Nanoscale Dynamics of Actin Filaments in the Red Blood Cell Membrane Skeleton

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(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.)

RE: Manuscript #E21-03-0107
TITLE: Nanoscale organization of actin filaments in the red blood cell membrane skeleton

Monitoring Editor (Remarks to Author):

Dear Velia,

Thank you for submitting your manuscript to MBoC. It has now been seen by two external reviewers with complementary expertise. Their comments follow below.

As you can see, both reviewers consider that you are addressing an important problem, and your results are potentially interesting for the general cell-biological audience of MBoC. But, there are issues that should be addressed by a revision.

From my perspective, I would especially draw your attention to the following points:

1) Reviewer 1 raises important points about the analysis of the STORM data, especially the use of DBSCAN. I think that you should endeavour to reanalyse your data with another approach (the reviewer gives some suggestions). The issue, of course, is whether what you have found is a consequence of the analysis method that you have used. Confirmation with a second method would increase confidence in your results.

2) Reviewer 2 suggests that the movement of F-actin nodes is a key message for the field, but has some recommendations to strengthen the data. If you agree with this suggestion, I wonder if the message of your manuscript could be productively refocused. To what extent to you wish your MS to principally stand as a challenge to the results of Pan et al (2018)? That seems to be an implication which Reviewer 1 drew from their reading.

3) I agree with Reviewer 2 that the Discussion should be shortened and focused.

We look forward to seeing your revised MS soon.

Best wishes,

Alpha

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Fowler,

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 haveopted 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.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this manuscript the author applied a number of fluorescence microscopy techniques to investigate the organization of F-actin in red blood cells (RBCs). F-actin was primarily visualized by phalloidin-based fluorophores in fixed cell or jasplakinolide-derivative (SiR-actin) in living cells. Due to spatial resolution limit, the authors largely focused their quantitative analysis of fixed cell images obtained by STORM super-resolution microscopy. Quantitative analysis was primarily performed using a clustering algorithm DBSCAN. The authors conclude that the F-actin nodal structures in RBC membrane are non-uniformly distributed and question the conclusion by a previous study (Pan et al. Cell Reports 2018 which found that F-actin nodes are distributed with ~80 nm spacing on average, indicative of relaxed spectrin geometry).
Over all, the reviewer finds that while the study is potentially interesting, the results and analysis presented are on the side of being preliminary at this stage. Particularly, technical concerns are registered on the imaging/image analysis aspects. These shortcomings make it difficult to evaluate the biological claims of this study. Furthermore, while this manuscript directly question the findings in Pan et al. Cell Reports 2018, the presentation and analysis of their data is completely different, which makes it impossible to make an informed comparison. Detailed comments can be found below:
1. The choice of DBSCAN for analysing STORM data is dubious and in my opinion the numerical results that one obtained from DBSCAN is completely irrelevant to the question of long-range organization of F-actin nodes. While it is true that DBSCAN is commonly used to analyse single-molecule super-resolution datasets, most of their use has been to do cluster analysis for local short-ranged spatial distribution. Mathematically speaking, DBSCAN gives absolutely no information on long-range spatial organization.
The methods that one can use for long-range organization quantification would be the correlation analysis such as those used by Pan et al., which is well-grounded in a method developed by Sengupta..Lippincott-Schwartz, Nature Methods 2011. Alternatively Ripley function, or Fourier analysis would likely be viable techniques that can be implemented without too much difficulty. This will go a long way towards allowing a direct comparison between this study and the previous Cell Reports paper.
2. The technical description of STORM data is inaccurate (e.g. Fig. 5 and elsewhere). The author appears to refer to each coordinate as "molecule", when the more accurate term would be "localizations". It is well established that many common STORM fluorophores exhibit very extensive blinking which lead to severe overcounting. In other words, the numbers of "localizations" cannot be directly converted to actual number of "molecules" without careful calibration of the blinking properties. The "molecules" count discussed by the authors very likely overestimate the actual number of molecules by a significant margin.

