Fluorescence Microscopy

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DNA Origami as a Nanoscopic Ruler for Super-Resolution Microscopy**

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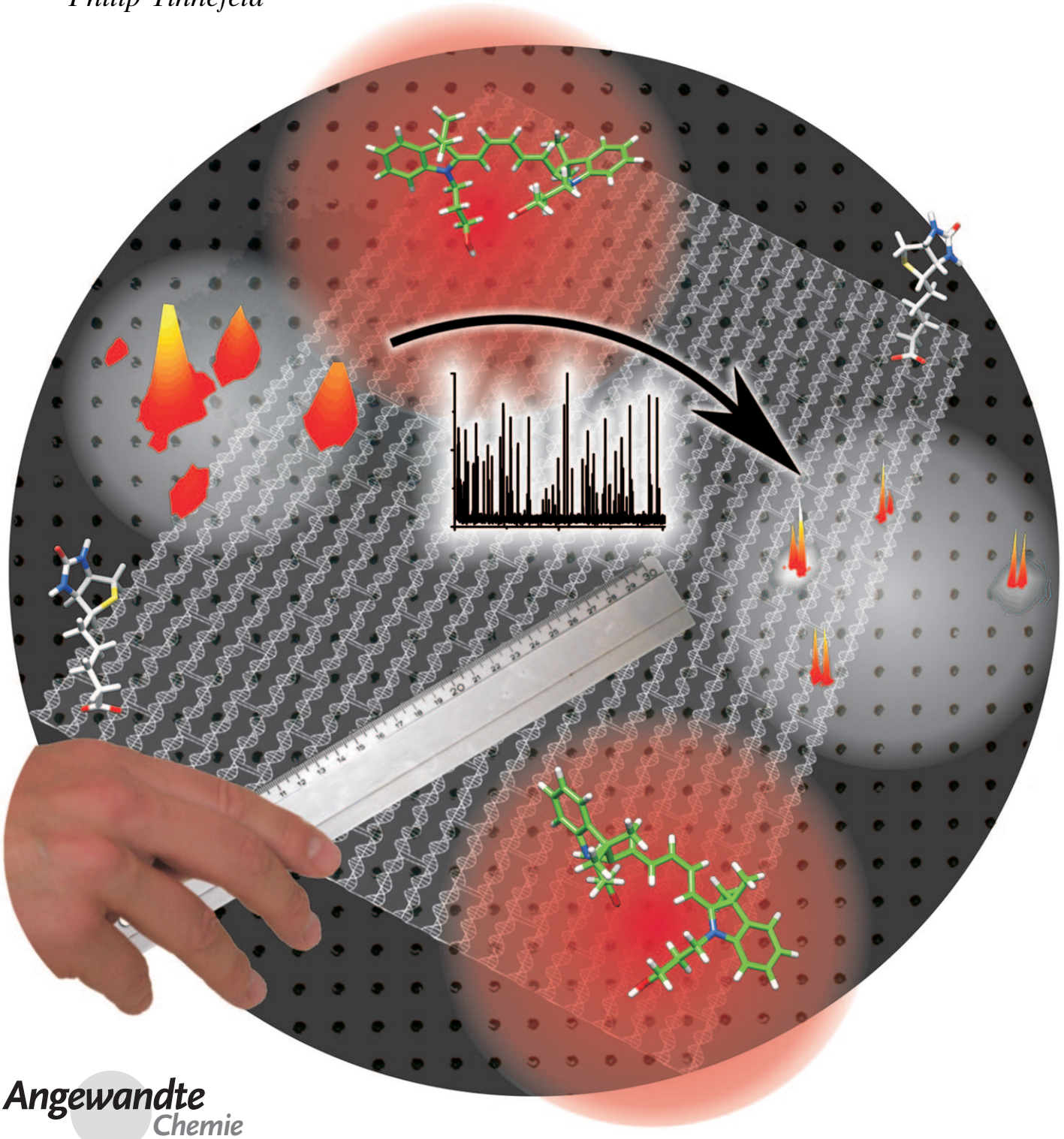

