DNA Bipedal Motor Achieves a Large Number of Steps Due to Operation Using Microfluidics-Based Interface

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ABSTRACT: Realization of bioinspired molecular machines that can perform many and diverse operations in response to external chemical commands is a major goal in nanotechnology, but current molecular machines respond to only a few sequential commands. Lack of effective methods for introduction and removal of command compounds and low efficiencies of the reactions involved are major reasons for the limited performance. We introduce here a user interface based on a microfluidics device and single-molecule fluorescence spectroscopy that allows efficient introduction and removal of chemical commands and enables detailed study of the reaction mechanisms involved in the operation of synthetic molecular machines. The microfluidics provided 64 consecutive DNA strand commands to a DNA-based motor system immobilized inside the microfluidics, driving a bipedal walker to perform 32 steps on a DNA origami track. The microfluidics enabled removal of redundant strands, resulting in a 6-fold increase in processivity relative to an identical motor operated without strand removal and significantly more operations than previously reported for user-controlled DNA nanomachines. In the motor operated without strand removal, redundant strands interfere with motor operation and reduce its performance. The microfluidics also enabled computer control of motor direction and speed. Furthermore, analysis of the reaction kinetics and motor performance in the absence of redundant strands, made possible by the microfluidics, enabled accurate modeling of the walker processivity. This enabled identification of dynamic boundaries and provided an explanation, based on the “trap state” mechanism, for why the motor did not perform an even larger number of steps. This understanding is very important for the development of future motors with significantly improved performance.

Our universal interface enables two-way communication between user and molecular machine and, relying on concepts similar to that of solid-phase synthesis, removes limitations on the number of external stimuli. This interface, therefore, is an important step toward realization of reliable, processive, reproducible, and useful externally controlled DNA nanomachines.

KEYWORDS: DNA nanotechnology, DNA motors, DNA machines, single-molecule fluorescence, sm-FRET, microfluidics

Biological molecular motors made of proteins, which play major roles in many biological processes, often operate with remarkably high chemical yield and speed. For example, the kinesin bipedal motor can perform hundreds of steps per second before dissociating from the microtubule track.1,2 These motors are autonomous, directional, processive, and fast and, like enzymes, do not chemically and irreversibly change during operation (no “burnt-bridge” mechanism3,4).

Received: January 24, 2017
Accepted: April 12, 2017
Published: April 12, 2017
For bipedal walkers, such as kinesin, to avoid dissociation from the track, the trailing leg must somehow “know” not to detach from the track before the leading leg is stably anchored. Developing such an internal coordination mechanism for an autonomous synthetic molecular motor that is directional, processive, and free of the burnt-bridge effect is, however, a very difficult task. To the best of our knowledge, only two such motors have been demonstrated so far, but these remarkable motors have low processivity (only two steps) or are slow (one rotation in 12 h). By sacrificing autonomy and providing control and, therefore, coordination, from the outside, directionality, processivity, speed, and avoidance of the burnt-bridge effect can be more easily achieved. This approach was demonstrated for DNA motors that operate by responding to a sequence of externally introduced commanding DNA strands, often called “fuel” and “antifuel,” that also provide chemical energy for the motors. Following this approach, our group developed a DNA bipedal motor that strides on the DNA origami track by responding to fuel and antifuel strands. Although theoretically DNA stability and DNA interaction yields should allow many operations, externally controlled motor and machines typically performed only a small number of steps, and our motor performs only seven steps. Because commanding strands accumulate in the solution, it is reasonable to suspect that these strands eventually interfere with the motor operation, reducing motor processivity. Puriﬁcation methods such as gel electrophoresis and magnetic beads can remove such DNA strands from the motor solution. These methods, however, disrupt continuous operation, may harm the integrity of the motors, and are slow and tedious, making them inadequate for performing more than a few sequential operations. External input of strands and other reactants is important for many functions and applications. These include enzymatic synthesis of chemical compounds using molecular assembly lines, structural manipulation and maneuvering of externally controlled substrate molecules, and DNA-based molecular computing, to name a few. We, therefore, set out to develop a method that allows unlimited introduction and removal of commanding strands and other reactants without disturbing the molecular motor and machines or interrupting their operation.