Without the correction of these blinking, this effect likely undermine the interpretation of Fig. 6 analysis. Large cluster seen in RBC data likely comes from the frequent blinking of the fluorophores.
3. From close inspection of sample preparation protocol between this study and Pan et al., it is clear that there are many differences. Given that the intent of this study appears to be to challenge Pan et al., at the very least, this reviewer would expect that the authors would try to replicate the exact same specimen preparations used in Pan et al., and compared to their own methods. At present, this is not done, and thus the contribution of different specimen condition cannot be ruled out.
4. One specific concern regarding STORM imaging of actin and specimen preparation is that the high concentration of Phalloidin is extremely important for high quality imaging as the authors of Pan et al. demonstrated in their earlier study, Xu et al. Nature Methods 2013. In this manuscript, the concentration of Phalloidin used seems to be lower than in Pan et al. by multiple fold. Incomplete or suboptimal labelling would certainly affect the observation of so-called 'uniform' distribution and easily leads to the observation of 'non-uniform' distribution.
5. In this present study, only F-actin was imaged, whereas in Pan et al., a more complete array of molecules beyond F-actin, .e.g spectrin, 4.1, TMOD, adducing, etc. were imaged, and all showed a distribution pattern consistent with their conclusion. It would be interesting to see what are the distributions of these F-actin nodes component.

Reviewer #2 (Remarks to the Author):

See Attached.

December 19, 2021

Dear Dr. Yap,

Thank you for the thoughtful and constructive review of our manuscript MBoC: E21-03-0107, now entitled, "Nanoscale dynamics of actin filaments in the red blood cell membrane skeleton". We have carefully considered your comments as well as those of the reviewers and are submitting a revised version of our manuscript along with this letter and a Response to the Reviewers. Note that we modified the title of the manuscript based on your suggestion to refocus the manuscript to emphasize F-actin node dynamics and better reflect the advances of our study over that of previous work in the field.

As we discussed previously, it was impossible for us to perform new experiments with fresh human red blood cells due to the COVID19 pandemic, which caused the University of Delaware to close the normal donor blood collection service. Given this limitation, we followed the revision strategy that I had discussed previously with you, and are grateful that you agreed that this plan was reasonable.

Briefly, this included reframing the manuscript to emphasize our novel observations about F-actin node dynamics, rather than as a challenge to Pan et al 2018. We were able to address Reviewer 1's major concern by reanalyzing our existing STORM data using a second statistical analysis method they suggested, Ripley's *K* function, which demonstrates that the Alexa 647-phalloidin fluorescence localizations are present in non-random clusters of varying sizes in the RBC membrane, in agreement with the DBSCAN analysis. We addressed Reviewer 1's concerns about F-actin dynamics in live RBCs by including a control with fixed RBCs, and by including additional data we had already collected before the pandemic, showing effects of actin depolymerizing drugs on the locations of F-actin nodes, emphasizing their dependence on actin polymerization, an ATP-dependent process.

On the subsequent pages, you will find point-by-point responses to your comments, as well as to comments from each reviewer. Our responses are presented in the same sequence as the original comments, which we indented and reprinted in *italics*. We hope that these revisions will satisfy the reviewers' concerns so that our article will be suitable for publication in *MBoC*.

Sincerely,

Velia M. Fowler

Response to Editor

1) Reviewer 1 raises important points about the analysis of the STORM data, especially the use of DBSCAN. I think that you should endeavour to reanalyse your data with another approach (the reviewer gives some suggestions). The issue, of course, is whether what you have found is a consequence of the analysis method that you have used. Confirmation with a second method would increase confidence in your results.

We reanalyzed our STORM data using the normalized form of Ripley's *K* function (Ripley's *H* function) (Kiskowski *et al.*, 2009). New Figure 3 shows that the Ripley *H* test indicates a high degree of clustering over radii up to ~600 nm, with distribution random at length scales greater than r~600 nm. New Supplemental Figure 2 presents analysis of Ripley *H* test results for individual RBCs, and shows that in each case the experimental data falls above the randomly distributed data indicated by the simulation envelope. Therefore, we conclude that the fluorescence localizations are non-random and are present in clusters of varying sizes in the RBC membrane, in agreement with the DBSCAN approach. However, in view of this Reviewer's concerns with limitations of DBSCAN, we simplified and shortened that section and moved the DBSCAN data to new Supplemental Figure 3.

