Practical Course Biophysics Program

Super-Resolution Microscopy: Imaging the Nuclear Pore Complex with DNA-PAINT

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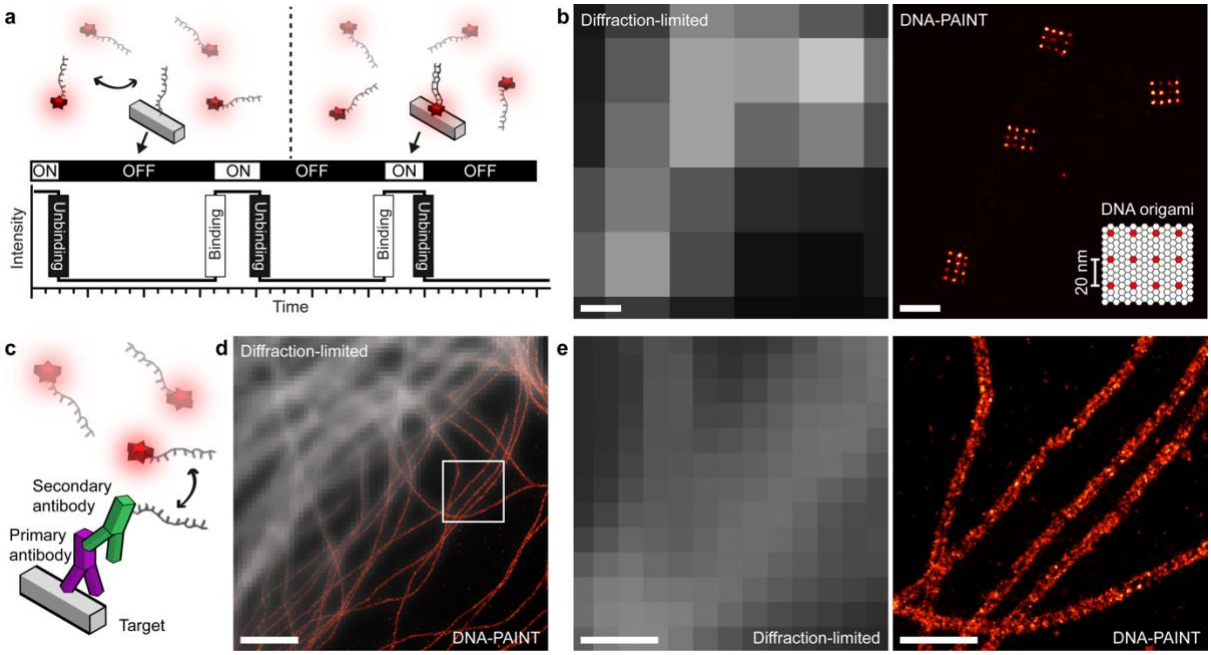

Figure 1: ***Jungmann et al.*** *Super-resolution microscopy with DNA-PAINT,* ***Nature Protocols*** *2017*

Description

In this one-day lab course you will use the super-resolution microscopy technique DNA-PAINT to study the structure of nuclear pore complexes (NPC) on the nucleus of a cell. The experiment will start with the sample preparation of the cells. Afterwards, the NPCs are measured with a custom TIRF microscope. In the end, you will analyse DNA-PAINT data using our lab software 'Picasso'.
The course constitutes intensive training in sample preparation, super-resolution imaging with DNA-PAINT and post-processing.

Preparation

In advance of the course, the lab manual should be read carefully. Particular steps during the course will be supervised, nevertheless it is assumed that participants are familiar with the basics of super-resolution microscopy. For the post-processing, the participants are expected to use the software 'Picasso'.

DNA: the Source of Life

Deoxyribonucleic acid (DNA) has long been studied due to its biological relevance and function. DNA is widely known as the molecule, which carries genetic information in living organisms. Genetic information can be seen as the blueprint of cellular components like proteins etc.. Early research, where DNA could be isolated for the first time, was done in 1869 by the young Swiss doctor Friedrich Mischer [Dah08]. In 1919, Phoebus Levene was able to identify the main components of DNA, sugar deoxyribose, phosphate groups and four nucleobases: adenine, guanine, thymine and cytosine [Lev19], shown in figure 2b. Until 1953 the question about the structural composition of DNA was still up for debate. Using an X-ray diffraction image of DNA taken by Rosalind Franklin and Raymond Gosling in May 1952 [FG53] James Watson and Francis Crick proposed the first correct double helix model of DNA [WC53] shown in figure 2a.

