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Micropattern-Guided Assembly of Overlapping Pairs of Dynamic Microtubules

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Abstract

Interactions between antiparallel microtubules are essential for the organization of spindles in dividing cells. The ability to form immobilized antiparallel microtubule pairs *in vitro*, combined with the ability to image them via TIRF microscopy, permits detailed biochemical characterization of microtubule cross-linking proteins and their effects on microtubule dynamics. Here, we describe methods for chemical micropatterning of microtubule seeds on glass surfaces in configurations that specifically promote the formation of antiparallel microtubule overlaps *in vitro*. We demonstrate that this assay is especially well suited for reconstitution of minimal midzone overlaps stabilized by the antiparallel microtubule cross-linking protein PRC1 and its binding partners. The micropatterning method is suitable for use with a broad range of proteins, and the assay is generally applicable to any microtubule cross-linking protein.

1. INTRODUCTION

Microtubule-cross-linking proteins are important for microtubule organization in living cells. During cell division, cross-linkers help to connect the two halves of the spindle apparatus by mediating antiparallel microtubule contacts (Duellberg, Fourniol, Maurer, Roostalu, & Surrey, 2013; Glotzer, 2009). A crucial cross-linker in anaphase is PRC1 (protein required for cytokinesis 1) (Janson et al., 2007; Mollinari et al., 2002; Verni et al., 2004). PRC1, a homodimeric molecule with microtubule-binding sites at opposite ends of the dimer (Subramanian, Ti, Tan, Darst, & Kapoor, 2013), cross-links antiparallel microtubules with considerably higher affinity than parallel microtubules (Bieling, Telley, & Surrey, 2010; Gaillard et al., 2008; Janson et al., 2007; Subramanian et al., 2010). During anaphase, it localizes selectively to the central spindle where it contributes to spindle

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stability and recruits several other proteins with critical functions for the central spindle (Duellberg et al., 2013). The PRC1 homologs in plants also play important roles in microtubule bundling in interphase (Gaillard et al., 2008).

Microtubule cross-linking activity has often been assayed *in vitro* by mixing purified cross-linkers and microtubules to form microtubule bundles. The large and variable number of microtubules and their mixed orientation can make it difficult to determine the properties of the proteins bound to the bundle. Therefore, *in vitro* experiments with pairs of microtubules with known orientation have been developed. For the study of microtubule-cross-linking and sliding motors (such as kinesin-5 or kinesin-14), microtubule pairs are formed from two stabilized microtubules, one of which is surface immobilized and the other of which is tethered to the immobilized microtubule by the cross-linking motors, either in the absence or presence of other cross-linkers (Braun et al., 2011; Hentrich & Surrey, 2010; Kapitein et al., 2005; Roostalu et al., 2011; van den Wildenberg et al., 2008). The goal of this assay is to determine how motors slide two microtubules with respect to each other.

A complementary assay was designed to form microtubule pairs that exhibit dynamic polymerization and depolymerization behavior (Bieling et al., 2010). This assay is a variation of a commonly used microtubule dynamics assay in which short, stabilized microtubules are bound to a glass surface and then extended by the addition of free tubulin (Telley, Bieling, & Surrey, 2011). When two growing microtubules oppose each other in this assay, antiparallel encounters lead to the formation of antiparallel microtubule overlaps that can be used to study PRC1 binding, PRC1-dependent recruitment of other proteins to these overlaps, and their effect on the dynamic properties of the microtubules themselves. This assay has been used to study the combined effects of Xenopus PRC1 and kinesin-4 Xklp1 on setting the length of antiparallel microtubule overlaps (Bieling et al., 2010; Nunes Bastos et al., 2013).

A technical challenge in this type of assay is how to orient microtubules and control the density of immobilized seeds so that the chance of antiparallel overlap is relatively high. Recent developments in techniques for micropatterning glass surfaces now enable more spatially controlled microtubule nucleation or seed immobilization (Aoyama, Shimoike, & Hiratsuka, 2013; Ghosh, Hentrich, & Surrey, 2013; Portran, Gaillard, Vantard, & Thery, 2013; Waichman, You, Beutel, Bhagawati, & Piehler, 2011). Patterning enables the growth of microtubules from distinct foci with well-defined positions and dimensions, and it has recently been used to reconstitute bipolar microtubule bundles (consisting of several microtubules) *in vitro* (Portran et al., 2013; Su et al., 2013).

We describe here a high contrast micropatterning method and demonstrate its use to chemically micropattern microtubule seeds on glass surfaces to guide formation of antiparallel microtubule pairs with defined seed-to-seed distance (Fig. 19.1). We produce micropatterns of maleimide functionalization on polyethylene glycol (PEG) brushes covalently linked to glass (Waichman et al., 2011). Maleimide is then used to covalently link either thiol-biotin or cystein-tagged streptavidin to the maleimide-functionalized areas, generating biotin-PEG or streptavidin-PEG micropatterned glass. Both methods achieve selective immobilization of biotinylated microtubule seeds via a Cys-streptavidin or a

biotin–neutravidin sandwich. In combination with using fluid flow for seed orientation, this method allows the generation of pairwise antiparallel microtubule overlaps with controlled seed-to-seed distance.

2. REAGENTS AND EQUIPMENT

The rationale and practical details for TIRF microscopy have been described elsewhere (Gell et al., 2010). Here, we focus on the glass treatment, patterning process, and the sample preparation for the dynamic microtubule overlap assay.

2.1. Reagents for glass treatment

- NaOH (3 *M* solution).
- Hydrogen peroxide (30% stabilized).
- Sulfuric acid (Sigma; concentrated, 95–97%).
- (3-Glycidyloxypropyl)-trimethoxysilane (GOPTS; Sigma #440167-100ML).
- H_2N-PEG-NH_2 2000 (DAPEG; Rapp-Polymere #11 2000-2).
- *N*-[β-maleimidopropyloxy] succinimide ester (BMPS; Thermo Scientific, 50 mg #22298).
- Acetone (Sigma, Chromasolv Plus).
- Dimethylformamide (DMF, Sigma, Chromasolv).
- Dimethylsulfoxide (DMSO, Sigma, ACS grade).
- Coverslips (Zeiss, High Precision, 22 × 22 mm, 170 μm thick, No. 1.5H).
- Microscope slides (Thermo Scientific, 76 × 26 mm).
- Poly-L-lysin-PEG (PLL-PEG; SuSoS, PLL-g-PEG).
- Double-sided tape (Tesa).
- Inverted tweezers (Dumont, N2A Inox.).
- Coverslip washing racks (ceramic or Teflon).
- Diamond pen.
- Kimwipes (lint free).
- Coverslip spinner or nitrogen gas (for drying).
- Oven (75 °C).
- Hamilton syringe #710 (100 μL). Used for GOPTS exclusively.
- Hamilton syringe #1001 (1 mL). Used for acetone and DMF.
- Beakers 1 L, 600 mL, 100 mL.
- Closed weighing jars.

- Disposable culture tubes, borosilicate glass grade 3.3 (VWR #212-0028).
- Kimtech gloves grade 3 (residue free, #99237).

  Note: all glassware should be cleaned with 3 *M* NaOH.

2.2. Material and reagents for patterning and functionalization

- 0.5 mL desalting columns (Thermo Scientific, Zebaspin 7K MWCO).
- Carbon-fiber tipped tweezers (Agar Scientific).
- Dry seal desiccator (Wheaton #02-913-360).
- Quartz masks with positive chromium stripe pattern (NB Technologies). Custom design, with stripes spaced 30, 20, or 10 μm apart and 2, 5, or 10 μm wide.
- Newport Solar Simulator with 300 W xenon arc lamp.

  Note 1: Make sure all optical components transmit UV wavelengths down to 300 nm, as these short wavelengths are most efficient for photodestruction of maleimide groups.

