

# Journal of Visualized Experiments

## Assembling molecular shuttles powered by reversibly attached kinesins

--Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE59068R1
<b>Full Title:</b>	Assembling molecular shuttles powered by reversibly attached kinesins
<b>Keywords:</b>	nanobiotechnology, molecular shuttles, microtubules, kinesin motors, reversible binding, dynamic self-assembly.
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<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Manhattan, New York (NY), USA

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DEPARTMENT OF BIOMEDICAL ENGINEERING

Jialan Zhang  
JoVE Science Editor  
JoVE

Wednesday, September 5, 2018

Dear Dr. Zhang,

We would like to submit our manuscript entitled “Assembling molecular shuttles powered by reversibly attached kinesins” to JoVE to be considered for publication as a video journal article.

The behavior of active nanosystems have so far been characterized by long-lived, nearly irreversible bonds between their components and the surface with which they interact. For instance, in the well-studied example of the microtubule-kinesin system, gliding microtubules are typically propelled by irreversibly surface-bound kinesin motors. Our manuscript presents a protocol that describes how to create kinesin-powered molecular shuttles with a weak and reversible attachment of the kinesins to the surface. To achieve this, we use complex laboratory techniques including UV-Vis spectrophotometry; silanization of coverslips; and total internal reflection fluorescence (TIRF) microscopy. By following this protocol, one will observe that as microtubules glide in a flow cell, they accumulate kinesin motors from solution. Furthermore, as the microtubules move forward, a trail of kinesin motors is left behind them. This trails then slowly desorbs from the surface.

This work continues our investigation into the applications of kinesin powered molecular shuttles and follows our previous publication in JoVE (Jeune-Smith, Y., Agarwal, A., Hess, H. Cargo Loading onto Kinesin Powered Molecular Shuttles. J. Vis. Exp. (45), (2010)), which has already been viewed more than 4,000 times.

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Thank you for giving our manuscript your fullest consideration!

With best regards,

A handwritten signature in black ink, appearing to read "Henry Hess". The signature is fluid and cursive, with the first name "Henry" and last name "Hess" clearly distinguishable.

Henry Hess, Ph.D.  
Professor  
Editor-in-Chief, IEEE Transactions on NanoBioscience

**TITLE:****Assembling Molecular Shuttles Powered by Reversibly Attached Kinesins****AUTHORS & AFFILIATIONS:**

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**KEYWORDS:**

nanobiotechnology, molecular shuttles, microtubules, kinesin motors, reversible binding, dynamic self-assembly.

**SUMMARY:**

We present a protocol to build molecular shuttles, where surface-adhered kinesin motor proteins propel dye-labelled microtubules. Weak interactions of the kinesins with the surface enables their reversible attachment to it. This creates a nanoscale system which exhibits dynamic assembly and disassembly of its components while retaining its functionality.

**ABSTRACT:**

This protocol describes how to create kinesin-powered molecular shuttles with a weak and reversible attachment of the kinesins to the surface. In contrast to previous protocols, in this system, microtubules recruit kinesin motor proteins from solution and place them on a surface. The kinesins will, in turn, facilitate the gliding of the microtubules along the surface before desorbing back into the bulk solution, thus being available to be recruited again. This continuous assembly and disassembly leads to striking dynamic behavior in the system, such as the formation of temporary kinesin trails by gliding microtubules.

Several experimental methods will be described throughout this experiment: UV-Vis spectrophotometry will be used to determine the concentration of stock solutions of reagents, coverslips will first be ozone and ultraviolet (UV) treated and then silanized before being mounted into flow cells, and total internal reflection fluorescence (TIRF) microscopy will be used to simultaneously image kinesin motors and microtubule filaments.

**INTRODUCTION:**

The interactions governing the behavior of active nanosystems have always been characterized by long-lived, nearly irreversible bonds<sup>1-8</sup>. A well-studied example of this is the microtubule-kinesin system, where gliding microtubules are propelled by irreversibly surface-bound kinesin motors<sup>1-5</sup>. Systems in which the components are reversibly connected to one another have been studied theoretically<sup>9,10</sup> and achieved at the macroscale<sup>11,12</sup>, but scaling these systems down to the nanoscale has been challenging. One of the major reasons for this is that breaking and reforming bonds between components often requires a large change in the environmental conditions. Even though such changes have been implemented in the past<sup>13-15</sup>, they would rely on modifying the system itself rather than adapting it to its environment. Designing molecular-scale systems in which components continually assemble and reorganize into structures without disturbing the overall environment in which the experiments take place will open the door to the exploration of a wide range of dynamic behaviors<sup>16,17</sup>.

Here, we describe and demonstrate the detailed protocol for creating a dynamically assembling and disassembling system functioning at the nanoscale. The system and its general behavior has been introduced earlier<sup>18</sup>: microtubule filaments are propelled by tracks of *reversibly* surface-bound kinesin-1 motors. These kinesin motor proteins are recruited from the solution to help propel microtubules forward, before desorbing again shortly afterwards. Once back in solution, they can be recruited again to propel a new microtubule. In the past<sup>13-15</sup>, the breaking and reforming of bonds required environmental modifications; in contrast, the environment of our flow cell remains unchanged while the kinesin motors interact with the surface.

This protocol will help interested researchers to (1) visualize all the steps of the protocol, and (2) assist with troubleshooting this type of assay. It has been derived from the procedures described in Howard et al. 1993<sup>19</sup>.

## PROTOCOL:

### 1. Solution preparation

CAUTION: Three of the reagents used in this protocol (toluene, dimethyldichlorosilane, and dithiothreitol) are highly toxic. Please consult the relevant material safety data sheets (MSDS) before use. Furthermore, parts of this protocol need to be performed under a higher level of protection, wearing safety glasses and two sets of protective gloves as indicated. Unless otherwise advised, the experiments can be performed on lab benches while using all the appropriate personal protective equipment (safety glasses, gloves, lab coat, full length pants, and closed-toe shoes).

