Nanoscale imaging in DNA nanotechnology

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DNA nanotechnology has developed powerful techniques for the construction of precisely defined molecular structures and machines, and nanoscale imaging methods have always been crucial for their experimental characterization. While initially atomic force microscopy (AFM) was the most widely employed imaging method for DNA-based molecular structures, in recent years a variety of other techniques were adopted by researchers in the field, namely electron microscopy (EM), super-resolution fluorescence microscopy, and high-speed AFM. EM is now typically applied for the characterization of compact nanoobjects and three-dimensional (3D) origami structures, as it offers better resolution than AFM and can be used for 3D reconstruction from single-particle analysis. While the small size of DNA nanostructures had previously precluded the application of fluorescence microscopic methods, the development of super-resolution microscopy now facilitates the application of fast and powerful optical methods also in DNA nanotechnology. In particular, the observation of dynamical processes associated with DNA nanoassemblies—e.g., molecular walkers and machines—requires imaging techniques that are both fast and allow observation under native conditions. Here single-molecule fluorescence techniques and high-speed AFM are beginning to play an increasingly important role. © 2011 Wiley Periodicals, Inc.

INTRODUCTION

Our ability to produce structures on the nanoscale is intimately linked to our ability to characterize them. Without a possibility to ‘check’ whether we have produced the molecular structure aimed for, we would not receive the experimental feedback needed to make progress in our nanoscale endeavors. In the past, researchers most often had to rely on ‘indirect’ methods to collect evidence for the correct assembly of a desired target structure. Of course, the whole range of analytical chemical methods belongs here, or powerful scattering techniques that have been developed for the elucidation of atomic structure of materials or macromolecules. In many cases, however, these methods do not provide all of the information one would like to have from a structure, and most of them do not allow for monitoring dynamic processes. Moreover, many of these methods are not even applicable because the molecule numbers (or concentrations) in nanoscience are often too small to produce a measurable signal.

Direct imaging techniques such as scanning probe microscopy (SPM) and electron microscopy (EM) can, in principle, be used to study single nanoobjects, and therefore naturally overcome some of the problems associated with ensemble measurements. Imaging techniques therefore generally play a prominent role in nanoscale science and technology, and they have also been decisive for the development of DNA nanotechnology.

When Seeman started this field in 1982,1 he envisioned the utilization of artificial branched DNA structures for the arrangement of nanoobjects attached to the DNA—e.g., proteins—in three-dimensional (3D) space. Such ‘DNA crystals’ would be extremely useful for structural studies on proteins that are hard to crystallize otherwise. For crystallographic studies,
however, large structures with perfect order would be required, and it took almost 30 years until Seeman achieved his original goal. In the decade following Seeman’s proposal, a variety of DNA objects were produced in his lab, but were mainly characterized using biochemical methods, most often gel electrophoresis. A major breakthrough for the field came with the first application of an imaging technique for the analysis of two-dimensional (2D) DNA assemblies: in 1998, Winfree in collaboration with Seeman for the first time used an atomic force microscope (AFM) to image 2D lattices made from so-called ‘double-crossover’ (DX) DNA tiles originally developed in Seeman’s lab.2 This is just an example how influential the interplay of novel assembly methods and characterization techniques can be. The AFM has become one of the standard tools in the field ever since and is the ‘workhorse’ for most of the groups working on DNA nanotechnology.

In this review, we will give a short introduction into the imaging techniques typically used in the field of DNA nanotechnology. Apart from the ‘standard’ techniques such as AFM and EM, we also cover optical techniques such as super-resolution fluorescence microscopy.

TECHNIQUES

Atomic Force Microscopy

The era of SPM began in 1982 with the invention of the scanning tunneling microscope (STM).3 This microscope allowed researchers for the first time to visualize matter on the level of single atoms. In STM, a sharp tip is raster scanned over a surface and its distance is controlled using the tunneling current from tip to surface as a control signal. Experiments are usually performed in vacuum and at low temperatures—requirements, which preclude most applications in biology. Scanning probe imaging of biomolecules on the nanoscale became possible with the invention of the AFM in 1986.4 Using the STM’s concept of raster scanning a sharp probe across a surface, AFMs detect surface topography by directly measuring forces between probe and surface. The measurements can be performed under ambient conditions and samples do not have to be conductive. Further technical improvements such as imaging in liquids5 and dynamic imaging modes6–9 quickly made AFM one of the most versatile tools for the characterization of biological samples—and of DNA-based nanostructures in particular—at high-spatial resolution and near native conditions.

