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TITLE:

Controlling Flow Speeds of Microtubule-Based 3D Active Fluids Using Temperature

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SUMMARY:

The goal of this protocol is to use temperature to control the flow speeds of three-dimensional active fluids. The advantage of this method not only allows for regulating flow speeds in situ but also enables dynamic control, such as periodically tuning flow speeds up and down.

ABSTRACT:

We present a method for using temperature to tune the flow speeds of kinesin-driven, microtubule-based three-dimensional (3D) active fluids. This method allows for tuning the speeds in situ without the need to manufacture new samples to reach different desired speeds. Moreover, this method enables the dynamic control of speed. Cycling the temperature leads the fluids to flow fast and slow, periodically. This controllability is based on the Arrhenius characteristic of the kinesin-microtubule reaction, demonstrating a controlled mean flow speed range of 4–8 µm/s. The presented method will open the door to the design of microfluidic devices where the flow rates in the channel are locally tunable without the need for a valve.

INTRODUCTION:

Active matter is differentiated from conventional passive matter due to its capability to convert chemical energy into mechanical work. A material that possesses such capability can consist of living or non-living entities such as bacteria, insects, colloids, grains, and cytoskeletal filaments[1-10]. These material entities interact with their neighbors. At a larger scale, they self-organize into either turbulent-like vortices (active turbulence) or material flows[11-20]. An understanding of self-organization of active matter has led to various applications in molecular shuttles, optical devices, and parallel computation[21-23]. To bring applications to the next level requires control beyond self-organization. For example, Palacci et al. developed a hematite-encapsulated colloid that self-

propelled only when exposed to manually controlled blue light, which led to the emergence of living crystals[24]. Morin et al. established the control of rolling Quincke colloids by using a tunable external electric field, resulting in colloidal flocking in a racetrack-like channel[25]. These previous works demonstrate the role of local control in applications and advance the knowledge base of active matter.

In this article, we focus on the controllability of kinesin-driven, microtubule (MT)-based 3D active fluids. The fluids consist of three main components: MTs, kinesin molecular motors, and depletants. The depletants induce a depletion force to bundle the MTs, which are later bridged by motor clusters. These motors walk along the MTstoward the plus end. When a pair of bridged MTsis antiparallel, the corresponding motors walk in opposite directions. However, the motors are bound in a cluster and are unable to walk apart, so they cooperatively slide apart pairs of MTs (interfilament sliding, **Figure 1A**). These sliding dynamics accumulate, causing bundles of MTsto extend until reaching their buckling instability point and break (extensile bundles, **Figure 1B**)[26]. The broken bundles are annealed by the depletion force, which subsequently extends again, and the dynamics repeat. During the process of the repeating dynamics, the bundle movements stir the nearby liquid, inducing flows that can be visualized by doping with micron-scale tracers (**Figure 1C**). Sanchez et al. and Henkin et al. have characterized the mean speeds of tracers, finding that the speeds were tunable by varying the concentrations of adenosine triphosphate (ATP), depletants, motor clusters, and MTs[19,27]. However, such tunability existed only prior to active fluid synthesis. After synthesis, the tunability was lost, and the fluids self-organized in their own way. To control active fluid activity after synthesis, Ross.et al. reported a method using the light-activated dimerization of motor proteins, allowing fluid activity to be tuned on and off using light[28]. While light control is convenient in terms of locally activating the fluids, the method requires redesigning the structures of motor proteins, along with modifying the optical paths in a microscope. Here, we provide an easy-to-use method for locally controlling fluid flows without microscope modification while keeping the motor structure intact.

Our method of locally tuning active fluid flow is based on the Arrhenius law because the kinesin-MT reaction has been reported to increase with temperature[29-32]. Our previous studies showed that the temperature dependence of the mean speed of an active fluid flow followed the Arrhenius equation: $v = A \exp(-E_a/RT)$, where A is a pre-exponential factor, R is the gas constant, E_a is the activation energy, and T is the system temperature[33]. Therefore, fluid activity is sensitive to the temperature environment, and the system temperature needs to be consistent to stabilize the motor performance, and consequently, the fluid flow speed[34]. In this article, we demonstrate the use of the motor's temperature dependence to continuously tune the flow speeds of active fluids by adjusting the system temperature. We also demonstrate the preparation of an active fluid sample, followed by mounting the sample on a microscope stage whose temperature is controlled via computer software. Increasing the temperature from 16 °C to 36 °C speeds up the mean flow speeds from 4 to 8 μm/s. Additionally, the tunability is reversible: repeatedly increasing and decreasing the temperature sequentially accelerates and decelerates the flow. The demonstrated method is applicable to a wide range of systems where the main reactions obey the Arrhenius law, such as the MT gliding assay[29-32].

PROTOCOL:

1. Preparation of MTs

CAUTION: In this step we purify tubulins from bovine brain tissue. Bovine brain may cause variant Creutzfeldt-Jakob disease (vCJD)[35]. Therefore, the brain waste and related solutions, bottles, and pipette tips should be collected in a biowaste bag and disposed of as biohazardous waste according to the rules of the institution.

1.1. Purify tubulins from bovine brain (modified from Castoldi et al.[36]).

1.1.1. Transport approximately 1.5 kg of fresh bovine brains from a local slaughterhouse to a university cold room. During transport, store the brains in phosphate buffer (20 mM NaH_2PO_4, 150 mM NaCl, pH 7.2) on ice.

NOTE: To maximize the final yield of tubulin, the initial amount of functional tubulin in the fresh brain tissue is key. Fresher brains contain more functional tubulin. To obtain the freshest brains from the slaughterhouse, we recommend asking the butcher to provide brains from the most recently slaughtered cows. Brains should be no older than 3 h when starting the procedure, because reducing the time between slaughter and procedure start will produce better yields.

1.1.2. Homogenize and clarify the brains.

1.1.2.1. Clean the brain by using scalpels to cut blood vessels and connective tissue into smaller pieces and remove them from the brain by hand.

NOTE: The cleaned brains should be pink.

1.1.2.2. Immerse the cleaned brain tissue in 1 L of depolymerization buffer (DB: 50 mM 2-(N-morpholino) ethanesulfonic acid, 1 mM $CaCl_2$, pH 6.6) per kilogram of brain.

1.1.2.3. Homogenize the brains with a kitchen blender.

1.1.2.4. Centrifuge the homogenized brain solution at 10,000 x *g* and 4 °C for 150 min.

1.1.3. Polymerize MTs (first polymerization).

1.1.3.1. Collect and mix the supernatant with equal volumes of the following solutions at 37 °C: glycerol, high-molarity PIPES buffer (HMPB: 1 M PIPES, 10 mM $MgCl_2$, 20 mM ethylene glycol-bis(β-aminoehyl ether)-N,N,N',N'-tetraacetic acid [EGTA], pH 6.9)

1.1.3.2. Add 1.5 mM ATP and 0.5 mM guanosine triphosphate (GTP) to the mixture.

1.1.3.3. Incubate the mixture at 37 °C for 1 h.

1.1.4. Depolymerize MTs (first MT depolymerization).

1.1.4.1. Pellet MT by centrifuging at 151,000 x *g* and 37 °C for 30 min.

1.1.4.2. Discard supernatant and resuspend each MT pellet in 10 mL of 4 °C DB.

1.1.4.3. Incubate on ice for 30 min.

1.1.4.4. Agitate the mixture every 5 min with a pipette tip to avoid MT sedimentation.

NOTE: The mixture should turn clear in 30 min, indicating completion of MT depolymerization.

1.1.5. Polymerize MTs (second MT polymerization).

1.1.5.1. Clarify the solution by centrifuging at 70,000 x *g* and 4 °C for 30 min.

1.1.5.2. Collect and mix the supernatant with equal volumes of 37 °C glycerol and HMPB.

1.1.5.3. Add 1.5 mM ATP and 0.5 mM GTP to the mixture.

1.1.5.4. Incubate the mixture at 37 °C for 30 min.

1.1.6. Repeat step 1.1.4, replacing HMPB with Brinkley reassembly buffer (BRB) 80 (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8), followed by clarifying the cleared mixture by centrifuging at 79,000 x *g* and 4 °C for 30 min.

1.1.7. Collect the supernatant. Measure the 280 nm absorbance with a spectrometer. Determine the tubulin concentration using the Beer-Lambert law (extinction coefficient of tubulin: 1.15 $(mg/mL)^{-1}cm^{-1}$)[37,38].

1.1.8. Store the protein at -80 °C.

1.2. Recycle tubulins (modified from Castoldi et al.[36]).

NOTE: To enhance tubulin purity, the purified tubulin is polymerized and depolymerized again.

1.2.1. Polymerize the MTs by mixing the purified tubulin (step 1.1.7) with 500 µM dithiothreitol (DTT) and 20 µM GTP, followed by 30 min of incubation at 37 °C.

1.2.2. Pellet the MTs.

1.2.2.1. Load 1 mL of glycerol cushion (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 60% v/v glycerol, pH 6.8) in the bottom of a centrifuge tube.

1.2.2.2. Lay the polymerized MTs on top of the cushion.

1.2.2.3. Centrifuge at 172,000 x *g* for 90 min at 37 °C.

1.2.3. Depolymerize the MTs.

1.2.3.1. Remove the supernatant and cushion.

1.2.3.2. Resuspend each MT pellet in 150 µL of 4 °C MT buffer (M2B: 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 6.8).

1.2.3.3. Incubate the mixture on ice for 30 min.

1.2.3.4. Agitate the mixture with a pipette tip every 5 min. The mixture should turn clear.

1.2.4. Clarify the mixture by centrifuging at 172,000 x *g* and 4 °C for 30 min.

1.2.5. Collect the supernatant. Measure the protein concentration. Store at -80 °C.

1.3. Label the tubulin with fluorescent dye[39].

1.3.1. Polymerize the MTs. Mix the purified tubulin (step 1.1.7) with 500 µM DTT and 16.7 µM GTP, and incubate the mixture at 37 °C for 30 min.

1.3.2. Pellet the MTs.

1.3.2.1. Place 1 mL of 37 °C high-pH cushion (0.1 M NaHEPES, 1 mM $MgCl_2$, 1 mM EGTA, 60% v/v glycerol, pH 8.6) into a centrifuge tube.

1.3.2.2. Lay the polymerized MTs on the cushion.

1.3.2.3. Centrifuge at 327,000 x *g* for 50 min at 37 °C.

1.3.3. Label tubulin.

1.3.3.1. Resuspend each MT pellet with 700 µL of 37 °C labeling buffer (0.1 M NaHEPES, 1 mM $MgCl_2$, 1 mM EGTA, 40% v/v glycerol, pH 8.6).

1.3.3.2. Mix the suspension with a 10–20 molar excess of far-red fluorescent dye functionalized with a succinimidyl ester.

1.3.3.3. Incubate the mixture at 37 °C for 30 min in the dark to allow the MTs to react with the ester of the fluorescent dye.

1.3.3.4. Stop the labeling reaction by saturating the ester of the suspending dye with 50 mM K-glutamate, incubating for 5 min at 37 °C.

1.3.4. Pellet the labeled MTs: Repeat step 1.3.2, replacing the high-pH cushion with low-pH cushion (80 mM pipes, 2 mM $MgCl_2$, 1 mM EGTA, 60% v/v glycerol, pH 6.8).

