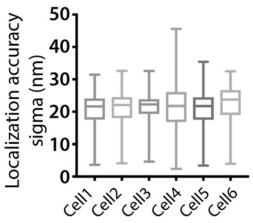
# Supplemental Materials

*Molecular Biology of the Cell*

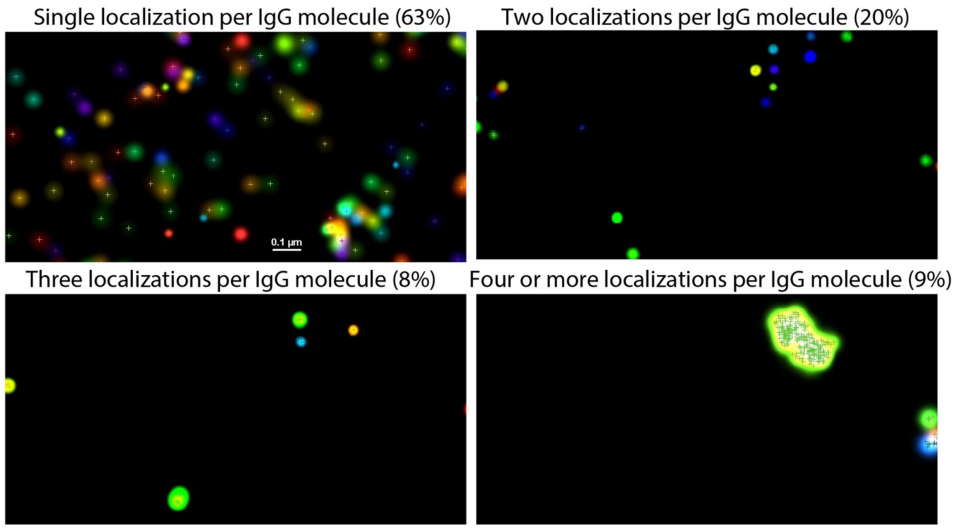
Nowak *et al*.