NOTE: The concentrations of the ATP, kinesin, and microtubules that are used in this protocol can be modified given the needs of each experiment. However, if modified, please ensure that the final concentrations of the other reagents remain the same as given below. All experiments were performed at room temperature, (~25 °C).

## **1.1. Preparation of stock solutions**

NOTE: Prepare and aliquot the following solutions beforehand. All aliquots can be prepared at room temperature (~25 °C).

### **1.1.1. BRB80 buffer**

NOTE: The buffer for most of the solutions used in this protocol is BRB80. BRB80 can be prepared in large quantities and stored in a -20 °C freezer.

1.1.1.1. Dissolve 24.2 g of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and 3.1 g of potassium hydroxide (KOH) in 800 mL in deionized water to make 800 mL of 100 mM PIPES. Add 100 mL of 10 mM magnesium chloride ( $\text{MgCl}_2$ ) and 100 mL of 10 mM ethylene glycol tetraacetic acid (EGTA) to get a final volume of 1 L. Raising the pH to 8 with potassium hydroxide (KOH) can help in the dissolution of PIPES and EGTA.

NOTE: The final concentrations of chemicals in the BRB80 buffer are 80 mM PIPES, 1 mM  $\text{MgCl}_2$ , and 1 mM EGTA.

1.1.1.2. Adjust the pH of the buffer to 6.9 using KOH and hydrochloric acid (HCl).

### **1.1.2. ATP**

1.1.2.1. Prepare a 1 mL solution of 100 mM ATP in ultrapure water. Pipet the solution into 10  $\mu\text{L}$  aliquots. Store the aliquots in a -80 °C freezer.

### **1.1.3. GTP**

1.1.3.1. Prepare a 500  $\mu\text{L}$  solution of 25 mM GTP in ultrapure water. Pipet the solution into 5  $\mu\text{L}$  aliquots. Store the aliquots in a -80 °C freezer.

### **1.1.4. $\text{MgCl}_2$**

1.1.4.1. Prepare a 1 mL solution of 100 mM  $\text{MgCl}_2$  in ultrapure water. Pipet the solution into 10  $\mu\text{L}$  aliquots. Store the aliquots in a -20 °C freezer.

### **1.1.5. Casein**

1.1.5.1. Weigh 1 g of dry casein and transfer it to a 50 mL conical centrifuge tube. Add 35 mL of BRB80 buffer to the tube to dissolve the casein powder.

1.1.5.2. Place the solution in a tumbler in a cold room to dissolve overnight. At this point, the solution will look thick and viscous.

1.1.5.3. Leave the tube upright in a 4 °C fridge to allow for any large undissolved clumps to settle. Transfer the supernatant to a new tube.

1.1.5.4. Spin the tube in a centrifuge at 1,000 x *g* to pellet out more precipitates. Once again, transfer the supernatant to a new tube.

1.1.5.5. Repeatedly filter the solution using 0.2 µm (7 bar maximum pressure) filters. The solution being thick, many filters will get clogged, so repeat this step until the filter is clear and unclogged.

1.1.5.6. Determine the casein concentration in the resulting solution using UV/Vis spectrophotometry, using casein's extinction coefficient of 19 mM<sup>-1</sup>·cm<sup>-1</sup> at 280 nm<sup>20</sup>. Assuming a molecular weight of 23 kDa for casein, dilute the solution to a concentration of 20 mg·mL<sup>-1</sup> in BRB80.

1.1.5.7. Pipet the solution into 20 µL aliquots and store the aliquots in a -20 °C freezer.

#### 1.1.6. D-Glucose

1.1.6.1. Prepare a 1 mL solution of 2 M D-glucose in ultrapure water. Pipet the solution into 10 µL aliquots. Store the aliquots in a -20 °C freezer.

#### 1.1.7. Glucose oxidase

1.1.7.1. Prepare a 1 mL solution of 20 mg·mL<sup>-1</sup> glucose oxidase in BRB80. Pipet the solution into 10 µL aliquots. Store the aliquots in a -20 °C freezer.

#### 1.1.8. Catalase

1.1.8.1. Prepare a 1 mL solution of 0.8 mg·mL<sup>-1</sup> catalase in BRB80. Pipet the solution into 10 µL aliquots. Store the aliquots in a -20 °C freezer.

#### 1.1.9. Dithiothreitol (DTT)

NOTE: Since DTT is slightly volatile and toxic, please perform the following steps under a fume hood.

1.1.9.1. Prepare a 1 mL solution of 1 M DTT diluted in ultrapure water. Pipet the solution into 10 µL aliquots. Store the aliquots in a -20 °C freezer.

#### 1.1.10. Paclitaxel

1.1.10.1. Prepare a 1 mL solution of 1 mM paclitaxel diluted in DMSO. Pipet the solution into 10 µL aliquots. Store the aliquots in a -20 °C freezer.

### **1.1.11. Dimethyl sulfoxide (DMSO)**

NOTE: The DMSO used in these experiments is pure.

1.1.11.1. Pipet 1 mL of pure DMSO into 10  $\mu$ L aliquots. Store the aliquots in a -20 °C freezer.

### **1.1.12. Creatine phosphate**

1.1.12.1. Prepare a 1 mL solution of 0.2 M creatine phosphate in ultrapure water. Pipet the solution into 10  $\mu$ L aliquots. Store the aliquots in a -20 °C freezer.

### **1.1.13. Creatine phosphokinase**

1.1.13.1. Prepare a 1 mL solution of 200 units·L<sup>-1</sup> creatine phosphokinase in ultrapure water. Pipet the solution into 10  $\mu$ L aliquots. Store the aliquots in a -20 °C freezer.

