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TITLE:

Simultaneous Visualization of the Dynamics of Crosslinked and Single Microtubules *In Vitro* by TIRF Microscopy

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SUMMARY:

Here, a TIRF microscopy-based *in vitro* reconstitution assay is presented to simultaneously quantify and compare the dynamics of two microtubule populations. A method is described to simultaneously view the collective activity of multiple microtubule-associated proteins on crosslinked microtubule bundles and single microtubules.

ABSTRACT:

Microtubules are polymers of αβ-tubulin heterodimers that organize into distinct structures in cells. Microtubule-based architectures and networks can consist of subsets of microtubule arrays that differ in their dynamic properties. For example, in dividing cells, stable bundles of crosslinked microtubules coexist in close proximity to dynamic non-crosslinked microtubules. TIRF-microscopy-based *in vitro* reconstitution studies enable the simultaneous visualization of dynamics of these different microtubule arrays. In this assay, an imaging chamber is assembled with surface-immobilized crosslinked microtubule bundles and non-crosslinked single microtubules. Introduction of tubulin, nucleotides, and protein regulators allows direct visualization of the dynamic properties of and protein localization on single and crosslinked microtubules. Furthermore, changes that occur as dynamic single microtubules organize into a bundle can be monitored in real-time. The method described here allows for a systematic evaluation of the activity and localization of individual proteins, as well as synergistic effects of protein regulators on two different microtubule subsets under identical experimental conditions, thereby providing mechanistic insights that are inaccessible by other methods.

INTRODUCTION:

Microtubules are biopolymers that form structural scaffolds essential for multiple cellular

processes, ranging from intracellular transport and organelle positioning to cell division and elongation. To execute these diverse functions, individual microtubules are organized into micron-sized arrays, such as mitotic spindles, ciliary axonemes, neuronal bundles, interphase arrays, and plant cortical arrays. A ubiquitous architectural motif found in these structures is a bundle of microtubules crosslinked along their lengths[1]. An intriguing feature of several microtubule-based structures is the coexistence of bundled microtubules and non-crosslinked single microtubules in close spatial proximity. These microtubule subpopulations can display starkly different polymerization dynamics from each other, as needed for their proper function[2–5]. For instance, within the mitotic spindle, stable crosslinked bundles and dynamic single microtubules are present within a micron-sized region at the cell center[6]. Studying how the dynamic properties of coexisting microtubule populations are specified is, therefore, central to understanding the assembly and function of microtubule-based structures.

Microtubules are dynamic polymers that cycle between phases of polymerization and depolymerization, switching between the two phases in events known as catastrophe and rescue[7]. The dynamics of cellular microtubules are regulated by myriad Microtubule Associated Proteins (MAPs) that regulate the rates of microtubule polymerization and depolymerization and the frequencies of catastrophe and rescue events. It is challenging to investigate the activity of MAPs on spatially proximal arrays in cells, owing to the high density of microtubules in micron-sized regions, and the limitations of spatial resolution in light microscopy. Moreover, the presence of multiple MAPs in the same cellular region hinders interpretations of cell biological studies. These difficulties are circumvented by *in vitro* reconstitution assays performed in conjunction with Total Internal Reflection Fluorescence (TIRF) microscopy. Here, the dynamics of microtubules assembled *in vitro* are examined in the presence of one or more MAPs under controlled conditions[8–10]. However, conventional reconstitution assays are typically performed on single microtubules or on one type of array, which do not permit the visualization of coexisting populations.

Here, *in vitro* reconstitution assays are presented to enable the simultaneous visualization of two microtubule populations under the same solution conditions[11]. A method is described to simultaneously view the collective activity of multiple MAPs on single microtubules, and on microtubule bundles crosslinked by the mitotic spindle-associated protein PRC1, which preferentially binds to and cross-links anti-parallel microtubules. Briefly, this protocol consists of the following steps: (i) preparation of stock solutions and reagents, (ii) cleaning and surface treatment of coverslips used to create the imaging chamber for microscopy experiments, (iii) preparation of stable microtubule “seeds” from which polymerization is initiated during the experiment, (iv) specification of TIRF microscope settings to visualize microtubule dynamics, (v) immobilization of microtubule seeds and generation of crosslinked microtubule bundles in the imaging chamber, and (vi) visualization of microtubule dynamics in the imaging chamber through TIRF microscopy, upon addition of soluble tubulin, MAPS, and nucleotides. These assays enable the qualitative evaluation and quantitative examination of MAP localization and their effect on the dynamics of two microtubule populations. Additionally, they facilitate the evaluation of synergistic effects of multiple MAPs on these microtubule populations, across a wide range of experimental conditions.

PROTOCOL:

1. Prepare reagents

1.1. Prepare buffers and reagents as outlined in **Table 1** and **Table 2**. During the experiment, keep all solutions on ice unless otherwise noted.

[Place **Table 1** here]

[Place **Table 2** here]

2. Prepare Biotin-PEG slides

NOTE: Prepare imaging chambers as close to the start of an experiment as possible, and not more than 2 weeks in advance.

2.1. Clean coverslips

[Place **Figure 1** here]

2.1.1. Position an equal number of 24 x 60 mm and 18 x 18 mm #1.5 coverslips (**Figure 1F**) in slide-staining jars and slide-washing racks, respectively (**Figure 1A,B**). Place the slide-washing racks containing 18 x 18 mm coverslips in a 100 mL beaker.

2.1.2. Rinse all coverslips 5–6 times in ultrapure water (18.2 MΩ-cm resistivity) and remove excess liquid after each rinse with a pipette tip attached to a vacuum tube (**Figure 1C**).

2.1.3. Fill beakers and slide-staining jars containing the coverslips with ultrapure water, seal with parafilm, and sonicate for 10 min.

2.1.4. Fill two 150 mL beakers with 200-proof ethanol. Using tweezers, dip each coverslip into one beaker filled with ethanol, and then the other.

2.1.5. Using tweezers, transfer coverslips to the slide-drying rack (**Figure 1D**), dry them under nitrogen gas stream and incubate at 37 °C until completely dry (~15 min).

2.1.6. Place the dried coverslips in a single layer inside the plasma cleaner. Form vacuum seal, and then set the Radio Frequency (RF) level of the plasma cleaner to ~8 MHz.

2.1.7. Once the plasma is generated, leave coverslips in plasma cleaner for 5 min. Switch off the plasma cleaner and release the vacuum slowly.

2.1.8. Once the vacuum seal is released, flip the coverslips over and repeat the plasma cleaning

for 5 min for the other side of the coverslips.

2.1.9. Alternative to plasma cleaning: In place of steps 2.1.2–2.1.3, sonicate coverslips in a warm solution of 2% detergent (in ultrapure water) for 10 min. Then, thoroughly wash coverslips with ultrapure water and sonicate in ultrapure water 2–3 times (10 min each). Next, wash in ethanol and dry as in steps 2.1.4–2.1.5. Skip steps 2.1.6–2.1.8.

2.2. Biotin-PEG treatment

2.2.1. Immediately before use, dissolve 400 µL of 3-Aminopropyltriethoxysilane in 40 mL of acetone. Using tweezers, move plasma-cleaned coverslips into the slide-washing rack and slide-staining jars. Submerge coverslips in 3-Aminopropyltriethoxysilane solution and incubate for 5 min[12,13].

2.2.2. Wash all coverslips 5–6 times with ultrapure water.

2.2.3. Transfer coverslips to the slide-drying rack, dry them under a nitrogen gas stream and incubate at 37 °C until completely dry (~20 min).

2.2.4. Lay the dried coverslips on delicate task wipes and label each coverslip on one corner, e.g., 'p' on each 18 x 18 mm coverslip and 'b' on each 24 x 60 mm coverslip (see **Figure 2**).

2.2.5. On the day of the experiment, prepare a fresh 0.1 M sodium bicarbonate solution by dissolving 0.84 mg of $NaHCO_3$ in 10 mL of ultrapure water.

2.2.6. Bring aliquots of mPEG-Succinimidyl Valerate (PEG-SVA) and Biotin-PEG-SVA to room temperature, immediately before use. See notes on Polyethylene Glycol (PEG) aliquot preparation in **Table 2**.