**Supplemental Fig 1**

**A**

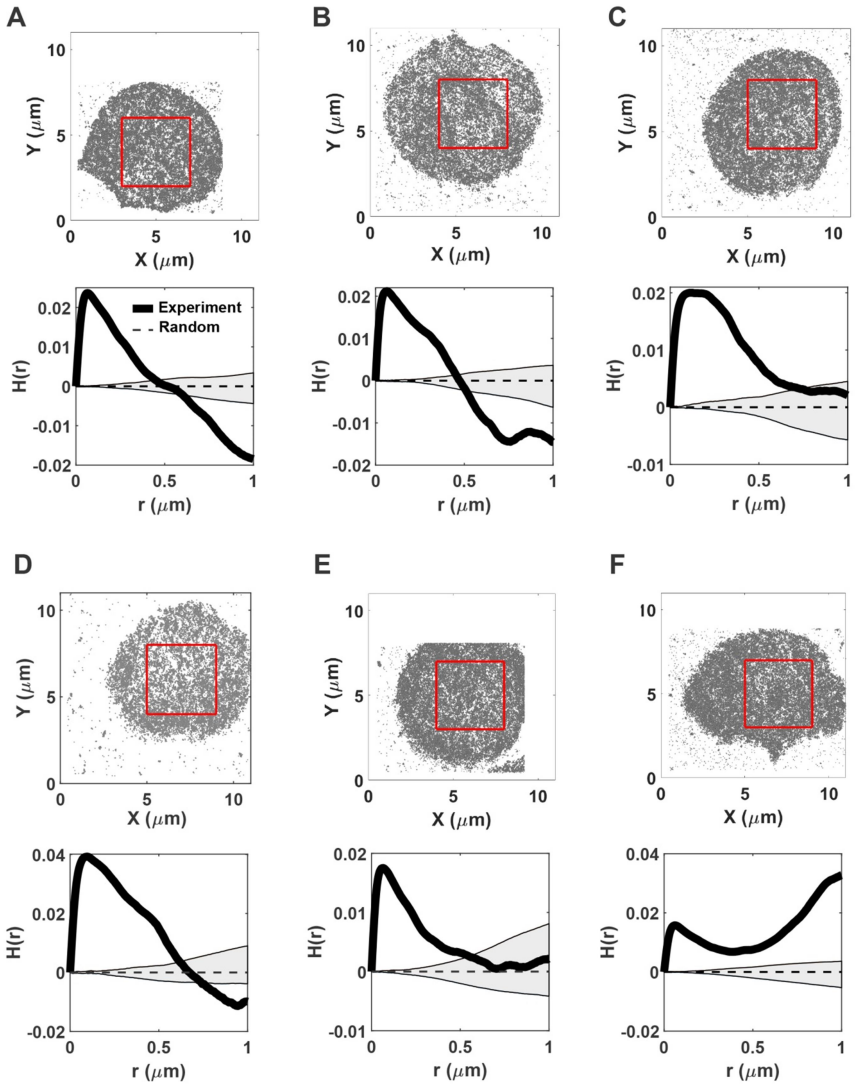



**B**



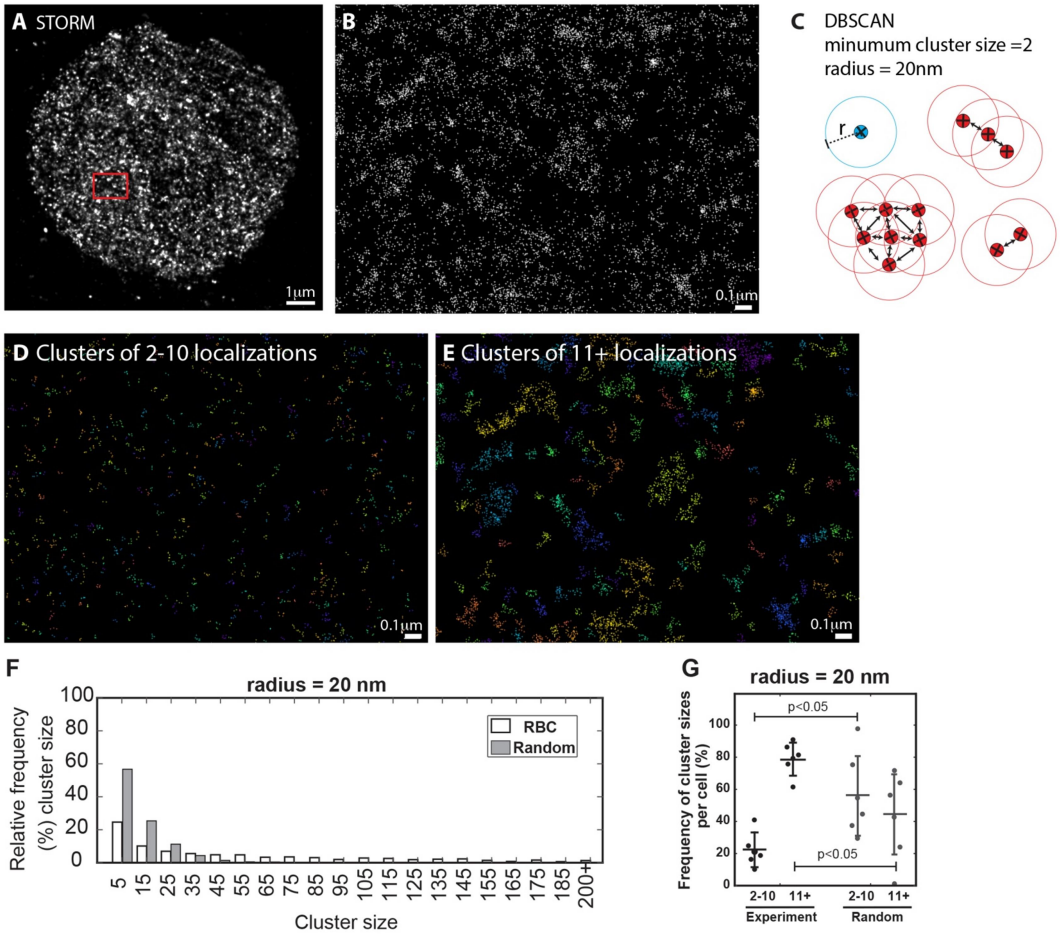
**Supplemental Figure S1.** (A) Localization accuracy for Alexa 647-phalloidin signals in each of 6 individual RBCs. The median values of the localization precision (sigma, $\sigma$) for each cell were plotted: Cell 1, 20 +/- 4 nm, n = 101,139; Cell 2, 21 +/- 4 nm, n = 107,836; Cell 3, 21 +/- 4 nm, n = 45,800; Cell 4, 21 +/- 5 nm, n = 128,838; Cell 5, 21 +/- 5 nm, n = 121,983; Cell 6, 22 +/- 5 nm, n = 117,846. The line in the box indicates the median, and the box shows the upper 25% and lower 75% and the whiskers the minimum and maximum. The Full Width Half-Maximum (FWHM) of the Gaussian for each localization is calculated as 50 to 53 (2.355 $\sigma$; 2.355 X 21 or 23). Thus, the centroids of fluorescence signals >25-26 nm apart can be from two individual molecules, while signals <25-26 nm apart may be from the same molecule. (B) STORM calibration of the blinking behavior of control Alexa-647-conjugated IgG molecules imaged by TIRF. The IgG molecules were well separated by limit dilution onto poly-L-lysine coated coverslips so that the distance between them exceeded their overall size (plus the added envelope of error based on the localization accuracy). The observed blinking behavior is depicted by the colored spots showing the Gaussian rendered localization, with each cross marking the centroid of an individual localization. After imaging for the same length of time under identical conditions used for RBCs, the majority (>60%) of the IgG molecules show only one cross indicative of a single localization. A lesser number of IgG molecules show 2-10 localizations, likely because each IgG molecule is conjugated to an average of 5 Alexa 647 molecules. A few large aggregates of IgG were also observed showing multiple localizations. Bar, 0.1 $\mu$m.

