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Completing the Canvas: Advances and Challenges for DNA-PAINT Super-Resolution

Imaging

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Abstract

Single-molecule localization microscopy is a potent tool to examine biological systems with unprecedented resolution, enabling the investigation of increasingly smaller structures. On the forefront of these developments is DNA-based point accumulation in nanoscale topography (DNA-PAINT), which exploits the stochastic and transient binding of fluorescently labeled DNA probes. In its early stages, the implementation of DNA-PAINT was burdened by low-throughput, excessive acquisition time and difficult integration with live-cell imaging. However, recent advances are addressing these challenges, expanding the range of applications of DNA-PAINT. Here we review the current *state-of-the-art* of DNA-PAINT in light of these advances and contemplate what developments are still indispensable to realize live-cell imaging.

Introduction

Over the past decade, insights in wave optics have enabled the development of fluorescence super-resolution microscopy, allowing researchers to image with a resolution beyond the diffraction limit [1]. The high resolution is used to visualize structures at the molecular scale and unravel the complexity of cells. Super-resolution imaging can be achieved by a variety of techniques, including stimulated emission depletion (STED) [2], photo-activated localization microscopy (PALM) [3,4], and stochastic optical reconstruction microscopy (STORM) [5], which rely on a universal working principle; namely, limiting the number of simultaneously emitting fluorophores within a diffraction-limited sample. Although many fluorophores may be present within a nanometer-sized sample, only a few of them are excited at each given moment. This restricted excitation and identification can be realized in two distinct manners: reversible saturable optical fluorescence transitions (RESOLFT) [6] and **single-molecule localization microscopy (SMLM)** (see **Glossary**) [7], although recently they have been combined into a single method [8].

The key concept of SMLM is the switching between on and off states of fluorescent probes, such as in PALM [3,4] and STORM [5] (**Box 1**). An alternative approach for SMLM is point accumulation in nanoscale topography (PAINT) [9]. Repetitively and transiently binding fluorescent probes are used for the detection of blinking events in PAINT. In the first demonstration of PAINT in 2006, a lipophilic stain bound the membrane of large unilamellar vesicles (LUVs) transiently and non-specifically [9]. Recently, DNA nanotechnology has revolutionized PAINT imaging via DNA-based PAINT (or **DNA-PAINT**) [10]. DNA-PAINT uses short fluorescently labeled oligo's that can bind transiently to their complementary labeled targets to achieve blinking.

The early days of DNA-PAINT primarily focused on bringing the resolution down to the molecular level, and having achieved this, recent developments have improved other

aspects of the technique while exploring the plethora of potential applications (**Figure 1A**). DNA-PAINT has been implemented to measure piconewton forces in living cells, allowing the simultaneous quantification of mechanical force and visualization of cellular structures and thereby bridging the gap between structural biology and mechanobiology [11]. It is also increasingly being used in the medical realm, as both a more accurate and versatile tool to monitor biomarkers for disease diagnosis [12] and to study patient histology at the highest detail [13]. Preceding these applications is a wide range of ongoing developments, greatly expanding the versatility, applicability and ease of use of DNA-PAINT. While novel multiplexing strategies and advances in acquisition speed are addressing a crucial limitation of lengthy acquisition times, progress in labeling probes and alternative PAINT methods also pave the way for live-cell imaging. Here, we discuss these advances and contemplate remaining challenges before the DNA-PAINT canvas gets completed and live-cell imaging can be realized.

Single-molecule localization microscopy with DNA-PAINT

DNA-PAINT uses base-pairing between short fluorescently labeled DNA oligonucleotides [10]. A target is labeled with a short DNA **docking strand**, while the complementary, fluorescently labeled **imager strands** diffuse freely in solution. Upon hybridization, an increase in fluorescence intensity is observed (ON) for several hundreds of milliseconds after which the imager strand unbinds (OFF) and leaves the docking strand unoccupied (**Figure 1B**). As imager strands bind and unbind, the pool of imaged fluorophores is continuously replenished, eliminating concerns over the photon-budget in DNA-PAINT. In recent work photo-induced depletion of docking strands has been observed [14], which implies that the binding and unbinding cycle of imager strands is finite. Furthermore, DNA-PAINT allows for high target specificity and programmability since the length and sequence

of imager strands can be tuned [15,16]. Another advantage over other SMLM methods is that the choice of fluorophore is unrestricted because they do not need to be photo-switchable.

