

# Assembling microtubule-based active matter

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## Running head: Microtubule active matter

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### **Abstract**

Studied for more than a century, equilibrium liquid crystals provided insight into the properties of ordered materials, and led to commonplace applications such as display technology. Active nematics are a new class of liquid crystal materials that are driven out of equilibrium by continuous motion of the constituent anisotropic units. A versatile experimental realization of active nematic liquid crystals is based on rod-like cytoskeletal filaments that are driven out of equilibrium by molecular motors. We describe protocols for assembling microtubule-kinesin based active nematic liquid crystals and associated isotropic fluids. We describe the purification of each protein and the assembly process of a 2-dimensional active nematic on a

water-oil interface. Finally, we show examples of nematic formation and describe methods for quantifying their non-equilibrium dynamics.

## **1. Introduction**

Microtubules and their associated proteins play a vital role in a variety of essential cellular functions, ranging from long-ranged intercellular transport to formation of self-organized dynamical structures such as the mitotic spindle [1]. Quantifying these *in vivo* processes is challenging due to the inherent complexity of many interdependent pathways that simultaneously take place within a cell. Minimal *in vitro* microtubule systems aim to recreate the above-described biological processes with a minimal number of essential components, thus allowing for quantitative study and modeling of the mechanical processes that drive the cell motility and division. Furthermore, these reconstituted systems provide an experimental platform for developing biologically inspired materials with tunable dynamical properties that mimic some basic biological functionalities. From this perspective reconstituted microtubule-molecular motors fluids have served as versatile model experimental systems for the study of active matter – materials which are collectively driven away from equilibrium by energy consuming microscopic constituents [2, 3]. Microtubule based active matter systems exhibit diverse emergent behaviors ranging from dynamical aster formation [4], extensile isotropic active networks that generate turbulent flows [5] to orientationally ordered active nematic liquid crystals in both 2 and 3 dimensions [6, 7]. Active nematic liquid crystals have been of particular interest because of a wealth of theoretical work on systems with a well-defined director field [3, 8–14]. In this chapter, we describe experimental protocols for assembling microtubule based active isotropic fluids and nematic liquid crystal [6]. Being composed of well-studied biological components these materials allow for systematic control of the emergent mechanical and dynamical properties [9, 11, 15, 16].

## 2. Tubulin

Filamentous microtubules spontaneously assemble from alpha-beta tubulin dimers in the presence of GTP. Tubulin purification consists of several consecutive steps of temperature sensitive microtubule polymerization- and depolymerization cycles followed by differential centrifugation [17]. We divide the purification process into three steps: **2.1** tubulin purification, **2.2** tubulin recycling and **2.3** tubulin labeling with fluorescent dye or other functionalities. These three steps yield high purity tubulin monomers that readily polymerize *in vitro*.

### 2.1 Tubulin Purification

We describe the purification of tubulin from calf brain using two cycles of temperature sensitive polymerization-depolymerization [17]. The protocol yields pure alpha-beta tubulin heterodimers that can be stored at -80 °C for many years. This protocol takes **16-24 hours**. It is not advisable to pause at any point.

## MATERIALS

Obtain calf, cow or pig brains directly from a slaughterhouse. It is *vital* to put the brains *immediately* on ice in PBS buffer after the cow is slaughtered. Every minute counts [18]. It is helpful to find a slaughterhouse that has a scientific liaison and will work with you. The speed of this transfer will influence the final tubulin yields.

### Buffers

1. **Phosphate Buffered Saline (PBS)** - Make 2 L per kg of brain tissue. Used for storage of the brain tissue during transport from the slaughterhouse. Dissolve 20 mM Na-phosphate and 150 mM NaCl in water. Adjust pH to 7.2 with NaOH. Store at 4 °C.
2. **Depolymerization Buffer (DB)** - Make 1 L per kg of brain tissue. Dissolve 50 mM MES (2-[N-morpholino]ethanesulfonic acid) and 1 mM CaCl<sub>2</sub> in water. Adjust pH to 6.6 with 10 N HCl. Store at 4 °C.
3. **High Molarity PIPES Buffers (HMPB)** - Make 2 L per kg of brain tissue. Mix 1 M PIPES, 10 mM MgCl<sub>2</sub>, and 20 mM EGTA. The PIPES will not fully dissolve until the pH is adjusted. Begin by adjusting the pH with solid KOH (~50 g) and then 10N KOH solution. Store at 4 °C.

### Reagents

1. **100 mM Adenosine triphosphate (ATP)** - Use 61 mL 100 mM ATP per kg of brain tissue (3.3 g ATP/kg of brain tissue). Dissolve 100 mM ATP in water. Adjust pH to 7.4 with NaOH. ATP has a short half-life in solution, so prepare this solution the night before or the day of the purification. Store at -20 °C overnight, 4 °C after thaw.

2. **200 mM Guanosine triphosphate (GTP)** - Use 10 mL GTP per kg of brain tissue (1 g GTP/kg of brain tissue). Dissolve 200 mM GTP in water. Adjust pH to 7.4 with NaOH. GTP has a short half-life in solution, so make this suspension the night before or the day of the purification. Store at  $-20\text{ }^{\circ}\text{C}$  overnight,  $4\text{ }^{\circ}\text{C}$  after thaw.
3. **Glycerol** - 2 L per kg of brain tissue.

### Equipment

1. Serological pipette, 50 mL and 15 mL douncers (optional), heat bath, spectrometer, cold room, liquid nitrogen,  $-80\text{ }^{\circ}\text{C}$  freezer.
2. Rotor 1: Centrifuge with capacity 1 L per kg of brain and minimum centrifugation force of 17,700 g at  $r_{\text{max}}$  with temperature control ( $4\text{ }^{\circ}\text{C} - 37\text{ }^{\circ}\text{C}$ ), e.g. Beckman Coulter Avanti J-25 centrifuge with JA-10 rotor.
3. Rotor 2: Ultracentrifuge with capacity of  $\sim >400$  mL and minimum centrifugation force of 151,000 g at  $r_{\text{max}}$  with temperature control ( $4\text{ }^{\circ}\text{C} - 37\text{ }^{\circ}\text{C}$ ), e.g. Beckman Coulter Optima LE-80K with Ti-45 rotor.
4. Appropriate centrifuge tubes for high-speed spins. Consult the manual for your rotor.

### METHODS

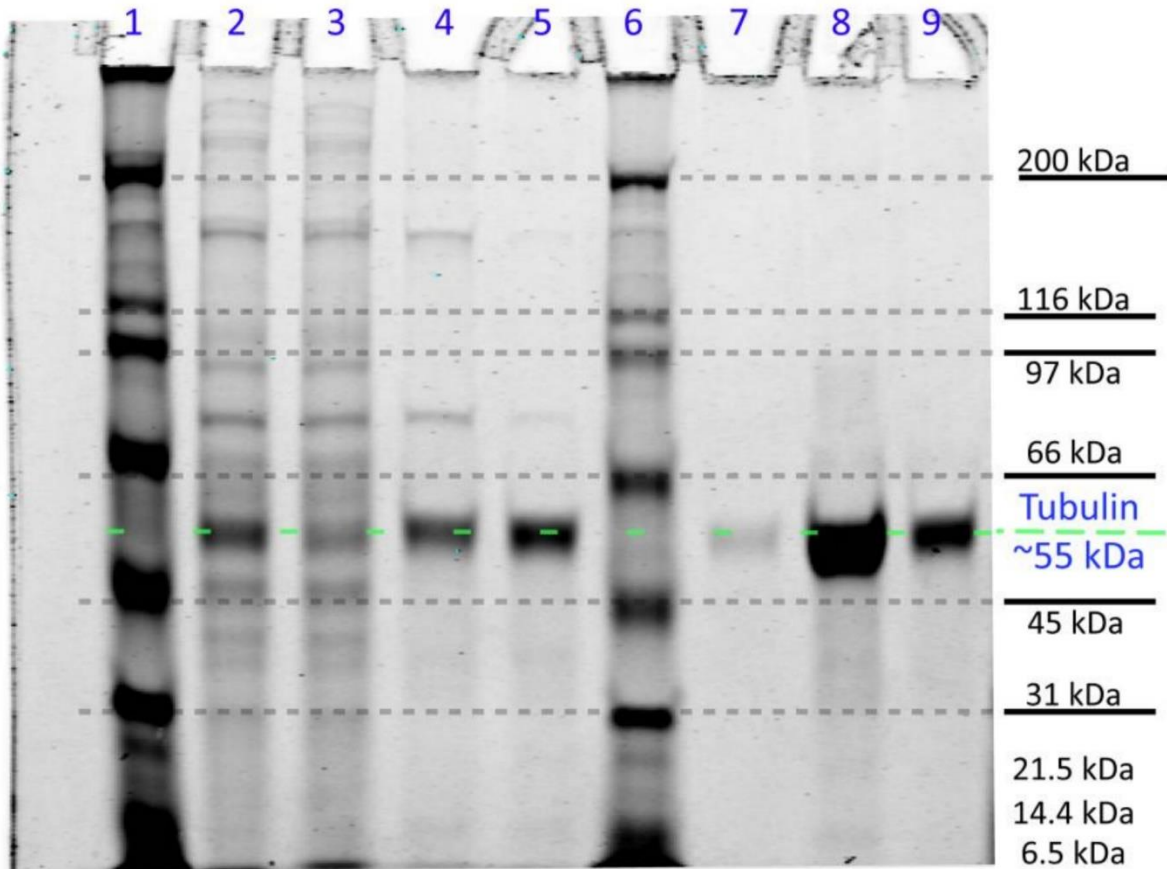
1. Prepare brains for centrifugation. This step needs to be done in a cold room at  $4\text{ }^{\circ}\text{C}$ . Keep the brains cold while handling. **(COLD)**
  - a. Clean the brains by removing any membrane material, blood vessels and cutting away fat. Razor blades and paper towels are very useful at this step.
  - b. Weigh the cleaned brains. Add 1 L of DB/ 1 kg of clean brain. Blend for 30 s in a blender until smooth.
2. Pellet large contaminants. **(COLD)**
  - a. Have a rotor 1 chilled to  $4\text{ }^{\circ}\text{C}$ . Pour pureed brains into centrifuge tubes. Ensure bottles are weight-balanced. Spin at 17,700 g for 45 min (rotor 1).
  - b. Recover supernatant into a fresh centrifuge tube using a serological pipette. The pellet will be extremely soft—avoid agitation.
  - c. Spin the recovered supernatant at 17,700 g for 20 min (rotor 1).
  - d. Optional: keep 5  $\mu\text{L}$  of supernatant in a tube labeled “step 2” for SDS-PAGE gel.
3. Polymerize microtubules (MTs). **(WARM)**
  - a. Recover supernatant into a beaker from the final spin above. It should be a mostly clear pink solution.
  - b. Add equal volume of warm HMPB (37C). Add ATP to 1.5 mM final concentration. Add GTP to 0.5 mM final. Add supernatant volume of warm glycerol (37C).
  - c. Incubate at  $37\text{ }^{\circ}\text{C}$  for 1 hr. The mixture should turn cloudy after the addition of ATP and GTP.
  - d. Spin the mixture at 150,000 g for 30 min at  $37\text{ }^{\circ}\text{C}$  to pellet the MTs. Often this will require multiple spins. Continue to pellet into the same tubes, throwing away the supernatant after each spin (rotor 2).
  - e. Optional: keep 5  $\mu\text{L}$  of supernatant in a tube labeled “step 3” for SDS-PAGE gel.
4. Depolymerize MTs. **(COLD)**

