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Ca^{2+} elevations disrupt interactions between intraflagellar transport and the flagella membrane in *Chlamydomonas*

Cecile Fort, Peter Collingridge, Colin Brownlee and Glen L. Wheeler
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MS TITLE: Flagella Ca2+ elevations regulate pausing of retrograde intraflagellar transport trains in adherent Chlamydomonas flagella

AUTHORS: Glen L Wheeler, Cecile Fort, Peter Collingridge, and Colin Brownlee
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

While broadly enthusiastic, the reviewers highlight the close overlap with your previously published work (Collingridge, 2013, Current Biology). They go as far as to question the novelty of the new findings while fully appreciating the quality of the data and consistent conclusions. This is a key point that you would need to address in any rebuttal. Furthermore, they suggest that you might consider a reorganization of the results to simplify the narrative. I do consider that this might help you articulate the novelty of the current work. Given the supportive comments of the reviewers I would like to invite you to revise and resubmit but restate the importance of defining the novelty of this new work in the context of your previous publication. The reviewers do raise other significant concerns and we would expect you to attend to all of those in your response.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so

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within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Calcium regulates many facets of flagella related functions such as flagellar beating, intraflagellar transport and flagellar assembly, and flagellar surface motility. Thus, detailed characterization of the dynamic changes of calcium in a flagellum and to learn how this dynamics is related to flagellar functions is important. This work employing a genetically encoded calcium sensor studied spatiotemporal characteristics of calcium in Chlamydomonas flagella, and how calcium affect IFT and flagellar surface motility, which is a further extension of the authors' previous work and is interesting.

Previously, the authors have used dexan-conjugated dye to study flagellar calcium and IFT. Compared to that work, this work revealed more detailed information about calcium and IFT. One of the new findings as presented is that elevated calcium detaches IFT from the membrane, which stops IFT dependent bead motility on the flagellar surface. This provides an explanation why the trailing flagella during cell gliding has elevated calcium. I think this is an important finding.

Comments for the author

Calcium regulates many facets of flagella related functions such as flagellar beating, intraflagellar transport and flagellar assembly, and flagellar surface motility. Thus, detailed characterization of the dynamic changes of calcium in a flagellum and to learn how this dynamics is related to flagellar functions is important. This work employing a genetically encoded calcium sensor studied spatiotemporal characteristics of calcium in Chlamydomonas flagella, and how calcium affect IFT and flagellar surface motility, which is a further extension of the authors' previous work and is interesting.

Previously, the authors have used dexan-conjugated dye to study flagellar calcium and IFT. Compared to that work, this work revealed more detailed information about calcium and IFT. One of the new findings as presented is that elevated calcium detaches IFT from the membrane, which stops IFT dependent bead motility on the flagellar surface. This provides an explanation why the trailing flagella during cell gliding has elevated calcium. I think this is an important finding.

However, I had a problem in understanding this statement: we demonstrate that intraflagellar Ca2+ elevations act primarily to regulate the accumulation of paused retrograde IFT particles, by promoting their dissociation from the flagella membrane. I thought calcium plays two roles: elevation of calcium suppresses the tip accumulation of IFT by triggering its movement and somewhere along the flagellum, it causes detachment of IFT trains with membrane. The accumulation of IFT trains should not be regulated by membrane detachment. If I understand correctly, you also showed that increased frequency of calcium spikes correlates with high frequency of IFT and no IFT accumulation. I could be wrong, please explain or clarify.

The calcium spikes can be initiated from both ends of the flagellum, and calcium elevation can be seen along the whole flagellum. It appears that only the distal end spikes can trigger retrograde IFT of the paused IFT trains at the tip. Does that mean the calcium spike at the proximal flagella can not generate a wave towards the distal end of the flagellum and thus is unable to trigger retrograde IFT at the tip?

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From the title, I could not see how the finding in this ms is different from previous publications of the lab. In previous publication, the authors have demonstrated that elevated calcium regulate IFT by triggering restart of paused IFT trains. I thought the critical finding that the elevated calcium induces detachment of IFT is not reflected. I could have misunderstood the findings.

IFT was first observed in the flagella of Chlamdyomonas. Using DIC video microscopy moving particles were observed, and termed IFT particles. Later cryo-EM studies show that the so-called particles are composed of linear array of protein complexes and renamed IFT trains. I thought that IFT particles and IFT trains mean the same thing. Thus, the sentence in the introduction "The anterograde IFT particles are assembled into trains" may not be accurate.

It is claimed that the tip accumulated IFT trains is from retrograde IFT trains. I do not think the evidence can support it. Intuitively, only the trains that leave from the tip, then can be called retrograde trains. The accumulation may reflect the remodeling process of IFT complexes at the tip. The trains arrived at the tip may not be dissociated from the kinesin-II motor, or not be properly remodeled to activate dynein, which may lead to accumulation. I thought that it is good enough just to say there is an IFT pause at the tip.

For IFT and calcium imaging, I could not see in the methods how fast the videos were taken. Please indicate frames per second.

Figure 2B legend. Bar= 5um should be removed as there is no bar.

Figure 2D, please provide explanation for the blue and red bars.

Reviewer 2

Advance summary and potential significance to field

The authors convincingly identify paused retrograde IFT particles and show, using flagella specific Ca imaging using a genetically encoded Ca reporter, that these pauses are released upon a Ca flux. In addition they show that these Ca fluxes coincide with release of adhesion to a surface.

