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Tau forms dynamic hot spots that persist after microtubule perturbation and cholesterol depletion

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Dear Jürgen,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees. A third referee had agreed to review the manuscript for us, but I haven't received the report and I think at this stage that I will not. I will therefore go with the two reports on hand.

As you can see from the comments below, the referees appreciate the reported findings and support publication here. They also raise a number of constructive comments that I would like to ask you to address in a revised version.

I think it would be helpful to discuss the raised points further and I am available to do so via email or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further with you.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to prepare the revised version.

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (4th Aug 2022).

As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

If you require more time to complete the revisions let me know as we can grant an extension.

Use the link below to submit your revision:

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Referee #1:

In this work, Padmanabhan et al. have described a new technology, single-molecule-based super-resolution microscopy to analyze the organization of Tau near the membrane of cultured cells. They found the formation of nanometer sized tau spots than lasted tens of seconds, a long time compared to the dwell time of tau on microtubules. Looking at how these tau spots arise, they depolymerized microtubules or depleted, the analyzed cells, of cholesterol, but not changes in spot formation were found. On the other hand, the role of these dynamic tau spots, associated to cell membrane, in any cellular function remains to

be clarified.

Specific points:

1. To visualize individual tau molecules, they expressed ON4R tau isoform. However, an old paper by Binder's group (J. Neurochem., 74 (2000), 1749) shows that tau lacking exons 2 and 3 formed small aggregates and are unable to aggregate in larger polymers, that are found when those exons are present in tau molecule. This point should be discussed.
2. There is heterogenous tau mobility pattern near the plasma membrane showing three motion states: immobile, confined and freely diffusive molecules. After microtubule depolymerization, tau spots dynamics is increased. However, cholesterol depletion does not have any effect on tau spots dynamics. It may suggest an absence of cholesterol molecule on tau spot formation or dynamics and that other different molecules should play a role on those processes.
3. Also, Binder et al. and other groups suggested a role for arachidonic acid for tau aggregation. Since arachidonic acid is present at cell membrane, the effect of arachidonic acid (presence or depletion) on the formation or dynamics of tau spot should be also analyzed, or discussed.

In summary, the positive data is the novel description of a technology that allows to detect single molecules using a super resolution microscopy analysis. This is important and the obtained results are clear and relevant.
On the other hand, little is indicated about the function of tau spots.

Referee #2:

