Protein–DNA Chimeras for Nano Assembly

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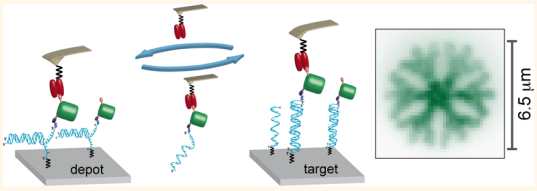
ABSTRACT In synthetic biology, "understanding by building" requires exquisite control of the molecular constituents and their spatial organization. Site-specific coupling of DNA to proteins allows arrangement of different protein functionalities with emergent properties by self-assembly on origami-like DNA scaffolds or by direct assembly *via* Single-Molecule Cut & Paste (SMC&P). Here, we employed the ybbR-tag/Sfp system to covalently attach Coenzyme A-modified DNA to GFP and, as a proof of principle, arranged the chimera in different patterns by SMC&P. Fluorescence recordings of individual molecules proved that the proteins remained folded and fully functional throughout the assembly process. The high coupling efficiency and specificity as well as the negligible size (11 amino acids) of the ybbR-tag represent a mild, yet versatile, general and robust way of adding a freely programmable and highly selective attachment site to virtually any protein of interest.



KEYWORDS: protein–DNA chimera · single-molecule cut & paste · AFM · spatial arrangement · patterning · single-molecule fluorescence

To study protein networks at the single molecule level, precise targeting and localization of its constituents are indispensable prerequisites. To this end, we developed the Single-Molecule Cut & Paste (SMC&P) technique,[1,2] which combines the angstrom level precision of the scanning probe microscope with the selectivity of bio–molecular interactions for the assembly of molecules in arbitrary arrangements. It allows individual molecules to be picked up from a depot area and assembled one by one at a chosen position in a "construction site" in the target area (Scheme 1).

SMC&P is based on noncovalent, but thermally stable, bonds for storage (depot), handling (AFM cantilever), and deposition (target). These bonds are chosen such that the force required to release the storage interaction is lower than the force required to overcome the handle attachment, which again is lower than the deposition bond ($F_s < F_h < F_d$). For one-by-one assembly, the functionalized AFM cantilever tip is allowed to bind a transfer molecule in the depot area *via* the specific handle. Upon retraction the storage bond ruptures, the transfer molecule remains attached to the cantilever and is then transferred to the construction site. There, the AFM tip is lowered and the transfer molecule forms a deposition bond and is thus placed at a chosen position in the construction site. Upon retraction of the tip, the handle bond ruptures, while the transfer molecule remains at its position, and the AFM tip is free again to pick up a new transfer molecule from the depot area. Remarkably, the system is now in the same state as prior to the first pick-up so that the SMC&P-process may be repeated with the same tip in a cyclic manner. The rupture forces in this hierarchical system, which allow this cut and paste process to be run over thousands of cycles, may either be programmed by the selection of the binding partners or predetermined by the force loading rates.[3–6] Note that for each of these bond-rupture processes a force *versus* distance curve is recorded to verify that indeed individual molecules were handled or, in the case of high density tip functionalization, to provide an estimate of the number of transferred molecules per cycle.

During recent years, this method was improved and taken from the initial DNA-based stage *via* the functional assembly of RNA aptamers[7] to the much more complex protein level.[8,9] The first approach in protein

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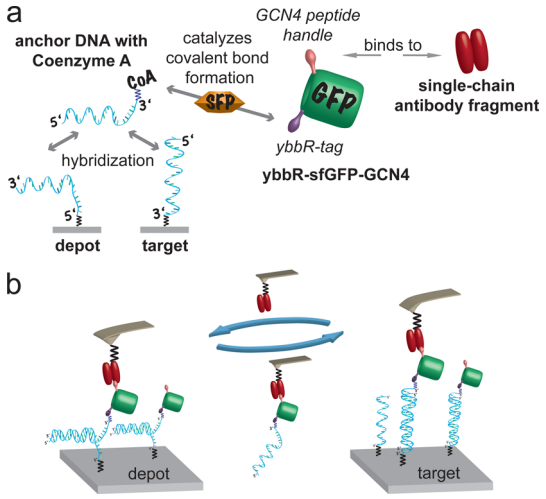