2) Reviewer 2 suggests that the movement of F-actin nodes is a key message for the field, but has some recommendations to strengthen the data. If you agree with this suggestion, I wonder if the message of your manuscript could be productively refocused. To what extent to you wish your MS to principally stand as a challenge to the results of Pan et al (2018)? That seems to be an implication which Reviewer 1 drew from their reading.

We welcome this suggestion and have reframed and rewritten the manuscript to emphasize our novel observations about F-actin node dynamics, so as to better reflect the advances of our study over that of previous work in the field. Therefore, we also changed the title to: "Nanoscale dynamics of actin filaments in the red blood cell membrane skeleton".

As we had discussed previously, it was impossible for us to perform new experiments with fresh human red blood cells due to the COVID19 pandemic, which caused the University of Delaware to close the normal donor blood collection service. Given this limitation, we followed the revision strategy that I had discussed previously with you, and are grateful that you agreed that this plan was reasonable.

3) I agree with Reviewer 2 that the Discussion should be shortened and focused.

We agree that the Discussion was too long, and have shortened and refocused it.

Response to Reviewer 1.

In this manuscript the author applied a number of fluorescence microscopy techniques to investigate the organization of F-actin in red blood cells (RBCs). F-actin was primarily visualized by phalloidin-based fluorophores in fixed cell or jasplakinolide-derivative (SiR-actin) in living cells. Due to spatial resolution limit, the authors largely focused their quantitative analysis of fixed cell images obtained by STORM super-resolution microscopy. Quantitative analysis was primarily performed using a clustering algorithm DBSCAN. The authors conclude that the F-actin nodal structures in RBC membrane are non-uniformly distributed and question the conclusion by a previous study (Pan et al. Cell Reports 2018 which found that F-actin nodes are distributed with ~80 nm spacing on average, indicative of relaxed spectrin geometry).
Over all, the reviewer finds that while the study is potentially interesting, the results and analysis presented are on the side of being preliminary at this stage. Particularly, technical concerns are registered on the

imaging/image analysis aspects. These shortcomings make it difficult to evaluate the biological claims of this study. Furthermore, while this manuscript directly question the findings in Pan et al. Cell Reports 2018, the presentation and analysis of their data is completely different, which makes it impossible to make an informed comparison.

We thank the reviewer for their thoughtful comments. As explained below in our response to Point 2, we have addressed the issues regarding the image analysis of the STORM data by correcting and clarifying the terminology and methods (Points 2-4) and by applying a new statistical analysis to the localizations (Ripley's *K* function) (Point 1).

We have also reconsidered the detailed comparisons of our data with those in Pan et al 2018. We agree with the Reviewers and the Editor that it was unproductive to emphasize this comparison. Furthermore, in the Discussion, we now mention that Pan et al (2018) had actually observed frequent irregularities or 'holes', in the periodic lattice which were depleted of F-actin nodes and the F-actin associated proteins (see our Discussion, page 17) (Pan *et al.*, 2018). It is likely that these 'holes' correspond to the dimmer regions of F-actin staining we observe by TIRF and Airyscan microscopy, and to the sparser Alexa 647-phalloidin fluorescence localizations detected by STORM. Additionally, we have refocused our manuscript to emphasize the F-actin foci dynamics visualized by TIRF microscopy.

1. The choice of DBSCAN for analysing STORM data is dubious and in my opinion the numerical results that one obtained from DBSCAN is completely irrelevant to the question of long-range organization of F-actin nodes. While it is true that DBSCAN is commonly used to analyse single-molecule super-resolution datasets, most of their use has been to do cluster analysis for local short-ranged spatial distribution. Mathematically speaking, DBSCAN gives absolutely no information on long-range spatial organization.
The methods that one can use for long-range organization quantification would be the correlation analysis such as those used by Pan et al., which is well-grounded in a method developed by Sengupta..Lippincott-Schwartz, Nature Methods 2011. Alternatively Ripley function, or Fourier analysis would likely be viable techniques that can be implemented without too much difficulty. This will go a long way towards allowing a direct comparison between this study and the previous Cell Reports paper.

