Dynein-dependent collection of membranes defines the architecture and position of microtubule asters in isolated, geometrically confined volumes of cell-free extracts

Abdullah Sami and Jesse Gatlin

Corresponding author(s): Jesse Gatlin, University of Wyoming

Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | 2022-03-01 |
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Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Dr. Gatlin,

Thank you for sending us your manuscript, which has now been seen by two reviewers, whose comments follow below.

As you can see, both reviewers find your manuscript interesting (as do I). However, they raise issues that warrant further experiments and revision before they could be published in MBoC. They also give some clear suggestions as to how you might proceed.

From the editorial point of view, I note that you have submitted this as a Brief Report, but it may be better to submit the revision as a full manuscript, which will give you the opportunity to characterize the system more fully (for example, the partial membrane depletion method, as suggested by Reviewer 2) and introduce further analysis. Both reviewers indicated that manipulations over longer length scales would be informative. I appreciate that the manuscript may endeavour to capture the physical features of self-organization, rather than detailed molecular/cellular mechanism. If so, then issues such as length scales and quantitative imaging (as per Reviewer 1) become very relevant for the revision.

I note that you have submitted this for our Special Issue on Forces. If you choose to revise the manuscript, it is likely that you will miss the deadline for this issue. However, if approved by the reviewers, we would include your report in one of the regular issues of MBoC.

Best wishes,

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gatlin,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.
Review #1 (Remarks to the Author):

In this manuscript the authors show that centrosomal microtubule networks assembled in confined egg extracts can self-center by exerting pushing forces on physical boundaries but adopt radial morphologies depending on the activity of dyneins that cluster membranous organelles toward the center of the aster. This work sheds light on the possibility that networks in homogeneous compressional states can adopt various counter-intuitive morphologies, including a radial aster with straight microtubules, which reminiscent of microtubules under tension. This radial structure is shown to be linked to vesicle aggregation rather than to the mechanical process leading to the self-centering of the microtubule array. Importantly, this description could extend to more complex systems such as interphasic cells and explain the paradox that exists between networks that seem to be under tensional load but that could be positioned and maintained through compressional forces. As such this work is of great value and deserves publication. However, some key elements require further investigation to be fully convincing.

- The observations are made on short time scale and the complete description of the actual steady-state would require longer observation. Several descriptions are performed as soon as the system reach a given morphology with no mention of its lifespan. It is therefore difficult to assess if this is indeed a true steady state. What happen to the straight microtubules once the membrane organelles have been clustered at the center of the array and tension forces vanish?
- The spatial boundaries are also not very much explored although this parameter is key to set the contribution of pushing forces. Could the tensional forces play a more significant role in larger wells? The system would be better described if the authors could perform the same experiments in larger wells in order to better define the spatial range in which the model is valid.
- The work is rather descriptive but not so many examples are shown. It would more convincing if the authors could provide additional examples of the morphologies adopted by the microtubule networks. It is indeed difficult to get a precise idea of the generality of their conclusions.
- A major point that definitely needs to be improved is the image analysis. Quantification are rather approximative and imprecise. In particular the shape of individual microtubules, or least of microtubule bundles should be reported. Could the author use appropriate tool to improve the segmentation of the microtubule networks? Could they describe further the shapes of microtubules in the various regions of the bull's eye? This would also lead to more accurate microtubule orientation maps.
- Finally, it seems that the authors overlooked the literature regarding the mechanics and morphologies of the microtubule networks, in particular they should refer more exhaustively to pre-existing studies on the respective contribution of pushing and pulling forces on aster positioning and conformation.

Review #2 (Remarks to the Author):

This paper addresses the role of organelles and dynein in organizing microtubules in an artificial cell system comprised of frog egg extract trapped in cylindrical enclosures. The size of the enclosures is comparable to sea urchin eggs. The microtubule organizing centers (MTOCs) center in this system in a manner that depends strongly on microtubules but less strongly on membranes and dynein. The organelles move inwards under the influence of dynein but not all the way to the center. The architecture of the microtubules depends on dynein and partial removal of organelles has a similar effect to inhibiting dynein. The authors propose a qualitative model for how the MTOC centers and the role of membrane and dynein in organizing the system.

