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Engineering Circular Gliding of Actin Filaments Along Myosin-Patterned DNA Nanotube Rings to Study Long-Term Actin-Myosin Behaviors

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Abstract

Nature has evolved molecular motors that are critical in cellular processes occurring over broad timescales, ranging from seconds to years. Despite the importance of the long-term behavior of molecular machines, topics such as enzymatic lifetime are underexplored due to the lack of a suitable approach for monitoring motor activity over long time periods. Here, we developed an “O”-shaped Myosin-Empowered Gliding Assay (OMEGA) that utilizes engineered micron-scale DNA nanotube rings with precise arrangements of myosin VI to trap gliding actin filaments. This circular gliding assay platform allows the same individual actin filament to glide over the same myosin ensemble (50–1000 motors per ring) multiple times. First, we systematically characterized the formation of DNA nanotubes rings with 4, 6, 8, and 10 helix circumferences. Individual actin filaments glide along the nanotube rings with high processivity for up to 12.8 revolutions or 11 minutes in run time. We then show actin gliding speed is robust to variation in motor number and independent of ring curvature within our sample space (ring diameter of 0.5–4 μm). As a model

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Author Contributions

R.F.H., A.J.A., and S.S. planned and designed the experiments. R.F.H. and A.J.A. performed experiments and analyzed the results. R.F.H. wrote the Mathematica analysis codes. R.F.H., A.J.A., and S.S. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Additional methods, tables, and figures related to the nanotube ring design and characterization and gliding assay behavior (PDF)

Movie S1 (MPG)

Movie S2 (MPG)

Movie S3 (MPG)

Movie S4 (MPG)

Movie S5 (MPG)

Movie S6 (MPG)

application of OMEGA, we then analyze motor-based mechanical influence on “stop-and-go” gliding behavior of actin filaments, revealing that the stop-to-go transition probability is dependent on motor flexibility. Our circular gliding assay may provide a closed-loop platform for monitoring long-term behavior of broad classes of molecular motors and enable characterization of motor robustness and long timescale nanomechanical processes.

Graphical Table of Contents

Keywords

DNA nanotechnology; molecular motors; actin gliding

The myosin family of motor proteins powers movement along actin filaments using ATP hydrolysis. Each of the myosin isoforms plays various roles in key cellular functions. Myosin VI has been shown to affect endocytosis,¹ to maintain the Golgi morphology,² and to act as an anchor in filopodia.³ Certain myosin isoforms, such as myosin II found in cardiac muscle, must be robust to cyclic mechanical stress over long spans of time (Table S1).^{4–7} Understanding the long-term behavior of myosin motors is crucial because these long-lived proteins are responsible for functions that occur throughout the life cycle of a cell.⁸ Despite the importance of the long-term performance of myosin activity, nearly all single molecule biophysical investigations focus on short-term performance, and relatively little is known regarding long timescale characteristics of myosin motors and their interactions with actin.

Actin gliding assays are commonly used to measure myosin activity.⁹ In the classical actin gliding assay, actin filaments glide over a myosin-coated glass surface. One recognized limitation with these assays is the lack of control over the number, type, and spatial organization of myosin motors. Moreover, classical gliding assays, although able to support long run lengths and run times using many motors, can only probe the interactions between individual actin filaments and small groups of myosin motors over a short timescale, since single filaments are not confined and can travel out of the field of view. To address this challenge, we exploited the positional control of DNA nanotechnology, which has been used in the biophysical characterization of molecular motors, such as in the precise patterning of myosin motors in gliding assays¹⁰ and for controlling the ensemble of kinesin and dynein in motility assays.^{11,12} Specifically, we engineered DNA nanotube rings as scaffolds for circular one-dimensional arrays of myosin motors. DNA nanotube rings are well suited to function as actin gliding scaffolds as they allow high precision patterning of myosin and effectively provide an “endless track” for actin to glide over. This ring-based approach increases both the time period over which we can study these molecular motors and the number of repetitive interactions between the same gliding actin filament and defined myosin motor ensemble.

In this study, we introduce a ring-shaped assay that can be used to monitor long-term interactions between a single actin filament and myosin motors. We show a reliable method to produce DNA nanotube rings with up to 4 μm in diameter from less than 30 strands. Actin filaments glide around the myosin-labeled DNA nanotube rings for up to 12.8 revolutions or 11 minutes (40 μm total run length). By analyzing the gliding movement of individual filaments along a broad range of ring diameters, we found that actin gliding speed was independent of both motor number and ring curvature. To demonstrate one model application of the assay, we investigate actin filament alternation between motility and arrest. This gliding behavior is particularly suited to be studied using our assay since individual filaments can revisit the same motors multiple times and due to these repeating interactions, influences other than specific motor issues may be investigated a cause for the stop-and-go phenomena. We found that the stop-to-go transition probability increases as linker flexibility increases, suggesting that the alternating gliding behavior is influenced by motor mechanics. Our work also suggests that this nanotube ring approach may eventually be utilized as a platform for investigating long-term motor protein functions, enzymatic lifetime, and wear, which are underexplored topics in molecular motor biology.