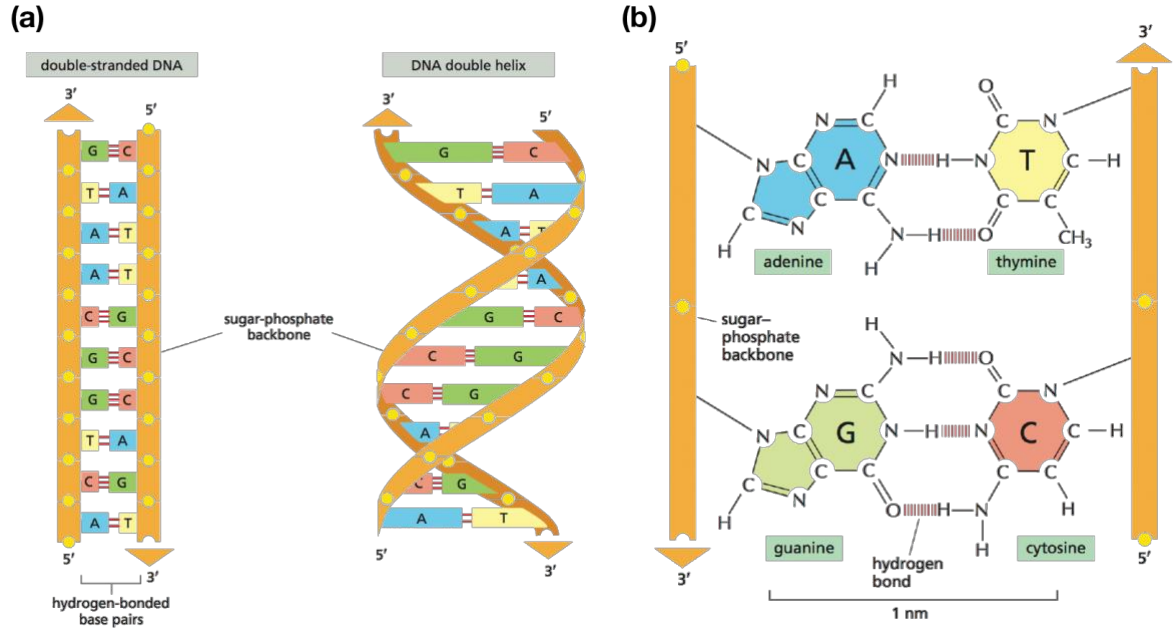

Figure 2: ***(a)*** *Double-stranded DNA: A normal DNA molecule consists of two individual strands. Each strand is a chain made up of repeating units called nucleotides, which are linked covalently to each other. Every nucleotide consists of a base (adenine (A), thymine (T), guanine (G) or cytosine (C)) and a sugar-phosphate backbone. The single strands hybridize together at the nucleobase site. Every strand has a characterizing direction from 5' to the 3' end. DNA double helix: Two single strands form a double helix. One full turn is reached after 10,5 base pairs (bp).* ***(b)*** *According to the Watson-Crick base pairing nucleobases are linked together via hydrogen bonds. Base A and T form two hydrogen bonds, whereas C and G form three hydrogen bonds.) [AJL+07]*

A DNA double helix is a polymer made up of two antiparallel strands of repeating units called nucleotides. One nucleotide consists of one out of four nucleobases (adenine, guanine, thymine and cytosine) and the backbone section, which is formed from the phosphate group and the sugar deoxyribose. Nucleotide monomers of a strand are linked together covalently by phosphodiester bonds connecting the 3'-hydroxyl (-OH) group of one sugar and the 5'-hydroxyl group of the subsequent sugar group. Due to these 3'-5' bonds, the strand carries an intrinsic chemical polarity or direction.

The two individual strands are connected, i.e. hybridized, through the nucleobases, which form hydrogen bonds. Successful base pairing of individual nucleotides can only occur between adenine (A) and thymine (T) with the formation of two hydrogen bonds and guanine (G) and cytosine (C) via three hydrogen bonds, shown in figure 2b. This is called the Watson-Crick base pairing rule.

In the past 35 years DNA, aside from its genetic importance, is also used as a building material in nanotechnologies. The field of DNA nanotechnology was initiated in 1982 by Nadrian Seeman's idea to use the base pairing rule as a tool to engineer DNA nanostructures [See82].

Optical Microscopy

Fluorescence

Shiny white spots of a freshly washed dark T-shirt under blacklight, for example in a nightclub, is a well-observed phenomenon. It is often not known that this is caused by the fluorescence of residual laundry detergent, which gets excited from the UV light. Besides giving a plain T-shirt a special effect, fluorescence plays an important role in biological research. The most attractive feature of fluorescence microscopy and many modern imaging and analytical techniques is the ability to image and quantitatively study not only the structure but also the function of intact cells in situ and in vivo.

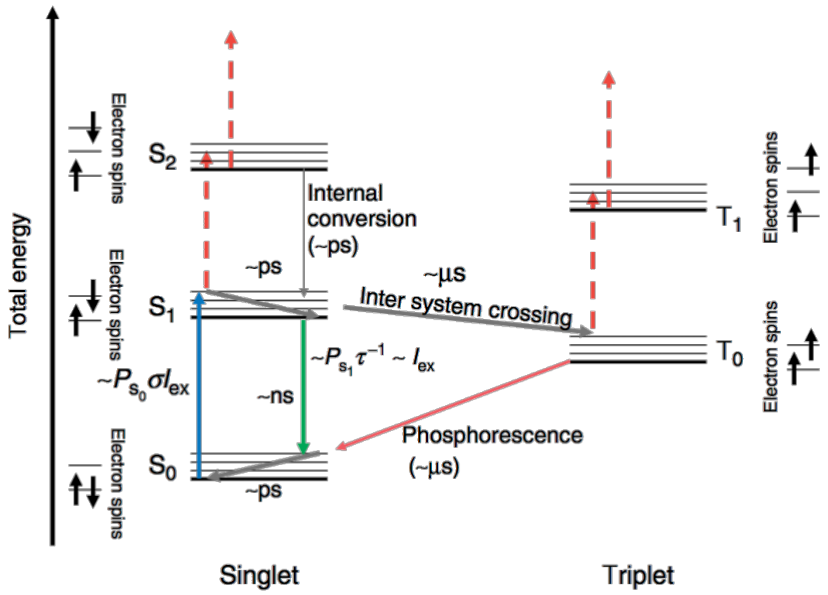

Figure 3: ***Jablonski Diagram:*** *Diagram of the total energy of a molecule, given by the electronic state of a molecule. The groups of levels dedicate the electronic states and the thin lines describe the multiple vibrational levels. Transitions between different energy levels are highlighted with arrows. During fluorescence, the molecule is excited due to the absorption of a photon from the ground state S0 into the first excited state S1, illustrated by the blue arrow. After internal conversion, meaning relaxation into the lowest excited state, the molecule relaxes into its ground state S0 via the emission of a photon, indicated by the green arrow. Internal conversion happens in the time scale of picoseconds. The time from excitation to the relaxation into the ground state is in the order of nanoseconds. [Kub13]*