The manuscript addresses several topical issues. How MTOCs center remains a topic of great interest in the field and the question of how organelles shape microtubules is under-researched and of considerable interest. There is also great interest in cell-mimetic systems. The confinement system described is an advance on previous methods that have been used to confine egg extract, which is a nice feature of the paper that will interest specialists.
The paper as a whole, reads as somewhat preliminary and it is not clear that it provides a significant mechanistic advance. The figures raise multiple interesting issues that are not addressed, see below. The large number of un-addressed questions and the somewhat thin nature of the results provided leave the reader somewhat frustrated. Also, the cartoon model at the end seems inaccurate (see below) and the relevance to real cells is far from clear.

Specific points
1) The authors vary radius of their enclosure, but only over a very narrow range. This seems like a missed opportunity to probe scaling relationships

2) The organelles move part way inward to the center of the aster but then appear to stop or at least reach some sort of steady state. This is very interesting and may model lack of ER movement to the center of large egg and blastomere cells. No experiments are provided to address the reason for this partial inwards movement or what sets the radius of the ER disc at steady state. Scaling relationships could be highly informative on what sets the radius of the membrane disc. Partial membrane depletion could also be highly informative. If membrane crowding blocks inward movement, there might be more inwards movement when membrane density is reduced. Partial dynein inhibition would also be potentially informative.

3) The radial density function for microtubules is quite strange in the intact system, with an interesting ring of microtubules just outside the membrane enriched disc. The cartoon in Fig 4A proposes that all the microtubules emanate from the MTOC and appear denser on the outside of the membrane because they bend more. The images in Fig 1 do not support this cartoon view. It appears much more likely that microtubules are nucleated on the surface of the membrane disc and this effect increases their density on the outside. This would be interesting and should be better characterized, eg by EB1 comet counting. If organelles nucleate microtubule in interphase asters, that would be a significant step forward. The cartoons, which show very long microtubules, are not consistent with previous work on interphase asters on egg extract, which showed that asters are made of short, dynamic microtubule nucleated away from the MTOC and this discrepancy is not discussed.

4) The partial membrane depletion method introduced in this paper is elegant and would likely be adopted by others in the field. It would be useful if it were better characterized, for example, does it deplete ER and mitochondria equally, or does the depleted extract end up relatively enriched in one population?

5) The paper is unclear re the status of F-actin in the figures and methods. Authors reference Field et al 2017 which discusses actin-intact extract and then add a statement about why one would add an actin poison but do not state they have. They should clearly state the type of extract here--not just give a reference. Is this the correct one? Later they reference Field et al 2011 (twice) and discuss it as if this is a different type of extract-however this is also actin intact. Is F-actin inhibited in all the experiments, or is it left intact in some? Please clarify. If actin is intact in the initial experiments and then poisoned in the those with membrane removed they cannot be compared ---- authors are removing two conditions at the same time. Also note-the extract for Figures 1 and 2 is in interphase and the extract in Figure 3 is CSF arrested. This also complicates a direct comparison.

If the F-actin is left intact in any of the analyses, it seems important to characterize where it is and whether it contributes to any of the phenomena. It would be interesting to know whether F-actin makes a contribution to altering the distribution of organelles and their effect on microtubules.
The current draft of the manuscript represents a substantially revised version in which we have attempted to address the concerns expressed by the reviewers. Improvements include several items that were missing from the original due to time constraints, limitations imposed by the brief report format (which now no longer exist), and our own unfortunate omissions. Some of the more salient items that this new manuscript now includes are:

- a longer introduction which describes in more detail aster centration models
- new data which explore the effects of membrane depletion on aster centration
- spatial analysis of microtubule curvature
- spatial analysis of microtubule nucleation
- analysis of pattern persistence over longer observation times

What follows is a point-by-point response to all reviewers’ comments. Regardless as to the eventual outcome, we wanted to express our thanks to the reviews for their time and effort and believe that in addressing your comments, the manuscript is much improved.