In this study, Padmanabhan et al. used quantitative single-molecule imaging to study Tau's organization and its dynamics near the plasma membrane. They used murine neuroblastoma cells transiently expressing the 0N4R isoform of Tau fused to the photoactivatable fluorescent protein mEos3.2 and imaged Tau in live cells using single molecule tracking PALM (spt-PALM). They then compared the mobility of Tau in live versus fixed cells using mean square displacement (MSD) computations and determined that there are both mobile and immobile pools of Tau molecules near the plasma membrane of those cells. They performed further computations to characterize the three diffusive states of tau (immobile, confined, freely diffusive). Moreover, they identified regions where there was high Tau localization density and confined diffusion of Tau molecules, which they named "hot spots". They characterized those "hot spots" quantitatively and found that their heterogeneous mobility was also present in other cell types. Microtubule perturbation resulted in an increase in the mobility of Tau molecules near the plasma membrane, a behavior also validated using a microtubule-binding deficient mutant of Tau. However, the formation of Tau hot spots was not affected by such perturbations or by cholesterol depletion.
This is an interesting and useful study of the dynamic behavior of Tau near the plasma membrane. It complements the available literature and identifies novel dynamic behaviors of Tau. However, the conclusions are not fully supported by the data provided and either need to be better supported or toned down. Further, the existing super-resolution literature on tau is not properly discussed and the findings are not put into the context of this existing literature. These issues should be addressed before the manuscript is acceptable for publication. See below for a detailed list of major concerns:
Major concerns:
1. The authors do not provide sufficient evidence that either the tagging or the over-expression is not responsible for the "hot spot" formation. Their only evidence for tagging not influencing the hot spots is to use another tag (Halo-tag instead of mEos2). This is not a good control since it still introduces a tag. In the absence of a proper control, it is hard to conclude whether the hot spots are an artefact of tagging or over-expression and the authors should address this issue either by a proper control or by toning down their conclusions and properly discussing the shortcomings.
2. Along the same lines, the authors provide very little evidence that the hot spots are membrane associated or that the hot spots have anything to do with interaction with the membrane as opposed to interaction with other structures near the membrane. Their main evidence is the use of TIRF illumination, which suggests that the hot spots are *near* the membrane but does not prove that the hot spots are membrane associated. Again, either direct evidence needs to be provided or the authors need to tone down their conclusions.
3. The microtubule depolymerization and cholesterol depletion did not impact hot spots. The authors conclude that the hot spots do not depend on microtubules or cholesterol. Once again, I don't think they can make this conclusion based on this data. The hot spots may depend on microtubules and cholesterol for their initial formation, but they may not require microtubules or cholesterol for their maintenance once they are formed. For example, tau can interact with free tubulin and this interaction maybe sufficient to maintain "hot spots" once they are formed on polymerized microtubules near the membrane.
4. It is not clear if the authors have accounted for repeated blinking of mEos2 in their experiments, which would make things look like "hot spots" artificially. There are several algorithms for correcting for repeat blinking such as the Distance Distribution Correction (DDC) algorithm by Bohrer et al., Nature Methods, 2021 and the authors should employ these correction methods to ensure that the formation of hot spots is not a result of repeated mEos2 blinking.
5. What is the nature of the tau hot spots if not condensates? Are they tau oligomers? It would be nice if the authors can discuss this point in more detail. In particular, it is important that the authors put their results into the context of existing literature. For example, Merezhko et al, Cell Reports, 25 2027-2035, 2018 showed that tau can be secreted via an unconventional non-vesicular mechanism, which involves its oligomerization. In this paper, the authors use dSTORM super-resolution and electron microscopy to show that tau accumulates in small puncta near the membrane. While this paper is cited in the current manuscript,

it is not discussed in the right context. The authors should compare their hot spots to the accumulation of tau in small puncta shown in the Merezhko paper and discuss their findings in the context of this paper. Additionally, Gyparaki et al, PNAS, 118, e2021461118, 2021 used STORM super-resolution microscopy to show that tau forms small oligomers on microtubules. This paper is not cited or discussed. Could the hot spots be these small oligomers on microtubules near the plasma membrane? Once again, the authors need to put their results in the context of these previous findings.

Minor comments:
1. In the last section of the results, when reporting the burst duration and size of the Tau hot spots, the authors can consider changing the 13-15 s and 160-180 detection counts to individually report what happens in treated and untreated cells. The way it is written now makes it look like it's referring to a range of values.

RESPONSE TO REVIEWERS' COMMENTS

on our manuscript with the tracking number EMBOJ-2022-111265 entitled, "Tau forms dynamic hot spots that persist after microtubule perturbation and cholesterol depletion"

We are grateful to the two reviewers for their insightful comments and observations, which have helped to significantly improve our manuscript. Please find our response to specific comments below:

Reviewer #1:

In this work, Padmanabhan et al. have described a new technology, single-molecule-based super-resolution microscopy to analyze the organization of Tau near the membrane of cultured cells. They found the formation of nanometer sized tau spots than lasted tens of seconds, a long time compared to the dwell time of tau on microtubules. Looking at how these tau spots arise, they depolymerized microtubules or depleted, the analyzed cells, of cholesterol, but not changes in spot formation were found. On the other hand, the role of these dynamic tau spots, associated to cell membrane, in any cellular function remains to be clarified.

Response: We thank the reviewer for their encouraging remarks and have addressed their concerns as follows.

Specific points:

1. To visualize individual tau molecules, they expressed ON4R tau isoform. However, an old paper by Binder's group (J. Neurochem., 74 (2000), 1749) shows that tau lacking exons 2 and 3 formed small aggregates and are unable to aggregate in larger polymers, that are found when those exons are present in tau molecule. This point should be discussed.