Comments for the author

In this manuscript, Fort et al. analyze paused IFT particles in Chlamydomonas. Using a genetically encoded Ca sensor targeted to the flagella, they show a correlation between Ca flux in the trailing flagellum and gliding. Interestingly, the Ca fluxes coincide with clearance of paused distal IFT particles, but not with other IFT events. They provide evidence that suggests that the Ca fluxes regulate restart of paused retrograde IFT particles. In addition, they show that the Ca fluxes play a role in attachment of the flagellum to a surface.
The manuscript is in principle well written although the overall rationale of the results section is not so clear. Some parts focus on the distal pausing particles, some on sliding and some on attachment, but the link of all of these to the interaction between the IFT machinery and cargo is not always very clear. This is sometimes confusing. Perhaps the manuscript has been shuffled around, as also some conclusions are too strong at that moment in the text, but later on supported by additional results. The authors should have a careful look at the overall structure of the results section and reorder some parts. Results are mostly clearly presented, although some lettering in the figures is very small (eg "tip" and "base" in Fig 2D). Some results would be more convincing when shown in higher magnifications and when quantified. Furthermore, no statistical analysis has been performed. This should be included in a revised version of the manuscript.

On page 6 and Fig 1A the authors identify two categories of paused IFT particles. They state several characteristics of the categories, which I don't find immediately apparent from the images provided: first category remains highly localized, may disrupt movement of anterograde trains but do not interfere with retrograde trains. The second category appear more diffuse and slowly expand in size and intensity. Could the authors provide more high magnification images of examples of the paused particles and make their statements more quantitative (remains highly localized (how

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long?), may disrupt movement of anterograde trains (how often? at least show examples) but do not interfere with retrograde trains (show in higher magnification). The second category appear more diffuse (quantify) and slowly expand in size and intensity (quantify).

On page 6 and Fig 1C the authors use FRAP to characterize the paused IFT trains. The authors state that individual anterograde IFT trains were largely unimpeded by the accumulation of paused IFT trains. It's not clear to me what they mean: the processivity, the speed, the frequency of trains? Please clarify and quantify and show magnifications of examples. In addition, the authors claim the accumulations are formed by retrograde particles. I agree with this conclusion based on the presence of dynein subunits and the lack of kinesin subunits, but I don't see how the data presented in Fig 1C makes the authors conclude this. It would be useful to quantify fluorescence intensity of anterograde, pausing and retrograde particles.

In figure 3D the authors show that depletion of external Ca inhibited repetitive Ca elevations in the flagella. Does this also inhibit gliding on non-treated glass coverslips?

On page 8 the authors conclude "that the primary role of [Ca2+]fla elevations in adherent flagella is to initiate the movement of paused retrograde IFT trains.". I agree that the Ca fluxes correlate with the paused particles to resume retrograde motility, but the authors have not shown that this is a causative event, nor do they know of any other functions of the Ca fluxes. Many Ca fluxes do not correlate with release of pausing, so why call it a primary function? To show that Ca fluxes cause release of pausing the authors could deplete external Ca and see if this results in continued pausing. Such experiments are shown in Sup Fig 4, which I think should be included in the main text. This figure also includes a quantification of distal pausing. As it is presented now it is just one experiment. The authors would need to repeat these and also display the average effect.

On page 8 and Fig 5, the authors conclude that in periods of high frequency Ca fluxes no distal accumulations were observed, whereas anterograde IFT trains did pause and restart. Please quantify these results.

Page 10. The authors conclude that "The results indicate that light-dependent flagella detachment requires the Ca2+-dependent release of FMG-1B from paused retrograde IFT trains. This allows FMG-1B to move in an unrestricted manner in the flagella membrane, facilitating the lifting movements." In the light of previously published data the authors propose a very likely model, but as they do not include FMG-1B in their experiments here e.g. by showing FMG-1B relocalization this conclusion is too strong and should be tuned down. On page 11, however, the authors provide evidence that the Ca fluxes indeed regulate attachment of microspheres (probably via FMG-1B) to the IFT machinery. Please reorder these sections and rephrase the conclusion.

I'm a but puzzled by the experiments presented in Fig 7, where the authors nicely show that the Ca fluxes coincide with release of the FMG-1B binding microspheres. Previously in this manuscript, the authors showed that these paused retrograde trains are only present at the distal tip. Is that also the case for the experiments presented in Fig 7? To me it seems these microspheres are not at the tip. Please explain.

In the discussion, page 12, the authors state that "The primary role of the [Ca2+]fla elevations is to disrupt the interaction between FMG-1B and paused retrograde IFT trains..". I agree the Ca fluxes play a role, but it might not be their primary role. Please rephrase. The same goes for a similar statement on page 13, first line.

Minor
In figure 2D the position of the flagellum is clear, but in Fig 3A and especially 3B this is not so clear. Could the authors also plot the position/motility of the flagellum, e.g. in the same plot that depicts the F/F0?

Please indicate in Fig. 5A and B (in addition to the legend) which is on poly-lysine and which is not.