**Reviewer #1 (Remarks to the Author):**
In this manuscript the authors show that centrosomal microtubule networks assembled in confined egg extracts can self-center by exerting pushing forces on physical boundaries but adopt radial morphologies depending on the activity of dyneins that cluster membranous organelles toward the center of the aster. This work sheds light on the possibility that networks in homogeneous compressional states can adopt various counter-intuitive morphologies, including a radial aster with straight microtubules, which reminiscent of microtubules under tension. This radial structure is shown to be linked to vesicle aggregation rather than to the mechanical process leading to the self-centering of the microtubule array. Importantly, this description could extend to more complex systems such as interphasic cells and explain the paradox that exists between networks that seem to be under tensional load but that could be positioned and maintained through compressional forces. As such this work is of great value and deserves publication. However, some key elements require further investigation to be fully convincing.

**Concern #1.1** - The observations are made on short time scale and the complete description of the actual steady-state would require longer observation. Several descriptions are performed as soon as the system reach a given morphology with no mention of its lifespan. It is therefore difficult to assess if this is indeed a true steady state. What happen to the straight microtubules once the membrane organelles have been clustered at the center of the array and tension forces vanish?

**Response:** We agree with the reviewer regarding the duration of the observation to assess if the pattern achieved represents a true steady-state. To better assess the lifespan of this bullseye pattern with compacted membrane at the aster center, we increased the observation time up to 40 min after the aster centration in the enclosures. We summarized our observations of these longer observation times in new Fig. S3 and new Supplementary movie 5 with a description in the text. Our observations indicate that compacted membranes near aster center come to a pseudo steady-state shortly after aster centration in the enclosure (see Fig. S3B), and that the
A microtubule ring exists around the membrane compacted zone for the duration of the observation time. However, the spoke-like central array of straight microtubules is more transient, becoming undetectable sometime between the 16- and 32-minute time points after aster centration. Due to this revelation, we no longer use “steady-state” as an adjective to describe the observed bullseye pattern and have removed it from the manuscript.

**Concern #1.2:** The spatial boundaries are also not very much explored although this parameter is key to set the contribution of pushing forces. Could the tensional forces play a more significant role in larger wells? The system would be better described if the authors could perform the same experiments in larger wells in order to better define the spatial range in which the model is valid.

**Response:** We agree that our original set of experiments was limited in terms of enclosure size and that larger enclosures would be informative as to the limits of the centering mechanism that we’ve proposed in our discussion. To address this concern, the revised manuscript now includes experiments in larger cylindrical enclosures (up to 220 µm diameter). Interestingly, we found that asters were able to center in these larger enclosures, albeit with slower dynamics, and that the membrane dense central core and bullseye microtubule pattern again emerged after aster centration (Fig. S5). The revised version of the manuscript also now includes a critical set of new data (Fig. 3) in which we explored the spatial range of our model in larger enclosures. Interestingly, we found that centration can occur after membrane depletion in our 110 µm diameter devices, but not in larger diameter devices, suggesting that in agreement with our model, the presence of membranes extends the distance over which centration can occur.

**Concern #1.3:** the work is rather descriptive but not so many examples are shown. It would more convincing if the authors could provide additional examples of the morphologies adopted by the microtubule networks. It is indeed difficult to get a precise idea of the generality of their conclusions.

**Response:** This is a valid point and we apologize for this omission. In the revised manuscript, we have included a new supplementary figure (Figure S1) to represent which shows many examples of microtubule asters and membrane patterns in 110 µm and 150 µm diameter cylindrical enclosures in untreated control conditions.