1.3.5. Depolymerize MTs.

1.3.5.1. Discard the supernatant and the cushion.

1.3.5.2. Resuspend the pellet in 700 µL of 4 °C M2B.

1.3.5.3. Incubate the suspension on ice.

1.3.5.4. Agitate every 5 min with a pipette tip until the solution is clear.

1.3.6. Clarify the cleared solution by centrifuging at 184,000 x *g* and 4 °C for 35 min.

1.3.7. Enhance the purity of the labeled tubulin solution by repeating steps 1.2.1–1.2.4.

1.3.8. Measure the concentration of proteins and fluorescent dye. Use these measurements to determine the tubulin concentration and the fractions of labeled tubulin, defined as the ratio of concentrations of fluorescent dye to tubulin.

1.3.9. Store the labeled tubulin solution at -80 °C.

1.4. Polymerize the MTs (adopted from Sanchez et al.[19]):

1.4.1. Mix the recycled tubulin (step 1.2.5) with labeled tubulin (step 1.3.9) in a ratio that yields a 3% labeled tubulin fraction.

1.4.2. Mix the 8 mg/mL tubulin mixture with 1 mM DTT and 0.6 mM guanosine-5'[(α,β)-methyleno]triphosphate (GMPCPP), followed by 30 min of incubation at 37 °C.

1.4.3. After the incubation, anneal the MTs at room temperature in the dark for 6 h.

1.4.4. Aliquot and store at -80 °C.

2. Synthesize kinesin clusters

NOTE: Bacteria exist ubiquitously and can grow in the media and contaminate the preparation process. To prevent contamination, actions involving contact with the cell cultures (e.g., pipetting) MUST be performed near a flame. Tools such as flasks, pipettes, pipette tips, media,

and plates MUST be autoclaved before use.

2.1. Express kinesin motors in *Escherichia coli*[40].

2.1.1. Transform the cells.

2.1.1.1. Pipette 1 µL of the K401-BCCP-H6 plasmid into 10 µL of competent cells.

2.1.1.2. Incubate on ice for 5 min.

2.1.1.3. Heat-shock at 42.5 °C for 45 s.

2.1.1.4. Incubate the cells on ice for 2 min to allow recovery.

2.1.1.5. Mix the cells with 300 µL of antibiotic-free 2XYT (5 g/L NaCl, 10 g/L yeast extract, 16 g/L tryptone).

2.1.1.6. Incubate the cells at 37 °C for 1 h.

NOTE: Unless specified, monitoring the cell growth is not required during incubation.

2.1.2. Inoculate plate media.

2.1.2.1. Spread the cell culture on a 2XYT plate (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, 15 g/L agar, 100 µg/mL ampicillin, 25 µg/mL chloramphenicol).

2.1.2.2. Incubate the plate upside down at 37 °C overnight.

2.1.3. Inoculate liquid media.

2.1.3.1. From the overnight plate, harvest one isolated colony with a pipette tip.

2.1.3.2. Eject the tip into a flask containing 50 mL of 2XYT.

2.1.3.3. Incubate and shake the culture media at 37 °C and 200 rpm for 12–16 h.

2.1.4. Expand the cells.

2.1.4.1. Mix 2.5 mL of the cell culture in 500 mL of 2XYT.

2.1.4.2. Incubate and shake the 500 mL culture at 37 °C and 250 rpm for 3–6 h.

2.1.5. Induce protein expression.

2.1.5.1. During the incubation, monitor cell growth by measuring the absorbance at 600 nm, using 2XYT as a reference. Measure the absorbance every 60 min, until it reaches OD_{600} = 0.3. Then measure the absorbance every 30 min until it reaches 0.5–0.6.

2.1.5.2. Allow the cells to grow until the absorbance reaches OD_{600} = 0.5–0.6

2.1.5.3. Add 24 µg/mL biotin and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cell culture.

NOTE: IPTG is used to induce protein expression of the kinesin motors, which are tagged with biotin carboxyl carrier protein (BCCP) and six histidines (H6). The H6 tag is used in the purification process (step 2.2), while BCCP binds to the added biotin molecules to biotinylate the expressed kinesin motors.

2.1.5.4. Incubate and shake the cell culture at 20 °C and 250 rpm for 12–20 h.

2.1.6. Harvest the cells by centrifuging at 5,000 x *g* and 4 °C for 10 min. Discard the supernatant. Store the cell pellets at -80 °C.

2.2. Purify the kinesin motor protein (modified from Spriestersbach et al.[41]):

2.2.1. Suspend the cell pellet (step 2.1.6) with an equal volume of lysis buffer (50 mM PIPES, 4 mM $MgCl_2$, 50 µM ATP, 10 mM 2-mercaptoethanol (βME), 20 mM imidazole, pH 7.2), followed by adding one tablet of protease inhibitor, 2 mg of phenylmethyl sulfonyl fluoride (PMSF), and 2 mg of lysozyme.

2.2.2. Lyse the cells by flash freezing in liquid nitrogen (LN) 3x and thawing the cell mixture.

NOTE: After lysing, the mixture should become viscous.

2.2.3. Clarify the lysed cell mixture by centrifuging at 230,000 x *g* and 4 °C for 30 min.

2.2.4. Collect the supernatant and flow it through a gravity column, followed by washing the column with 10 mL of lysis buffer.

NOTE: Proteins with an H6 tag, such as kinesin K401-BCCP-H6, should remain in the column.

2.2.5. Eluate the tagged protein with 5 mL of elution buffer (50 mM PIPES, 4 mM $MgCl_2$, 50 µM ATP, 500 mM imidazole, pH 7.2). Collect the flow-through sequentially in 1 mL fractions. To determine the protein-containing fractions, mix 3 µL of each fraction with 100 µL of triphenylmethane dye. The protein-containing fractions should turn blue. Combine these fractions and dilute by 5x with lysis buffer.

2.2.6. Concentrate the protein solution.

2.2.6.1. Load the solution in a centrifugal filter tube.

2.2.6.2. Centrifuge at 3,000 x *g* for 10 min at 4 °C.

2.2.6.3. Shake the tube gently and centrifuge again until the solution volume is <3 mL.

2.2.7. Measure the protein concentration with a spectrometer (extinction coefficient: 0.549 $(mg/mL)^{-1}cm^{-1}$)[42,43]. Dilute the protein to 1 mg/mL while adding 35% w/v sucrose.

2.2.8. Store at -80 °C.

2.3. Measure the kinesin concentrations with an electrophoresis gel (modified from Taylor et al.[44]).

NOTE: To measure the kinesin concentration with an electrophoresis gel, tubulin is an ideal concentration ladder because of its high purity and its measurable concentration via a spectrometer (**Figure 2A**)[36].

2.3.1. Prepare tubulin samples with concentrations of 0.25, 0.5, 0.75, 1.00, and 1.25 mg/mL. Mix 15 µL of each tubulin sample and kinesin sample (step 2.2.8) separately with 5 µL of sample buffer (200 mM Tris-HCl, 8% sodium dodecyl sulfate (SDS), 400 mM DTT, 0.2% bromophenol blue, 40% glycerol, pH 6.8) and incubate at 90 °C for 3 min.

2.3.2. Prepare the electrophoresis.

2.3.2.1. Lock an electrophoresis gel into the gel box.

2.3.2.2. Fill the box with running buffer (50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.7).

NOTE: Ensure that the buffer level is above the top opening of the gel.

2.3.3. Load 10 µL of protein standard ladder, tubulin samples, and kinesin sample into separate wells and apply 200 V across the gel for 45 min.

2.3.4. Gel staining.

2.3.4.1. Incubate and rock the gel with boiled (approximately 95 °C) stain solution A (0.5 g/L triphenylmethane dye, 10% v/v acetic acid, 25% isopropanol) for 5 min, then rinse the gel with deionized (DI) water.

2.3.4.2. Repeat with stain solutions B (0.05 g/L triphenylmethane dye, 10% v/v acetic acid, 10% isopropanol), C (0.02 g/L triphenylmethane dye, 10% v/v acetic acid), and D (10% v/v acetic acid),

sequentially. Incubate and rock the gel in DI water overnight.

2.3.5. Scan the gel. Convert the gel image to a grayscale image, followed by a black and white inversion and contrast enhancement to reveal bright protein bands in the black background.

2.3.6. Measure the brightness of each band by summing the pixel values. Apply the linear fit $C = aB + b$, where B is the brightness of a tubulin band, C is the corresponding concentration, and a and b are fitting parameters. Determine the concentration of kinesin C_k by using the brightness of the kinesin band B_k to calculate the linear equation: $C_k = aB_k + b$.

2.4. Cluster the kinesin motors.

2.4.1. Mix 1.5 µM kinesin (step 2.2.8) with 120 µM DTT and 120 nM streptavidin. Incubate on ice for 30 min.

2.4.2. Store at -80 °C.

3. Prepare polyacrylamide-coated glass slides and coverslips (modified from Lau et al.[45])

3.1. Load new glass slides and glass coverslips in corresponding containers. Submerge the slides and coverslips in 1% v/v detergent in DI water and then boil the water in a microwave.

3.2. Sonicate the slides and coverslips for 5 min and then rinse with DI water to remove detergent.

3.3. Submerge and sonicate the slides and coverslips in ethanol for 5 min. Rinse with DI water.

3.4. Submerge and sonicate the slides and coverslips in 100 mM potassium hydroxide (KOH) for 5 min. Rinse with DI water.

3.5. Incubate the slides and coverslips in a silane solution (1% acetic acid and 0.5% 3-(trimethoxysilyl)propyl methacrylate in ethanol) for 15 min and then rinse with DI water.

3.6. Incubate the slides and coverslips in acrylamide solution (2% w/w acrylamide, 0.7 mg/mL ammonium persulfate, and 0.0035% v/v tetramethylethylenediamine in DI water) for ≥3 h.

3.7. Store the slides and coverslips in the acrylamide solution.

4. Prepare kinesin-driven, MT-based active fluids

4.1. Prepare active fluids (modified from Sanchez et al.[19]).

NOTE: The following steps demonstrate the process of preparing 100 µL of an active fluid using example stocks. The final volume is scalable, and the concentrations of the example stocks can

be adjusted as long as the final concentration of each component is maintained.

4.1.1. Mix 16.7 µL of 8 mg/mL MTs (step 1.4.4) with 6.7 µL of 1.8 µM kinesin motor clusters (step 2.4.2), and 1.1 µL of 500 mM DTT in high-salt M2B (M2B + 3.9 mM $MgCl_2$).

4.1.2. Bundle MTs by adding 11.4 µL of 7% w/w polyethylene glycol (PEG).

4.1.3. Activate kinesin motors by adding 2.8 µL of 50 mM ATP.

4.1.4. Maintain the ATP concentrations by adding 2.8 µL of stock pyruvate kinase/lactate dehydrogenase (PK/LDH) and 13.3 µL of 200 mM phosphenol pyruvate (PEP).

4.1.5. Reduce the photobleaching effect by adding 10 µL of 20 mM Trolox, 1.1 µL of 3.5 mg/mL catalase, 1.1 µL of 20 mg/mL glucose oxidase, and 1.1 µL of 300 mg/mL glucose.

4.1.6. Track the motion of the fluid by adding 1.6 µL of 0.025% v/v tracer particles.

4.1.7. Add high-salt M2B to achieve a total volume of 100 µL.

NOTE: The mixing orders in steps 4.1.1–4.1.6 are interchangeable. However, once ATP, MTs, and the motors are mixed, the motors start to consume ATP while stepping along the MTs. The sample is activated with a finite lifetime due to the limited fuels (ATP and PEP), so the experiment should be started promptly. The final active fluid should contain 1.3 mg/mL MT, 120 nM kinesin motor clusters, 5.5 mM DTT, 0.8% w/w PEG, 1.4 mM ATP, 2.8% v/v PK/LDH, 27 mM PEP, 2 mM Trolox, 0.038 mg/mL catalase, 0.22 mg/mL glucose oxidase, 3.3 mg/mL glucose, and 0.0004% v/v colloid in high-salt M2B.