Scheme 1. SMC&P with a chimeric GFP−DNA moiety. (a) To ensure a hierarchical force distribution, DNA duplex interaction is utilized in depot and target region, with the DNA in zipper ($F_{rupture} \sim 20$ pN)[3] and shear conformation ($F_{rupture} \sim 65$ pN),[4] respectively. The intermediate force for the transport handle was achieved using an anti-GCN4-peptide single-chain antibody fragment ($F_{rupture} \sim 50$ pN).[5] (b) Principle of repeatable transfer cycling in protein SMC&P experiments.

SMC&P relied on the use of Zincfinger fusion proteins.[9] The Zincfinger moiety and its specifically bound DNA transfer strand acted as a shuttle for other proteins of interest, combining the advantages and reproducibility of DNA-only SMC&P with the ability to selectively collect and deposit single proteins without loss of functionality. The need for an even more versatile protein transport system arises from the size of the Zincfinger, which imposes a rather big alteration to the protein of interest; its poor solubility, especially in combination with more complex protein candidates; and the noncovalent nature of its DNA interaction.

Minimal modification of the proteins of interest, as well as covalent attachment to the DNA carrier, is greatly desirable. Moreover, there is a general need for robust strategies to selectively couple DNA to proteins. Such chimeras are extremely useful in immunobiological applications[10,11] as well as nanobiotechnology,[12] *e.g.*, for the DNA origami technology.[13] Since the various options to couple DNA-oligonucleotides to proteins harbor certain drawbacks, no gold standard exists hitherto.

Click-chemistry,[14] *e.g.*, while being very specific and selective itself, requires less selective modification of amino acid side chains[15] or the incorporation of nonnatural amino acids into proteins.[16,17] The latter is often laborious in terms of expression system and yield.[18] Furthermore, reaction conditions can be rather harsh for proteins or relatively inefficient.[19] Coupling strategies involving bifunctional cross-linkers are less specific. Attachment can be achieved *via* either primary amino groups in proteins or thiol groups, which often requires incorporation of a single accessible cysteine and mutation of others. Thus, full integrity and functionality of the modified proteins is not guaranteed or even unlikely. Furthermore, suicide enzymes, *e.g.*, HaloTag or SNAP-tag (hAGT), could be employed as fusion protein tags for site-specific immobilization reactions.[20−22] However, their respective sizes of 33 and 20 kDa diminish their attractiveness for single-protein manipulation.

We thus chose to employ the 11 amino acid ybbR-tag that, assisted by the Phosphopantetheinyl Transferase Sfp,[23] allows for the site-selective attachment of Coenzyme A (CoA)-modified DNA to proteins of interest[24] (Scheme 1). Coenzyme A is easily reacted to commercially available Maleimide-modified oligonucleotides *via* its intrinsic thiol group, and the already-coupled construct is available upon request for purchase from several companies. The ybbR-tag technology is widely used for labeling proteins with, *e.g.*, biotin or fluorescent dyes and works efficiently on either N- or C-terminus or accessible unstructured regions of proteins.[25] The ybbR-tag/Sfp system can be further employed in the immobilization of proteins on Coenzyme A-functionalized solid carriers or surfaces.[26−28]

RESULTS AND DISCUSSION

We expressed GFP with an N-terminal ybbR-tag and a C-terminal short GCN4-tag and reacted the construct with Coenzyme A-modified transfer-DNA with high yield (Supporting Information Figure S1). The purified chimera was then successfully incorporated in SMC&P experiments. Transport processes were extremely efficient, and the GFP remained intact and fluorescent throughout the SMC&P procedure (Figures 1a,b, and 2). The number of transported molecules can be easily tuned by using either different cantilever sizes and/or varying functionalization densities at the cantilever tip (Figures 1 and 2). Glass surface functionalization is kept as dense as possible to allow for a homogeneous distribution of transfer-DNA:protein complex binding sites in the depot and target area. The number of deposited protein molecules is thus solely dependent on the number of GCN4-binding antibody anchors on the cantilever tip.