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Reviewer 3

Advance summary and potential significance to field

This is a paper that is very likely to be of considerable interest to the readership of the Journal of Cell Science. Following up on their previous paper on calcium transients in trailing flagella in gliding cells of Chlamydomonas, the authors present a wealth of data regarding calcium pulses in the trailing flagella of gliding cells and relate these calcium pulses spatially and temporally to the behavior of anterograde and retrograde IFT particles. Previously, the Yildiz lab (Shih et al., 2013) showed that IFT is responsible for gliding motility (and microsphere movement). In particular, retrograde IFT particles interact with the transmembrane flagellar protein FMG-1B, causing an arrest of the retrograde IFT particle, during which time, the anterior flagellum moves relative to the substrate, dragging along the cell body and the other flagellum. Either flagellum can serve as the leading flagellum responsible for the traction force for whole cell gliding motility. At any one time, many of the cells that are in the gliding configuration (with the flagella oriented 180o to each other) are not gliding and it has long been thought that this is due to the fact that both flagella are using IFT to exert traction forces in opposite directions and hence there are balanced forces on the cell body. How does the cell overcome this problem? The authors of this paper provide a clear answer to this conundrum (as they do in a previous paper). A calcium transient in one of the flagella "disconnects" the retrograde IFT particles from the flagellar membrane protein (FMG-1B) adherent to the substrate and hence eliminates traction forces at the surface of one flagellum allowing the other flagellum (now the leading flagellum) to drag the cell body and the flagellum experiencing the calcium transients. This is a well written paper with generally very convincing and extensive data; it does a good job of referencing and discussing the literature in this field.
I am concerned about overlap with the previous paper on this topic from this lab: Peter Collingridge, Colin Brownlee and Glen L. Wheeler. (2013) Compartmentalized Calcium Signaling in Cilia Regulates Intraflagellar Transport. Current Biology 23, 2311–2318 (Reference #20 in this manuscript). http://dx.doi.org/10.1016/j.cub.2013.09.059 The abstract to this paper reads (in part): "Here we demonstrate that intraflagellar Ca2+ elevations act to directly regulate the movement of IFT particles. IFT-driven movement of adherent flagella membrane glycoproteins in the model alga Chlamydomonas enables flagella-mediated gliding motility. We find that surface contact promotes the localized accumulation of IFT particles in Chlamydomonas flagella. Highly compartmentalized intraflagellar Ca2+ elevations initiate retrograde transport of paused IFT particles to modulate their accumulation. Gliding motility induces mechanosensitive intraflagellar Ca2+elevations in trailing (dragging) flagella only, acting to specifically clear the accumulated microtubule motors from individual flagella and prevent a futile tug-of-war." There is no re-use of any figures but they do some very similar experiments coming to the same conclusions, such as "Ca2+ elevations initiate retrograde transport of paused IFT particles". The data in this paper are much more extensive and of higher quality than in the previous paper.

Comments for the author

1. The initial data in Fig 2C (and the authors' previous paper, Ref 20), showing that calcium transients are only seen in the trailing flagellum during gliding motility is, of necessity, obtained using gliding cells. However, much of the rest of the data on calcium pulses in this paper were obtained using poly-L-lysine "adherent" flagella, which I presume are not being allowed to glide because of the increased adherence of the flagella due to the poly-L-lysine. In this case, are the calcium pulses observed in both flagella on any particular cell, or only one?

2. My biggest concern about the data in this paper is that all of the research data on rapid, repetitive calcium transients are generated using a somewhat artificial situation, by increasing the adhesiveness of the flagella to the glass substrate using poly-L-lysine to the point of inhibiting gliding motility. Indeed, it appears that the rapid calcium pulses are not seen in the absence of the poly-L-lysine, although isolated calcium pulses certainly have been shown to occur in trailing flagella in gliding cells (Ref 20).

3. The Discussion, while detailed and extensive, fails to deliver one of the most important messages of the paper, the relationship of the data sets to whole cell gliding motility. In the Discussion, the authors need to explicitly point out something like this:
a. The leading flagellum powers gliding motility (Bloodgood lab).

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b. Retrograde IFT powers gliding motility by putting force onto FMG-1B that is adherent to the substrate (Yildiz lab)
c. If both flagella are trying to glide at the same time, the traction forces generated by each flagellum will be balanced by the other, leading to the gliding configuration but no net gliding movement (something often seen).
d. How then does one disrupt the balance of forces allowing net movement of the cell?
e. You do this by disrupting the traction forces in only one of the flagella.
f. This is done by means of a rise in intraflagellar calcium that inhibits the mechanical contact between the FMG-1B flagellar membrane protein and the retrograde IFT complexes in only one of the flagella.
g. The calcium rise inhibits gliding motility only in the trailing flagellum so that it does not retard the effect of gliding in the leading flagellum

4. The authors should then go on in the Discussion to highlight some of the big unanswered questions, such as:
a. How does one initiate a rise in intracellular calcium in only one of the two flagella?
b. How does an increase in flagellar adhesion increase the frequency of the calcium transients?
c. How does calcium uncouple retrograde IFT complexes from the cytoplasmic domain of the FMG-1 membrane proteins?

5. On p. 13, the author's state: "Whilst mastigonemes are not thought to be involved in microsphere movements or gliding motility…". While this is certainly true in Chlamydomonas (the organism used for their studies), it should be noted that mastigonemes have been implicated in flagella-dependent gliding motility in Peranema. Saito et al. (2003) Gliding movement in Peranema trichophorum is powered by flagellar surface motility. Cell Motil Cytoskeleton 55(4):244-53 DOI: 10.1002/cm.10127

First revision

Author response to reviewers' comments

Calcium elevations disrupt interactions between intraflagellar transport and the flagella membrane in *Chlamydomonas*

Fort et al

General Response: We thank the Editor and the three reviewers for their helpful comments, which have helped us substantially improve the manuscript.

Two reviewers have raised the issue of how this manuscript differs from a previous manuscript (Collingridge et al 2013). This is an important point and we have revised the manuscript to highlight the novelty of our findings. We have summarised the major novelty of our manuscript below.