4.2. Prepare a sample in a flow channel (modified from Chandrakar et al.[46]):

4.2.1. Rinse a polyacrylamide-coated glass slide and coverslip (step 3.7) with DI water. Dry the glasses with pressurized air. Place on a clean, flat surface.

4.2.2. Cut two strips of wax films with a 3 mm width and the same length as the glass coverslip (20 mm). Insert the strips between the slide and coverslip as channel spacers.

4.2.3. Adhere the glass to the wax film by placing the glass-wax complex on an 80 °C hot plate to melt the wax. During the melting, press the coverslip gently with a pipette tip to uniformly adhere the wax film to the glass surfaces. After adhesion, cool the glass complex to room temperature.

4.2.4. Load the active fluids (step 4.1) to the flow channel. Seal the channel with UV glue.

5. Control sample temperature

5.1. Build a temperature control setup (modified from designs in Lowensohn et al. and Wu et

al.[47-49]).

5.1.1. Prepare an aluminum cooling block.

5.1.1.1. Mill an aluminum plate with dimensions of approximately 30 mm × 30 mm × 5 mm.

5.1.1.2. Drill an internal channel through the plate (**Figure 3A**) and install hose fittings at the channel ends.

5.1.1.3. Hook each fitting to a water tube.

5.1.1.4. Connect one tube to a fish tank pump in a water reservoir while extending the other tube to the reservoir.

NOTE: The pump will circulate reservoir water through the aluminum internal channels to maintain the block temperature at approximately room temperature.

5.1.2. Wire a thermoelectric cooler (TEC) and a thermosensor to a temperature controller. Connect the controller to a computer using a USB port (**Figure 3B**).

5.1.3. Wire the temperature controller to a direct current (DC) power supply. Turn on the controller by plugging the power supply to an electrical outlet.

5.1.4. Perform the initial set up of the TEC following the controller manufacturer's guide. Test the TEC output. It is recommended to control the temperature controller via manufacturer-provided software, allowing thermosensor data recording and easier manipulation of the temperature controller.

5.1.5. Identify the heating and cooling sides of the TEC.

5.1.6. Attach the TEC's cooling side onto the cooling block (step 5.1.1) using thermal paste.

5.1.7. Attach a sapphire disk to the TEC's heating side using thermal paste.

5.1.8. The setup is complete. The sapphire surface and thermosensor can contact a sample to cool and heat the sample based on its temperature and target temperature.

NOTE: It is recommended that the TEC and cooling block have an aligned central hole for imaging the samples using bright-field microscopy (**Figure 3C**).

5.2. Use the temperature control setup to control the sample temperature[33].

5.2.1. Mount the sample to the setup.

5.2.1.1. Place the active fluid sample (step 4.2.4) on the sapphire surface with the slide side contacting the surface.

5.2.1.2. Secure the glass slide with paper tape.

5.2.1.3. Attach the thermosensor to the coverslip surface using copper tape.

5.2.2. Mount the setup on a microscope stage with the coverslip side facing toward the objectives. For example, on an inverted microscope, the coverslip side should face down. Secure the setup with paper tape and the microscope stage needle clamps if applicable.

NOTE: The presented temperature stage should work with common microscopes that are either inverted or upright. To ensure that the temperature stage is not moved during the experiment, it is best to secure the temperature stage to the microscope stage with tape.

5.2.3. Control the sample temperature.

5.2.3.1. Turn on the temperature controller and fish tank pump.

5.2.3.2. Follow the manufacturer's guide to set the target temperature and enable temperature control. The controller will adjust the heating or cooling power based on the target temperature and the sample temperature, as assessed by the thermosensor.

5.2.4. Record sample temperatures.

5.2.4.1. Follow the manufacturer's guide to record thermosensor temperature data during the experiment.

NOTE: The sample temperature should now be controlled by the controller and recorded by the computer (**Figure 3D**).

6. Characterize the active fluid activity (modified from methods by Henkin et al. and Wu et al.[20,27])

NOTE: The previous sections are used to prepare active fluid samples (sections 1–4) and control their temperature (section 5). To demonstrate the use of temperature to control the active fluid activity, observe the fluid behaviors, analyze their activities, and characterize their response to temperature.

6.1. Monitor tracers.

6.1.1. Image the sample with a constant interval Δt using green fluorescent protein (GFP) fluorescence to capture the movement of the tracer particles.

NOTE: Δ*t* should be chosen to allow the tracer movement to be tracked. Large Δ*t* values, such as 100 s, result in losing the tracer trajectories, whereas short Δ*t* values, such as 0.1 s, prevent the tracking algorithm from detecting the tracer movement between frames. A working Δ*t* should allow the tracer displacement between frames to be within ~9 pixels. For imaging tracers moving at 10 µm/s using a 4x objective, the Δ*t* is recommended to be 1–5 s.

6.1.2. Save the images as TIFF files, name the files based on frame number, and store them in a separate folder. These processes are necessary to ensure that the acquired images will be correctly analyzed with the MATLAB script provided (step 6.2.2).

6.2. Track tracers adopting the tracking software developed by Ouellette et al.[50,51]):

6.2.1. Download the tracking software from the Environmental Complexity Laboratory, Stanford University (https://web.stanford.edu/~nto/software.shtml). Ensure that each MATLAB file is in the same folder.

6.2.2. Track tracers using a custom MATLAB script: particle_tracking.m. The script reads tracer images (step 6.1) and tracks tracer movement using the software of Ouellette et al.[50,51]. It outputs two files: 1) background.tif, representing the image background, and 2) Tracking.mat, containing the particle trajectories and velocities in each frame (**Figure 4A**).

6.3. Analyze the tracer mean speeds using a custom MATLAB script: analysis.m. The script reads the tracking file (Tracking.mat) and outputs the mean speed of tracers vs. time, along with a time-averaged mean speed with specified averaging windows (**Figure 4B**)[33].

6.4. Record the time-averaged mean speed.

6.5. Measure the time-averaged mean speed by repeating the experiment (steps 4, 5.2 and 6.1–6.4) at 10–40 °C. Use the recorded mean speeds to plot the mean speed vs. temperature (**Figure 4C**)[33].

REPRESENTATIVE RESULTS:

Preparing the kinesin-driven, MT-based active fluids requires both kinesin and MTs. The MTs were polymerized from labeled tubulins (steps 1.3 and 1.4) that were purified from bovine brains (step 1.1, **Figure 2A**), followed by recycling to enhance purity (step 1.2, **Figure 2B**). The kinesin motor proteins were expressed in and purified from *E. coli* (steps 2.1 and 2.2, **Figure 2B**)[41,52]. The concentration of the prepared kinesin stock was measured with an SDS gel by comparing the main band brightness with that of recycled tubulins with known concentrations (step 2.3, **Figure 2B inset**). The brightness values of the tubulin bands (B) were linearly fit to their corresponding concentration (C) using the equation $C = \alpha B + \epsilon$, yielding $\alpha = 3.1 \times 10^{-2}$ mg/mL and $\epsilon = 8.7 \times 10^{-4}$ mg/mL (**Figure 2C**). The fitted equation was used to determine the concentration of a kinesin band by applying the measured band brightness, B_k = 1,060, yielding a kinesin concentration of C_k = 0.95 mg/mL. MT and kinesin samples with known concentrations were used to prepare active fluid samples. The active fluids were synthesized, loaded into a polyacrylamide-coated flow cell,

and the cell was sealed with UV glue (sections 3 and 4)[46].

To demonstrate the control of the active fluid activity with the temperature, the active fluid sample was mounted on the homemade temperature stage (step 5.2, **Figures 3A–C**). The sample temperature was monitored and controlled by the controller according to a proportional-integral-derivative (PID) algorithm[53]. Samples controlled at 10 °C, 20 °C, 30 °C, and 40 °C appeared to fluctuate in temperature within 0.1–0.3 °C for 4 h, demonstrating the stability and reliability of this temperature control setup (**Figure 3D**).

To observe the sample, the setup was mounted onto an epifluorescence microscope. The sample was doped with Alexa 488-labeled tracers, which were imaged with fluorescence microscopy via a GFP channel (step 6.1). The tracers were imaged every Δt = 2 s. The sequential images allowed for tracking tracer trajectories $\boldsymbol{r}_i$, where i represents the tracer index (step 6.2, **Figure 4A**). The trajectories revealed a mean speed $\boldsymbol{v}(t) \equiv \langle |\boldsymbol{r}_i(t) - \boldsymbol{r}_i(t-\Delta t)|/\Delta t \rangle_I$ (step 6.3). The mean speeds measured at 20–36 °C appeared to be nearly time-independent at t = 0–2 h, whereas at 10 °C and 40 °C the mean speeds decayed quickly (**Figure 4B**). The decay at 10 °C was caused by MT depolymerization below 16 °C. The decay at 40 °C was due to the kinesin clusters malfunctioning above 36 °C. According to our previous studies these kinesin motor clusters lose the ability to drive pairs of MTs after preincubation at >36 °C[33]. To characterize the mean speeds for each temperature while reflecting the decay induced by these factors, the mean speeds were averaged between t = 1–2 h (steps 6.3 and 6.4)[33]. The time-averaged mean speeds were measured between 10 °C–40 °C (step 6.5, **Figure 4C**). Below 16 °C and above 36 °C the mean speeds decayed quickly due to MT depolymerization and malfunctioning kinesin clusters[33], whereas increasing temperatures from 16 °C to 36 °C accelerated the mean speeds from 4 to 8 μm/s, demonstrating the feasibility of tuning the mean speed of an active fluid flow using temperature. To further demonstrate the capability of the temperature control, the system temperatures were alternated between 20 °C and 30 °C every 30 min. The mean speeds of the active fluids did not only accelerate and decelerate accordingly, but they also responded to the temperature change within 10 s (**Figure 5**). Such a reversible and quick response of the active fluid demonstrates the workability of using temperature to dynamically control fluid activities.

FIGURE AND TABLE LEGENDS:

Figure 1. Introduction of kinesin-driven, MT-based 3D active fluids. (**A**) Schematics of interfilament sliding. Pairs of antiparallel MTs were bundled by depletants and driven apart by motor clusters. (**B**) Motor clusters collectively drove pairs of MTs, leading MT bundles to extend. (**C**) The extensile bundles constituted a MT-based active gel (green) that stirred the surrounding liquid to induce a flow. To track the flow, the liquid was doped with tracers (red). This figure was adapted from Bate et al.[33].

Figure 2. Images of SDS gels from the tubulin and kinesin purifications. (**A**) Image of an SDS gel of purified tubulin. (**B**) Image of an SDS gel of recycled tubulins and purified kinesin. The lanes from left to right were protein standards, blank, 1.25–0.25 mg/mL recycled tubulins, blank, and kinesin stock. (**C**) The concentration of purified kinesin was determined by comparing the band brightness with the sequential bands of tubulins with measured concentrations (red and blue

dashed rectangles in inset). The brightness of each band was measured by summing the pixel values within a cropped band image. To reduce the background noise, before cropping the gel image was transferred to grayscale, black-and-white inverted, and then contrast-enhanced to reveal a black background (inset).