Fluorescence belongs to the subgroup of luminescence effects called photoluminescence. It describes the molecular process of the absorption of energy and subsequent emission. A typical fluorescence process starts with the absorption of a photon by a molecule, which consists of several atomic nuclei and an electron cloud, where the electrons have different spatial probabilities of presence called orbitals. Because the nuclei are far heavier than the electrons, they can be regarded as stationary. When the photon gets absorbed by the molecule, one electron is being displaced from one orbital into an unoccupied orbital. This happens almost instantaneously, on the order of femtoseconds. The displacement of the electron causes a change in the charge distribution around the nucleus and brings the molecule out of the equilibrium state into an excited state. Due to the net force resulting from the unbalanced charge distribution the molecule starts to oscillate, i.e. vibrate.
After internal conversion, thus rearrangement of the charge distribution the molecule settles in a new excited equilibrium state, which is the lowest excited electronic state. For a few nanoseconds, the molecule rests in this lowest excited state, before relaxing back into the ground state via the emission of a photon. Compared to the absorbed photon, the emitted photon is red-shifted, hence has a lower energy, because the vibrational relaxation also consumes energy. The difference in energy is called the Stokes-Shift. This whole process of radiation is illustrated in the Jablonski diagram, in figure 3. Fluorescence molecules can undergo this radiation process a few thousand times, before they suffer a structural change, resulting in the loss of fluorescence. This is called photobleaching.
Regarding the fact that the transition between ground state and excited state can happen not only at specific energy lines but rather at a range of energies, the photo-physical properties of a fluorescent molecule are described by an excitation-emission spectrum. A typical spectrum is shown in figure 4.

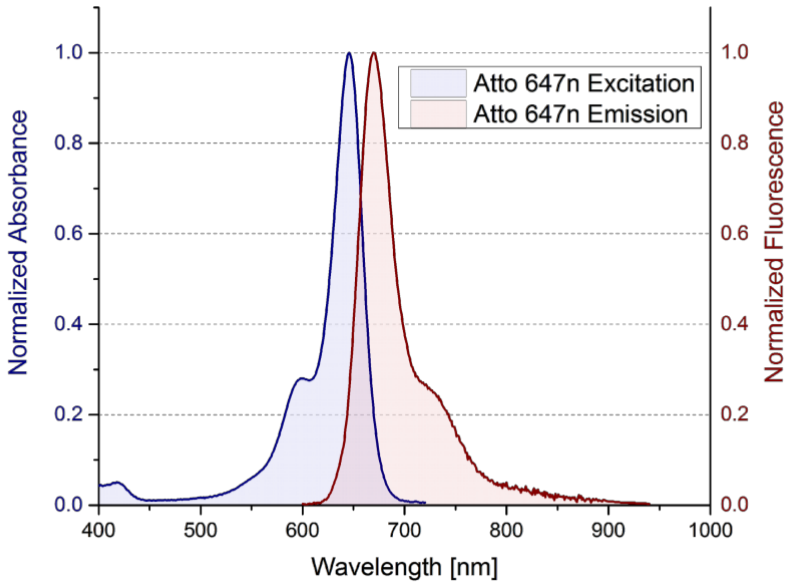

Figure 4: ***Fluorescence Spectrum of Atto 647N****: The excitation and emission spectrum of the fluorescent dye Atto 647N from Atto-Tec GmbH. Excitation maximum at a wavelength of 646nm and the emission maximum at 664nm. [AT16b]*

Bypassing the Diffraction Limit

For a long period of time optical microscopy was restricted by an alleged physical limitation. Ernst Abbe postulated 1873 that spatial resolution of optical microscopes is limited by the physical nature of light, the diffraction of light [Abb73]. 1903 Lord Rayleigh expressed the phenomenological observation of Abbe into mathematical equations [Ray03]. A fairly good approximation of achievable resolution is half the wavelength of the observed light. Using violet visible radiation with a wavelength of 400nm for example, it is not possible to distinguish two violet light emitting point sources, when they are placed closer together than 200nm.
Eric Betzig, Stefan Hell and William E. Moerner received the Nobel Prize in Chemistry 2014 for developing methods surpassing the resolution limit. Their achievements in optical microscopy opened a new window into the nanoworld. With their powerful new tools, they pushed the boundaries in biological research.

