

Actin restricts FcεRI diffusion and facilitates antigen-induced receptor immobilization

Nicholas L. Andrews^{1,4}, Keith A. Lidke^{2,3,4}, Janet R. Pfeiffer¹, Alan R. Burns³, Bridget S. Wilson¹, Janet M. Oliver¹ and Diane S. Lidke^{1,5}

The actin cytoskeleton has been implicated in restricting diffusion of plasma membrane components. Here, simultaneous observations of quantum dot-labelled FcεRI motion and GFP-tagged actin dynamics provide direct evidence that actin filament bundles define micron-sized domains that confine mobile receptors. Dynamic reorganization of actin structures occurs over seconds, making the location and dimensions of actin-defined domains time-dependent. Multiple FcεRI often maintain extended close proximity without detectable correlated motion, suggesting that they are co-confined within membrane domains. FcεRI signalling is activated by crosslinking with multivalent antigen. We show that receptors become immobilized within seconds of crosslinking. Disruption of the actin cytoskeleton results in delayed immobilization kinetics and increased diffusion of crosslinked clusters. These results implicate actin in membrane partitioning that not only restricts diffusion of membrane proteins, but also dynamically influences their long-range mobility, sequestration and response to ligand binding.

Signal transduction from the external environment to the cell interior is typically mediated by ligand-bound transmembrane receptors embedded in a lipid bilayer. In many systems, receptor activation is associated with changes in receptor dynamics and membrane topography^{1–3}. Among these are the multi-chain immune recognition receptor family members that include the B and T cell receptors, and the high affinity IgE receptor (FcεRI) of mast cells and basophils, which are crucial to the execution of key events in the immune response. Crosslinking of these transmembrane receptors induces receptor oligomerization, protein and lipid kinase activation, and Ca²⁺ mobilization, leading in turn to cytoskeletal reorganization, receptor trafficking and cell-specific responses, including altered gene expression^{4–6}. These signalling events have been well studied using biochemical techniques, but the precise mechanism by which oligomerization initiates these events has remained unclear. Full understanding of these complex signalling cascades will require a more complete description of receptor movements in the membrane, including restrictions that may limit receptor diffusion and accessibility.

A rich literature on single-particle tracking (SPT) methods to follow the lateral diffusion of transmembrane and membrane-associated proteins^{7–10} has revealed nanometer-scale ‘confinement zones’ that restrict lateral diffusion, and supports the general notion that plasma membrane organization is more structured than originally postulated by the fluid-mosaic model¹¹. A membrane-skeleton fence (picket fence) model has been proposed to explain confined diffusion^{12,13} at the level of nanometers and microseconds. In this model, a static meshwork

of actin-defined domains transiently retain diffusing membrane proteins and lipids, leading to long time-scale diffusion rates that are much slower than those measured in artificial lipid bilayers. A recent comparison of high-resolution electron microscopy (EM) images of cytoskeletal ‘ghosts’ with high-speed SPT data demonstrates that nanometer-sized transient confinement zones measured by SPT correlate well with regions delimited by membrane-associated cortical cytoskeleton observed by EM¹⁴. However, technical limitations preclude simultaneous SPT and direct observation of nanometer-size actin structures in living cells, leaving the precise mechanism of restricted diffusion at the nanometer level open to debate. Specifically, other structural features in membranes, such as lipid rafts or protein islands^{15–18}, have also been suggested to affect lateral diffusion.

Here, we directly characterize transmembrane receptor diffusion with respect to actin by simultaneous fluorescence imaging of micron-scale features of the cortical actin cytoskeleton and FcεRI. Quantum dot (QD)-labelled IgE was used to tag FcεRI. IgE binds tightly to the α subunit of FcεRI, essentially becoming another subunit of the receptor^{19,20}. Exploiting this tight binding enables labelling of the resting receptor with minimal disruption to normal cell physiology, providing a highly specific and relevant probe of receptor dynamics and membrane topography. Micron-sized features of the actin-based cytoskeleton have been described in detail using EM of detergent-extracted platelets²¹. We sought to observe large membrane-proximal actin bundles in RBL-2H3 cells using GFP-tagged actin with confocal and total internal reflection

¹Department of Pathology and Cancer Research and Treatment Center and ²Department of Physics and Astronomy, University of New Mexico, Albuquerque, New Mexico 87131, USA. ³Sandia National Laboratories, Albuquerque, New Mexico 87185-0351, USA.

⁴These authors contributed equally to this work.

⁵Correspondence should be addressed to D.S.L. (e-mail: dlidke@salud.unm.edu)

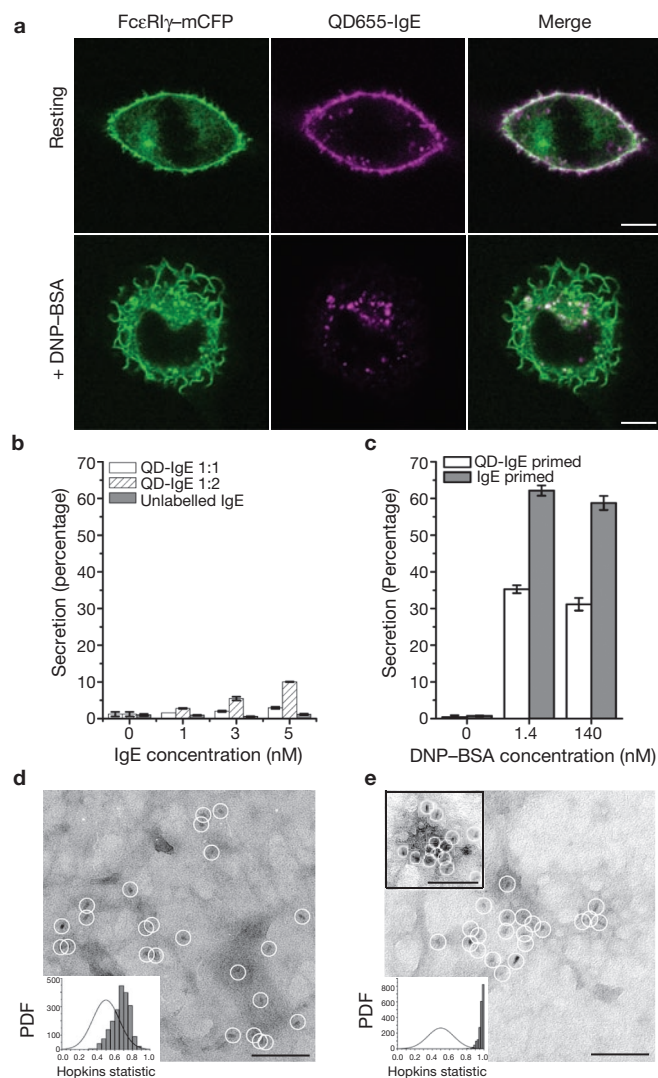


Figure 1 QD-IgE serves as a non-perturbing probe of Fc ϵ RI diffusion. **(a)** RBL-2H3 cells stably expressing Fc ϵ RI- γ -monomeric cyan fluorescent protein (mCFP) were labelled with 1 nM QD655-IgE and then imaged (top panels) or crosslinked with 14 nM DNP-BSA for 10 min before imaging (bottom panels). **(b, c)** Degranulation assay plots showing the percentage of total β -hexosaminidase content secreted in response to 30 min incubation at 37 °C with with unlabelled or QD-labelled IgE **(b)** or after priming with QD-IgE or IgE in response to various amounts of DNP-BSA **(c)**. **(d, e)** Individual QD655-IgE-Fc ϵ RI are indicated by white circles in electron micrographs from membrane sheets prepared from RBL-2H3 cells labelled with QD655-IgE **(d)** and stimulated for 10 min with 14 nM DNP-BSA **(e)** before fixation. A right shift in the experimental data (inset, grey bars) away from a random distribution (inset, solid line) in the Hopkins plot indicates a slightly clustered distribution of QD655-IgE-Fc ϵ RI in the resting state **(d)** and a highly clustered distribution after crosslinking **(e)**³. Scale bars are 10 μ m **(a)** and 100 nm **(d, e)**.

fluorescence (TIRF) microscopy. We provide direct evidence that actin cables near the membrane define regions that limit receptor diffusion. These structures do not provide a static meshwork of enclosed domains, but instead form a dynamic labyrinth defined by micron-sized actin barriers that reorganize over time scales of 1–10 s. In contrast to the putative transient confinement zones of the picket fence model, we describe a phenomenon that occurs in time scales of seconds and over distances of microns. In addition to restriction of membrane protein

diffusion, we provide evidence that actin-based membrane partitioning dynamically influences long-range mobility, sequestration and response to ligand binding.

RESULTS

QD-IgE is functionally monovalent

A monovalent QD-IgE probe was developed to study the properties of the resting IgE receptor, Fc ϵ RI. The reaction conditions were carefully controlled to produce functionally monovalent complexes of QD with anti-DNP IgE (QD-IgE, see Methods). Several approaches were taken to evaluate valency (see Methods; Supplementary Information, Fig. S1). For functional evaluation, we determined that QD-IgE did not induce substantial activation of RBL-2H3 cells assessed by confocal microscopy as a lack of ruffle formation and QD-IgE internalization (Fig. 1a), and by degranulation assay as a lack of β -hexosaminidase release (Fig. 1b). In addition, QD-IgE-primed cells were capable of responding to stimulation with the multivalent antigen DNP-BSA. This was demonstrated visually as cell ruffling and QD-IgE internalization (Fig. 1a; Supplementary Information, Movie 1) and biochemically as significant β -hexosaminidase release (Fig. 1c). The blunted secretory response of QD-IgE-primed cells (Fig. 1c) is probably due to steric limitations that the QD label imposes on the IgE-Fc ϵ RI aggregates²².

As QDs can serve as probes in both EM and fluorescence microscopy imaging²³, we analysed the topography of QD-IgE-tagged receptors on native membrane sheets by transmission EM. Visual inspection of QD distribution on the sheets shows many singlets as well as small clusters of 20–40 nm in size (Fig. 1d), verified as statistically non-random by the Hopkins test (Fig. 1d). These results are consistent with previous studies using immunogold^{3,24} and confirm that previously observed clustering of resting receptors cannot be explained as an artefact of the multivalent gold probes used to label fixed cell membranes (described in ref. 13). Brief treatment of QD-IgE-labelled cells with polyvalent antigen resulted in the formation of large clusters of receptors (Fig. 1e), as well as the appearance of QD-labelled receptors in clathrin-coated pits (Fig. 1e). These studies demonstrate that QD-IgE behaves comparably to unlabelled IgE and therefore serves as a reliable probe of IgE-Fc ϵ RI dynamics.

QD-IgE-Fc ϵ RI demonstrates characteristic membrane protein diffusion

Estimations of membrane protein diffusion are variable, depending on differences in acquisition rates, temperature and the method of measurement. For example, imaging at ambient temperatures is simpler, but the diffusion rate is slower than at physiological temperatures. The frequent use of cold (4 °C) labelling conditions in studies of Fc ϵ RI dynamics^{25,26} is of particular concern because a long duration of cold exposure has been shown to decrease measured diffusion of other transmembrane proteins²⁷. We compared the diffusion of QD-IgE-Fc ϵ RI at room temperature (22 °C) and at 35 °C and observed a faster diffusion coefficient (D) and larger restricted region of diffusion (L) at the higher temperature (Supplementary Information, Fig. S2). To avoid these potentially confounding effects, we labelled cells and acquired data at physiological temperatures (34–37 °C). The median diffusion coefficient (D_{1-3}), measured at video rate (33 frames s⁻¹), was 0.074 μ m² s⁻¹ (Table 1). This value is consistent with previous measurements made at lower temperatures (D = 0.02–0.05 μ m² s⁻¹; refs 25, 26, 28–30).

Table 1 Median diffusion coefficients and actin overlap

Imaging condition	Cell treatment	<i>n</i>	D_{1-3} ($\mu\text{m}^2 \text{s}^{-1}$) ^a	IQR ^c	Relative actin overlap ^d
Apical	Resting	236	0.074	0.065	
Apical	Latrunculin B	138	0.10	0.087	
Apical	DNP-BSA	647	0.010	0.038	
Apical	Latrunculin B + DNP-BSA	403	0.020	0.044	
TIRF	Resting	303	0.077 ^b	0.074	1.43
TIRF	Latrunculin B	265	0.059 ^b	0.067	1.30
TIRF	PMA	102	0.075 ^b	0.078	1.32

^a D_{1-3} values were calculated as described in Methods. Values are reported as median.