  Note 2: A 150-W lamp can be used as an alternative to the 300 W lamp, though illumination time must be doubled.
- Ethanol (Sigma, Chromasolv).
- Ammonium hydroxide (Sigma, ACS grade).
- Microscope slides (Fisher Scientific) with two plasma-bonded 3 mm, wide PDMS stripes aligned along the long axis edges. PDMS stripes are manually cut from a thin PDMS sheet (SSP-M823, Specialty Silicone Products).
- Vacuum-sealed illumination chamber made with quartz microscope slide, acrylic plates, sponge gasket, UV glue, and acrylic cement (Fig. 19.2A).
- Teflon holder for masks. Custom made from Teflon sheets, screws, and nuts (Fig. 19.2B).
- Vacuum pump (2511B-01, Gardner Denver Welch Vacuum Technology).
- Humidifier.

  Note: Alternatively, a hot water beaker and air pump (Aquarium 212, Petco) can be used.
- Sonicator bath with heating function.
- Humid storage container for slide incubation (e.g., an empty pipet tip container filled with MilliQ (MQ) water at the bottom, sealed with parafilm).
- *Maleimide coupling buffer*: 20 m*M* HEPES pH 7.4, 300 m*M* NaCl, 1 m*M* EDTA.
- Thiol-biotin (Prochimia, #FT 005-0.2).

2.3. Proteins

- Pig brain tubulin in BRB80, purified as described (Castoldi & Popov, 2003), flash-frozen, and stored in single-use aliquots at a concentration of 150–200 μ*M* at liquid nitrogen temperature.
- Biotinylated-tubulin in BRB80, labeled as described (Hyman et al., 1991), stored in 2 μL aliquots at 150 μ*M*, in liquid nitrogen.
- Alexa568- and/or Alexa647-tubulin, labeled as described (Hyman et al., 1991), stored in 2 μL aliquots at 150 μ*M*, in liquid nitrogen.
- PRC1 from *Xenopus laevis* (Bieling et al., 2010) stored in 2 μL aliquots at 18 μ*M*, in liquid nitrogen.
- Streptavidin-Cys. We introduced a single cysteine by site-directed mutagenesis into a previously described construct (Sørensen, Sperling-Petersen, & Mortensen, 2003), containing domain I of the *Escherichia coli* translation initiation factor 2 (IF2) as an N-terminal solubility tag. The IF2(I)-streptavidin-His6-Cys protein was expressed in BL21 pRIL cells overnight at 18 °C after induction with 0.5 m*M*IPTG. Bacterial pellets were lysed in 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 4 m*M* imidazole, 1 m*M*EDTA, 1 m*M* $MgCl_2$, 0.2 m*M* PMSF, 10 m*M* β-mercaptoethanol, supplemented with Complete Protease Inhibitors and DNase I (Roche). The lysate was ultracentrifuged 30 min at 50,000 × *g*, at 4 °C, and the clear lysate then incubated with 2 g protino Ni-TED resin (Macherey-Nagel). The resin was loaded on a gravity column and washed with 80 mL wash buffer (50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 4 m*M* imidazole, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol), before elution with 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 500 m*M* imidazole, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol. The imidazole was removed using desalting PD10 columns (GE Healthcare) equilibrated with 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol. The protein was aliquoted (50 μL, 72 μ*M* monomeric concentration) and flash-frozen in liquid nitrogen.
- Constructs were made for the expression of wild-type mCherry (Shaner et al., 2004) and a nonfluorescent variant (Tyr72Ser) fused to an N-terminal His10-Lys-Cys-Lys-tag using a modified pETMz vector. The protein was expressed in Rosetta cells overnight at 18 °C and purified by IMAC over a 5-mL HiTrap Chelating column (GE Healthcare) followed by TEV cleavage, desalting over a HiPrep XK26/10 Desalting column (GE Healthcare) to remove imidazole and a second round of IMAC to remove the z-tag. The eluate after the second round of IMAC containing His10- and Cys-tagged mCherry was gel filtered over a Superdex 200 XK26/60 PG column (GE Healthcare). The protein was flash-frozen in aliquots in liquid nitrogen.

  Note: In contrast to most other fluorescent proteins, mCherry does not contain a cysteine in its wild-type sequence.

2.4. Microtubule polymerization and overlap assay

- BRB80 buffer: 80 m*M* K-PIPES pH 6.8 at RT, 1 m*M* $MgCl_2$, 1 m*M* EGTA. Kept maximum 6 weeks at 4 °C.
- Guanosine-5′-[(α,β)-methylene]triphosphate (GMPCCP; Jena Biosciences). 10 m*M*, 5 μL aliquots stored in liquid nitrogen.
- Guanosine-5′-triphosphate (GTP; Sigma). 200 m*M*, 10 μL aliquots stored in liquid nitrogen.
- Kappa casein (Sigma, #C0406-100MG). 5 mg/mL in BRB80, 90 μL aliquots stored in liquid nitrogen. Before use, ultracentrifuge at 80,000 rpm for 10 min, at 4 °C.
- Glucose 20% stock solution, kept at 4 °C.
- Glucose oxidase (Serva, #22778.01). 40 mg/mL, 30 μL aliquots stored in liquid nitrogen. Before use, ultracentrifuge at 279,000 × *g* for 10 min, at 4 °C.
- Pluronic F-127, 5% solution (in water), kept at 4 °C.
- Catalase (Sigma, #C40–100MG). 6.9 mg/mL, 30 μL aliquots stored in liquid nitrogen. Before use, ultracentrifuge at 279,000 × *g* for 10 min, at 4 °C.
- *Midzone buffer*: 80 m*M* K-PIPES pH 6.8 at RT, 85 m*M* KCl, 85 m*M* K-acetate, 4 m*M* $MgCl_2$, 1 m*M* EGTA, 0.25% (v/v) Brij-35. Kept for maximum 6 weeks at 4 °C.
- Methylcellulose 2% solution (viscosity 4,000 cP; Sigma). Kept maximum about 6 weeks at 4 °C.

3. GLASS TREATMENT AND SURFACE CHEMISTRY

To generate a micropatterned streptavidin or neutravidin surface for the patterned immobilization of biotinylated microtubule seeds, we first generate a maleimide-PEG micropattern on glass. Diamino-PEG is covalently coupled to glass using silane chemistry followed by coupling maleimide to the PEG. UV-irradiation through a photolithographic mask is used to destroy the maleimide locally in the irradiated areas, generating a micropattern of areas with thiol-reactive maleimide-PEG separated by PEG areas. We describe two methods that can be used to produce either neutravidin-PEG or streptavidin-PEG micropatterns (Fig. 19.3). In the first method, maleimide is allowed to react with thiol-biotin, producing a biotin-PEG pattern to which commercially available neutravidin is bound. In the second method, a cysteine-tagged recombinant streptavidin is allowed to react with the maleimide, producing a streptavidin-PEG pattern. Both methods produce high contrast patterns (see Fig. 19.4) of biotinylated microtubule seeds in geometries defined by the photolithographic mask. The highly specific interactions between the protein and surface-bound PEG result in very good control of seed binding. The uniform PEG coating of the glass surface greatly reduces non-specific protein adsorption, thus limiting protein depletion and improving the imaging quality. To ensure that protein was not depleted by other parts of the chamber, the unpatterned glass surface opposite of the patterned surface was passivated using poly-L-lysine-PEG (PLL-PEG). Careful attention to surface passivation

is important for the correct quantitative determination of biochemical parameters from the analysis of fluorescence signals.

3.1. Coverslip treatment and functionalization

3.1.1 PEG-maleimide coating

Glass cleaning

- Using a diamond pen, mark each coverslip with an asymmetric triangular cut in the top right corner. Arrange the coverslips in racks and place the racks in a large beaker.

  Note: We typically process 36 coverslips at a time.

- Cover the coverslips with 3 *M* NaOH and sonicate for 15 min. Rinse them with plenty of MQ—purified water. At this stage, rinse the weighing jars with 3 *M* NaOH and MQ water and dry them off in the oven.

  Note: The coverslips can stand in NaOH several hours.

- Drain the coverslips and, under a fume hood, gently pour in two parts of hydrogen peroxide and three parts of sulfuric acid (“piranha” solution). Sonicate the glass in piranha for 30 min.

  Note: This piranha solution is extremely corrosive and volatile—it is essential to keep it under the hood; wear a labcoat and protective goggles. Discard the piranha in the appropriate acid waste (do not screw the cap tightly as gas accumulation might result in an explosion).

