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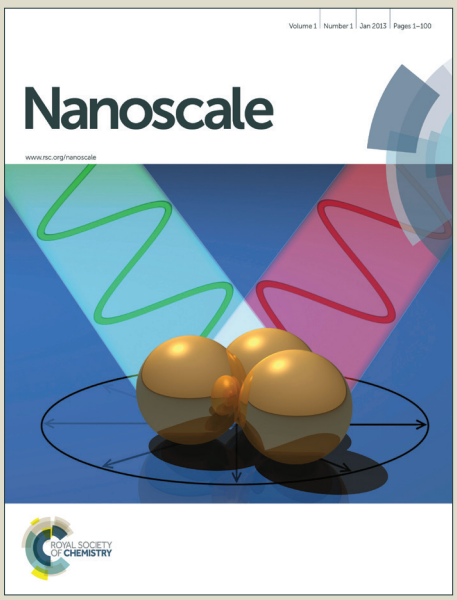

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Orthogonal Enzyme Arrays on a DNA Origami Scaffold Bearing Size-Tunable Wells

Takahiro Yamazaki,[1] Jonathan Gardiner Heddle,[2] Akinori Kuzuya,[3,4]* Makoto Komiyama[1,5]*

[1]RCAST, The University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8904, [2]Heddle Initiative Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, JAPAN, [3]Dept. Chem. Mater. Eng., Kansai University, 3-3-35 Yamate, Suita, Osaka 564-8680, JAPAN, [4]PRESTO, JST, [5]Life Science Center of Tsukuba Advanced Research Alliance, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8577, JAPAN.

Abstract

A new waffle-like DNA origami assembly (DNA waffle) with nine nanometer-scale wells in a 3x3 matrix pattern has been successfully constructed and used as a scaffold for selective nano-patterning of individual protein molecules. The folding pattern of the scaffold was specially designed so that the dimensions of each well could be independently tuned according to the dimensions of the guest nanoparticles. We demonstrated that two distinct proteins, streptavidin (SA) tetramer (d = 5 nm) and anti-fluorescein antibody (IgG) (inter-paratope distance ~ 14.0 nm), could be selectively captured in size-variable wells of dimensions 6.8 × 12 × 2.0 nm for SA and 6.8 × 12 × 2.0 nm or 10.2 × 12 × 2.0 nm for IgG respectively, through the attachment of two biotins or two fluoresceins at the two edges of each well. This allowed formation of a heterogeneous protein nanoarray of individual molecules. The position of SA or IgG capture can be fully controlled by placement of biotins or fluoresceins in the nanoarray well. Moreover, a hetero-nanoarray consisting of two kinds of enzyme: horseradish peroxidase–labeled streptavidin (HRP-SA) and alkaline phosphatase-labeled anti-FITC antibody (AP-IgG) was successfully constructed through selective attachment of biotin or fluorescein in any desired wells. Successful enzyme-heteroarray formation was confirmed by enzymatic activity analyses after purification of mixtures of enzymes and DNA waffles.

Introduction

Recent progress in structural DNA nanotechnology,[1] which is based on programmed assembly of branched DNA helices, has enabled precise nanopatterning of protein molecules. DNA origami,[2] in which long single-stranded DNA is folded into designed planar nanostructures with the aid of many short staple strands, is a promising nano-scaffold candidate.[3-7] Recently, we proposed an effective

strategy to fix proteins to DNA nanostructures that leads to robust and precise protein nanoarrays.[8-10] This strategy is based on our previous finding that a nanometre-sized cavity embedded in a tape-like DNA nanostructure can serve as a well capable of size-selectively capturing a single protein molecule. The accommodation of the proteins is so stable that, even under repetitive AFM scanning, consistently clear images of individual molecules were obtained.[11,12] We previously prepared a stick-like DNA origami containing 9 periodically spaced wells with dimensions of 7 nm x 14 nm x 2 nm.[8] When two staple strands placed at opposite edges of a well (anchor strands) are biotinylated, exactly one streptavidin (SA; tetramer diameter 5 nm) is size-selectively captured in the well to form part of a SA nanoarray. The captured SA shows remarkable stability because of an "anchoring" effect due to bidentate binding as well as steric protection by the 2-nm deep DNA well. Although homogeneous SA nanoarrays have been successfully obtained with the above strategy, heterogeneous nanoarrays with multiple diverse protein guests are also crucially important when considering practical applications of protein nanoarrays such as single-molecular analyses of cascade reactions.[13,14] We have recently reported stepwise attachment of SA to DNA origami for this purpose.[9] However, it requires time-consuming annealing of anchor strands and tedious removal of unbound SA from the solution at every step, and the final yield of DNA origami motifs is often not high.

