Biomotor-based Nanotransport System Constructed by Pick-and-Place Assembly of Individual Molecules

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Abstract—Despite the benefits of miniaturized devices, handling of tiny amount of molecules became a great challenge. Direct transport, similar to the one in intracellular transport, is a way to cope with the problem of transporting tiny amount of materials. Using motor proteins, i.e. kinesin, and the corresponding rail structures, i.e. microtubules, provides powerful building blocks for a nanotransport system. However, it is extremely difficult to construct a complex transport network to allow arbitrary design. Here, we have developed a MEMS-based molecular handling system for the pick-and-place assembly of individual microtubules. The technology enabled us to build a bio/MEMS hybrid system composed of multidirectional microtubule networks along which kinesin molecules move. Using MEMS tweezers to manipulate a single microtubule enables precise positioning and full control in functional orientation and stacking order. As a result, kinesin-coated beads can be transported along the route specifically assembled for them to follow. Direct transport of target molecules can be achieved by loading the beads with the molecules to carry. “Bottom-up” functionalities of biomaterials are incorporated with micro fluidic devices by the handling capabilities of the “top-down” approach.

I. INTRODUCTION

In the last decades, with the developments in the miniaturization of devices, smaller, faster and cheaper equipments have become available. Integration of biological materials with the miniaturized devices is extremely promising for healthcare and environmental monitoring. With smaller dimensions, necessary amounts of sample volume can be decreased. This is especially important in experiments where the target molecules are scarce. However, conventional continuous-flow-type micro fluidic devices have limitations in handling small amount of molecules due to diffusion in flowing medium against extremely high viscous drag in sub-micrometer channels [1].

Direct transport of molecules is a possible way to cope with the problems caused by miniaturization. In living organisms, intracellular transport widely uses direct transport provided by the motor proteins moving along rail structures. Direct transport has advantages on carrying small amount of molecules, because no liquid manipulation is necessary as in the case of microfluidics. Target molecules can be captured on the carriers via highly selective molecular recognition. Then, the carriers can be directly, and separately if needed, transported to the desired location for detection or further processing.

Being the main components of cell cytoskeleton, microtubules have very important roles in intracellular transport as rail structures (Fig. 1). The polymeric structure of microtubules is assembled from tubulin monomers [2]. The polymerization processes with a fast growing (+) end and a slow growing (-) end. Once completed the polymerization process, microtubules can be found as hollow tubes of tens of micrometers long with a diameter of about 25 nm; they serve as a rail for kinesin molecules.

Kinesin, a linear motor protein, moves along microtubules by hydrolyzing adenosine tri-phosphate (ATP) [3]. Having two heads to bind to microtubules, conventional kinesin can move unidirectionally according to the functional polarity of the microtubules from (-) end toward (+) end (Fig. 1). The tail of kinesin can be used to attach target molecules directly or through intermediate carriers [4].

There have been several attempts to build transport systems based on motor proteins [5-8]. One of the main issues is controlling the direction of kinesin motion along immobilized microtubules. As kinesin motion is unidirectional depending on the polarity of underlying microtubules, functional orientation of microtubule polarity determines the direction of motion. However, orientation process is quite challenging and requires complex procedure with additional setup [9-12]. Even with the existing techniques, it is extremely challenging (if not impossible) to build a rail network with multidirectional and multilayered
microtubule with proper orientation. Obtaining multidirectional orientation is the key for realizing complex transport systems working at micro/nano scale to handle extremely tiny amount of molecules.

Although pick-and-place assembly is a well-known technique, it is not widely used for handling biological materials. Such an assembly technique can be used as a supreme method for building microtubule networks to use for nanotransport systems. Manipulation of nanostructures by integrating manipulators into scanning electron microscope and transmission electron microscope has already been performed [13-15]. However, such methods require vacuum environment, which is not applicable to biological assay. On the other hand, using optical tweezers for manipulation is another well-known method [16]. Yet, manipulating 25-nm-diameter microtubules with optical tweezers remains a great challenge as well.

In this work, we have developed a heterogeneous integrated system, i.e. a bio/MEMS hybrid system by a micro robotic method. We have picked and placed a single microtubule to assemble a complex network to enable a unique nanotransport system. We have combined bottom-up functionalities, e.g. specificity and throughput of the bio-world with top-down fabrication and handling capabilities of MEMS technology. As a result, microtubules were captured individually by silicon micro tweezers and placed precisely to assemble the desired network for kinesin-coated beads to move along.

