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Super-resolution microscopy reveals arrangement of inner-membrane protein complexes in mammalian mitochondria.

Catherine Palmer, Jieqiong Lou, Bety Kouskousis, Elvis Pandzic, Alexander Anderson, Yilin Kang, Elizabeth Hinde and Diana Stojanovski
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Original submission

First decision letter

MS ID#: JOCES/2020/252197

MS TITLE: Super-resolution microscopy reveals arrangement of inner-membrane protein complexes in mammalian mitochondria.

AUTHORS: Catherine Palmer, jieqiong Lou, Bety Kouskousis, Elvis Pandzic, Alexander Anderson, Yilin Kang, Elizabeth Hinde, and Diana Stojanovski
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.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. 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 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

In this manuscript, Palmer and colleagues demonstrate, that single molecule localization microscopy (SMLM) combined with density-based spatial clustering of applications with noise (NBSCAN) is a powerful tool to study mitochondrial inner membrane proteins. At this point, this area of research still relies mainly on biochemical approaches and this study demonstrates how visual approaches could advance the field. However, the biological findings presented in this manuscript are less comprehensive and there are a number of issues that reduce the impact or are of technical concern.

Comments for the author

1. The STORM images of TOM20 shown in Figure 1C do not show the expected outer-membrane staining, that is visible on the confocal image and that has also been reproduced with STORM or PALM in various other publication (e.g. Douglass et al.Nat Photonics, 2016;Rosenbloom et al., PNAS, 2014). It would be helpful to show a corresponding wide-field (or confocal) image of the STORM examples and to verify that the ideal settings for localization precision were chosen.

2. To get more insight into the relative position of inner membrane proteins to each other, could the authors perform dual color STORM images and use either TOM20 or COXIV as reference protein?

3. In Figure 2 B-D, the average value and sd are not always visible, since both lines and points are black.

4. In Figure 3 A, STORM images for TIM23, Tim44 and TOM20 should be shown for (wildtype and knockouts) and added to the quantification to verify if only Complex IV is affected.

5. In Figure 3B, error bars should be shown (biological replicates?).

6. Several studies have shown previously that mitochondrial inner membrane proteins can be resolved and quantified by STED microscopy (e.g. Stoldt et al., PNAS, 2019). The authors should at least discuss why they chose this new approach (resolution, localization precision, photobleaching etc).

Reviewer 2

Advance summary and potential significance to field

Palmer et al. use the superresolution fluorescence microscopy technique STORM to examine the distribution/clustering of 4 mitochondrial membrane proteins (Tom20, COXIV, Tim23, Tim44). The authors are aware that Tom20 and COXIV have previously been imaged and analyzed for cluster density via STED and STORM (Jans et al 2013, Wurm et al 2011; van de Linde et al 2008). They find a punctate distribution of Tim and complex IV, which mirrors recent observations in the literature about the spatial distribution of the cristae-organizing MICOS complex. Overall, the new information presented by this study is limited and the authors have not provided sufficient evidence that their method is capable of reliably reporting the subtle effects of Tim8 KO shown, and this is the only experimental condition they test.

Comments for the author

This story would be improved if they could confirm that proteins in complexes co-cluster as predicted, that antibodies are accurate probes, and identify a factor that convincingly alters this arrangement. Specific comments are outlined.

Major:
1. The rationale for using STORM should be better explained. The issue is whether labeled complexes of interest are sufficiently spaced apart to be resolvable by STORM; it does not matter what size the complex is.
The authors state that the localization precision of N-STORM is ~20-30 nm, which is why you can resolve TIM/TOM complexes. By the author's rationale, they would be unable to resolve 2 complexes right next to one another because TIM and TOM complex are less than 10x20nm.

2. Related, does STORM have the resolution to resolve diffuse from defined complexes?

3. How many times have these experiments been repeated? Is there variability between staining? Data should come from 3 biological replicates (i.e., independently grown, stained, and imaged samples). Conclusions rely entirely on the consistency of their labeling and imaging strategies. Wurm et al 2011 states that Tom20 clustering is highly variable between cell type and cell density.