In this report we employed an antibody-antigen interaction in addition to SA-biotin binding in order to achieve more convenient heterogeneous assembly of protein nanoarrays, and place different proteins in a single reaction step. These protein heteroarrays could be constructed with only a single purification step. We used fluorescein as the antigen and anti-Fluorescein IgG as the antibody.[15] DNA origami can be decorated with fluorescein, which can be localized in any arbitrarily chosen well and to which IgG can subsequently be attached. Because IgG is considerably larger than SA, the distance between bound fluorescein groups (inter-epitope distance) has to be larger than that used between biotin and SA. For this reason, a new waffle-like DNA origami (DNA waffle) with optimized large wells of the appropriate size was produced. Nine such wells were placed in the origami, forming a lattice pattern. The size of the well was fixed at 4 duplexes in the direction orthogonal to the DNA helix axis and 2, 3 or 4 helical turns in length along the direction of the helical axis. Firstly the appropriate well size for immobilization of IgG was determined by using a DNA origami where the dimensions of the wells were systematically widened from line to line. Subsequently, wells appropriate for SA were added to construct an origami suitable for heterogeneous arraying. Finally, an IgG and alkaline phosphatase (AP) complex (AP-IgG) and a SA and horseradish peroxidase (HRP) complex (HRP-SA) were used to construct a heterogeneous

enzyme array. If different antibodies can be fixed onto DNA origami, then it should be possible to produce heterogeneous arrays of the corresponding antigen molecules. Furthermore, due to the wide availability of various enzyme complexes, the system can be adapted for a range of potential applications.

Results and Discussion

Design of DNA waffles

Figure 1 shows the folding pattern of the M13 scaffold in DNA waffles. In total, 28 duplexes are bundled in a plane and nine wells (#1–9) of 4 duplexes long and 4 helical turns wide are embedded in 3 × 3 arrangement (Figure 1a). One unique feature of the present DNA waffle is that the size of each well is individually tunable from two to four helical turns. Scaffold portions in both sides of each well (gray parts in Figure 1a) are called "tunable blocks", which can be altered to produce differently sized wells. To make the well #2 two-turns wide, for example, scaffold portions running along the side of the well are drawn out of the original position as shown in Figure 1b. Staple strands arranged in the tunable blocks are accordingly replaced. Fill strand **1**, which is 40-nt long, inserts into the scaffold portions and causes partial filling of the cavities. Fill strand **2** (80 nt) is used when both of the portions in a tunable block are drawn as shown in Figure 1b between wells #1 and #4.

We employed two differently patterned DNA waffles in this study. Diagonally patterned DNA waffles with three-turn wells in diagonal positions and two-turn wells in the others is shown in Figure 1b and is formed by introducing short fill strand **1** to the left side of well #2 and the right sides of wells #2, #4, #6–#9 together with long fill strand **2** on the right sides of the wells #1, #3, and #5. Similarly, DNA waffles with a columnar pattern of triadic two-, three-, and four-turn wells (Figure 1c) can be prepared by using these fill strands at appropriate positions.

Determination of appropriate well dimensions for each protein target

The most suitable size of well for each protein was determined by using columnar-patterned DNA waffles. To emphasize the discriminatory ability of differently sized wells, only wells #2, #4, #6, #7, and #9 were chemically modified with ligands to capture target proteins, and other wells were kept empty. Introduction of the ligands was done by using appropriate biotinylated anchor strands for SA capture and FAM-modified anchor strands for IgG capture in the annealing stage of columnar-patterned DNA waffles.

AFM imaging of captured SA on columnar-patterned DNA waffles confirmed that a two-turn well is the best to capture exactly one SA tetramer in a well (Figure 2). The proportion of wells

containing SA tetramers did not alter substantially as well size changed. However, the *number* of SAs per well did change as well size was altered. The yield of single-SA capture in two-turn wells was 93%, but for three- and four-turn wells it was only 70 and 40%, respectively. In fact, the proportion of wells containing two SA tetramers increased as the size of the well became larger. The yields were 4.3%, 26%, and 59% for two-, three-, and four-turn wells, respectively. Such a trend agrees well with the expected distance between the ligands in a well. The calculated distance between the ends of the DNA portion in anchor strands is 6.8 nm for two-turn wells, 10.2 nm for three-turn wells, and 13.6 nm for four-turn wells, while each of the biotin residues is connected to the DNA portions via a 2.3 nm-long linker. The ligand-ligand distance in two-turn wells is thus expected to be 2.2 nm. This number is sufficiently small for the distance between the biotin binding sites (ca. 2 nm)[16] on a SA tetramer, to allow bidentate co-operative binding of a tetramer in a well. Expected ligand-ligand distances in three-turn wells (5.6 nm) and four-turn wells (9.0 nm), in contrast, seem to be too large for such bidentate 1:2 SA-biotin binding and more appropriate for 1:1 binding.

In comparison with SA, the capture yield of IgG was not as dependent on the size of the wells. Both two and three-turn wells showed 77-78% capture yields, and that of four-turn wells was approximately 67%. In IgG the antigen binding domain, Fab and the body of IgG, Fc are connected via a hinge structure, and thus the distance between the epitopes is flexible. According to a previous report,[17] the binding constant of IgG is above 10^8 when the epitope-epitope distance is 4.8–11.1 nm whereas it decreases to 10^7 when the distance is increased to 13.7 nm. This may be the main reason for the decrease in the capture yield observed above, since the calculated distance between the introduced fluorescein is below 11.1 nm in two- and three-turn wells, but is just 13.6 nm in a four-turn well. Few wells were found containing two IgG molecules while a significant number of four-turn wells captured two SA molecules. This may be also a consequence of the flexibility between the epitopes.