- a. Discard supernatant. Add cold DB to each tube so that the pellet can be immersed in buffer.
  - b. Allow the pellets to chill on ice for 30 min. Using a 1ml pipette with 1/3 of the tip cut-off resuspend the pellet. If available, a dounce homogenizer can be used to aid resuspension as well. Be cautious to not generate bubbles from either method. The solution should be clear when the tubulin is fully depolymerized.
  - c. Spin the tubulin at 70,000 g for 30 min at 4 °C (rotor 2).
  - d. Optional: keep 5 µL of supernatant in a tube labeled “step 4” for SDS-PAGE gel.
5. Polymerize MTs. (**WARM**)
- a. Recover supernatant into a container from the spin in #4. It should be a clear solution.
  - b. Add equal volume of warm HMPB (37 °C). Add ATP to 1.5 mM final concentration. Add GTP to 0.5 mM final concentration. Add supernatant volume of warm glycerol (37 °C).
  - c. Incubate at 37 °C for 1 hr. The mixture should turn cloudy after the addition of ATP and GTP.
  - d. Spin the mixture at 150,000 G for 30 min at 37 °C to pellet the MTs. Often this will require multiple spins. Continue to pellet into the same tubes if needed, throwing away the supernatant after each spin (rotor 2).
  - e. Optional: keep 5 µL of supernatant in a tube labeled “step 5” for SDS-PAGE gel.
6. Depolymerize MTs (**COLD**)
- a. Discard supernatant. Add cold DB to each tube so that the pellet can be immersed in buffer.
  - b. Allow the pellets to chill on ice for 30 min. Using a pipette with a tip cut so that the diameter is large (5-10 mm), resuspend the pellet. If available, a dounce homogenizer can be used to aid resuspension as well. Be cautious to not generate bubbles from either method. The solution should be clear when the Tubulin is fully depolymerized.
  - c. Spin the tubulin at 100,000 g for 30 min at 4 °C (rotor 2 or rotor 3).
  - d. Optional: keep 5 µL of supernatant in a tube labeled “step 6” for SDS-PAGE gel.
7. Measure concentration and store raw tubulin (**COLD**)
- a. Recover supernatant and pool into one container. Keep on ice. Mix gently.
  - b. Measure the concentration using a spectrometer. The extinction coefficient of tubulin at 280nm is 115,000 M<sup>-1</sup>cm<sup>-1</sup>. Tubulin is stable at -80 °C even at concentrations >30 mg/mL. However, depending on your future needs, you can dilute it to a convenient concentration for your experiments. We recommend 10-25 mg/mL. \*Tip: It is important to blank the spectrophotometer with a tubulin buffer .
  - c. Aliquot into conical 1.5 mL Eppendorf tubes at convenient volumes (typically 1 mL). Use liquid nitrogen to flash freeze and store at -80 °C. Under these conditions tubulin remain stable indefinitely.

## NOTES

- For troubleshooting, run an SDS-PAGE gel on the supernatant obtained after each spin cycle. A representative example is shown in Fig. 1.
- This protocol uses temperature as well as buffer conditions to cycle the tubulin between polymerized and depolymerized state. Make sure to have centrifuge rotors and buffers pre-chilled/pre-warmed for each step.
- To minimize centrifuging time, use minimal volumes for resuspension.
- A high-quality blender is advised to minimize heat introduced at this critical step.
- From 1 kg of brain tissue, we typically obtain 600-700 mg tubulin.

- Previous protocols included ion-exchange chromatography to remove MAPs. However, using the above protocol is more efficient and has no discernible effect in our systems.



**Figure 1: SDS Page gel of tubulin purification process.** (1) Protein standard. (2) Supernatant from step 2. (3) Supernatant from step 3. (4) Pellet from step 3 resuspended in DB. (5) Supernatant from clarification step 4. (6) Protein standard. (7) Supernatant from step 5. (8) Pellet from step 5 resuspended in M2B. (9) Supernatant from clarification step 6.

## 2.2 Tubulin Recycling

Tubulin recycling is an additional cycle of polymerization-depolymerization that removes aggregates and tubulin monomers that are unable to polymerize and will contaminate the sample [19]. This protocol takes **3 hours**.

### MATERIALS

Raw tubulin from a tubulin purification or ordered from a supplier (PurSolutions or others).

### Buffers

1. **Magnesium 2X Buffer (M2B)** - Need 20 mL per 1 mL of raw tubulin. Dissolve 80 mM PIPES, 2 mM  $\text{MgCl}_2$  and 1 mM EGTA in water. Adjust pH to 6.8 with KOH. Use a 0.2  $\mu\text{m}$  filter to sterilize the buffer. Store at 4 °C.
2. **Cushion** - 1 mL for each centrifuge tube used during spinning. The cushion will help keep small, unpolymerized tubulin aggregates in the supernatant above the glycerol cushion from the final pellet. Mix 60% (v/v) glycerol with M2B.

### Reagents

1. **Dithiothreitol (DTT)** - Need 1  $\mu\text{L}$  per 1mL raw tubulin. Dissolve 0.5 M DTT in M2B. Store at -20 °C.
2. **GTP** - Need 20  $\mu\text{L}$  per 1mL of raw tubulin. Dissolve 100 mM GTP in M2B. Store at -20 °C. GTP has a short solution half-life, so make this the night before or the day of recycling.

### Equipment

1. Ultracentrifuge capable of speeds of 350,000 g at  $r_{\text{max}}$  with temperature control (4 °C to 37 °C), e.g. Coulter Beckman Optima with TLA-100.4.
2. UV-Vis Spectrometer.

### METHODS

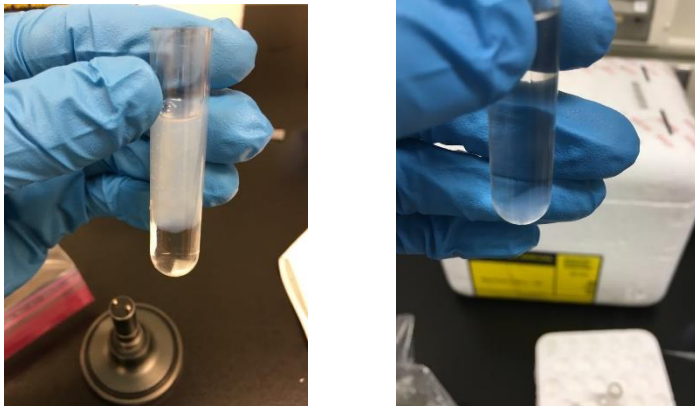
1. Polymerize microtubules (MTs). (**WARM**)
  - a. Combine 1 mL of tubulin with 1  $\mu\text{L}$  of 0.5 M DTT and 20  $\mu\text{L}$  of 100 mM GTP. Mix thoroughly with a pipette or gentle vortexing.
  - b. Place in a 37 °C water bath for 30 min.
2. Pellet the MTs. (**WARM**)
  - a. Prepare a centrifuge tube with 1 mL of warm cushion.
  - b. Gently pipette the MTs on top of the cushion (Fig. 2).
  - c. Prepare a counterweight.
  - d. Spin for 30 min at 350,000 RCF at 37 °C.
3. Rinse away any contaminants.



- a. Remove supernatant and cushion (Fig. 2).
- b. Rinse the pellet with 2 mL of warm M2B 2-3 times to remove contaminants on tube walls.
4. Depolymerize MTs. **(COLD)**
  - a. Add cold M2B to cover the pellet (~100  $\mu$ L).
  - b. Place rotor in 4 °C fridge to chill.
  - c. Keep the pellet on ice and mix with a cut pipette time every 5 min for 15 min. Switch to an uncut tip when the pellet is thin and continue mixing every 5 min until the solution is clear, usually another 15 min. \*If pellet does not thin, add more cold M2B.
5. Clarification spin. **(COLD)**
  - a. Place the depolymerized tubulin in a centrifuge tube in the cold rotor.
  - b. Prepare a counterweight.
  - c. Spin for 10 min at 350,000 RCF at 4 °C.
6. Measure concentration and store. **(COLD)**
  - a. Recover supernatant and pool into one container. Keep on ice. Mix gently.
  - b. Measure the concentration using a spectrometer. The extinction coefficient at 280nm, of tubulin is 115,000  $cm^{-1}M^{-1}$  Tubulin can be stored >30 mg/mL. If desired, dilute to convenient concentration.
  - c. Aliquot into freezer tubes at convenient volumes (typically 50  $\mu$ L). Use liquid nitrogen to flash freeze and store at -80 °C indefinitely.

## NOTES

At all points in this protocol, it is important to minimize bubbles. Be especially careful during resuspension.



**Figure 2:** (left) Polymerized microtubules on a glycerol cushion before spinning. (right) Pelleted microtubules after spin.

