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Site-specific labeling of Affimers for DNA-PAINT microscopy

Thomas Schlichthaerle, Alexandra S. Eklund, Florian Schueder, Maximilian T. Strauss, Christian Tiede, Alistair Curd, Jonas Ries, Michelle Peckham, Darren C. Tomlinson, and Ralf Jungmann*

Abstract: Optical super-resolution techniques allow fluorescence imaging below the classical diffraction limit of light. From a technology standpoint of view, recent methods are approaching molecular-scale spatial resolution. However, this remarkable achievement is not easily translated to imaging of cellular components, as current labeling approaches are limited by either large label sizes (antibodies) or the sparse availability of small and efficient binders (nanobodies, aptamers, genetically-encoded tags). Here, we combine recently developed Affimer reagents with site-specific DNA modification for high-efficiency labeling and imaging using DNA-PAINT. We assay our approach using an actin Affimer. The small DNA-conjugated affinity binders could provide a solution for efficient multi-target super-resolution imaging in the future.

Super-resolution (SR) microscopy techniques[1] are evolving to become standard characterization tools in the life sciences. Due to the use of target-specific fluorescent labels, super-resolution – in contrast to electron microscopy (EM) – enables high-contrast imaging in complex 3D cellular architectures[2], and thus combines the advantages of increased spatial resolution with molecular affinity probes. While the use of labels with high target specificity such as antibodies is one of the biggest advantages of fluorescence microscopy over e.g. EM, it is at the same time also one of the most severe limitations for SR approaches to date. As current SR methods reach localization precisions on the order of only a few nanometers[3], this achievement does not translate to achievable image resolution in biological specimen, due to the relatively large size of commonly used labeling probes. In the case of primary and secondary antibodies, the size of the probes easily adds an additional linkage error of ~10-15 nm to the actual position of the molecule of interest[1]. This results in the fact that high-performance super-resolution techniques actually report the location of the probe rather than the one of the biomolecule under investigation.

The ideal labeling probe would need to be small and target-specific, thus reducing the linkage error and allowing for high labeling densities due to minimal sterical hindrance. Furthermore, quantitative imaging approaches[4] demand stoichiometric labeling, i.e. ideally one reporter molecule should be bound to exactly one copy of the target molecule.

The issue of efficient labeling using small probes has recently been taken on by the development of novel affinity reagents including nanobodies[5], aptamers[6], as well as genetically-encoded self-labeling tags such as SNAP or HALO[7]. Besides labeling probe size, it is equally important to be able to engineer these binders against a large library of target molecules in a rapid and straightforward fashion. This has traditionally been difficult for e.g. nanobodies, as the selection process of novel binders usually relies on immunization of animals[8], which makes it both time-consuming and expensive. While aptamers are promising candidates for the rapid development of small and efficient labeling probes, only a few candidates have been shown to work for extracellular target molecules. Self-labeling tags such as SNAP (19.4 kDa) or HALO (33 kDa) allow for efficient target labeling, however require genetic engineering of cell lines e.g. via CRISPR/Cas[9], Zinc fingers[10] or TALEN[11]. The same argument holds true for the large amount of GFP-tagged cell lines which can be targeted using GFP nanobodies[5a, 12]. In addition, the GFP fusion (25 kDa) adds an additional linkage error to the one from the nanobody (15 kDa).

Affimers[13] are a recently developed class of labeling probes (~10–12 kDa, ~2 nm) that are isolated from large phage display libraries (~10^{10}). The ability to quickly isolate Affimers[14] with high specificity and affinity that can be used in a range of applications[15] highlights their potential as alternatives to traditional antibodies. In SR microscopy, Affimer reagents have been used to image tubulin and a receptor tyrosine kinase by site-specific labeling with a fluorophore[13b]. Interestingly, the anti-tubulin Affimer labels interphase microtubules in a similar way to a widely used antibody, but also labels the central region of the cytokinetic furrow, from which antibodies are usually excluded. This highlights a further advantage of using smaller probes, as their small size allows them to penetrate dense structures that exclude antibodies.