Some IgG molecules were found at the edges of DNA waffles where the ends of the helices are aligned. This is probably due to nonspecific interactions between IgG and multiple T4 loops attached to the edges to prevent stacking interactions between the motifs. Omission of the T4 loops consistently reduced nonspecific IgG binding.

Overall, we conclude that the best size for single-molecule SA-capture in a well is two helical turns and for IgG, three helical turns are adopted.

Orthogonal Heteroarraying of Two Proteins

We next examined orthogonal heteroarraying of SA and IgG on biotin and FAM dual-modified diagonally patterned DNA waffles (Figure 3). These DNA waffles have three-turn wells in five diagonal positions (wells #1, 3, 5, 7, and 9), and two-turn wells in the remaining four rhombic positions (wells #2, 4, 6, and 8). The three-turn wells were modified with FAM for IgG capture, and the two-turn wells with biotin for SA capture. Figure 3a is a typical AFM image of a SA nanoarray formed on the DNA waffle. As expected, bound SA tetramers were only found in biotinylated wells in rhombic positions with comparable yield with that in Figure 2a. Similarly, IgG nanoarrays forming diagonal patterns were clearly imaged by AFM (Figure 3b). Combination of biotin modification in the rhombic positions and FAM modification in the diagonal positions gave orthogonal heteroarraying of SA and IgG (Figure 3c). Here, SA and IgG were simultaneously added to the solution of dual-modified DNA waffles. The yield of IgG (ca. 55%) was lower than those in Figure 3c and the corresponding system in Figure 2b, possibly because of steric hindrance from multiple proteins.

Construction of Enzyme Heteroarrays on DNA Waffles

The advantage of using multiple kinds of protein-ligand interactions is that it also enables one-step arraying of two or more kinds of enzymes on a DNA origami scaffold. To show a practical application of the present system, we prepared an enzyme-heteroarray of horseradish peroxidase (HRP) and alkaline phosphatase (AP) by using HRP-SA and AP-IgG conjugates together with the biotin and FAM dual-modified diagonally patterned DNA waffles used above.

Four kinds of mixture in total were prepared: a mixture of biotin and FAM dual-modified DNA waffles and the enzyme conjugates (sample H), a mixture of unmodified DNA waffles and the enzyme conjugates (N), a simple mixture of the enzyme conjugates (E), and a blank buffer solution (Figure 4a). After excess staple strands were removed from a 10 nM solution of appropriately modified DNA waffles by using micro spin columns packed with GPC media (Sephacryl S-400HR), 1.2 eq. of HRP-SA and AP-IgG conjugates to the wells were added to the solution. Unbound enzyme conjugates were then removed from the solution by again using micro spin columns. The resulting solution was divided into two portions and either of the specific substrates (Amplex UltraRed for HRP or *p*-nitrophenylphosphate (*p*NPP) for AP) was added. Enzymatic activity was monitored by observing fluorescence (for HRP, Figure 4b) or absorbance (for AP, Figure 4c). In both of the systems, sample H exhibited the highest activity, showing that an enzyme heteroarray was successfully formed on the DNA waffles. While, as expected, the HRP activities of N, E, and blank were very low representing successful removal of unbound enzyme conjugates, the AP activities of

N and E were relatively high compared to the blank, probably due to nonspecific binding of AP-IgG to the edges of DNA waffles in the case of sample N (vide ante). Alternatively, the high molecular weight of AP-IgG (244 kDa) may prevent complete removal from the systems in the gel-filtration step in the case of samples N and E.

Conclusions

A new DNA origami motif with nanometer-sized variable wells has been successfully constructed. The most suitable well size depends on the target protein; the best size for SA capture was two turns, whereas two- or the three-turns was the best for IgG. Capture of each protein is a completely independent process, and heteroarraying of SA and IgG on a DNA waffle was successfully achieved by using appropriate anchor strands. Introduction of IgG for protein nanoarraying on DNA origami scaffolds opens up vast opportunities in the field since antibodies for almost any kind of biochemical compounds are available. Arraying diabodies, in which two Fv fragments, one recognizing a target enzyme and the other an antigen on DNA waffles, are fused, may allow us to realize direct heteroarraying of unmodified enzymes on DNA waffles. Application to rapid and simple detection and imaging of toxins or marker proteins may be feasible.

Methods

Materials.

Unmodified staple strands were purchased from Integrated DNA Technologies (IA, USA) and used without further purification. Fluorescein and Biotin-TEG modified anchor strands were chemically synthesized using appropriate phosphoramidite monomers (Glen Research, VA), and purified by Glen-Pack (Glen Research, VA). M13mp18 ssDNA (Takara, Japan) was used for the DNA origami scaffold. Amino acids for synthesizing the His tag peptide were purchased from Novabiochem (CA, USA). Streptavidin (SA) was purchased from Sigma (MO, USA), horseradish peroxidase–streptavidin (HRP-SA) conjugate was purchased from KPL (MA,USA), and anti-FITC antibody (IgG) and alkaline phosphatase-anti-FITC antibody (AP-IgG) conjugate were purchased from Jackson ImmunoResearch (PA,USA) . 4-Nitrophenylphosphoric Acid Disodium Salt (pNPP) was purchased from TCI (Japan), and Amplex UltraRed was purchased from Invitrogen (UK). SYBR Green I nucleic acid gel stain was purchased from Roche (Switzerland).

