MOLECULAR DYNAMICS IMAGING OF MAST CELL MEMBRANE
NANOSTRUCTURE IN IMMUNORECEPTOR SIGNALING

A Dissertation in
Chemistry
by
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ABSTRACT

Plasma membrane domains enriched in cholesterol, saturated phospholipids and sphingolipids are hypothesized to play functionally important roles in cell signaling, biomolecular trafficking and disease pathogenesis. However, the precise composition, dimension and life span of these domains in biological membranes are still under debate. Detergent extraction and biochemical characterization of membrane domains, including cholesterol depletion studies, have been widely used but are often plagued by various problems and do not allow for direct visualization of domains in live, intact cell membranes. Various microscopic and spectroscopic methods have been developed to provide the spatial and temporal information required to better understand domain formation and function. Still, many of these methods are not amenable to live cells, do not provide single cell data, are spatially limited by optical diffraction, or provide only seconds to microseconds temporal resolution. Under physiological conditions, membrane domains are likely transient and smaller than the diffraction limit of optical microscopy and therefore defy detection using conventional imaging methods.

To overcome these limitations and obtain molecular-level, spatio-temporal information on membrane domains, we developed an ultrafast fluorescence dynamics imaging assay that includes time-resolved fluorescence lifetime and polarization anisotropy imaging. Supported lipid bilayer model membranes and suspended and adherent RBL mast cells under non-physiological and physiological conditions of IgE receptor cross-linking were used as model systems in these studies. The excited-state dynamics and rotational diffusion (picoseconds to nanoseconds timescale) obtained with this assay are inherently sensitive to the immediate surroundings of a fluorescently
labeled molecule and allow for real-time monitoring of membrane structure, organization and heterogeneity in individual, live cells.

In this Dissertation, the feasibility of this ultrafast fluorescence dynamics assay is demonstrated on simple model lipid membranes labeled with a fluorescent phospholipid analog. The fluorescent probe exhibits longer lifetime, higher order and longer overall rotational correlation time in more ordered, gel phase membranes as compared to fluid, or liquid-disordered, phase. These site-specific observations agree with the literature for model membranes analyzed via traditional ensemble spectrofluorimetric lifetime and anisotropy experiments.

The fluorescence dynamics assay is then used to detect more ordered, cholesterol enriched domains and relate their nanostructure and dynamics to IgE receptor signaling, in the plasma membrane of single, live RBL mast cells under non-physiological and physiological conditions. In mast cells, antigen-mediated cross-linking of the high affinity IgE receptor (FcεRI) results in movement of FcεRI into cholesterol-rich domains in the plasma membrane, where it is phosphorylated by the Src kinase, Lyn, to initiate the exocytotic release of histamine in the allergic response. Lyn-induced phosphorylation of FcεRI occurs in a cholesterol-dependent manner, leading to the hypothesis that cholesterol-rich membrane domains may act as functional receptor signaling platforms.

When IgE-FcεRI is non-physiologically cross-linked with anti-IgE at 4°C, molecules associated with cholesterol-rich microdomains (e.g., saturated lipids [the lipid analog 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine (diI-C₁₈) or glycosphingolipids]) and lipid-anchored proteins co-redistribute with cross-linked IgE-FcεRI. We find an enhancement in diI-C₁₈ and Alexa Fluor 488-labeled IgE-FcεRI fluorescence lifetime and
anisotropy in optically resolvable microdomains where these molecules co-localize. These results suggest that fluorescence lifetime and, particularly, anisotropy permit us to measure lipid molecule recruitment into more ordered domains that serve as IgE-mediated signaling platforms.

In a collaborative effort, using time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging of intact, individual mast cells, lipid chemical identity and distribution are also investigated as a function of extensive cross-linking. Multiple lipid species are visualized at the sub-cellular level, with the results suggesting that IgE-FceRI cross-linking induces very subtle changes in cholesterol distribution, necessitating exquisitely sensitive analysis methods such as fluorescence lifetime and polarization anisotropy.

Under more challenging physiological conditions, excited-state fluorescence dynamics are used to correlate sub-resolution nanostructural changes in the diI-C<sub>18</sub>-labeled plasma membrane with IgE-FceRI cross-linking in adherent mast cells stimulated with multivalent antigen at ~20°C. Time-dependent fluorescence lifetime imaging of diI-C<sub>18</sub> shows changes in lifetime that agree with the kinetics of stimulated FceRI tyrosine phosphorylation under the same conditions. Lifetime imaging of Alexa Fluor 488-labeled IgE-FceRI indicates that fluorescence resonance energy transfer (FRET) occurs with diI-C<sub>18</sub> with similar kinetics. These live cell studies provide direct evidence for IgE-FceRI associations with specialized cholesterol-rich domains within ~4 nm at maximal association during IgE receptor signaling.
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LIST OF ABBREVIATIONS

1P    one photon
2P    two photon
α-IgE polyclonal rabbit anti-IgE
∆⁹-diI-C₁₈ 1,1’-dioleyl-3,3,3’,3’-tetramethylindocarbocyanine
A488 Alexa Fluor 488
A488-IgE Alexa Fluor 488-labeled IgE
A488-IgE-FcεRI Alexa Fluor 488-labeled, receptor-bound IgE
AFM atomic force microscopy
BODIPY-PC 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine
BSS buffered saline solution
BSA bovine serum albumin
CCD charge coupled device
DAG diacylglycerol
di-4-ANEPPDHQ 1-[2-hydroxy-3-(N,N-di-methyl-N-hydroxyethyl)ammoniopropyl]-4-[β-[2-(di-n-butylamino)-6-napthyl]vinyl] pyridinium
DIC differential interference contrast
diI-C₁₂ 1,1’-didodecyl-3,3,3’,3’-tetramethylindocarboxycyanine
diI-C₁₈ 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarboxycyanine
DMPC 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMSO dimethylsulfoxide
DNP-BSA 2,4-dinitrophenyl bovine serum albumin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-&lt;i&gt;sn&lt;/i&gt;-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPH-PC</td>
<td>2-[3-((diphenylhexatrienyl) propanoyl]-1-hexadecanoyl-&lt;i&gt;sn&lt;/i&gt;-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-&lt;i&gt;sn&lt;/i&gt;-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
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<tr>
<td>FLIM</td>
<td>fluorescence lifetime imaging microscopy</td>
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<tr>
<td>FCCS</td>
<td>fluorescence cross-correlation spectroscopy</td>
</tr>
<tr>
<td>FceRI</td>
<td>high affinity receptor for IgE</td>
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<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FWHM</td>
<td>full-width at half-maximum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GUV</td>
<td>giant unilamellar vesicle</td>
</tr>
<tr>
<td>ICS</td>
<td>image correlation spectroscopy</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>IgE-FceRI</td>
<td>receptor-bound immunoglobulin E</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>laurdan</td>
<td>6-dodecanoyl-2-dimethylaminonaphthalene</td>
</tr>
<tr>
<td>Lᵦ</td>
<td>gel phase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>( L_d )</td>
<td>liquid-disordered or fluid phase</td>
</tr>
<tr>
<td>( L_o )</td>
<td>liquid-ordered phase</td>
</tr>
<tr>
<td>MCP</td>
<td>microchannel plate-photomultiplier tube</td>
</tr>
<tr>
<td>( m/z )</td>
<td>mass to charge ratio in mass spectrometry</td>
</tr>
<tr>
<td>NA</td>
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</tr>
<tr>
<td>ND</td>
<td>neutral density filter</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered solution</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>PCH</td>
<td>photon counting histogram</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PIP(_2)</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC(_\gamma)</td>
<td>phospholipase C(_\gamma)</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-( sn )-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RBL</td>
<td>rat basophilic leukemia</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>SIMS</td>
<td>secondary ion mass spectrometry</td>
</tr>
<tr>
<td>SPT</td>
<td>single particle tracking</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>TCSPC</td>
<td>time-correlated single-photon counting</td>
</tr>
<tr>
<td>TIRFM</td>
<td>total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>ToF-SIMS</td>
<td>time-of-flight secondary ion mass spectrometry</td>
</tr>
<tr>
<td>VALAP</td>
<td>Vaseline:lanolin:paraffin</td>
</tr>
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</table>
**LIST OF SYMBOLS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>amplitude fraction associated with fluorescence lifetime</td>
</tr>
<tr>
<td>$\beta$</td>
<td>pre-exponential factor (or amplitude fraction) associated with rotational correlation time</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>chi square</td>
</tr>
<tr>
<td>$\tilde{\chi}^2$</td>
<td>reduced chi square</td>
</tr>
<tr>
<td>$X$</td>
<td>experimental result in z-score calculation</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>molecular brightness from PCH analysis</td>
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<tr>
<td>$\varepsilon_A$</td>
<td>acceptor extinction coefficient</td>
</tr>
<tr>
<td>$\varphi$</td>
<td>rotational correlation time</td>
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<tr>
<td>$\Phi_{fl}$</td>
<td>fluorescence quantum yield</td>
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<tr>
<td>$\kappa^2$</td>
<td>orientation parameter</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>fluorescence wavelength</td>
</tr>
<tr>
<td>$\lambda_{ex}$</td>
<td>wavelength of light used for fluorescence excitation</td>
</tr>
<tr>
<td>$\mu$</td>
<td>most probable result in z-score calculation</td>
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<tr>
<td>$\theta_c$</td>
<td>wobbling-in-cone angle</td>
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<tr>
<td>$\sigma$</td>
<td>standard deviation</td>
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<tr>
<td>$\tau$</td>
<td>time interval in autocorrelation function</td>
</tr>
<tr>
<td>$\tau_{fl}$</td>
<td>average fluorescence lifetime</td>
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<tr>
<td>$\tau_i$</td>
<td>fluorescence lifetime</td>
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<tr>
<td>$\tau_d$</td>
<td>translational diffusion time</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>( \tau_D )</td>
<td>fluorescence lifetime of donor</td>
</tr>
<tr>
<td>( \tau_{DA} )</td>
<td>fluorescence lifetime of donor in the presence of acceptor</td>
</tr>
<tr>
<td>( \tau_f )</td>
<td>average fluorescence lifetime</td>
</tr>
<tr>
<td>( \omega )</td>
<td>axial-to-lateral dimension ratio of FCS detection volume</td>
</tr>
<tr>
<td>( \omega_b )</td>
<td>structural parameter</td>
</tr>
<tr>
<td>( c )</td>
<td>concentration</td>
</tr>
<tr>
<td>( d )</td>
<td>degrees of freedom</td>
</tr>
<tr>
<td>( D )</td>
<td>lateral diffusion coefficient</td>
</tr>
<tr>
<td>( E_T )</td>
<td>energy transfer efficiency</td>
</tr>
<tr>
<td>( f )</td>
<td>amplitude fraction associated with translational diffusion time</td>
</tr>
<tr>
<td>( F_D )</td>
<td>fluorescence intensity of the donor</td>
</tr>
<tr>
<td>( G(0) )</td>
<td>amplitude of autocorrelation curve</td>
</tr>
<tr>
<td>( G(\tau) )</td>
<td>autocorrelation function</td>
</tr>
<tr>
<td>( h )</td>
<td>photon counts in PCH analysis</td>
</tr>
<tr>
<td>( I_{547} )</td>
<td>fluorescence intensity acquired with magic angle detection</td>
</tr>
<tr>
<td>( I_\bot )</td>
<td>perpendicular fluorescence intensity</td>
</tr>
<tr>
<td>( I^0_\bot )</td>
<td>background-corrected perpendicular fluorescence intensity</td>
</tr>
<tr>
<td>( I_\parallel )</td>
<td>parallel fluorescence intensity</td>
</tr>
<tr>
<td>( I^0_\parallel )</td>
<td>background-corrected parallel fluorescence intensity</td>
</tr>
<tr>
<td>( J(\lambda) )</td>
<td>spectral overlap between donor emission and acceptor absorption</td>
</tr>
</tbody>
</table>
\( k_p \) excited-state fluorescence decay rate
\( k_{FRET} \) energy transfer rate
\( k_{nr} \) non-radiative decay rate
\( k_r \) radiative decay rate
\( m \) number of diffusion components in autocorrelation function
\( n \) refractive index
\( N \) average number of fluorescent molecules in detection volume
\( p \) probability in PCH analysis
\( P \) Pearson product-moment correlation coefficient for a sample
\( Q_D \) quantum yield of donor
\( r \) anisotropy
\( r_{\infty} \) residual anisotropy
\( r_0 \) initial anisotropy
\( R \) donor-acceptor separation distance
\( R_0 \) Förster distance between donor and acceptor
\( R^2 \) square of linear regression correlation coefficient
\( S \) order parameter
\( t \) time
\( T_m \) phase transition melting temperature
\( V_{PSF} \) volume of 3D-Gaussian point spread function
\( z \) z-score
ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Erin Sheets, for leading this chemist into the world of cell biology and biophysics by providing me with the opportunity to study IgE receptor-mediated allergic signaling in mast cells. I owe my greatest professional respect and appreciation to my co-advisor, Dr. Ahmed Heikal, for teaching me most of what I know about biophotonics, encouraging and motivating me with his positive attitude, and always being available as a mentor and confidant. Valuable conversations about research often included coffee and discussions about the latest movies or what Kevin had been up to lately.

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I also owe my gratitude to my other committee members, Dr. Christine Keating, Dr. Tae-Hee Lee, Dr. Peter Butler, and former member Dr. Juliette Lecomte, for taking the time to learn about my research and offer thoughtful advice along the way. Thanks also to Dr. David Holowka for providing reagents for studying IgE receptor signaling and recommendations about their use. Finally, I would like to thank Dr. Dan DiLella, Dr. Jack Schmidt and Dr. Judith Bischoff for their inspiration and steering me toward chemistry and research during my undergraduate career.
1. INTRODUCTION

1.1 The history of domains in biological membranes

The plasma membrane provides a barrier between the cell interior and its extracellular environment, allowing the passage of essential nutrients, and other solutes, and the transduction of various signals. For proper function, it has long been accepted that a certain amount of lateral heterogeneity must exist within the plasma membrane (Jacobson et al., 1995). The plasma membrane itself is a lipid bilayer, which provides fluidity and selective permeability, with many types of lipids and associated proteins that are involved in transport, catalysis, and signal transduction. In the early 1970s, the Singer-Nicolson fluid-mosaic model predicted that phospholipids and membrane proteins are homogeneously distributed within the plasma membrane, with random, unrestricted lateral freedom of motion (Singer and Nicolson, 1972). More recently, however, many studies have provided evidence of nonrandom associations of membrane lipid and protein components on various spatial scales (e.g., nm–µm) (Vereb et al., 2003), suggesting important roles for lipid-lipid, protein-protein and especially lipid-protein interactions. Thus, the plasma membrane is no longer considered a dilute solution of protein in a fluid, two-dimensional lipid solvent, but rather a complex and crowded mixture of lipids and proteins (Jacobson et al., 2007). Further, various modes of lateral diffusion of membrane-associated biomolecules have been observed, including random diffusion, directed motion, and hindered diffusion via transient confinement due to dynamic restructuring of obstacle clusters (Vereb et al., 2003) or the underlying cytoskeleton (Jacobson et al., 1995).
Studies of lipid heterogeneity within model membranes of defined composition led to the development of the raft hypothesis (Simons and Ikonen, 1997), which proposes that different lipid species have different tendencies to associate with each other, thus driving lipid segregation or domain formation. Specifically, the formation of cholesterol-rich domains is attributed to the affinity of rigidly-structured cholesterol for more ordered, saturated phospholipids and the desire to be separate from disordered, unsaturated phospholipids (Shaw, 2006) to minimize the free energy of the two phases. A comparison of the phases occurring in biomembranes is depicted in Fig. 1.1. Some proteins exhibit an affinity for more ordered membrane domains (such as glycosyl-phosphatidylinositol (GPI)-anchored proteins), while others prefer disordered domains. In the plasma membrane, the proposed organization of lipid species into liquid-ordered and liquid-disordered domains is believed to be critical to the mechanisms of vesicular trafficking and cell signaling.

