

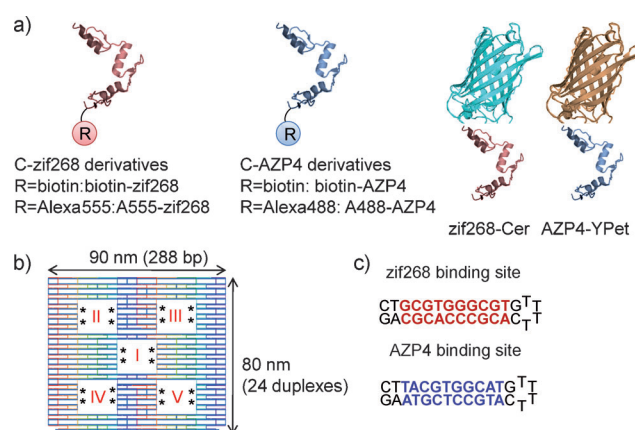
# Zinc-Finger Proteins for Site-Specific Protein Positioning on DNA-Origami Structures\*\*

Eiji Nakata, Fong Fong Liew, Chisana Uwatoko, Shigeki Kiyonaka, Yasuo Mori, Yousuke Katsuda, Masayuki Endo, Hiroshi Sugiyama, and Takashi Morii\*

Structural DNA nanotechnology,<sup>[1]</sup> which includes DNA origami,<sup>[2]</sup> enables the rapid production of self-assembled nanostructures. One of the key features of this technology is that fully addressable nanoarchitectures of various shapes and geometries are easily designed and constructed. By taking advantage of their addressable nature, DNA nanostructures have been used as scaffolds for the site-directed assembly of functional entities, such as small molecules and nanoparticles.<sup>[1,2]</sup> As well as these functional entities, proteins are a particularly interesting class of molecules to assemble because of their huge functional variability.<sup>[3]</sup> Methods to attach proteins at specific locations on DNA scaffolds have been reported and include those based on antibody–antigen interactions,<sup>[4]</sup> aptameric binding,<sup>[5]</sup> Ni-NTA–hexahistidine interactions,<sup>[6]</sup> and biotin–avidin interactions.<sup>[7,4c]</sup> Orthogonal targeting of specific locations can also be achieved by hybridization with DNA-tethered proteins,<sup>[8]</sup> sequence-specific DNA binding of pyrrole-imidazole polyamides,<sup>[9]</sup> and self-ligating protein tags.<sup>[10]</sup> Many of these methods require modification of the protein. Therefore, a method that is fully based on protein components would accelerate the specific assembly of proteins on the DNA nanoarchitecture. Herein, we report that different locations within DNA-origami structures are site-specifically and orthogonally targeted by using sequence-specific DNA-binding proteins as an adaptor, and demonstrate that adaptor-fused functional proteins are assembled at specific locations within DNA-origami structures. Zinc-finger proteins (ZFPs) are one of the best-

characterized classes of DNA-binding proteins;<sup>[11,12]</sup> designed, artificial ZFPs bind to a wide variety of DNA sequences.<sup>[13]</sup> Each zinc-finger domain is capable of recognizing a tract of four base pairs in the major groove of a DNA duplex. A three-fingered protein recognizes a tract of ten base pairs with nanomolar affinity.

Two well-characterized ZFPs, zif268<sup>[14]</sup> and AZP4,<sup>[15]</sup> each with an affinity for a unique sequence of ten base pairs in the low nanomolar range, were chosen as the orthogonal adaptors for specific locations in the DNA-origami structures. Each ZFP was engineered to possess an N-terminal cysteine residue (C-zif268 and C-AZP4) as a selective chemical modification site (Figure 1 a). Modification of C-zif268 with the fluorophore Alexa555 or biotin gave A555-zif268 and biotin-zif268, respectively; modification of C-AZP4 with the fluorophore Alexa488 or biotin gave A488-AZP4 and biotin-AZP4, respectively (see the Supporting Information).<sup>[16]</sup>



**Figure 1.** a) Illustrations showing the structure of the zinc-finger adaptors and adaptor-fused proteins. b) A structural image of the DNA-origami structures. The addressable cavities are indicated by the numbers I, II, III, IV, and V. The positions of the addresses (binding sites) of zinc-finger adaptors are indicated by asterisks. Bp = base pairs. c) Nucleotide sequences for the specific binding site of the zinc-finger adaptors.