Imaging Modes

In the most direct AFM imaging mode—contact mode AFM—probe and sample are in continuous contact with each other during the raster-scan process. Although contact mode allows for relatively high-image speeds and resolution, the constant contact of probe and surface leads to high-lateral forces that can damage soft samples like DNA. The limitations of contact mode for imaging biological samples led to the invention of non-contact imaging in 19876 and finally intermittent contact or tapping mode imaging in 1993.7 In these dynamic modes, the cantilever is excited by a piezocrystal to oscillate at a distance of a few nanometers above the surface and interactions with the substrate are detected as changes in the oscillation amplitude. Intermittent contact or tapping mode combines the advantages of contact and non-contact imaging by ‘tapping’ the sample surface during each oscillation, thus obtaining tip-limited resolution while maintaining low lateral forces. Although tapping mode imaging highly reduces destructive forces in the lateral direction, forces perpendicular to the sample are relatively high due to capillary forces induced by a water layer on the sample surface in ambient conditions. These destructive forces can be highly reduced when imaging in liquid, which was first demonstrated in 1992 for contact8 and 1994 for tapping mode.9,9 AFM operation in liquid has the important advantage that biological samples like DNA can be imaged under native conditions, i.e., in buffer solutions, and therefore tapping mode in liquid today is the most widely used imaging mode in DNA nanotechnology.

High-Speed AFM

Compared with other imaging techniques such as electron or fluorescence microscopy (see below), high-resolution AFM imaging is a rather slow and painstaking process. Conventional AFMs need approximately 10 min to capture a high-resolution image of 5 μm size of, e.g., DNA origami structures using tapping mode in liquid. While this is usually sufficiently fast for imaging static nanostructures, most dynamic biomolecular processes occur on a faster time scale and are thus out of reach for conventional AFM systems. In order to speed up AFM imaging, a variety of technical improvements have been made in the past decades toward so-called high-speed AFMs.10–12 These instruments are similar in design to conventional AFMs but various components such as scanners, cantilevers, amplitude detectors, and feedback electronics have been optimized for higher imaging speeds. Endo et al. first demonstrated imaging of DNA nanostructures using high-speed AFM in
2010. For a more detailed review of biological applications of high-speed AFM, see Ando et al. Imaging speeds of up to several frames per second at resolutions below 10 nm in combination with only little sample preparation effort make high-speed AFM one of the most attractive imaging techniques for DNA nanotechnology today.

**Electron Microscopy**

EM has played an extremely important role for the development of nanotechnology in general. It was first demonstrated in 1931 by Ruska and Knoll, and previous to the invention of SPM it was the only imaging method available for structures with dimensions well below the wavelength of visible light. EM utilizes electron beams for imaging, which are extracted in high vacuum from a special cathode using high voltage or heat or a combination of both. For imaging, the electron beam is shaped and focused using magnetic lenses. The wavelength of the electrons is given by the de Broglie relation and is on the order of 1 nm. This determines the theoretical resolution limit of EM, but in reality this is governed by other factors such as the diameter of the electron beam and interactions with the sample. EM is sub-divided into scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Each has its peculiarities and also different fields of application.

**Scanning Electron Microscopy**

Because of its special requirements and limitations, SEM has only played a minor role for DNA nanotechnology. In SEM, an electron beam is scanned over a solid sample, and back-scattered electrons are recorded in a detector. The image is constructed from the signals obtained from the different ‘pixels’ of the scanning area. While SEM is ideally suited for studying solid state devices—for which a resolution of approximately 1 nm can be achieved—it is only of restricted utility for DNA nanotechnology. Among the disadvantages of SEM are its requirement for conductive samples, its operation under vacuum and its relatively low resolution. Structural features of DNA nanoassemblies are typically smaller than the SEM resolution that can be achieved with these samples, and the metallization required for imaging masks the finest features. SEM becomes interesting, when DNA nanostructures are combined with inorganic materials such as quantum dots or carbon nanotubes, or when they are arranged on solid substrates using lithographic methods. In these cases, one is not so much interested in the finest details of the DNA structures, but rather in their large-scale arrangement.

**Transmission Electron Microscopy**

The situation is quite different for TEM. Although TEM also requires high vacuum and complicated sample treatment, in terms of resolution it beats all other methods discussed here. For this reason, it has become increasingly popular in DNA nanotechnology in recent years, in particular in the context of 3D DNA nanoobjects. In contrast to SEM, TEM is performed on very thin samples, and only electrons transmitted through these are collected with a detector. This makes the technique similar to ordinary transmitted light microscopy, albeit with waves of smaller wavelength. Depending on a variety of factors such as the acceleration voltage (typically 100–200 kV), the resolution of TEM can be as low as 0.1 nm. Applications of EM on biological samples have a long history in structural biology and many of the procedures developed there can be adapted to the requirements of DNA nanotechnology.