NOTE: Work quickly because the hydrolysis half-life of the Succinimidyl Valerate (SVA) moiety is ~30 min.

2.2.7. Add 102 µL of 0.1 M $NaHCO_3$ to 34 mg of PEG-SVA, spin in a benchtop microcentrifuge at 2,656 x *g* for 20 s, and then mix by pipetting up and down. Dissolve 3 mg of Biotin-PEG-SVA in 27 µL of 0.1 M $NaHCO_3$ by pipetting up and down. Adjust dilution volumes according to the exact weight of PEG noted on the tubes (see **Table 2**).

2.2.8. Prepare 100:1 w/w PEG:biotin-PEG mixture by combining 75 µL of PEG-SVA solution and 2.25 µL of Biotin-PEG-SVA solution for 20 coverslips, 100 µL and 3 µL for 30 coverslips, or 125 µL and 3.75 µL for 40 coverslips.

2.2.9. Construct a hydration chamber by placing wet paper towels beneath the tip rack in the bottom of an empty 10 µL tip box (**Figure 1E**). This will prevent evaporation of the PEG solutions.

2.2.10. Pipette 6 µL of 100:1 PEG-SVA:biotin-PEG mixture onto the center of one 24 x 60 mm coverslip on the labeled side. Place another 24 x 60 mm coverslip on top of the first coverslip such that the pair forms an X-shape, with the sides labeled 'b' facing each other. Place the pair on an empty tip rack in the hydration chamber and repeat for the remaining 24 x 60 mm coverslips.

2.2.11. Pipette 6 µL of PEG-SVA onto the center of one 18 x 18 mm coverslip on the labeled side. Place another 18 x 18 mm coverslip on top of the first coverslip, with the sides labeled 'p' facing each other. Place the pair on an empty tip rack in the hydration chamber and repeat for the remaining 18 x 18 mm coverslips.

2.2.12. Close the hydration chamber and incubate for 3 h or overnight.

2.2.13. Separate the pairs of coverslips and rinse in ultrapure water.

2.2.14. Dry coverslips with a nitrogen stream and place them in a 37 °C incubator to fully dry.

2.2.15. To construct the imaging chamber, stick three strips of double-sided tape on a 24 x 60 mm coverslip on the side labeled 'b'. To the other side of tape strips, attach an 18 x 18 mm coverslip with its side labeled 'p' facing the larger coverslip. This forms two lanes for microscopy experiments, with treated surfaces facing each other (**Figure 2 and Figure 1G**).

[Place **Figure 2** here]

3. Polymerize microtubules

3.1. Prepare GMPCPP seeds

NOTE: Prepare GMPCPP seeds in a cold room, keeping all reagents, tips, and tubes at 4 °C. GMPCPP seeds can be prepared in advance and stored at -80 °C for up to 1 year.

3.1.1. Resuspend lyophilized tubulin (**Table 2**) to ~10 mg/mL in 1x BRB80, immediately before use.

3.1.2. Mix components of GMPCPP seeds as described in **Table 3**.

NOTE: Keep all tubulin components on ice as much as possible to minimize the polymerization of soluble tubulin.

[Place **Table 3** here]

3.1.3. Clarify mix in a fixed-angle ultracentrifuge rotor at 352,700 x *g* for 5 min at 4 °C.

3.1.4. Separate the supernatant into 5 µL aliquots, snap freeze them in liquid nitrogen, and store

them at -80 °C.

3.2. Polymerize seeds on the day of the experiment

3.2.1. Warm 1–2 mL of BRB80-DTT to 37 °C.

3.2.2. Place 5 µL aliquot of GMPCPP seeds (-80 °C) from step 3.1.4 on ice and immediately dissolve in 20 µL of warm BRB80-DTT. Spin at 2,000 x *g* for 5 s at room temperature and tap to mix.

NOTE: Initial dilution volume may vary between 13 µL and 21 µL and is empirically determined for each batch of seeds. If seeds fail to polymerize, troubleshoot by supplementing initial dilution buffer (step 3.2.2) with 0.5 µM GMPCPP.

3.2.3. Protect from light and incubate at 37 °C for 30–45 min.

NOTE: The length of the microtubules depends on the duration of incubation. For short microtubules, the incubation time can be as short as 15 min. For long microtubules, the incubation time can be as long as 2 h. Biotinylated microtubules tend to require longer incubation times than non-biotinylated microtubules.

3.2.4. Place fixed-angle ultracentrifuge rotor, containing 500 µL centrifuge tubes, in ultracentrifuge and pre-warm to 30 °C.

3.2.5. After incubation, add 50 µL of warm BRB80-DTT (step 3.2.1) to the polymerized GMPCPP seeds and transfer the mixture to a 500 µL centrifuge tube. Add 50 µL of warm BRB80-DTT to an empty tube, pipette up and down, and add this buffer to the centrifuge tube containing the mixture.

3.2.6. Before spinning, mark the rim of the centrifuge tube to indicate where the pellet will be (the pellet will be too small to see). Spin for 10 min at 244,900 x *g* at 30 °C[12].

3.2.7. Discard the supernatant and resuspend the pellet in 100 µL of BRB80-DTT. Tap to mix.

3.2.8. Spin for 10 min at 244,900 x *g* at 30 °C, aligning the marking with the rotor to pellet in the same place.

3.2.9. Remove the supernatant and resuspend the pellet in 16 µL of BRB80-DTT. Transfer the microtubule solution to a clean 0.6 mL microcentrifuge tube. Protect from light and keep at or above room temperature.

NOTE: After polymerization, keep microtubules at or above room temperature. If they get cold, they will depolymerize. Incubate at 28 °C for added stability.

3.3. Check microtubules *via* TIRF microscopy

3.3.1. Pipette a mixture of 4.5 µL of BRB80-DTT and 1 µL of microtubule solution (step 3.2.9) onto a microscope slide. Cover with an 18 x 18 mm coverslip and seal the edges with either clear nail polish or a 1:1:1 mixture of petrolatum, lanolin, and paraffin (valap sealant), which is solid at room temperature and liquid at 95 °C.

3.3.2. Position the TIRF objective beneath the coverslip (see step 4 for recommended microscope settings) and visualize the newly polymerized microtubules at wavelength appropriate for the fluorescently labeled tubulin in the Bright mix (**Table 3**), to determine what dilution of microtubules to use in the upcoming experiments.

4. Microscope settings

4.1. Temperature: Set the microscope temperature to 28 °C to view dynamic microtubules.

4.2. Filters: Use the best combination of filter cubes and emission filters, depending on the fluorescent channels to be imaged. To visualize 488 nm, 560 nm, and 647 nm wavelengths in the same experiment, use a 405/488/560/647 nm Laser Quad Band Set, coupled with emission filters for the designated wavelengths.

4.3. Align Lasers: Ensure that the TIRF illuminator is aligned. Determine the Laser intensity for the experiment empirically, such that all fluorescent proteins are illuminated, but do not undergo significant photobleaching over the time course of the experiment.

4.4. Objective: Use lens paper to clean a 100x objective with 70% ethanol. Prior to imaging, add a drop of microscope immersion oil to the objective.

4.5. Set up an imaging sequence

4.5.1. For an experiment with 647 nm fluorophore-labeled biotinylated microtubules, 560 nm fluorophore-labeled non-biotinylated microtubules and soluble tubulin, and GFP-labeled protein of interest, image for 20 min. Image the 560 nm and 488 nm channels every 10 s, and the 647 nm channel every 30 s.

4.5.2. To capture a reference image of bundles before the addition of soluble tubulin and MAPs, set up a sequence with one image each in 560 nm and 647 nm wavelengths.

5. Generate surface-immobilized microtubule bundles

NOTE: For the following steps, flow all solutions into the imaging chamber by pipetting into one open side, while placing a filter paper against the other side. Protect the imaging chamber from light to reduce photobleaching of fluorescently labeled proteins. Tape the prepared imaging chamber to a slide holder (**Figure 1G,H**). Follow the steps in **Table 4**, which correspond to protocol

steps 5.2–6.4.

5.1. Prepare soluble tubulin mix according to **Table 5** and keep it on ice.

[Place **Table 5** here]

NOTE: Soluble tubulin must always be placed on ice to prevent polymerization. Prepare a fresh soluble tubulin mix approximately every 2 h, or when microtubules are no longer polymerizing.