RESULTS AND DISCUSSION

Design and characterization of DNA nanotube rings

Here, we form DNA nanotubes by designing the complementarity between different domains of a 42-base Single Stranded Tile (SST) made from unpurified DNA strands.¹³ During nanotube formation, the SSTs self-assemble and nucleate into rigid crystalline structures (Figure 1a and Figure S1a–l), and then elongate into crystalline tubular structures with prescribed diameters (Figure 1a, Tables S2 and S3). End-to-end joining of complementary sticky ends can occur between two DNA nanotubes resulting in longer non-ring DNA nanotubes¹⁴ (Figure 1a; L_i in Figure 1b), as well as between both ends of the same DNA nanotube forming DNA nanotube rings (Figure 1a; R_i in Figure 1b). Our strategy differs from previously reported techniques of forming SST-based DNA rings.¹⁵ First, in the previous design, Yang *et al.* guided the formation of DNA nanotube rings by using non-uniform domain length for their SSTs to program their local curvature. Because the local curvature was directly controlled by the SST domain length, their DNA ring assembly was expected to require purified DNA strands. Additionally, their approach led to small DNA rings with diameters of 50–200 nm, which are sufficiently large for atomic force microscopy assays but not suitable for diffraction-limited fluorescence microscopy experiments.

Fluorescence imaging was conducted on Cy3-labeled DNA nanostructures after annealing a set of SSTs at 2.5 nM tile concentration (Tables S2–S4). In all experiments, non-ring DNA nanotubes (L_i in Figure 1b) abundantly outnumbered DNA nanotube rings (R_i in Figure 1b) by a ratio of >20:1. After overnight incubation, nanotubes with smaller helix numbers generally had a higher density of DNA nanotube rings, which is defined as the total number of DNA nanotube rings found on all images analyzed divided by the total number of images analyzed (Table S5).

To utilize DNA nanotube rings as gliding assay scaffolds, a range of SST nanotube designs¹³ were screened based on two considerations. First, we screened for nanotube designs with

high cyclization rate, in order to maximize the ring density (Table S5). The high ring density increases the data throughput by reducing the search time for finding DNA nanotube rings with suitable diameters. Secondly, we sought DNA nanotube rings with a broad range of diameters, D . Broad size distribution provides a large data set to study the effect of ring curvature on actin gliding speed. To optimize these conditions, we varied two factors in our nanotube designs: helix number n and the number of SSTs per number of helices n (Single Layer, S), $2n$ (Double Layer, D), and $3n$ (Triple Layer, T), where $n = 4, 6, 8$, and 10 (Figure 2a). We report the effect of the number of helices (n) and layer number (S, D, or T) on the ring diameter below.

Increasing helix number increases diameter of nanotube rings

The mean ring diameter $\langle D \rangle$ systematically increases with helix number n , regardless of the layer number (Figure 2b and Figure S2; Table S6). The range of the mean ring diameter is $0.71 \pm 0.43 \mu\text{m}$ ($n = 4$ -helix, single layer; $N = 31$ rings) to $2.67 \pm 0.80 \mu\text{m}$ ($n = 10$ -helix, triple layer; $N = 28$ rings). It is expected that increasing the helix number n will increase the size of the nanotubes formed, and thereby, the diameter D of the rings formed. The positive correlation between helix number and ring diameter is based on the positive correlation between helix number and persistence length p of the nanotubes, which has been shown previously by thermal fluctuation analysis of nanotubes with helix number ranging from five to ten.^{16,17} In addition, the observed helix number-dependent ring size is in agreement with the persistence length-dependent closure model,^{16,18,19} a theoretical model that is later tested using 8-helix double layer (8D) DNA nanotubes (Figure 3a and b).

