Supplemental Information

The Membrane Skeleton Controls Diffusion Dynamics and Signaling through the B Cell Receptor

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Supplemental Experimental Procedures

Position accuracy

To determine the position accuracy an estimate based on the theory in (Thompson et al., 2002) and a direct measurement were used. The contribution of the photon noise to the position accuracy for a given number of $N$ photons recorded with pixel size $a$ and a spot size of standard deviation $s$ is

$$\langle \Delta x^2 \rangle = \frac{s^2 + a^2/12}{N}$$

The EMCCD camera does not give a readout in photon counts, so we estimate the number of detected photons during one frame as 100 (Elf et al., 2007). The pixel size is 0.107 µm and the half width of the peaks was measured to be 0.25 µm resulting in a root mean squared (RMS) position accuracy of 0.025 µm due to photon noise. The contribution due to background noise of standard deviation $b$ is given by

$$\langle \Delta x^2 \rangle = \frac{8\pi^4 b^2}{a^2 N^2}$$
The relation between peak intensity $I$ in one pixel and total intensity $N$ for a Gaussian peak of standard deviation $s$ is

$$I = \frac{a^2 N}{2\pi s^2}$$

This allows calculating the peak intensity for a signal of 100 counts to 2.9 photons and together with the measured signal to noise ratio of 7.2, the standard deviation of the background noise will be 0.4 photons. The resulting RMS error of the position accuracy due to background noise is then 0.012 µm. Together with the contribution from photon noise we have a total position accuracy of 0.037 µm.

Furthermore it is possible to determine the position accuracy from the intercept $A$ of the linear fit to the mean square displacement (MSD) curve (Martin et al., 2002). The relation is

$$\langle (\Delta r)^2 \rangle = A / 2$$

This results in a RMS error of 0.061 µm for the measurement of IgM particles on live cells and 0.036 µm for anti-IgM Fab immobilized on glass. The same result was obtained for the immobilized particles by directly calculating the position accuracy by using the mean position of the track as reference. We conclude that we can determine the position of single particles under our experimental conditions with a precision of at least 0.061 µm, which is below the diffraction limited optical resolution of the microscope but comparable to other single molecule TIRFM experiments (Bruckbauer et al., 2007; Elf et al., 2007; Mashanov and Molloy, 2007; Umemura et al., 2008).
Single particle tracking

We used the well-established single particle-tracking algorithm by J. Crocker and D. Grier (Crocker and Grier, 1996) in the Matlab (The MathWorks, Natick, MA) implementation by Daniel Blair and Eric Dufresne (http://physics.georgetown.edu/matlab/). The raw images were band pass filtered to reduce noise and background of a size larger than the particle size. Particle positions were then determined in two steps, first by finding local maxima and then by measuring the centroid position with sub-pixel accuracy. Finally the particle positions were linked to generate tracks. A minimum track length of 10 was used to discard short tracks, which can be caused by statistical noise, and to have sufficient positions for Mean Squared Displacement (MSD) analysis.

Data Analysis

For individual trajectories of N points \((x_i, y_i)\) measured at time intervals \(\Delta t\), the mean square displacement \(MSD\) for a time lag \(n\Delta t\) was calculated using a running average along the trajectory (Ritchie et al., 2005).

\[
MSD(n\Delta t) = \frac{1}{N-n-1} \sum_{i=1}^{N-n-1} (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2
\]

In the case of Brownian diffusion the \(MSD\) increases linearly with time lag. The diffusion coefficient can be obtained from the slope of the MSD vs. time-lag curve, for diffusion in two dimensions the relation is:

\[
MSD = 4 \, D \, n\Delta t
\]
Diffusion coefficients for individual trajectories were determined from the first three data points of the MSD curves of the individual trajectories. The restriction to just three points was used because the error in MSD increases with time lag as the number of independent measurements decreases (Qian et al., 1991).

