

June 6, 2011

Re: Manuscript #201105007

Dr. Daniela Nicastro
Brandeis University
Rosenstiel Center, MS 029
415 South Street
Waltham, MA 02454-9110

Dear Dr. Nicastro,

Thank you for submitting your manuscript entitled “Cryo-electron tomography reveals novel interactions and doublet specific structures in the II dynein” to *The Journal of Cell Biology*. We have now received reports from two reviewers, and these are appended below. As you will see, there are two key issues raised by both referees. First, there are numerous technical and scientific issues raised in regard to the interpretation of the structural studies. The major issue in this regard, carefully described by Referee 1, is how to clearly prove a “connection” versus a “proximity” when analyzing these structures. Additional issues are also raised, including the interpretation of the intermediate chain structure. The second major issue is the lack of integration between the structural and biochemical aspects of the work. Neither referee felt that the biochemical analysis, as it stands, provides a strong component of the manuscript. Finally, both referees provided advice on more clearly communicating the structural information in this work.

After careful consideration, we have decided that the points raised by the reviewers preclude publication of the manuscript in *The Journal of Cell Biology*. I am therefore, with regret, returning the manuscript to you. However, if you feel that you are able to address the reviewers’ comments in full with additional data and analysis, we would be willing to rereview a completely revised manuscript. A revised manuscript would either need to better integrate the structural and biochemical aspects of the work or to follow the advice of Referee 2 and focus on the structural aspects only. However, in the latter case, the revised manuscript may not be viewed by the referees as providing a sufficiently important contribution to merit publication in *The JCB*.

If you decide to resubmit your manuscript, please submit a cover letter describing the changes made and addressing the reviewers’ comments point by point. Please be aware that the revised manuscript may be treated as a new submission if received more than three months after the date of this letter.

Thank you for your interest in *The Journal of Cell Biology*.

Yours sincerely,



Monitoring Editor

Reviewer #1

The work investigates the structure of inner dynein arm 1 (I1) in *Chlamydomonas* flagella using a combination of cryo-electron tomography (cryo-ET) of wild-type and subunit-lacking mutants as well as biochemical analyses to examine, among other things, the level of phosphorylation of subunits.

The tomography work on I1 is a valuable contribution to the field of ciliary and flagellar structure and function. It reveals interesting and novel structures the authors call the ‘tether’ and ‘tether head’, that lie between one of the I1 motor domains (I1a) and the cargo microtubule (doublet A). They also show an unexpectedly small connection between I1 and the cargo microtubule (connection 1). These findings are robust. **However, several other aspects of the work are (1) questionable, (2) flawed or (3) provide only vague conclusions that do little to advance understanding, as follows.**

1) The authors greatly emphasize what they call ‘connections’ (numbered 2-7) made between I1 and various other neighboring structures. **While connecting features can certainly be seen in isosurface renderings and slices of the averaged tomograms, their attribution as ‘connections’ (implying structural continuity or physical contact) is speculative at best. Any two neighboring unconnected objects can be made to appear connected when imaged at an appropriately low resolution.** Therefore, the validity of these six connections cannot be known. This contrasts with ‘connection 1’ made between I1 and the cargo (A-) microtubule because it is known that these two structures must form a connection (I1 is located at precisely 96nm intervals along the A-microtubule) and therefore the structure of this connection is of great interest, especially given its unexpected small size. **The remaining ‘connections’ are dubious** and more appropriately termed ‘proximities’ or some such less assertive or definitive term. This would still permit the existence of the implied regulatory pathways. Finally, it is inconsistent and puzzling that the authors do not mention other features that could, by their same reasoning, be termed ‘connections’. For example, features can be seen between the ‘tether’ structure and the A-microtubule (Fig. 5 M, N, O) and become substantially weakened in the *bop-5* mutants (Fig. 5 N and 5 O). Are we not to believe these? If not, why not? Interpretation based on isosurfaces or slices alone, without any supporting functional data of connectivity, should in all cases be avoided, for the reason given above. The weight in the text attributed to these should be commensurately reduced.

2) The authors make an attempt to identify the locations of intermediate chains within the tail of I1 using comparisons with two mutants. **However there is a serious flaw in their experimental approach which undermines the validity of this result.** Specifically, an interesting observation is made about the structural variability between I1 tails from the nine doublet microtubules (Fig. 3). Having established this, however, the authors go on to compare a combined I1 tail structure (from all nine doublets merged together) with similarly combined tails from the *bop-5-1* and *bop-5-2* strains. Combining structurally different tails must produce artifactual structures. It is therefore puzzling that the authors do not repeat the doublet-specific analysis for the *bop-5* mutants too to make the relevant comparisons with the wild-type. **As a result of this flawed strategy, the claimed identification of the intermediate chains cannot be supported as is.** This must be corrected (or removed).

3) The conclusions drawn from the ‘connections’ (preferably ‘proximities’) between I1 and neighboring structures and those from the novel phosphorylation states of the various mutant strains are vague and do not in themselves reveal any substantive new insights about flagellar function. It is difficult even to summarize what are the authors’ conclusions on this part of their work. Nor is it clear how the structural (tomography) work relates specifically to the biochemical work in any substantive way.

Specific comments:

P7

For the pWT (pseudo Wild-Type) strain the authors explain that they have used a cross between two strains: NDRC pf2 with GFP-tagged WT PF2 without describing what these strains are (e.g. what components and functions they lack), nor the rationale for examining them. While it is clear that the resolution of this strain is higher than the wild-type (WT) this at least suggests something different about these axonemes that the authors do not describe.

