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Ratchet patterns sort molecular shuttles

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ABSTRACT Molecular shuttles based on microtubules propelled by motor proteins can be guided on surfaces by adsorbing motors in chemical patterns or by using open guiding channels. While chemical patterns can guide microtubules based on a Brownian ratchet mechanism, the rigidity of the microtubules limits guiding to features with dimensions on the order of their persistence length (5 mm). To achieve guiding on micron-scale dimensions, physical barriers are required which can exploit the forces exerted by multiple motors to bend tubules into tight radii of curvature. Microtubule guiding is illustrated for the case of a special ratchet pattern that is capable of sorting microtubules on the basis of the direction of their motion.

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1 Introduction

Organizing transport on the nanoscale is a challenge for nanotechnology. Transport by diffusion is very effective on small scales but requires a concentration gradient and is stochastic in nature. Molecular shuttle systems [1] are being developed as an alternate means of transporting molecules and nanoscale objects under user control. Such systems must include molecular motors, means for guiding the shuttles, strategies for loading and unloading cargo, and methods for commanding the shuttles to start or stop moving [2]. Molecular shuttles may find applications as nanoscale probes [3], in novel sensors, and in nanoelectromechanical systems (NEMS).

Our shuttle system is based on the interactions between motor proteins and cytoskeletal fibers, which provide a wide range of transport functions in living cells [4]. Motor proteins such as myosin and kinesin have nanometer dimensions and generate pN forces from the hydrolysis of ATP. In cells, the motor proteins "walk" along pathways provided by actin filaments or microtubules to carry or move other objects. We prefer to utilize the motor kinesin in a "gliding geometry", where the motors are tethered to a surface and move the microtubules. An overview of shuttle designs based on a combination of either myosin motors with actin filaments or kinesin motors with microtubules is given in [5].

This paper deals with the complex issue of guiding the shuttles. Guiding can be achieved by selective adsorption of

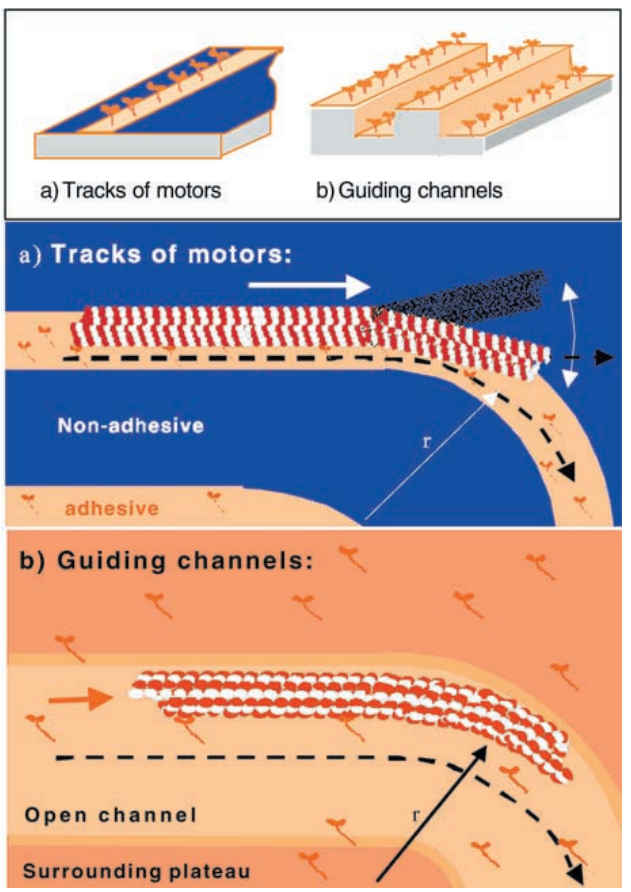

FIGURE 1 Guiding of molecular shuttles based on kinesin and microtubules can be achieved by **a** selectively adsorbing motors to adhesive tracks or **b** providing open, micron-scale guiding channels. Brownian motion causes the tip of the advancing microtubule to swivel to the left and to the right, which enables the microtubule to bind to off-axis motors on a curved track (**a**). In open guiding channels the microtubule is bent into the new direction when pushed against the sidewall (**b**). Due to the high flexibility of the tail region, the motor can bind to microtubules approaching from all directions. Dozens of motors bind to a single microtubule at typical motor surface densities in adhesive regions. In **b** also the sidewalls adsorb motors, which allow some microtubules to slowly climb up and escape the channel

