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Applications of DNA origami in super-resolution microscopy

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DNA origami enables the bottom-up fabrication of structures at the nanoscale. Using modified staple strands that guide the structure in place, functional groups can be placed with nm-scale precision onto the so-called origami breadboard. Super-resolution microscopy is a technique in which images are reconstructed from multiple datasets to allow for resolutions below the diffraction limit. Here, we will be looking at three different ways the origami breadboard has been used as a testing ground for super-resolution imaging: DNA-points accumulation in nanoscale topography (DNA-PAINT), FRET-based imaging, and how DNA-nanoantennas have been used to place nanoparticles to enhance fluorescent output.

1 Introduction

DNA origami is a novel way of making shapes out of DNA. First proposed by Seeman in 1982 [1], DNA origami makes use of the specificity of non-covalent interactions between nucleobases (Watson-Crick pairing) to rationally design and self-assemble structures at the nanoscale. In 2006, Rothemund expanded on this idea by using the single stranded DNA (ssDNA) of the M13mp18 virus in combination with DNA staples to create a one-pot synthesis approach to make DNA structures [2] (Figure 1). DNA staples are small strands of ssDNA that are complementary to a specific part of the scaffold, thereby guiding the scaffold into the desired shape depending on where they bind. Due to their relatively short length, DNA staples can be easily synthesized, and no stoichiometric considerations have to be taken into account during folding

due to their orthogonality [2].

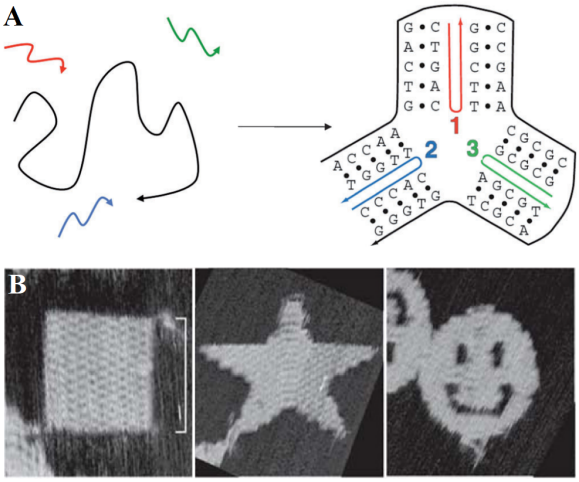

Figure 1: **A)** Schematic overview of three staples binding to a ssDNA scaffold. By tuning the length of the DNA staples such they are orthogonal to both each other and the scaffold, the scaffold can be made to precisely fold into the desired shape. Figure adapted from [3]. **B)** AFM images of several 2D origami structures. Panel size 165 nm × 165 nm. Figure adapted from [2].

Although simple DNA origami structures had been made previously as a proof of concept [4, 5], the genius of the one-pot synthesis approach led to an explosion of interest in the field. Now, almost two decades later, DNA origami has seen a variety of (potential) applications, including: DNA computing [6], DNA assisted electronics [7], drug delivery [8], catalysis [9], and super-resolution microscopy.

Super-resolution microscopy is an optical imaging technique that allow for resolutions beyond the diffraction limit (approximately 200 nm for visible light) [10]. Over the years different super-resolution techniques have been developed, such as stochastic optical reconstruction microscopy (STORM) [11] and photoactivated localization microscopy (PALM) [12]. Both work on a similar principle: By imaging only a fraction of the fluorescent dyes at once, and repeating this multiple times, an image can be reconstructed over many illuminations with a higher spatial resolution than possible with an ordinary optical microscope. Figure 2 shows a schematic overview of the reconstruction principle.

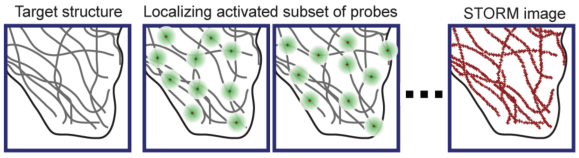

Figure 2: Basic principle of STORM: A densely labeled target structure is taken, but only a select number of fluorophores are activated at one time (green circles). Repeatedly activating a changing subset of fluorophores allows high localization precision of individual fluorophores, in turn allowing for a high-resolution image to be reconstructed. Figure adapted from [13].

Super-resolution microscopy does not come without limitations: The fluorescent dyes used have to be able to withstand many illumination cycles without photobleaching, and the emphasis on spatial resolution comes at the cost of temporal resolution. In this review, we will delve further into how DNA origami can be applied in super-resolution microscopy to overcome these limitations.