1.2 Biochemical characterization of domains in biological membranes and their functional roles

Although the raft hypothesis was initially based on model membrane and intracellular lipid trafficking studies in polarized epithelial cells (Simons and Ikonen, 1997; Simons and van Meer, 1988), its inception brought forth many early biochemical efforts to isolate and characterize domains from various biomembranes to help determine their function. Namely, solubilization of membranes with cold, non-ionic detergent (e.g., Triton X-100 at 4°C) revealed that an extractable detergent-resistant membrane fraction floated on sucrose density gradients and was enriched in cholesterol, sphingolipids, GPI-anchored proteins (Jacobson et al., 2007) and doubly-acylated Src family tyrosine
Figure 1.1. Comparison of lipid bilayer phases. The liquid-disordered, or fluid ($L_d$) phase (A), is populated with unsaturated lipids that are characterized by low acyl chain order and fast lateral diffusion. Conversely, saturated lipids and sphingomyelin tend to comprise the gel ($L_{β}$) phase (B), which is highly ordered and exhibits slow lateral diffusion. An intermediate phase, known as the liquid-ordered ($L_o$) phase (C), is formed when lipids are enriched with cholesterol and sphingolipids, resulting in high order, yet fast lateral and rotational diffusion. The plasma membrane of cells is believed to have co-existing $L_d$ and $L_o$ domains.
kinases. In model membrane systems, the liquid-ordered phase, but not the liquid-disordered phase, was similarly resistant to detergent solubilization (London and Brown, 2000). These comparative studies imply that phase separation in model membranes, or lipid heterogeneity in biomembraness, is due to thermodynamically preferential interactions between various types of lipids. Furthermore, upon cholesterol disruption with methyl-β-cyclodextrin, detergent-insoluble membrane fractions were abrogated and proteins formerly localized to these fractions were disassociated from the domains.

Because a range of detergents, detergent concentrations, incubation times and temperatures have been used for biochemical isolation and characterization of biomembrane domains various problems have plagued these methods (Shaw, 2006). Studies suggested that detergent treatment induced lipid reorganization, and therefore artifactual membrane domain formation and localization of proteins into those domains (Giocondi et al., 2000; Heerklotz, 2002). Mass spectrometry analysis revealed that detergent-resistant fractions were enriched in saturated lipids, while those fractions extracted without detergent were enriched in unsaturated lipids (Pike et al., 2002). Moreover, cholesterol chelation with methyl-β-cyclodextrin has been shown to affect cell morphology, disrupt the actin cytoskeleton and key steps in endo- and exocytosis, and delocalize signaling molecules (Edidin, 2003). These results highlight the limitations of biochemical analyses of membrane domains and emphasize the need for direct visualization of these domains in intact cell membranes.

Despite controversy associated with the nature of cell membrane domains, their roles in functional cell pathophysiology have been hypothesized. Many signaling molecules are concentrated in cholesterol-enriched membrane domains, suggesting that
these domains serve to organize signaling proteins and perhaps even enhance or protect from interactions with other molecules. For example, both B-cells (Sproul et al., 2000) and T-cells (Balamuth et al., 2001) exhibit differential localization of key receptors to these domains, depending on their developmental or functional stage. In addition to signal transduction, membrane domains have been implicated in protein and lipid transport and sorting from the beginning of the raft hypothesis. GPI-anchored proteins (Bamezai et al., 1992; Brown and Rose, 1992) and saturated lipid analogs (Mukherjee et al., 1999) tend to localize in late endosomes, with their return to the plasma membrane inhibited due to domain association, whereas transmembrane proteins and unsaturated lipid analogs are concentrated in the early endosomes where they are more rapidly recycled to the plasma membrane. Ordered cell membrane domains have further implications in the pathogenesis of diseases such as influenza and HIV (Luo et al., 2008; Nayak and Hui, 2004), as well as Alzheimers (Taylor and Hooper, 2007).

1.3 The current view of domains in biological membranes

Membrane domain composition, dimension and life span within cell membranes are still unclear. Macroscopic phase separation is readily observed in various model membranes, including substrate-supported planar bilayers, giant unilamellar vesicles (GUVs) and plasma membrane vesicles (Brown and London, 2000; Mukherjee and Maxfield, 2004). In contrast, domains have not been observed in live, intact biomembranes under resting conditions; although large-scale (micron-sized) domains can be formed by cross-linking with antibodies (Simons and Toomre, 2000). This general observation suggests that if membrane domains exist, they must be smaller than the resolution of light microscopy (<250 nm). High spatial resolution fluorescence resonance
energy transfer data (Sharma et al., 2004), electron microscopy images (Prior et al., 2003) and laser trapping experiments (Pralle et al., 2000) describe cholesterol-rich membrane domains that are 5–20 nm in diameter. There is additional controversy about how much of the plasma membrane is comprised of cholesterol-enriched domains, with estimates upwards of 30–50 mol% (Hao et al., 2001).

Without substantial perturbation, macroscopic scale phase separations likely do not occur in non-equilibrium structures such as the cell membrane due to dynamic mixing, yet it is still debated as to whether the transient formation or existence of membrane domains exist provides sufficient stability for any biological function to take place. Both electron paramagnetic resonance (Kawasaki et al., 2001) and single-molecule energy transfer (Kenworthy et al., 2000) experiments provide a half-life of ~100 ns for lipids in cholesterol-rich domains. Smaller domains could also be stabilized (or larger domains destabilized) in the cell membrane by proteins and cholesterol that act to decrease the thermodynamic line tension or by the cell membrane being at the very edge of a sort of phase boundary where only a substantial perturbation can induce large scale domain formation (Sengupta et al., 2007a). Others propose that membrane domains are constantly dissolving and reforming due to the entropic instability of lipid associations (Almeida et al., 2005).

Since the formulation of the raft hypothesis, membrane domains were recently described as “small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes… and are sometimes stabilized to form larger platforms through protein-protein and protein-lipid interactions” (Pike, 2006; Pike, 2008). A simple schematic of membrane domain organization is
Figure 1.2. Membrane domain organization in biological membranes. The liquid-ordered phase ($L_o$) is enriched in cholesterol and sphingolipids (shaded areas) and surround by liquid-disordered phase ($L_d$) lipids. The arrows indicate clustering of proteins with an affinity for more ordered membrane domains into larger domains, and suggest that clustering may occur via coalescence of small, $L_o$ domains (A). Alternatively, protein attraction for $L_o$ phase may be increased sufficiently for recruitment to the larger domains (B). Instead of lipids recruiting proteins, it has also been hypothesized that proteins may themselves control the formation of $L_o$ membrane domains (Edidin, 2003). Studies have suggested that proteins may exist in immobile or mobile $L_o$ domains for long times (e.g., seconds), or that proteins may dynamically partition into and out of these domains (Kenworthy et al., 2004). Figure is taken from Brown and London (2000).
shown in Fig. 1.2. Domain formation is fundamentally driven by intermolecular van der Waals forces, hydrogen bonding and hydrophobic forces (Mayor and Rao, 2004). Cholesterol experiences favorable van der Waals forces with $L_\beta$ lipid acyl chains, disrupting these forces among the acyl chains themselves, and increases van der Waals forces when incorporated into $L_d$ phase lipids (Mayor and Rao, 2004). Cholesterol can also hydrogen bond with sphingolipids and experiences hydrophobic shielding beneath hydrated phospholipid headgroups due to steric concerns (Mayor and Rao, 2004). However, the precise physical and chemical nature of membrane domain formation is still unknown. For instance, evidence exists from model membrane studies that preexisting domains can recruit proteins, such as GPI-anchored proteins (Silvius, 2003), though it has also been suggested that proteins induce domain formation via protein interactions with lipid acyl chains (Edidin, 2003). These questions regarding biomembrane domains cannot be addressed with biochemical analyses and further stress the need for direct visualization with high spatial and temporal resolution.

1.4 Methods for studying domains in biological membranes

Defining and characterizing cell membrane domains by sucrose gradient ultracentrifugation of detergent-lysed cells and sensitivity to cholesterol depletion does not provide the spatial and temporal information needed to adequately describe the formation and function of these features. This Section will describe research that has addressed these needs by applying advanced microscopic and spectroscopic methods for evaluating the spatial organization, composition and dynamic properties of domains in intact cell membranes. Ensemble methods that have been used to characterize membrane structural order, but provide either no spatial or no temporal information, such as
spectrofluorimetry (Gidwani et al., 2001), and electron spin resonance (Ge et al., 1999; Ge et al., 2003; Swamy et al., 2006) and nuclear magnetic resonance (Veatch et al., 2004; Veatch et al., 2007) spectrosopies, will not be discussed here. This Section will lastly describe a novel combination of these modalities, in the form of an ultrafast, excited-state fluorescence dynamics imaging assay based on the principle of time-correlated single-photon counting (TCSPC) (see Section 2.4.1), to overcome the detection limitations of traditional microscopic and spectroscopic techniques. The major experimental approaches for characterizing membrane domains discussed in this Section are compared in Table 1.1.

1.4.1 Imaging approaches that assess domain spatial organization and composition

Direct methods for observing membrane domains have been focused around fluorescence microscopy, mainly because it is amenable to live cell imaging, although domains have yet to be observed in the plasma membrane of resting cells using conventional optical microscopy. These direct observation methods have spawned the development of various fluorescent membrane probes for studying domains in both model membranes and biomembranes.

The most common probe used to interrogate domains in model membranes and cell membranes is the B subunit of cholera toxin, which is pentameric and specifically binds to the ganglioside GM1 that is localized to the cholesterol-enriched $L_n$ phase, and can be cross-linked, if needed, with a fluorescently-labeled secondary antibody (Owen et al., 2007). The localization of fluorescently labeled proteins to these large domains can then be assessed. Micron-sized domains are stabilized due to the high valency of this probe complex (nM dissociation constant (Fishman, 1982)), which likely misrepresents
Table 1.1. Various methods for characterizing domains in model and biological membranes.

<table>
<thead>
<tr>
<th>Method</th>
<th>Primary observables</th>
<th>Spatial / temporal resolution</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical analysis of detergent lysates</td>
<td>Putative domain association of molecules</td>
<td>– / –</td>
<td>Easy and commonly used</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not amenable to live cells and prone to artifacts</td>
</tr>
<tr>
<td>Fluorescence microscopy</td>
<td>Putative domain association of molecules</td>
<td>~250 nm / –</td>
<td>Easy and commonly used</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fluorescent labeling needed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Extensive cross-linking needed to observe membrane domains in live cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cell fixation required for immunofluorescence</td>
</tr>
<tr>
<td>Atomic force microscopy (AFM)</td>
<td>Membrane topography</td>
<td>~1 nm / –</td>
<td>Commonly used on model membranes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No extrinsic probes required</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not amenable to live cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not sensitive to small height differences</td>
</tr>
<tr>
<td>Electron microscopy (EM)</td>
<td>Spatial distribution of probe density</td>
<td>~1–10 nm / –</td>
<td>Not applicable to live cells</td>
</tr>
<tr>
<td>Secondary ion mass spectrometry imaging (SIMS)</td>
<td>Spatial distribution of mass species</td>
<td>~100 nm / –</td>
<td>No exogenous probes required</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not applicable to live cells</td>
</tr>
<tr>
<td>Fluorescence resonance energy transfer (FRET)</td>
<td>Donor-acceptor proximity</td>
<td>~1–10 nm / No</td>
<td>Amenable to live cells</td>
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<td></td>
<td></td>
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<td>Cluster identification on nanometer scale</td>
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<td>Various detection methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Must carefully choose donor and acceptor probes</td>
</tr>
<tr>
<td>Method</td>
<td>Measurement</td>
<td>Time Resolution</td>
<td>Characteristics</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>--------------------------------------------------</td>
<td>-----------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| Fluorescence recovery after photobleaching (FRAP) | Translational dynamics of fluorophore             | ~250 nm / ~1 µs | Applicable to live cells  
  Ensemble-averaging  
  Weakly sensitive to subpopulations of diffusion |
| Fluorescence correlation spectroscopy (FCS)  | Translational dynamics and concentration of fluorescent species | ~250 nm / ~1 µs | Low fluorophore concentration  
  Applicable to live cells  
  Sensitive to clustering and subpopulations of diffusion  
  Can be performed with multiple colors (FCCS) and in imaging mode (ICS) |
| Single particle tracking (SPT)               | Translational trajectory and diffusion of probe particle or fluorescent molecule | ~250 nm / ≤25 µs (≤10 nm precision in detecting centroid) | Single particle sensitivity  
  Detects various modes of diffusion  
  Amenable to live cells  
  Probe valency must be carefully determined |
| Time-correlated single-photon counting (TCSPC) | Fluorescence lifetime, anisotropy and rotational dynamics of fluorophore, and donor-acceptor proximity | ~250 nm (~1–10 nm for FRET) / ≤100 ps | Applicable to live cells  
  Sensitive to environmental heterogeneity  
  Independent of probe concentration  
  Can be performed in single point or imaging (scanning) mode  
  Polarized emission can be used to obtain rotational dynamics and order parameters  
  Time-resolved FRET can be performed on dually labeled samples  
  Need a femtosecond pulsed laser |
the size of biomembrane domains. Smaller fluorescently-labeled GPI anchored proteins that do not require cross-linking, such as GPI-anchored folate receptor (Varma and Mayor, 1998) and Thy-1 (Dietrich et al., 2002) are often used as alternative membrane domain probes.

Other typical membrane probes are fluorescent phospholipid analogs, such as 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE) that intercalate into the outer leaflet of the plasma membrane. BODIPY-PC, which is acyl chain-labeled, preferentially partitions into the $L_d$ phase and has been used to study domains in GUVs (Korlach et al., 1999). Headgroup-labeled NBD-DPPE, on the other hand, partitions into the $L_o$ phase and has been used to label domains in planar bilayers (Dietrich et al., 2001a) and to assess cholesterol-dependent ordering in mast cell membrane via spectrofluorimetric measurements (Gidwani et al., 2001).

6-dodecanoyl-2-dimethylaminonaphthalene (laurdan) is an example of a fluorescent probe that is sensitive to the ordering of lipid acyl chains (and therefore water penetration into the bilayer), with emission that is blue-shifted in the $L_o$ phase as compared to the $L_d$ phase, and also has a phase-dependent orientation that can be probed with fluorescence polarization (Owen et al., 2007). Large-scale membrane domains have accordingly been visualized in laurdan-labeled GUVs comprised of an equimolar mixture of phosphatidylcholine (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC), sphingomyelin (brain) and cholesterol (Dietrich et al., 2001a). 1-[2-hydroxy-3-(N,N-di-methyl-N-hydroxyethyl) ammoniopropyl]-4-[β-[2-(di-n-butylamino)-6-naphthyl] vinyl] pyridi-
nium (di-4-ANEPPDHQ) exhibits a blue-shift similar to that of laurdan and increases its fluorescence lifetime in $L_0$ (egg $n$-palmitoyl-sphingomyelin:cholesterol, 0.7:0.3, mol/mol) as compared to $L_d$ (DOPC) large unilamellar vesicles (Jin et al., 2006). Although laurdan (Gaus et al., 2006) and di-4-ANEPPDHQ have been used to monitor the distribution of micron-sized membrane domains in cells (Owen et al., 2006), they have not successfully identified small-scale domains in resting cells.

Techniques such as atomic force microscopy (AFM), electron microscopy (EM) and secondary ion mass spectrometry (SIMS) imaging allow for the direct observation of membrane domains with higher spatial resolution than light microscopy. Because AFM and SIMS do not require exogenous labeling, physiological relevance is better maintained. Using AFM, both nanoscale-sized and microscale-sized membrane domains have been detected in cholesterol-containing model bilayers, with more ordered domains having a 1.4 nm greater height than the surrounding bilayer (Tokumasu et al., 2003). Membrane domains that contain immunogold labeled raft markers and are sensitive to cholesterol extraction have been detected via AFM of plasma membrane sheets and directly correlated with cholesterol-enriched dark patches observed with EM of the same sheets (Frankel et al., 2006). It is not yet clear whether AFM can be successfully applied to lipids within the plasma membrane of intact living cells. Because EM samples can only be 100 nm thick at most, plasma membrane sheets are the best approximation of an intact cell (Lagerholm et al., 2005). SIMS imaging has been used to determine the chemical composition of micron-sized membrane domains within model bilayers, demonstrating localization of cholesterol and sphingomyelin to the domains and exclusion of unsaturated phosphatidylcholine (McQuaw et al., 2007). AFM, EM and
SIMS imaging are limited because they do not allow for live cell imaging or for dynamics measurements of membrane domains, thus limiting their application to model membranes or cell membrane sheets, rather than intact cells.

