

MOLECULAR PERSPECTIVE OF ANTIGEN-MEDIATED MAST CELL SIGNALING

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Running head: Multiscale Dynamics of IgE Receptor Activation

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Antigen-mediated crosslinking of the high-affinity receptor for IgE (FcεRI) in the plasma membrane of mast cells is the first step in the allergic immune response. This event triggers the phosphorylation of specific tyrosines in the cytoplasmic segments of the β and γ subunits of FcεRI by the Src tyrosine kinase Lyn, which is anchored to the inner leaflet of the plasma membrane. Lyn-induced phosphorylation of FcεRI occurs in a cholesterol-dependent manner, leading to the hypothesis that cholesterol-rich domains, or 'lipid rafts', may act as functional platforms for IgE receptor signaling. Testing this hypothesis under physiological conditions remains challenging due to the notion that these functional domains are likely transient and much smaller than the diffraction limit of optical microscopy. Here, we use ultrafast fluorescence dynamics to investigate the correlation between nanostructural changes in the plasma membrane (labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine [diI-C₁₈]) and IgE-FcεRI crosslinking in adherent RBL mast cells stimulated with multivalent antigen. Time-dependent two-photon fluorescence lifetime imaging microscopy (2P-FLIM) of diI-C₁₈ shows changes in lifetime that agree with the kinetics of stimulated tyrosine phosphorylation of FcεRI under the same conditions. In addition, 2P-FLIM of Alexa Fluor 488-labeled IgE indicates that Förster resonance energy transfer occurs with diI-C₁₈ in the plasma membrane. Our live cell studies provide direct evidence for the association of IgE-

FcεRI with specialized cholesterol-rich domains within ~4 nm-proximity and with an energy transfer efficiency of 0.22 ± 0.01 at maximal association during IgE receptor signaling.

INTRODUCTION

Cholesterol-rich lipid domains in the plasma membrane, also known as 'lipid rafts', are believed to be engaged in various cellular functions, such as IgE receptor (FcεRI) signaling in mast cells and basophils (1-5). Antigen-mediated crosslinking of these cell surface receptors leads to their translocation into these domains prior to phosphorylation by the Src family kinase, Lyn, in a process that ultimately initiates the exocytotic release of histamine in the allergic response (1,6). Due to the inherent complexity of cellular membranes, there has been major interest in understanding the thermodynamics and kinetics regulating the formation of lipid domains in model systems, such as giant unilamellar vesicles (7-9) and supported bilayers and monolayers (10-12), as well as in cells or blebs derived from plasma membranes (13-23). Forming model membranes with physiological proportions of cholesterol and various lipids, however, has produced conflicting evidence of whether segregated lipid domains actually form at 37°C (24). In addition to imaging such lipid microdomains with optical microscopy (7-12,21), translational diffusion (ms-s) has been the main observable in various studies using single particle tracking (17,18), fluorescence recovery after photobleaching (FRAP) (10,20,21), and fluorescence correlation spectroscopy (FCS) (7,16,20).

Many studies of cholesterol-rich lipid domains associated with IgE receptor signaling have mainly

focused on RBL mast cells in suspension that are substantially (i.e., non-physiologically) perturbed by crosslinking with a secondary antibody at 4°C (6,20,21,25,26). In such studies, ordered lipid microdomains within the plasma membrane (labeled with a fluorescent lipid analog such as 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine, or diI-C₁₈ (25)) have been found to co-redistribute with patches of crosslinked receptor-bound IgE (labeled, for example, with Alexa Fluor 488 (A488-IgE) (25)). Previously, we reexamined existing spatial relationships between lipid microdomains and crosslinking-induced IgE-FcεRI patches at the single cell level by probing the spatio-temporal dynamics (ps–ns) of the molecules that comprise these domains (25). Using ultrafast excited-state dynamics and rotational diffusion (25), we observed that lipid order (i.e., packing) or nanostructure of the plasma membrane is enhanced within crosslinking-induced lipid microdomains, or patches, as evidenced by increases in both fluorescence lifetime and rotational diffusion of diI-C₁₈. Similar trends in the same dynamic properties were also observed for crosslinked, co-localized A488-IgE-FcεRI. These results supported the hypothesis that lipid molecules are recruited into more ordered domains that serve as platforms for IgE-FcεRI signaling. Further, the gross perturbations inherent under non-physiological crosslinking conditions provided a simple platform on which to test and optimize our fluorescence dynamics imaging assay (25,27) for probing molecule-molecule interactions and structural changes relevant to cell signaling.

Nonetheless, under physiological conditions, cholesterol-rich lipid domains in live cells have been hypothesized to vary in composition, dimension and life span, making them a challenge to directly interrogate with relevant spatial and temporal resolution (28,29). The spatial scale of diffraction-limited imaging and the timescale of translational diffusion-based spectroscopic techniques (μs–s) present an obstacle toward understanding the molecular bases regulating domain formation and biological function. FRAP and FCS have been used to monitor the translational dynamics of carbocyanine probes in adherent RBL cells as a function of cholesterol depletion (16) and in suspended cells subjected to non-physiological IgE-FcεRI crosslinking (20,21). Further,

electron-spin resonance spectroscopy (23) and Förster resonance energy transfer (FRET) (22) have been used to investigate the phase or order of suspended cell membrane lipids and their lateral distributions, but not within the context of IgE-FcεRI crosslinking and signaling.

Here, in individual mast cells under more physiological conditions, we test the hypothesis that IgE-FcεRI signaling occurs in specialized, cholesterol-rich regions of the plasma membrane, using the same fluorescence dynamics assay (25,27). Our rationale is that membrane-associated molecular events on the ps–ns timescales translate to interrogating structural changes on the spatial order of 100 nm (at 100 ps resolution) (30), which is below the diffraction limit of traditional optical microscopy. By interrogating the fast molecular dynamics and order of the plasma membrane as mast cells are stimulated with multivalent antigen at room temperature (23°C), where domains may be transient and smaller than the diffraction limit, we could relate these changes directly to stimulated tyrosine phosphorylation of FcεRI under identical conditions. To the best of our knowledge, the work described here represents the first time that these ultrafast dynamics methods have been used to monitor membrane-protein relationships during physiological cell signaling.

EXPERIMENTAL PROCEDURES

Cell preparation — RBL-2H3 mast cells were maintained and harvested as previously described (6,31). Adherent cells (1.5×10^5 cells/mL) were plated onto glass-bottomed 35-mm Petri dishes (MatTek) and sensitized overnight with 0.75 μg of mouse anti-2,4-dinitrophenyl IgE (α-DNP IgE, provided by Dr. David Holowka, Cornell University) per 1 mL of cell suspension. IgE was used either unlabeled or conjugated to Alexa Fluor 488 (A488-IgE) via a commercially available protein labeling kit (Invitrogen) (dye/IgE = 4). The day of the experiment, the cells were washed 3× with bovine serum albumin (BSA)-containing buffered saline solution (BSA/BSS: 20 mM HEPES pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 1 mg/mL BSA). Cells were then labeled for 3 min at 37°C with 1 mg/mL diI-C₁₈ (Invitrogen) stock solution prepared in dimethylsulfoxide (DMSO) that had been diluted 100-fold in BSS, and then washed 3× with

BSA/BSS. RBL cells were stimulated on the microscope at room temperature (23°C) with 1 µg/mL multivalent DNP-BSA antigen. Cells were either imaged as only diI-C₁₈ labeled, only A488-IgE labeled, or dually labeled with both probes.

For solution experiments, A488-IgE was diluted in phosphate-buffered saline (PBS: 10 mM Na₂HPO₄, pH 7.4, 150 mM NaCl) and diI-C₁₈ in DMSO. Absorption spectra of 10 µM Alexa Fluor 488 in water, 0.052 µM A488-IgE in PBS, and 10 µM diI-C₁₈ in DMSO were measured with a diode array spectrophotometer (Hewlett Packard 8453A) equipped with Chemstation software (Agilent). A fluorimeter (HORIBA Jobin Yvon Fluorolog FL3-21) was used to record the fluorescence emission spectra of 0.1 µM Alexa Fluor 488 in water, 0.01 µM A488-IgE in PBS, and 2.5 µM diI-C₁₈ in DMSO.