5.2. To immobilize microtubules *via* a biotin-neutravidin-biotin linkage, first flow in Neutravidin (NA) solution until the chamber is filled (~7.5 µL) and incubate for 5 min.

5.3. Wash with 10 µL of MB-cold.

5.4. Flow in 7.5 µL of the blocking protein κ-casein (KC) and incubate for 2 min.

5.5. Wash with 10 µL of MB-warm to prepare the chamber for the introduction of microtubules.

5.6. Dilute the stock of biotinylated microtubules (according to observations in step 3.3.2) in 1X BRB80-DTT and add 1 µL of this dilution to 9 µL of MB-warm. Flow the mixture into the chamber and incubate for 10 min. Use a higher concentration of microtubules for more bundles.

5.7. Wash away non-immobilized microtubules with 10 µL of MB-warm.

5.8. Flow 7.5 µL of warm KC into the chamber and incubate for 2 min.

5.9. During the incubation, prepare a 2 nM solution of the cross-linker protein PRC1 in KC. Flow 10 µL of this solution into the imaging chamber and incubate for 5 min.

NOTE: Recombinant PRC1 is expressed and purified from bacterial cells as previously described[13].

5.10. To make bundles, flow 10 µL of non-biotinylated microtubules into the chamber and incubate for 10 min. PRC1 will cross-link the non-biotinylated and immobilized biotinylated microtubules[15,16] (**Figure 3**).

NOTE: See step 6.1 for preparing the assay mix during the incubation time.

5.11. Wash the chamber twice with 10 µL of MB-warm. The attached microtubules are stable for around 20 min from this point.

[Place **Table 4** here]

[Place **Figure 3** here]

6. Image microtubule dynamics

6.1. During the 10 min incubation time in step 5.10, prepare 10 µL of assay mix containing proteins of interest, soluble tubulin, nucleotides, oxygen scavengers[14], and antioxidants according to **Table 6**. Keep the mix on ice.

[Place **Table 6** here]

6.2. Load the prepared imaging chamber, taped to slide holder, on the 100x TIRF objective. Use the 560 nm and 647 nm channels to find a field of view that contains an optimum number and density of single microtubules and bundles.

NOTE: If both biotinylated and non-biotinylated microtubules are labeled with the same fluorophore, line-scan analyses of fluorescence intensities can distinguish between single microtubules and bundles.

6.3. Once a field of view is identified, take a reference image.

6.4. Carefully flow in the assay mix without disturbing the imaging chamber.

6.5. Seal the open ends of the chamber with valap sealant.

6.6. Start the imaging sequence as described in step 4.5.1.

REPRESENTATIVE RESULTS:

The experiment described above was performed using 647 nm fluorophore-labeled biotinylated microtubules, 560 nm fluorophore-labeled non-biotinylated microtubules, and 560 nm fluorophore-labeled soluble tubulin mix. Microtubules were crosslinked by the crosslinker protein PRC1 (GFP-labeled). After surface-immobilized bundles and single microtubules were generated (step 5.11), the imaging chamber was mounted on a 100x TIRF objective and viewed in the 560 nm and 647 nm fluorescence channels. Single microtubules were identified by their fluorescence signal in the 647 nm channel. Microtubules with fluorescence signals in both channels were identified as pre-formed bundles (**Figure 4**). If experiments are performed with biotinylated and non-biotinylated microtubules with the same fluorescent label, detected fluorescence intensities for bundles will be around two-fold or higher than that of single microtubules. Based on the proportion and density of each population, the concentration of microtubule seeds in steps 5.6 and 5.10 can be optimized.

[Place **Figure 4** here]

[Place **Video 1** here]

After an imaging sequence has been acquired, analyze the video to ensure that microtubules are dynamic (**Video 1**). Adjust assay components (tubulin volume in soluble tubulin mix, nucleotide

stocks, protein concentrations) according to observations. For example, if microtubules do not polymerize, increase concentration of soluble tubulin and/or GTP in soluble tubulin mix. Similarly, increase working concentrations of fluorescently labeled MAPs if they are not visible on microtubules, and decrease concentrations if their background fluorescence intensity in the field of view is comparable to their intensity on microtubules. When visualizing motile motor proteins, increase ATP concentrations if motors do not exhibit motility on microtubules. Adjust Laser intensity for the excitation channels corresponding to microtubule fluorescence to ensure that differences in fluorescence intensity between single microtubules and bundles can be captured within the dynamic range of the detector.

[Place **Video 2** here]

In the representative example shown in **Video 2**, a field of view containing single microtubules and crosslinked bundles is shown. It is found that under these assay conditions (assay mix containing 0.5 nM PRC1, 50 mM KCl, and MAPs of interest: 200 nM GFP-labeled CLASP1, 10 nM Kinesin Kif4A), single microtubules elongate over the course of the assay, whereas the growth of crosslinked microtubules is stalled.

For quantitative analysis of microtubule dynamics, open microscopy files in the FIJI software, and select single microtubules and bundles for analysis. Use the following criteria to exclude single microtubules and bundles from further analysis: exclude microtubules or bundles (i) found at the edges of the field of view, (ii) obscured by protein aggregates, or (iii) whose filaments move in the z-direction out of the TIRF range. Parameters of microtubule dynamics, such as length, growth rate, rescue frequency, and catastrophe frequency, can be obtained by constructing and analyzing kymographs for each single microtubule or microtubule pair[11,15].

FIGURE AND TABLE LEGENDS:

Figure 1: **Equipment for coverslip treatment and imaging chamber preparation.** (**A**) slide-staining jar for 24 x 60 mm coverslips, (**B**) slide-washing racks for 18 x 18 mm coverslips, (**C**) vacuum set-up, (**D**) slide-drying rack, (**E**) hydration chamber, (**F**) coverslips, (**G**) imaging chamber, (**H**) slide holder.

Figure 2: **Schematic for preparation of imaging chambers using double-sided tape (gray) and PEG/Biotin-PEG treated coverslips**. Created with BioRender.com.

Figure 3: **Schematic of addition of assay components to make and image fluorescently labeled bundles and single microtubules.** Biotinylated seeds are shown in blue, non-biotinylated seeds and soluble tubulin in red, PRC1 in black, and protein of interest in cyan. Step numbers in figure correspond to those in **Table 4**. Panel corresponding to step 9 shows a pre-formed bundle (lower left); step 11 shows a newly formed bundle (upper left). Created with BioRender.com.

Figure 4: **Identification of single microtubules and crosslinked microtubules in the field of view.** Representative field of view showing 647 nm (left), 560 nm (center), and merged (right) channels. Single microtubules (yellow arrowheads) and bundles (white arrowheads) are indicated in the

merged channel. Scale bar represents 2 µm.

Table 1: **List of Buffers used in this protocol and their components.** See the "Recommended Storage Duration" column for guidance on how far in advance each buffer can be prepared.

Table 2: **List of reagents used in this protocol**. Included are the recommended storage conditions and concentrations, working concentrations used during the experiment, and final concentration in the imaging chamber. Additional notes are given in the far-right column.

Table 3: **GMPCPP seed mix**. Components of GMPCPP microtubule seeds, including volume and order of addition. Prepare 5 µL aliquots and store for up to 1 year at -80 °C.

Table 4: **Assay steps.** List of reagents added to the imaging chamber, with indication of wash or incubation time.

Table 5: **Soluble tubulin mix components**. Mix at the start of the experiment and keep on ice.

Table 6: Assay mix components. Mix, flow into imaging chamber, and image microtubule dynamics, within 30 min.

Video 1: **Dynamics of single microtubules and PRC1-crosslinked bundles.** Representative video showing microtubule dynamics, with 647 nm fluorophore-labeled biotinylated microtubule seeds (blue), 560 nm fluorophore-labeled non-biotinylated microtubule seeds and 560 nm fluorophore-labeled soluble tubulin (red), and GFP-labeled PRC1 (green). Single and crosslinked microtubules are indicated by white and yellow arrows, respectively. The movie was recorded over 10 min (61 frames) and displayed at a rate of 12 frames/second. Assay conditions: 0.5 nM GFP-PRC1, 50 mM KCl and 37 °C. Scale bar: 5 µm.