These advantages have expanded the use of DNA hybridization beyond the field of DNA-PAINT to other imaging methods, such as spectroscopy [17], STED [18–20], structured illumination microscopy (SIM) [19,20] and STORM [19]. Likewise, relying on DNA hybridization, rather than intensity overlap, to measure colocalization has allowed the determination of target proximity unconstrained by the optical resolution [21–23]. Early developments of DNA-PAINT improving both the localization precision of single molecules and the signal-to-noise ratio (SNR) have allowed discrete molecular imaging with <5 nm spatial resolution [20] (**Figure 1C**).

Despite the high resolution of DNA-PAINT, the quantification of the absolute number of target-bound fluorophores, especially for a large number of target molecules in densely packed clusters, remains a challenge. An attractive approach for the quantification of these complexes is quantitative DNA-PAINT (or qPAINT). qPAINT relies on the predictability of DNA hybridization, where the imager strand **association rate** linearly increases with the number of docking strands, thereby reporting on the number of molecular targets within a region of interest [25]. For conventional qPAINT, a calibration step was required, which might not be possible in complex heterogeneous biological samples. Recently, calibration was made redundant with the development of localization-based fluorescence correlation spectroscopy (lbFCS), which employs a post-imaging algorithm capable of autocorrelation [26].

Advances in visualizing distinct species through multiplexing with DNA-PAINT

Novel advances have focused on **multiplexing**, that is the visualization of multiple distinct molecular species within a single sample [27–31]. Recently, the number of dimensions

through which multiplexing is achieved expanded to include sequence, kinetic and spectral barcoding.

In sequence multiplexing, orthogonal sequences are used to label distinct cellular targets and the level of multiplexing is only limited by the number of orthogonal sequences that can be designed. Sequence multiplexing is the working principle of Exchange-PAINT [28]. These approaches achieve high resolution single-color multiplexing by using orthogonal DNA sequences to label and image different structures. However, because only a single type of fluorophore is used and pseudocolors are assigned to each orthogonal DNA sequence, imaging has to take place in sequential imaging cycles (**Figure 2A**). The acquisition time thus scales with the number of structures, making the imaging of a large number of structures in a single sample a long process. To reduce the time between sequential imaging cycles, the washing step can be eliminated by adding "quencher" strands prior to each new imaging round. These quencher strands are complementary to the imager strands from the previous round and upon hybridization the quencher strand prevents binding to the target and eliminates background fluorescence [32].

Effort has been placed in alternative multiplex approaches that can allow for the detection of multiple species in a single round of imaging. One such approach is kinetic fingerprinting, which is able to probe different species simultaneously. Multiplexing is achieved by varying both the **binding time** and **binding frequency** for different species (**Figure 2B**). While the former is tuned by the number of basepairs that are formed between docking and imager strand, the latter is modulated by the number of binding site repeats on a docking strand. The two orthogonal approaches can thus be varied combinatorically. The concept was demonstrated with 4-fold multiplexing, however to reach higher levels of multiplexing, different dyes had to be integrated. This allowed 124-fold multiplexing on DNA origami constructs [31] yet reaching this level of multiplexing requires up to 44 sequence

repeats, which might not be possible in more complex systems, where labeling efficiency is lower and the number of labeling sites is limited.

A third dimension of multiplexing exploits the spectral properties of dyes. Multiplexing by using different dyes is the most easily implemented approach (**Figure 2C, top panel**), but it is inherently limited by the number of distinguishable dyes. To minimize color cross-talk, the dyes are typically excited sequentially at different wavelengths. However, recently the number of required excitation lasers for spectral multiplexing was reduced by frequency modulation, allowing for the detection of five different dyes [33].

