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DNA origami structures as calibration standards for nanometrology

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Abstract

In this work we have studied the feasibility of DNA origami nanostructures as dimensional calibration standards for atomic force microscopes (AFMs) at the nanometre scale. The stability of the structures and repeatability of the measurement have been studied, and the applicability for calibration is discussed. A cross-like Seeman tile (ST) was selected for the studies and it was found suitable for repeatable calibration of AFMs. The height of the first height step of the ST was 2.0 nm. Expanded standard uncertainty ($k = 2$) of the measurement U_c was 0.2 nm. The width of the ST was 88 nm and width of its arm was 28 nm with $U_c = 3$ nm. In addition, prepared dry samples were found out to be stable at least for 12 months.

Keywords: atomic force microscopy, DNA nanotechnology, DNA origami, self-assembly, calibration

(Some figures may appear in colour only in the online journal)

1. Introduction

Metrological traceability to the SI units is needed in all quantitative measurements. Typically atomic force microscopes (AFMs) are calibrated using calibrated grating pitch and step height standards [1–3]. Standards are commercially available for large scale, step height >6 nm and pitch >150 nm. For measurements truly on nanometre scale, new types of standards are needed. Self-assembled objects such as crystalline structures, polymers and DNA nanostructures (see below) are interesting new types of structures for calibration of AFMs. In addition to increased accuracy they could provide completely new calibration methods. For example, if the calibration structures with known dimensions can be mixed with other samples having unknown dimensions, the measurements can be carried out even with uncalibrated instruments, and *in situ* calibration can be performed during the analysis.

DNA molecules can be used for bottom-up design of custom and accurate nanoscale structures [4]. During recent years, the structural DNA nanotechnology has witnessed a great expansion of the shape space, and for example platonic solids, lattice-like structures, hollow containers and functional objects have been introduced [4–6]. One of the most common techniques to build with DNA is dubbed a DNA origami, which enables fabrication of complex 2D [7] and 3D [8–11] DNA structures with precisely defined dimensions. The software and techniques for designing such structures have also evolved, and very recently, effortless and fully automated procedures have been introduced (progress in software reviewed in [12]). Furthermore, the origami method allows complex molecular patterns to be created with an outstanding resolution from few nanometres even down to tens of picometres [5, 13, 14]. Due to the structural addressability, DNA origamis could serve as templates for example in molecular electronics [15, 16], plasmonics [17] and in various biological applications, for

example as drug-delivery vehicles or molecular devices [6, 18–20]. DNA origamis with specific and accurate fluorophore attachment techniques (such as DNA-PAINT and Exchange-PAINT [21]) could be utilized in calibration of optical microscopes to achieve super-resolution images [22, 23]. In general, bottom-up methods are urgently needed for the development of novel and refined calibration standards. There are also commercially available simple DNA test samples for AFMs, for example DNA01, which is merely a linearized plasmid that can be used as a rough tester in imaging of biological samples [24]. However, these samples have not been tested for actual calibration of AFMs, and apparently the samples do not have traceability to the metre, or the traceability chain is unknown.

In general, the DNA origami technique enables formation of almost arbitrary and readily functionalizable two (2D) and three-dimensional (3D) shapes on nanoscale via molecular self-assembly of DNA strands (Watson–Crick base-pairing of complementary DNA sequences) [5]. In detail, the scaffolded origami method is based on folding a long viral single-stranded DNA (scaffold strand) into a desired shape by stapling it with tens of short single-stranded DNA oligonucleotides. Thus, in fact, the structure effectively consists of interconnected double-stranded DNA (dsDNA) domains. It has been shown that molecules can be placed with Bohr radius resolution using specific DNA origami device [14] and that the degree of structural order can reach the similar level to that found in proteins [13, 14].