Response: We chose 0N4R isoform of Tau because it is the most prevalent isoform in the mouse brain. We thank the reviewer for making us aware of this seminal study, which we now discuss in the manuscript (page 12):

"*Previous studies have shown that different recombinant forms of Tau have different aggregation rates and filament formation capabilities in cell-free conditions (Goedert & Jakes, 1990, King et al., 2000). For instance, in the presence of arachidonic acid, Tau isoforms (0N3R and 0N4R) lacking N-terminal exons 2 and 3 aggregate into smaller, globular oligomers, whereas those containing these exons (1N3R and 2N3R) form very long filaments (King et al., 2000). Moreover, missense mutations found in frontotemporal lobar degeneration (FTLD), such as P301L and R406W, exert differential effects on Tau aggregation kinetics and accelerate aggregation of Tau into filaments (Combs & Gamblin, 2012, Mutreja et al., 2019, Nacharaju et al., 1999, Sonawane & Chinnathambi, 2020). How this is reflected in vivo and whether different isoforms and FTLD mutations display distinct hot spot dynamics remains to be investigated. Future work is therefore needed to determine how different factors work together to regulate Tau hot spot dynamics.*"

2. There is heterogenous tau mobility pattern near the plasma membrane showing three motion states: immobile, confined and freely diffusive molecules. After microtubule depolymerization, tau spots dynamics is increased. However, cholesterol depletion does not have any effect on tau spots dynamics. It may suggest an absence of cholesterol molecule on tau spot formation or dynamics and that other different molecules should play a role on those processes.

Response: We agree that multiple factors could influence the dynamics of individual Tau molecules and hot spots and discuss this in the revised manuscript (page 12):

"*How do the observed hot spots form? … Protein clusters can form either through self-association or interaction with binding partners. Therefore, one possibility is that preexisting plasma membrane-associated clusters of Tau's interaction partner(s), such as Fyn and annexin, may serve as hubs to capture and trap Tau molecules as they pass by. Considering that many membrane lipids have been shown to directly interact with Tau (Bok et al., 2021, Sallaberry et al., 2021) and that anionic compounds such as arachidonic acid (which are present in these lipids) promote Tau nucleation in a cell-free system (King et al., 2000), Tau/lipid interactions at the plasma membrane could possibly generate assemblies that resemble the Tau hot spots observed in our study. Alternatively, Tau might itself form assemblies at the plasma membrane and interact with membrane-associated proteins and lipids*."

3. Also, Binder et al. and other groups suggested a role for arachidonic acid for tau aggregation. Since arachidonic acid is present at cell membrane, the effect of arachidonic acid (presence or depletion) on the formation or dynamics of tau spot should be also analyzed, or discussed.

Response: We have chosen to discuss the potential role of arachidonic acid in the discussion (page 12; reproduced above in our response to comments #1 and #2).

4. In summary, the positive data is the novel description of a technology that allows to detect single molecules using a super resolution microscopy analysis. This is important and the obtained results are clear and relevant. On the other hand, little is indicated about the function of tau spots.

Response: We again thank the reviewer for referring to our results as being clear and important. In the revised version, we now also discuss the potential functions of Tau hot spots, while trying not to be too speculative (page 13):

"*What could be the functions of these hot spots? An intriguing possibility is that they are the sites where vesicle-free Tau is released into the extracellular space (Katsinelos et al., 2018, Merezhko et al., 2018). Given that Tau interacts with actin and microtubules (Magnani et al., 2007), it is also plausible that hot spot formation is necessary for Tau to link the cytoskeleton to the plasma membrane. Alternatively, Tau hot spots could be molecular depots that recruit specific membrane-associated kinases and phosphatases to regulate signaling pathways in the local environment (Li & Götz, 2017, Mueller et al., 2021). An exciting next step forward would therefore be to characterize the local environment (such as lipid and protein composition) that influences or is influenced by Tau hot spots and to determine the functions of Tau hot spots.*"

Reviewer #2:

In this study, Padmanabhan et al. … cholesterol depletion.