In the proposed method, prior to the capture, polarity-marked microtubules are isolated on a device with parallel walls (Fig. 2a). Then, micro tweezers pick a single microtubule (Fig. 2b). Next, the captured microtubule is placed on a docking zone in proper position and orientation. Repeating the picking and placing steps, a complex microtubule network is assembled with full control in the stacking order and relative position (Fig. 2c). Finally, the assembled microtubules are used as a rail network for kinesin-coated bead transport (Fig. 2d). Such engineered networks can be well integrated with our previously achieved motor-protein based molecular sorting device [8] where beads were loaded with target molecules for direct transport.

Up to our knowledge, such a multidirectional, multilayered transport network is achieved for the first time in a controlled way. The pick-and-place assembly of individual microtubules provides the necessary freedom to assemble any complex network scheme.

II. MATERIALS

Biological materials were used for the transport mechanism of the proposed system. Polarity-marked microtubules were the rail structures for the molecular motors, i.e. kinesin, to carry beads.

A. Polarity-marked Microtubules

Tubulin was purified from four porcine brains and stored at a concentration of 4 mg/ml in liquid nitrogen. Tubulin purification protocols are explained elsewhere [17, 18]. For fluorescence observations, rhodamine labeled tubulin was obtained. Purified tubulin was polymerized into
microtubules in BRB80 buffer (80 mM PIPES-NaOH pH=6.9, 1 mM MgCl₂, 1 mM EGTA) containing 1 mM MgSO₄ and 1 mM GTP incubating at 37°C for 30 minutes.

Polarity-marked microtubules have one side densely labeled and the rest sparsely labeled so that the functional polarity of the microtubule can be determined by examining the fluorescence image [19]. Obtaining such marking on the microtubules requires a two-step polymerization [17]. This two-step polymerization process was accomplished by using different concentrations of rhodamine-labeled tubulin during each of the polymerization steps (Fig. 3). In the first step, high concentration of labeled tubulin was used to obtain bright seeds. Then, in the next step, a lower concentration of labeled tubulin was used. Inhibiting the (-) end growth, the second step provided sparsely labeled (+) end. As a result, the polarity-marked microtubules had brighter (-) ends and darker (+) ends when observed via fluorescence microscopy (Fig. 3).

B. Kinesin

Conventional kinesin was used in the experiments. GST-tagged kinesin was expressed in E.coli. Purification process of conventional kinesin is explained elsewhere [11].

Commercially available carboxylated silica beads (Bangs Labs, 0.33 µm in diameter) were used in the transport experiments. Beads were first washed, suspended in caseinated BRB80 solution and then incubated in kinesin solution for coating based on non-specific binding. Bead transport is examined on microtubules fixed on poly-L-lysine (PLL)-coated glass surface (Fig. 4) [7].

III. Fabrication

There are two main devices used in the experiments: parallel walls device and micro tweezers. Micro fabrication techniques were used for building parallel walls to isolate and micro tweezers to capture microtubules.

A. Parallel Walls

Microtubule isolation required a device with parallel walls for suspending microtubules between them as shown in Fig. 2a. The pick-and-place manipulation of microtubules should be performed on the same device. Thus, the device consisted of two parts: a repeating structure of parallel walls and a planar docking zone.

Because the inverted microscopy was used, transparency was necessary to carry out transport experiments after completing the assembly process. Furthermore, the material used for the parallel walls and the docking zone should not auto fluoresce since this would disturb imaging during assembly.

The height of the walls was extremely important to provide the necessary maneuvering space for the micro tweezers. Thus, the material should be thick enough to ensure necessary height for the structures, especially for the parallel walls.

Among several possible materials, CYTOP (CTL-809M, Asahi Glass), a fluoropolymer, was chosen because it had some excellent characteristics on high optical transparency, and easy fabrication possibilities.

Fabrication of the CYTOP structures was started with spin coating of CYTOP solution on a glass slide (Fig. 5a). This step was repeated depending on the desired thickness. For example, two coating cycles provided a layer of about 4 µm with baking at 100°C for 30 minutes in between. After the last cycle, the glass slide was baked above the glass transition temperature at 185°C for 90 minutes (Fig. 5b). Then, aluminum layer was thermally deposited and patterned as a mask layer for the following CYTOP etching (Fig. 5c). The etching was done by oxygen plasma to expose the bottom glass surface (Fig. 5d).