Here, we employed a microﬂuidics device that, as in solid-phase synthesis, allowed convenient user- and computer-controlled introduction and, most importantly, removal of chemical inputs to and from molecular machines immobilized...
Figure 2. Immobilization and assembly of the motor inside the microfluidics device. Motor schematics and typical TIRF images (donor and acceptor channels are shown on the left and right side of the image, respectively) recorded at different assembly steps. To avoid accumulation of fluorophore bleaching, each of the TIRF images was recorded at different positions along the working chamber. (a) Introduction of biotinylated bovine serum albumin and NeutrAvidin. The TIRF images demonstrate the cleanliness of the working chamber. (b) Introduction of acceptor-labeled origami track. Track concentration and uniformity were monitored by the acceptor signal. (c) Introduction of FS and donor-labeled walker resulted in binding of the walker to TS, as was indicated by the low FRET (E) values. (d) Introduction of F1 followed by AFS resulted in the walker parking on T1 (state 1) as was indicated by the high FRET values (see also Figure 3a). Direct binding of the walker to T1 would result in binding to TS and T9 (which have identical sequences to that of T1). Therefore, it was necessary to bind to TS, which has a unique sequence, followed by walking to T1 (state 1). The traffic-light-like symbols indicate whether the donor laser or the acceptor laser was on (see Supplementary Figure 3 for scheme of the optical setup). The size of each TIRF image is 60 × 60 μm.

inside the microfluidics device. Specifically, we demonstrated, using the single-molecule fluorescence-based interface, the assembly and operation of a bipedal DNA walker that strides on a DNA origami track in response to DNA fuel and antifuel strand commands. First, the motor was assembled inside the microfluidics using an optimized, computer-programmed procedure, and the microfluidics-driven motor performed 6-fold more steps than an identical motor operating without removal of the redundant command strands (which we will call the “nonwashed” motor). Moreover, to explain why the microfluidics-driven motor performed only 32 steps and not more, we measured the motor reaction kinetics with high time resolution made possible by the microfluidics fast solution exchange rate and also measured the motor operational yields (defined as the percentage of motors that respond as designed) at different fuel concentrations and incubation times. Using this information, which could not have been obtained without the removal of redundant strands, we developed a mechanistic model that considers the binding of two fuels to a leg (which we call the “trap state” model) that predicts the operational yield and explains the dependency of operational yield on motor speed. Finally, we propose a design for a bipedal motor that avoids the principle limitations of the current motor.

RESULTS AND DISCUSSION

In our design, an integrated microfluidics device is mounted on a single-molecule total internal reflection fluorescence (sm-TIRF) setup (Figure 1a–c and Supplementary Sections 1 and 2). The device includes 16 input channels, which convey solutions containing the molecular components for motor immobilization, assembly, and operation. The pneumatic valves that regulate flow are embedded in the device in a design that enables rapid and precise computer-controlled exchange of small-volume solutions at a programmable and reproducible sequence and timing. The motor (Figure 1d–g) is a bipedal walker (made of two legs, L1 and L2) that moves on a DNA origami-based asymmetric track with 10 branching strands that serve as footholds for the walker (TS, T1-T9). The legs attach to the footholds by base pair complementarity and reproducible sequence and timing. The motor speed of the walker. Motor status readout is enabled by labeling L1 with a donor fluorophore and two footholds (T1 and T9) with acceptor fluorophores (Figure 1g), such that different Förster resonance energy transfer (FRET) values (E, eq S1) are obtained when L1 is lifted or parked at different positions along the track. The motor design and DNA sequences are detailed in the Supplementary Section 3 and Supplementary Table 1.

Motor Assembly and Immobilization inside the Microfluidics Device. The motor was assembled and immobilized using our microfluidics and single-molecule fluorescence-based interface. We immobilized the origami track on the coverslip within the working chamber, attached the walker to the track, and then advanced it to the initial state 1 (Figure 2). The assembly procedure was initially optimized using information acquired by single-molecule fluorescence measurements. Optimization included tuning the motor density, ensuring that the walkers faced upward, minimizing fluorescence contamination in the chamber, and ensuring that all walkers were positioned in state 1 (for more details, see Supplementary Section 4). The optimized procedure, which included a total of 36 microfluidics commands (Supplementary Table 2), resulted in almost 100% of the motors positioned in state 1 (Figure 3a). To maintain reproducibility, the optimized assembly procedure was thereafter performed via an automatic computer algorithm. Alternatively, the motor was prepared with the walker attached to the starting foothold (TS) during the...
annealing procedure (Supplementary Figure 4a). This approach was not as efficient due to some dissociation of the walker from the single TS foothold on which it was standing. The difference in efficiencies of these two methods was not very large in this particular case; however, we demonstrate the benefits and potential of the solid-phase chemistry-like, precise, step-by-step molecular machine assembly that is assisted in real-time by feedback from single-molecule fluorescence.