To image samples with a TEM, they have to be prepared in a special way. To improve the scattering of electrons at the object to be imaged, ‘staining’ with elements of high atomic number has become a standard method to generate contrast in EM. Often uranium salts such as uranyl acetate or formate are used, whose uranyl ions interact electrostatically with (poly)anions like DNA. Two different staining modes are distinguished. In positive staining, material is deposited onto the objects under investigation, while in negative staining, the objects are surrounded by the material. In the latter case, objects appear bright in the electron micrograph, while positive staining results in dark objects. Staining modifies the sample and hence decreases the resolution of the image and may also change the structure of biomolecules compared with their native conformation. For preservation of the solution conformation and for ultimate resolution structural studies, cryo-EM is used. Here, the samples are not stained, but frozen and kept at low temperatures during imaging.

As the TEM image of a single biomolecular object is usually very noisy, image analysis, and single-particle reconstruction methods play an important role in EM imaging. To improve image quality, typically images of many objects are super-imposed and then averaged. As the 2D EM images represent projections of the 3D objects under study, the images first have to be sorted into different classes. From these, one can actually reconstruct a 3D model of the object. In recent years, single-particle reconstruction methods have been frequently used to generate 3D representations of self-assembled DNA nanoobjects.

TEM image reconstruction may actually turn out as an important field of application for DNA
nanotechnology. It has been recently shown that DNA nanoassemblies of known structure can be used as a ‘frame of reference’ for other biomolecules, whose structure is yet unknown, and thereby assist the image analysis process significantly. This has been used to determine the structure of a non-crystalline membrane receptor, a G protein, and a complex of both molecules.20 Many researchers in the field believe that such utilization of DNA nanoassemblies for structural biology represents a real ‘killer app’ for DNA nanotechnology.

**Fluorescence Microscopy**

Fluorescence is the spontaneous emission of light from atoms or molecules that have been previously excited with light of a smaller wavelength. A large variety of fluorescent molecules—‘fluorophores’—are available today as fluorescent labels and many bioconjugate methods have been developed to specifically attach these to biomolecules of interest. Fluorescence techniques are ubiquitous in biological and biophysical research today, where they are used for the study of molecular interactions, the investigation of conformational dynamics, and, of course, for imaging.21

Image quality in light microscopy is characterized by several factors such as resolution, brightness, and contrast. In classical microscopy, the resolution of an image is limited by the diffraction of light and is given by Abbe limit of approximately \[ \frac{\lambda}{2 \times \text{NA}} \], where \( \lambda \) is the wavelength of light and NA is the numerical aperture of the objective. Thus with visible light, the minimum distance between two objects that can still be resolved is about 200 nm. To be able to discern structural features within an image, contrast—variations in brightness or color—is required. As biological samples often have low contrast, a variety of contrast-generating methods have been developed, for instance, phase contrast imaging. In fluorescence microscopy contrast is generated by selectively staining of biomolecular structures with fluorescent molecules. Using appropriate filters, the molecules under study can be excited and fluorescence light can be collected to generate an image. In bioimaging, using multiple fluorophores and filters allows to image a variety of cellular structures together and study their inter-relations.

Until recently, ordinary fluorescence imaging has not played a major role in the investigation of DNA nanostructures, simply because these structures were usually much smaller than the diffraction limit. This has changed, however, with the advent of super-resolution microscopy or ‘nanoscopy’.

**Nanoscopy**

Starting in the early 1990s, a variety of techniques have been developed that allow imaging beyond the classical diffraction limit using far-field fluorescence microscopy.22–27 Most implementations of ‘super-resolution’ imaging share the general principle of switching molecules between fluorescence ON and OFF states, so that individual molecules can be localized consecutively. On the basis of the mechanism used for localizing single fluorescence emitters, super-resolution microscopy may be divided into targeted and stochastic readout schemes.28 In the first case, locations of single fluorophores are optically ‘targeted’ to actively define ON and OFF states. A prominent example of such a technique is stimulated emission depletion (STED) microscopy.22 Using a laser-scanning microscope, each spot of an image is illuminated by an excitation laser overlaid by a second, red-shifted laser with a doughnut-shaped intensity profile. This second laser de-excites fluorophores in the doughnut area and thus ‘focuses’ the active excitation area below the diffraction limit. STED microscopy currently allows imaging with a lateral resolution of approximately 20 nm. As the sample has to be scanned, image acquisition time varies from milliseconds to minutes.

