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An optimized toolbox for the optogenetic control of intracellular transport

Wilco Nijenhuis, Mariëlle Van Grinsven, and Lukas Kapitein

Corresponding Author(s): Lukas Kapitein, Utrecht University

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August 29, 2019

Re: JCB manuscript #201907149

Prof. Lukas Kapitein
Utrecht University
Padualaan 8
Utrecht 3533 CH
Netherlands

Dear Lukas,

Thank you for submitting your manuscript entitled "An optimized toolbox for the optogenetic control of intracellular transport". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We sincerely apologize for the delay in communicating our decision to you. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Your work was well received by both reviewers and is indeed an exciting new toolbox for control of molecule motors. In your revision, please address all of the reviewer comments. In particular, it would be good to see an expanded discussion of this system compared to previous systems, many of which come from your group. Ideally, it would be nice to see a direct comparison, as suggested by Reviewer #2, with at least one of these other approaches. Others in the field will be trying to decide whether to switch methods. In addition, a thorough discussion of possible applications should be added and proof-of-principle data where you present a new application is desirable to show the value of the method for the field -- in line with the expectations for a Tools paper at JCB.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Sam

Samara Reck-Peterson, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, Nijenhuis and Kapitein et al described an optimized toolbox for the optogenetic control of intracellular transport. Kinesin and dynein microtubule motors transport intracellular cargoes away or toward the perinuclear region. In a previous work, Kapitein had engineered light-inducible recruitment of these molecular motors to desired cargoes. This approach allows dynamic control of intracellular transport. In this current work, authors presented an optogenetic toolkit that improves cellular responses and limits side effects. This work combined two opto-proteins iLID and a mutant VVD for manipulating aterograde transport. For retrograde transport, this work used a reverse-walk kinesin instead of dynein. Both are very clever approaches and also seem to work out well. The new tools reduces dark-state activation and enhances cell responses. I agree with the authors that these more robust control systems will aid dissecting the intracellular transport process. Overall, the experiments were carefully designed and carried out with proper controls. I recommend the publication with minor modifications.

1. For Fig. 2E and F reversibility studies, the authors measured peripheral intensity of opto-Kinesin. Upon turning off the light, these kinesins will dissociate, unbind, then diffuse away and re-bind to other cellular places. Of these steps, is VVD dissociation the limiting step?
2. Regarding the reversibility, is the cargo location also reversible, i.e. does the cellular location of cargoes reverse upon turning off the light? What is the time duration? How does it differ from

different types of cargoes?
3. For the ppKin14 constructs and cargoes, are they also time reversible?
4. In the discussion section, can the authors discuss a few potential applications of this technique?

Reviewer #2 (Comments to the Authors (Required)):

Repositioning of organelles by the recruitment of motor proteins has been a popular tool of late. Original systems utilized the chemically induced dimerization of FKBP and FRB whereas more recent methods have taken advantage of optogenetic tools and much of this has been driven by the senior author's laboratory. Here the authors describe an "improved" optogenetic toolkit for recruitment of microtubule-based motors to a variety of cellular organelles or cargos. The system utilizes a different light-regulated protein-protein interaction that previous systems. However, without direct experimental comparisons, it is difficult to discern if this system is really better. If the authors cannot perform experimental comparisons to previous systems, then at a minimum the manuscript requires a more thorough discussion of the advantages/disadvantages of this system as compared to those of van Bergijk et al 2015, Duan et al 2015, Balister et al 2015, and Guardia et al 2019. As it stands, it is difficult for the reader to assess whether this is an improved system and is worth switching to if he/she is already using a different system. In addition, most of the findings would benefit from a more thorough presentation of all of the pertinent data so that the reader can better evaluate the benefits and drawbacks to this system.

1. P 7 line 2: "previously used kinesin-3 mutant, KIF1A(1-383)". It seems that this is not a mutant but a truncated version of the wild-type protein?

2. In figure 1, the authors introduce the iLID system for peripheral enrichment of Rab11 membranes by KIF1A kinesin motors. The significant activation of the system in the dark state is a problem and the authors go on to explore methods to decrease this background. How much does the residual activation depend on the level of expression of the motor and cargo components? Same for Figure 3C and 5H,I.