**Concern #1.4:** A major point that definitely needs to be improved is the image analysis. Quantification are rather approximative and imprecise. In particular the shape of individual microtubules, or least of microtubule bundles should be reported. Could the author use appropriate tool to improve the segmentation of the microtubule networks? Could they describe further the shapes of microtubules in the various regions of the bull’s eye? This would also lead to more accurate microtubule orientation maps.
Response: We appreciate the reviewer’s concerns regarding our image analyses and made two major changes to our microtubule pattern analysis to address them:

1) The revised manuscript now includes microtubule curvature analysis via the “Kappa” ImageJ plug-in (developed by the Brouhard lab) to measure microtubule curvatures in two distinct regions of the bullseye pattern, the first near the aster center and second more proximal, near enclosure wall (Fig. S2). These results support the claim that microtubules near the enclosure wall exhibit more curvature, likely because they are under compressive loads, whereas microtubules in the central region are indeed straighter. See also Supplementary movie 4, where we attempt to better convey spatial differences in microtubule dynamics and curvature within the bullseye pattern.

2) Admittedly, we weren’t completely satisfied with our previous characterization and analysis of the spatial relationship between compacted membranes and the underlying microtubule pattern. Specifically, the radiality measurement employed, in which the intensities of both the microtubule and membrane channels were averaged for all intensity profiles along radial lines extending from the aster center to the enclosure edge and 0 to 360 degrees, tended to smooth out the boundaries of the central membrane core and blurred the relationship between the membrane edge and the high density microtubule ring encircling it. This was particularly pronounced for non-circular membrane aggregates. We adopted a new analysis (see Fig. S4 and Materials and Methods) which circumvents this issue and better reflects the spatial relationship between the membrane edge and the distal microtubule ring (see new plots in Figs. 2 & S5).

Concern #1.5: Finally, it seems that the authors overlooked the literature regarding the mechanics and morphologies of the microtubule networks, in particular they should refer more exhaustively to pre-existing studies on the respective contribution of pushing and pulling forces on aster positioning and conformation.

Response: We originally submitted the manuscript to adhere to the word limits imposed by MBoC’s brief report format and perhaps made a poor choice in omitting a more detailed and thorough description of aster centration models and their underlying force generation mechanisms. In the revised manuscript, which we are submitting as a full-length article at the bequest of the editor, we have addressed this concern with a more extended introduction.
Reviewer #2 (Remarks to the Author):
This paper addresses the role of organelles and dynein in organizing microtubules in an artificial cell system comprised of frog egg extract trapped in cylindrical enclosures. The size of the enclosures is comparable to sea urchin eggs. The microtubule organizing centers (MTOCs) center in this system in a manner that depends strongly on microtubules but less strongly on membranes and dynein. The organelles move inwards under the influence of dynein, but not all the way to the center. The architecture of the microtubules depends on dynein and partial removal of organelles has a similar effect to inhibiting dynein. The authors propose a qualitative model for how the MTOC centers and the role of membrane and dynein in organizing the system.

The manuscript addresses several topical issues. How MTOCs center remains a topic of great interest in the field and the question of how organelles shape microtubules is under-researched and of considerable interest. There is also great interest in cell-mimetic systems. The confinement system described is an advance on previous methods that have been used to confine egg extract, which is a nice feature of the paper that will interest specialists.

The paper as a whole, reads as somewhat preliminary and it is not clear that it provides a significant mechanistic advance. The figures raise multiple interesting issues that are not addressed, see below. The large number of un-addressed questions and the somewhat thin nature of the results provided leave the reader somewhat frustrated. Also, the cartoon model at the end seems inaccurate (see below) and the relevance to real cells is far from clear.