### **1.1.14. Nickel (II) sulfate**

1.1.14.1. Prepare 500 mL of a solution of 50 mM nickel (II) sulfate in ultrapure water. Store the solution at room temperature (~25 °C).

### **1.1.15. Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEG-PPG-PEG) solution**

1.1.15.1. Weigh out 2 mg of PEG-PPG-PEG (number average molecular weight: 14,600 g·mol<sup>-1</sup>)-NTA powder on weighing paper. PEG-PPG-PEG-NTA is the triblock copolymer functionalized with a nitrilotriacetic acid (NTA) group. Refer to the **Table of Materials** for more details about the PEG-PPG-PEG-NTA.

1.1.15.2. Transfer the powder into a 1.5 mL microcentrifuge tube. Add 1 mL of the stock nickel (II) sulfate solution to the tube. Vortex until powder is dissolved and no visible clumps remain.

1.1.15.3. Store for up to one month at room temperature.

## **1.2. Before starting an experiment**

1.2.1. Fill a bucket with ice.

1.2.2. Take one aliquot from each of the 13 reagents described in sections 1.1.1 to 1.1.13 above and add them to the bucket, thus keeping them on ice. Thaw each of the reagents before use.

## **1.3. Microtubule Preparation**



NOTE: Microtubules were polymerized from a 20  $\mu\text{g}$  aliquot of dye-labeled lyophilized tubulin. The excitation wavelength of the dye is of 647 nm.

### **1.3.1. Preparation of the microtubule growth buffer**

1.3.1.1. Pipet 21.8  $\mu\text{L}$  of BRB80 buffer into a small (0.6 mL) microcentrifuge tube. Add 1  $\mu\text{L}$  of the  $\text{MgCl}_2$  stock solution, 1  $\mu\text{L}$  of the GTP stock solution, and 1.2  $\mu\text{L}$  of the DMSO stock solution.

NOTE: Thus, the final concentrations of the reagents in the BRB80 buffer are 4 mM  $\text{MgCl}_2$ , 1 mM GTP, and 5% (v/v) dimethyl sulfoxide.

### **1.3.2. Microtubule polymerization**

1.3.2.1. Add 6.25  $\mu\text{L}$  of microtubule growth buffer directly into a 20  $\mu\text{g}$  aliquot of labeled lyophilized tubulin. Vortex the aliquot for 5 s at 30 rps.

1.3.2.2. Cool the aliquot on ice for 5 min before incubating it at 37  $^{\circ}\text{C}$  for 45 min and proceed to section 1.3.3.

### **1.3.3. Microtubule stabilization**

1.3.3.1. Add 5  $\mu\text{L}$  of the aliquoted paclitaxel solution to 490  $\mu\text{L}$  of BRB80 buffer. Vortex the solution for 10 s at 30 rps.

1.3.3.2. Once the 45 min of incubation for the microtubules are up, add 5  $\mu\text{L}$  of the polymerized microtubule solution to the BRB80/paclitaxel solution.

NOTE: This 100-fold diluted, stabilized, microtubule solution will hereafter be referred to as MT100. It can be used for up to 5 days, and it can be diluted to obtain the desired microtubule density for each experiment.

## **1.4. Motility solution**

### **1.4.1. Enzymatic antifade, ATP regenerating system, and ATP**

1.4.1.1. Add 9.0  $\mu\text{L}$  of the aliquoted casein solution to 291  $\mu\text{L}$  of BRB80 buffer.

1.4.1.2. If the desired ATP concentration for the experiment is lower than 1 mM, pipet 83  $\mu\text{L}$  of the BRB80/casein solution into a new 0.6 mL microcentrifuge tube. Otherwise, pipet 85  $\mu\text{L}$  of that solution into a new 0.6 mL microcentrifuge tube.

1.4.1.3. Add 1  $\mu\text{L}$  of D-glucose, 1  $\mu\text{L}$  of glucose oxidase, 1  $\mu\text{L}$  of catalase, and 1  $\mu\text{L}$  of DTT to that tube.

NOTE: These chemicals constitute an enzymatic antifade cocktail<sup>21</sup> that will reduce photobleaching by removing dissolved oxygen and quenching reactive radicals. This reduces photobleaching and microtubule disintegration caused by the excitation illumination during fluorescence imaging<sup>22,23</sup>.

1.4.1.4. For experiments in which the ATP concentration is chosen to be significantly lower than 1 mM, add the following reagents to the solution to create an ATP-regenerating system: 1  $\mu\text{L}$  of the aliquoted creatine phosphate solution and 1  $\mu\text{L}$  of the aliquoted creatine phosphokinase solution.

1.4.1.5. Add 1  $\mu\text{L}$  of the stock ATP solution to the motility solution. Flick or vortex the aliquot to homogenously distribute the chemicals.

NOTE: Thus, the final concentration of chemicals in the solution are 10  $\mu\text{M}$  paclitaxel, 0.5  $\text{mg}\cdot\text{mL}^{-1}$  casein, 20 mM D-glucose, 200  $\mu\text{g}\cdot\text{mL}^{-1}$  glucose oxidase, 8  $\mu\text{g}\cdot\text{mL}^{-1}$  catalase, 10 mM dithiothreitol, 2 mM creatine phosphate (if added), 2  $\text{units}\cdot\text{L}^{-1}$  creatine phosphokinase (if added), and 1 mM ATP. This solution will hereafter be referred to as the motility solution.