In vivo tyrosine phosphorylation assays — Adherent, IgE-sensitized RBL cells were plated overnight in 48-well culture plates. The cells were washed 3× with BSA/BSS and stimulated at 23°C for the indicated times with 1 µg/mL DNP-BSA, lysed by addition of SDS sample buffer (10% glycerol, 0.05 M Tris, pH 6.8, 1% SDS, 0.1% bromophenol blue), boiled for 5 min and centrifuged for 5 min at 13,000 × g. Equal numbers of cell equivalents of lysate (typically 3.5 × 10³ cell equivalents) were electrophoresed on 12% nonreduced SDS polyacrylamide gels, transferred to BioTrace PVDF membrane (PALL Gelman Laboratory), and blotted with horseradish peroxidase-conjugated anti-phosphotyrosine (4G10-HRP) (Millipore). Enhanced chemiluminescence (Pierce Biotechnology) was used to detect phosphorylated proteins and quantified from scanned blots using Un-Scan-It (Silk Scientific) and IgorPro (WaveMetrics) (6,26).

Confocal fluorescence microscopy — The experimental setup and data analysis have been described in more detail elsewhere (25,27). Briefly, a confocal microscope consisting of a fiber-coupled laser system, a scanner (Olympus FluoView300), differential interference contrast (DIC) optics, an inverted microscope (Olympus IX81), and an Olympus 60×, 1.2 NA water-immersion objective was used for three-channel imaging. A488-IgE was imaged using 488 nm argon ion laser excitation and a 525/30 bandpass filter, while diI-C₁₈ was imaged with 543 nm HeNe laser exci-

tation and a 605/70 bandpass filter. DIC imaging was used to monitor cell viability before and after all experiments to ensure that no photodamage had occurred. Unlabeled RBL cells showed negligible autofluorescence (data not shown).

Fluorescence lifetime imaging — A femtosecond laser system (~120 fs, 76 MHz) provided 960 nm excitation, which was conditioned and steered toward the sample, through the confocal scanner and Olympus 60×, 1.2 NA water-immersion objective for 2P-FLIM. The epi-fluorescence was isolated from the excitation laser (~0.5–5 mW, or ~10–100 pJ/pulse at the sample) using a dichroic mirror and filters (690SP for all samples, plus a 600LP to detect only diI-C₁₈ or plus a 525/50 bandpass to detect only A488-IgE, Chroma) prior to detection using microchannel plates (Hamamatsu R3809U-50). For 2P-FLIM, measured at the magic angle (54.7°), and polarization imaging, measured at polarizations parallel and perpendicular relative to the excitation laser, the epi-fluorescence polarization was analyzed using a Glan-Thompson polarizer before each of two detector channels. The signal was amplified and fluorescence lifetime images were constructed with a time-correlated single-photon counting (TCSPC) module (Becker & Hickl SPC830) (27). 2P-FLIM images were recorded using 256 × 256 pixels with 65 time bins per pixel (i.e., 259 ps/bin) and a total acquisition time of 120 s, which is slower than membrane ruffling of antigen-stimulated cells. However, these studies remain relevant due to the three-dimensional resolution inherent in 2P-microscopy, the polarized excitation selectivity, and the distinct differences in time-scales of ruffling (s) versus excited-state dynamics (ps–ns). Experiments were performed over several days on different cell preparations. Complementary 1P (480 nm, 4.2 MHz) time-resolved fluorescence measurements, were carried out to enhance the time resolution and signal-to-noise ratio as described previously (25,27). In these studies, the laser beam was strategically positioned at areas of interest on the cell membrane. In some cases, single-cell averaging measurements using 2P-FLIM (pseudo-single point measurements) were also carried out to enhance the signal-to-noise ratio and temporal resolution using low repetition rate laser pulses (960 nm, 4.2 MHz) scanned over the entire cell.

Fluorescence polarization anisotropy imaging — Steady-state and time-resolved fluorescence anisotropy measurements were performed using 2P- and 1P-excitation, respectively. For steady-state 2P-anisotropy imaging, the parallel (0°) and perpendicular (90°) fluorescence polarizations were recorded simultaneously in two-channel, laser scanning mode. For time-resolved fluorescence anisotropy, 1P-laser pulses were positioned on regions of interest on the cells, or a single cell was averaged via 2P-FLIM, and the epi-fluorescence polarizations were resolved and detected simultaneously as described earlier. Both polarization images and time-resolved fluorescence polarization decays were constructed using the SPC830 module.

Theory and data analysis — The fluorescence lifetime of a fluorophore provides information about its molecular structure and the surrounding local environment (32,33). Generally, the magic-angle fluorescence decay of a fluorophore, located at pixel (x,y), can be described as a sum of exponentials with time constants (τ_i) and amplitudes (α_i) as follows (27):

$$I_{54.7}(t; x, y) = \sum_{i=1}^3 \alpha_i(x, y) \exp[-t / \tau_i(x, y)] \quad (1)$$

The magic-angle fluorescence decays were analyzed by a non-linear, least-squares fitting routine with deconvolution of the system response function (FWHM ~60 ps) in the SPCImage software package (Becker & Hickl). The average fluorescence lifetime was calculated as $\langle \tau_{fl} \rangle = \sum_i \alpha_i \tau_i / \sum_i \alpha_i$, and the residual and reduced chi square ($\tilde{\chi}^2$) were used to assess the goodness of fit, with $\tilde{\chi}^2 = 1.0$ –1.3 considered satisfactory. The error of the average lifetime value for a given 2P-FLIM image was determined by fitting the total image histogram to a Gaussian with IgorPro to obtain the width, or standard deviation. Unpaired, two-tailed Student's *t*-tests were performed using Excel (Microsoft) to determine whether variations in fluorescence decay parameters as a function of antigen stimulation were statistically significant ($p \leq 0.05$).

Following photoselective excitation of a fluorophore at pixel coordinates (x,y) in a cell, the time-resolved fluorescence polarization anisotropy, $r(t; x, y)$, can be described as (27):

$$r(t; x, y) = \frac{[I_{\parallel}(t; x, y) - I_{\parallel}^0] - G \cdot [I_{\perp}(t; x, y) - I_{\perp}^0]}{[I_{\parallel}(t; x, y) - I_{\parallel}^0] + 2G \cdot [I_{\perp}(t; x, y) - I_{\perp}^0]} \quad (2)$$

$$= \sum_{j=1}^3 \beta_j(x, y) \exp[-t / \phi_j(x, y)]$$

where the parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) fluorescence emission intensities per pixel were corrected for background signals ($I_{\parallel}^0$ and $I_{\perp}^0$, respectively). The *G*-factor (0.74) was calculated using the tail matching approach (32,33) to account for the polarization dependence of the detection efficiency (33). The rotational correlation time (ϕ) depends on the hydrodynamic volume of the fluorophore and the surrounding viscosity (33). The pre-exponential factor (β_j), whose sum is equal to the initial anisotropy, r_0 , can be used to assess the population fractions of fluorophores under different environmental restrictions (33). Depolarization due to the high NA objective was previously determined to be negligible when using an underfilled objective, as is the case for 1P-measurements here (25). The degree of orientational constraint of a fluorophore is defined by the ratio, r_{∞} / r_0 , which describes its range of angular rotation (34,35). Using MATLAB (The Mathworks), we developed an image processing algorithm to calculate steady-state initial anisotropy images ($r_0(t; x, y)$) (25,27) from parallel and perpendicularly polarized 2P-fluorescence images. The error in the average initial anisotropy value for a given image was determined by the method described for 2P-FLIM images above. For single point, time-resolved fluorescence polarization anisotropy, non-linear least-squares fitting (Eq. 2) of these decays was carried out using either OriginPro 7 (OriginLab) or IgorPro without deconvolution of the system response function. The goodness of the fit was evaluated using chi-square (χ^2) calculated by OriginPro 7 or IgorPro, with $\chi^2 = 0$ –0.01 considered satisfactory.

To probe lateral heterogeneity in the plasma membrane associated with IgE-FcεRI stimulation, we also carried out time-resolved FRET (24,36–38). Based on the spectral overlap between the membrane (diI-C₁₈) and IgE (A488), we

examined the potential of these fluorophores to be a donor-acceptor pair for FRET. The efficiency of energy transfer, E_T , is defined as $E_T = 1 - (\tau_{DA} / \tau_D)$, where τ_{DA} is the lifetime of the donor (A488-IgE) in the presence of acceptor (diI-C₁₈) and τ_D is the donor lifetime in the absence of acceptor, both of which are obtained from time-resolved fluorescence decays (33). Using E_T in combination with the spectral overlap, the donor-acceptor separation distance, R , was determined following $R = R_0 \left[(1/E_T) - 1 \right]^{1/6}$, where R_0 (in nm) is calculated as $R_0 = 0.0211 \left[\kappa^2 J(\lambda) \eta^{-4} Q_D \right]^{1/6}$ (33). The orientation parameter, κ^2 , describes the relative donor-acceptor dipole orientations and has an estimated value of 2/3 for randomly distributed fluorophores (33). We used time-resolved fluorescence polarization anisotropy to examine the validity of the dipole moment randomization of both the donor and acceptor (24). η is the refractive index of the medium (1.4 for biomolecules in aqueous solution (33)), and Q_D is the quantum yield of the donor Alexa Fluor 488 (0.6 (39)). $J(\lambda)$, the spectral overlap between the donor emission and acceptor absorption, is expressed as $J(\lambda) = \int \epsilon_A(\lambda) F_D(\lambda) \lambda^4 d\lambda$, where ϵ_A is the acceptor extinction coefficient (0.0148 M⁻¹ nm⁻¹), and F_D is the fluorescence emission intensity of the donor as a fraction of the total integrated intensity. Further, the rate of energy transfer (in ns⁻¹) is defined as $k_{FRET} = \left([\tau_D] \left[(1/E_T) - 1 \right] \right)^{-1}$ (33).