**Distribution of diffusion coefficients**

Histograms of diffusion coefficients can be compared to a probability distribution $p(D_e)$ of apparent diffusion coefficients $D_e$ for a given true diffusion coefficient $D_0$ and a given number of independent data points $N = N_{total}/(n\Delta t)$ calculated according to (Vrljic et al., 2002)

$$p(D_e)dD_e = \frac{1}{(N-1)!} \left( \frac{N}{D_0} \right)^N (D_e)^{N-1} \exp\left( -\frac{N D_e}{D_0} \right) dD_e$$

This distribution is only valid if tracks of the same length are used and only independent data points are used to calculate the diffusion coefficients. However we found no apparent difference in the histograms when we analyzed the tracks with different length as described above. Therefore a comparison of the distribution with the data as analyzed throughout the paper should be valid. For the data set from Figure 1c (IgM), this comparison is shown in Supplementary Figure 1d with the following parameter: Track length of 21 steps, true diffusion coefficient is the measured median of 0.032 $\mu$m$^2$s$^{-1}$ and three independent data points. From the curves
it is clear that no diffusion coefficients in the lowest bin and no values above 0.06 µm²s⁻¹ are expected for IgM if the histogram would have originated from a single diffusion coefficient. This strongly indicates that the data has different contributions of slow and fast diffusion.

**Image and sub-trajectory analysis**

To identify regions of high actin or ezrin content, TIRFM images were cropped and a spatial band-pass filter applied to remove noise of 1 pixel size and larger scale features (typically 6 pixels). A binary image was obtained by application of a threshold determined by Otsu’s method (Otsu, 1979). White pixels in the binary image are regions above the threshold and are used to identify areas of high fluorescence in the original image. This creates a mask to identify sub-trajectories of at least 10 steps inside and outside these regions. These were analyzed separately using MSD analysis to determine the diffusion coefficient. Furthermore an edge detection algorithm was used to visualize the boundaries of the binary image (Figure S4).
**Supplementary References**

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Figure S1. Single particle visualization of Cy3-labelled anti-IgM Fab
(A) Single step photobleaching of Cy3-labeled anti-IgM Fab. The intensity profile of a single particle imaged for 1 s shows characteristic single step photobleaching. (B) Schematic to illustrate the calculation of the mean square displacement (MSD) for a given time lag. The lines show the displacements, which are squared and averaged to calculate the MSD of a given time lag (black: time interval 1 frame, green time interval 2 frames). The MSD was calculated as a running average, so that e.g. for a time lag of 2 frames the squares of the displacements 2-4, 4-6, 6-8 and 8-10 were also taken into account. (C) Averaged MSD vs. time lag curves. In blue, MSD vs. time lag averaged over 54 trajectories of IgM single particle from one live cell. The graph shows mean and standard error as well as a linear fit, the good agreement indicates random diffusion on this time scale. The mean diffusion coefficient from this fit is 0.055 µm²s⁻¹. In red, time lag averaged over 49 trajectories of single particle of anti-IgM Fab immobilized on glass. The mean diffusion coefficient from this fit is 5.5x10⁻⁴ µm²s⁻¹. (D) Relative frequencies of single molecules of IgM (black squares) with diffusion coefficients in the indicated diffusion bins from Figure 1c and theoretical distribution of diffusion coefficients of IgM (red line), see supplementary methods. Red symbols are values after binning.
Figure S2. Diffusion of MHC class I is similar to IgM-H2
(A) Schematic representation of the various IgM BCRs: wild-type IgM (IgM-WT) associates with Igα (green) and Igβ (yellow); a chimeric IgM with extracellular domain of IgM (blue) fused to the transmembrane and intracellular domains of MHC class I (black) (IgM-H2); and a chimeric IgM with the extracellular domain of IgM fused to the transmembrane domain of MHC class I and the intracellular domain of Igβ (yellow) (IgM-Mutβ). (B-C) Comparison of the diffusion coefficients (B) and distribution histogram (C) of single molecules of IgM and MHC class I in the A20 B cell line. 300 representative diffusion coefficients from a total of 500-1000 from 2 independent experiments. (D) Schematic representation of the two Hel proteins: a chimeric Hel (magenta) fused to the transmembrane and intracellular domains of MHC class I (black) (Hel-H2); and a chimeric Hel fused to the transmembrane domain of MHC class I and the intracellular domain of Igβ (yellow) (Hel-Igβ).
Figure S3. IgM molecules diffuse linearly within actin-rich filopodia. (A) TIRFM images showing the diffusion of IgM (red) in A20 B cells expressing Lifeact-GFP (green) on fibronectin-coated coverslips. White circles in the right panels highlight the tracking of IgM along actin-rich filopodia extending from the periphery of the cell contact. Scale bar: 2 μm. (B) Individual BCR tracks shown in different colours. (C) Overlay of IgM tracks on TIRFM image of actin network. Tracks are color coded red for inside actin-rich region and yellow for outside actin-rich region.
Figure S4. Masks of actin/ezrin rich regions.