Increasing layer number does not affect diameter of nanotube rings

We varied the layer number, which is equal to the number of SSTs divided by the number of helices, to be single (S), double (D), or triple (T). We found that the layer number had no significant effect on the ring diameter for all helix numbers tested ($n = 4, 6, 8$, and 10 ; Figure 2b; Table S6). Circularization, in addition to elongation and end-to-end joining, of nanotubes only occurs when complementary SST layers hybridize. In single layer nanotubes, all tiles are complementary (only blue tiles) so every end-to-end collision can result in a ring closure event; in triple layer nanotubes, only collisions between two specific tile types (blue and green tiles) in a structure made of three different tile types (blue, red, and green tiles) allow for the possibility of ring closure (Figure 2a). Increasing the layer number is expected to decrease the rate of successful intra-molecular end-to-end joining required for ring formation. As the result of a decreased probability for rings “snapping” closed, in the presence of free monomers, there would be more time for nanotube elongation, and so, it is expected that larger rings would form. The observed layer-independent ring sizes suggest that the ring formation occurs when the elongation rate is negligible which occurs once free monomer concentration is close to zero. Effectively, adding layer number generally decreases the ring density ($N_{\text{rings}}/N_{\text{analyzed images}}$) without significantly affecting the ring diameters (Figure 2b; Tables S5 and S6).

We used 8-helix double layer (8D) nanotubes for circularization analysis (Figure 3) and in gliding assays (Figures 4 and 5). Two nanotube designs, namely 8S and 8D, satisfied both of the criteria we set for a suitable ring-shaped gliding assay platform. Both designs have

comparable ring densities to other nanotube designs (Table S5) and a wide array of ring diameter sizes (Figure 2b). One may be concerned that the specific set of strands for the 8S and 8D nanotube designs had particularly poor synthesis or unpredictable sequence determinants that resulted in a systematic twist that encouraged higher probability of ring closure than 4-, 6-, and 10-helix nanotubes. However, the likelihood that one specific design had a unique problem is very low since a majority of the strands in all of the designs share common base sequences (Tables S2 and S3). Finally, 8D nanotubes were chosen over 8S nanotubes due to their 14 nm spacing between repeating layers (*versus* 7 nm spacing for 8S), which matches the spacing between neighboring myosin II motors in the thick filaments of muscle.^{20,21}

Ring formation follows the Yamakawa-Stockmayer ring formation model

To investigate the formation of DNA nanotube rings, we measured the contour lengths of non-ring DNA nanotubes (Figure 3a and Figure S3) and the diameter of DNA nanotube rings (Figure 3b and Figure S4). By Bayesian fit (Supporting Materials and Methods), the distribution of nanotube length after overnight incubation matches the Schulz-Flory model for distribution of one-dimensional polymers during an end-to-end joining process (Figure 3a, black line).²² The probability distribution function for nanotube of length ℓ is

$$W(\ell) = \frac{\ell}{\lambda^2} e^{-\ell/\lambda},$$

where λ is half of the mean length. Characterization of nanotube rings with different layer numbers show that the ring formation occurs after maturation of the nanotube system, as noted previously. Moreover, the ring density is less than 5% of the observed DNA structures. Consequently, ring formation does not significantly alter the length distribution. Based on these two observations, we can use the length distribution (ℓ) to calculate the distribution of the nanotube rings by diameter. The ring closure probability^{18,19} is modeled by Yamakawa and Stockmayer to be

$$G(\ell, p) = 497r^{-1}e^{-7.03/r}(1 - 0.812r), \text{ for } r < 0.961$$

$$G(\ell, p) = 0.330r^{-1.5}(1 - 0.625r^{-1} - 0.123r^{-2}), \text{ for } r \geq 0.961,$$

where $r = \ell/2p$ is the normalized length. Finally, the ring circumference distribution was calculated to be $R = W \bullet G$. Simultaneous fits, by Bayesian probability, of the length (Figure 3a) and diameter (Figure 3b) distributions show that the ring formation of 8D nanotubes follows the Yamakawa–Stockmayer model as expected. From the global fit, the mean length $\langle \ell \rangle = 2\lambda$ of the 8D nanotubes was estimated to be $4.2 \pm 0.4 \mu\text{m}$ and the persistence length of the 8D nanotubes was estimated to be $2.3 \pm 0.2 \mu\text{m}$.

Circularity was not affected by ring diameter

To gain insight into the shape of the rings formed, the images of closed-loop ring structures were fitted as ellipses (Supporting Materials and Methods). The circularity C was defined as

h/w , where h and w are the lengths of the major and minor axes, respectively, of a fitted ellipse (Figure 3c). A perfect circle ($C = 1$) is the ideal condition for nanotube rings used in gliding assays, as this condition would minimize potential curvature dependence of the gliding speed. No correlation was found between ring diameter and circularity (Pearson correlation coefficient $r = -0.069$; $N = 323$; Figure 3c and Table S7). Mean ring images for small, medium, and large rings also show that the ring circularity did not correlate with ring diameter (Figure 3d–f). It was found that $53 \pm 1\%$ of all rings had $1.0 < C < 1.2$ (Figure 3c, right, black bins), indicating more than half of the 8D nanotube rings were sufficiently circular for use in gliding assays. Despite the fact that the perfect circle ($C = 1$) would be the lowest energy configuration, we observed a large range of circularities ranging from 1.01 to 3.97 (Figure 3c). One possibility for this spread could be stochastic and irreversible deformation of rings during biotin-neutravidin-mediated immobilization. Even if rings form as perfect circles without any lattice defects during annealing, the impact of the ring on the glass slide during imaging could cause alterations in the shape. Thermal fluctuations occurring after ring formation could also lead to a less circular ring shape.