To circumvent this constraint and still multiplex spectrally, the **Förster Resonance Energy Transfer (FRET)** between a donor and an acceptor fluorophore has been used in correlative FRET multiplexing. By varying the position of the donor fluorophore on the imager strand, the separation between the dye pair will alter, and different FRET efficiencies (E) will be obtained (**Figure 2C, bottom panel**). However, while FRET efficiency is bounded between 0 and 1, so far only 3-FRET efficiencies could be distinguished given that their distributions cannot overlap to remain discernible [29,30].

These multiplexing approaches have enabled the detection of several targets of interest in a single imaging round, thereby increasing the imaging speed compared to conventional Exchange-PAINT. However, also for these approaches the overall low binding frequency is an intrinsic limitation of the imaging time. In the next section we will discuss the most recent advances in acquisition speed, bringing the acquisition time for super-resolution imaging with DNA-PAINT down from multiple hours to just several minutes.

Advances in the acquisition speed of DNA-PAINT

A long acquisition time, rooted in the requirement to collect many photons in order to pinpoint the center location of a fluorophore, is a fundamental limitation of all SMLM techniques [34–36]. Since distinct targets within a diffraction-limited region should not blink simultaneously in order to be super-resolvable, each individual docking position is unoccupied most of the time, increasing the acquisition time up to several hours [10,16,24,37]. The acquisition time of DNA-PAINT is affected by the number of required localizations, the number of docking positions within a diffraction-limited area and the binding frequency.

DNA-PAINT uses ~8 nucleotide long imager strands, which have an association rate of $\sim 2 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ under standard DNA-PAINT experimental conditions [10], but this parameter has a wide range depending on buffer composition, strand length and sequence. The freely diffusing imager strands contribute to background intensity, thus their concentration (c) is limited by the minimal required SNR. The imager strand concentration typically varies between 0.5 and 10 nM, depending on experimental conditions [15]. Recent advances have focused on accelerating image acquisition through increasing the binding frequency (f_b) of a target molecule either by enhancing the **permissive strand concentration** or by increasing the association rate of individual imager strands ($f_b = k_{on} \cdot \text{concentration}$).

Increasing the permissive imager strand concentration

The constraint on imager strand concentration originates from the fact that the fluorescent probes are non-**fluorogenic**, that is, both the hybridized and the freely diffusing probes emit photons, with the latter increasing background signals.

Acceleration methods for DNA-PAINT have focused on alleviating this concentration constraint by reducing the detected background intensity. To this end, approaches have been designed in which fluorescence from freely diffusing imager strands is not detected, either through various implementations of FRET or photoactivation [38–40].

In FRET-PAINT, donor labeled imager strands bind to an acceptor labeled docking strand, allowing for energy transfer between them (**Figure 3A**). By detecting only the acceptor fluorescence, while exciting the donor fluorophore, donor labeled imager strands do not contribute to background signal and their concentration can be increased to 1200 nM, consequently reducing the acquisition time to less than a minute [40].

In another scheme, fluorogenic DNA-PAINT adopts imager strands that contain a dye and a matching quencher linked to opposite ends of a single imager strand [41] (**Figure 3A**). In the unbound state the imager strands coils, placing the dye and quencher in close proximity and causing quenching of fluorescence signal. However, when bound to the docking strand, the imager strand is linearized and fluorescence emission is detectable. Using this design, the probes become practically fluorogenic and the associated increase in permissive imager strand concentration accelerate the image acquisition 26-fold.

Lastly, with photoactivatable DNA-PAINT, imager strands are chemically reduced and activation with UV illumination is required prior to photon emission [42], effectively integrating the concept of photo switchable fluorophores (**Figure 3A**), which underpins PALM and STORM with DNA-PAINT [3–5]. Through evanescent illuminations only the imager strands that are close to the surface, being those that are hybridized with a docking strand, are activated, alleviating background signal and allowing for higher imager strand concentration.

All speed optimization approaches discussed here increase the binding frequency, leaving room for improvement of other aspects governing the acquisition time. Notably, the

duration and number of localizations can be optimized by increasing fluorophore brightness [43], reducing fluorophore bleaching [44] and developing more advanced analysis algorithms [45].