Figure 3. Temperature control setup. (**A**) Schematics of the aluminum block for the temperature control setup. The block contains an internal channel for water to flow through it and carry away the TEC-generated heat. The central hole allows the sample to be illuminated by bright field microscopy on one side and imaged with an objective on the other side. (**B**) Schematics of the temperature control setup. Silicon thermal paste is applied between the aluminum cooling block and the TEC and between the TEC and the sapphire disc. (**C**) Image of a sample mounted on the temperature-controlled stage. Water tubes are connected to a pump immersed in a water reservoir. (**D**) Recorded sample temperatures vs. time for target temperatures 10 °C, 20 °C, 30 °C, and 40 °C, respectively. Sample temperatures were maintained at temperatures with a fluctuation of 0.1–0.3 °C. **B** and **D** were adapted from Bate et al.[33].

Figure 4. Tuning active fluid flows via temperature. (**A**) Imaging tracers (white dots) allowed for tracking the flow trajectories sequentially (miscellaneously colored curves). The tracers were monitored every 5 s (Δt = 5 s) at room temperature (~20 °C). (**B**) Tracer mean speed vs. time at 10 °C, 20 °C, 30 °C, 36 °C, and 40 °C, respectively. (**C**) Tracer mean speed vs. temperature. Each point represents the average tracer mean speed during the first and second hours. Below 16 °C the MTs depolymerized, and above 36 °C, kinesin clusters malfunctioned. Therefore, the working temperature is between 16–36 °C, where mean speeds varied from 4–8 μm/s. The error bars represent the standard deviation of the time-averaged mean speeds. **B** and **C** were adapted from Bate et al.[33].

Figure 5. Alternating the flow speed of active fluids by periodically alternating the system temperature. Switching the temperature between 20 °C and 30 °C every 30 min accelerated and decelerated flow speeds repeatedly, demonstrating local control of active fluid activities with the temperature. This figure was adapted from Bate et al.[33].

DISCUSSION:

Controlling active matter in situ opens the door to directed self-organization of active matter[4,5,24,28,54]. In this article, we present a protocol for using temperature to control kinesin-driven, MT-based active fluids in situ, based on the Arrhenius characteristic of the system[29-31]. Because the system is protein-based, maintaining protein functionality throughout the experiment is key to successfully applying the protocol. The main proteins in the system are MTs and kinesin clusters. The former depolymerize below 16 °C and the latter malfunction above 36 °C[33]. Maintaining the system temperature between 16–36 °C is therefore vital for active fluids to develop steady dynamics and to enable their response to temperature reversibly (**Figure 4** and **Figure 5**). However, the temperature is controlled based on a PID algorithm, which tends to overshoot the target temperature[53]. To reduce this overshooting, we recommend setting multiple intermediate target temperatures before setting the final target temperature. For example, to heat up the sample from room temperature (approximately 20 °C) to 35 °C, rather

than setting the target temperature directly at 35 °C, we recommend an intermediate target temperature of 30 °C to reduce the chance that the temperature increases above 36 °C, which would irreversibly damage the proteins[33]. Similarly, when cooling the sample from room temperature to ~16 °C, it is recommended to set an intermediate target temperature of 18 °C, because before reaching a steady state, the PID may cool the sample below 16 °C, depolymerizing the MTs[33].

The presented temperature control method relies on cooling and heating using a TEC. The use of the TEC ensures that the sample reaches the target temperature within seconds, whereas a conventional temperature control setup using a temperature-controlled water bath takes minutes to reach the desired temperature (**Figure 5**)[55]. The TEC generates nonzero net heat that is dissipated by an aluminum internal water circulation system. However, the water allows mold to grow, which will eventually clog the channel[56]. A clogged channel inhibits water flow, and the heat will accumulate in the aluminum block, eventually melting the water tubes. The melted tubes cause water to spill over the microscope and camera, damaging the electronic instruments. Therefore, to adopt the presented temperature control setup, we recommend adding 0.1% hydrogen peroxide to the water to inhibit mold growth[57-59]. We expect that this step will ensure that the aluminum internal channel remains clog-free and prevent water damage to nearby electronic devices.

Manipulating the temperature, coating the flow cell surfaces with polyacrylamide, and synthesizing active fluids are three critical steps to realizing this in situ-controlled active fluid. However, this controllability is limited to the temperature range where the involved proteins can function normally. In the active fluid system, the primary proteins are MTs and kinesin clusters, which function normally between 16–36 °C[33]. Within this temperature range, active fluids vary their mean flow speeds from 4–8 μm/s (**Figure 4C**). Mean speeds outside this range are beyond the limit of the control method presented. In contrast, Ross et al. reported an alternative that allows for the active fluid activities to be switched on and off using light[28]. The light control also allows for activating active fluids in a 50 μm scale optically-defined boundary. However, such an alternative requires modifying the kinesin structure along with tuning an optical path in a microscope. In comparison, the advantages of adopting the method presented in this article are 1) the active fluids do not need to be redesigned, 2) the microscopes do not need to be modified, 3) the temperature control setup is low-cost and easy to use, and 4) the method is transferrable to other temperature-dependent systems such as the gliding assay[29-32] or more generally enzyme-based systems[60]. We also expect that the presented method will open the door to designing microfluidic systems where channel flows are controlled locally without valves.

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DISCLOSURES:

The authors have nothing to disclose.

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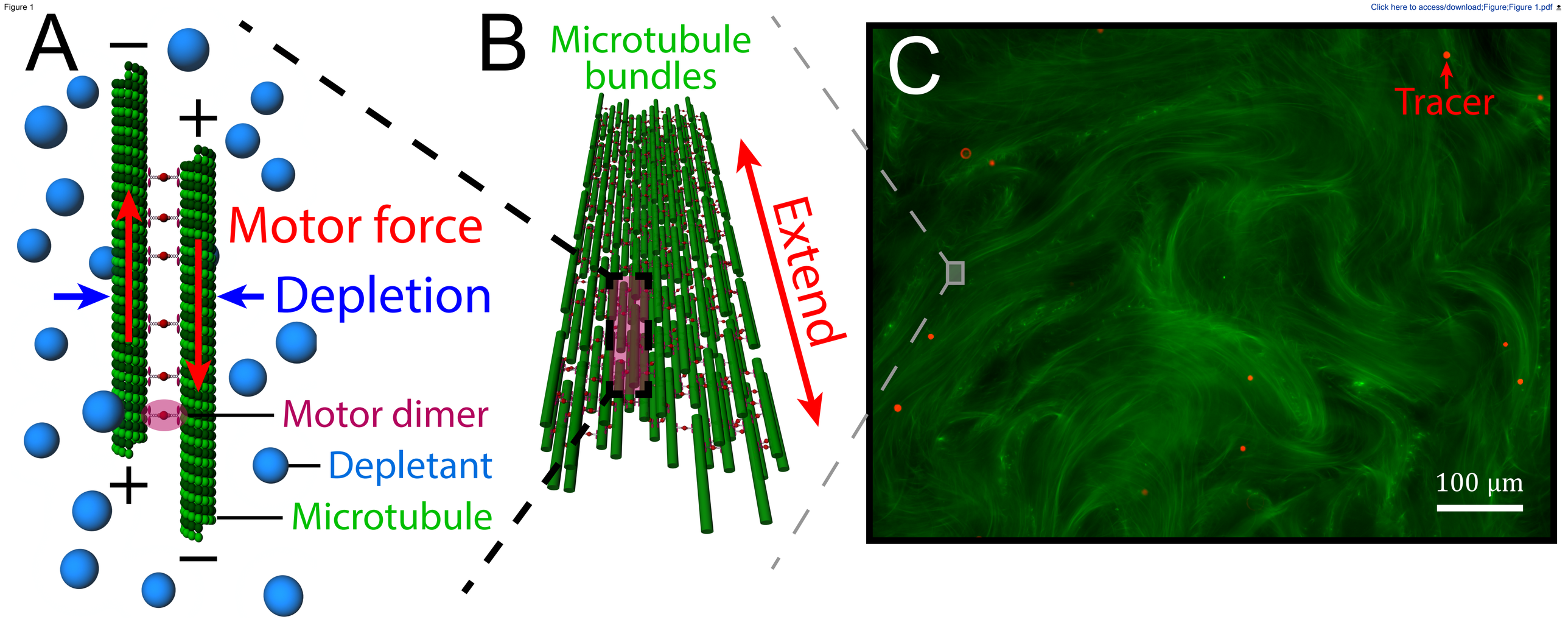