Actin gliding assay

To perform gliding assays, we modified 8D nanotubes to facilitate surface attachment (biotin), imaging (Cy5) and Cy3-labeled myosin VI attachment (Tables S2–S4; Supporting Materials and Methods). Time-lapse images and kymographs of Alexa 488-phalloidin stabilized actin (green) gliding along nanotube rings (red) illustrate movement of single actin filaments along the circular scaffold with high processivity (Figure 4a–c; Movie S1). The multiple revolutions indicate the engagement of the gliding actin filament with each myosin motor multiple times. The mean gliding speed over myosin VI-labeled DNA nanotubes rings was measured to be 116 ± 7 nm/sec, which is qualitatively consistent with both the gliding speed measured using myosin VI-labeled non-ring DNA nanotubes (119 ± 5 nm/sec) and the previously reported gliding speed measured using myosin VI-labeled non-ring DNA nanotubes (80 ± 14 nm/sec).¹⁰

Motor number does not affect gliding speed

We next investigated how gliding speed v varies with changes in motor number N_{myo} and ring curvature κ . Movement of isolated actin filaments were analyzed and gliding speed v was not affected by filament length ℓ_{actin} or motor number $N_{myo} = \ell_{actin}/s$, where $s = 14$ nm is the spacing between neighboring myosin motors (Figure 4d; $N = 153$ movies and 14,469 analyzed frames). The motor number-independent speed is also consistent with published reports on traditional gliding assays of actin filaments on myosin-coated surfaces^{23,24} and recent reports on gliding assays using myosin-patterned DNA nanotubes.¹⁰

The data presented in this paper provides additional supporting evidence to the model of gliding actin filament on a myosin ensemble.^{10,23} According to this model, actin filament movement is caused by instantaneous tension equilibration after each myosin step. The generated tension per myosin step is inversely proportional to the ensemble size. Consequently, the reduced tension in a larger ensemble decreases the negative impact on the tension-dependent stepping rate of all engaged myosin proteins. On the other hand, more motors have to take a step in order for a larger ensemble to travel over a myosin step size.

This interplay between the decrease in myosin stepping rate and the increase in number of myosin stepping events per unit distance results in gliding speed that is independent of motor number.

Gliding speed does not depend on ring diameter

To address the potential effect of track curvature κ , which was defined as the inverse of the diameter, on the gliding speed, we divided the data into 3 bins based on the ring sizes (Figure 3d–f). Surprisingly, gliding assays on DNA nanotubes with different ring sizes show that gliding speed v was not affected by ring diameter (Figure 4e). When curvature was accounted for, no significant correlation between the gliding speed and motor number was detected. Additionally, when motor number was accounted for, no significant correlation between the gliding speed and curvature was detected (Table S7). This curvature-independent gliding speed is consistent with the semi-flexible lever arm hypothesis of myosin VI²⁵ and the relative flexibility of the actin filament.^{26–28} This result can be explained by the interplay between the bending energy experienced by actin filament and the mechanical energy per unit step. First, the bending energy of an actin filament of length ℓ_{actin} along an arc of a nanotube ring of diameter D is given by

$$G_{\text{actin}}^{\text{bend}} = \frac{2p\ell_{\text{actin}}}{D^2} k_B T,$$

where $k_B T$ is the thermal energy and p is the actin persistence length (6–25 μm).^{26–28} Within the range of ring diameter used in this work ($D = 0.5\text{--}4\text{ }\mu\text{m}$), the additional bending penalty per unit length (14 nm) due to the curved track was calculated to be 0.01–3.0 $k_B T$, which is smaller than the inferred mechanical energy per step of the acto-myosin cross-bridge ($\sim 7 k_B T$).¹⁰ Consequently, myosin motor domains are not significantly affected by off-axis load due to the misaligned actin filament. In regard to the experimental assay in this paper, the curvature-independent gliding speed implies that for gliding assays with myosin VI, the measurements on a circular track are comparable to gliding assays on linear myosin VI-labeled DNA nanotubes ($119 \pm 5\text{ nm/sec}$). Therefore, our platform has the potential to monitor long timescale actin-myosin interactions without the circular system imparting additional, unaccounted influences on protein mechanics and behavior.