#### 1.4.2. Kinesin

NOTE: The stock kinesin solution used in these experiments is prepared by G. Bachand at the Center for Integrated Nanotechnologies at Sandia National Laboratories and made available under a user agreement (<https://cint.lanl.gov/becoming-user/call-for-proposals.php>). The buffer used here consists of 40 mM imidazole, 300 mM NaCl, 0.76  $\text{g}\cdot\text{L}^{-1}$  EGTA, 37.2  $\text{mg}\cdot\text{L}^{-1}$  EDTA, 50  $\text{g}\cdot\text{L}^{-1}$  sucrose, 0.2 mM TCEP, and 50  $\mu\text{M}$  Mg-ATP. For this series of experiments, the rkin430eGFP kinesin construct was used. This is a kinesin consisting of the first 430 amino acids of rat kinesin heavy chain fused to eGFP and a C-terminal His-tag at the tail domain<sup>24</sup>. It was expressed in Escherichia coli and purified using a Ni-NTA column. The concentration of the GFP-kinesin stock solution was  $1.8 \pm 0.3 \mu\text{M}$  as determined by UV/Vis spectrophotometry, using an extinction coefficient for GFP of  $55 \text{ mM}^{-1}\cdot\text{cm}^{-1}$  at 489 nm<sup>25</sup>, and taking into account that kinesin is a dimer.

1.4.2.1. Determine the desired kinesin concentration or surface density for the experiment. For typical assays, this concentration is 20 nM. If the kinesin concentration needs to be diluted more than a hundred-fold, dilute it in a solution of BRB80 that contains 0.5  $\text{mg}\cdot\text{mL}^{-1}$  of casein.

1.4.2.2. Add 1  $\mu\text{L}$  of kinesin solution to the motility solution in order to get a final concentration of approximately 20 nM.

#### 1.4.3. Microtubules

1.4.3.1. Add 10  $\mu\text{L}$  of the MT100 solution prepared in section 1.3 to the motility solution.

NOTE: This motility solution can be used for up to 3 hours. After that time, the antifade system loses its effectiveness because the glucose in the solution is depleted by the enzymatic reaction.

## **2. Assembling the flow cells**

### **2.1. Washing the coverslips**

2.1.1. For flow cells, use a large coverslip (dimension: 60 mm x 25 mm) and a small one (dimensions: 22 mm x 22 mm)<sup>19</sup>.

2.1.2. Rinse all coverslips twice with ethanol and twice with ultrapure water. Sonicate the coverslips in ultrapure water for 5 min. Dry them in an oven at a temperature of 50–75 °C.

2.1.3. Using a UV/ozone cleaner (see **Table of Materials** and manufacturer's instructions), treat one side of each coverslip for 15 min. Perform this step can at room temperature (~25 °C) and in normal atmospheric condition (pressure of 1 atm).

2.1.4. Carefully turn each coverslip to its other side (using tweezers) and UV/ozone treat that side as well.

2.1.5. Sonicate the coverslips in ultrapure water again for 5 min before drying them again in the oven at a temperature of 50–75 °C.

### **2.2. Treating the coverslips to enable PEG-PPG-PEG coating**

NOTE: The dimethyldichlorosilane and the toluene used in this part of the protocol being highly toxic, perform the following steps under a fume hood, while taking the following precautions: wear two sets of protective gloves and a long-sleeved shirt under their lab coat. Tuck the edges of the gloves inside the sleeves of the shirt so that no skin from the forearms is directly exposed to the chemicals in case of a spillage. Wear protective glasses.

2.2.1. Dilute 25 mL of pure dimethyldichlorosilane in 475 mL of toluene. Immerse each coverslip in the dimethyldichlorosilane and toluene solution for 15 seconds.

2.2.2. Wash the coverslips twice in toluene and three times in methanol. Dry the coverslips using pressurized nitrogen.

### **2.3. Assembling coverslips into flow cells**

2.3.1. Once the coverslips are dry, cut a 2 cm x 2.5 cm piece of double-sided tape vertically into two 1 cm x 2.5 cm stripes.

2.3.2. Put the large coverslip on a delicate task wiper and stick the tape stripes lengthwise along the edges of the coverslip to create a 1 cm x 2.5 cm area between the pieces of tape.

2.3.3. Stick the small coverslip on top of the tape stripes to finish the flow cell assembly.

### 3. Flowing the solutions into a flow cell

**3.1.** Flow approximately 20  $\mu\text{L}$  of the PEG-PPG-PEG solution into the assembled flow cell. The volume that is flown in the cell has to be large enough to fill the chamber. In the next steps, use the same volume when exchanging the solutions.

**3.2.** Allow for the PEG-PPG-PEG solution to absorb on the surface for 5 minutes. Exchange the PEG-PPG-PEG solution with BRB80 buffer 3 times by flowing the buffer in. Flow the motility solution into the flow cell.

**3.3.** Seal the edges of the flow cell with grease to prevent evaporation if the planned experiment is longer than an hour.

NOTE: The flow cell is now ready to be imaged.

### 4. Imaging a flow cell

**4.1.** Perform the imaging using an objective-type total internal reflection fluorescence (TIRF) setup for both the microtubules and the kinesin motors (see **Table of Materials**).

NOTE: Here, we used a microscope with a 100x/1.49 numerical aperture objective lens, using two lasers, one with a wavelength of 642 nm and a maximum power of 140 mW, and another with wavelength of 488 nm and a maximum power of 150 mW.

**4.2.** Place a drop of immersion oil on the objective.

**4.3.** Place the flow cell on the microscope platform and bring the objective up until there is contact between the oil on the objective and the flow cell.

**4.4.** Use the microscope's interlock cover system to block all laser light from escaping.

**4.5.** Turn on the laser and focus on the lower surface of the flow cell. The microtubules are fluorescently labelled and excited at a wavelength of 647 nm. They will be imaged using a 642 nm laser while a 488 nm laser is used for the GFP-kinesin motors.

**4.6.** Record the images or videos of interest. Typically, the laser power is of about 30 mW and an exposure time of 50 ms for both laser channels. Images can be recorded for as long as there is motility in the flow cell.