2 The DNA breadboard

Soon after introduction of the one-pot DNA origami technique by Rothemund [2], it was discovered that the staple strands could be modified to have functional groups sticking out of the 2D origami scaffold, yielding a breadboard with nm-scale control over the distance between functional groups (Figure 3) [14]. By hinging two of such breadboards together, Funke and his colleague managed to show that they could rationally place groups attached to the staple strands with atomic-scale precision [15]. Despite substantial success at the nm-scale, claims of the technique being able to achieve displacements smaller than the Bohr radius (< 0.04 nm) have so far remained unverified.

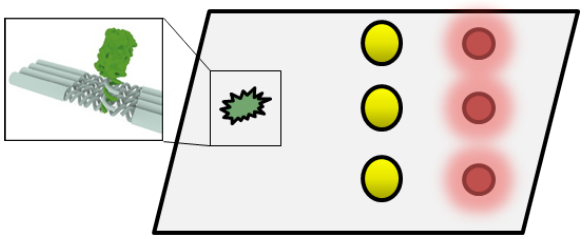

Figure 3: Schematic of the DNA breadboard. By modifying the staple strands to stick out of the breadboard, groups such as proteins (green), nanoparticles (yellow), or fluorophores (red), can be placed with molecular precision. Here, a DNA-conjugated protein is attached to a complementary sequence in one of the staples. Figure adapted from [16].

The breadboard modified with fluorescent staples has also been used as a nanoscale ruler [14, 17, 18]. The a-priori knowledge of where a dye will be found has been used to determine the true localization accuracy [14, 17] and precision [19] of microscopes in the sub-10 nm range. Now, we will look at how the origami breadboard has been used as a testing bed for different approaches in developing super-resolution microscopy techniques.

3 DNA-PAINT

Jungmann et al. first used a functionalized DNA-origami scaffold in combination with DNA-point accumulation for imaging in nanoscale topography (DNA-PAINT) [20]. Instead of directly linking their fluorophores to the staple strands, they designed their staple strands to stick out with a ssDNA docking strand. They then fabricated an imager strand, which is a fluorophore attached to a strand of ssDNA complementary to the docking strand (Figure 4). The imager strand binds either permanently or transiently, depending on the length of the complementary sequence. Using DNA-PAINT, they found the association of the imager strand to be weakly dependent on strand length ($k_{on} \sim 10^6$ $\mathrm{M}^{-1}\,\mathrm{s}^{-1}$), but the dissociation to be strongly dependent on strand length ($k_{off} < 1\ \mathrm{s}^{-1}$). As a proof of concept, they were able to achieve a localization precision of 20 nm at a time resolution of 500 s per image.

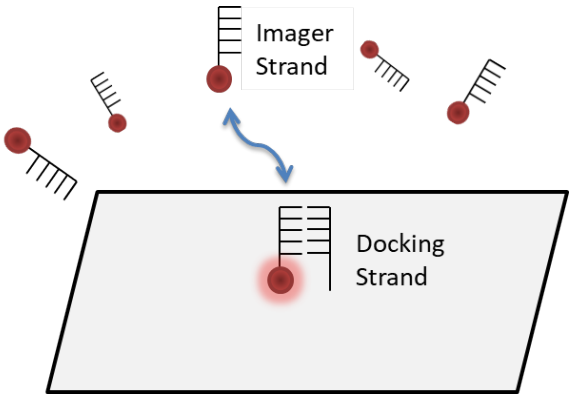

Figure 4: Schematic overview of DNA-PAINT. The imager strand is functionalized with a fluorophore and has a complementary sequence to the docking strand, allowing it to bond to the docking strand. The length of the complementary sequence determines how strongly the imager strand is bound [20]. Figure inspired by [21].

The advantage of DNA-PAINT based microscopy is the following: Ordinary super-resolution dyes have to be carefully chosen such that they have the right fluorescent spectrum for the chosen application, *and* that they can withstand many excitation-emission cycles before photobleaching. The transient binding of DNA-PAINT overcomes this limitation by constantly replenishing fluorescent dyes from solution. Using DNA-PAINT, Raab et al. were able to resolve binding sites on their origami breadboard 6 nm apart with a time resolution of 1800 s [22]. Expanding on this, Dai et al. were able to shave this down another nm to 5 nm (Figure 5) by lowering their imaging strand concentration [23]. Albeit at the cost of reducing the time resolution to 16 000 s.