- Rinse the glass in two successive baths of 1 L MQ water.

Silanization

- Spin-dry the coverslips and lay them on dust-free Kimwipes. Place half of them functional side up in weighing jars.

- Take GOPTS out with the 100 μL Hamilton syringe. Put 2.5 drops of GOPTS onto each slide. Form a sandwich by putting another slide face-to-face onto the GOPTS. Repeat until all coverslips are sandwiched. Incubate the closed weighing jars 30 min at 75 °C.

  Note: Once opened, a bottle of GOPTS can be kept for years at RT; however, the concentration of unreacted molecules will slowly decrease and you may have to adjust the incubation time accordingly, in order to get similar surface densities of silane groups.

- Get the weighing jars out of the oven and let them cool down for 15 min on the bench.

- Open the jars and quickly transfer the closed coverslip sandwiches into a 100-mL beaker filled with dry acetone (80 mL) and a Teflon rack. Separate the coverslips in acetone. Dip-wash the rack into successive acetone baths (80, 400, and 200 mL in a 1-L beaker). Repeat with as many racks as necessary to hold the coverslips.

- Spin-dry the acetone off from each coverslip, as quickly as possible. Store them functionalized side up on Kimwipes.

  Note: The GOPTS reaction with the PEG is the most sensitive step of the procedure. GOPTS is extremely reactive, and it has to be kept water-free at all times to remain active. Use a freshly opened bottle of acetone. Dry the coverslips quickly; otherwise, acetone evaporation will cool down the glass and cause water condensation.

PEGylation

- Prepare a DAPEG mix in acetone in a glass tube. Use 150 mg of PEG in 500 μL acetone. Vortex at medium speed for 2 min, while warming up the tube between your fingers (PEG should dissolve fully if vortexed long enough).
- Arrange half of the coverslips functionalized side up into clean weighing jars.
- Pipet 25 μL of the DAPEG solution on each coverslip and form sandwiches. Repeat until all coverslips are sandwiched.
- Close the jars and incubate for 4 h.

  Note: Longer incubation can result in oxidation of the PEG (brown material), which ultimately will reduce the surface density of functional groups.

- On a hot plate, separate the sandwiches using a razor blade and tweezers. Dip-wash each coverslip in two successive 1-L beakers of MQ water and store in racks in another beaker with MQ water. Sonicate for 3 min. Spin-dry each coverslip and store in a box in between layers of lens paper, at 4–8 °C.

  Note: This is a possible break point as the DAPEG-functionalized coverslips are chemically stable for several days/weeks.

Maleimide functionalization

- Arrange the slides in the weighing jars and form a sandwich with 7.5 μL of the 1 *M* β-mercaptoethanol in DMF solution in between two slides.
- Incubate for 10 min at RT.
- In the hood, transfer the slide sandwiches in a fresh beaker containing dry DMF (80 mL) and a Teflon rack. Separate the coverslips in DMF, dip wash the racks in two more baths of DMF (80, 400 mL), and store the slides in racks in a beaker containing dry DMF.

  Note: Make sure to remove β-mercaptoethanol fully. Use fresh gloves, etc.

- Prepare a solution of BMPS in dry DMF (use a fresh 50 mg aliquote, weigh in a glass tube, and add 500 μL of DMF per 50 mg BMPS). Vortex until fully dissolved.

- Spin-dry the slides in the hood and collect them on Kimwipes outside of the hood. Then form a sandwich with 25 μL of the BMPS solution in between two glass pieces in clean weighing jars.
- Incubate for 30 min at RT.
- In the hood, transfer the slide sandwiches in a fresh beaker containing dry DMF and a Teflon rack. Separate the slides in DMF, dip wash once more in DMF, and store the slides in racks in a beaker containing dry DMSO.
- Immediately prior to UV illumination, spin-dry each coverslip to remove DMSO. Dip in EtOH 2× and spin-dry.

3.1.2 UV micropatterning

Preparation of chrome-on-glass photomasks

- Mount the individual photomasks in the custom-built Teflon holder using carbon-fiber tipped tweezers (all mask handling must be done using these tweezer types to avoid mechanical damage to the mask). The chrome sides of the masks should face the same direction. Put the Teflon holder containing the masks into a 1-L beaker with the chrome layers facing upward.
- Under a fume hood, gently pour in two parts of hydrogen peroxide and three parts of sulfuric acid. Sonicate the masks in piranha for 30 min at RT.
- Carefully decant the piranha solution and wash the Teflon holder containing the masks in the beaker under running MQ water for 5 min. Disassemble the Teflon holder and dip wash the masks individually in a clean 1-L beaker containing MQ water. Individually dry the masks under a clean nitrogen stream and keep them in vacuum in the desiccator.
- Directly before illumination, immerse the individual photomask into a clean beaker containing heated (50 °C), diluted SC1 solution (diluted Standard Clean 1 solution: 1 part ammonium hydroxide (28% solution): four parts hydrogen peroxide (30% solution): 50 parts MQ water). Sonicate for 12 min, wash in a clean beaker under running MQ water for 2 min, dip wash in two subsequent beakers containing EtOH, and dry under a stream of clean nitrogen.

UV illumination

- Place a spin-dried, PEG-maleimide coverslip with the functionalized side up in the custom-built vacuum illumination device.
- Briefly pass a freshly SC1-cleaned quartz photomask through a stream of water vapor generated from a humidifier with the chrome layer facing the vapor. The water vapor should leave a minimum uniform layer of condensed water on the mask.
- Immediately, right before the vapor layer has completely gone, put the mask with the chrome side down on the PEG-maleimide coverslip forming a sandwich.

- Close the custom-built vacuum illumination device by mounting the top assembly and apply vacuum with the vacuum pump. The residual water on the photomask should form a visible adhesive layer between the mask and coverslip surface covering at least 10% of the overall contact area (see Fig. 19.2A). Failure to form this adhesive layer will result in patterns with less sharp edges and less homogenous ligand densities in the photoprotected area.

  Note: The extent of the adhesive layer depends on the cleanliness of the glass and mask, and on the surface properties conferred by the successive cleaning and functionalization steps.

- Mark the center position of the UV beam emitted from the Newport Solar Simulator. Close the illumination shutter and position the vacuum illumination device with the chrome patterns of the photomask in the beam center. Open the illumination shutter and illuminate with UV light for 6 min.

3.1.3 Maleimide coupling

3.1.3.1 Coverslip preparation before coupling

- After UV illumination, store coverslips in a rack covered with DMSO.
- Transfer the rack into EtOH and sonicate for 3 min.
- Transfer into water and sonicate for 3 min.

3.1.3.2 Cys-mCherry and streptavidin-Cys coupling

- Prereduce a sufficient amount (40 μL of a 10 μ*M* solution per individual sample) of Cys-containing protein by adding fresh β-mercaptoethanol to 1 m*M* final concentration. Incubate for 30 min on ice.
- Desalt the protein into maleimide coupling buffer not containing reducing agents.
- Determine protein concentration by UV absorbance at 280 nm. Adjust protein concentration to 10 μ*M* with maleimide coupling buffer.
- Spin-dry coverslips and mount them on the microscope slides with plasma-bonded PDMS stripes with the functionalized slide down. This should form a central flow chamber of approximately 40 μL volume covering the whole patterned area of the coverslip. The adhesion between the PDMS and the coverslip should be strong enough to keep the coverslip in place but sufficiently weak to allow later removal of the coverslip with tweezers.
- Add 40 μL of the 10 μ*M* protein solution to the flow chamber. Incubate for 25 min at RT in a humid storage container.
- Wash with 250 μL maleimide coupling buffer containing 5 m*M* β-mercaptoethanol. Incubate for 5 min to quench the residual maleimide moieties.
- Wash with 250 μL maleimide coupling buffer containing 2 m*M* TCEP. Store in a cooled humid storage container for up to 2 weeks.

Note: This method can achieve a high contrast of mCherry bound on photoprotected versus UV-illuminated areas (Fig. 19.4).