## 2.3 Tubulin Labelling

We describe a protocol to label the primary amine of tubulin monomers using the common reactional group N-Hydroxysuccinimide (NHS) [19]. The same protocol enables labelling with a variety of tags for different functional purposes. Most commonly, one uses a fluorescent tag to visualize MTs with optical microscopy. One can also use NHS linked to biotin to enable attachment of streptavidin beads [20]. Finally, one can use the same protocol to label microtubules with benzylguanine to subsequently attach diverse SNAP-tag labeled proteins, such as kinesin motors [21].

This protocol takes **4 hours**.

### MATERIALS

1. NHS ester fluorescent dye or other tag (e.g. Alexa Fluor 647 Carboxylic Acid Succinimidyl Ester, Alexa Fluor 568 Carboxylic Acid Succinimidyl Ester).
2. Anhydrous DMSO.
3. 1 mg dye per ~10 mg raw tubulin.

### Buffers

1. **10X Magnesium 2X Buffer (M2B)** - Dissolve 800 mM PIPES, 20 mM MgCl<sub>2</sub> and 10 mM EGTA in water. Adjust pH to 6.8 with KOH. Use a 0.2 μm filter to sterilize the buffer. Store at 4 °C.
2. **Labeling buffer** - Need 30 μL/mg tubulin. Mix 40% (v/v) glycerol with water. Dissolve 0.1 M NaHEPES, 1 mM MgCl<sub>2</sub> and 1 mM EGTA into the 40% glycerol mixture. Adjust pH to 8.6 with KOH.
3. **High pH cushion** - Need 1 mL for each centrifuge tube needed for pelleting. The cushion aids in buffer exchange during the pelleting spin and helps to separate small unpolymerized tubulin aggregates in the supernatant above the glycerol cushion from the final pellet. Mix 60% (v/v) glycerol with water. Dissolve 0.1 M NaHEPES, 1 mM MgCl<sub>2</sub> and 1 mM EGTA into the 60% glycerol mixture. Adjust pH to 8.6 with KOH.
4. **Quench buffer** - Need 1 mL for each centrifuge tube. The cushion aids in effective buffer exchange during the pelleting spin. Dilute 10X M2B to 2X M2B in water. Dissolve 100 mM K-Glutamate in 2X M2B. Mix 40% (v/v) glycerol and K-Glutamate solution.
5. **Low pH cushion**- Need 1 mL/centrifuge tube. Mix 60% (v/v) glycerol with M2B.

### Reagents

1. **Dithiothreitol (DTT)** - Dissolve 0.5 M DTT in M2B. Store at -20 °C.
2. **GTP** - Need 40 μL per 1 mL raw tubulin. Dissolve 100 mM GTP in M2B. Store at -20 °C. GTP has a short half-life in solution, so make this the night before or the day of recycling.

## Equipment

1. Ultracentrifuge capable of speeds of 350,000 RCF at  $r_{\max}$  with temperature control (4 °C to 37 °C), e.g. Coulter Beckman Optima with TLA-100.4.
2. UV-Visible spectrometer.

## METHODS

1. Polymerize microtubules (MTs). (**WARM**)
  - a. Combine raw tubulin with DTT to 0.5 mM final and GTP to 0.1 mM final. Mix thoroughly with a pipette or gentle vortexing.
  - b. Place in a 37 °C water bath for 30 min.
2. Pellet the MTs. (**WARM**)
  - a. Prepare centrifuge tube(s) with 1 mL of warm high pH cushion.
  - b. Gently pipette the MTs on top of the cushion.
  - c. Prepare a counterweight.
  - d. Spin for 30 min at 350,000 RCF at 37 °C.
3. Rinse pellet and exchange buffers. (**WARM**)
  - a. Remove supernatant above the cushion(s) using a pipette.
  - b. Rinse the cushion(s) with 1 mL of warm labeling buffer 2-3 times, changing tips each time.
  - c. Remove the cushion(s).
  - d. Rinse the pellet(s) with 1 mL of warm labeling buffer 2-3 times, changing tips each time.
4. Label MTs. (**WARM**)
  - a. Resuspend the pellet(s) in a minimal volume (~30  $\mu$ L/ mg of tubulin) of warm labeling buffer using a cut pipette tip and vortexing if necessary. Keep MTs warm as they will not re-polymerize at the pH of the labeling buffer. Combine all MTs into an Eppendorf tube.
  - b. Suspend dye in 70  $\mu$ L DMSO/ 1 mg of dye.
  - c. Add dye to resuspended MTs.
  - d. Vortex MTs gently every 2 min for 30 min. Keep warm in a water bath at 37 °C when not vortexing.
  - e. Stop the reaction by adding an equal volume of quench buffer to the labeling mixture.
5. Pellet the MTs. (**WARM**)
  - a. Prepare centrifuge tube(s) with 1 mL of warm low pH cushion.
  - b. Gently pipette the MTs on top of the cushion.
  - c. Prepare a counterweight.
  - d. Spin for 30 min at 350,000 RCF at 37 °C.
6. Depolymerize MTs. (**COLD**)
  - a. Add cold M2B to cover the pellet (~100  $\mu$ L).
  - b. Place rotor in a 4 °C fridge to chill.
  - c. Keep the pellet on ice and mix with a cut pipette time every 5 min for 15 min. Switch to an uncut tip when the pellet is thin and continue mixing every 5 min until the solution is clear, usually another 15 min. \*If pellet does not thin, add more cold M2B.
7. Clarification spin. (**COLD**)
  - a. Place the depolymerized tubulin in a centrifuge tube in the cold rotor.

- b. Prepare a counterweight.
- c. Spin for 10 min at 350,000 RCF at 4 °C.
8. Pellet the MTs. (WARM)
  - a. Prepare centrifuge tube(s) with 1 mL of warm low pH cushion.
  - b. Gently pipette the MTs on top of the cushion.
  - c. Prepare a counterweight.
  - d. Spin for 30 min at 350,000 RCF at 37 °C.
9. Depolymerize MTs. (COLD)
  - a. Add cold M2B to cover the pellet (~100 uL).
  - b. Place rotor in a 4 °C fridge to chill.
  - c. Keep the pellet on ice and mix with a cut pipette time every 5 min for 15 min. Switch to an uncut tip when the pellet is thin and continue mixing every 5 min until the solution is clear, usually another 15 min. \*If pellet does not thin, add more cold M2B.
10. Clarification spin. (COLD)
  - a. Place the depolymerized tubulin in a centrifuge tube in the cold rotor.
  - b. Prepare a counterweight.
  - c. Spin for 10 min at 350,000 g at 4 °C. Tip: typical pellet is shown in figure 3.
11. Measure concentration and store.
  - a. Recover supernatant and pool into one container. Keep on ice. Mix gently.
  - b. Measure the concentration using a spectrometer. The extinction coefficient of tubulin is 115,000 M<sup>-1</sup>cm<sup>-1</sup>. Tubulin can be stored >30 mg/mL. If desired, dilute to convenient concentration.
  - c. If using a fluorescent tag, the concentration of the protein and the degree of labeling (DOL) of the dye can be calculated using the formulas below.

$$A_{protein} = A_{280} - A_{647} \cdot CF$$

$$DOL = \frac{\text{mols of dye}}{\text{mols of protein}} = \frac{A_{647} \cdot MW}{C_{protein} \cdot \epsilon_{647}}$$

Where  $A_{280}$  is the absorbance of the labeled protein,  $A_{647}$  is the absorbance at the dye wavelength at 647 nm, CF is the correction factor specific for each dye for Alexa 647 CF = 0.03, MW is the molecular weight of the protein and  $C_{protein}$  is the concentration of the protein,  $\epsilon_{647}$  is the absorbance coefficient of the dye for alexa647  $\epsilon = 239,000 \text{cm}^{-1} \text{M}^{-1}$ . The correction factor and the extinction coefficient can be found from the dye manufacturer. For more details see Thermo Fisher guide (<http://tools.thermofisher.com/content/sfs/brochures/TR0031-Calc-FP-ratios.pdf>).

- d. Aliquot into conical freezer tubes at convenient volumes (typically ~50  $\mu\text{L}$ ). If volume allows, use thin-walled PCR tubes to aid in flash freezing. Use liquid nitrogen to flash freeze and store at -80 °C indefinitely.

#### Notes:

- For experiments in which two-color microtubule imaging is necessary, we recommend labeling tubulin with NHS-Alexa 647 and NHS-Azide. After polymerization (Section 2.6), one can use CLICK chemistry to label Azide microtubules with DBCO-Alexa 488. This

labeling yield microtubules that are more photostable than those directly labelled with NHS-Alexa 488.

- NHS-Alexa 568 is another photostable label for tubulin. However, the excitation and emission spectra of Alexa 647 and Alexa 568 are not well separated so bleed-through is a significant challenge.



**Figure 3:** Pellet of 647 labeled microtubules.

## 2.4 Polymerizing Microtubules

Microtubule-based active nematics are assembled with MTs stabilized with a non-hydrolyzable GTP analog, GMPCPP, which suppresses any tubulin turnover [22]. GMPCPP is a non-hydrolysable GTP analog that allows for long lifetime (days) of polymerized microtubules without tubulin turnover. Tubulin concentration determines the length distribution of the microtubules. For active nematics short microtubule with a typical average length of  $\sim 2\mu\text{m}$  are used. The protocol described here is optimized for polymerizing 8 mg/mL tubulin. It yields MTs with an average length anywhere between 1 and 3  $\mu\text{m}$  [6, 23]. Each polymerization yield MTs with different properties, including their length distribution, which can greatly affect the dynamics of active nematics. Therefore, we recommend using MT from a single polymerization to obtain a set of self-consistent experiments.

## MATERIALS

### Protein

1. Recycled tubulin
2. Labeled tubulin

### Reagents

1. 20 mM DTT in M2B
2. Guanosine-5'-[( $\alpha,\beta$ )-methylene]triphosphate, Sodium salt (GMPCPP) - 10 mM from Jena Scientific.

### Equipment

Water bath (37 °C)

## METHODS

To image MTs with fluorescent microscope, 3-6% of the tubulin for a polymerization should be labeled tubulin.