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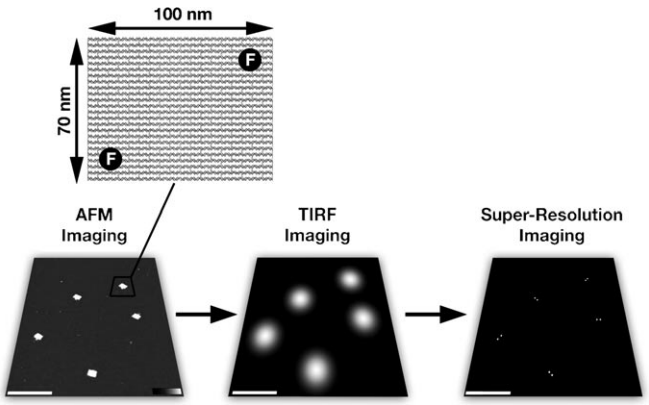
The highly parallel formation of nanostructures through the self-assembly of DNA molecules provides a powerful tool for bottom-up fabrication.[1] The DNA origami technique[2] is a striking example of DNA self-assembly that involves folding a long single-stranded DNA scaffold with short DNA staple strands that can only bind at particular points along this scaffold. With this technique, large numbers (billions or more) of identical structures can be assembled simultaneously in a single experiment. One of the most salient features of the origami technique is the precise addressability of the DNA structures formed. Each staple strand can serve as an attachment point for many different kinds of molecules or other nanoobjects, which are either directly attached to a staple strand or hybridized through a complementary DNA strand.

DNA nanostructures are commonly imaged using atomic force microscopy (AFM) or electron microscopy.[3] Recent advances in far-field fluorescence microscopy below the diffraction limit (super-resolution microscopy) has resulted in structures in the sub-200 nm regime becoming amenable to optical analysis.[4] By using different super-resolution techniques based on the subsequent localization of single molecules, namely, single-molecule high-resolution imaging with photobleaching (SHRImP), direct stochastic optical reconstruction microscopy (dSTORM), and blink microscopy,[5–7] we show that fluorescently labeled staple strands (labeled with either Cy5 or ATTO655 fluorophores) bound at specific positions of rectangular DNA origami structures exhibit a defined separation. As there are more than 200 addressable positions on the origami which can be labeled individually, this technique provides a highly versatile calibration standard for super-resolution microscopy based on the subsequent localization of single molecules (for example, PALM, STORM, FPALM, dSTORM, PAINT, and blink microscopy).[8,9]

Until now, inhomogeneous filamentous structures such as actin filaments or microtubules have been used to demonstrate optical resolution. Short double-stranded DNA has also served as a nanoscale ruler,[5,9–11] but is disadvantageous because of its non-negligible flexibility, which already becomes noticeable at short distances.[12] Furthermore, it is difficult to immobilize the DNA in a fixed orientation under relevant conditions. A defined standard is desired to quantify and demonstrate the resolution that can be obtained, to verify the optical magnification, to correct for aberrations, and to study and calibrate the photophysical and photochemical properties of the fluorescent probes under defined conditions.[6,7]

Our concept is depicted in Scheme 1. As a proof of principle, two staple strands in diagonally opposing corners of the rectangular DNA origami were labeled with fluorescent probes. The overall size of the origami was (100×70) nm, which results in fluorophore distances well below the diffraction limit. After formation, the origami samples were purified to remove excess staple strands and then imaged by AFM in a liquid cell to check that the correct structure had been formed (see Figure S1 in the Supporting Information). For the fluorescence microscopy, the origami samples were immobilized on a glass surface and imaged using total internal reflection fluorescence (TIRF) microscopy (see the Supporting Information for immobilization protocols and experimental methods). The TIRF micrograph yields a diffraction-limited image in which the emission patterns of the two fluorophores overlap. Super-resolution imaging techniques then allowed identification of the positions of the individual fluorophores.



Scheme 1. The rectangular DNA origami structure with two fluorescently labeled staple strands (F in black circle) at a specific distance (top). After preparation, immobilized origami samples are imaged using AFM (bottom left) to check that the correct structures have been formed. Fluorescence images are then recorded in TIRF mode (bottom middle). Single fluorophores on the origami sample are identified by using different methods of super-resolution fluorescence microscopy (bottom right). Scale bars: 500 nm, AFM height scale: 6 nm.

