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van Wee, Raman; Filius, Mike; Joo, Chirlmin

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

Raman G. van Wee*, Mike Filius*, and Chirlmin Joo[#]

Department of BioNanoScience, Kavli Institute of Nanoscience, Delft University of Technology, van der Maasweg 9, 2629HZ Delft, The Netherlands.

* These authors contributed equally

Correspondence should be addressed to c.joo@tudelft.nl

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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.

25 **Introduction**

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

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

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

50 aspects of the technique while exploring the plethora of potential applications (**Figure 1A**).
51 DNA-PAINT has been implemented to measure piconewton forces in living cells, allowing
52 the simultaneous quantification of mechanical force and visualization of cellular structures
53 and thereby bridging the gap between structural biology and mechanobiology [11]. It is also
54 increasingly being used in the medical realm, as both a more accurate and versatile tool to
55 monitor biomarkers for disease diagnosis [12] and to study patient histology at the highest
56 detail [13]. Preceding these applications is a wide range of ongoing developments, greatly
57 expanding the versatility, applicability and ease of use of DNA-PAINT. While novel
58 multiplexing strategies and advances in acquisition speed are addressing a crucial limitation
59 of lengthy acquisition times, progress in labeling probes and alternative PAINT methods also
60 pave the way for live-cell imaging. Here, we discuss these advances and contemplate
61 remaining challenges before the DNA-PAINT canvas gets completed and live-cell imaging
62 can be realized.
63

64 **Single-molecule localization microscopy with DNA-PAINT**

65 DNA-PAINT uses base-pairing between short fluorescently labeled DNA
66 oligonucleotides [10]. A target is labeled with a short DNA **docking strand**, while the
67 complementary, fluorescently labeled **imager strands** diffuse freely in solution. Upon
68 hybridization, an increase in fluorescence intensity is observed (ON) for several hundreds of
69 milliseconds after which the imager strand unbinds (OFF) and leaves the docking strand
70 unoccupied (**Figure 1B**). As imager strands bind and unbind, the pool of imaged fluorophores
71 is continuously replenished, eliminating concerns over the photon-budget in DNA-PAINT. In
72 recent work photo-induced depletion of docking strands has been observed [14], which
73 implies that the binding and unbinding cycle of imager strands is finite. Furthermore, DNA-
74 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

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

102 In sequence multiplexing, orthogonal sequences are used to label distinct cellular
103 targets and the level of multiplexing is only limited by the number of orthogonal sequences
104 that can be designed. Sequence multiplexing is the working principle of Exchange-PAINT
105 [28]. These approaches achieve high resolution single-color multiplexing by using orthogonal
106 DNA sequences to label and image different structures. However, because only a single type
107 of fluorophore is used and pseudocolors are assigned to each orthogonal DNA sequence,
108 imaging has to take place in sequential imaging cycles (**Figure 2A**). The acquisition time thus
109 scales with the number of structures, making the imaging of a large number of structures in a
110 single sample a long process. To reduce the time between sequential imaging cycles, the
111 washing step can be eliminated by adding "quencher" strands prior to each new imaging
112 round. These quencher strands are complementary to the imager strands from the previous
113 round and upon hybridization the quencher strand prevents binding to the target and
114 eliminates background fluorescence [32].

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

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

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

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

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

146

147

148 **<u>Advances in the acquisition speed of DNA-PAINT</u>**

149 A long acquisition time, rooted in the requirement to collect many photons in order to
150 pinpoint the center location of a fluorophore, is a fundamental limitation of all SMLM
151 techniques [34–36]. Since distinct targets within a diffraction-limited region should not blink
152 simultaneously in order to be super-resolvable, each individual docking position is
153 unoccupied most of the time, increasing the acquisition time up to several hours
154 [10,16,24,37]. The acquisition time of DNA-PAINT is affected by the number of required
155 localizations, the number of docking positions within a diffraction-limited area and the
156 binding frequency.

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

166

167 ***Increasing the permissive imager strand concentration***

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

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

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

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

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

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

196 duration and number of localizations can be optimized by increasing fluorophore brightness
197 [43], reducing fluorophore bleaching [44] and developing more advanced analysis algorithms
198 [45].