Another advantage of DNA-PAINT is the ability for multi-colour imaging. Instead of dyes with different absorption and emission spectra, orthogonal docking sites are used. By adding only one complementary imaging strand at a time and washing after each measurement, multicolour imaging can be done se-

quentially. This method therefore requires only one laser and dye, allowing for the optimal selection of each. Then, during reconstruction an arbitrary colour can be assigned to each measurement, and all images can be combined into one multicolour image. As a proof of concept, Jungmann et al. were able to design a ten-colour super-resolution image on just one substrate (Figure 6) [24].

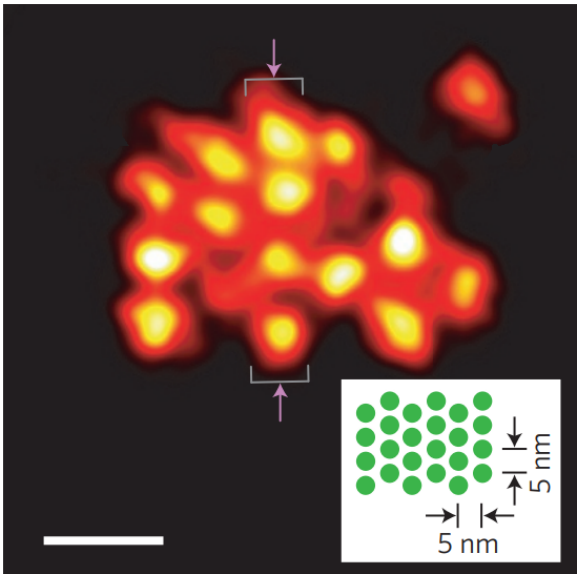

Figure 5: Super-resolution image of a 5 nm origami grid acquired with DNA-PAINT. Pink arrows indicate the projection direction along which the authors did their localization assessment. Inset: design schematic in which each green dot represents a docking strand. The scale bar corresponds to 10 nm. Figure taken from [23].

Several variations on DNA-PAINT have been developed [1]. Quantitative PAINT (qPAINT) is a variation on DNA-PAINT that determines the number of target sites by looking at blinking kinetics [27, 28]. At a constant concentration of imager strands, the blinking of fluorophores in a region of interest is only determined by the concentration of docking strands. By comparing blinking times between structures, quantitative assessments can be made about the (origami) structure [28, 29] or concentration [30].

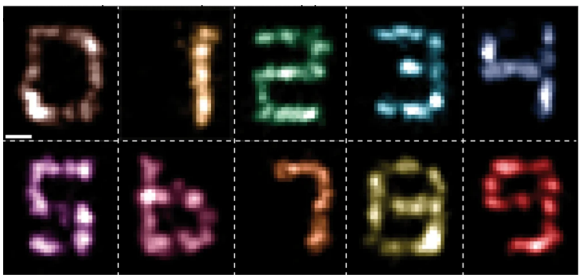

Figure 6: Multicolour image taken using Exchange-PAINT on a single origami breadboard. Using orthogonal docking sites, measurements could be done sequentially by removing previous fluorophores before introducing the next imaging strand. This washing step allowed the usage of the same fluorophore for all ten steps. After reconstruction, each image was assigned a pseudocolour and all images were combined to create a multicolour image. The scale bar in the top left panel corresponds to 25 nm. Figure adapted from [24].

Another variation worth mentioning is 3D imaging using DNA-PAINT. Using astigmatism, the axial position of a fluorophore can be determined by looking at the ellipticity of the normally circular point spread function of the fluorophore [31]. Applying these principles to DNA-PAINT, a sub-15-nm lateral resolution with a sub-50-nm axial resolution has been achieved on origami tetrahedra (Figure 7) [32]. Other groups found slightly worse results using either tetrahedra [33], or DNA-nanorods [28].

Several works have used the DNA-PAINT principle to test new fluorophores [34, 35]. Instead of incorporating these dyes directly into the staples, they use very long (∼20nt) imager strands. Directly incorporating the dyes into the staples is often not beneficial to the nucleation of the origami scaffold. By increasing the nucleotide sequence between the docker and imager strand, the binding of the imager is no longer transient, and individual fluorophores can still be studied.

[1] For the sake of brevity, I have chosen to focus on the main variations and to skip smaller proof-of-concept variations such as action-PAINT [25] or peptide-PAINT [26].