For the scanning probe calibration purposes, DNA origami is an interesting candidate. As discussed above, it has accurate dimensions truly on nanoscale, it is straightforward to fabricate with simple equipment, the structures are formed through a self-assembly process enabling highly parallel fabrication of the numerous structures, and moreover, the method allows also larger periodic structures to be created. In general, for the calibration of AFM, the size of a DNA origami object plays an important role. The main factor that limits the overall size of the common scaffolded origami is the length of the long single-strand. In principle, origamis could be formed using various single-stranded scaffolds as sources, but so far the most reliable results have been obtained with a commercially available genome of a virus M13mp18 (7249 nucleotides) or slightly extended versions of it [5, 10]. To give an idea about the dimensions of a single DNA origami structure based on a M13mp18 scaffold, a folded cube would be roughly 25 nm × 25 nm × 25 nm in size. In addition, the geometry of a double-stranded DNA itself sets fundamental structural limitations for the calibration purposes. The plain dsDNA helix diameter of a B-form DNA is 2.0 nm and the helix rise per each base pair (bp) is 0.34 nm. However, the effective dsDNA helix diameter in a multi-layered origami structure is theoretically 2.25 nm on average (calculated using transmission electron microscopy (TEM) data and simulation models [10]), because the negatively charged helices tend to repel each other. Thus, the theoretical minimum volume unit in a simple DNA origami design is a cylinder with a diameter of 2.25 nm and a length of 0.34 nm. In addition, the theoretical 'sculpting resolution' of origami architectures depends on the

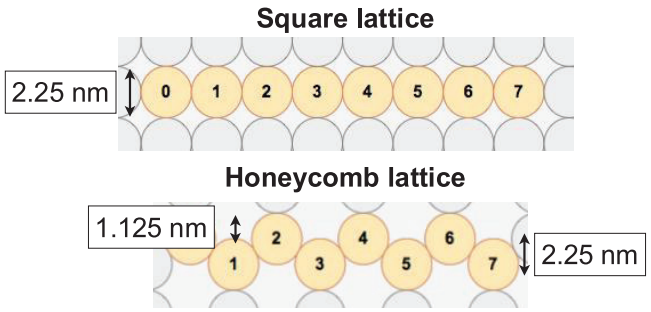

Figure 1. Dimensions and step sizes in square- and honeycomb DNA lattices. Each cylinder represents a cross-section of a double-stranded DNA helix.

packing of the adjacent helices in lattices (see figure 1): In simple cases, if one considers a plate-like structure consisting of multiple layers of helices, each layer is exactly 2.25 nm thick in the case of square lattice packing, or on average 2.25 nm in the case of honeycomb lattice with the 'step size' between adjacent helices being half of that, i.e. 1.125 nm (hexagonal symmetry of the helices: neighboring helices at 120° angles). Single-layer structures, for one, should appear as 2.0 nm thick on the substrate, given by the helix diameter of a B-form DNA.

The real challenge in exploiting DNA-based structures as calibration standards is the softness and flexibility of DNA molecules. By forcing a long scaffold strand into a well-defined shape via crossovers between the dsDNA domains, one can build rigid and stable DNA-based objects, but in any case, the structure constantly fluctuates in a solution. For calibration purposes the structure should be as rigid and hard as possible, but on the other hand the increasing rigidity obtained by making the object thicker may reduce the length of the single object. In AFM imaging, a large and nearly uniform step area provides plenty of measuring points for calibration, and therefore the dimensions of the flat area (step) of an origami-based structure should be much larger than the tip apex radius (area preferably $> 20\,\text{nm} \times 20\,\text{nm}$) in order to get reliable and statistically valid results. Therefore, the demand regarding a large enough step area sets rather strict limitations for the height of the possible 3D origami structure.

In addition, DNA is an environment sensitive molecule; DNA can adopt various conformations depending on the pH and humidity level, which also might limit its use as a calibration standard. For single DNA molecules it has been observed that some amount of salt ions and water molecules are usually needed in order to sustain the B-form of the double-stranded helix. If one considers a DNA structure lying on an AFM substrate, also the substrate itself can induce twisting, bending or other deformation of the helices. However, DNA origami objects can be utilized in producing custom-shaped, entirely metallic nanostructures [25]. These structures are naturally more resistant on wear, and by further optimization of the fabrication procedure, the current patterning resolution could be increased. Such structures could be then as well used for lateral calibration purposes (however, the temperature has to be controlled in order to avoid dilation).

