Supporting Material

Visualization of HRas Domains in the Plasma Membrane of Fibroblasts

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S1 Time course analysis of clustering

As reported by Annibale et al, [1] molecular clustering observed in PALM experiments can be the results of subsequent reactivation of the same molecule. To correct for possible artefacts we introduced a tolerance time of 12 s, corresponding to 100 subsequent frames. The signals appearing within our positional accuracy during this time lag were assumed to arise from the same molecule. To test the validity of our assumption we performed a time series analysis of clustering on one cell. We applied Ripley's K analysis and DBSCAN algorithm to the full dataset and after merging peaks appearing within 1, 50 and 100 frames ,corresponding to 120ms, 1.2 s, 6 s and 12 s. Visual inspection of the resulting PALM images show clustering in all the resulting images, although the clusters appear to be better defined and more spaced with increasing time tolerance. Scarce or none differences are visible between 6 and 12 s merging (Fig. S1 A). Ripley's K function confirmed the visual inspection. Although we observed a difference in the absolute value of $L(r) - r$ (Fig.S1 B), particularly a drop after merging peaks within 6 s, the function lies above the one for a random distribution. The localization of the the maximum is unaltered within the error in the different conditions (Fig. S1 C). To confirm the results we performed the analysis with DBSCAN. The unclustered fraction increases from around 10% when considering each signal as arising from one molecule to 50% when merging molecules within 100 frames (Fig.S1 D). However this large discrepancy can be explained when looking at the histogram of the clusters size distribution. When merging only up to 1.2 s the algorithm detects very large clusters, including more than 2000 molecules, which account for up to 30% of the total. We interpret these extremely large aggregates as an artefact resulting from the blinking of the same molecules multiple times.
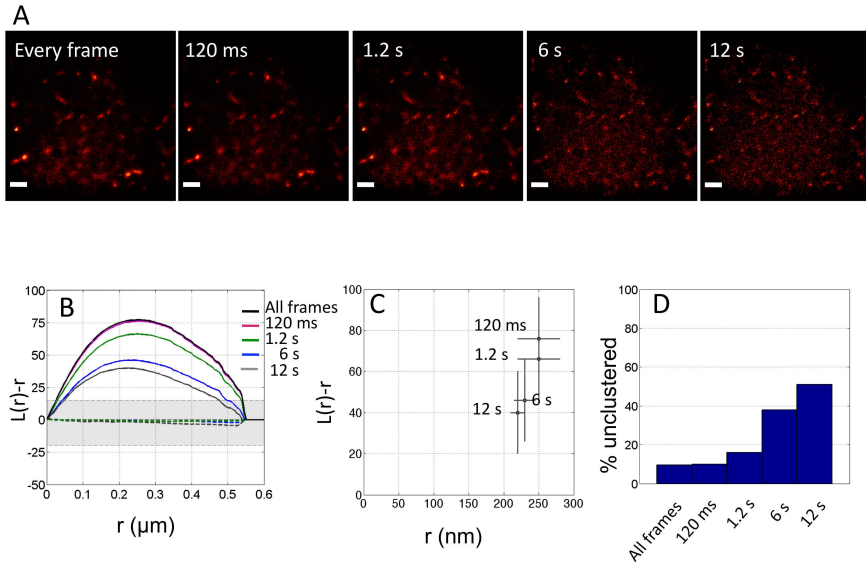
Figure S1:**A)** Palm image of HRas. Scale bar 1 μm. From left to right, PALM images were reconstructed using all the peaks, and merging peaks appearing every subsequent frame, and then every 10,50 and 100 frames.**B)** Solid lines: Ripley's K function for each of the resulting images. Dashed lines: Ripley's K function calculated from simulated random point patterns containing the same number of points. **C)** Positions and values of the maximum for the different tolerance times. **D)**Percentage of unclustered molecules as calculated from DB-SCAN for the different tolerance times.

S2 Estimation of Radius of Maximum clustering

As reported by Kenworthy et al. [2], the position of the maximum of $L(r) - r$ is a good estimate for the radius of maximum clustering. To validate this assumption, we performed a series of simulation varying the clustering radius. Fixed parameters in the simulations were: randomly distributed molecules Nr = 5000 molecules/cluster, number of clusters Nc = 600, and the number of protein per cluster Np = 15 molecules/cluster. The radius of maximum clustering was varied between 22 and 200 nm. As shown in figure S2 b-c, the position of the maximum of $L(r) - r$ tends to slightly overestimate the real cluster size, in agreement with [2].
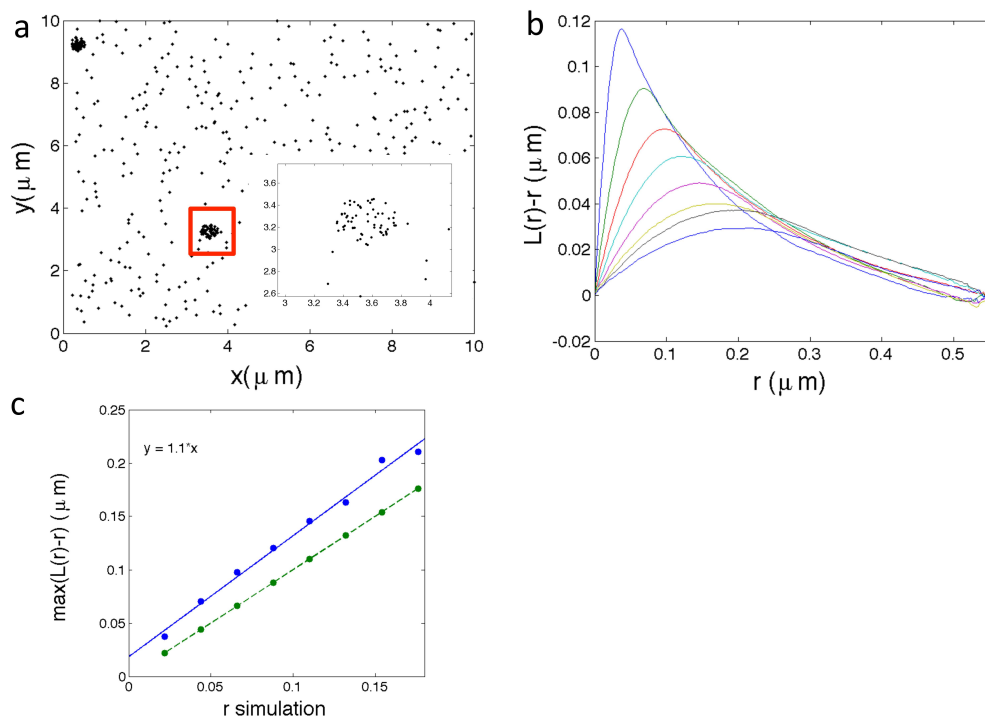
Figure S2: **a)** Example of a simulated point pattern. Two clusters with ~ 60 molecules are visible. Clustering radius r=200nm. From graphical inspection we confirm that the chosen radius corresponds to outcome of the simulation.**b)** $L(r)-r$ was evaluated for each simulation. As the radius increases the maximum of the curves shift toward higher radii and lower value of L_{max}.**c)** Blue solid line: position of the maximum of $L(r)-r$ as a function of the clustering radius. Green dotted line: actual domains radius, as set in the simulations.

References

[1] Annibale P., Vanni S., Scarselli M.,Rothlisberger U., Radenovic A. 2011. Identification of clustering artifacts in photoactivated localization microscopy. *Nat. Meth.* 8:527-528.

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