Video 2: **Differences in dynamics of single microtubules and PRC1-crosslinked bundles in the presence of the MAPs, CLASP1, and Kif4A.** Representative video showing microtubule dynamics, with 647 nm fluorophore-labeled biotinylated microtubule seeds (blue), and 560 nm fluorophore-labeled non-biotinylated microtubule seeds and 560 nm fluorophore-labeled soluble tubulin (red). Single and crosslinked microtubules are indicated by white and yellow arrows, respectively. The movie was recorded over 20 min (121 frames) and displayed at a rate of 20 frames/second. Assay conditions: 200 nM CLASP1-GFP, 0.5 nM PRC1, and 10 nM Kif4A. Scale bar: 2 µm. Video is reproduced from reference[11].

DISCUSSION:

The experiment described here significantly expands the scope and complexity of conventional microtubule reconstitution assays, which are traditionally performed on single microtubules or on one type of array. The current assay provides a framework to simultaneously quantify and compare the regulatory MAP activity on two populations, namely, single microtubules and crosslinked bundles. Further, this assay allows for the examination of two types of bundles: those that are pre-formed from stable seeds before the initiation of dynamics, and those that are newly

formed when two growing ends encounter each other and crosslink (**Figure 3**). Moreover, in addition to conventional experimental variables, such as protein concentrations and buffer conditions, these assays enable the evaluation of the effects of geometrical features of microtubule arrays, such as the lengths and angles between adjacent filaments in a bundle, which are emerging as important determinants of microtubule dynamics and MAP activity[16].

In order to extend this experimental method for the *in vitro* reconstitution of multiple microtubule-based structures, the following key issues need to be addressed: (i) PRC1 preferentially crosslinks microtubules that are oriented anti-parallel to each other. While such bundles are found at the cell center during mitosis, bundles of parallel microtubules are a common feature in other microtubule-based structures within neuronal axons and the mitotic spindle. The protocol described above can be readily adapted to generate cross-linked parallel microtubules using recombinant crosslinkers such as Kinesin-1 and TRIM46[10,17]. (ii) In these reconstitution assays, differences in fluorescence intensity can be used to distinguish between single microtubules and pairs of microtubules[11,15]. Under the experimental conditions used here, intensity analyses indicate that most bundles contain two cross-linked microtubules, and line scan analyses provide information on their relative positioning. However, when there are more than two or three filaments in a bundle, the spatial resolution of standard TIRF-based imaging systems hinders identification of the ends and polarity of individual microtubules (~25 nm diameter)[18]. Moreover, while it is possible to identify the plus-ends of cross-linked anti-parallel microtubules from the direction of their growth, distinguishing the ends of cross-linked parallel microtubules growing in the same direction is hindered by the spatial resolution of optical microscopy. An extension of the experiment described here is to use polarity-marked microtubules or microtubule tip-binding proteins to position individual microtubules. For bundles with tens of microtubules, complementing the high temporal resolution of TIRF-based assays with techniques that have high spatial resolution, such as Atomic Force Microscopy, promises to yield new insights into the dynamics of individual microtubules within a bundle.

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DISCLOSURES:

The authors declare no competing interests.

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Figure 1: Equipment for coverslip treatment and imaging chamber preparation