A rectangular DNA-origami structure that has five addressable cavities (90 nm × 80 nm) was designed as previously reported (Figure 1 b).<sup>[2a]</sup> Such addressable cavities have been shown to be useful for monitoring the DNA binding of proteins.<sup>[17]</sup> Each addressable cavity was designed to hold up to four ZFP-adaptor binding sites for zif268 and/or AZP4 (Figure 1 c). Various types of DNA-origami structures with binding sites for ZFP-adaptors were prepared (Table 1; see

[\*] Dr. E. Nakata, Dr. F. F. Liew, C. Uwatoko, Prof. Dr. T. Morii  
Institute of Advanced Energy, Kyoto University  
Uji, Kyoto 611-0011 (Japan)  
E-mail: t-morii@iae.kyoto-u.ac.jp

Dr. S. Kiyonaka, Prof. Dr. Y. Mori  
Department of Synthetic and Biological Chemistry  
Graduate School of Engineering, Kyoto University (Japan)

Y. Katsuda, Prof. Dr. H. Sugiyama  
Department of Chemistry, Graduate School of Science  
Kyoto University (Japan)

Dr. M. Endo, Prof. Dr. H. Sugiyama  
Institute for Integrated Cell-Material Sciences  
Kyoto University (Japan)

Dr. E. Nakata, Dr. S. Kiyonaka, Prof. Dr. Y. Mori, Dr. M. Endo,  
Prof. Dr. H. Sugiyama, Prof. Dr. T. Morii  
CREST, JST (Japan)

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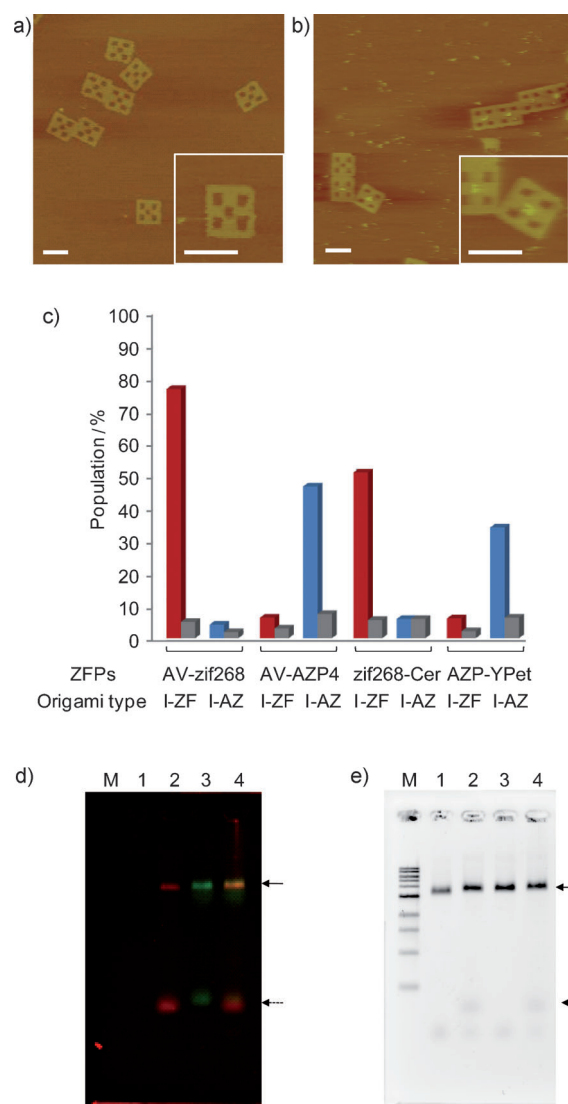
**Table 1:** Types and binding sites of DNA-origami structures.

| DNA-origami structure | Cavity I                      | Cavities II–V   |
|-----------------------|-------------------------------|-----------------|
| NB                    | no binding site               |                 |
| I-ZF                  | zif268 binding sites          | no binding site |
| I-AZ                  | AZP4 binding sites            | no binding site |
| 5ZF                   | zif268 binding sites          |                 |
| 5AZ                   | AZP4 binding sites            |                 |
| 5ZF/AZ                | zif268 and AZP4 binding sites |                 |

also Figure S1 in the Supporting Information). The folding of an M13mp18 single-stranded DNA through the use of 159 staple strands was analyzed by AFM, as shown in Figure 2a (see the Supporting Information).