1) Requirement to confirm previous findings. It is important to note that there have been conflicting reports in the literature on the role of Ca^{2+} in the regulation of IFT in *Chlamydomonas*. Our previous findings using fluorescent dyes suggested that flagella Ca^{2+} elevations initiated the retrograde movement of accumulated IFT trains (Collingridge et al 2013). In contrast, other researchers have suggested that Ca^{2+} elevations act to promote rather than initiate pausing (Shih et al eLife 2013). As these two conflicting reports were published at a similar time, an important aspect of our manuscript was to resolve this uncertainty and provide a detailed investigation of the interaction between Ca^{2+} and a wider range of different IFT processes (Fig 4). The current manuscript strongly supports our previous findings, even though we used a different approach from our previous study (i.e. by developing genetically encoded fluorescent Ca^{2+} reporters). However, the current manuscript also provides a number of important novel findings as listed below.

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2) Nature of paused IFT particles. Whilst we previously noted that IFT particles accumulate in gliding *Chlamydomonas* flagella (Collingridge 2013), we did not explore the nature of these particles further. In the current study, we examine the localisation and nature of paused IFT particles, showing that they are comprised of retrograde IFT particles that pause primarily in the distal region of the flagellum. We have also used transmission electron microscopy of adherent flagella to show that the inhibition of Ca^{2+} signalling results in an accumulation of IFT particles at the distal end of the flagellum, similar in phenotype to mutants defective in retrograde IFT.

3) Spatial and temporal specificity in the role of Ca^{2+}. Our initial manuscript focused on how Ca^{2+} elevations contribute to gliding motility. In the current manuscript, we primarily utilise immobilised flagella to provide a detailed examination of the spatial and temporal interactions between Ca^{2+} elevations and IFT. We show that high-frequency repetitive Ca^{2+} elevations prevent the pausing and accumulation of retrograde IFT particles (Fig 5&6). We also identify that flagella Ca^{2+} elevations have distinct spatial properties and must co-localise with the paused IFT particles in order to initiate movement (Fig 8).

4) Interaction between Ca^{2+}, IFT and flagella surface motility. An important aspect of the current manuscript is the demonstration that Ca^{2+} acts to disrupt an interaction between retrograde IFT and flagella membrane glycoproteins, observed through the movement of beads on the flagella surface (Fig 7). This is the first demonstration of a direct link between Ca^{2+}, IFT and flagella surface motility.

5) Tool development for flagella signalling. *Chlamydomonas* is an important model organism for flagella function, but the study of signalling in *Chlamydomonas* flagella has been hampered by a lack of available tools to visualise Ca^{2+}. Our previous manuscript used biolistic delivery of fluorescent dyes, which is an effective but specialised and labour-intensive technique. Our current manuscript represents the first use of genetically encoded fluorescent Ca^{2+} indicators in *Chlamydomonas* (in fact the first use in any alga, aside from our recent report in diatoms; Helliwell et al Current Biology 2019). It also represents to our knowledge the first report of a flagella targeted calcium reporter in any non- metazoan. The techniques described within the manuscript therefore represent an important breakthrough in this field that has the potential to support many further studies.

We have now substantially revised the manuscript to emphasise these novel findings. We have included a list of the major unknowns from our previous study in the Introduction (lines 89-96). We have also revised many aspects of the results to clarify what was observed in our previous study and how the current study builds upon this. We have also changed the title to ensure that this message is conveyed more clearly.

Reviewer 1 Comments for the Author:

Calcium regulates many facets of flagella related functions such as flagellar beating, intraflagellar transport and flagellar assembly, and flagellar surface motility. Thus, detailed characterization of the dynamic changes of calcium in a flagellum and to learn how this dynamics is related to flagellar functions is important. This work employing a genetically encoded calcium sensor studied spatiotemporal characteristics of calcium in Chlamydomonas flagella, and how calcium affect IFT and flagellar surface motility, which is a further extension of the authors' previous work and is interesting.

Previously, the authors have used dexan-conjugated dye to study flagellar calcium and IFT. Compared to that work, this work revealed more detailed information about calcium and IFT. One of the new findings as presented is that elevated calcium detaches IFT from the membrane, which stops IFT dependent bead motility on the flagellar surface. This provides an explanation why the trailing flagella during cell gliding has elevated calcium. I think this is an important finding.

Response: We agree that this is an important finding and have now highlighted this in the revised manuscript title to emphasise the novelty in the manuscript.

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However, I had a problem in understanding this statement: we demonstrate that intraflagellar Ca^{2+} elevations act primarily to regulate the accumulation of paused retrograde IFT particles, by promoting their dissociation from the flagella membrane. I thought calcium plays two roles: elevation of calcium suppresses the tip accumulation of IFT by triggering its movement and somewhere along the flagellum, it causes detachment of IFT trains with membrane. The accumulation of IFT trains should not be regulated by membrane detachment. If I understand correctly, you also showed that increased frequency of calcium spikes correlates with high frequency of IFT and no IFT accumulation. I could be wrong, please explain or clarify.

Response: We apologise for a lack of clarity. Retrograde IFT trains may pause at any point along the flagellum, but this occurs primarily in the distal region of the flagellum. As Ca^{2+} elevations cause these paused IFT particles to dissociate from the flagella membrane glycoproteins, we propose that they regulate the accumulation of paused IFT particles. Ca^{2+} therefore does not play two roles; detachment from the membrane is the trigger that causes movement. To avoid confusion, we have revised the 1st section of the Results to clarify that the IFT particles pause in the distal region, rather than at the tip (line 122-125).

The calcium spikes can be initiated from both ends of the flagellum, and calcium elevation can be seen along the whole flagellum. It appears that only the distal end spikes can trigger retrograde IFT of the paused IFT trains at the tip. Does that mean the calcium spike at the proximal flagella can not generate a wave towards the distal end of the flagellum and thus is unable to trigger retrograde IFT at the tip?