Motor Operation and Performance. The operation phase of the motor was also executed by a computer-programmable procedure (Supplementary Section 5). We introduced 64 fuel and antifuel strands in sequence, resulting in 32 steps performed by the walker, including three preprogrammed changes in direction (Figure 3a). Overall, the microfluidics device conveyed 192 solutions (including washing solutions) through 384 computer commands (Supplementary Tables 3 and 4), which would have been impractical if conducted manually. During operation, we used FRET to monitor the status of the motor at six selected states. Each state yielded the expected FRET values (Figure 3a,b and Supplementary Sections 6 and 7). Forty-four percent of the motors remained intact after 32 steps, traversing 370 nm in total (Figure 3c). This result indicates that only \( \sim 2.5\% \) of the motors dissociated in each step, a record processivity for controlled DNA motors and for motors that do not damage the track (those that damage the track are known as “burnt-bridge” motors\(^{22,33} \)).

Comparison with Nonwashed Motor Performance. To demonstrate the contribution of the microfluidics to the motor operational yield and processivity, we measured the operational yield of an identical motor operated without removal of fuel or antifuel after each step and that was freely diffusing in a solution\(^{17} \) (Supplementary Sections 8 and 13). With this operational scheme, redundant fuels and antifuels accumulate in the solution. We found that the operational yield of the microfluidics-driven motor was 6-fold higher than that of the nonwashed motor (Figure 3c). Furthermore, incorrect populations were observed in the nonwashed motor (Supplementary Figure 6) but not in the microfluidics-driven motor (Figure 3a and Supplementary Figure 5). These results indicate...
that the nonwashed motors interact with the redundant strands: redundant fuels can command the walker to a wrong position, and redundant antifuels can disconnect the walker from the track. By enabling removal of these redundant strands, the microfluidics enabled correct operation of the motor and a significant increase in operational yield.

**Trap State Mechanism Limits Motor Performance.** Despite the removal of redundant strands, some microfluidics-driven walkers dissociated in each step (Figure 3c). Use of the microfluidics system enabled us to determine the mechanism that results in this dissociation. We previously showed that increasing the concentration of fuels decreases the yield of the leg-placing reaction, and we explained that this phenomenon results from binding of one fuel strand to the foothold and another to the leg, forming a stable “trap state.” Such a state prevents the placing of the leg on the foothold and leads to the dissociation of the walker upon interaction with the consecutive antifuel. We showed that this effect can be mitigated by using fuel strands with a hairpin structure (for the operation principle of hairpin fuels, see Supplementary Section 10). However, because the operation with hairpin fuels was demonstrated on a nonwashed motor where the redundant strands reduce the operational yield, we could not use operational yield information to determine to what degree the hairpin fuels prevented the formation of trap states. Using the microfluidics, we were able to answer this question. We measured the operational yield of a microfluidics-driven motor at different fuel and antifuel concentrations and incubation times. The results showed that the operational yield decreased with the increase of hairpin fuel concentration (e.g., for long incubation times, Figure 4a). This indicates that hairpin fuels did not fully prevent the formation of trap states, most likely because some hairpin fuels bind the legs without binding the foothold first (Figure 4b). We developed a trap-state-based kinetic model that calculates the operational yield (Supplementary Section 11). The model is based on the rates of leg-lifting and leg-placing reactions (Supplementary Section 9).

The high temporal resolution that was required for the kinetic measurements was achieved by the fast solution exchange rate of the microfluidics device (~0.07 s, Supplementary Figure 8). The good agreement between the experimental operational yield and operational yield calculated based on the trap state model (Figure 4a) indicates that use of hairpin fuels limits, but does not completely prevent, formation of the trap state and validates the model for hairpin fuel. In addition, the agreement between the experimental results and the model indicates that walker dissociation was primarily a result of the trap state and not errors related to the operation of the microfluidics device. Therefore, we expect that, providing an externally controlled DNA machine that is free of trap state effects can be designed, our interface will enable many more externally controlled operations than demonstrated here. In addition, our model indicates that regular fuels (fuels that lack hairpin structure and bind the leg and the foothold at the same rate) would result in 2- to 6-fold decrease (corresponding to 0.2 to 10 μM fuel concentrations, respectively) in operational yield.