In addition to the direct methods described above, the most common indirect method of membrane domain observation is fluorescence resonance energy transfer (FRET) imaging. FRET measures interactions between fluorescently-labeled molecules that are 1–10 nm in proximity in live cells. FRET is typically measured at the steady-state level as a decrease in the ratio of donor and acceptor fluorescence (Owen et al., 2007); however, it can also be measured via a decrease in donor fluorescence lifetime, which can yield molecular dynamics information (see Section 1.4.3). FRET between variously partitioned probes in model membranes and the plasma membrane of live cells has detected nanometer-scale $L_0/L_d$ domains that are sensitive to cholesterol depletion, enhanced by cross-linking of $L_0$-associated proteins, and correlate with lipid probe partitioning into detergent-resistant membranes (Sengupta et al., 2007b). FRET between GPI-anchored proteins labeled with the same probe (homo-FRET) on the plasma membrane of live cells has also been assessed via a decrease in steady-state (Varma and Mayor, 1998) or time-resolved anisotropy (Sharma et al., 2004). The latter study determined that ~20–40% of GPI-anchored proteins were localized to cholesterol-rich membrane domains <10 nm in diameter and contained as few as five protein molecules. These FRET studies (Varma and Mayor, 1998), and various others (e.g., Sengupta et al., 2007b), provide only a steady-state view of lipid and protein heterogeneity in biomembranes and suggest that cholesterol-rich domains exist on the nanoscale in resting
cells, with only a small portion of the proteins classified as detergent-resistant found within their boundaries.

1.4.2 Diffusion-based methods

Several fluorescence microscopy-based methods have been developed for interrogating the spatio-temporal dynamics of various fluorescent membrane probes to gain more detailed information about biomembrane domain structure and function. One of the earliest established methods for measuring lipid and membrane-associated protein lateral diffusion is fluorescence recovery after photobleaching (FRAP). After photobleaching, the time required for fluorescent molecules to diffuse back into the bleached area (i.e., fluorescence recovery), reflects the translational mobility of the probed molecules. This recovery time is dependent upon the diffraction-limited beam size, fluorescent probe size, local viscosity, and many other physiological factors such as molecular diffusional barriers and crowding, transient cytoskeletal associations and membrane turnover (Lagerholm et al., 2005).

FRAP has been applied to influenza hemagglutinin proteins with different membrane anchors to the plasma membrane in live cells and showed that putative raft-associated proteins diffuse in a cholesterol-dependent manner and more slowly (lateral diffusion coefficient, $D$, $\sim 6 \times 10^{-10}$ cm$^2$/s) than non-raft-associated proteins ($D \sim 1 \times 10^{-9}$ cm$^2$/s) (Shvartsman et al., 2003). A similar trend was also observed for inner leaflet Ras proteins with different membrane anchors, providing information about the role of cholesterol-rich membrane domains in Ras signaling in vivo (Niv et al., 2002). Another comprehensive FRAP study concluded that protein diffusion coefficients in COS-7 cell membranes were dependent upon the way that they were anchored to the
membrane rather than upon their tendency to partition into cholesterol-rich domains, as determined by detergent resistance (Kenworthy et al., 2004).

Fluorescence correlation spectroscopy (FCS) has been used to study molecular diffusion in live cells and offers sensitivity to various diffusion subpopulation and measures diffusion within a restricted observation volume. FCS measures the fluorescence intensity fluctuations as a function of time caused by the diffusion of single molecules through a diffraction-limited observation volume. These fluctuations are temporally autocorrelated to obtain the characteristic molecular diffusion time and average number of molecules in the observation volume and to classify the diffusion mode (Haustein and Schwille, 2007; Lagerholm et al., 2005; Magde et al., 1974). FCS revealed an ~11-fold cholesterol-dependent decrease in lateral diffusion of a lipid microdomain-associated probe (Alexa Fluor 488-labeled, GM1-bound cholera toxin B) versus a non-domain-associated probe (1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine, diI-C16) (Bacia et al., 2004) in DOPC:sphingomyelin:cholesterol (1:1:1, mol/mol) GUVs. Using FCS, fluorescently-labeled GPI-anchored proteins in COS-7 cells were shown to be confined to sub-resolution, cholesterol-rich domains for tens of microseconds, whereas non-microdomain markers (e.g., transferring receptor and dipeptidyl peptidase IV) exhibited cytoskeleton-dependent confinement and diffusion (Lenne et al., 2006).

If more than one type of molecule is labeled with spectrally distinct fluors, fluorescence cross-correlation spectroscopy (FCCS) can be used to infer associations between the different types of molecules while simultaneously obtaining lateral diffusion of both species. FCCS has been used to measure the co-redistribution of various
fluorescently labeled inner and outer leaflet components of rat basophilic leukemia (RBL) cells as a function of immunoglobulin E (IgE) receptor cross-linking (Larson et al., 2005). The results of these studies supported the hypothesis that interactions between Lyn and cross-linked IgE receptor result from their co-association with cholesterol-rich domains in the plasma membrane of mast cells (Pyenta et al., 2003). Image correlation spectroscopy (ICS) is the imaging analog of FCS in which the spatial correlation function is calculated to assess cluster density and degree of aggregation (Kolin and Wiseman, 2007; Nohe and Petersen, 2007). ICS has been used, for example, to study the clustering and diffusion of GPI-anchored proteins in the plasma membrane of live cells, revealing reduced clustering and increased diffusion at higher temperatures (i.e., 37°C versus room temperature) (Nohe et al., 2006).

While FCS averages thousands of single molecule diffusion events within the acquisition time (Marguet et al., 2006), single particle tracking (SPT) can be used to follow the lateral diffusion of individual molecules in live cells. Membrane proteins or lipids are specifically labeled with a small particle (often 40–100 nm diameter gold or quantum dots) and their trajectories tracked with 10–20 nm precision in image series or movies acquired by optical microscopy (Levi and Gratton, 2007) for s–min. SPT of both a raft-associated GPI-anchored protein (CD59) and a non-raft-associated phospholipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DOPE) described random, Brownian diffusion in transient confinement zones, corralled by cytoskeletonally-anchored transmembrane proteins, with infrequent hop-diffusion between zones (Subczynski and Kusumi, 2003). Lipid restriction within confinement zones was poorly correlated with membrane cholesterol content or with lipid affinity for a particular phase, indicating that
confinement is not exclusively attributed to partitioning into or out of cholesterol-rich membrane domains (Subczynski and Kusumi, 2003). Kusumi and co-workers also observed cholesterol-rich domains consisting of only a few molecules and able to diffuse through spaces between cytoskeletal proteins (~2–9 nm (Kusumi and Suzuki, 2005)), with lifetimes less than a millisecond (Suzuki et al., 2001). Although these domains are believed to be small and transient, when CD59 is cross-linked, for example, larger immobile cholesterol-rich domains develop (Subczynski and Kusumi, 2003).

Other SPT/laser trapping studies have demonstrated that raft-associated GPI-anchored (e.g., placental alkaline phosphatase, PLAP) and transmembrane proteins (e.g., influenza virus hemagglutinin, HA) have reduced cholesterol-dependent mobility compared to non-raft transmembrane (e.g., the artificial yellow fluorescent protein, LFPGT46) proteins (Pralle et al., 2000). In this study, mobility was measured in terms of local viscous drag, with LFPGT46 experiencing 3-fold less drag than raft-associated proteins. The raft-associated GPI-anchored proteins remained in cholesterol-rich membrane domains for up to 10 min, with the complexes estimated at 26 ± 13 nm in diameter (Pralle et al., 2000). Such cholesterol-rich membrane domain-associated lipid or protein molecules are less likely to undergo hop-diffusion and thus more likely to remain in the confinement zones discussed above (Dietrich et al., 2002). In SPT studies, the large size of the particle relative to the labeled molecule, potential multi-valency of labeling, and various non-specific interactions can distort the observed diffusion. Thus, labeling molecules with smaller fluorescent proteins (Douglass and Vale, 2008) or quantum dots and using total-internal-reflection fluorescence microscopy (TIRFM) to
eliminate out-of-focus fluorescence hold promise for studying lipid and protein associations with cholesterol-rich membrane domains via SPT (Lidke et al., 2007).

Conventional methods for obtaining translational diffusion of membrane-associated molecules are limited by optical diffraction, ensemble averaging, probe valency and \( \mu s\text{–s} \) time resolution. In addition to measuring lateral diffusion using FRAP, FCS and SPT, rotational diffusion and molecular order (e.g., wagging motions) can be detected by electron spin resonance (ESR) and fluorescence anisotropy. However, these methods are typically ensemble in nature, provide no spatio-temporal information, and are not amenable to imaging of individual live cells. Thus, to obtain molecular dynamics information while overcoming these various limitations, we have used an ultrafast fluorescence dynamics imaging assay that includes time-resolved fluorescence lifetime and polarization anisotropy imaging.

1.4.3 **Excited-state fluorescence dynamics imaging approaches**

Fluorescence lifetime imaging microscopy (FLIM) and polarization anisotropy imaging determine the lifetime and rotational dynamics, respectively, of a fluorescently-labeled molecule at different spatial positions (Suhling et al., 2005). These excited-state dynamics imaging methods report on molecular structural conformation, which is influenced by the surrounding environment. The surrounding environment affects the fluorescence quantum efficiency of the fluorophore; thus lifetime measurements are relatively independent of probe density, or concentration (Lakowicz, 1999). Fluorescence lifetime and polarization anisotropy imaging are based on optical microscopy and are therefore limited by optical diffraction, but because these techniques have ultrafast (i.e., ps–ns) time resolution, they reflect sub-diffraction molecular structure, order and
dynamics (Davey et al., 2008; de Almeida et al., 2008; Suhling et al., 2005). Fluorescence lifetime and anisotropy can monitor time-resolved hetero-FRET and homo-FRET to assess molecular interactions in a dually labeled sample below the diffraction limit of optical microscopy. Hetero-FRET is reported by a decrease in the lifetime of the donor species the presence of acceptor (de Almeida et al., 2008), while homo-FRET is observed by a decrease in the rotational dynamics of a single species (Clayton et al., 2002; Sharma et al., 2004).

The fluorescent probes used for dynamics imaging must be sensitive to changes in their environment. As described in Section 1.4.1, probes such as laurdan and di-4-ANEPPDHQ have fluorescence lifetimes that reflect the phase of the membrane in which they are incorporated by increasing in a more ordered environment (Owen et al., 2007). Other molecules, such as carbocyanine dyes (e.g., diI-C_18) have fewer conformational degrees of freedom for relaxation from the excited state in a more ordered environment, thus increasing their lifetimes (Davey et al., 2008; Davey et al., 2007; Packard and Wolf, 1985). It is critical for polarization anisotropy measurements that the fluorescent probe has a well-defined molecular structure and specific attachment to the molecule of interest so that various components of rotational diffusion can be accurately assigned to each structural degree of freedom. If the fluorescent probe is too large, for example, subtle dynamics information could be hidden.

Various techniques, such as those described above, have been used to assess the spatial organization and dynamics of cholesterol-rich membrane domains and suggest that these domains are transient and smaller than the diffraction limit of optical microscopy. Fluorescence lifetime and polarization anisotropy offer to challenge the current
spatial and temporal limits of domain detection provided by such techniques. This Dissertation describes the development of a fluorescence dynamics imaging assay that includes FLIM, polarization anisotropy imaging, and time-resolved FRET. The assay was tested on model membranes (Chapter 3) and then used to obtain spatio-temporal information on lipid/lipid, protein/protein and lipid/protein interactions associated with IgE receptor signaling in the plasma membrane of individual, live mast cells under non-physiological (Chapter 4) and physiological (Chapter 6) conditions.

1.5 Role of membrane domains in IgE receptor signaling in mast cells

1.5.1 The allergic response and IgE receptor signaling

As shown in Fig. 1.3, the immune system is sensitized upon exposure to an antigen or allergen. As a result of this exposure, antibodies (i.e., immunoglobulin E, or IgE) that are specific for the antigen are produced by B cells. These antibodies attach to high affinity receptors for IgE (FcεRI) present on the surface of mast cells and basophils in various tissues (Rivera et al., 2008). When these mast cells and basophils are subsequently challenged with the antigen, a signaling cascade is initiated (Fig. 1.4) that leads to the release of histamine and other inflammatory mediators and cytokines that trigger the allergic response. IgE-mediated allergic reactions can lead to asthma and food and drug allergies that in some cases lead to anaphylaxis (Kraft and Kinet, 2007). Thus, it is of particular interest to determine the precise mechanism for the allergic response initiated by antigen cross-linking of IgE receptors (Rivera et al., 2008).

IgE receptor signaling has been studied extensively in the rat basophilic leukemia (RBL) mast cells (Fig. 1.4). When antigen-specific IgE that is bound to the α subunit of
Figure 1.3. Mechanism of the allergic response. Upon exposure to an antigen for the first time, B cells produce plasma cells that secrete antibodies specific to the antigen. These antibodies attach to the high affinity IgE receptors on mast cells. When challenged with antigen again, the mast cells release histamine- and cytokine-filled granules, causing characteristic allergy symptoms.
Figure 1.4. Mechanism of IgE receptor signaling. Antigen cross-linking of IgE bound to its receptor (FcεRI) results in tyrosine phosphorylation of FcεRI by Lyn, which recruits Syk. Syk phosphorylates and activates LAT and PLCγ. PLCγ catalyzes PIP2 hydrolysis (not shown) into IP3 and DAG, activating Ca^{2+} mobilization and PKC that are required for the release of secretory granules in the allergic response. Figure courtesy of Erin D. Sheets and Rebecca M. Williams.
FcεRI is cross-linked via multivalent antigen, a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) in the β subunit of FcεRI is phosphorylated by the Src family kinase, Lyn, which is anchored to the inner leaflet of the plasma membrane by dual acylation (Holowka et al., 2005). Lyn then phosphorylates ITAMs on the γ2 subunit of FcεRI to recruit and activate another tyrosine kinase, Syk, which phosphorylates and activates LAT (the linker for activation of T cells; an adaptor protein) and phospholipase Cγ (PLCγ). LAT-associated PLCγ hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), thus elevating Ca2+ levels and activating protein kinase C (PKC), which are required for the secretory response (Holowka et al., 2007; Honda, 2006; Sengupta et al., 2007a; Torres et al., 2008). Cross-linking of two or more FcεRI is required for sufficient propagation of these signals to culminate in degranulation, or the exocytotic release of histamine (Pribluda et al., 1994). Although these key IgE receptor signaling proteins and their basic functions have been identified, primarily via biochemical and fluorescence microscopy studies, there is still much to be learned about how these protein molecules assemble and interact dynamically and how the structure of the plasma membrane may mediate or facilitate these dynamic processes.

1.5.2 Evidence for membrane domain involvement in IgE receptor signaling

As described in Section 1.2, a key membrane domain function that has been proposed is the regulation of signal transduction. It is hypothesized that certain signaling molecules have an affinity for recruitment into L0 regions of the plasma membrane based on certain post-translational modifications (e.g., GPI-anchors, myristoylation or palmitoylation (Lucero and Robbins, 2004)), where they may be concentrated to enhance
signaling (Baird et al., 1999). However, concentration of signaling molecules implies that $L_o$ domains comprise a small fraction of the plasma membrane, which may or may not be the case (Hao et al., 2001; Swamy et al., 2006). There is substantial evidence that protein compartmentalization is essential for the interaction of signaling molecules, and thus for the regulation of immune signaling (Holowka et al., 2000). Indirect biochemical and fluorescence microscopy approaches that are sensitive to cholesterol depletion and protein modifications have been used to provide evidence of the role of membrane organization in IgE receptor signaling (Sengupta et al., 2007a).

1.5.2.1 Biochemical analyses

Ligand-mediated cross-linking of IgE-FcεRIIs on RBL-2H3 mast cells initiates stimulated tyrosine phosphorylation of FcεRI via Lyn and results in FcεRI association with detergent-resistant membranes upon solubilization with Triton X-100, (Sheets et al., 1999a). Studies reveal that FcεRI transmembrane sequences that permit associations with $L_o$ domains upon cross-linking are critical for initial receptor phosphorylation and downstream events such as degranulation (Gosse et al., 2005). Although Lyn is constitutively associated with $L_o$ regions of membrane due to its acylation, IgE-FcεRI cross-linking increases the percentage of Lyn recovered from detergent-resistant membranes (Field et al., 1995). Independent of IgE receptor cross-linking, Lyn has ~5-fold higher specific activity in detergent-resistant membranes than in $L_d$ phase regions of the plasma membrane, corresponding to higher tyrosine phosphorylation in the active site loop (Young et al., 2003). Cholesterol depletion via methyl-β-cyclodextrin abrogates antigen-mediated phosphorylation of FcεRI by Lyn and the association of cross-linked FcεRI and Lyn with detergent-resistant membranes (Sheets et al., 1999a). These results
suggest that association of cross-linked FcεRI with active Lyn in cholesterol-rich membrane domains is necessary to initiate FcεRI phosphorylation (Holowka et al., 2005) and that the active site tyrosine of Lyn is more protected from deactivation by various phosphatases in a more ordered membrane environment (Young et al., 2005).

