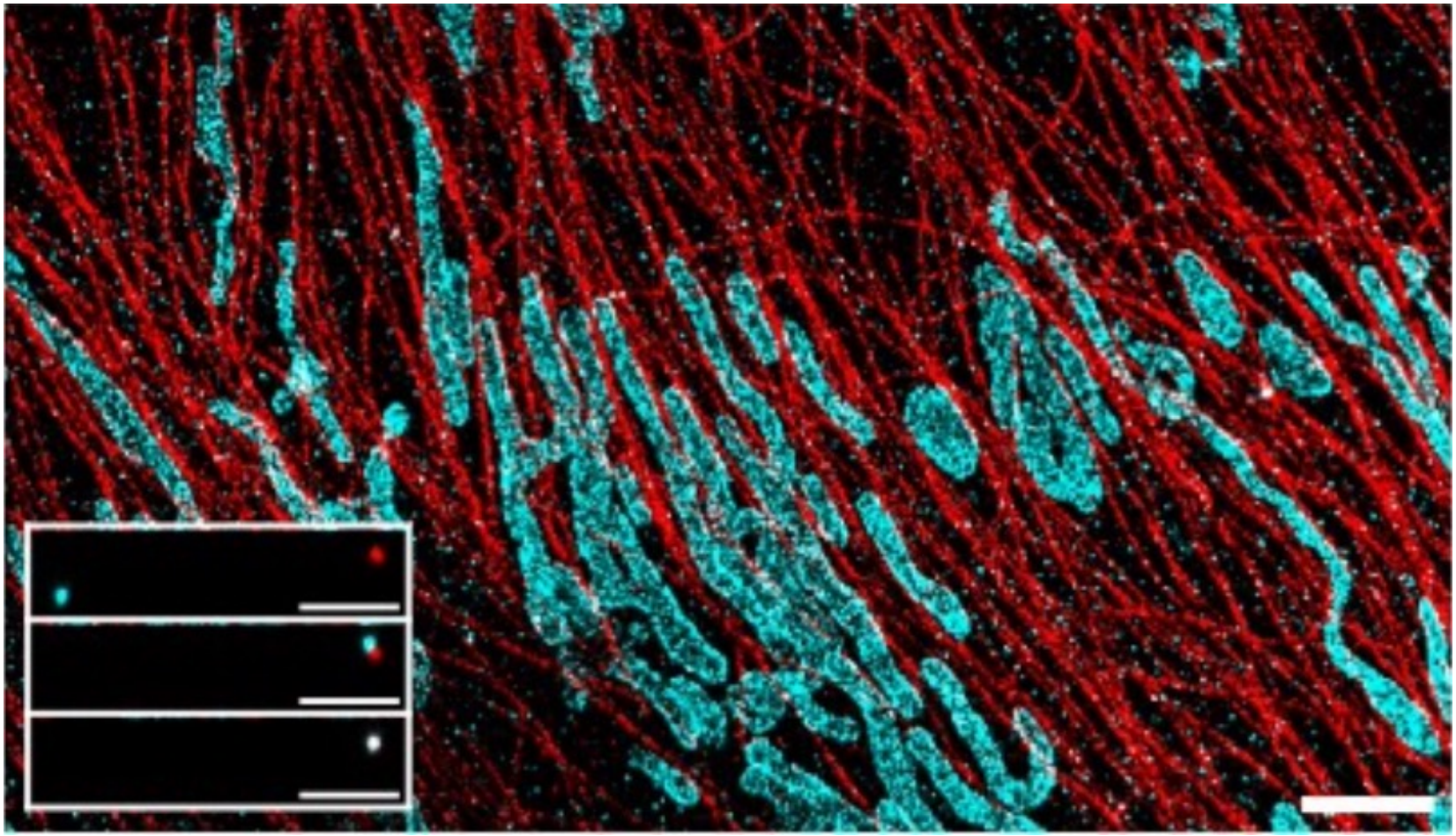
Exchange-PAINT (2014): Another DNA-Based Super-Resolution Imaging Technique

Exchange-PAINT images of DNA Origami:



Exchange-PAINT (2014): Another DNA-Based Super-Resolution Imaging Technique

In situ Exchange-PAINT image of protein targets a-tubulin (red) and Tom20 (cyan)



Exchange-PAINT (2014)

- **Wins:**
  - Overcame a DNA-PAINT limitation of spatial sub-10nm resolution demonstration by collection more photons per binding event.
  - Also, demonstrated that technique is not limited to DNA by imaging cells.
  - Increased multiplexing by sequential dye-washing and orthogonal DNA strands.