Concern #2.1: The authors vary radius of their enclosure, but only over a very narrow range. This seems like a missed opportunity to probe scaling relationships

Response: We have expanded the size range of enclosures used and the revised manuscript now includes data in enclosures double the size of our original 110 µm diameter cylindrical enclosures (see also response to a similar concern expressed by Reviewer #1, Concern #1.2 above). We should note that attempts to make even larger devices were experimentally difficult as (i) the generation of larger hydrogel enclosures requires a piece-by-piece modular construction and (ii) oil crossflow over larger hydrogel enclosures frequently resulted in oil/surfactant droplets mixed in with the enclosed extract.

Concern #2.2: The organelles move part way inward to the center of the aster but then appear to stop or at least reach some sort of steady state. This is very interesting and may model lack of ER movement to the center of large egg and blastomere cells. No experiments are provided to address the reason for this partial inwards movement or what sets the radius of the ER disc at steady state. Scaling relationships could be highly informative on what sets the radius of the membrane disc. Partial membrane depletion could also be highly informative. If membrane crowding blocks inward movement, there might be more inwards movement when membrane density is reduced. Partial dynein inhibition would also be potentially informative.
**Response:** We acknowledge that a more comprehensive analysis of the inward movement of membranes (including titration of the dynein-inhibitor p150-CC1) would be interesting and informative, however, we feel that this is beyond the scope of current work. This avenue of future research is now mentioned at the end of the discussion. As far as scaling is concerned, new data is included in the revised manuscript in which we have addressed the relationship between the amount of membrane present in the system, the size of the resulting internal membrane disk that forms, and effects on the microtubule pattern (Fig. 2). These data suggest that as the amount of membrane in the system is reduced, so is the size of the central membrane disk and the diameter of the high-density microtubule ring (e.g., compare Figs. 2A and 2D).

**Concern #2.3:** The radial density function for microtubules is quite strange in the intact system, with an interesting ring of microtubules just outside the membrane enriched disc. The cartoon in Fig 4A proposes that all the microtubules emanate from the MTOC and appear denser on the outside of the membrane because they bend more. The images in Fig 1 do not support this cartoon view. It appears much more likely that microtubules are nucleated on the surface of the membrane disc and this effect increases their density on the outside. This would be interesting and should be better characterized, eg by EB1 comet counting. If organelles nucleate microtubule in interphase asters, that would be a significant step forward. The cartoons, which show very long microtubules, are not consistent with previous work on interphase asters on egg extract, which showed that asters are made of short, dynamic microtubule nucleated away from the MTOC and this discrepancy is not discussed.

**Response:** We agree with the reviewer that microtubules could be nucleated on the surface of the membrane disc by organelle dependent microtubule nucleation and this effect may increase local density on the outside of the membrane disk. To more thoroughly evaluate the source of microtubules in this region, we measured the flux rate of EB1 comets through two circles, each concentric with the aster center and with each other, one positioned inside the high density microtubule ring, the other within the dense region (see new Fig. S7 and Supplementary movie 6). Indeed, as you suggested, the higher flux of EB1 comets through the outer circle indicates that at least some of the increased microtubule density in the membrane proximal region is the result of a localized increase in microtubule nucleation there. However, the underlying noncentrosomal microtubule nucleation mechanism at play remains unknown and we are hesitant to ascribe it solely to one or the other. This being said, the results are interesting as they differ from what is observed for aMTOC asters in bulk extract, which show no bullseye pattern in their microtubules and exhibit near constant EB1 flux rates (see Ishihara et al. 2014 PNAS, Ishihara et al. 2021 MBoC). Note that we have updated our cartoon model (Figure 4) such that it now includes...
short microtubules nucleated at the surface of the central membrane disk.

**Concern #2.4:** The partial membrane depletion method introduced in this paper is elegant and would likely be adopted by others in the field. It would be useful if it were better characterized, for example, does it deplete ER and mitochondria equally, or does the depleted extract end up relatively enriched in one population?