Click here to access/download;Figure;EquipmentFig2.png

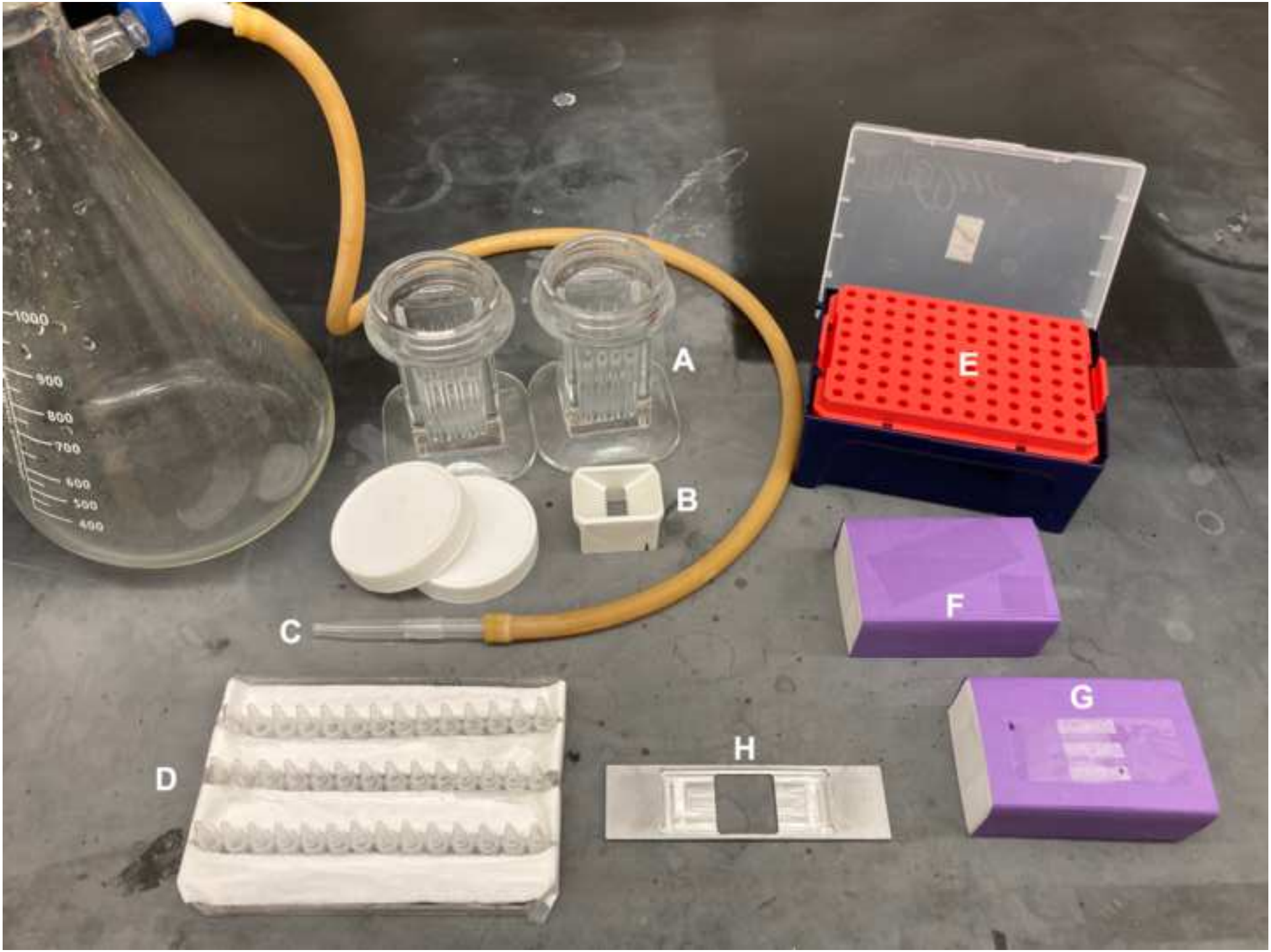


Figure 2: BioPEG Imaging Chamber

Click here to access/download;Figure;BioPEGslideconstruction.png

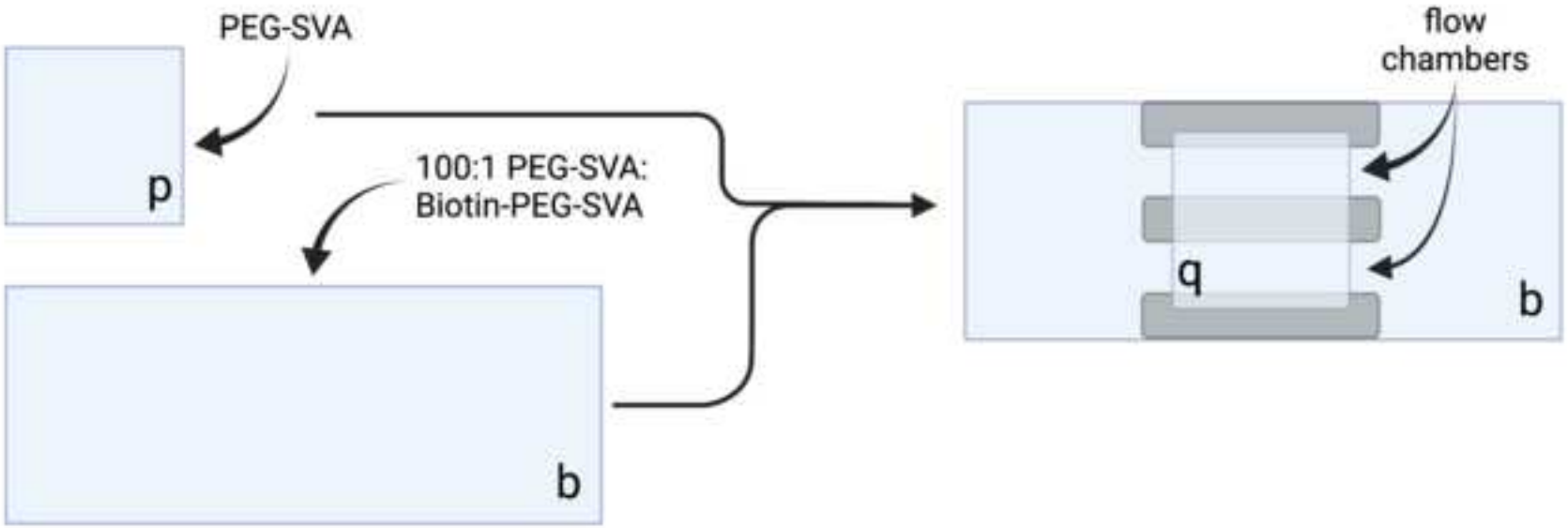


Figure 3: Assay Schematic

Click here to access/download;Figure;MTDynamicsSchematic1 (2).png

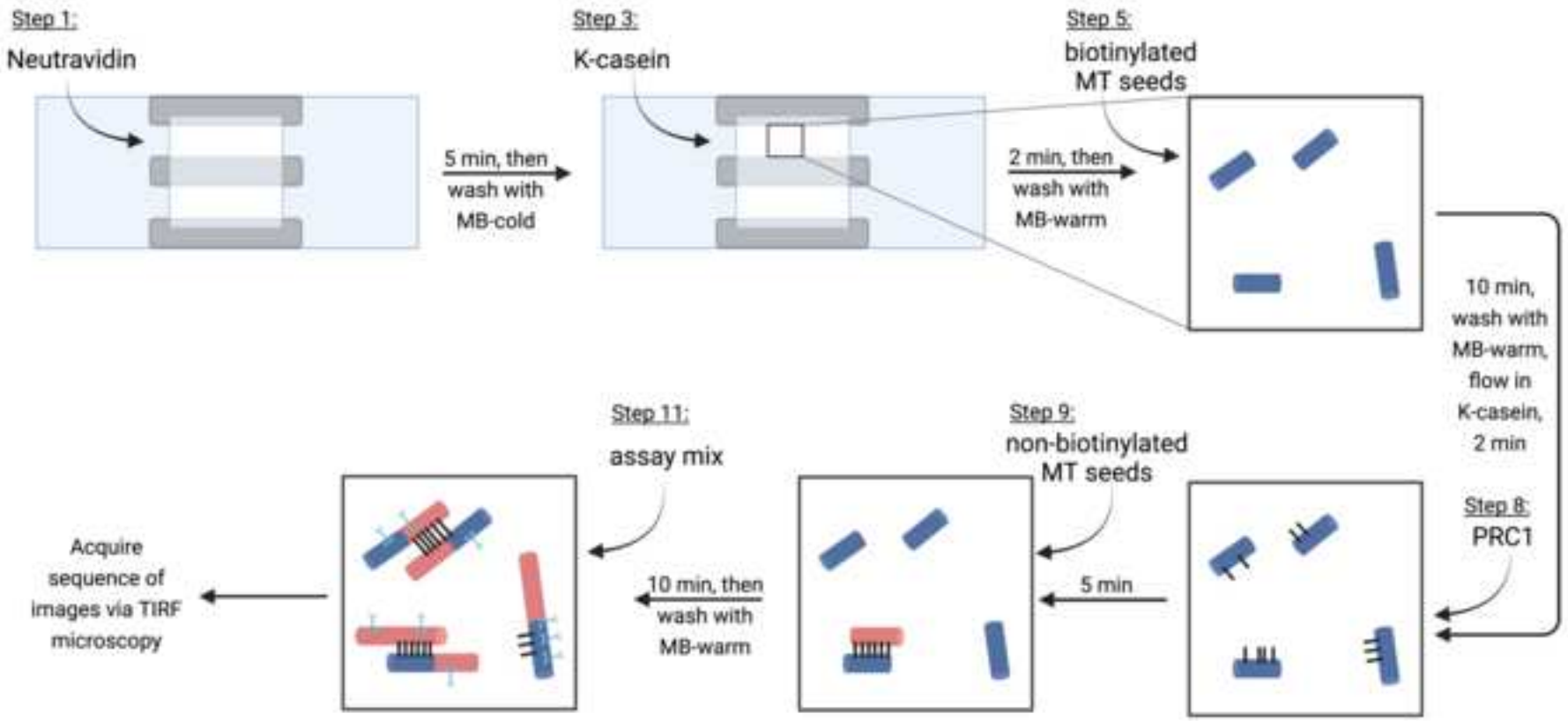
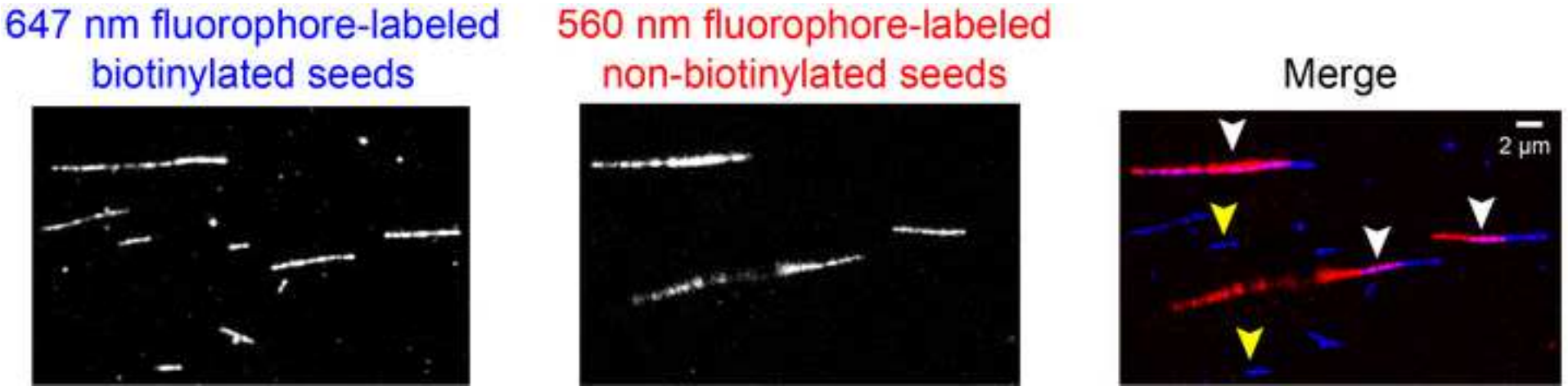