After assembly, origami structure I-ZF, with the zif268 adaptor binding site at the central cavity I (Figure 1b), was incubated with biotin-zif268 and then with an equal molar concentration of streptavidin (termed AV-zif268) to enlarge the molecular size of the adaptor. The mixture was adsorbed onto mica and analyzed by AFM at the single-molecule level, as shown in Figure 2b (see the Supporting Information). The AFM images showed that AV-zif268 binding occurred exclusively at the central cavity I of I-ZF, at over 70%, whereas little or no AV-zif268 binding was observed at cavities which lack the zif268 binding site or on the DNA-origami scaffold (Figure 2c; see also Table S1 in the Supporting Information). The height of I-ZF after binding to AV-zif268 increased to almost 3 nm from the mica surface (Figure S2 in the Supporting Information). Incubation of I-AZ (Table 1; see also Figure S1 in the Supporting Information) with streptavidin that was attached biotin-AZP4 (AV-AZP4) also showed specific binding at cavity I, and has an average binding efficiency of 45% (Figure 2c; see also Table S1 in the Supporting Information). Conversely, incubation of I-AZ with AV-zif268 or incubation of I-ZF with AV-AZP4 resulted in minimal occupation (less than 10%) of the binding site at the central cavity I (Figure 2c; see also Table S1 in the Supporting Information). The equilibrium dissociation constants for biotin-zif268 and biotin-AZP4 for the binding site,  $63 \pm 18$  and  $138 \pm 34$  nM, respectively (Figure S3 and Table S2 in the Supporting Information), seem to correlate well with the observed occupation of the target sites of I-ZF and I-AZ. These results clearly show the selective and orthogonal binding of AV-zif268 and AV-AZP4 adaptors to their expected locations.

The selectivity and orthogonality of ZFP adaptors were further assessed by gel electrophoresis (Figures 2d and e; see also Figure S4 in the Supporting Information). Four types of DNA-origami structures, each with a different binding site, namely, 5ZF with the binding site for zif268, 5AZ for AZP4, 5ZF/AZ for zif268 and AZP4, and NB without the target sequence binding site (Table 1; see also Figure S1 in the Supporting Information) were incubated with both fluorophore-attached adaptors A555-zif268 and A488-AZP4 and analyzed by gel electrophoresis (Figure 2d).<sup>[18]</sup> For 5ZF and 5AZ, only the fluorescence color that corresponds to the matched adaptor, A555-zif268 and A488-AZP4, respectively, was observed. Merged color was observed for 5ZF/AZ, which



**Figure 2.** AFM images of a) unmodified I-ZF and b) I-ZF modified with avidin-attached biotin-zif268 (AV-zif268). Insets: magnified images (scale bars = 100 nm). c) Selective binding of ZFP adaptors to the target site was estimated by counting the number of ZFP-bound origami structures in the AFM images. d) Gel-electrophoretic analysis of the binding ability of A555-zif268 and A488-AZP4 to different types of DNA-origami structures (1% agarose) visualized by Alexa555 (red), Alexa488 (green) fluorescence, and by e) ethidium bromide staining. In panels d) and e): lane M: 1000 bp DNA ladder; lanes 1–4: A555-zif268 and A488-AZP4 with NB, 5ZF, 5AZ, and 5ZF/AZ, respectively. The solid arrows and dashed arrows indicate bands that correspond to DNA-origami structures and excess staple DNA, respectively.

contains the binding sites for both adaptors, and no fluorescence was detected for NB. Furthermore, the fluorescence of 5ZF/AZ was diminished by the addition of DNA that contains the zif268-binding sequence (zif268-ODN) and the AZP4-binding sequence (AZP4-ODN) as competitors (Figure S4 in the Supporting Information). These results demonstrate specific and orthogonal targeting of locations on the DNA-origami structures by the ZFP adaptors.

To investigate the applicability of ZFP adaptors to engineered proteins, a cyan fluorescent protein variant

(Cerulean)<sup>[19]</sup> and a yellow fluorescent protein variant (YPet)<sup>[19]</sup> were fused to the C terminal of zif268 and AZP4, respectively, by using an appropriate linker to give zif268-Cer and AZP4-YPet. These adaptor-fused, auto-fluorescent proteins were expressed in *Escherichia coli* and purified by using conventional procedures (see Supporting Information). The adaptor-fused proteins zif268-Cer and AZP4-YPet were incubated with DNA-origami structures that contain the respective binding sites, and were analyzed by AFM and gel electrophoresis (Figure 3 a; see also Figures S6, S7, and S8 in

that originated from Cer in the band that corresponds to the complex with I-ZF disappeared only in the presence of an excess amount of zif268-ODN (Figure S6 in the Supporting Information). The same was true for AZP4-YPet (Figure S7 in the Supporting Information). These results indicate that these proteins, which were engineered with the adaptor, specifically target the expected location on the DNA-origami structure.