1.5.2.2 Fluorescence co-localization experiments

Due to the potential artifacts associated with the biochemical characterization of membrane domains, fluorescence imaging experiments have also been performed using intact RBL-2H3 mast cells that were extensively cross-linked at 4°C to create micron-sized domains of IgE receptors and ordered lipids. Early fluorescence co-localization experiments demonstrated co-redistribution of cross-linked IgE receptor with detergent-resistant membrane components such as saturated phospholipid analogs (Thomas et al., 1994) and the GD_{1b} ganglioside (Pierini et al., 1996; Sheets et al., 1999a). Similar to the biochemical analyses, microscopy studies also revealed cholesterol-sensitive interactions between cross-linked IgE receptors and Lyn (Sheets et al., 1999a), the GPI-anchored protein Thy1, and the actin cytoskeleton (Holowka et al., 2000). Together, these data provided evidence that cholesterol is required for the functional coupling of Lyn with cross-linked IgE receptor. It was further suggested that cholesterol-enriched membrane domain formation may be regulated by stimulated actin polymerization (Holowka et al., 2000).

When receptor-bound IgE on RBL-2H3 mast cells was bound to patterned supported lipid bilayers containing a ligand for IgE, micron-sized clusters of IgE receptor complexes were observed via fluorescence microscopy at ~20°C and 37°C (Torres et al., 2008; Wu et al., 2007; Wu et al., 2004). Similar to global stimulation by soluble antigen,
the receptors are phosphorylated, and Lyn and other inner leaflet raft components are subsequently recruited to the localized clusters in an actin polymerization-dependent manner. Interestingly, various outer leaflet raft components, such as a saturated phospholipid analog and the GD\textsubscript{1b} ganglioside, do not co-redistribute with cross-linked IgE receptor. These results demonstrate that inner and outer raft components can localize independently of one another and that the actin cytoskeleton mediates lipid and protein organization into cholesterol-rich domains (Holowka et al., 2005).

Taken together, these biochemical studies and fluorescence co-localization experiments led to the hypothesis that cholesterol-rich membrane domains facilitate the initiation of IgE receptor signaling (Fig. 1.5). Thus, it is essential to examine the function of \( L_0 \) plasma membrane domains in the IgE receptor signaling mechanism using techniques amenable to cells under more biologically relevant conditions, where dynamic molecular interactions can occur below the spatial and temporal resolution of conventional optical microscopy.

1.5.3 Monitoring mast cell membrane structure and molecular dynamics as a function of IgE receptor cross-linking or signaling

This Dissertation describes the study of changes in the molecular structure and dynamics of the RBL-2H3 mast cell membrane due to IgE receptor cross-linking using an ultrafast fluorescence dynamics imaging assay (see Section 1.4.3). The goal of these studies is to test the hypothesis that cholesterol-rich membrane domains facilitate the initiation of immunoreceptor signaling, and thus the allergic response, and to determine how plasma membrane organization may facilitate protein assembly and dynamic interactions. These approaches also probe the dynamic and transient interactions
Figure 1.5. Hypothesized role of cholesterol-rich membrane domains in IgE receptor signaling. In resting mast cells (A), Lyn is often associated with small liquid-ordered ($L_o$) domains where autophosphorylation occurs. Outside of these domains, Lyn is not protected from phosphatases and is readily dephosphorylated, thereby lowering Lyn activity. Upon antigen stimulation of receptor-bound IgE (B), larger domains are transiently stabilized. With Lyn present in these domains and protected from phosphatases, it readily phosphorylates the receptor to initiate the downstream signaling cascade. Figure is adapted from Brown (2006).
occurring between cholesterol-rich membrane domains and other molecules involved in IgE receptor signaling, and lend insight into how domain formation is mediated in vivo. Experimental methods were developed on mast cells cross-linked under non-physiological conditions to create optically resolvable membrane domains (Chapter 4) and subsequently applied to cells that were cross-linked and stimulated under physiological conditions where cholesterol-rich domains are too small and transient to image optically (Chapter 6). Excited-state fluorescence dynamics imaging provides molecular-level environmental sensitivity with ultrafast time resolution to assess sub-diffraction limit molecular structure, order, dynamics and interactions at the single cell level. Thus, dynamics imaging overcomes the restrictions imposed by the methods typically used to study the role of membrane domains in various signaling processes, yet data can still be correlated with functional information obtained via biochemistry.
2. EXPERIMENTAL METHODS

This Chapter details the preparation of model lipid membranes, as well as RBL mast cells subjected to non-physiological and physiological treatments. The microscopy and spectroscopy techniques used to probe these samples, with theoretical perspectives and data analysis procedures, are also described here.

2.1 Small unilamellar vesicle (SUV) and planar supported lipid bilayer preparation

The day prior to an experiment, SUVs were formed as described previously (Kyoung et al., 2007; Pearce et al., 1992; Vats et al., 2008). These SUVs were composed of either $L_{\beta}$ phase (1,2-dipalmitoyl-sn-glycerol-3-phosphocholine; DPPC; $T_m = 41^\circ$C) or $L_d$ phase (1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; POPC; $T_m = -2^\circ$C) lipids (Avanti Polar Lipids), with addition of a fluorescent lipid analog (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); NBD-DPPE) (Avanti Polar Lipids). Specifically, lipid and fluorescent probe at the desired composition (e.g., 99.5 mol% DPPC:0.5 mol% NBD-DPPE) were added to an ethanolic-KOH-cleaned test tube and dried under nitrogen. The lipids were then resuspended in 50 mM Tris, 100 mM NaCl, pH 7.4, and probe sonicated until clarified and subjected to ultracentrifugation (Airfuge, 30 psi, 178,000 $\times$ g, 1 h). The top quarter of the supernatant was collected and stored overnight at room temperature and used within 24 h. On the day of an experiment, the SUV solution was pipetted between a sandwich, composed of a detergent-cleaned 3” $\times$ 1” glass slide and a 22 mm $\times$ 22 mm glass coverslip, both of which were cleaned in an argon plasma immediately before applying the solution. The SUVs spontaneously fuse to form uniform lipid bilayers. After 30 minute incubation in a humidified chamber, samples were rinsed with 50 mM Tris, 100 mM NaCl, pH 7.4, to remove unfused
vesicles. Bilayer samples were sealed with VALAP (Vaseline:lanolin: paraffin [2:1:1, wt/wt]) and measurements carried out immediately.

2.2 Cell culture and labeling

2.2.1 Suspended mast cells

RBL-2H3 mast cells were maintained and harvested as previously described (Pierini et al., 1996; Sheets et al., 1999a). Cells (3 × 10^6 cells/mL) suspended in 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) were sensitized with a 10-fold molar excess of mouse anti-2,4-dinitrophenyl IgE (α-DNP IgE, provided by Dr. David Holowka, Cornell University). IgE was used either unlabeled or conjugated to Alexa Fluor 488 (A488-IgE) via a commercially available protein labeling kit (Invitrogen), which labels the monomeric IgE at exposed primary amines (the dye/IgE ratio was ~5, as determined by absorption spectroscopy). Cells were labeled with a 50-fold dilution of 0.2 mg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (diI-C₁₈) (Invitrogen) stock solution prepared in dimethylsulfoxide (DMSO). For this protocol, the dye/lipid ratio in cells was previously estimated to be ~1:220 (or 0.46 mol %) (Thomas et al., 1994), which is lower than the concentration range required for considerable self-quenching (Packard and Wolf, 1985).

The chromophoric portion of diI-C₁₈ resides in the interfacial region of the plasma membrane, between the surface lipid headgroups and the core lipid acyl chains (Gullapalli et al., 2008; Krishna and Periasamy, 1998). RBL cells were incubated (2 h, 4°C) either in the presence or absence of 10 µg/mL polyclonal rabbit anti-IgE (α-IgE) (provided by Dr. David Holowka, Cornell University), as described previously (Thomas
et al., 1994), to form extensive patches of receptor-bound IgE, i.e., IgE-FceRI, (cross-linked cells), or not (uncross-linked or monomeric cells). Before imaging, 25 µL of cell suspension was pipetted onto a 22 mm × 22 mm glass coverslip, covered with a 3” × 1” glass slide, sealed with VALAP, and incubated for 10–15 min at room temperature (~20°C) for cell immobilization.

2.2.2 Adherent mast cells

RBL-2H3 mast cells were maintained and harvested as noted above (Pierini et al., 1996; Sheets et al., 1999a). Adherent cells (1.5 × 10⁵ cells/mL) were plated onto glass-bottomed 35-mm Petri dishes (MatTek) and sensitized overnight with 0.75 µg of α-DNP IgE per 1 mL of cell suspension. IgE was used either unlabeled or as A488-IgE, as described above, with a dye/IgE ratio of ~4. The day of the experiment, the cells were washed 3× with BSA/BSS. Cells were then labeled for 3 min at 37°C with 1 mg/mL diI-C₁₈ stock solution prepared in DMSO that had been diluted 100-fold in BSS, and then washed 3× with BSA/BSS. For some control experiments, cells were labeled in the same manner, but with 1 mg/mL 1,1'-didodecyl-3,3',3',3'-tetramethylindocarbocyanine (diI-C₁₂, Invitrogen) or 1,1'-dioleyl-3,3',3'-tetramethylindocarbocyanine (Δ⁹-diI-C₁₈, Invitrogen) stock solution in DMSO that had been diluted 500-fold in BSS.

RBL cells were stimulated on the microscope at room temperature (~20°C) with 1 µg/mL multivalent antigen, i.e., 2, 4-dinitrophenyl bovine serum albumin (DNP-BSA). As a control, cells were monitored as a function of time, in the absence of DNP-BSA. To verify that observed changes were specific to IgE receptor-mediated signaling, some samples were treated with 80 nM phorbol 12-myristate-13-acetate (PMA) (Alexis
Biochemicals), a protein kinase C activator that stimulates actin polymerization and leads to cell ruffling (Xu et al., 1998b), instead of DNP-BSA.

2.2.3 Tyrosine phosphorylation assays

Adherent, IgE-sensitized RBL cells (1 × 10^6 cells/mL + 1 µg/mL IgE) were plated overnight in 48-well culture plates (250 µL/well). Prior to an experiment, the cells were washed 3× with BSA/BSS and stimulated at ~20°C for the indicated times with 1 µg/mL DNP-BSA, lysed by sodium dodecyl sulfate (SDS) sample buffer (10% glycerol, 0.05 M Tris, pH 6.8, 1% SDS, 0.1% bromophenol blue) addition, boiled for 5 min and centrifuged for 5 min at 13,000 × g. Equal numbers of cell equivalents of lysate (typically 3.5 × 10^3 cell equivalents) were electrophoresed on 12% nonreduced SDS polyacrylamide gels, transferred to BioTrace PVDF membranes (PALL Gelman Laboratory), and blotted with horseradish peroxidase-conjugated anti-phosphotyrosine (4G10-HRP, Millipore) diluted 1:10^4 in TBST (0.1% Tween-20 in 1× Tris-buffered solution (0.2 M Tris base, 1.4 M NaCl, 38 mM HCl, pH 7.6):stabilizer (50:50). Enhanced chemiluminescence (Pierce Biotechnology) was used to detect phosphorylated proteins and quantified from scanned blots using Un-Scan-It (Silk Scientific) and IgorPro (WaveMetrics) (Holowka et al., 2000; Sheets et al., 1999a).

2.3 Confocal fluorescence microscopy

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 numerical aperture (NA) water immersion objective was used for three-channel imaging (see Materials and methods sections in each Chapter for laser excitation wavelengths and filters used) (Fig. 2.1)
Figure 2.1. System for quantitative fluorescence microscopy and spectroscopy. The system is capable of fluorescence confocal and DIC imaging in conjunction with time-resolved or steady-state two-photon fluorescence microscopy, or time-resolved one-photon fluorescence microscopy (Heikal lab, Penn State, Bioengineering). M: mirror, FM: flip mirror, G: glass window, ND: neutral density filter, L: lens, PHD: fast photodiode, MCP: microchannel plate-photomultiplier tube (PMT), P: polarizer, BS: beam splitter, DM: dichroic mirror, AMP: amplifier, OBJ: microscope objective, PH: confocal pinhole, and GMS: galvanometer scanning mirrors (Ariola et al., 2006; Yu et al., 2008).
(Ariola et al., 2006; Davey et al., 2007; Yu et al., 2008). The scanner was also modified to allow two-photon (2P) laser scanning required for fluorescence lifetime imaging microscopy (2P-FLIM) applications (Fig. 2.1). DIC imaging was used to monitor cell viability before and after all experiments to ensure that no photodamage had occurred. Imaging and spectroscopy experiments were carried out at room temperature (~20°C).

2.4 Fluorescence lifetime imaging

2.4.1 Time-correlated single photon counting (TCSPC)

All of the time-resolved fluorescence spectroscopy methods detailed herein are based on the principle of TCSPC (Fig. 2.2). In TCSPC-based techniques, a fluorophore ensemble is excited by a high repetition rate (e.g., 4.2 MHz or 76 MHz), ultrafast (e.g., femtosecond) pulsed laser. The rising edge of the excitation pulse triggers a fast photodiode that generates an electronic signal pulse (start or sync pulse) to initiate a voltage ramp of the time-to-amplitude converter. In a given excitation-detection cycle, the first fluorescent photon detected stops the time-to-amplitude converter voltage ramp (hence stop pulse). Based on the arrival time of the fluorescence photon, the corresponding time-to-amplitude converter output voltage is stored in time channels via an analog-to-digital converter (Lakowicz, 1999) as sorted by a multichannel analyzer. During the data acquisition, excitation-detection (or start-stop) cycles are repeated and a probability histogram of photon counts per time channel, or fluorescence intensity decay, is built. The probability of detecting one photon per pulse is much less than one due to low light intensity, resulting from high repetition rate excitation pulses (Lakowicz, 1999). After a single excitation pulse, only the first arriving photon is registered (hence single photon counting), therefore the accuracy of the intensity decay is bolstered by using high
Figure 2.2. Principle of time-correlated single photon counting and its relationship with fluorescence lifetime. A femtosecond laser excitation pulse triggers a fast photodiode, or constant-fraction discriminator (CFD), to provide a start signal to the time-to-amplitude converter (TAC). When the first emitted photon arrives at the detector, the TAC receives a stop signal. The TAC converts the voltage that is proportional to the time between the stop and start signals into units of time using an analog-to-digital converter (not shown). A multichannel analyzer (not shown) sorts the time counts into bins, forming a probability histogram or fluorescence decay. For a given fluorescence event, only one photon is detected, and the time between the laser pulse arrival and the detection time is recorded as one count in the time bin corresponding to that time interval (x-axis), as indicated by the gray bars (ps–ns timescale). Here, the y-axis represents the probability that a photon will be counted for a given time bin. When the fluorescence intensity has decayed to 1/e of its original value (indicated by the uppermost, horizontal dashed line), the lifetime of the fluorescent species (τ) is defined with respect to the excitation pulse time. Figure courtesy of Ahmed A. Heikal.
speed detectors such as microchannel plate-photomultiplier tubes that function as multiple, separate detectors (Lakowicz, 1999).

2.4.2 Time-resolved fluorescence lifetime measurements

2.4.2.1 2P-photon fluorescence lifetime imaging microscopy (2P-FLIM)

A femtosecond laser system (~120 fs, 76 MHz), consisting of a solid-state Ti:sapphire laser (Coherent Mira 900F) pumped with a 10 W diode laser (Coherent Verdi), provided 700–1000 nm excitation (Fig. 2.1). The laser beam 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 (see Materials and methods sections in each Chapter for filters used) prior to detection using microchannel plates (Hamamatsu R3809U-50). For 2P-FLIM, measured at the magic angle (54.7°), the epi-fluorescence polarization was analyzed using a Glan-Thompson polarizer before the detector. The signal was amplified and fluorescence lifetime images were constructed with a TCSPC module (Becker & Hickl SPC830) (Ariola et al., 2006). The module was synchronized using a fast photodiode, which was triggered with ~5% of the laser pulses. 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, without photodamage or substantial cell movement.

2.4.2.2 Single point and pseudo-single point fluorescence lifetime

Complementary one-photon (1P) time-resolved fluorescence measurements using 1024 time channels with 16 ps/channel were carried out to enhance the time resolution and signal-to-noise ratio as described previously (Ariola et al., 2006; Davey et al., 2007).
In this case, 1P-laser pulses were generated (480 nm, 4.2 MHz) using a pulse picker (Coherent Mira 9200) and second harmonic generation (Coherent SHG-4500), and the laser beam was steered toward the sample via the microscope rear exit port and strategically positioned at areas of interest on the cell membrane, without scanning. Single point fluorescence decays were then measured by a single detector set to the magic angle (54.7°). In some instances, single-cell averaging measurements using 2P-FLIM 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, with all photons acquired to form a single fluorescence decay. In either case, the time-resolved fluorescence decays were constructed using the SPC830 module.