3.1.3.3 Thiol-biotin coupling

- Spin-dry coverslips and arrange half of them in weighing jar, store the rest functionalized side up on Kimwipes.
- Prepare 6 mL of 1:1 mix of DMF and 50 m*M* HEPES pH 7.4 aqueous solution.
- Weigh the thiol-biotin in a glass tube and make a 5-m*M* suspension in the previous mix (e.g., 5 mg in 2.5 mL). Vortex well. Then by serial dilution prepare a 10 μ*M* solution—the thiol-biotin should fully dissolve.
- Use 25 μL of the 10 μ*M*thiol-biotin solution per coverslip sandwich and incubate for 30 min at RT.
- Arrange the sandwiches and separate the coverslips in a rack covered in EtOH. Transfer the rack into a fresh beaker of EtOH and sonicate for 3 min.
- Spin-dry and store the coverslips in between layers of lens paper, in the fridge.

Note: The biotin-PEG coverslips can keep for several months.

3.2. PLL-PEG counterglass preparation

- For each glass slide, fix two parallel strips of double-sided tape (15 × 3 mm each, 5 mm apart). Apply pressure and let stand for 5 min to allow bonding.
- Pipet 3 μL PLL-PEG 2 mg/mL aqueous solution and spread in between the strips of tape, by pressing the pipet tip on the glass. Let it dry for 20 min.
- Rinse with plenty of MQ water and let the slides fully dry.

4. MICROTUBULE OVERLAP ASSAY ON MICROPATTERN

In the assay described here, immobilized seeds are aligned perpendicular to patterned functionalized stripes by flow. This increases the likelihood that microtubules extending from these seeds will make head-on encounters so that antiparallel overlaps can form. To promote oriented seed immobilization to the neutravidin or streptavidin micropatterns, long GMPCPP seeds are bound to the surface under strong laminar flow.

4.1. Microtubule seeds preparation

- On ice, prepare a 40-μL mix containing 1 m*M* GMPCPP, 20 μ*M* biotinylated-tubulin (30%), 15 μ*M* Alexa568- or Alexa647-tubulin (25%), and 25 μ*M* unlabelled tubulin, in BRB80.
- Incubate 5 min on ice, to allow nucleotide exchange.
- Transfer to a water bath at 37 °C for 30 min.
- Dilute with 400 μL warm BRB80. Pipet up and down. Spin for 7 min at 17,000 × *g* in a benchtop centrifuge at RT.

- Discard the supernatant. Pipet gently 400 μL warm BRB80 into the tube, making sure not to disturb the pellet (pink dot). Discard the solution and resuspend the pellet in 50 μL warm BRB80—pipet up and down 20 times slowly, using a cut pipet tip to limit the shearing of the seeds.

  Note 1: GMPCPP microtubule seeds keep for 1–2 days at RT (they are cold-sensitive).

  Note 2: Tubulin labeling can affect differently seed nucleation and elongation. We found that Cy5-tubulin tends to cause more nucleation and therefore much shorter seeds than, for example, Alexa647 or Alexa568-tubulin. A low labeling ratio is advisable (e.g., we used a 0.4 ratio Alexa647/tubulin).

  Note 3: You may need to adjust tubulin concentrations given here. Keep in mind that lowering tubulin concentration will result in fewer microtubule nuclei and longer seeds. To double the amount of seeds without changing their length, double the volume of the polymerization mix.

  Note 4: GMPCPP seeds fuse over time. You can take advantage of this to obtain longer seeds.

4.2. Flow chamber assembly and seed attachment

4.2.1 Protein-coupled patterns

- Flow 250 μL water into the glass-PDMS-coverslip flow chamber. Carefully lift the coverslip with tweezers, briefly spin-dry it, and fix it on the counter glass. Flow BRB80 immediately.

  Note: The protein-coupled patterns can tolerate brief dehydration, but it is advisable to be quick during this step.

- Flow 100 μL BRB80 supplemented with glycerol (we used 7–21%).

  Note: Seeds will align in a laminar flow, which can be readily obtained using a viscous buffer.

  Note: When using 21% glycerol, you might want to build a chamber with a double layer of tape to increase its volume and thus facilitate flowing the viscous buffer.

- Prepare 50 μL diluted seeds (e.g., 10 μL seed mastermix in 40 μL BRB80 + 21% glycerol). Mix by pipetting up and down with a cut pipet tip.

- In a P200, pipet 150 μL BRB80. Set the P200 to 200 μL and pipet in the seeds so that the pipet tip contains 50 μL seeds + 150 μL BRB80 wash solution stacked on top of each other.

- Flow steadily the 200 μL through the chamber.

  Note: To flow steadily, one can wick out the solution using a long filter paper strip that is steadily swiped at the outlet of the chamber. One can

alternatively use a vacuum pump to suck the liquid out of the chamber at constant speed.

- *Optional step*: flow 50 μL BRB80 + oxygen scavengers (1% glucose, glucose oxidase 0.32 mg/mL, catalase 0.055 mg/mL) to visualize the seeds in the TIRF microscope (Fig. 19.5A).

4.2.2 PEG-biotin pattern

- Using a diamond pen, cut a coverslip into two pieces (22 × 11 mm).
- For each counter glass, fix a coverslip piece across the two stripes of tape so that its functionalized side is inside the chamber. Apply pressure and let stand for 5 min to ensure the coverslip is tightly bound.
- Flow 50 μL pluronic 5% and kappa casein 100 μg/mL in MQ water, at RT. The amphiphilic molecule pluronic and the blocking protein kappa casein will, in addition to the PEG coating, further prevent adsorption of the proteins of interest to the chamber surface.
- Wash with 100 μL kappa casein 50 μg/mL in midzone buffer with 10 m*M* β-mercaptoethanol, on ice.
- Flow 50 μL neutravidin 50 μg/mL and kappa casein 50 μg/mL in midzone buffer with 10 m*M* β-mercaptoethanol, on ice. Incubate for 4 min.

  Note: neutravidin concentration can be varied to achieve different PEG-biotin–neutravidin densities. We found that the maximal density was achieved with 50 μg/mL and incubated for 4 min on ice.
- Wash with 100 μL BRB80 + 7% glycerol.
- Prepare and flow a seed mix as in Section 4.2.1.
- *Optional step*: flow 50 μL BRB80 + oxygen scavengers (1% glucose, glucose oxidase 0.32 mg/mL, catalase 0.055 mg/mL) to visualize the seeds in the TIRF microscope (Fig. 19.5B).

4.3. TIRF microscopy

- For the dynamic assay, prepare the following 50 μL mix on ice: tubulin 17 μ*M* (from a pre-mix of freshly thawed 10 μL unlabelled tubulin and 1 μL Alexa647- or Alexa568-tubulin), and PRC1-SNAP-Alexa647 5 n*M* (concentration expressed for the monomer) in midzone buffer supplemented with 10 m*M* β-mercaptoethanol, 1 m*M* GTP, 0.1% methylcellulose, and oxygen scavengers (1% glucose, glucose oxidase 0.32 mg/mL, catalase 0.055 mg/mL).
- Pre-warm the mix and flow it in a glass chamber loaded with seeds.
- Seal the chamber with nail varnish or Valap (1:1:1 mix of Vaseline, lanolin, and paraffin wax heated to 50 °C).

- Image immediately in the TIRF microscope. To capture the formation of antiparallel overlaps, timelapse movies are typically recorded with a rate of 1 frame per 10 s (Fig. 19.6A and B).

5. DISCUSSION

The assay described here is a modification of a previously reported assay where microtubules were grown from surface-immobilized seeds in order to form dynamic antiparallel microtubule overlaps (Bieling et al., 2010). The novel aspect introduced here is the controlled immobilization of biotinylated microtubule seeds in precisely defined areas of the glass surface, using micropatterning, to promote the formation of antiparallel microtubule interactions with controlled seed spacing. This is achieved by photolithographic surface patterning that produces a thiol-reactive maleimide-pattern on a PEG-passivated glass surface (Waichman et al., 2011). This is a versatile method for the covalent immobilization of thiol-containing small functional groups or proteins to which we have added detailed sample preparation steps. Here, we use this method to produce biotin-PEG or streptavidin-PEG patterns with high contrast. These functionalized patterns allow the localized and specific immobilization of biotinylated microtubule seeds via biotin–streptavidin or biotin–neutravidin bonds on a PEG brush-passivated glass. This method is designed to create reproducible protein immobilization and preserve protein activities on micropatterned surfaces, because it relies on defined and oriented protein immobilization via a PEG-linker rather than non-specific adsorption to a glass surface.