3. There are several instances where the authors compare the effects of monomer/dimer state of KIF1A on transport: comparison of VVD52C to VVDfast for motor localization (Figure 2), comparison of VVD52C to VVDfast to dimer for mitochondrial transport (Figure 3D), and comparison of VVD52C to VVDfast to monomer for Rab5 membranes (Figure 4A-E). It is hard to compare these results since different types of data and analysis are shown for each situation. Does the motor come off of the microtubules in Figure 2 and off of the cargoes in Figures 3 and 4 when imaging goes back to the dark state? Please show images of all channels over time. For mitochondrial transport in Figure 3, the data demonstrate that the native KIF1A dimer is better than the VVDfast at all illumination levels and the authors conclude that the VVDfast KIF1A is acting primarily as a monomer. For Rab5 membranes in Figure 4, the data show that the VVD modules are better than the native KIF1A monomer and the authors conclude that the VVD KIF1As are acting as dimers. This is confusing. To make these statements, it would be better to compare all motors (monomer, dimer. VVD52C, VVDfast) for transport of the same cargos.

4. The images in Supplemental Figure 1 part B compare different KIF1A motors and none of these appear to have redistributed the Rab11-membranes to the periphery. Are these images in part B in the dark state? Is the quantification in part C in the illuminated state?

5. In Figure 2, the authors show that the VVDfast KIF1A leaves the periphery when the illumination ends. Is this because it goes back to a monomer? It would be good to show this perhaps biochemically. In contrast, the VVD52C motor stays at the periphery. Does this suggest that this motor remains a dimer? Is it just monomer or dimer or do the results of Guedes-Dias et al 2019 that KIF1A detaches from microtubule plus ends influence the behavior of the motors?

6. The big advantage to the optogenetic control system is the reversibility. In this study, the reversibility of cargo distribution is shown in Figure 4 and more data is needed to assess the usefulness of the system. Is the motor falling off the cargo (please show images)? How do the different monomer/dimer/VVD constructs behave? How much does reversibility depend on the cargo (Rab5 vs Rab11 vs mitochondria)?

7. P 12, line 6: please quote Guardia et al 2019 who showed that the BICD2 can act as a dominant negative in these assays.

8. For the minus end motor ppKin14-VIb, please show images of the localization of both the dimerized and tetramerized constructs (Figure 5B shows dimer but text also refers to tetramerized version without indicating any data).

9. The multi-cistronic plasmid (Figure 6) seems to be a big advantage. How well does the self-cleaving PP2A peptide work to create the cargo and motor components? If it doesn't work to 100%, this could explain the high background in the dark state. Please display the data as in Figure 1 so the dark state activation can be directly compared for the two plasmid vs one plasmid systems. Is there less cell-to-cell variability in this system?

Response to the reviewer's comments

Reviewer #1:

In this manuscript, Nijenhuis and Kapitein et al described an optimized toolbox for the optogenetic control of intracellular transport. Kinesin and dynein microtubule motors transport intracellular cargoes away or toward the perinuclear region. In a previous work, Kapitein had engineered light-inducible recruitment of these molecular motors to desired cargoes. This approach allows dynamic control of intracellular transport. In this current work, authors presented an optogenetic toolkit that improves cellular responses and limits side effects. This work combined two opto-proteins iLID and a mutant VVD for manipulating aterograde transport. For retrograde transport, this work used a reverse-walk kinesin instead of dynein. Both are very clever approaches and also seem to work out well. The new tools reduces dark-state activation and enhances cell responses. I agree with the authors that these more robust control systems will aid dissecting the intracellular transport process. Overall, the experiments were carefully designed and carried out with proper controls. I recommend the publication with minor modifications.

- We thank the reviewer for his positive evaluation of our manuscript and for recommending publication.