- **Loses:**
  - Number restricted to orthogonal DNA strands.
  - Every wash takes 1 - 2 mins.
  - For cellular structure, resolution was not sub 10nm.

Sequential super-resolution imaging using DNA strand displacement (2018)

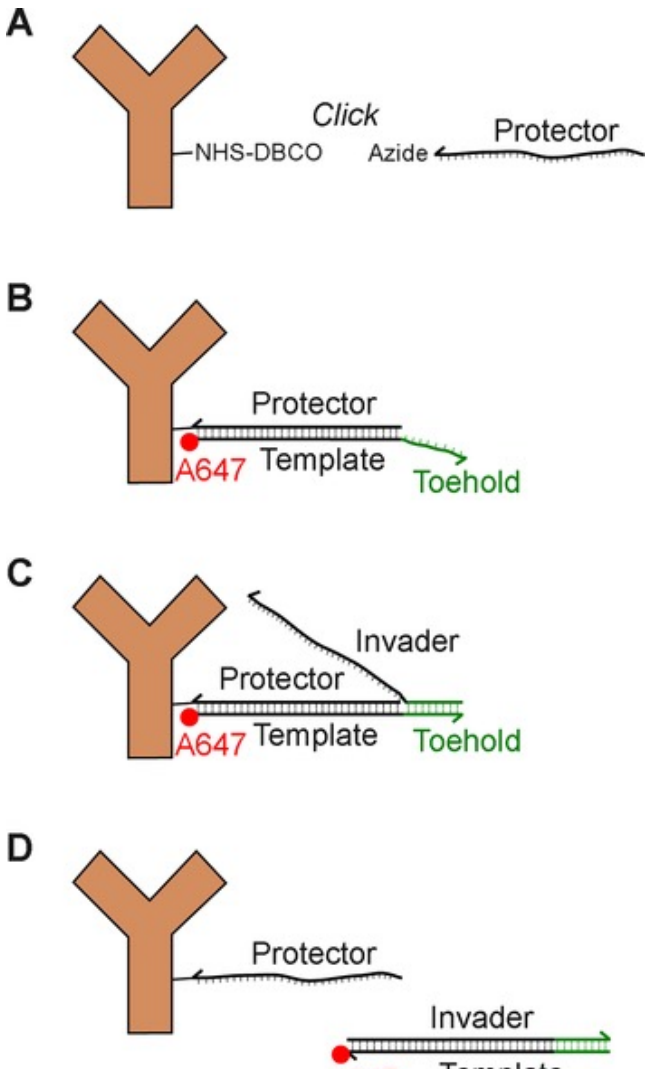
Concept of DNA strand displacement Imaging:

(A) A protector strand is covalently conjugated to an antibody.

(B) After labeling the cell with all antibodies of interest, a specific dye-labeled template strand is introduced to the buffer and binds to its matched protector strand. This sequence is long enough to prevent spontaneous dissociation.

(C) After imaging, the template is removed by addition of an invader strand. The invader gains a toe-hold on the overhang section of the template and since individual base pairs can dissociate, the invader eventually out-competes the protector.

(D) The dissociated double stranded invader/template is washed away.