Response: Ca^{2+} elevations that initiate at the proximal end of the flagellum can trigger the movement of paused IFT trains in the distal region, but this only occurs when the Ca^{2+} elevation reaches the distal region (Fig 8). We have now clarified this section of the manuscript (lines 317-324).

From the title, I could not see how the finding in this ms is different from previous publications of the lab. In previous publication, the authors have demonstrated that elevated calcium regulate IFT by triggering restart of paused IFT trains. I thought the critical finding that the elevated calcium induces detachment of IFT is not reflected. I could have misunderstood the findings.

Response: This is an important point, raised also by Reviewer 3, and we apologise for not making this clearer in our initial manuscript. We have provided a detailed list of the novel findings of the manuscript in the general response to all reviewers above, and have substantially revised the manuscript throughout to make it clear what was known previously and how the current study builds on these findings. We have also revised the title to reflect the progression from previous findings and to emphasise the interaction with the flagella membrane.

IFT was first observed in the flagella of Chlamdyomonas. Using DIC video microscopy, moving particles were observed, and termed IFT particles. Later cryo-EM studies show that the so-called particles are composed of linear array of protein complexes and renamed IFT trains. I thought that IFT particles and IFT trains mean the same thing. Thus, the sentence in the introduction "The anterograde IFT particles are assembled into trains" may not be accurate.

Response: The terminology used in our manuscript is widespread in the literature (e.g. Vannucinni 2016 JCS; Wingfield et al eLife 2017). 'IFT particle' is usually used to describe the individual biochemically stable protein complex that comprises of the IFT-A and IFT-B sub-complexes. These particles are assembled into trains of different lengths (i.e. comprising of different numbers of IFT particles).

It is claimed that the tip accumulated IFT trains is from retrograde IFT trains. I do not think the evidence can support it. Intuitively, only the trains that leave from the tip, then can be called retrograde trains. The accumulation may reflect the remodeling process of IFT complexes at the tip. The trains arrived at the tip may not be dissociated from the kinesin-II motor, or not be properly remodeled to activate dynein, which may lead to accumulation. I thought that it is good enough just to say there is an IFT pause at the tip.

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Response: We believe that the paused particles in the distal region have left the tip and travelled a short distance in a retrograde direction before pausing. This can be observed most clearly in the TEM studies showing accumulation of IFT particles in the distal region (rather than the tip itself) (Supplementary Fig 5C). As these paused particles only subsequently move in a retrograde direction and do not contain kinesin-II, we propose that they are retrograde IFT particles. For the FRAP analysis, we agree that we cannot state that paused IFT particles in the tip region are retrograde IFT particles, because we cannot resolve whether they have left the tip. However, it must be remembered that there is already a large accumulation of IFT particles in the distal region in these flagella prior to FRAP bleaching. We show that the anterograde IFT particles can move through this accumulation to reach the tip, but particles then accumulate at the tip as they cannot move through the existing accumulation in a retrograde direction. We have revised this section to clarify this point (lines 134- 150).

For IFT and calcium imaging, I could not see in the methods how fast the videos were taken. Please indicate frames per second.

Response: All videos of G-GECO and IFT54-mScarlet were recorded at 100 ms per frame. Videos of Oregon Green BAPTA and IFT20-mCherry were recorded at 300 ms per frame. These details have now been added to the Methods (line 567-569).

Figure 2B legend. Bar= 5um should be removed as there is no bar.

Response: removed

Figure 2D, please provide explanation for the blue and red bars.

Response: Blue bars were meant to indicate trailing movements and red bars to indicate forward (leading) movements, but we have now removed them from the figure and replaced them with a trace showing the position of the flagella tip, as suggested by reviewer 2.

Reviewer 2 *Advance Summary and Potential Significance to Field:*

The authors convincingly identify paused retrograde IFT particles and show, using flagella specific Ca imaging using a genetically encoded Ca reporter, that these pauses are released upon a Ca flux. In addition, they show that these Ca fluxes coincide with release of adhesion to a surface.

Reviewer 2 *Comments for the Author:*

In this manuscript, Fort et al. analyze paused IFT particles in Chlamydomonas. Using a genetically encoded Ca sensor targeted to the flagella, they show a correlation between Ca flux in the trailing flagellum and gliding. Interestingly, the Ca fluxes coincide with clearance of paused distal IFT particles, but not with other IFT events. They provide evidence that suggests that the Ca fluxes regulate restart of paused retrograde IFT particles. In addition, they show that the Ca fluxes play a role in attachment of the flagellum to a surface. The manuscript is in principle well written although the overall rationale of the results section is not so clear. Some parts focus on the distal pausing particles, some on sliding and some on attachment, but the link of all of these to the interaction between the IFT machinery and cargo is not always very clear. This is sometimes confusing. Perhaps the manuscript has been shuffled around, as also some conclusions are too strong at that moment in the text, but later on supported by additional results. The authors should have a careful look at the overall structure of the results section and reorder some parts.

Results are mostly clearly presented, although some lettering in the figures is very small (eg "tip" and "base" in Fig 2D). Some results would be more convincing when shown in higher magnifications and when quantified. Furthermore, no statistical analysis has been performed. This should be included in a revised version of the manuscript.

Response: We have added statistical analyses where appropriate (e.g. Fig 3,4, 5) and revised fonts

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in all figures. The order of the Results has been amended, moving the description of bead movements earlier in the text (now Fig 7) as suggested. Revisions to the clarity of individual figures are described below.