The advantage of micropatterned seed attachment for the antiparallel overlap assay described here is that the distance between microtubule seeds can be well controlled by the geometry of the chosen micropattern, improving the efficiency and reliability of the assay. Biotinylated seeds are attached to functionalized stripes with perpendicular orientation using flow. This causes microtubules to grow over PEG-passivated areas of the glass where they meet “head-on” and start forming antiparallel overlaps in the presence of the antiparallel cross-linker PRC1. This assay can easily be extended to study other homologs of PRC1, such as the various MAP65 homologs in plants that are responsible for interphase microtubule bundling (Gaillard et al., 2008). Another natural extension of the assay is the study of the role of the other anaphase midzone proteins in microtubule overlap formation that are known to be recruited by PRC1 (Duellberg et al., 2013; Glotzer, 2009), leading toward the reconstitution of a more complex “minimal midzone.” Since the biotin patterns are quite versatile, further applications with other proteins can also be envisaged.

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REFERENCES

Aoyama S, Shimoike M, Hiratsuka Y. Self-organized optical device driven by motor proteins. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(41): 16408–16413. [PubMed: 24065817]

Bieling P, Telley IA, Surrey T. A minimal midzone protein module controls formation and length of antiparallel microtubule overlaps. Cell. 2010; 142(3):420–432. http://dx.doi.org/10.1016/j.cell.2010.06.033, S0092-8674(10)00723-3 [pii]. [PubMed: 20691901]

Braun M, Lansky Z, Fink G, Ruhnow F, Diez S, Janson ME. Adaptive braking by Ase1 prevents overlapping microtubules from sliding completely apart. Nature Cell Biology. 2011; 13(10):1259–1264. http://dx.doi.org/10.1038/ncb2323, ncb2323 [pii].

Castoldi M, Popov AV. Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. Protein Expression and Purification. 2003; 32:83–88. [PubMed: 14680943]

Duellberg C, Fourniol FJ, Maurer SP, Roostalu J, Surrey T. End-binding proteins and Ase1/PRC1 define local functionality of structurally distinct parts of the microtubule cytoskeleton. [Review]. Trends in Cell Biology. 2013; 23(2):54–63. [PubMed: 23103209]

Gaillard J, Neumann E, Van Damme D, Stoppin-Mellet V, Ebel C, Barbier E, et al. Two microtubule-associated proteins of Arabidopsis MAP65s promote antiparallel microtubule bundling. Molecular Biology of the Cell. 2008; 19(10):4534–4544. http://dx.doi.org/10.1091/mbc.E08-04-0341, E08-04-0341 [pii]. [PubMed: 18667529]

Gell C, Bormuth V, Brouhard GJ, Cohen DN, Diez S, Friel CT, et al. Microtubule dynamics reconstituted in vitro and imaged by single-molecule fluorescence microscopy. Methods in Cell Biology. 2010; 95:221–245. [PubMed: 20466138]

Ghosh S, Hentrich C, Surrey T. Micropattern-controlled local microtubule nucleation, transport, and mesoscale organization. ACS Chemical Biology. 2013; 8(4):673–678. [PubMed: 23294267]

Glotzer M. The 3Ms of central spindle assembly: Microtubules, motors and MAPs. Nature Reviews Molecular Cell Biology. 2009; 10(1):9–20. http://dx.doi.org/10.1038/nrm2609, nrm2609 [pii].

Hentrich C, Surrey T. Microtubule organization by the antagonistic mitotic motors kinesin-5 and kinesin-14. Journal of Cell Biology. 2010; 189(3):465–480. [PubMed: 20439998]

Hyman A, Drechsel D, Kellogg D, Salser S, Sawin K, Steffen P, et al. Preparation of modified tubulins. Methods in Enzymology. 1991; 196:478–485. [PubMed: 2034137]

Janson ME, Loughlin R, Loiodice I, Fu C, Brunner D, Nedelec FJ, et al. Crosslinkers and motors organize dynamic microtubules to form stable bipolar arrays in fission yeast. Cell. 2007; 128(2):357–368. http://dx.doi.org/10.1016/j.cell.2006.12.030, S0092-8674(07)00048-7 [pii]. [PubMed: 17254972]

Kapitein LC, Peterman EJ, Kwok BH, Kim JH, Kapoor TM, Schmidt CF. The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. Nature. 2005; 435(7038):114–118. [PubMed: 15875026]

Mollinari C, Kleman JP, Jiang W, Schoehn G, Hunter T, Margolis RL. PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. Journal of Cell Biology. 2002; 157(7):1175–1186. http://dx.doi.org/10.1083/jcb.200111052, jcb.200111052 [pii]. [PubMed: 12082078]

Nunes Bastos R, Gandhi SR, Baron RD, Gruneberg U, Nigg EA, Barr FA. Aurora B suppresses microtubule dynamics and limits central spindle size by locally activating KIF4A. Journal of Cell Biology. 2013; 202(4):605–621. [PubMed: 23940115]

Portran D, Gaillard J, Vantard M, Thery M. Quantification of MAP and molecular motor activities on geometrically controlled microtubule networks. Cytoskeleton. 2013; 70(1):12–23. [PubMed: 23027541]

Roostalu J, Hentrich C, Bieling P, Telley IA, Schiebel E, Surrey T. Directional switching of the kinesin Cin8 through motor coupling. Science. 2011; 332(6025):94–99. [PubMed: 21350123]

Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nature Biotechnology. 2004; 22:1567–1572.

Sørensen HP, Sperling-Petersen HU, Mortensen KK. A favorable solubility partner for the recombinant expression of streptavidin. Protein Expression and Purification. 2003; 32(2):252–259. [PubMed: 14965771]

Su X, Arellano-Santoyo H, Portran D, Gaillard J, Vantard M, Thery M, et al. Microtubule-sliding activity of a kinesin-8 promotes spindle assembly and spindle-length control. Nature Cell Biology. 2013; 15(8):948–957.

Subramanian R, Ti SC, Tan L, Darst SA, Kapoor TM. Marking and measuring single microtubules by PRC1 and kinesin-4. Cell. 2013; 154(2):377–390. [PubMed: 23870126]

Subramanian R, Wilson-Kubalek EM, Arthur CP, Bick MJ, Campbell EA, Darst SA, et al. Insights into antiparallel microtubule crosslinking by PRC1, a conserved nonmotor microtubule binding protein. Cell. 2010; 142(3):433–443. http://dx.doi.org/10.1016/j.cell.2010.07.012, S0092-8674(10)00781-6 [pii]. [PubMed: 20691902]

Telley IA, Bieling P, Surrey T. Reconstitution and quantification of dynamic microtubule end tracking in vitro using TIRF. Methods in Molecular Biology. 2011; 777:127–145. [PubMed: 21773926]

van den Wildenberg SM, Tao L, Kapitein LC, Schmidt CF, Scholey JM, Peterman EJ. The homotetrameric kinesin-5 KLP61F preferentially crosslinks microtubules into antiparallel orientations. Current Biology. 2008; 18(23):1860–1864. [PubMed: 19062285]

Verni F, Somma MP, Gunsalus KC, Bonaccorsi S, Belloni G, Goldberg ML, et al. Feo, the Drosophila homolog of PRC1, is required for central-spindle formation and cytokinesis. Current Biology. 2004; 14(17):1569–1575. http://dx.doi.org/10.1016/j.cub.2004.08.054, S0960982204006578 [pii]. [PubMed: 15341744]

Waichman S, You C, Beutel O, Bhagawati M, Piehler J. Maleimide photolithography for single-molecule protein-protein interaction analysis in micropatterns. Analytical Chemistry. 2011; 83(2): 501–508. [PubMed: 21186833]

