Rapid and efficient C-terminal labeling of nanobodies for DNA-PAINT

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Abstract

Single molecule localization-based approaches to super-resolution microscopy (SMLM) create images that resolve features smaller than the diffraction limit of light by rendering them from the sequentially measured positions of thousands of individual molecules. New SMLM approaches based on the transient binding of very bright dyes via DNA–DNA interaction (DNA-PAINT) allow the resolution of dyes only a few nanometers apart in vitro. This imaging of cellular structures requires the specific association of dyes to their targets, which results in an additional ‘linkage error’. This error can be minimized by using extremely small, single-domain antibody-based binders such as nanobodies, but the DNA-oligomers used in DNA-PAINT are of significant size in comparison to nanobodies and may interfere with binding. We have developed an optimized procedure based on enzymatic labeling and click-chemistry for the coupling of DNA oligomers to the nanobody C-terminus, which is located on the opposite side of the epitope-binding domain. Our approach allows for straightforward labeling, purification and DNA-PAINT imaging. We performed high efficiency labeling of two different nanobodies and show dual color multiplexed SMLM to demonstrate the general applicability of our labeling scheme.

Keywords: nanobodies, DNA-PAINT, copper free click chemistry, super-resolution microscopy

Supplementary material for this article is available online
(Some figures may appear in colour only in the online journal)
due to the flexibility of such a fluorescent ‘chain’). Clearly, the smaller the detected label, the better. Recently, small binders called nanobodies have been used to deliver dyes to within a few nm of the target structure [8] and indeed this improves resolution significantly in comparison to traditional immuno-labelling [8]. Nanobodies are generalized single-domain binding modules derived from the heavy-chain only immunoglobulins produced by camels [9]. Their combination of an enormous potential binding repertoire, comparable and complementary to that of IgGs, in a robust domain obtainable via low cost and straightforward production has made them powerful tools for basic research and biotechnological applications [10–14]. Because these advantages come in a very small package (~15 kDa), nanobodies are quickly becoming indispensable for super-resolution microscopic studies. The small size and compact structure of nanobodies (~2–3 nm diameter, compared to ~10 nm for an IgG) makes them very attractive as labels in super-resolution experiments—particularly if fluorophores can be site-specifically attached via minimal length linkers. Their compact form also has a positive impact on the potential labeling density that can be achieved with them, particularly for continuous filamentous structures (e.g. microtubules), minimizing the steric interference inherent to the use of much larger probes [15].

A recent improvement to both the ease and flexibility of labeling and detection techniques is provided by the ‘DNA points accumulation for imaging in nanoscale topography’ method (DNA-PAINT) (figure 1), in which the target species, or a specific target detection module, is modified with a short DNA oligonucleotide (the ‘docking’ strand) [16, 17]. The position of the labeled species is then detected via addition of a complementary oligonucleotide harbouring a fluorophore (the ‘imager’ strand). The low melting temperature (~25 °C) of the short, corresponding duplex ensures a high imager strand koff [17, 18] and since the fluorophore can only be localized during the brief period the duplex exists, fluorophores are rapidly exchanged, thus constantly allowing new fluorophores to illuminate the target, thereby providing the necessary ‘blinking’ for SMLM (figure 1). This technique allows the use of very bright, non-blinking dyes and antifade reagents, leading to significantly brighter localizations and thus higher resolution. DNA-PAINT using nanobodies has been reported for the anti-GFP nanobody, but the labeling was based on poorly selective succinimidyl ester chemistry combined with click-chemistry [19]. As shown in figure 1(f), the DNA-oligomer required for DNA-PAINT is of significant size in comparison with the nanobody and thus coupling directly to the C-terminus as the site furthest away from the epitope-binding domain would be highly desirable. Here we report C-terminal labeling of anti-tubulin and anti-GFP nanobodies for DNA-PAINT. We have developed a streamlined procedure for the site-specific labeling of nanobodies based on the Sortase A (StaA) reaction [20] and the copper-free strain promoted alkyn–azide cycloaddition (SPAAC) [21] that improves both the ease of handling and the orthogonality of previously reported labeling schemes. The optimized procedure enables a great flexibility of label choice, allowing for multiplexed DNA-PAINT by coupling different oligo binder sequences to different nanobodies. Given their broad applicability we expect these contributions will substantially augment the current super-resolution toolbox.