To achieve the highest precision possible and to prove that individual molecules can be transported, we performed SMC&P of the GFP-DNA chimera employing BioLever Mini (BLM) cantilevers. Such cantilevers harbor extremely sharp and small, but still functionalizable, tips (10 nm nominal tip radius of curvature; sharpened from the initial pyramidal shape by an oxidation process) and hence, offer the highest accuracy in molecule deposition. Grid patterns of 8 × 8 distinct transfer sites (10.5 × 10.5 μm in size, 1.5 μm in each direction between each grid point) were assembled (Figure 1 and Supporting Information Figure S2). The transport process was followed directly by recording force distance curves with the AFM during SMC&P

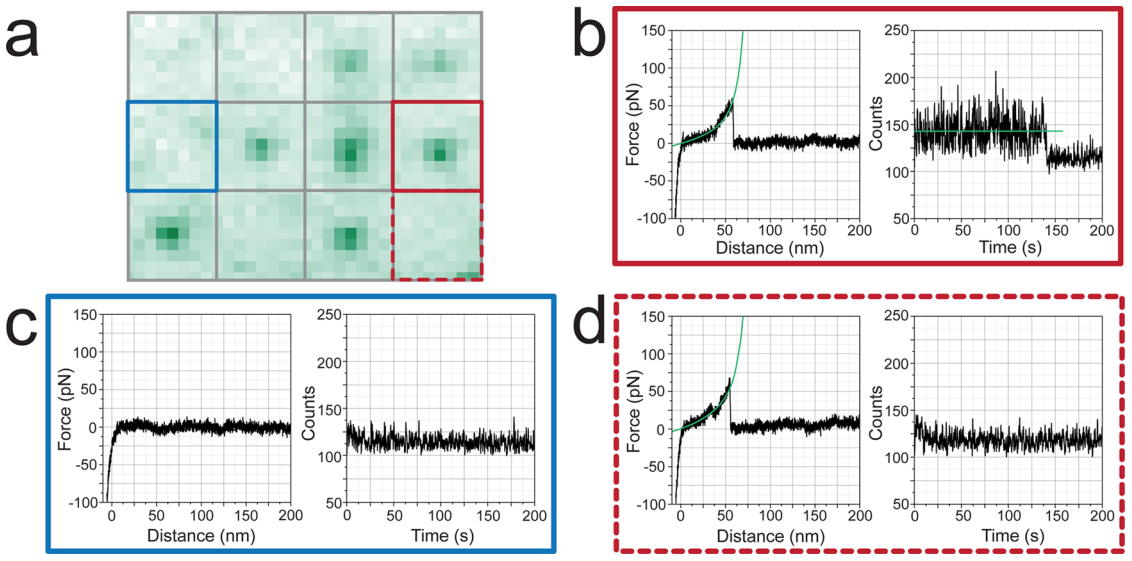

Figure 1. Individual GFP molecules can be transported with AFM cantilevers. (a) Representative 3 × 4 deposition point grid section obtained by SMC&P of GFP molecules employing a BLM cantilever (standard deviation of the fluorescence signal over 100 s, ImageJ) with 7 observable GFP signals out of 12 transfer cycles. (b) Rupture forces around 50 pN (at loading rates around 300 pN/s) correspond to single deposition events in the target area and correlate with a single bleaching step in the fluorescence signal over time at the distinct deposition point (2 × 2 pixel area). (c) Target force curves showing no force built-up correspond to cycles where no molecule could be deposited, which is also evident from the lack of a fluorescence signal at the respective grid position. (d) Due to its limited photostability, a fraction of the GFP molecules is expected to already be bleached throughout the purification and SMC&P preparation process. Yet, the dual mode of transport observation—directly following force–distance curves while performing SMC&P and subsequent fluorescence imaging—allows the detection of single GFP deposition events, even in the absence of a fluorescence signal.