4. How do we know that the foci in reconstructed STORM images are bona fide protein complexes (this terminology is used throughout the paper), and how do we know that all complexes are detected using the antibody? There are two separate issues: (1) are the antibodies labeling the expected structures; and (2) is the labeling sufficiently dense to exclude the possibility that puncta shown are actually due to sparse labeling and not the underlying protein architecture? Test by methods such as (1) colocalization of two subunits with two-color imaging; or (2) test additional subunits of the complexes shown. The expectation is that statistically similar numbers of clusters should be detected per μm^2 among subunits in the same complex (related to the analysis in Figure 2B); or (3) titrate primary antibody to show that labeling is saturated at the dilutions used (which is not disclosed).

5. How can the authors compare the number of molecules per area between proteins if it is not shown that the antibody used is at saturating concentrations? The authors should do an antibody titer for all POIs and test whether there is a maximal # of molecules per area reached. Another way to compare molecules of protein between several different proteins would be to endogenously tag them. For example, there are more Tim44 clusters per μm^2 (Figure 2B) than Tim23 clusters per μm^2. But it is expected that all Tim44 proteins would be in complex with Tim23 and should have the same or fewer clusters. This makes me question whether any comparisons can be made between different proteins and antibodies (Fig 1, Fig 2, Fig S1).

6. Data in Fig 4 show a statistically significant but very weak effect. It does not appear that Tim8a/b are major determinants of Tim complex arrangement. This result seems over-sold.

7. Related, If there is no difference in number of clusters and avg number of molecules per cluster in Tim8a KO cells, how can the authors explain that there is an increase in avg cluster area if COXIV should be reduced in these cells?

Minor:
1. In the abstract, the STORM acronym is incorrectly defined. It should be "Stochastic Optical Reconstruction Microscopy."
2. For consistency and by community standards it is better to use "MICOS" throughout instead of mixing MINOS and MICOS.
3. The authors should mention how many molecules are in 1 cluster in the main text. Instead of less clusters should be fewer clusters.

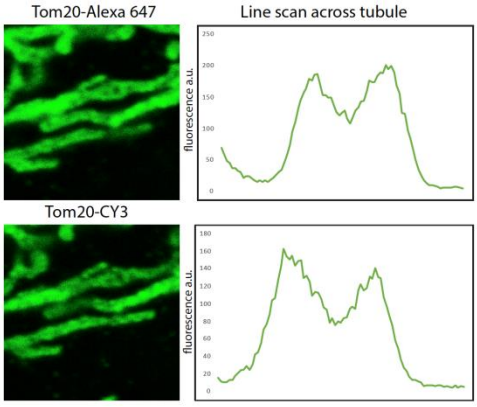
First revision

Author response to reviewers' comments

Reviewer 1:

1. The STORM images of TOM20 shown in Figure 1C do not show the expected outer-membrane staining, that is visible on the confocal image and that has also been reproduced with STORM or PALM in various other publication (e.g. Douglass et al. Nat Photonics, 2016;Rosenbloom et al., PNAS, 2014). It would be helpful to show a corresponding wide-field (or confocal) image of the STORM examples and to verify that the ideal settings for localization precision were chosen.

Here a sample labelled for N-STORM imaging was subsequently taken to a confocal microscope, and images of the Tom20 labelled protein signal taken for each reporter molecule, Alexa Fluor 647 and CY3 (false coloured green here for ease of view). A fluorescence profile was done to show signal distribution. It can be clearly seen that the primary/secondary antibody pair can be localised to the mitochondrial outer membrane for each reporter molecule. Confocal examples of the same primary antibodies are shown in Figure 1, while the NSTORM secondary antibody is shown below.