Pallikkuth S, Martin C, Farzam F, Edwards JS, Lakin MR, et al. (2018) Sequential super-resolution imaging using DNA strand displacement. PLOS ONE 13(8): e0203291. https://doi.org/10.1371/journal.pone.0203291
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0203291

Sequential super-resolution imaging using DNA strand displacement (2018)

- Sequential labeling and imaging in fluorescence microscopy allows the imaging of multiple structures in the same cell using a single fluorophore species.
- In super-resolution applications, the optimal dye suited to the method can be chosen, the optical setup can be simpler and there are no chromatic aberrations between images of different structures.
- Uses a method based on DNA strand displacement that can be used to quickly and easily perform the labeling and removal of the fluorophores during each sequence.
- Site-specific tags are conjugated with unique and orthogonal single stranded DNA. Labeling for a particular structure is achieved by hybridization of antibody-bound DNA with a complimentary dye-labeled strand.
- After imaging, the dye is removed using toehold-mediated strand displacement, in which an invader strand competes off the dye-labeled strand than can be subsequently washed away.
- Labeling and removal of each DNA-species requires only a few minutes.
- Demonstrated the concept using sequential dSTORM super-resolution for multiplex imaging of subcellular structures

Pallikkuth S, Martin C, Farzam F, Edwards JS, Lakin MR, et al. (2018) Sequential super-resolution imaging using DNA strand displacement. PLOS ONE 13(8): e0203291. https://doi.org/10.1371/journal.pone.0203291
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0203291

Sequential super-resolution imaging using DNA strand displacement (2018)

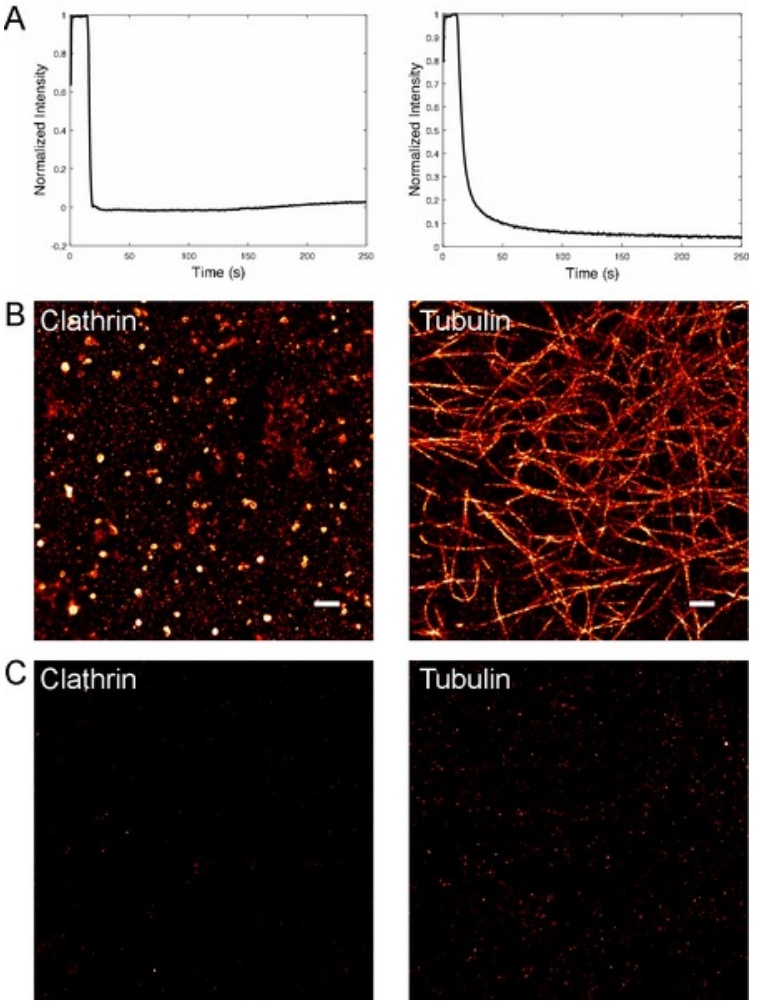
Invader time course and residuals:

(A) **Fluorescence intensity of a labeled cell sample as a function of time after invader addition. The cell is imaged for ~ 20 s before adding invader**

(B) Super-resolution image before invader.

(C) Super-resolution image after invader.

The residual fluorescence for Set A and Set B are 2% and 5% respectively.