As a first model system, we investigated an origami structure which contains two staple strands labeled with ATTO655 at their 5′ ends and located in the lower left and upper right corner of the rectangular origami structure (see Scheme 1 and Figure S1 in the Supporting Information). The distance between the fluorophores was designed to be 89.5 nm (assuming 10.67 bases per turn and a 3 nm interhelical distance in the origami design).[2] Figure 1 a shows a TIRF image of the origami sample immobilized through biotin

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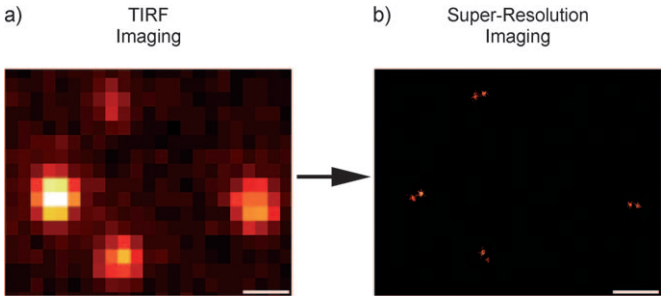

Figure 1. a) TIRF image of surface-immobilized DNA origami containing two ATTO655-labeled staple strands. The positions of the single fluorophores cannot be determined because of their overlapping point-spread functions. b) Super-resolution image of the same region using blink microscopy: Single fluorophore positions are clearly resolved. Scale bar: 500 nm.

linkers on to a glass substrate. As the distance between the two fluorophores is smaller than the diffraction limit, the image appears as a single blurred intensity spot for each origami structure with two fluorophores. We studied these structures with three super-resolution techniques: blink microscopy, SHRImP, and dSTORM.

The dark (OFF) states required for blink microscopy are engineered through redox reactions that yield metastable radical ions.[7] The technique is characterized by simplicity (requiring only one laser), high speed, and the possibility to use several common fluorophores, some even in the presence of oxygen.[13,14] As a consequence of the induced dark states, which are controlled by a reducing and oxidizing system (ROXS) comprising 50 μM ascorbic acid and 50 μM methylviologen (MV) in the presence of oxygen, only a sufficiently small fraction of molecules is fluorescent at any given time.[13,15] This is illustrated in Figure 2a, which displays a fluorescence transient of a single origami structure immobilized on a glass substrate through electrostatic interactions. The transient shows frequent blinking of the dyes with ON times matching the frame rate of the camera of 4 ms and OFF times of about 200 ms.

Subsequent localization of all the fluorophores enables reconstruction of super-resolution images, as depicted in Figure 1b. The distances between the two molecules on each origami rectangle were determined from these images, which yielded a distance distribution as shown in Figure 2b. A distance of (88.2 ± 9.5) nm was determined for the 84 spots measured, which is in good agreement with the designed distance of 89.5 nm. Single molecules were localized with a precision of ± 5.9 nm and the distances between two fluorophores with ± 8.3 nm.[16] Hence, the precision of the localization almost completely accounts for the widths of the distribution obtained. This finding indicates that origami structures represent a robust and reproducible nanoscopic ruler.

We also compared different immobilization strategies, as it may be important to use origami rulers under different conditions. Immobilizing an origami rectangle to a BSA-passivated surface through a single, centrally attached biotin linker resulted in a very poor yield (only 20%) of resolvable structures, which exhibited a separation of only (62.2 ± 10.4) nm (see Figure S3 in the Supporting Information). We presume that the sample can rotate or bend around the attachment point so that only a small fraction of the origami can be resolved. By attaching three biotin linkers in a row perpendicular to a line connecting the two dye molecules (see Scheme S1 in the Supporting Information)—hence restricting motion—71% of the constructs could be resolved and found to exhibit a separation of (76.4 ± 8.7) nm. Considering that 79% of the origami structures initially carry two fluorescent molecules (measured by photobleaching analysis; data not shown) as a consequence of inactive fluorophores or imperfect labeling, this finding indicates that approximately 90% of the correctly formed structures could be resolved. For the electrostatically immobilized origami that exhibited the expected distance (as described above), the yield of spots where two fluorophores could be resolved was 50%. This lower yield can be explained by altered photophysics, in this case because of fluorophore–glass interactions, and a fraction of the excess staple strands sticking unselectively to the nonpassivated surface.