Decreasing the motor attachment stiffness decreases probability of actin filament arrest

As a model application of our assay, we investigated the “stop-and-go” behavior that was observed in actin gliding kymographs (Figure 5a and Figure S5; Movie S2). Filaments switched between an arrested state (“stop”) and a motile state (“go”), in which there was directed motion along the DNA nanotube track. We also observed that actin filaments would stochastically stop at different points on the nanotube track during different revolutions (Figure 5a and Figure S5; Movie S2). This finding, because of the circular shape of our assay which enables myosin motors to be revisited by the same filaments multiple times, suggests that stop-and-go behavior may not only be influenced by filaments engaging with dead motors. Another plausible explanation for this behavior involves the mechanical interactions between motors. To further explore this hypothesis, we performed experiments

with a flexible motor attachment linker in addition to the rigid linker we used previously (Figure 5b, left). This flexible linker was created by extending the rigid 20 base pair dsDNA nanotube-myosin attachment strands with two additional 20 nucleotide flexible ssDNA regions (Table S2), which lowers the net stiffness of the myosin linker.¹⁰ We measured the stop fraction (χ_{stop}), which is defined as the fraction of time intervals in which filaments were not motile over the total number of time intervals during which the filaments are on the track, where the time of each interval $t = 2.5$ seconds or 5 frames (Supporting Materials and Methods). We first observed stop fraction to be filament length-dependent, which supports observations made in previous studies (Figure S6).²³ We then treated the measurements from actin filaments of different lengths as one homogeneous dataset. The stop fractions were comparable for the two linker types, with $\chi_{\text{stop}} = 20 \pm 3\%$ for the rigid linker (Figure 5b, top right; $N = 31$ movies, 116 filaments, and 30,467 analyzed frames) and $\chi_{\text{stop}} = 14 \pm 4\%$ for the flexible linker (Figure 5b, bottom right; $N = 20$ movies, 62 filaments, and 24,017 analyzed frames).

Motor attachment stiffness affects filament transition probabilities

Rather than switching rapidly between the stop and go state, filaments would generally alternate between periods of sustained motility or sustained arrest (Figure 5a and Figure S5). Thus, we modeled filament gliding behavior as a Markov process, with the time between states $t = 2.5$ seconds or 5 frames (Figure 5c). In this model, there are two states, “stop” ($v < 0.043 \mu\text{m/sec}$) and “go” ($v \geq 0.043 \mu\text{m/sec}$), two “stay” transitions (go $\rightarrow$ go and stop $\rightarrow$ stop), and two “switch” transitions (go $\rightarrow$ stop and stop $\rightarrow$ go). Transition probabilities for actin gliding were obtained from kymograph analysis (Figure 5a and Figure S5; Supporting Materials and Methods; Tables S8 and S9). We observed that a filament was more likely to be in the go state if it was in the go state in the previous time interval, with go-to-go probability $P(\text{go} \rightarrow \text{go}) = 0.92 \pm 0.01$ for the rigid linker ($N = 11$ movies and 1,536 analyzed transitions) and 0.90 ± 0.01 for the flexible linker ($N = 15$ movies and 1,728 analyzed transitions; Tables S8 and S9). Similarly, a filament was more likely to be in the stop state if it was in the stop state in the previous time interval, with stop-to-stop probability $P(\text{stop} \rightarrow \text{stop}) = 0.77 \pm 0.01$ for the rigid linker ($N = 11$ movies and 437 analyzed transitions) and 0.64 ± 0.01 for the flexible linker ($N = 15$ movies and 359 analyzed transitions; Tables S8 and S9).

In order to determine how frequently actin filaments switch between the go and stop states, we formulated a metric to quantify the average number of myosin heads traversed by filaments per switch transition event. This frequency parameter M is defined as

$$M = \frac{N_{\text{go}}}{N_{\text{switch}}} \frac{x v \Delta t}{s},$$

where N_{go} is the number of go $\rightarrow$ go transition events, N_{switch} is the number of switch transition events, $x = 2$ is the number of myosin heads per myosin dimer, v is the average gliding velocity of motile filaments, $t = 2.5$ sec is the interval time, $s = 14$ nm is the spacing between neighboring myosin motors. We found M to be comparable for the rigid

(407 ± 80 myosin heads traveled/switch transition; $N = 11$ movies) and flexible (326 ± 91 myosin heads traveled/switch transition; $N = 15$ movies; Figure 5d). These large values suggest that the stop-and-go switch transition events occur relatively rarely along the myosin-patterned track. Thus, in order to study these rare events over time, an assay should provide a long run length for individual actin filaments, such as the theoretically-endless track provided by our assay.