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motor proteins to a patterned surface [1, 6, 7] (Fig. 1a), by using open channels as physical barriers to confine shuttle motion [2, 8] (Fig. 1b), or a combination of the two techniques [9]. In our work, we have produced linear "tracks" of motor proteins via self-assembly on protein-adsorbing patterns surrounded by adsorption-resistant regions; microtubules then follow these tracks of adsorbed kinesins. Guiding channels have been fabricated by replica molding in a thin polyurethane film. Microtubules moving on the bottom of the channel are guided by the sidewalls [10]. While motor protein tracks and guiding channels force the shuttles to follow the pattern, the direction of motion is still undefined. Random adsorption of the polar microtubules results in half of the shuttles being transported in one direction, while the others move in the opposite direction.

To sort shuttles according to their direction, Hiratsuka et al. introduced arrowhead-shaped guiding channels that act as directional rectifiers [9]. These arrowhead-shaped channels invert the direction of microtubules moving against the arrow direction with a probability of up to 70% while maintaining the direction of movement for microtubules moving in the direction of the arrow. Hiratsuka et al. presented a series of beautiful experiments using different arrowhead geometries, and demonstrating devices employing the directional rectifiers, but did not analyze the sorting mechanism in detail.

Here we discuss the underlying mechanism of a new ratchet-like device for directional sorting of molecular shuttles. In this device the shuttle is given a choice of two paths oriented at different angles to the direction of motion. The energy required to reorient the shuttle can be supplied by thermal energy, in which case we have a Brownian ratchet, or by harnessing the work done by motor proteins. Our analysis provides vital information for optimizing the size and geometry of directional sorters and other guiding devices.

2 Theory of a ratchet-like sorting device

Adsorbing motors in tracks is the simplest mechanism for guiding shuttles (Fig. 1a). This mechanism relies on Brownian motion to reorient the shuttle into the intended direction. As a shuttle consisting of a microtubule or an actin filament is transported along a featureless surface, the leading tip of the shuttle will swivel to the left and the right under the influence of Brownian motion. When the tip encounters the next motor, it attaches to it in a strong bond. If the motors are not uniformly distributed, but are patterned to describe an arc, the probability of attachment is biased towards one side. With each attachment the shuttle changes its direction of motion slightly and is guided along the arc. The requirement for guiding is that there be sufficient thermal energy ($kT/2$) to overcome the flexural rigidity of the shuttle, allowing it to bend to a sufficient extent to encounter the next motor in the arc. If the shuttle is too stiff, the shuttle will escape the pattern and be propelled in a straight line by the motors still in contact with the shuttle. After the last motor has reached the end of the shuttle, the shuttle will detach from the surface and diffuse freely in the solution.

Interestingly, shortly before the shuttle detaches and is held solely by the last motor, it has a second opportunity to reach for the next motor by twisting the flexible tail of the last motor. In this case the shuttle does not have to bend but reorients due to rotational diffusion around the last motor. Since this mechanism only allows the guiding of very short shuttles (microtubules shorter than 1 μm) that have a high rotational diffusion coefficient ($D_{\mathrm{rot}} = 3kT/cL^3$, where c is the drag coefficient per unit length [11]), we will not consider it further due to the high probability that the shuttle cargo will impede the rotation of the whole shuttle.

Based on the "swiveling tip" model, the minimum radius of curvature for guidance using motor protein patterns can be estimated to be on the order of the persistence length of the microtubules or actin filaments ($L_{\mathrm{p}} = EI/kT$, where EI is the flexural rigidity, E the modulus of elasticity, and I the geometric moment of the beam cross-section), since with an energy of $kT/2$ a filament can be bent into a 45° turn with radius

$$r_{kT/2} = \frac{\pi EI}{4kT} = \frac{\pi}{4} L_{\mathrm{p}} \,. \tag{1}$$

The persistence length of microtubules and actin filaments has been measured to be 5 mm and 20 μm, respectively [12].