**Methods**

**Expression and purification of nanobodies**

Expression constructs coding for the anti-tubulin nanobody [22] and anti-GFP nanobody [3, 23] were previously described. The anti-tubulin nanobody construct was synthesized by GeneArt Gene Synthesis (Thermo Fisher Scientific) and the anti-GFP nanobody construct was obtained by a polymerase chain reaction (PCR) of pGEX-6P1-containing anti-GFP-nanobody-4K [23]. PCR-amplified constructs were digested with Ncol and BstEII restriction enzymes and ligated in frame in similarly cut pHEN29 plasmid [15]. The pHEN29 plasmid comprises an N-terminal pelB signal sequence, directing the nanobody to the periplasm, followed by a C-terminal SrtA recognition sequence (LPETGG) upstream of a His6 tag and an EPEA tag. Resulting constructs pHEN29-anti-GFP-LPETGG-His6-EPEA and pHEN29-anti-tubulin-LPETGG-His6-EPEA were confirmed by sequencing and transformed into Escherichia coli WK6 for expression. Bacteria were grown in 1 l of Terrific Broth medium (Carl Roth) supplemented with 1 mM MgCl2, 0.1% glucose, 0.4% glycerol and 100 µg ml⁻¹ ampicillin at 37 °C, to an OD600 of 0.6–0.9 in baffled shaking flasks. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by incubation for 16–18 h at 28 °C. Cells were harvested by centrifugation (10 min, 11 800 g, 4 °C) and the resulting pellets were resuspended in 12 ml ice-cold TES buffer (0.2 M Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose) containing one protease inhibitor cocktail tablet (Roche) per 10 ml of TES. After shaking for 1 h at 4 °C, and 200 rpm, 18 ml ice-cold TES/4 (0.05 M Tris (pH 8.0), 0.125 mM EDTA, 0.125 M sucrose) was added and the cells were further incubated for 1 h at 4 °C. After centrifugation (11 800 g, 30 min), the supernatant was collected, and the TES extraction repeated. The resulting periplasmic extracts were pooled and diluted 1:1 in wash buffer (20 mM HEPES (pH 7.5), 300 mM NaCl, 5 mM imidazole 10% glycerol) and applied to a 5 ml HisPur cobalt column (Thermo Fisher Scientific), pre-equilibrated in wash buffer at a flow rate of 3 ml per minute using a peristaltic pump. The resin was washed with ten bed volumes of wash buffer and His-tagged nanobodies were eluted with 20 mM HEPES (pH 7.5), 300 mM NaCl, 500 mM imidazole and 10% glycerol. Protein concentrations in eluted fractions were determined by absorbance at 280 nm using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific) using the calculated extinction coefficients. The nanobody was further purified and buffer exchanged by size-exclusion chromatography over a Superdex 75 10/300 GL-column (GE Healthcare) in 20 mM HEPES (pH 7.5), 300 mM NaCl and 10% glycerol on
Expression and purification of SrtA

Expression of the SrtA pentamutant (eSrtA, hereafter SrtA) in pET29 (a kind gift from David Liu (Addgene plasmid #75144)) was performed essentially as described with minor changes [24].

Briefly, E. coli BL21 DE3 were transformed with pET29-SrtA pentamutant-His6, and grown in 1 l of LB medium, supplemented with 50 μg ml⁻¹ kanamycin at 37 °C to an OD₆₀₀ of ~0.5. Expression was induced with 0.5 mM IPTG and the culture incubated at 30 °C for 16 h. Cells were harvested by centrifugation (6000 x g, 4 °C, 15 min) and resuspended in 50 ml cold binding buffer (20 mM HEPES pH 7.5, 150 mM NaCl) and centrifuged again. Bacteria were lysed by resuspension in 25 ml ice cold lysis buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 5 mM imidazole, 10% glycerol, 1 mg ml⁻¹ DNase I, 1 mg ml⁻¹ lysozyme) and sonication on ice. The lysate was cleared by centrifugation (30 min, 16000 x g, 4 °C) and purified via HisPur cobalt resin and size-exclusion chromatography as for nanobody purification. SrtA containing fractions were pooled and concentrated with Amicon concentrators (3000 MWCO) to 25 mg ml⁻¹ in 20 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol. The protein was analyzed by reducing SDS PAGE, snap-frozen in 100 μl aliquots in liquid nitrogen and stored at −80 °C until further use.