On page 6 and Fig 1A the authors identify two categories of paused IFT particles. They state several characteristics of the categories, which I don't find immediately apparent from the images provided: first category remains highly localized, may disrupt movement of anterograde trains but do not interfere with retrograde trains. The second category appear more diffuse and slowly expand in size and intensity. Could the authors provide more high magnification images of examples of the paused particles and make their statements more quantitative (remains highly localized (how long?), may disrupt movement of anterograde trains (how often? at least show examples) but do not interfere with retrograde trains (show in higher magnification). The second category appear more diffuse (quantify) and slowly expand in size and intensity (quantify).

Response: We have now revised Fig 1 to show much clearer examples of pausing anterograde and retrograde trains and included a schematic illustration of each. We have also provided a quantitative assessment of the duration and localisation of the paused particles (Supplementary Fig 1A-B). The statement in the text '*may disrupt movement of anterograde trains but do not interfere with retrograde trains'* mentioned by the reviewer refers to a publication by Stepanek and Pigino (Science, 2016). We have now clarified this in the text (line 120-122).

On page 6 and Fig 1C the authors use FRAP to characterize the paused IFT trains. The authors state that individual anterograde IFT trains were largely unimpeded by the accumulation of paused IFT trains. It's not clear to me what they mean: the processivity, the speed, the frequency of trains? Please clarify and quantify, and show magnifications of examples. In addition, the authors claim the accumulations are formed by retrograde particles. I agree with this conclusion based on the presence of dynein subunits and the lack of kinesin subunits, but I don't see how the data presented in Fig 1C makes the authors conclude this. It would be useful to quantify fluorescence intensity of anterograde, pausing and retrograde particles.

Response: The FRAP data primarily indicates how additional IFT trains contribute to the accumulation of paused IFT trains. Anterograde IFT trains travel through the region of bleached accumulated IFT trains all the way to the flagella tip before additional paused IFT trains can be observed. Therefore anterograde IFT trains do not contribute to the accumulation, but we agree that we cannot definitively say that the paused IFT trains are retrograde trains from this evidence. We have now added additional data to clarify the FRAP analysis, including a quantitative assessment of the accumulation of IFT particles, and substantially revised the text to modify our interpretation of the findings (line 134-150).

In figure 3D the authors show that depletion of external Ca inhibited repetitive Ca elevations in the flagella. Does this also inhibit gliding on non-treated glass coverslips?

Response: The inhibition of gliding by removing external Ca^{2+} was previously demonstrated by Bloodgood and Salomonsky 1990 JCS. We have also previously shown that inhibition of Ca^{2+} signalling inhibits gliding (Collingridge et al 2013). We have clarified this in the text (line 187).

On page 8 the authors conclude "that the primary role of [Ca^{2+}]fla elevations in adherent flagella is to initiate the movement of paused retrograde IFT trains.". I agree that the Ca fluxes correlate with the paused particles to resume retrograde motility, but the authors have not shown that this is a causative event, nor do they know of any other functions of the Ca fluxes. Many Ca fluxes do not correlate with release of pausing, so why call it a primary function? To show that Ca fluxes cause release of pausing the authors could deplete external Ca and see if this results in continued pausing. Such experiments are shown in Sup Fig 4, which I think should be included in the main text. This figure also includes a quantification of distal pausing. As it is presented now it is just one experiment. The authors would need to repeat these and also display the average effect.

Response: We described it as a primary function as our analysis indicates that Ca^{2+} elevations are not correlated to other IFT processes. However, we agree that Ca^{2+} elevations may have other functions and have amended the text accordingly. We have also moved the Ca^{2+} depletion

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experiments to the main text (Fig 6) and added additional examples with quantitation of the average effect (Supplementary Fig 4), as suggested by the reviewer.

On page 8 and Fig 5, the authors conclude that in periods of high frequency Ca fluxes no distal accumulations were observed, whereas anterograde IFT trains did pause and restart. Please quantify these results.

Response: We have now added a figure indicating that distal accumulations of retrograde IFT trains do not occur in periods of high frequency Ca^{2+} elevations, whilst anterograde IFT trains can continue to pause (Fig 5D). In addition, the Ca^{2+} depletion experiments (Fig 6, Supplementary Fig 4) also show that IFT does not accumulate during high frequency Ca^{2+} elevations, but rapidly accumulates following the inhibition of Ca^{2+} signalling.

Page 10. The authors conclude that "The results indicate that light-dependent flagella detachment requires the Ca2+ -dependent release of FMG-1B from paused retrograde IFT trains. This allows FMG-1B to move in an unrestricted manner in the flagella membrane, facilitating the lifting movements." In the light of previously published data the authors propose a very likely model, but as they do not include FMG-1B in their experiments here e.g. by showing FMG-1B relocalization this conclusion is too strong and should be tuned down. On page 11, however, the authors provide evidence that the Ca fluxes indeed regulate attachment of microspheres (probably via FMG-1B) to the IFT machinery. Please reorder these sections and rephrase the conclusion.

Response: We thank the reviewer for these suggestions. We have now reordered these sections to highlight the role of Ca^{2+} in disrupting the interaction between IFT and microspheres prior to describing the results relating to flagella adhesion.

I'm a but puzzled by the experiments presented in Fig 7, where the authors nicely show that the Ca fluxes coincide with release of the FMG-1B binding microspheres. Previously in this manuscript, the authors showed that these paused retrograde trains are only present at the distal tip. Is that also the case for the experiments presented in Fig 7? To me it seems these microspheres are not at the tip. Please explain.

Response: The paused IFT trains are found primarily in the distal region 0-2 µm from the tip, rather than at the tip itself (see Supplementary Fig 5C). We believe that retrograde IFT trains can pause at any point along the flagellum (examples in Fig 1), but they accumulate primarily in the distal region because this is where they first encounter FMG-1B following their return from the flagella tip. The bead experiments show that Ca^{2+} can act on the interaction between IFT and FMG-1B anywhere along the flagellum. We have now provided clearer examples of the IFT accumulations in Fig 1 to clarify this point and have revised the text to note that microspheres are acted on in any region of the flagellum (line 300).