RESULTS

Antigen-mediated changes in cell morphology and IgE distribution are demonstrated by confocal microscopy — We monitored changes in cell morphology, IgE-FcεRI clustering and plasma membrane structure as a function of antigen stimulation using three-channel confocal microscopy. RBL cells labeled with the lipid analog, diI-C₁₈, and sensitized with A488-IgE were imaged immediately prior to the addition of soluble multivalent antigen to establish an initial baseline (“0 min”), and then subsequently imaged after the cells have

been stimulated for 5, 10, 20 and 30 min. Fig. 1 shows a representative time series, in which panel A depicts cell morphology and panels B and C show the corresponding diI-C₁₈ and A488-IgE-FcεRI fluorescence images, respectively. (Note that the fluorescence background observed only in Fig. 1B is due to diI-C₁₈ nonspecifically adhering to the glass coverslip, despite extensive cell washing or varying labeling conditions.) Activated cells spread and ruffle in an actin-dependent manner as observed previously (26,40).

Phosphorylation of IgE-FcεRI has been hypothesized to result from crosslinking-induced coalescence of nanoscopic, cholesterol-rich membrane domains (41) that are smaller than the diffraction limit of optical microscopy ($\sim \lambda/2$), thereby explaining the absence of clear diI-C₁₈-labeled membrane domains (Fig. 1B) that colocalize with A488-IgE-FcεRI (Fig. 1C). This physiological observation contrasts with non-physiological studies on RBL cells at 4°C (6,20,21,25,26), plasma membrane blebs below 20°C (42) and model systems such as GUVs (7-9,16), in which micron-sized domains were clearly observed. Although antigen-mediated changes in cell morphology and antigen-induced receptor crosslinking and their associated dynamics can easily be visualized with fluorescence microscopy (Fig. 1 and Supplemental Movie 1), the putative *sub*-diffraction changes of membrane nanostructure are not readily apparent in conventional imaging. To overcome this limitation, we performed excited-state dynamics experiments and 2P-FRET on diI-C₁₈ and/or A488-IgE labeled mast cells, which report on the immediate local environment of the fluorophore (≤ 10 nm) (33).

Antigen-mediated IgE-FcεRI crosslinking alters the membrane nanostructure as reported by ultrafast excited-state dynamics — We investigated changes in 2P-fluorescence lifetime and time-resolved FRET that occur as IgE receptor signaling proceeds. It is worth noting that stimulated plasma membrane ruffling by the actin cytoskeleton is much slower (s–min timescale) than the excited-state lifetime (ps–ns), allowing us to effectively separate these two processes. Even though the acquisition times for 2P-FLIM images (~ 120 s) are on the same timescale as membrane ruffling, the inherent three-dimensional resolution of 2P-excitation and the photoselectivity associ-

ated with polarized excitation validates the potential of this approach for probing molecular events of the plasma membrane and associated proteins during the cellular response to antigen stimulation (i.e., ruffling).

2P-FLIM images of diI-C₁₈ in RBL cells, which were dually labeled with diI-C₁₈ and A488-IgE, were fit to a triexponential and are shown before and after antigen stimulation in Fig. 2A. Corresponding A488-IgE confocal images were sequentially recorded after each diI-C₁₈ FLIM image to observe how changes in IgE-FcεRI distribution correlate with changes in membrane nanostructure, as reported by the fluorescence lifetime. FLIM images of diI-C₁₈ reveal morphological changes similar to those observed with confocal microscopy (Fig. 1). Based on these images, three apparent populations of diI-C₁₈ can be readily identified in the corresponding pixel-lifetime histogram (Fig. 2B), with lifetime components centered at ~0.65 ns and ~1.2 ns, and a fraction at ~1.7 ns. The fast (~0.65 ns) component undergoes the most notable shift upon antigen stimulation.

Complementary pseudo-single point time-resolved fluorescence studies (4.2 MHz laser pulses, λ_{ex} = 960 nm) on RBL cells labeled with diI-C₁₈ (Fig. 3A; Table 1) reveal a statistically significant increase in the average fluorescence lifetime of diI-C₁₈ within the first 5 min of stimulation (from 0.26 ± 0.2 ns to 0.32 ± 0.4 ns [*p* = 0.020; *n* = 4]) followed by a plateau in lifetime at later stimulation times (Table 1). The very short time constant (τ₁ ~60 ps) does not vary considerably with antigen stimulation and is likely due to aggregated, free diI-C₁₈ on the coverslip. Note that the pseudo-single point lifetime data shown in Table 1 of diI-C₁₈ labeled cells at 0 min (*n* = 4) agree with data from cells that were not subsequently stimulated with antigen (*n* = 17; data not shown).

Conventional 1P-single point lifetime measurements of diI-C₁₈ labeled cells (Table 1 and Fig. 3B) were carried out to confirm the results of 2P-FLIM and pseudo-single point experiments and reveal that diI-C₁₈ fluorescence decays as a triexponential. Compared with the results prior to antigen stimulation ($\langle \tau_{fl} \rangle = 1.1 \pm 0.1$ ns), the average fluorescence lifetime of diI-C₁₈ increases significantly (as determined by unpaired, two-tailed Student's *t*-tests) within the first 5 min after

stimulation, with $\langle \tau_{fl} \rangle = 1.25 \pm 0.09$ ns (*p* = 0.035; *n* = 5). As signaling continues, $\langle \tau_{fl} \rangle$ returns to its original value for the experimental duration (Fig. 3C). Further, the data shown in Table 1 for single point lifetime measurements of diI-C₁₈ labeled cells at 0 min (*n* = 5) agree with additional data from cells that were not subsequently stimulated (*n* = 25; data not shown).

Stimulated tyrosine phosphorylation of FcεRI supports fluorescence dynamics observations — The functional relevance of the transient increase in the lifetime of diI-C₁₈ was examined by following the time course of tyrosine phosphorylation of cells stimulated with the same conditions as the micro-spectroscopy experiments (1 μg/mL antigen, 23°C). The first identifiable biochemical step that occurs upon crosslinking of IgE-FcεRI by multivalent antigen is the phosphorylation of the β and γ subunits of FcεRI by the tyrosine kinase Lyn (6). Fig. 4A shows a representative immunoblot (*n* = 4) of time-dependent cell stimulation, and Fig. 4B shows the corresponding densitometric trace for the phosphorylation of FcεRI β. Under these conditions, phosphorylation is maximal at 5 min and remains sustained for the experimental duration. The phosphorylation kinetics agree with the kinetics of the changes in diI-C₁₈ fluorescence lifetime (Fig. 3C) and further strengthen the relevance of the lifetime results.

Time-resolved FRET between A488-IgE and diI-C₁₈-labeled membrane reveals lateral, nanoscale heterogeneity associated with antigen stimulation — To examine the potential interaction between ordered membrane domains (diI-C₁₈) and IgE receptor (A488-IgE) during antigen-induced stimulation, we carried out time-resolved FRET measurements on dually-labeled RBL cells. This approach enabled us to probe lateral heterogeneities at the nanoscale (i.e., 0.1–10 nm), well below the diffraction-limited resolution (~λ/2) inherent in optical microscopy. Steady-state spectroscopy of A488-IgE and diI-C₁₈ (Supplemental Fig. 1) indicated the potential of these fluorescent labels as a donor and acceptor, respectively, in a FRET pair, and based on the spectral overlap of the donor emission and acceptor absorption, the estimated Förster distance (*R*₀) is 3.33 ± 0.04 nm.

