Molecular motor-driven filament transport across three-dimensional, polymeric micro-junctions

Cordula Reuther, Sönke Steenhusen, Christoph Robert Meinecke, Pradheebha Surendiran, Aseem Salhotra, Frida W Lindberg, Alf Månsson, Heiner Linke, and Stefan Diez

1 B CUBE – Center for Molecular Bioengineering, Technische Universität Dresden, Germany
2 Fraunhofer Institute for Silicate Research ISC, Würzburg, Germany
3 Center for Microtechnologies, Technische Universität Chemnitz, Chemnitz, 09126, Germany
4 NanoLund and Solid State Physics, Lund University, S-22100 Lund, Sweden
5 Dept. Chemistry and Biomedical Sciences, Linnaeus University, S-39182 Kalmar, Sweden
6 Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

* Author to whom any correspondence should be addressed.
7 Shared first authors.

E-mail: stefan.diez@tu-dresden.de

Keywords: molecular motors, biocomputation, polymeric nanostructure, 3D junctions

Abstract

Molecular motor-driven filament systems have been extensively explored for biomedical and nanotechnological applications such as lab-on-chip molecular detection or network-based biocomputation. In these applications, filament transport conventionally occurs in two dimensions (2D), often guided along open, topographically and/or chemically structured channels which are coated by molecular motors. However, at crossing points of different channels the filament direction is less well determined and, though crucial to many applications, reliable guiding across the junction can often not be guaranteed. We here present a three-dimensional (3D) approach that eliminates the possibility for filaments to take wrong turns at junctions by spatially separating the channels crossing each other. Specifically, 3D junctions with tunnels and overpasses were manufactured on glass substrates by two-photon polymerization, a 3D fabrication technology where a tightly focused, femtosecond-pulsed laser is scanned in a layer-to-layer fashion across a photo-polymerizable inorganic–organic hybrid polymer (ORMOCER®) with μm resolution. Solidification of the polymer was confined to the focal volume, enabling the manufacturing of arbitrary 3D microstructures according to computer-aided design data. Successful realization of the 3D junction design was verified by optical and electron microscopy. Most importantly, we demonstrated the reliable transport of filaments, namely microtubules propelled by kinesin-1 motors, across these 3D junctions without junction errors. Our results open up new possibilities for 3D functional elements in biomolecular transport systems, in particular their implementation in biocomputational networks.

1. Introduction

Filament transport driven by surface-attached biomolecular motors has been widely explored for various biomedical and nanotechnological applications [1–6]. Biomolecular motor proteins such as kinesins or myosins provide self-driven and directional transport of corresponding cytoskeletal filaments, i.e. microtubules and actin filaments, respectively. Using the energy derived from ATP hydrolysis, such filament transport can be orders of magnitude faster than passive diffusion [7]. Moreover, motor-driven filaments can carry cargo or detect different antigens if they have been specifically modified beforehand [8–13]. Most commonly, filament transport is spatially controlled by microscale networks, in which guiding channels and
directional rectifiers are etched into planar surfaces [5, 14, 15]. Despite these topographical surface structures, actual transportation occurs in two dimensions (2D), conventionally on the channel floor.

A recently reported application of molecular motor-driven filament transport is space-encoded network-based biocomputation [16]. Within this application, motor-driven filaments solve combinatorial problems by exploring all allowed paths in a 2D physical network. The filaments pass a number of different junctions along their paths through the network. A key requirement for biocomputation is a negligible error rate [17] at so-called pass junctions. These junctions are channel crossings where filaments are required to move in a straight direction along the same channel; i.e. they are not allowed to take turns into the crossing channel. However, intrinsic design limitations in the 2D junction geometry are prone to increase the total error rate when up-scaling the physical networks for biocomputation. Therefore, it is imperative to explore novel design approaches where, ideally, the crossing channels are completely separated from one another. Here, we demonstrate molecular motor-driven filament transport within two separate crossing channels of 3D polymeric micro-junctions on planar glass substrates without junction errors. Beyond their application in network-based biocomputation, the here presented 3D junctions, can also be implemented into other advanced devices based on motor-driven filament transport, for example, in sensor or lab-on-a-chip systems with channel crossings.