SrtA-mediated coupling of clickable moieties (DBCO-amine)

SrtA-mediated functionalization of sortagged nanobodies with dibenzocyclooctyne-NH₂ (DBCO-amine) (Sigma-Aldrich # 761540) was carried out by reacting 50 μM nanobody, 150 μM SrtA and 10 mM DBCO-amine (from a 50 mM dimethyl sulfoxide (DMSO) Stock) in Sortase buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM CaCl₂) for 1 h at 24 °C. During the reaction, the GG-His₆-EPEA peptide was cleaved from the nanobody to form a nanobody:SrtA complex.
covalent intermediate linked at the threonine in the LPET-sequence. Subsequent addition of DBCO-amine resolved the intermediate, substituting DBCO for SrtA at the LPET threonine residue. SrtA-His6, -GG-His6-EPEA and unreacted His-tagged nanobody were separated from DBCO-nanobody conjugate using HisPur cobalt resin. For this, the reaction mixture was diluted 1:1 in 20 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol, 5 mM imidazole, supplemented with 10 mM EDTA to stop the Sortase reaction. Resin was washed with wash buffer until UV absorption at 280 nm reached baseline. Nanobody-DBC0-containing flowthrough and wash fractions were pooled and remaining DBCO-amine was removed using an Amicon concentrator (3000 MWCO). Nanobody-DBC0-conjugate concentrations were determined by Bradford assay and successful conjugation and purity were assessed by SDS-PAGE. The degree of labeling (DOL) was determined by absorbance at 280 nm (nanobody) and 309 nm (DBC0) using a Nanodrop1000 spectrophotometer (Thermo Fisher Scientific) (see online supplementary table 2 available at stacks.iop.org/JPhysD/51/474005/mmedia). The conjugate was stored at 4 °C or immediately used for click-reactions. Optimal molar ratios of DBCO-amine:nanobody and reaction duration were determined in test reactions with 50 µM nanobody with 150 µM SrtA pentamutant in Sortase buffer 300 mM NaCl and 10% glycerol and incubated for 12 h. The reaction mixture was diluted 1:1 in 20 mM HEPES (pH 7.5), 1 mM MgCl2, 10 mM EGTA, 3.2% parafomaldehyde, 0.1% glutaraldehyde) and cells were incubated for 10 min at 37 °C. Fixation buffer was removed by washing with PBS and 10 mM freshly prepared sodium borohydride in PBS was then added for 7 min followed by a 10 min incubation in 100 mM glycine. Fixed cells were repeatedly washed with PBS and blocked with Image iT FX signal enhancer (Invitrogen) for 1 h and then with 5% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 30 min at room temperature. After washing with PBS three times, cells were stained with a 2.5 µg ml−1 solution of nanobodies, diluted in 5% BSA and 0.1% Triton X-100 in PBS. After 1 h at room temperature, cells were washed three times with PBS and immediately used for imaging.

**Optical setup**

Images were acquired with a Vutara 352 super-resolution microscope (Bruker) equipped with a Hamamatsu ORCA Flash4.0 sCMOS for super-resolution imaging and a 60× oil immersion TIRF objective with numerical aperture 1.49 (Olympus).

**Super-resolution dual color DNA-PAINT imaging**

Imager strand oligonucleotides (Imager 1: CTAGATGTAT and Imager 3: GTAATGAGA), modified with a 3′ Atto 655 dye, were synthesized by Eurofins Genomics (Germany), aliquoted in PBS at a concentration of 100 µM and stored at −20 °C until imaging. For each sample, imager strand concentrations were empirically determined to ensure sufficiently high on-off ratios. Fixed samples were placed in a one-well magnetic chamber (Live Cell Instrument, South Korea) and covered in PBS (pH 7.4) supplemented with 0.5 M NaCl and 500 pM Atto 655-labeled Imager 1. A 250 pM solution of Atto 655 labeled Imager 3 was then added. Ideal exposure times for both binder–imager pairs were calculated with the Picasso Render tool [26]. Data were acquired with TIRF/HILO-illumination at a laser-power density of 2.5 kW cm−2 using a 639 nm laser. Images were collected with a 100 ms acquisition time and typically 10000 images were used to reconstruct the super-resolution composites.

**Super-resolution data processing**

Raw data files were analyzed using the Picasso software package (https://github.com/jungmannlab/picasso). Individual binding events were identified by Picasso:Localize (Identification) with a box side length of 7 and a minimum net gradient of 1500 and 2000 for the microtubule and caveolae localizations, respectively. Subsequently, the localizations...
were loaded into Picasso:Render to correct for microscopic drift (Undrift by RCC), align microtubule and caveolin fluorescence and obtain binding kinetics. For our complementary oligos, we determined a $t_{\text{on}} = 600\, \text{ms}$ and $t_{\text{off}} = 118\, \text{s}$ for binder–imager pair 1 and $t_{\text{on}} = 520\, \text{ms}$ and $t_{\text{off}} = 153\, \text{s}$ for binder–imager pair 3. Microtubule (250 nm straight sections without overlaps, aligned and processed as in [8]) and caveolin cross sections were taken in ImageJ and fitted to a Gaussian function to determine their respective full width at half maximum and interpeak distance.