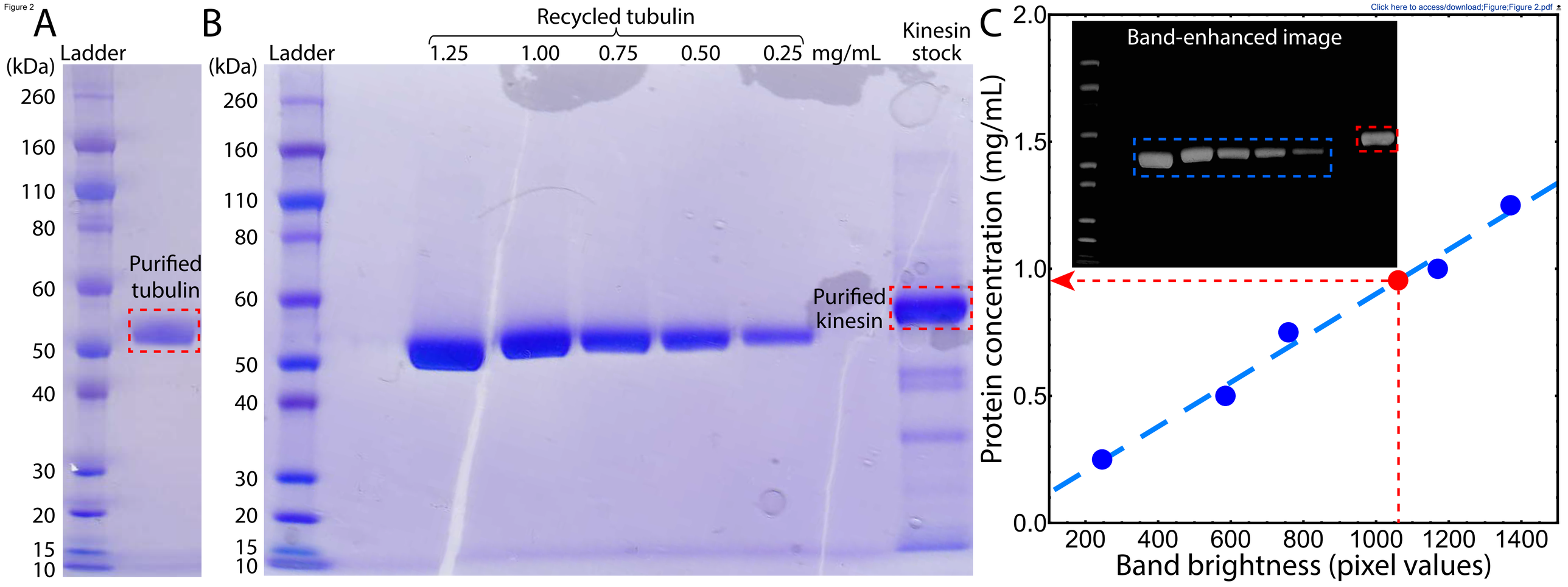
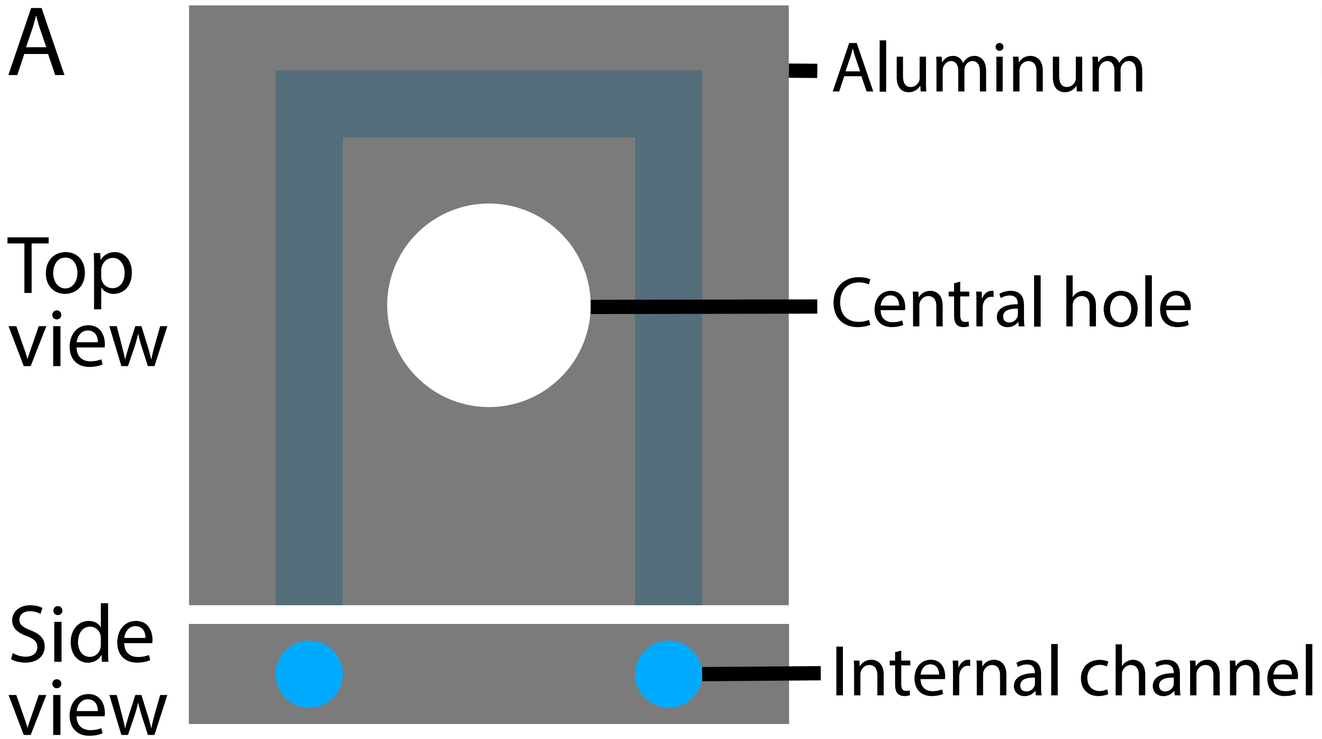
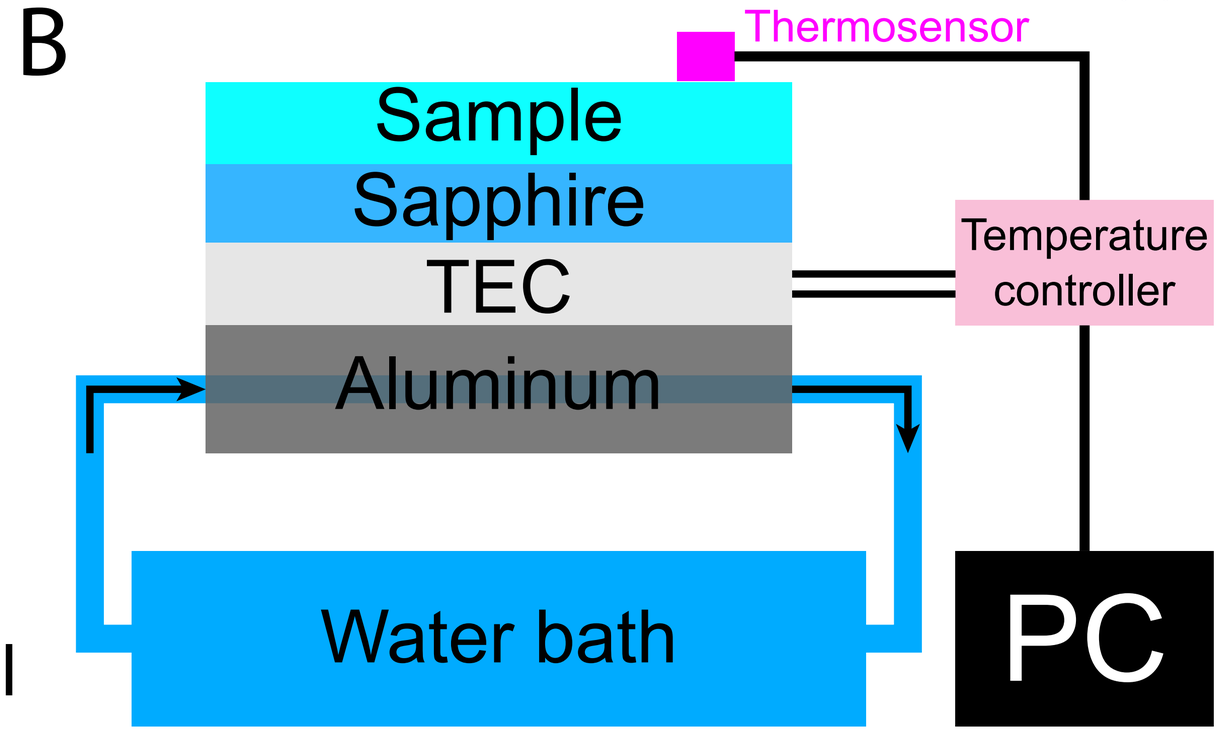
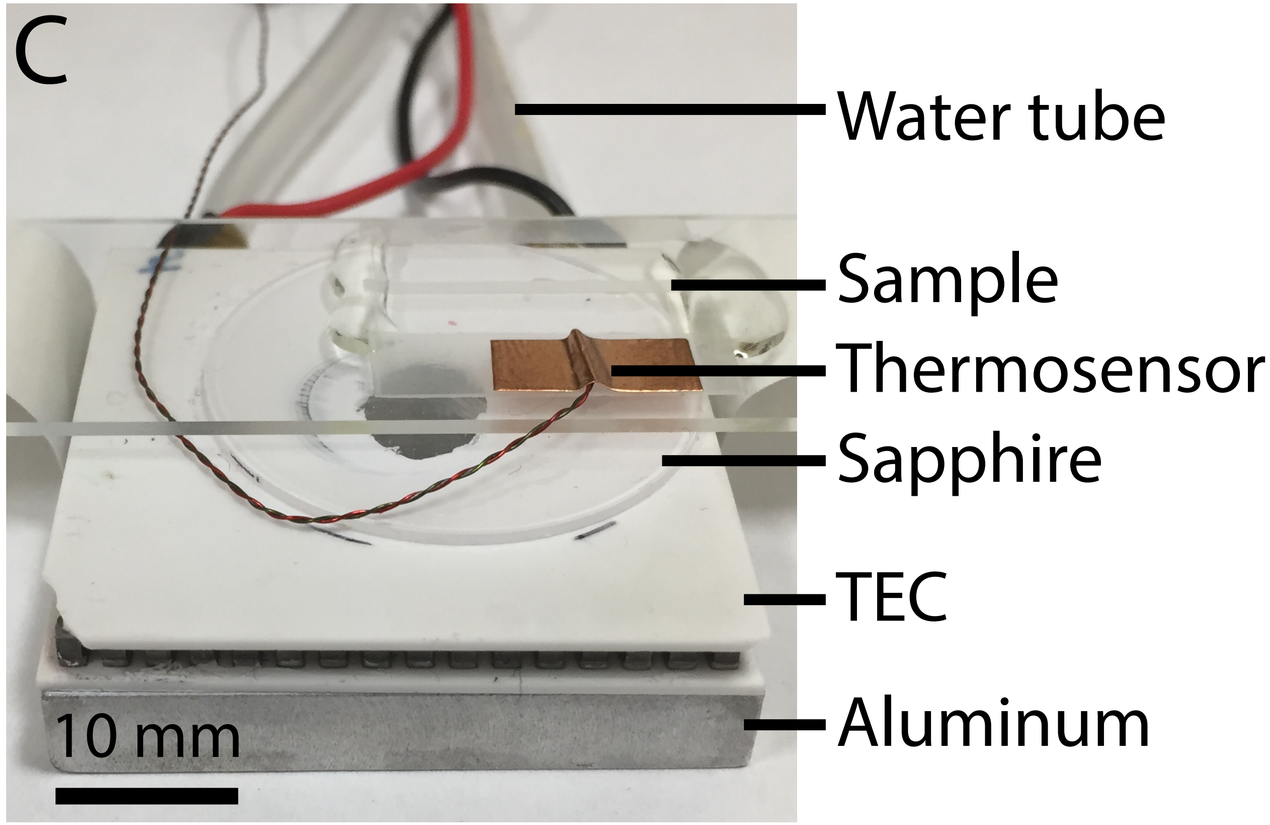