**Stochastic Reconstruction Techniques**

Stochastic reconstruction methods such as photoactivated localization microscopy,23 stochastic optical reconstruction microscopy (STORM),24 point accumulation for imaging in nanoscale topography (PAINT),25 or blink microscopy (BM)26 rely on stochastic switching of fluorophores between bright and dark states and localization of single molecules within a diffraction-limited area (cf. Figure 1). In fact, localization of single molecules can be achieved with almost arbitrarily high precision—e.g., in 2003, Yildiz et al. utilized a localization technique termed ‘fluorescence imaging with one nanometer accuracy’ (FIONA) to investigate the motion of myosin V on actin filaments.29 FIONA works under conditions, where only a single molecule emits light within a diffraction-limited area. Then the resulting point-spread function (PSF) of the emitter can be fit by a 2D Gaussian and its center position gives an estimate of the molecule’s position. The localization precision is mainly influenced by the number of collected photons N and scales like.30

For reconstruction of distances below the diffraction limit, a sufficient fraction of molecules has to be switched to a fluorescent dark state, while only very few molecules are allowed to reside in a bright state during the acquisition of each image
Precise localization of a single molecule

No localization possible

Temporal separation of fluorescence emission

**FIGURE 1** Stochastic readout principle for super-resolution fluorescence microscopy. The localization of a single fluorophore in a diffraction-limited area is possible with high precision by fitting a two-dimensional (2D) Gaussian. If the point-spread function (PSF) of two or more emitters within a diffraction-limited area overlap, localization is not possible. Using temporal separation of fluorescence emission by stochastically switching fluorescence between ON and OFF states, the locations of the molecules can be reconstructed in a super-resolved image.

Frame. This ensures that typically only a single molecule emits fluorescence within a diffraction-limited spot. Fluorescence ON and OFF times and the number of emitted photons per activation are the most important parameters determining the resolution that can be obtained. For detailed reviews of current super-resolution techniques see Refs 28, 31, and 32. As will be discussed below, the utilization of super-resolution microscopy for DNA nanotechnology has started only recently.33,34 In fact, their nanometer-precise addressability make DNA nanostructures an extremely attractive substrate for super-resolution imaging as well as for the observation of dynamic processes using single-molecule fluorescence techniques.

**VISUALIZATION OF 2D DNA ASSEMBLIES**

**Tile-Based Nanoassembly**

As mentioned in the introduction, one of the early breakthroughs of structural DNA nanotechnology was the realization of 2D DNA crystals by Winfree et al. in 1998.5 These crystals were based on so-called ‘DX’ tiles, in which two parallel DNA double helices were linked by strand crossovers similar to Holliday junctions that occur in genetic recombination (Figure 2). These tiles had dimensions of 4 × 16 nm with a height of 2 nm (the diameter of dsDNA). Via complementary ‘sticky-end’ extensions, these tiles could associate with each other to form nicely ordered 2D lattices with extensions of several hundreds of nanometers. One of the major technological advances in this work was the first-time utilization of AFM under liquid to image DNA nanostructures. AFM turned out to be a method matched perfectly to the requirements of DNA nanotechnology—as explained above, it allows high-resolution imaging on the typical length-scale of DNA assemblies and it can be operated under native conditions. For this reason, AFM has remained the main characterization method for DNA assemblies ever since. AFM has been used to characterize many other tile-based lattices, such as tilings based on triple-crossover molecules,35 ‘4 × 4’,36 ‘tensegrity triangle’ tilings,37 and so forth. Only in a few cases fluorescence microscopy was used for characterization—e.g., in Ref 38, where almost millimeter-sized lattices were assembled using a variation of the crossbar tiling structure. Here fluorescence microscopy was used to demonstrate the extraordinarily large size of the whole assembly.

Tile-based assemblies were also used as templates for the arrangement of metallic nanoparticles into lattices,39 or for the localized deposition of metal from solution.36 As metals give good contrast in EM, TEM, or SEM could be used for visualization of these structures, respectively.

**Scaffolded DNA Origami**

Scaffolded DNA origami, first introduced by Rothemund in 2006,40 marked another breakthrough in structural DNA nanotechnology. In DNA origami, a long single-stranded viral DNA molecule is ‘folded’ into a specific shape using many short ‘staple’ strands (cf. Figure 3(a)). As for tile-based assemblies, AFM is the method of choice for imaging of flat origami structures, as it offers operation under native conditions and sub-10 nm resolution. A variety of shapes from Rothemund’s original publication imaged by tapping mode AFM under liquid are shown in Figure 3(b).

One important feature of DNA origami structures is the fact that each staple strand can be used for the attachment of other molecular components with a ‘resolution’ of approximately 6 nm—which is the distance between neighboring ‘molecular pixels’. To demonstrate the addressability of origami structures, Rothemund used staple strands with dumbbell-shaped hairpin extensions to highlight their position. The rigid hairpin structures produce height contrast in AFM
images and are visible as bright spots compared to the unmodified staple strands of an origami structure.