1. Equilibrate water bath to 37 °C.
2. Make polymerization mixture: Microtubules are polymerized in a solution of 0.6 mM GMPCPP and 1.2 mM DTT in M2B with the tubulin added last. Tip: rapid thaw tubulin, ahead of time mix the unlabeled recycled tubulin and labeled tubulin and add to the polymerization mixture.
3. Polymerize MTs.
  - a. Combine tubulin and polymerization mixture. Mix thoroughly, avoiding bubbles.
  - b. Place in water bath at 37 °C for 30 min.
  - c. Leave at room temperature 26 °C for 4 hours. The length of time spent at room temperature determines the MT length distribution.

### 3. Kinesin

Kinesin-1 is an ATP dependent molecular motor that steps along a microtubule. This protocol describes a purification of a kinesin-1 fragment consisting of 401 amino acids of the N-terminal *Drosophila* kinesin (K401) linked to 87 amino acids C-terminal of the biotin carboxyl carrier protein subunit of *Escherichia coli* acetyl-CoA carboxylase [24, 25]. We describe the expression and purification of kinesin motors and assembly of streptavidin-biotin motor clusters that drive microtubule inter-sliding in active nematics.

#### 3.1. Kinesin Protein Expression

This step describes the expression of a kinesin fused to a biotin carboxy protein tag (BCCP tag).

#### MATERIALS

1. Bacteria strain containing plasmid for K401-BCCP (addgene id: 15960)
2. Antibiotics: for bacteria growth 1000x concentrated.
3. Chloramphenicol
4. Rifampicin
5. 1M IPTG
6. Biotin

#### Bacteria growth Media (2XYT)

16 g/L tryptone, 10 g/L yeast, 5 g/L NaCl, for BCCP plasmids, add 24mg/L (100 $\mu$ M) biotin.

#### Equipment

1. Incubators (37 °C, 18 °C)
2. UV-Vis spectrophotometer
3. Centrifuge with minimum 5000 g and 0.5 L capacity.
4. -80 °C freezer

#### METHODS

##### Expression of kinesin-401 protein for 1 l of culture

##### 1. Day 1- Small starter culture (10 ml),

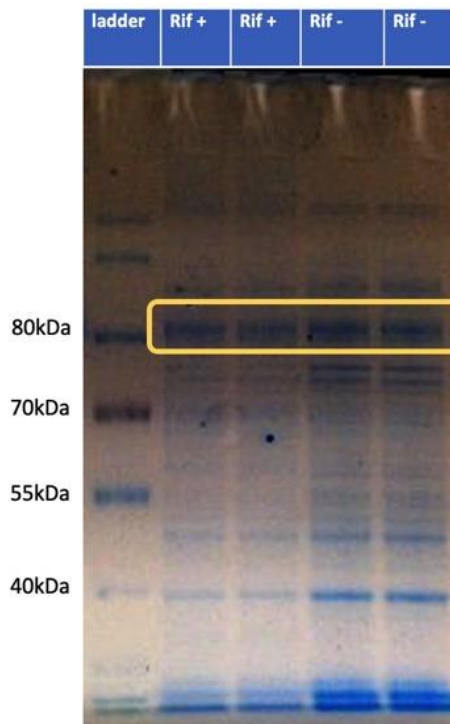
- a. Prepare media and autoclave for 45 min.
- b. Add to 10 ml of media antibiotic 1000X concentrated. Preferably use a 50 ml falcon tube.
- c. Add chloramphenicol at a final concentration of 25ug/ml. Rosetta DE3 cell line contains a plasmid coding for tRNAs rarely used in *E.coli* on a chloramphenicol resistant plasmid.
- d. Use a sterile tip to scrape Rosetta DE3 bacteria stock containing the plasmid coding for kinesin 401, and drop the tip in the falcon tube.
  - i) Do not thaw the bacteria stock.
  - ii) Alternatively scrape a colony from a plated culture and leave tip in culture.
- e. Do not seal completely the falcon tube to allow air in the tube. (**Tip:** tape the cap to prevent it from falling while shaking in the incubator).
- f. Grow culture overnight at 37 °C while shaking ~200 RPM.

## 2. Day 2- Growth and induction (0.5-1 liter, recommended 0.5 l in each flask)

- If necessary, make a 1 ml glycerol stock. 25% glycerol final. Freeze at  $-80\text{ }^{\circ}\text{C}$  for future use.
- Add 1/200 of starter culture to final culture, 2.5 ml for 0.5 L Media.
- Grow main culture in incubator at  $37\text{ }^{\circ}\text{C}$  250 RPM until it reaches  $OD_{600} = 0.6$ 
  - Do not overgrow culture.
  - If culture is overgrown dilute it to  $OD_{600} = 0.6$
- Remove flasks and place in a bin with ice.
- Add 1/1000 of IPTG 1 M, Final concentration 1 mM
- Change incubator temperature to  $18\text{ }^{\circ}\text{C}$ .
- Shake overnight at  $20\text{ }^{\circ}\text{C}$ .
- Optional** – Add 1/200 volume of 40mM Rifampicin 2 hrs after induction. Rifampicin will increase purity of the protein but decrease the yield (Fig. 4).

## 3. Day 3- Harvest cells

- Pour cells into centrifuge bottles and centrifuge at 5000 g,  $4\text{ }^{\circ}\text{C}$  for 30 min.
- Pour off most of the supernatant, keep 40 ml for every 500 ml of culture.
- Resuspend pellet in 40 ml media. This part is time consuming, and will take longer the more pellets you have.
- Transfer to 50 ml falcon tubes.
- Spin in swinging bucket centrifuge at top speed,  $4\text{ }^{\circ}\text{C}$  for 30 min.
- Pour the supernatant and keep pellets at  $-80\text{ }^{\circ}\text{C}$  until ready to use.
- Rinse flasks with 25% bleach suspension.



**Figure 4 :** Data taken after cell lysis by SDS and heat purification of protein at 85 kDa, yellow box, with Rifampicin (column 2-3) and without rifampicin (column 4-5)



### 3.2. Kinesin purification using FPLC

This step describes the purification of the kinesin from bacteria pellet generated in section 3.1 on a HisTrap column. This step is generic and similar to any His-tag protein purification with slight variations in the buffers that prevent kinesin from aggregating by adding ATP and DTT [26, 27].

#### MATERIALS

##### Reagents

1. Pierce Protease Inhibitor Mini Tablet (Thermo #88665)
2. PMSF (MP Biomedicals, # 195381)
3. Lysozyme, Chicken, RZ3 Muramidase (US Biological # L9200)
4. Nickel Column—HisTrap HP

##### Buffers

1. **Wash Buffer:** 50mM PIPES, 4mM MgCl<sub>2</sub>, 50μM ATP, 10mM βME (4o), 20mM Imidazole. pH with KOH to 7.2
2. **Elution Buffer:** 50mM PIPES, 4mM MgCl<sub>2</sub>, 50μM ATP, 2mM DTT (4o), 500mM Imidazole. pH with HCl to 7.2

##### Equipment

1. Centrifuge at 100kg
2. Ultrasonic Homogenizer (Omni International Sonic Ruptor or equivalent)
3. AKTA or equivalent model.

#### METHODS

##### Cell lysis

1. Remove cells from freezer and thaw in a beaker with water at room temp until it is 80% thawed.
2. Take 1.5-2 pellet volumes of wash buffer and put on ice.
  - a. Dissolve 1 tablet of protease inhibitor for 1 liter of culture in of wash buffer. Resuspend thoroughly. Tip you can crush the tablet with a heavy weight before adding it to the buffer.
  - b. Add 1mg/ml of PMSF
  - c. Add 1mg/ml of Lysozyme.
3. Add wash buffer to cell pellet and resuspend cells either by gently vortexing or by resuspending using a cut pipette tip. Keep cells cold and put on ice.
4. Put in a tube rotator at 4 °C for 30-60 min.
5. Lyse cell using an ultrasonic sonicator. Parameters might vary between different equipment. 50% power 10 s on 5 0s off, total of 6 cycles. **Tip:** make sure that falcon tube is in an ice bucket and well supported. After sonication the suspension appears less viscous.
6. Centrifuge at ~100,000g for 30 min at 4 °C to remove cells debris.
7. Collect the supernatant.
8. Filter the suspension through a 0.2 μm filter using a syringe. **Notice** that you might need to use multiple filters as they get clogged from leftover cell debris. You will lose some volume due to the filtration

##### Affinity chromatography purification

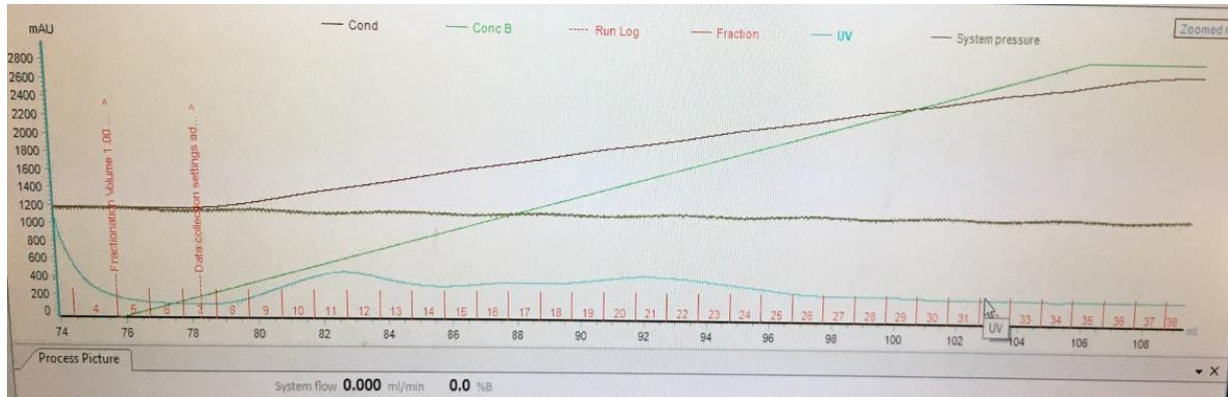
1. Start by flowing ethanol through the system.