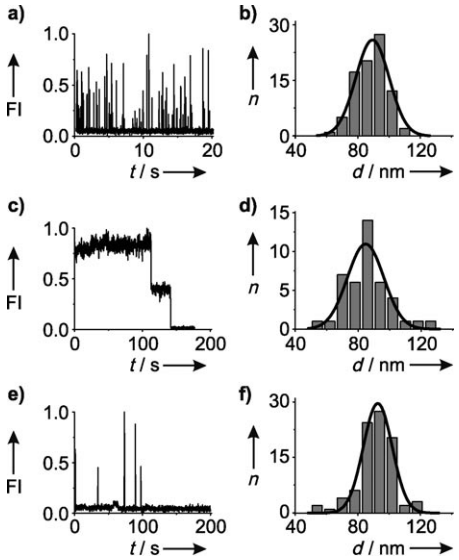

Figure 2. Super-resolution microscopy of doubly labeled DNA origami. Intensity (Fl) versus time (*t*) profiles (a,c,e) of one region of interest and statistical distribution of measured distances *d* (b,d,f). Method: Blink microscopy (a,b), SHRImP (c,d), and dSTORM (e,f).

The SHRImP/NALMS[5,10] technique has the advantage that it can be used with all single-molecule-compatible fluorophores, but it is limited in the number of molecules that can be colocalized within a diffraction-limited area. Since ATTO655 shows a stable emission in phosphate-buffered saline (PBS) buffer, no special care, such as oxygen removal, had to be taken. Movies of origami structures immobilized on BSA surfaces through three biotin linkers were recorded until all the molecules were photobleached. Fluorescent spots were identified and their fluorescence transients were analyzed with respect to the photobleaching steps (Figure 2c). In this example, the first molecule was photobleached after about 115 s and the second molecule about 25 s later. Single molecules were localized in reversed order of their photobleaching. From the time frames, the second molecule is

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localized between 115 and 140 s. Its intensity distribution is then subtracted from the previous frames, thereby allowing localization of the first molecule. Statistical analysis of the data yields a distance of (85.2 ± 14.2) nm (42 spots; Figure 2d), which again is in good agreement with the theoretical distance of 89.5 nm, given the slightly shorter distance measured with the biotin attachment points.

The dSTORM technique was used to experimentally detect distances between two Cy5 molecules attached to an origami rectangle at a designed distance of 99.1 nm.[6] dSTORM exploits the fact that in the presence of thiols such as β-mercaptoethylamine (MEA) the fluorescence of Cy5 can be switched ON and OFF using blue/green and red laser excitation.[17] dSTORM can be applied with different di- and tricarbocyanine derivatives, thus allowing multicolor imaging, and is flexible with respect to imaging speed. To switch Cy5, oxygen was removed enzymatically, 50 μM MEA added, and an additional laser operating at a wavelength of 532 nm was used for switching ON. Figure 2e shows a fluorescent transient of Cy5-labeled origami, thus demonstrating ON/OFF blinking with simultaneous excitation at 532 and 650 nm. The experimentally obtained distance is (91.6 ± 12.2) nm (90 spots; Figure 2f), which is smaller than the designed distance, as expected when using biotin anchors.

Combining DNA origami as a molecular breadbord and super-resolution far-field fluorescence microscopy as an analytical tool will allow the construction of new kinds of bottom-up nanoscale structures and the study of molecular interactions on them. Here we used DNA origami structures labeled with fluorescent probes at defined positions as nanoscopic rulers for the calibration of super-resolution microscopy. Designed distances were verified experimentally in the case of electrostatic immobilization, while smaller but also reproducible distances were determined if immobilization was carried out through biotin linkages. Therefore, origami molecular rulers might serve as quantification standards for super-resolution microscopes and other spectroscopic techniques such as plasmon coupling.[18] Recent advances in the origami technique[19] will enable our concept to be extended to three dimensions and to super-resolution techniques requiring photoswitchable fluorescent proteins.[20] Single-molecule methods can thus cover the full critical length scale from a few nanometers using fluorescence-resonance energy transfer (FRET) to the length scales of conventional optical microscopy. Such advances are also crucial for the study of dynamic processes such as diffusive or directed transport occurring on DNA-based nanostructures. For example, Brownian DNA "walkers"[21] placed onto an origami grid could be tracked in real time with fluorescence microscopy by using super-resolution methods.

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