As suggested, we reanalyzed our STORM data using the normalized form of Ripley's *K* function (Ripley's *H* function) (Kiskowski *et al.*, 2009). Figure 3 presents the results of the Ripley *H* test, which suggests a high degree of clustering with a peak at r~70 nm and a shoulder extending up to r~600 nm, with the distribution becoming random at length scales greater than r~600 nm. Supplemental Figure 2 presents analysis of Ripley *H* test results for individual RBCs, and shows that in each case the experimental data falls above randomly distributed data indicated by the simulation envelope. Therefore, we conclude that the fluorescence localizations are non-random and are present in clusters of varying sizes in the RBC membrane, consistent with the uneven fluorescent-phalloidin intensity distributions observed in TIRF and Airyscan images of RBC membranes (Figures 1 and 2). In view of this Reviewer's concerns with limitations of the DBSCAN approach, we shortened and simplified that section and moved the DBSCAN data to Supplemental Figure 3.

2. The technical description of STORM data is inaccurate (e.g. Fig. 5 and elsewhere). The author appears to refer to each coordinate as "molecule", when the more accurate term would be "localizations". It is well established that many common STORM fluorophores exhibit very extensive blinking which lead to severe overcounting. In other words, the numbers of "localizations" cannot be directly converted to actual number of "molecules" without careful calibration of the blinking properties. The "molecules" count discussed by the authors very likely overestimate the actual number of molecules by a significant margin. Without the correction of these blinking, this effect likely undermine the interpretation of Fig. 6 analysis. Large cluster seen in RBC data likely comes from the frequent blinking of the fluorophores.

We thank the reviewer for pointing this out, and have corrected the technical description of the STORM data to refer to "localizations" and not "molecules" throughout. We also were concerned about the potential re-blinking of the Alexa 647-phalloidin which could lead to overcounting. We have now included a calibration experiment using Alexa 647-conjugated IgG molecules adsorbed to poly-L-lysine coated coverslips. Under our imaging conditions, we found that the majority (>60%) of the IgG molecules showed a single localization, ~20% had two, and ~17% had 3 or greater. This is described in the Results and the data is included in a new Supplemental Figure 1B. Since the commercial IgG-Alexa 647 conjugates have several (3-8) dye molecules per IgG molecule (average 5 per IgG for the lot used), the total blinks observed likely underestimate the number of Alexa 647-phalloidin molecules under our experimental conditions. Consequently, the localizations that we see for Alexa 647-phallloidin in RBCs most likely correspond to only 1 out of 5 phalloidin molecules blinking a single time for the duration of the acquisition. This may be due to our use of Vectashield as a mounting medium which quenches Alexa-647 dyes, leading to bleaching and resulting in reduced re-blinking (Arsic *et al.*, 2020). Therefore, rather than overcounting, it appears that the observed fluorescence localizations may be undercounting the numbers of Alexa 647-phalloidins. This is discussed on page 10 in the Results.

3. From close inspection of sample preparation protocol between this study and Pan et al., it is clear that there are many differences. Given that the intent of this study appears to be to challenge Pan et al., at the very least, this reviewer would expect that the authors would try to replicate the exact same specimen preparations used in Pan et al., and compared to their own methods. At present, this is not done, and thus the contribution of different specimen condition cannot be ruled out.

We apologize for the confusion. The procedures we followed to prepare the RBC membranes and label them with Alexa 647-phalloidin were the same as that of Pan et al 2018, as far as we were able to determine from a careful reading of the Methods in that publication (Pan *et al.*, 2018).

4. One specific concern regarding STORM imaging of actin and specimen preparation is that the high concentration of Phalloidin is extremely important for high quality imaging as the authors of Pan et al. demonstrated in their earlier study, Xu et al. Nature Methods 2013. In this manuscript, the concentration of Phalloidin used seems to be lower than in Pan et al. by multiple fold. Incomplete or suboptimal labelling would certainly affect the observation of so-called 'uniform' distribution and easily leads to the observation of 'non-uniform' distribution.