2. Samples

2.1. The requirements for calibration standards

There are several special requirements for the structures if they are used as dimensional calibration standards. The structures should have the designed shape and defective or deformed structures should be easily distinguishable in AFM images. The geometrical structure should be suitable for calibration purposes and the calibration units should be evenly deposited on the substrate. For step height calibration, large enough flat areas are required in order to enable sufficient number (>20 points per area) of independent measurement points for analysis. The characterized material should be rigid enough and not affected by the measurement. For *XY* scale calibration, large periodic defect-free structures with sharp edges are needed. The structures should be uniform and the structures should allow repeated measurements without significant wear of the structures.

For calibration purposes the stability of the structures is indeed one of the most important requirements. Prepared samples should be stable for several days to allow repeated measurements. To allow commercial production and delivery, the samples should be stable for more than a year in liquid media. DNA origami samples could be frozen (snap-freezing with liquid nitrogen) and on the other hand heated up to 50–60 °C [26] without losing their structural integrity. Moreover, DNA origami structures (in aquatic solution) are stable for months at room temperature, and therefore the deposition can be freshly performed before each calibration. In general, fabrication of DNA origami nano-objects is relatively expensive (1 g costs roughly 100 000 euros), but for the calibration purposes, sparse coating of small substrates can be considered as a low-cost method. Coating of the substrates can be furthermore enhanced by spray-coating [27], resulting in the price of ~10 euros m^{-2} (with relevant coating densities for the calibration purposes).

Most of the AFM users are not necessarily experts on preparing samples of DNA nanostructures. Therefore, the samples should be relatively easy to handle, i.e. with generally available instruments and written instructions, and without specific knowledge about fabrication techniques of DNA objects. Moreover, the sample preparation should be highly reproducible.

By taking all the abovementioned issues into account, we selected a so-called Seeman tile (ST) [28] for testing the feasibility of DNA structures as calibration standards. The cross-like structure is shown in figure 2. The structure has two height steps of 2.0 ± 0.1 nm for *Z*-axis calibration (assuming the helices in the structures maintain their helix diameter on the substrate as in [28, 29]). The flat areas are ~30 nm × 30 nm.

2.2. Production

The DNA origami structures were fabricated using commercially available DNA strands (scaffold strands and synthetic staple strands). All the folding parameters are given in the [27, 28]). Briefly, the origamis were fabricated in 100 μl quantities including 20 nM M13mp18 scaffold strand (Tilibit Nanosystems) and 200 nM of staple strands (IDT) in 1 × TAE buffer (40 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid (EDTA), and acetic acid for adjusting pH to 8.3) with 12.5 mM Mg^{2+}. The sample solution was slowly annealed from 90 °C to 27 °C using a following thermal ramp: (1) From 90 °C to 70 °C: 0.2 °C decrease/8 s. (2) From 70 °C to 60 °C: 0.1 °C decrease/8 s. (3) From 60 °C to 27 °C: 0.1 °C decrease/2 min. (4) Store at 12 °C. After this, the quality of folding was verified by using agarose gel electrophoresis and/or TEM imaging. Furthermore, the excess staple strands can be efficiently removed from the solution by spin-filtering (1 × TAE buffer with 20 mM Mg^{2+}). This results in purified high-quality samples with intact DNA origami nanostructures.

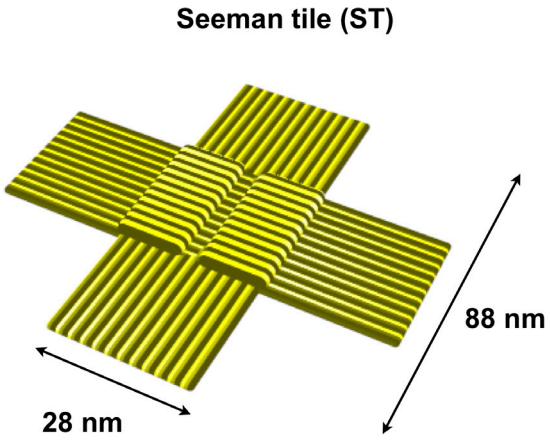

Figure 2. A schematic view and the dimensions of the Seeman tile (ST) [28]. Figure is reproduced from [27]. Published by Nature Publishing Group, 2015.

