Response to review

We thank the editors and reviewers for their careful and considerate review of our manuscript. With the suggested revisions, we believe the manuscript will better serve the community and aid in the adoption of these techniques. We particularly appreciate the investment of time and energy to review, and we are grateful that the methods we have developed will be made more accessible through the production of these detailed protocols. We have addressed each point made by the editors and reviewers, and enumerate these changes on a point-by-point basis below. Both our responses to the reviewers and major changes in the manuscript are in blue text. By addressing all of these points, we believe we have made this protocol both stronger and more clear. (Please note that some changes we made based on the reviewers' comments have been moved out of the protocol itself and into the ***Introduction*** as per the style requirements of *JoVE*.)

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/ substeps.
3. Please ensure that all references appear as numbered superscripts after the appropriate statement(s).
4. 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: Beckman Coulter, GE Healthcare, ProTEV, Millipore, 3M, Sephacryl,

We have made these needed edits.

Protocol:

1. Everything in the protocol should be in a numbered step in the imperative tense and of no more than 4 sentences, header, or 'Note'. Please move the introductory paragraphs of the protocol sections to the Introduction, Results, or Discussion (as appropriate) or break into steps. Please make recipes (2.4, 3.4, 4.3, 5.3, 7.5) into imperative steps or, alternatively, make into tables.

We have made these needed edits.

2. 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 believe we have made these needed edits. Please let us know if we have missed anything.

Figures, Tables, and Figure Legends:
1. Please remove the titles and Figure Legends from the uploaded figures. Please instead include all the Figure Legends together at the end of the Representative Results in the manuscript.

We have made these needed edits.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
In this methods article, Hu and Deer provide detailed protocols for the purification of the microtubule (MT)-associated motor proteins, kinesin and cytoplasmic dynein, and the generation of polymerized MTs and DNA origami. I believe the provided protocols are sufficient to reproduce the discussed experiments. The presented procedures are straightforward and relatively easy to implement in a lab. The protocol is written with care and I feel that it will be a timely and well-cited contribution. In conclusion, I believe the methods article is appropriate for JoVE and would recommend publication, subject to the following minor changes:

Minor Concerns:

1. The authors write in line 47 that "By converting the chemical energy of ATP into productive work and force generation, […]". As work is the product of force times the distance over which the force is applied, I recommend to write either "By converting the chemical energy of ATP hydrolysis into productive work, […]" or "By converting the chemical energy of ATP hydrolysis into force generation, […]".

Thank you for catching this imprecise language. We have made the suggested edit.

2. In lines 623 to 626, the authors write that "The kymographs (Figure 6) of flexible chassis conjugated to seven dynein proteins ("7D" ensembles) show highly processive runs at relatively consistent and high velocities, which demonstrate the coordination and cooperation of multiple dynein motors in transporting one cargo." This statement is not correct. As the authors have shown in their Science paper (Fig. 2B in Derr et al. Science 2012), the velocity of the chassis with the 7 dynein motors is significantly lower than the velocity of a single dynein. This result suggests that yeast dynein motors, which are highly processive on their own, are not coordinating with each other and instead interfere. That the processivity of the multi-motor chassis is increased compared to the processivity of a single dynein, can be easily explained by the reduced likelihood of the chassis to detach from the MT as a result of the multiple MT-chassis dynein linkages. I recommend that the authors modify this sentence.

We agree and thank you for catching this. We have corrected it.

Reviewer #2:

Manuscript Summary:

This manuscript describes the procedure for assembling a complex hybrid nanostructure, consisting of a DNA-origami 'chassis' and purified molecular motors kinesin and dynein, and undertaking microscopy studies of the resulting nanostructures to elucidate motor mechanisms. It covers all the necessary steps, from yeast motor protein expression and purification, DNA origami assembly, loading of proteins onto the origami, and TIRF imaging.

This is an extremely comprehensive range of skills, in a very interdisciplinary project area, and so a JOVE video would be highly useful in broadening the applicability of this method. The efficacy of the protocol is well demonstrated, with reference to a number of recent high-impact publications. This protocol will be useful to scientists working in both motor protein and DNA origami areas. It is unlikely that many groups have sufficient background in both areas to develop such a protocol independently, so this video would act to bridge the expertise gap between the two areas. As many applications of DNA origami involve protein functionalization, this video would also serve as a useful example of such a protocol that could be adapted for other proteins.

Generally the discussion of background context and merits of the technique are unbiased and suitable. However, the protocol contains a large range of skills, and there is a high likelihood of scientists from diverse expertise backgrounds undertaking the protocol. Thus, some additional background discussion is required, as specified below. There are also a number of specific points that should be clarified.

Major Concerns:
None

Minor Concerns:

General discussion:

Abstract, Line 21: 'mechanisms the lead to emergent motile behavior'
Add a more specific example of the types of property that could be measured, eg 'how run length and velocity can vary with motor number'.

Thank you for this suggestion. We have added a phrase about motile properties to be measured.

Introduction, Line 78: 'segmented DNA origami with tunable compliance'
This sections needs more discussion on different DNA origami chassis/cargo designs and properties. For example, some examples of the different shapes, and a brief discussion of why

one would be chosen over another. As written it would not be clear to someone who is not already familiar with the referenced DNA origami papers.