This is an interesting and useful study of the dynamic behavior of Tau near the plasma membrane. It complements the available literature and identifies novel dynamic behaviors of Tau. However, the conclusions are not fully supported by the data provided and either need to be better supported or toned down. Further, the existing super-resolution literature on tau is not properly discussed and the findings are not put into the context of this existing literature.

These issues should be addressed before the manuscript is acceptable for publication. See below for a detailed list of major concerns:

Response: We are pleased that the reviewer finds the study interesting and useful. We appreciate the concerns raised by this reviewer and have addressed them as follows.

Major concerns:

1. The authors do not provide sufficient evidence that either the tagging or the over-expression is not responsible for the "hot spot" formation. Their only evidence for tagging not influencing the hot spots is to use another tag (Halo-tag instead of mEos2). This is not a good control since it still introduces a tag. In the absence of a proper control, it is hard to conclude whether the hot spots are an artefact of tagging or over-expression and the authors should address this issue either by a proper control or by toning down their conclusions and properly discussing the shortcomings.

Response: We appreciate this concern of the reviewer. When we designed our study, we aimed to avoid potential artefacts due to tagging and overexpression as follows:

(i) An important point is that we used mEos3.2 and not mEos2 (as indicated by the reviewer). To mitigate tagging-induced aggregation, Zhang et al (Nature Methods, 2012, PMID: 22581370) solved the crystal structure of mEos2 and then developed mEos3.1 and 3.2 that are truly monomeric.

(ii) As noted by this reviewer, we have used two different tags (HaloTag and mEos3.2) and observed heterogenous Tau dynamic behaviour and Tau hot spots in both conditions.

(iii) Tau interacts with the plasma membrane through its N-terminus, in addition to the microtubule-binding region. Tau also folds back with its N-terminus onto the microtubule-binding region. For this reason, we tagged Tau in the C-terminus. As is evident from the low-resolution images (Fig. 1), this tagging did not prevent Tau from localising on the microtubules, increasing our confidence that Tau's properties were not overtly impacted by the tagging.

(iv) We avoided imaging cells with high expression levels (green signal from mEos3.2 at the footprint of cells), as a routine strategy in our studies.

Transient Tau overexpression studies, such as ours, have provided valuable insights into Tau-membrane interactions, including vesicle-free Tau release (PMID: 30463001), the phosphorylation state of plasma membrane-associated Tau (PMID: 10685603), and the loss of frontotemporal dementia-linked R406W mutant Tau's interactions with the plasma membrane (PMID: 21339331). In light of these studies, despite the potential overexpression caveat, we believe our findings provide significant new insights into the spatiotemporal dynamics of Tau.

Following this reviewer's suggestion, however, we now added a section in our discussion describing the limitations of our study (page 14):

"*We recognise the limitations of our study. Firstly, our approach relies on transient overexpression of Tau in N2a and HEK293T cell lines. However, using the intensity of the cell footprint as a proxy for Tau expression, we did not image cells with very high expression levels. Secondly, although we have used different tags (mEos3.2 and HaloTag) to investigate the dynamic behavior of Tau, the possibility of a role of the tag in Tau hot spot assembly and stability cannot be fully ruled out. Addressing these limitations would require the development of novel molecular and imaging tools to quantify the dynamics of label-free, endogenous Tau in live cells.*"

2. Along the same lines, the authors provide very little evidence that the hot spots are

membrane associated or that the hot spots have anything to do with interaction with the membrane as opposed to interaction with other structures near the membrane. Their main evidence is the use of TIRF illumination, which suggests that the hot spots are *near* the membrane but does not prove that the hot spots are membrane associated. Again, either direct evidence needs to be provided or the authors need to tone down their conclusions.

