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*Commentary and Perspective (Invited)*

# DNA nanomachine tutorial

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DNA nanotechnology exploits the programmable specificity bestowed by base-pairing to custom-build macromolecular objects with desired shapes, and sometimes with machine-like functions. These synthetic nanomotors are self-assembled and controllable by signaling molecules or environmental changes. They are routinely designed and made to mimic natural biological molecular motors or perform various human-desired tasks. With potential applications in molecular processing, molecular computing, drug delivery, biological imaging, diagnosis, detection and more, they are powerful tools to add to any laboratory's arsenal. This perspective article serves as a short introduction to the field and a quick start to designing your do-it-yourself DNA nanomachines. The content is mainly based on the "Hands-on training program D on DNA nanomachine tutorial", part of the 21st International Union for Pure and Applied Biophysics and the 62nd Biophysical Society of Japan Joint Congress.

## A Program for Young Researchers and Students

The International Union for Pure and Applied Biophysics (IUPAB) is an international non-governmental organization whose mission is to assist in the worldwide development of biophysics, to foster international cooperation in biophysics, and to help in the application of biophysics toward solving problems of concern to all humanity. As a commitment to promoting education and capacity-building programs for students and young researchers, several Hands-on programs happened across Japan before and after the main 21st IUPAB Congress. The "Hands-on program D on DNA nanomachine tutorial" took place on the beautiful campus of Kansai University, Osaka, Japan, on the 22nd of June 2024. The university is home to two well-regarded professors in the DNA nanotechnology field, Dr. Masayuki Endo and Dr. Akinori Kuzuya. Joined by Dr. Yuki Suzuki (Mie University) and Dr. Ibuki Kawamata (Tohoku University), the four lecturers effectively covered the basics of DNA nanomachine design, evaluation and analysis; along with practical tools for designing, simulation and visualisation to ten participants. The students came from various backgrounds, career stages, genders and nationalities, and had yet to gain experience in the field. After an afternoon, they could directly apply the tools to create their first designs and present them in front of the group. A quick lab tour gave a glimpse into the practical set-up of a DNA Nanomachine lab and an evening reception allowed interesting exchanges on different cultures. The event was organised by Dr. Sinn-ichiro M. Nomura and volunteers and sponsored by Grant-in-Aid for Transformative Research Areas (A) "Molecular cybernetics".

## Basics of DNA Nanomachines

After a warm welcome, the participants were introduced to the general basics of DNA nanomachines by Dr. Masayuki Endo (Kansai University). The content of his talk mainly comes from the review paper on Molecules in 2018 [1] along with new updates. As an outsider without much prior knowledge, the author was fascinated with the creativity of the inventors who created many DNA nanomachines in different shapes, forms and functions. Roughly, DNA nanomachines can be classified into four main groups based on their functions (Figure 1A-D). Noticeably, to add those different functions

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to DNA motors, the most used mechanism is called toehold-mediated strand displacement (Figure 1E). An example is in zipping motion of DNA tweezers which are nanodevices that can capture and release targeted strands [2]. Other examples are DNA walkers, a group of motors that walk on origami pathways [3,4], mimicking biological processive molecular motors such as kinesins. Using toehold-mediated strand displacement as a gating mechanism for branch migration, these motors are capable of walking on 100 nm track and navigating between different routes.

Another notable group of motors is the DNA dynamic switches. They can alter between two states: "open" vs "locked", depending on the presence of the toehold signaling strands [5]. Advancements in new ways to control DNA motors leads to similar two-state DNA dynamic switches that responds to its environment, such as salt and temperature [6]. Eliminating the need to add extra molecules (such as toehold strands or additional salt) to control molecular motors is highly desirable. Hence, Dr. Endo and his team developed similar systems that can be controlled by UV and visible lights [7,8]. Based on the photoisomerisation of azobenzene moiety, photoswitches of this kind has become the most common strategy to create reversible and enclosed nanomachine systems.