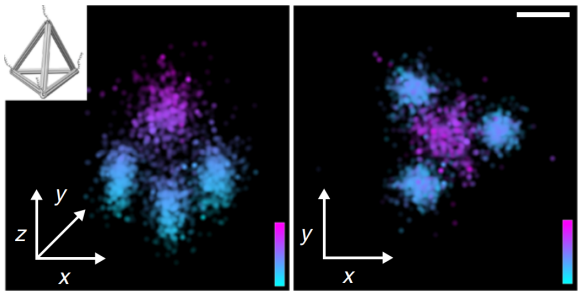

Figure 7: Sum of 42 tetrahedra with sub-15-nm lateral and sub-50-nm axial resolution resolved with DNA-PAINT. Inset: design of the tetrahedron. Each edge was designed to be 100 nm. 3D imaging was achieved using astigmatism to determine the axial position. Scale bars: 200 nm. Figure taken from [32].

As with other techniques, DNA-PAINT does not come without its challenges and limitations. At high concentrations ($\sim$ 10nM) of imager strands, the fluorescent background from unbound imager strands in solution becomes a significant problem [36–38]. As a consequence, DNA-PAINT imaging has been done with nM concentrations of fluorophores, resulting in a slow imaging speed.

In the past years, several groups have made efforts to overcome this slow temporal resolution.

For example, Chung et al. were able to increase k_{on} and k_{off} fluorophore rates resulting in the generation of more blinking events per second [30]. Other works have optimized their imager strands to be as dark as possible when in the unbound state by self-quenching [30, 37, 38]. Another work concatenated docking strands to increase the probability of binding and thus imaging speed [39].

In recent years, there have been increased efforts to improving image quality through software. Narayanasamy et al. combined higher fluorophore concentrations with a deep-learning algorithm to predict fluorophore positions [40]. They managed to reduce their imaging time by a factor of 25 compared to normal super-resolution reconstructed imaging, decreasing their temporal resolution down to 60 s per image. However, resolution decreased from 35 to 45 nm, which they attribute to rendering differences between software and increased background due to the higher fluorophore density. Similarly, Zhu et al. demonstrated that they could use a neural network to reconstruct microtubules with less than 10% of the raw frames they would otherwise need to achieve a 40 nm resolution [41]. As a last example, Heydarian et al. designed an algorithm that fuses (incomplete) images from multiple breadboards into one localization image, thereby saving otherwise unusable datasets and ultimately lowering experiment time [42].

4 FRET

DNA-Förster resonance energy transfer (DNA-FRET), also known as FRET-PAINT, works on similar principles as DNA-PAINT. Just like in normal FRET, two dyes are brought into proximity of each other. The excited donor dye transmits its energy to the acceptor dye, which subsequently releases a photon. In DNA-FRET, the donor and acceptor are conjugated to an imager strand. Both of these imager strands are complementary to sections of one single docking strand. Similarly to DNA-PAINT, FRET-PAINT prevents any photobleaching by constantly refreshing dyes from solution. The main benefit of FRET-PAINT over DNA-PAINT is the absence of a fluorescent background, as the fluorescence of the acceptor is only present when a donor is nearby to form a donor-acceptor pair. The absence of a fluorescent background allows for much higher concentrations of fluorophores to be used during imaging as compared to DNA-PAINT, leading to potentially faster imaging times. Figure 8 shows a schematic overview of the FRET-PAINT principle.

Auer et al. were the first to apply the principles of FRET-PAINT on an origami breadboard [21]: First, they used a docking

strand functionalized with their acceptor molecule (thus fixing the acceptor in place). Although achieving high FRET efficiency, they quickly photobleached all their acceptor dyes. Second, they used both transiently binding donor and acceptor strands to bind to the docking strand. Testing the second approach on the DNA breadboard resulted in a resolution of 20 nm in a mere 35 s. Furthermore, they applied the first technique to microtubules stained with secondary antibodies. On the antibody sat a docking strand with an acceptor dye fixed in place. They were able to resolve a resolution of 46 nm in just 28 s, being only limited by the bulky antibody. They did not go into detail however as to why the acceptor did not photobleach as it did in their first experiment. Lee et al. repeated the microtubules experiment, finding similar results [36]. Figure 9 shows a comparison in imaging speed of DNA-PAINT vs FRET-PAINT on microtubules.