This suggests that the flexural rigidity of the shuttle element has a large impact on the guiding process. While tracks of myosins will be able to guide the flexible actin filaments in patterns with feature sizes larger than 20 μm, chemically defined patterns of motors will be insufficient if the shuttle element is relatively stiff, such as a microtubule, or small feature sizes are desired. One strategy for overcoming the limitations of bending by Brownian motion is to supply more energy to bend the stiff shuttle.

Although the energy requirement to bend a microtubule into a 90° turn with 10 μm radius is high ($\sim 200kT$), such energy can be supplied from the work done by the motor proteins [13]. Since each kinesin can do about $10kT$ of work per 8-nm step, the energy provided by just one motor ($\sim 10^4 kT$) is more than sufficient to guide a microtubule through a turn having a radius of curvature of 10 μm (which requires > 1000 steps to complete). However, in order to use the work of the motors for bending, the solid sidewall of a guiding channel is needed to redirect the pushing force into a bending force. This conversion mechanism makes open guiding channels an attractive option for directing microtubules at small length scales.

After understanding the general relations between shuttle rigidity, guiding mechanism, and scale of the pattern, as outlined above, we can now search for useful pattern geometries.

A geometric pattern designed to sort molecular shuttles according to their direction of movement is shown in Fig. 2. Shuttles, which approach the structure from one of the side arms, will be trapped and directed to move in a clockwise direction in the left circle and in a counter-clockwise direction in the right circle. At the intersection between the two circles the shuttles can cross over and change the direction of movement.

In such a pinwheel pattern the actual path of the shuttles is octagonal rather than circular. The mismatch between the actual direction and the circular one shows a ratchet-like dependence on the angle of revolution (Fig. 3). At each corner of the octagon the shuttles are confronted with the choice between turning 45° towards the intended direction or 135° to escape into a sidearm.

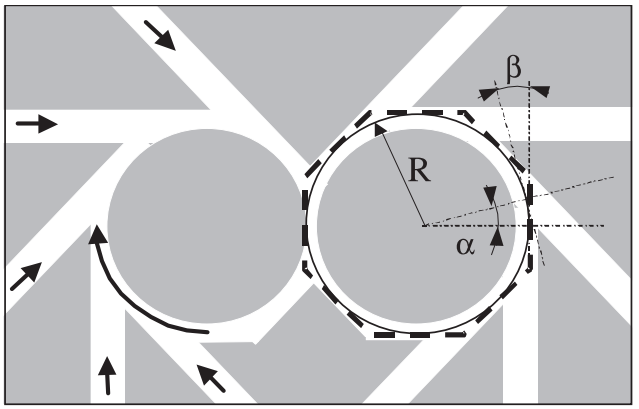

FIGURE 2 A geometric pattern which sorts molecular shuttles according to their direction of motion. Shuttles, guided to the pinwheel by side arms, move clockwise in the left pinwheel and counterclockwise in the right pinwheel

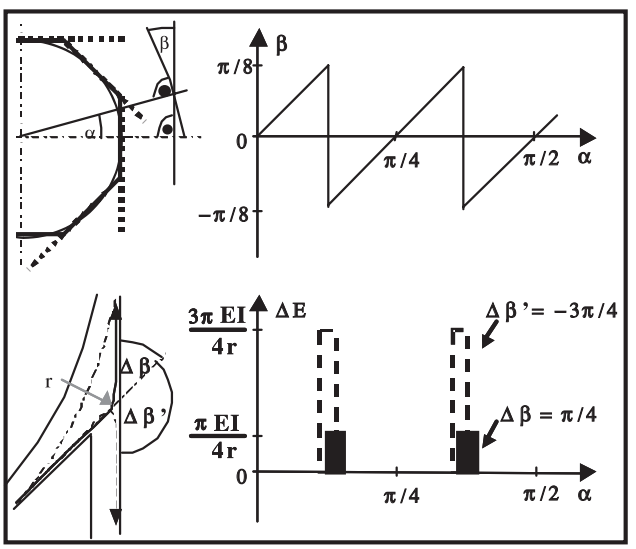

FIGURE 3 *Top*: The angle, β between the direction of the shuttle on the actual octagonal path and the tangent of the intended circular motion has a sawtooth-like dependence on α (should define alpha). *Bottom*: When the shuttle approaches the boundary of the track, it can make a wide turn and follow the intended path or make a sharp turn and escape (*dashed path*). Since sharper turns require more work, ΔE, to bend the filament, the choice is biased