^bMedian D_{1-3} values from TIRF experiments were calculated after excluding immobile trajectories, defined as $D_{1-3} \leq 0.0009 \mu\text{m}^2 \text{s}^{-1}$.

^cInterquartile range (IQR), see Methods.

^dRatio of actin crossings between simulated and real trajectories. A value > 1 indicates that simulated, freely diffusing particles cross actin more frequently than real trajectories. The differences in behaviour between real and simulated data are statistically significant.

As documented in previous studies of other membrane proteins^{8,25,27,31–33}, we observed four distinct modes of FcεRI diffusion in unstimulated cells: free, directed, restricted and immobile (Supplementary Information, Fig. S3). Our observation of restricted and immobile receptors is consistent with the general notion that the plasma membrane contains structures that limit lateral diffusion, which are not present in artificial bilayers.

Receptors are co-confined in micron-scale membrane domains

Despite labelling conditions that resulted in only a few QD-IgE-labelled receptors per cell, we often observed prolonged overlap between the emissions of two or three tracked receptors (Supplementary Information, Movie 2). These observations suggest that weak attractive interactions may exist between non-crosslinked receptors. However, when two QDs with the same emission spectra overlap, they cannot be easily distinguished until they separate. Thus, analysis of close interactions between receptors is limited in protocols using only one colour of fluorophore.

We overcame this limitation by labelling cells with a mixture of QD585-IgE and QD655-IgE and simultaneously tracking both QDs. This allowed us to independently localize each molecule even when their emissions overlapped. In the example shown in Fig. 2a–c, two QD-labelled receptors repeatedly approach each other to within our localization limits (see Methods) while never moving farther than 500 nm apart before coming back together (Fig. 2c). Despite their close proximity, the trajectories of the two molecules do not seem to be correlated (Fig. 2b). In the second example (Fig. 2d–f), a maximum separation of 2 μm occurs before the two molecules come back together (Fig. 2f). The observation that QD-IgE–FcεRI complexes can move microns apart before seeming to reverse direction to rejoin each other cannot be explained by attractive forces between the observed receptors.

To determine whether protein–protein interactions (that is, transient dimerization) could explain the prolonged colocalization of QD-IgE–FcεRI complexes, we developed mathematical analyses to quantify coordinated movement between receptors at separation distances less than 500 nm (see Supplementary Information Methods). In order to capture these potentially highly dynamic events, we used total internal reflection fluorescence (TIRF) microscopy with a faster acquisition rate of 100 frames s^{-1} . This involves measuring the receptor displacement vector magnitude (jump magnitude) and the extent of correlated motion between nearby receptors (uncorrelated jump distance). If two receptors form a transient dimer, their diffusion is expected to be slowed¹³, producing a decrease in their jump magnitude. Additionally, if two receptors

form a transient dimer, they would move together during the life of this dimer; that is, the motion of the two interacting receptors would be correlated, producing a decrease in the uncorrelated jump distance. By plotting the jump magnitude and the uncorrelated jump distance as a function of distance between receptor pairs, we can detect the presence of transient dimers, provided their lifetime is longer than the acquisition time for each image (10 ms).

To model the observed behaviour of prolonged colocalization, we performed simulations of diffusing particles which formed transient dimers for 200 ms when two particles approached within 25 nm of each other (Supplementary Information, Fig. S4). This produced a marked decrease in the uncorrelated jump distance as receptors came close enough to interact (Supplementary Information, Fig. S4). In contrast to the simulations, analysis of real trajectories produced only gradual decreases in the jump magnitude and the uncorrelated jump distance (Fig. 3). The weak dependence of these values on separation distance is consistent with measuring a slower diffusion coefficient when particles are confined to a small region⁹. These analyses do not support transient dimer formation as a mechanism for the prolonged colocalization we observed and instead suggest co-confinement by membrane structure as the most likely explanation.

Dynamic actin structures restrict FcεRI diffusion

To determine the role of the actin cytoskeletal network in the lateral diffusion and observed co-confinement of QD-IgE–FcεRI complexes, RBL-2H3 cells were stably transfected with GFP-actin. Two-colour time-series of QD-IgE–FcεRI motion with respect to the actin cytoskeleton were acquired. Because of its superior signal-to-noise ratio, TIRF microscopy was primarily used to track QD-IgE–FcεRI and GFP-actin movements on the adherent cell surface at 33 frames s^{-1} .

In initial experiments, cells were treated with the protein kinase C agonist, phorbol myristate acetate (PMA), to induce actin filament bundles resembling stress fibres³⁴. QD-IgE–FcεRI diffusion was mainly limited to actin-poor regions of the membrane, often remaining confined within a single actin-defined compartment for several seconds of tracking (Fig. 4a, b; Supplementary Information, Movie 3). The size of these compartments (L) can be estimated from the plateau value on the y axis of the mean square displacement (MSD) plot. A typical restricted trajectory and its MSD plot are shown in Fig. 4a (inset), and fit by $\text{MSD} = \text{offset} + (L^2/3)(1 - \exp(-\Delta t/\tau))$ (ref 35), where $D = L^2/12\tau$. From the fit we find that $L = 1.1 \mu\text{m}$, which correlates with the actual, observed actin-defined region in Fig. 4a.

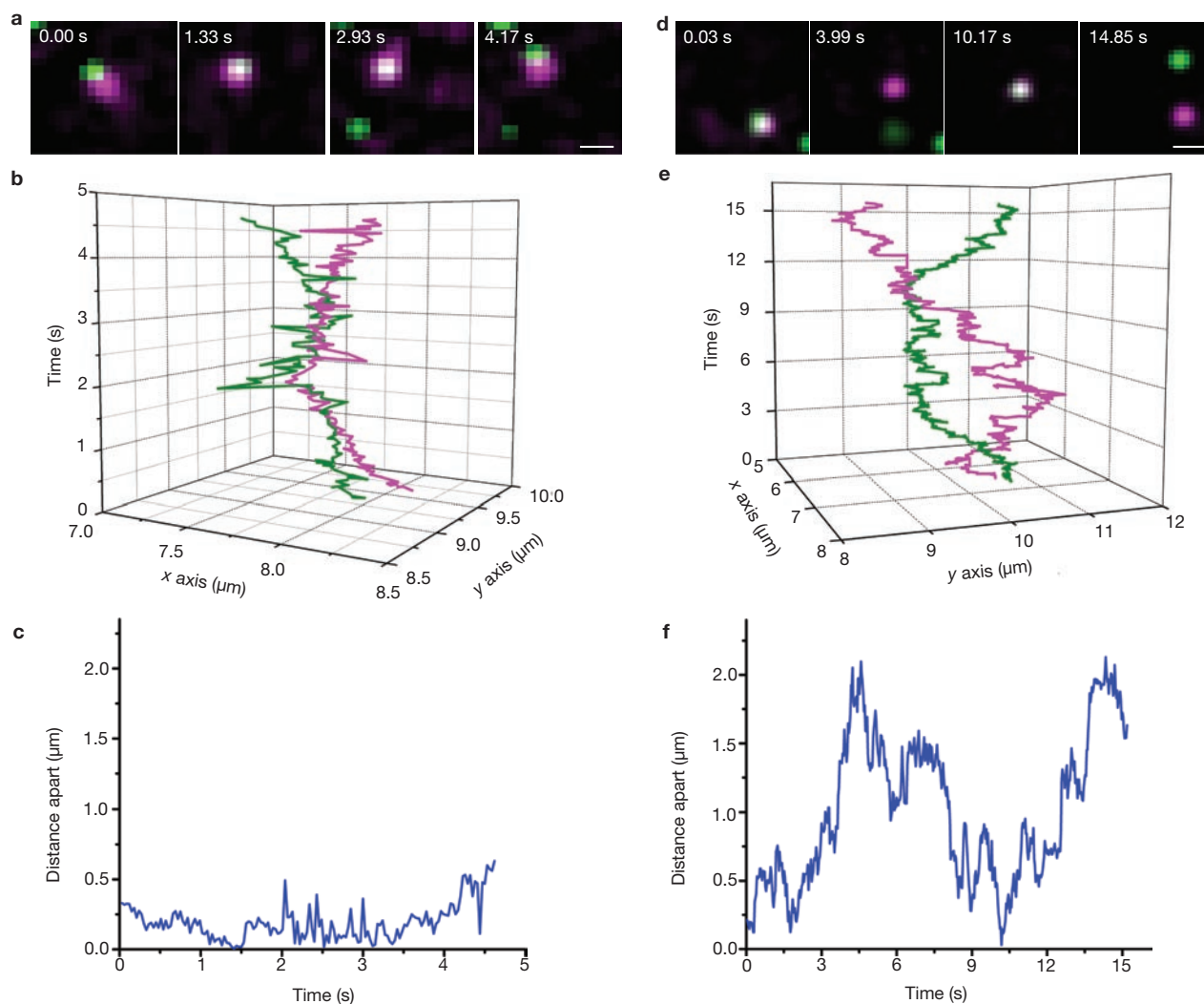


Figure 2 Fc ϵ RI are co-confined. (a) Sample images from a time-series showing two QD-IgE-Fc ϵ RI complexes co-confined within a domain of about 500 nm. (b) xy versus time plot of trajectories of QD655-IgE-Fc ϵ RI (magenta) and QD585-IgE-Fc ϵ RI (green) from time-series shown in a. (c) Plot of interparticle distance versus time for particles shown in a. (d) Sample images from a time-series showing two QD-IgE-Fc ϵ RI

complexes co-confined within a domain of about 2 μ m. (e) xy versus time plot of trajectories of QD655-IgE-Fc ϵ RI (magenta) and QD585-IgE-Fc ϵ RI (green) from time-series shown in d. (f) Plot of interparticle distance versus time for particles shown in d. Images in a and d have been Gaussian-filtered. Images were acquired on the apical surface at 33 frames s $^{-1}$. Scale bars are 1 μ m (a, d).

Imaging of untreated RBL-GFP-actin cells revealed continuous re-organization of the actin network, resulting in a nearly complete change in the pattern of GFP-actin fluorescence on a 10-s time-scale (Supplementary Information, Movie 4). Nevertheless, diffusion of QD-IgE-Fc ϵ RI remained limited to actin-poor regions (Fig. 4c). Mathematical analysis of receptor/actin crossing frequency was performed to verify that receptors were avoiding actin structures. Comparison of the real data trajectories with simulated, freely diffusing particles showed a significant difference in actin/trajectory overlap, confirming that the real trajectories cannot be modelled by free diffusion that is unaffected by actin structures (Table 1 and Supplementary Information Methods).

The data in Fig. 4a–c were acquired from the relatively flat, adherent cell surface in TIRF mode, which images structures within about 200 nm of the coverslip. We performed multiple control experiments to ensure that interactions between the plasma membrane and the coverslip were

not responsible for the observed behaviour of QD-IgE-Fc ϵ RI complexes (Supplementary Information, Fig. S5). The observation of similar behaviour on the apical cell surface provides perhaps the most compelling evidence supporting the TIRF data showing actin-restricted Fc ϵ RI diffusion. Confocal microscopy was used to eliminate the cytoplasmic GFP-actin signal and image the actin structure in a 1 μ m slice at the apical cell surface. In confocal mode, the frame rate had to be reduced to 1 frame s $^{-1}$ to image single QDs. Despite the slower frame rate, we were able to follow the trajectories of QD-labelled receptors over a period of 100 s while simultaneously imaging GFP-actin. Receptors on the apical surface did not cross actin-rich regions (Fig. 4d). As seen at the adherent cell surface, dynamic actin re-organization accompanies receptor movement into previously restricted areas (Fig. 4d; Supplementary Information, Movie 5).