As explained above, we actually did follow the sample preparation procedure of Pan et al 2018, including using 0.4 μM Alexa 647-phalloidin for the STORM experiments (Pan *et al.*, 2018). This information was buried in the Methods, and we have now included this information in the Results section as well. The Reviewer was likely misled since we used 0.14 μm fluorescent-phalloidin for the TIRF and Airyscan imaging experiments. For those latter experiments, we tested a wide range of phalloidin concentrations, and observed the same irregular, patchy distribution of F-actin staining by TIRF and Airyscan regardless of the phalloidin concentration from 0.14 to 0.4 μM. We chose to use the lower concentration for those experiments, but did use the higher concentration for the STORM experiments, as in Pan et al., 2018.

5. In this present study, only F-actin was imaged, whereas in Pan et al., a more complete array of molecules beyond F-actin, .e.g spectrin, 4.1, TMOD, adducing, etc. were imaged, and all showed a distribution pattern consistent with their conclusion. It would be interesting to see what are the distributions of these F-actin nodes component.

We are very interested in investigating the distributions of additional components of the RBC membrane skeleton, especially with respect to their dynamics. However, this will have to await a future study as we have been unable to perform any new experiments due to the COVID-19 shutdown of the normal donor blood collection service at the University of Delaware.

Response to Reviewer 2.

1. The title should convey the major findings (especially local motion of F-actin nodes) and distinguish this paper from the Pan et al. 2018 paper. Also, for readers unfamiliar with the RBC membrane, the term "actin filaments" may imply conventional extended actin polymers.

We have revised the title to: "Nanoscale dynamics of actin filaments in the red blood cell membrane skeleton", to emphasize the RBC actin dynamics presented in our manuscript, as suggested. We have also clarified wording throughout, including describing the short RBC actin filaments as "F-actin nodes", and specifying their length and numbers of subunits.

2. Figure 3 showing local movement of Sir-labeled actin in intact RBCs is the most important finding in the paper. The following points would further increase the impact of these experiments:

a. include a control with fixed RBCs

We included a control with fixed RBCs from a prior experiment as the bottom panel of a revised figure, new Figure 5.

b. address whether movements are active; ex determine effects of ATP depletion,

We included the results of an experiment performed previously at Scripps showing that pre-treatment of RBCs with actin depolymerizing drugs, Latrunculin A or Cytochalasin D, affected the distribution and intensity of the F-actin foci observed in TIRF microscopy (new Figure 4). These results suggest that formation and maintenance of the F-actin foci depend on actin filament polymerization, which is an ATP-dependent process. However, we were unable to perform new experiments with ATP-inhibitors, due to the University of Delaware canceling the normal blood donor service, as explained above.

c. present the data to better appreciate local dynamics. One suggestion: color t=0 red, t-2 sec green and then overlay the images; there should be some red and green where movement occurred along with yellow where the images overlap

This is a great idea. We revised the images as suggested, to better reveal the lateral movements and appearance and disappearance of F-actin foci in live RBCs. The red and green spots apparent in the images of live RBCs clearly show local movements, compared to the overall yellow color of the spots in the fixed RBC, indicating no movements after fixation (new Figure 5).

d. *consider moving the airy scan data to supplemental in order to focus more attention to* Figure 3.

We elected to retain the Airyscan data in the main text as Figure 2, to clearly establish the irregular pattern of F-actin staining in bright foci. In addition, several researchers in the RBC field have proposed enrichment of F-actin at the rim vs the dimple, and thus we felt it was important to establish the overall similar intensity of F-actin staining along the entire RBC membrane, at the rim and at the dimple. Other RBC researchers have proposed that F-actin is in the cytoplasm, which this imaging imaging clearly shows is not the case.

3. This study, using a combination of TIRF and STORM resolves individual actin monomers within actin nodes, which I believe has not been previously reported. The authors should highlight this new level of resolution, both by contrasting it with other reports and by explicit presentation of data. For example, sets of linear arrays of dots could be displayed and the number of dots/array and lengths of arrays quantitated.