Increasing the imager strand association rate

The imager strand association rate is largely dependent on the sequence of the imager strand, as freely diffusing ssDNA strands can coil up into secondary structures, which is one of the main causes of comparatively low association rates. A 5-fold speed increase has been accomplished by optimizing the imager strand sequence [46] (**Figure 3B**). First, the formation of secondary structures that decelerate binding was prevented by refraining from including complementary bases within a strand. In addition, the sequence was chosen such that the free energy of the hybridized duplex resulted in optimal binding times, which were as short as possible, to limit simultaneous binding in dense regions, but long enough to collect sufficient photons. Alternatively, by varying the ethylene carbonate concentration in the buffer, the probe **dissociation rate** and thereby the binding time can be tuned an order of magnitude [47]. Buffer composition can also affect the imager strand association rate through increased electrostatic screening, as variations in the magnesium concentration allow for a two-fold change [46]. The approach of protein-assisted DNA-PAINT [48] reduces the entropic barrier of hybridization through preforming of the imager strand. The concept was first demonstrated with the Argonaute (Ago) protein, which is a naturally occurring protein that uses an RNA or DNA guide strands to bind complementary RNA or DNA targets [49]. Ago-assisted DNA-PAINT (Ago-PAINT) can facilitate a 10-fold acceleration and has the major advantage of being sequence independent.

Optimization of the docking strand sequence also increases the imager strand association rate, which was shown recently when a docking strand with repeated DNA

binding site motifs produced a predicted 100-fold acceleration [36]. The increase in the number of binding sites on a single docking strand led to an equal linear increase in binding frequency and acquisition speed and the concept has been verified repeatedly [31,47,50]. Furthermore, by using periodic binding motifs with partial overlap, the required docking strand size was minimized. Although one might expect the spatially distinct binding motifs to blur signal and reduce resolution, this has been shown not to be the case [50].

A second method how docking sequence design can increase the imager strand association rate is incorporating a spacer between the target and the binding sequence [47]. Incorporating a polymer spacer has been shown to increase the association rate by 60%, which has been ascribed to a reduction in steric hindrance between the imager strand and the target molecule [47]. **Figure 3C** compares the relative acceleration of different techniques, and their compatibility with multiplexing approaches. Several approaches have been integrated to accelerate acquisition speed in a synergistic manner [36] and we anticipate that new combinations will further reduce acquisition time.

Next generation PAINT probes towards live-cell imaging

Despite tremendous advances in the field of DNA-PAINT, their sensitive and dynamic environment makes high-resolution imaging in living cells very challenging. In addition, the extended total acquisition time restricts the range of biological samples that can be measured [36]. Therefore, *state-of-the-art* DNA-PAINT concepts and labeling protocols (**Figure 4**) are typically validated on distinguishable cellular structures, such as microtubules, mitochondria and nuclear pore complexes in more controllable fixed environments instead [28,36,40,41,46,51–54]. Furthermore, nucleic acids are routinely visualized in fixed cells with Fluorescence In Situ Hybridization (FISH), but the long sequences required to attain a certain target specificity are hard to unite with the transient binding required for DNA-PAINT [55].

Recently, DNA-PAINT has been used to visualize, multiplex and quantify short RNA (sRNA) fragments with 10-nm resolution inside fixed cells [56]. To ensure sufficient specificity despite these short targets, the incorporation of Locked Nucleic Acid (LNA) bases was vital, as it increases stability, specificity and hybridization efficiency [57]. The static environment in fixed cells ensures that labeled targets are immobile over the course of an experiment. In addition, the membrane is permeabilized, which eases the removal of interfering proteins and oligonucleotides and allows the introduction of staining molecules. Live-cell imaging does not benefit from these simplifications, and even when nucleic acid imager strands are successfully introduced inside the cell via perfusion, they may be rapidly degraded. These challenges have until recently limited live cell imaging to surface proteins [51,58].

Additionally, an arbitrary DNA imager strand will have thousands of complementary binding sequences with cellular DNA and RNA, which results in an abundance of false-positives and elevated background levels, reducing resolution. These challenges are surmounted by the recent approach using left-handed DNA (L-DNA) for transient binding instead, which is non-natural and thus cannot hybridize with cellular nucleic acids [59].