2.3. Sample preparation

For the AFM characterization, the samples were deposited on mica substrates. The sample preparation is straightforward: The stock solution (~10 nM origami, 20 mM magnesium in the buffer after spin-filtering) was diluted 10–20 fold in order to get a suitable coverage. A droplet of the diluted solution was pipetted on a freshly cleaved mica substrate. The sample was incubated 1 min on the substrate, and after that the sample was rinsed 1–3 times with a droplet of Milli-Q or double-distilled water. Finally the samples were dried using nitrogen gas or compressed air. After the samples were dried, they were ready for AFM imaging. A typical prepared sample is shown in figure 3.

3. Measurements and results

3.1. Measurement instrument and traceability

The measurements were done with a calibrated PSIA XE-100 AFM [3] using non-contact mode (so called true non-contact mode). Intermitted contact mode was also tested, and the obtained differences are discussed below (see section 3.3). Several different types of AFM tips were tested for the

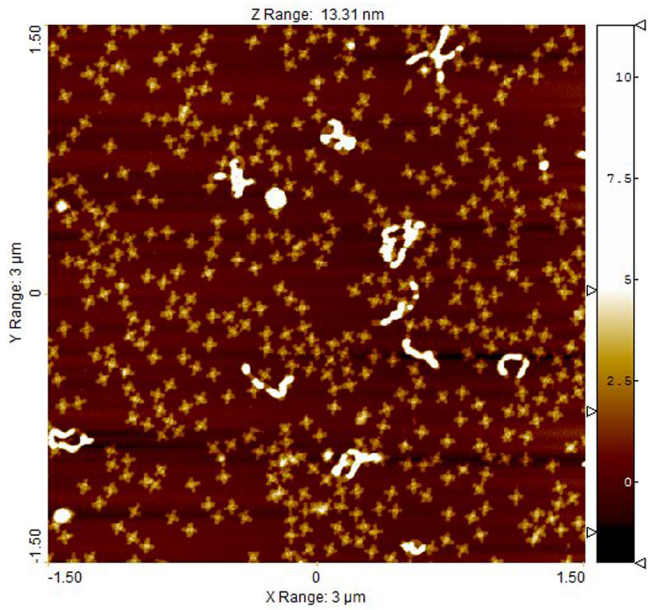

Figure 3. AFM image of a typical sample with fairly homogeneous coating.

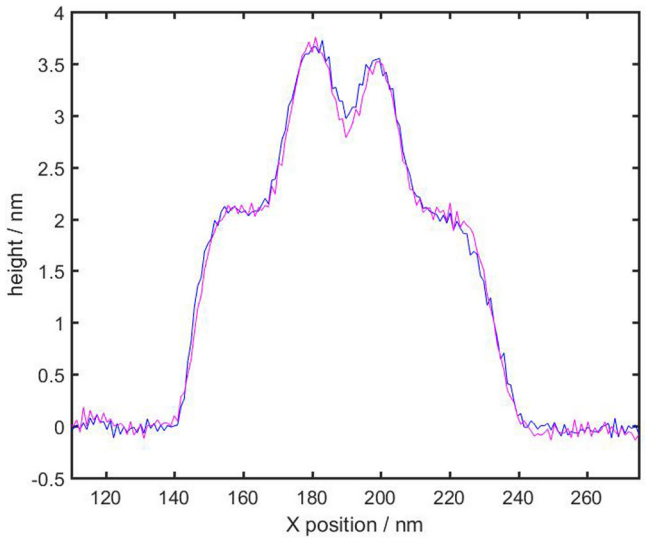

Figure 4. Two line profiles from the repeated measurements. Blue line is measurement number 1 and pink line is measurement number 20.

measurements. The results given in this paper are measured with super sharp SSS-NCHR and diamond AFM Probe ART D160. The noise level in the measurements was minimized using optimized parameters (set point 217 nm–227 nm, amplitude 1.5×10^3 nm–1.6×10^3 nm). The samples are soft and thus not suitable for contact mode measurements.

Tip shape was estimated by combining different methods: Measurements of a tip characterizer with sharp peaks (TGT), assuming the edges of DNA origami being cylindrical and using information given by the manufacturer. The uncertainty of the tip shape is the main uncertainty component in the measurements of the lateral dimensions. Differences in probe-sample interaction on substrate and DNA were not studied.