Here, we combine site-specific DNA labeling of Affimers with the recently developed DNA-PAINT super-resolution technique[16]. In DNA-PAINT, the apparent “switching” between bright and dark states of dye molecules used for super-resolution reconstruction is achieved by the transient interaction of a dye-labeled imager strand with its complementary docking strand linked to a target molecule of interest. In order to perform DNA-PAINT on proteins in a cellular context, the docking strand needs to be attached to an affinity reagent (e.g. antibodies). To achieve quantitative, site-specific one-to-one labeling of Affimers with DNA-PAINT docking strands, we make use of the c-terminal cysteine modification of the Affimer to site-specifically attach a single DNA strand using cysteine-maleimide chemistry[17]. We assay the achievable imaging resolution of an actin Affimer in

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comparison to phalloidin, a small molecule commonly used for actin labeling and super-resolution imaging, and achieve comparable results[18].

To site-specifically modify Affimers, we first labeled a reduced c-terminal cysteine residue with Maleimide-DBCO which was then linked to Azide-functionalized DNA using a strain-promoted Azide-Alkyne cycloaddition (SPAAC) reaction (Figure 1, see also Material and Methods for details). Subsequently, the Affimer was purified via a HisTrap column to remove the free unreacted DNA (Supplementary Figure 1). The successful conjugation was evaluated using SDS-PAGE gel analysis. In comparison to the unconjugated Affimer, the molecular weight increased by ~5 kDa, suggesting a stoichiometric 1:1 labeling of DNA to Affimers (Supplementary Figure 2).

The DNA-PAINT docking strand was additionally labeled with a fixed Atto488 dye (Figure 1), which allowed us to rapidly identify a specific cellular phenotype using diffraction-limited widefield microscopy before subsequent DNA-PAINT image acquisition was performed. We then labeled actin filaments in fixed Cos7 cells with our purified DNA-Affimer conjugate and performed 3D DNA-PAINT microscopy (Figure 2a). Comparison of the diffraction-limited image acquired using the fixed Atto488 dye shows a clear improvement in resolution and highlights the high labeling specificity and efficiency of our DNA-conjugated actin Affimer (Figure 2b and c, see also Supplementary Figure 3 for comparison with Phalloidin labeling). To quantify the achievable resolution, we overlaid thirteen single actin fibers by the center of each filament and measured a line width of ~18 nm (FWHM). In comparison, we also performed the same analysis on a Phalloidin dataset and measured a line width of ~13 nm. Both values are in good agreement with earlier studies[18-19] (Supplementary Figure 4), taking the different binder size of Phalloidin vs. Affimers into account.

Additionally, we performed a copper-catalyzed cycloaddition reaction (CuAAC) as alternative labeling strategy (Supplementary Figure 5), achieving similar labeling and imaging performance (Supplementary Figure 6). While labeling efficiency and image quality is similar, we recommend the use of SPAAC, as it is less time consuming and involves fewer reagents. In addition, we also performed direct Stochastic Optical Reconstruction Microscopy (dSTORM) imaging using an Alexa647-labeled Affimer (Supplementary Figure 7) to further demonstrate the wide applicability of Affimers as labeling reagents for super-resolution methods.

Next, we assayed the achievable 3D resolution of our DNA-conjugated Affimers and DNA-PAINT by evaluating the actin network in Cos7 cells in three dimensions (Figure 3a–b). We were able to confirm two distinct layers of actin in 3D (Figure 3d) with an apparent thickness of ~40 nm and a distance of ~130 nm (Figure 3e), as previously reported using dual-objective STORM imaging[18] (See Supplementary Figure 8 for additional 3D data). The combination of the efficient labeling of the actin Affimers with the high-resolution capability of DNA-PAINT allowed us to achieve similar imaging performance (localization precision ~5 nm) using a standard inverted fluorescence microscope without the need for dual objective detection. In the DNA-PAINT case, the higher resolution in 2D as well as 3D is due to the increased photon budget available from the repetitive and transient binding of probes, rather than improved instrumentation.

In conclusion, site-specific conjugation of single DNA strands to Affimer reagents provides an attractive route for DNA-based super-resolution techniques due to their small label size and quantitative labeling capability. We envision applications towards absolute quantitative microscopy approaches such as qPAINT and multiplexed target detection using orthogonal Affimer binders coupled to distinct DNA sequences for e.g. Exchange-PAINT microscopy. In general, cysteine-based DNA labeling of affinity reagents could be an efficient way for obtaining quantitative DNA-PAINT reagents and it should be possible to apply it also for other probes such as nanobodies[5a], FAB fragments[20] or Darpins[21].

