Pair Correlation Analysis Maps the Dynamic Two-Dimensional Organization of Natural Killer Cell Receptors at the Synapse

Per Niklas Hedde,1,2,# Elina Staaf,3 Sunitha Bagawath Singh,3 Sofia Johansson,3 and Enrico Gratton1,4,#

1Laboratory for Fluorescence Dynamics, University of California Irvine, Irvine, California 92697, United States.
2Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96813, United States.
3Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm 17177, Sweden.
4Lead Contact

#Correspondence should be addressed to phedde@uci.edu and egratton@uci.edu

Supporting Information

| Supplementary Figure S1. | 2D-pCF analysis of simulated image data of molecules diffusing in a mesh at different pCF distances. |
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| Supplementary Figure S2. | 2D-pCF analysis of simulated image data of molecules diffusing inside and outside of a narrow channel as a function of the pCF distance. |
| Supplementary Figure S3. | 2D-pCF analysis of simulated image data of 5,000 molecules diffusing in a mesh with the same and different numbers of molecules per compartment with and without bleaching. |
| Supplementary Figure S4. | Creating connectivity maps from 2D-pCF data to visualize effective molecular flow. |
| Supplementary Figure S5. | Immobile fractions do not interfere with 2D-pCF analysis. |
| Supplementary Figure S6. | 2D-pCF analysis of simulated image data as a function of molecule concentration and number of frames. |
| Supplementary Figure S7. | 2D-pCF analysis of simulated image data where barriers appear during the measurement. |
| Supplementary Figure S8 | 2D-pCF analysis of Anti-KIR antibody labeled with Abberior STAR 635 in cultured cells expressing B51-YFP and Cw4-YFP. |
| Supplementary Figure S9. | 2D-pCF analysis at different pCF distances of NK cell inhibitory receptor Ly49A labeled via YE1/48 antibody conjugated to MFP488-NHS. |
To be able to resolve structures of interest, the pair correlation distance must be carefully adjusted. To visualize the effect, we analyzed the data shown in Figure 2A at different pair correlation distances from 2-8 pixels (0.2-0.8 µm). The simulation plane was subdivided into 25 compartments that molecules that were otherwise freely diffusing cannot cross. Data was simulated as described in the Methods section. Although the simulated barriers were of zero thickness, at a pair correlation distance of 2 pixels (0.2 µm) the compartments are barely visible. This is because even for pixels close to the barrier most pixel pairs are located in the same compartment. At pair correlation distances of 4 and 6 pixels (0.4 µm and 0.6 µm) the barriers are clearly visible in the corresponding anisotropy maps. At 8 pixels (0.8 µm) pair correlation distance, the barriers are still visible but washed out. In this case of freely diffusing molecules, the larger the distance between pixel pairs, the less likely it is to observe the same molecule in both pixels, resulting in a reduced signal-to-noise ratio of the corresponding correlation functions at large pCF distances.
Figure S2. 2D-pCF analysis of simulated image data of molecules diffusing inside and outside of a narrow channel as a function of the pCF distance.

When molecule motion is highly directional, such as inside a channel, the probability to find the same molecule is high even at larger pCF distances.
Figure S3. 2D-pCF analysis of simulated image data of 5,000 molecules diffusing in a mesh with the same and different numbers of molecules per compartment with and without bleaching.

To study the effect, if any, of differences in concentrations between neighboring compartments as well as changes in intensity, for example, due to bleaching, we repeated the simulation of the data shown in Figure 2A (left column) with varying number of molecules per compartment.
(center column) as well as adding a molecule off-rate to simulated bleaching (right column). Data was simulated as described in the Methods section. Single frames, averages of all frames and anisotropy maps from 2D-pCF analysis are shown from top to bottom. Insets show the average intensity as a function of time. No difference can be observed between the simulation with the same numbers of molecules per compartment and the simulation with different numbers of molecules per compartment due to random seeding. The slight reduction of the overall anisotropy that can be observed for the data set with simulated bleaching is not an effect of the intensity change per se but is due to less molecules being present in the simulated frames due to off-switching resulting in a reduced signal-to-noise ratio. Nonetheless, barriers between compartments are clearly visible.
Figure S4. Creating connectivity maps from 2D-pCF data to visualize effective molecular flow.