1. *For Fig. 2E and F reversibility studies, the authors measured peripheral intensity of opto-Kinesin. Upon turning off the light, these kinesins will dissociate, unbind, then diffuse away and re-bind to other cellular places. Of these steps, is VVD dissociation the limiting step?*

- We indeed believe it is. In these experiments, motors are not recruited to cargoes, but only transition from being monomeric to dimeric. The individual dimeric motors that are generated will run processively along microtubules with a characteristic run length. Assuming that run length and velocity are within the typical ranges of 3-5 μm and 1-3 μm/s, respectively, each motor will be bound to microtubules for just 1-5 seconds. Thus, even in the presence of blue light, motors will cycle between microtubule-bound and -unbound intervals and the final distribution will be determined by the on-rate, off-rate/run length, motor speed and diffusion coefficient. Because the average microtubule-bound time per run is much shorter than the typical reversal time of even the fast VVD, which is around 30 s, the time scale with which the distribution reverses to homogeneous is set by the dissociation of the VVD domain.

2. *Regarding the reversibility, is the cargo location also reversible, i.e. does the cellular location of cargoes reverse upon turning off the light? What is the time duration? How does it differ from different types of cargoes?*

- We thank the reviewer for raising this interesting point. In the revised manuscript, we have explored how the positioning reversibility differs between different organelles and devoted a new figure to this (Figure 8). When comparing lysosomes/late endosomes, early endosomes, and recycling endosomes, we found that early endosomes completely regain their original distribution within 500-1000 seconds. (Note that this time scale is much longer than the time scale of motor dissociation (~50 seconds, new Fig. 4K and new Fig. 5F).) In contrast, recycling endosomes also regain their distribution, but this takes 2-3 times longer. Finally, lysosomes show some distribution restoration, but appear to settle on a new base line. In future work, we aim to further develop these findings and explore the biological mechanisms that underlie positional sensing and restoration.

3. *For the ppKin14 constructs and cargoes, are they also time reversible?*

- The dissociation of the Kin14 motor is shown in the new Fig. 5F, whereas the reversibility of cargoes is shown in Figure 8. As discussed above, the motors quickly dissociated when blue light illumination is stopped, whereas the reversal of cargo positioning was much slower and depended on the exact cargoes.

4. *In the discussion section, can the authors discuss a few potential applications of this technique?*

- We have added Figure 8 to demonstrate that this assay can be used to examine how different organelles respond to mislocalization. In future work, we aim to further develop these findings and explore the biological mechanisms that underlie positional sensing and restoration. In addition, we are currently exploring how repositioning one type of organelles affect the positioning of other organelles, as proposed in the final sentence of the discussion. These experiments provide an alternative strategy to explore connections between various organelles and add mechanical information to the organelle connectome.

Reviewer #2:

Repositioning of organelles by the recruitment of motor proteins has been a popular tool of late. Original systems utilized the chemically induced dimerization of FKBP and FRB whereas more recent methods have taken advantage of optogenetic tools and much of this has been driven by the senior author's laboratory. Here the authors describe an "improved" optogenetic toolkit for recruitment of microtubule-based motors to a variety of cellular organelles or cargos. The system utilizes a different light-regulated protein-protein interaction that previous systems. However, without direct experimental comparisons, it is difficult to discern if this system is really better. If the authors cannot perform experimental comparisons to previous systems, then at a minimum the manuscript requires a more thorough discussion of the advantages/disadvantages of this system as compared to those of van Bergijk et al 2015, Duan et al 2015, Balister et al 2015, and Guardia et al 2019. As it stands, it is difficult for the reader to assess whether this is an improved system and is worth switching to if he/she is already using a different system. In addition, most of the findings would benefit from a more thorough presentation of all of the pertinent data so that the reader can better evaluate the benefits and drawbacks to this system.

- We thank the reviewer for the constructive review of our manuscript. In the revised version, we now include a more extensive discussion on the advantages and disadvantage of various optogenetic or chemogenetic approaches. In addition, we have modified the presentation of our data following the reviewer's recommendations.

1. *P 7 line 2: "previously used kinesin-3 mutant, KIF1A(1-383)". It seems that this is not a mutant but a truncated version of the wild-type protein?*

- That is correct. We changed the sentence to "previously used truncated kinesin-3 construct, KIF1A(1-383)."