Results

Nanobody production

Recombinant anti-tubulin and anti-GFP-LPETGG-His$_6$-EPEA nanobody constructs were produced in E. coli, extracted from the bacterial periplasm via osmotic shock and purified via immobilized metal ion and size exclusion chromatography to near homogeneity (>95% by SDS-PAGE). Typical final yields were between 15 and 20 mg l$^{-1}$ culture for both constructs (supplementary figure 1). SrtA (~40 mg l$^{-1}$ culture) was similarly produced and purified.

SrtA-mediated conjugation and copper-free SPAAC via DBCO-amine

For derivatizing nanobodies with short oligonucleotides to use as labels in DNA-PAINT, we initially attempted a peptide exchange strategy using SrtA, similar to that of Massa et al [15]. We used a SrtA pentamutant [24] to label two different probes: an anti-tubulin [22], and an anti-GFP nanobody [27]. Our substrate peptide carried a C-terminal alkyne moiety providing a specific reaction point for azide labeled ‘docking’ oligonucleotides using click chemistry via Cu(I) catalysed alkyne–azide cycloaddition (CuAAC) [28]. Although the reactions were successful (data not shown), we encountered several difficulties with the procedure (see discussion), not least of which was the poor yield of final labeled nanobodies due to the need to remove Cu(I) from the reaction mix. In order to simplify the labeling procedure and maximize the yield, we sought to devise an efficient Cu(I) independent reaction scheme. Cu(I) free click reactions with biomolecules based on strain promoted alkyne–azide cycloaddition (SPAAC) using dibenzocyclooctyne (DBCO) have been reported and can be performed under physiological conditions [28–30]. Building on this idea, we tested whether an amine modified
DBC0 could directly function as the SrtA releasing nucleophile (figure 2(a)). We again prepared SrtA nanobody intermediates and subsequently added DBC0 amine in excess. We observed nearly quantitative conversion of the SrtA nanobody intermediate and confirmed the association of one DBC0 molecule per nanobody by absorption spectroscopy (figures 2(c) and (f); supplementary table 2) and Bradford assay. To confirm that the resulting constructs were competent for label addition, we optimized the labeling reaction using azide-modified mPEG ($M_W = 2000$ Da) at different concentrations and varying incubation times and monitored the results by electrophoretic mobility shift on SDS polyacrylamide gels. Nearly quantitative labeling was observed after 12 h incubation with a 1:1 molar ratio of azide-modified reaction partner (supplementary figure 2). To assess whether the reaction yields conjugates with only one label per protein, Alexa Fluor 647-azide was reacted with DBC0−nanobody. Addition of Alexa Fluor 647-azide to the DBC0 nanobodies in a 1:1 ratio resulted in association of one fluorophore per nanobody, again confirmed by absorption spectroscopy (figures 2(d) and (e); supplementary table 2). The nanobodies performed well in conventional (d)STORM SMLM (not shown).

We then repeated our labeling reaction using azide-oligo docking strands. Again, we observed high efficiency addition of the oligos to the nanobodies based on electrophoretic mobility shift on SDS gels (figures 2(b) and (d)).

**Dual color DNA-PAINT labeling**

The anti-tubulin- and anti-GFP-LPETGG-His$_6$-EPEA nanobody constructs were coupled to previously reported oligo sequences binder 3 and binder 1, respectively [16]. The high efficiency of the SPAAC reaction of DBCO-functionalized nanobodies with the azide-modified docking strands allowed easy removal of unreacted oligonucleotides by simple buffer exchange.

We tested the viability of our nanobodies as super-resolution labeling probes by staining fixed Hela cells stably expressing CAV1-GFP with anti-tubulin nanobody coupled to binder 3 and anti-GFP nanobody coupled to binder 1. Sequential addition of dye-labeled imager 3 and imager 1 strands allowed us to acquire dual color DNA-PAINT images of cells (figure 3). The microtubules were resolved with a FWHM of $31 \pm 4$ nm and the plasma membrane caveolae with a diameter of $61 \pm 17$ nm (supplementary figures 4 and 5). These distances are far lower than the diffraction limit of light and agree well with previously reported microtubule [22] and caveolae diameters [31].