P8-9

Descriptions of the structure jump repeatedly between Figures 2 and 4 which is deeply irritating for the reader.

P20

The authors refer to the delta heavy chain of the outer dynein arm (dHC of ODA), whereas this should refer to the gamma heavy chain.

P25

The authors do not state the nucleotide condition of the dynein ATPase motors in their specimens. Since nucleotide is known to alter the conformation of the dynein ATPases this is an omission. It would also help if the authors stated the likely phosphorylation state(s) of their I1s under their experimental conditions. Even if the nucleotide and/or phosphorylation status is uncertain, this should be stated clearly for the reader in the main text and in the Methods section.

Tomogram deposition:

While perhaps not necessarily a publication policy of *JCB* itself, in the structural biology world it is very strongly encouraged, if not imperative, that authors deposit their tomograms in the appropriate public databases for the community and authors are required by many journals to declare their commitment to such by initiating upload (for preliminary accession codes) at the time of submission.

Figures:

The figures of the tomogram structures contain a wealth of data but are arranged and labeled in such a way as to be rendered difficult and sometimes impossible to understand, even for readers familiar with the structure of the axoneme. The panels are often too closely cropped, crowded with over-labeling, have rotation symbols overlapping the edges of boxes and contain so many panels that they are extremely difficult to follow from the legends. I recognize the enthusiasm of the authors to show as much information as possible,

which is to be applauded. Simply using a key instead of labels would improve matters. The orientations of tomogram slices are almost impossible to figure out because ‘front’ and ‘side’ views are not defined sufficiently clearly, nor are the (z-) position of the slices indicated. A cartoon representation of these (valuable) panels, illustrating the position of the slice, would help.

Importantly a new development in presentation of complex 3D structures is the use of 3D PDF files (available free for readers using Acrobat Reader 9) that allows the user to rotate, enlarge and translate the object, as well as to switch off (author-defined) objects, features and densities and for author-defined views to be created. These are amazing new features that should be embraced by these authors. The use of 3D PDFs is increasing greatly because they dramatically improve the reader’s understanding of the results and their use here should be exploited.

Reviewer #2

In this paper, the authors analyzed the axonemal inner-arm dynein I1 using cryo-electron tomography and biochemical methods to understand how the two-headed dynein interacts with other axonemal components.

From the electron tomograms, they found seven connections between I1 dynein and neighboring structures, which suggest the regulatory roles of the I1 dynein. They also discovered unique features of the I1 complex, such as differences specific to the location in the axoneme and a large complex that links the motor domain of the I1a heavy chain (HC) to the A-tubule (Tether and Tether head). Furthermore, they revealed the localization of intermediate chain (IC) 138 and its sub-complex within the intermediate chain and light chain complex (ICLC) by comparing wild type and *bop5* mutants. **This part (Fig.1~5) is technically advanced and solid.** Although the structures themselves are mostly descriptive, the new findings are essential for understanding the regulatory mechanisms of axoneme.

In addition to the structural studies, they examined the phosphorylation conditions of I1 subunits, which are thought to affect the dynein activities. Although novel phosphorylated isoforms of several subunits were found, it is difficult to correlate the phosphorylation with the structures obtained by cryo-electron tomogram or with signaling pathway of axoneme.

In summary, the reviewer’s recommendation is to shorten the manuscript to include only strong data by omitting some of the biochemical analysis. With such revisions, the paper will be worthy of publication in *J. Cell Biology*.

Specific comments:

1) Table I. The authors briefly describe the swimming phenotypes for strains used in this study, like “fast” or “slow”. More detailed description of the phenotypes is necessary for understanding the in vivo function of I1 subunits. Although wild type can swim faster than *ida2-7* (+ β tail), the authors describe both phenotypes as “fast”. The mutant *bop5-2* probably cannot swim forward owing to the second mutation (*mbo*) (Bower et al., 2009). Therefore, the phenotype is not “slow”, but “mixed motility” (Bower et al., 2009).

2) The second paragraph on p19. A unique structure associated with the ICLC on doublet MTs (dMTs) 3 and 4 was identified by the doublet-specific averages. The authors suggested that the structure plays a role in generating the asymmetric distribution of dynein activity required for effective ciliary-type motility and indicated that the flagellar waveform analysis of *bop5* mutants (VanderWaal et al., 2011) supports this hypothesis. Since the reference has not been published yet, the brief explanation of the experimental results will be helpful for readers. If the hypothesis is correct, the structure may be affected in the *mbo* mutant, which displays symmetric flagellar-type motility. Discussion about the structure in the *mbo* mutant is valuable.

3) The second paragraph in p20. The authors tried to find a polypeptide corresponding to the tether head, but they could not. The large size of the polypeptide (~150kD) may not be analyzed by 2D gel electrophoresis. If tether head remains bound to 1a HC during I1 complex purification, the component will be purified with I1a HCs. If so, the authors will be able to find an extra 100-150 kD polypeptide in the purified fraction on 1D gel. I would like to recommend the authors to re-evaluate the SDS-PAGE gel of the purified I1 dynein.

4) According to the JCB's editorial policy "Free exchange of materials", deposition of the electron density maps to the public database should be a requirement and the manuscript should include the database accession number. In this case, EMDB is appropriate, like this entry: http://emsearch.rutgers.edu/atlas/1696_summary.html

Minor points:

1) The first paragraph in p20. ODA d HC is incorrect. ? HC.