Formation of DNA origami.

DNA waffles were formed with M13mp18 ssDNA as the scaffold (5 nM for AFM imaging and 10 nM for enzyme nanoarray) and staple strands (50 nM and 100 nM of each strand including anchor strand, for AFM imaging and for enzyme nanoarrays, respectively) in a solution containing 40 mM Tris, 20 mM acetic acid, 2 mM EDTA and 12.5 mM magnesium acetate (1X TAE/Mg^{2+} buffer, 50 μL). This mixture was cooled from 90 **°C** to 25 **°C** at a rate of -1.0 **°C**/min using a PCR thermal cycler.

Preparation of streptavidin or antibody nanoarrays.

The annealed mixtures (20 μL) were purified using a gel-filtration microspin column equilibrated with 1 × TAE/Mg^{2+} buffer (Microspin S-400HR, GE Healthcare, UK) to remove excess staple strands, and SA (3 eq. to the number of biotinylated wells) or IgG (1.5 eq. to the number of fluorescein labeled wells) were then added to form the protein nanoarray. For the IgG array, the mixture was kept at r.t. for at least 20 min after the addition of IgG.

AFM imaging.

AFM imaging of the DNA origami was performed on a SPA-300HV system (SII, Japan). The DNA origami solution (2 μL) was deposited onto freshly cleaved mica, and additional 1X TAE/Mg^{2+} buffer (200 μL) was added. Imaging was carried out using the fluid DFM scanning mode.

Purification of enzymes nanoarrays.

The annealed mixture of DNA waffles with or without anchor strands were purified by using a gel-filtration microspin column equilibrated with 1 × TAE/Mg^{2+} buffer (Microspin S-400HR, GE Healthcare, UK) to remove excess staple strands, AP-IgG (for 1, 3, 5, 7, 9 wells) or HRP-SA (for 2, 4, 6, 8 wells) conjugates (1.2 eq. to the number of biotinylated wells) were added to the solution and the mixture was kept at r.t. for 1h. DNA waffles were purified using a gel-filtration microspin column equilibrated with 1 × TAE/Mg^{2+} buffer (Microspin S-400HR, GE Healthcare, UK) to remove non-immobilized enzymes. The enzymatic activity was examined at r.t. with 10 μL of filtrated solution and 20 μL of substrate solution (5 μM pNPP in 1X TAE/Mg^{2+} for AP, and 5 μM Amplex UltraRed and 5 μM H_2O_2 in 1X TAE/Mg^{2+} for HRP). The reaction mixture was quantified using a microplate reader (SpectraMax M4 (Molecular Devices, Japan)) with absorption wavelength of 405 nm for pNPP and a combination of excitation/emission 530 nm/590 nm for Amplex UltraRed. The activity of the filtrated solution from the mixture of enzymes containing DNA waffles without anchor strands was taken as unity. The enzymatic activity was normalized with the amount of purified origami quantified by the fluorescent intensity of SYBR Green.

Acknowledgements

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Figure Legends.

Figure 1. Scaffold folding pathway of a DNA waffle. a) Wells are numbered 1-9. Gray areas on both sides of the wells are "tunable blocks" that can accommodate fill strands. b) Folding pathway for diagonally patterned DNA waffle. Pink and blue strands indicate fill strands **1** and **2** respectively. Fill strand **1** is introduced to the left side of well #2, and the right side of wells #2, #4, #6–#9, whereas **2** is introduced to the right side of wells #1, #3, and #5. c) Folding pathway for column-patterned DNA waffle.

Figure 2. Determination of appropriate size of wells for single-molecule SA and IgG capture. a) An AFM image of a SA nanoarray on column-patterned DNA waffles. Wells #2, #4, #6, #7 and #9 are biotinylated. b) Percent of wells containing indicated number of SA particles. Green, red, and blue portions in the bars represent wells with no protein, two proteins, and one protein, respectively. c) An AFM image of IgG nanoarrays on column-patterned DNA waffles. Fluorescein was attached to the wells in place of biotins in (a). d) Percent of wells containing indicated number of IgG particles. The color codes are the same as in (b).

Figure 3. Protein nanoarray on diagonally patterned DNA waffles. a) Rhombic SA nanoarray obtained with biotinylation in wells #2, #4, #6, and #8. b) Diagonally patterned IgG nanoarray with fluorescein modification in wells #1, #3, #5, #7, and #9. c) SA and IgG heteroarray with simultaneous modifications shown in (a) and (b).

Figure 4. Assembly of enzyme heteroarray on DNA waffles. a) Schematic illustration of the purification process. A mixture of modified DNA waffles and HRP and AP (H) was compared with a mixture of unmodified DNA waffles and the enzymes (N) or with a simple mixture of the enzyme conjugates (E). b) Time-dependent fluorescence changes as a result of the reaction of Amplex UltraRed by HRP after gel filtration. Blank represents a control reaction in the enzyme-free buffer solution. c) Time-dependent absorbance change as a result of the reaction of pNPP by AP after purification.

