Accurate polarity control and parallel alignment of actin filaments for myosin-powered transport systems†

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An actin filament is a micrometer-long biological filament, which serves as a track of transport systems in cells. The filament exhibits polarity while myosin transports a cargo along it unidirectionally. Here, we established a method to align many actin filaments parallel with each other on a substrate with uniform (>99%) polarity as assessed by myosin movements. This actin array is an ideal candidate for the construction of a nano-scale unidirectional transport system powered by myosin.

Introduction

The micro total analysis system (µTAS) is a device that miniaturizes several experimental systems for chemical or biochemical analysis on the micrometer scale and integrates them on a single chip.1–4 For instance, real-time PCR (polymerase chain reaction),5 cell sorting,6 immunoassay,7 and drug discovery8 assays are performed on such a chip. Such a device enables high-throughput analysis and thus it is utilized in a wide range of fields from molecular biology to medical research.

The main component of the device is a micrometer-scale flow channel. Target molecules are transported inside the channel by accurate control of the fluid pressure using a syringe pump, sub-picoliter volumes of sample may be quickly and precisely transported in the channel. To increase the device throughput, further downsizing of the flow channel has been desired. However, the conventional transport method utilizing a syringe pump is inappropriate for smaller (nanometer) scales because the large fluid resistance makes the flow speed abruptly slow on the nano-scale.9,10

To transport target molecules in a nano-scale channel efficiently, reconstituting a biological transport system in a fluidic device has been regarded as a possible approach because nano-scale transport is well performed within cells.11–14 The biological transport system essentially consists of vesicles, motor proteins, and protein filaments. Target molecules are encapsulated in submicron-sized vesicles and nanometer-sized motor proteins transport the vesicles along micrometer-long protein filaments, which is driven by ATP, an energy source in the cell. The protein filament exhibits structural polarity, and the motor protein recognizes the polarity and moves unidirectionally along the filament. In cells, the alignment and polarity of the filaments control the direction of transport. To apply this biological function for the nano-scale transport system, in which many target molecules are transported unidirectionally along multiple filaments, the following technique is essential: polarity control of protein filaments and parallel immobilization of the multiple filaments on a substrate without the loss of their biological function as a track used by the motor protein.

Both, actin filaments and microtubules are known to be suitable tracks for motors protein movement.14,15 An actin filament is a semi-flexible polymer of 6 nm in diameter.15 The filament is formed by self-assembly of actin monomers and exhibits twisted two-stranded configuration. Flexibility of the filament is explained by the persistent length: \( l_p = 15–20 \mu m \).16–19 In contrast, a microtubule is a rigid tube-like rod of 25 nm in diameter, co-assembled by \( \alpha \) and \( \beta \) tubulin subunits15 and with \( l_p = 5000–6000 \mu m \).20 Both filaments have “plus” and “minus” ends, which determine the structural polarity. Recently, isopolar arrays of microtubules have been successfully constructed, and unidirectional transport systems powered by microtubule-associated motor protein kinesin have been proposed.9,10,21–24 Therefore, development of actin-myosin transport systems is essential to integrate two independent biological transport systems (actin- and microtubule-based systems) on a single chip, which allows nano-scale sorting of target molecules by the specific interaction between motor proteins and their tracks.

In this article, we propose an efficient method to fabricate isopolar parallel arrays of actin filaments that act as a track for myosin. The idea of this method is based on the following three reports. Huang et al.25 controlled the polarity of actin filaments by anchoring the filaments on the substrate through gelsolin, an actin capping protein that severs the actin filament and caps the generated plus end of the filament.26
Spudich et al. utilized another capping protein, severin, to anchor the filaments via one end of the filament on a surface. Then, they applied buffer flow to trail the filaments parallel to the image plane and studied actin-myosin interactions under continuous flow. Recently, Yuan et al. used poly-L-lysine, a polymer which has a counter (plus) charge against actin filaments, to immobilize oriented actin filaments on a glass surface via electrostatic interaction. However, the motor activity was diminished because of the effect of poly-L-lysine. The durability and stability of this actin array based on the electrostatic interaction were unknown as well. In our study here, we utilized gelsolin to anchor the filaments on a glass substrate via the plus ends, buffer flow to orient the filaments in parallel, and stable biotin-avidin conjugations to immobilize the filaments on the substrate (Fig. 1). From ATP-dependent unidirectional motion of myosin V, we confirmed that almost all (>99%) the filaments were oriented in the expected direction, and the immobilized filaments retained intact speed of myosin. Advantages of the actin-myosin system compared to the microtubule-kinesin system are discussed as well.