2.4.2.3 Fluorescence resonance energy transfer (FRET)

With sufficient spectral overlap between the emission of one fluorescent species (donor) and the excitation of another fluorescent species (acceptor), the two fluorophores can be a donor-acceptor pair for FRET. In the presence of acceptor, the donor fluorescence decays more quickly than it would in the absence of acceptor as a result of energy transfer. For FRET studies, single point or pseudo-single point fluorescence decays (see Section 2.4.1.2) were obtained for dually-labeled cells, but the emission was filtered such that only the donor species contributed to the detected signal. Donor decays in the presence of acceptor were compared with donor decays obtained from singly-labeled cells. As a control, cells labeled only with the acceptor species were measured under the same conditions to ensure that no acceptor emission could be detected.

2.4.3 Theory and data analysis

The fluorescence lifetime of a fluorophore provides information about its molecular structure and the surrounding local environment (Hess et al., 2003; Lakowicz,
1999). 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 $(\tau_i)$ and amplitudes $(\alpha_i)$ as follows (Ariola et al., 2006; Yu et al., 2008):

$$I_{\text{sa}}(t; x, y) = \sum_{i=1}^{3} \alpha_i(x, y) \exp\left[-t / \tau_i(x, y)\right].$$  

(2.1)

These fluorescence decays are measured at the magic angle to eliminate any rotational effects on the observed excited-state dynamics. The magic-angle fluorescence decays were analyzed by a non-linear, least-squares fitting routine with deconvolution of the system response function (full-width at half-maximum, FWHM ~60 ps) in the SPCImage software package (Becker & Hickl). The response function was generated by SPCImage based on the rise time of the measured decay, and the average fluorescence lifetime was calculated as $\tau_{\mu} = \sum_i \alpha_i \tau_i / \sum_i \alpha_i$. The residual and reduced chi square ($\tilde{\chi}^2 = \chi^2 / d$, where $\chi^2 = \sum_i (y - y_i / \sigma_i)^2$, $y$ is the original data value for the point, $y_i$ is the fitting function at a given point, $\sigma_i$ is the standard error for the point and $d = \text{degrees of freedom}$, i.e., the number of fitting parameters) were used to assess the goodness of fit, with $\tilde{\chi}^2 = 1.0–1.3$ considered satisfactory. Unpaired, two-tailed Student’s $t$-tests were performed using Excel (Microsoft) to determine whether variations in fluorescence decay parameters as a function of cross-linking (suspended cells) or antigen stimulation (adherent cells) were statistically significant ($p \leq 0.05$). For lifetime imaging experiments, the error of the average lifetime value for a given 2P-FLIM image was determined by exporting the total image histogram from SPCImage and fitting the histogram to a Gaussian with IgorPro to obtain the width, or standard deviation.
The fluorescence quantum yield (\(\Phi_\beta\)) and excited-state fluorescence decay rate (\(k_\beta = 1/\tau_\beta\)) of a given fluorophore depend on the radiative (\(k_r\)) and non-radiative (\(k_{nr}\)) rates, following \(\Phi_\beta = k_r / k_\beta = k_r / (k_r + k_{nr})\). Thus, considering the complexity of cellular environments, various non-radiative pathways are likely to compete with fluorescence emission of a given fluorophore. For example, when a fluorophore is incorporated into a lipid membrane, its excited-state lifetime is affected by the overall membrane organization or order (Owen et al., 2006), lipid compositional diversity (heterogeneity), lipid polarity and solvent-fluorophore interactions. Isomerization, which explains the enhanced fluorescence properties of diI-C_{18} upon restriction in an organic phase (i.e., the membrane), is a primary example of such a pathway. As a result, the nature of the fluorescence decays enables us to quantify the excited-state dynamics and assign the competing non-radiative pathways present under given environmental conditions.

Time-resolved FRET allows for probing the lateral interactions between fluorescent molecules within 0.1–10 nm, well below the diffraction-limited resolution (\(\sim \lambda / 2\)) inherent in optical microscopy (Lagerholm et al., 2005; Navratil et al., 2006; Suhling et al., 2005). The energy transfer efficiency, \(E_T\), is defined as \(E_T = 1 - \left(\tau_{DA} / \tau_D\right)\), where \(\tau_{DA}\) is the donor lifetime in the presence of acceptor and \(\tau_D\) is the donor lifetime in the absence of acceptor, both of which are obtained from time-resolved fluorescence decays (Lakowicz, 1999). Using the energy transfer efficiency combined with the spectral overlap, the donor-acceptor separation distance, \(R\), was determined following \(R = R_0 \left[\left(1/E_T\right) - 1\right]^{1/6}\), where \(R_0\) (the Förster distance in nm) is calculated as \(R_0 = 0.0211 \left[\kappa^2 J(\lambda) n^{-4} Q_D\right]^{1/6}\) (Lakowicz, 1999). The Förster distance is the distance be-
tween the donor and acceptor at which the FRET efficiency is 50% (Lakowicz, 1999). The orientation parameter, $\kappa^2$, describes the relative donor-acceptor dipole orientations and has an estimated value of $2/3$ for randomly distributed fluorophores (Lakowicz, 1999), which may not be a valid assumption in a highly organized membrane environment. Accordingly, we used time-resolved fluorescence polarization anisotropy to examine the validity of both donor and acceptor dipole moment randomization. $J(\lambda)$, the spectral overlap between the donor emission and acceptor absorption, is expressed as

$$J(\lambda) = \int e_A(\lambda) F_D(\lambda) d\lambda,$$

where $e_A$ is the acceptor extinction coefficient (0.0148 M$^{-1}$ nm$^{-1}$), and $F_D$ is the donor fluorescence emission intensity as a fraction of the total integrated intensity. $n$ is the medium refractive index and $Q_D$ is the donor quantum yield. Further, the energy transfer rate, $k_{\text{FRET}}$ (in ns$^{-1}$), is given by

$$k_{\text{FRET}} = \left( \frac{1}{Q_D} - 1 \right)^{-1},$$

(Lakowicz, 1999).

2.5 Fluorescence polarization anisotropy imaging

2.5.1 Steady-state anisotropy imaging

2.5.1.1 1P-anisotropy imaging via wide-field fluorescence microscopy

A multimodal, inverted microscope (Nikon TE2000U) capable of simultaneous DIC and wide-field fluorescence was used to image steady-state anisotropy (Kyoung et al., 2007; Sheets lab, Penn State, Chemistry) and is described briefly in Fig. 2.3. Excitation was provided by a vertically polarized (via a custom-mounted polarizer) mercury arc lamp and emission collected by a Nikon 40×, 0.8 NA water immersion objective. Excitation and emission filterwheels were controlled by a MAC5000 controller (Ludl Electronic Products). A Dual-View (Optical Insights) aperture imaging
Figure 2.3. Microscope system for 1P-anisotropy imaging via wide-field fluorescence. This multimodal system is based on a Nikon TE2000U inverted microscope, capable of both wide-field and epi-fluorescence for microscopy and spectroscopy applications (Sheets Lab, Penn State, Chemistry). Because epi-fluorescence was not used for the steady-state anisotropy imaging experiments discussed in this Dissertation, the laser excitation pathways are not shown in detail, but are discussed in Appendix B, as is a second detection pathway. For anisotropy imaging, wide-field fluorescence from a mercury (Hg) arc lamp is polarized before reaching the sample and the emission is collected by the objective. Excitation and emission filter wheels provide spectral control. After leaving the objective via a dichroic mirror, the emission passes through the emission filter wheel to the Dual-View aperture imaging device, which allows separation, and simultaneous imaging, of parallel and perpendicular emission on the CCD detector. The polarization images were used to calculate anisotropy images using ISee Imaging software (PC 1), as described in Section 2.5.3. BS: beam splitter, DM: dichroic mirror, FW: filter wheel, M: mirror, P: polarizer. Figure is adapted from Kyoung et al (2007).
device, placed between the microscope and the charge coupled device (CCD) detector (Photometrics CoolSnap HQ), enabled parallel and perpendicular fluorescence emission separation into two spatially identical, but spectrally distinct, images of the sample on the CCD. Following simultaneous imaging, the two, non-overlapping polarization images were separated and used to calculate a single anisotropy image (see Section 2.5.3). ISee Imaging software (ISee Imaging) was used for data acquisition and analysis.

2.5.1.2 1P-anisotropy imaging via confocal fluorescence microscopy

For steady-state anisotropy imaging, filters were placed in front of each of two detectors to select fluorescence polarized either parallel (0°) or perpendicular (90°) to the excitation polarization. The corresponding 1P-steady-state polarization images were recorded simultaneously in two-channel, laser scanning mode by the FluoView300 module and the anisotropy images were calculated using MATLAB (The Mathworks) (see Section 2.5.3 below).

2.5.1.3 2P-anisotropy imaging

2P-polarization images were acquired in the same way as 2P-FLIM images (see Section 2.4.2.1), except that a 50/50 beamsplitter or polarized beam cube is placed in the emission pathway, such that the epi-fluorescence polarization (parallel and perpendicular relative to the excitation laser) can be analyzed using a Glan-Thompson polarizer before each of two detector channels, rather than a single channel. The 2P-polarization images were constructed using the SPC830 module and anisotropy images were calculated using MATLAB (see Section 2.5.3 below).
2.5.2 Single point and pseudo-single point time-resolved fluorescence anisotropy measurements

Time-resolved fluorescence anisotropy measurements were performed similarly to time-resolved fluorescence lifetime measurements (see Section 2.4.2.2), with 1P-laser pulses (480 nm, 4.2 MHz) positioned on regions of interest on the cells (single point) or 2P-laser pulses (960 nm, 4.2 MHz) scanned over the entire cell (pseudo-single point). However, two polarized fluorescence decays were simultaneously acquired in separate detectors, with parallel and perpendicular polarization selection, as described above for 2P-anisotropy imaging (see Section 2.5.1.3).

2.5.3 Theory and data analysis

Anisotropy \((r)\) is the measure of the extent of polarized fluorescence emission after photoselective excitation of fluorescent molecules with linearly polarized light. Following photoselective fluorophore excitation at pixel coordinates \((x,y)\) in a cell, the time-resolved fluorescence polarization anisotropy, \(r(t; x, y)\), can be described as (Ariola et al., 2006; Yu et al., 2008):

\[
\begin{align*}
\frac{1}{I_{\parallel}(t; x, y) - I_{\parallel}^0} \left( I_{\perp}(t; x, y) - I_{\perp}^0 \right) - G \left( I_{\parallel}(t; x, y) - I_{\parallel}^0 \right) \\
\frac{1}{I_{\parallel}(t; x, y) - I_{\parallel}^0} \left( I_{\perp}(t; x, y) - I_{\perp}^0 \right) + 2G \left( I_{\perp}(t; x, y) - I_{\perp}^0 \right),
\end{align*}
\]

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

(2.2)

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). Fluorophores preferentially absorb photons whose electron vectors are aligned parallel to the absorption transition dipole moments of the fluorophore, such that the anisotropy reflects any angular
displacement between the fluorophore absorption and emission dipoles during the excited-state lifetime, yielding a measure of the local environment surrounding the fluorophore (i.e., rotation, viscosity, etc.). The denominator in Eq. 2.2 is equivalent to the magic-angle fluorescence decay, but with the advantages of direct correlation between fluorophore excited-state and rotational dynamics at the same cellular location and minimal cellular photodamage. For co-linear (zero angular displacement) absorption and emission dipoles resulting from single photon excitation of multiple fluorophores in isotropic solution, $r$ has a maximum theoretical value of 0.4 or 0.57 for 1P- and 2P-excitation, respectively (Lakowicz, 1999). In our experiments, the measured initial anisotropies were always lower than the projected theoretical values, which implies a non-zero inter-dipole angle between the absorbing and emitting dipoles (Gryczynski et al., 1995; Lakowicz, 1999; Volkmer et al., 2000). In fact, the 1P-initial anisotropies of diI-C$_{18}$ ($r_0 = 0.381$, as calculated from a polarization of 0.480, measured by Axelrod (1979)) and Alexa 488 ($r_0 = 0.376$, as measured by Rusinova et al (2002)) in highly ordered environments (encapsulated in Sylgard 182 silicone elastomer and 100% glycerol, respectively) suggest that, while the absorption and emission dipoles are not completely parallel, these fluorophores have the structural range to report on the order of their surroundings.

To account for the polarization dependence of the detection efficiency (Lakowicz, 1999), the $G$-factor was calculated for 2P-steady-state anisotropy imaging (and for 1P-time-resolved anisotropy measurements) using the tail matching approach (Hess et al., 2003; Lakowicz, 1999) for free fluorescein (Sigma) in water, Alexa Fluor 488 (A488) (Invitrogen) in water, or diI-C$_{18}$ in DMSO. For 1P-steady-state anisotropy imaging, the
$G$-factor was estimated, using reference fluorophores, by comparative measurements of steady-state anisotropy carried out in a spectrofluorimeter with polarization capabilities (HORIBA Jobin Yvon Fluorolog FL3-21; Keating lab, Penn State, Chemistry) and on our confocal microscope. In these control experiments, free fluorophore molecules (~1 µM) in solution (e.g., green fluorescent protein (GFP), DsRed, rhodamine green or rhodamine 6G in water) and in viscous media (e.g., rhodamine green or rhodamine 6G in 100% glycerol) were excited by either 488 nm or 543 nm excitation. As an example, we obtained steady-state anisotropies of 0.31 ± 0.01 and 0.30 ± 0.01 for 1 µM GFP in water using spectrofluorimetry and confocal microscopy, respectively, which agree with Rocheleau et al. (2003). Depolarization due to the high NA objective was thus determined to be negligible when using an underfilled objective, as is the case for 1P-measurements here (Davey et al., 2007). Similar calibrations were used to determine the $G$-factor for steady-state anisotropy measurements based on wide-field microscopy (see Section 2.5.1.1).

Using MATLAB as a platform, we developed a custom image processing algorithm to calculate steady-state initial anisotropy images ($r_0(t;x,y)$) (Ariola et al., 2006; Davey et al., 2007) from simultaneously recorded parallel and perpendicularly polarized fluorescence images. The error in the average initial anisotropy value generated for a given image was determined by the same method described for 2P-FLIM images above.

During the excited-state lifetime, a fluorophore undergoes rotational diffusion, such that the rotational correlation time ($\phi_j$) is sensitive to fluorophore hydrodynamic volume and the surrounding viscosity (Lakowicz, 1999). The pre-exponential factor
(\beta_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 (Lakowicz, 1999). In some cases, \( \phi_j \) approaches infinity so that \( \beta_j \) is referred to as the residual anisotropy, \( r_\infty \), which is due to hindered fluorophore rotation and is present at times that are long compared to the probe fluorescence lifetime (Lakowicz, 1999). The degree of fluorophore orientational constraint is defined by the ratio, \( r_\infty / r_0 \), which describes its angular rotation range and is related to the order parameter, \( S \), with \( S = (r_\infty / r_0)^{1/2} \) (Kinosita et al., 1982; Kinosita et al., 1977).

For single point time-resolved fluorescence polarization anisotropy, non-linear least-squares fitting (Eq. 2.2) of these decays was carried out using either OriginPro 7 (OriginLab) or IgorPro without deconvolution of the system response function. Because the anisotropy decays were calculated from time-resolved parallel and perpendicular fluorescence decays, such deconvolution is rather difficult. As an alternative approach, the first few time channels from the zero-time of these calculated decays were omitted and the fit was started beyond the FWHM (~60 ps) of the system response function. The fit goodness was evaluated using chi-square (\( \chi^2 \)) calculated by OriginPro 7 or IgorPro, \( \chi^2 = \sum_i (y_i - y_i / \sigma_i)^2 \), where \( y_i \), \( y_i \), and \( \sigma_i \) are as previously described). In this case, decays fit with \( \chi^2 = 0–0.01 \) were considered satisfactory. Unpaired, one-tailed or two-tailed Student’s \( t \)-tests were performed using Excel (Microsoft) to determine whether variations in fluorescence anisotropy decay parameters as a function of cross-linking (suspended cells) or antigen stimulation (adherent cells) were statistically significant (\( p \leq 0.05 \)).
2.6 Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

ToF-SIMS was conducted in collaboration with Paul Piehowski, under the supervision of Professor Nicholas Winograd (Penn State, Chemistry), and is included here for a complete picture of the studies that contribute to understanding the role of RBL mast cell membrane structure in IgE receptor signaling. The Winograd group carried out experiments and performed data analysis, whereas we provided the biological system and question, and facilitated data interpretation. A further description of this work will be discussed in Paul Piehowski’s Dissertation.