2. Results

For manufacturing biocompatible 3D-junctions with sufficient structure resolution we chose two-photon polymerization (2PP). 2PP, an inherently 3D micropatterning technology, is well-established in the academic domain, allows feature sizes down to less than 100 nm [18, 19], and has recently been applied in microoptics [20, 21], microfluidics [22–24], micromechanics [25, 26], tissue engineering and drug delivery [27–29]. The 2PP manufacturing process is carried out similarly to 3D printing in a layer-to-layer fashion. The light of a tightly focused femtosecond laser (typically wavelengths around 780–800 nm or 515 nm [30, 31]) is usually not absorbed by the photopolymerizable resin. However, if the light intensity within the focal volume is sufficiently high to trigger two-photon absorption, resin solidification is induced, similarly to conventional UV-lithography. Scanning the focal volume across the resin followed by washing with solvent to remove the unexposed resin lead to the desired 3D microstructures. In order to test filament transport across 3D junctions the layout was designed to guide the filaments from different directions toward a 3D junction (figure 1(a)). Walls structured the surface into two pairs of a closed and an open reservoir, each connected by a channel. When crossing each other in the center of the layout, the two channels were spatially separated by a tunnel and an overpass. After designing this 3D junction using a computer-aided design software, the 3D data were translated into positioning and laser control commands, represented as contour and filling vectors (supplementary figure 1) (https://stacks.iop.org/NJP/23/125002/mmedia). The design parameters (figure 1(b)) were not only selected for successfully realizing the 3D junctions but also for achieving reliable filament transport.

In our work, we applied microtubules as filaments (with a diameter of 25 nm, an average length of 5 to 10 μm and a persistence length of about 1 mm) which are propelled about 20 nm above the surface by kinesin-1 motor proteins [32]. For optimal performance of kinesin-1 driven motility, surface properties such as hydrophilicity and roughness are decisive parameters [33, 34]. In addition, the materials used need to be non-toxic to the biological system. With respect to microtubule transport across 3D junctions, the interaction of the employed material with the biological system is crucial, as the structures are intended to act as surface substrates and physical guiding barriers. Thus, the 3D junctions were realized using

Figure 1. Design of 3D junction. (a) 3D design of a junction with tunnel and overpass, each connecting a closed and an open reservoir. (b) The junction design is illustrated in schematic images (lower panel) and the relevant parameters are provided in the table (upper panel).
Figure 2. Fabrication layout and optical characterization of 3D junctions. (a) Fabrication layout for 2PP patterning process of a sample. The laser power as well as the Z-offset of the initial layer relative to the substrate was varied in X- and Y-direction, respectively. Increasing negative values of dZ mean that the 3D junction will protrude from the substrate. In addition to the 7 × 5 array of 3D junctions, identifier marks labeled ‘1a’ to ‘7c’ were inserted to associate the processing parameters to individual 3D junctions during fluorescence microscopic imaging. (b) Laser scanning microscopy images of the resulting array of 3D junctions. Optical image (left) and height characterization (right). (c) Laser scanning microscopy images of a fabricated 3D junction (2.25 mW and −1.5 μm Z-offset). Optical image (left) and height characterization (right). (d) Fluorescence images of 3D junctions with blocked (left) and open (right) tunnel.

inorganic–organic hybrid polymers (ORMOCER®s) that show good mechanical and thermal stability (glass-like properties). ORMOCER®s are composed of inorganic oxidic structures, that can be cross-linked photochemically by organic groups, and represent a well-known resin class for 2PP microfabrication [21, 31, 35]. Varying the selection of precursors and adjusting the synthesis conditions modifies the physical and chemical properties of the material and allows a wide range of applications [36–38]. In our experiments we tested two different ORMOCER® material systems: a standard system, labeled OC-I, based on methacrylates as polymerizable moieties and an acrylic system, OC-V, that has been used for microoptical applications in the past [39–41]. In initial tests, both materials revealed smooth kinesin-1 driven transport of microtubules on patterned ORMOCER®s as well as on the surrounding glass surface. Microtubules were guided along the structure walls and did not get stuck. Thus, both ORMOCER®s were compatible for the experiments on 3D junctions.