On the basis of the same principle, in 2008, Ke et al. demonstrated the utilization of DNA origami structures as sensors for the detection of RNA molecules. To this end, DNA origami structures were modified with an index region containing hairpin-modified staple strands acting as a unique ‘barcode’. A single detection site for RNA consisted of single-stranded extensions of two neighboring staple

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**FIGURE 2** | A DNA lattice formed from double-crossover (DX) tiles. (a) Architecture of a DX tile: two DNA double helices are connected at two crossover points. (b) DX tiles can be connected by ‘sticky-end hybridization’ to form extended molecular lattices in two dimensions (2D). (c,d) Atomic force microscopy (AFM) images in liquid of a 2D DX crystal, scale bars: 300 nm. (Reprinted with permission from Ref 2. Copyright 1998 Macmillan Publishers Ltd)

**FIGURE 3** | Design and imaging of single-layer DNA origami structures. (a) Formation of rectangular DNA origami by thermal annealing. (Reprinted with permission from Ref 41. Copyright 2010 Macmillan Publishers Ltd) (b) Schematic representations and atomic force microscopy (AFM) images in liquid from DNA origami structures. In the rightmost structure, single-staple strands are highlighted using dumbbell-shaped hairpin extensions to represent the map of the Americas on an origami scaffold. (Reprinted with permission from Ref 40. Copyright 2006 Macmillan Publishers Ltd)
strands. Each extension was chosen complementary to one half of the RNA target sequence to detect. Upon binding of the RNA target to the probe, a structurally rigid RNA/DNA hybrid was formed which could be visualized using AFM due to its increased height.

In 2009, Steinhauer et al. for the first time used super-resolution fluorescence microscopy to optically image single-layer DNA origami structures. In this study, rectangular DNA origami structures were modified with two single fluorophores at a distance of approximately 90 nm (cf. Figure 4). The fluorophores were arranged on the origami structure by simply modifying two staple strands at the 5' end, i.e., facing ‘up’. The origami structures also contained biotinylated staple strands protruding to the opposite side, i.e., ‘down’. Folded origami structures were immobilized on a streptavidin-modified glass slide and imaged using total internal reflection fluorescence microscopy (TIRFM). A typical diffraction-limited image of these structures is shown in Figure 4(a). In Ref 33, a variety of super-resolution techniques were used to resolve the 90 nm sub-wavelength distance of the two fluorophores, among them dSTORM, a simplified implementation of the original STORM method, and BM. In BM, the required dark states of the fluorophores for localization-based reconstruction are engineered using a reducing and oxidizing system (ROXS). In ROXS, a reducing agent reduces the fluorophore (e.g., the oxazine dye ATTO655) to a non-fluorescence state and an oxidizing agent activates fluorescence again. By adjusting the ratio of oxidizing and reducing agent the required fluorescence ON and OFF times for super-resolution reconstruction can be obtained. Figure 4(b) shows a typical diffraction-limited image of single origami structures carrying two fluorophores with the super-resolved reconstruction overlaid. The magnified view reveals two single fluorophores within each diffraction-limited area. Using BM, the distance between the two fluorophores on the origami (nominally 89.5 nm) could be determined to be 88.2 ± 9.5 nm. As the variation of the measured distances can be almost completely attributed to the localization precision of the method, DNA origami can be used as a reliable nanoscopic ruler for super-resolution microscopy techniques.

In 2010, Jungmann et al. developed a single-molecule assay, termed DNA-PAINT, which allows for direct observation of dynamic processes on DNA.

**FIGURE 4** | Super-resolution microscopy with DNA origami. (a) Total internal reflection fluorescence (TIRF) image of surface-immobilized DNA origami containing two ATTO655-labeled staple strands. The positions of the single fluorophores cannot be determined because of their overlapping point-spread functions (PSFs). The positions of the fluorophores on the origami nanostructure are shown in the scheme at the bottom, their distance is 89.5 nm. (b) Using blink microscopy (BM) super-resolution microscopy, the positions of the two fluorophores can be well resolved. The image shows an overlay of the diffraction-limited TIRF image with a super-resolved reconstruction. The fluorescence time trace obtained from one origami molecule is shown below the micrograph, highlighting the blinking of the fluorophores. Two magnified views of several structures from (b) reveal two single fluorophores. The distance distribution shown at the bottom yields (88.2 ± 9.5 nm).
origami scaffolds using single-molecule fluorescence microscopy. The method is based on specific, transient binding of short fluorescent DNA oligonucleotides to single-stranded ‘docking’ strands protruding from DNA origami structures (cf. Figure 5(a)). Repetitive binding events of fluorescently labeled ‘imager’ strands are monitored using TIRFM. In the unbound state, only background fluorescence is observed. Upon binding of a fluorescently labeled strand from solution, the fluorescence emission from this strand is detected (Figure 5(b)). This can be used to determine association and dissociation rates of single-molecule DNA hybridization events. The fact that transient binding in DNA-PAINT is based on DNA hybridization also allows for the precise control over fluorescence ON and OFF times—an important prerequisite for stochastic super-resolution microscopy. The fluorescence ON time can be adjusted by the length of the imager-docking strand duplex and the fluorescence OFF time can be controlled by the concentration of imager strands in solution. Figure 5(c) shows the viability of DNA-PAINT super-resolution microscopy by imaging three docking sites on an origami substrate with a distance of approximately 130 nm. The binding sites cannot be distinguished using diffraction-limited TIRFM imaging, but after stochastic reconstruction the three binding sites are clearly resolved. At its current state, DNA-PAINT is able to resolve distances as low as 30 nm.