To determine whether Fc ϵ RI alters its diffusion properties when encountering actin structures or is simply deflected by the physical barrier, we calculated the mean square single-jump length of Fc ϵ RI

as a function of distance from actin (Fig. 5). Although the data analysis in Fig. 5 suggests a slight reduction in mean jump distance as the receptor approaches the actin (solid line), simulations (dashed line) show that this can be explained by a combination of geometric effects and finite detector integration time⁹. Thus, there is no indication of a real change in diffusion as close as 100 nm from underlying actin structures. These results support the interpretation that membrane-associated actin structures or proteins bound to actin act on FcεRI as a physical barrier that deflects the receptor.

To further discern the influence of the actin cytoskeleton on FcεRI diffusion, we treated cells with 500 nM latrunculin B for 10 min before imaging. This concentration of latrunculin disrupts cytoskeletal architecture (Supplementary Information, Fig. S6) without causing significant damage to cells³⁶. Latrunculin produced a small but significant ($P = 1.9 \times 10^{-5}$, Kolmogorov-Smirnov test) increase in FcεRI diffusion on the apical membrane (Fig. 6a, Table 1), consistent with previous observations of less confined motion¹³. Unexpectedly, latrunculin treatment decreased diffusion at the adherent surface (Table 1). Although the mechanism is unclear, we note that non-actin components of the cortical cytoskeleton (possibly spectrin) are still visible in EM as a fine meshwork on the adherent surface of treated cells (Supplementary Information, Fig. S7).

Cytoskeletal disruption slows immobilization kinetics

Activation of RBL-2H3 cells is known to induce substantial cytoskeletal remodelling and an increase in F-actin content³⁶. There is also a marked decrease in FcεRI mobility within minutes of cross-linking³⁷. To determine the kinetics of receptor immobilization, we devised an assay to measure the mobility of QD-IgE-FcεRI complexes during cross-linking with 30-ms time resolution.

On addition of 14 nM DNP-BSA, FcεRI diffusion decreased rapidly. The mobility transition was evident in trajectories of individual receptors (Fig. 6b). Averaging the behaviour of many receptors over time gave the mean instantaneous diffusion coefficient plot (Fig. 6c). Note that diffusion reaches a baseline within seconds after crosslinking. An exponential decay model was used to fit the data after crosslinker addition, returning a characteristic decay time (τ) of 11.0 ± 0.19 s for untreated cells. In the presence of latrunculin, which disrupts actin structures of both nanometer and micron size, τ increased to 30.0 ± 1.3 s (error indicates 95% confidence interval), suggesting a role for the actin structure in the process of antigen-mediated receptor immobilization. From these data, we were also able to calculate median diffusion coefficients both before and immediately after crosslinking. Latrunculin treatment significantly ($P = 1.8 \times 10^{-13}$, Kolmogorov-Smirnov test) increases the final diffusion rate after crosslinker addition (Fig. 6a; Table 1), confirming that the actin cytoskeleton has an important role in crosslink-induced immobilization of FcεRI.

DISCUSSION

Our results provide evidence for micron-scale restriction of membrane protein diffusion by dynamic actin structures and are particularly relevant to studies where membrane molecules are tracked on relatively long time-scales (seconds). We frequently observed two or more QD-IgE-FcεRI complexes remaining in close proximity for several seconds (Fig. 2; Supplementary Information, Movie 2). However, close inspection of this behaviour revealed an absence of detectable

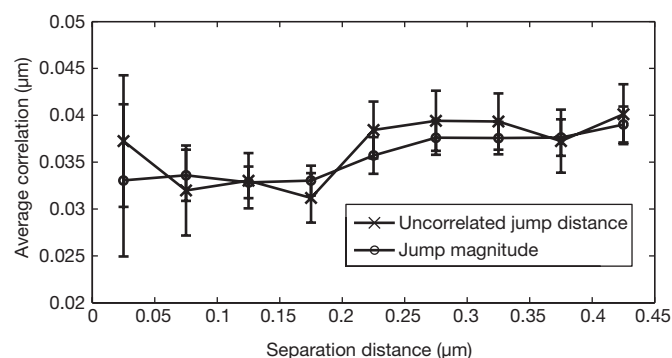


Figure 3 Motion of QD-IgE-FcεRI is consistent with co-confinement not attraction. Mean distance of uncorrelated jump distance and jump magnitude plotted as a function of distance between QD-IgE-FcεRI complexes. Data mean $\pm$ s.e.m. of 1,128 instances of QD-IgE-FcεRI complexes approaching within 0.5 μ m.

correlated motion between individual receptors (Fig. 3). We suggest that this behaviour represents co-confinement of multiple receptors within the same membrane domain and supports the idea that the micron-scale domains of co-confinement are defined by barriers of filamentous actin. These shifting actin barriers subdivide the membrane, producing transiently co-confined subpopulations of membrane proteins. Actin could restrict diffusion through various mechanisms, including acting as a physical barrier (actin itself or associated proteins), directly binding FcεRI or interacting with lipids and increasing membrane viscosity. Our analysis of FcεRI diffusion with respect to actin bundles (Fig. 5) supports the physical barrier explanation.

In addition to the micron-scale actin-mediated restriction we have observed, we recognize that there is a finer level of membrane organization at work that we cannot directly observe. This level of organization has been described as a meshwork composed not only of actin, but also a wide variety of cell-type specific intermediate filament proteins (e.g., spectrin^{38,39}). This nanometer scale organization may account for the small confinement zones apparent in higher frame rate SPT measurements^{7–10} and may have a role in forming the smaller clusters (~50 nm) of resting FcεRI observed in electron micrographs (Fig. 1d). We speculate that the finer cytoskeletal meshwork may be involved in producing a heterogeneous distribution of membrane components on a nanometer scale. The literature offers several representations of this heterogeneity, which exists within the boundaries of large-scale actin-defined regions described in this work, including the protein islands model^{15,18}, the lipid raft model^{40,41} and the membrane skeleton model^{12,13} (Supplementary Information, Fig. S8).

Although differing in their descriptions of the roles played by specific proteins in the cytoskeletal meshwork, the published models are universally dependent on an intact cortical cytoskeleton^{13,18,41}. Each of these putative membrane features are reported to be highly dynamic. In the membrane skeleton model, tracked membrane components reside in individual domains on millisecond time-scales¹³. Lipid rafts are also reported to be very dynamic^{40,41}. From our data, the lack of correlated motion at small separation distances (Fig. 3) suggests that receptors remain only briefly in the same nanometer-scale domain. These data suggest that the small clusters of resting FcεRI observed by EM (Fig. 1d) are highly dynamic, with exchange between neighbouring small domains

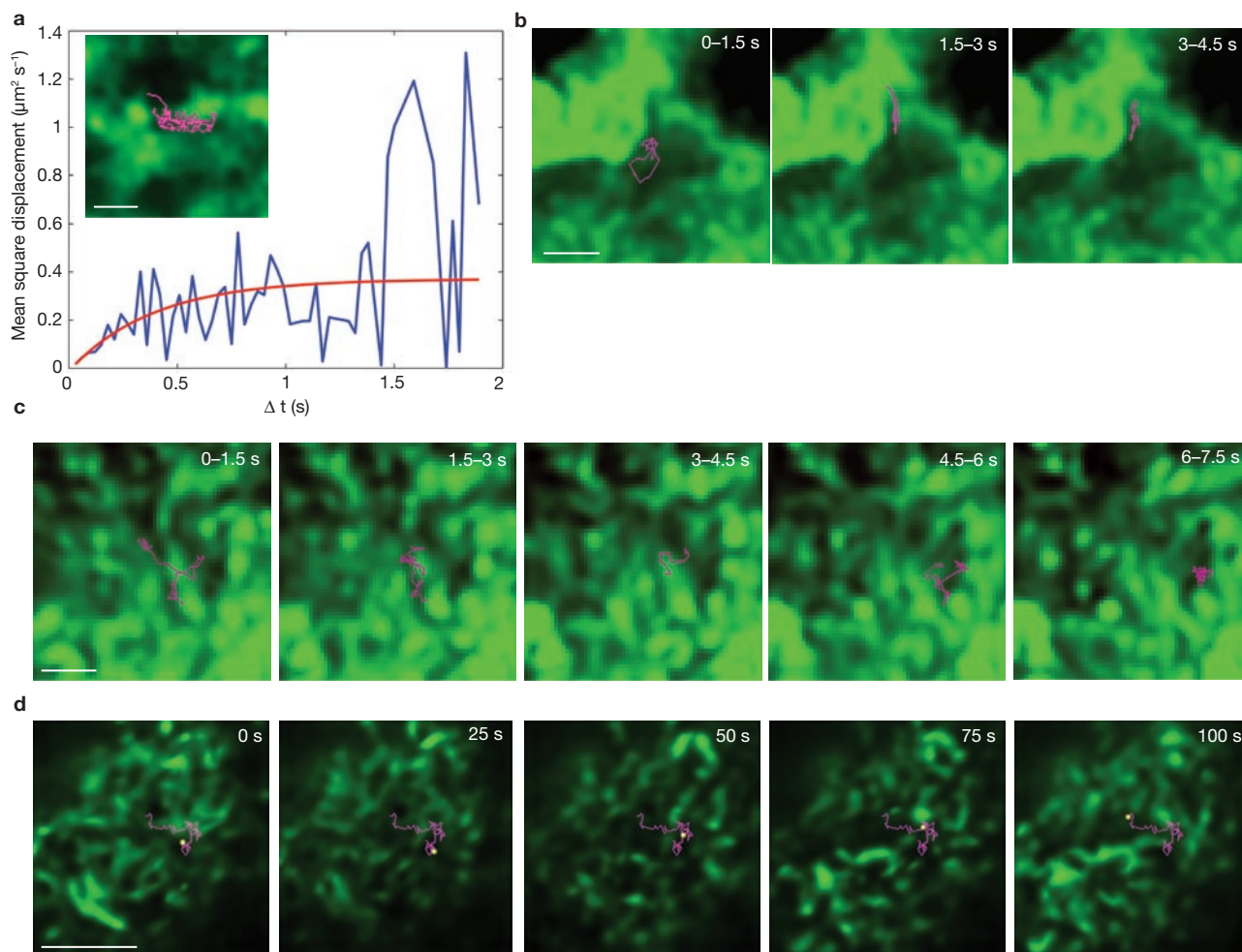


Figure 4 Actin defines regions of Fc ϵ RI motion in the membrane. (a) MSD plot of the trajectory shown in inset, demonstrating diffusion restricted to an area consistent with that delineated by the actin structures. (a, inset) QD-IgE-Fc ϵ RI trajectory (magenta) overlaid with the deconvolved GFP-actin image (green) from the adherent surface of a PMA-treated RBL-2H3 cell. (b, c) Image series of deconvolved GFP-actin (green) and 1.5-s-long segments of a single QD655-IgE-Fc ϵ RI trajectory (magenta) on the

adherent surface of a PMA-treated (b) or untreated (c) RBL-2H3 cell. (d) Full 100 s QD-IgE-Fc ϵ RI trajectory (magenta) with position at 25-s intervals highlighted (white) overlaid with the deconvolved GFP-actin image (green) on the apical membrane of an RBL-2H3 cell. Panels a–c were acquired with TIRF microscopy at 33 frames s⁻¹. Panel d is from a 1 frame s⁻¹ confocal time series with 1- μ m slice thickness. Scale bars are 1 μ m (a–c) and 5 μ m (d).

occurring on time-scales much more rapid than the diffusion of the domains themselves.

The immobilization of IgE-Fc ϵ RI complexes in response to treatment with crosslinking agents has been previously studied by fluorescence recovery after photobleaching (FRAP)^{28,29,37,42} and by SPT²⁵. Unique features of our study include labelling and tracking at physiological temperatures and acquisition of real-time kinetics. A previous attempt to measure the time-course of crosslinking-induced immobilization by FRAP was limited to events occurring at least 90 s after addition of the crosslinking agent, at which time immobilization had already reached a maximum³⁷. We used our real-time assay to evaluate the kinetics of crosslinking from the precise moment of crosslinker addition. Our results demonstrate that diffusion of QD-IgE-Fc ϵ RI decreases within seconds of adding a crosslinking agent (Fig. 6b), and that the rate of immobilization is slowed by actin disruption with latrunculin (Fig. 6a, Table 1). Combining our kinetic data with the SPT and EM results reveals that small, dynamic clusters of Fc ϵ RI transition to large, stable clusters by an actin-dependent mechanism within

seconds of crosslinking. Disruption of actin by latrunculin caused an increase in the apical diffusion coefficient of both resting and crosslinked receptors (Fig. 6a, Table 1). Interestingly, treatment with latrunculin is reported to prolong Fc ϵ RI γ phosphorylation and to increase degranulation³⁶, suggesting that the more mobile clusters may have an increased signalling lifetime. Our observation that crosslinked receptors are not as immobile in the presence of latrunculin suggests that immobilization is an important regulatory mechanism of Fc ϵ RI signalling.