Because fabrication of the 3D junctions was very sensitive to the processing conditions and the detected position of the ORMOCER®-substrate-interface did not exactly match the Z-position of the focal spot, an array of 3D junctions was created with varying processing parameters on each sample (figure 2(a)). Specifically, the laser power was varied in X-direction whereas the position of the initial layer relative to the substrate (‘Z-offset’, dZ) was varied in Y-direction. Laser scanning microscopy images of the fabricated 3D junctions show that the structures ‘fade away’ with decreasing laser power (figure 2(b)). We attribute this finding to the fact that decreased polymerization rates at lower laser powers led to smaller line widths and poorer crosslinking causing a lack of mechanical stability. The corresponding height profiles confirm that the 3D junctions tend to protrude more out from the substrate with increasing negative values of dZ. Figure 2(c) depicts a closer view on the topography of a 3D junction fabricated with a laser power of 2.25 mW and a Z-offset of −1.5 μm. When imaged by fluorescence microscopy, we found that the 3D junctions exhibited strong autofluorescence. In particular, the optical ORMOCER® OC-V was autofluorescent across the entire wavelength range of visible light, whereas the standard ORMOCER® OC-I only showed autofluorescence when excited by blue and green light, but not by red light. Therefore, only OC-I was used for motility experiments: blue or green light was applied to locate the 3D junctions, while red light was used to record the filament motility. Importantly, fluorescent imaging allowed us to check if the 3D junctions contained continuous (i.e. open) or blocked tunnels (figure 2(d)). Open tunnels could be identified for at least three 3D junctions on each sample, mainly located in rows d and (dZ = −1.5 or −2 μm).
Figure 3. Electron microscopical evaluation of 3D junctions. (a) Top view of a 3D junction with channels to and from the reservoirs. (b) Magnification of 3D junction shown in (a). Overpass floor and rims are clearly formed by ORMOCER® with a smooth surface. (c) Top view of a 3D junction for which a tunnel was optically observed. The 3D junction was coated with platinum in tunnel direction and across the overpass. The dashed lines indicate the cut positions of the FIB that resulted in the cross sections shown in (d) and (e). (d) Close-up of the platinum coated 3D junction shown in (c). The FIB cut shows the cross-section of the channel walls and the floor as well as the rims of the overpass (black structures). (e) Cross-section of the 3D junction parallel to the tunnel and perpendicular to the overpass direction verified the continuity of the tunnel under the overpass. The tunnel had an average height of approx. 600 nm (design: 1.5 μm). The overpass had a rim height (1) of 2.6 μm (design: 2.5 μm), a floor thickness (2) of 1.1 μm (design: 0.5 μm) and a rim width (3) of 1.8 μm (design: 2 μm).

Additionally, we examined a number of 3D junctions with open tunnels by scanning electron microscopy (SEM) (figure 3). The channel walls, as well as the overpass floors and rims, were clearly formed by ORMOCER® and had smooth surfaces. For some 3D junctions a small step between the channel floor and the start of the ramp leading to the overpass was observed (figures 3(a) and (b)). The entrance of the tunnel could also be identified but this was no proof for its continuity. Therefore, we used focused ion beam (FIB) milling to obtain a cross-section of the 3D junction. FIB sections were prepared with the help of a field emission-SEM/FIB apparatus equipped with a Kleindiek MM3A micromanipulator and a platinum gas injection system. When a 3D junction (after platinum coating) was cut parallel to the tunnel and perpendicular to the overpass direction a continuous tunnel with an average height of about 600 nm was revealed (figures 3(c)–(e)). When comparing the measured structure dimensions with the design, we found the rim height and width of the overpass matching within the fabrication accuracy, while the floor thickness and thus the tunnel height differed significantly. This was likely due to the elongation of thin structures in the Z direction as a consequence of the extended voxel length in that direction. Thus, polymerization occurs a few hundred nanometers outside the bounding box in the design.

In order to investigate the performance of the 3D junctions for biomolecular transport using the kinesin-microtubule system the samples were assembled into flow channels and a casein solution was applied for 5 min. Subsequently, the casein solution was exchanged for a kinesin-1 solution and the motor proteins were allowed to bind out of solution onto the casein-coated surface. Finally, an ATP-containing motility solution with Atto 647-labeled microtubules was introduced. Transport of the microtubules across four to seven different 3D junctions (with and without open tunnels) of a sample (figure 4(a)) was imaged for 2 to 3 h, ensuring that the viability of the assay lasted at least that long. Microtubules were propelled smoothly across the whole surface of the sample and their otherwise random movement was for the most part guided by the ORMOCER® walls. Occasionally, individual microtubules crossed the walls, but this did not impair the performance of the 3D junction. In fact, the walls were mainly designed to direct
Figure 4. Molecular motor-driven filament transport across 3D junctions. (a) Schematic illustration of a kinesin-1 microtubule gliding motility assay. Kinesin-1 motor molecules are adsorbed to the whole surface, i.e. the glass substrate as well as the ORMOCER® structures. Microtubules are transported across the surface by kinesin-1, guided by the ORMOCER® walls in channels, across the overpass, and through the tunnel. (b) Autofluorescence image of a 3D junction with open tunnel (left). Corresponding time-lapse maximum projection image of fluorescently labeled microtubules that were transported across the surface, through the tunnel and across the overpass (right). (c) Time series of fluorescence images showing microtubule transport across the 3D junction of the structure shown in (b). Images are overlays of a still image of the 3D junction (gray) with images of the microtubules (green) at given time points. White pointers indicate microtubules moving across the overpass and magenta pointers indicate microtubules moving through the tunnel.