The two choices require a different amount of bending energy [12], which depends on the flexural rigidity, EI, the radius of curvature, r (which is much smaller than the radius R of the pattern), and the bending angle, $\Delta\beta$, and can be estimated as

$$E_\mathrm{b} = \frac{EI\Delta\beta}{2r} = \frac{kT}{2} \times \frac{L_\mathrm{p}\Delta\beta}{r} \,. \qquad (2)$$

If E_b is on the order of $kT/2$, the pattern can be formed by tracks of motors, and the device constitutes a Brownian ratchet (Fig. 4, left). If E_b is much smaller than $kT/2$, no significant preference of the shuttle for either path exists, and the thermal forces will randomize the direction of the shuttle. If E_b is much larger than $kT/2$, a pattern formed by tracks of motors will not guide the shuttle in either direction, and the shuttle will escape (Fig. 4, center). However if guiding channels form the pattern, the work of the motor proteins is converted into bending energy, and successful sorting is achieved

FIGURE 4 *Left*: An actin filament advancing over the boundary of a track of motor proteins will begin to swivel with the free tip and reach for the next available motor. *Center*: A microtubule in the same situation will swivel only slightly due to its 300-times-greater flexural rigidity. *Right*: Guiding channels with high sidewalls can force the microtubule into a turn provided the motors push the microtubule from behind

(Fig. 4, right). Because the difference between the bending energies required for guiding and escape is also large, Brownian motion does not affect the sorting process.

While a Brownian ratchet mechanism can be used in principle for a sorting pattern, the minimum size R of a pinwheel-shaped sorting device is limited to ~ 0.1 mm for shuttles based on actin filaments and more than 1 cm for shuttles based on microtubules, since the radius, r, of the turn at each octagonal corner is limited to ~ 20 μm for actin filaments and ~ 5 mm for microtubules, their respective persistence lengths. Smaller devices based on a ratchet pattern, but using controlled guiding instead of relying on Brownian motion, can be built using guiding channels.

3 Experimental realization of sorting device

Because a shuttle system based on kinesin and microtubules has advantages with respect to loading of cargo over an actin/myosin system (for example, actin filaments rotate around their long axis while moving [14]), our experimental goal was to create a sorting device with micrometer dimensions for shuttles based on kinesin and microtubules.

From our observations (Fig. 5) of microtubule behavior at the interface between motor protein-rich and motor protein-poor regions, we can deduce that our ideas regarding the guiding by tracks are in general accurate: The microtubule crosses the boundary between the two regions, and the free front end of the microtubule begins to swivel. The acquisition time of each camera frame (100 ms) is much longer than the characteristic time of the swiveling motion (2.5 ms for a 10-μm-long microtubule – (32) in [12]); thus, the region sampled by the microtubule corresponds to the blurred image of the microtubule. The swiveling tip of the microtubule bends only slightly due to its high stiffness (see Figs. 4 and 5), which limits the radius of curvature of a turn. This results in the inability of the tip to reach the motor-rich region again and detachment of the advancing microtubule.

In the previous theory section we concluded that a pinwheel pattern based on adhesive tracks of motors is unfeasible for small pattern dimensions. This also is supported by the current experiment, which focuses solely on the boundary between motor protein-rich and motor protein-poor regions and not a complex pattern.

Therefore, we tested the suggested sorting geometry with a pattern of open guiding channels. Using the technique of

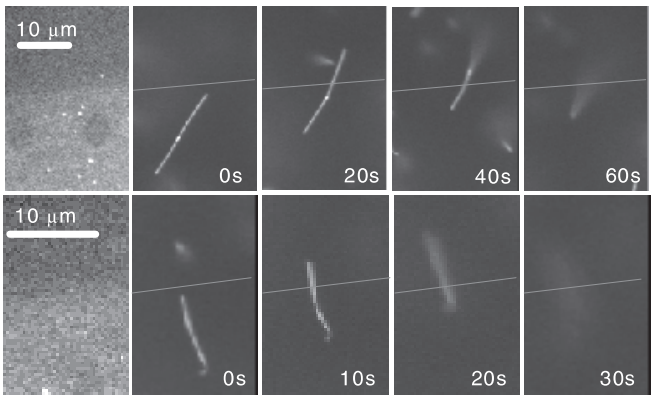

FIGURE 5 Two examples of microtubules crossing from a motor protein-rich region of the surface to a motor protein-poor region of the surface. The *left frame* shows the distribution of the GFP-kinesin fusion protein. A visible drop in GFP-fluorescence intensity occurs at the boundary between the adhesive region (*bright*) and the non-adhesive region (*dark*) with different surface chemistries (see Sect. 5). Each *row* shows a different microtubule crossing a boundary moving upwards. The image of the microtubule blurs when the microtubule is not attached to a motor and undergoes Brownian motion (acquisition time 100 ms). The *last frame* shows how the microtubule leaves the surface and drifts away