We measured the fluorescence lifetime of A488-IgE in RBL cells as a function of antigen stimulation, in the presence and absence of diI-C₁₈ (acceptor). Using pseudo-single point measurements, we observed a reduction in the excited-state fluorescence lifetime of A488-IgE in the presence of diI-C₁₈, as compared to A488-IgE in the absence of diI-C₁₈. (When detecting A488-IgE lifetime in the presence of diI-C₁₈, we ensured that the crosstalk between the donor and acceptor emission was negligible by using the appropriate emission filters prior to detection.) Representative fluorescence decays with two exponential components are shown in Fig. 5A, and the lifetimes (τ_i) and amplitudes (α_i) of all decay components are summarized in Table 1. In unstimulated cells labeled solely with A488-IgE, $\langle\tau_{fl}\rangle$ is 2.94 ± 0.05 ns ($n = 5$) and 2.41 ± 0.07 ns ($p = 2.73 \times 10^{-7}$) in the presence of diI-C₁₈ ($n = 6$). Using higher time resolution (4 ns timescale), the reduction in A488-IgE $\langle\tau_{fl}\rangle$ in the presence of acceptor is further emphasized (data not shown). Based on the measured reduction of the donor fluorescence lifetime in resting cells (i.e., prior to stimulation), in the presence of acceptor, the estimated E_T , R , and k_{FRET} were determined to be 0.18 ± 0.01 , 4.3 ± 0.1 nm, and 0.07 ± 0.02 ns⁻¹, respectively (Table 2).

Following antigen stimulation, the reduction of donor (A488-IgE) $\langle\tau_{fl}\rangle$ is significant in the presence of diI-C₁₈, going from 2.7 ± 0.1 ns to 2.1 ± 0.1 ns ($p = 8.88 \times 10^{-4}$) at 5 min of stimulation, and from 2.5 ± 0.2 ns to 2.15 ± 0.01 ns ($p = 5.93 \times 10^{-3}$) at 10 min. By 20 min, the average lifetime of A488-IgE in the presence of diI-C₁₈ is no longer statistically significantly different from that with A488-IgE alone. Interestingly, $\langle\tau_{fl}\rangle$ decreases significantly between 0 min and 5 min, both for cells labeled only with A488-IgE (2.94 ± 0.05 ns at 0 min and 2.7 ± 0.1 ns [$p = 5.52 \times 10^{-3}$] at 5 min) and for those dually labeled with A488-IgE and diI-C₁₈ (2.41 ± 0.07 ns at 0 min and 2.1 ± 0.1 ns [$p = 3.16 \times 10^{-3}$] at 5 min). This trend of decreasing $\langle\tau_{fl}\rangle$ as a function of stimulation time is substantiated by our observations of A488-IgE labeled cells obtained from 2P-FLIM at lower

signal-to-noise and temporal resolution. When the 2P-FLIM images of A488-IgE are fit under conditions of high binning to improve histogram signal-to-noise and better resolve lifetime components, the pixel image histograms reveal a decrease in $\langle\tau_{fl}\rangle$ by 5 min and at all subsequent time points relative to 0 min (Supplemental Fig. 2). (At low binning, there were no resolvable, significant shifts in $\langle\tau_{fl}\rangle$ of A488-IgE with antigen stimulation; data not shown.) The major peak of $\langle\tau_{fl}\rangle$ at ~ 2.5 ns (Supplemental Fig. 2) further agrees with the $\langle\tau_{fl}\rangle$ acquired via pseudo-single point measurements (Table 1). When we calculate E_T as a function of stimulation time (Fig. 5B), we find that the FRET efficiency increases from 0.18 ± 0.01 prior to stimulation to 0.22 ± 0.01 after 5 min of stimulation, followed by a gradual decrease with 0.14 ± 0.04 at 10 min, 0.11 ± 0.04 at 20 min and 0.09 ± 0.01 at 30 min (see Table 2 and Fig. 5B). The kinetics of energy transfer efficiency agree with those of stimulated tyrosine phosphorylation and changes in the average lifetime of diI-C₁₈.

These pseudo-single point measurements, with high temporal resolution as compared to 2P-FLIM, indicate that A488-IgE interacts with the membrane label, diI-C₁₈ (acceptor), via energy transfer. Such interactions only occur when A488-IgE and diI-C₁₈ are within 10 nm proximity, which is below the diffraction limit, and may lend insight into the transient nature of cholesterol-rich nanodomains that may act as signaling platforms.

From the acceptor perspective of FRET, we would expect that the fluorescence of diI-C₁₈ would increase at a rate of k_{FRET} and decay with a time constant corresponding to the excited-state lifetime(s) of diI-C₁₈ (33). To directly measure k_{FRET} , we measured the fluorescence decay of diI-C₁₈ at lower (16 ps/channel) and higher (5 ps/channel) temporal resolutions (data not shown). The acceptor fluorescence decay in resting RBL cells builds up instantaneously (i.e., within the FWHM of the system response) without a rise in either the presence ($n = 8$; data not shown) or absence ($n = 5$; Table 1) of donor. These results may be attributed to the presence of a large population of acceptor thereby causing the emission from directly excited diI-C₁₈ to surpass the emission due

to FRET. Another possibility is that k_{FRET} is much faster than our temporal resolution. In our FRET studies, we assume that the dipole moments of the donor and acceptor are fully randomized and thus an orientation parameter (κ^2) of 2/3 was used. To test whether the randomization assumption for the donor and acceptor was valid, we carried out fluorescence polarization anisotropy imaging of RBL cells as a function of antigen stimulation.

Antigen-mediated variations in membrane and protein order are probed by fluorescence polarization anisotropy — To assess further whether antigen-induced IgE-FcεRI crosslinking leads to accompanying changes in lipid order and intermolecular interactions, we carried out both steady-state and time-resolved fluorescence polarization anisotropy measurements. 2P-steady-state anisotropy images of diI-C₁₈ as a function of stimulation time (0, 10, 20 and 30 min) reveal that the initial anisotropy (r_0) of diI-C₁₈ does not change as a result of stimulation or the accompanying morphological changes ($n = 7$, data not shown). Similarly, little difference in r_0 was observed for A488-IgE over the entire cell, although individual A488-IgE-FcεRI puncta that form as a result of ongoing crosslinking do have higher initial anisotropies than uncrosslinked A488-IgE-FcεRI ($n = 9$, data not shown). These results suggest that lipid and protein undergo negligible changes with respect to anisotropy as a function of stimulation and indicate the degree to which the membrane constrains rotational, or tumbling, motions of the probes, which could act as a fluorescence depolarization mechanism.

Rotational diffusion of molecular dipoles directly probes fast conformational changes and possible restriction of the surrounding environment. Further, these measurements also allow us to examine the validity of the randomization assumption in FRET calculations and, therefore, of using $\kappa^2 = 2/3$. We performed 1P-time-resolved fluorescence polarization anisotropy measurements on diI-C₁₈ labeled cells (Table 3) to avoid sampling any free aggregated dye on the coverslip. With 1P-excitation, the focal volume along the z-axis is larger than the ruffling amplitude of antigen stimulated cells. Thus, to avoid averaging over dipole moment ruffling and obscuring the interpre-

tation of diI-C₁₈ rotational dynamics within the plane of the plasma membrane, 1P-time-resolved anisotropy measurements were performed without respect to stimulation. High signal-to-noise single point measurements reveal that diI-C₁₈ decays as a single exponential ($\phi_1 = 0.65 \pm 0.09$ ns, $\beta_1 = 0.04 \pm 0.02$), and an additional residual anisotropy component (Table 3). The fast rotational correlation time reflects rapid depolarizations within our timescale of observation, suggesting temporal dipole-moment randomization of diI-C₁₈ as a FRET acceptor. The degree of orientational constraint ($r_\infty / r_0 = 0.8 \pm 0.1$) is high because diI-C₁₈ is intercalated in the membrane.

The fast rotational correlation time of A488-IgE at all time points of stimulation (Table 3), in combination with that of diI-C₁₈, validates the randomization assumption in our FRET calculations (i.e., $\kappa^2 = 2/3$). Prior to antigen stimulation, 2P-pseudo-single point anisotropy decays of A488-IgE are somewhat slower in the presence of diI-C₁₈ (Supplemental Fig. 3). Upon stimulation, the rotational correlation time of A488-IgE in the presence of diI-C₁₈ decreases by 5 min (as determined by unpaired, two-tailed Student's *t*-tests), indicating enhanced association of the donor and acceptor (43) (Supplemental Fig. 3). For A488-IgE on the membrane surface, the degree of orientational constraint is considerably less (e.g., 0.40 ± 0.07 prior to stimulation) than for diI-C₁₈ in the membrane. Single point anisotropy decays of free fluorophores in solution (data not shown) generated fit parameters that agree with Davey et al. (25), verifying the consistency in our experimental setup.