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Keywords: Small Binders • Super-resolution Microscopy • DNA-PAINT • Quantitative Microscopy

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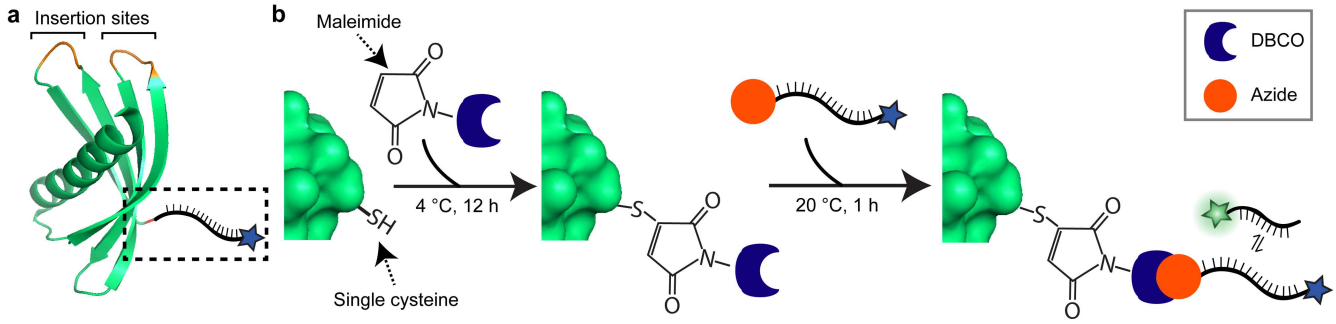

Figure 1. (a) Site-specific DNA labeling of Affimer reagents. Insertion sites for binder evolution against target proteins are highlighted in orange. Site-specifically attached DNA strand (dotted box) contains an additional Atto488 fluorophore for fast diffraction-limited imaging. (b) Labeling of single cysteines using Maleimide-DBCO (left). Subsequently, Azide-labeled DNA is added and binds covalently to the DBCO (center). Finally, DNA-labeled Affimers can be used for DNA-PAINT imaging (right). Affimer cartoons modified from PDB entry 4N6T.

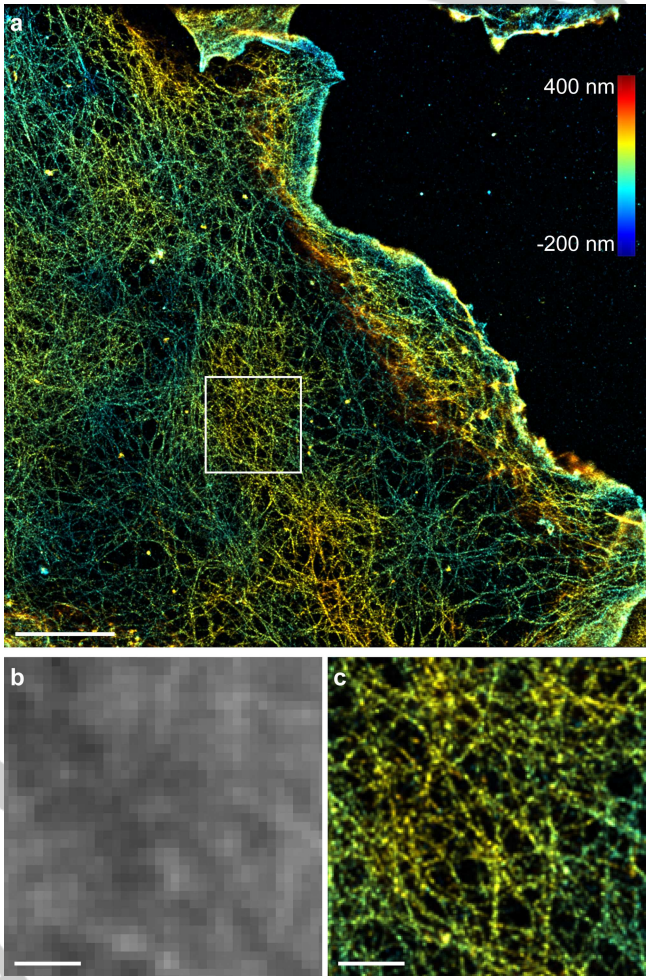

Figure 2. (a) DNA-PAINT imaging of Affimer-labeled actin network in fixed Cos7 cells (color indicates height). (b) Diffraction-limited zoom-in of the highlighted area in a). (c) Super-resolved zoom-in of the highlighted area in a) shows increased spatial resolution and dense actin labeling. Scale bars: 5 µm (a), 1 µm (b, c).