In fixed cells, DNA docking strands could be successfully linked to antibodies that bind intracellular targets, or genetically fused tags [60]. Yet the use of an antibody resulted in a linkage error, the distance between position of the fluorophore and the actual target position [61], of at least 10 nm [62–65] (**Figure 4**). This error not only introduces a localization bias, but also reduces the maximum labeling density owing to steric hindrance and possibly impedes imaging of denser cellular structures that are impermeable for the probe [61].

Nanobodies (a single-domain antibody) do not suffer from these problems and have reduced the linkage error to 4 nm, whilst achieving a resolution of 20 nm on various organelles in fixed cells [66]. However, the number of nanobodies that has sufficient affinity with endogenous proteins is limited, making this approach challenging. Therefore, proteins of

interest have to be genetically tagged with epitopes that can be recognized by the available nanobodies [54,66].

The need for simpler labels, not requiring genetically encoded protein tags, has pushed the development of affimer labeling [67] and slow off-rate modified aptamers (SOMAmers) [51], both of which use small (<30 kDa) target-specific probes to which a DNA docking strand is attached. Affimers are small, naturally occurring proteins that have been screened for target protein affinity and that have a DNA docking strand fused to their cysteine amino acids [68]) (**Figure 4**). Their use has been validated on intracellular targets in live cells, yielding ~15 nm resolution [67]. In contrast, SOMAmers are DNA structures that contain a region with modified bases with hydrophobic residues to increase target affinity and specificity (**Figure 4**). SOMAmers have achieved an impressive resolution of ~8 nm [51], but so far SOMAmers have only been used in fixed cells. The limited ability of aptamers to bind intracellular targets might further confine applications [51]. Both SOMAmers and affimers are limited by unpredictable target binding affinity, necessitating laborious high-throughput screening and selection to find suitable probes.

A forthright approach to overcome some of the mentioned challenges inherent to DNA oligos is to replace them with proteins or peptides (**Figure 4**). In protein-PAINT, synthetic cell-permeable fluorophores are added extracellularly and upon cell entry, the fluorophores transiently bind genetically encoded protein tags that are fused to target proteins [69]. More recently, the heterodimeric E/K coiled-coil peptide pair has been used for transient and tunable binding *in vitro* with peptide-PAINT, where the docking peptide was conjugated to the target protein via secondary antibodies [70]. This peptide counterpart of DNA-PAINT has a roughly double association rate because the electrostatic interactions are less repulsive than for DNA, accelerating imaging acquisition. Furthermore, peptide-PAINT labels more efficiently and has a smaller linkage error, since the docking strand is genetically fused to the

protein of interest, thereby removing the need for antibodies or nanobodies. These advances set the stage for live-cell imaging with LIVE-PAINT, which relies on similar peptide-protein interactions [71]. Here, also the imager peptides were genetically encoded and endogenously expressed inside living yeast, circumventing extracellular introduction. Owing to the limited predictability and specificity of peptide interactions, these alternative backbones have not yet been widely adopted for PAINT imaging, but this may change in the near-future, as coiled-coil interactions are becoming increasingly programmable [72].

Challenges for live-cell imaging

Novel variations of DNA-PAINT have expanded the super-resolution imaging toolbox, enabling research in previously uncharted directions. Advances have enabled a resolution down to the molecular level [24] and spectrally unrestricted multiplexing [27–31]. While traditionally being considered the Achilles' heel of DNA-PAINT, the lengthy acquisition time has now been reduced by several orders of magnitude to the point where super-resolution images can be acquired within several minutes [36,38–42,46–48,50]. If the approaches for speed optimizations perform well inside cells, a crucial obstacle for live-cell imaging will be surmounted. The underlying SMLM super-resolution concept of DNA-PAINT is in principle compatible with living systems, as another SMLM approach, STORM, has been used to image living eukaryotic cells [7]. Other important advances have also been made for cell imaging for the past few years. Strategies that use peptides [70,71] or proteins [69] have successfully eliminated problems like probe introduction and degradation, and intracellular target labeling has been demonstrated with affimer- [67] and aptamer-based [51] approaches. Nevertheless, several outstanding challenges remain.