NOTE: The laser illumination is harmful for the naked eye and can cause irreparable damage. Please make sure that the illuminated area is completely covered with an opaque lid.

### REPRESENTATIVE RESULTS:

In these experiments, we used a 1000 times dilution of the microtubules prepared in section 1.3.2. The kinesin concentration was 20 nM, and the ATP concentration was 1 mM. Imaging was performed using TIRF microscopy. Gliding microtubules were separately imaged from the kinesin motors: microtubules were visible upon excitation with a 647 nm laser (**Figure 1**, red), and the GFP-kinesin was visible when excited with a 488 nm laser (**Figure 1**, green). The time between excitation with the red and green light was less than 1 s. The time between frames was 10 s. Microtubules displayed stable gliding. The average microtubule gliding velocity was approximately 800 nm/s. The microtubule surface density was 400  $\mu\text{m}^{-2}$ . Tracks of kinesin (green) appeared to extend beyond the trailing end of the microtubules (red) for several micrometers.

#### FIGURE LEGENDS:

**Figure 1: Gliding microtubules propelled by weakly surface-bound kinesin motors.** As microtubules move forward, they accumulate kinesin motors from solution. These motors alternate between two states: single-bound to the microtubule, and double-bound to both the microtubule and the surface. When a double bound motor reaches the end of the microtubule, the motor is left behind and slowly desorbs from the surface with an off rate of approximately  $0.1 \text{ s}^{-1}$ . As a result, trails of kinesin motors remain behind the microtubules. Top row: red (microtubule) channel. Middle row: green (kinesin) channel. Bottom row: combined red (microtubule) and green (GFP-kinesin) channel. Scale bar: 20  $\mu\text{m}$ .

#### DISCUSSION:

In this work, we present an active nanoscale system which self assembles weakly-binding building blocks to construct its own track. As shown in **Figure 1**, gliding microtubules accumulate kinesin motors from solution and deposit them on the surface. The kinesin motors remain in the wake of the microtubule for a short period of time before returning to solution. Thus, in this experiment, kinesin motors alternate between 3 states:

- (1) A microtubule single-bound state: this is when a kinesin first binds to a microtubule. It exists in equilibrium with state (2).
- (2) A double-bound state: in this case, a microtubule single-bound kinesin also binds to the surface *via* its His-tag. This double-bound state allows for microtubule propulsion.
- (3) A single surface-bound state: a double-bound kinesin that has walked off the end of the microtubule and has not yet desorbed from the surface is in this state. These motors can be observed in **Figure 1** (combined and green channels): they extend behind the tail of the microtubule for several micrometers and form its diminishing trail.

The most critical step of this protocol is the formation of the hydrophobic surface on the slide. Not only does it use dangerous chemicals, but it also allows the PEG-PPG-PEG functionalized with the NTA group to coat the surface, which then allows the kinesin to reversibly bind to the surface. Another important step is sealing the flow cell with grease. This allows for prolonged imaging without the liquid in the flow cell evaporating.

The primary modifications to this technique consist of changing microtubule concentration, kinesin concentration, and ATP concentration. Changing microtubule concentration will change

the number of microtubules gliding on the surface. Changing kinesin concentration will change the number of kinesin molecules that can bind to the microtubule. However, increasing the kinesin concentration above the amounts already defined in this experiment could increase background fluorescence, making it more difficult to see the kinesin trails left behind gliding microtubules. Meanwhile, lowering ATP concentrations below 10  $\mu$ M will significantly decrease microtubule gliding velocity. If this effect is desired, it is necessary to utilize an ATP regenerating system consisting of creatine phosphatase and phosphokinase.

A possible limitation of this technique is that, due to the large active kinesin content of the system, the ATP can be rapidly consumed, and experiments may last less than an hour in certain conditions. This would for example be the case if one used a twofold higher kinesin concentration and five-fold higher microtubule concentration than what is presented in this protocol.

In our previous work<sup>18</sup>, we studied the spatial distribution of kinesin motors along the microtubules, proving that gliding microtubules accumulate kinesin motors from solution, resulting in an increase of the density of motors along the length of the microtubule. We also found that the microtubules' gliding stability demonstrated a nonlinear dependence on the solution kinesin concentration and microtubule velocity.

The presented protocol paves the way for a more efficient use of protein motors in nanoscale engineered systems and for further investigation in the design of active nanosystems that are in dynamic equilibrium. Furthermore, the dynamic nature of this system allows it to serve as a model system for studying self-healing and dynamic replacement of molecular components, closing part of the gap between engineered and natural structures.

#### ACKNOWLEDGMENTS:

The authors gratefully acknowledge financial support under NSF grant NSF-DMR 1807514. The authors thank G. Bachand and V. Vandelinder for providing the GFP-kinesin protein. This work was performed, in part, at the Center for Integrated Nanotechnologies, an Office of Science User Facility operated for the US Department of Energy (DOE) Office of Science by Los Alamos National Laboratory (contract no. DE-AC52-06NA25396) and Sandia National Laboratories (contract no. 97 DE-AC04-94AL85000). The authors thank Dr. Jennifer Neff and AllVivo Vascular for their gift of PEG-PPG-PEG functionalized with NTA.

#### DISCLOSURE:

The authors have nothing to disclose.