Microtubules into the channels toward the 3D junction. In the future, the junction elements can be implemented into optimized, chemically selective network structures. For each 3D junction that had already revealed an open tunnel during previous optical inspection by fluorescence microscopy, movement of the labeled microtubules through the 5 μm long tunnels was actually observed. We also detected overpass events on the majority of 3D junctions. Importantly, for about half of the investigated 3D junctions with an open tunnel we observed both, microtubules moving through the tunnel as well as across the overpass. Examples of these events are illustrated in figures 4(b) and (c).

The average velocities of microtubules moving through tunnels or across overpasses of the 3D junctions were both similar and in the same range as those moving on the surrounding areas (table 1). When evaluating the performance of all 3D junctions for which tunnel and overpass events were observed, we followed every filament moving in a channel either toward the tunnel or toward the overpass. Thereby, we found that none of them ended up in the wrong reservoir. Thus, most importantly, the 3D junction showed zero junction errors. However, not all microtubules always reached the opposing reservoir. 87% of the microtubules moving from one reservoir toward the tunnel reached the connected reservoir. In contrast, only 39% of the microtubules moving toward the overpass arrived at the connected reservoir (table 1). The majority of microtubules which did not make it across the overpass detached from the surface when moving the ramp downward toward the connected reservoir. This likely happened due to the stiffness of the microtubules: Motor molecules interacting with the rear end of the microtubule continue to propel it forward whereas the fluctuating leading tip of the microtubule is hardly able to attach to the motors on the downward ramp due to the increasing distance between motors and filaments [42]. In some 3D junctions microtubules turned around inside the channel just before entering the ramp to the overpass. This behavior was probably observed when there was a physical step at the transition between the glass surface and the ORMOCER® ramp of the overpass (see figure 3(b)). Compared to the performance of 2D pass-junctions, where 0.2%–0.4% of microtubules took a wrong path [16], the actual error-free performance of the 3D junctions is a major improvement. Admittedly, these error rates do not include any loss of filaments due to detachment or sticking. However, while these events will obviously affect the overall performance of an
Table 1. Analysis of microtubule motility across 3D junctions.

|                         | Overpass | Tunnel | Surrounding surface |
|-------------------------|----------|--------|--------------------|
| Average velocity ± Stdv [nm s⁻¹] | 612 ± 75 | 614 ± 65 | 622 ± 64 |
| Total number of microtubules moving toward a 3D junction | 61      | 68     |         |
| Microtubules that passed the 3D junction               | 24      | 59     |         |

application, they importantly do not result in functional errors, such as errors in the calculation of network-encoded mathematical problems.

3. Conclusions

In conclusion, 3D micro-junctions suitable for advanced biomolecular transport devices, such as biocomputational networks, were designed and realized by 2PP of ORMOCER®, an organically modified hybrid material. The 3D junctions spatially separated two perpendicularly oriented channels in the form of an overpass and a tunnel. Both structural elements were verified by optical and electron microscopical imaging and showed a high structure fidelity. In experiments with kinesin-1 driven microtubules, the compatibility of the applied ORMOCER® structures with the biological system was verified and the guiding of microtubules across the overpasses and through the about 5 μm long tunnels was demonstrated. While the motion through the tunnel element of the 3D junctions was highly reliable, the design of the overpasses may profit from further optimization. One promising alteration to increase the overpass efficiency could be to cover the overpasses. This would prevent microtubule detachment when moving down the ramp by directing them back toward the motor proteins on the floors of the overpasses.

Confined, tunnel-like transport has earlier been demonstrated for microtubules moving through coverslip-sealed submicron channels [32] and actin filaments moving through hollow nanowires [43]. However, our work is, to our knowledge, the first demonstration of simultaneous, spatially overlapping 2D molecular motor-driven filament transport along guiding structures separated in the third dimension. Importantly, the presented error-free junctions constitute a major advancement for the implementation in network designs of future upscaled biocomputing devices [16]. Furthermore, our approach of patterning surfaces with hybrid polymer structures including 3D elements is expected to open up new possibilities also in biomolecular transport applications like lab-on-a-chip devices.

In many nanotechnological applications with molecular motors as well as in network-based biocomputation, actin filaments propelled by myosin motors are explored extensively in parallel to the microtubule-kinesin system because each of the two systems has its own advantages [6]. Thus, the successful application of ORMOCER® 3D junctions for actin–myosin would further enhance their potential. However, due to specific surface chemistry requirements for reliable actin motility (especially with respect to the hydrophobicity), the applied ORMOCER® material and possible surface treatments (e.g. oxygen plasma ashing, silanization) will have to be chosen and optimized separately. Besides 3D junction designs supporting motility on both, the ORMOCER® and the substrate surface, as presented in this work, designs that allow motility only on the ORMOCER® might be worth exploring for the actin–myosin system in future efforts.