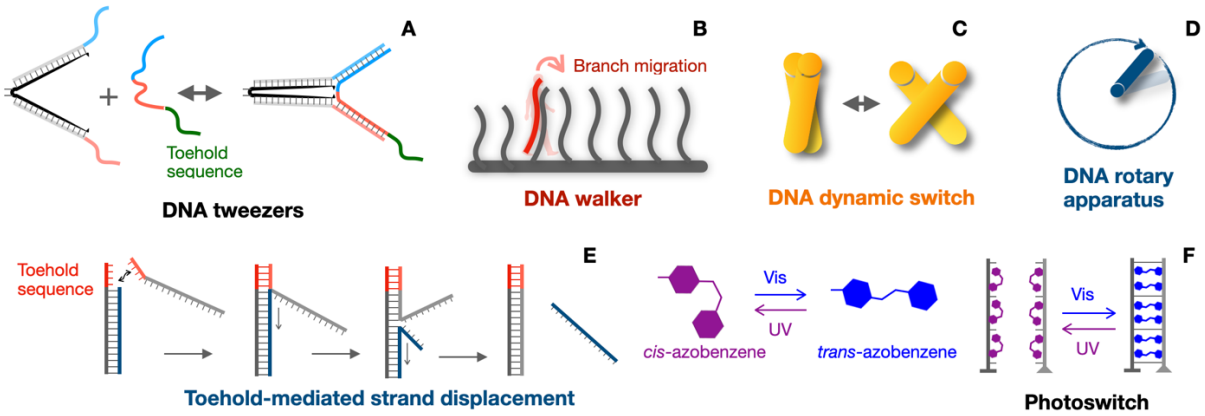


**Figure 1** (A-D) Four big groups of DNA nanomachines: DNA tweezers (A), DNA walker (B), DNA dynamic switch (C), and DNA rotary apparatus (D). (E-F) Two commonly used design strategies to add functions to DNA nanomachines: Toehold-mediated strand displacement (E) and photoisomerisation of azobenzene moiety as photoswitch (F).

To investigate DNA nanomachines' shapes, atomic force microscopy (AFM) is routinely performed. Designing DNA motors with additional functions opened up opportunities to employ other single-molecule analyses. For instance, plasmonic circular dichroism (CD) spectra were used to determine different states of hybridisation corresponding to different states of DNA switches [7]. By combining DNA motors with two gold nanorods (AuNR), the signals of the locked and relaxed states can be repeatedly "written", "erased", and "read" as an enclosed light-driven 3D plasmonic nanosystem. Other examples are from the group of rotary motors. The first DNA rotary motors were originally reported in [9]. A more sophisticated version mimicking biological motors such as ATPase is lately reported in [10]. Recently, a DNA origami-motor protein hybrid using RecBCD is also developed [11]. With this new mode of motion, direct rotation of the motors can be observed using single-molecule fluorescence microscopy.

## Design Principles of DNA Nanostructures

All DNA nanomachines are made of DNA nanostructures, arbitrary shapes and forms made of folding DNA strands. In order to understand the design principles of DNA nanostructures, Dr. Akinori Kuzuya introduced us to the two fathers of the field: Ned Seeman and Paul Rothemund along with the basic design principles they laid out. For details, readers can refer to two origami books: "DNA Origami: Structures, Technology, and Applications" and "Introduction to DNA Origami" (in Japanese only).

In a nutshell, all DNA nanomachines are self-ensembled from DNA-based materials. In normal conditions, DNA is stable in the B-form as a long double helix, with a 10.67 basepairs per pitch of 3.6 nm and a 3.4 Å distance between two consecutive basepairs. The only native branched conformation that appears in biology is the Holiday Junction (HJ), formed by two homologous DNA double helices. However, HJ's branching point can be shifted, and strands can be switched. Seeman came up with the first design of a fixed branching point in his famous 1982 paper [12] (Figure 2A). At the time, inspired by Maurits Cornelis Escher's art, Seeman wrote about applications of his design as a part of an infinite branching DNA network in the context of assisting crystallisation and structure-solving problems. Nowadays, his proposal is normally referred to as the birth of the field of DNA nanotechnology.

Following the initial proposal, Seeman quickly assembled multiple branched DNA strands into DNA cube [13] and

DNA octahedron [14]. He also realised that when two double-stranded DNA (dsDNA) are aligned and connected via single crosslinking, the fixed-point branch structure forms a cross shape instead of maintaining the polarities of the two initial helices. In addition, the junction can switch between legs. So Seeman added a second cross-linking [15] in a design normally referred to as a "double crossover" tile, or DX tile (Figure 2B). Double crossover molecules are DNA structures containing two Holliday junctions connected by two double-helical arms. In nature, they occur as intermediates in recombination processes involving double-strand breaks. To eliminate distortions and maintain stability, Seeman broke it down into five components which can be ordered from commercial companies. The initial DX tile was proposed in 1993 [15] and later, assembled into 2D sheets and directly verified with AFM imaging in 1998 [16].