Adaptor-fused proteins are routinely expressed by *E. coli*. It would be convenient to locate the protein of interest directly onto the specific site of the DNA-origami structure by using *E. coli* lysate, in which DNA-origami structures were reported to be stable and functional after extended exposure.<sup>[20]</sup> *E. coli* lysate containing expressed zif268-Cer was subjected to size-exclusion gel chromatography, incubated with I-ZF, subjected to size-exclusion chromatography again, then analyzed by AFM. The AFM images show that the central cavity I was occupied (Figure 3 b; see also Figure S9a in the Supporting Information). A height analysis of the bound origami structure (Figure S9b in the Supporting Information) shows the same pattern as that obtained for purified zif268-Cer (Figure S6b in the Supporting Information). Furthermore, selective occupation through zif268-adaptor binding was confirmed by electrophoretic competition analysis (Figures 3 c and d). The band that corresponds to I-ZF emitted fluorescence from Cer. A selective reduction in Cer-derived fluorescence occurred only when zif268-ODN was used as a competitor (Figure 3 c, lane 3). These results indicate that the selective arrangement of an engineered protein on DNA-origami structures is feasible by simply using overexpressed cell lysate.

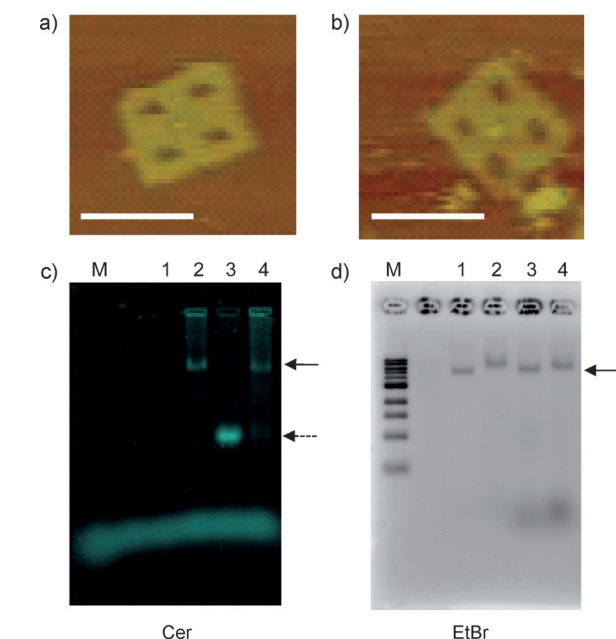
In conclusion, we have demonstrated that ZFPs are convenient and site-selective adaptors for targeting specific locations within DNA-origami structures. The diversity of target DNA sequences and the semi-programmable design of ZFPs offers orthogonal adaptors, thereby enabling the placement of multiple engineered proteins at different locations onto DNA-origami structures. Nature uses multiple proteins and/or enzymes in close proximity to efficiently carry out chemical reactions and signal transductions. Such assemblies of multiple proteins may be realized in vitro by using DNA-origami structures that have defined binding sites and various kinds of ZFP adaptor-fused proteins.

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**Figure 3.** An AFM image of I-ZF treated with a) purified zif268-Cer or b) a lysate that contains zif268-Cer (scale bar = 100 nm). c) Gel-electrophoretic analysis of the binding of zif268-Cer to I-ZF in lysate (1% agarose) visualized by Cer fluorescence and d) ethidium bromide staining. In panels c) and d): lane M: 1000 bp DNA ladder; lane 1: I-ZF, lanes 2–4: I-ZF and lysate in the absence (lane 2) and presence of zif268-ODN (lane 3) or AZP4-ODN (lane 4). The solid arrows and the dashed arrow indicate bands that correspond to free DNA-origami structures or DNA-origami structures in complex with zif268-Cer, and to excessive zif268-ODN in complex with zif268-Cer, respectively.

the Supporting Information). The AFM images showed that zif268-Cer and AZP4-YPet bound to the origami structures selectively at the central cavity with the corresponding binding site with a binding efficiency of over 50% (I-ZF) and 30% (I-AZ, Figure 2c; see also Table S1 in the Supporting Information). The height of I-ZF after binding to zif268-Cer increased to almost 2 nm from the mica surface (Figure S6 in the Supporting Information), which is lower than that of I-ZF after binding to AV-zif268 (almost 3 nm).

These results correspond directly to protein size, where AV is a tetramer of 53 kDa and Cer is 27 kDa. Little nonspecific binding was detected in other cavities of the target origami or on the origami structures that have no corresponding binding site (less than 10%; Table S1 in the Supporting Information). In the agarose gel, the fluorescence

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