Our direct and simultaneous observations of Fc ϵ RI motion and actin dynamics demonstrate that IgE receptors diffuse within micron-sized membrane domains defined by actin bundles and that confinement is dynamic over length-scales of microns and time-scales of seconds. Restricted motion has been observed for a wide variety of membrane proteins, including the erbB family³³, glycine receptors⁴³ and G-protein-coupled receptors²⁷. The ubiquitous nature of restricted diffusion, coupled with the our observations reported here, suggest that actin-mediated restriction of membrane protein diffusion has an important regulatory role in a wide variety of signalling pathways. □

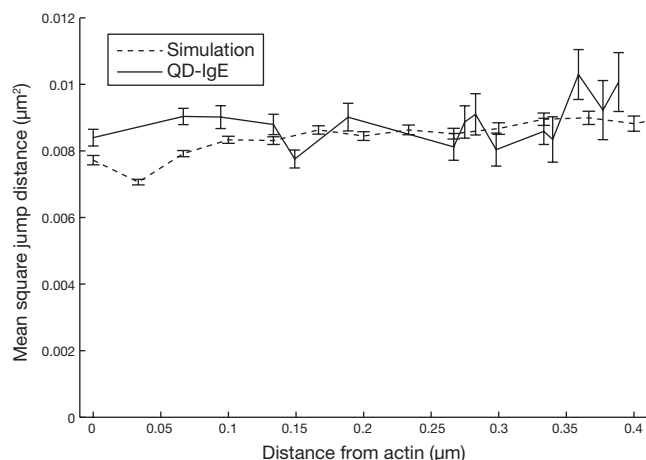


Figure 5 Effect of actin proximity on FcεRI trajectories. All QD-IgE trajectories found by TIRF-SPT at 33 frames s⁻¹ in PMA-treated cells were used to calculate single time-step mean square jump distances as a function of distance from an actin structure (solid line). For comparison, simulations of particles diffusing near a reflecting boundary with constant membrane viscosity and finite localization accuracy, modelled after the real data, were used to calculate mean square jump distances as a function of distance from the reflecting boundary (dashed line).

METHODS

Reagents. Mouse monoclonal anti-DNP IgE was prepared as described previously⁴⁴. Monovalent biotin-IgE was prepared through modification of the FluoReporter mini-biotin-XX protein labelling kit protocol (Invitrogen): a biotin-succinimidyl ester to IgE ratio of 4:1 and a 15 min reaction time was used to generate biotin-IgE with an average biotin:IgE ratio of 0.83:1, as determined with the FluoReporter biotin quantitation assay kit (Invitrogen). Biotin-IgE was combined in a 1:1 stoichiometry with Qdot 655 or Qdot 585 streptavidin conjugate (Invitrogen) in PBS containing 1% BSA to generate 20–60 nM stock solutions of monovalent QD-IgE. Stock solutions were stored at 4 °C and used within four weeks. To facilitate comparisons between unlabelled IgE and QD-IgE, all concentrations are reported in nM, with 5 nM IgE or 14 nM DNP-BSA being equivalent to 1 μg ml⁻¹. Latrunculin B was from Sigma-Aldrich, PMA was from Invitrogen and DNP-BSA was from Molecular Probes.

Cell culture. Rat basophilic leukaemia (RBL-2H3) cells were grown as adherent monolayers in minimum essential medium with 10% fetal calf serum (Invitrogen) as described previously²⁴. For microscopy, cell monolayers were cultured in 8-well Lab-Tek chambers (Nunc) 24 h before experiments.

Degranulation assay. Cell monolayers were grown in 24-well tissue culture plates for 24 h. Typically, FcεRI were primed by adding 5 nM anti-DNP-IgE to cultures overnight. After washing away excess IgE, cells were activated with 0.014–140 nM DNP-BSA. Release of β-hexosaminidase was measured as described previously²².

Cell treatment for SPT experiments. Cells in Lab-Tek chambers were labelled with 50 or 100 pM QD-IgE in Hanks' balanced salt solution (HBSS) for 10 min at 37 °C, then washed with HBSS before imaging. This produced an average of ten QD-IgE-FcεRI complexes per cell. For TIRF imaging, washing was omitted. All imaging was obtained at 34–36 °C. PMA-treated samples were pre-incubated for 30 min and imaged in the presence of 50 nM PMA. Latrunculin B-treated samples were pre-incubated for 10 min and imaged in the presence of 500 nM latrunculin B. Where indicated, cells were stimulated with 14 nM DNP-BSA.

Cell treatment for immobilization. Cells were labelled with 500 pM QD-IgE for 10 min at 37 °C as above, then incubated at 37 °C for 30 min with 140 nM unlabelled IgE. Cells were washed and imaged by wide-field microscopy at 35 °C in 200 μl HBSS for about 10 s, followed by addition of DNP-BSA to a final concentration of 14 nM. Where described, cells were treated with 500 nM latrunculin B during the last 10 min of IgE priming and latrunculin B was maintained in the

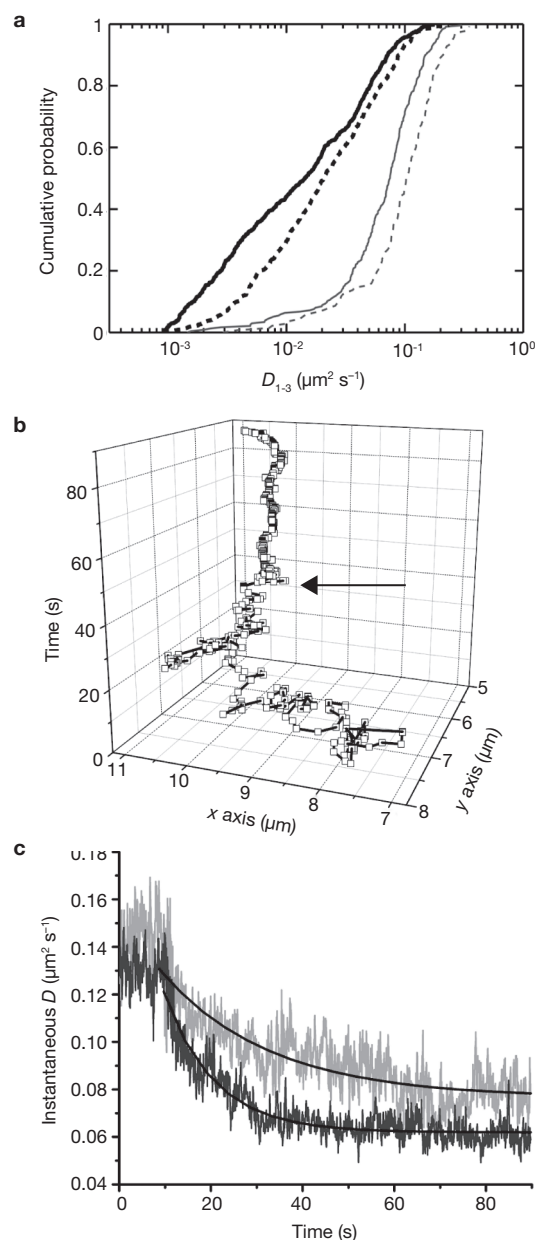


Figure 6 Actin facilitates crosslink-induced immobilization of FcεRI. (a) Cumulative probability distribution plot of diffusion coefficients for QD-IgE in the absence (solid lines) and presence (dashed lines) of latrunculin B, and before (thin lines) and after (thick lines) crosslinking with DNP-BSA. (b) XY versus time plot of a single QD-IgE-FcεRI. Arrow indicates time of DNP-BSA addition. (c) Kinetics of crosslink-induced immobilization in the presence (light grey, $n = 12$) and absence (dark grey, $n = 17$) of latrunculin B. Black lines are fit to the exponential decay model: $D(t) = D_0 e^{-(t/\tau)}$, where D is the instantaneous diffusion coefficient and τ is the decay constant.

medium during activation. The presence of 500 nM latrunculin B did not affect the rate or amount of IgE binding (data not shown).

Electron microscopy. RBL-2H3 cell monolayers on glass coverslips were labelled with 30 nM QD655-IgE for 30 min at 37 °C. Cells were fixed for 7 min in 0.5% PFA and membrane sheets prepared as described previously²⁴. Electron micrographs were acquired using a transmission electron microscope (Hitachi H600). Probe identification and Hopkins statistical analysis were performed as described previously³.

Fluorescence microscopy. Confocal imaging was performed on a Zeiss LSM 510 META system equipped with a $\times 63$ 1.4 N.A. oil objective. Single 1 μm z-sections were acquired at 1 frame s^{-1} . GFP and QDs were excited simultaneously at 488 nm and emission collected using a 545 nm dichroic mirror with 505 nm LP filter and the 625–689 nm range of the META detector, respectively.

Wide-field imaging for SPT was performed using an Olympus IX71 inverted microscope equipped with a $\times 60$ 1.3 N.A. water, $\times 100$ 1.4 N.A. oil, or $\times 150$ 1.45 N.A. oil TIRF objective. Wide-field excitation was provided by a mercury lamp with either a 436 nm BP or 543 nm BP excitation filter. Objective-based TIRF microscopy was performed using excitation from a 472-nm continuous wave laser (CrystaLaser), which was expanded and entered the microscope through a laser side-port of the microscope filter turret. Fluorescence emission was filtered with a 473-nm long pass filter (LP01-473RU, Semrock). Emission was collected by an electron-multiplying CCD camera (Andor iXon 887 for widefield; Andor Luca for TIRF). In both cases, an image splitter (Cairn Research OptoSplit II) was used to simultaneously image two spectrally distinct QDs or QD655 and GFP. Images were registered using a calibration image of multi-fluorophore fluorescent beads (0.2 μm Tetraspek, Invitrogen) that have an emission spectrum covering the two spectral windows. For further details see Supplementary Information Methods. QD emission was collected using the appropriate QD (20 nm bandpass) emission filters (Chroma). GFP emission was collected using a 510/40 BP emission filter. The sample temperature (34–36 °C) was maintained by an objective heater (Bioscience Tools).

Single-particle tracking. Images were acquired at either 33 or 100 frames s^{-1} for a total of 3,000 frames. Analysis of the acquired image series was performed as described previously^{43,45} to obtain trajectories, which were used to generate MSD plots⁴⁶ (see Supplementary Methods for more detail). Diffusion coefficients were found by fitting the first three points of the MSD plots to $\text{MSD} = \text{offset} + 4D_{1-3}\Delta t$ (ref 33). Localization accuracy for each individual trajectory is described by the offset from the origin of the resulting fit. Localization accuracy of the system was determined by imaging QD-IgE-FcεRI in cells treated with 14 nM DNP-BSA for 5 min, then fixed in 2% paraformaldehyde for 20 min at room temperature. Imaging under various experimental conditions produced localization accuracies of 11.86 and 13.81 nm for QD655 and QD585, respectively, imaged at 100 frames s^{-1} in TIRF mode; 9.32 and 10.37 nm for QD655 and QD585, respectively, imaged at 33 frames s^{-1} in TIRF mode; and 50.00 nm for QD655 imaged on the apical surface at 33 frames s^{-1} in wide-field mode. Note that all interaction analyses were performed on data acquired under TIRF imaging conditions, where the localization is most accurate. Instantaneous diffusion coefficients are average diffusion coefficients calculated over all tracked QDs and using all points within a sliding window of 10 frames (0.3 s). The instantaneous diffusion coefficient is given by the following equation:

$$D = (1/N) \sum_{i,j} ((r_{i+\tau} - r_i)^2 - \text{offset}) / 4\tau$$
 where r is a trajectory position, offset is found by a fit to the longest trajectory, and the sum is over all N valid trajectory coordinate pairs in the time window.

