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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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).

Figures:
1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

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).