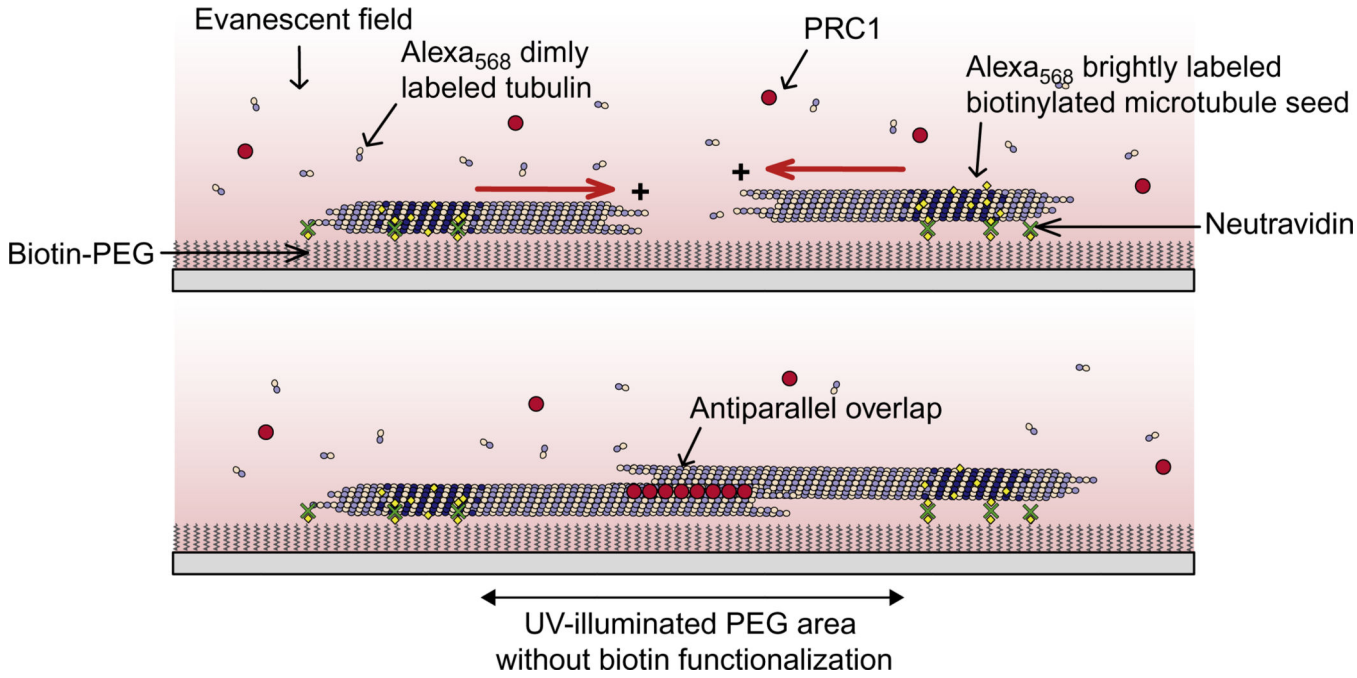

Figure 19.1.

Schematic overview of the dynamic antiparallel microtubule assay on micropatterned coverslips. Brightly labeled microtubule seeds are immobilized on the functionalized areas of the coverslip surface by a biotin–neutravidin sandwich (or alternatively by a streptavidin-Cys directly coupled to the glass—not shown in this scheme) and are visualized using TIRF microscopy. From these seeds, dynamic microtubule extensions are grown with dimly labeled tubulin. When antiparallel microtubules meet, PRC1 accumulates in the overlap region. (*Adapted from* Bieling et al. (2010)).

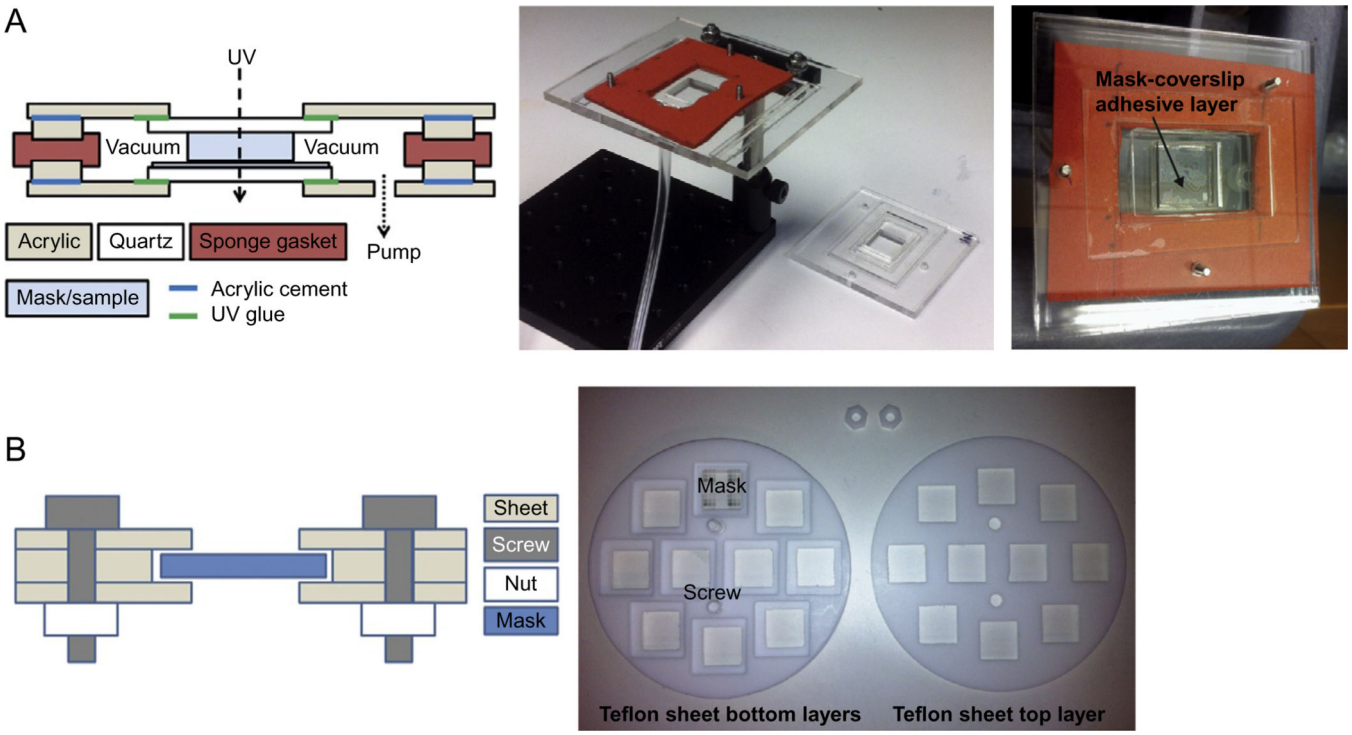

Figure 19.2.

Custom-built sample preparation devices for UV micropatterning. (A) Schematic diagram and photographs of the UV illumination vacuum-sealed chamber; Photos: left: empty chamber with lid off; right: sample-loaded and vacuum-sealed chamber. Dark areas seen at the coverslip-mask interface in the loaded chamber correspond to regions of tight adhesion. (B) Schematic diagram and photo of the Teflon holder for piranha cleaning of the masks. Photo: *quartz and chromium mask loaded in the open Teflon holder*.