Response: We appreciate this concern, which we believe reflects a fundamental limitation of the current technology. We now mention this caveat in the discussion (page 14):

"*Finally, because the width of the plasma membrane is <10 nm, which is much smaller than the ~100 nm axial resolution of TIRF illumination, it is not possible to determine whether the Tau hot spots associate directly with the plasma membrane or whether they are linked indirectly through other proteins or structures. Further work with an improved axial resolution is needed to clarify the precise location of these hot spots in this compartment.*"

3. The microtubule depolymerization and cholesterol depletion did not impact hot spots. The authors conclude that the hot spots do not depend on microtubules or cholesterol. Once again, I don't think they can make this conclusion based on this data. The hot spots may depend on microtubules and cholesterol for their initial formation, but they may not require microtubules or cholesterol for their maintenance once they are formed. For example, tau can interact with free tubulin and this interaction maybe sufficient to maintain "hot spots" once they are formed on polymerized microtubules near the membrane.

Response: We agree with this reviewer and have now revised our interpretation, such that we no longer suggest that Tau hot spots are resistant to microtubule and cholesterol perturbations, but rather that they persist after these perturbations. We have modified the title and the text accordingly.

The revised title of the manuscript is "*Tau forms dynamic hot spots that persist after microtubule perturbation and cholesterol depletion.*"

On page 4: "*Moreover, these hot spots persisted after the depletion of cholesterol, which is known to reduce Tau pathology in transgenic mice (Boimel et al., 2009).*"

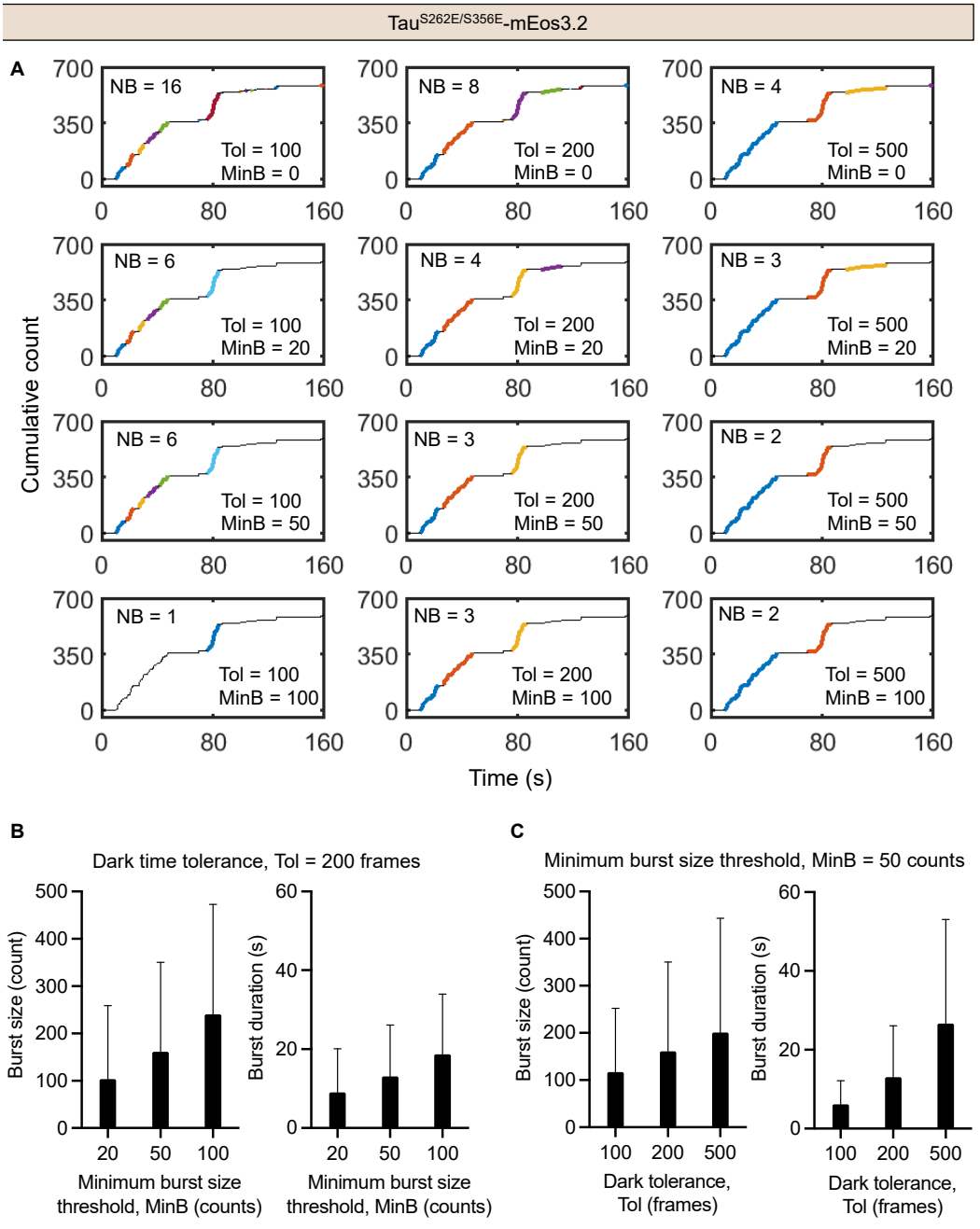
On page 10: "*Together, these findings suggest that Tau hot spots persist after microtubule perturbations and cholesterol depletion.*"