**Response:** We thank the reviewer for their positive comments and agree that we could have done a better job in characterizing the depletion. The revised manuscript now includes a characterization of ER (DiO-labeled membranes) before and after partial depletion and of mitochondria (TMRE-labeled membranes; see Figure S6 C-F).

**Concern #2.5:** The paper is unclear re the status of F-actin in the figures and methods. Authors reference Field et al 2017 which discusses actin-intact extract and then add a statement about why one would add an actin poison but do not state they have. They should clearly state the type of extract here--not just give a reference. Is this the correct one? Later they reference Field et al 2011(twice) and discuss it as if this is a different type of extract however this is also actin intact. Is F-actin inhibited in all the experiments, or is it left intact in some? Please clarify. If actin is intact in the initial experiments and then poisoned in the those with membrane removed they cannot be compared ---- authors are removing two conditions at the same time. Also note-the extract for Figures 1 and 2 is in interphase and the extract in Figure 3 is CSF arrested. This also complicates a direct comparison.

**Response:** We apologize for any confusion about the type of extracts used in our experiments (and that we referenced Field et al., 2011 twice, which has been corrected!). All of our aster experiments were performed in cytochalasin-treated, actin-free interphase egg extracts. This is now stated unequivocally near the beginning of our results section. We note that membrane-depleted supernatant was prepared from CSF-arrested F-actin intact egg extract. Collected supernatant from actin-contraction and gelation was then treated with cytochalasin D and later with Ca$^{2+}$ to induce it into interphase.

**Concern #2.6:** If the F-actin is left intact in any of the analyses, it seems important to characterize where it is and whether it contributes to any of the phenomena. It would be interesting to know whether F-actin makes a contribution to altering the distribution of organelles and their effect on microtubules.

**Response:** Based on studies of confined, f-actin intact extracts, we agree that it would be interesting to investigate the affect that the f-actin cytoskeleton might have on aster centration, however, we feel it is beyond the scope of the current studies.
Dear Dr Gatlin,

Thank you for sending us your revision, which has now been seen by the external reviewers. As you can see, they appreciate your efforts to respond to their comments. Arguably, the in vivo significance of your findings remains somewhat elusive, but I don't think that this is the principal contribution of your MS. It uses a rather unique system to describe interesting phenomena that will provide guides to biological experiments. So, I am happy to accept in principle your MS for MBoC.

However, Reviewer 2 had some further questions of detail that would be informative, if you have the data to hand. So, before we formally accept the MS, let me ask you to see if you can address these. I will handle your response and it does not need to go back to the reviewers.

Best,

Alpha

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Gatlin,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,
Reviewer #1 (Remarks to the Author):

In this revised version of their manuscripts, authors have taken into account the comments formulated by the two reviewers. The conclusions are interesting but the study failed at providing clear answers to the main questions that are addressed.

I am left unsure about the conclusions on the relative role of pushing and pulling forces on aster positioning (which is poorly characterized in this study in general) and on their respective spatial distribution patterns (near the border, limited to a specific central area).

In the new data the authors found that the centering phase is followed by a decentring phase. They decided not to describe the centered state as a steady state but they did not take advantage of the existence of these two phases. Looking in details at microtubule shapes and membrane distribution during these two phases might have led to some interesting conclusions about the mechanisms of centering and decentering.

The new experiment about EB1 asked by reviewer 2 provided exciting results that are under-exploited. The linear density of EB1 comets should be much lower on the outer ring and it is even higher than on the central ring. This suggest that there is a massive nucleation of microtubule in the intermediate region. How this contribute to aster geometry and position is not studied.

Reviewer #2 (Remarks to the Author):

The authors performed additional experiments to address a majority of our questions and clarified others. This reviewer appreciated the effort to increase the size of the enclosure. This appears to identify an upper limit for the distance that an MTOC can travel away from an enclosure wall (an upper limit to pushing forces). This is new and useful data. A couple experiments were perhaps beyond the scope of this manuscript and at this point no further experiments are asked by this reviewer. However, a bit more data/information would be useful.