4. Materials and methods

2PP patterning instrumentation. 3D junction design was accomplished with the CAD-program Autodesk Inventor Professional 2020. The computer-aided manufacturing process was carried out in proprietary software (SliceLas (from Lightfab) running in Rhinoceros 3D (from Mc Neel)). A custom-built 2PP patterning setup [44–46] consisting of a femtosecond laser oscillator (amplitude systems, t-pulse 200), which is frequency doubled to 515 nm and is operating at 10.1 MHz repetition rate and 350 fs pulse duration, was used to fabricate 3D junctions. The positioning of the focal spot in 3D space was performed by a galvoscanner (XY-direction) and a 300 μm travel piezostage (Z-direction) that included the mount for the focusing optics (both part of a writing head developed by Lightfab GmbH, Aachen—Germany). In order to allow stitching for 3D junctions exceeding the field of view of the focusing optics or for large area positioning, the scanner and the sample were mounted to high-precision linear stages (Aerotech ABL in XY-direction and Aerotech ATS in Z-Direction). We used a 100x, NA = 1.4 microscope lens (Zeiss Plan-Apochromat) to focus the incoming beam into the resin.

2PP patterning of ORMOCER®. A small amount of ORMOCER® resin including 2 wt.% photoinitiator Irgacure 369 [46, 47] was applied by drop casting onto a glass coverslip. In the next step, an
automated interface recognition procedure (‘autofocus’) was carried out to locate the exact position of the first layer on the glass substrate prior to the exposure. The glass-ORMOCER®-interface on the backside of the coverslip represented the best focusing conditions for the employed microscope lens (according to the design of the manufacturer). Thus, the 3D junctions were fabricated in a ‘hanging’ fashion and light for the exposure of the \((n + 1)\)th layer had to penetrate the \(n\)th exposed layer. Within the array of 3D junctions on each sample, the laser power was varied in \(X\)-direction from \(P = 3.25\, \text{mW}\) to \(1.75\, \text{mW}\) in \(−0.25\, \text{mW}\) increments (7 steps) and the position of the initial layer relative to the substrate (‘\(Z\)-Offset’, \(dZ\)) was varied slightly in the \(Y\)-direction from \(0\, \mu\text{m}\) to \(−2.0\, \mu\text{m}\) in increments of \(−0.5\, \mu\text{m}\) (5 steps) to ensure optimal alignment of the channel floor with the ramp at the beginning of the overpass. The positioning velocity was set to \(5\, \text{mm s}^{-1}\) for all 3D junctions. Additional parameters regarding the exposure are given in table 2 and were chosen in a way that the resulting photon flux ensures sufficient crosslinking of the ORMOCER® resin. Finally, after fabrication, all samples were developed for 20 min using a 1:1 solution of isopropanol and methyl-isobutyl-ketone followed by rinsing with pure isopropanol.

**Characterization of 3D junctions.** The topography of the 3D junction and preliminary optical characterization (figures 2(a)–(c)) were carried out with a laser scanning microscope (Keyence VK-X210). SEM characterization (figures 3(a) and (b)) was performed on a scanning electron microscope from JEOL (JSM 7800F) using the lower electron detector. For the field emission-SEM/FIB and electron backscatter imaging experiments we applied a cross-beam scanning electron microscope from Zeiss (AURIGA®.CrossBeam workstation) equipped with a Kleindiek MM3A micromanipulator and a platinum gas injection system. First, we extracted pieces (few mm\(^2\)) from the glass-substrate prior to the exposure. The glass-ORMOCER®-CrossBeam®-samples. Then, a platinum layer was deposited on top of the sample to reduce charging. A \(10 \times 10 \times 10\, \mu\text{m}^3\) cubic shaped part of the sample was removed using Ga\(^+\) ion milling with a current and voltage of 50 \(\text{pA}\) and 30 \(\text{kV}\), respectively. Subsequently an InLens-EsB detector was used to reveal the different materials (Pt, ORMOCER®) in the region of interest (figures 3(c) and (d)). For detailed images the sample was tilted by 36°.