DISCUSSION

Antigen-stimulated phosphorylation of FcεRI by Lyn has been shown to occur in a cholesterol-dependent manner during the initial biochemical step of the allergic response in RBL-2H3 mast cells (6), and these cholesterol-rich membrane domains have also been shown to protect Lyn from tyrosine phosphatases, thereby enhancing its activity (44). However, little direct information about the formation and chemical and physical nature of these domains within complex biomembranes is known, due to the transient nature of domains with respect to space and time. In this work, we used a fluorescence dynamics assay to measure changes

in membrane structure that occur as RBL mast cells are stimulated with multivalent antigen. Our experiments are performed at room temperature, which reduces the rate of signaling as compared to that occurring at 37°C, and previous *in vivo* tyrosine phosphorylation assays performed on suspended RBL cells at 37°C revealed maximal FcεRI phosphorylation between 2 min and 5 min after stimulation with the same concentration of DNP-BSA used in these studies (6,26). Although ongoing crosslinking of IgE-FcεRI continues throughout the experiment, its phosphorylation decreases with time as the receptors compete for limited amounts of Lyn (45), and as stimulated actin polymerization separates Lyn from IgE-FcεRI as signaling proceeds (26,46), resulting in downregulation. We found that changes in the fluorescence lifetime of diI-C₁₈ (Fig. 3) and the efficiency of energy transfer between IgE-FcεRI and diI-C₁₈ (Fig. 5) agree with the kinetics of the earliest biochemical step in the pathway—that is, the stimulated tyrosine phosphorylation of FcεRI (Fig. 4). This agreement leads us to suggest that these changes in the fluorescence dynamics are functionally relevant to antigen-mediated stimulation in mast cells.

The first indication of the association of IgE-FcεRI with low-density cholesterol enriched membrane domains upon crosslinking was provided by sucrose gradient ultracentrifugation experiments of RBL cells lysed with Triton X-100 detergent (47). Further, cholesterol depletion with methyl-β-cyclodextrin was shown to reduce the degree of stimulated tyrosine phosphorylation of FcεRI as assessed by immunoblotting (6). When suspended mast cells are crosslinked with secondary antibody at 4°C, colocalization of a fluorescent lipid analog, which partitions preferentially into cholesterol-rich regions, with fluorescently labeled IgE-FcεRI into micron-sized patches has been observed using conventional optical microscopy (21,25). However, imaging cholesterol-rich domains *in vivo*, without substantial non-physiological perturbation (i.e., low temperatures or extensive IgE-FcεRI crosslinking (6,21,25,26)) has remained a challenge and, therefore, the functional role of these domains in signaling is poorly understood and speculative at best. Such difficulty in characterization of these domains arises from their perceived

small size, transient nature and the diffraction-limited resolution of optical microscopy. Fortunately, time resolved FRET can overcome the spatial limitation of conventional optical microscopy by probing nanoscale (0.1–10 nm) lateral heterogeneities of the membrane (i.e., associations of IgE-FcεRI with cholesterol-rich domains) in real time. Recently, using steady-state FRET between carbocyanine probes that preferentially partition into the liquid-ordered phase based on their alkyl chains, Sengupta et al. demonstrated nanoscale lipid heterogeneity in the plasma membrane of RBL cells that was enhanced by non-physiological crosslinking of IgE-FcεRI (22).

To circumvent the temporal resolution of translational diffusion-based spectroscopic techniques (μs–s) (16–18,20,21), we used a fluorescence dynamics assay capable of probing molecular dynamics (fluorescence lifetime and rotational correlation time) on a ps–ns timescale to interrogate antigen-mediated changes in cell morphology (Supplemental Movie 1). This fluorescence dynamics assay was previously tested on massively crosslinked, suspended mast cells, demonstrating increased fluorescence lifetime and rotational diffusion of diI-C₁₈ and A488-IgE, and thus providing evidence for nanostructural changes in the plasma membrane within the non-physiologically induced microdomains (25).

At room temperature, diI-C₁₈-labeled membrane domains are transient, thermodynamically unstable and smaller than the diffraction limit of DIC and confocal microscopy (Fig. 1). Despite the lack of visible domain formation during the course of antigen stimulation or colocalization between diI and the IgE receptor, 2P-FLIM revealed a distinct change in the membrane nano-environment of diI-C₁₈ (Fig. 2), as observed by averaging over the entire FLIM images (binning = 1). The transiently increasing $\langle\tau_{fl}\rangle$ becomes more obvious when we interrogate lifetime with higher signal-to-noise and temporal resolution (Fig. 3 and Table 1), which follows the kinetics of stimulated tyrosine phosphorylation (Fig. 4). The effects of membrane structure on the photophysics of diI-C₁₈ likely results from increased local concentrations of saturated and mono-unsaturated phospholipids (48), or solvent protection by crosslinked membrane proteins and the underlying actin cytoskeleton (1,49).

When the dipole-moment distribution of diI-C₁₈ was monitored in cells as a function of antigen stimulation via steady-state 2P-fluorescence polarization anisotropy imaging, no gross changes in membrane order were observed over time, which is not surprising considering that there are no optically resolvable membrane domains. Changes in cellular morphology, which occur on the seconds timescale, may also obscure minor changes in the orientational order of the diI-C₁₈ dipole-moment during the ~120 s necessary for acquisition. At all time points of stimulation monitored, the average initial anisotropies obtained by 2P-fluorescence polarization anisotropy imaging were greater for adherent RBL cells (data not shown) than for cells in suspension (25), suggesting that lipid in adherent cell membranes may have an intrinsically higher packing order than those in the spherical, suspended cell membranes. Collectively, these diI-C₁₈ results imply that there are no functional domains at the steady-state, or resting, level under physiological conditions, but rather that such domains are small, dynamic assemblies, as proposed by Mayor and Rao (50). It is possible that the longer fluorescence and rotational dynamics of diI-C₁₈ observed in stimulated adherent cells, as compared to crosslinked suspended cells (25), results from increased restriction due to confinement of diI-C₁₈ into many nanoscopic domains with higher line tension (51) than the large microdomains of suspended cells.

The modest antigen-mediated changes in average A488-IgE fluorescence lifetime observed with 2P-FLIM can be attributed to the fact that the binding of DNP-BSA to IgE-FcεRI follows a transient hapten exposure model (45,52), in which the antigen and antibody interact at fast on/off rates. Populations of A488-IgE that are variously bound by DNP-BSA are suggested by the multi-Gaussian fits of pixel-lifetime histograms from total FLIM images (Supplemental Fig. 2). At various time-points of DNP-BSA stimulation, the average lifetimes of A488-IgE obtained with 2P-FLIM (Supplemental Fig. 2) are longer than those we had observed in suspended cells that were extensively crosslinked with a secondary antibody ($\langle\tau_{fl}\rangle = 0.9$ ns and 1.35 ns (25)). The increase in environmental restriction for adherent cells may be explained by the steric hindrance of the DNP-BSA molecules transiently and electrostatically binding to

IgE-FcεRI, as compared to the more stable binding of secondary antibody to suspended cells. Increased restriction due to differences in cell treatment is supported by slightly higher average initial anisotropies obtained by 2P-fluorescence polarization anisotropy imaging of A488-IgE in antigen-stimulated adherent cells (data not shown), as compared to extensively crosslinked suspended cells (25). Notably, using either 2P-FLIM with high binning or single point lifetime methods, the average fluorescence lifetime ($\langle\tau_{fl}\rangle$) of A488-IgE decreases within the first 5 min of antigen stimulation, in direct contrast with the transient increase of diI-C₁₈'s lifetime, and may be explained by the increased environmental disorder due to adding multivalent antigen to the cells.

Using spectrophotometric resonance energy transfer methods on RBL cell-derived membrane vesicles (i.e., ensemble averaging in a cuvette), Baird and Holowka (53) determined distances between various regions of IgE and the *outer* cell membrane surface ranging from ~4.5–10 nm. Their findings, in combination with the R_0 obtained here (3.33 ± 0.04 nm), suggested that energy transfer would be feasible between A488-IgE and diI-C₁₈. Here, the reduction of A488-IgE (donor) fluorescence lifetime in the presence of diI-C₁₈ (acceptor) prior to stimulation, as reported by single point methods, can be attributed to FRET (Fig. 5A; Table 1) that occurs only when the donor and acceptor are within 10 nm and indicates that A488-IgE and diI-C₁₈ are within 4.3 ± 0.1 nm (Table 2). Mathematical modeling studies of FRET data and electron microscopy images from cell membranes have implied that functional membrane domains are between ~5–20 nm in diameter (54).