Two key obstacles for DNA based imaging inside living cells are the stability of the DNA and the potential non-specific interactions with cellular nucleic acids. The photo-induced depletion of DNA docking strands can be minimized by using a lower excitation

power [14], increasing the spacing between the fluorophore and the docking strand [14] or increasing the number of imager binding sites in a docking strand [50]. Furthermore, increased DNA stability against DNases may be achieved by protecting the imager strands with Ago-PAINT [48], but to permit live-cell imaging, a smaller, truncated version of the protein might be required [73]. Alternatively, oligos may be protected through chemical modifications of the DNA, for example through the usage of LNA in sRNA-PAINT [56,74]. The use of LNA also reduces non-specific interactions with other nucleic acids, while L-DNA eliminates any interaction with cellular nucleic acids [56,59].

In addition, the labeling of targets of interest in living cells continues to be one of the biggest challenges in the super-resolution community [31,52,53,56]. Currently, most approaches in fixed cells rely on the use of docking sequence labeled antibodies that bind specifically to a target protein. However, this may not be suitable for live-cell imaging due the challenge of introducing these sizeable antibodies inside the cell. Several chemistry-based approaches have been developed, which rely on the incorporation of unique functional groups via unnatural amino acids [75,76] or self-labeling protein tags, thereby reducing the size of the probe that has to be introduced into the cell (e.g., SNAP [77], HALO [78] and FGE [79] tags). Although most of these labeling methods require engineering of the target molecules and can thus not be directly applied to unperturbed cells or tissues, we envision these strategies to be important for early proof-of-concept experiments. We invite biophysicists and chemists to further develop protocols for efficient and specific labeling strategies to boost super-resolution for live cell imaging.

Another hurdle for live cell imaging is the variation in cellular content (e.g. protein concentration) among different cells in a single sample, which precludes uniform and up-front labeling of cellular targets. An outcome might be Action-PAINT [80], in which cellular targets are first probed and then labeled after visualization, allowing the labeling to be tuned

to the composition of each individual cell. In Action-PAINT, the imager strands in the first round are chemically modified, such that they can be rapidly crosslinked to a complementary docking strand upon UV-illumination [81]. Additionally, these imager strands contain a sequence that functions as a new binding site for a subsequent round of DNA-PAINT imaging with new imager strands. In this second round, only the user-selected cellular components that were labeled through crosslinking in the first round are imaged with DNA-PAINT. With Action-PAINT, cellular targets can thus be labeled with high-resolution after visualization on a per-cell basis.

Concluding Remarks

To conclude, the field of DNA-PAINT has seen tremendous advances in multiplexing, acquisition speed and resolution *in vitro*, however, it will be challenging to achieve the same performance in living cells (see **Outstanding Questions**). We envision that live-cell imaging with DNA-PAINT will first be demonstrated in its most primitive form. Once a capable methodology has been developed, we expect that more sophisticated concepts, such as multiplexing and quantitative analyses, can be implemented with relative ease. These concepts are subject to the same barriers as conventional DNA-PAINT and have already been demonstrated in fixed cells. As soon as live-cell imaging with DNA-PAINT becomes a routine experiment, elemental aspects like the dynamics of intracellular protein localization and protein interaction might be addressed and the cellular concentrations of proteins and nucleic acids may be quantified in real time with super-resolution, answering fundamental questions about the rate and regulation of translation and transcription.

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552

GLOSSARY

Association rate (on-rate, k_{on}): The number of times a particular imager strand binds to a docking strand per second per mol. A typical ~ 8 nucleotide DNA imager strand has a k_{on} of $\sim 2 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Scales with the affinity of an imager strand for its docking strand and the number of binding sites on a docking strand.