In the experiments, the walls were fabricated with a height of 4 µm considering imaging quality and micro tweezers integration.

B. MEMS Tweezers

MEMS micro tweezers were fabricated using an SOI wafer. Fabrication was based on reactive ion etching, local oxidation and anisotropic etching of silicon as explained detailed elsewhere [20]. First, a thin Si₃N₄ layer was deposited by low-pressure chemical vapor deposition
(LPCVD) and patterned. Then, Si$_3$N$_4$ and the Si over layer were etched by reactive ion etching (RIE). A local oxidation of silicon process was used to grow SiO$_2$ on the top and sidewalls of the uncovered Si surface. The Si$_3$N$_4$ layer was then removed and a KOH wet anisotropic etching of Si from the exposed area is performed to obtain {111} facets. The intersection of three planes, i.e. the bottom <001> plane protected by the buried oxide, the vertical <010> plane protected by LOCOS oxide, and the <111> plane obtained by KOH etching, made sharp opposing tips. As the last step, the buried oxide was removed using vapor HF, and the handling Si was structured due to deep reactive ion etching.

An important point and design issue of the micro tweezers was that they should have sharp, protruding tips. Sharpness was necessary for the precise capturing and relocation of the microtubules. Protrusion was necessary for maneuvering the tips between the parallel walls without touching the walls or glass surface. As a result, the distance between the tips should be smaller than the distance between the parallel walls. The reason of this design issue will be understood better in the next section. A photo of micro tweezers with sharp protruding tips can be seen in Fig. 6.

The length of the individual rail structures for the building network was decided by the gap between the tips of the tweezers. Thus, micro tweezers should be fabricated considering the desired rail network. In the experiments, we have used a gap of around 20 µm between the tips of the tweezers.

IV. PICK-AND-PLACE ASSEMBLY PROCESS

Pick-and-place assembly of individual microtubules can be divided into three steps: isolation, capturing and relocation. Isolation is the step where microtubules are bridged between the parallel walls. For the capturing step, micro tweezers are used for approaching to the bridging microtubules. After capturing the microtubule, the third step, relocation is performed by carrying the microtubule captured between the tips of the tweezers using a micromanipulator.

A. Setup

Fluorescence microscopy was used throughout the pick-and-place assembly process to determine the position and polarity of polarity-marked microtubules. On the other hand, after the assembly, the microtubule network was used for transport experiments where kinesin-coated beads moved along the network. A DIC (differential interference contrast) setup was used to observe the kinesin-coated bead motion.

An inverted microscope (Olympus IX-71) was used to provide the necessary working distance for micro tweezers to approach to the glass surface and maneuver after capturing the microtubule. Micro tweezers were attached to a micromanipulator arm providing the necessary 3-degree of freedom in x-, y- and z-axes.

B. Microtubule Isolation

The first step of the pick-and-place assembly process was the isolation of the microtubules. The isolation process should provide good imaging possibilities of microtubules. Furthermore, microtubules should be isolated with adequate space for micro tweezers to approach. The isolation process should also provide good alignment of microtubules with the micro tweezers to permit a handy capture. One other important point was to provide sufficient space for micro tweezers to maneuver in close proximity of the microtubules without touching the glass surface that could result in damage to the tweezers tips.

One of the best geometries to satisfy all the requirements was to bridge microtubules between parallel walls, which were made of transparent material (Fig. 2a). For microtubules to bridge the gap, microtubules had to be aligned perpendicular to the walls. This is efficiently achieved by flow induced drag force perpendicular to the wall. Microtubules aligned with the flow and bridge between the parallel walls (Fig. 7a). As the micro tweezers were also positioned perpendicular to the walls, a good alignment with the microtubules was achieved. Furthermore, the set-up provides an adequate maneuvering space for capturing microtubules without the risk of damage due to collision with the glass slide.

As the distance between the tips of the tweezers were decided to be 20 µm, in order to provide good maneuvering capabilities, the distance between the walls of the isolation device was chosen as 30 µm in the experiments.

C. Microtubule Capturing

Isolated microtubules were captured using fabricated silicon micro tweezers. Attached to a micromanipulator arm, the tweezers could be manipulated with 3-degree of freedom in x-, y- and z-axes to easily approach to the isolated microtubules suspended between the walls. The capturing process consisted of coating of the tips of the tweezers, checking the polarity of the microtubules and capturing them between the tips of the tweezers.