Several other DNA tiles are worth quickly mentioning (Figure 2C-D). A PX tile is made from paranemic crossover in which all meeting points between two dsDNA helices are crossover. PX tile contains 4 strands instead of 5 as in DX tile. A JX2 tile also has two pairs of DNA strands that are wind together but have one cross-over skipped in comparison to the PX design. The JX2 tile was applied to make the first rotary device in 2002 [9]. Other than tiles, Seeman also designed the six-helix bundle (6HB) motif [17]. These are the building blocks for all different 2D sheets and 3D structures, including the largest DNA tile which is termed "DNA tape" created by Dr. Kuzuya [18], and the Sierpinski triangles and nanotube created by Paul Rothemund [19,20]. All in all, Seeman has laid out the conceptual foundations for DNA nanotechnology.

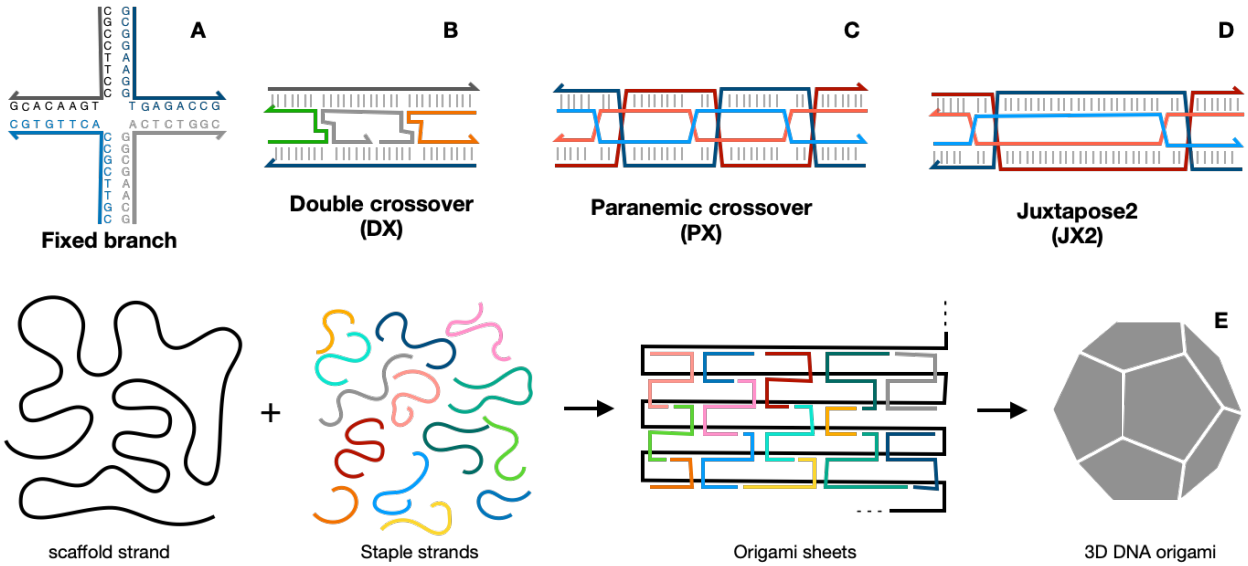


**Figure 2** (A-D) Basic elements of DNA nanostructures designed by Seeman. For clarity, the twisting effects of dsDNA helices are not explicitly shown in the diagrams. They are implicitly implied as crossover positions are naturally meeting points of any pair of strands. Thin bars are marked where dsDNA is formed, not reflecting exact numbers of basepairs. (E) The "one-pot" strategy of "scaffolded DNA origami" designed by Rothemund: long DNA strands (scaffolds) is mixed with a hundred-fold excess of ~ 200 shorter DNA strands (staples and remainders) and slowly annealed to self-assemble into 2D patterns and 3D shapes.

In 2006, Paul Rothemund developed a technique to fold a single long DNA strand into different shapes and forms using hundreds of staple strands, termed "scaffolded DNA origami" [21]. The technique is based on DX crossovers placed at every 16 basepairs, which correspond to 1.5 turns of DNA helical pitch (Figure 2E). It is simple and reliable as a "one pot" method and is quickly adopted by almost all scientists when they want to make DNA objects. The readers are encouraged to not only read the main text of his paper but also to check details in the supplement information. Based on Rothemund's design rules, Shih and his team designed general rules to make 3D origami structures using building blocks of honeycomb lattice [22] and carved honeycomb structures along with much larger 3D structures [23]. This completed the method development of 3D DNA origami.