Statistical analysis. Because of the wide range of values and non-parametric distribution of D_{1-3} , values are reported as medians, and interquartile range is provided as a measure of statistical dispersion. Statistical comparisons between data sets were made using the Kolmogorov-Smirnov test, unless otherwise noted, and significance was defined as $P < 0.05$.

Image processing. All image processing was performed using Matlab (The MathWorks) in conjunction with the image processing library DIPImage (Delft University of Technology). For descriptions of specific analysis routines, see Supplementary Information Methods.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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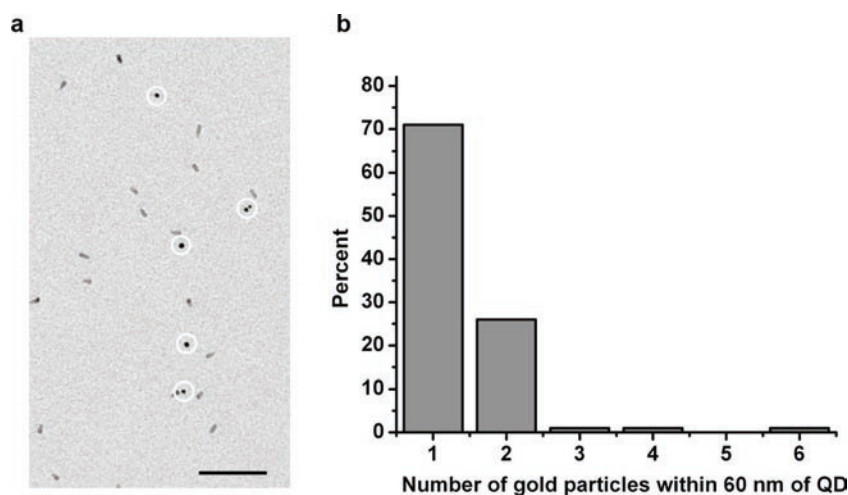


Figure S1 QD-IgE is predominantly monovalent. A number of analyses were performed to assess the valency of QD-IgE. In addition to the degranulation and biotinylation assays described in the main text, we carried out EM experiments (see Supplementary Methods for more detail) as shown here. (a) Representative electron micrograph of QD-IgE + DNP-BSA-gold. Gold particles (5 nm) are marked with white circles. Scale bar represents 100

nm. (b) Graph showing the distribution of the number of DNP-BSA-gold probes within 60 nm of QD-IgE ($n = 107$). We reason that if QD-IgE were predominantly monovalent, then each complex would have a maximum of two available binding sites for DNP-BSA-gold (IgE valency for DNP = 2). In fact, we find that the majority of the QDs have only one gold particle nearby, further supporting the monovalent nature of QD-IgE.

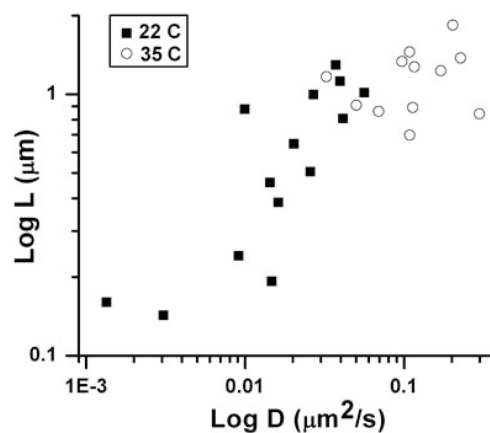


Figure S2 Influence of temperature on diffusion. Log-log plot of diffusion coefficient (D) versus restricted region size (L) for trajectories fitting the restricted diffusion model at 22°C (filled squares) and 35°C (open circles). The plotted D and L values were determined by fitting the restricted diffusion equation $MSD = offset + (L^2/3)(1 - \exp(-\Delta t / \tau))$ (ref ¹), where $D = L^2/12\tau$ to the first 10% of the data points in each MSD plot. It is apparent that at physiological temperature, D is faster and L is larger than at ambient temperature.

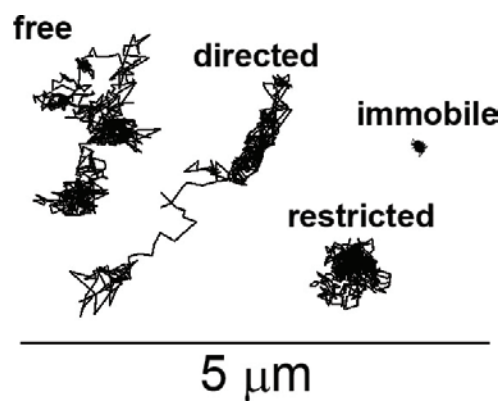


Figure S3 QD-IgE-FcεRI exhibits four motional modes. Representative trajectories of QD-IgE-FcεRI are shown. We observed four types of diffusion for FcεRI in resting cells at 35°C: free (65%), restricted (23%), immobile (4%) and directed (8%). Trajectories were classified by a slight modification of the method described in ².

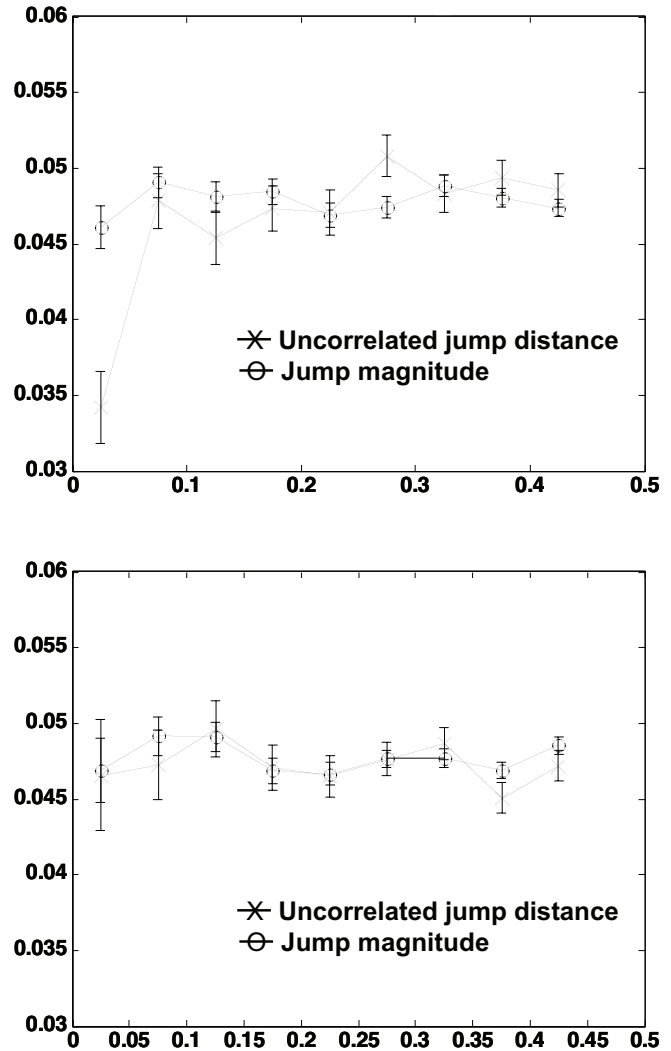
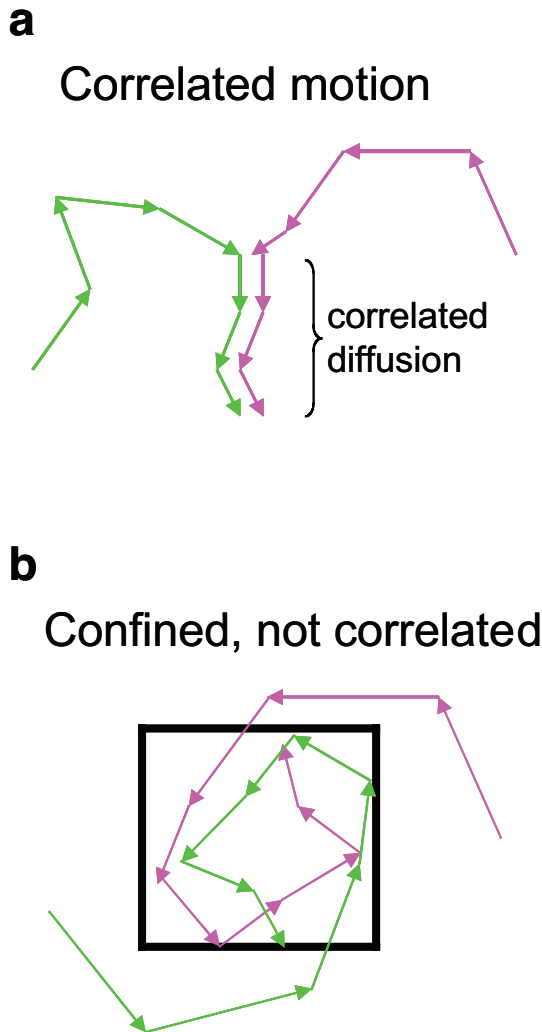


Figure S4 Analysis of simulated receptor dimerisation or co-confinement. Particles undergoing free Brownian diffusion ($D = 0.07 \mu\text{m}^2/\text{s}$) were tracked under dimerising (**a**) and non-dimerising (**b**) conditions. Parameters for the simulation included probability of binding (0.5), binding interaction distance (25 nm), localisation accuracy (15 nm) and QD blinking. (**a**) Left: Schematic showing the displacement vectors of two receptors that form a transient (up to 200 ms) dimer and undergo correlated motion. Right: Analysis of simulations of transient dimer formation. Note the pronounced decrease in the uncorrelated jump distance as diffusing particles approach each other to within the interaction distance. (**b**) Left: Schematic depicting

the displacement vectors of two particles that maintain proximity due to confinement in a region (black square) but do not show correlated motion. Right: Analysis of simulations under the same conditions as **a**, except that the probability of binding was set to zero. Note that the uncorrelated jump distance does not vary significantly as a function of separation distance. Thus, even when these particles are close enough to show emission overlap, the analysis shows that their motion is not correlated. Given that the mechanism of decreased diffusion rate upon oligomerisation is poorly characterised in live cells, we did not attempt to include this phenomenon in our simulations and therefore the jump magnitude does not vary in these analyses.