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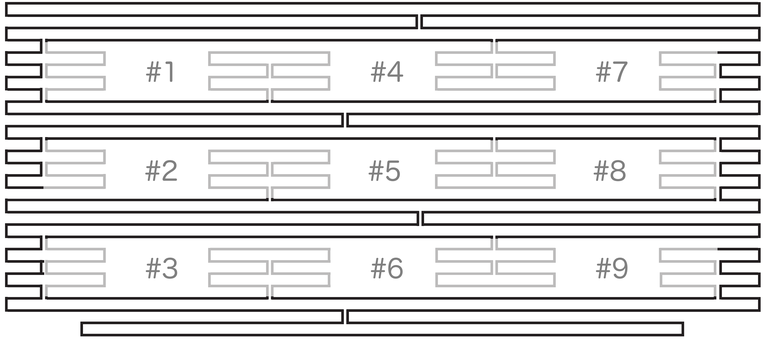

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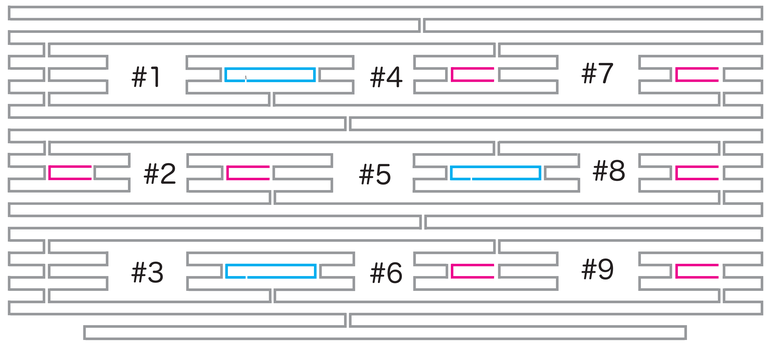

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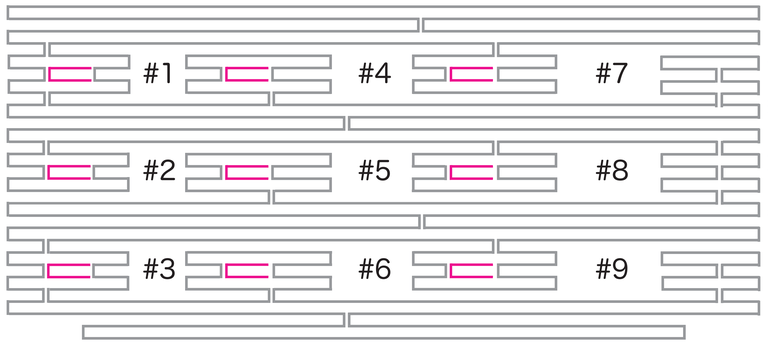

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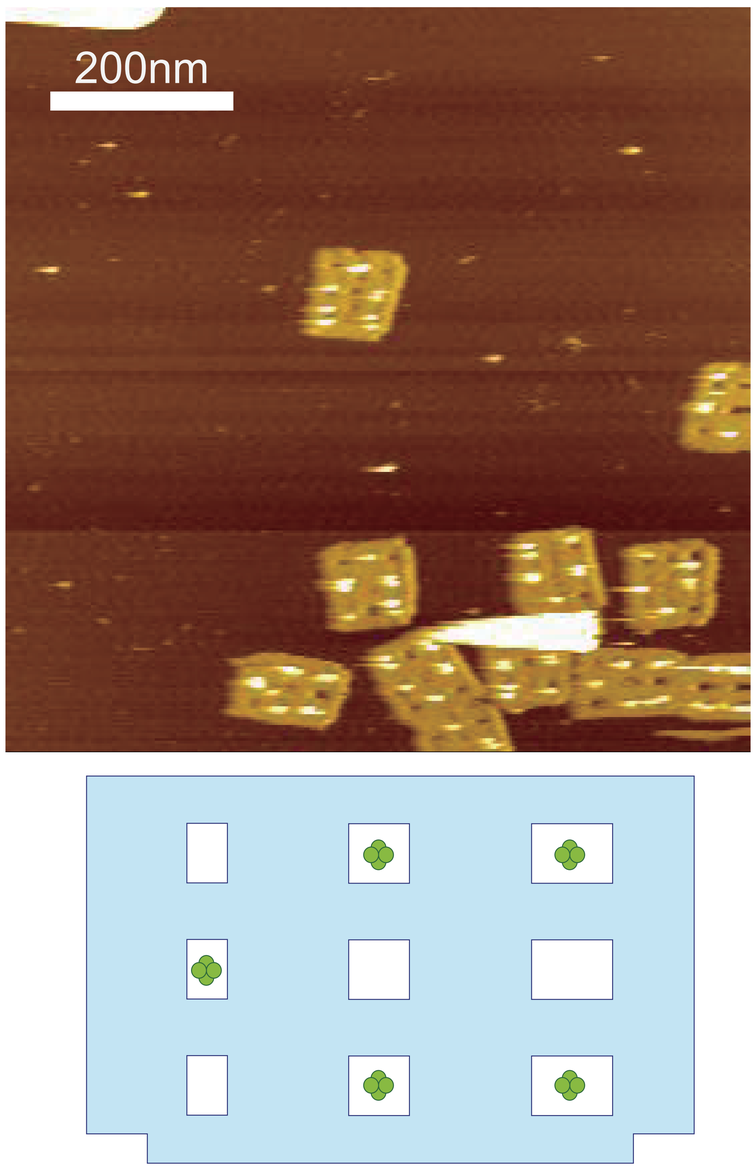