2. Hookup the column and flow ethanol through the column.
3. Wash column with 5 column volume of ethanol.
4. Wash the column with 5 volumes of water.
5. Repeat step 3 and 4 and wash pumps.
6. Wash column with 5 volumes of elution buffer and 5 volumes of wash buffer
7. Wash pumps
8. Once there is a stable baseline in absorbance and conductivity are stable load the clarified lysate, either by manually loading with a syringe or automatically loading through one of the pump channels.
9. After the sample is loaded run 20 column volumes, or until there is a stable absorbance.
10. Put collection tubes in fraction collector.
11. Change setting to not include loading loop.
12. Elute using a gradient of wash buffer and elution buffer at 1 ml/min for 30 min. and collect all fractions.
13. Run an SDS acrylamide 8% Mops gel (Invitrogen NuPage). Add MOPS buffer and then load samples of 15 uL of denatured protein samples. Run for 32 minutes at 200 V.
14. Choose the desired fractions (Fig. 5) and dialyze in desired buffer for 2hrs. **Tip:** it is important to dialyze to remove imidazole which interferes with determining concentration of the protein. Dialyze in a buffer containing ATP (wash buffer or M2B with ATP) to prevent kinesin aggregation.
15. Repeat step 14 in a new buffer bath for additional 2 hours-overnight.
16. Concentrate protein as desired using a filter concentrator (10kDa). Recommendation to get to final concentration of ~0.7 mg/ml for kinesin 401-BCCP.
17. Store in 30% final sucrose. Aliquot in thin-walled PCR tubes and flash freeze in liquid nitrogen.
18. Clean up the chromatography setup and column according to manufacturer properties.

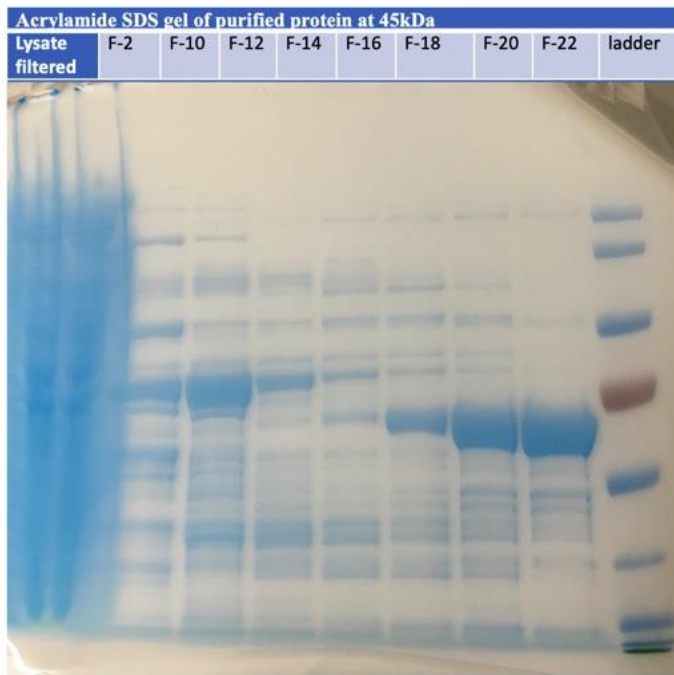
## NOTES

look at the column information and set a pressure alarm accordingly. For 1 ml Nickel HisTrap HP max pre-column pressure 0.5 MPa.

a



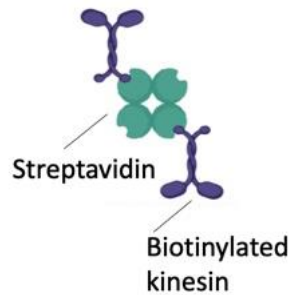
b



**Figure 5:** a. FPLC screenshot of a protein expression absorbance profile (blue curve) , and conductivity profile (green curve), as a function of elution volume in ml, and fraction (red). b. acrylamide SDS gel of different fractions compatible with the red notation in a. fraction chosen in this purification 16-24.

### 3.3. Kinesin-Streptavidin Motor clusters

Microtubules inter-filament sliding is powered by clusters of kinesin motors that are held together by tetrameric streptavidin. The ratio of streptavidin to kinesin can be varied. Test experiments demonstrated that maximum efficiency is obtained for clusters that were assembled by two biotinylated kinesins and one streptavidin (5).



**Figure 6-:** Illustration of KSA, kinesin-streptavidin motor clusters. Image generated using BioRender.

## MATERIALS

### Buffers

M2B (see microtubule section)

### Reagents

1. DTT 5mM in M2B.
2. Streptavidin diluted in M2B to 0.35 mg/ml. (Invitrogen, Cat. # S-888)
3. kinesin with BCCP tag purified with Biotin (usually stored at -80 °C at 0.7mg/ml)

## METHODS

In this section we will assemble kinesin motor clusters. The stoichiometric ratio of the molecules is designed to have two dimeric kinesin motors on a streptavidin molecule (Figure 6).

1. Thaw purified Biotin labeled kinesin-401 stored at a concentration of ~0.7 mg/ml.
2. Mix kinesin and streptavidin at a molar ratio of 2:1 dimer kinesin: streptavidin motors. Notice that Kinesin-401 with a BCCP tag is a dimer, with a monomer Molecular weight of 56 kDa. Molecular weight of streptavidin is 53 kDa
  - a. 10  $\mu$ l of kinesin-401-BCCP.
  - b. 4.6  $\mu$ l of streptavidin 0.35 mg/ml.
  - c. 0.5  $\mu$ l of DTT 5 mM in M2B.
3. Incubate on ice for 30 min.
4. Flash freeze using liquid nitrogen and store at -80 °C.

## NOTES

- This protocol can be altered for any type of kinesin motor containing the BCCP tag and purified with Biotin, by adjusting for the difference molecular weight and keeping the molar ratio between the kinesin and streptavidin.
- Each kinesin monomer contains a biotin, and the streptavidin protein has 4 biotin binding sites, therefore the kinesin-401 motor clusters can potentially form multimer protein clusters.
- At sufficiently high concentrations kinesin-401 forms clusters due to nonspecific interactions. As a result, kinesin-401 can result in microtubule sliding even without streptavidin or at different stoichiometric ratios.
- There are no quantitative investigations that studied the average cluster size that results from this protocol. Early work suggested that streptavidin clusters might have up to a dozen kinesin motors [4]. This is not entirely unexpected as kinesin-401 clusters exhibit tendency to aggregate (see note above) and these aggregates can bridge multiple streptavidin thus yielding higher order clusters. Fluorescent microscopy of labelled streptavidin clusters provides some further evidence for the presence of such clusters.

### 3.4 Motor types

There are multiple motor types that can efficiently drive non-equilibrium dynamics of microtubule based active matter [28]. Motor name is followed by a number indication the number of amino acids of the N-terminal motor domain of *D. melanogaster* kinesin. The first 340 residues are highly conserved and are monomeric, dimerization is made by approximately next 40 residues termed the neck.

1. Kinesin 401, dimeric motor, prevalently used in generation of active nematic [6].
2. Kinesin 365, a monomeric motor containing 25 residues of the neck domain. This Motor can surprisingly maintain an active nematic even though it is non-processive [21].
3. Kinesin 560, this motor forms clusters of unknown order due to non-specific interactions, and does not require the addition of streptavidin to generate an active nematic, even at low motor concentrations [29].

#### 4. ATP Regeneration System and Antioxidants

Stepping of kinesin motor is powered by the hydrolysis of ATP. Consequently, their velocity as a single kinesin is determined by the ATP concentrations. To keep system dynamics constant requires fixed concentration of ATP. This is accomplished by a biochemical chemical ATP regeneration system. Kinesin hydrolyses ATP into ADP. The regeneration system quickly regenerates ATP by transferring a phosphate from PhosphoenolPyruvic acid (PEP) to ADP by an enzyme pyruvate kinase (4, 5).

#### MATERIALS

##### Buffers

1. M2B - Dissolve 80 mM PIPES, 2 mM MgCl<sub>2</sub>, 1 mM EGTA in DI water. Adjust pH to 6.8 using KOH.
2. Phosphate buffer - Dissolve 20 mM K<sub>2</sub>HPO<sub>4</sub> in DI water. Adjust pH to 7.2 with KOH.

##### Reagents

1. 200 mM Phosphoenylpyruvate acid monopotassium salt (PEP) - Dissolve 200 mM PEP in M2B. Adjust pH to 6.8 using KOH.
2. Pyruvate kinase/lactic dehydrogenase (PKLDH)
3. 50 mM ATP - Dissolve 50 mM ATP in M2B. Adjust pH to 7 using KOH.
4. 0.5 M DTT - Dissolve 0.5 M DTT in M2B.
5. 20 mM Trolox - Dissolve 20 mM Trolox in phosphate buffer. Adjust pH to 7.2 with KOH. Trolox will not dissolve completely
6. 12% Polyethyleneglycol (PEG) - Dissolve 12% w/v 35 kDa PEG in M2B.
7. Glucose - Dissolve 300 mg/mL of glucose in phosphate buffer.
8. Glucose catalase - Dissolve 3.5 mg/mL of glucose catalase in phosphate buffer.
9. Glucose oxidase - Dissolve 20 mg/mL of glucose oxidase in phosphate buffer.
10. Magnesium Chloride - Dissolve 67  $\mu$ M of MgCl<sub>2</sub> in M2B.

#### NOTES

- The active nematic system is highly sensitive to the purity and quality of the PEP. We recommend Beantown Chemicals product 129745.

- It is important to pH with KOH rather than NaOH as Na ions can interfere with microtubules.
- Other molecular weights of PEG can be used, particularly several early publications make use of PEG - 20 kDa  
2.

## **METHODS**

The final active mixture which includes the ATP regeneration system and antioxidant system (above) as well as kinesin motors and microtubules will have 0.8% w/v PEG, 26.6 mM PEP, 1.4 mM ATP, 6.7 mg/mL glucose, 0.4 mg/mL glucose catalase, 0.08 glucose oxidase, 5 mM MgCl<sub>2</sub>.

For a 60  $\mu$ L final sample, mix the above stock solutions:

0.67  $\mu$ L glucose, glucose oxidase, glucose catalase, and DTT

1.7  $\mu$ L ATP

1.7  $\mu$ L PKLDH

2.9  $\mu$ L magnesium chloride

4  $\mu$ L PEG

6  $\mu$ L trolox

8  $\mu$ L PEP.

This mixture can be kept at 4 °C and used within a day, or flash frozen in liquid nitrogen and stored at -80 °C indefinitely.