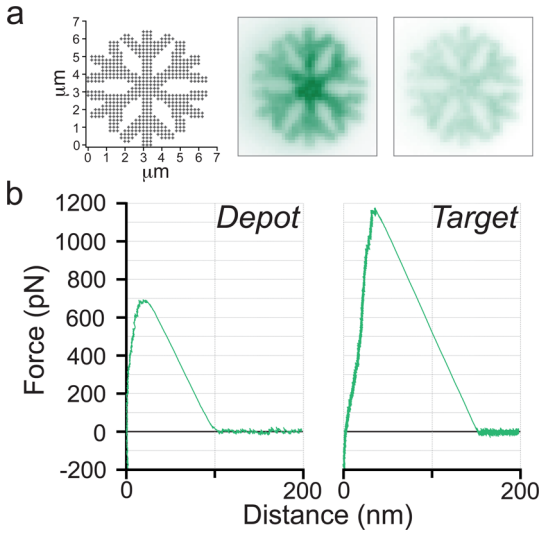

Figure 2. High transport efficiency protein SMC&P. (a) After exposure of the 552-point deposition snowflake pattern for 60 s (0.1 s exposure time at ~10 W/cm^2), it still appears homogeneous and clearly discernible. The pattern template and the average fluorescence over the first (bright) and last (faint) 20 frames of the TIRFM acquisition (600 frames at 0.1 s exposure time) are depicted. (b) Judging from extremely high rupture forces, several (>20) GFP molecules were transported in each cycle.

cycling (Figure 1 and Supporting Information Figure S3). The pattern was subsequently imaged by TIRF microscopy (Figure 1a and Supporting Information Figure S2a). The number of deposited GFP molecules arises from the fluorescence signal over time at a distinct grid point (Figure 1b). We could thus show that indeed single molecules were transferred. Notably, SMC&P utilizing such sharp-tipped cantilevers can also result in force curves devoid of any rupture event and thus no GFP deposition (Figure 1c). In some cases, even though single rupture events were observed, no fluorescence signal could be detected at the corresponding grid position (Figure 1d). A likely cause is the limited photostability of GFP. A fraction of the GFP molecules can be expected to already undergo photobleaching during the purification and SMC&P preparation process. Thus, nonfluorescent GFP molecules would be occasionally transported as well. Furthermore, the rupture events underlying the SMC&P procedure only have a certain probability to lie in the expected force range. In rare cases, the observed rupture event for the deposition process could therefore theoretically originate from a rupture of the shear DNA deposition bond (a most probable rupture force ~65 pN would be expected for the utilized 40 bp duplex at the observed loading rates around 300 pN/s)[4] instead of the desired antibody fragment/GCN4-peptide dissociation ($F_{rupture}$ ~ 50 pN at the observed loading rates around 300 pN/s).[5] This would result in the GFP-DNA chimera remaining on the cantilever and could hence also account for the absence of a fluorescence signal in the respective grid position.

In a typical SMC&P experiment where a 64-point distinct deposition pattern was assembled, an average of 0.89 molecules per cycle were picked up from the

depot area, judging from the according force spectroscopy data. More relevantly, an average of 0.84 molecules were deposited per cycle, based on rupture force evaluation. A fluorescence-based assessment of the number of transported and actually deposited molecules gives rise to an average value of 0.5 molecules per cycle (Supporting Information Figure S2). For comparison, in former DNA-only SMC&P experiments, employing AFM probes with broader tips, around 0.5 molecules per cycle were transported.[29] Further, in earlier Zincfinger-based protein-SMC&P approaches, where larger numbers of molecules should be transferred with densely functionalized broad-tipped cantilevers, efficiencies ranged around 2 molecules per cycle.[9]