Figure 3







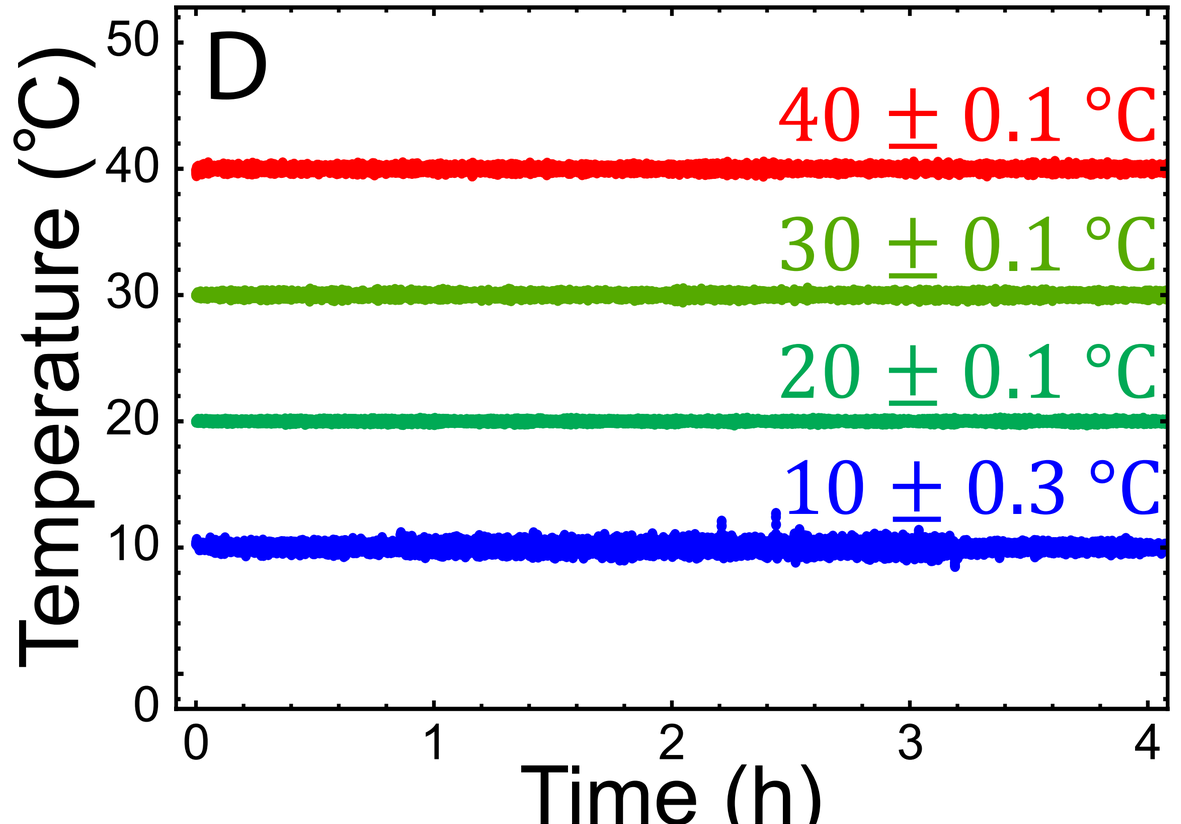


Figure 4

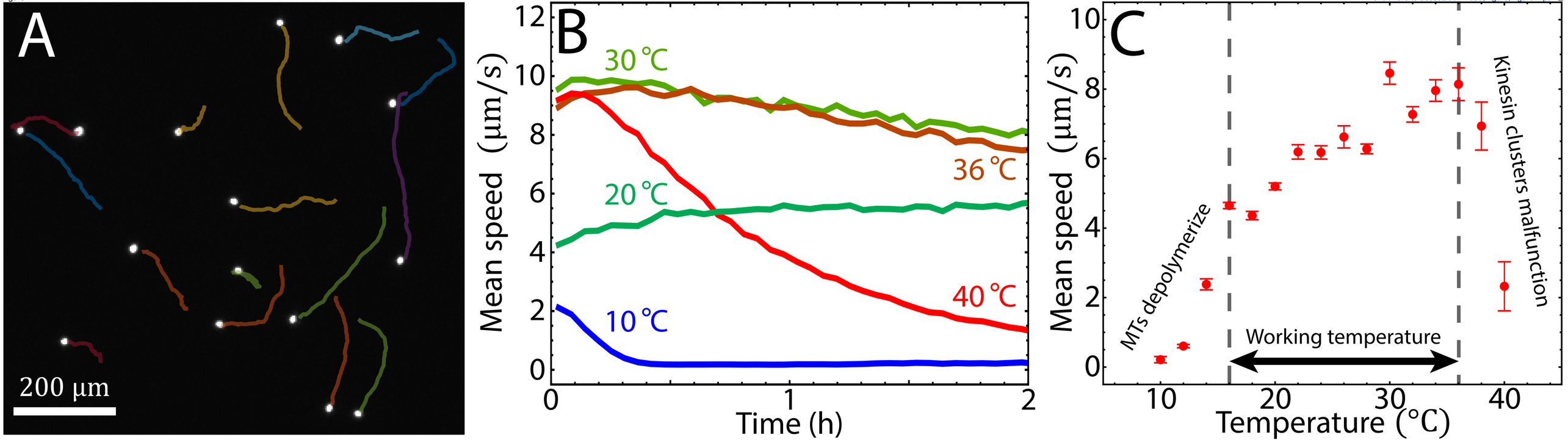


Figure 5

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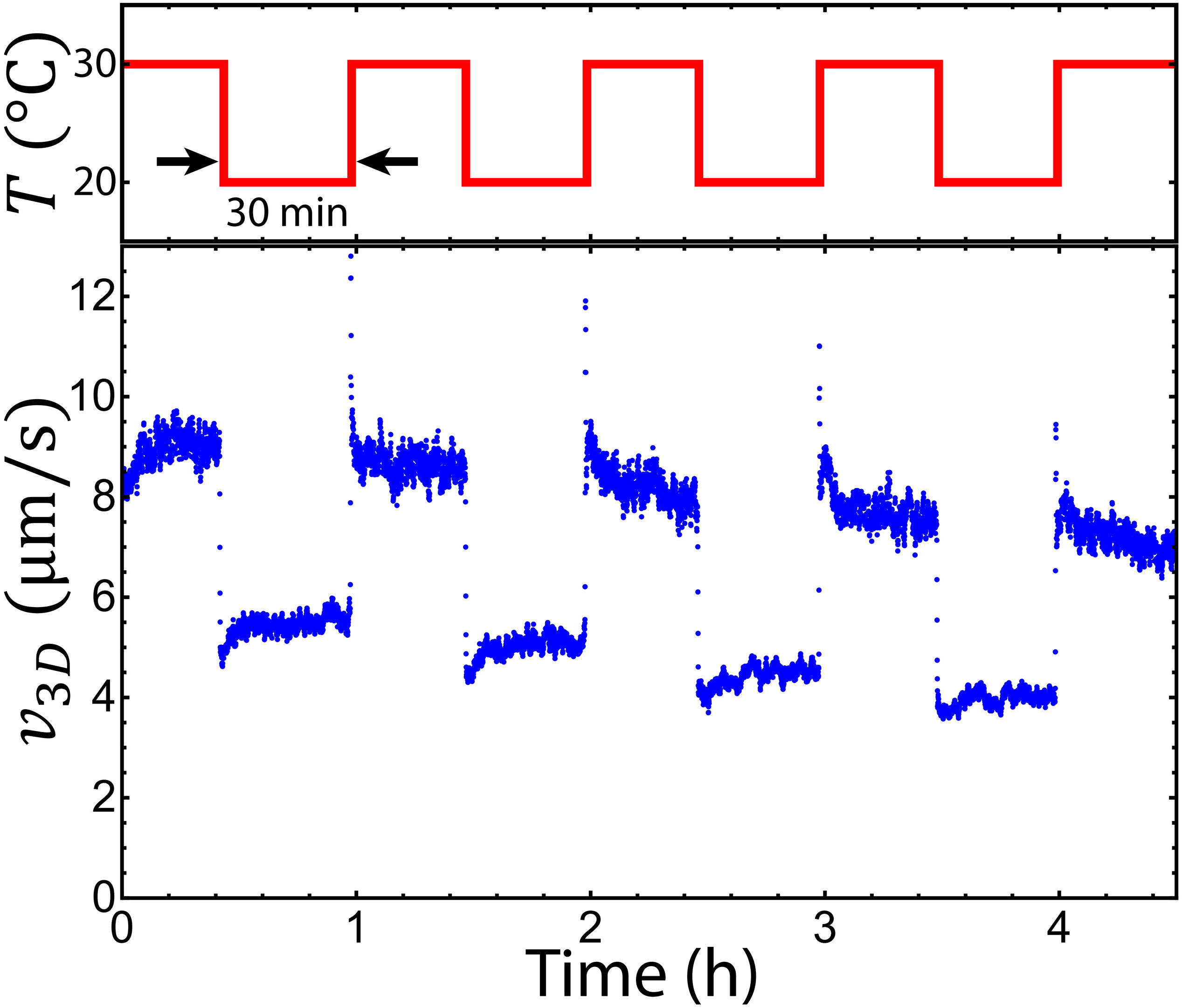

Name of Material/ Equipment	Company	Catalog Number
(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid	Sigma-Aldrich	238813
2-Mercaptoethanol	Sigma-Aldrich	M6250
3-(Trimethoxysilyl)propyl methacrylate, 98%, ACROS Organics	Fisher Scientific	AC216550050
3.2mm I.D. Tygon Tubing R-3603	HACH	2074038
31.75 mm diameter uncoated, sapphire window	Edmund Optics	43-637
3M 1181 Copper Tape - 1/2 IN Width X 18 YD Length - 2.6 MIL Total Thickness - 27551	R.S. HUGHES	054007-27551
Acetic Acid	Sigma-Aldrich	A6283
Acrylamide Solution (40%/Electrophoresis), Fisher BioReagents	Fisher Scientific	BP1402-1
Adenosine 5’-triphosphate dipotassium salt hydrate	Sigma-Aldrich	A8937
Alexa Fluor 647 NHS Ester (Succinimidyl Ester)	Thermo Fisher Scientific	A20006
Amicon Ultra-4 Centrifugal Filter Unit	Sigma-Aldrich	UFC801024
Ammonium Persulfate, 100g, MP Biomedicals	Fisher Scientific	ICN802829
Ampicillin Sodium Salt (Crystalline Powder), Fisher BioReagents	Fisher Scientific	BP1760
Antivibration Table	Nikon	63-7590S
Avanti J-E Centrifuge	Beckman Coulter	369001
Bacto Agar Soldifying Agent, BD Diagnostics	VWR	90000-760
Biotin	Alfa Aesar	A14207
Bucket-plastic white - 2 gallon	Bon	84-715
Calcium Chloride	Sigma-Aldrich	746495
Catalase from bovine liver	Sigma-Aldrich	C40
CFI Plan Apo Lambda 4x Obj	Nikon	MRD00045
C-FLLL-FOV GFP HC HC HISN ero Shift	Nikon	96372
CH-109-1.4-1.5	TE Technology	CH-109-1.4-1.5
Chloramphenicol, 98%, ACROS Organics	Fisher Scientific	C0378

Cooling block	N/A	N/A
Coomassie Brilliant Blue R-250 #1610400	Bio-Rad	1610400
D-(+)-Glucose	Sigma-Aldrich	G7528
Dimethyl Sulfoxide (Certified ACS), Fisher Chemical	Fisher Scientific	D128
DL-1,4-Dithiothreitol, 99%, for biochemistry, ACROS Organics	Fisher Scientific	AC165680050
DOWSIL 340 Heat Sink Compound	Dow	1446622
ETHYL ALCOHOL, 200 PROOF ACS/USP/NF GRADE 5 GALLON POLY CUBE	Pharmco by Greenfield Global	111000200CB05
Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid	Sigma-Aldrich	E3889
Ethylenediaminetetraacetic acid	Sigma-Aldrich	798681
Fisher BioReagents Microbiology Media Additives: Tryptone	Fisher Scientific	BP1421
Fisher BioReagents Microbiology Media Additives: Yeast Extract	Fisher Scientific	BP1422
Fluoresbrite YG Microspheres, Calibration Grade 3.00 µm	Polysciences	18861
Glucose Oxidase from *Aspergillus niger*	Sigma-Aldrich	G2133
Glycerol	Sigma-Aldrich	G5516
GpCpp	Jena Bioscience	NU-405L
GS Power's 18 Gauge (True American Wire Ga), 100 feet, 99.9% Stranded Oxygen Free Copper OFC, Red/Black 2 Conductor Bonded Zip Cord Power/Speaker Electrical Cable for Car, Audio, Home Theater	Amazon	B07428NBCW
Guanosine 5’-triphosphate sodium salt hydrate	Sigma-Aldrich	G8877
Hellmanex III	Sigma-Aldrich	Z805939
HEPES Sodium Salt (White Powder), Fisher BioReagents	Fisher Scientific	BP410
High performance blender machine	AIMORES	AS-UP1250
His GraviTrap	GE Healthcare	11003399
Imidazole	Sigma-Aldrich	I5513
IPTG	Sigma-Aldrich	I6758