2. *In figure 1, the authors introduce the iLID system for peripheral enrichment of Rab11 membranes by KIF1A kinesin motors. The significant activation of the system in the dark state is a problem and the authors go on to explore methods to decrease this background. How much does the residual activation depend on the level of expression of the motor and cargo components? Same for Figure 3C and 5H,I.*

- We have now analyzed how residual activation depends on the expression level of the motor and cargo (Fig. 1D, Fig. 3D, Fig. 6F). This show that when using the dimeric motor, very few cells with expression levels that are <25% of maximum levels display dark-state activation (Fig. 1D). For the opto-kinesin, the few cells that have altered distributions in the dark are among the cells that express both components at >60% of the maximum levels.

  We have also tried to experimentally compare the level of dark state activation in the iLID system with the TULIP system that we used previously, but were unable to compare these systems directly in this assay because (1) for repositioning of RAB proteins, the TULIP system requires the use of a third construct to couple the endosome to KIF1A and (2) the affinity of the activated TULIP system lies in the lower end of the dynamic range of this assay (K_d: 72 µM, Hallett et al., ACS Synthetic Biology 2015) and is comparable to SSPB(milli) in the light (56 µM, Zimmerman et al., Biochemistry 2016).

3. *There are several instances where the authors compare the effects of monomer/dimer state of KIF1A on transport: comparison of VVD52C to VVDfast for motor localization (Figure 2), comparison of VVD52C to VVDfast to dimer for mitochondrial transport (Figure 3D), and comparison of VVD52C to VVDfast to monomer for Rab5 membranes (Figure 4A-E). It is hard to compare these results since different types of data and analysis are shown for each situation. Does the motor come off of the*

microtubules in Figure 2 and off of the cargoes in Figures 3 and 4 when imaging goes back to the dark state? Please show images of all channels over time. For mitochondrial transport in Figure 3, the data demonstrate that the native KIF1A dimer is better than the VVDfast at all illumination levels and the authors conclude that the VVDfast KIF1A is acting primarily as a monomer. For Rab5 membranes in Figure 4, the data show that the VVD modules are better than the native KIF1A monomer and the authors conclude that the VVD KIF1As are acting as dimers. This is confusing. To make these statements, it would be better to compare all motors (monomer, dimer. VVD52C, VVDfast) for transport of the same cargos.

- We apologize for the confusion and have tried to improve consistency in the revised manuscript. We used different experiments and analyses in Figure 2, 3 and 4, because we were characterizing different properties of the optoKinesins. Figure 2 studies the behavior of the two optoKinesins themselves and shows that both VVD52C and VVDfast accumulate at the cell periphery upon illumination with blue light. Since the VVD modules induce homodimerization and single-headed motors cannot effectively move over microtubules inside living cells, we interpret these results as evidence for light-induced dimerization of motor domains. Because reversal of dimerization in the dark is known to be much faster for VVDfast, we furthermore tested how the distribution of optoKinesins would change after exposure to blue light has ceased. This revealed that for VVDfast, motors quickly regained their original, diffuse localization, whereas motors dimerized using VVD52C remained accumulated at the cell periphery. The quick reversal seen with VVDfast is consistent with the interpretation that kinesin dimerization is rapidly reversed in the dark, resulting in monomeric motors that can interact much less effectively with microtubules and cannot generate directional movement. The lack of distribution changes seen with VVD52C is consistent with the very slow reversal of dimerization in this construct. As a result the motors remain dimeric and are able to effectively interact with microtubules and move along it (by alternating strong binding and powerstrokes).

  In Figure 3, we examined how the behaviors of the two opto-Kinesins compare to that of a constitutive dimeric motor. We designed optoKinesin to limit dark-state activation by introducing a second layer of control and examined its behavior in the same way as we did for the dimer in Figure 1C. This revealed that we have indeed less dark-state activation when using the optoKinesin. We agree that it would have been better to also compare monomeric kinesin in the same way. For the revised manuscript, we have therefore performed these experiments and analyzed the monomeric kinesin using the same cargo and quantification. This revealed that monomeric kinesin shows less dark-state activation than dimeric kinesin, but is also much less effective in redistributing cargoes (Fig. 1C and Fig. 3C). In contrast, opto-Kinesins also show less dark-state activation, but these motors can still effectively redistribute the endosomes. We have added this to Figure 3 and we removed the analysis of mitochondrial redistribution. From these results we conclude that the dimerization of optoKinesins is required to drive effective cargo transport.