In the discussion, page 12, the authors state that "The primary role of the [Ca^{2+}]fla elevations is to disrupt the interaction between FMG-1B and paused retrograde IFT trains..". I agree the Ca fluxes play a role, but it might not be their primary role. Please rephrase. The same goes for a similar statement on page 13, first line.

Response: We agree that Ca^{2+} may play further roles in flagella biology. We have rephrased accordingly throughout to emphasise that we were referring to the role in regulating IFT.

Minor

In figure 2D the position of the flagellum is clear, but in Fig 3A and especially 3B this is not so clear. Could the authors also plot the position/motility of the flagellum, e.g. in the same plot that depicts the F/F0?

Response: We have now added a trace to these figures to indicate the position of the flagella tip to highlight flagella movements. In doing so, we found that an error had occurred in the assembly of Fig 3B that led to the inversion of the kymograph (this why the flagella tip was so unclear in this image). We apologise for this error and for any confusion it may have caused. This error occurred

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solely at the stage of figure assembly and did not affect our quantitative assessment of Ca^{2+} signalling.

Please indicate in Fig. 5A and B (in addition to the legend) which is on poly-lysine and which is not.

Response: We have now attached the appropriate labels.

Reviewer 3 Advance Summary and Potential Significance to Field:
This is a paper that is very likely to be of considerable interest to the readership of the Journal of Cell Science. Following up on their previous paper on calcium transients in trailing flagella in gliding cells of Chlamydomonas, the authors present a wealth of data regarding calcium pulses in the trailing flagella of gliding cells and relate these calcium pulses spatially and temporally to the behavior of anterograde and retrograde IFT particles. Previously, the Yildiz lab (Shih et al., 2013) showed that IFT is responsible for gliding motility (and microsphere movement). In particular, retrograde IFT particles interact with the transmembrane flagellar protein FMG-1B, causing an arrest of the retrograde IFT particle, during which time, the anterior flagellum moves relative to the substrate, dragging along the cell body and the other flagellum. Either flagellum can serve as the leading flagellum responsible for the traction force for whole cell gliding motility. At any one time, many of the cells that are in the gliding configuration (with the flagella oriented 180o to each other) are not gliding and it has long been thought that this is due to the fact that both flagella are using IFT to exert traction forces in opposite directions and hence there are balanced forces on the cell body. How does the cell overcome this problem? The authors of this paper provide a clear answer to this conundrum (as they do in a previous paper). A calcium transient in one of the flagella "disconnects" the retrograde IFT particles from the flagellar membrane protein (FMG-1B) adherent to the substrate and hence eliminates traction forces at the surface of one flagellum allowing the other flagellum (now the leading flagellum) to drag the cell body and the flagellum experiencing the calcium transients. This is a well written paper with generally very convincing and extensive data; it does a good job of referencing and discussing the literature in this field.

I am concerned about overlap with the previous paper on this topic from this lab: Peter Collingridge, Colin Brownlee and Glen L. Wheeler. (2013) Compartmentalized Calcium Signaling in Cilia Regulates Intraflagellar Transport. Current Biology 23, 2311-2318 (Reference #20 in this manuscript).

http://dx.doi.org/10.1016/j.cub.2013.09.059

The abstract to this paper reads (in part): "Here we demonstrate that intra intraflagellar Ca2+ elevations act to directly regulate the movement of IFT particles. IFT-driven movement of adherent flagella membrane glycoproteins in the model alga Chlamydomonas enables flagella-mediated gliding motility. We find that surface contact promotes the localized accumulation of IFT particles in Chlamydomonas flagella. Highly compartmentalized intraflagellarintraflagellar Ca2+ elevations initiate retrograde transport of paused IFT particles to modulate their accumulation. Gliding motility induces mechanosensitive intraflagellar Ca2+ elevations in trailing (dragging) flagella only, acting to specifically conclusions, such as "Ca2+ elevations initiate retrograde transport of paused IFT particles". The data in this paper are much more extensive and of higher quality than in the previous paper.

Response: We have provided a detailed response to these comments in the general response to all authors. The Collingridge et al (2013) manuscript did not determine 1) the role of Ca^{2+} in other IFT events, 2) the nature of the accumulated IFT particles, 3) the spatial and temporal organisation of Ca^{2+} elevations in relation to IFT events and 4) the relationship between Ca^{2+} and flagella surface motility. We have now revised the manuscript, clearly referring to our previous results where appropriate, to emphasise these points and highlight the novelty of the current study.

Reviewer 3 Comments for the Author: *1.The initial data in Fig 2C (and the authors' previous paper, Ref 20), showing that calcium transients are only seen in the trailing flagellum during gliding motility is, of necessity, obtained using gliding cells. However, much of the rest of the data on calcium pulses in this paper were obtained using poly- L-lysine "adherent" flagella, which I presume are not being allowed to glide because of the increased adherence of the flagella due to the poly-L-lysine. In this case, are the calcium pulses observed in both flagella on*

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any particular cell, or only one?

Response: The use of poly-lysine to increase adhesion is discussed in more detail in the response to point 2 (below). It is important to note that the behaviour of the cells is relatively similar on the two surfaces, but the degree of adhesion is different. High frequency Ca^{2+} elevations are observed on untreated surfaces, but only in a small proportion of cells (Fig 3C). On an untreated surface, the majority of cells lift their flagella and swim away within a short period of time, so TIRF imaging is consequently focused on the less active cells that remain. On a poly-lysine treated surface, cells still glide or lift their flagella to some extent, but as the resistive forces are greater, some cells are immobilised and we are to image them for a longer period. We have now amended the text to clarify this point (lines 171-180).
Cells on a poly-lysine surface show Ca^{2+} elevations in both flagella, although they are not coordinated between flagella. We showed an example of a cell exhibiting Ca^{2+} elevations in both flagella in our previous publication (Collingridge 2013).