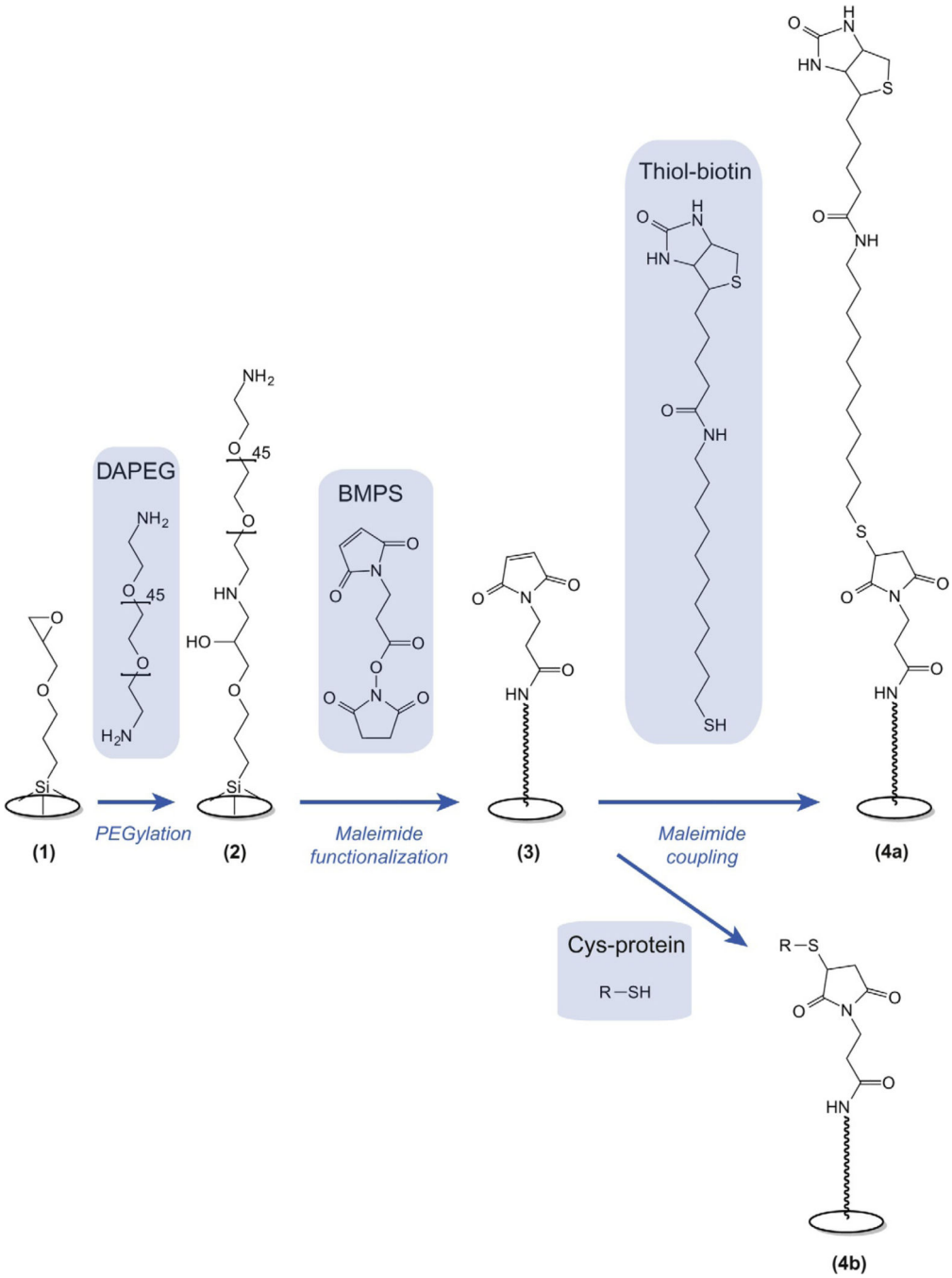

Figure 19.3.

Surface chemistry for coverslip functionalization. The glass is silanized (1), PEGylated (2), and reacted with the maleimide derivative BMPS (3). At that stage, the coverslip, functionalized with a uniform layer of maleimide-PEG, can be patterned using a UV source and a quartz/chromium mask. UV illumination destroys the maleimide groups. In contrast, the maleimide-PEG groups remain intact in the photoprotected areas and will react with thiols (here: thiol-biotin (4a), or cysteine-containing proteins (4b)) to generate covalently linked biotin-PEG or protein-PEG chains.

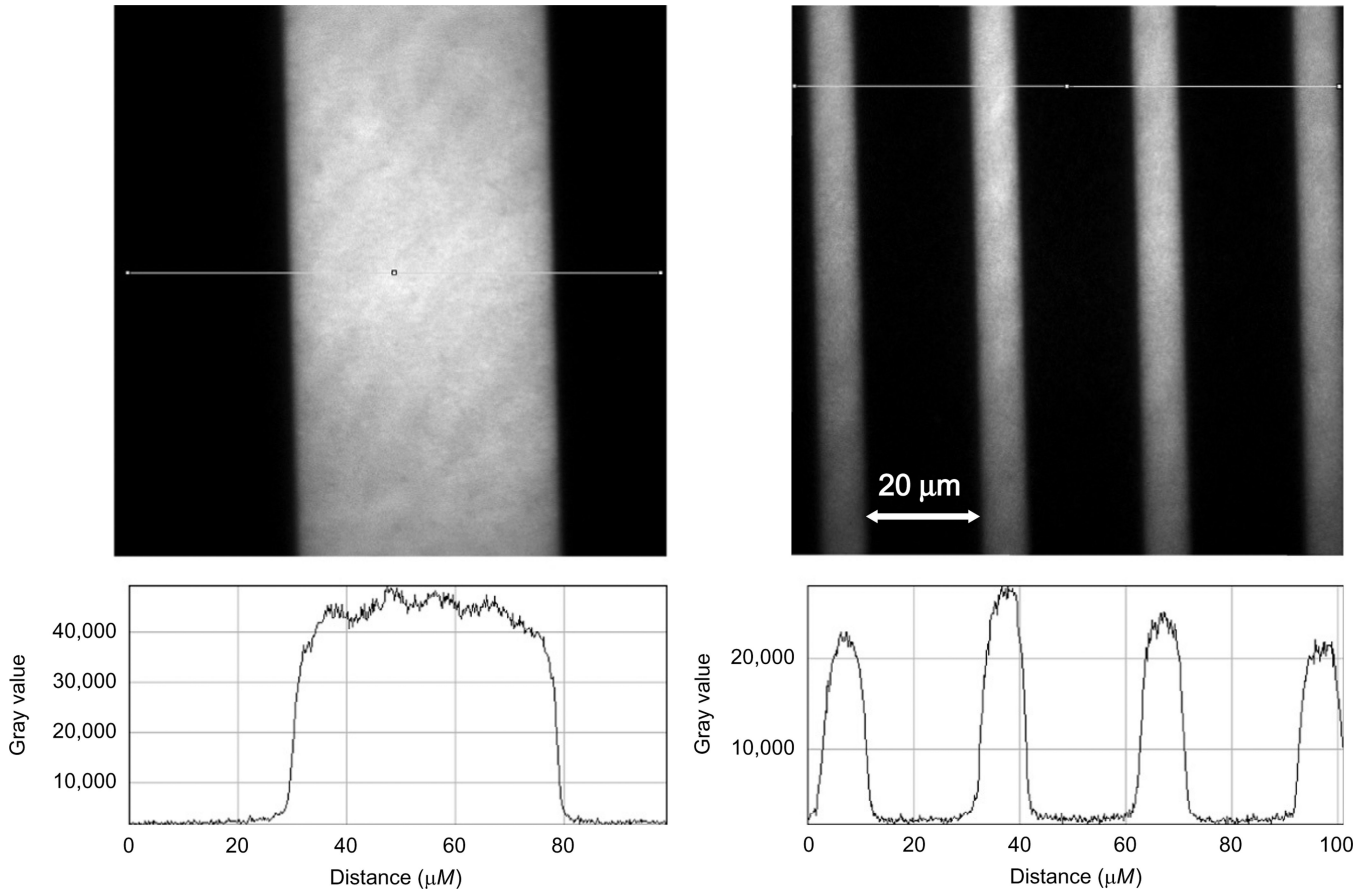

Figure 19.4.

High contrast micropatterning of cross-linked Cys-mCherry. Micropatterned coverslips coupled to a cysteine-containing mCherry and imaged by TIRF microscopy. Two fields of view and fluorescent intensity line profiles are shown. Comparison of fluorescence intensity of the photoprotected and illuminated areas reveals the highly selective patterning of proteins that can be achieved with this method.