2) Figure 1E. Please label each inner dynein arm structure to help reader to understand the surrounding components of I1 dynein, as in Fig. 10.



Brandeis University

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10th of August 2011

Re: Manuscript #201105007

Dear Dr. [REDACTED]

Thank you for your feedback and your explanations to the reviewers' comments, which identified some weak points in our original manuscript. We have taken this criticism to heart and reworked our paper. We are now resubmitting a completely revised version, in which we incorporated many of the suggestions provided by the reviewers.

Please find here below **our answers (in red font)** to the points you highlighted **in your letter (blue font)**. In addition, we have attached below a detailed point-by-point responses to the reviewer's comments (black font).

Thank you for considering our manuscript for revision.

Thank you for your consideration.

Sincerely,

A handwritten signature in black ink, appearing to read "D. Nicastro", written over a horizontal line.

Daniela Nicastro, Ph.D.
Brandeis University
415 South Street, Waltham, MA 02454

ANSWERS TO MONITORING EDITOR ONLY:

... are two key issues raised by both referees.

1) First, there are numerous technical and scientific issues raised in regard to the interpretation of the structural studies. The major issue in this regard, carefully described by Referee 1, is how to clearly prove a “connection” versus a “proximity” when analyzing these structures. Additional issues are also raised, including the interpretation of the intermediate chain structure.

As a point of clarification, issues regarding technique and interpretation were only raised by Reviewer #1. In contrast, reviewer # 2 wrote: ***“This part (Fig.1~5) is technically advanced and solid. Although the structures themselves are mostly descriptive, the new findings are essential for understanding the regulatory mechanisms of axoneme.”*** And ***“In summary, the reviewer’s recommendation is to shorten the manuscript to include only strong data by omitting some of the biochemical analysis. **With such revisions, the paper will be worthy of publication in J. Cell Biology.**”***

We have included a detailed point-by-point response to Reviewer #1’s critiques in order to clarify some of the Reviewer’s misconceptions about interpretation of structural data and averages. These are relatively long because the reviewer’s comments question fundamental methods used in the cryo-EM/electron tomography field.

I would like to emphasize, that the data presented here are the best and highest resolution data of intact axonemes to date (and among the best of cellular specimen in general). Similar to our N-DRC paper (Heuser et al 2009), we document many steps much more thorough than is common in structural papers, such as data quality, or how we identified the doublet specific difference (by showing the average of each doublet in the supplements). This June, Thomas Heuser presented a poster of this work at the Gordon Research Conference for Three-dimensional Electron Microscopy – as you know a conference attracting the major experts in a field. Although I was an invited speaker at the same conference, the poster committee felt so positive about Thomas’s work that he was one of only a few poster presenters selected at the conference to give a 10 min. talk (which was very well received)!

Therefore, if the editors of JCB still feel hesitation concerning the technical quality of our data and validity of our interpretations, I would urge you to please consult an EM expert as additional reviewer, rather than basing your decision on an incorrect critique. Any speaker (or participant) of the recent Gordon Research Conference for Three-dimensional Electron Microscopy or International Congress on Electron Tomography would be qualified.

<http://www.grc.org/programs.aspx?year=2011&program=threedim>

<http://www.embl.de/training/events/2011/TOM11-01/programme/>

2) The second major issue is the lack of integration between the structural and biochemical aspects of the work. Neither referee felt that the biochemical analysis, as it stands, provides a strong component of the manuscript.

This was a very useful critique raised by the Reviewers. Sparked by our new structural findings we invested a lot of work into trying to identify the tether head/tether protein(s) and/or additional I1-components in general. Reading the reviewers’ comments I realized that we were too attached to this body of biochemical work that ultimately fell short of providing many new insights. I believe in our revised submission, we have corrected this by both shortening the biochemical part significantly and by integrating the remaining biochemical results more completely with the structural data.

Finally, both referees provided advice on more clearly communicating the structural information in this work. [...] A revised manuscript would either need to better integrate the structural and biochemical aspects of the work or to follow the advice of Referee 2 and focus on the structural aspects only. However, in the latter case, the revised manuscript may not be viewed by the referees as providing a sufficiently important contribution to merit publication in *The JCB*.

I hope the editors of JCB will agree with Reviewer #2: “*In summary, the reviewer’s recommendation is to shorten the manuscript to include only strong data by omitting some of the biochemical analysis. With such revisions, the paper will be worthy of publication in J. Cell Biology.*”

September 22, 2011

Re: Ms# 201105007R

Dr. Daniela Nicastro
Rosenstiel Center, MS 029
415 South Street
Waltham, MA 02454-9110

Dear Dr. Nicastro,

Your revised manuscript entitled “Cryo-electron tomography reveals novel interactions and doublet specific structures in the II dynein” has now been seen by two reviewers, whose reports you will find below. One of these reviewers (#2) assessed the first version submitted to our journal, whereas the other (#3) was added in this round because original Reviewer #1 was unavailable. After careful consideration, **we have decided that the points raised by these reviewers preclude publication of the manuscript in *The Journal of Cell Biology*.**

As you will see, **the two referees differed in their opinions of the work.** While Reviewer #2 feels that the work has improved sufficiently, **Reviewer #3 raises major concerns about the interpretations of some of these data.** Some of these points were also raised by Reviewer #1 in the first round of review, and clearly these concerns were not satisfactorily addressed upon revision.