Isopropyl Alcohol 99%	Pharmco by Greenfield Global	231000099
JA-10 rotor	Beckman Coulter	369687
L-Glutamic acid potassium salt monohydrate	Sigma-Aldrich	G1501
Lysozyme from chicken egg white	Sigma-Aldrich	L6876
Magnesium chloride hexahydrate	Sigma-Aldrich	M2670
MES sodium salt	Sigma-Aldrich	M5057
MOPS	Sigma-Aldrich	M1254
MP-3022	TE Technology	MP-3022
N,N,N',N'-Tetramethylethylenediamine 99%, ACROS Organics	Fisher Scientific	AC138450500
Nanodrop 2000c UV-VIS Spectrophotometer	Thermo Fisher Scientific	E112352
Nikon Ti2-E Nikon Inverted Microscope	Nikon	MEA54000
Norland Optical Adhesive 81	Norland Products	NOA81
Novex Sharp Pre-stained Protein Standard	Thermo Fisher Scientific	LC5800
NuPAGE 4-12% Bis-Tris Protein Gels, 1.5 mm, 10-well	Thermo Fisher Scientific	NP0335BOX
Optima L-90K Ultracentrifuge	Beckman Coulter	365672
Parafilm PM996 Wrap , 4" Wide; 125 Ft/Roll	Cole-Parmer	EW-06720-40
Pe 300 ultra Illumination System Single Band , 3mm Light Guide control Pod power supply	Nikon	PE-300-UT-L-SB-40
Phenylmethanesulfonyl fluoride	Sigma-Aldrich	78830
Phosphoenolpyruvic acid monopotassium salt, 99%	BeanTown Chemical	129745
Pierce Coomassie (Bradford) Protein Assay Kit	Thermo Fisher Scientific	23200
Pierce Protease Inhibitor Mini Tablets	Thermo Fisher Scientific	A32953
PIPES	Sigma-Aldrich	P6757
Pluronic F-127	Sigma-Aldrich	P2443

Poly(ethylene glycol)	Sigma-Aldrich	81300
Potassium Hydroxide (Pellets/Certified ACS), Fisher Chemical	Fisher Scientific	P250-500
PowerEase 300W Power Supply (115 VAC)	ThermoFisher Scientific	PS0300
PS-12-8.4A	TE Technology	PS-12-8.4A
Pyruvate Kinase/Lactic Dehydrogenase enzymes from rabbit muscle	Sigma-Aldrich	P-0294
Quiet One Lifegard Fountain Pump, 296-Gallon Per Hour	Amazon	B005JWA612
Rosetta 2(DE3)pLysS Competent Cells - Novagen	Millipore Sigma	71403
Sharp Microwave ZSMC0912BS Sharp 900W Countertop Microwave Oven, 0.9 Cubic Foot, Stainless Steel	Amazon	B01MT6JZMR
Sodium Chloride (Crystalline/Certified ACS), Fisher Chemical	Fisher Scientific	S271-500
Sodium dodecyl sulfate	Sigma-Aldrich	L3771
Sodium phosphate monobasic	Sigma-Aldrich	S8282
Streptavidin Protein	Thermo Fisher Scientific	21122
Sucrose	Sigma-Aldrich	S7903
TC-720	TE Technology	TC-720
Tris Base, Molecular Biology Grade - CAS 77-86-1 - Calbiochem	Sigma-Aldrich	648310
Type 45 Ti rotor	Beckman Coulter	339160
Type 70 Ti rotor	Beckman Coulter	337922
Type 70.1 Ti rotor	Beckman Coulter	342184
VWR General-Purpose Laboratory Labeling Tape	VWR	89097-916
VWR Micro Cover Glasses, Square, No. 1 1/2	VWR	48366-227
VWR Plain and Frosted Micro Slides, Premium	VWR	75799-268
XCell SureLock Mini-Cell	ThermoFisher Scientific	EI0001
ZYLA 5.5 USB3.0 Camera	Nikon	ZYLA5.5-USB3

	Comments/Description
Trolox	
Water tubes	
Sapphire disc	
Copper tape	
ATP	
	Far-red fluorescent dye. Alexa 647 can be pre suspended in dimethylsulfoxide (DMSO) before mixing with microtubules (1.3.3.2.)
	Centrifugal filter tube. Cutoff molecular weight: 10 kDa
APS	
Ampicillin	
Agar	
Water bucket	
$CaCl_2$	
4x air objective	
GFP filter cube	
Thermoelectric Cooler (TEC)	

Custom milled aluminum
Triphenylmethane dye

DMSO

DTT

Thermal paste

Ethanol

EGTA

EDTA

Tryptone

Yeast extract

Tracer particles

Guanosine-5’[(α,β)-methyleno]triphosphate (GMPCPP)

Copper wire

GTP
Detergent
NaHEPES
Blender
Gravity Column

Isopropyl β-D-1-thiogalactopyranoside

Isopropanol

K-Glutamate

$MgCl_2 \bullet 6H_2O$

2-(N-Morpholino)ethanesulfonic acid sodium salt

3-(N-Morpholino)propanesulfonic acid

Thermocouple

TEMED

Spectrometer

UV glue

Protein standard ladder

SDS gel

Wax film

Cool LED Illuminator

PMSF

PEP

1,4-Piperazinediethanesulfonic acid

PEG. Average molecular weight 20,000 Da

KOH

DC power supply of the gel box

DC power supply of the temperature controller

PK/LDH

Fish tank pump
Competent cells

Microwave for boiling the water

NaCl

SDS
NaH_2PO_4

Temperature controller

Tris-HCL

Paper tapes
Glass coverslips
Glass slides

Gel box

Monochrome CCD camera

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Author(s):	Teagan E. Bate, Edward J. Jarvis, Megan E. Varney, Kun-Ta Wu

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Title:	Assistant Professor
Signature:	Wu, Kunta
Date:	June 7, 2019

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WORCESTER POLYTECHNIC INSTITUTE

August 29, 2019

Dr. Phillip Steindel
Review Editor
Journal of Visualized Experiments
1 Alewife Center
Suite 200
Cambridge, MA 02140

Dear Dr. Steindel,

Please find attached our manuscript entitled "Controlling Flow Speeds of Microtubule-Based 3D Active Fluids Using Temperature," which we are resubmitting for publication to *Journal of Visualized Experiments*. We thank the editor and reviewers for the insightful and constructive suggestions. Please find our point-by-point responses in the attachment of this letter. We have revised the manuscript based on the editor's and reviewers' comments and suggestions. We believe that our manuscript is now clearer and more readable for a wider audience, and therefore justifies publication in the *Journal of Visualized Experiments*. Please do not hesitate to contact us if you have questions. We thank you for your time and consideration.

Sincerely,

Wu, Kunta

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Assistant Professor
Department of Physics
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Email: kwu@wpi.edu

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the editor for their reminder. We have sent the manuscript to a professional language editor to proofread the documents to ensure that the manuscripts have no spelling or grammar issues.

2. Please avoid using Equation Editor for inline text as such text will be formatted differently than the surrounding text. Please include equations on their own line.

We have eliminated the usage of Equation Editor and are now using plain text to describe the equations.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Rosetta 2, NanoDrop, Parafilm.

We thank the editor for the reminder about the JoVE policy. We have removed Rosetta 2, NanoDrop, and Parafilm in the manuscript, and replaced them with competent cells, spectrometers, and wax films, respectively. We believe all the commercial products used in the protocol are referenced in the Table of Materials.

Protocol:

1. For each protocol step, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We thank the editor for the correction. We have corrected each step, and split long steps into sub-steps. Most of our steps were from a reference so we cited the protocol in the beginning of the section. For example, the protocol in Step 1.1 of purifying tubulins from bovine brains was adopted from Castoldi *et al.* so we cited the protocol in the beginning of the step (Step 1.1).

Specific Protocol steps:

1. 1.1.1: How fresh are the brains, exactly?

We have clarified the freshness of the brains in a NOTE following 1.1.1. and specify time between slaughter and procedure start in hours.

2. 1.1.2: How are blood vessels cleared?

We used scalpels to cut the vessels to smaller pieces and remove them from the brains. We have addressed this detail in the manuscript (Step 1.1.2.1)

3. 1.1.7/2.2.7: Do you have an extinction coefficient?

We have provided the extinction coefficients of tubulins and kinesin motor proteins along with corresponding references (Steps 1.1.7 and 2.2.7).

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We thank the editor for the reminder of confirming the copyright permission. The figures we used are from our previously published work in *Soft Matter*. According to the Royal Society of Chemistry (RSC), we can reuse the figure without permission as long as the RSC is acknowledged. Therefore we acknowledge RSC in the acknowledgement section to comply with the RSC copyright rule. We attached this information along with our email conversation with RSC as a supplementary file.

2. Figure 3C, 4A, 5: Please use ‘h’ instead of ‘hr’.

We thank the editor for the correction. We have corrected these figures.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have examined the Table of Materials. We believe that all materials and equipment used are now listed in the Table of Materials

2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

We have examined the Table of Materials. It does not contain any trademark and registered symbols.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

The manuscript by Bate et al. presents a detailed protocol of the preparation of microtubule-based active fluids pioneered by the Dogic's group and a temperature control setup that enables control of the active fluid flow speed. The authors also present the methodology for characterizing active fluid activity as influenced by temperature.

While I believe MT active fluid preparation would be of great interest to the scientific community - from those in basic biology to physicist or engineers interested in active fluids, it is unclear how much of the tutorial will be focused on active fluid preparation versus the construction of a Peltier temperature control setup. Also, the claims of local control of fluid flow in the abstract and intro seems overreaching, considering no data on the influence of convection via temperature gradient is discussed. There are many good tips in the method description. However, there are several issues with erroneous phrasing that does not conform to general terminology within the field (examples to be listed below). Some instructions are overly specific to the exact instrumentation listed in the manuscript and does not provide the readers general guidelines that are easy to follow.

We thank the reviewer for the detailed comments and suggestions that have guided us to improve the manuscript. Please find the details of our revision in the following responses.

Major Concerns:

1. In the intro, it is mentioned that a minor microscope modification was done (line 71) - yet there no mention of this in the protocol.

We apologize to the reviewer for describing the temperature stage for the microscope unclearly. Our initial meaning was that our method required a temperature stage, but the microscope did not need to be modified. To clarify our meaning, we corrected the manuscript and specified that adopting our method did not require microscope modification.

1. section 2.1.2-2.1.5
It seems to suggest that standard sterile technique is optional. Plate the cell, grow cells, grow starter, shake mixture, are very ambiguous phrasing - suggest using standard phrasing such as inoculate, expand cells, shaking culture, etc.

We thank the reviewer for the correction. The standard sterile technique is an important process during the cell growth and protein expression. We have corrected the manuscript and specified that the process of growing the cell must be under standard sterile condition (the note after Step 2). We have also corrected the phrasing such as the use of ‘Inoculate plate media’ rather than ‘Plate media’. We believe that the phrasing in Step 2.1 is more standard.

This entire section is very confusion - it is not clear when cell growth should be monitored, and when the reader should adhere to using a incubation time range without monitoring.

We are sorry for the confusion about the need of monitoring in the protocol. During the cell growth, monitoring is not required unless specified such as in Step 2.1.5.2. We clarified the concern for monitoring the cell growth in the note after Step 2.1.1.6.

2. instructions in section 4 should be re-written for clarification. The authors should use example stock solutions and give the final target concentration as opposed to 'add ___ mM or ___ v/v' of certain reagents.

We thank the reviewer for the suggestion. We have rewritten this section by demonstrating an example of preparing 100 µL from example stocks such as specifying 2.8 µL of 50 mM ATP along with a note clarifying that the recipe is scalable as long as the final concentrations of each components are the same (after Step 4.1). We also specify the final concentrations of each components (the note before Step 4.2). We believe that the protocol is now sufficiently transparent for the readers who want to synthesize this active fluid.

3. In section 5, the instructions too detailed and very specific to the exact instrumentation: 'Press button 18 times' 'navitage controller setting to 'output enable'', 'press button 3 times'

We are sorry for over-specifying the process. We have eliminated the sentences that are too detailed and redundant such as the number of times to press the menu button.

4. Since the tutorial in section 5 is specific to the authors' home-build setup, a CAD drawing could be provided to help visualize the description in 5.1.1.