Our results also show that A488-IgE and diI-C₁₈ experience maximal association 5 min after stimulation begins, indicated by the increase in energy transfer efficiency (from 0.18 ± 0.01 prior to stimulation to 0.22 ± 0.01 at 5 min; Table 2) and also by the decrease in rotational correlation time of A488-IgE in the presence of diI-C₁₈ between 0 min and 5 min (Supplemental Fig. 3; Table 3). The time of maximal FRET agrees with maximal tyrosine phosphorylation of FcεRI by membrane domain-associated Lyn, suggesting that IgE-FcεRI is recruited into ordered cholesterol-

rich domains for high efficiency signaling. Even if such domains only coalesce transiently, as suggested by steady-state FRET studies by Kenworthy et al. (55), the ultrafast time resolution of our FRET studies provides an advantage for resolving sub-diffraction association of various molecules (e.g., IgE-Fc ϵ RI) with cholesterol-rich domains (56). The FRET results, together with the diI-C₁₈ lifetime results, support the hypothesis that crosslinked IgE-Fc ϵ RI transiently translocates into regions of membrane with greater nanostructure to facilitate signaling; however, our results do not rule out the possibility that receptor engagement may recruit functional lipid nanodomains (54).

The lack of FRET by 20 min (Table 1) indicates a reduction in lateral interactions between IgE-Fc ϵ RI and diI-C₁₈-labeled membranes and may be related to the hypothesis that actin microfilaments help to separate Fc ϵ RI from ordered membrane domains (26,46), resulting in the down-regulation of signaling. Antigen-induced IgE-Fc ϵ RI crosslinking at 37°C results in endocytosis of ~50% of the receptor complexes with a half-time of 5 min (57,58), so it is unlikely that the reduced FRET observed here by 20 min is due to IgE-Fc ϵ RI internalization, even with its much slower kinetics at room temperature. Moreover if IgE-Fc ϵ RI is internalized, the reduction in FRET suggests that diI-containing domains are not co-internalized with the IgE receptor.

In summary, we report a molecular perspective of the molecule-molecule interactions associated with antigen-stimulated IgE receptor signaling in RBL mast cells. Using confocal microscopy, we demonstrated antigen-mediated changes in cell morphology and IgE-Fc ϵ RI crosslinking and reorganization that occur on the seconds time-scale (Fig. 1; Supplemental Movie 1). Fluorescence lifetime imaging of diI-C₁₈ reveals nanostructural changes of the plasma membrane as a function of antigen-stimulation time (Fig. 2 and 3; Table 1). These changes correlate with the kin-

etics of tyrosine phosphorylation of Fc ϵ RI as assessed by immunoblotting (Fig. 4). These results suggest an IgE-Fc ϵ RI stimulation-dependent change in the membrane nanostructure at early times of the signaling process, which is further supported by the transient increase in FRET between A488-IgE-Fc ϵ RI and diI-C₁₈ within a 0.1–10 nm spatial resolution (Fig. 5; Table 2). The results presented here suggest that IgE-Fc ϵ RI is recruited into transient, ordered membrane domains that serve as platforms for IgE-Fc ϵ RI signaling. Furthermore, these studies offer an experimental approach that is applicable to other signaling pathways that depend upon localized membrane ordering.

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FIGURE LEGENDS

Figure 1: Confocal microscopy demonstrates antigen-mediated changes in morphology (A) and distribution of the lipid analog, diI-C₁₈ (B), and A488-IgE-FcεRI (C) over 30 min of stimulation with antigen at room temperature. No obvious colocalization of diI-C₁₈ with crosslinked A488-IgE is observed. The crosshairs (and arrows) indicate the position of the focused laser beam for single point time-resolved fluorescence decay measurements (decays shown in Fig. 3, with corresponding data in Table 1). Bar, 20 μm.

Figure 2: Nanostructural changes in the plasma membrane occur upon antigen stimulation. (A) 2P-FLIM imaging of diI-C₁₈ shows antigen-stimulated changes in membrane nanostructure and A488-IgE-FcεRI distribution (binning = 1). When compared with diI-C₁₈ in the membrane, the background features in the diI-C₁₈ FLIM images have a distinctively shorter lifetime (~0.3 ns), which can be fit to a single exponential (25), confirming that these features are unincorporated free diI-C₁₈ aggregates on the coverslip surface. (B) Total FLIM image histograms, with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple) time points, are shown to represent the overall changes in average lifetime distributions as a function of antigen stimulation. The most noteworthy shift in average lifetime is between 0 and 5 min. Bar, 10 μm.

Figure 3: Time-resolved fluorescence decays indicate variations in membrane and protein environment as a result of antigen-mediated stimulation. (A) Pseudo-single point time-resolved fluorescence decays of diI-C₁₈, in which the entire cell is scanned and the signal is binned into one “pixel”, are shown as a function of antigen stimulation time (0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple)) and serve as a comparison to the single point measurements. Note that the specific spatial information is lost due to the binning although overall changes that occur upon antigen stimulation are accounted for. Table 1 shows data for several of these pseudo-single point time series, revealing a transient increase in the average fluorescence lifetime of diI-C₁₈ within 5 min of stimulation. (B) Single point time-resolved fluorescence decays of diI-C₁₈ are displayed as a function of stimulation time (0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple)). The location of the focused laser beam on the dually labeled cell is indicated by the arrow and crosshairs shown in Fig. 1. The average fluorescence lifetime transiently increases after 5 min. The statistics for several of these time series are shown in Table 1. (C) The average lifetime ($\langle\tau_{fl}\rangle$) from single point measurements (Table 1) are plotted as a function of stimulation time.

Figure 4: The kinetics of antigen stimulation agree with nanostructural changes in the plasma membrane. RBL cells are stimulated with antigen under the same conditions as the micro-spectroscopy experiments (1 μg/mL DNP-BSA, 23°C). (A) The representative anti-phosphotyrosine immunoblot shows stimulated *in vivo* tyrosine phosphorylation, and the band corresponding to the β subunit of FcεRI (6) is indicated. (B) The level of tyrosine phosphorylation of FcεRI β for the immunoblots shown in panel A is quantified using densitometry.

Figure 5: A488-IgE and diI-C₁₈ interact transiently as RBL are stimulated with antigen, as measured by the excited-state dynamics of A488-IgE. (A) The pseudo-single point fluorescence decays of the donor A488-IgE decays more quickly in the presence of the acceptor diI-C₁₈ (red) as compared to the absence of acceptor (blue). The representative decays were acquired prior to antigen stimulation, however the fluorescence of A488-IgE decays faster in the presence of diI-C₁₈ at all time points of stimulation (see Table 1). (B) The efficiency of energy transfer (E_T) is plotted as a function of stimulation time to show that the probes experience maximal association 5 min after stimulation, corresponding to the largest

increase in energy transfer efficiency (from 0.18 ± 0.01 before stimulation to 0.22 ± 0.01 at 5 min). The standard deviation of E_T is determined by the error in τ_{DA} ($n = 6$; Table 1) and τ_D ($n = 5$; Table 1).

TABLES

TABLE 1

Summary of fluorescence lifetime parameters obtained from pseudo-single point and conventional single point, time-resolved fluorescence decays of diI-C₁₈ and A488-IgE in adherent mast cells

	τ_1 /ns	α_1	τ_2 /ns	α_2	τ_3 /ns	α_3	$\langle \tau_{fl} \rangle$ /ns	n
diI-C₁₈								
<u>pseudo-single point:</u>								
0 min (pre-stimulation)	0.05(1)*	0.7(1)	0.39(2)	0.2(1)	1.7(2)	0.1(1)	0.26(2)	4
5 min	0.06(1)*	0.6(1)	0.39(4)	0.3(1)	1.66(2)	0.1(1)	0.32(4) [†]	4
10 min	0.048(7)*	0.7(1)	0.42(7)	0.2(1)	1.85(8)	0.1(1)	0.28(6)	4
20 min	0.06(1)*	0.6(1)	0.45(3) [†]	0.3(1)	2.0(2)	0.1(1)	0.36(9)	4
30 min	0.06(2)*	0.6(1)	0.41(6)	0.3(1)	1.9(4)	0.1(1)	0.38(8)	4
<u>single point:</u>								
0 min (pre-stimulation)	0.4(2)	0.4(1)	1.1(3)	0.5(1)	2.8(4)	0.1(1)	1.1(1)	5
5 min	0.8(2) [†]	0.5(1)	1.4(3)	0.4(1)	3.2(6)	0.1(1)	1.25(9) [†]	5
10 min	0.7(2)	0.4(1)	1.2(1)	0.5(1)	2.7(3)	0.1(1)	1.2(1)	5
20 min	0.8(2) [†]	0.6(1)	1.3(3)	0.3(1)	3.0(6)	0.1(1)	1.2(1)	5
30 min	0.8(1) [†]	0.5(1)	1.2(2)	0.4(1)	2.9(9)	0.1(1)	1.16(8)	5
A488-IgE in the absence of diI-C₁₈								
<u>pseudo-single point:</u>								
0 min (pre-stimulation)	—	—	1.4(4)	0.3(1)	3.50(6)	0.7(1)	2.94(5)	5
5 min	—	—	1.3(2)	0.3(1)	3.3(4)	0.7(1)	2.7(1) [†]	5
10 min	—	—	1.3(4)	0.3(1)	3.1(3) [†]	0.7(1)	2.5(2) [†]	5
20 min	—	—	0.7(3) [†]	0.3(1)	3.1(3) [†]	0.7(1)	2.4(2) [†]	5
30 min	—	—	0.9(2) [†]	0.3(1)	3.0(2) [†]	0.7(1)	2.4(1) [†]	5
A488-IgE in presence of diI-C₁₈								
<u>pseudo-single point:</u>								
0 min (pre-stimulation)	—	—	0.9(4) [†]	0.3(1)	3.0(1) [†]	0.7(1)	2.41(7) [†]	6
5 min	—	—	0.5(2) [†]	0.3(1)	2.8(2)	0.7(1)	2.1(1) ^{††}	6
10 min	—	—	0.7(3)	0.3(1)	2.9(3)	0.7(1)	2.15(1) ^{††}	6
20 min	—	—	0.5(1)	0.3(1)	2.6(2) [†]	0.7(1)	2.14(8) [†]	6
30 min	—	—	0.9(3)	0.3(1)	2.8(1)	0.7(1)	2.19(5) [†]	6