Binding frequency (f_b): The number of times a target molecule hybridizes with an imager strand per second. Inversely proportional to the unbound time. $f_b = \frac{1}{\tau_u} = k_{on} \cdot c$

Binding time (τ_b): The average duration for which an imager strand remains hybridized to a docking strand. Generally ~ 2 seconds $\tau_b = \frac{1}{k_{off}}$

Dissociation rate (off-rate, k_{off}): The rate at which an imager strand dissociates from the docking strand. Inversely proportional to the binding time.

DNA-based Point Accumulation in Nanoscale Topography (DNA-PAINT): Single-Molecule Localization Microscopy method that relies on transient binding of fluorescently labeled DNA imager strands to their complementary docking strands that are fused to a molecular target. Attainable resolution $< 5 \text{ nm}$.

Docking strand: DNA sequence that serves as the landing site for the imager strands and is attached to the point of interest in DNA-PAINT imaging. A single docking strand can have multiple imager strand binding sites.

Imager strand: Fluorescently labeled DNA sequence (~ 10 nucleotides) that is complementary to the docking sequence and transiently binds to it.

Fluorogenic probe: An imager strand that only emits fluorescence when hybridized with a docking strand and not while freely diffusing in solution.

Förster Resonance Energy Transfer (Efficiency) (FRET (E)): Event in which a donor fluorophore in the excited state transfers energy to an acceptor fluorophore via dipole-dipole coupling. Typical range is 1-10 nm. The efficiency equals the acceptor intensity divided over the sum of the intensity of both donor and acceptor.

Localization: Datapoint consisting of one or several frames that is used to determine the center position of fluorescence signal through Gaussian fitting.

581 **Localization Precision:** Metric to quantify the deviation in estimated position of multiple
582 subsequent localizations of a single fluorescent molecule. Scales with the square root of the
583 number of photons.

584 **Multiplexing:** The concept of probing various distinct targets in a single experiment, while
585 having the capacity to distinguish the signal from each.

586 **Permissive concentration (c):** Maximum concentration of fluorescently labeled imager
587 strands at which the Signal-to-Noise-Ratio is still sufficient to achieve super-resolution.
588 Typical value in conventional DNA-PAINT is 10 nM.

589 **Photoswitchable Fluorophore:** Fluorescent dye that can cycle between a dark state and a
590 bright state, while excitation and fluorescence emission are only possible in the latter state.
591 Photoswitching is typically induced by illumination with another wavelength than the
592 excitation wavelength.

593 **Single Molecule Localization Microscopy:** Classification of various super-resolution
594 methods, such as PALM, STORM and PAINT. Super-resolution is achieved by
595 spatiotemporal separation of fluorescence emission of single fluorophores, which allows
596 Gaussian fitting to each single-molecule, drastically reducing the uncertainty in fluorophore
597 position and thereby attaining a higher resolution.

598 **Unbound time (τ_u):** The average time in between subsequent binding events on a single
599 target molecule.

Box 1: Principles of super resolution microscopy

The RESOLFT class, representing the deterministic avenue, relies on controlled and selective illumination of many fluorophores within a region. STED is a prominent example of this approach and uses a depletion laser to selectively suppress excited fluorophores on the edge of a region of interest. Upon illumination with the excitation laser, signal is collected only from the non-depleted fluorophores in the center of the region [2]. This reduces the effective point spread function of the laser below the diffraction limit to achieve super-resolution. While STED has the benefit of being compatible with conventional fluorophores, complicated illumination setups are required [2].

SMLM uses widefield illumination and relies on the stochastic cycling between bright and dark states of fluorophores. By ensuring that a sufficiently small fraction of the molecules is in the bright state at each moment, emission is collected from a single molecule within each diffraction-limited area. This subsequently allows high-precision fitting, thereby achieving a highly accurate **localization** for each single molecule. The cycling of fluorophores can be achieved by using **photoswitchable fluorophores**, a principle that underlies PALM [3,4] and STORM [5]. For these techniques, blinking behavior of photoswitchable fluorophores is controlled through a low-level or pulsed activation beam [3–5]. The **localization precision** of a single molecule increases with the number of detected photons ($\sigma = \frac{1}{\sqrt{N}}$) [82], while the number of blinking cycles a single fluorophore can undergo is limited.

FIGURE CAPTIONS

Figure 1 Concept of DNA-PAINT.