**Preparation of flow channels.** Glass coverslips (22 \(\times\) 22 mm\(^2\) or 24 \(\times\) 60 mm\(^2\), Menzel-Gläser) were cleaned by sonication in Mucasol/water (1:20; \(v = v\)) for 15 min followed by rinsing in deionized water for 2 min. Further, coverslips were sonicated in ethanol/water (1:1; \(v = v\)) for 10 min, rinsed in deionized water for 2 min, rinsed in MilliQ-water for 2 min and finally dried using a nitrogen airflow. The kinesin-1 gliding motility experiments were performed in 3 mm-wide flow-channels consisting of a cleaned glass coverslip, an ORMOCER® sample and two stripes of parafilm as spacers.

**Kinesin-1 gliding motility experiments.** All solutions were prepared in Brinkley Reassembly Buffer 80 mM (BRBB0; adjusted to pH = 6.9 with KOH) that was composed of 80 mM 1,4-piperazinediethanesulfonic acid (PIPES), 1 mM EGTA and 1 mM MgCl\(_2\). Microtubules were polymerized from 4 mg ml\(^{-1}\) porcine brain tubulin [48], labeled with Atto647, in BRBB0 with 5 mM MgCl\(_2\), 1 mM GTP, 5% dimethyl sulfoxide (DMSO) at 37 °C for 30 min. The microtubules were stabilized and diluted 60-fold in BRBB0 containing 10 \(\mu\text{M}\) Taxol. Full-length *Drosophila melanogaster* kinesin-1 molecules were used as motor proteins that were expressed in insect cells and purified as described earlier [49]. A solution containing casein (0.5 mg ml\(^{-1}\)) was perfused into the flow-cell and allowed to adsorb to the surface for 3 min. This solution was exchanged for a 10 \(\mu\text{g ml}^{-1}\) kinesin-1 solution in BRBB0 with 0.2 mg ml\(^{-1}\) casein, 1 mM ATP as well as 10 mM dithiothreitol and incubated for 5 min. A BRBB0 solution containing microtubules (33 mM polymerized tubulin), 10 \(\mu\text{M}\) Taxol, 1 mM ATP, 40 mM D-glucose, 55 \(\mu\text{g ml}^{-1}\) glucose oxidase, 11 mg ml\(^{-1}\) catalase and 10 mM dithiothreitol was added to the flow-cell. After the ORMOCER® 3D junctions had been localized, image acquisition was started.

**Imaging of gliding motility assays and data analysis.** Image acquisition was performed using an inverted fluorescence microscope Zeiss Axiovert 200M (Zeiss, Germany) with a 40x air objective Plan-Apochromat NA = 0.95 (Zeiss, Germany). For excitation a LED lamp SOLA SE (Lumencor) was applied. The data were recorded with an electron multiplying charge-coupled device (EMCCD) camera (iXon + EMCCD, DU-897E, Andor) having a pixel size of 16 \(\mu\text{m}\). If not stated differently, images were

| Parameter       | Symbol | Unit       | Exposure parameters                                      |
|-----------------|--------|------------|---------------------------------------------------------|
| Velocity        | \(v\)  | mm s\(^{-1}\)| 5                                                       |
| Line interval   | \(\Delta XY\) | nm | 50                                                     |
| Slice interval  | \(\Delta Z\)  | nm | 50                                                     |
| Filling mode    |        |            | Diagonal hatching (45° w.r.t. XY-axes), alternating direction from layer to layer, filling only, unidirectional (no meandering) |

Table 2. Exposure parameters for the fabricated 3D junctions.
acquired every 2 s with an exposure time of 100 ms using MetaMorph (Molecular Devices, LLC., USA). The data was analyzed using Fiji. Each microtubule moving toward and leaving a junction was followed frame by frame in a time-lapse movie. Thereby their number was counted. Then the path of each microtubule crossing the junction was manually tracked, the length of the path was measured and divided by the time between the first and the last frame of the track. Finally, the obtained velocities of individual microtubules were averaged.

Acknowledgments

We thank Till Korten for fruitful discussions regarding the 3D junction design. This work was funded by the European Union Horizon 2020 FET Program under Contract 732482 (Bio4Comp) and the Technische Universität Dresden.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

ORCID iDs

Aseem Salhotra https://orcid.org/0000-0003-4835-0598
Alf Månsson https://orcid.org/0000-0002-5889-7792
Heiner Linke https://orcid.org/0000-0002-4451-4006
Stefan Diez https://orcid.org/0000-0002-0750-8515