(A) Image analysis to obtain the regions. The cropped original actin/ezrin TIRFM image is band-pass filtered and a threshold applied to obtain a binary image, which serves as a mask for further analysis, white pixels show regions of high actin/ezrin concentration. An overlay of the edges of the mask with the original image to visualize the result is also shown.

(B) Simulated random walks with $D = 0.1 \, \mu m^2 s^{-1}$ and circular zones of different sizes (white circles, size indicated above the image) which are generated independently from the walks.

(C) Error by analysing sub-trajectories in small regions. Shown is the measured diffusion coefficient of tracks inside the zones as function of the zone diameter (mean and standard error of 20 simulations, the solid line is a guide to the eye). Dashed lines show the decrease to 0.082 $\mu m^2 s^{-1}$ at 1 $\mu m$ zone diameter indicating a maximum error of 18% in our experiments using this type of analysis. Scale bars 1 $\mu m$. 
Figure S5. Ezrin-GFP recovers more rapidly than Actin-GFP following photobleaching
(A) Fluorescence recovery after photobleaching of Actin-GFP and Ezrin-GFP. Representative images of Actin-GFP (top row) and Ezrin-GFP (lower row) pre-bleach, immediately after 50 ms bleach, and 1.8 s of recovery. (B) Plot of fluorescence recovery of Actin-GFP (black line) and Ezrin-GFP (red line). Mean ± SEM from at least 20 cells from two independent experiments.
Figure S6. Jasplakinolide induces F-actin polymerisation.
(A) Jasplakinolide induces F-actin polymerisation in patches at the plasma membrane and intracellular regions. A20 B cells stably expressing Actin-GFP (green) were imaged by confocal microscopy before and after treatment with 1 µM jasplakinolide (JP). Cells were pre-labelled with B220 (red) to delineate the plasma membrane. Scale bar: 5 µm. (B) Primary naive B cells were treated or not with 1 µM jasplakinolide, incubated for the indicated time and then cells were lysed. Cell lysates were separated into soluble (S) and insoluble (P) fractions, subjected to SDS-PAGE and western blotted for actin. Increases in actin within insoluble fraction is indicative of F-actin polymerisation. (C) Quantification of F-actin content was determined by total intensity of insoluble/ soluble + insoluble fractions represented as percentage.
Figure S7. Representational model of actin-induced BCR signaling. Steady-state actin dynamism may permit low intensity BCR signaling (red) which may then feedback (indicated by black arrows) into alterations of the actin cytoskeleton. Gross alteration of the actin cytoskeleton induces increased BCR diffusion and robust BCR signaling. Two possible models are depicted which may account for how an increase in BCR diffusion upon disruption of the actin cytoskeleton is related to signaling. One model is that the actin cytoskeleton may immobilize BCR together with phosphatases during the steady-state and disruption of the actin cytoskeleton releases this inhibitory interaction as BCRs diffuse away (left model). Alternatively, disruption of the diffusion barrier defined by the actin cytoskeleton increases the mobile fraction of the BCR and may thus increase the probability that the BCR will encounter an activated kinase or coreceptor (right model).