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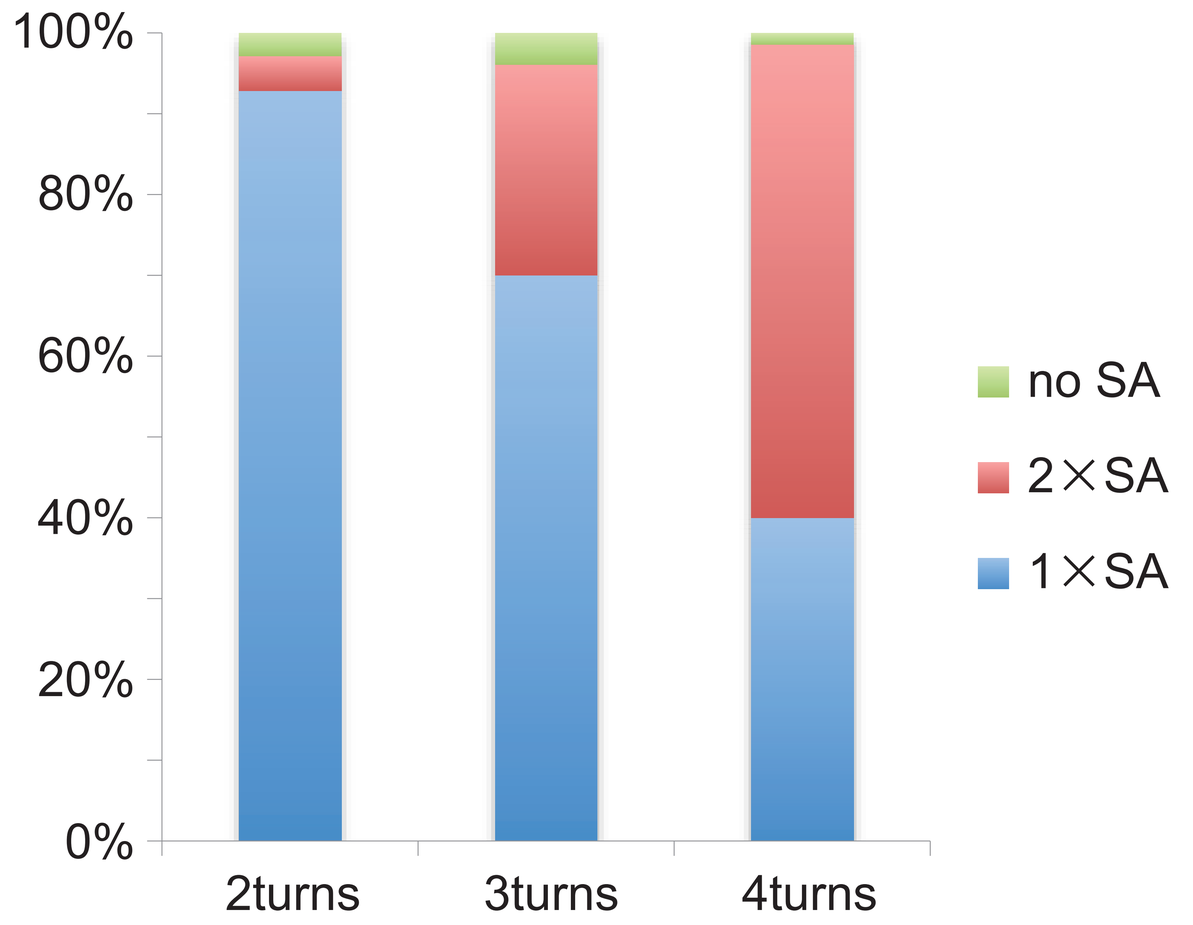

