Review of: "Focal adhesion membrane is dotted with protein islands and partitioned for molecular hop diffusion"

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Focal adhesion membrane is dotted with protein islands and partitioned for molecular hop diffusion

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Advance Summary and Potential Significance to Field:

This manuscript presents a substantial amount of new information concerning the model of focal adhesion and the theories developed for analyzing the movements of molecules undergoing hop diffusion in the compartmentalized plasma membrane. The topic addressed is interesting in attempting to delineate the structures and distributions of the residency times within a compartment of FA. The findings presented are exciting, and the use of an ultrafast camera system strengthens them. Finally, I want to acknowledge the quality of the figures in this paper, even though some of them, like Fig. 2, need more attention to be adequately understood. Although this paper could provide a valuable contribution, it would require addressing issues before publication.

Comments for the Author:

In this revised manuscript, the authors have provided substantial amounts of new data, revised conclusions, and clarifications that have greatly strengthened this study to the extent that it may now ultimately become acceptable for publication after appropriate revisions.

1. The authors refer to the property of the basal PM outside the FAs, showing that the “basal PM is compartmentalized like the apical PM and that the dwell lifetimes of TfR and Cy3-DOPE within compartment in the basal PM outside the FAs are the same as those in the apical PM”, yet the data remain somewhat confusing. Specifically, even though the quantification in Figures 2B, C, and D appear

convincing, the quantitative loss of p values. Furthermore, searching for significance in the Table 1 is even more problematic due to the loss of consistency between Figures 2B, C, and D and the data order in Table 1.

2. The authors *claimed at the top of page 10* "Assuming that 60% of mEos3.2 is fluorescent (Baldering et al., 2019) and that 30% of mEos3.2 paxillin is recruited to the basal PM, then 1,650,000 copies of mEos3.2-paxillin would be expressed in a cell." However, each transfection method creates different conditions, which may impact the expression of the tagged protein. Did authors experimentally assess the expression efficiency of fluorescently tagged paxillin compared with the total abundance of paxillin in the cell? At a minimum, the authors should acknowledge this.

3. The authors claimed that "The paxillin islands occupy 15 ± 0.81% of the FA area." Can the authors rule out the possibility that the mean size of paxillin islands detected by fluorescence intensity might be more prominent than referred ~15% of the FA area occupancy due to recruitment of untagged paxillin?

4. On the bottom of page 10, the authors revealed that "the median compartment size was reduced to 74 nm (from 109 nm in the bulk basal PM, Fig. 6 B). Namely, the compartment area size was reduced by a factor of 2.2." Based on provided data, the compartment area size was reduced by a factor ~ 1.5 or by 32%.

5. Figure 6F shows a new FA adhesion model consisting of many FA islands combined in the "archipelago", which is an exciting view of FA architecture. The authors suggested that "the rapid diffusion of FA proteins inside the FA domain would facilitate their rapid exchanges with those located in the bulk domain, allowing the simultaneous formation and disintegration of the FA-islands (including paxillin islands) everywhere in the FA region". Do authors have any experimental evidence to support this statement?

6. At the bottom of page 13, the authors point out that the protein compositions of the FA-protein islands could vary, and some FA-protein islands may contain only a few paxillin molecules. In addition, it would be good to mention the differences between focal complexes and focal adhesion. These are two different stages of FA formation, which differ in the size and the content of adhesive proteins. At a minimum, the authors should acknowledge this.

7. The authors may also consider that the adhesion molecules' structure and mobility may be modified based on whether or not they are adhering to fixed or soluble matrix proteins.

8. Discussion is well written, and the authors clearly pointed out the main findings. However, in view of the novel microscopic methods that they are describing in the paper for looking at molecular hopping on the plasma membrane and the organization of proteins in adhesion structures, it may be helpful to discuss this new technology and discuss, in particular, what further information this methodology brings and what might be some of its limitations.

9. In conclusion, the authors stressed the physical property of the FA islands in the novel archipelago model. Anyway, there is a noticeable lack of conclusion on the data's biological function and how the paper really advances the field.

10. In the Introduction paragraph, the authors explained the ultra-fast camera system, referring to the

companion paper (Fujiwara et al., 2021). Unfortunately, the Discussion section did not correctly consider their parallel ultra-fast PALM paper that I think is intended to complement the current paper.

Minor points:

1. The authors compare the behavior of cells FA, but besides the Material and Methods paragraph, they never clarified whether equal amounts of fibronectin were present on the surface.
2. In the legend of Figure 6C is “Statistically significant difference between before and after stimulation with P = 4.9 x 10-4, using the log-rank test.” Is this sentence correct? If yes, that kind of stimulation was used in this experiment?
3. Discussion. It is not usual to provide the numerical data in the text if it is also shown in the Figures and results. It might be better to state what is the % level of reduction.