2.6.1 Cryogenic preparation of suspended mast cells

Cells were prepared as described above (Section 2.2.1) and in Davey et al. (2007). Instead of pipetting cells onto a glass coverslip and covering with a glass slide, the cells were diluted 3-fold (to $1 \times 10^6$ cells/mL) and pipetted onto silicon shards ($\sim 0.5$ cm $\times$ 0.5 cm) and incubated for 10 min at 37°C to promote cellular adhesion to the substrates. The shards were rinsed for 5 s in water and a second silicon shard placed on top. The sandwich structure was immediately plunge-frozen in liquid ethane to minimize structural and chemical reorganization due to ice crystallization and stored under liquid nitrogen until analysis. The sample shards were maintained at liquid nitrogen temperatures and introduced into the ultrahigh vacuum environment of the ToF-SIMS spectrometer (Kratos Prism) (Piehowski et al., 2008). After fracturing the sample sandwich to remove the top shard, leaving cells covered with ice, the samples were heated quickly (5°C/min, from approximately –200°C to –80°C) to remove excess water with minimal crystallization and then returned to liquid nitrogen temperatures at the sample stage (Kore Tech. Ltd.) (Piehowski et al., 2008). Finally, a water layer was redeposited onto the sample in
vacuum below −100°C to increase ionization while maintaining spatial resolution. This method, referred to as freeze-etching, enhances biomolecule sputter yields and produces a uniform analysis surface (as assessed by lipid film controls (Piehowski et al., 2008)) without adversely affecting cell morphology or molecular distribution.

2.6.2 Mass spectrometry

A ToF-SIMS spectrometer, equipped with an In⁺ liquid metal primary ion source (FEI), was used to obtain mass spectrometry images. The ion beam was focused to an ~350 nm diameter and directed at the analysis surface, providing 1 nA of current in 50 ns pulses (Piehowski et al., 2008) (the shorter the pulse, the higher the mass resolution (Sodhi, 2004)). A mass spectrum was acquired for every pixel by raster-scanning the pulsed primary ion beam across the sample stage. The sputtered secondary ions were collected by an extraction lens, directed along the flight path of the ToF mass analyzer and detected at a microchannel plate detector (Galileo Co.) (Piehowski et al., 2008). Mass specific images were created by selecting a mass peak from the total ion mass spectrum and plotting the intensity of that mass at every image pixel via in-house imaging software written in Visual C++ (Piehowski et al., 2008). Intensity images were pseudo-colored for display and signal intensities compared among various mass images using a line scan function of the software. As a control, mass spectra of total lipid extracts from RBL cells were acquired on a custom-built ToF-SIMS spectrometer equipped with a pulsed (50 ns) C₆₀⁺ primary ion source (IOG 40-60, Ionoptika) (Zheng et al., 2008). Because the Winograd group has not yet successfully used C₆₀⁺ primary ions to resolve subcellular lipid distributions in single, cryogenically prepared mammalian cells, the In⁺ source was used instead.
2.6.3 Theory and data analysis

In ToF-SIMS imaging, it is important to determine whether one image pixel is statistically significantly different from the next image pixel (Piehowski et al., 2008). Thus, to determine the useful lateral resolution of the measurements and therefore the appropriate binning amount in mass spectrometry image analysis, Poisson statistics were used to model the imaging data. Specifically, a z-score was determined as a function of the lateral resolution limit for various lipid ionization efficiencies while holding the change in lipid concentration constant at 100%, or as a function of percent change in lipid concentration for various lipid ionization efficiencies, while holding the ToF-SIMS image pixel size constant at 1.2 μm. A z-score expresses the divergence of an experimental result, \(X\), from the most probable result, \(\mu\), as a number of standard deviations, \(\sigma\), following 
\[ z = \frac{(X - \mu)}{\sigma} \] (Koosis, 1997). For example, three standard deviations provides ~95% confidence that pixels are different in terms of lipid concentration and do not simply vary due to counting statistics. So, given the ionization efficiency of a particular lipid species, the concentration difference (or lateral resolution limit) required to conclude that the concentration is significantly different between two pixels with 95% confidence can be determined.

In some cases, the significance of linear relationships between various membrane components was quantitated using Pearson’s correlation test. The correlation coefficient for a sample, \(P\), is equal to 
\[ P = \frac{\sum_{i=1}^{n} z_{ai}z_{bi}}{n - 1} \] (McKillup, 2006), where \(z\) is calculated as described above, and \(a\) and \(b\) represent the two components under comparison. \(P\) ranges from –1 to 1, with –1 denoting a perfect negative linear relationship (i.e., anti-correla-
tion), 0 indicating no relationship and 1 signifying a perfect positive linear relationship (i.e., correlation) between variables (McKillup, 2006).
3. VISUALIZING LOCALIZED STRUCTURAL ORDERING AND DYNAMICS IN MODEL MEMBRANES

This Chapter describes the development of an ultrafast fluorescence dynamics imaging assay that includes fluorescence lifetime and anisotropy, for probing the order and dynamics of model lipid membranes. These studies are compared with lifetime and anisotropy methods described in the literature and provide a fundamental basis for the interpretation of studies of the role of the cell membrane structure in immunoreceptor signaling.

3.1 Introduction

Since the introduction of the Singer and Nicolson fluid mosaic model (1972), biological membranes have been observed to be more heterogeneous in structure and lipid species than originally envisioned (Edidin, 2003; Jacobson et al., 1995; Vereb et al., 2003). The lipids comprising these membranes are typically characterized by their relative acyl chain order. Gel ($L_{\beta}$) phase lipids possess rather rigid acyl chains, while the acyl chains of fluid or liquid-disordered ($L_{d}$) phase lipids are marked by higher levels of unsaturation and freedom of motion. An intermediary level of order is found in liquid-ordered ($L_{o}$) phase lipids. Structural heterogeneities, such as cholesterol-rich domains within the plasma membrane, are thought to exist in the $L_{o}$ phase and have been hypothesized to play important cell signaling and membrane trafficking roles (Anderson and Jacobson, 2002; Brown and London, 1998; Jacobson et al., 2007; Sengupta et al., 2007a; Simons and Ikonen, 1997). These domains are proposed to be enriched in saturated phospholipids, sphingolipids, and cholesterol, as compared with the bulk plasma membrane, which is relatively enriched in unsaturated phospholipids. The combination of
long, tightly packed, saturated sphingolipid acyl chains and cholesterol results in a higher melting temperature and resistance to solubilization in non-ionic detergents at low temperature. Consequently, domains have been isolated as detergent-resistant membranes.

Much controversy still exists over domain size and shape, and whether domains are transient or exist for a fixed lifetime. Detergent-resistant membranes, which are isolated from cells, unfortunately do not accurately model domain organization in intact cellular membranes, considering that cellular lipids exist in much greater diversity, and are affected by their protein content. While it has been shown that aggregates of many domains can be detected with high-resolution confocal microscopy (Vereb et al., 2003), noncaveolar domains have not yet been observed individually in living cells. Often, these larger domains cannot be observed in resting cells and can be seen only upon cross-linking membrane proteins with antibodies or toxins (Harder et al., 1998). Yet, it is still unclear how macroscopic phase separation is related to domain formation in molecularly complex, crowded biomembranes of the cell. Thus, the long-term goal of my research has been to develop an approach to observe specialized domains and relate domain dynamics to their biological function (in our case, IgE receptor signaling). Fluorescence lifetime can provide insight into membrane organization and composition. In addition, fluorescence anisotropy is useful for this purpose because it can not only give an initial overall snapshot of lipid order, but it can also reveal information about lipid rotational motions that are exquisitely sensitive to environmental rigidity (Lakowicz, 1999). Thus, these quantitative fluorescence microscopy methods are ideal probes of membrane structure, and therefore biological function.
In this Chapter, we describe the development of time-resolved fluorescence lifetime, steady-state anisotropy imaging and time-resolved fluorescence anisotropy as tools for identifying localized structural ordering in biomimic lipid membranes, specifically small unilamellar vesicles (SUVs) and supported planar bilayers labeled with the phospholipid analog 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE). Although NBD-DPPE was not subsequently used in the cell membrane studies (Chapter 4 and 6), it was initially chosen for its well-characterized sensitivity to lipid composition and ordering in model membranes (Mesquita et al., 2000), and for direct comparison with previous spectrofluorimetric lifetime and anisotropy measurements by other researchers (Araiso and Koyama, 1995; Gidwani et al., 2001). In addition, various researchers have used NBD-DPPE to fluorescently label the \( L_o \) phase (Dietrich et al., 2001a; Gidwani et al., 2001; Mesquita et al., 2000). Our ultrafast excited-state fluorescence dynamics imaging methods offer the advantage of spatial resolution and higher temporal resolution than existing spectrofluorimetry-based lifetime and anisotropy assays.

3.2 Materials and methods

3.2.1 SUV and planar supported lipid bilayer preparation

Biomimic planar lipid bilayers are easily prepared with lamellar-preferring lipids via the vesicle fusion method (Kyoung et al., 2007; Thompson and Palmer, 1988; Vats et al., 2008) and can also contain various proteins or have different lipid compositions at each leaflet to mimic natural asymmetry (Sinha et al., 2003). Due to their planar geometry, these supported bilayers are amenable to surface techniques such as quantitative microscopy and are commonly used as model systems for studying lateral and rotational
diffusion (Chan and Boxer, 2007; Crane and Tamm, 2007). SUV and planar supported lipid bilayer (e.g., 99.5 mol% DPPC or POPC:0.5 mol% NBD-DPPE) preparation is detailed in Chapter 2. For spectrofluorimetric measurements, SUVs (2 mM lipid) were pipetted into a plastic cuvette, rather than pipetted between a glass sandwich to form a planar lipid bilayer. Lipid species and fluorescent lipid analog structures are shown in Fig. 3.1.

3.2.2 1P-time-resolved fluorescence lifetime

1P-single point time-resolved fluorescence lifetime experiments were performed as described in Chapter 2, with 480 nm excitation and filtering to isolate scattered light (BGG22, Chroma). Fluorescence lifetime describes the characteristic time that a molecule remains in an excited state prior to returning to the ground state and is modified by environmentally-dependent, non-radiative processes during the excited-state lifetime that compete with the fluorescence emission process (Lakowicz, 1999). For fluorophore molecules in a lipid membrane, lifetime is affected by the overall membrane organization, lipid compositional diversity, as well as interactions with solvent surrounding or adjacent to the molecules (Williams and Stubbs, 1988). By fitting a fluorescence intensity decay over time to a single exponential or multi-exponential variation of Eq. 2.1, the characteristic lifetime(s) ($\tau_i$) can be determined. Fluorescein (1 µM in PBS) and 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoic acid (NBD, 10 µM in water) solutions were used as lifetime measurement controls.

3.2.3 1P-steady-state anisotropy imaging

Steady-state anisotropy imaging was set up and optimized on a multimodal wide-field fluorescence microscope (Kyoung et al., 2007; Vats et al., 2008), which involved
Figure 3.1. Lipid species and fluorescent lipid analog used for model membrane studies. Structures of DPPC, $L_\beta$ phase lipid (A), POPC, $L_d$ phase lipid (B), and NBD-DPPE, head-group labeled fluorescent phospholipid analog (C).
installing and aligning a Dual-View aperture imaging device between the microscope and CCD detector, as described in Chapter 2. The $G$-factor was estimated as 1.16 (see Section 2.5.3), and experiments were performed at $\sim 20^\circ \text{C}$. NBD-DPPE-labeled SUVs and planar lipid bilayers were excited at 485 nm (485DF15) by a vertically polarized mercury arc lamp and emission collected using a Nikon 40×, 0.8 NA water-immersion objective via a 505DRLP/510ALP dichroic mirror. Anisotropy values are expected to be near the maximum for highly ordered $L_\beta$ phase lipids, and approaching the minimum for more fluid lipids (Sinha et al., 2003). Comparative spectrofluorimetric measurements were carried out (at $\sim 20^\circ \text{C}$) using a polarization capable HORIBA Jobin Yvon Fluorolog FL3-21 fluorimeter (Keating lab, Penn State, Chemistry).

### 3.2.4 1P-time-resolved anisotropy

1P-single point time-resolved anisotropy measurements were performed as described in Chapter 2, with 480 nm excitation and filtering to isolate scattered light (BGG22, Chroma). The $G$-factor was calculated as 1.01 (see Section 2.5.3), and experiments were performed at $\sim 20^\circ \text{C}$. By fitting a time-resolved anisotropy decay to a single exponential or multi-exponential version of Eq. 2.2, the rotational correlation time(s) ($\phi_j$) associated with a sample can be derived. $\beta_j$ represents the fractional amplitudes related to each $\phi_j$. The wobbling-in-cone angle, $\theta_w$, which describes the angular range of rotational motion is calculated as

$$
\beta_J \left(1 + \cos \theta_w \right) = \left[0.5 \cos \theta_w \left(1 + \cos \theta_c \right) \right]^2
$$

(Hess et al., 2003). A single exponential anisotropy decay is characteristic of a spherical molecule, such as a free dye molecule rotating in solution, while a more complicated, multi-exponential anisotropy decay is expected for non-spherical molecules, or those that are somehow restricted, such as a fluorescent molecule embedded in a lipid bilayer.
(Lakowicz, 1999). Here also, fluorescein (1 µM in PBS) and free NBD (10 µM in water) solutions were used as controls.

3.3 Results and discussion

3.3.1 Fluorescence lifetime reports on the phase-specific environment of NBD-DPPE in planar bilayers

Fig. 3.2 shows representative fluorescence lifetime decays for NBD-DPPE-labeled \( L_\beta \) phase DPPC and \( L_d \) phase POPC bilayers, as well as corresponding solution controls, with a summary of key parameters obtained from fitting lifetime decays shown in Table 3.1. These simple lipid compositions were used as a proof-of-concept that fluorescence lifetime, and the anisotropy techniques described below, are sensitive to phase. The bilayer lifetime decays were fit to a biexponential, giving \( \tau_1 \) and \( \tau_2 \) values indicative of a fluorophore existing in an environment with some level of heterogeneity. \( \alpha_1 \) and \( \alpha_2 \), the decay component amplitudes at \( t = 0 \), represent the fractional fluorophore amount in each environment (Lakowicz, 1999). The longer lifetimes observed for DPPC bilayers (\( \tau_1 = 2.39 \pm 0.05 \) ns \( [p = 0.031] \) and \( \tau_2 = 8.51 \pm 0.04 \) ns \( [p = 0.018] \), with \( \tau_{\beta} = 6.24 \pm 0.03 \) ns \( [p = 0.022] \)) versus POPC bilayers (\( \tau_1 = 1.80 \pm 0.03 \) ns and \( \tau_2 = 7.28 \pm 0.02 \) ns, with \( \tau_{\beta} = 5.52 \pm 0.01 \) ns) can be explained by increased interactions between the NBD moiety and the glycerol backbone of well-packed \( L_\beta \) phase DPPC. Conversely, NBD-DPPE in POPC experiences increased interactions with the solvent, reducing the overall lifetimes. The two-component fluorescence lifetime of NBD-DPPE in supported bilayer compositions indicates some level of environmental heterogeneity, whereas the
Figure 3.2. Representative time-resolved fluorescence decays of NBD-DPPE in various supported lipid bilayer environments with comparative solution measurements. The biexponential decays of NBD-DPPE in $L_{\beta}$ phase (99.5 mol% DPPC:0.5 mol% NBD-DPPE) and $L_{d}$ phase (99.5 mol% POPC:0.5 mol% NBD-DPPE) bilayer compositions are designated by curves (1) and (2), respectively. Single exponential lifetime decays of NBD and fluorescein aqueous solutions are respectively labeled as curves (3) and (4). The fluorescence lifetime of membrane-associated molecules is affected by the overall membrane organization, lipid compositional diversity, and interactions with the solvent surrounding or adjacent to the molecules. As a result, restricted fluorophores have longer, more complex lifetimes than the corresponding fluorophore in solution.
Table 3.1. Fluorescence lifetime of NBD-DPPE in DPPC and POPC bilayers\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Supported bilayers</th>
<th>$\tau_1$/ns</th>
<th>$\alpha_1$\textsuperscript{b}</th>
<th>$\tau_2$/ns</th>
<th>$\alpha_2$</th>
<th>$\tau_\beta$/ns</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.5 mol% DPPC:</td>
<td>2.39(5)</td>
<td>0.371(9)</td>
<td>8.51(4)</td>
<td>0.629(7)</td>
<td>6.24(3)</td>
<td>9</td>
</tr>
<tr>
<td>0.5 mol % NBD-DPPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99.5 mol% POPC:</td>
<td>1.80(3)\textsuperscript{c}</td>
<td>0.321(4)</td>
<td>7.28(2)\textsuperscript{c}</td>
<td>0.679(3)</td>
<td>5.52(1)\textsuperscript{c}</td>
<td>9</td>
</tr>
<tr>
<td>0.5 mol % NBD-DPPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Free markers in solution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescein (PBS\textsuperscript{d})</td>
<td>3.915(2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.915(2)</td>
<td>3</td>
</tr>
<tr>
<td>NBD (water\textsuperscript{d})</td>
<td>3.075(3)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.075(3)</td>
<td>3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Experiments were conducted over three different days and decays were fit according to Eq. 2.1. Averaged results over the three experiments are shown with propagated standard deviation (± SD). \textsuperscript{b} $I_{547} = \alpha_1 + \alpha_2$, normalized to unity after fitting. \textsuperscript{c} $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests, indicating a statistically significant difference in the means of POPC versus DPPC bilayers. \textsuperscript{d} The pH of PBS is 7.4, and the pH of water is ~5–5.5.
one-component lifetime of NBD is indicative of a free fluorophore in solution. Agreeing with this trend of increasing fluorescence lifetime with increasing lipid order, Araiso and Koyama (1995) previously performed spectrofluorimetric time-resolved lifetime measurements on various SUV solutions and analogously found that the average fluorescence lifetime ($\tau_\beta$) was longer for $L_\beta$ phase DPPC, as compared to $L_d$ phase 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC).