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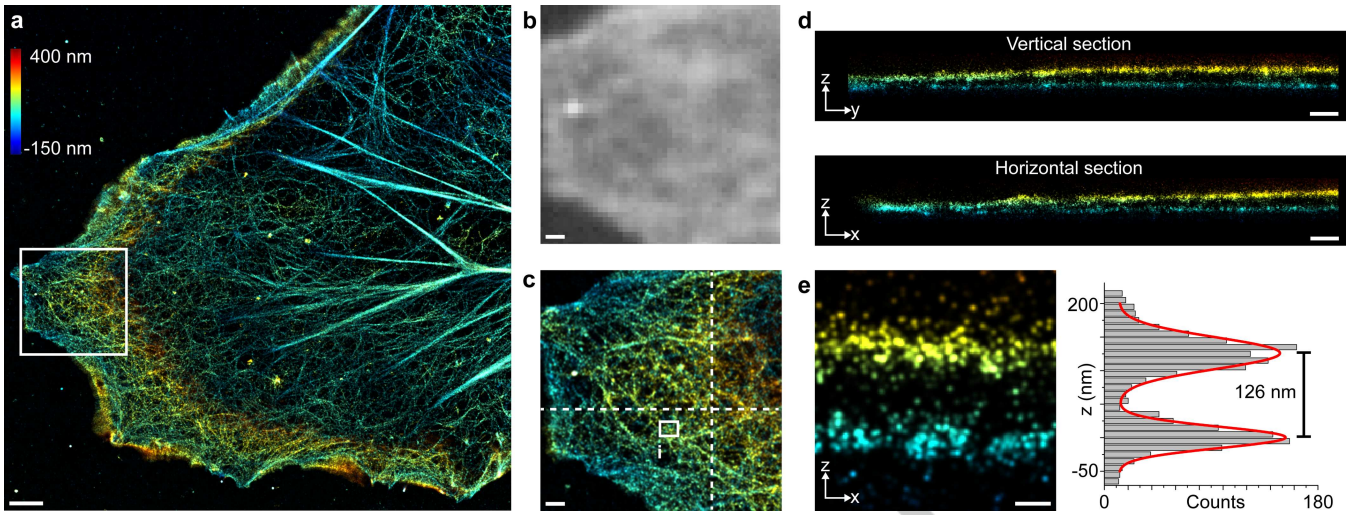

Figure 3. Actin Affimer imaging of Cos7 cell in 3D reveals the dual layer organization of actin. (a) Overview DNA-PAINT image of actin filaments in Cos7 cell (color indicates height). (b) Diffraction-limited zoom-in of the highlighted area a). (c) Zoom-in of super-resolved image of highlighted area in a). (d) Vertical and horizontal z-sections indicated as dashed lines in c). (e) Z-section of highlighted area (i) in c) indicating the two-layer organization of the actin filaments. Cross-sectional histogram analysis shows a layer-to-layer distance of 126 nm in a single cell. Scale bars: 2 µm (a), 500 nm (b, c), 300 nm (d), 50 nm (e).

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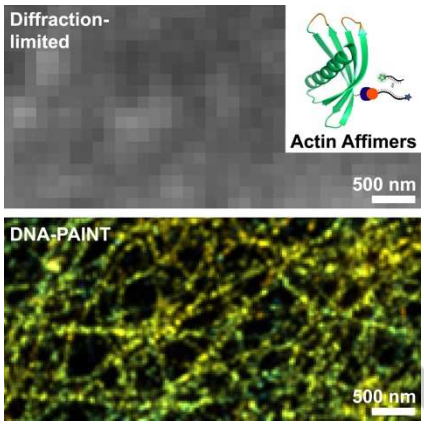
Site-specific labelling of Affimer reagents enables high-performance DNA-PAINT microscopy



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