Results and discussion

Preparation of the gelsolin-actin complex

We first prepared the gelsolin-actin complex. For gelsolin, we prepared a Ca²⁺ insensitive mutant, which caps the plus end of actin filament even in the absence of Ca²⁺. This mutant enables one to use a variety of solution conditions. While gelsolin severs actin filaments and caps their plus ends, it is known that, in the presence of actin monomers, gelsolin recruits two actin monomers and nucleates actin assembly. We utilized this property; we first mixed gelsolin with actin monomers in solution and increased the salt concentration to promote actin polymerization. A small amount of biotinylated actin monomer was also added to this mixture to prepare sparsely biotinylated actin filaments. We stabilized the polymerized actin filament by adding fluorescence-labeled phalloidin. We confirmed this complex is stable on ice for more than one month.

Parallel alignment of actin filaments

A flowchamber was assembled by placing two ~50 μm thick spacers onto a KOH-washed coverslip (32 × 24 mm²) with another coverslip (18 × 18 mm²) on top. The gap between the two spacers was ~5 mm and the inner volume of the chamber was ~15 μL. To exchange the solution (or make flow described below), paper filters cut into a wedge shape were used. In the following procedures, the solution was infused into one side of the chamber and excess volume was drawn off by the paper filter from the outlet. First, the biotinylated-BSA solution was infused to coat the inner surface. Next, the gelsolin-capped actin filaments that had been sparsely biotinylated and fluorescently-labeled were injected (Fig. 1a). After a 3 min incubation, we found that the gelsolin-capped filaments bound to the surface at one end (Fig. 2a and the beginning of Movie S1, ESI). We confirmed that the filaments were anchored to the surface through gelsolin (see below). Then, we imposed buffer flow containing streptavidin, which binds to four biotin molecules tightly (Kₐ ~ 10⁻¹⁴ M)²² (Fig. 2c and Movie S1, ESI). The shear flow made the filaments stretch and lie down on the surface (Fig. 1c). Subsequently, the filaments got crosslinked to the surface via biotin-avidin conjugations (Fig. 1d). The flow rate was ~15 μL s⁻¹, which was estimated by the total inlet volume divided by the exchange time. Finally, unbound streptavidin was washed out. Thus, we succeeded in orienting anchored filaments in parallel and immobilizing them on the glass surface. The length of the immobilized filament was 5.4 ± 3.8 μm (mean ± s.d., n = 134) (Fig. 2a and 2b captions). We confirmed that the gelsolin-capped actin filaments were attached to the surface through gelsolin as follows. We prepared actin filaments in the absence of gelsolin, and performed the same procedure as the actin orientation process. As mentioned, the gelsolin-capped actin filaments were bound to the surface at one end (Fig. 2a), while absorption of the uncapped filaments on the surface was not observed for almost all the parts of the surface (Fig. 2b). By comparing the density of gelsolin-capped and uncapped actin filaments attached on the surface (see Fig. 2a and 2b captions), we estimated that the probability of nonspecific absorption was ~0.1%. Although the specificity of the connection between gelsolin and the surface is unclear, the connection was tight. In the flow rate (~15 μL s⁻¹) and time period (~5 s), less than 0.1% of actin filaments were detached from the surface. Moreover, we have never found the filaments detaching from the substrate on the usual observation time scale (~15 min) in the absence of flow.
For applications of this method, the time required to construct the system is important. The previously proposed method to assemble similar arrays of microtubules requires expertise in microfluidics to maintain constant flow over several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} In their system, the fabrication process includes three biochemical/chemical reactions and takes several minutes.\textsuperscript{9,10} However, our method allows one to construct the desired density of actin arrays easily by controlling the concentration of actin in solution. To show the capability to control the density, we first prepared different concentrations of gelsolin-capped actin filaments (0.1–5 μM actin, the molar ratio between actin and gelsolin was fixed) and then performed the same procedure as the surface anchoring process (Fig. 1a and 1b). We confirmed that the density of surface-anchored filaments was almost proportional to the concentration of the gelsolin-capped actin filament solution perfused into the flow chamber (Fig. 3).