  Finally, as requested, we have now also imaged how the motor dissociates from the cargoes when blue-light illumination is stopped. These results are shown in Fig. 4K for the inducible dimers, as well as the monomer.

4. *The images in Supplemental Figure 1 part B compare different KIF1A motors and none of these appear to have redistributed the Rab11-membranes to the periphery. Are these images in part B in the dark state? Is the quantification in part C in the illuminated state?*

- Indeed, B and C were both showing the distribution before illumination to illustrate that the difference in the distribution of the various motors relative to the Rab11. In C, the distribution of the cargo (first column) and the different motors (column 2-4) is analyzed, again without optogenetic stimulation. For clarity, we have added the header “Without heterodimerization” to both panels.

5. *In Figure 2, the authors show that the VVDfast KIF1A leaves the periphery when the illumination ends. Is this because it goes back to a monomer? It would be good to show this perhaps biochemically. In contrast, the VVD52C motor stays at the periphery. Does this suggest that this motor remains a dimer? Is it just monomer or dimer or do the results of Guedes-Dias et al 2019 that KIF1A detaches from microtubule plus ends influence the behavior of the motors?*

- Before illumination, both VVDfast-KIF1A and VVD52C-KIF1A have a distribution that is similar to monomeric KIF1A (Fig. 2C, S1B,C). Upon illumination, both motors accumulate in the periphery, similar to dimeric KIF1A (Fig. S1B). It has been shown previously that the reversal of VVD52C dimerization in the dark is very slow, while it is much faster for VVDfast (Zoltowksi et al., Nature Chemical Biology 2009). Together, these results support the interpretation that the different distributions observed after stimulation for the two VVD motors is caused by the difference in dimerization status. We do not think that observations of Guedes-Dias would apply differently to different dimeric KIF1A constructs.

6. *The big advantage to the optogenetic control system is the reversibility. In this study, the reversibility of cargo distribution is shown in Figure 4 and more data is needed to assess the usefulness of the system. Is the motor falling off the cargo (please show images)? How do the different monomer/dimer/VVD constructs behave? How much does reversibility depend on the cargo (Rab5 vs Rab11 vs mitochondria)?*

- We now added new data that directly shows that motors detach from cargoes when stimulation is stopped (Fig. 4K, Fig. 5F). Nonetheless, detachment of the motors does not necessarily need to result in reversal of cargo distributions to the original state. If certain cargoes were largely immobile before stimulation, this would indicate that motors were not actively positioning these cargoes and were perhaps not even bound to these cargoes. Optogenetic motor recruitment would reposition these cargoes, but no mechanism might be in place to actively reposition these cargoes. Alternatively, cells might sense cargo mislocalization and activate pathways to ensure redistribution. As requested by the reviewer, we have now explored how the positioning reversibility differs between different organelles and devoted a new figure to this (Figure 8). When comparing lysosomes/late endosomes, early endosomes, and recycling endosomes, we found that early endosomes completely regain their original distribution within 500-1000 seconds. (Note that this time scale is much longer than the time scale of motor dissociation (~50 seconds, new Fig. 4K and new Fig. 5F).) In contrast, recycling endosomes also regain their distribution, but this takes 2-3 times longer. Finally, lysosomes show some distribution restoration, but appear to settle on a new base line. In future work, we aim to further develop these findings and explore the biological mechanisms that underlie positional sensing and restoration.

7. *P 12, line 6: please quote Guardia et al 2019 who showed that the BICD2 can act as a dominant negative in these assays.*

- We now reference this work in addition to the original report that we cited (Hoogenraad et al. 2001).

8. *For the minus end motor ppKin14-VIb, please show images of the localization of both the dimerized and tetramerized constructs (Figure 5B shows dimer but text also refers to tetramerized version without indicating any data).*

- We now show images of the localization of both the dimerized and tetramerized constructs (new Fig. 5C).