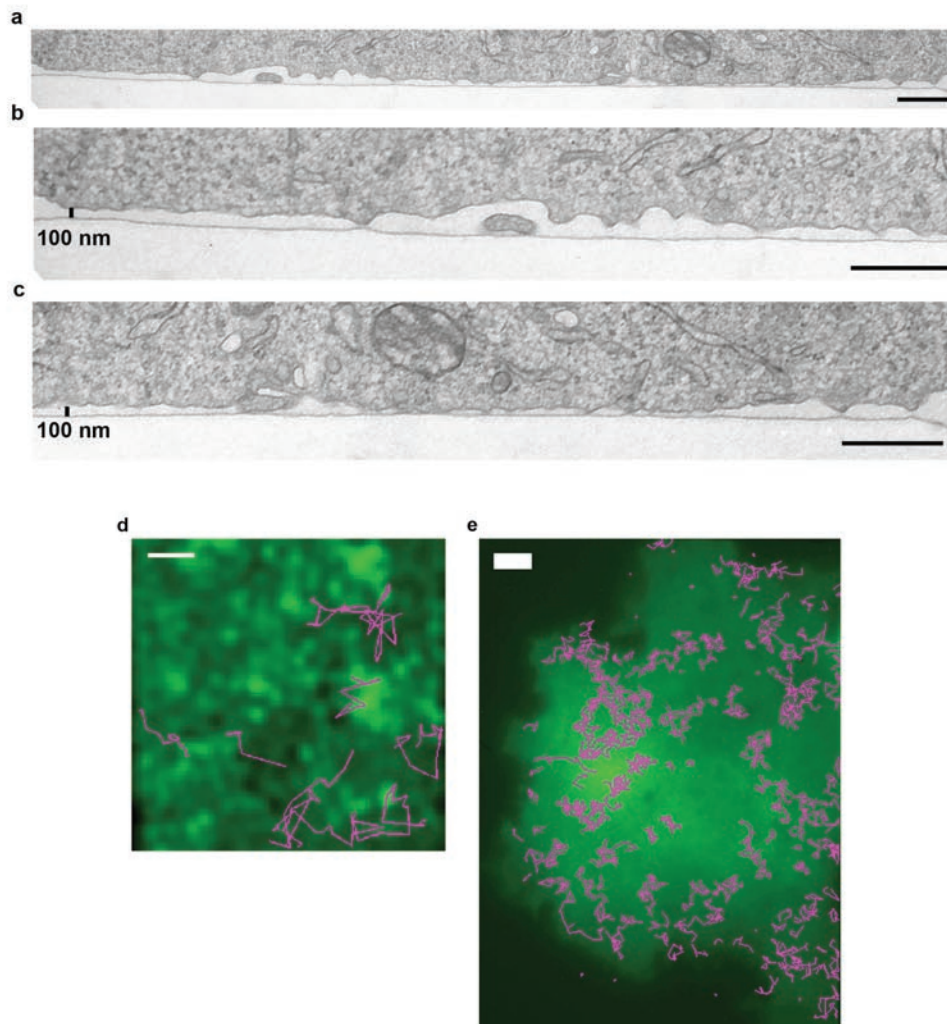


Figure S5 Membrane-coverslip distance does not dictate QD-IgE-FcεRI diffusion. (a-c) TEM image of an adherent RBL-2H3 cell taken perpendicular to the plane of adhesion (see Supplementary Methods). Images in b and c are the left and right half, respectively, of the region depicted in a. (d) TIRF image of GFP-actin (green) overlaid with trajectories of *free* QDs diffusing under the cell (magenta). (e) TIRF image of cytoplasmic GFP (green) overlaid with QD655-IgE-FcεRI trajectories (magenta) (see Supplementary Methods). Even regions closest to the coverslip (brightest GFP signal) do not exclude QD-IgE-FcεRI. Also note the highly dissimilar patterns of GFP fluorescence in d and e; indicating that GFP-actin intensity does not correlate with membrane-coverslip distance as measured by cytoplasmic GFP intensity. (f-q) TIRF images of adherent, RBL-2H3 cells expressing GFP-actin (f, g, j, k, n, and o) or cytoplasmic GFP (h, i, l, m, p, and q) in the presence of a soluble, extracellular dye. First and third columns show images of GFP-actin (f, j, and n) and cytoplasmic GFP (h, l, and p) signal, respectively. Second and fourth columns (g, i, k, m, o, and q) show signal

from a soluble fluorophore which diffuses underneath the cells and is brighter in regions where there is more space between the coverslip and cell membrane and dimmer in regions of close contact. Images are a mean of 50 time frames. A cell mask was generated from the GFP image and applied to f-i to remove the high background dye fluorescence surrounding the cell. Note that the large (~300kD) fluorophore has access to nearly all of the area underneath the cell. Selected GFP-actin structures (f, j, and n) or regions of cytoplasmic GFP intensity (l-q) are outlined in white and superimposed on both the GFP image and the image of the soluble fluorophore to facilitate comparisons between the two images. All images have been background subtracted. (r-w) Confocal images of fixed RBL-2H3 cells expressing GFP-actin enabling comparison of GFP-actin fluorescence (r, u, and green in t and w) with β3 or VLA4 (α4) integrin staining (s, v, and magenta in t and w) at the adherent surface. Confocal slice thickness is 1 μm. Images have been Gaussian filtered. Scale bars represent 1 μm in a-d and r-w, 2 μm in e, and j-q and 5 μm in f-i.

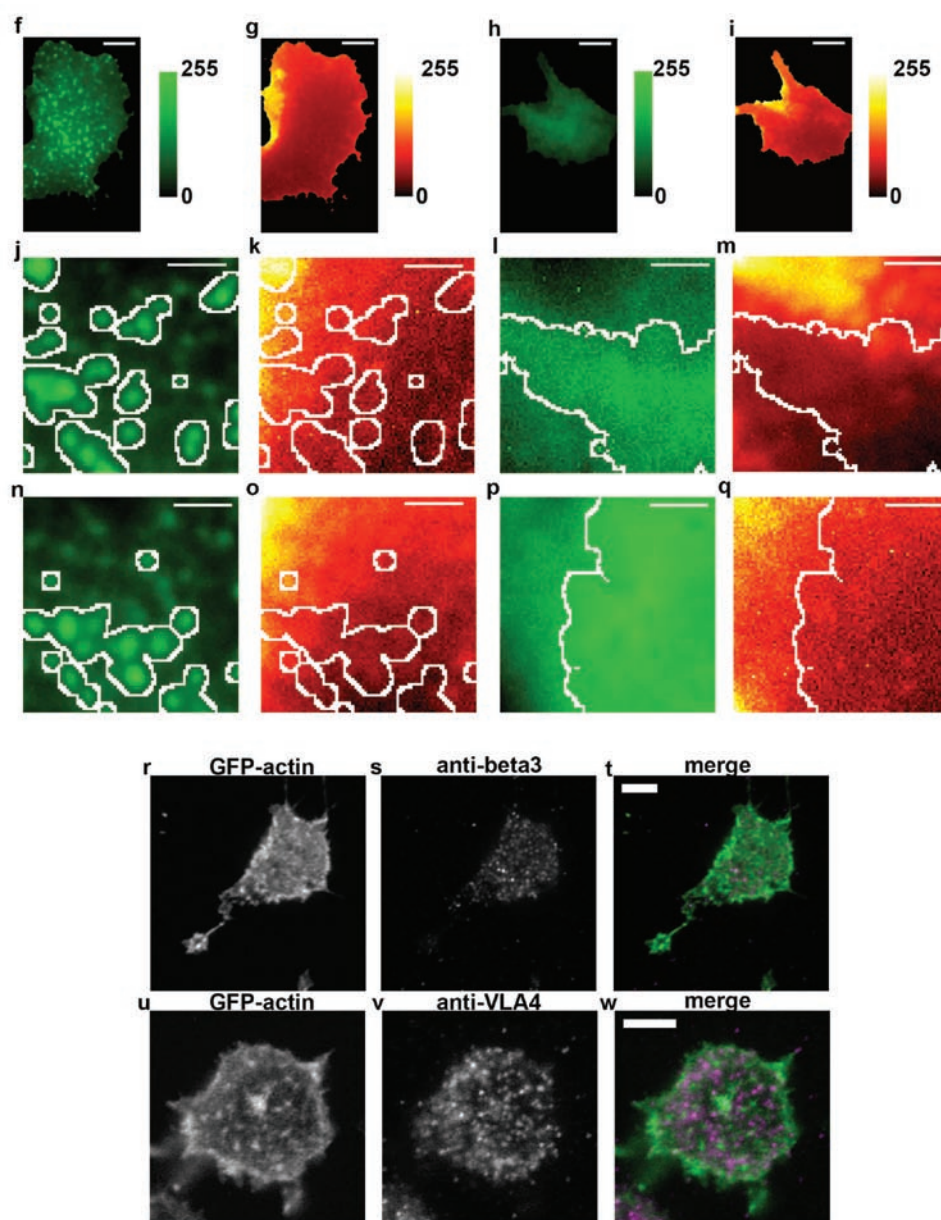


Figure S5 continued

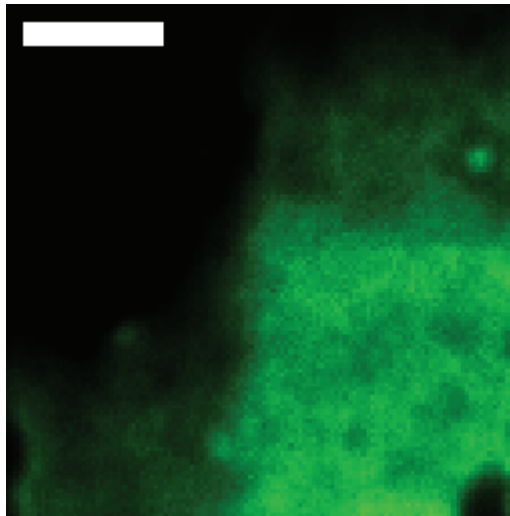


Figure S6 GFP-actin structure is visibly disrupted by latrunculin treatment. Deconvolved TIRF image of an RBL GFP-actin cell after 10 minute incubation with 500 nM latrunculin B. Note that less structure is apparent than in untreated cells (Figure S5a; Figure 4c in main text, and Supplementary Video 4). Scale bar represents 2 μm .

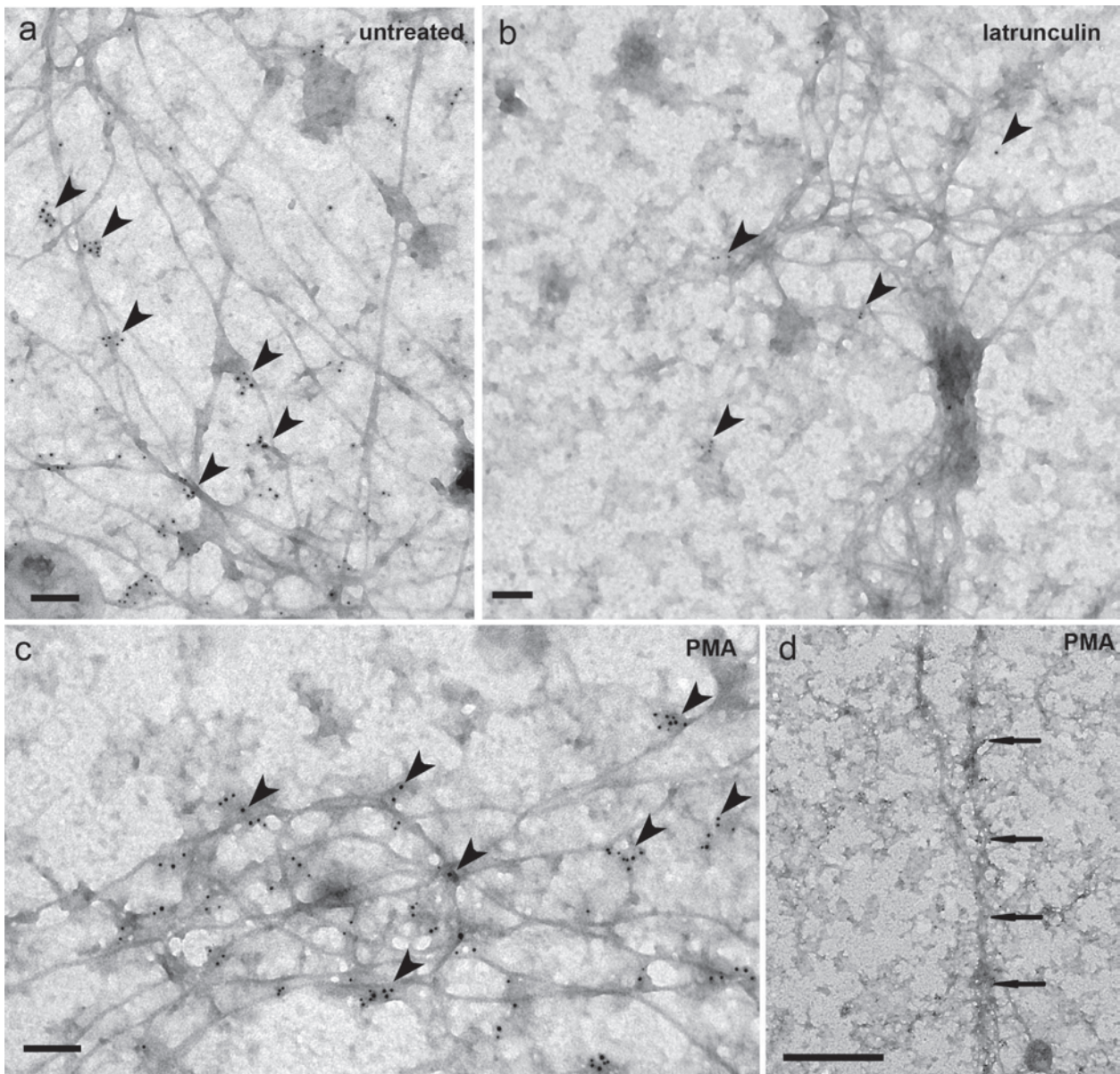


Figure S7 Electron microscopy of membrane sheets. Membrane sheets from the adherent surface of RBL cells (prepared as in³ with the exception that cells were grown on EM grids) expressing GFP-actin. Gold particles label GFP-actin (arrowheads in **a-c**), which is located primarily at cytoskeletal junctions in the untreated (**a**) and PMA treated (50 nM for 30 min) (**c, d**) cells. This pattern is consistent with earlier observations⁴. Large actin

bundles (arrows in **d**) likely correspond to GFP-actin structures visible by fluorescence microscopy. After treatment with latrunculin B (10 µg/ml for 60 min), GFP labelling is dramatically decreased (indicating a disruption of the actin filaments), but cable-like structures are still present (presumably composed of an intermediate filament protein) (**b**). Scale bars represent 100 nm in **a-c** and 500 nm in **d**.