Finally, we analyzed the transition probabilities for the switch transition events. The go-to-stop probability for a filament, $P(\text{go} \rightarrow \text{stop})$, was similar for both the rigid motor linker (0.08 ± 0.01 ; $N = 11$ movies and 130 analyzed transitions) and flexible linker (0.10 ± 0.01 ; $N = 15$ movies and 202 analyzed transitions; Figure 5e, left). However, when motor linker stiffness was decreased, the stop-to-go probability for a filament increased, with $P(\text{stop} \rightarrow \text{go}) = 0.23 \pm 0.01$ for the rigid linker ($N = 11$ movies and 129 analyzed transitions) *versus* 0.36 ± 0.01 for the flexible linker ($N = 15$ movies and 200 analyzed transitions; Figure 5e, right; $p < 0.001$). This significant increase of stop-to-go probability with increased linker flexibility, considered together with the position-independent stopping behavior observed in Figures 5a and S5, suggests that there may be other factors influencing stop-and-go gliding behavior of actin filaments besides just specific motor issues. Our findings with OMEGA suggest that motor-based mechanical processes may be one of these factors.

CONCLUSION

Molecular motors, such as myosin VI, perform their functions in groups²⁹ with each motor operating at high turnover numbers. For example, the lifetime of myosin II in rat cardiac muscle,⁴ rat skeletal muscle,⁵ chicken skeletal muscle,⁶ and rabbit skeletal muscle⁷ was measured to range from ~2 days up to ~30 days, which translates to anywhere from 10^5 to 10^7 cycles per lifetime, assuming 1 cycle per second. Our ring-shaped, myosin-labeled gliding assay, in addition to providing additional supporting evidence to the model of a gliding actin filament on a myosin ensemble,^{10,23} serves as a potential tool to explore long timescale dynamics of interactions between single actin filaments and small groups of myosin by providing an infinite maximum run length for motility. In this manner, the OMEGA gliding assay has the potential to monitor actin-myosin interactions until the point of failure.

Moreover, single molecule analysis has been shown as an approach to study rare molecular events, such as the previously reported back steps in myosin motors^{30,31} and the stop-and-go events during actin gliding. Since each myosin ensemble is activated by the same actin filament multiple times in our circular assay, we can monitor multiple interactions between the same actin filament and defined myosin ensemble in order to pinpoint and study these stop-and-go events. This application is just one example of how our assay may allow investigation into relatively underexplored aspects of myosin motors. Our goal is to use this platform to monitor molecular motor processes over longer timescales, which is not feasible with existing gliding assays. Currently, the timespan of our gliding assay was limited by collision between multiple filaments on the same nanotube ring (Figure S7a and Movie S3), actin dissociation (Figure S7b and Movie S4), actin fragmentation (Figure S7c and Movie S5), actin filament directional change (Figure S7d and Movie S6), and photobleaching. If

these limitations can be addressed, and if the construction of circular myosin arrays can be scaled up in terms of ring sizes and ring densities, perhaps by patterning the myosin VI attachment strands on a microscope slide,³² OMEGA may serve as a robust, high throughput tool for understanding long-term molecular motor behavior, function, and lifetime.

MATERIALS AND METHODS

Buffer and reagents

1x TAE Mg⁺⁺ – 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, 12.5 mM MgCl₂; 1x Assay Buffer (AB) – 25 mM imidazole (pH 7.5), 4 mM MgCl₂, 1 mM EGTA, 25 mM KCl, 10 mM DTT; 1x AB.BSA buffer – AB buffer + 1 mg/mL BSA; 1x AB.BSA.DNA – 1x AB.BSA + 10 nM random nucleotide mix (5'-NNNNNNNNNN-NNNNNNNNNN-NNNNNNNNNN-NNNNNNNNNN-NNNNNNNNNN-NN-3').

DNA nanotube assembly

DNA nanotubes were prepared from individual SSTs in 1x TAE Mg⁺⁺. Unmodified SSTs, UM-11-RC-oligo-a, and oligo-a-Cy3 for Cy3-labeling of nanotubes were mixed during characterization. Unmodified SSTs, UM-11-RC-oligo-a for myosin VI-attachment, and UM-03-Cy5 for Cy5-labeling of nanotubes were mixed for use in gliding assays with the rigid linker. UM-11-RC-oligo-a was replaced with UM-11-Flex-RC-oligo-a for gliding assays with the flexible linker. All strands were combined to a final total tile concentration of 100 μM and then were annealed, as previously described.¹⁰ Nanotubes were then diluted in 1x TAE Mg⁺⁺ and incubated at 4 °C before imaging. Details are given in Supporting Materials and Methods.

Myosin expression, purification, and labeling

Myosin VI was constructed, expressed in Sf9 insect cells, purified through FLAG affinity, and conjugated with Cy3-Benzyl-Guanine double-labeled single-stranded DNA (BG-Cy3-oligo-a' for rigid linker or BG-Flex-Cy3-oligo-a' for flexible linker) as outlined in previous reports.^{10,33} Details are given in Supporting Materials and Methods.