Because of this continuing issue of conflicting interpretations and of inconsistent support for publication, and because aspects of this work remain somewhat speculative, we must regretfully decline to consider this work further. This decision reflects the specific comments of the reviewers as well as the priority that the manuscript was assigned in relation to competing submissions to this journal. (*JCB* currently publishes less than 15% of submitted manuscripts.) I am therefore, with regret, returning your manuscript to you with the hope that you will find our reviewers’ comments helpful in revising the manuscript for submission elsewhere.

I am sorry that our answer on this occasion must be negative and hope that it will not dissuade you from submitting other manuscripts to this journal in the future.

Thank you for your interest in *The Journal of Cell Biology*.

Sincerely,


Monitoring Editor

Reviewer #2

In this revised manuscript, the authors have reconsidered the interpretations of the results obtained from the structural and biochemical studies and rewritten the manuscripts according to the reviewers' comments. They omitted most of the biochemical data on the phosphorylation states of several intermediate and light chains (ICs and LCs) of II/f dynein. The change is reasonable because the functional roles of IC/LC phosphorylation could not be directly related to the structure of II/f dynein. In contrast, the biochemical data remained in the revised manuscript and can support the results obtained from structural studies. The revisions will help the readers of *JCB* to understand the important regulatory roles performed by II/dynein f in the ciliary and flagellar motility. **Thus, the paper is now worthy of publication in this journal.** Some remaining minor errors are listed below.

1) The first paragraph, page 16.

"Some of the "missing" structure in the averages is actually present, but its size is underestimated due to increased flexibility of the remaining ICLC when stabilizing connections."

I believe the "its" in the second sentence refers to the missing structure, thus its size is "overestimated" instead of "underestimated"

2) The last sentences of the first paragraph, page 20.

"Thus tethering may serve as part of a mechanical feedback mechanism for dynein coordination. Alternatively, the substantial connection between the 1a motor domain and the A-tubule may stabilize the II complex."

The authors proposed that the tether structure might have regulatory roles for the motor activity of II/f dynein. Similar regulatory functions have been already discussed in LC1 of ODA gamma HC by Patel-King and King (2009). Thus, the authors should refer to the studies again here.

Reviewer #3

Heuser et al. studied the II dynein (dynein f) by structural analysis using cryo-electron tomography and proteomic analysis using 2D SDS PAGE and mass spectroscopy. They presented the 3D structure of ICLC (intermediate- and light-chains of dynein II) of WT, pWT (which they reasonably claim is a mutant that will enable them to analyze intact structure at higher resolution), pf9-3 mutant (lacking entire II dynein), and bop5-1 and bop5-2 (lacking a part of ICLC). **Their graphical presentation is beautifully prepared and impressive and, except some points to improve (below in minor points), acceptable enough.** Based on the comparison of structures from these strains, they proposed (1) "connection 1" between ICLC and the A-tubule (probably the same monomer tubulin as MIP1 binds), (2) two domains proximal to II dynein (tether and tetherhead), and (3) structural difference between ICLCs of WT and bop5 mutants, which they assigned to IC138/IC97/FAP120 subunits. **These three are convincing and are original results. However, other conclusions in this manuscript must be re-examined by further analysis and/or rewriting the logistics in the manuscript as I show below. Therefore I recommend review of the manuscript by referees after intense revision of the manuscript and, if necessary, re-analysis of the data.**

Major concerns:

(1) Significance of connections:

I share the concern of Reviewer 1 about the validity of the connections proposed in this manuscript. **While connections #1,2,6 look like stable connections, connections #3,5 are dim.** In the response letter (p.5, l.10) the authors claimed “the reviewer recognizes the significance of connection #1, which in fact is one of the smaller connections” to justify the validity of other connections. This is a completely inappropriate comment. It is obvious that a blurred density (because of low resolution or flexibility) looks, depending on the threshold level, wider than the tight solid density. Grey broad density of connection #5 (Fig.4F), while connection #1 looks very tight solid density (fig.4D), strongly suggests this is the case here. The same for connections #3, #4 and #7 (Fig.4EGI). The authors must demonstrate that these features appear reproducibly. Is it possible to show connections in WT and other mutants (I believe they have structures of a number of strains) to prove the reproducibility of these connections? For example, bop5 mutants should have #3 (#2 is obviously seen in Fig.5EF).

(2) Doublet specificity:

Their description on doublet specific structures in ICLC (green circles in Fig.3CD) is interesting but needs careful examination. In Fig.S3, features which seem part of this extra density of DMT#3,4 appear also in DMT #6,9 (top part in the green dotted circle in F and right side in the green dotted circle in I). Do the authors interpret them as noise, or part of the ICLC? There are also a number of doublet-specific features observed in Fig.S3 but not described in the manuscript; twin intense dots in DMT#1 (other DMTs have only one) (just next (right) to the green dotted circle), strong density at the top of N-DRC in DMT #2,5,6 (top right close to the B-tubule in Fig.S3BEF), while DMT #4,7,8 have one strong contact and the other contact weak (DGH), and DMT #3,8 have faded density. Are they due to DMT-specific DRC structure (which was not described in Heuser et al. (2009)) or noise? If they are noise, how can the authors prove that the difference in ICLC is real and other differences are not? The shape of dynein I1 ring also looks quite different among nine DMTs (rings at the left of each panels in Fig.S3); dynein I1 in DMT #1 is thick in the part close to the A-tubule, while dynein I1 in DMT #2 is thickest in the distal part. Is this difference reality? If so, it must be described in this manuscript (which is I1 paper). Or do they think this is noise? Or are they the identical dynein I1 3D structure with slightly different orientation (and thus sliced at the different sections)? If the authors claim so, they must prove that the 3D structures of nine I1s are identical. I am afraid that these tiny differences among nine DMTs could come from the non-isotropic data collection; although they collected data from five tomograms (720 repeats) in total, the number of average for each DMT is only ~80 repeats. If corresponding DMTs are located in the similar position of the axonemes by chance, the number of data is not uniform (isotropic) and sparse in one direction, which makes deconvolution difficult and cause non-isotropy problem. Can the authors show it is not the case here?