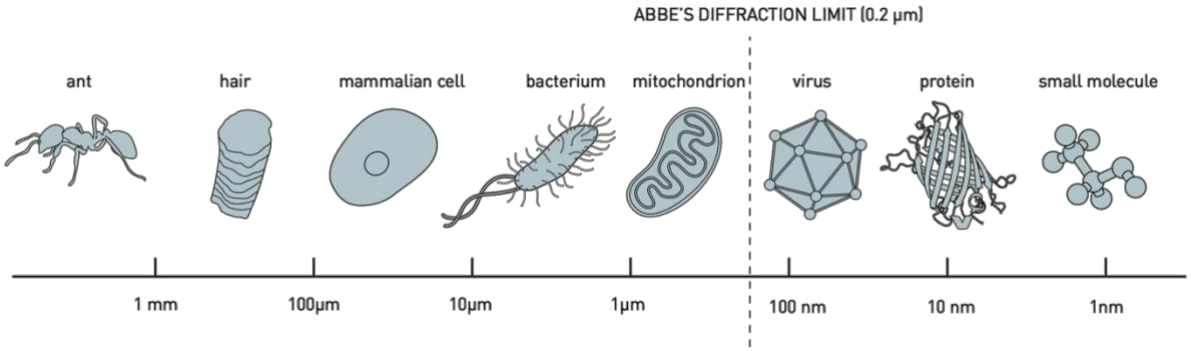

*Figure 5: **Length scales in biology**: From macroscopic animals like ants in the order of millimetres, into the nanoscopic world through the Abbe diffraction limit at around 200nm down to small molecules like fluorescent dyes in the order of nanometres. [JJ14]*

A look at the length scales in biological research (see figure 5) indicates that the vast majority of molecular processes in living organisms are hidden from observation using conventional light microscopy because of the diffraction (or Abbe) limit.

Ernst Abbe postulated in his work 1873 that due to the diffraction limit it is not possible to resolve two light points sources with wavelength λ if they are placed closer together as the distance d, given by the formula [Abb73]:

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

NA is the numerical aperture of the optical device.

The point spread function (PSF) is the mathematical description of the imaging process of a point light source. For a lens and a point light source at the optical axis of the lens the PSF is approximated by the Airy function, illustrated in figure 6. The image of the point emitter gets broadened on the camera screen with the distribution of an Airy disc.

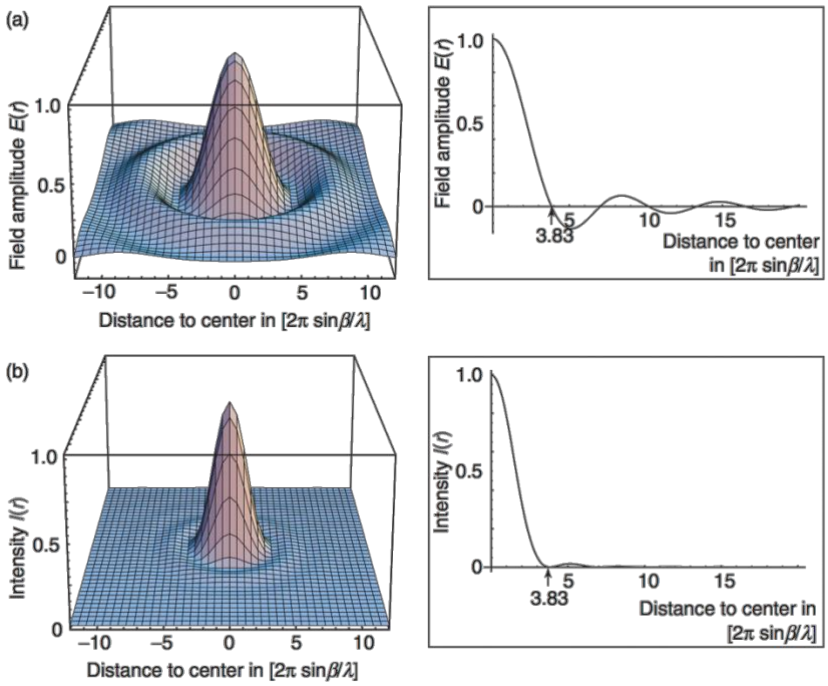

Figure 6: ***(a)*** *Left: Three-dimensional plot of a 2D Airy function. Shown in the normalised electric field distribution E(r) in the object plane of a point source as a function of the distance to the optical axis in optical units.* ***(b)*** *Intensity distribution I(r), proportional to the square of the absolute values of the electronic field distribution E(r). [Kub13]*

Betzig and Hell invented techniques to surpass the diffraction limit.
Stefan Hell presented in 1994 the theory for a new scanning fluorescence microscope called STED-microscope (Stimulated Emission Depletion), which uses the principle of RESOLFT microscopy [HW94]. RESOLFT denotes REversible Saturable Optical Linear Fluorescence Transitions. Using inhomogeneous illumination, fluorescence molecules are switched into a dark state, where the emission of photons is prevented. By excluding a small spot from the depletion illumination, the achievable resolution can be enhanced and the diffraction limit surpassed. During imaging the whole sample is scanned point by point.

Betzig et al. published in 2006 a method for fluorescence microscopy called PALM, Photo-Activated Localization Microscopy, where they exploit these circumstances [BPS06]. Unlike STED-microscopy, the sample is not scanned. In fact, through stochastic activation using weak laser pulses, a small subset of photoactivatable fluorescent proteins, which are genetically added to the target of interest, are switched from the dark state into the bright state. Photons from this subset of fluorophores are collected until they photobleach. In an ongoing experiment, a time series of images is recorded, where different subsets of fluorophores were activated, shown in figure 7.