Figure 4: Representative Field of View

Click here to access/download;Figure;Fig4-Rev1.tif



Video 1

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Video or Animated Figure

Video1_rev1.mov

Video 2

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Video or Animated Figure

Video2.mov

Table 1: Buffers

Solution	**Components**	**Recommended Storage Duration**
5X BRB80	400 mM K-PIPES, 5 mM $MgCl_2$, 5 mM EGTA, pH 6.8 with KOH, filter sterilize	up to 2 years
1X BRB80	80 mM K-PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8	up to 2 years
BRB80-DTT	1X BRB80, 1 mM DTT	up to 2 days
Assay Buffer	80 mM K-PIPES, 3 mM $MgCl_2$, 1 mM EGTA, pH 6.8, 5% sucrose (OR 1X BRB80, 5% sucrose, 2 mM $MgCl_2$)	up to 1 year
Master Buffer (MB)	Assay Buffer, 5mM TCEP	1 week
Master Buffer with MethylCellulose (MBMC)	1X BRB80, 0.8% methylcellulose, 5 mM TCEP, 5 mM $MgCl_2$	1 week
Protein Dilution Buffer (DB)	MB, 1 mg/mL Bovine Serum Albumin (BSA), 10 µM ATP	1 day, on ice
Oxygen Scavenging Mix (OSM)	MB, 389 µg/mL catalase, 4.44 mg/mL glucose oxidase, 15.9 mM 2-mercaptoethanol (BME)	1 day, on ice
Oxygen Scavenging Final (OSF)	MB, 350 µg/mL catalase, 4mg/mL glucose oxidase, 14.3 mM BME, 1.5 mg/mL glucose	use within 30 min

Notes
Store at 4 °C
Store at 4 °C
Store at 4 °C
Prepare on the day of experiment; Separate into two tubes: MB-warm at room temperature and MB-cold on ice; include 1 mM DTT if using fluorescent dyes
Prepare on the day of experiment; include 1 mM DTT if using fluorescent dyes
Prepare on the day of experiment; include 1 mM DTT if using fluorescent dyes
Prepare on the day of experiment
Prepare immediately before use by adding 1 µL of glucose to 9 µL of OSM

Table 2: Reagents

Reagent	Storage Concentration	Storage Solvent	Storage Temperature	Working Concentration
Neutravidin (NA)	5 mg/mL	1X BRB80	-80°C	0.2 mg/mL
Kappa-casein (KC)	5 mg/mL	1X BRB80	-80°C	1 mg/mL
Bovine Serum Albumin (BSA)	50 mg/mL	1X BRB80	-20°C	1 mg/mL (in DB)
Catalase	3.5 mg/mL	1X BRB80	-80°C	350 µg/mL (in OSF)
Glucose oxidase	40 mg/mL	1X BRB80	-80°C	4 mg/mL (in OSF)
Tubulin	Lyophilized	N/A	4°C	10 mg/mL
Adenosine Triphosphate (ATP)	100 mM	ultrapure water	-20°C	10 mM
Guanosine Triphosphate (GTP)	100 mM	ultrapure water	-20°C	10 mM
Guanosine-5'-[(α,β)-methyleno]triphosphate (GMPCPP)	10 mM	ultrapure water	-20°C	10 µM
Dithiothreitol (DTT)	1 M	sterile water	-20°C	1 mM
Tris(2-carboxyethyl)phosphine (TCEP)	0.5 M	filter-sterilized water	Room temperature	5 mM
Methylcellulose	1%	sterile water	Room temperature	0.8% (in MBMC)
Beta-mercaptoethanol (BME)	143 mM	sterile water	Room temperature	14.3 mM (in OSF)

Glucose	150 mg/mL	1X BRB80	-80°C	15 mg/mL (in OSF)
(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)	10mM	1X BRB80	-80°C	10 mM
mPEG-Succinimidyl Valerate, MW 5,000	powder	N/A	-20°C	333 mg/mL (in 0.1 M sodium bicarbonate)
Biotin-PEG-SVA, MW 5,000	powder	N/A	-20°C	111 mg/mL (in 0.1 M sodium bicarbonate)

Final Concentration	Recommended Storage Duration	Notes
0.2 mg/mL	up to 1 year	Used to immobilize microtubules via a biotin-neutravidin-biotin linkage; store in small aliquots
1 mg/mL	up to 2 years	Used to block the slide's surface; Store in small aliquots; On day of experiment, set a small volume aside at room temperature
N/A	up to 2 years	store in small aliquots
35 µg/mL	up to 2 years	component of oxygen scavenging mix; store in small aliquots
0.4 mg/mL	up to 2 years	component of oxygen scavenging mix; store in small aliquots
2.12 mg/mL (in tubulin mix)	up to 1 year	Once tubulin is in solution, keep it cold to avoid polymerization.
1 mM	6 months	Prepare solutions in filter-sterilized water, adjust pH to ~7.0, and freeze in small aliquots.
1.29 mM (in tubulin mix)	6 months	Prepare solutions in filter-sterilized water, adjust pH to ~7.0, and freeze in small aliquots.
0.5 µM	6 months	
N/A	up to 2 years	
N/A	up to 2 years	
0.21% (in tubulin mix)	up to 1 year	Dissolve methylcellulose by slowly adding it to near-boiling water. Allow to cool while stirring continuously.
1.43 mM	up to 5 years	143 mM is a 1:100 dilution of stock BME

1.5 mg/mL	up to 2 years	Add to OSM immediately before use
1 mM	up to 1 year	Does not fully dissolve. Add some NaOH, stir for ~4 hours, and filter sterilize before use
324 mg/mL (in 0.1 M sodium bicarbonate)	6 months	Prepare ~34 mg aliquots, marking each tube with an exact weight of powder (tare the empty tube). Pass nitrogen gas over the solid, seal tubes with parafilm, and store at -20°C in a container with desiccant.
3.24 mg/mL (in 0.1 M sodium bicarbonate)	6 months	Prepare ~3 mg aliquots, marking each tube with an exact weight of powder (tare the empty tube). Pass nitrogen gas over the solid, seal tubes with parafilm, and store at -20°C in a container with desiccant.

Table 3: GMPCPP Seed Mix

Click here to access/download;Table;Table3_GMPCPPseedmix.xlsx

Reagent	**Bright mix (µL)**	**Order of addition**	**Bright mix + biotin (µL)**	**Order of addition**
Fluorescent tubulin, 10 mg/mL	2	6	2	7
Biotin-tubulin, 10 mg/mL	0	N/A	2	6
Unlabeled tubulin, 10 mg/mL	18	5	18	5
GMPCPP, 10 mM	30	4	30	4
DTT, 0.2 M	0.7	3	0.7	3
5X BRB80	26.4	2	26.4	2
sterile water	54.9	1	52.9	1
Total Volume (µL)	**132**		**132**	

Table 4: Assay Steps

Step	Reagent	Volume (µL)	Incubation time (minutes)
1	Neutravidin	7.5	5
2	MB-cold	10	-
3	κ-casein	7.5	2
4	MB-warm	10	-
5	Biotinylated microtubule (diluted in MB-warm)	10	10
6	MB-warm	10	-
7	Warm κ-casein	7.5	2
8	2 nM PRC1 diluted in κ-casein	10	5
9	Non-Biotinylated Microtubule	10	10
10	MB-warm x 2	10	-
11	Assay mix	10	-
Attached seeds are stable for around 20 minutes at this point			

Table 5: Soluble Tubulin Mix

Click here to access/download;Table;Table5_SolubleTubulinMix.xlsx

Reagent	**Volume (µL)**
Recycled tubulin, 10 mg/mL	10
MB-Cold	10.3
MBMC	13.7
BRB80-DTT	3.4
GTP, 10 mM	6.7
ATP, 10 mM (If using kinesins)	6.7
Fluorescently labeled tubulin, 10 mg/mL	1 (Resuspend lyophilized labeled tubulin in cold BRB80-DTT)

Table 6: Assay Mix

Reagent	**Volume (µL)**
Soluble tubulin mix	4
OSF	1
Trolox (if using microtubules labeled with a readily-photobleaching fluorophore)	1
ATP, 10 mM (If using kinesins)	1
PRC1 (or crosslinker of choice)	1
Proteins of interest	X
MB-cold	2-X

Dear Dr. Subramanian,

Your manuscript, JoVE63377 "Simultaneous visualization of the dynamics of crosslinked and single microtubules by TIRF microscopy," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by Nov 19, 2021.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Vidhya Iyer, Ph.D.
Review Editor
JoVE
vidhya.iyer@jove.com
617.674.1888
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We thank the editor and the reviewers for reviewing the manuscript, and for their overall positive assessment of our protocol. Their suggestions and comments have been very useful in improving the clarity of our instructions.

Please revise the manuscript to thoroughly address the reviewers' concerns and all the editorial comments. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:
Changes to be made by the Author(s):

Thank you for the formatting clarifications. All the points raised below have now been addressed in the revised manuscript.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please provide an email address for each author.
3. The Protocol should contain only discrete action items that direct the reader to do something without large paragraphs of text between sections or references to figures and tables that are

- not accompanied by other actions.