As mentioned by reviewer 1, Rosenbloom et al (2014) used PALM for imaging of the outer membrane and inner membrane (figure below), however these were exogenously expressed constructs, which is in contrast to the endogenous protein labelled in our study. Despite this, the data obtained in our study is reminiscent of the labelling patterns observed by Rosenbloom et al (2014) (below).

[Figure provided for reviewer has been removed. It showed Fig. 4 from **Rosenbloom et al.** (2014) Super resolution imaging of Drp1 by Dronpa variant. *Proc Natl Acad Sci U S A* **111** (36) 13093-13098. (doi: 10.1073/pnas.1320044111)]

We suggest the N-STORM data presented here is in agreement with conclusions drawn from STED imaging of Tom20 presented by Wurm et al (2011) PNAS (see below), displaying a diffuse and punctate arrangement in the membrane.

[Figure provided for reviewer has been removed. It showed Fig. 1b from **Wurm et al.** (2011) Nanoscale distribution of mitochondrial import receptor Tom20 is adjusted to cellular conditions and exhibits an inner-cellular gradient. *Proc Natl Acad Sci U S A* **108** (33) 13546-51. (doi: 10.1073/pnas.1107553108)]

2. To get more insight into the relative position of inner membrane proteins to each other, could the authors perform dual color STORM images and use either TOM20 or COXIV as reference protein?

Preliminary dual colour imaging has been completed in HeLa cells of the combinations available and presented in Figure S2. This is further discussed in text to elaborate on the complexes that contain Tim44 and/or Tim23. Due to COVID19 restrictions and subsequent lockdown events we had limited access to the specialised N-STORM equipment, as such a limited sample of dual colour images were obtained, limiting our ability to quantify these findings. To address this query, we have provided representative images as supplemental data.

3. In Figure 2 B-D, the average value and sd are not always visible, since both lines and points are black.
This has been corrected in all figures to enhance the visibility of error bars and mean.

4. In Figure 3 A, STORM images for TIM23, Tim44 and TOM20 should be shown for (wildtype and knockouts) and added to the quantification to verify if only Complex IV is affected.
Our study (Kang et al., 2019 eLife) undertook extensive proteomic and biochemical analysis on the HEK cells utilised in Figure 3 to show isolated defects in Complex IV. For this reason, we only included Complex IV as part of this analysis to capture the biochemical alteration using STORM and to showcase use of the technique in characterisation of biochemical changes.

Two colour imaging has been done in HeLa cells Fig. S2. In spite of testing a number of additional Complex IV antibodies these were not suitable for N-STORM (not specific enough).

5. In Figure 3B, error bars should be shown (biological replicates?).
Figure 3B has been adjusted to present the data in an objective manner. Error bars have been provided, and information on the replicates and number of independent samples has been included in the figure legends. All experiments have been conducted with 3 independent experiments.

6. Several studies have shown previously that mitochondrial inner membrane proteins can be resolved and quantified by STED microscopy (e.g. Stoldt et al., PNAS, 2019). The authors should at least discuss why they chose this new approach (resolution, localization precision, photobleaching etc).
Addressed in text, further justification has been discussed p6.

Reviewer 2:

Major:
1. The rationale for using STORM should be better explained. The issue is whether labeled complexes of interest are sufficiently spaced apart to be resolvable by STORM; it does not matter what size the complex is. The authors state that the localization precision of N-STORM is ~20-30 nm, which is why you can resolve TIM/TOM complexes. By the author's rationale, they would be unable to resolve 2 complexes right next to one another because TIM and TOM complex are less than 10x20nm.
We have added further clarification in text to address this concern. In particular, we now present the size of the complexes to demonstrate the need for super resolution imaging rather than conventional imaging and we point out that STORM is advantageous over other super-resolved methods such as SIM or STED since it offers the highest spatial resolution beyond the diffraction limit via use of a relatively simple optical steup. Molecules resolved by STORM have a localisation precision on average of 20-30 nm, compared to STED that has a spatial resolution of ~60-80 nm laterally.