*The very short time constant (~ 60 ps) for pseudo-single point magic angle decays of diI-C₁₈ is due to aggregated free dye on the coverslip being scanned along with the cell. [†] $p < 0.05$, as determined from unpaired, two-tailed Student's t -tests. For decays of either diI-C₁₈ ($n = 5$ or $n = 4$), A488-IgE ($n = 5$) or A488-IgE in the presence of diI-C₁₈ ($n = 6$), this indicates that the mean of the given fit parameter is statistically significantly different from the mean prior to antigen stimulation, i.e. at 0 min. [‡] $p < 0.05$, as determined from unpaired, two-tailed Student's t -tests. For decays of A488-IgE, this indicates that the mean of the given fit parameter is statistically significantly different in the presence of diI-C₁₈, as compared to in the absence of diI-C₁₈, at the same time point of stimulation.

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TABLE 2

Summary of fluorescence resonance energy transfer parameters obtained from steady-state spectrophotometry of diI-C₁₈ and A488-IgE and pseudo-single point time-resolved fluorescence decays of A488-IgE in adherent mast cells

	E_T *	R /nm	k_{FRET} /ns ⁻¹
0 min	0.18(1)	4.3(1)	0.07(2)
5 min	0.22(1)	4.1(1)	0.10(2)
10 min	0.14(4)	4.5(4)	0.06(5)
20 min	0.11(4)	4.7(5)	0.04(5)
30 min	0.09(1)	4.9(1)	0.03(1)

*The donor (A488-IgE) fluorescence lifetime in the presence of acceptor (diI-C₁₈) (τ_{DA}) and the donor fluorescence lifetime in the absence of acceptor (τ_D), used in the calculation of energy transfer efficiency (E_T), were 2.41 ± 0.07 ns and 2.94 ± 0.05 ns, respectively (Table 1). R_0 was calculated as 3.33 ± 0.04 nm and used to determine R , as described in Experimental Procedures.

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TABLE 3

Summary of rotational anisotropy parameters obtained from single point and pseudo-single point, time-resolved anisotropy decays of diI-C₁₈ and A488-IgE in adherent mast cells

	ϕ_1 /ns	β_1	ϕ_2 /ns	β_2	r_∞	r_0	r_∞ / r_0	n
diI-C₁₈								
<u>single point:</u>								
0 min (pre-stimulation)	0.65(9)	0.04(2)	—	—	0.18(6)	0.22(5)	0.8(1)	8
A488-IgE								
<u>pseudo-single point:</u>								
0 min (pre-stimulation)	0.6(1)	0.17(3)	—	—	0.11(1)	0.28(2)	0.40(7)	7
5 min	0.6(2)	0.14(2)	—	—	0.12(2)	0.26(1)	0.46(9)	4
10 min	0.6(2)	0.17(4)	—	—	0.11(2)	0.28(2)	0.4(1)	4
20 min	0.7(3)	0.17(3)	—	—	0.11(2)	0.28(4)	0.40(4)	4
30 min	0.5(2)	0.16(4)	—	—	0.14(4)	0.30(3)	0.5(1)	4
A488-IgE in presence of diI-C₁₈								
<u>pseudo-single point:</u>								
0 min (pre-stimulation)	0.8(2)*	0.18(3)	—	—	0.13(3)	0.31(5)	0.4(1)	7
5 min	0.61(1) [†]	0.19(1)*	—	—	0.11(1)	0.30(1)*	0.36(4)	3
10 min	0.6(1)	0.1(1)	—	—	0.13(1)	0.2(1)	0.6(2)	3
20 min	0.50(8)	0.15(1)	—	—	0.15(1)	0.30(1)	0.50(1)* [†]	3
30 min	0.56(8)	0.17(4)	—	—	0.12(3)	0.29(6)	0.43(1)	3

* $p < 0.05$, as determined from unpaired, two-tailed Student's t -tests, meaning that the mean of the given fit parameter are statistically significantly different in the presence of diI-C₁₈, as compared to in the absence of diI-C₁₈, at the same time point of stimulation. [†] $p < 0.05$, as determined from unpaired, two-tailed Student's t -tests, indicating that the mean of the given fit parameter is statistically significantly different from the mean prior to antigen stimulation, i.e. at 0 min.