We have added several new sentences to illustrate these ideas more fully.

5.1, Line 405: 'The compliance of the chassis structure is determined by the presence or absence of "linker" staples'
Similar to above comment, term 'Linkers' isn't very obvious in this conext without looking up reference 7. Could describe it more specifically here, eg 'alternating regions of rigid DNA-origami helix bundles and regions of flexible ss or dsDNA, which act as linkers'

Thank you for noticing this. We have more fully explained these ideas as well.

1.1, Line 92: 'streak desired frozen yeast strain'
Additional detail needs to be added either here or before this on the yeast strain. This section would not be clear to those without detailed protein production experience, and could use some more references. For example, how is the strain selected? Does the yeast strain already contain the construct of interest? If so, where was this obtained from?

We have more thoroughly explained about the yeast strain and referenced a Methods paper that details how to create modified yeast proteins for single molecule assays.

4, Line 336: 'Microtubule (MT) affinity purification'
Would be good to give some background rationale for this step. Why do only non-functional motors bind to MTs under these conditions? 1-2 sentences would be suitable. Are there other potential affinity purifications that could be helpful?

Thank you for the advice. We have elaborated on the background rationale for this purification method. (We are unaware of other affinity purification methods that could be helpful at this step, so have not elaborated on that point.)

6.1.3, Line 504: 'A high absolute and relative concentration of motors enables saturation of the chassis' motor binding sites.'
Accurate control of motor stoichiometry is one of the key benefits of using DNA origami as a chassis for these experiments. Given this, it is necessary to give further discussion on how accurate binding site occupancy is at this point in the method, and in the background section. How are motor and chassis concentration estimated? What are appropriate ranges equivalent to 'high' and 'low' for good preparations? What is expected occupancy of motor binding sites, and how robust are these values?

We have added more details to address these concerns. We have added more details in sections 4.2.5 on PAGE analysis of motors and in section 5.2.10 on quantification of chassis concentration. We have also discussed the occupancy in section 6.1.3 as suggested.

6.4.3, line 557: ' generating kymographs from the chassis movies.'

Should provide suggestion of software or methods for this. Could provide references for methods papers covering this. This is mentioned in Figure caption for Figure 6, but should be mentioned earlier.

We have added more details on kymograph generation and analysis in Protocol section 6.4.3, suggested ImageJ as the software, and cited a method paper.

Specific points to clarify:

1.4, Line 103: 'When the culture is between an OD 1.5 and 2'
Can you suggest a time frequency for checking OD, eg. Every hour? For those not familiar with yeast culture, is shape of growth curve known? Is it better to under or overshoot the suggested OD range? Do you know how sensitive yield is to OD at this point?

We do not have data on all of these points ,but we have some more information here as suggested so as to facilitate adoption of this protocol by others.

2.1.4, Line 141: 'add PMSF to the buffer'
What would be an appropriate time between addition of PMSF to lysis buffer and addition of lysis buffer to yeast powder? 1 min? <5 min? Should clarify what time frame is meant by 'immediately' here.

We have clarified the time frame here.

2.1.5, Line 142: 'pre-chilled glass beaker on ice'
What is appropriate size for beaker?

We have included the beaker size that we typically use in our experiment.

2.1.7 Line 148: 'estimate the lysate volume, and add more 4x lysis buffer'
Add an example of typical/expected volumes for this point

We have added the expected volumes for this step.

2.1.8 Lines 153-156: 'The bottles must be carefully balanced in the rotor'
Balancing of centrifuge is key step, can you reference another JOVE video that shows this step if it is not shown directly in this video?

This will be in our video.

2.2.3, Line 171: '4x buffer prepared previously'
Does this require fresh PMSF?

We have clarified this point as suggested.

2.3.1, Line 193: '100 l of 1x TEV buffer containing ~15 uM of the purified BG-oligo'

You gives some indication of reaction yield dependence on reaction time, can you give some information on dependence on oligo concentration? How sensitive is it to oligo concentration, would be better to specify, eg. 10-20 uM instead of ~15 uM.

We agree 10-20 uM is a better instruction for this step and have changed it accordingly. We have added a comment on concentration as well.

2.3.2, Line 299: 'do not mix by pipetting'
Add a brief dicussion of why this is important, does pipetting/vortexing disrupt MT nucleation?

We have added this discussion.

5.1.1, Line 408: 'at 250 M concentration in Tris buffer'
Plate oligos can also be ordered dry and normalised, then resuspended, this is a more commonly available shipping option for many locations.

We have amended this step as suggested.

5.1.5, Line 418: '600 nM sore staple pool'
Typo, should be 'core' not 'sore'

This typo has been fixed.

5.1.7, Line 430 & 5.2.7, Line 456: 2% agarose gel in 0.5x TBE buffer supplemented with 11 mM MgCl2'
Should specificy that gel running buffer also requires 11 mM MgCl2.

Thank you for noticing these omissions. We have made the suggested edits.

5.2.10, Line 463: 'Concentrations of selected fractions can be quantified using appropriate spectroscopic methods.'
Give some examples here, eg. UV absorption at 260 nm.

We have added this suggestion.