The AFM was traceably calibrated [3]. The *Z*-axis was calibrated using step height standards of three different heights. The calibration standards were calibrated with MIKES interferometrically traceable metrological AFM (IT-MAFM) [30]. *XY*-scales were calibrated using grating pitch standards. The standards were calibrated with MIKES laser diffractometer [31].

3.2. Stability of the structures

The stability of the structures was studied by measuring the samples repeatedly. Due to a slight drift ($<$20 nm h^{-1}) caused by thermal expansion in the instrument frame the maximum number of repeated measurements was limited to 20. The effect of the drift on the measured dimensions is negligible, but in time, structures drift out from the measurement area. The shape or dimensions did not change during the repeated measurements. A line profile of one of the ST structures is shown in figure 4. Measurements number 1 and 20 are shown in the same figure.

The prepared samples were measured just after the preparation and again 6 and 12 months later. The samples were stored in a closed box in temperature and humidity controlled laboratory conditions (temperature 20 °C $\pm$ 0.1 °C, relative humidity 46% $\pm$ 1%). The DNA structures characterized after 6 and 12 months were randomly selected from the same sample that was initially measured; in other words, find-me-structures were not used on the substrate. However, one can assume that all the deposited DNA structures have same properties throughout the substrate. Indeed, no differences were seen in the number of defective structures between the measurements, and the differences in origami dimensions were insignificant; the differences were actually smaller than the standard uncertainty of the measurements, and especially for lateral dimensions they were smaller than the uncertainty caused by the determination of tip size (different tips were used in the measurements).

3.3. Step height measurement

Step height of the ST structures was analyzed using a histogram method. This method was selected, because the ISO 5436 method cannot correctly detect the double-steps. Tilt and drift was corrected on substrate without the ST structures. Gaussian filtering was used in order to reduce the effect of noise in the measurement. One of the analyzed AFM images is shown in figure 5. The height histogram calculated from the image is shown in figure 6. A clearly visible peak is seen on the height of the first step. The height of the first step is 2.0 nm with expanded standard uncertainty ($k = 2$) $U_c = 0.2$ nm, which matches well with the theoretical value of 2.0 nm (helix diameter in B-form DNA) The main uncertainty component is statistical noise in the measurement. The uncertainty caused by the instrument calibration is $<$0.02 nm. Intermitted contact mode results are about 0.2 nm lower than noncontact mode results. The effect of the probe sample interactions or measurement force is assumed to be clearly smaller than the difference between the non-contact and intermitted contact mode results. Guidelines given in the Guide to the expression of Uncertainty

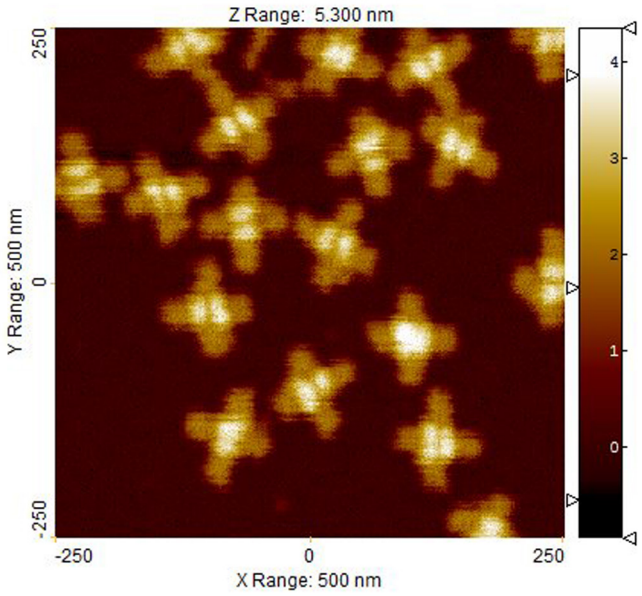

Figure 5. An AFM image of the ST structures.