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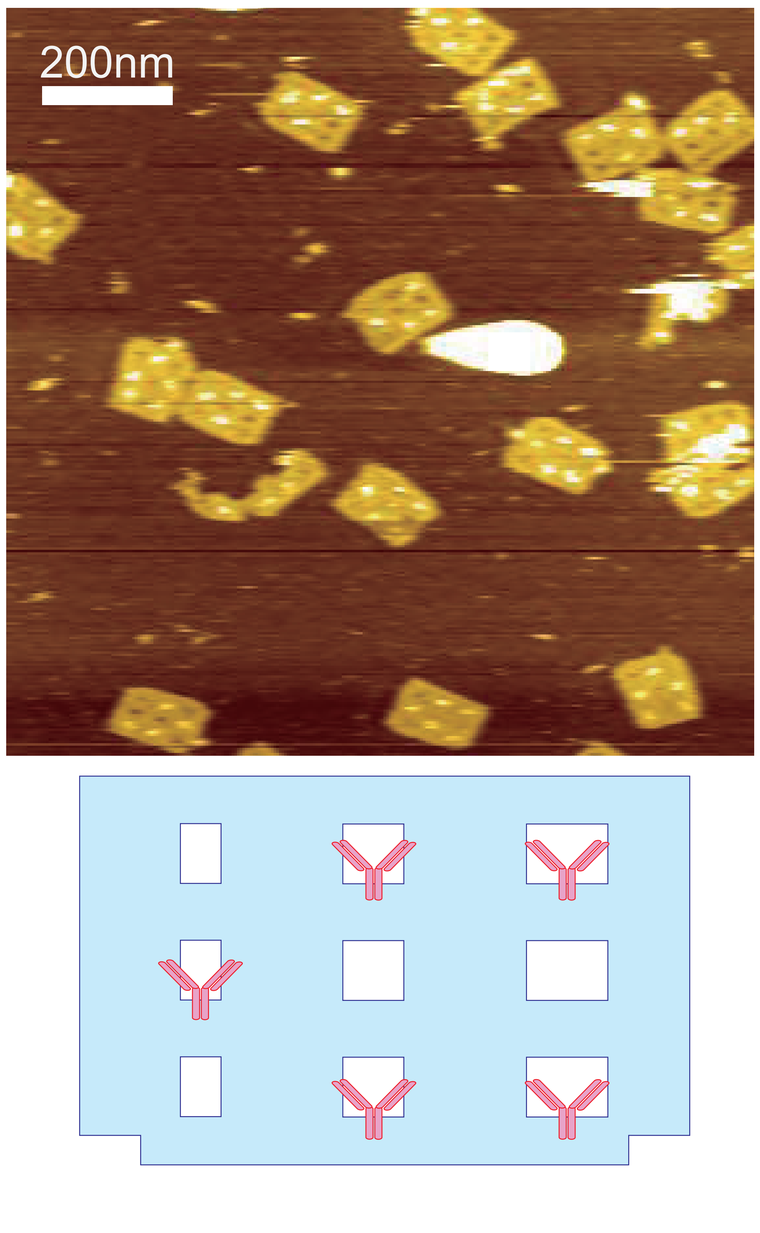

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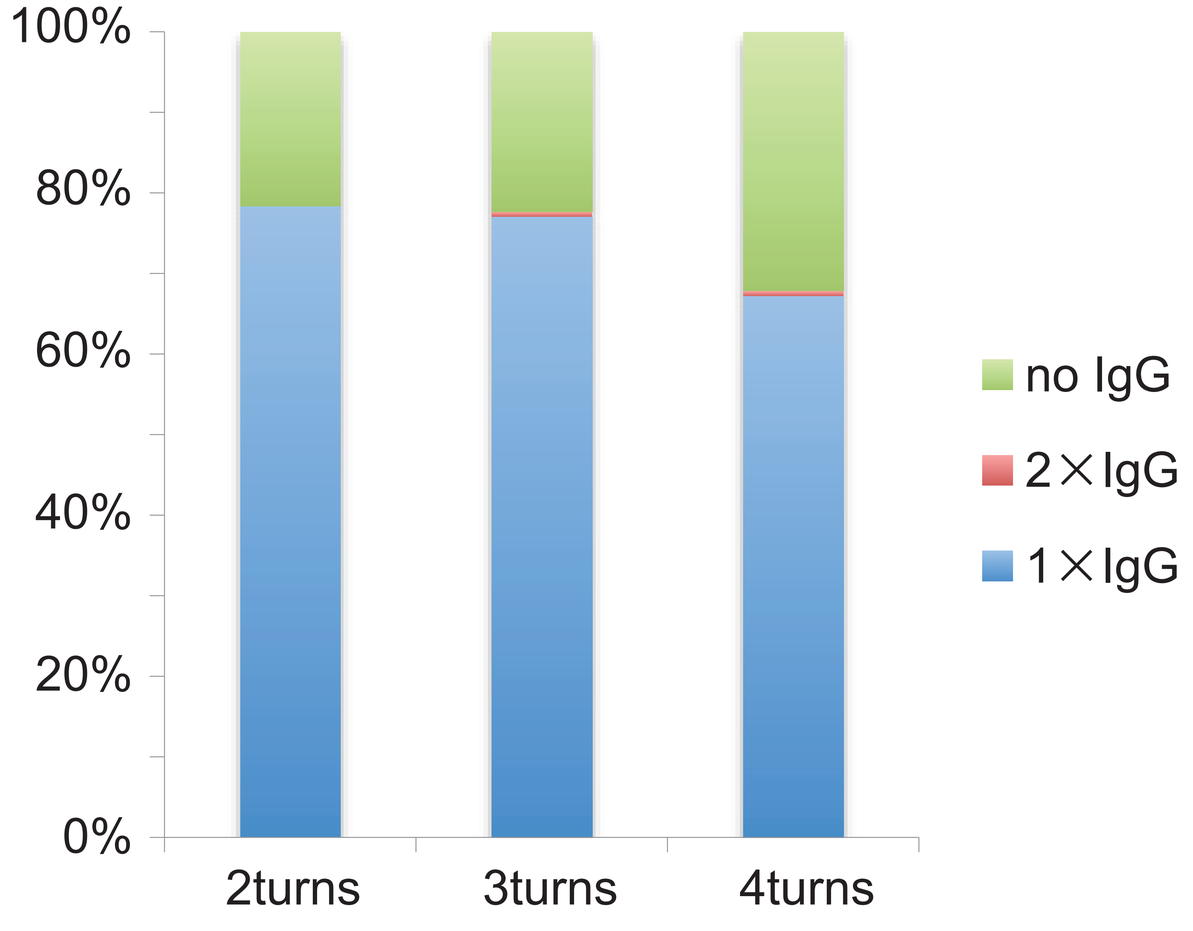