- Ensure that you refer to figures, tables, and video in the text.

If you want to draw the reader's attention to the tables with solutions, buffers etc, introduce just one note in the beginning of the protocol and say something like "See Table 1, Table 2, and Table 4 for buffers, protein reagents, and chemical reagents, respectively."

- Delete the blank headings like 1.1 and 1.4 that have only have the table references but no action steps. Leave the table and figure references in the step if you also have an action step like you have for 1.3. In this case, please remember to cite all tables and figures in the order of appearance and renumber your tables and figures if you move the reference to the beginning of the protocol.

We have now done this.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: milli-Q; Hellmanex III detergent; Vectabond; Kimwipes; Jena Bio; Cytoskeleton XYZ; TLA120.2 rotor; Beckman-Coulter 343776; Valap etc

We have now done this.

5. Please transfer all materials in step 3.1.1 to the appropriate table and delete this section from the text.

We have now done this.

6. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm), e.g., step 3.2.6, 3.2.8….

We have now done this.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have now done this.

8. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

We have now done this.

9. In case you would like to include the polymerization steps in the video and still ensure that you don't exceed three pages of highlighted text, please combine some of the shorter highlighted Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step and don't highlight references to and placeholders for figures and tables.

We have highlighted steps 3.3 through 6.6 for inclusion in the video.

10. Please include table legends in the figure and table legends section after the representative results section.

We have now done this.

11. Consider combining some of the smaller, related tables IF POSSIBLE to reduce the number of tables. If you do this, remember to change table references.

Tables 2, 3, and 4 have been combined into Table 2, and Tables 5, 6, 7, and 8 have been renumbered, respectively, to Tables 4, 3, 5, and 6.

12. Please obtain explicit copyright PERMISSION to reuse figures or tables or videos from a previous publication, in this case, your video from the Nature Chemical Biology 2021 paper. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have requested copyright permission, and it will be uploaded as soon as it is received.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have now added more details about challenges, limitations, and ways to extend the protocol. These modifications also address concerns and comments of reviewer 1.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

The references section has been adjusted to match the requested citation style.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this protocol, Mani et al describe the procedures of imaging microtubule dynamic instability of single and crosslinked microtubules simultaneously using TIRF microscopy. Currently, many laboratories are using TIRF microscopy to study microtubule dynamics in vitro. However, the protocol presented here differentiates form conventional protocols by the formation of microtubule-seeds in the presence of a microtubule bundling protein (in this specific case: PRC1), which in turn results with a fraction of the surface-immobilized microtubules forming microtubule bundles. Addition of fluorescently labeled free tubulin dimers and microtubule associate proteins of interest to this imaging chamber allow to study regulation of microtubule

dynamic stability both of single and crosslinked microtubules. The variation of the protocol presented here is physiologically relevant because in the cell different population of microtubules (single and crosslinked) coexist in very close proximity distances. Importantly, authors describe this protocol with high experimental details, so I highly recommend this manuscript for publication in JoVE. However, there are few points that authors should address for publication:

We thank the reviewer for their overall positive assessment of our manuscript, and for recommending it for publication.

1. PRC1 favors the formation of microtubule bundles with antiparallel orientation. This is the common microtubule orientation found in the midzone of the mitotic spindle. However, in interphase, microtubules can be bundled by other MAPs with uniform orientation (i.e microtubules in axons). Authors need to describe and discuss the orientation properties of PRC1-mediated microtubule bundles and the limitations that this can cause in data interpretation. To overcome this limitation, the authors can speculate the use of alternative MAPs as microtubule-bundle tools that potentially could generate uniform-oriented microtubule bundles.

We have now added the following lines in the Introduction (Lines 68-71):

"We describe a method to simultaneously view the collective activity of multiple MAPs on single microtubules, and on microtubule bundles crosslinked by the mitotic spindle-associated protein PRC1, which preferentially binds to and cross-links anti-parallel microtubules."

In the Discussion, we speculate on the use of alternate MAPs to generate bundles of parallel microtubules (Lines 468-488):

"In order to extend this experimental method for the *in vitro* reconstitution of multiple microtubule-based structures, the following key issues need to be addressed: (i) PRC1 preferentially crosslinks microtubules that are oriented anti-parallel to each other. While such bundles are found at the cell center during mitosis, bundles of parallel microtubules are a common feature in other microtubule-based structures such as neuronal axons and the mitotic spindle. The protocol described above can be readily adapted to generate cross-linked parallel microtubules using recombinant crosslinkers such as Kinesin-1 and TRIM46. (ii) In these reconstitution assays, differences in fluorescence intensity can be used to distinguish between single microtubules and pairs of microtubules. Under our experimental conditions, intensity analyses indicate that most bundles contain two cross-linked microtubules, and line scan analyses provide information on their relative positioning. However, when there are more than two or three filaments in a bundle, the spatial resolution of standard TIRF-based imaging systems hinders identification of the ends and polarity of individual microtubules (~25 nm diameter). Moreover, while it is possible to identify the plus-ends of cross-linked anti-parallel microtubules from the direction of their growth, distinguishing plus-ends of cross-linked parallel microtubules growing in the same direction presents a challenge. An extension of the experiment described here is to mark the ends of microtubules using standard microtubule tip-binding proteins to position individual microtubules. For bundles with tens of microtubules, complementing the high temporal resolution of TIRF-based assays with techniques that have high spatial resolution, such as Atomic Force Microscopy, promises to yield new insights into the dynamics of individual microtubules within a bundle."

2. What is the number of expected microtubules in PRC1-mediated microtubule bundles? I guess that the experimental conditions (low PRC1 concentration) were selected to preferentially generate microtubules bundles containing 2 microtubules coexisting with single microtubules. Do the authors find microtubule bundles formed with more than 2 microtubules using their experimental conditions? If so, authors should explain how they differentiate among microtubule bundles containing different number of microtubules.

Under our experimental conditions, the majority of bundles do indeed contain 2 anti-parallel microtubules, based on intensity analyses. This is optimized through titrations of PRC1 and non-biotinylated microtubule concentrations.

We have indicated the limitations of TIRF-based experiments and analyses in identifying the relative positions and orientations of individual microtubules within a bundle containing more than 2 or 3 microtubules, in Lines 476-488 in the Discussion:

"Under our experimental conditions, intensity analyses indicate that most bundles contain two cross-linked microtubules, and line scan analyses provide information on their relative positioning. However, when there are more than two or three filaments in a bundle, the spatial resolution of standard TIRF-based imaging systems hinders identification of the ends and polarity of individual microtubules (~25 nm diameter). Moreover, while it is possible to identify the plus-ends of cross-linked anti-parallel microtubules from the direction of their growth, distinguishing the ends of cross-linked parallel microtubules growing in the same direction is hindered by the spatial resolution of optical microscopy. An extension of the experiment described here is to use polarity-marked microtubules or microtubule tip-binding proteins to position individual microtubules. For bundles with tens of microtubules, complementing the high temporal resolution of TIRF-based assays with techniques that have high spatial resolution, such as Atomic Force Microscopy, promises to yield new insights into the dynamics of individual microtubules within a bundle."

We have also added the following instructions in Lines 380-383 of Representative Results:
"Adjust LASER intensity for the excitation channels correspond to microtubule fluorescence, to ensure that differences in fluorescence intensity between single microtubules and bundles can be captured within the dynamic range of the detector."

3. Purified PRC1 is a critical component responsible to form microtubule bundles in this protocol. The authors do not describe the source of this protein. Is it commercially available or is it purified in their lab? If purified, they should describe the purification protocol. If this protocol is out of the scope of this paper, at least they should reference articles which describe the purification protocol of PRC1 highly detailed.

We have now cited a reference with a detailed protocol for the expression and purification of PRC1 in step 5.9 of the protocol:

"Recombinant PRC1 can be expressed and purified from bacterial cells as preciously described. [13]"

Reference 13: Subramanian, R. *et al.* Insights into antiparallel microtubule crosslinking by PRC1, a conserved nonmotor microtubule binding protein. *Cell.* **142** (3), 433-443, doi:10.1016/j.cell.2010.07.012, (2010).