Based on the comments of Reviewer 1 regarding the STORM data, we re-evaluated the statistical analyses used to evaluate clustering of the fluorescence localizations. Our new computational analyses using the Ripley *H* test supported our original conclusions that localizations are clustered (new Figure 3, new Supplemental Figures 2). However, as we scrutinized the data, we realized that the localization accuracy for the Alexa-647 phalloidins in our experiments (σ = 21 to 23 nm; Gaussian distribution Full Width Half Maximum ~ 50 nm) is insufficient to measure the lengths of the individual short actin filament nodes accurately (~37 nm from electron microscopy). Additionally, our calibration experiment to evaluate the Alexa 647-phalloidin blinking frequency (Supplemental Figure 1B) showed that the observed localizations under-reported the number of dye molecules, with only ~1 in 5 dye molecules blinking at all (see response above to Reviewer 1, point 2). We also have no way to determine whether the 1/5 of Alexa 647-phalloidins that do blink are in the middle or at the ends of the filaments, thus leading to another source of error in a length measurement for the short ~37 nm filaments. Thus, while we can conclude that small clusters of Alexa 647-phalloidin localizations (2-11) likely originate from individual filaments and larger clusters from groups of filaments (11+) (new Supplemental Figure 3), we do not think we can use the positions of individual localizations to accurately measure filament lengths. Future studies with higher resolution single molecule imaging will be required to address this question.

4. The Pan et al paper actually presents experimental data that are consistent with this study. For example, 200 nm gaps in the spectrin network were noted. Moreover, the number of F-actin nodes/square micron was around 80 (corresponding to about 11000/cell, which is less than the predicted ~ 30,000/cell). This was interpreted as partial labeling, but is could also be explained by clusters of nodes that may not have been resolved by 3D STORM. These points could be addressed in the discussion.

We thank the reviewer for these important insights. The discussion has been rewritten to make these points (pages 17-18).

5. The discussion gets lost in details and does not mention the finding of node dynamics. A suggestion is to start with a bullet statement of the principal new discovery of locally dynamic behavior of F-actin nodes which by definition would be expected to disrupt polygonal patterning of the spectrin-actin membrane skeleton. Then proceed, without subheadings, to place the findings in context with the literature.

The discussion has been extensively revised and shortened to emphasize the F-actin node dynamics, and subheadings removed, to place the findings in context with the literature.

References

Arsic, A., Stajkovic, N., Spiegel, R., and Nikic-Spiegel, I. (2020). Effect of Vectashield-induced fluorescence quenching on conventional and super-resolution microscopy. Sci Rep *10*, 6441.

Kiskowski, M.A., Hancock, J.F., and Kenworthy, A.K. (2009). On the use of Ripley's K-function and its derivatives to analyze domain size. Biophys J *97*, 1095-1103.

Pan, L., Yan, R., Li, W., and Xu, K. (2018). Super-Resolution Microscopy Reveals the Native Ultrastructure of the Erythrocyte Cytoskeleton. Cell Rep *22*, 1151-1158.

RE: Manuscript #E21-03-0107R
TITLE: "Nanoscale Dynamics of Actin Filaments in the Red Blood Cell Membrane Skeleton"

Dear Velia,

Thank you for sending us your revised manuscript, which I am now happy to accept for MBoC.
I note that you've made a good-faith attempt to address the reviewers' comments, especially given that Covid protocols limited further experimentation for you. You will note that one reviewer is supportive. Unfortunately, the other reviewer was unable to see the MS again, but it seemed to me that you'd endeavoured to address their comments by reanalysis and rewriting. I think that the community is better served by having your report available, rather than delaying the publication process by enlisting a new reviewer.

Best wishes,

Alpha

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Fowler:

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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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #2 (Remarks to the Author):

The revised manuscript addresses most of the reviewers' concerns and is acceptable for publication. A few comments for the authors to consider in presentation:
1. The title "Nanoscale Dynamics of Actin Filaments in the Red Blood Cell Membrane Skeleton" does a better job of conveying

the new findings but still could be improved. The authors should consider changing "actin filaments" to "actin filament nodes" to clarify for general readers that these are not the standard micron-length filaments typically imaged in cells. Also, the title implies that actin filament dynamics were already known, but now are imaged on the nanoscale. My understanding is that actin filament dynamics. Perhaps "Actin filament nodes exhibit nanoscale dynamic behavior in erythrocyte membranes" would convey the new findings?

2. The discussion is improved but still gets lost in detail. The authors should consider beginning with stating all of the principal findings in the first paragraph. Then move on to interesting implications, such as the possibility the motion is driven by myosin. They also could mention earlier findings of membrane flickers etc. The details now in the discussion could be presented in the results and do not need to be repeated.