2. Related, does STORM have the resolution to resolve diffuse from defined complexes?
Within the text, the dynamic nature of the membrane complexes being imaged has been further described. As these are large multimeric protein complexes, they are found in a variety of arrangements including subcomplexes as well as the mature complex. Clarification has been added to the text and the analysis in Fig. 3 has been redone to ensure a clear discussion of the data. The analysis no longer discusses defined vs diffuse but the number of intensity points. This includes data from 3 independent experiments. In addition, when we apply DBSCAN to STORM data we can obtain actual cluster size. As such, if there was a diffuse point pattern due to a random distribution of proteins, then there would be a change in the cluster parameters such as size, density or number (depending on the parameters used to define a cluster).

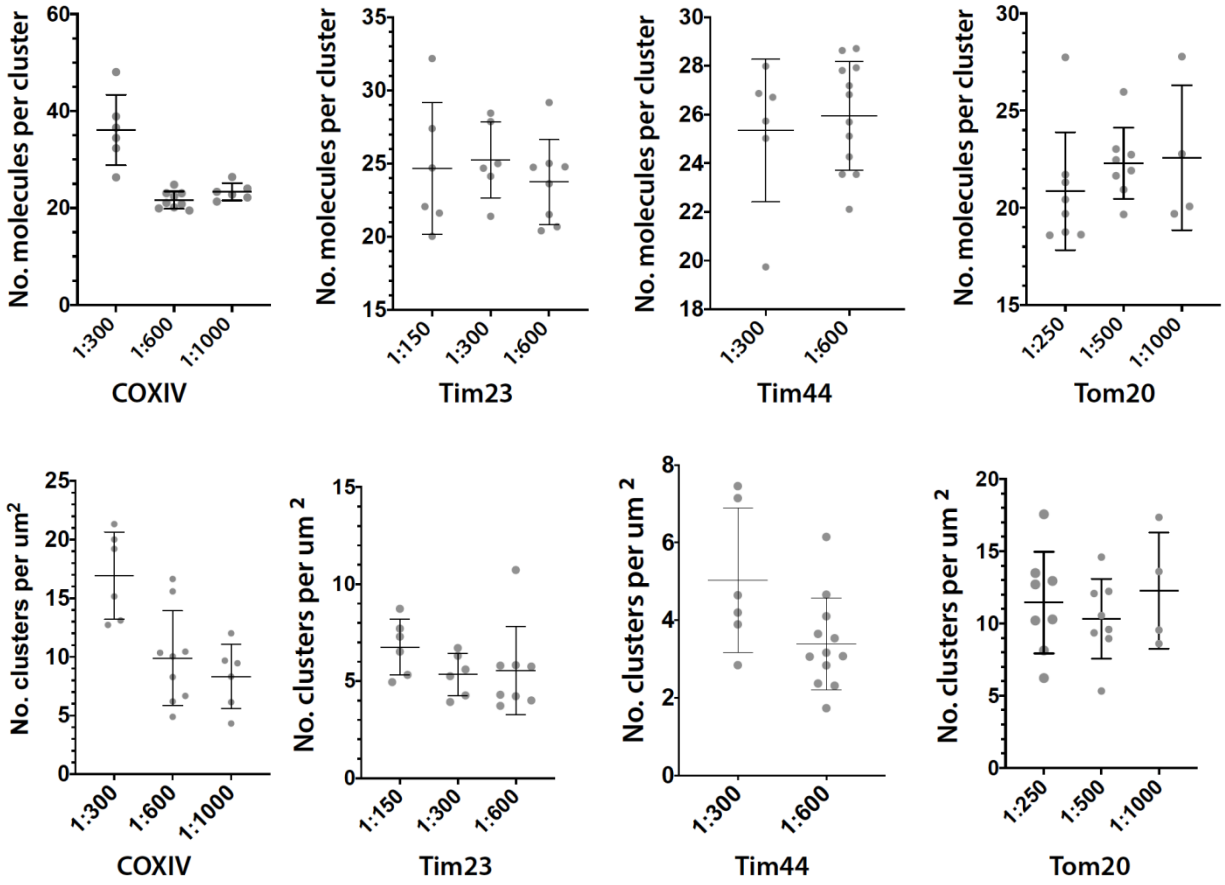
3. How many times have these experiments been repeated? Is there variability between staining? Data should come from 3 biological replicates (i.e., independently grown, stained, and imaged samples). Conclusions rely entirely on the consistency of their labeling and imaging strategies. Wurm et al 2011 states that Tom20 clustering is highly variable between cell type and cell density.