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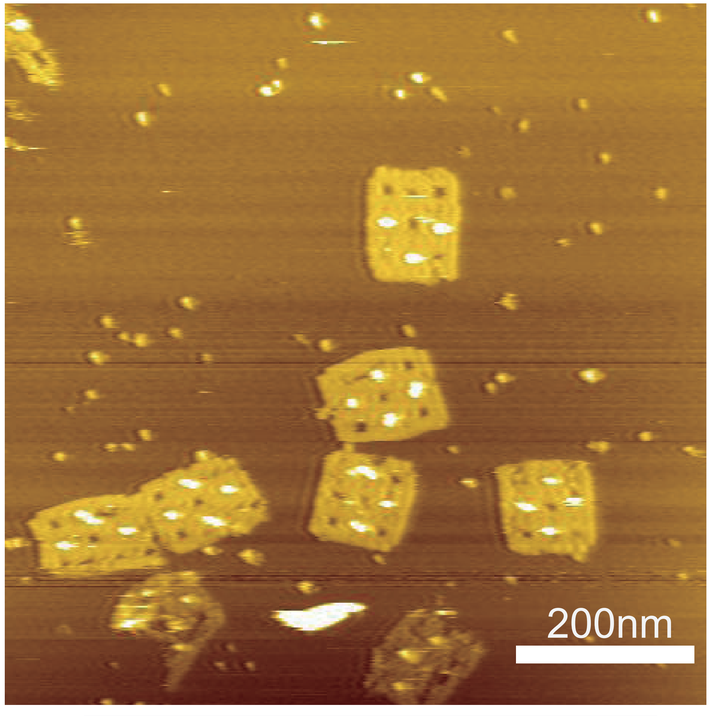

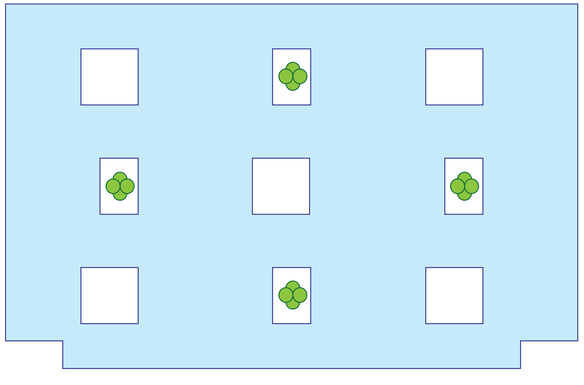

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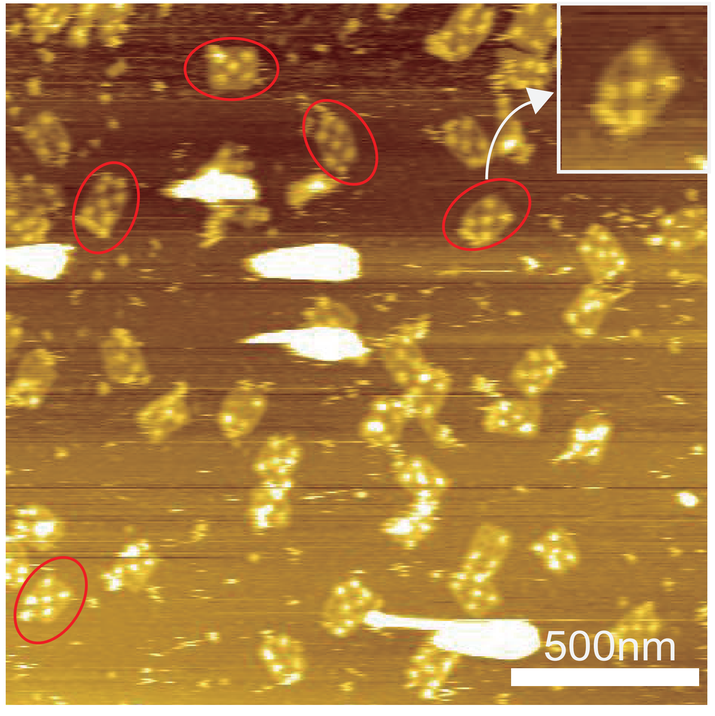

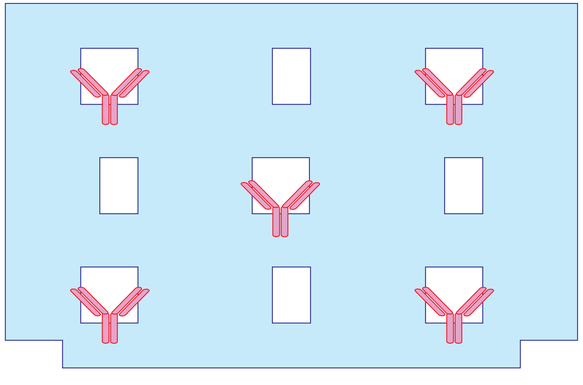

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