9. *The multi-cistronic plasmid (Figure 6) seems to be a big advantage. How well does the self-cleaving PP2A peptide work to create the cargo and motor components? If it doesn't work to 100%, this could explain the high background in the dark state. Please display the data as in Figure 1 so the dark state activation can be directly compared for the two plasmid vs one plasmid systems. Is there less cell-to-cell variability in this system?*

- In the revised manuscript, we have further analyzed the experiments with the self-cleaving construct. We biochemically demonstrate the efficiency of cleavage (Fig. 6B), we characterize the constructs in the same assay as an Fig. 1 and Fig. 3, display the data in the same way, show cell-to-cell variability in motor/adaptor expression, and analyzed dark-state activation as a function of expression level. These results demonstrate that the P2A peptide is efficiently cleaved, that motor-adaptor ratios are less variable than when expressing two constructs independently, and that dark state activation is comparable to experiments in which opto-kinesin and Rab11 are expressed independently. Importantly, the biggest advantage of these plasmids is that they enable packaging both constructs (motor and adaptor) in one vector, which greatly simplifies selecting stable cells in which the

expression levels of both construct are as desired. This enabled the robust population-wide repositioning shown in Fig. 6I.

January 6, 2020

RE: JCB Manuscript #201907149R

Prof. Lukas Kapitein
Utrecht University
Padualaan 8
Utrecht 3533 CH
Netherlands

Dear Prof. Kapitein:

Thank you for submitting your revised manuscript entitled "An optimized toolbox for the optogenetic control of intracellular transport". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, you should also include the additions requested by the reviewer, which need to be highlighted upon resubmission of your final files.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
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d. Imaging medium
e. Fluorochromes
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h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Tools may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Samara Reck-Peterson
Monitoring Editor
Journal of Cell Biology

Andrea L. Marat
Scientific Editor
Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

The authors have carried out experiments and rewriting to improve the manuscript. I appreciate the new analysis on motor expression levels and the attempts to improve consistency in data analysis and presentation. More could be done to show images of both motors and cargoes for all experiments. More could be done in terms of comparisons to previous methods and a better discussion of the benefits of this system over previous ones. There is also little added about how the assays could be used to investigate cell biological questions. I think the authors could have done a much better job. I am generally in favor of publication although I have some suggestions to further improve the writing:

1. The new results in Figure 8 are quite interesting in terms of the differences between motors and cargoes in their repositioning and the authors should spend more time discussing their findings and how they fit into the field. For example, for the optokinesin, there is efficient redistribution of RAB11 but this organelle does not return to baseline upon return to the dark state whereas neither RAB5 nor LAMP1 is efficiently redistributed and LAMP1 does not return to baseline. What do these results tell us about the motor and/or organelles? How do the results here compare to the previous results on RAB5 (Fig 4) and the redistribution of other organelles by this motor (Schimert et al 2019)?

2. The argument for choosing moss kinesin-14 is not clear. The authors state that "most kinesin-14 motors are either weakly processive as single motors..." but so is the ppKin14-VIb they have chosen to use "although non-processive as a native dimer". The authors state that they chose ppKin14-VIbbecause it is "highly processive and fast motility when clustered as dimer of dimers" but the other kinesin-14 motors are also highly processive when clustered. It would be fine to say that kinesin-14 motors are processive when clustered and we chose to use the moss motor. Did the authors try any of the mammalian kinesin-14s? It would be helpful for the reader to know this.

3. The experiment in Fig 5A is described in a confusing way in the text: "To uncouple such dominant-negative effects from potential dark-state activation resulting from the optogenetic modules, we first made use of a chemically-induced heterodimerization system that allows inducible recruitment of an FKBP12 domain to an FRB domain upon addition of the cellpermeable small molecule AP21967 (rapalog hereafter). Indeed, whereas FKBP-mCherry- RAB11 labeled recycling endosomes were efficiently recruited to the perinuclear region after the addition of rapalog, we occasionally observed that the distribution of recycling endosomes was perturbed in BICDN-expressing cells that were not exposed to rapalog (Fig. 5A)."

4. p14: "We first validated that the P2A plasmid was efficiently cleaved by immunoblotting". It is not the plasmid that is being cleaved but rather the resulting fusion protein.