199

200 ***Increasing the imager strand association rate***

201 The imager strand association rate is largely dependent on the sequence of the imager strand,
202 as freely diffusing ssDNA strands can coil up into secondary structures, which is one of the
203 main causes of comparatively low association rates. A 5-fold speed increase has been
204 accomplished by optimizing the imager strand sequence [46] (**Figure 3B**). First, the formation
205 of secondary structures that decelerate binding was prevented by refraining from including
206 complementary bases within a strand. In addition, the sequence was chosen such that the free
207 energy of the hybridized duplex resulted in optimal binding times, which were as short as
208 possible, to limit simultaneous binding in dense regions, but long enough to collect sufficient
209 photons. Alternatively, by varying the ethylene carbonate concentration in the buffer, the
210 probe **dissociation rate** and thereby the binding time can be by tuned an order of magnitude
211 [47]. Buffer composition can also affect the imager strand association rate through increased
212 electrostatic screening, as variations in the magnesium concentration allow for a two-fold
213 change [46]. The approach of protein-assisted DNA-PAINT [48] reduces the entropic barrier
214 of hybridization through preforming of the imager strand. The concept was first demonstrated
215 with the Argonaute (Ago) protein, which is a naturally occurring protein that uses an RNA or
216 DNA guide strands to bind complementary RNA or DNA targets [49]. Ago-assisted DNA-
217 PAINT (Ago-PAINT) can facilitate a 10-fold acceleration and has the major advantage of
218 being sequence independent.

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

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

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

235

236 **Next generation PAINT probes towards live-cell imaging**

237 Despite tremendous advances in the field of DNA-PAINT, their sensitive and dynamic
238 environment makes high-resolution imaging in living cells very challenging. In addition, the
239 extended total acquisition time restricts the range of biological samples that can be measured
240 [36]. Therefore, *state-of-the-art* DNA-PAINT concepts and labeling protocols (**Figure 4**) are
241 typically validated on distinguishable cellular structures, such as microtubules, mitochondria
242 and nuclear pore complexes in more controllable fixed environments instead
243 [28,36,40,41,46,51–54]. Furthermore, nucleic acids are routinely visualized in fixed cells with
244 Fluorescence In Situ Hybridization (FISH), but the long sequences required to attain a certain
245 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

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

273 The need for simpler labels, not requiring genetically encoded protein tags, has pushed
274 the development of affimer labeling [67] and slow off-rate modified aptamers (SOMAmers)
275 [51], both of which use small (<30 kDa) target-specific probes to which a DNA docking
276 strand is attached. Affimers are small, naturally occurring proteins that have been screened for
277 target protein affinity and that have a DNA docking strand fused to their cysteine amino acids
278 [68]) (**Figure 4**). Their use has been validated on intracellular targets in live cells, yielding
279 ~15 nm resolution [67]. In contrast, SOMAmers are DNA structures that contain a region
280 with modified bases with hydrophobic residues to increase target affinity and specificity
281 (**Figure 4**). SOMAmers have achieved an impressive resolution of ~8 nm [51], but so far
282 SOMAmers have only been used in fixed cells. The limited ability of aptamers to bind
283 intracellular targets might further confine applications [51]. Both SOMAmers and affimers
284 are limited by unpredictable target binding affinity, necessitating laborious high-throughput
285 screening and selection to find suitable probes.

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

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

303 **Challenges for live-cell imaging**

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

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

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

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

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

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

354 **Concluding Remarks**

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

367

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373

374

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552

553 **GLOSSARY**

554 **Association rate (on-rate, k_{on})** The number of times a particular imager strand binds to a
555 docking strand per second per mol. A typical ~8 nucleotide DNA imager strand has a k_{on} of ~
556 $2 \cdot 10^6$ M^{-1} s^{-1} . Scales with the affinity of an imager strand for its docking strand and the
557 number of binding sites on a docking strand.

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

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

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

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

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

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

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

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

579 **Localization:** Datapoint consisting of one or several frames that is used to determine the
580 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.