On page 11: "*Moreover, these hot spots lasted tens of seconds and persist after microtubule perturbation and cholesterol depletion.*"

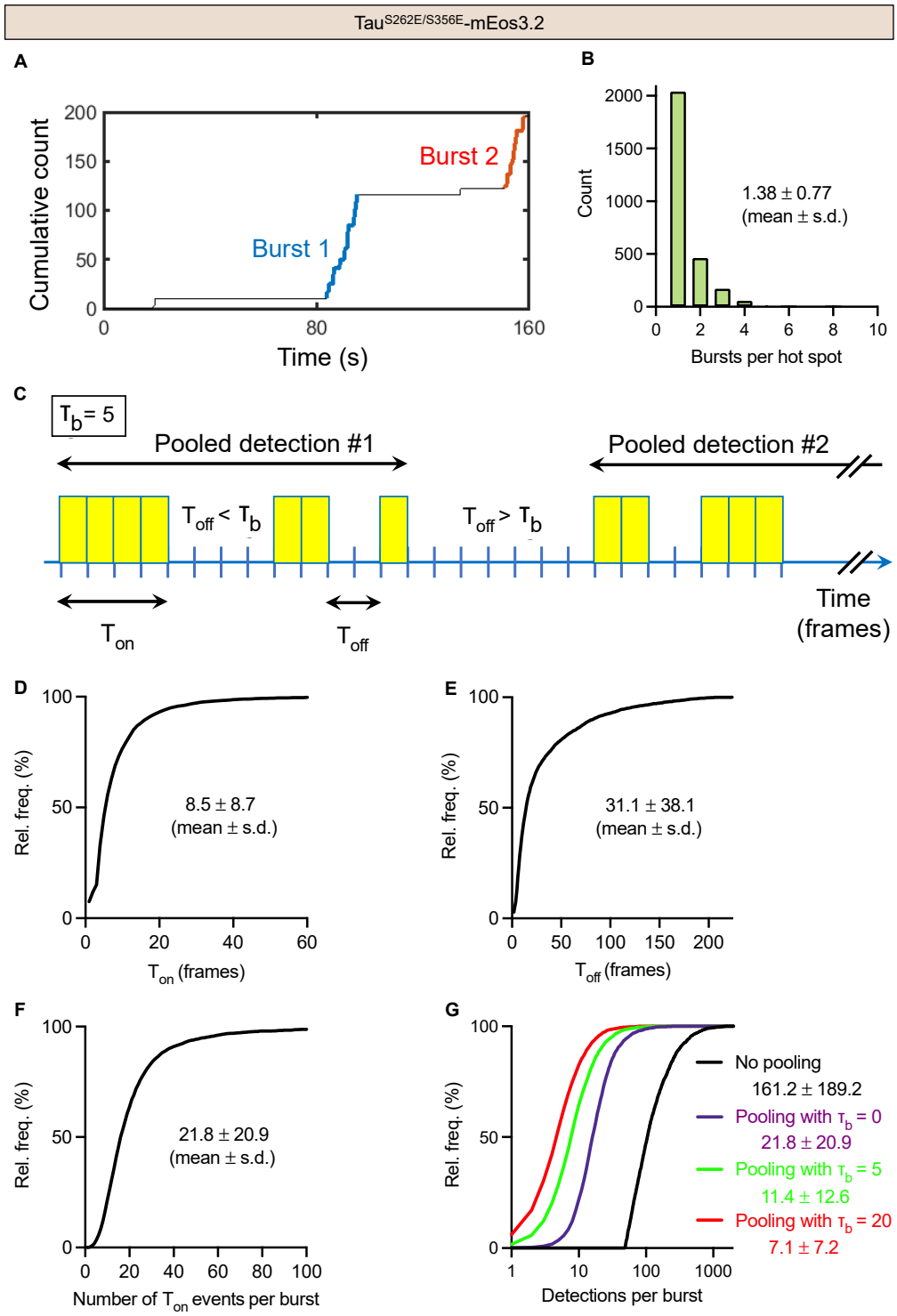
4. It is not clear if the authors have accounted for repeated blinking of mEos2 in their experiments, which would make things look like "hot spots" artificially. There are several algorithms for correcting for repeat blinking such as the Distance Distribution Correction (DDC) algorithm by Bohrer et al., Nature Methods, 2021 and the authors should employ these correction methods to ensure that the formation of hot spots is not a result of repeated mEos2 blinking.