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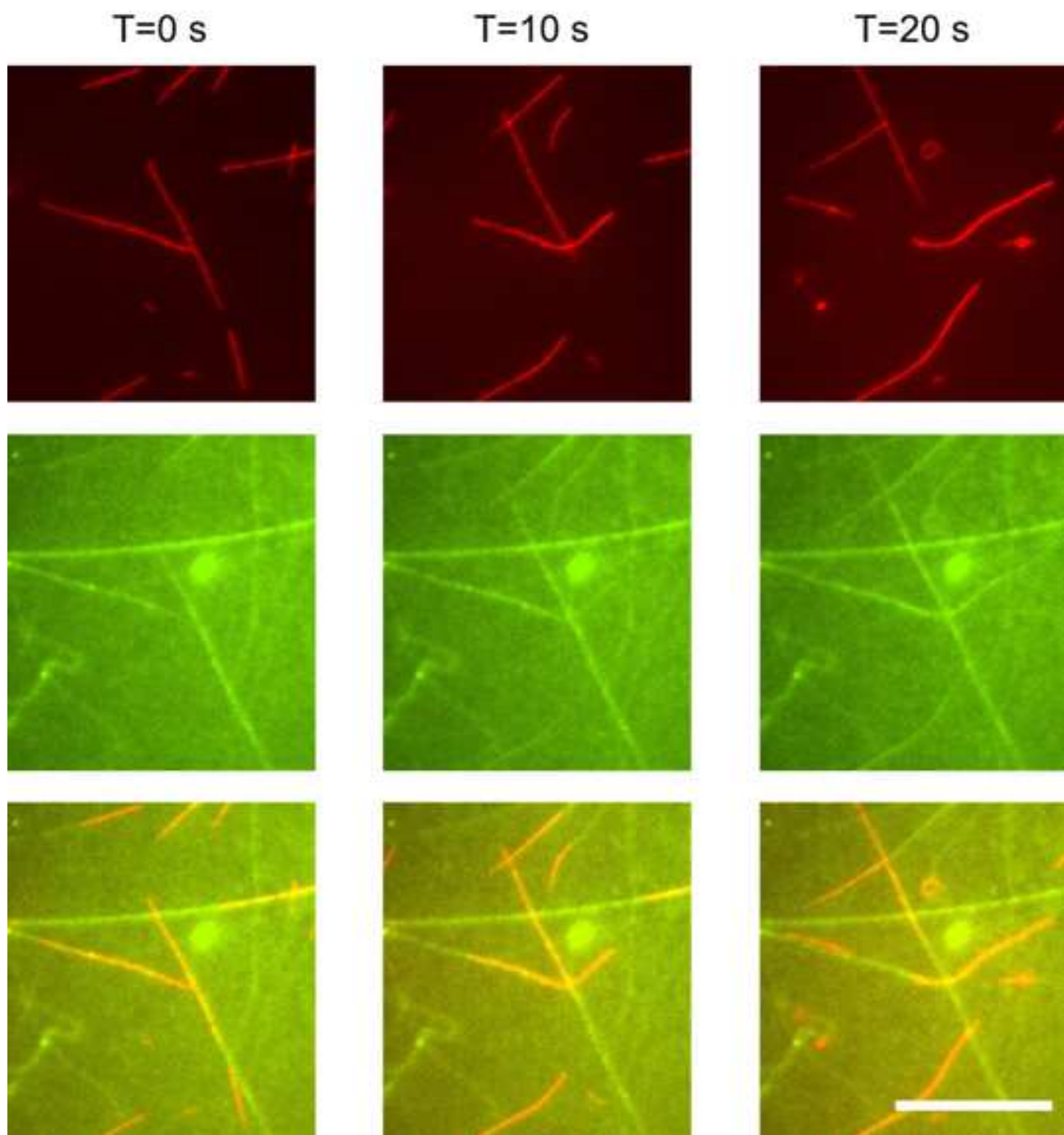
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
488 nm laser	Omicron Laserage	LuxX 488-150	
642 nm laser	Omicron Laserage	LuxX 642	
Casein	Sigma	C7078-500G	
Catalase from bovine liver	Sigma	C40-500MG	
Creatine Phosphate	Sigma	P-7936	
Creatine Phosphokinase	Sigma	C3755-500UN	
D-Glucose	Sigma	G2133-50KU	
Dichlorodimethylsilane solution	Sigma	40140-25ML	Toxic
Dimethyl Sulfoxide	Sigma	34869-100ML	
Dithiothreitol	Sigma	D0632-5G	Toxic
Eclipse TI	Nikon Instruments		
eGFP rkin430	Provided by George Bachand		
EGTA	Sigma	E4378-25G	
Falcon 50 mL Conical Centrifuge Tubes	Fisher Scientific	14-959-49A	
Glucose Oxidase	Sigma	G0543-10KU	
Guanosine Triphosphate	Sigma	G8877-10MG	
Kimwipes Delicate Task Wipers	Sigma Pharmaceuticals	8089	
Magnesium Chloride	Sigma	M1028-100ML	
Methanol	Fisher Chemical	A412	Toxic
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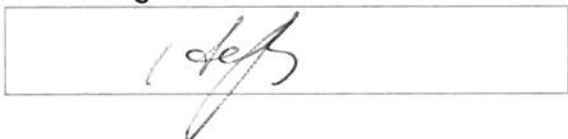
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*Editorial comments:*

*Changes to be made by the author(s) regarding the manuscript:*

**Author Response:**

We thank the editors for their comments.

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

**Author Response:**

We have proofread the manuscript.

*2. Please revise lines 281-283 to avoid previously published text.*

**Author Response:**

We have revised these lines as follows:

“For this series of experiments, the rkin430eGFP kinesin construct was used. This is a kinesin consisting of the the first 430 amino acids of rat kinesin heavy chain fused to eGFP and a C-terminal His-tag at the tail domain<sup>24</sup>. It was expressed in Escherichia coli and purified using a Ni-NTA column.”

*3. Please provide an email address for each author.*

**Author Response:**

The email addresses for all authors are given on the first page of the manuscript.

*4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.*

**Author Response:**

We have renumbered the protocol.

*5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).*

**Author Response:**

We have proofread the protocol and removed uses of personal pronouns.

*6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*

**Author Response:**

We have proofread the protocol to only contain action items.