4. It will be very useful if the authors include a representative example of this assay when no additional MAPs (no CLASP1 and Kif4A) are added. It would be very useful for other researches if authors describe their quantification strategy used to analyze the movies generated using this protocol.

We have now added a representative video (Video 1) showing the dynamics of single and cross-linked microtubules with no additional MAPs (no CLASP1 or Kif4A) present.

We have also added a paragraph about our quantification strategy in the Representative Results section (Lines 392-398):

"For quantitative analysis of microtubule dynamics, open microscopy files in the FIJI software, and select single microtubules and bundles for analysis. Use the following criteria to exclude single microtubules and bundles from further analysis: exclude microtubules or bundles (i) found at the edges of the field of view, (ii) obscured by protein aggregates, or (iii) whose filaments move in the z-direction out of the TIRF range. Parameters of microtubule dynamics, such as length, growth rate, rescue frequency, and catastrophe frequency, can be obtained by constructing and analyzing kymographs for each single microtubule or microtubule pair."

Reviewer #2:

Manuscript Summary:

This manuscript describes a clever approach to simultaneously visualize different microtubule populations by TIRF microscopy, in particular ones that are crosslinked versus single ones. Most of the protocol is very detailed, the complicated mixing steps are well explained, and the tables are very helpful. I think this topic is well suited for a JoVE article.

We thank the reviewer for their overall positive assessment of our manuscript.

Major Concerns:

None.

Minor Concerns:

1. The manuscript would benefit from a short overview, probably in the introduction, about what the major steps or the protocol are and why they are being done. I was a bit lost about the direction this was taking as I read the manuscript and had to discover from context why certain steps were being done.

We agree with the reviewer that providing a short overview of the experiment at the beginning adds more clarity to the protocol. We have now added a paragraph in the introduction (Lines 71-78) summarizing the various steps of the entire protocol:

"Briefly, this protocol consists of the following steps: (i) Preparation of stock solutions and reagents; (ii) Cleaning and surface treatment of cover-slips used to create the imaging chamber for microscopy experiments; (iii) Preparation of stable microtubule "seeds" from which polymerization is initiated during the experiment; (iv)

Specification of TIRF microscope settings to visualize microtubule dynamics; (v) Immobilization of microtubule seeds and generation of crosslinked microtubule bundles in the imaging chamber; (vi) Visualization of microtubule dynamics in the imaging chamber through TIRF microscopy, upon addition of soluble tubulin, MAPS, and nucleotides."

2. It would be helpful to have a description somewhere which steps can be done ahead of time and for how long. For example, how long ahead of time can the coverslips be cleaned? And I would like the note after 2.2.6 to come earlier. Otherwise, if you follow the procedure step by step, you will suddenly learn that you should have prepared those aliquots ahead of time.

We have now provided information about how far in advance each reagent can be prepared and stored. This information is contained in a new column labeled "Recommended Storage Duration" in Tables 1 and 2. Further, we have highlighted which steps can be done ahead of time in *Steps 2, 3.1, 5.1*.

3. There is a possible confusion about slides versus coverslips. If I understand correctly, the imaging chamber is assembled from different sizes of coverslips and that apart from 3.3.1 there are not traditional glass slides used. However, the text refers to "slides" multiple times, in cases that seem to refer to either coverslips or the imaging chamber. The authors should make sure that their nomenclature is consistent throughout.

We now use consistent nomenclature throughout the manuscript. We refer to both sets of glassware used to prepare imaging chambers (24x60 mm #1.5 coverslips and 18x18 mm #1.5 coverslips) as coverslips, and the glassware used to visualize and check microtubules after polymerization (Microscope Slides, Diamond White Glass, 25 x 75mm, 90° Ground Edges, WHITE Frosted), as microscope slide. Coverslips are pictured in Figure 1F.

4. The following instances require a bit more detail:

2.1.1. It would be helpful to know what this slide holder looks like. It is not part of the reagent/materials list.

The slide holder where the imaging chamber is placed for the experiment is shown in Figure 1H.

2.1.2. How is excess liquid removed by vacuum? In an evacuated chamber?

We have clarified: "Rinse all coverslips five to six times in ultrapure water of resistivity 18.2 MΩ-cm, removing excess liquid after each rinse with a pipette tip attached to a vacuum tube (Figure 1C)."

2.1.3 What does "containers sealed" refer to? There is no mention of containers before.

The slide-washing racks and slide-staining jars are pictured in Figure 1A-1B. We have clarified what we mean by sealed containers with the line: "Fill beakers and slide-staining jars with water, seal with parafilm, and sonicate for 10 minutes."

2.1.4 How big are the beakers/volume of ethanol used? How are the coverslips held while being dipped? Via tweezers or gloved hands or in the slide holder?

We have clarified: "Fill two 150 mL beakers with 200-proof ethanol. Use tweezers to dip each cover slip into one beaker filled with ethanol and then the other."

2.1.5 How do you dry with nitrogen gas and what is the incubator? Is it heated? How long a drying time is needed?

We have clarified: "Transfer coverslips to slide-drying rack, spray with nitrogen gas stream, and incubate at 37°C until completely dry (~15 minutes)."

We added the model of benchtop incubator to our Materials list.

2.1.6 It would be helpful to have a bit more context here. If the reader doesn't have access to the exact same plasma cleaner, the reference to "once purple light is visible" is not helpful. Consider explaining why this is done so that a reader has a chance to accomplish the same thing with a different brand of plasma cleaner.

We have clarified: "Once dry, place coverslips in a single layer inside the plasma cleaner. Form vacuum seal, then set the Radio Frequency (RF) level of the plasma cleaner to Low. Once plasma is generated, leave coverslips in plasma cleaner for five minutes."

2.2.1: In what type of container are the coverslips incubated in? Are they still in a holder or are they transferred individually? If individually, how are they grabbed to move around?

We have clarified: "Immediately before use, dissolve 400 µL of 3-Aminopropyltriethoxysilane in 40 mL of acetone. Use tweezers to move individual plasma-cleaned coverslips into slide-washing rack and slide staining jars. Submerge coverslips in 3-Aminopropyltriethoxysilane solution and incubate for five minutes"

2.2.3. What is this rack, how are they sprayed with nitrogen, and where are they incubated?

The slide-drying rack is pictured in Figure 1D.

We have clarified: "Transfer coverslips to slide-drying rack, spray with nitrogen gas stream, and incubate at 37°C until dry (~20 minutes)."

We added the model of benchtop incubator to our Materials list.

2.2.7: What type of centrifuge is being used?

We have clarified "benchtop microcentrifuge." We added the model used to our Materials list.

2.2.11: What is this "tip rack"?

Step 2.2.9 now describes the preparation of a hydration chamber from an empty tip box, as pictured in Figure 1E, and we have clarified that the tip rack mentioned is part of the hydration chamber.

Step 2.2.9: "Prepare a hydration chamber. One way to construct a hydration chamber is by placing wet paper towels beneath the tip rack in the bottom of an empty 10µL tip box (Figure 1E)."

2.2.15: Viewers would benefit from seeing this step in the video.

We have now rewritten step 2.2.15 for more clarity: "To construct imaging chamber, stick three strips of double-sided tape on 24x60 mm coverslip, on side labeled 'b'. To the other side of tape strips, attach a 18x18 mm coverslip with its side labeled 'P' facing the larger coverslip. This forms two lanes for microscopy experiments, with treated surfaces facing each other (Figure 2 and Figure 1G)."

Additionally, this step is now displayed in Figure 2 and Figure 1G.

Line 213: What are old seeds? I.e., does this refer to the time they were stored at -80°C or time they spent after being thawed?

We have now clarified: "NOTE: If seeds fail to polymerize, troubleshoot by supplementing initial dilution with 0.5 µM GMPCPP."

5.1 "Tape a prepared glass slide to a slide holder" Is this "slide" one of the imaging chambers? And I suspect that the "slide holder" in this step is different from the slide holder in step 2.1.1. Please clarify.

We have now used consistent nomenclature throughout the manuscript. The slide-holders mentioned in 2.1.1 are now referred to as slide-staining jars and slide-washing rack to distinguish them from the slide-holder mentioned in 5.1. These items are pictured in Figure 1.

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