Response: We have added a new figure (Appendix Fig S13; also reproduced below) to clarify how we have analysed the dynamics of Tau hot spots. Briefly, the time series of detections from each Tau hot spot is computed. Then, using dark time tolerance of 200 frames, detections within the hot spots are grouped into individual bursts. In line with previous studies

(PMIDs: 23828889, 29930094, 33214152), bursts with at least 50 detections were considered for further analysis to avoid hot spots arising from multiple appearances of single Tau-mEos3.2 molecules. In the revised version, we have also performed a sensitivity analysis to determine how burst time and duration vary with dark time tolerance and minimum burst size (Appendix Fig S13B and C). Moreover, we have now computed fluorescence on-time, T_{on}, fluorescence off-time, T_{off}, and number of T_{on} events within each burst (Appendix Fig S14; also reproduced below). Following Levet et al. (Nature Methods, 2015, PMID: 26344046), using the blinking tolerance interval, τ_b (5 to 20), we grouped multiple T_{on} events into a single pooled detection. Despite grouping, we observed multiple pooled detections per burst (see Appendix Fig S14G). Together, these analyses indicate that the observed Tau hot spots are unlikely due to repeated appearances of mEos3.2 molecules.



Appendix Figure S13. Sensitivity analysis. A, Representative time series of detections from one Tau$^{S262E/S356E}$-mEos3.2 hot spot. NB is the number of different bursts detected per hot spot (color coded) by varying dark time tolerance, Tol, and minimum burst size, MinB. **B, C,** The burst size and duration as a function of MinB with fixed Tol (**B**) and as a function of Tol with fixed MinB (**C**). The mean ± s.d. is shown in B and C. This analysis corresponds to corresponding to data in Fig. 6L-N.