The precise control over fluorescence ON and OFF times combined with the specific binding of DNA strands makes DNA-PAINT applicable over a wide range of samples and imaging conditions. In fact, DNA-PAINT imaging is not restricted to DNA nanostructures and can be also applied, e.g., for imaging of cellular structures by using DNA-antibody conjugates.

CHARACTERIZATION OF 3D DNA NANOSTRUCTURES

It is still one of the major challenges for DNA nanotechnology to create structures extending in 3D. This is caused by the lacking structural integrity of many of the structures and by their frequently strong interactions with surfaces (there might actually be 3D order in solution that is disrupted upon deposition on a surface). Hence, most of the 3D assemblies characterized so far are relatively small geometrical objects, or compact multihelix packings such as in 3D origami structures.

DNA Objects

Early DNA objects such as Seeman’s famous DNA cube lacked structural stability and only had the...
Because of their intrinsic structural stability, tetrahedral structures were natural target objects for 3D assemblies that could actually be visualized. One of the first examples in this direction was the realization of a DNA-based octahedron by Shih and Joyce in 2004, which was based on intramolecular folding of a long single-stranded DNA molecule (in a sense a predecessor of Rothemund’s scaffolded origami method). Because of its small diameter of only 20 nm, the structure was visualized by cryo-EM and single-particle reconstruction methods. In 2005, Goodman et al. created a simple DNA tetrahedron composed of only four strands of DNA, with a side length of 10 nm. In the original publication, the structure was visualized using AFM, and also its mechanical properties were probed with the AFM. However, due to the small size of the tetrahedron, the AFM was clearly at its resolution limit. Later, the same structure was investigated in detail using cryo-EM (Figure 6). To date this is actually the smallest biomolecular structure that was reconstructed from cryo-EM images at a resolution of 1 nm.

In recent years, researchers demonstrated other impressive 3D objects such as platonic bodies made from DNA or DNA/RNA hybrids, and also rigid RNA cubes, and it has become common to characterize these structures both using AFM and TEM. Because of strong interactions with the charged mica surfaces that are typically used as substrates for AFM imaging, 3D objects are often ‘flattened’ out in AFM, while they retain their structure in EM.

DNA Helix Bundles
DNA helix bundles represent, simply speaking, rolled up 2D lattices and only have small extensions in two of their dimensions. There have been demonstrations of nanotubular structures composed of DNA tiles, or simply DNA helices with diameters ranging between 10 and 50 nm and lengths of several micrometers. Most of these structures were characterized by AFM and sometimes also TEM. One notable exception is the work by Fygenson and coworkers, in which video microscopy was applied to study the dynamics of fluorescently labeled DNA nanotubes. Similar as in biophysical experiments on dsDNA or cytoskeletal filaments, studies of the Brownian dynamics of DNA nanostructures can be used to determine, e.g., their persistence length. In addition to ‘tile-based’ DNA nanotubes, a variety of other helix bundle types were realized recently using the DNA origami technique.

3D Origami
In 2009, Douglas et al. demonstrated that DNA origami can be extended to the third dimension by ‘stacking’ of 2D DNA origami slices. In contrast to Rothemund’s 2D patterns, the new design was based on a ‘honeycomb lattice’, in which neighboring DNA helices were connected together at 120° angles (cf. Figure 7(a)). It turned out that the resulting massive blocks of DNA were best imaged by TEM—several TEM micrographs of such DNA monoliths are shown in Figure 7(a).

Later, Shih and Yan published an even more compact 3D origami design based on a square lattice. Several multilayer structures were built with this technique, ranging from 2 × 21 to 8 × 8 blocks (number of layers × number of helices). A schematic of a 6 × 12 block is shown in Figure 7(b) along with corresponding TEM micrographs. In addition to TEM imaging of negatively stained particles, in Ref 60 also cryo-EM was used to investigate the origami structures under ‘native’ conditions.
Also in 2009, Andersen et al. took another approach toward 3D structures by arranging planar DNA origami sheets into the form of a nanoscale box\(^6\) (Figure 7(c)). Notably, they designed the box with a controllable lid, which could be opened and closed upon addition or removal of ‘lock’ and ‘key’ strands via a strand displacement mechanism. Andersen et al. used a whole battery of analytic techniques to study the properties of the box, e.g., dynamic light scattering and also small-angle X-ray scattering. As nanoscale imaging techniques, AFM, cryo-EM, and also single-molecule fluorescence microscopy were applied.\(^62\) AFM could be used to image both open and closed states of the box, whereas cryo-EM facilitated single-particle reconstruction of the box. As shown in Figure 7(c), a 3D map generated from the cryo-EM data showed good correspondence with a theoretical model.