### 3.3.2 Steady-state anisotropy imaging of NBD-DPPE measures differences in phase and curvature

1P-anisotropy imaging via wide-field fluorescence microscopy was used to compare the steady-state anisotropy of $L_\beta$ phase (DPPC) and $L_d$ phase (POPC) supported planar bilayers and SUVs, labeled with fluorescent lipid analog (NBD-DPPE), analogous to the SUV compositions used in spectrofluorimetric studies by Gidwani et al. (2001). Anisotropy imaging was calibrated using a GFP solution standard, with steady-state anisotropy near the theoretical limit of 0.4, as described in Chapter 2. Table 3.2 contains representative steady-state bilayer anisotropy data obtained using this calibration method and proper background correction. At room temperature, $L_\beta$ phase DPPC exhibits higher anisotropy ($r_0 = 0.371 \pm 0.004$ [$p = 6.51 \times 10^{-9}$]) than $L_d$ phase POPC ($r_0 = 0.347 \pm 0.002$) because headgroup-labeled NBD-DPPE is more confined, and thus dynamically restricted, upon association with $L_\beta$ phase lipids. The average anisotropy that we measured for DPPC vesicles with 0.5 mol % NBD-DPPE agrees with Gidwani et al. (2001), and is likely lower than the anisotropy measured for supported bilayer of the same composition due to the high degree of SUV curvature, and therefore reduced lipid packing or order.
Table 3.2. Steady-state anisotropy of NBD-DPPE in DPPC and POPC bilayers and SUVs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( r_0 )^a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Supported bilayers</strong></td>
<td></td>
</tr>
<tr>
<td>99.5 mol% DPPC:0.5 mol % NBD-DPPE</td>
<td>0.371(4)</td>
</tr>
<tr>
<td>99.5 mol% POPC:0.5 mol % NBD-DPPE</td>
<td>0.347(2)^b</td>
</tr>
<tr>
<td><strong>Vesicles (SUVs)</strong></td>
<td></td>
</tr>
<tr>
<td>99.5 mol% DPPC:0.5 mol % NBD-DPPE</td>
<td>0.15(2)</td>
</tr>
<tr>
<td>99.5 mol% DPPC:0.5 mol % NBD-DPPE^c</td>
<td>0.15(2)</td>
</tr>
<tr>
<td>99.5 mol% POPC:0.5 mol % NBD-DPPE</td>
<td>0.10(2)^b</td>
</tr>
<tr>
<td><strong>Buffer only</strong>^d</td>
<td>0.01(1)</td>
</tr>
</tbody>
</table>

^a (± SD), \( n = 10 \) for each sample.  ^b \( p < 0.05 \), as determined from unpaired, two-tailed Student’s \( t \)-tests, indicating a statistically significant difference in the means of POPC versus DPPC bilayers or SUVs.  ^c Taken from spectrofluorimetry data acquired by Gidwani et al. (2001) at 37°C.  ^d Buffer only is 50 mM Tris, 100 mM NaCl, pH 7.4.
Using spectrofluorimetry, Gidwani et al. (2001) similarly observed that NBD-DPPE-labeled labeled $L_\beta$ phase DPPC (37°C) exhibited higher steady-state anisotropy than NBD-DPPE-labeled $L_d$ phase DPPC (45°C).

### 3.3.3 Time-resolved anisotropy reveals multiple phase-dependent modes of NBD-DPPE rotational diffusion in planar bilayers

While steady-state anisotropy imaging is a powerful method for obtaining a static picture of overall lipid order, it cannot resolve dynamic depolarizations caused by lipid molecule rotational motions on the picosecond to nanosecond timescale. Fig. 3.3 shows representative anisotropy decays obtained for $L_\beta$ phase DPPC and $L_d$ phase POPC bilayers, as well as solution controls. The key parameters extracted from fitting anisotropy decays are summarized in Table 3.3.

Initial anisotropy ($r_0$) is substantially greater for the DPPC $L_\beta$ phase bilayer composition ($r_0 = 0.322 \pm 0.003 \ [p = 0.032]$), as compared to the POPC $L_d$ phase composition ($r_0 = 0.289 \pm 0.005$), consistent with our hypothesis. The fluorescein rotational correlation time ($\phi_1$) is near the expected value (~150 ps) (Hess et al., 2003) and is slightly greater than that of NBD (MW 294 Da), which may be partially due to the somewhat larger molecular weight of fluorescein (376 Da) (Lakowicz, 1999). DPPC exhibits faster $\phi_1$ (0.41 ± 0.04 ns [$p = 0.026$]) than POPC ($\phi_1 = 0.56 \pm 0.04$ ns), indicating restricted internal or segmental NBD-DPPE motion within the more confining $L_\beta$ phase DPPC environment. However, $\phi_2$ is slower for DPPC (5.6 ± 0.2 ns [$p = 0.024$]) than POPC ($\phi_2 = 4.5 \pm 0.2$ ns), due to the slower overall rotational motion or diffusion of
Figure 3.3. Representative time-resolved fluorescence anisotropy decays of NBD-DPPE in various supported lipid bilayer environments with comparative solution measurements. The biexponential decays of NBD-DPPE in $L_{\beta}$ phase (99.5 mol% DPPC:0.5 mol% NBD-DPPE) and $L_{d}$ phase (99.5 mol% POPC:0.5 mol% NBD-DPPE) bilayer compositions are labeled as (1) and (2), respectively. Single exponential anisotropy decays of NBD and fluorescein aqueous solutions are designated respectively by (3) and (4). Fluorophores in solution exhibit a rapid monoexponential decay to zero (indicated by the horizontal line), while lipid bilayer-associated fluorophore shows a rapid, picosecond decay component ($\phi_1$) due to internal segmental motion and a slower, nanosecond decay component ($\phi_2$) from overall rotational diffusion throughout the bilayer environment.
Table 3.3. Time-resolved anisotropy of NBD-DPPE in DPPC and POPC bilayers.

<table>
<thead>
<tr>
<th>Supported bilayers</th>
<th>$\phi_1$/ns</th>
<th>$\beta_1$</th>
<th>$\phi_2$/ns</th>
<th>$\beta_2$</th>
<th>$r_0^b$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.5 mol% DPPC:</td>
<td>0.41(4)</td>
<td>0.053(3)</td>
<td>5.6(2)</td>
<td>0.107(1)</td>
<td>0.322(3)</td>
<td>1</td>
</tr>
<tr>
<td>0.5 mol % NBD-DPPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>99.5 mol% POPC:</td>
<td>0.56(4)c</td>
<td>0.100(4)</td>
<td>4.5(2)c</td>
<td>0.156(3)</td>
<td>0.289(5)c</td>
<td>1</td>
</tr>
<tr>
<td>0.5 mol % NBD-DPPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Free markers in solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescein (PBSd)</td>
<td>0.143(7)</td>
<td>0.204(7)</td>
<td>–</td>
<td>–</td>
<td>0.204(7)</td>
<td>4</td>
</tr>
<tr>
<td>NBD (waterd)</td>
<td>0.108(6)</td>
<td>0.152(5)</td>
<td>–</td>
<td>–</td>
<td>0.164(5)</td>
<td>4</td>
</tr>
</tbody>
</table>

a Experiments were conducted over three different days and decays were fit according to Eq. 2.2. Averaged results over the three experiments are shown with propagated standard deviation ($\pm$ SD).

b $r_0 = \beta_1 + \beta_2$ for fluorescein and $r_0 = y_0 + \beta_1 + \beta_2$ for all other samples (Hess et al., 2003).

c $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests, indicating a statistically significant difference in the means of POPC bilayers versus DPPC bilayers.

d The pH of PBS is 7.4, and the pH of water is ~5–5.5.
NBD-DPPE in the $L_\beta$ phase environment of DPPC. The wobbling-in-cone angle of the fluorescent moiety of NBD-DPPE, $\theta_c$, is calculated as $\sim 29 \pm 1^\circ$ and $\sim 32 \pm 0.5^\circ$ in DPPC and POPC, respectively, further suggesting that NBD-DPPE rotation is somewhat more restricted in a $L_\beta$ phase lipid environment. Araiso and Koyama (1995) previously performed ensemble spectrofluorimetric time-resolved anisotropy measurements (at 25°C) on $L_\beta$ phase (DPPC) and $L_d$ phase (DMPC) SUVs labeled with 1 mol% NBD-DPPE, similarly finding that DPPC, as compared to $L_d$ phase DMPC, SUVs exhibited higher initial anisotropy (0.17 versus 0.14) and slower rotational correlation time (7 ns versus 2.5 ns). In contrast to their work, our approach offers the advantage of location-specific, spatio-temporal dynamics information.

3.4 Conclusions

These single-composition model bilayer experiments represent initial technique development for monitoring lipid order and dynamics photophysically via a phospholipid analog (i.e., NBD-DPPE), demonstrate feasibility and offer chemically and spatially well-defined models. Chapter 4 describes analogous studies of the fluorescent probe diI-C$_{18}$ in $L_d$ phase and more biologically relevant $L_\alpha$ phase giant unilamellar vesicle (GUV) model membranes. These controls allow us to interpret how the diI-C$_{18}$ dynamics reflect the surrounding membrane environment in subsequent cell membrane studies. Because the fluorescence lifetime and rotational mobility of a molecule are exquisitely sensitive to the membrane phase, fluorescence lifetime and anisotropy methods allow us to identify local heterogeneities or cholesterol-rich domains in biological systems and determine their dynamics within biomembranes as a function of IgE-FcεRI cross-linking under both non-physiological (Chapter 4) and physiological (Chapter 6) conditions.
4. MOLECULAR DYNAMICS PERSPECTIVE OF MEMBRANE DOMAINS ASSOCIATED WITH EXTENSIVE IGE RECEPTOR CROSS-LINKING IN RBL MAST CELLS: NON-PHYSIOLOGICAL CONDITIONS

This Chapter describes the use of ultrafast excited-state fluorescence dynamics, including lifetime and anisotropy, to assess the structure and dynamics of the mast cell plasma membrane as a function of extensive IgE receptor crosslinking, in which micron-sized membrane domains are formed. These studies verify the recruitment of lipid molecules into more ordered domains that serve as platforms for IgE-mediated signaling and serve as a foundation for analogous studies performed under more physiological signaling conditions. The work detailed in this Chapter was published, in part, as Davey et al., “Membrane order and molecular dynamics associated with IgE receptor cross-linking in mast cells”, Biophysical Journal 92:343–355 (2007).

4.1 Introduction

Cholesterol-rich microdomains within the plasma membrane have been implicated in essential cell functions such as signaling and membrane trafficking (Edidin, 2003; Mukherjee and Maxfield, 2004; Simons and Vaz, 2004) and are proposed to exist in a liquid-ordered ($L_o$) phase that is enriched in saturated phospholipids and sphingolipids (Simons and Vaz, 2004). However, non-caveolar cholesterol domains have not been directly observed in vivo without substantial perturbation (e.g., long term incubation in the cold or extensive cross-linking of cholesterol-rich microdomain markers) (Holowka et al., 2000; Sheets et al., 1999a; Thomas et al., 1994; Vereb et al., 2003); thus, much controversy about their size, shape, lifetime and molecular composition remains. In model membrane systems such as giant unilamellar vesicles
(Feigenson and Buboltz, 2001; Veatch and Keller, 2002) and supported lipid bilayers and monolayers (Crane and Tamm, 2004; Dietrich et al., 2001a; Dietrich et al., 2001b; Stottrup et al., 2004), \( L_0\) has been widely visualized and characterized, but these models lack the full complexity and reactivity associated with the plasma membrane of living cells.

A variety of spectroscopic and microscopic techniques have been used to investigate lipid microdomain structure and dynamics in both model and cellular systems. Spectrofluorimetry (Gidwani et al., 2001), single point dynamics with tens of nanoseconds temporal resolution (Sinha et al., 2003), ESR (Ge et al., 1999; Ge et al., 2003) and nuclear magnetic resonance (NMR) (Veatch et al., 2004) spectroscopies have provided valuable insights into the structural order of SUVs, GUVs and isolated detergent-resistant membranes extracted from various cell types. Nevertheless, these ensemble measurements offer either no location-specific dynamics or no spatio-temporal information at the single-cell level. For single cell experiments, FRAP (Thomas et al., 1994), FCS (Bacia et al., 2004), and SPT (Dietrich et al., 2002; Sheets et al., 1997) have been used to analyze lipid microdomain biophysical properties. These translational diffusion-based techniques are limited by optical diffraction and ensemble averaging (FRAP and FCS) or by probe valency (SPT), as well as by the timescale (\(\mu s\text{–s}\)) associated with translational diffusion. In contrast as described in Chapter 3, fluorescence lifetime and rotational diffusion approaches are exquisitely sensitive to molecular structure (i.e., organization and conformation) and rigidity of the surroundings (Harms et al., 1999; Lakowicz, 1999) and may provide insight into the short-range dynamics (ps-ns timescale) and molecular order of the membrane nanostructure. This molecular-level environmental
sensitivity provides an opportunity to bypass the diffraction limit of the intensity-dependent measurements typically used to characterize cholesterol-rich membrane domains in vivo.

In this Chapter, we investigate specialized cholesterol-rich domains in individual RBL-2H3 mast cells and probe the influence of cross-linking-induced changes on membrane nanostructure, and therefore biological function. Our integrated biophotonics techniques include confocal and 2P-fluorescence microscopies, 2P-FLIM and fluorescence polarization anisotropy, with complementary 1P-measurements. When the high affinity immunoreceptor for IgE (FcεRI) on mast cells and basophils is cross-linked with multivalent antigen, it is phosphorylated by the Src family tyrosine kinase, Lyn, in a cholesterol-dependent manner (Holowka et al., 2005; Sheets et al., 1999a). We are able to visualize large-scale IgE-FcεRI domains when these complexes are cross-linked with secondary antibody (α-IgE) in the cold (Holowka et al., 2000; Sheets et al., 1999a; Thomas et al., 1994). Although this extensive cross-linking represents a gross, non-physiological perturbation, it permits clear comparison of cross-linked and uncross-linked cells and ease in identifying regions of interest for single point measurements. Thus, these conditions establish a fundamental basis for more physiological studies, while allowing correlation between dynamic membrane nanostructure and the location of specific signaling molecules (i.e., IgE-FcεRI). Table 4.1 outlines the various experimental controls that will be discussed in this Chapter.
Table 4.1. Experimental controls performed on variously labeled suspended RBL cells, with and without IgE-FcεRI cross-linking by α-IgE.

<table>
<thead>
<tr>
<th>fluorescent molecule monitored, treatment</th>
<th>FLIM</th>
<th>single point lifetime</th>
<th>1P-anisotropy imaging</th>
<th>2P-anisotropy imaging</th>
<th>single point anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>diI-C18, – α-IgE</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>diI-C18, + α-IgE</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>A488-IgE, – α-IgE</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>A488-IgE, + α-IgE</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>diI-C18 + A488-IgE, – α-IgE</td>
<td>not shown</td>
<td>–</td>
<td>not shown</td>
<td>not shown</td>
<td>–</td>
</tr>
<tr>
<td>diI-C18 + A488-IgE, + α-IgE</td>
<td>not shown</td>
<td>–</td>
<td>not shown</td>
<td>not shown</td>
<td>–</td>
</tr>
</tbody>
</table>

a X designates the experimental controls that are described in this Chapter.
4.2 Materials and methods

4.2.1 Cell preparation

Suspended mast cell preparation is detailed in Section 2.2.1. Cells dually labeled with diI-C$_{18}$ and A488-IgE were used for steady-state confocal imaging. For all other experiments, cells were fluorescently labeled with either diI-C$_{18}$ (in the presence of unlabeled IgE for possible cross-linking) or A488-IgE. For solution experiments, A488-IgE was diluted to \(~1\) µM in phosphate-buffered saline (PBS, 10 mM Na$_2$HPO$_4$, pH 7.4, 150 mM NaCl) and diI-C$_{18}$ to \(~10\) µM in DMSO. (Other complementary solution controls included free A488 diluted in water to \(~10\) µM and fluorescein diluted in water or PBS to \(~1\) µM.)