2.My biggest concern about the data in this paper is that all of the research data on rapid, repetitive calcium transients are generated using a somewhat artificial situation, by increasing the adhesiveness of the flagella to the glass substrate using poly-L-lysine to the point of inhibiting gliding motility. Indeed, it appears that the rapid calcium pulses are not seen in the absence of the poly-L-lysine, although isolated calcium pulses certainly have been shown to occur in trailing flagella in gliding cells (Ref 20).

Response: This is definitely an important consideration, although it should be noted that many published studies of IFT in *Chlamydomonas* have used a poly-lysine treated surface to promote gliding. Concentrations ranging from 0.01-1% poly-lysine have been used routinely to treat surfaces (e.g. Zhu et al 2017 PLoS Genetics 13(2): e1006627), although many studies do not state the concentration used. Low concentrations of poly-lysine (0.01%) only marginally slow gliding motility, making it difficult to image flagella over any length of time. Larger concentrations (1%) can completely immobilise cells but can lead to deflagellation if used over prolonged periods (discussed in Dentler et al Methods Cell Biol. 2009; 93: 145–155 and Engel et al Methods Cell Biol, 2009, 93:157-177). We previously used 0.01% poly-lysine treated coverslips in our study of gliding motility (Collingridge 2013), but chose 0.1% poly-lysine for the present study to enable us to reduce motility sufficiently to image flagella over longer periods, without inducing toxicity. Note that not all cells are completely immobilised at either of these concentrations, but cells that move extensively or detach from the surface cannot be easily included in quantitative studies of IFT. One of the greatest challenges when imaging *Chlamydomonas* flagella is that they rarely sit still for long. Our results show that the methods commonly used to immobilise flagella by researchers studying IFT have an important impact on this process itself. We have included more detail on the use of poly-lysine in the text (lines 171-175).

We should also point out that the degree of adhesion used isn't necessarily an artificial situation. In a natural environment, *Chlamydomonas* is likely to encounter a wide range of surfaces, many of which are likely to have a greater adhesiveness than a clean glass slide e.g. bacterial biofilms or organic substrates. Moreover, our previous publication indicated that gliding flagella can exhibit repetitive Ca^{2+} elevations during movement, so the repetitive Ca^{2+} elevations do not relate specifically to being immobilised, but are more likely to reflect an increase in membrane tension encountered during resistance to movement.

3.The Discussion, while detailed and extensive, fails to deliver one of the most important messages of the paper, the relationship of the data sets to whole cell gliding motility. In the Discussion, the authors need to explicitly point out something like this:

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a.The leading flagellum powers gliding motility (Bloodgood lab).
b.Retrograde IFT powers gliding motility by putting force onto FMG-1B that is adherent to the substrate (Yildiz lab)
c.If both flagella are trying to glide at the same time, the traction forces generated by each flagellum will be balanced by the other, leading to the gliding configuration but no net gliding movement (something often seen).
d.How then does one disrupt the balance of forces allowing net movement of the cell?
e.You do this by disrupting the traction forces in only one of the flagella.
f.This is done by means of a rise in intraflagellar calcium that inhibits the mechanical contact between the FMG-1B flagellar membrane protein and the retrograde IFT complexes in only one of the flagella.
g.The calcium rise inhibits gliding motility only in the trailing flagellum so that it does not retard the effect of gliding in the leading flagellum

Response: We agree that the Discussion requires a clear summary of how these processes contribute to gliding motility and have added a paragraph according to the suggestions made the reviewer (lines 372-385).

4.The authors should then go on in the Discussion to highlight some of the big unanswered questions, such as:
a.How does one initiate a rise in intracellular calcium in only one of the two flagella?
b.How does an increase in flagellar adhesion increase the frequency of the calcium transients?
c.How does calcium uncouple retrograde IFT complexes from the cytoplasmic domain of the FMG-1 membrane proteins?

Response: We have also added a paragraph highlighting the major unanswered questions in the Discussion (lines 405-411).

5.On p. 13, the author's state: "Whilst mastigonemes are not thought to be involved in microsphere movements or gliding motility…". While this is certainly true in Chlamydomonas (the organism used for their studies), it should be noted that mastigonemes have been implicated in flagella-dependent gliding motility in Peranema. Saito et al. (2003) Gliding movement in Peranema trichophorum is powered by flagellar surface motility. Cell Motil Cytoskeleton 55(4):244-53 DOI: 10.1002/cm.10127

Response: We have modified the text to include the citation noted by the reviewer (line 472).

Second decision letter

MS ID#: JOCES/2020/253492

MS TITLE: Calcium elevations disrupt interactions between intraflagellar transport and the flagella membrane in Chlamydomonas

AUTHORS: Cecile Fort, Peter Collingridge, Colin Brownlee, and Glen L Wheeler
ARTICLE TYPE: Research Article

Thank you for your careful revisions to this work. I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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Reviewer 1

Advance summary and potential significance to field

I have provided a summary in the last review.

Comments for the author

I have seen the response of the authors. I am satisfied with the revision.

Reviewer 2

Advance summary and potential significance to field

I'm happy with the revisions of the manuscript and support publication in JCS.

Comments for the author

I'm happy with the revisions of the manuscript and support publication in JCS.

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