To better visualize the results obtained with 2D-pCF analysis, connectivity maps can be created. Calculation of the anisotropy is followed by calculation of the angle of the major axis for all SPRITEs. Instead of just color coding the pixels according to the angle, segments with the direction indicative of the angle and length proportional to the anisotropy value can be drawn on top of the fluorescence image to highlight areas of strongly directional molecule movement. To avoid that many tiny segments overcrowd the connectivity map, segments are plotted only for anisotropies above a given threshold (e.g., $A > 0.1$). This does not mean that molecules are less mobile in regions not containing segments. In fact, the opposite is usually the case as reduced molecule mobility and high segment density occur near obstacles.
Figure S5. Immobile fractions do not interfere with 2D-pCF analysis.

To study the effect, if any, of immobile features in the image such as caused, for example, by cellular autofluorescence, we repeated the simulation of the data shown in Figure 2B and added areas of higher intensity (immobile fraction) to each frame. Data was simulated as described in the Methods section. Map of the locations of simulated microdomains and immobile fractions, single frames, averages of all frames and anisotropy maps from 2D-pCF analysis are shown from left to right. No effect of the immobile features can be observed in the anisotropy map demonstrating that the 2D-pCF method is selective for barriers to the motion of molecules and not affected by static spatial variations in intensity.
Because our simulated data does not contain signal fluctuations that correlate other than the fluctuations from moving molecules (the added shot noise does not correlate), there is no effect of the concentration on the anisotropy maps. Yet, it can be clearly seen that collecting a number of frames adequate for the given molecule brightness is important to generate high contrast 2D-pCF maps.
Figure S7. 2D-pCF analysis of simulated image data where barriers appear during the measurement.

To study the effect, if any, of differences in concentrations between neighboring compartments as well as changes in intensity, for example, due to bleaching, we repeat
Figure S8. 2D-pCF analysis of Anti-KIR antibody labeled with Abberior STAR 635 in cultured cells expressing B51-YFP and Cw4-YFP.

(A,B) Fluorescence images of Abberior STAR 635 labeled Anti-KIR in B51-YFP (A) and Cw4-YFP (B) expressing cultured 721.221 cells. (C,D) Anisotropy maps of the same cells shown in (A,B) calculated for a pair correlation distance $\delta r = 0.4$ μm. (E) Histograms of the anisotropy maps shown in panels (C,D). (F) Average anisotropies of Abberior STAR 635 labeled Anti-KIR in 6 (B51-YFP) and 5 (Cw4-YFP) cells. Compared to the data of Figure 3 showing a significantly higher presence of barriers for Cw4-YFP versus B51-YFP, a similar trend in the anisotropy distributions of KIRs can be found although not statistically significant for the antibody due to a lack of statistical power. Boxes represent the median and the first and third quartiles, notches represent the 95% confidence intervals, whiskers indicate the 95% ranges. $P > 0.05$ (Mann-Whitney test). Scale bar, 3 μm.
Figure S9. 2D-pCF analysis at different pCF distances of NK cell inhibitory receptor Ly49A labeled via YE1/48 antibody conjugated to MFP488-NHS.

As with the simulated data, we varied the pCF distance for a subset of the NK cell data. It can be seen that the overall trend of higher anisotropy values for the 2D-pCF maps of the inhibitory receptor Ly49A labeled with the YE1/48 antibody conjugated to MFP488-NHS in educated NK cells is conserved for pair correlation distances of 2, 4 and 6 pixels (0.2, 0.4 and 0.6 µm). Yet, the significance of differences between educated and uneducated cells is reduced with larger pCF distances as the signal-to-noise ratio declines for larger pixel pair distances as observed for the simulated data shown in Figure S1. Each point represents the average anisotropy of a single cell. The boxes represent the median and the first and third quartiles, and the notches represent the 95% confidence intervals. The whiskers show the 95% ranges. **P ≤ 0.01, ****P ≤ 0.0001, ns P> 0.05 (Mann-Whitney test).