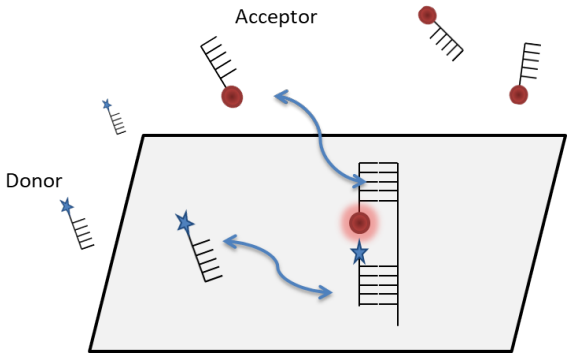

Figure 8: Schematic illustrating principle of FRET-PAINT. Both donor and acceptor strands can independently bind to different parts of a single docking strand, but only when both are bound at the same time is a fluorescent signal from the acceptor observed. Figure inspired by [21].

In their next paper. [43] Lee et al. further improved upon the work of Auer: By tuning their spectral filters they managed to reduce bleed-through of the donor into the acceptor channel, allowing them to increase their probe concentration and excitation intensity [43]. Additionally, they used shorter donor strands to increase dissociation rates, increasing their imaging frame rate. All these changes combined into an 8-fold localization rate compared to their previous work. Although improved imaging rates were achieved, the increased laser intensity resulted in damage to the docking strands over time, eventually preventing further binding of the fluorophores.

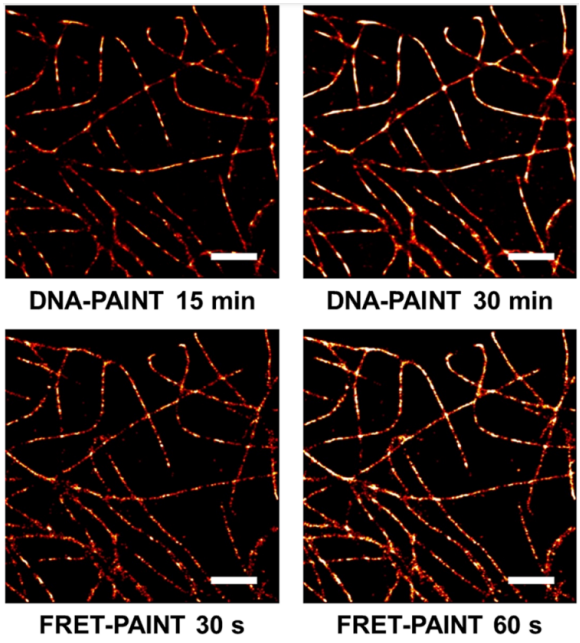

Figure 9: Comparison of microtubules imaged with DNA-PAINT and FRET-PAINT. Each image was recorded at a 10 Hz frame rate. FRET-PAINT shows a 30-fold increase in image acquisition speed compared to DNA-PAINT. Scale bars: 2 µm. Figure taken from [36].

Aside from imaging, the breadboard has also been used to test other FRET parameters such as efficiencies of FRET radii. By changing the distance between dyes with nm precision on the origami scaffold, FRET efficiencies have been determined for either dye pairs [18, 44, 45] or ensembles [46]. Deußner-Helfmann et al. then used the efficiency measurements of multiple dye pairs as an alternative parameter for multi-colour imaging [45].

5 Fluorescent enhancement via nanoparticles

So far, we have looked at the ability of DNA origami to direct fluorophores for super-resolution microscopy. In this section, we examine how DNA origami can also be used to place nanoparticles close to fluorescent dyes, thereby increasing fluorescent yield.

Acuna et al. were the first to use origami nano-antennas to direct nanoparticles to either quench [47] or enhance fluorescent intensity [48]. To enhance fluorescent intensity, they attached gold NPs to the origami structure and incorporated docking sites for fluorophores between the nanoparticles (Figure 10). The nanoparticles produce highly enhanced local fields known as plasmonic hotspots. By placing a fluorophore in the plasmonic hotspot of the nanoparticles, Acuna et al. observed a 28-fold increase in fluorescent intensity [48].

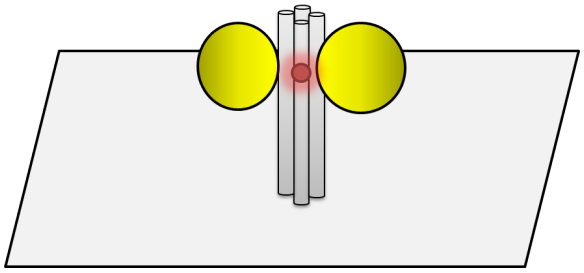

Figure 10: Schematic design of the fluorescent enhancement via nanoparticles as performed by Acuna et al. [48]. Gold nanoparticles (yellow) are placed onto an origami nanoantenna near the fluorophore (red). This creates a plasmonic hotspot in the volume where the fluorophore sits and greatly increases fluorescent output. Figure inspired by [48].