## 5. Assembling microtubule based active matter.

Microtubules can form either 3D isotropic gels or 2D nematics. The final dynamical state is determined by the surface properties of the experimental chamber. Three-dimensional isotropic gels require hard-wall boundaries that suppress adsorption of various protein components. The hydrophilic surface is made with an poly-acrylamide brush that coats the coverslip [30]. Alternatively, presence of a surfactant stabilized liquid-liquid interface promotes assembly of dense 2D active nematic liquid crystals as microtubule spontaneously adsorb onto such surface [6]. To stabilize liquid-liquid interface the two sides of the chamber have different surface treatments. The hydrophobic surface, formed using rain-x or aquapel, promotes the wetting of the thin oil layer. As in 3D gel, the hydrophilic surface is coated with a poly-acrylamide brush. We discuss each surface treatment and flow cell assembly.

### 5.1 Polyacrylamide coating

In this step we assemble an acrylamide brush on the coverslip. The acrylamide is a sterically repulsive brush that prevents proteins from sticking to the surface. The resulting surface is hydrophilic.

## MATERIALS

### Reagents

1. Hellmanex soap
2. 0.1 M NaOH or 0.1 M KOH in water,
3. Ethanol
4. Tetramethylethylenediamine (TEMED)
5. ammonium persulfate (APS).
6. 3-(Trimethoxysilyl)propylmethacrylate
7. acetic acid
8. 40% acrylamide solution. (you can prepare a 40% acrylamide stock or buy an acrylamide solution.  
Note: if working with acrylamide powder it is important to work in a hood. Refer to lab safety manual for MSDS info.

### Solutions

1. **Silane** - Mix 98.5% ethanol, 1% acetic acid and 0.5% 3-(Trimethoxysilyl)propylmethacrylate (silane agent). This solution is highly unstable. Combine immediately before use.
2. **Acrylamide** - Mix 2% acrylamide solution from 40% stock in water. Degas 2% acrylamide. Acrylamide is a known neurotoxin and carcinogen; refer to the MSDS for details and dispose of appropriately.

### Equipment

1. Sonicator
2. degasser,
3. slide racks and containers,
4. Glass coverslips 22x22 mm preferably #1.5.



## **METHODS**

### **Cleaning slides**

1. Arrange coverslips in rack (Wash-N-Dry Coverslip Rack Sigma or equivalent) and put in container.
2. Fill container with DI water.
3. Add 1.5% volume Hellmanex soap.
4. Microwave coverslips until to properly disperse the soap. Tip: do not boil.
5. Sonicate for 5-10 min.
6. Rinse 3 times in DI water.

### **Additional slide cleaning, recommended for acrylamide coating**

1. Fill container with ethanol.
2. Sonicate for 5-10 minutes.
3. Rinse 3 times in DI water.
4. Fill container with 0.1M NaOH (or KOH) incubate 10 minutes to etch surface.
5. Rinse 3 times in DI water.

### **Acrylamide surface coating**

**Tip:** make a 2% acrylamide solution and start degassing it before preparing silane solution.

### **Silane coating for acrylamide binding to the surface.**

1. Dry coverslips from water. 90% dry.
2. Prepare silane coupling solution, the solution is unstable prepare right before use. For 300ml solution
  - a. 300ml ethanol
  - b. 3ml Acetic acid (1%)
  - c. 1.5ml Silane agent (0.5%)

Use stir bar to properly mix the solutions.

3. Pour silane coupling solution onto coverslips immediately after it is prepared and incubate for 15 minutes.
4. Rinse 3 times in DI water Acrylamide polymerization

### **Acrylamide polymerization**

1. Make a 2% acrylamide solution in DI water.
2. for 300 ml, 15 ml of 40% acrylamide stock in 285 ml of DI water.
3. Pump solution under vacuum for 30 minutes. This removes oxygen which can inhibit acrylamide polymerization.
4. Put acrylamide solution on stir plate and add 105  $\mu$ l of TEMED, and 210 mg of APS. Mix properly. (it is important to first add TEMED and then APS).
5. Pour solution over silane coated slides.
6. Coverslips are ready within 2 hours, and can be used up to 2 months.
7. Keep in solution and when ready to use remove one slide from container rinse in water and dry with nitrogen. Coverslip can last for several months.

## 5.2 Assembling a 3D isotropic gel

### Materials

1. Active mixture with microtubules (Section 4).
2. Acrylamide slides.

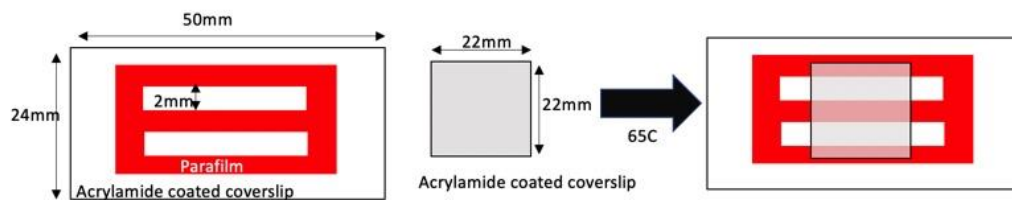
### Equipment

1. Hot plate
2. Optional silhouette cameo for cutting parafilm channels. It is also possible to cut channels with scissors.

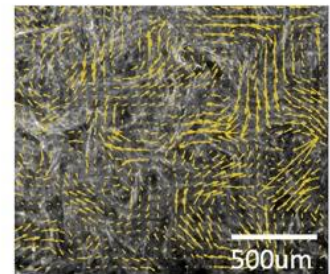
### Assembling a flow chamber (Fig. 7):

1. Cut either manually or using silhouette a channel into a parafilm sheet. Recommended dimensions: 2 mm width and of 3 cm or longer length. Parafilm thickness is  $\sim 120 \mu\text{m}$ .
2. Place the parafilm channel on the larger acrylamide slide.
3. Center a 22 x 22 mm acrylamide coated coverslip on top of the parafilm channel.
4. Put on heat plate at  $65^\circ\text{C}$  for few minutes, and press two coverslips against each other. Make sure there are no air bubbles or potential holes for leaks.
5. Using a razor blade, cut the exposed parafilm ends.

#### a Flow channel for 3D isotropic gels



b



**Figure 7:** (a) illustration of a hydrophilic flow cell assembled from two acrylamide coated coverslips. (b) Image of 3D isotropic gel overlaid with velocity field vectors, for more details see section 7.

## 5.3 Hydrophobic coating forming oil water interface

### MATERIALS

#### Reagents

1. Rain-X or Aquapel (Available commercially).
2. Isopropanol
3. Parafilm channels
4. HFE7500 oil (fluorinated oil)
5. 008-FluoroSurfactant (RAN biotechnologies)

## Solution

1. 50 g of 2 weight % 008-FluoroSurfactant in HFE7500

## Equipment

1. Hot plate
2. Optional silhouette cameo for cutting parafilm channels. It is also possible to cut channels with scissors.

## METHODS

### Hydrophobic slide

#### Treat clean 24X50mm microscope slide Aquapel

1. Spread 100  $\mu$ l of Aquapel on a clean microscope slide.
2. Drop a coverslip over the Aquapel drop, to spread evenly.
3. Incubate for 30-60 s.
4. Remove top coverslip and dry with nitrogen.
5. Immerse the slide in water.
6. Optional: sonicate for 2 minutes in a beaker containing water.
7. Rinse and dry the slide

#### Option 2: Treat clean 24X50mm microscope slide with Rain-X

1. Spray rain-X on both sides of a coverslip.
2. Wash coverslip from rain-X residues with ethanol.
3. Rinse in DI water.
4. Dry coverslip.

#### Assembling a flow cell (Fig. 8)

1. Cut either manually or using silhouette a channel into a parafilm sheet. Recommended dimensions: 2 mm width and of 3 cm or longer length. Parafilm thickness is  $\sim 120 \mu\text{m}$ .
2. Place the parafilm channel on the hydrophobic slide.
3. Center a 22 x 22 mm acrylamide coated coverslip on top of the parafilm channel.
4. Put on heat plate at 65 °C for few minutes, and press two coverslips against each other. Make sure there are no air bubbles or potential holes for leaks.
5. Using a razor blade, cut the exposed parafilm ends.

## 5.4 Assembling an oil water interface.

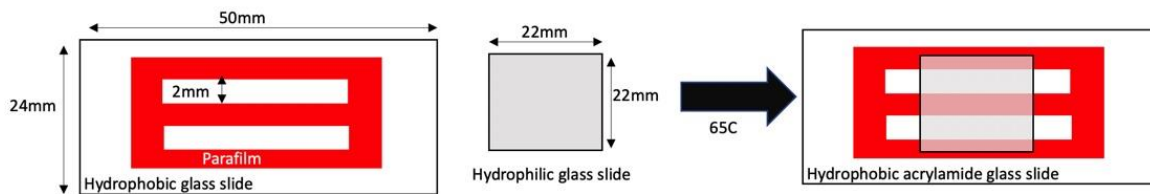
1. Flow the oil- surfactant mixture. Pipette near the entrance and the fluid will flow by capillary. (Fig. 8b)
2. Prepare a reaction mixture containing: 1  $\mu$ L of 50 mM ATP (final 2 mM), 11  $\mu$ L of ATP regeneration system and antioxidants (chapter 4), 2.5  $\mu$ l of PEG 35 kDa 12% (final 1% PEG), 1  $\mu$ L of KSA (chapter 3), and 9.5  $\mu$ L of M2B.
3. Take 10  $\mu$ l of reaction mixture, add 2  $\mu$ L of labeled microtubules at concentration of 8 mg/mL.
4. Once the chamber is filled with oil flow the reaction mixture containing microtubules kinesins and energy regeneration system and slowly displace the oil (Figure 8c). **Tip:** use a Kimwipe at

- one end of the chamber to wick out the oil while pipetting in the water phase at the other end. You should see an oil/water interface moving across the channel.
- Seal the chamber using UV curable glue (Norland adhesive). Draw a thin bead of glue all around the device for best sealing and cure under a UV lamp.
  - Optional: centrifuge the sample at 25 °C 1000 g for 10 minutes. If the sample is not centrifuged it will take about an hour to settle to the interface depending on the kinesin and ATP concentration. Sample that is not centrifuged will take time to settle into a nematic (Figure 8).
  - A proper nematic will have similar defects size across the sample (Fig 9).