Conditions are optimized in a way that mostly individual molecules are transported. Rarely, the transport of two molecules per cycle is observed, whereas SMC&P cycles devoid of a deposition event are much more likely to occur. A transport efficiency of less than one molecule per cycle is acceptable for the benefit of being able to frequently transport truly individual protein constructs. Under the given conditions, one SMC&P cycle takes less than 3 s. This is mainly affected by the chosen pulling speeds that are optimized with respect to apparent loading rates and thus probable rupture forces. These parameters require careful adjustment to ensure functional and structural integrity of the transported protein as well as balancing the hierarchical rupture force "triangle" the SMC&P principle builds-up on. Binding kinetics of the interacting molecules are not expected to be limiting under the experimental conditions (see Supporting Information, p S7).

To further demonstrate the robustness of the SMC&P setup, we additionally utilized a pyramidal shaped MLCT cantilever probe with a nominal tip curvature radius of approximately 20 nm to assemble the pattern of a snowflake in 552 transfer cycles (Figure 2). GFP fluorescence of the pattern was extremely strong, and after laser exposure at 10 W/cm^2 for 60 s, the homogeneous pattern was still clearly discernible (Figure 2). Considering GFP's limited photostability, this indicates high transport efficiency. Judging from AFM rupture force curves of this experiment, more than 20 molecules were transported per cycle.

CONCLUSIONS

In conclusion, we have largely improved protein-based SMC&P in terms of versatility, precision, efficiency and robustness. The adaptability of the system will in the future allow tackling of any protein of interest in single-molecule studies or in complex protein networks, spatially arranged by means of SMC&P. Moreover, protein SMC&P can be utilized to for example place individual enzymes in the center of bow-tie nanoantenna structures[30] or Zero-Mode Waveguides (ZMW), as has been demonstrated for DNA.[31] In favor of this, the applicability of cantilever tips with a high aspect ratio is especially crucial for protein SMC&P as the cantilevers with larger pyramidal shaped tips exceed the dimensions of the nanometer-sized holes of ZMWs. The precision and spatial control achieved with protein SMC&P will thereby significantly improve enzymatic studies in the presence of high concentrations of fluorescent substrates that are unmet by other single-molecule fluorescence methods.[32]

Importantly, the protein–DNA coupling strategy employing Coenzyme A-modified DNA and the ybbR-tag/Sfp system proved to be high-yielding, straightforward (also with other protein constructs, data not shown), and relatively inexpensive in terms of material costs and time. It thus promises to be a useful tool in providing protein–DNA chimeras, which should also be advantageous for other fields of nanobiotechnology and protein engineering.

EXPERIMENTAL SECTION

SMC&P experiments were carried out on a combined AFM/TIRFM setup, as described previously[1] and detailed information can be found in the Supporting Information. In short, GFP harboring an N-terminal Hexa-His-tag, followed by an 11 amino acid ybbR-tag[25] and a C-terminal GCN4-tag[5] was expressed in *Escherichia coli* BL21 DE3 CodonPlus and purified according to standard protocols. The construct was then reacted with Coenzyme A-modified transfer-DNA (biomers.net GmbH, Ulm, Germany) in the presence of Sfp. The progress of the coupling reaction was assessed by SDS-PAGE and subsequent fluorescence scanning as well as Coomassie staining of gels. The GFP–DNA chimera was then purified by anion exchange chromatography. The construct was bound to the DNA-depot area on a functionalized glass surface *via* a custom-built microfluidic system. SMC&P was achieved by means of anti-GCN4 antibody functionalized cantilever tips, delivering GFP–DNA molecules from the depot area to the construction site in the target area. BLM cantilevers were used to transport individual GFP–DNA chimeras. MLCT cantilevers were utilized for comparison and high transport efficiencies. Molecule pick-up and deposition was followed directly by AFM force–distance curves, and the assembled pattern was imaged by TIRF microscopy subsequent to the writing process. Simultaneous detection of AFM curves and fluorescence is also possible; however, it was not feasible for GFP due to its relatively low photostability.

Conflict of Interest: The authors declare no competing financial interest.

Supporting Information Available: Further details on experimental methods and supplementary results. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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