replica-molding a PDMS stamp was created from a lithographically patterned silicon wafer and stamped into a thin polyurethane film on a coverslip, recreating the pattern of 1-μm-deep and 2.5-μm-wide guiding channels. The movement of the microtubules on this surface, which was uniformly covered with motor proteins, is shown in Fig. 6. The microtubules can enter the pattern from the elevated regions surrounding the channels and are then guided in the channels. They frequently escape by slowly crawling up the sidewalls, a problem which can be overcome by selectively coating only the bottom of the channel with motors [9]. However, as the two examples shown in detail demonstrate, the sorting geometry functions as described: The tip of the microtubule is pressed against the sidewall and bent in a sharp turn ($r < 2$ μm) into the intended direction.

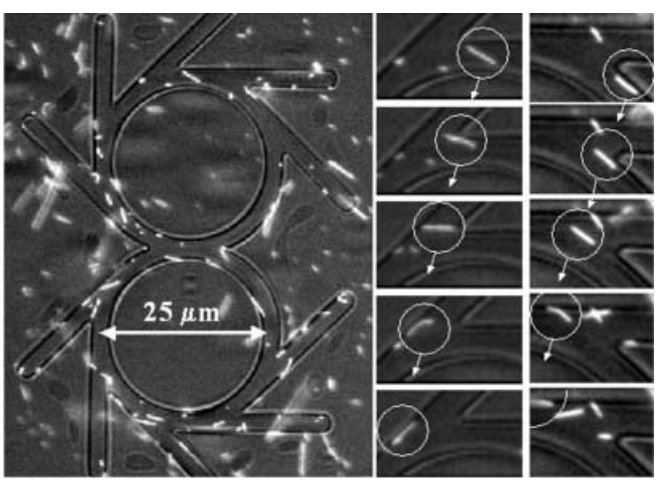

FIGURE 6 Microtubules moved by kinesin on a surface with 1-μm-deep open channels in pinwheel. *Left*: the whole pattern with an inner diameter of 25 μm. The width of the channels is 2.5 μm. *Right*: two examples of microtubules moving around a corner (frame interval = 5 s). The radius of curvature of the microtubule at the bending point is less than 2 μm (*fourth frame* from *top*). The pattern is imprinted into a thin film of polyurethane by replica-molding and imaged using bright field microscopy

4 Conclusion

Directional sorting of molecular shuttles can be achieved by providing a pinwheel pattern of adhesive tracks of motors or open guiding channels. A Brownian ratchet mechanism underlies the directional sorting if the pattern consists of motor protein-rich and motor protein-free surface regions, and Brownian motion is responsible for a change of direction of the molecular shuttle. Depending on the flexural rigidity of the filament, Brownian motion may cause only small changes in direction. This limits the size of the pattern to several times the persistence length of the filament (actin filaments – 20 μm, microtubules – 5 mm). Smaller sorting devices based on the same geometric pattern can be built if the molecular shuttles are forced to move in guiding channels, which can convert the push of the motors into a bending force on the advancing tip of the filament. However, if we rely on guiding channels, Brownian motion ceases to be an essential part of the sorting devices.

Since our goal is to build a nanoscale transport system, we focus on engineering the smallest possible devices. Moreover, for reasons related to loading of cargo, we also prefer a shuttle system based on the kinesin motor and the stiff microtubules, thus making a "Brownian ratchet sorter" an unfavorable choice over a device based on guiding channels.

Nevertheless, the discussed example of a "Brownian ratchet" is potentially applicable to an actin/myosin system and is fundamentally interesting in the respect that here Brownian motion does not cause lateral diffusion but bending of a filamentous structure.