Over time, the structure of the nano-antennas has been improved to better orient and space the NPs with respect to the fluorophore [49–51]. Applying these optimizations to their experiments, Puchkova et al. reached an average 306-fold fluorescent enhancement [50].

More recently, it was found that using nanorods (NRs) instead of NPs, a fluorescent enhancement of more than three orders of magnitude could be achieved [52]. Furthermore, it was found that the emission spectrum of the associated fluorophore can be tuned by modifying the length of the NRs [53].

The potential of fluorescent enhancement on DNA origami is not hard to see; it would allow us to conduct and measure bio-assays at much lower concentrations than previously possible. Fluorescent enhancement may also prove useful to achieve higher temporal resolutions in single-molecule studies, as the higher fluorescent intensity would allow for shorter integration times per frame. Although plasmonic enhancement with nanoantennas to increase imaging speed has been tried as a proof of concept [54], no one seems to have tried applying it to DNA-based nanoantennas. Applying plasmonic enhancement to DNA-based nanoantennas therefore seems an exciting avenue for further research.

6 Final remarks

In this paper, we have seen how DNA origami can be used as a breadboard with nm-scale addressability to develop three techniques in super-resolution microscopy: DNA-PAINT, FRET, and the fluorescent enhancement of fluorophores. We have seen that using these techniques on the breadboard nm-scale super-resolution microscopy is possible. However, this was only on the breadboard. Many groups have tried and succeeded in resolving microtubules in both 2D [21, 36] and 3D [24, 30, 32] using the aforementioned techniques. However, all these works first coupled antibodies to the microtubules and then func-

[2]The length of the docking strand does introduce some flexibility, and with that some limitations on the exact positioning of the dyes. This uncertainty, however, is in the order of nanometres and does not have any significant impact resolution [36].

tionalized the antibodies with docking strands for imaging. These bulky antibodies severely limit resolution to > 40 nm in their works [2]. To be able to truly harness the nm-scale resolutions we have seen on origami structures, significant efforts should be focused on creating smaller linkers.

One obvious application of DNA nanotechnology I felt was missing in the literature is the detection of RNA. Strangely enough, only in 2023 did the first paper on RNA detection using the origami breadboard and DNA-PAINT come out [55] [3]. Here, the authors designed a breadboard with staple strands that were complementary to half of a miRNA strand. The other half was then designed to be complementary to a bridge strand, which was in turn complementary to an imager strand. Using this approach, they were able to detect RNA in the blood plasma of breast cancer patients at concentrations as low as 11 fM. They further determined the specificity of their sensor to be within only one nucleotide mismatch. I believe that this technology could greatly benefit from the techniques to enhance fluorescent yield as described in Section 5. For example, increases in fluorescent yield brought on by plasmonic enhancement could enable the detection of RNA at sub-fM concentrations, thereby providing an amplification-free alternative to the polymerase chain reaction (PCR) detection technique.

Although the aforementioned developments in super-resolution microscopy techniques seem promising, one drawback is that modern microscopy hardware can be prohibitively expensive. Luckily, the future holds great promise. One group overcame the high costs by using cheaper off-the-shelf hardware to design their own microscope [33]. Another group implemented DNA-PAINT with a spinning disc confocal system to overcome this limitation [32]. I believe that image reconstruction aided by artificial intelligence (AI) may also allow us to image with less specialized (and thus cheaper) equipment. As alluded to earlier, applying neural networks to super-resolution microscopy techniques could indeed allow us to have our cake and eat it too: achieve both nanometre resolution and high speed imaging. Given the rapidly developing field of deep learning image recognition models, I foresee that these advancements will characterize the improvements we will be seeing in optical super-resolution microscopy in the coming years.

Finally, it seems we have come full circle. As super-resolution techniques get refined by using DNA origami as a testing bed, these microscopy techniques are now being used to study the assembly of the origami structures themselves. Strauss et al. used DNA-PAINT to look at the incorporation efficiency and accessibility of staple strands in origami structures [57]. Other groups have used DNA-PAINT to study DNA nanostructures formed by the assembly of origami structures [58–60]. A symbiosis between the fields of DNA origami and super-resolution microscopy has formed, and I believe that - together with AI - DNA origami will become the preferred testing and developing platform for super-resolution microscopy.

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[3] In fairness, some research was done on RNA detection using DNA origami with AFM. See for instance [56]. Their methods required RNA concentrations in the 100s of nM, making it clinically unviable.

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