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https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0203291

Sequential super-resolution imaging using DNA strand displacement (2018)

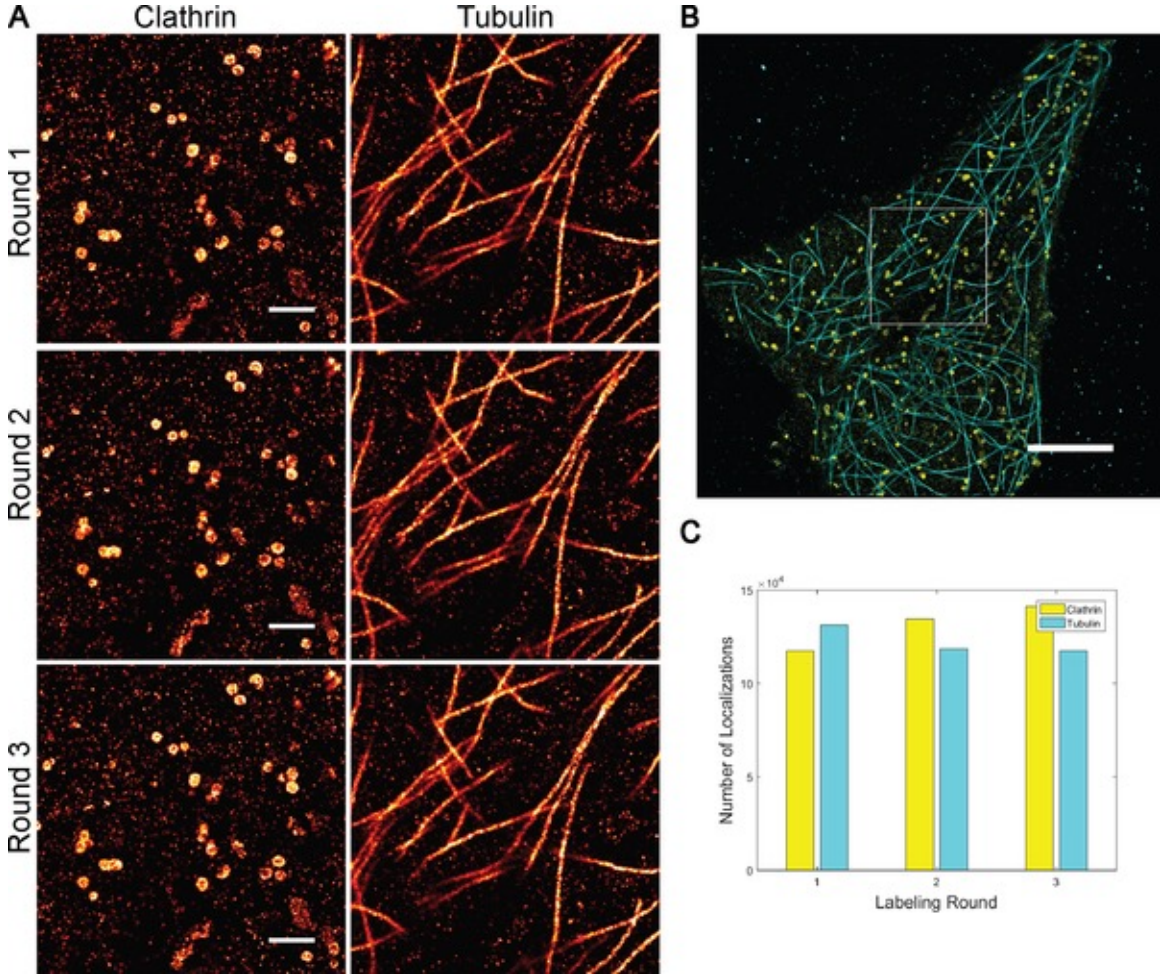
Sequential imaging results with clathrin and tubulin.

Clathrin and tubulin are labeled and imaged using Set A and Set B, respectively.

(A) Super-resolution images acquired after 3 rounds of the label-image-remove process.

(B) Overlay of all tubulin (cyan) and clathrin (yellow) imaging rounds. White box indicates regions shown in (A).

(C) Comparison of the number of localization for each structure/set per imaging round.



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