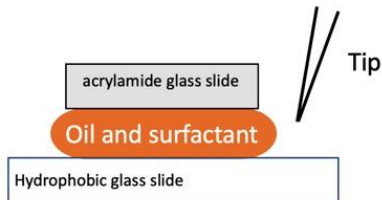
**NOTES:**

- If channel dimensions are too wide the oil layer that forms is not uniform and results in inhomogeneities in defect size across the nematic. Recommended channel dimensions are  $\sim 100 \mu\text{m}$  thickness, 2 mm width or less and 22 mm length.
- The above-described conditions yield quasi 2D active nematics with  $\sim 150 \text{ nm}$  thickness. The thickness can be varied and is determined by the microtubule concentration[6].

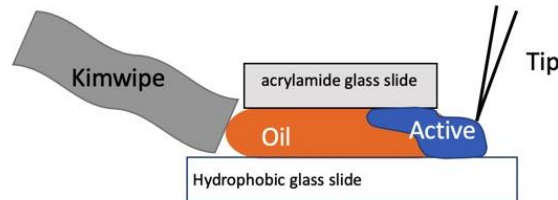
**a. Make a flow channel (top view)**



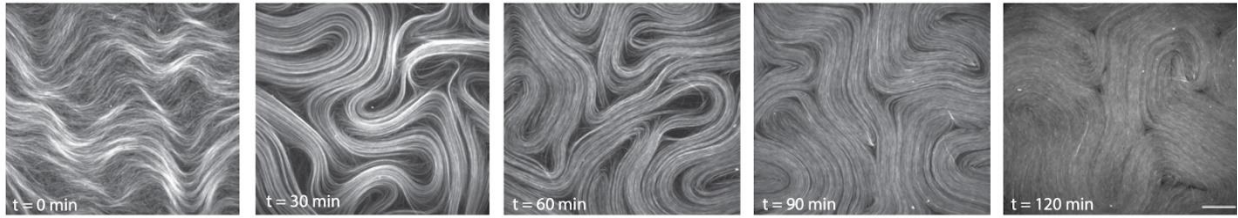
**b. Flow oil (side view)**



**c. Exchange oil with active reaction (side view)**



**Figure 8 :** Illustration of different steps for assembling an oil water interface.



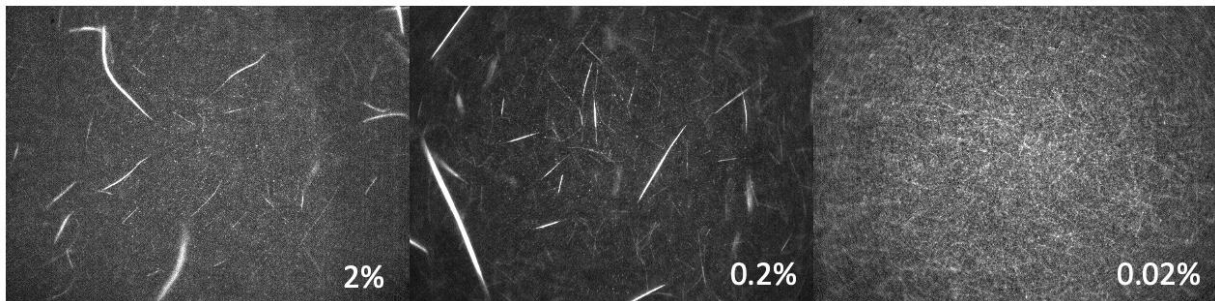
**Figure 9:** Spontaneous assembly of MTs into a dense 2D nematic at the oil-water interface. The process of active microtubules settling onto the oil-water interface into a dense 2D nematic. The chamber was spun at 300 g for 10 min prior to the first frame. This process is slowed down in these images by reducing  $[ATP]$  to  $18 \mu M$ . Scale bar,  $100 \mu m$ .

## 6. Depletion Interactions

Depletion forces arise between large assemblages, when these are suspended in a dilute solution of solutes that are smaller and are excluded from the vicinity of the large particles [20, 31]. Microtubules bundles are generated by adding poly(ethylene glycol) PEG which causes formation of motor driven extensile bundles. The PEG concentration and the molecular weight determines the bundle structure. Active nematic will start forming at a critical concentration required for bundle formation. One can test if depletion interactions are strong enough by simply mixing microtubules and PEG solution at desired concentration. For assembly of active nematics we recommend using 20-35 kDa PEG at a final concentration of 1-2% (Fig. 10).

### NOTES

- One can also use Pluronic, a non ionic triblock copolymer, as a depletant, however Pluronic can form micelles and can potentially cage kinesin molecules generating high order motor cluster.
- Salt concentration changes the onset of bundle formation by affecting the cohesion energy of microtubules [20]. However, kinesin interaction with MTs is salt dependent and is only functional at physiological conditions (80-150 mM). At these concentrations the ionic strength does not influence bundling significantly (up to a factor of 2).



**Figure 10:** Changing the percentage of depletant PEG 35 kDa in buffer with no motors in a thin chamber between two acrylamide coverslips. At high PEG concentrations microtubules form large bundles, whereas at low PEG concentrations individual microtubules diffuse in the chamber.

## 7. Characterizing active materials

### 7.1 Imaging 2D active nematic.

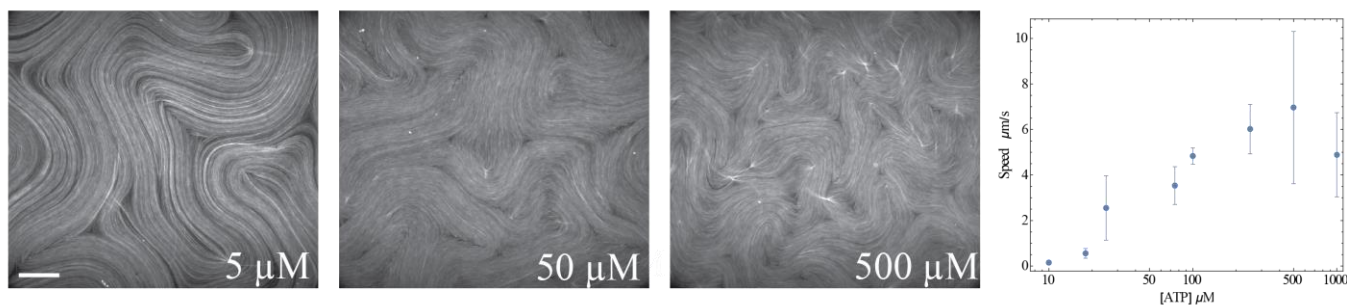
The active nematic can be imaged either using a fluorescent or brightfield optical microscope. The thickness of the nematic layer will depend on the microtubule concentration as well as on the thickness of the channel [6]. For a  $100\ \mu\text{m}$  thick channel with  $1\ \text{mg/ml}$  tubulin concentration, the thickness of the nematic layer is estimated to be  $\sim 200\ \text{nm}$ . The thickness of the oil sublayer is  $\sim 1\ \mu\text{m}$  thick. Upon formation of a well-defined layer there are no measurable microtubules in the aqueous solution above the layer. It is recommended to image from the hydrophobic side of the chamber. At saturating ATP concentrations, the expected velocity of the nematic at  $21\ ^\circ\text{C}$  is  $\sim 3\ \mu\text{m/s}$ . For velocity analysis it is recommended to acquire consecutive images every 2 s. The nematic can be easily visualized with a 2x objective and higher. The antioxidants in the premix solution described in section 4 suppresses photodamage.

#### Note

When using high NA lens, it is expected to observe photobleaching over time. Low ATP concentrations reduce velocities thus enhancing any photobleaching effects.

### 7.2 ATP Dependence

Changing the ATP concentration controls the speed of the material as well as the defect density. The amount of ATP in the system dictates the stepping rate of kinesin motors [32]. Additionally, since kinesin will bind MTs in the absence of ATP, decreasing ATP also increases the amount of passively crosslinking clusters. The combination of reduced activity and increased elasticity results in an active nematic with lower speeds and defect density. The system velocity saturates at  $\sim 300\ \mu\text{M}$  ATP.