**Supplemental Figure 2**



**Supplemental Figure S2.** STORM images of Alexa-647 phalloidin labeled RBCs, analyzed by Ripley *H* test. (A-F, upper) Scatter plot of distribution of Alexa 647-phalloidin localizations at the RBC membrane of 6 different cell samples obtained from STORM images. We performed the Ripley analysis over a 4 μm X 4 μm square region of each RBC, shown by a red square in each panel. (A-F, lower) Ripley's *H* function as a function of distance r for 6 different cell samples. Dashed line indicates the expected value for the homogeneous Poisson process (known as complete spatial randomness) and the gray domain represents the simulation envelope constructed based on 100 realizations from a uniform distribution. A positive value of *H(r)* and above the simulation envelope indicates clustering, a negative value of *H(r)* and below the simulation envelope indicates dispersion, and the value of *H(r)* within the envelopes represents randomness over the spatial scale.

**Supplemental Fig 3**



**Supplemental Figure S3.** DBSCAN analysis of RBC STORM images versus randomly generated synthetic data reveals a non-random, clustered distribution of Alexa-647 phalloidin localizations in RBCs. (A) STORM image of Alexa 647-phalloidin localizations in an RBC (same RBC as shown in Figure 3A). Red box shown at higher magnification in panel B. Bar, 1 μm. (B) High magnification image of boxed region in panel A. Bar, 0.1 μm. (C) DBSCAN parameters used to define classification of a cluster. The minimum cluster size was set to 2, and localizations are included in a cluster only if they are within 20 nm. (D, E)) Visual depiction of clusters identified by DBSCAN, with localizations in the same cluster coded the same color, and adjacent clusters a different color. Bars, 0.1 μm. (D) Clusters with 2-10 localizations. (E) Clusters with 11 or more (11+) localizations. (F) Scatter plot of distribution of Alexa 647-phalloidin localizations at the RBC membrane obtained from TIRF/STORM images as in Panel A. The red circle marks the RBC perimeter. Inset shows a clustered distribution in the zoomed area. (G) Scatter plot of randomly generated uniform data distributed within the RBC area (red circle in panel A). The number of data points in random distributions is set equal to the number of Alexa 647-phalloidin localizations obtained from STORM images of RBCs as in panel A. (H) Histograms of cluster sizes and frequency in an individual representative RBC (open bars) compared to random data (shaded bars) for the same total number of localizations, with a minimum cluster size of 2 and radius of 20 nm. A range of cluster sizes from 2-10 up to >200 is observed, with larger clusters more abundant in experimental as compared to random data sets. Cluster sizes are grouped into bins of 2-10, 11-20, 21-30 etc., and centered on 5, 15, etc., for purposes of display. (I) Frequency of clusters with 2-10 and >11 (11+) Alexa 647-phalloidin localizations in RBCs, versus random data. The frequency of clusters with $\geq$11 localizations in RBCs is significantly larger than for the random data. We report the frequency of cluster sizes for each distribution as Mean ± SD for N = 6 RBCs. Each point represents an individual RBC.