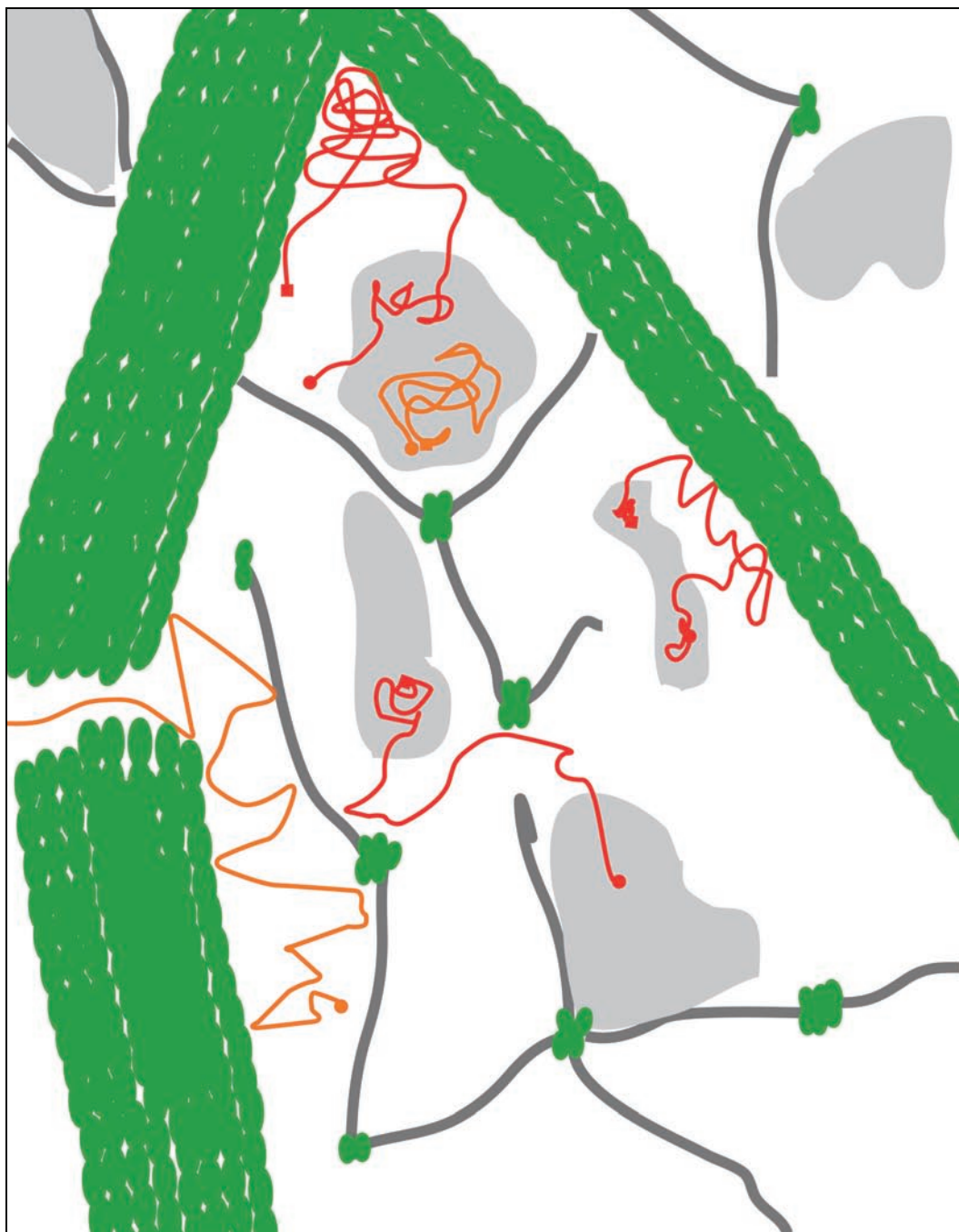


Figure S8 Cartoon model of receptor movement through the membrane architecture. Large actin bundles (green filaments), as observed in fluorescence microscopy, partition the membrane into large, micron-sized regions. Within this larger structure is a fine meshwork of cytoskeleton composed of intermediate filaments (dark grey) with actin at the junctions (as seen in EM)

that form nano-scale domains. Additionally, membrane rafts (lipid rafts/protein islands, light grey) form specialised domains. Sample receptor trajectories (red, orange) are overlayed on this landscape, to demonstrate ways that these various components can restrict diffusion. Though a single snapshot of the membrane landscape is depicted here, in reality it is dynamically changing.

Supplementary Movie Legends

Movie S1 RBL-2H3 cell activation through QD-IgE. RBL-2H3 cell stably expressing FcεRIγ-mCFP (left panel), primed with 1 nM QD-IgE (middle panel) and stimulated with 14 nM DNP-BSA. Receptor aggregation and internalisation are readily apparent in the fluorescence channels, and cell ruffling and spreading can be seen in the DIC image (right panel). Images acquired at 37°C at 2 frames/min; playback is 5 frames/s. Fluorescence channels have been Gaussian filtered. Scale bar represents 10 μm.

Movie S2 Co-confinement of multiple QD-IgE-FcεRI. RBL-2H3 cell labelled with 100pM QD655-IgE for 10 min at 37°C. Images acquired at 35°C at 33 frames/s, playback is 33 frames/s. Scale bar represents 5 μm.

Movie S3 RBL-2H3 cells were transfected with GFP-actin, labelled with QD655-IgE and treated with PMA. Images of the two spectral channels were collected simultaneously on the same CCD camera at 33 frames/s and the two channels were made coincident by shifting the GFP-actin channel (green) using a Fourier based shift algorithm. SPT was performed on the QD-IgE images as described in the text. A small ~ 4x4 micron area centered on the region containing a QD-IgE trajectory was selected for further processing. The “green” channel containing the GFP-actin signal was de-noised and deconvolved as described. For improved visual clarity, the found positions of the tracked QD-IgE were used to generate a 2D Gaussian representation of the particle (magenta) and were combined as a colour overlay with the actin time series to generate a two-colour movie. Playback is 10 frames/s. Scale bar represents 1 micron.

Movie S4 Restricted receptor diffusion on untreated RBL-GFP-actin cells. Same image processing details as in Video 3. Playback is 10 frames/s. Scale bar represents 1 micron.

Movie S5 As described in the text, a 100 frame time series image was collected by imaging the top of the cell with a Zeiss 510 confocal microscope. RBL-2H3 cells were transfected with GFP-actin (green) and labelled with QD-IgE (magenta) and were otherwise untreated. Emission was collected with a 545 nm dichroic mirror splitting emission to a 505 LP emission filter for GFP and the META detector was used as a bandpass filter (625-689 nm) to collect QD655 emission. Images were taken with 2× averaging, giving a final rate of 1 frame/s. Each frame of the GFP-actin image was de-noised using a wavelet based filter as described for the initial de-noise step in⁵. SPT was performed on the QD-IgE images as described in the text. For improved visual clarity, the found positions of the tracked QD-IgE were used to generate a 2D Gaussian representation of the particle (magenta) and were combined as a colour overlay with the actin time series to generate a two-colour movie. Playback is 10 frames/s. Scale bar represents 5 μm.

Supplementary Methods

EM analysis of QD-IgE valency

Solutions of 1nM QD-IgE or QD were prepared in PBS, 1% BSA and incubated on EM grids (formvar and carbon coated, then glow-discharged) for 15 min at room temperature. Grids were rinsed with PBS, then incubated with DNP-BSA-5nM gold (in PBS, 1% BSA) for 30 min at room temperature, followed by a PBS wash and fixation with 2% glutaraldehyde. Electron micrographs were acquired using a transmission electron microscope (Hitachi H600). From these images, we calculated averages of 1.3 and 6.5 DNP-BSA-gold particles per μm^2 for QD only ($43.5 \mu\text{m}^2$ total area) and QD-IgE ($12.8 \mu\text{m}^2$ total area) samples, respectively; indicating 20% non-specific binding of the DNP-BSA-gold probe. The average distances between each gold particle and the nearest QD were 92.7 and 51.4 nm for QD (n=57 gold particles) and QD-IgE (n=83 gold particles) samples, respectively.

TIRF controls

To ensure that interactions with the coverslip were not dictating QD-IgE-Fc ϵ RI motion, we performed several control experiments. We monitored diffusion of unbound streptavidin QD underneath GFP-actin cells (Fig. S6a), acquired cross-sectional EM images of adherent RBL-2H3 cells (Fig. S5), tracked QD-IgE-Fc ϵ RI complexes on the top (non-adherent) surface of RBL GFP-actin cells using confocal microscopy (Fig. 4 in main text) and on the bottom of RBL-GFP cells using TIRF microscopy (Fig. S6b), imaged RBL-GFPactin and RBL-GFP cells in the presence of soluble fluorophore (Fig. S7 and S8, respectively) using TIRF microscopy, and acquired confocal images of the adherent surface of fixed GFP-actin cells stained for the $\alpha 4$ and $\beta 3$ integrins (Fig. S9). All of these experiments indicate that there is enough room for diffusion of the QD-IgE-Fc ϵ RI complex on the adherent cell surface and that the observed restriction of diffusion by actin structures is not merely a result of steric limitations due to membrane-coverslip proximity.

Free QD diffusion. SPT conditions were the same as for QD-IgE-Fc ϵ RI, except that unlabelled QDs that diffused between the cell and coverslip were tracked.

EM sections. Cells were grown as monolayers on plastic tissue culture dishes, and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. Cells were embedded in EPON and allowed to harden. The EPON-embedded cells were separated from the plastic surface and then mounted perpendicular to the plane of adhesion for sectioning. Sections were then processed and imaged as described previously⁶.

RBL-GFP cells. RBL-GFP cells provided a means to visualise the topography of the adherent cell surface. Regions of the cell closer to the coverslip would have a larger volume within the TIRF field and therefore appear brighter, whereas regions further from the coverslip would have less volume within the TIRF field and appear dimmer. These cells were then labelled with 1 nM QD655-IgE and the QDs were tracked at 10 frames per second while simultaneously acquiring the cytoplasmic GFP signal.

Free dye. RBL-GFPactin or RBL-GFP cells were imaged at 5 frames/s in the presence of 20 nM Alexa Fluor® 647-R-phycoerythrin streptavidin (Invitrogen, Carlsbad, CA) in the imaging buffer. Regions of close contact between the cell and the coverslip exclude more of the soluble fluorophore and therefore have less fluorescence compared to regions which were further from the coverslip. By simultaneously acquiring the GFP-actin signal and the soluble fluorophore signal, we could compare the distribution of GFP-actin with the distance between the membrane and the coverslip.

Integrin staining. RBL-2H3 cells expressing GFP-actin were grown on 15 mm round glass coverslips and fixed with 2% paraformaldehyde for 20 minutes at room temperature. Cells were then permeabilised by 10 minute incubation in 1% Triton and stained with anti-VLA4 (anti- $\alpha 4$; Endogen, Boston, MA) or anti- $\beta 3$ (H-96; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies, followed by incubation with Alexa555-conjugated secondary antibodies (Alexa Fluor® 555 F(ab')₂ fragment of goat anti-mouse IgG (Invitrogen, Carlsbad, CA) for anti-VLA4 and Alexa Fluor® 555 donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA) for anti- $\beta 3$). Coverslips were then mounted in ProLong Gold® anti-fade reagent (Invitrogen, Carlsbad, CA) and imaged on a Zeiss LSM510 META confocal imaging system.