**Motor Processivity and Speed Limits.** Several factors contribute to the motor dynamic boundaries and limits on yield and speed of the walker. Our kinetic measurements showed that the leg-lifting rate was linearly dependent on the antifuel concentration (Supplementary Figure 7). This demonstrates that fast and complete leg-lifting reactions can be achieved simply by increasing antifuel concentrations (e.g., ~99.99% leg lifting in 0.4 s for a 100 μM antifuel concentration). Contrary to that, the leg-placing rate increased only to a certain value, corresponding to the intrinsic intramolecular stepping rate, which therefore constitutes a fundamental walking speed limit for our motor. To illustrate, based on this stepping rate, 50% of the walkers can advance at a speed of 2 nm/s (for foothold spacing of 11.6 nm). However, to enable such speed, it is necessary to use high concentrations of fuels, which, because of the trap state effect, results in reduced processivity. A reasonable compromise between speed and yield can be achieved using 1 μM fuel and antifuel concentrations and 100 s incubation times (Figure 4a). With these parameters, one motor cycle (from state 1 to state 17) takes 1 h and has an operational yield of 60% and a speed of 0.1 nm/min. To achieve much better performance, however, the trap state mechanism must be solved or somehow avoided.

**Proposal of Mechanisms for Externally Controlled DNA Machines with Significantly Improved Performance.** Parallel binding of two strands that results in the trap state is a known problem,11,17,34 for which there is no simple solution. The use of several pairs of legs and footholds may offer a remedy. Because one leg attached to a foothold prevents dissociation, the probability that the walker will dissociate (that is, no legs are attached to the track) exponentially decreases with an increase in the number of legs. Thus, several pairs of
legs and footholds should allow an exponential increase in the concentration of the command strands and, therefore, motor speed, without compromising operational yield and performance. Another possible solution is reversing the order of commanding fuel and antifuel strands such that when the leg is lifted the consecutive fuel is already placed on the corresponding foothold and the redundant fuels are already washed away. This will result in unwanted, but unavoidable, partial binding of antifuel with the preceding fuel (e.g., half of AF1 binds half of F3). This problem, however, can be resolved by shortening the antifuel strands and/or by waiting for the unwanted fuel/antifuel duplex to dissociate thermally. We are currently testing these two mechanisms, and the results are promising. We postulate that success with these efforts should result in motors and machines with operational yields and speeds significantly higher than those demonstrated in this work and higher than that for any DNA machine demonstrated thus far.

CONCLUSIONS

The conjunction of the integrated microfluidics and single-molecule fluorescence techniques establishes a robust interface that enables communication and feedback between users and molecular machines. As in solid-phase synthesis, the microfluidics device allows computer-controlled, automatic introduction of an essentially unlimited number of external inputs and, therefore, an essentially unlimited number of assembly and operation steps. Further, removal of excess materials and waste products is achieved with minimal effort and without disrupting motor assembly and operation, as is the case for gel electrophoresis and magnetic-bead purification methods. Furthermore, we demonstrate a single-molecule fluorescence study of molecules immobilized inside a microfluidics device, and a use of our interface enabled capture of reaction kinetics with high time resolution and on a single-molecular machine level, which is crucial for rational development of complex machines. Specifically, by removing the redundant strands, use of the microfluidics resulted in a 6-fold increase in operational yield and processivity relative to the motor operated without removal of command strands. This, together with the ability to measure fast kinetics, enabled elucidation of the trap state mechanism, which limits the performance of our motor, including under conditions when hairpin fuels were used. This knowledge is very important for rational design of improved motors. Assuming that DNA motors or other machines can be designed free of the trap state effect, we expect that our interface will allow hundreds and possibly thousands of operations. Thus, our approach constitutes a conceptual step toward the realization of “next generation” complex bioinspired molecular machines that respond, in real-time, to an essentially unlimited number of instructions and perform numerous and potentially parallel processes.

METHODS

Microfluidic design and fabrication, computer control of the microfluidics, TIRF and diffusion-based single-molecule setups, single-molecule data analysis, analysis of kinetic profiles, numeric simulation of the trap state model, principle of hairpin fuel, origami and bipedal motor design and preparation, DNA sequences, DNA labeling procedures, optimization of motor immobilization and assembly, and motor operation procedures are described in detail in the Supporting Information.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b00547.

Supplementary Sections 1–13, supplementary Figures 1–12, and supplementary Tables 1–4 (PDF)

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by grants from the Israel Science Foundation (1578/13, E.N., and 715/11, D.G.) and ERC (Grant No. 309600, D.G.). T.E.T. and Y.B. are supported by the Negev Fellowship, and M.L. is supported by the Darom Fellowship.

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