All samples were collected from 3 independently grown stained and imaged samples. Additional information has been added in text to clarify the number of experiments in figure legends for clarity. Labelling strategies were maintained throughout experiments, with optimal conditions determined prior to collecting N-STORM data. Imaging strategies were also maintained throughout each experiment. To ensure reproducibility of these experiments, replicates of initial experiments were also conducted on a separate instrument to ensure consistency of data. Secondary antibodies were also tested with different fluorophore combinations (data not shown) during the initial testing phase. For example - Tim23 (primary antibody) data was recorded using N-STORM with the secondary anti-mouse-CY2/647 or anti-mouse-CY3/647. For each protein or cell line investigated, data was obtained from 3 independently grown, stained and imaged samples prepared at different times. Cell density was consistent for each experiment with cells seeded to a confluency of approximately 80% prior to fixation. In addition; in order to account for possible variability within cells, multiple regions were examined within a single cell.

4. How do we know that the foci in reconstructed STORM images are bona fide protein complexes (this terminology is used throughout the paper), and how do we know that all complexes are detected using the antibody? There are two separate issues: (1) are the antibodies labeling the expected structures; and (2) is the labeling sufficiently dense to exclude the possibility that puncta shown are actually due to sparse labeling and not the underlying protein architecture? Test by methods such as:
(1) colocalization of two subunits with two-color imaging;
This has been added to Fig. S2 and discussed below. Dual labelled HeLa cells of the antibody combinations available were imaged by STORM and representative cell sections are shown in Fig. S2B. Cells sections of the TIM23 complex components (Tim23 and Tim44), and controls Tom20 and Tim44, and COXIV and Tim23 were imaged. Due to the COVID19 pandemic, access to specialised equipment including the STORM microscope was restricted. As such, a representative sample of these images was obtained, however statistical analysis was not able to be conducted. These images are used to support the bimodal cluster size, and variation in Tim23 and Tim44 cluster number. This is discussed on p11.

or
(2) test additional subunits of the complexes shown. The expectation is that statistically similar numbers of clusters should be detected per μm^2 among subunits in the same complex (related to the analysis in Figure 2B);
During our analysis we tested a large panel of antibodies. The ones selected proved best for STORM imaging due to limited background staining. Unfortunately, the additional antibodies available to us were not suitable for analysis of different components of Complex IV or the TIM23 complex, however two colour imaging has been done in HeLa cells to demonstrate the relative relationship between the proteins examined here. The two colour imaging does not demonstrate complete colocalization of the two subunits of the TIM23 complex, however what can be seen is a proportion of clusters that are localised in close proximity or co-localised, while other clusters contain either Tim23 or Tim44 alone. This is not surprising as these proteins exist in separate complexes as well as a Complex conformation containing both proteins.

This point has been expanded in-text along with an additional data and a diagram to demonstrate this in figure S2.
or (3) titrate primary antibody to show that labeling is saturated at the dilutions used (which is not disclosed).
Titrations have been done for each antibody and the number of clusters per micron, and the number of molecules per cluster as shown below. Antibody dilutions used for the data presented in this manuscript are appropriate based on this titration data. The dilutions used for each antibody for this study are: COXIV 1:300, Tim23 1:300, Tim44 1:300 and Tom20 1:500. Analysis of lower antibody dilutions for COXIV and Tim44 were not successful. Further clarification has been added to the method. This data has not been included in the manuscript, however the specific dilutions used for labelling have been noted in text.