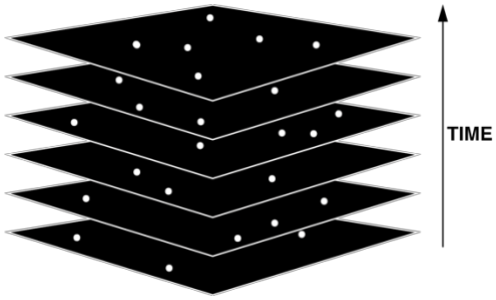

Figure 7: Localization Microscopy movie. A stack of images, where in every frame only a subset of single fluorophores are activated. The distance between the activated fluorophores is larger than the diffraction limit.

Using software algorithms every diffraction-limited image is analysed in the following way that single molecule signals are identified and the position of the molecule localised. Therefore, the diffraction broadened spot is de-convolved with a two-dimensional Gaussian function. Strictly speaking a two-dimensional Airy function, shown in figure 6, needs to be considered, however an Airy disc is well approximated by a 2D Gaussian function [Kub13]. The underlying principle of single molecule localization microscopy (SMLM) is illustrated in figure 8.

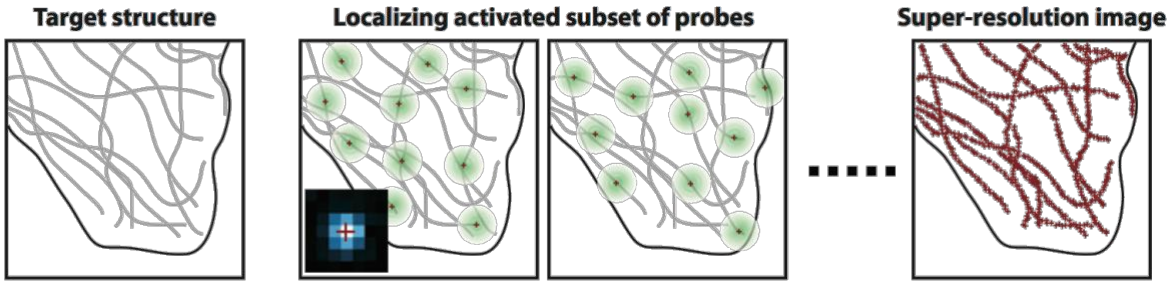

Figure 8: ***Principle of single molecule localization microscopy:*** *The target structure is decorated with fluorophores, for example with fluorescent proteins. Only a subset of fluorescent probes are activated at different time points, allowing fractions of fluorophores to be imaged without spatial overlap and localised with high precision. By recording a whole stack of images, where different subsets of probes are activated, a super-resolution image can be constructed from a large number of localised probe molecules [HBZ09].*

The precision of the localization described by the standard deviation σ_{Loc} of an individual fluorophore from the diffraction limited image using the 2D gaussian function is proportional to the number of photons N and the standard deviation of the point spread function σ_{PSF} [TLW02]:

$$\sigma_{Loc} \sim \frac{\sigma_{PSF}}{\sqrt{N}}$$

This pushes the achievable spatial resolution from formula of Abbe by a factor of $1/\sqrt{N}$ resulting in:

$$d \sim \frac{\lambda}{2 \cdot \sqrt{N} \cdot NA}$$

DNA-PAINT and Exchange-PAINT

Besides the methods PALM and STORM a different approach in localization microscopy called DNA-PAINT was published by Jungmann et al. in 2010 [JSS10]. It is based on the concept of PAINT, Points Accumulation for Imaging in Nanoscale Topography, where in contrast to PALM or STORM imaging is carried out using diffusing fluorescent molecules that interact transiently with the sample [SH06]. Key limitation of PAINT is achieving specificity of targeting molecules of interest, as the interaction relies on molecular effects like electrostatic coupling or hydrophobic interactions, which are challenging to control. The idea behind DNA-PAINT is the use of hybridization reactions of short single-stranded DNA oligonucleotides functionalized with fluorescence dyes, called imagers, to create programmable interactions with high specificity in targeting molecular components. The activation and deactivation of fluorophores called blinking is not pursued using photo-physical processes as it is done in PALM or STORM. The so-called on- and off-states of a fluorescent probe at the target is achieved by the repetitive, transient binding of imagers to a single-stranded complementary DNA docking strand, see figure 9. Unbound imagers in solution diffuse too fast for being localized during the camera exposure times, hence it only increases background signal. Once the imager hybridized to the docking site, the fluorophore is immobilized on the target and photons are emitted and recorded. Looking at the equation above, the more the better.