We thank the reviewer for the suggestion. We have revised Figure 3 and added a schematic of our home-built aluminum stage (part A), and referenced the figure in the protocol (Step 5.1.1.2). We believe that this figure will help readers to better understand our temperature control setup.

5. In section 5, there is no mention of how this setup fit with common microscopes (inverted vs upright)- and whether alternative temperature control system could be used. It will be useful to comment on the consideration for imaging purpose as it is the key step to the characterization to follow.

We thank the reviewer for addressing such a concern. We have clarified in the manuscript that our setup fits to common microscopes that are either inverted or upright. We also described the alternative temperature control system using temperature-controlled water bath along with discussing the advantages of using our presented setup (2nd paragraph of the Discussion section). We commented our setup design for the purpose of imaging in the note after Step 5.1.8. While our manuscript describes the process for imaging fluorescent tracers in the sample mounted in our temperature setup (Step 6.1.1), the setup is compatible with bright-field imaging as well. The setup has a central hole that allows a sample to be illuminated by bright field from one side while being imaged on the other side with an objective (Figure 3B).

6. section 6 instructions again reads as an instruction manual without context. Notes in section 6.1. 'working delta t should allow tracer movement to be traced by naked eyes' - this is very confusing.

We are sorry for the lack of context in Section 6. In the manuscript, we have added a note to clarify the connection between Steps 5 and 6, along with the goal of Step 6. We are also sorry for our non-scientific language of 'tracked by naked eyes'. We have corrected the manuscript and specified that the tracer displacement between frames should be within ~9 pixels.

Minor Concerns:
1. There is no mention of biosafety or compliance issue with using animal parts.

We thank the reviewer for addressing the concern about biosafety and compliance issues. In the manuscript, we have addressed these issues in the beginning of the protocol (the caution after Step 1).

2. section 1.1.7. 'Measure the concentration of proteins with a spectrometer, which should be mainly tubulins.' Do the author mean to say use a spectrometer to measure the 'total protein concentration or just that of tubulins'? a simple description of how the concentration is obtained should be included (abs wavelength).

We are sorry for the confusion of the measurement description. In the manuscript, we have clarified the abs wavelength, the use of the Beer-Lambert Law along with the extinction coefficient of tubulin. We expect that these added contents should provide sufficient information for readers to measure the tubulin concentrations.

3. section 3.
Several use of 'glasses' in place of 'glass slides' or 'slides'. 'rinsing' instead of 'rinse' on line 297, 302.

We apologize for our incorrect grammar and inconsistent wordings. We have replaced the 'glasses' as 'slides and coverslips' to avoid the confusion. For the grammar, we hired a professional editor to proofread the grammar through the manuscript. We believe that the grammar used in the manuscript should be error free.

4. What do the authors mean by 'malfunctioning' - line 498, second results page

We are sorry for the ambiguous description. According to our previous studies (Bate et al. *Soft Matter* **15**, 5006), the motor clusters lost the ability to drive pairs of microtubules (malfunctioning) and therefore the fluid flows were inhibited. We have described in more detail the performance of kinesin motor clusters above 36 °C in the manuscript (the 3rd paragraph in the Representative Results section).

5. 'instantly (~10s)' author should clarify the relevant timescale as most would not consider 10 second an 'instant' response time - line 503, second results page

We are sorry for the ambiguous description. We have corrected the manuscript and stated that the active fluid was responsive to temperature change within 10 s.

6. adding chemicals to a liquid should not be termed 'dope with hydrogen peroxide'

We thank the reviewer for the correction. We have corrected the manuscript as “adding 0.1% hydrogen peroxide to water”.

Reviewer #2:

Manuscript Summary:
Nice and detailed protocol for an emerging method in the field. I learned something new, and got more detail on established procedures.

We thank the reviewer for the assessment. Please find our responses to the reviewer's comments below.

Major Concerns:
None.

Minor Concerns:

- 2. Where is the kinesin plasmid coming from? How is it biotinylated?

The plasmid was from Dr. Zvonimir Dogic who is a principle investigator in Brandeis MRSEC. The facility provides their plasmid to whoever requests. We acknowledge Dr. Zvonimir and Brandeis MRSEC in the acknowledgement section.

We added biotin in Step 2.1.5.3. which binds to kinesin motor protein through its BCCP tag. We thank the reviewer for pointing out the confusion. We clarify the biotinylation in the note of the step.

- 2.1. state that the expression is in E.coli

We specified the cells for expressing our proteins as *Escherichia coli*.

- 2.3.3.-6. The fact that the tubulin is just used to calibrate the concentration is not well explained.

We thank the reviewer for pointing out the confusion. We clarified the reason of loading tubulins in the gel in the note after Step 2.3.

- 2.2.6. What is the centrifugal filter tube? Later referred to as "centrifugal filter unit". What is the cutoff Mw for the one used?

We thank the reviewer for addressing this issue. Centrifugal filter unit and centrifugal filter tube are identical. The cutoff Mw is 10 kDa. We clarified the naming and cutoff Mw in the Table of Materials.

- "s" not "sec", "h" not "hr" - use units according to SI conventions and with consistency.

We thank the reviewer for the correction. We have corrected the use of units according to SI conventions.

- Tracking software: I assume that you got Ouellette et al. gave permission for you passing on their software. Nevertheless, it would be helpful to give a link to their website, in case new and improved versions become accessible or problems are discovered by the authors of the software.

We thank the reviewer for the reminder of Ouellette's tracking software. We specified the link to the Ouellette's group website (https://web.stanford.edu/~nto) which provided the software. To direct the reader to the software better, we provided the link to the software directly (Step 6.2.1): https://web.stanford.edu/~nto/software.shtml. We believe that this link provides readers a clear instruction for downloading the software.

- Just as a comment: In R. Tucker, A.K. Saha, P. Katira, M. Bachand, G.D. Bachand, and H. Hess: "Temperature Compensation for Hybrid Devices: Kinesin's Km is Temperature Independent", Small, 5(11), 1279-1282 (2009), the reverse question is discussed: How can velocity be stabilized against temperature fluctuations. It also provides the temperature-dependence of the kinesin Km, which is useful to know when using sub-saturating ATP concentrations.

We thank the reviewer for suggesting a relevant article. We have introduced and cited the paper in the introduction section, to provide readers a broader perspective of the role of temperature in the enzyme-based systems.

Reviewer #3:

Manuscript Summary:
In this work, Bate et. al. report a new method of tuning flow speeds of kinesin-driven, microtubule-based 3D active fluids with temperature. The described method allows for tuning the speeds in situ without the need of remaking samples for reaching different desired speeds and dynamical control of it. Cycling temperatures leads the fluids to flow fast and slow periodically. According to the authors, this controllability is based on Arrhenius characteristic of kinesin-microtubule reaction, which leads to a control range of 4 to 8 μm/s in flow mean speeds. In my view of point, through this work authors share a new idea of controlling speeds based on varying temperature. They described the work in a narrative way as the work is understood very well. Some issues may be considered for the better version of the manuscript.

We thank the reviewer for assessing our manuscript and providing comments to improve the manuscript. Please find our response to the reviewer's comments below.

Major Concerns:
* The MTs decay at 10 °C was caused by microtubule depolymerization below 16 °C, the decay at 40 °C was by malfunctioning kinesin clusters above 36 °C. At reference 33 (Bate et al), it is mentioned that, heating above 38 oC inhibited K401-associated activities. Is it possible to hint the exact destiny of the K401 above 38 oC, how K401 activities inhibited or malfunctioned?

We apologized for the unclear description of malfunctioning kinesin clusters. In our previous studies (Bate *et al. Soft Matter* **15**, 5006), we performed a series of experiments to examine the performance of our kinesin clusters after pre-incubation in various temperatures before being used (in the supplementary information). We found that the kinesin clusters were not able to drive the microtubules after being pre-incubated at >36 °C for 30 minutes. We conjectured that the reason was either because the kinesin clusters disintegrated or because the kinesin denatured. Nevertheless, we did not have experimental evidence to demonstrate the underlying mechanisms for such malfunctioning behaviors. We stated the unresolved issue and invited interested research groups to take on further investigation.

We thank the reviewer for addressing this issue. For the readers who want to know the performance of kinesin clusters above 38 °C, in the manuscript, we invited the readers to read our previous work published in *Soft Matter*.

* In section 5.1.5, details heating and cooling system is described. In section 5.2.4 the same method is applied. The details description may be skipped in section 5.2.4.

We thank the reviewer for the suggestion. We have shortened Step 5.2.3 by referencing the Steps in 5.1.5.

* In section 6.1, it is told to save images in TIFF, name the files based on frame number and store files in separate folder. Is it really need to mention? The last line is enough to describe about Δt in the NOTE of section 6.1.

We are sorry for the detailed description in this section. We realize that this description may appear to be redundant, but to ensure that the readers who want to use our MATLAB script will be able to analyze the acquired images without error, we chose to describe the process step by step so the readers can avoid unnecessary issues when running our MATALB script. We thank the reviewer for pointing out this issue. In the manuscript, we further informed the readers about the importance of following these steps, if they choose to use our MATLAB script to analyze the data.

* In representative results section, experimental works are described. Almost all the works described in details in previous sections, so the description may be briefly presented in this section.

We thank the reviewer for the suggestion. We avoided the duplicated content and reduced the section from 53 to 44 lines.

* In the 2nd para of the discussion section drawbacks of mineral containing tap water is described in detail. Detailed description is better to skip.

We have eliminated the description about tap water.

* In figure 4 (A) trajectory images are shown but no detail information is given. Time interval is mentioned 2 s but the corresponding temperatures are not described.

We are sorry for the confusion of the figure. We have specified the detailed information about the experiment. The tracers were imaged every 5 seconds; the experiment was performed at room temperature (~20 °C).

Dear Editor,

In the manuscript, we adopted figures from a paper on *Soft Matter* that we previously published (Bate *et al. Soft Matter* **15** 5006 (2019)). Since we are the authors of the paper, according to their policy, we can reuse the published figures as long as the Royal Society of Chemistry is acknowledged. Please find policy about reusing the figures on the website: https://www.rsc.org/journals-books-databases/journal-authors-reviewers/licences-copyright-permissions/ along with attached screenshots and emails from the Roayl Society of Chemistry. If you have further concern about the copyright permission, please do not hesitate to contact us. Thank you.

Sincerely,

Kun-Ta Wu
Assistant Professor
Department of Physics
Phone: 508.831.6057
Email: kwu@wpi.edu

Email with the Royal Society of Chemistry:

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CONTRACTS-COPYRIGHT (shared) <Contracts-Copyright@rsc.org> Tue, Jun 25, 2019 at 2:48 AM
To: "kwu@wpi.edu" <kwu@wpi.edu>

Dear Kun-Ta

I have added the answers to your questions in the email below.

Regards

Gill Cockhead

Publishing Contracts & Copyright Executive

Gill Cockhead

Publishing Contracts & Copyright Executive

Royal Society of Chemistry,

Thomas Graham House,

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Sent: 20 June 2019 21:14
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Subject: Not CCC Re: Automatic reply: Inquiry of Adopting Figures in a Soft Matter paper

Dear Chloe & Gill,

Greetings! This is Kun-Ta from Worcester Polytechnic Institute. I write this email regarding reusing the figures we published on Soft Matter recently. We are working on a manuscript that is to be submitted to *Journal of Visualized Experiments* as a method paper. We would like to adopt or adapt **Figs, 1, 2, and 5**. I have read the instructions online (attached pics). However, we would like to check with you in case we misunderstand the policy. Our questions are:

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Best,

Kunta

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