5. How can the authors compare the number of molecules per area between proteins if it is not shown that the antibody used is at saturating concentrations? The authors should do an antibody titer for all POIs and test whether there is a maximal # of molecules per area reached. Another way to compare molecules of protein between several different proteins would be to endogenously tag them. For example, there are more Tim44 clusters per µm^2 (Figure 2B) than Tim23 clusters per µm^2. But it is expected that all Tim44 proteins would be in complex with Tim23 and should have the same or fewer clusters. This makes me question whether any comparisons can be made between different proteins and antibodies (Fig 1, Fig 2, Fig S1).
The antibody titration is provided above. The arrangement of Tim23 and Tim44 observed here supports extensive biochemical data from many groups showing a dynamic existence of the TIM23 complex. Tim23 and Tim44 exist in separate complexes that dynamically associate. The biology of these complexes is well defined in-text. As such, not all Tim44 proteins will be in complex with Tim23. This has been further addressed in text p11 and p12.

6. Data in Fig 4 show a statistically significant but very weak effect. It does not appear that Tim8a/b are major determinants of Tim complex arrangement. This result seems over-sold.
Here we are not looking at the arrangement of the TIM complexes, but the arrangement of COXIV (a component of Complex IV of the respiratory chain). Our biochemical work on these cells (Kang et al., 2019) showed that loss of Tim8a/b results in a destabilisation of Complex IV, while the level of the component COXIV is not affected. The N-STORM data aligns well with the biochemical observations suggesting Tim8b has a greater influence on Complex IV assembly in HEK293 cells. We have extended our discussion on this point.

7. Related, If there is no difference in number of clusters and avg number of molecules per cluster in Tim8a KO cells, how can the authors explain that there is an increase in avg cluster area if COXIV should be reduced in these cells?

This has been addressed in text. Additional STORM imaging was conducted of these cell lines to ensure data from 3 independent experiments is recorded. The increase in cluster area indicates an alteration in the spatial arrangement of the complex, potentially due to reduced stability of the Complex IV in these cells. Destabilisation of Complex IV was observed by BN-PAGE in the Tim8bKO HEK cells, but not in Tim8aKO HEK cells (Kang et al., 2019). The information gained here by STORM analysis supports a different effect on the stability of Complex IV upon loss of Tim8a or Tim8b. These results are in agreement with the biochemical analysis conducted previously (Kang et al., 2019). Based on previous biochemical analysis of these cells, we do not expect a reduction in the protein COXIV (Kang et al; 2019).

Minor:
1. In the abstract, the STORM acronym is incorrectly defined. It should be "Stochastic Optical Reconstruction Microscopy."
Addressed in text
2. For consistency and by community standards it is better to use "MICOS" throughout instead of mixing MINOS and MICOS.
Addressed in text
3. The authors should mention how many molecules are in 1 cluster in the main text.
The number of molecules per cluster for the proteins examined in HeLa cells (COXIV, Tim23, Tim44 and Tom20) are now included in Supplemental Fig. S2B.

Instead of less clusters, should be fewer clusters. *Addressed in text*

Second decision letter

MS ID#: JOCES/2020/252197

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AUTHORS: Catherine Palmer, Jieqiong Lou, Bety Kouskousis, Elvis Pandzic, Alexander Anderson, Yilin Kang, Elizabeth Hinde, and Diana Stojanovski
ARTICLE TYPE: Research Article

The reviewers are generally pleased with your revised paper. They suggest a coupleminor revisions for clarification. Please address these concerns in a final revised paper. I will then be happy to accept your paper for publication.

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.

Reviewer 1

Advance summary and potential significance to field

Overall the authors have addressed the previously raised points of concern sufficiently. The findings they present are novel and interesting and demonstrate the advantages of their STORM approach over other conventional approaches in the field.