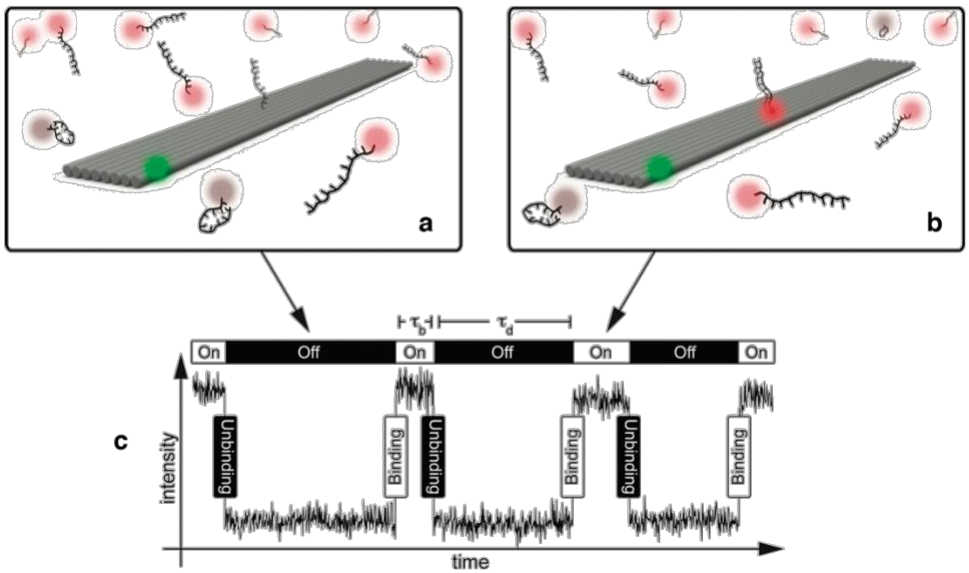

Figure 9: ***DNA-PAINT principle:*** *Stochastic switching between fluorescence on- and off-states (blinking) is pursued using DNA fluorescent probes called imagers. The short single-stranded oligonucleotides labelled with fluorescent molecules interact transiently with single-stranded complementary DNA strands at the target, called docking strands. This creates artificial fluorescent on- and off-states at the target of interest.* ***(a)*** *Planar DNA nanostructure decorated with one DNA-PAINT docking strand in the centre of the structure. As no imagers are hybridised to the docking site, no fluorescent signal from the docking site can be observed.* ***(b)*** *An imager is hybridised to the docking site, enabling the collection fluorescent signal at target. Depending on the stability of the duplex, the imager detaches after a given time, leaving the docking site available for subsequent reactions of different imagers.* ***(c)*** *Typical time trace of the intensity of a single docking site. A bound imager produces the fluorescent on-state. The time between the stochastic interactions is the off-state. Kinetic parameters of this repetitive reaction are completely controllable. The bright time t_{bright} for a given imaging buffer system at a specific temperature, depends on the length of the formed duplex of the imager and docking strands, whereas the dark time t_{dark} is controlled by the concentration of the imager. [JSS10]*

One benefit of using hybridization reactions of DNA to create this artificial blinking is that by sequence engineering kinetic parameters of the reactions are completely controllable. The on-time, i.e. bright time t_{bright} , is the period the imager is bound to the docking strand is given by the length of the imager sequence, meaning the number of complementary bases. The stability of the duplex is described by the dissociation rate k_{off} and inverse proportional to t_{bright}:

$$t_{bright} = \frac{1}{k_{off}}$$

Whereas the off-time, dark time t_{dark} is controlled by the concentration of imagers c in solution, for a given salt concentration of the imaging buffer. k_{on} is called the association rate and can be determined experimentally for a given imaging buffer:

$$t_{dark} = \frac{1}{c \cdot k_{on}}$$

Another notable advantage of DNA-PAINT is the free choice of fluorophores. Fluorescent probes do not need to be genetically added to the sample, moreover photo-physical or photo-chemical properties for stochastic blinking do not need to be considered in the selection of the fluorophores.
In 2014 Jungmann et al. published an extension called exchange-PAINT, where they showed that DNA-PAINT can be used for multiplexed imaging in a sequential fashion [JAW14]. The principle of exchange-PAINT is shown in figure 10. Initially different targets are labelled with orthogonal docking strands. Beginning with one imager sequence, the first target is imaged. As the imagers bind only transiently, after gathering enough localizations, the first imager can be removed with washing rounds and the next imager with a different sequence introduced. Subsequent washing and imaging rounds are carried out until all targets are imaged. In contrast to spectral multiplexing exchange-PAINT uses pseudocolors, which are encoded in the nucleotide sequence of the imager strand. This enables the usage of the same dye in every imaging round.

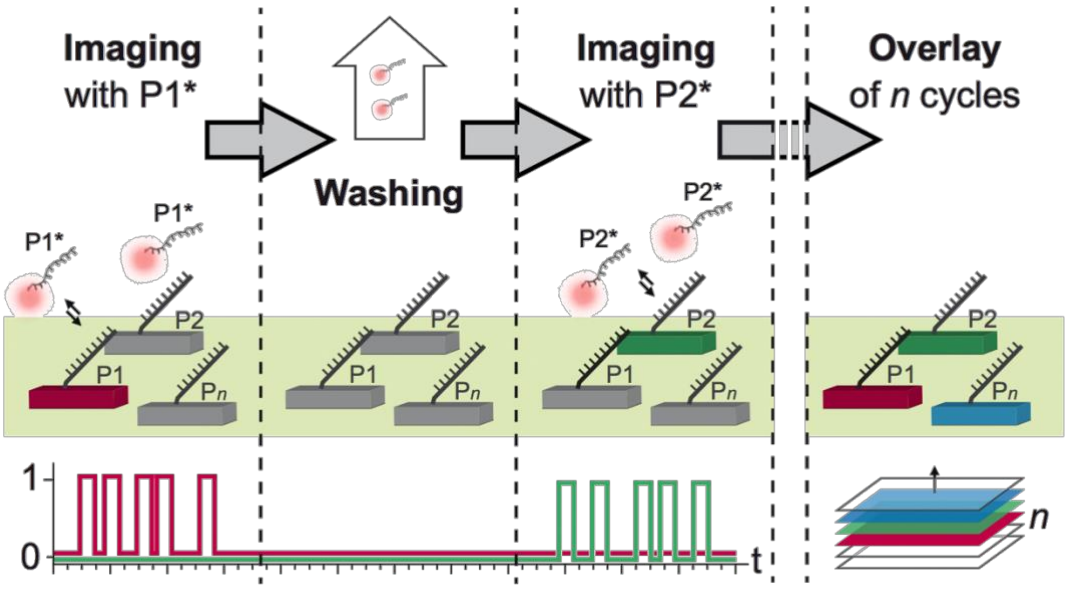

Figure 10: ***Exchange-PAINT principle****: Multiplexing approach using pseudocolours. The sample is labelled with single-stranded docking sites, where every target has an unique docking sequence. In sequential imaging rounds with imagers complementary to the docking sequences and subsequent washing rounds multiple targets can be imaged. [JAW+14]*