With the current format of figures, I don't know if we can really trust their statement that ICLC structure is different DMT by DMT, while other structures are the same. They must show controls which have identical structure in all the nine doublets. Currently, since all of the dynein I1 ring, N-DRC, and other structures in Fig.S3 show differences among nine DMTs, they cannot be a control to prove that the difference in ICLC is not noise, but reality. Since small detailed structures and structural differences in each DMT are described in this manuscript,

reproducibility of the analysis must be demonstrated; for example, density maps at the same sections from other strains will demonstrate the reproducibility).

(3) Validity of mutant structures:

Regarding bop5 mutants, in p.12, l.7, it is written “Other I1 structures, such as, as well as other axonemal structures, such as the ODAs appear unchanged compared to WT (Fig.5)”. Clearly it is wrong. If you see Fig.5E, you will realize that half of the ODA alpha head disappeared. IDA2 and the dynein next to IDA2 are smaller in Fig.5E than Fig.5D (one dynein disappears in Fig.5F). Do the authors claim the lack of ICLD affect the stability of ODA alpha? I cannot believe so. How do the authors explain this phenomenon?

To justify the validity of their structures, the authors repeatedly insist their data is “the best and highest resolution data” (p.2, l.21 of the response letter). **This is another inadequate statement.** First, even if the structures presented here are better than works by other groups, it does not guarantee the correct conclusion on detailed structure. Second, although the FSC presented in the table (31A) indicates the high resolution (Table I), this describes the resolution of the whole structure (including microtubule, dyneins and all the other components) in average and does not mean the local resolution at ICLC is also 31A; they could earn resolution at the DMT and lose resolution at ICLC. Indeed they claim “high resolution” mixing the entire datasets (from all the DMTs) and claims at the same time that these subtomograms do not share the identical structure (DMT specificity). Is it not the case “DMT is an apple, while ICLC is a leaf” (p.6, l.24 of their letter)? If they like to justify the description of their ICLC based on resolution, they must box out the subtomograms which cover only ICLC and calculate FSCs.

Comment [DN1]: Note, this comment clearly shows, that the confidential comments in the revised submission that were addressed to the monitoring editor were shared with reviewer #3 !

(4) Schematic diagram (Fig.7A-D):

I cannot find any reason to place IC138 and IC97 at the current locations and not in the opposite way. Since both IC138 and IC97 decrease in bop5-1 and completely disappear in bop5-2 (Table III), extra density in bop5-1 (red arrow in Fig.7G) could be assigned to IC138 or IC97. Both interpretations should be possible: IC138 left and IC97 right as in Fig.7A-D) or IC138 right and IC97 left. If there is any basis (of their own or in the past research) to assign IC138 left, it must be mentioned. Otherwise, this diagram must be modified.

(5) ICLC and N-DRC:

Their discussion on the similarity between ICLC and N-DRC is made without basis. They propose that ICLC “might” contact to the adjacent DMT in the DMTs #3,4 (p.22, l.3), without showing any data (they admit that they have not seen connection in p.10, l.13), although strangely they seem sticking the idea of connection between ICLC and B-tubule. Under this condition, I would say it is more reasonable to think that ICLC and N-DRC have different way of regulation; ICLC regulates ATP-driven motility of I1 heavy chains while N-DRC modulates connections between adjacent DMTs. There is no sense to spare one paragraph (p.21 bottom) for their speculative hypothesis of “parallel” functioning of ICLC and N-DRC. They must either remove this discussion or describe the other possibility (for example, as mentioned above) as well.

Minor points:

Page 3, l.15: “through RSs to IDAs and ODAs”. Reference (there should be a good review) must be cited.

Page 17, l.17: “because it is reduced in I1 mutants”. The reference must be shown.

-----Original Message-----

From: Daniela Nicastro [mailto:nicastro@brandeis.edu]

Sent: Sunday, October 02, 2011 3:09 AM

Subject: RE: Ms. No. 201105007, The Journal of Cell Biology

Dear Dr. [REDACTED]

I have now re-read your rejection letter after thinking about it for a few days - unfortunately I can still not follow why you have rejected our manuscript without giving us the opportunity to address the comments of the new reviewer #3?

Please find attached to this email your decision letter where I comment on the questions raised by the reviewer #3 (reviewer #2 I deleted as he was in favor of publications so no need to discuss his comments). I hope you will see that it would be fairly easy for us to address ALL issues raised by reviewer #3. Reviewer #3's comments are DIFFERENT IN NATURE from what reviewer #1 raised, thus I'm puzzled by your decision to reject the manuscript based on "these concerns were not satisfactorily addressed upon revision" - how could we have anticipated different issues from a new reviewer?

Thank you for taking the time to read my comments.