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Figure 1

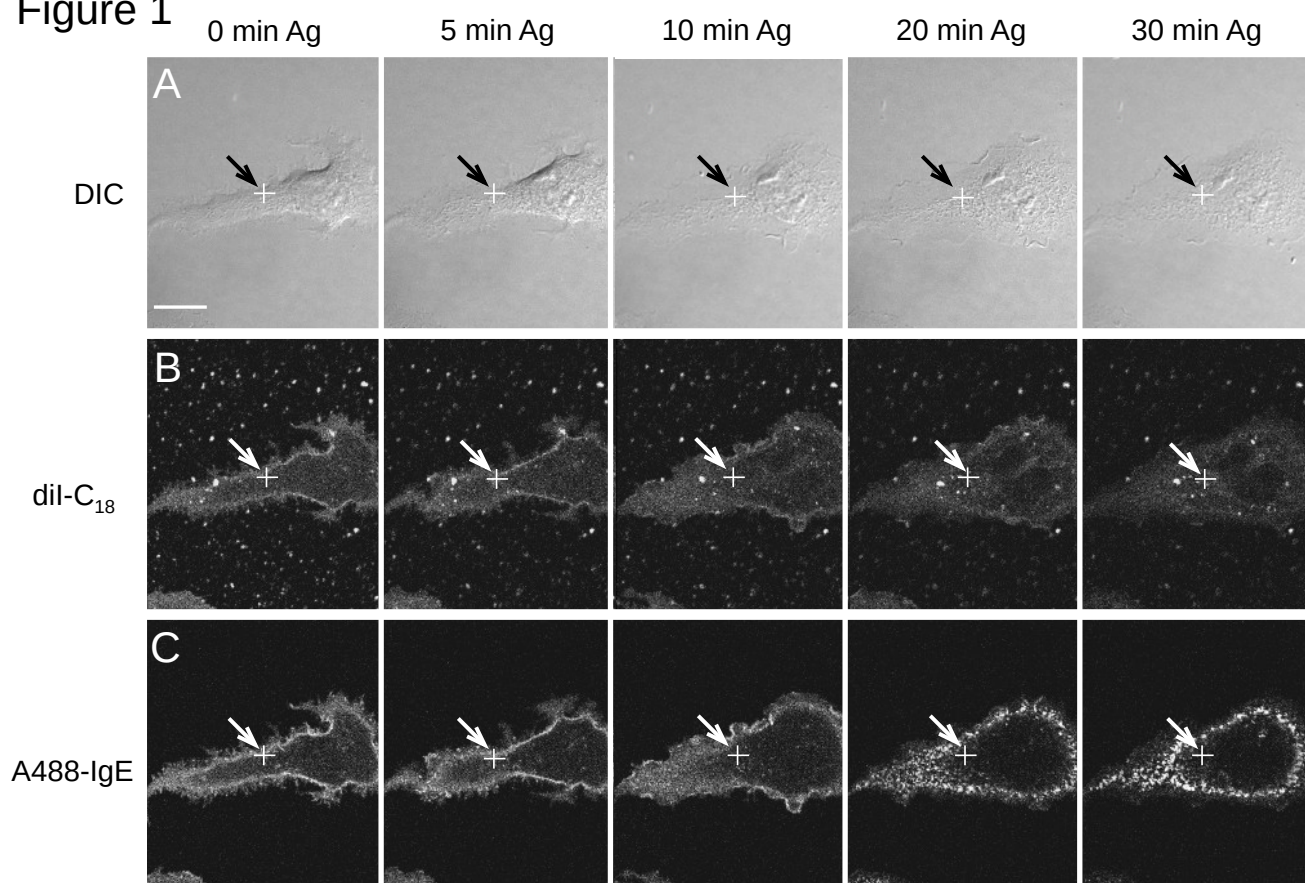
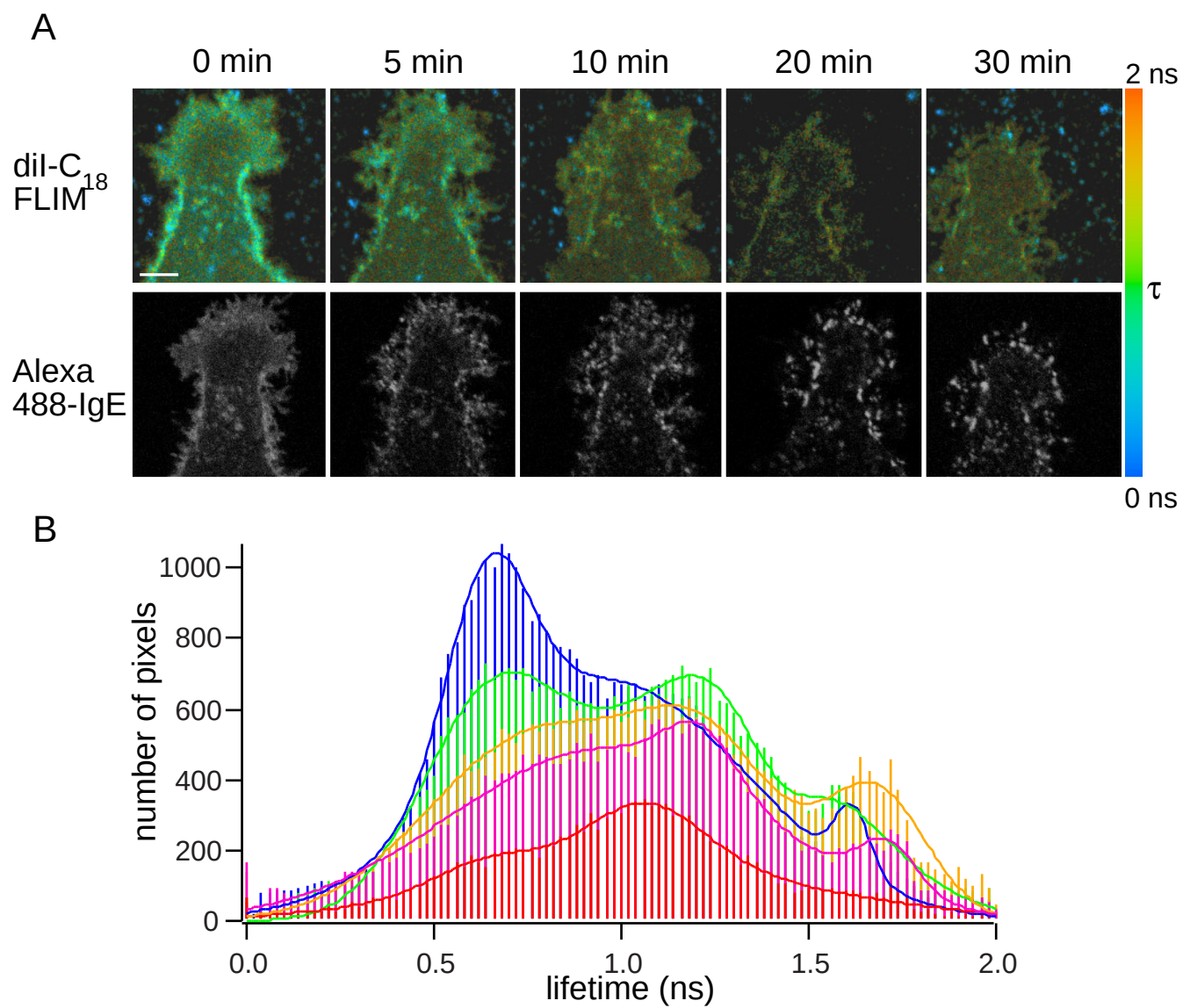


Figure 2



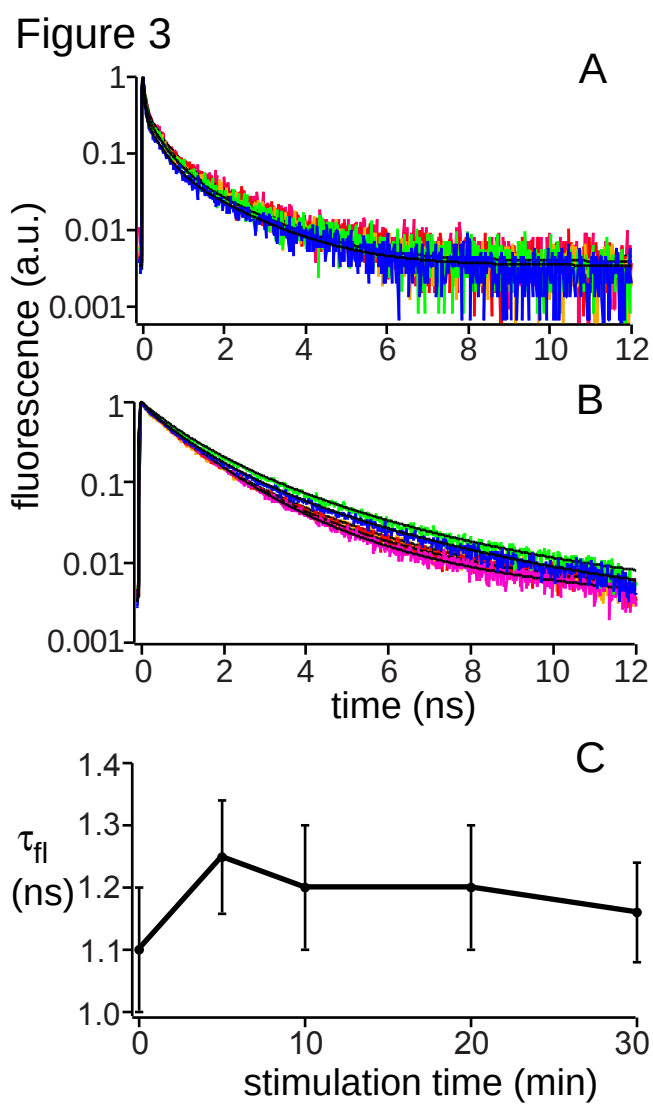


Figure 4

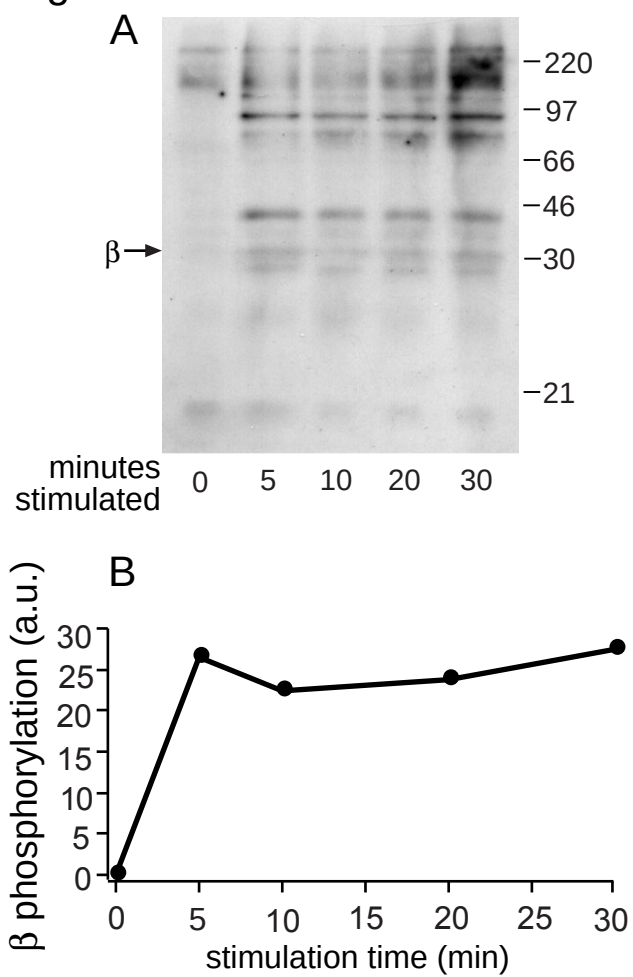


Figure 5