References

[1] Korten T, Månsson A and Diez S 2010 Towards the application of cytoskeletal motor proteins in molecular detection and diagnostic devices Curr. Opin. Biotechnol. 21 477–88
[2] Hess H 2011 Engineering applications of biomolecular motors Annu. Rev. Biomed. Eng. 13 429–50
[3] Bachand G D, Rivera S B, Carroll-Portillo A, Hess H and Bachand M 2006 Active capture and transport of virus particles using a biomolecular motor-driven, nanoscale antibody sandwich assay Small. 2 381–5
[4] Bachand G D, Hess H, Ratna B, Satir P and Vogel V 2009 ‘Smart dust’ biosensors powered by biomolecular motors Lab Chip 9 1661–6
[5] Chaudhuri S, Korten T, Korten S, Milani G, Lana T, te Kronnie G and Diez S 2018 Label-free detection of microvesicles and proteins by the bundling of gliding microtubules Nano Lett. 18 117–23
[6] Reuther C et al 2021 Comparison of actin- and microtubule-based motility systems for application in functional nanodevices New J. Phys. 23 075007
[7] Phillips R, Kondov J, Theriot J and Garcia H 2012 Physical Biology of the Cell edn 2 (Boca Raton: Garland Science) https://doi.org/10.1201/9781134111589
[8] Brunner C, Wahn C and Vogel V 2007 Cargo pick-up from engineered loading stations by kinesin driven molecular shuttles Lab Chip 7 1263–71
[9] Chaudhuri S, Korten T and Diez S 2017 Tetrazine-trans-cyclooctene mediated conjugation of antibodies to microtubules facilitates subpicomolar protein detection Bioconjugate Chem. 28 918–22
[10] Kumar S, ten Siethoff L, Persson M, Lard M, te Kronnie G, Linke H and Månsson A 2012 Antibodies covalently immobilized on actin filaments for fast myosin driven analyte transport PLoS One 7 e46298
[11] Malcos J L and Hancock W O 2011 Engineering tubulin: microtubule functionalization approaches for nanoscale device applications Appl. Microbiol. Biotechnol. 90 1–10
[12] Persson M, Gullberg M, Tolf C, Lindberg A M, Månsson A and Kocer A 2013 Transportation of nanoscale cargoes by myosin propelled actin filaments PLoS One 8 e55931
[13] Kumar S and Månsson A 2017 Covalent and non-covalent chemical engineering of actin for biotechnological applications Biotechnol. Adv. 35 867–88
[14] van den Heuvel M G L, Butcher C T, Smeets R M M, Diez S and Dekker C 2005 High rectifying efficiencies of microtubule motility on kinesin-coated gold nanostructures Nano Lett. 5 1117–22
[15] Clemmens J, Hess H, Doot R, Matzke C M, Bachand G D and Vogel V 2004 Motor-protein ‘roundabouts’: microtubules moving on kinesin-coated tracks through engineered networks Lab Chip 4 83–6
[16] Nicolau D V et al 2016 Parallel computation with molecular-motor-propelled agents in nanofabricated networks Proc. Natl Acad. Sci. USA 113 2591–6
[17] Zhou Y et al 2021 Physical requirements for scaling up network-based biocomputation New J. Phys. 23 105004
[18] Fischer J and Wegener M 2013 Three-dimensional optical laser lithography beyond the diffraction limit Laser Photon. Rev. 7 22–44
[19] Heldt G et al 2018 Approach to combine electron-beam lithography and two-photon polymerization for enhanced nano-channels in network-based biocomputation devices Proc. SPIE 10775 1077517
[20] Gissibl T, Thiele S, Herkommer A and Giessen H 2016 Two-photon direct laser writing of ultracompact multi-lens objectives Nat. Photon. 10 554–60
[21] Steenhusen S, Burmeister F, Groß M, Domann G, Houbertz R and Nolte S 2018 Heterogeneous microoptical structures with sub-micrometer precision Thin Solid Films 668 74–80
[22] Liu Y-J, Yang J-Y, Nie Y-M, Lu C-H, Huang E D, Shin C-S, Baldeck P and Lin C-L 2015 A simple and direct reading flow meter fabricated by two-photon polymerization for microfluidic channel Microfluid Nanofluid 18 427–31
[23] Mayer F et al 2019 Multimaterial 3D laser microprinting using an integrated microfluidic system Sci. Adv. 5 eaax9160
[24] Sina F, Wu D, Xu J, Midorikawa K and Sugitaka K 2015 Ship-in-a-bottle integration by hybrid femtosecond laser technology for fabrication of true 3D biochips Proc. SPIE 9350 93500F
[25] Kim D, Han Z, Ueda J and Ansari A 2019 A 5 mg micro-bristle-bot fabricated by two-photon lithography J. Micromech. Microeng. 29 105006