In order to enable the microtubule-capturing process, the tips of the tweezers should be coated with PLL, a polycation that can bind to any negatively charged proteins. The coating process was simply dipping the tips of the tweezers into a PLL droplet and waiting for 2 minutes inside the droplet.
Depending on the desired microtubule network for the transport system, microtubules with a suitable polarity should be selected. Using the micromanipulator, tweezers should be aligned well with the selected microtubule in the x- and y-axis. Then, using the z-axis motion of the micromanipulator, tweezers were brought closer to the surface and in contact with the selected microtubule. The polycation coating of the tips of the tweezers easily attached to the negatively charged microtubules. Using the z-axis motion of the micromanipulator once again, the tweezers were removed away from the surface (Fig. 2b). Microtubules were usually cut at the binding points and moved between the tips of the tweezers (Fig. 7). All these manipulations were manually performed under visualization control.

D. Microtubule Relocation

The next step consisted of carrying the captured microtubule together with the tweezers to the location where the transport network was built and precisely relocating the microtubule (in terms of location and stacking order).

An important point to mention in the relocation process was the removal of microtubules from the tips of the tweezers. The docking zone was coated with PLL similar to the tips of the tweezers. As a result, when the captured microtubule made contact with the docking zone, the contact area between the microtubule and the PLL coated surface was much larger than the area between the tips and the microtubule and the microtubule sticked on the substrate.

Repeating the capturing and relocation steps several times, different microtubules arrangements could be obtained (Fig. 2c). One of the most important advantages of the proposed method was to give high degree of freedom to build the transport system. Complex shape and precise location of the constructed transport system were important benefits. Furthermore, one-by-one relocation provided a full-control on the stacking order.

Fig. 8 shows a demonstration of repeated capturing and relocation process for building different transport schemes. x shape, ≠ shape, # shape could easily be achieved as shown. Successive images clearly show how the stacking order of the relocation process was decided.

V. TRANSPORT SYSTEM

To investigate the kinesin motion along the relocated microtubules, kinesin-coated beads were used. After preparing the desired network system, a flow cell was assembled over the docking zone. Kinesin-coated beads were then injected into the flow cell. After attachment of some beads to the relocated microtubules, unbound beads were washed out using BRB80 solution. Finally, flushing ATP solution activated kinesin molecules transporting beads along the designed microtubule network (Fig. 2d). The motion of the beads was observed by DIC microscopy.

Successful transport can be seen with successive images of the kinesin-coated bead motion (Fig. 9) as in the case of usual bead assay (Fig. 4). Carboxylated polystyrene beads with a diameter of 0.33 µm were coated with full-length kinesin before injection into a flow cell fabricated using the glass slide with the relocated microtubules. The bead marked with the left-most arrow initially attached to the vertically oriented microtubule (top image in Fig. 9b). It moved up to the crossing point (middle image). Then, it moved rightward (bottom image) along the horizontally oriented microtubule. It means that the kinesin-coated bead switched the microtubules from the vertical one to the horizontal one at the crossing because the horizontal one was located on top of the vertical one. The stacking order provided the necessary layer information and determined the moving direction of beads.

VI. CONCLUSION

Although pick-and-place manipulation is a well-known technique, it was not used for single microtubule assembly until now. The proposed method, based on single microtubule manipulation, is superior to other existing orientation methods due to its high-degree of freedom.
Figure 9: Kinesin motion along relocated microtubules. a) Photos showing microtubule relocation before & after each relocation step b) Kinesin coated beads’ motion along the relocated microtubules. White lines correspond to the microtubules seen with fluorescent setup. Red line is a trajectory for the left-most bead.

provided by full control in the stacking order and precise relocation. Easy picking and placing of microtubules were an important merit of using PLL-microtubule interaction. In this work, using a pick-and-place technique, a multilayered and multidirectional microtubule network was assembled. Furthermore, the assembled microtubule network was used as a part of a transport system where kinesin-coated beads were transported along. In other words, the handling capabilities of the “top-down” approach were used to assemble a microtubule network for building a transport system based on the “bottom-up” functionalities of biomaterials. The result is a significant example of the integration of the “bottom-up” and the “top-down” approaches.

As a future direction, automation of microtubule relocation integrated with the proposed method is very important to decrease the assembly time and increase the efficiency of the assembly. Combining an image-processing unit with a motorized stage provides an excellent tool to minimize the necessary user input.

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