Comments for the author

Comments:
- It is not clear to me, why the authors have decided to use an unpaired t-test with Welch's correction. Are the data sets normally distributed and if not, why did the authors not chose a non-

parametric test (e.g. Mann-Whitney-U test). A brief explanation should be included into the methods section.
Minor comments:
- The authors should be consistent in using upper case letters for proteins (TIM vs Tim) throughout the text and figures
- Mathematical symbols in the Image Analysis section (Methods part) are in a wrong format

Reviewer 2

Advance summary and potential significance to field

The main scientific conclusion of this paper is that Tim8 plays a role in regulating clustering of complex IV – I still wonder if this is a new conclusion since it would have been expected based on their previous eLife paper where they instead used biochemical methods to study the same process. But it may be that complementing their biochemical conclusions with complementary structural data warrants publication in JCS.

Otherwise, the authors have addressed my main technical concerns. They have provided supporting data that validate their immunolabeling protocols for STORM. These technical details are also now better documented in the methods text.

Comments for the author

In the final revision, I suggest tweaking the language slightly where describing the sizes of mitochondrial membrane complexes (related to my major point #1 in the initial review). It would be useful to explicitly state that the complexes exist on a highly crowded membrane. This crowding of unresolvable (sub-diffraction limited) complexes is what motivates using STORM in this study.

The fact that the complexes themselves are sub-diffraction in size does not by itself preclude using conventional microscopy. (For example, you can easily resolve microtubules with conventional microscopy – at 25 nm in size – because microtubules are generally well spaced apart from one another). I think this presentation will also aid the reader in understanding the cluster measurements and dispersion phenotypes reported later.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

Comments:
- It is not clear to me, why the authors have decided to use an unpaired t-test with Welch's correction. Are the data sets normally distributed and if not, why did the authors not chose a non-parametric test (e.g. Mann-Whitney-U test). A brief explanation should be included into the methods section.

We have addressed this by including an additional statement and a reference for use of this statistical method for description of ClusDoc analyses. We have analysed the data using both parametric and non-parametric methods (non-parametric data not shown). This did not alter our conclusions or interpretation of the data discussed in this manuscript. For consistency with the published analysis used previously by Schmider et al; 2020 we have presented the t-test results in this manuscript.

Minor comments:
- The authors should be consistent in using upper case letters for proteins (TIM vs Tim) throughout the text and figures

As defined in the text – TIM refers to the complex, where Tim refers to the protein. This convention is used to differentiate between the two. For example – the TIM23 complex, and the Tim23 protein. The text has been reviewed for consistency; however, both these designations are necessary within the text.
- Mathematical symbols in the Image Analysis section (Methods part) are in a wrong format

This has been corrected.

Reviewer 2 Comments for the Author:
In the final revision, I suggest tweaking the language slightly where describing the sizes of mitochondrial membrane complexes (related to my major point #1 in the initial review). It would be useful to explicitly state that the complexes exist on a highly crowded membrane. This crowding of unresolvable (sub-diffraction limited) complexes is what motivates using STORM in this study. The fact that the complexes themselves are sub-diffraction in size does not by itself preclude using conventional microscopy. (For example, you can easily resolve microtubules with conventional microscopy – at 25 nm in size – because microtubules are generally well spaced apart from one another). I think this presentation will also aid the reader in understanding the cluster measurements and dispersion phenotypes reported later.

We thank the reviewer for this suggestion, and an additional statement has been added to the text as requested by reviewer 2 to describe the protein rich environment of the mitochondrial inner membrane.

Third decision letter

MS ID#: JOCES/2020/252197

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AUTHORS: Catherine Palmer, Jieqiong Lou, Bety Kouskousis, Elvis Pandzic, Alexander Anderson, Yilin Kang, Elizabeth Hinde, and Diana Stojanovski
ARTICLE TYPE: Research Article

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