Subsequently, we performed the same procedure as the surface orientation process as described above. The parallel relationship between the concentrations of gelsolin-capped actin filaments imposed into the flow chamber and the area density of actin filaments attached to the glass surface. The molar ratio between actin and gelsolin was fixed, and the concentration of actin was varied. KOH-washed glass with a biotinylated BSA coat (circle), collodion-coated glass with a biotinylated BSA coat (diamond), and collodion-coated glass with a biotinylated casein coat (triangle) were each evaluated. Error bars indicate standard deviations and the dotted lines indicate linear fittings (black: KOH-washed glass with a biotinylated BSA coat, gray: collodion-coated glass with a biotinylated BSA coat). To evaluate the area densities of actin filaments, we counted up the number of anchored filaments in every 5 μm square field of view and took the average. For all data points, n > 37. For more than 1 μM actin, it was difficult to distinguish the anchored points of each filament one by one due to the high density.

It is important to control the density of oriented actin filaments, especially for long distance transport. The reason is as follows. Single molecule studies on motor proteins have clarified that the run length of motor proteins (the moving distance of a single motor protein along the filament without detachment after the molecule encounters the filament) is a few micrometers at the longest. Therefore, to carry a cargo for a longer distance, multiple motor proteins attached to the same cargo have to interact with the oriented filaments simultaneously.\textsuperscript{12,33} In this situation, a dense array which allows multiple motors to interact simultaneously with more than one filament may support longer transport. This principle seems to be realized in living cells, where so-called actin cables (bundles of multiple actin filaments) appear to be involved in transport processes.\textsuperscript{33,34}
We confirmed polarities and cargo-transport activities of each filament immobilized on a substrate by ATP-dependent movement of myosin V that moves unidirectionally along the actin filament towards the plus end. Myosin V molecules were attached to small fluorescent beads (d = 0.01 μm), and the myosin V-coated beads were perfused into a flowchamber. Then, the chamber was sealed with silicon grease to prevent buffer flow. We utilized a thin array of actin filaments in order that individual actin filaments can be distinguished one by one. The beads moved along the filaments in one direction (Fig. 4). Since myosin V is a plus-end directed motor, the beads were expected to translocate in the upstream direction of the flow if the actin filaments were oriented in the correct direction. Indeed almost all of the beads translocated in the upstream direction. We counted the numbers of filaments that were oriented in the correct and incorrect directions over three different chambers and confirmed that the orientation accuracy was 99.6% (n = 264) (Table 1). In addition, we confirmed that the streptavidin molecules, which were sparsely bound to the immobilized actin filaments (see Fig. 1d), did not slow down the speed of myosin V. The velocity of myosin V-coated beads moving along the immobilized filaments was 0.62 ± 0.10 μm s⁻¹ (mean ± s.d., n = 29), which was almost the same value without streptavidin.

Since myosin V has two “feet” and translocates along an actin filament in a “hand-over-hand” fashion apparently like human walking, it can easily step over small obstacles such as streptavidin molecules on the actin filament.

Alternative methods for the surface immobilization of actin filaments and for the surface treatment of flow chambers

Providing alternative methods is important for the practical utility of this technique because optimal surface treatment of the flow channel and the cross-linker of actin filaments on the surface depend on the target molecules and the other parts of the chip.

For the immobilizing process of actin filaments on the surface, streptavidin could be replaced by α-actinin in the case of dense actin arrays. α-Actinin is an actin-binding protein that forms a dimer and crosslinks two actin filaments. We prepared non-biotinylated actin filaments capped with gelsolin, and anchored the filaments through gelsolin to the glass surface pre-coated with biotinylated BSA. Then, buffer flow containing α-actinin was imposed into the chamber. As a result, actin filaments were successfully immobilized in the downstream direction (Fig. 5a). The line density of this array perpendicular to the orientation of the flow was estimated to be 14.5 μm⁻¹ (see Fig. 5a caption). We note that the interaction between α-actinin and the surface is unclear.