TIRF Microscopy

The observation of single molecules to study biology in a quantitative fashion is often prohibited by the performance of wide-field and conventional laser-scanning microscopes, as the fluorescent probes are illuminated in an extensive section of the sample. This results in limited resolution capability, especially if molecules are imaged outside of the focal plane of the objective. TIRF microscopy provides an attractive alternative to wide-field and laser-scanning microscopy for single-molecules studies. Here, only a small fraction of the sample above the area of the cover glass is illuminated. TIRF abbreviates Total Internal Reflection Fluorescence and utilises the unique phenomenon of evanescent waves, which are created if light is totally reflected at the interface between two media of different refractive index. If a beam, a propagating electromagnetic wave, strikes a medium boundary, where the refractive index is lower at the other side of the boundary at an angle larger than a particular critical angle, the wave cannot pass the boundary and gets reflected. This phenomenon is heavily used in long-distance signal techniques, to transmit information with little attenuation compared to electric cables. As a side effect of the total reflection, an evanescent wave with an exponentially decaying electromagnetic field at the other side of the interface can be observed. The illumination in TIRF is based on the generation of the evanescent wave at the interface of the glass slide and the sample solution. Figure 11 illustrated the optical path inside the objective. A laser beam is shifted to the border of the objective lens parallel to the optical axis of the objective. This causes a tilted emission of the laser. If the angle is larger than the critical angle, the laser beam is totally reflected and an evanescent wave inside the sample is

created. The evanescent field can be used to excite fluorescent molecules close to the surface of the glass slide.

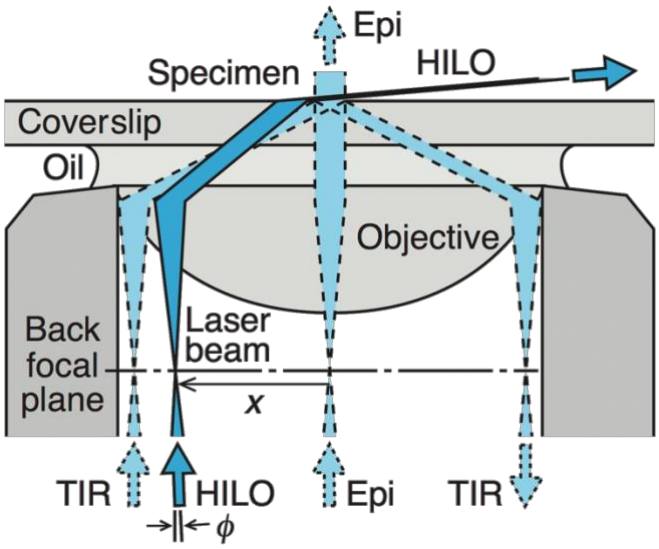

Figure 11: ***TIRF, HILO and EPI microscopy:*** *Fluorescent probes can be excited with the use of a laser in different modes. In EPI (epifluorescence) the laser beam is centred along the optical axis of the objective and transmitted into the sample. By shifting the beam parallel to the optical axes of the objective to the border of the objective lens, the illumination can be changed to HILO. Here, a highly inclined and laminated optical sheet is generated, which can be used to illuminate areas of the sample, while keeping background fluorescence of out-of-focus areas low. If the angle of the beam reaches the critical angle, the beam is totally reflected at the interface between glass and solution. This causes the generation of an evanescent electromagnetic field with an exponentially decaying field above the glass slide. This method is called TIRF and can be used to excite only a small volume of the sample, above the surface of the glass slide. TIRF provides a great method of illumination for single-molecule methods like DNA-PAINT. [TISS08]*

Besides TIR, figure 11 shows two additional modes to illuminate the sample. In EPI (epifluorescence) the laser beam propagates in the centre of the objective along the optical axis. The complete beam is transmitted into the sample.
To overcome the surface restriction of TIRF another approach to illuminate the sample is called HiLO (Highly Inclined and Laminated Optical sheet) can be used. It uses a highly inclined and laminated optical sheet, which is generated by the displacement of the incident laser beam near the objective edge, before reaching the critical angle. This allows the excitation of the sample in areas, above the TIRF range, while keeping the out-of-focus illumination low.
DNA-PAINT relies on the usage of TIRF and HiLO illumination, since the diffusing imagers create background fluorescence, which reduces the signal-to-background ratio, thus imaging quality.