1. Information on how the release from M-phase arrest is achieved (calcium addition?) and what the zero time point in the experiments correlates with---calcium addition? warming? This is useful information.
2. Regarding statement that the size of the MT aster pattern scales with the enclosure size (Sup Fig 5), while the aster size does appear to scale, the architecture does not. There is no bullseye structure formed in the largest enclosure size (no focused center or circumferential MT) and Figure 3 which also varies size does not look at later time points. Do authors have additional examples at the 220 um size? Does it become more like unconfined extract published by others? Authors do include later time points for 150 um. I ask this because as the authors state in the discussion, "the bullseye pattern of microtubule distribution produced by confined asters differs from the microtubule architecture seen in typical animal cells, and even from aster architectures observed in previous studies of unconfined asters in Xenopus laevis egg extracts. Since the entire paper is focused on this structure it would be good to see the scale limits of its formation.

The work performed here is interesting and well done and the methodology useful, but this reviewer is not sure that the biology being examined has relevance to anything in vivo. It is up to the editors to decide if that is required for publication.
We again want to thank the reviewers for their time and critical comments. Our responses are interspersed in the text below and indicated in blue text.

Reviewer #1 (Remarks to the Author):

In this revised version of their manuscripts, authors have taken into account the comments formulated by the two reviewers. The conclusions are interesting but the study failed at providing clear answers to the main questions that are addressed.

I am left unsure about the conclusions on the relative role of pushing and pulling forces on aster positioning (which is poorly characterized in this study in general) and on their respective spatial distribution patterns (near the border, limited to a specific central area).

In the new data the authors found that the centering phase is followed by a decentering phase. They decided not to describe the centered state as a steady state but they did not take advantage of the existence of these two phases. Looking in details at microtubule shapes and membrane distribution during these two phases might have led to some interesting conclusions about the mechanisms of centering and decentering.

Though we appreciate this comment, we feel that our previous work has definitively shown that pushing forces do indeed make a major contribution to aster centering, at least over distances up to 60 µm in our experimental system. As such, the purpose of this work was not to characterize the nature of the force (i.e. whether it is pushing or pulling), but rather to characterize the putative need for dynein’s involvement in centering the aster over longer distances (e.g. see Wuhr et al, 2010). We believe that our data are consistent with the proposed buttressing hypothesis and that this hypothesis, if correct, explains (i) how aster centering occurs over distances longer than 60 µm and (ii) why dynein’s involvement is required for long-distance translocation of the aster.

The “decentering” phase comment from the reviewer is likely in response to the aMTOC positioning observed in large (110 µm diameter enclosures) at longer time points (Fig. S3). It seems that in our system, at longer time points, the aMTOC becomes detached from the rest of the aster and begins to move independently from the rest of the structure. We attribute this to a decrease in microtubule nucleating activity from the aMTOC surface and feel that it is likely an artifact of our small and discrete extract volumes.
The new experiment about EB1 asked by reviewer 2 provided exciting results that are under-exploited. The linear density of EB1 comets should be much lower on the outer ring and it is even higher than on the central ring. This suggests that there is a massive nucleation of microtubule in the intermediate region. How this contributes to aster geometry and position is not studied.

We agree with the reviewer that this is an interesting result and it is one that we be explored in the future, however, we felt that this avenue of research was beyond the scope of the current work.

Reviewer #2 (Remarks to the Author):

The authors performed additional experiments to address a majority of our questions and clarified others. This reviewer appreciated the effort to increase the size of the enclosure. This appears to identify an upper limit for the distance that an MTOC can travel away from an enclosure wall (an upper limit to pushing forces). This is new and useful data. A couple experiments were perhaps beyond the scope of this manuscript and at this point no further experiments are asked by this reviewer. However, a bit more data/information would be useful.