DNA nanotube imaging

DNA nanotubes were imaged at 150× magnification on a Nikon TiE microscope equipped with a 100× 1.4 NA Plan-Apo oil-immersion objective, 1.5× magnifier, EMCCD camera (512 pixel × 512 pixel; Evolve; Photometrics), mercury arc lamp, Nikon Perfect Focus System, and Nikon NIS-Elements software, with 2 frame/sec frame rate. Flow chambers were cleaned using a Harrick Plasma plasma cleaner, and nanotubes were immobilized on the surface of the flow chambers using biotin-neutravidin links. Biotinylated BSA at 0.1 mg/mL in 1× AB buffer was flowed into the chamber and incubated for 5 minutes. Excess biotinylated BSA was washed out by flowing in 1× AB.BSA and incubating for 2 minutes. Neutravidin at 0.1 mg/mL in 1× AB.BSA was then flowed in and incubated for 5 minutes. Again, AB.BSA was used to wash out excess neutravidin. Nanotubes at 2.5 nM concentration in AB.BSA were then incubated for 10 minutes. 1× AB.BSA was then flowed in to wash out excess nanotubes. For characterization, Cy3-labeled nanotubes were used without adding myosin VI (Figures 1–3). For motility assays, Cy5-labeled nanotubes and

Cy3-labeled myosin VI were used (Figures 4–5). Details are given in Supporting Materials and Methods.

Ring-based gliding assay

Myosin VI was diluted in AB.BSA.DNA with 8 μ M calmodulin. After nanotubes were immobilized on a glass slide in a flow chamber, myosin VI was incubated in the flow chamber for 10 min, which was repeated twice. Excess motor was removed by washing with 1 $\times$ AB.BSA.DNA + calmodulin. Alexa 488 phalloidin-labeled F-actin was diluted with 1 $\times$ AB.BSA and gently sheared using a Hamilton syringe (22s gauge, 25 μ L volume). The actin was then added to a solution which consisted of 2 mM ATP, 1 mM phosphocreatine, 0.1 mg/mL creatine phosphokinase, 45 μ g/mL catalase, 25 g/mL glucose oxidase, 1% glucose, and 8 μ M calmodulin before being added to the flow chamber. Cy5-labeled nanotubes, Cy3-labeled myosin VI, and Alexa 488-labeled actin were then imaged. Details are given in Supporting Materials and Methods.

Data analysis

Nanotubes were analyzed using custom Mathematica codes. Histograms of linear nanotube contour lengths and ring perimeters were simultaneously fit using Bayesian analysis. Motility assays were also analyzed using a custom Mathematica code that tracked actin gliding speed and actin length. Kymographs were obtained using automated kymograph analysis software KymographClear,³⁴ and by manually tracing the z-projection of the image sequence using the segmented line tool in ImageJ. Filament behavior analysis was done using visual inspection of gliding movies and a custom Mathematica analysis of kymographs. The time interval used for this analysis was 2.5 sec or 5 frames. For Markov analysis, filament speeds < 0.043 μ m/sec (1 camera pixel/2.5 sec) were defined as arrest (stop state), while filament speeds $\geq$ 0.043 μ m/sec were defined as motile (go state). Significance was determined using unequal variance unpaired t-tests with $\alpha = 0.05$. Details are given in Supporting Materials and Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Formation of DNA nanotubes and DNA nanotube rings from single-stranded tiles (SSTs)

(a) DNA SSTs self-assemble into one-dimensional nuclei with precise circumferences. The nanotube nuclei elongate into long DNA nanotubes. The complementary ends of two DNA nanotubes of lengths ℓ_i and ℓ_j can end-to-end join to form longer nanotube of length $\ell_i + \ell_j$. Intra-molecular end-to-end joining results in the formation of a nanotube ring. (b) Representative fluorescence image of Cy3-labeled double layer 8D nanotubes taken after annealing and overnight incubation. L_i denotes linear nanotube i while R_j denotes nanotube ring j .