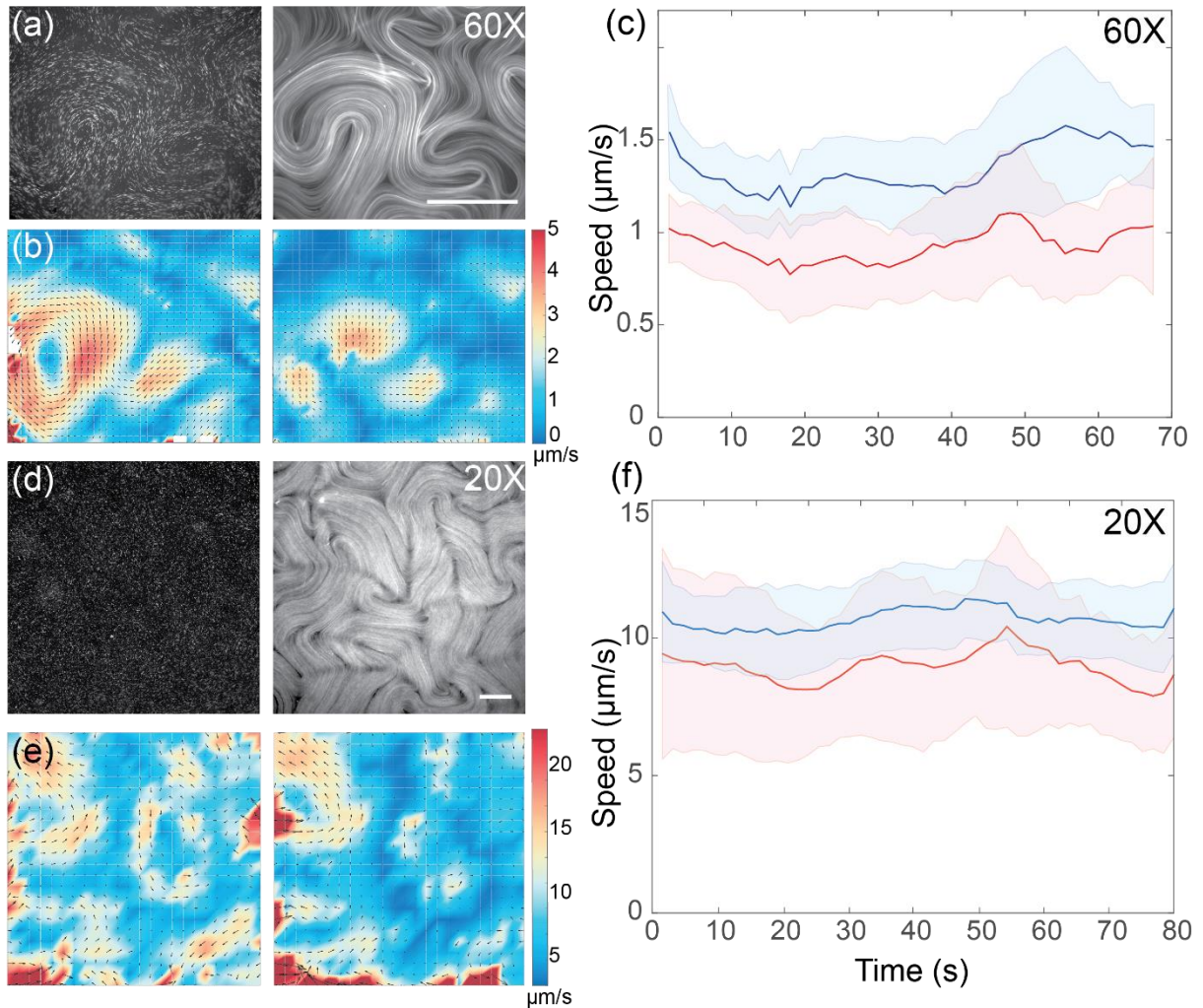


**Figure 11:** Active nematics assembled at different ATP concentrations. Speed as a function of ATP concentration measured by particle image velocimetry on lightly labeled nematics (Section 7.3). The speed and the defect density both increase with ATP. Scale bar,  $100\ \mu\text{m}$ .

### 7.3 Measuring the velocities of the nematic using Particle tracking and PIV

Active nematics flows can be quantified by either using particle image velocimetry (PIV) or particle tracking of beads embedded in the nematic. The PIV algorithm is optimized to measure gradients in intensity. In fully labelled nematics, this can underestimate velocities in region of uniformly aligned microtubules. To mitigate this effect, one can use sparsely labeled microtubules, where only one in every 1,000-10,000 is labeled with a fluorescent dye. The failure of PIV is especially pronounced at higher

magnification where there are few defects in the field of view (Fig. 12a-c). At low magnification where many defects are observed, the mean speed from the fully and lightly labeled nematics are in agreement (Fig. 12d-f).

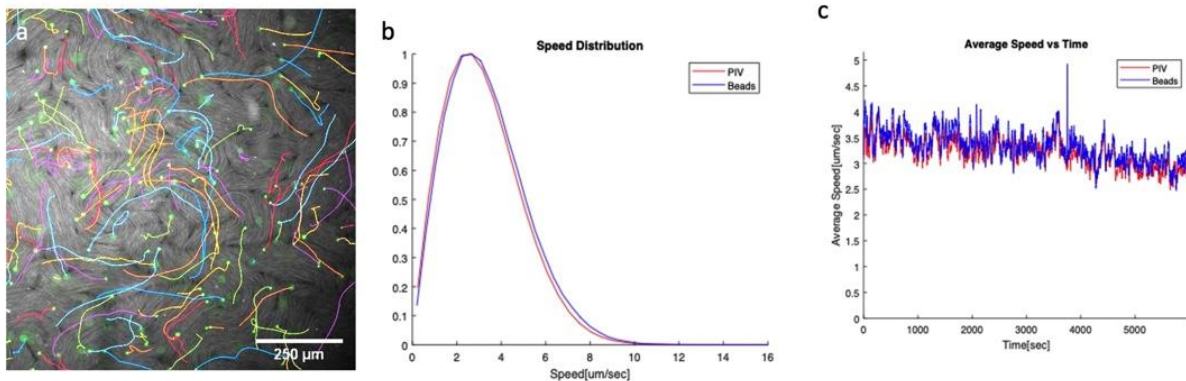


**Figure 12 : Quantifying active nematic velocity using PIV.** Scale bars  $100 \mu\text{m}$ . **(a)** Fluorescence images of an active nematic with 1/1000 MTs labeled in Azide-DBCO-488 (left) and fully labeled with Alexa 647 (right) at high magnification 60X. **(b)** The resulting velocity fields from particle image velocimetry on images from (a). Color shows the measured speed. The fully labeled flow field underestimates flows in regions where MTs are aligned. **(c)** Average speed over time from lightly labeled (blue) and from fully labeled (red) fluorescence images at high magnification. Shaded regions indicate the standard deviation of the speed at each time point. **(d)** Fluorescence images of an active nematic with 1/1000 MTs labeled in Azide-DBCO-488 (left) and fully labeled with Alexa 647 (right) at low magnification 20X. **(e)** The resulting velocity fields from particle image velocimetry on images from (d). Color shows the measured speed. The fully labeled flow field still underestimates flows along the director. However, the two flow fields are in closer agreement. **(f)** Average speed over time from lightly labeled (blue) and from fully labeled (red)



fluorescence images at low magnification 20X. Shaded regions indicate the standard deviation of the speed at each time point.

**Note:** Particle tracking can also be used for velocity measurements by embedding  $\sim 3 \mu\text{m}$  beads into the nematic layer. It is consistent with PIV measurements at low magnifications (Figure. 13).



**Figure 13 : Measuring nematic velocity using particle tracking.** (a) Image of  $3 \mu\text{m}$  beads trajectories embedded in a nematic film. (b) velocity distribution of analyzed from PIV measurements of fully labeled nematic film (red) and particle tracing of beads taken with a 10X lens. (c) Velocity profile as a function of time comparing PIV of fully labeled nematic and particle tracking of embedded beads.

#### 7.4 Imaging and characterizing flows of 3D active gels.

It is recommended to image a 3D active gel using fluorescent microscopy. One can measure the flows directly from these images or by using particle tracking to quantify the motion of beads that are advected by the spontaneously generated flows [21]. Velocities and flows depend on various parameters such as ATP concentration, kinesin concentration, depletant concentration, boundary conditions, and motor type [15, 21, 33].

Tip: in order to do particle tracking it is important to heavily dope the sample with beads as they frequently go out of plane.

## 8. Measuring Orientation field from Polarization Microscopy (LC-PolScope)

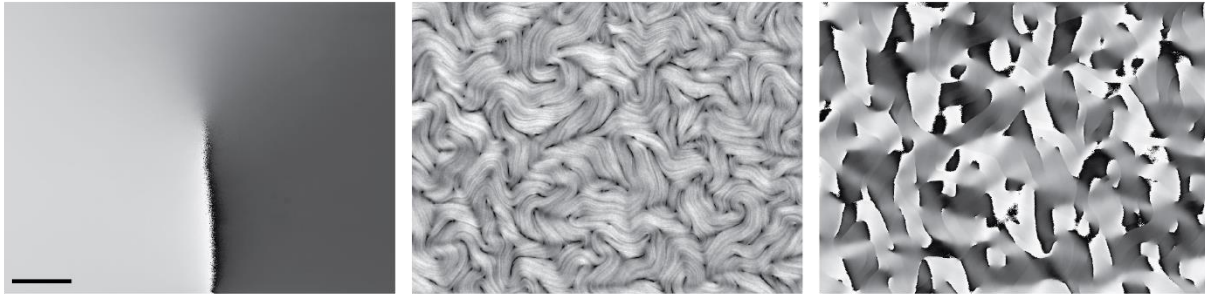
Quantitative polarization microscopy is a useful technique to quantify the orientation of the MTs, which is the defining feature of active nematics. Using an electronically controlled set of liquid crystals, one can measure the orientation field and the retardance or density field of the active nematic over time. This technique can be used at any magnification. This technique is the only way to get pixel-level resolution of the orientation field of the active nematic. We used LC-PolScope in conjunction with a freely available software OpenPolScope [34]. This device takes multiple pictures of the sample with different orientations of the internal liquid crystals. It then reconstructs the retardance and orientation fields using previously described techniques.

### Equipment

1. LC-PolScope- available for purchase at OpenPolScope ([https://openpolscope.org/pages/Info\\_Request\\_Form.htm](https://openpolscope.org/pages/Info_Request_Form.htm)).
2. Inverted optical microscope with an air or oil immersion condenser.
3. Micromanager compatible camera and computer with micromanager and OpenPolScope installed. It is recommended to get a license for 5-frame acquisition as this can improve image quality when time resolution is less important.

### Methods

1. Make a chamber as in Section 5.
2. Load the chamber with M2B. Leave it unsealed. Rub the glass with lens paper to remove any dust or dirt.
3. At the microscope, find the focal plane by focusing on the edge of the parafilm.
4. Set up Kohler alignment.
5. Find a region in the M2B channel that is free from contaminants, and calibrate the liquid crystals.
6. Take a background image in the same region. This image should appear like Fig. XX. If it looks blurry or distorted, recalibrate the liquid crystals, move to a cleaner region of the slide and/or check the alignment of the optical path.
7. Use N<sub>2</sub> to blow the M2B out of the chamber.
8. Load the nematic as in Section 5.3. Fill a neighboring chamber with M2B.
9. Sediment onto the interface by spinning in a swinging bucket centrifuge or by waiting. Rub the glass with lens paper to remove any dust or dirt and place on the microscope.
10. Take an image of the nematic using the background image acquired in 6. If the computed retardance and orientation images looks like Fig 13(b-c), the system is properly aligned and one can set up a long-term acquisition. If the images show aberrations or poor resolution, the system needs to be recalibrated. Use a neighboring chamber filled with M2B.



**Figure 14 : Imaging active nematics with LC-PolScope.** (a) Background orientation image of an empty region for well-calibrated LC-PolScope. The sharp contrast between light and dark is the signature of good calibration. (b) The retardance image of an active nematic taken with a well-calibrated LC-PolScope. (c) The corresponding orientation image of an active nematic taken with a well-calibrated LC-PolScope. Scale bar, 500  $\mu\text{m}$ .

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