With best regards, Daniela Nicastro

September 22, 2011

Re: Ms# 201105007R

Dr. Daniela Nicastro
Rosenstiel Center, MS 029
415 South Street
Waltham, MA 02454-9110

Dear Dr. Nicastro,

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Because of this continuing issue of conflicting interpretations and of inconsistent support for publication, and because aspects of this work remain somewhat speculative, we must regretfully decline to consider this work further. This decision reflects the specific comments of the reviewers as well as the priority that the manuscript was assigned in relation to competing submissions to this journal. (*JCB* currently publishes less than 15% of submitted manuscripts.) I am therefore, with regret, returning your manuscript to you with the hope that you will find our reviewers’ comments helpful in revising the manuscript for submission elsewhere.

I am sorry that our answer on this occasion must be negative and hope that it will not dissuade you from submitting other manuscripts to this journal in the future.

Thank you for your interest in *The Journal of Cell Biology*.

Sincerely,

Erika Holzbaur
Monitoring Editor

Reviewer #3

Heuser et al. studied the 11 dynein (dynein f) by structural analysis using cryo-electron tomography and proteomic analysis using 2D SDS PAGE and mass spectroscopy. They presented the 3D structure of ICLC (intermediate- and light-chains of dynein 11) of WT, pWT (which they reasonably claim is a mutant that will enable them to analyze intact structure at higher resolution), pf9-3 mutant (lacking entire 11 dynein), and bop5-1 and bop5-2 (lacking a part of ICLC). Their graphical presentation is beautifully prepared and impressive and, except some points to improve (below in minor points), acceptable enough. Based on the comparison of structures from these strains, they proposed (1) “connection 1” between ICLC and the A-tubule (probably the same monomer tubulin as MIP1 binds), (2) two domains proximal to 11 dynein (tether and tetherhead), and (3) structural difference between ICLCs of WT and bop5 mutants, which they assigned to IC138/IC97/FAP120 subunits. These three are convincing and are original results. However, other conclusions in this manuscript must be re-examined by further analysis and/or rewriting the logistics in the manuscript as I show below. Therefore I recommend review of the manuscript by referees after intense revision of the manuscript and, if necessary, re-analysis of the data.

Major concerns:

(1) Significance of connections:

I share the concern of Reviewer 1 about the validity of the connections proposed in this manuscript. While connections #1,2,6 look like stable connections, connections #3,5 are dim. In the response letter (p.5, l.10) the authors claimed “the reviewer recognizes the significance of connection #1, which in fact is one of the smaller connections” to justify the validity of other connections. This is a completely inappropriate comment. It is obvious that a blurred density (because of low resolution or flexibility) looks, depending on the threshold level, wider than the tight solid density. Grey broad density of connection #5 (Fig.4F), while connection #1 looks very tight solid density (fig.4D), strongly suggests this is the case here. The same for connections #3, #4 and #7 (Fig.4EGI). The authors must demonstrate that these features appear reproducibly. Is it possible to show connections in WT and other mutants (I believe they have structures of a number of strains) to prove the reproducibility of these connections? For example, bop5 mutants should have #3 (#2 is obviously seen in Fig.5EF).

Comment [DN1]: These are two completely different cases! Reviewer #1 fundamentally questioned if one can interpret continuous density as “connections”. Reviewers 3 finds connection 3 & 5 “dim”! The reviewer must have misunderstood connection #3, because it is massive (see magenta in figure 4A). I can only imagine that the reviewer thought we mean the very faint density above – maybe because the arrow in Fig. 4E was not ideally placed (easy to change). I can not comprehend why the reviewer questions #7 (see Fig. 4I !!!) ... that leaves the two dynein tail-linker connections 4 and 5 that the reviewer thought was not shown convincing – I could have worked on a better angle. But nobody before has doubted the dynein-dynein connections between the dynein head domains of ODAs or several IDAs shown by Ishikawa and my lab – at the same position to the tail-linker of the head domain!

Comment [DN2]: Only if one shows very isolated regions of the isosurface – which we do not! Anybody averaging structures knows that if you try to make a “blurred out structure” visible by changing the threshold, all remaining structures will start looking bloated - which again our isosurface-rendering is clearly not (Fig. 4A-C)!

Comment [DN3]: Yes, we could have easily done this – Fig. 5 G shows connection 4 pretty good in WT, Fig. 5H/I both show connection 5.

(2) **Doublet specificity;**

Their description on doublet specific structures in ICLC (green circles in Fig.3CD) is interesting but needs careful examination. In Fig.S3, features which seem part of this extra density of DMT#3,4 appear also in DMT #6,9 (top part in the green dotted circle in F and right side in the green dotted circle in I). Do the authors interpret them as noise, or part of the ICLC? There are also a number of doublet-specific features observed in Fig.S3 but not described in the manuscript; twin intense dots in DMT#1 (other DMTs have only one) (just next (right) to the green dotted circle), strong density at the top of N-DRC in DMT #2,5,6 (top right close to the B-tubule in Fig.S3BEF), while DMT #4,7,8 have one strong contact and the other contact weak (DGH), and DMT #3,8 have faded density. Are they due to DMT-specific DRC structure (which was not described in Heuser et al. (2009)) or noise? If they are noise, how can the authors prove that the difference in ICLC is real and other differences are not? The shape of dynein I1 ring also looks quite different among nine DMTs (rings at the left of each panels in Fig.S3); dynein I1 in DMT #1 is thick in the part close to the A-tubule, while dynein I1 in DMT #2 is thickest in the distal part. Is this difference reality? If so, it must be described in this manuscript (which is I1 paper). Or do they think this is noise? Or are they the identical dynein I1 3D structure with slightly different orientation (and thus sliced at the different sections)? If the authors claim so, they must prove that the 3D structures of nine I1s are identical. I am afraid that these tiny differences among nine DMTs could come from the **non-isotropic data collection**; although they collected data from five tomograms (720 repeats) in total, the number of average for each DMT is only ~80 repeats. If corresponding DMTs are located in the similar position of the axonemes by chance, the number of data is not uniform (isotropic) and sparse in one direction, which makes deconvolution difficult and cause non-isotropicity problem. Can the authors show it is not the case here?