**Discussion**

The efficient conjugation of labels to an antigen-specific antibody or nanobody is a crucial first step for super-resolution microscopy studies. Conventional protocols rely primarily on the use of N-hydroxysuccinimide mediated conjugation of dyes to the $\varepsilon$-amine groups of lysine side chains. Since most proteins harbor several solvent-exposed lysines, this precludes site-specific label introduction and leads to non-uniform labeling of the probe, although specific labeling of the N-terminus has been reported [19]. Lysine residues can also occur near the antigen-binding site in the labeled protein, which can result in loss of affinity or complete inhibition of binding the target after dye conjugation. Introducing an unpaired carboxy-terminal cysteine for conjugation via maleimide chemistry allows the site-specific introduction.
we substantially reduced the preparation time of our samples (peptide), thereby driving the desired reaction to completion. Since the oligonucleotide is quite large in comparison with the nanobody (see figure 1(f)), its coupling close to the epitope-binding site in combination with its high charge may significantly perturb target recognition.

The SrtA variant from Staphyloccocus aureus recognizes an LPXTG-sequence (where X = any amino acid) on the target protein which it cleaves at the terminal glycine to form a target-SrtA acyl-intermediate at the preceding threonine, simultaneously releasing the C-terminal fragment. Subsequent nucleophilic attack by an N-terminal amine-containing species then releases SrtA, generating a hybrid target. The reaction is exploited for labeling by using probes containing a primary amine that can resolve the target-SrtA intermediate [35–37]. This reaction scheme allows specific coupling of a wide range of functional molecules to the C-terminus of any LPXTG-containing protein, including ‘clickable’ alkyne residues.

While CuAAC has been successfully applied for a variety of biomolecules, it requires a multi-step protocol and has the disadvantage of Cu(I) toxicity, thereby requiring removal of the metal for live cell experiments; additionally, Cu(I) can have a denaturing effect on proteins. Due to the need to remove Cu(I) from our reaction mixtures, we found it difficult to obtain satisfying yields of labeled nanobody. We therefore sought to eliminate Cu(I) altogether and reduce the number of steps in the labeling procedure by using SPAAC.

Van Lith et al [38] used DBCO in SPAAC as a general reactive moiety attached to a variable length PEG amine to provide a functionalizable linker for derivatization using various reaction chemistries. While this flexibility in linker length is intended to be beneficial for preparing antibody–drug conjugates for in vivo applications, it confers no advantage for super-resolution fluorescence studies, where increasing linker length complicates the precise localization of the detected species. We therefore used DBCO-amine directly.

Baer et al [39], in testing different amine nucleophiles for resolving the SgtA intermediate, observed a clear preference for small amine groups for the most efficient reactions. In our hands, resolution of the SgtA reaction using DBCO-amine was nearly quantitative. The specificity and low cost of the reagent provides the additional benefit that it can be used in large excess in the reaction to out-compete other potential nucleophiles (including the initially cleaved NH2-GG-His6 peptide), thereby driving the desired reaction to completion.

By using DBCO-amine instead of Cu(I) in the reaction, we substantially reduced the preparation time of our samples while simultaneously increasing the efficiency of the labeling reaction and the overall yield of labeled probe. We suggest that the results here can be extended to accommodate the most diverse labeling strategies by utilizing small, bifunctional molecules having any compatible reactive group of interest coupled to an amine moiety that can complete the Sortase reaction. This approach facilitates the site-specific introduction of uniquely reactive tags into any peptide of interest, the hydrodynamic radius of whose subsequent reaction products need not exceed what the label itself adds. As the native Sortase reaction requires a small amine nucleophile (typically an N-terminal glycine for peptides), the high yields and simplification of preparation of our nanobody conjugates achieved using DBCO-amine are likely to be mimicked using other small amine nucleophiles, greatly improving the ease and flexibility of preparing probes for super-resolution studies. In our case, addition of the detection moiety to the C-terminus of the nanobodies ensures an accessible recognition sequence and that the modification has no detrimental effect on the binding interface, making the final constructs—always with a single label per molecule—ideal for a variety of quantitative studies (for example, single molecule brightness analyses, stepwise photobleaching, etc). Taken together we here present a simple and efficient method for quantitative labeling of nanobodies for DNA-PAINT that due to the versatility of copper-free click chemistry provides an available labeling site at the very C-terminus of nanobodies for a variety of labeling schemes.

One of the great advantages of DNA-PAINT is the possibility of multiplexed labeling with the same dye on different epitopes where the specificity is encoded in the DNA-oligomers [16]. The combination with nanobodies holds great promise as more and more such binders are developed. In the future, highly multiplexed labeling will be possible using secondary nanobodies [40], nanobodies against peptide tags [41] and RFPs [14]. Further optimization of the labeling procedure will likely allow for unprecedented resolution using DNA-PAINT in cells.

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