EM offers a wealth of structural information about 3D bio-nanostructures and is therefore used in essentially all recent publications on 3D origami. EM studies were performed on twisted and curved shapes,\(^63\) tensegrity structures,\(^64\) and curved spherical- or flask-like shells.\(^65\)

### DNA Crystals

As mentioned in the introduction, one of the original goals of the founder of the field, Nadrian Seeman, was to produce macroscopic crystals from DNA. In 2010, he and his coworkers came up with a simple design that generated millimeter-sized crystals that could be used for X-ray crystallographic studies.\(^66\) From the X-ray diffraction pattern, an all-atom model could be reconstructed with a resolution of 4 Å. The crystals were built from triangular structures that were formed by three crossover structures at the vertices. This structure is not planar and extension of its helices by sticky-end hybridization to other triangles leads to a 3D crystal structure. Important for the successful...
assembly of a highly ordered structure were two main ingredients: the rigid triangular building block itself, and the utilization of very short sticky ends of only 2 nt length. Notably, due to the macroscopic nature of the crystals they were actually visible to the naked eye!

**DYNAMICAL STRUCTURES AND PROCESSES**

One of the ultimate goals of nanotechnology is the realization of artificial molecular machines and ‘robots’, and their operation within autonomous, dynamical molecular systems. While there is already a large body of work on nucleic acid-based molecular switches and machines,57 most of the structural changes and movements realized so far were too small to be imaged directly. DNA origami now offers a versatile substrate for the construction of systems, in which the dynamics of biomolecules and artificial nanomachines may be studied on a larger scale, and in a well-controlled setting. However, real-time observation of dynamical processes involving biomolecules precludes many of the techniques traditionally used for the characterization of DNA nanostructures—these are either too slow, or they are not compatible with the reaction conditions required. Hence, the realization of large-scale dynamical DNA nanosystems not only represents the frontier of research regarding their construction, but it also requires the most advanced imaging techniques available.

**Imaging Chemical and Biochemical Processes on Origami**

Unlike reactions in well-stirred chemical reactors, chemical processes in live cells often occur localized, within small compartments and in a spatially orchestrated manner. It is therefore of considerable interest to study the influence of spatial organization on chemical reaction systems, and also to attempt the construction of artificial chemical ‘reaction centers’—e.g., to optimize synthetic pathways or energy transduction between neighboring components. One first example of a spatially organized system was recently demonstrated by Voigt et al.68 who monitored the progress of chemical reactions on DNA origami on the single-molecule level. To this end, streptavidin proteins were attached to origami structures via chemically cleavable linkers, and the effect of cleaving agents on the assemblies was investigated by AFM. With ‘traditional’ AFM, however, the reactions could only be assessed ‘in hindsight’, i.e., without temporal resolution.

In addition to the fabrication of spatially controlled reaction systems, DNA self-assembly also facilitates the controlled placement of binding sites for biomolecules, which may be used to study the dynamics of biomolecular processes under well-characterized conditions. In a series of ground-breaking experiments, Sugiyama and coworkers recently utilized high-speed AFM to image biochemical processes in real time. Using DNA origami ‘frame’ structures into which DNA molecules were incorporated that contained the recognition sequences for a DNA methyl transferase,13 they were able to directly image the attachment of the enzyme to DNA and its subsequent search for its binding site. With a similar setup they were able to monitor the dynamic formation and disruption of intermolecular G quadruplex structures upon addition or removal of potassium ions,69 and also the action of DNA-based excision repair enzymes.70

**DNA Walkers**

One of the most challenging goals of DNA nanotechnology is the realization of artificial molecular motors. Since 2004, a variety of molecular ‘walker’ concepts have been put forward,71 but typically their operation was only indirectly demonstrated by gel electrophoresis or fluorescence spectroscopy. It took until 2010 to see the first nanoscale images of DNA walkers, when Lund et al. presented a ‘molecular robot’ walking on a DNA origami-based track.72 The walker consisted of a streptavidin core with one capture and three catalytic legs made from DNA73 (Figure 8(a)). When the walker is bound to the DNA origami track via complementary substrate strands, the catalytic legs (containing the 8–17 deoxyribozyme) cleave the substrates and thereby reduce their lengths. This destabilizes the association of the walker with the track and it passes on to the next substrate. By patterning a ‘path’ of substrate molecules onto the DNA origami structure, the otherwise diffusive movement of the walker is made directional. The motion of the walker could be monitored by AFM imaging as shown in Figure 8(a), where its position is clearly visible in the height contrast image at different times. In Ref 72, the speed of the walker was also determined using single-molecule fluorescence measurements, from which position-time trajectories could be constructed.