1. Information on how the release from M-phase arrest is achieved (calcium addition?) and what the zero time point in the experiments correlates with---calcium addition? warming? This is useful information.

In all experiments, extracts were released from the M-phase arrest (CSF-extract) to interphase by adding calcium. This was mentioned in our Materials and Methods section (lines 435-436):

“Prepared CSF extracts (both normal, membrane-depleted supernatant and partial membrane-depleted) were induced into interphase by adding Ca2+ to a final concentration 0.4mM.”

Extract-filled microfluidic devices (with extract containing calcium, cycloheximide, and any additional treatments or fluorescent probes) was kept in on-ice to prevent MT nucleation. The ‘zero’ time point was defined as the time at which confined aMTOCs started to nucleate microtubules after being taken off ice. Typically, aMTOCs start to nucleate MTs when extract comes close to room temperature. This was described in lines 465-467 in the manuscript’s Materials and Methods section (lines 465-467).
Lines 465-467: “For time-lapse studies, the “zero” time point was defined as the time at which confined aMTOCs first began to nucleate microtubules after being taken off ice.”

2. Regarding statement that the size of the MT aster pattern scales with the enclosure size (Sup Fig 5), while the aster size does appear to scale, the architecture does not. There is no bullseye structure formed in the largest enclosure size (no focused center or circumferential MT) and Figure 3 which also varies size does not look at later time points. Do authors have additional examples at the 220 um size? Does it become more like unconfined extract published by others? Authors do include later time points for 150 um. I ask this because as the authors state in the discussion, “the bullseye pattern of microtubule distribution produced by confined asters differs from the microtubule architecture seen in typical animal cells, and even from aster architectures observed in previous studies of unconfined asters in Xenopus laevis egg extracts. Since the entire paper is focused on this structure it would be good to see the scale limits of its formation.

We acknowledge that example used in supplementary figure 5 of 220um diameter enclosure did not form a distinct bullseye pattern as observed in other smaller enclosure diameter. We argue that the requirement of longer times for the aster to center in such large enclosures does impact the overall aster morphology. The central part of the network seems to become less robust with time progression, which occurs even in smaller enclosures (see supplementary figure 3). However, we would argue that still there is an annular zone of circumferential microtubules around the region of compacted membrane. As requested, we have provided two more examples from such enclosures (see below). We would also like to reiterate (from our responses to the first-round of comments, that attempts to make even larger devices are experimentally difficult as (i) the generation of larger hydrogel enclosures requires a piece-by-piece modular construction and (ii) oil crossflow over lager hydrogel enclosures frequently results in oil/surfactant droplets mixed in with the enclosed extract.
Two examples of microtubule pattern formation in 220µm diameter enclosures. From left to right, the first column in each panel shows inverted LUT images of microtubule asters after centration in cylindrical hydrogel enclosures and extract with untreated control in 220 µm diameter. Second column in each panel shows the concurrent positions of DiO-labeled ER membranes at the same time point. Blue and green circles represent the inner edges of enclosures of microtubule and membrane channels respectively. The third column in each panel shows the merged representation of the microtubule channel (pseudo-colored red) and membrane channel (pseudo-colored green). All the images are average intensity projections of 10 consecutive confocal images each taken at 2.5 µm z-axis intervals above the coverslip surfaces to the oil-extract interface at the top of the enclosures. Scale bars (white) = 20 µm.

The work performed here is interesting and well done and the methodology useful, but this reviewer is not sure that the biology being examined has relevance to anything in vivo. It is up to the editors to decide if that is required for publication.
3rd Editorial Decision
August 4, 2022

RE: Manuscript #E22-03-0074RR
TITLE: "Dynein-dependent collection of membranes defines the architecture and position of microtubule asters in isolated, geometrically confined volumes of cell-free extracts"

Dear Jesse,

Thank you for your final responses. Everything seems in place and I am happy to formally accept your MS for MBoC.

Best wishes,

Alpha

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Gatlin:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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