Figure 2. Optimization of DNA nanotube design for ring formation

(a) Strand diagrams of 4-helix and 8-helix nanotubes with single (S), double (D), and triple layer (T) designs. (b) Ring diameter distribution for n -helix single, double, and triple layer nanotubes where $n = 4, 6, 8$, and 10 ($N = 12\text{--}323$ rings). 8-helix double layer tube has more data points ($N = 323$ rings) due to a more rigorous ring-formation analysis (Figure 3) and usage of rings in the gliding assay experiments (Figures 4 and 5). Red circles and error bars represent mean $\pm$ SD. The shaded grey areas are proportional to the best fit distribution based on the Shulz-Flory²² and Yamakawa-Stockmayer model.¹⁸

Figure 3. Characterization of DNA nanotube ring formation

(a) Histogram of contour lengths of linear nanotubes ($N = 167$ tubes; $\langle \ell \rangle = 4.2 \pm 0.4 \mu\text{m}$; Figure S3). Solid line represents the best-fit linear nanotube contour length distribution function W . (b) Diameter distribution of nanotube rings ($N = 323$ rings; Figure S4). The solid line represents the best-fit ring circumference distribution $R = W \cdot G$. (c) Circularity of nanotube rings as a function of ring diameter (left). Circularity was defined as the length of the major axis h divided by the length on minor axis w . Thus, a C value of 1 indicates a perfect circle. Dashed lines from left to right indicate ring diameters of $1.0 \pm 0.5 \mu\text{m}$ (small rings; $N = 79$ rings), $2.0 \pm 0.5 \mu\text{m}$ (medium rings; $N = 28$ rings), and $3.0 \pm 0.5 \mu\text{m}$ (large rings; $N = 10$ rings), respectively. Histogram of circularity of nanotube rings (right). Black bars indicate rings with $1.0 < C < 1.2$ ($53 \pm 1\%$ of rings). (d–f) Mean ring images for small (d; $D = 0.5\text{--}1.5 \mu\text{m}$), medium (e; $D = 1.5\text{--}2.5 \mu\text{m}$), and large rings (f; $D = 2.5\text{--}3.5 \mu\text{m}$).

Figure 4. Movement of actin filaments along DNA nanotube rings patterned with myosin VI
(a) DNA nanotube ring scaffold (red). Myosin VI motor protein (light brown) binds to attachment strands of DNA platform. Actin filaments (green) glide along the circular myosin VI track. **(b)** Time-lapse images of an OMEGA gliding assay with Alexa 488 phalloidin-labeled actin filament (green) and Cy3-labeled myosin VI (red; run time = 11 min; Movie S1). **(c)** Kymograph illustrating 5 revolutions of single actin filament around DNA nanotube ring with myosin VI motors attached (Movie S1). **(d–e)** Actin gliding speed as a function of filament length **(d)** and ring diameter **(e)**. Motor number is calculated as filament length/spacing between myosin motors ($\ell_{\text{actin}}/14$ nm). Gray area indicates the regime when the calculated bending penalty of the actin filaments is at least comparable to the estimated elastic penalty of the myosin motors. $N = 153$ filaments and 14,469 frames.

Figure 5. Stop-and-go behavior of actin filament gliding

(a) Kymograph of actin gliding along myosin VI-labeled DNA nanotube ring. Green segment indicates filament is in go state, while red segment indicates stop state. The movement was analyzed at 2.5 sec intervals. For clarity, the arrest threshold used here is 0.021 $\mu\text{m}/\text{sec}$. **(b)** Schematic of myosin – DNA linker for rigid linker with single 20 base pair dsDNA attachment strands (blue; top left) and flexible linker with extended DNA attachment strands (orange; bottom left). Stop fraction of actin filaments for rigid (top right; N = 31 movies, 116 filaments, and 30,467 total analyzed frames) and flexible (bottom right, N = 20 movies, 62 filaments, and 24,017 total analyzed frames) myosin linker. Stop fractions are reported as mean $\pm$ SEM. **(c)** Actin filament movement modeled as a two-state Markov chain process with $t = 2.5$ seconds. A motile filament ($v \geq 0.043 \mu\text{m}/\text{sec}$) at time t will either remain moving with go-to-go probability $P(\text{go} \rightarrow \text{go})$ or stop moving with go-to-stop probability $P(\text{go} \rightarrow \text{stop})$ at time $t + 2.5$ sec. An arrested filament ($v < 0.043 \mu\text{m}/\text{sec}$) at time t will remain arrested with stop-to-stop probability $P(\text{stop} \rightarrow \text{stop})$ or begin moving with stop-to-go probability $P(\text{stop} \rightarrow \text{go})$ at time $t + 2.5$ sec (Tables S8 and S9). $P(\text{go} \rightarrow \text{go}) = 1 - P(\text{go} \rightarrow \text{stop})$ and $P(\text{stop} \rightarrow \text{stop}) = 1 - P(\text{stop} \rightarrow \text{go})$. **(d)** The average number of myosin heads traversed per switch transition event for rigid (N = 11 movies) and flexible (N

= 15 movies) myosin linkers. Error bars indicate SEM and $\alpha = 0.05$. **(e)** Switch transition probabilities for actin filament movement for rigid (N = 11 movies) and flexible (N = 15 movies) myosin linkers. Error bars indicate SEM and $\alpha = 0.05$.

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