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**Author Response:**

We have removed commercial language from the manuscript and we have directed the reader to the table of materials where necessary.

*8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).*

**Author Response:**

We have converted the centrifuge speed to centrifugal force.

*9. Approximate volumes for all buffers, gradients, and stock solutions to be set up should be given.*

**Author Response:**

Approximate volumes for all buffers have been added to the manuscript.

*10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.*

**Author Response:**

We have simplified the protocol and made use of substeps.

*11. Lines 291: Please specify the amount of kinesin solution added and the final concentration.*

**Author Response:**

This step has been modified to include the amount of kinesin and the final concentration as follows:

“Add 1 µL of kinesin solution to the motility solution in order to get a final concentration of approximately 20 nM.”

*12. Please include single-line spaces between all paragraphs, headings, steps, etc.*

**Author Response:**



We have added single line spaces.

*13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

**Author Response:**

We have highlighted approximately 2.5 pages of the protocol.

*14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.*

**Author Response:**

We have highlighted complete sentences and made sure each highlighted section contains an action in imperative tense.

*15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

**Author Response:**

We have highlighted all relevant details that are required to perform the steps in highlighting.

*16. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:*

- a) Critical steps within the protocol*
- b) Any modifications and troubleshooting of the technique*
- c) Any limitations of the technique*
- d) The significance with respect to existing methods*
- e) Any future applications of the technique*

**Author Response:**

We have added three paragraphs to address comments 16.a)-c). Comments 16.d)-e) were already discussed in the previous version of the manuscript. The added paragraphs are:

“The most critical step of this protocol is the formation of the hydrophobic surface on the slide. Not only does it use dangerous chemicals, but it also allows the PEG-PPG-PEG functionalized with the NTA group to coat the surface, which then allows the kinesin to reversibly bind to the surface. Another important step is sealing the flow cell with grease. This allows for prolonged imaging without the liquid in the flow cell evaporating.

The primary modifications to this technique consist of changing:

- Microtubule concentration: this will change the number of microtubules gliding on the surface.
- Kinesin concentration: this will change the number of kinesin molecules that can bind to the microtubule. However, increasing the kinesin concentration above the amounts already defined in this experiment could increase background fluorescence, making it more difficult to see the kinesin trails left behind gliding microtubules.

- ATP concentration: lowering ATP concentrations below 10  $\mu\text{M}$  will significantly decrease microtubule gliding velocity. If this effect is desired, it is necessary to utilize an ATP regenerating system consisting of creatine phosphatase and phosphokinase.

A possible limitation of this technique is that, due to the large active kinesin content of the system, the ATP can be rapidly consumed, and experiments may last less than an hour in certain conditions. This would for example be the case if one used a twice-higher kinesin concentration and five-fold higher microtubule concentration than what is presented in this protocol.”

*17. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.*

**Author Response:**

We have sorted the table.

**Reviewer #1:**

*Manuscript Summary:*

*In this manuscript, the authors have elaborately described the experimental details of their newly tailored dynamic assembling and disassembling system. They have engineered the dynamic system utilizing molecular shuttles i.e., microtubule/kinesin nano-actuators. From the perspective of mimicking aforementioned method, I consider that this manuscript allowed adequate information and I warrant its' publication without further modification.*

*Major Concerns:*

NA

*Minor Concerns:*

*If the authors have information for pressure and gas condition used in the UV+ozone treatment (line 310), they should be specified.*

**Author Response:**

We thank the reviewer for their comments. Our instrument works at room temperature and pressure, so we re wrote the sentence:

“UV+ozone treat one side of each coverslip for 15 minutes. This step can be done at room temperature (~ 25 °C) and in normal atmospheric condition (pressure of 1 atm).”

**Reviewer #2:**

*Comments to the Authors*

*This present Invited Method Article is based on the authors' recent published article. (Nano Lett. 2018, 18, 1530-1534) In that article, authors reported a new motility where microtubules are propelled by reversibly bound kinesin-1. The important point is that the weak and temporary but stable binding of kinesin-1 to the surface was achieved by the interaction between His-tag in kinesin-1 and Ni-NTA functionalized surface.*

*This present video protocol surely helps researchers in construction of an in vitro gliding motility assay with microtubules propelled by reversibly bound kinesin-1.*

*Following is few minor points that author might consider to readdress/ change*

**Author Response:**

We thank the reviewer for their thoughtful evaluation of our work.

*Short Abstract*

*Line 36, functionalized microtubule*

*Comment: In the present method article, authors didn't use any functionalized microtubules, only use fluorescent dye-labelled microtubules.*

**Author Response:**

We have rewritten the sentence to specify that we use dye-labelled microtubules:

“We present a protocol to build molecular shuttles, where surface-adhered kinesin motor proteins propel dye-labelled microtubules.”

*Long Abstract*

*Line 43, In contrast to previous protocols*

*Comment: Better to cite few previous protocols.*

**Author Response:**

We have added references to previous protocols (Vale et al., 1985; Ray et al., 1993; Dennis et al., 1999; Kawamura et al., 2010; Korten et al., 2016)

*1.1.1 BRB80 buffer*

*Line 115, 1mL of 100 mM EGTA*

*Comment: It is better to mention how it was prepared. The EGTA (free acid) is not easy to be dissolved in Milli-Q water and they probably needed to increase the pH to dissolve EGTA.*

**Author Response:**

We have added more details to the dissolution of EGTA and PIPES for preparation of BRB80 buffer:

“Dissolve 24.2 g of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and 3.1 g of potassium hydroxide (KOH) in 800 mL in deionized water to make 800 mL of 100 mM PIPES. Add 100 mL of 10 mM magnesium chloride (MgCl<sub>2</sub>) and 100 mL of 10 mM ethylene glycol tetraacetic acid (EGTA) to get a final volume of 1 L. Raising the pH to 8 with KOH can help in the dissolution of PIPES and EGTA.”