With the current format of figures, I don't know if we can really trust their statement that ICLC structure is different DMT by DMT, while other structures are the same. They must show controls which have identical structure in all the nine doublets. Currently, since all of the dynein I1 ring, N-DRC, and other structures in Fig.S3 show differences among nine DMTs, they cannot be a control to prove that the difference in ICLC is not noise, but reality. Since small detailed structures and structural differences in each DMT are described in this manuscript, reproducibility of the analysis must be demonstrated; for example, density maps at the same sections from other strains will demonstrate the reproducibility).

Comment [DN4]: Again reviewer 1 and 3 differ in WHAT they criticize! Reviewer 1 thought showing averages of all doublets to describe the basic I1 structure was a *flawed approach* after showing that there is a doublet-specific difference. Reviewer 3 does not; instead the reviewer is concerned about *noise and other visible differences* in suppl. Figure S3 (which I address in the next comment). THESE ARE TWO COMPLETELY DIFFERENT ISSUES! I could have addressed all questions of reviewer #3 appropriately. So to say "*and clearly these concerns were not satisfactorily addressed upon revision*" is *unfair* because reviewer #3 raised DIFFERENT issues – how should we have anticipated those???

Comment [DN5]: Correct. There are several TRUE doublet specific differences that are NOT PART of the I1 complex and thus are not discussed. We could have easily pointed some of these true differences out and mention them in the figure legend saying that they are not further discussed because they are not part of I1. Doublet specific averages are pushing beyond what was possible so far and yes these averages are inherently noisier, but we HAVE THOROUGHLY analyzed this – I looked at our two next best strains (sup-pf4 and ida6-GFP-rescue) and this doublet specific difference is reproducibly present!

Comment [DN6]: There is a slight anisotropy in the data, but I could have added cross-sections showing that the missing wedge was nowhere large enough to explain the doublet specific differences. Doublets 3 and 4 are neighbors, which means they would have similar anisotropy – BUT so would be the opposite side of the axoneme around doublet #8, which clearly does not have the extra loop.

(3) Validity of mutant structures:

Regarding bop5 mutants, in p.12, l.7, it is written “Other I1 structures, such as . . . , as well as other axonemal structures, such as the ODAs . . . appear unchanged compared to WT (Fig.5)”. Clearly it is wrong. If you see Fig.5E, you will realize that half of the ODA alpha head disappeared, IDA2 and the dynein next to IDA2 are smaller in Fig.5E than Fig.5D (one dynein disappears in Fig.5F). Do the authors claim the lack of ICLD affect the stability of ODA alpha? I cannot believe so. How do the authors explain this phenomenon?

To justify the validity of their structures, the authors repeatedly insist their data is “the best and highest resolution data” (p.2, l.21 of the response letter). This is another inadequate statement. First, even if the structures presented here are better than works by other groups, it does not guarantee the correct conclusion on detailed structure. Second, although the FSC presented in the table (31A) indicates the high resolution (Table I), this describes the resolution of the whole structure (including microtubule, dyneins and all the other components) in average and does not mean the local resolution at ICLC is also 31A; they could earn resolution at the DMT and lose resolution at ICLC. Indeed they claim “high resolution” mixing the entire datasets (from all the DMTs) and claims at the same time that these subtomograms do not share the identical structure (DMT specificity). Is it not the case “DMT is an apple, while ICLC is a leaf” (p.6, l.24 of their letter)? If they like to justify the description of their ICLC based on resolution, they must box out the subtomograms which cover only ICLC and calculate FSCs.

Comment [DN7]: The ODA alpha are very peripheral and must be fairly flexible – so they are notoriously blurred out when the resolution gets worse. E.g. in our Heuser et al 2009 (N-DRC paper) in Fig. 4H & J in sup-pf3 and pf3 the ODA-alpha appear significantly reduced . . . this is something we could have again easily address, e.g. by making a supplementary figures similar to Figure S2 in our radial spoke paper that you also have on your desk - showing that this is a difference dependent on resolution. – as you can see in that RS suppl. figure the bop's are right down there with sup-pf3!

Comment [DN8]: No, it doesn't. As we state in the M&M “Resolution was measured at the base of RS1 in close proximity to the I1 complex. This density, which is present in all strains [including pf9-3 which is missing eth ICLC], permits a direct comparison between all averages.” We have not included the volume size of the resolution measurement, but we could have easily added it (24x24x24 nm³). Again easy to address!

The reviewer's assumption that we measured the resolution over the whole structure and that would be better than local measurements does not make much sense as such measurement would have included more peripheral structures (like ODA alpha) and those resolution measurements are >4nm!

Comment [DN9]: No!

Comment [DN10]: We did and for pWT and WT the numbers were almost identical to the RS base . . . the only reason why we used the measurements from the RS base was pf9 is missing the ICLC and the bops are missing parts.