Appendix Figure S14. Sensitivity of burst size to repeated appearances. A, A, Representative time series of detections from one $Tau^{S262E/S356E}$-mEos3.2 hot spot displaying two bursts. Parameters: Tol = 200 frames and MinB = 50 counts. **B,** The distributions of the number of bursts per $Tau^{S262E/S356E}$-mEos3.2 hot spot. **C,** Schematic of photophysical parameters such as fluorescence on-time, T_{on}, fluorescence off-time, T_{off}, and the blinking tolerance interval, τ_b. **D-F,** The cumulative distributions of T_{on} (**D**), T_{off} (**E**) and the number of T_{on} events per burst (**F**). **G,** The cumulative distribution of the number of detections per burst as a function of blinking tolerance interval, τ_b. This analysis corresponds to data in Fig. 6L-N.

5. What is the nature of the tau hot spots if not condensates? Are they tau oligomers? It would be nice if the authors can discuss this point in more detail. In particular, it is important that the authors put their results into the context of existing literature. For example, Merezhko et al, Cell Reports, 25 2027-2035, 2018 showed that tau can be secreted via an unconventional non-vesicular mechanism, which involves its oligomerization. In this paper, the authors use dSTORM super-resolution and electron microscopy to show that tau accumulates in small puncta near the membrane. While this paper is cited in the current manuscript, it is not discussed in the right context. The authors should compare their hot spots to the accumulation of tau in small puncta shown in the Merezhko paper and discuss their findings in the context of this paper. Additionally, Gyparaki et al, PNAS, 118, e2021461118, 2021 used STORM super-resolution microscopy to show that tau forms small oligomers on microtubules. This paper is not cited or discussed. Could the hot spots be these small oligomers on microtubules near the plasma membrane? Once again, the authors need to put their results in the context of these previous findings.

Response: We apologise for the oversight and thank the reviewer for pointing out these important studies. We have now added a section in our discussion to place our findings in the context of these studies (page 11):

"*Using direct stochastic optical reconstruction microscopy and immunoelectron microscopy of fixed N2a cells, Merezhko et al. (2018) observed Tau localization in ~50-80 nm sized microdomains, which were not in membrane-bound compartments but were positioned just below the plasma membrane. They suggested that these domains mark the sites of Tau release into the extracellular space. It is therefore plausible that a subset of the hot spots which we observed in live cells using the sptPALM technique represents the Tau microdomains detected in fixed cells. Further, by super-resolution imaging of Tau in fixed cell lines and hippocampal neurons, Gyparaki et al. (2021) reported the presence of Tau nanoclusters on microtubules. These contained oligomeric Tau and were much smaller than the Tau condensates on microtubules observed in a cell-free system (Siahaan et al., 2019). It is likely that in conditions where Tau/microtubule interactions are not perturbed, a subset of Tau hot spots is associated with microtubules. Determining whether the Tau hot spots in live cells and the Tau nanoclusters in fixed cells represent the same or different entities will be an important subject for future research.*"

Minor comments:

1. In the last section of the results, when reporting the burst duration and size of the Tau hot spots, the authors can consider changing the 13-15 s and 160-180 detection counts to individually report what happens in treated and untreated cells. The way it is written now makes it look like it's referring to a range of values.

Response: We now report the values expressed as mean ± s.d. instead of a range.

1st Revision - Editorial Decision **18th Jul 2022**

Dear Jürgen,

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the original referee #2 who is happy with the revised version.

I am therefore very pleased to let you know that I will accept the MS for publication here. Before sending you the formal accept letter, there are just a few editorial points to sort out:

We need 3-5 keywords

Please relabel Competing Interest as Disclosure and Competing Interests

Regarding the Data Availability section =>As far as I can see no data is generated that needs to be deposited in a database. If this is correct, please state: Data Availability: This study includes no data deposited in external repositories.

For the appendix please add a ToC with page numbers

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That should be all! Congratulation on a nice study

Best Karin

Karin Dumstrei, PhD
Senior Editor
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Use the link below to submit your revision:

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Referee #2:

The authors have addressed my concerns and the paper is now suitable for publication.

Dear Jürgen,

Thanks for submitting your revised version. I have now had a chance to take a look at it and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

with best wishes

Karin

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Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Numerical data have been provided in excel sheets as suggested.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	Open source computational tools used in the study are appropriately referenced.
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	