*1.1.5 Casein*

*Line 151, extinction coefficient is of 19 mM<sup>-1</sup>.cm<sup>-1</sup>*

*Line 152, solution to a concentration 17g/mL*

*Comment: The concentration can be written with the unit of a molar*

**Author Response:**

We changed the second step mentioned in this comment to:

“Assuming a molecular weight of 23 kDa for casein, dilute the solution to a concentration of 20 mg.mL<sup>-1</sup> in BRB80.”

#### *1.4.2 Kinesin*

*Line 284,  $1.8 \pm 0.3 \mu\text{M}$*

*Comment: What was the solution/buffer condition of the kinesin?*

#### **Author Response:**

We have added the following sentence stating the buffer condition of the kinesin:

“It consists of 40 mM imidazole, 300 mM NaCl, 0.76 g.L<sup>-1</sup> EGTA, 37.2 mg.L<sup>-1</sup> EDTA, 50 g.L<sup>-1</sup> sucrose, 0.2 mM TCEP, and 50  $\mu\text{M}$  Mg-ATP.”

#### *1.4.3 Microtubules*

*Line 295, step 1.1*

*Comment: it should be step 1.3*

#### **Author Response:**

We have changed “1.1” to “1.3.” The sentence is now:

“Add 10  $\mu\text{L}$  of the MT100 solution prepared in step 1.3 to the motility solution.”

#### **Reviewer #3:**

*Manuscript Summary:*

*The manuscript entitled: 'Assembling molecular shuttles powered by reversibly attached kinesins' by Bassir Kazeruni et al. presents a protocol to create kinesin-powered molecular shuttles with a weak and 43 reversible attachment of the kinesins to the surface. In their work they describe a system where the microtubules recruit the motor proteins from the solution and place them on the surface. They show a continuous assembly and disassembly that leads to dynamic behavior of the system. Systems in which the components are connected to one another reversibly have been studied theoretically or at the macroscale. Scaling it down to the nanoscale has been a challenge with significance to the timely development of nano-motors. In addition to the protocol, the authors describe in details important experimental methods. Thus, this report will be a good reference in the field, and attract much attention to the general readership of the Journal of Visualized Experiment. Therefore, it is highly recommended to be published in the Journal of Visualized Experiment after revision of a few minor concerns as following:*

#### **Author Response:**

We thank the reviewer for their insightful comments.

*Minor Concerns:*

*1. Which parts are highlighted in yellow? Why you chose these parts?*

#### **Author Response:**

The parts highlighted in yellow indicate the important steps of the protocol which will be shown in the video.

2. *More details of preparation of the materials are required especially for the unique materials and solutions of this protocol (for example BRB80 buffer, DMSO, tubulin etc.) - make sure the table is complete.*

**Author Response:**

We have updated the table of materials and added more details to the preparation of BRB80:

“Dissolve 24.2 g of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and 3.1 g of potassium hydroxide (KOH) in 800 mL in deionized water to make 800 mL of 100 mM PIPES. Add 100 mL of 10 mM magnesium chloride (MgCl<sub>2</sub>) and 100 mL of 10 mM ethylene glycol tetraacetic acid (EGTA) to get a final volume of 1 L. Raising the pH to 8 with KOH can help in the dissolution of PIPES and EGTA.

The final concentrations of chemicals in the BRB80 buffer are 80 mM piperazine-N,N'-bis(2-ethanesulfonic acid), 1 mM MgCl<sub>2</sub>, and 1 mM ethylene glycol tetraacetic acid.

Adjust the pH of the buffer to 6.9 using KOH and hydrochloric acid (HCl).”

3. *Line 145 - 'Transfer the supernatant to a new tube' any prior spinning is required?*

**Author Response:**

We have clarified these steps as follows:

“Place the solution in a tumbler in a cold room to dissolve overnight. At this point, the solution will look thick and viscous.

Leave the tube upright in a 4 °C fridge to allow for any large undissolved clumps to settle.

Transfer the supernatant to a new tube.”

4. *Line 148 - 'Repeatedly filter the solution using 0.2 µm filters' it seems to be a very small volume, is it enough for using this filter? Is the filter is 0.22 µm?*

**Author Response:**

We do use 0.2 µm filters. The filters are Whatman FP 300.2 CA-S, 0.2 µm, 7-bar max filters.

5. *Line 220 - 'Take one aliquot from each of the 13 reagents described above and add them to the bucket' there is a list of 15 please specify.*

**Author Response:**

We thank the reviewer for noticing the typo. We have rewritten the sentence:

“Take one aliquot from each of the 13 reagents described in sections 1.1.1 to 1.1.13 above and add them to the bucket, thus keeping them on ice.”

6. *Line 244 - 'polymerized microtubule solution' is that the name along the entire protocol? It is better to specify.*

**Author Response:**

We refer to the polymerized microtubule solution as MT100 after it is described. We have added a sentence to clarify this:

“This 100-fold diluted, stabilized, microtubule solution will hereafter be referred to as MT100.”

7. *Please check line 285 ('.cm-1')*

**Author Response:**

We have checked the value for the extinction coefficient of GFP, and it is  $55 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  at 489 nm.

8. *Please give more details on flow cells.*

**Author Response:**

We have added more details on flow cells:

**Assembling coverslips into flow cells**

Once the coverslips are dry, cut a 2 x 2.5 cm piece of double-sided tape vertically into two 1 x 2.5 cm stripes.

Put a large coverslip (60 mm x 25 mm) on a delicate task wiper and stick the tape stripes lengthwise along the edges of the coverslip to create a 1 x 2.5 cm area between the pieces of tape.

Stick a small coverslip (22 mm x 22 mm) on top of the tape stripes to finish the flow cell assembly.