Supplementary Information

AFM kymographs can provide robust conformational transition detection for pharmaceutically relevant membrane protein in lipid bilayers

Katherine G. Schaefer[1], Arthur G. Roberts[2,*], Gavin M. King[1,3,*]

[1]Department of Physics and Astronomy, University of Missouri, Columbia, MO 65211

[2]Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA 30602

[3]Joint with Department of Biochemistry, University of Missouri, Columbia, MO 65211

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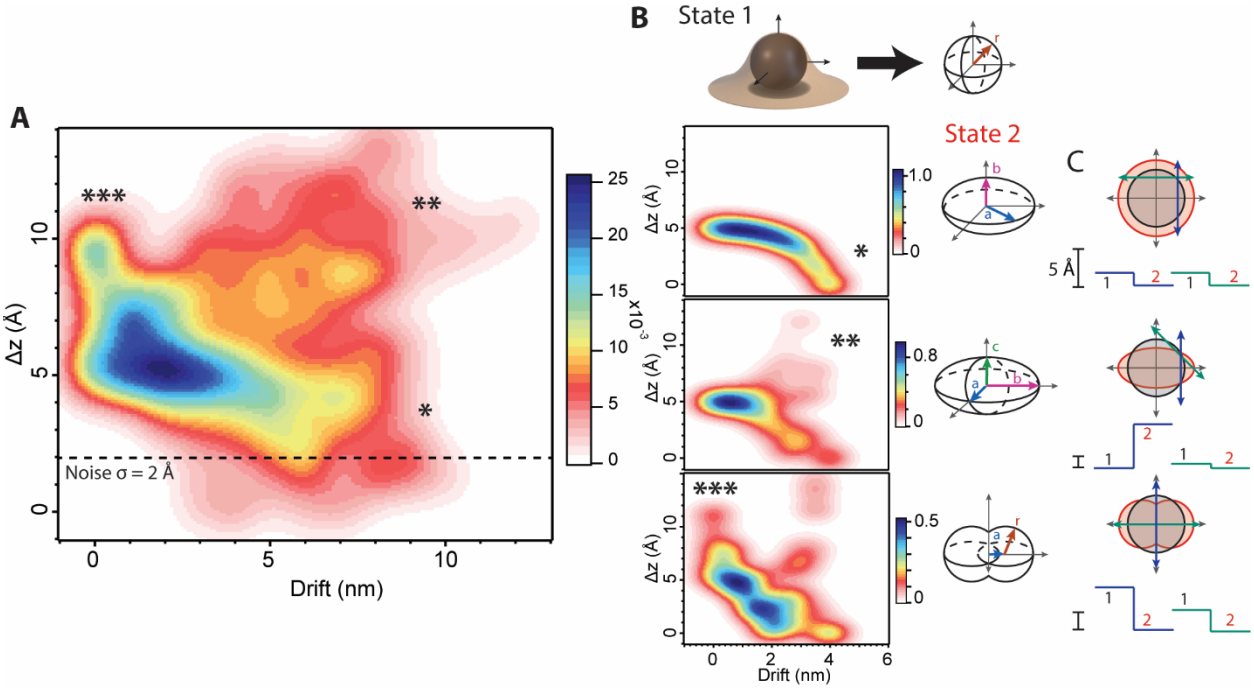
Generation and interpretation of Δz vs lateral drift heat map: We sought to quantify conformational height changes, Δz, in a meaningful way. A heat map of Δz versus drift was generated for all scan angles (**Fig. S1A**). Despite the relative simplicity of the two-state (open-closed) simulation, the data demonstrate a rich texture in the heat map. To aid interpretation, we further simplified the simulation from PDB coordinates to basic three-dimensional shapes. We used a single sphere of radius r = 4.8 Å to approximate the closed state as shown in **Figure S1B**, then geometries of increasing complexity as the open state: an oblate spheroid (a = 3.9 Å, b = 3.55 Å), an ellipsoid (a = 3.09 Å, b = 5 Å, c = 3.55 Å), and two overlapping spheres (r = 3.55 Å, a = 2 Å), representing the increased separation of the NBDs in the open state. Because of the radial symmetry of the oblate spheroid, the resulting heat map is simple. Every scan angle produces the same drift-dependent Δz, which decreases from a maximum at the center of mass (*). The drift is demonstrated in **Figure S1C**, where two scan angles, 0° and 90°, with the same drift produce the same Δz. However, the ellipsoid is narrow on one axis and wide on another, imposing two trends in drift-dependent Δz as the kymographs sweep through the different scan angles. For the set of angles normal to the major or minor axes of the ellipse, Δz increases with drift (**), whereas for the intermediate angles, Δz decreases (**Fig. S1B**). Again, this is evident for two scan lines with similar drift but different angles (**Fig. S1C**). This trend can be observed in the main simulation (**Fig. S1A**), indicating a similar change in geometries for the PDB structures. Finally, an overlapping sphere geometry gives insight into the spread in Δz at the zero drift position. The kymograph analysis software compares the maximum pixels in the measurement. For the overlapping sphere, a scan between the spheres will have a lower maximum height than a scan across the tops of the two spheres (**Fig. S1C**). Similarly, separated NBDs in the inward-facing state of Pgp will produce a different Δz at the center of mass depending on the scan angle (***). With a

better understanding of the complexities in the heat map, we can see that most scans have a Δz above the noise (S/N > 1). Based on our heat map, kymographs that drift >2 nm away from the center exhibit a non-zero likelihood of having Δz below the noise. This results in consequences for both the accuracy of state detection, and the dwell time of each state.



Supporting Information Figure S1. The effect of lateral drift on Δ*z*. (A) The height difference Δ*z* resulting from all scan angles is plotted in a heat map versus lateral drift. The vast majority of Δ*z*'s are above the 2 Å noise level (dashed line). Complex behavior can be isolated into three trends: (*) Δ*z* decreasing with drift, (**) Δ*z* increasing with drift, and (***) bimodal Δ*z* at the center of mass. **(B)** States are simplified into basic 2D geometries, a sphere for State 1, and three other shapes for State 2 listed in order from top to bottom: an oblate spheroid, an ellipsoid, and overlapping spheres. The resulting Δ*z* vs lateral drift heat maps correlate with behaviors observed in panel **A**. The false color scale bars are unitless, normalized probability density. **(C)** The two states are overlayed in a top view as a graphical representation of how different scan angles (blue and green) with similar drift can affect Δ*z*, giving rise to the behavior on corresponding heat maps. Transitions from state 1 to state 2 are shown as steps from the maximum *z*'s for each scan line. Note that despite changes in scale, the 5 Å value for the scale bar is preserved for each example.