***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 domain24. 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.

*7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Milli-Q, Falcon, Pluronic, Eppendorf, HiLyte, UV Ozone Procleaner, BioForce Nanosciences, Nikon Instruments, etc.*

**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 approximatively 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 approximatively 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 μM 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 rewrote 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 (MgCl2) 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-1.cm-1  
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-1 in BRB80.”

*1.4.2 Kinesin  
Line 284, 1.8 ± 0.3 µM  
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-1 EGTA, 37.2 mg.L-1 EDTA, 50 g.L-1 sucrose, 0.2 mM TCEP, and 50 μM 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 μL 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 (MgCl2) 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 MgCl2, 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 mM-1.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.