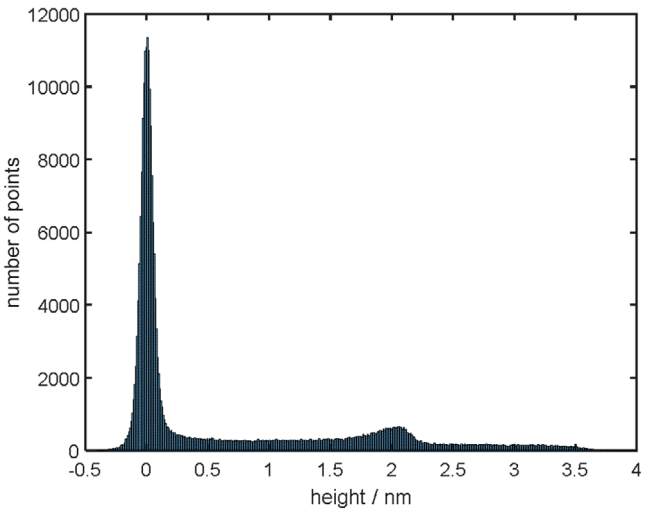

Figure 6. Histogram analysis of the AFM image shown in figure 5.

in measurement (GUM) [32] are used in analyses of the simplified uncertainty budget. The second step is a double-step (see figures 4 and 5) and its height seems to be 0.3–0.5 nm lower than the theoretical value. Therefore, the second step in ST structures is not suitable for accurate calibration.

3.4. Lateral dimensions

Width of the arms and width of the whole ST structure were also characterized. The width was measured manually using only structures which were aligned so that the measurement could be done in *X* direction to avoid the effect of drift in the measurement. Clearly defected structures were not included in the analysis. Selection of the defected and measured structures was partly subjective. Some of the arms appeared slightly narrower or wider but they were still measured. The selection of measured structures affects directly the standard deviation of the measurement. The selection was reasoned, because we did not have unambiguous criteria for defective structures, and underestimation of the effect of variations was deliberately avoided.

The width of the arms was 28 nm and the width of the whole ST structure 88 nm with expanded standard uncertainty ($k = 2$) $U_c = 3$ nm. The main uncertainty contribution was determination of the tip shape. Uncertainty component caused by instrument calibration was <0.1 nm. Standard deviation of the measurement was 3.5 nm, which is rather a standard deviation of the sample dimensions than a standard deviation of the measurement. The simplified uncertainty budget was analysed following guidelines given in the Guide to the Expression of Uncertainty in Measurement (GUM) [32].

4. Discussion

In this article, we have shown that single-layer DNA origami structures such as Seeman tiles could serve as feasible calibration standards with effortless sample preparation. No special instruments are needed for that, and the deposition routinely yields well-separated and intact DNA origami structures on the mica surface. The obtained sample uniformity was high; the number of defective structures was very low ($<10\%$) and these structures were easy to detect. In addition, the cross-like structure is partially self-referring. It can be easily observed if the arms do not have the same width. Improper folding of the DNA origami structures may yield to defected structures, but most probably the observed deformations are caused by the interaction between the flexible structure and the substrate, similarly as seen in [16, 28, 29], in which flexible 2D origamis (Seeman tiles [28] and rectangles [29]) and various 3D origamis (straight bricks in different lattice geometries, L-shaped and C-shaped origamis) [16] are AFM-imaged on different substrates (including mica). In some cases, even the rigid 3D origamis can bend due to the interactions between the substrate and the origami [16]. In this work, the studied ST structures were found to be relatively stable. The prepared dry samples can retain their shape at least for 12 months. The differences between measured dimensions after 6 and 12 months were smaller than standard uncertainty of the measurements. No degradation was observed in the measured samples when stored in temperature and humidity controlled laboratory conditions.

Usually lateral calibration of microscope is carried out using large periodic structures. Average pitch can be used to calibrate large scales, and also non-linearity of the scale can be detected. Single structures do not allow decent averaging, which limits their use in scale calibration of the instrument. Despite the quite large standard deviation of the lateral dimensions (errors in the dimensions are 10% or less), ST samples could be used in rough calibration, since the scale errors in non-calibrated instruments can be as high as 30% [33].

In addition to the scale errors, there are several other errors which affect the measurement accuracy and which are difficult to detect and correct from the measured image, especially the effect of drift. One solution is to mix the known origami structures with unknown samples, and measure these samples

simultaneously. Then the origami structures could be used to calibrate the scales, detect the drift and also correct the drift from the measured image. The ST is easy to distinguish from other structures, which makes it especially suitable structure for calibration purposes.

Acknowledgements

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