Nuclear Pore Complex

The nuclear pore complex (NPC) is a massive molecular structure embedded in the nuclear envelope of eukaryotic cells. It plays a crucial role in the selective transport of proteins and RNA between the nucleus and the cytoplasm. The NPC is composed of approximately 30 different proteins called nucleoporins, which are organised into three main substructures: the inner ring, cytoplasmic ring, and nucleoplasmic ring. These

rings surround the central transport channel, forming an aqueous passage through which molecules can move. [SX21Nat]
Nup96 is a specific nucleoporin that serves as a scaffold protein in both the cytoplasmic and nucleoplasmic rings of the NPC. Recent research using cryo-electron tomography has revealed that Nup96 plays a significant role in maintaining the overall structure and function of the NPC. This study also revealed that Nup96 is present in 32 copies per NPC – 16 in the nuclear ring and 16 in the cytoplasmic ring.

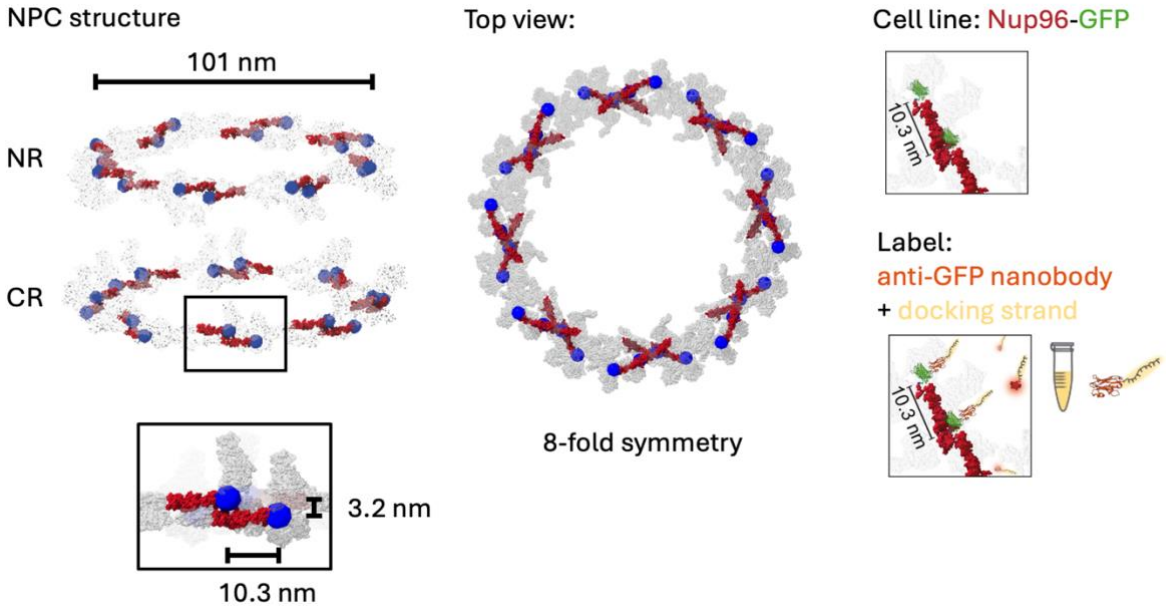

*Figure 12: **DNA-PAINT on Nup96:** Nup96 (red) is present in 32 copies per NPC. There are 16 Nup96 proteins in the nuclear ring (NR) and 16 copies in the cytoplasmic ring (CR). In one ring, there are eight pairs of adjacent copies of Nup96, spaced at 10.3 nm. To enable PAINT imaging, a cell line expressing Nup96 with a GFP tag (blue) is targeted with DNA-conjugated anti-GFP nanobodies. [RE23NAT]*

In the lab course, we will work with a human cell line that endogenously expresses Nup96 with a green fluorescent protein (GFP) tag. A DNA-conjugated anti-GFP nanobody binds to the GFP tag of the Nup96. The DNA strand on the anti-GFP nanobody is then used as a docking strand for DNA-PAINT, shown in figure 12 (right panel).

Experimental procedure

The lab course consists of the sample preparation and imaging of Nup96 in a human cell line which endogenously expresses Nup96-GFP.

Participants should carefully read the paper *Super-resolution microscopy with DNA-PAINT (*Schnitzbauer, J., Strauss, M., Schlichthaerle, T. *et al.* Super-resolution microscopy with DNA-PAINT. *Nat Protoc*ols (2017)), which explains DNA-PAINT in general. If it is not possible for participants to download the paper, please contact the supervisor as early as possible via email. You can receive a pdf version.

In addition, the software package Picasso (Windows 7 and higher) can be downloaded here:

https://github.com/jungmannlab/picasso/releases

During the practical course, the five following topics will be covered. Most of them are already explained by the Nature Protocols paper.
Particular steps during the course will be explained by the supervisor. Questions can be asked anytime, also via email beforehand.

Experimental Topics

1) DNA-PAINT simulation with 'Picasso Simulate'

Before starting with experimental data, super resolution data will be simulated with 'Picasso Simulate'. Parameters of the DNA-PAINT technique will be explored to fine tune experimental conditions.

2) Data processing with 'Picasso Localize' and 'Render'

The simulated single molecule localization data will be processed with the tool 'Picasso Localize'. Visualisation of the processed data will be done with 'Picasso Render'.

3) Sample preparation

Sample preparation of human cell line:

- Fixation and permeabilisation of cells
- Nanobody incubation
- Post-fixation
- Incubation with gold nanoparticles as fiducials for drift correction

4) DNA-PAINT imaging of Nup96 and data processing

The prepared sample will be visualised in super-resolution with DNA-PAINT imaging:

- Imaging with custom TIRF setup
- Analysis with 'Picasso Localize' and 'Picasso Render'

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