Gene constructs and transfection

The γ subunit of human Fc ϵ RI (Accession number NM 004106; GI 4758343) was initially cloned into the EcoRI/SalI site of pCMV6-XL5 (OriGene, Rockville, MD), followed by restriction digest-mediated transfer, in frame with mCFP, into pEF-DEST51 expression vector (Invitrogen, Carlsbad, CA). The pmaxGFP vector was supplied as part of the Cell Line Nucleofector Kit L (Amaxa, Gaithersburg, MD). Transfection was accomplished by introduction of the vector into early passage RBL-2H3 cells using the Amaxa Nucleofector II with Solution L and Program T-20 (Amaxa, Gaithersburg, MD). RBL-2H3 cells transiently transfected with pmaxGFP were imaged within 24 hours. Cells stably transfected with pEF-Fc ϵ RI γ -mCFP were selected with 2.5 μ g/ml Blasticidin (Invitrogen, Carlsbad, CA), followed by fluorescence associated cell sorting (FACS) for CFP emission.

Image Registration

For two colour imaging, fluorescence was separated into two spectrally distinct images by a dual colour image splitter (OptoSplit II, Cairn Research, UK), which were captured simultaneously and side-by-side on a single CCD camera (iXon 897 or Luca, Andor UK). The spectral windows of the captured images were defined by a dichroic filter and two emission filters in the image splitter (565DXCR, D510/40m, D655/40m, respectively, Chroma, Rockingham, VT). The relative translation of one image with respect to the other on the CCD camera was calibrated by imaging multi-fluorophore fluorescent beads (0.2 μ m Tetraspeck, Invitrogen, Carlsbad, CA) that have an emission spectrum covering the two spectral windows. The translation vector was found using an iterative cross-correlation routine providing sub-pixel accuracy⁷. The quality of the alignment (merging) of the two images was limited only by small aberrations present in one or both of the channels that lead to a typical 10 nm error towards the center of the image (where the alignment routine was biased) to a 30 nm error towards the edges of the images.

Image Processing

All data analysis and image processing was performed within the MATLAB (The Mathworks Inc.) environment, including the DIPImage⁸ image processing library. Single particle tracking algorithms were coded in the c language and called from within MATLAB through a mex interface. For essential speed optimisation (~20 X speedup over a MATLAB implementation), the Richardson-Lucy deconvolution code was written using the CUDA toolkit⁹ (NVIDIA, Santa Clara, CA) to allow calculation using a graphics processing unit (NVIDIA 8800 GTS), and was called from within the MATLAB environment through a mex interface. Confocal images were de-noised using a two dimensional wavelet based filter^{5,10}.

Single Particle Tracking

Single particle trajectories were determined from the raw data sets using a three step process: (1) identification of areas of interest; (2) Gaussian fitting; (3) building trajectories from coordinates. This approach is similar to those described previously¹¹⁻¹⁴.

1. Identification of areas of interest. Each 2D image from a 3D data set was processed independently to find QD coordinates. Areas of interest were contiguous regions of pixels that met two criteria: (a) pixels had intensities greater than three times the standard deviation of pixel intensities from areas defined as background (backgroundoffset algorithm,⁸) and (b) pixels were above a threshold, (threshold,⁸). Afterwards, a high pass filtering of the image was performed by subtracting from the image a 2D Gaussian filter with $\sigma = 5$. The result is a binary image of pixels that passed both criteria.

2. Gaussian fitting. The center of mass of each contiguous region in the binary image was used as a starting point in a Gaussian fitting routine. The raw 2D images were used being offset to zero. The highest intensity pixel in a small region around the starting point (typically 5 pixels square) was used as an updated starting point to the iterative “Gaussian mask”¹⁵ fitting routine. Fits were performed in a square region, of size $\sim 2 \times \sigma_{\text{psf}}$, around the updated starting point, where σ_{psf} defines the size of 2D Gaussian approximation to the

point spread function. After convergence of the fitting routine, defined as a change in location of less than 10^{-5} pixels, a normalised cross-correlation was calculated using the data and a 2D Gaussian with center given by the result of the fit. The found coordinates were only considered as positions of QDs and used in further analysis if they exceeded a cross-correlation value of 0.7.

3. *Building trajectories from coordinates.* The probability of finding a diffusing particle in two dimensions at a distance greater than r from its starting point after a time Δt is given by¹⁶

$$P(r, \Delta t) = \exp[-r^2 / (4D\Delta t)] \quad \text{eq. 1}$$

Trajectories were built from the set of 3D coordinates in two steps. First, coordinates identified at time t were compared with coordinates at time $t + \Delta t$ using eq. 1 where Δt is the inverse frame rate of data acquisition. If $P(r, \Delta t)$ was found to be greater than .05, the coordinate at $t + \Delta t$ is associated with the coordinate at t in a trajectory. This process builds short, un-interrupted trajectories. Due to the blinking of QDs, temporally separated trajectories may correspond to the movement of the same QD. The end coordinate of all trajectories are compared with all later starting coordinates of other trajectories using eq. 1, where Δt is now the time interval between the end of the first trajectory and the beginning of the second. The later trajectory with the smallest Δt that has a $P(r, \Delta t) > .01$ is connected with the first trajectory. This process is continued until there are no remaining pairs of trajectories that satisfy the criteria. If a trajectory contains more than four coordinates, a diffusion coefficient is estimated from the trajectory as

$$D_{\text{est}} = \text{MSD}(\Delta t) / 4\Delta t \quad \text{eq. 2.}$$

where MSD is the mean square displacement and Δt is the inverse frame rate. The diffusion coefficient, D , used in eq. 1 is then the mean diffusion coefficient from the two trajectories. If the diffusion coefficient of the two trajectories varies by more than a factor of ten, trajectories are not combined.

Short range interaction analyses

RBL-2H3 cells were labeled with 200 pM each QD655-IgE and QD585-IgE for 10 min at 37°C. TIRF imaging was performed on these cells at 100 frames/s at 35°C and the resulting QD-IgE-FcεRI time series were then tracked as described (see Supplementary Methods, Single Particle Tracking section) to generate sets of trajectories for each colour QD. All trajectories from one colour of QD were compared with all trajectories of the other colour. If QDs of different colour were within a cutoff distance (500 nm) in the same time frame, a set of parameters was calculated based on the found positions of the QDs in the next time frame. All calculated parameters were recorded as a function of initial separation. Uncorrelated jump distance and jump magnitude were calculated to determine if coordinated movement or transient dimerisation existed between the tracked QD-IgE-FcεRI complexes. These parameters were averaged over all frames and QD pairs into 50 nm separation bins and then plotted, with the error given as the standard error of the mean in each bin. The degree of correlation between the motions of two particles was obtained by determining the amount of uncorrelated motion between two nearby particles: $D_i = |\mathbf{J}_i - \mathbf{J}_j(\mathbf{J}_i \cdot \mathbf{J}_j) / (|\mathbf{J}_i||\mathbf{J}_j|)|$, where $\mathbf{J}_i = \mathbf{r}_{i+1} - \mathbf{r}_i$, $\mathbf{J}_j = \mathbf{r}_{j+1} - \mathbf{r}_j$, and $\mathbf{r}_i$ is the position of a particle in frame i . The magnitude of single time step displacements, $|\mathbf{J}_i|$, was calculated in a similar manner. Parameters without a particle index (1,2) are calculated for both QDs.

Deconvolution

Deconvolution of time series images involved several steps. First, the pixel regions in each three dimensional data stack that corresponded to actin regions were cropped to form smaller three dimensional data sets and filtered in the time dimension with a Gaussian filter with $\sigma = 10$ frames (0.3 seconds). For manageable processing of several hundred data sets, each data set containing 2,000 images, only 1 of 10 time filtered images were deconvolved, starting with frame 5 and ending with frame 1,995. These 200 images first had a pixel-wise camera offset subtracted from them and then were independently de-noised using a two dimensional wavelet based filter^{5,10}. To prevent edge related artifacts during deconvolution, each image was mirrored along each edge by 16 pixels that were then multiplied by a cosine function that went to zero at the outermost pixel. A two dimensional Gaussian was used to represent the microscope point spread function (PSF). The width of the PSF was determined by a fit to a high signal to noise image of a 100 nm bead (Tetraspeck, Invitrogen, Carlsbad,

CA). Two hundred iterations of a Richardson-Lucy deconvolution^{17,18} were performed on each image with an entropy regularisation¹⁹ after each iteration with regularisation parameter of 0.05. After deconvolution, images were cropped to their original size.

Binary Segmentation of Actin Structures

The deconvolved time series images were used for actin segmentation. Each image was filtered, and segmented using a four step process. First, a top hat filter (DIPImage function 'tophat') selects regions that are above a local minimum. Second, the top hat filtered image is eroded using grey scale erosion (DIPImage 'erosion') to better define the brightest regions. Third, the image is locally contrast stretched by dividing each pixel by the maximum value found in a 10 pixel (0.67 micron) radius around each pixel. Finally, the resulting image is thresholded (DIPImage function 'threshold') to create a binary image representing the location of actin structures.

Actin Trajectory Overlap

Coordinates found from single particle tracking were used to build binary trajectory images that were compared with actin structures. If a coordinate was valid (QD has not 'blinked' off) in both a time frame t and the next time frame at $t+1$, a binary image was created of this single time frame jump by drawing a pixelised line from the starting point to the end point. The linear pixel size used is the back-projected pixel size of the collected CCD images, which was 67 nm. For all valid single frame jumps in each trajectory found by single particle tracking, the total length of the linearised trajectory, as well as the number of pixels in the trajectory that overlapped with the binary actin image at time t were recorded. For each trajectory, the same calculation was made using 10 simulated particles that had the same starting coordinate, diffusion coefficient, trajectory length, and blinking behaviour as that of the single particle trajectory, but were otherwise unrestricted.

To test if the found trajectories had behaviour with respect to actin that was significantly different from the unrestricted, simulated particles with the same diffusion coefficient, we treated the system using a binomial model as follows. Within groupings for each cell treatment, all trajectories were combined to give a total trajectory length n , and a total actin overlap k . The simulated particle trajectories were used to define a probability for overlap p for the unrestricted case. We make the simplification that all pixels in a trajectory can be treated independently with respect to actin overlap in order to use the well defined binomial model. The cumulative distribution function of the binomial distribution is $F(k;n,p)=I_{1-p}(n-k,k+1)$ where I is the regularised incomplete beta function. F gives the probability that a randomly generated data set created from the binomial model with parameters n and p would have a value less than or equal to k . We reject the hypothesis that our trajectories can be modeled with unrestricted diffusion with respect to actin when F is less than .01. In all three cases, $F \ll 0.01$, with $F = 2.35 \times 10^{-286}$, 2.35×10^{-264} and 2.42×10^{-154} in the resting, latrunculin-treated, and PMA-treated cells, respectively.

Diffusion in proximity to actin

A pixelated distance map to the nearest actin structure was found by a distance transform (DIPImage function 'dt') of the inverse of the binary images created by the binary segmentation of the actin images. The mean square jump distance of single time step jumps was recorded as function of distance from actin for all SPT trajectories in cells treated with PMA.

An offset term was calculated by fitting individual particle trajectories to Eq.(1) using the first 3 data points of the MSD. This value is equivalent to the first point of a calculated MSD plot and can be related to the diffusion coefficient.

The mean observed jump size over some time interval of a freely diffusing particle (and thereby calculated diffusion coefficient) will appear to be reduced near a reflecting boundary due to the particle's inability to make large jumps in the direction of the boundary²⁰. In order to estimate the magnitude of this effect, simulations of a diffusing particle near a reflecting half space boundary were performed using the pixel size and frame rate of the experiment with parameters that correspond to the median experimentally observed D_{1-3} diffusion coefficient found as described above. To represent detector time averaging, 10 sub-frames at 10 times the frame rate were

added together to form one simulated observed frame. If a particle's position after a jump in one of the sub-frames was beyond the reflecting boundary ($x = 0$), its x -coordinate was multiplied by -1 and the next frame was calculated as a jump from the corrected position. The resulting time series were analysed with the same routines used for experimental data.

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