(4) Schematic diagram (Fig.7A-D):

I cannot find any reason to place IC138 and IC97 at the current locations and not in the opposite way. Since both IC138 and IC97 decrease in bop5-1 and completely disappear in bop5-2 (Table III), extra density in bop5-1 (red arrow in Fig.7G) could be assigned to IC138 or IC97. Both interpretations should be possible: IC138 left and IC97 right as in Fig.7A-D) or IC138 right and IC97 left. If there is any basis (of their own or in the past research) to assign IC138 left, it must be mentioned. Otherwise, this diagram must be modified.

Comment [DN11]: We explained our rationale in the discussion – IC97 has been shown to bind to tubulin . . . but here the reviewer has a point we could have made it more explicit in the legend to the model that IC97 is also in the mix . . .

(5) ICLC and N-DRC:

Their discussion on the similarity between ICLC and N-DRC is made without basis. They propose that ICLC “might” contact to the adjacent DMT in the DMTs #3,4 (p.22, l.3), without showing any data (they admit that they have not seen connection in p.10, l.13), although strangely they seem sticking the idea of connection between ICLC and B-tubule. Under this condition, I would say it is more reasonable to think that ICLC and N-DRC have different way of regulation; ICLC regulates

Comment [DN12]: Both share very similar sets of connections to neighboring structures (ODA, IDAs) and likely function as regulatory node. We did not mean to over-emphasize the putative linkage to the neighboring doublet – that could be revised in the discussion, but is not a major obstacle!

ATP-driven motility of I1 heavy chains while N-DRC modulates connections between adjacent DMTs. There is no sense to spare one paragraph (p.21 bottom) for their speculative hypothesis of “parallel” functioning of ICLC and N-DRC. They must either remove this discussion or describe the other possibility (for example, as mentioned above) as well.

Minor points:

Comment [DN13]: Easy to correct.

Page 3, l.15: “through RSs to IDAs and ODAs”. Reference (there should be a good review) must be cited.

Page 17, l.17: “because it is reduced in I1 mutants”. The reference must be shown.

October 24, 2011

Re: Manuscript #201105007

Dr. Daniela Nicastro
Brandeis University
Rosenstiel Center, MS 029
415 South Street
Waltham, MA 02454-9110

Dear Dr. Nicastro,

Your manuscript entitled "Cryo-electron tomography reveals novel interactions and doublet specific structures in the I1 dynein" and all of the editorial correspondence related to its consideration at *JCB* has now been seen by a second member of the Editorial Board. I thank you for your patience during this process, which was unusually long in part because reconsideration of our decision required particular care given the long history of this manuscript at our journal.

Our previous decision was based on all of the information available to our Editors, which included the reviewers' comments for the authors and their confidential comments for the Editors. From that information it is clear that many of the original concerns raised by reviewer #1 had not been addressed in the revised manuscript. Some of this was reflected in the comments to the authors from reviewer #3, who raised both shared and new concerns. It was clear from the advice we received that experts in the community continued to be concerned about some of the interpretations of your data. Our decision not to pursue your manuscript further was based on the fact that the revised manuscript did not appear to adequately address several fundamental issues raised during the first round of revision and was not based on any new issues raised during the second round of review.

Unfortunately, the opinion of the second Editor who now has assessed your manuscript is consistent with the opinions of the reviewers and of the original Monitoring Editor. **This second Editorial Board member feels that some of the averaged data have been overinterpreted and that presentation of some of your conclusions without sufficient evidence to support them would only hinder progress in the field.**

It is clear from all of the opinions we have solicited that your work has great potential. However, at this stage, we continue to feel the manuscript is not suitable for publication in *JCB*. Given the uncertainty of the outcome of further revision and *JCB*'s policy of limiting the number of rounds of revision, we remain unwilling to consider the manuscript further.

I am sorry that our response continues to be negative, but I thank you for your interest in *JCB* and hope this will not dissuade you from submitting to our journal in the future.

Sincerely,

A solid black rectangular box redacting the signature of the author.

The Journal of Cell Biology
The Rockefeller University Press

-----Original Message-----

From: Daniela Nicastro [mailto:nicastro@brandeis.edu]

Sent: Monday, October 24, 2011 2:56 PM

Subject: RE: Ms. No. 201105007, The Journal of Cell Biology

Dear [REDACTED]

Thank you for your message. It would have been helpful if this process hadn't taken another 3 weeks.

Usually JCB discloses the names of editors handling the manuscripts and thus I would greatly appreciate if you could please disclose the name of the second Editorial Board member (assuming this wasn't a fourth review, but rather an editorial evaluation of previous reviews). After all one of the strengths of JCB is the fact that scientists act as monitoring editors and manuscripts are matched to qualifications.

Thank you very much and best regards, Daniela

-----Original Message-----

Sent: Tuesday, October 25, 2011 10:04 AM

To: 'Daniela Nicastro'

Subject: RE: Ms. No. 201105007, The Journal of Cell Biology

Dear Daniela,

Appeals are handled differently from initial submissions to JCB. The appeal process is overseen by, including solicitation of a second opinion and consultation with any additional Editorial Board members required. The identity of the individual providing the second opinion is not revealed unless that individual chooses to reverse the original decision and continue consideration of the manuscript at the journal. I can assure you that individuals of the appropriate expertise are always consulted, and further expert advice beyond that available through members of the Editorial Board is sought when appropriate.

Thank you for your continuing interest in JCB. Please do not hesitate to contact me if you have any other questions.

Sincerely,

[REDACTED]

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