## Tools to Design DNA Objects

To assist with the designing process, Shih and his team created a design software called "caDNAno" [24] which is used and recommended by Rothemund himself. For the second half of the training section, the participants were introduced to how to use this tool by Dr. Yuki Suzuki and Dr. Ibuki Kawamata.

Dr. Yuki Suzuki first gave examples of how simply based on the above design concepts, complicated machines can be made even without sophisticated software. By breaking the nanomachines into key elements such as rigid bodies (double-

stranded DNA), flexible joints (single-stranded DNA region), fuel and anti-fuel strands, simple machines such as DNA tweezers can be made to capture small molecules [2] (Figure 1A). After the DNA origami design rules were available [21], Dr. Kuzuya applied them to create a more advanced version of DNA tweezers, termed "DNA origami forceps" [25]. In this design, the authors used only a single cross-over between the two rigid parts, which is unique compared to the common design with DXs. Impressively, he did not use design softwares for this work. With the assistance of new software, the designing process does get simpler. Combining stepping and rotating modes, Dr. Suzuki himself and his colleagues created a rotary DNA origami device that can also step [26]. Most recently, his team developed DNA origami nanolattices by implementing multiple flexible joints [27].

The participants then moved on to install caDNAno and tried to design their own patterns, under the instructions of Dr. Ibuki Kawamata. Readers are encouraged to follow instructions on the website https://cadnano.org to install and follow a series of videos on YouTube to try to design their first DNA sheet. Once getting used to the program, the readers can come back to the supporting information of the DNA origami paper [21] and try to create a design of one of Rothemund's original origamis (e.g. happy face) to understand better his design rules, including nick positions, seam positions, etc... Remember to save frequently since caDNAno is prone to crash. And most importantly, enjoy.

Once a design is created, it can be quickly put through Cando https://cando-dna-origami.org/ to gain quick computational feedback on the 3D structure of programmed DNA assemblies. Other computational platforms can also be used such as Dr. Kawamata's web server: oxDNA via GUI [28]. To visualise 3D structures, readers can use Cadnano2pdb from the Aksimentev lab to convert caDNAno files to .pdb format, which can be used in any visualisation program of choice (VMD, PyMOL, ChimeraX).

## Future Perspective

DNA is a versatile, stable, reliable and easy-to-access material. It is also well-studied and well-charactered, easy to make and combine with other materials. We are still in a boom period of DNA nanotechnology, in general, and DNA nanomachines, in particular. Many active research directions include but is not limited to DNA origami and nanoarchitecture, DNA computing, DNA-based therapeutics, bioimaging, artificial cells and smart condensates. Alternative materials, such as RNA origami, have just started to be explored. Other than developments in applications, advances in techniques used in DNA nanotechnology and DNA nanomachines are still yet to be fully explored. So far, the most used technique to directly visualise DNA objects is AFM. As powerful as they can be, classical AFM and high-speed AFM techniques still have many challenges and disadvantages. Current collaboration attempts to use other single-molecule techniques to monitor DNA nanomachines are excited but still limited. Future novel technologies capable of investigating vibrations of the DNA network, visualising movements or shapes of DNA objects away from surfaces and with proper spatial and temporal resolutions would be the next breakthroughs of the field. In addition, computational techniques capable of predicting and complementing current design strategies are still limited. They are particularly in need of when other less predictable and more expensive materials such as RNA are incorporated into the nanomachine designs. New cooperations and new perspectives from different fields would bring more unforeseen and more practical applications of DNA nanomachines in medicine and other fields in near future.

Lastly, there is no doubt what is covered here is just a scratch on the surface of the field of DNA nanomachines. Due to the size of this mini-tutorial, the author had to skip mentioning many other big names and big events in the field. Still, the author would like to express her gratitude for the opportunity to attend the original hands-on training and to gain such knowledge in such a short period of time. It provided her with so many new ideas and unexpected perspectives. The experience has been incredibly valuable for her career development as a young researcher. The author hopes this mini tutorial will continue to propagate the success of the program to more young researchers in a larger community.

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