[26] Reinhardt C, Osvianikov A, Passinger S and Chichkov B N 2007 Fabrication of micromechanical and microoptical systems by two-photon polymerization Proc. SPIE 6466 64660M
[27] Danilevicius P et al 2015 Burr-like, laser-made 3D microscaffolds for tissue spheroid encagement Biointerphases 10 021011
[28] Nguyen A K and Narayan R J 2017 Two-photon polymerization for biological applications Mater. Today 20 314–22
[29] Stichel T, Hecht B, Houbertz R and Sextl G 2010 Two-photon polymerization as method for the fabrication of large scale biomedical scaffold applications J. Laser Micro/Nanoeng. 5 209–12
[30] Burmeister F et al 2012 Materials and technologies for fabrication of three-dimensional microstructures with sub-100 nm feature sizes by two-photon polymerization J. Laser Appl. 24 042014
[31] Rys J, Steenhusen S, Schumacher C, Cronauer C and Dario C 2019 Locally addressable material properties in 3D micro-architectures Extreme Mech. Lett. 28 51–6
[32] van den Heuvel M G L, de Graaff M P and Dekker C 2006 Molecular sorting by electrical steering of microtubes in kinesin-coated channels Science 312 910–4
[33] Ying-Ming Huang Y M, Uppalapati M, Hancock W O and Jackson T N 2005 Microfabricated capped channels for biomolecular motor-based transport IEEE Trans. Adv. Packag. 28 564–70
[34] Martinez H, Martinez N J D, Guo J, Lujan V R, Depoy J, Brumbach M T, Brinker C J and Bachand G D 2020 Effects of surface chemistry and topology on the kinesin-driven motility of microtubule shuttles ACS Appl. Bio Mater. 3 7908–18
[35] Doraiswamy A, Patz T, Narayan R, Chichkov B, Osvianikov A, Houbertz R, Modi R, Auyeung R and Chrisey D 2004 Bio-compatibility of CAD/CAM ORMOCER polymer scaffold structures MRS Proc. 845 63–70
[36] Haas K-H, Ambreg-Schwab S and Rose K 1999 Functionalized coating materials based on inorganic-organic polymers Thin Solid Films 351 198–203
[37] Haas K-H and Wolter H 1999 Synthesis, properties and applications of inorganic-organic copolymers (ORMOCERS) Curr. Opin. Solid State Mater. Sci. 4 571–80
[38] Sanchez C, Belleville P, Popall M and Nicole L 2011 Applications of advanced hybrid organic-inorganic nanomaterials: from laboratory to market Chem. Soc. Rev. 40 696–753
[39] Burmeister F et al 2015 Optically Induced Nanostuctures: Biomedical and Technical Applications ed O A König (Berlin: De Gruyter)
[40] Fessel S, Schneider A M, Steenhusen S, Houbertz R and Behrens P 2012 Towards an atomistic model for ORMOCER-I: application of forcefield methods J. Sol-Gel Sci. Technol. 63 356–65
[41] Houbertz R et al 2003 Inorganic–organic hybrid materials for application in optical devices Thin Solid Films 442 194–200
[42] Kersemakers J, Ionov L, Queitsch U, Luna S, Hess H and Diez S 2009 3D nanometer tracking of motile microtubules on reflective surfaces Small 5 1732–7
[43] Lard M, ten Siethoff L, Generosi J, Månsson A and Linke H 2014 Molecular motor transport through hollow nanowires Nano Lett. 14 3041–6
[44] Houbertz R, Steenhusen S, Stichel T and Sextl G 2010 Coherence and Ultrafast Pulse Laser Emission (Croatia: IntechOpen) pp 583–608
[45] Steenhusen S, Houbertz R and Sextl G 2010 3D sub-diffraction limit patterning of hybrid polymers with visible and infrared laser pulses Proc. of LPM2010
[46] Steenhusen S, Stichel T, Houbertz R and Sextl G 2010 Multi-photon polymerization of inorganic–organic hybrid polymers using visible or IR ultra-fast laser pulses for optical or (opto-)electronic devices Proc SPIE 7591
[47] Schafer K J, Hales J M, Balu M, Belfield K D, Van Stryland E W and Hagan D J 2004 Two-photon absorption cross-sections of common photoinitiators J. Photochem. Photobiol. A 162 497–502
[48] Castoldi M and Popov A V 2003 Purification of brain tubulin through two cycles of polymerization–depolymerization in a high-molarity buffer Protein Expression Purif. 32 83–8
[49] Korten T, Chaudhuri S, Tavkin E, Braun M and Drie S 2016 Kinesin-1 expressed in insect cells improves microtubule in Vitro gliding performance, long-term stability and guiding efficiency in nanostructures IEEE Trans. Nanobiosci. 15 62–9