(A) Timeline indicating three main phases of the DNA-PAINT field: The development and improvement of the technique, recent advances that optimize and functionalize DNA-PAINT and future progress for novel applications and live-cell imaging.

(B) Transient binding of short dye labeled DNA oligos (imager strands) to the complementary target sequence (docking strands) causes an increase in fluorescent signal (ON) and is detected as a localization event.

(C) Computational simulation comparing diffraction limited imaging (left) and DNA-PAINT super-resolved imaging (right), of DNA origami nanostructures. The DNA origami was designed to have 12 docking sites that are arranged in a 20 nm grid pattern (see inlet in DNA-PAINT image). The simulation was performed with Picasso Software [16]. Scale bars are 100 nm.

Figure 2 Multiplexing with DNA-PAINT.

(A) In sequence-based multiplexing, different targets within a sample can be imaged sequentially. Each point of interest (POI) in a target sample is labeled with a unique docking sequence (1, 2, ..., N) and in a first round the imager strand for POI 1 will be introduced. After obtaining sufficient localizations, the imager strand for POI 1 will be washed away and the next imager strand can be introduced. This cycle can be repeated for N number of cycles, and pseudocolors are assigned to each imaging round.

(B) Kinetic multiplexing can achieve its discernibility through varying the length of the hybridized duplex and the subsequent difference in the dissociation rate and binding time of the imager strands (top). Alternatively, by having a distinct number of binding sites in a docking strand, the difference in binding frequency (bottom) adds another layer of multiplexing.

(C) Spectral multiplexing requires either orthogonal imager strands that are each labeled with a unique fluorophore to probe various targets in parallel (top) or a varying distance between a donor and acceptor FRET pair that results in a different FRET efficiency (bottom).

Figure 3 Approaches to increase the binding frequency of DNA-PAINT.

(A) Conventional DNA-PAINT suffers from a comparably high fluorescence background signal from the imager strands in solution, which limits their maximum concentration. Approaches that reduce background signal can thus increase the permissive concentration of imager strands, accompanying an equal acceleration in binding frequency and acquisition time. FRET-PAINT (left) blocks donor emission from the imager strands in solution and only detects acceptor emission. Fluorogenic DNA-PAINT (middle) has a quencher fused to the imager strand to quench fluorescence signal in solution, while upon binding, the imager strand linearizes and fluorescence signal can be detected. Photoactivatable DNA-PAINT (right) uses photoswitchable fluorophores that are in the dark-state while in solution to become activated only upon the UV-illumination (purple) near the surface.

(B) The association rate at which a particular imager strand binds a target molecule can be increased by electrostatic screening, for example by increasing the magnesium concentration of the buffer composition (left) or by increasing the number of bindings sites in a docking strand (middle). The rate also increases as secondary structures in the imager strand are removed through sequence design in which complementary bases are avoided to prevent self-interactions (top right) or with Ago-PAINT, which reduces the entropic barrier of hybridization through imager strand preforming (bottom right).

(C) Schematic table comparing the various acceleration methods on working principle, acceleration performance and compatibility with multiplexing approaches. Acceleration is defined as relative to conventional DNA-PAINT [10], with 1 dot = 1-4x, 2 dots = 5-9x, 3 dots = 10-19x, 4 dots = 20-100x, 5 dots = >100x. *These approaches have been integrated for up to 100x acceleration [36]. For an acceleration method to be compatible with a certain multiplexing approach, both the acceleration and the level of multiplexing must be as high as when used separately.

Figure 4 Probe design to label cellular target molecules for (DNA-)PAINT imaging.

Super-resolution imaging of cellular target structures requires labeling with DNA docking strands. DNA docking strands are attached to antibodies/nanobodies, affimers or SOMAmers and are introduced into fixed cells to allow immunostaining. Alternatively, a protein or peptide backbone, rather than a DNA backbone, may be used to create the blinking events for

684 PAINT imaging. Short peptide docking sequences are conjugated to an antibody in Peptide-
685 PAINT and introduced into the cell or intracellularly expressed with LIVE-PAINT.