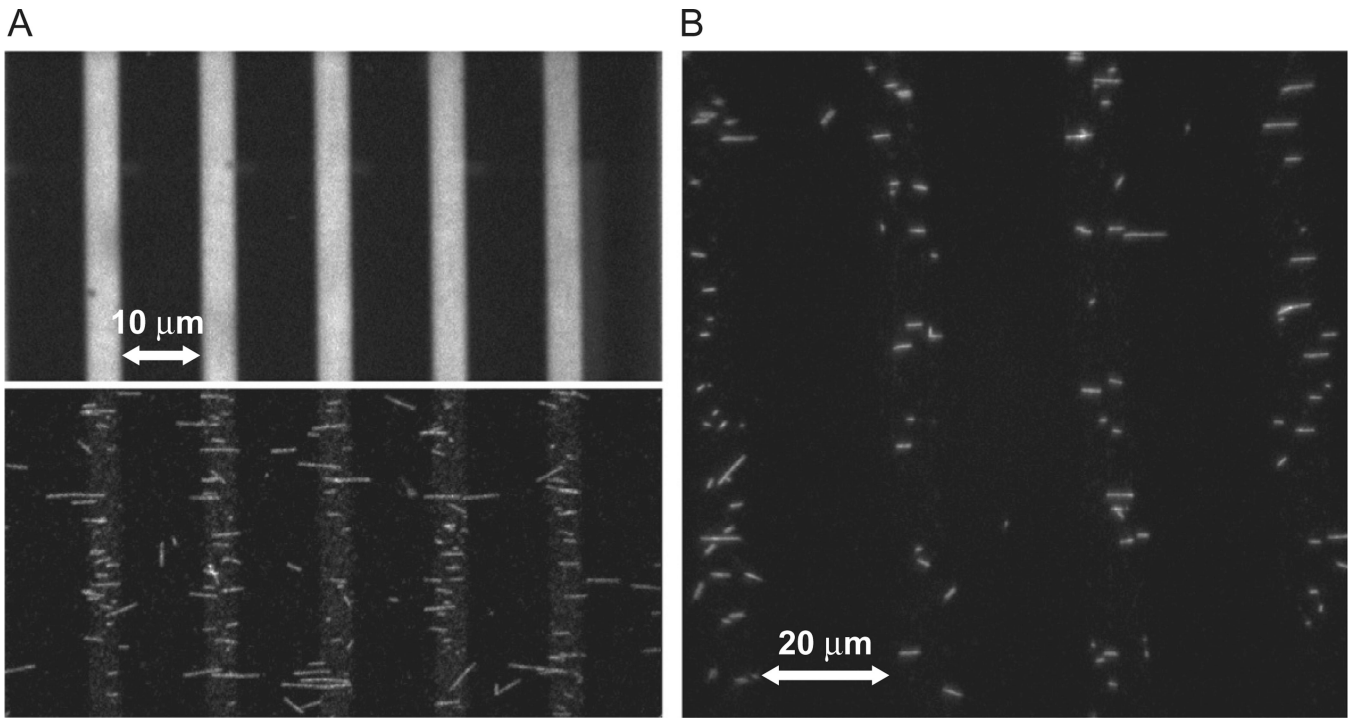

Figure 19.5.

Alignment of microtubule seeds on micropatterned surfaces under flow. (A) Alexa647-labeled GMPCPP microtubule seeds containing 30% biotinylated-tubulin in BRB80 + 21% glycerol were perfused into a chamber formed with a patterned coverslip coupled to 500 n*M* Cys-mCherry and 250 n*M* streptavidin-Cys (concentration expressed for the tetramer). (B) Alexa568-labeled GMPCPP microtubule seeds containing 30% biotinylated-tubulin in BRB80 + 7% glycerol were perfused into a chamber formed with a patterned coverslip coupled to thiol-biotin and previously incubated with 50 μg/mL neutravidin.

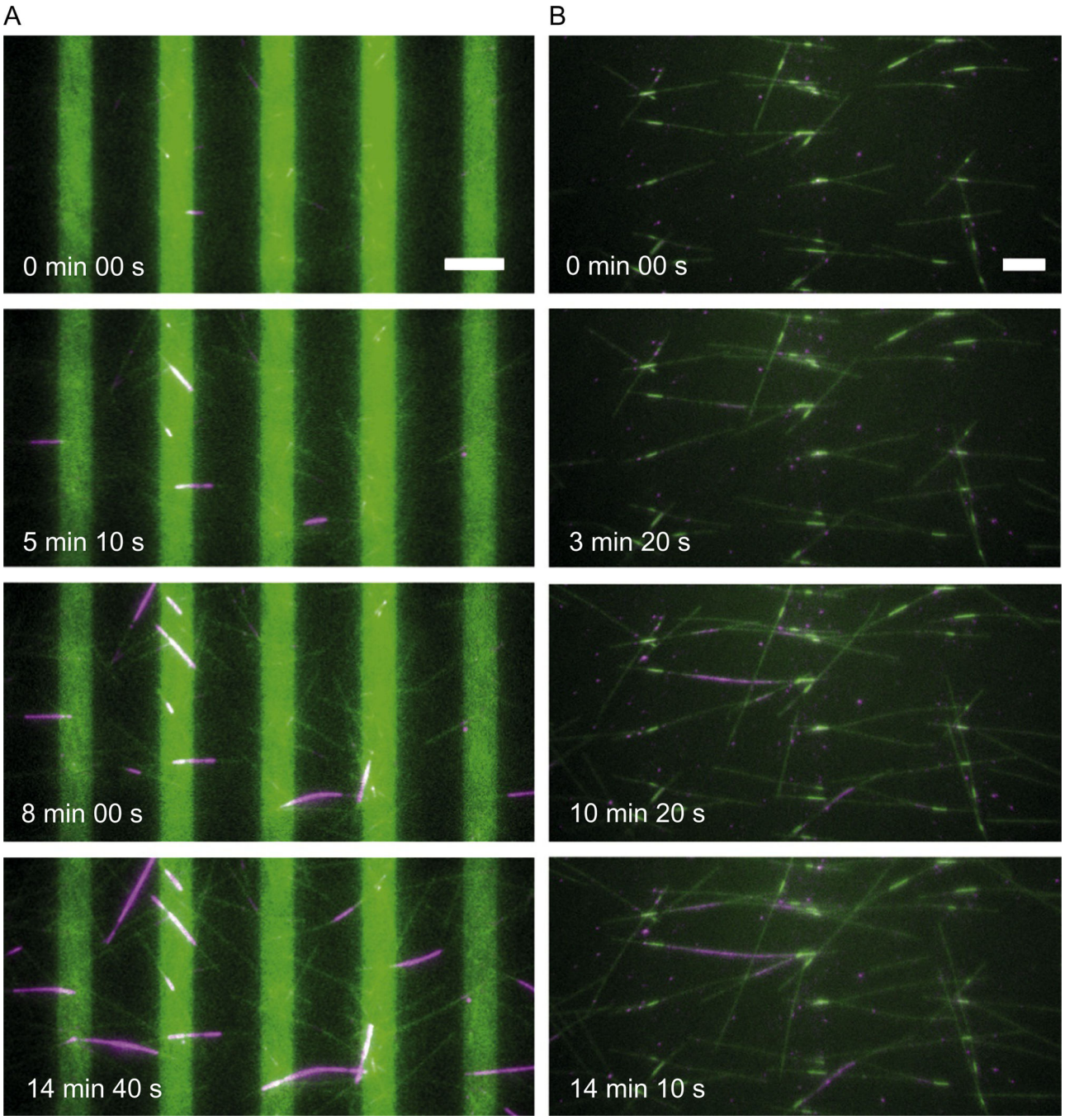

Figure 19.6.

TIRF imaging of antiparallel microtubule nucleated from micropatterned surfaces. (A) Time-course of dynamic microtubules grown from Alexa647-labeled microtubule seeds (dim magenta) aligned on Cys-mCherry (green) and streptavidin-Cys patterns, in the presence of 5 n*M* PRC1-SNAP-Alexa647 (bright magenta) and 22.5 μ*M* Alexa568-tubulin (green). Also see Video 1 (http://dx.doi.org/10.1016/B978-0-12-397924-7.00019-4). (B) Time-course of dynamic microtubules grown from Alexa568 brightly labeled microtubule seeds (bright green) aligned on PEG-biotin patterns, in the presence of 5 n*M* PRC1-SNAP-

Alexa647 (magenta) and 17 μ*M* Alexa568-tubulin (green). Scale bars are 10 μm. Also see Video 2 (http://dx.doi.org/10.1016/B978-0-12-397924-7.00019-4).