5 Materials and methods

5.1 *Kinesin and microtubules*

The kinesin and the microtubules were kind gifts of Prof. J. Howard.

5.1.1 Kinesin. A kinesin construct consisting of the wild-type, full-length *Drosophila melanogaster* kinesin heavy chain and a C-terminal His-tag [15] was expressed in *Escherichia coli* and purified using a Ni-NTA column. The eluent contained functional motors with a concentration of $\sim$ 100 nM, and it was used as stock solution after adding 10% sucrose.

5.1.2 GFP-kinesin. The 3′ end of the kinesin gene containing the his-tag and stop codon was excised from the pET plasmid using NarI and NotI restriction endonucleases. An oligonucleotide cassette was ligated to the 3′ end of the gene to replace the excised fragment. To prepare for EGFP ligation, the kinesin-cassette construct was digested with NheI and EcoRI. The EGFP gene was removed from pEGFP-C1 (Clontech, Palo Alto, CA) using NheI and EcoRI. EGFP was then ligated to the 3′ end of the kinesin-cassette construct in pET21b+ (Novagen, Madison, WI). The desirable coding sequence of the engineered construct has the following

sequence $5' - 3'$, wild-type kinesin, enhanced green fluorescent protein, and polyhistidine tag. The construct was then expressed in *Escherichia coli* and purified using a Ni-NTA column.

5.1.3 Microtubules. Tubulin was purified from bovine brain, fluorescently labeled with rhodamine and polymerized into microtubules according to [12]. The microtubules with a length between 2 and 20 μm were 100-fold diluted and stabilized in 10 μM Taxol (Sigma).

5.2 *Motility assays and microscopy*

The motility assays (Fig. 1) were performed in 60-μm-high and 1-cm-wide flow cells built from a microscopy slide, a coverslip and spacers according to [16] at a temperature of 20 °C. First casein (0.5 mg ml^{-1}, Sigma) dissolved in BRB80 (80mM PIPES, 1 mM MgCl, 1 mM EGTA, pH 6.9) was adsorbed for 5 min to reduce denaturation of kinesin. Then the kinesin solution diluted in BRB80 was adsorbed for 5 min and exchanged against a motility solution consisting of 1000-fold diluted rhodamine-labeled microtubules, 1 mM ATP, 0.2 mg ml^{-1} casein, 10 μM Taxol and an oxygen-scavenging system to reduce photobleaching [17] (20 mM glucose, 0.02 mg ml^{-1} glucose oxidase, 0.008 mg ml^{-1} catalase, 0.5% BME). Flow cells were either sealed with oil or kept hydrated in a moist environment. A Leica DMIRBE optical microscope with a 100× oil objective (NA 1.30), a Hamamatsu ORCAII camera and Openlab software (Improvision) were used to image rhodamine-labeled microtubules by epi-fluorescence microscopy, and patterned surfaces by brightfield microscopy.

5.3 *Patterned surfaces*

A coverslip with regions of different affinity for protein adsorption was fabricated by covering the surface partially with a PDMS stamp (Dow Chemical) and silanizing the exposed regions (TRIDECAFLUORO-1,1,2,2-TETRAHYDROOCTYL)-1-TRICHLOROSILANE, United Chemical Technologies). After removal of the stamp, the block copolymer F108 (BASF, Pluronic) was preferentially adsorbed to the silanized regions of the surface, and created an adsorption-resistant surface layer surrounding the region of bare glass surface previously covered by the stamp. The selective adsorption of a kinesin-GFP fusion protein was monitored with fluorescence microscopy.

To fabricate the surface covered by guiding channels, a 1-μm-thick polymer film of the photoresist SU8-2 (Microchem Corp.) on a silicon wafer was exposed to UV-light through a chrome mask (Photo Sciences). After removal of the film in the unexposed regions, the patterned polymer film on silicon was allowed to react (gas phase) for 30 min with a fluorocarbon-terminated silane (United Chemical Technologies) to help its release properties in the imprinting process. This master was then used to produce a PDMS (polydimethylsiloxane) stamp with the inverse pattern. A replica [18] of the master was created by stamping a thin film of polyurethane precursor (Norland Products, NOA73) on #1.5 coverslips with the PDMS stamp and curing the polyurethane with UV-light. In situ imaging of the pattern through polyurethane film plus coverslip was possible because the films were thin (20 μm) and transparent.

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