Review of: "Mass-Sensitive Particle Tracking to Characterize Membrane-Associated Macromolecule Dynamics"

Philipp Kukura[1]

1 University of Oxford

Potential competing interests: The author(s) declared that no potential competing interests exist.

The study by Steiert et al outlines a protocol for the use of mass-sensitive particle tracking (MSPT) to characterise the dynamics of protein-membrane interactions. Based on mass photometry, this technique measures the mass and location of single protein complexes on supported lipid bilayers label-free. The authors clearly describe a detailed stepwise approach to these measurements from the preparation of supported lipid bilayers to data acquisition and analysis parameters. The results and potential of MSPT are highlighted by applying it to the membrane-associating proteins annexin V and cholera toxin subunit B. The work is novel and of high value to the scientific community. Due to the lack of labelling requirements, this technique has the potential to fill gaps in our current knowledge on biophysical processes and mechanisms that other techniques struggle to address. Although, I did not find any significant issues with this manuscript, I do have suggestions on how it could be improved.

1. Line 32, “mass to contrast coversion” should be contrast to mass conversion
2. Lines 85-86: “… a more sophisticated image analysis approach had to be developed.” Temporal median filters are widely used in image analysis. I suggest rephrasing this to “an alternative image analysis approach had to be implemented” and to include some relevant papers.
3. Line 88, please add relevant references.
4. Line 108-109: Is there a specific reason to prepare the lipid mixture at 4 mg/mL?
5. Lines 111-112: Is there a specific reason to use positive displacement pipettes (PDPs) i.e. regular pipettes? They are the major source of the error for working with liquids with low surface tension. Lipid stocks are generally prepared in CHCl3 and/ or CHCl3-MeOH mix. With PDPs, there is an enhanced possibility of solvent evaporation and imprecise volume measurement. Have the authors considered gas-tight hamilton syringes should be used for lipid stock preparation.
6. Lines 123-124, is it necessary to keep the buffer the same for both bilayer formation and protein interactions? I assume that once bilayers have formed, buffers can be exchanged.
7. Lines 126-130, Is there a specific reason the authors use 5 mM MgCl2 in the buffer for lipid hydration and extrusion. Divalent cations like Mg2+ promote vesicle fusion. Would extruded vesicles not be stable in a buffer without Mg2+?

8. Lines 155-158: Have the authors compared the homo/heterogeneity of SUVs generated via sonication vs extrusion?
9. Lines 207-209: Regarding the assembly of flow chambers, is there an issue with loss of protein over time to the glass

surface of the 24x24mm coverslides? As this surface is not hydrophilized, it most likely will not form a lipid bilayer and proteins in solution may adsorb to it over time.

10. Lines 323-324: I would expect the concentration at which the particle density on the lipid bilayer becomes too high to reliably analyse to vary with the protein that is being used. Could the authors also provide a number in terms of particle density on the lipid bilayer, above which detection of single proteins becomes disturbed?

11. Lines 335-336: “If necessary, move the FOV to a position with a homogenous membrane using the lateral control.” Do the authors mean move the coverslide or move the FOV in the acquisition software?

12. A temporal median window of 1001 frames under the imaging parameters used here constitutes a period of approximately 5 s. At which point does long-term drift of the image become a problem, i.e. have the authors examined how the baseline noise of the images change as a function of the window size?

13. In Figure 4A, the authors highlight successfully fitted and rejected particle candidates. In these examples, the number or rejected candidates far outweighs the number of accepted ones. What determines whether a candidate particle is rejected?

14. Figure 4C: How does the slope of the mass-to-contrast calibration curve compare to that obtained in standard mass photometry experiments on glass? Also, why does the chosen minimum trajectory length vary across the figure panels?

15. Figure 5A-B: Have the authors recorded standard mass photometry measurements with the biotin-IgG and biotin-aldolase? Is the oligomeric composition in standard MP measurements similar to what is observed in MSPT measurements?

16. Line 641: “Only particles with a track length of at least 5 frames were included.”: In Supplementary Figure 1, the impact of background noise is shown using a minimum trajectory length of 7. Is there a significant change in this data when reducing the minimum trajectory length to 5?

17. Figure 6A: Are there any sources for the quoted molecular weights of AnV or CTxB? Also, why were these two proteins with such low masses chosen? Additionally, the results presented in Figure 6 seem counterintuitive. In panel A, the authors show mass and diffusion coefficients of AnV and CTxB. However, upon mixing two proteins on bilayer compositions that should ideally recruit both CTxB and AnV, resulting mass and diffusion histogram resemble either AnV (Figure 6B, left panel) or CTxB (Figure 6B, right panel). I would have expected to see broadening of both mass and diffusion for mixtures 1:1 and 1:2 mix as one would expect to see both proteins bound and diffusing independent of each other. Can the authors shed light on their results?

18. Lines 754-755: “However, macromolecule diffusion coefficients ranging between 0.05 and 10 μm2/s can clearly be resolved.” At D = 10 μm2/s and 200 Hz, the mean squared displacement of a particle during one frame is ~450 nm. Is the linking algorithm still able to perform reliably with particles diffusing so quickly? Additionally, other effects such as blurring of the particles due to motion will become more significant and result in underestimation of both mass and diffusion coefficient, so I would expect the quality of the tracking data to deteriorate significantly. Do the authors have any data to base this claim on?

19. In supplementary figure 1C, the authors show histograms of contrast detected on bilayers alone and in the presence of Streptavidin. The contrast detected in these conditions, show significant overlap between bilayers alone and in the presence of streptavidin. a) Have the authors checked the variability of detected contrast across different bilayers? b) In

this figure, the amount of streptavidin on the bilayer seems much higher than in typical MSPT experiments (Fig. 4a). How do the numbers of particles detected due to background fluctuations on empty SLBs compare to the number of STP particles detected at lower particle densities (e.g. at densities similar to the images shown in Fig. 4a)?