The groups of Turberfield and Sugiyama showed another walker system that also utilized a planar DNA origami structure as track74 (Figure 8(b)). The walker is based on a single DNA strand binding to complementary stator strands on the track moving by toehold-mediated strand displacement. The toehold is generated by a nicking enzyme, which cuts the stator strands as soon as a walker-stator-duplex...
is built. Beside ensemble fluorescence measurements they used AFM imaging to show the movement of the walker (Figure 8(b)). As conventional AFM is too slow to detect single steps of the walker they applied high-speed AFM allowing the observation of discrete steps between two neighboring stators in a kymograph. Assuming the highest point on each AFM profile as the walker position yields distances of

FIGURE 8 | DNA walkers and assembly lines. (a) A DNA spider walks on a prescriptive track on a planar DNA origami structure. High-resolution fluorescence microscopy is used to monitor the movement of the walker (top). Atomic force microscopy (AFM) allows the observation of specific actions at the start, on the track and at the stop site (bottom, sites marked with the green arrow respectively). (Reprinted with permission from Ref 72. Copyright 2010 Macmillan Publishers Ltd) (b) A DNA walker proceeds by toehold-mediated strand displacement on a linear DNA track (top). Kymographs constructed from AFM images at different times allow analysis of the progress of the walker. The histogram obtained from the height profiles reveals the walker’s stepping motion (bottom). (Reprinted with permission from Ref 74. Copyright 2011 Macmillan Publishers Ltd) (c) Molecular assembly line. A transporter made from DNA can take up cargo from three loading sites. The loading sites consist of two-state DNA machines that can either present or hide their cargo. The cargos themselves are combinations of differently sized gold nanoparticles (left). AFM imaging in air shows the uptake of all three cargos by the transporter (right), whereas transmission electron microscopy (TEM) micrographs show the results for all eight possible combinations of the cargo (bottom). (Reprinted with permission from Ref 75. Copyright 2010 Macmillan Publishers Ltd)
7.4 ± 1.0 nm between two adjacent stators. This value is in good accordance with the value of 6 nm expected from the origami design.

Nanomechanical Devices and Assembly Lines

In addition to ‘molecular robots’ and machines, another vision of nanotechnology is the realization of molecular-scale ‘assembly lines’. Most impressively, one first example of such a system could recently be demonstrated by Seeman and coworkers. The assembly line was composed of a DNA transporter and three switchable two-state DNA machines placed onto a planar DNA origami substrate (Figure 8(c)). By switching any of the DNA machines into the ‘ON’ state, cargo (here differently sized gold nanoparticles) could be transferred from machine to transporter. By moving from machine to machine, the transporter was able to pick up any of eight possible combinations of the cargos. Figure 8(c) shows AFM images of the uptake and steps of the transporter. The results of the eight possible assembly programs are shown as TEM micrographs.

CONCLUSION

In the past years, we have witnessed an exciting new development in DNA nanotechnology—DNA origami. DNA origami is a powerful technique for the assembly of almost arbitrarily shaped nanoscale objects. These objects may be modified sequence specifically with organic or inorganic materials, and hence customized for the requirements of the applications at hand. Origami is currently adopted by a growing number of researchers who aim at applications as diverse as nanophotonics, nanochemistry, drug delivery, structural biology, synthetic biology, or basic biophysics.

While AFM has been the dominant nanoscale imaging technique in DNA nanotechnology for almost a decade, many of the new structures and applications require additional characterization tools. Multilayer (3D) origami is mainly investigated with EM, and several groups are starting to realize the potential of modern super-resolution fluorescence microscopic methods. Interestingly, DNA nanotechnology is beginning to ‘pay back’ to imaging technology, and this may actually be one of the major applications for DNA-based nanostructures. The precise control over matter on the nanometer scale allows the construction of objects with known shape and dimensions that may serve as calibration standards for nanoscale imaging techniques. For example, it has been shown that origami structures can be used to calibrate the distances between fluorophores for optical super-resolution methods. The structure of a DNA object has been reconstructed from cryo-EM at an extraordinary accuracy, and DNA objects may in the future assist in the determination of yet unknown molecular structures.

The new frontier for DNA nanotechnology is the realization of dynamic, spatially extended nanoscale systems such as artificial reaction centers, molecular robotic systems, molecular transporters, and assembly lines. For these, fast imaging techniques are required, for which the samples can be kept under native conditions. It is clear that single-molecule fluorescence and super-resolution techniques as well as video rate AFM will play an increasingly important role in DNA nanotechnology in the years to come.

ACKNOWLEDGMENT

We gratefully acknowledge funding by the Nanosystems Initiative Munich (NIM).

REFERENCES