However, the dense isopolar array might be assembled by the filament crosslinking alone in the presence of flow; when the density of actin filaments is high enough so that the trailing end can crosslink with other filaments trailing in the downstream direction, the filaments (except for those at the end point of arrays) should be immobilized on the surface. For surface treatment, a KOH-washed cover slip coated with biotinylated-BSA can be replaced by a collodion-coated cover slip coated with biotinylated-BSA, or a collodion-coated cover slip coated with biotinylated-casein. In both cases, actin filaments were anchored to the surface via gelsolin; the densities were proportional to the concentration of gelsolin-capped actin filaments in the solution perfused into the flowchamber (Fig. 3), while the absorption of uncapped actin filaments to the surface was blocked almost equally compared

### Table 1 Accuracy of the polarity alignment of actin filaments

<table>
<thead>
<tr>
<th>Chamber no.</th>
<th>Correct</th>
<th>Incorrect</th>
<th>Accuracy (%)</th>
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<tr>
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<tr>
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<tr>
<td>Total</td>
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<td>1</td>
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to the case of a KOH-washed cover slip coated with biotinylated-BSA (Fig. 2b). In all cases, actin filaments were successfully aligned on the surface by buffer flow with streptavidin. Moreover, the histidine-tag on our gelsolin mutant could be utilized to specifically fix the molecule on the surface. Indeed, binding of gelsolin-capped actin filaments to the surface was controlled by an anti-His antibody (Fig. 5c and 5d). A different tag or anti-gelsolin antibody may also be utilized.

Collectively, we demonstrated two ways of the surface immobilization of the actin filament and four ways of the surface treatment of flow channels. Such variations allow one to choose the best method for one’s application.

In addition, surface binding of gelsolin-capped actin filaments, namely both gelsolin and actin filaments, was blocked by the coating of (non-biotinylated) BSA on a KOH-washed glass (Fig. 5b) or by the coating of (non-biotinylated) casein on a collodion-coated glass (Fig. 5d). Therefore, based on these methods, a patterned array of actin filaments can be fabricated;25 using a microprinting technique of coating only a part of a chip surface by biotinylated-BSA or biotinylated-casein and the rest by normal BSA or casein enables one to anchor gelsolin-capped actin filaments to desired locations.

Advantages of the actin-myosin system

Compared to the microtubule-kinesin system, the actin-myosin system may have several advantages for motor-protein driven transport systems. First, the actin filament allows faster transport because myosin XI is known as the fastest molecular motor (60–100 μm s⁻¹) among all actins or microtubule associated motor proteins that have been previously characterized (as far as we know).40,41 Second, the actin filament allows one to downsize a fluidic device. As mentioned, the actin filament is 100 times more flexible than the microtubule. Therefore, precise wiring in a serpentine nano-channel with a small curvature is easier, which helps the sophisticated integration of a flow circuit onto a smaller chip. Finally, as discussed above, the actin filament is more suitable than the rigid microtubule for long distance transport because high flexibility of the actin filament may be suited for the assembly of dense arrays of tracks of motor proteins. Indeed, dense actin arrays up to 2.9 μm⁻¹ (immobilized by streptavidin; Fig. 2d) and 14.5 μm⁻¹ (immobilized by α-actinin; Fig. 5a) were successfully fabricated. Thus, at least, submicron-sized cargos can be transported by multiple motor proteins along different filaments simultaneously.

Conclusion

In this study, we established a method to assemble an isopolar array of actin filaments applicable to motor-protein driven transport systems. The polarities of aligned filaments and cargo-transport capability were evaluated by the unidirectional movement of myosin molecules. Indeed, 99.6% of the filaments were oriented in the correct direction, and the array retained the speed of myosin previously reported.37,38 Our method is quick: the fabrication process requires less than ten minutes. Moreover, this method allows one to optimize the transport capability by controlling the actin density. Finally, multiple alternative methods were provided for the fabrication process which is essential for diverse applications requiring different conditions; two alternative ways of the surface immobilization of the actin filament, four alternative ways of the surface treatment of flow channels, and two ways of the surface treatment to block binding of gelsolin-actin complex were demonstrated.

Our study is a fundamental step toward the development of myosin driven transport systems inside flow channels. Since our actin array may have great advantages for downsizing of a fluidic device to the nanoscale, we hope our actin array contributes to further development of a nano total analysis system.

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