4.2.2 Confocal fluorescence microscopy

Three-channel detection confocal microscopy (~20°C, see Section 2.3) was used to image A488-IgE via 488 nm argon ion laser excitation and a 525/30 bandpass filter and diI-C$_{18}$ via 543 nm HeNe laser excitation and a 605/70 bandpass filter. Unlabeled RBL cells showed negligible autofluorescence (data not shown). DIC imaging was simultaneously used to monitor cell viability before and after all measurements.

4.2.3 Fluorescence lifetime imaging

Excited-state dynamics were used to reveal the effect of IgE cross-linking on local membrane nanostructure and protein-protein interactions in RBL cells, as probed by the lipid analog diI-C$_{18}$ and A488-IgE, respectively. 2P-fluorescence and lifetime imaging were performed as described in Section 2.4.2.1, with the fluorescence signal filtered (BGG22, Chroma) to isolate further scattered laser light. For 2P-FLIM of RBL cells, both A488-IgE and diI-C$_{18}$ were excited at 760 nm, based on the 2P-excitation spectra of
these dyes (data not shown and Xu et al., 1996). At this wavelength, autofluorescence from unlabeled RBL cells was negligible. To further eliminate the possibility of autofluorescence, we also conducted additional 2P-FLIM experiments at 960 nm excitation to avoid intrinsic autofluorescence from NADH and FAD, and comparable results for both A488-IgE and diI-C\textsubscript{18} were obtained. 2P-FLIM images were recorded in a single detector channel (set at the magic angle, 54.7°) at both the equatorial plane and the top cell surface. Experiments at 760 nm were performed over several days on different cell preparations that had \((n = 12\) cells for A488-IgE, 6 cells for diI-C\textsubscript{18}) or had not \((n = 12\) cells for A488-IgE, 12 cells for diI-C\textsubscript{18}) been incubated with α-IgE. The fluorescence decays per pixel in 2P-FLIM images measured at magic angle were analyzed as described in Section 2.4.3.

To enhance the signal-to-noise ratio and temporal resolution, we also conducted complementary single point measurements (i.e., the laser was parked without scanning, see Section 2.4.2.2). Because the fluorescent labels lack perfect symmetry centers in (diI-C\textsubscript{18}) or on (A488-IgE) the membrane, the first excited state can be excited with either one or two photons, thus our comparative 1P-single point time-resolved lifetime and 2P-FLIM measurements are likely equivalent. For all single point experiments, a uniformly labeled location on the cell equator of uncross-linked cells was chosen, whereas the beam was positioned on an equatorial membrane patch for cross-linked cells. Single point time-resolved lifetime experiments were performed, on three separate days, on cells that had \((n = 21\) cells for diI-C\textsubscript{18}, 19 cells for A488-IgE) or had not \((n = 23\) cells for diI-C\textsubscript{18}, 18 cells for A488-IgE) been incubated with α-IgE. The single point fluorescence decays measured at magic angle were analyzed as described in Section 2.4.3.
ed unpaired, two-tailed Student’s *t*-tests using Excel to assess whether the variation in fluorescence decay parameters observed for diI-C\textsubscript{18} and A488-IgE upon cross-linking was statistically significant (*p* ≤ 0.05).

In some cases, the single point fluorescence decays were calculated from simultaneously measured parallel and perpendicularly polarized time-resolved fluorescence decays (because the denominator of Eq. 2.2 is equal to the magic angle fluorescence decay). Non-linear least-squares fitting of these calculated decays were carried out using either OriginPro 7 or IgorPro without deconvolution of the system response function, particularly when the decay time constants were larger than the FWHM of the response function (i.e., for A488-IgE). The fit goodness was evaluated using \( \chi^2 \) calculated by OriginPro 7 or IgorPro, with \( \chi^2 = 0-0.01 \) considered satisfactory. Comparative lifetime measurements of diI-C\textsubscript{18} in cells and various free fluorophore solutions (data not shown) indicated negligible time constant differences using both techniques (with or without deconvolution).

### 4.2.4 Fluorescence polarization anisotropy imaging

To better understand the observed dependence of excited-state dynamics on membrane nanostructure, we performed 1P- and 2P-steady-state and time-resolved fluorescence anisotropy measurements as described respectively in Sections 2.5.1.2, 2.5.1.3 and 2.5.2. The *G*-factor was estimated as 0.96 for 1P-steady-state confocal anisotropy imaging, and determined as 0.7 for complementary 2P-steady state anisotropy imaging and single point time-resolved anisotropy measurements. 1P-steady-state anisotropy images of diI-C\textsubscript{18} were acquired at the equatorial plane and at the top cell surface, over four individual days on cells that had \((n = 27\) cells) or had not \((n = 20)\) been
incubated with $\alpha$-IgE, and analyzed with the custom MATLAB program detailed in Section 2.5.3.

Complementary single point time-resolved anisotropy experiments were performed with high signal-to-noise, on three separate days, on cells that had ($n = 11$ cells for diI-C$_{18}$, 12 cells for A488-IgE) or had not ($n = 11$ cells for diI-C$_{18}$, 13 cells for A488-IgE) been incubated with $\alpha$-IgE. The theory and analysis for single point time-resolved fluorescence polarization anisotropy measurements is also specified in Section 2.5.3. Our a priori expectation was that membrane anisotropy (as probed by diI-C$_{18}$) and the anisotropy of A488-IgE would increase upon IgE receptor cross-linking, so unpaired, one-tailed Student’s $t$-tests were performed in Excel on all anisotropy decay fit parameters to determine whether the mean values were significantly different ($p \leq 0.05$) for cross-linked versus uncross-linked cells.

4.3 Results and discussion

The so-called raft hypothesis, which attributes functional roles in signaling and trafficking to cholesterol-rich domains, has remained controversial, in part due to the difficulty of observing domains in vivo without substantial non-physiological perturbations (e.g., low temperatures or extensive cross-linking). The elusive nature of these domains is likely due to lipid-lipid and lipid-protein interactions that are short-lived and thermodynamically unstable in the unstimulated state and to the possible transient domain coalescence to nucleate functional domains upon receptor engagement (e.g., antigen-induced IgE-FceRI cross-linking) and allow signal initiation. Baird, Holowka and co-workers have investigated FceRI phosphorylation by the tyrosine kinase, Lyn, which occurs upon IgE-FceRI cross-linking by antigen (Sheets et al., 1999a). Their
studies have demonstrated that such phosphorylation proceeds in a cholesterol-dependent manner (Sheets et al., 1999a), which is due to the protective nature of cholesterol against tyrosine phosphatases (Young et al., 2003). As a first step toward our goal of following the association of IgE receptor and other fluorescently labeled signaling molecules with changes in membrane nanostructure as signaling proceeds, we used RBL mast cells that were extensively cross-linked with α-IgE to form micron-sized patches or domains. Although these conditions are non-physiological, this system allows for testing whether lifetime and anisotropy changes correlate with the formation and location of these receptor-containing domains.

4.3.1 Confocal microscopy reveals IgE cross-linking-induced co-redistribution of a lipid analog with IgE receptor in RBL cells

Under resting conditions, A488-IgE-FcεRI complexes are preferentially localized on the plasma membrane of live RBL mast cells, which is labeled with diI-C18 (Fig. 4.1). We used appropriate excitation wavelengths and emission filters to prevent crosstalk between the two imaging channels (A488 and diI-C18). When IgE-sensitized RBL mast cells are extensively cross-linked with α-IgE for long periods in the cold, we observe the formation of large IgE-FcεRI domains (Fig. 4.1D). The majority of the lipid analog diI-C18 (our lipid microdomain marker) co-redistributes with IgE-FcεRI into these well-defined patches (Fig. 4.1E), as observed in previous studies (Sheets et al., 1999a; Thomas et al., 1994). The patches are several microns in size and markedly different from the more uniform distributions of IgE-FcεRI (Fig. 4.1A) and diI-C18 (Fig. 4.1B) without cross-linking via secondary antibody. Upon cross-linking-induced domain formation, fluorescence intensity is enhanced. These results agree with prior work on co-
Figure 4.1. Confocal fluorescence imaging and steady-state fluorescence anisotropy imaging of RBL-2H3 cells. dil-C$_{18}$ ($\lambda_{ex} = 543$ nm) in the plasma membrane (B and E) colocalizes with A488-IgE ($\lambda_{ex} = 488$ nm) on the cell surface (A and D) upon extensive cross-linking by $\alpha$-IgE. Steady-state anisotropy images of dil-C$_{18}$ (C and F) are shown in the absence or presence of $\alpha$-IgE, respectively. Higher anisotropy, or membrane order, increases toward the red end of the colorscale. Bar, 5 $\mu$m.
redistribution of a variety of raft markers such as gangliosides (Pierini et al., 1996; Sheets et al., 1999a), GPI-anchored proteins (Holowka et al., 2000), kinases (Sheets et al., 1999a) and lipid analogs (Thomas et al., 1994) with IgE-FcεRI.

These steady-state images, nevertheless, lack information concerning the molecular dynamics associated with membrane nanostructural changes in the vicinity of specific signaling molecules (e.g., IgE-FcεRI). Furthermore, the observed fluorescence intensity enhancement at cross-linking-induced patches can be attributed to either increased local fluorophore concentration upon cross-linking and/or enhanced fluorescence quantum yield due to changes in fluorophore immediate surroundings. To understand the underlying structural bases of the observed fluorescence enhancement, we conducted 2P-FLIM where the excited-state fluorescence lifetime is independent of fluorophore concentration. In addition, we carried out polarization anisotropy imaging to visualize the orientation distribution of the diI-C18 dipole moment in the plasma membrane. These noninvasive techniques that are best-suited for short-scale dynamics (both in space and time) increase our chances for real-time monitoring of these fleeting microdomains in vivo.

4.3.2 Ultrafast excited-state dynamics report environmental changes with increased lifetime of diI-C18 and A488-IgE upon IgE receptor cross-linking

Fluorescence lifetime (i.e., quantum yield) is sensitive to conformational changes in both fluorophore molecular structure and surrounding environment. We exploited this property to probe potential membrane nanostructural differences as a function of receptor cross-linking. 2P-FLIM ($\lambda_{\text{ex}} = 760$ and 960 nm, 76 MHz) was performed with high spatial and temporal resolution on RBL cells labeled with diI-C18 or A488-IgE-FcεRI, in
the presence and absence of extensive cross-linking with α-IgE. To our knowledge, this work represents the first application of 2P-FLIM to IgE-FcεRI association with cholesterol-rich membrane domains. To overcome the inherently low signal-to-noise (256 × 256 pixels) and temporal resolution of 2P-FLIM decays (65 time channels, 195 ps/channel), we performed complementary single point lifetime experiments (1024 time channels, 16 ps/channel), using 1P-excitation (λ_{ex} = 480 nm, 4.2 MHz), in which the laser was focused on an equatorial region of interest. The relative size of the membrane patches in cross-linked cells and the 1P-excitation using an underfilled 1.2 NA objective makes it challenging to directly compare 1P- and 2P-FLIM results; however, we observe the same trend in our results with both excitation types.

Upon IgE cross-linking, 2P-FLIM reveals bright equatorial patches of either diI-C_{18} or A488-IgE-FcεRI (Fig. 4.2), similar to our confocal images. Corresponding DIC images (Fig. 4.2A, C, E and G) were captured to examine cell morphology and viability after 2P-FLIM. The fluorescence decays per pixel in these FLIM images were fit to a triexponential for diI-C_{18} and a biexponential for A488-IgE labeled cells, revealing longer fluorescence lifetimes for both probes in FcεRI cross-linked cells (Fig. 4.2F and H, respectively), as compared with uncross-linked cells (Fig. 4.2B and D). Representative data from the selected areas (noted by the arrows in Fig. 4.2) were acquired per pixel and the average lifetime, \(\tau_p\), was calculated.

The measured average diI-C_{18} lifetime is 0.71 ± 0.02 ns (area highlighted by an arrow in Fig. 4.2H) and 0.57 ± 0.03 ns (highlighted area in Fig. 4.2D) in the presence and absence of α-IgE, respectively. Fig. 4.2J shows average lifetime distribution histograms for diI-C_{18} in the cells depicted in Fig. 4.2H and D. For cross-linked cells (curve 3, Fig.
Figure 4.2. 2P-FLIM of RBL-2H3 cells. Representative 2P-FLIM images of dil-C18 (D and H) and A488-IgE (B and F) in the plasma membrane of cells that have been cross-linked (F and H) with α-IgE or not (B and D) (λex = 760 nm). The average lifetime, τfl, is indicated by the colorscale. Fitting the lifetime decays at the pixels (binning = 1) highlighted by arrows, yields the following average lifetimes: A488-IgE (– α-IgE) = 1.14 ± 0.09 ns; A488-IgE (+ α-IgE) = 1.34 ± 0.03 ns; dil-C18 (– α-IgE) = 0.57 ± 0.04 ns; and dil-C18 (+ α-IgE) = 0.71 ± 0.03 ns. Corresponding DIC images (A, C, E and G) show cell viability after 2P-FLIM. Histograms demonstrate increased dil-C18 (J) and A488-IgE (I) average lifetime distributions for cross-linked (curves 1 and 3) versus uncross-linked cells (curves 2 and 4). Free fluorophore solutions decay as a single exponential with average lifetimes of 0.43 ± 0.04 ns (n = 3) for 10 µM dil-C18 in DMSO and 3.1 ± 0.2 ns (n = 3) for 10 µM A488 in water (images not shown). Bar, 5 µm.
the average lifetime increases as compared to uncross-linked cells (curve 4, Fig. 4.2J [histogram peak at ~0.50 ns]). Recently, using 1P-FLIM (128 × 128 pixels and 180 s acquisition, with $\lambda_{ex} = 473$ nm), Owen et al. (2006) measured a lower lifetime distribution for the fluorescent probe, di-4-ANEPPDHQ, in $L_d$ phase (DOPC) large unilamellar vesicles, as compared with $L_o$ phase (egg n-palmitoyl-sphingomyelin:cholesterol, 0.7:0.3, mol/mol) vesicles. They also observed decreased probe lifetime and reduction of ordered phase in HEK293 cells upon temperature increase and cholesterol depletion. Together, our results and those of Owen et al. (2006) suggest that the higher diI-C18 fluorescence lifetime that we observe upon IgE cross-linking is likely a result of the constrained probe moving into more ordered regions in the mast cell plasma membrane. Our results further indicate that this redistribution may cause increased diI-C18 fluorescence lifetime (or quantum yield), and thus increased fluorescence intensity, as we observed with confocal imaging (Fig. 4.1). As a result, we conclude that cross-linking-induced membrane nanostructural changes can be reported by 2P-FLIM.

A488-IgE-FcεRI FLIM images show cross-linking-induced equatorial patching and overall increased fluorescence lifetime as well (Fig. 4.2). The average A488-IgE-FcεRI lifetime in cross-linked cells is $1.34 \pm 0.07$ ns (area highlighted by an arrow in Fig. 4.2F), as compared with $1.14 \pm 0.03$ ns (highlighted area in Fig. 4.2B) for the monomeric receptor. The bimodal distribution observed for the average lifetime of A488-IgE on cross-linked cells (see Fig. 4.2F and curve 1, Fig. 4.2I [histogram peaks at ~0.90 ns and 1.35 ns]) indicates one A488-IgE subpopulation with considerably longer lifetime and a second population with a lifetime distribution similar to that of cells not cross-linked with secondary antibody (Fig. 4.2B and curve 2, Fig. 4.2I [histogram peak at ~0.85 ns]).
These differences demonstrate that changes resulting from protein-protein interactions can also be identified using FLIM and suggest that α-IgE, which is a polyclonal antibody with various affinities, does not saturate the available A488-IgE cell surface binding sites. Because FLIM is a concentration-independent measurement, the enhanced lifetimes observed for both probes report a change in fluorophore local environment, rather than a cross-linking-induced increase in local fluorophore concentration. The broad FWHM of the pixel histogram lifetime distributions indicate a heterogeneous environment of both the lipid and IgE labels in living cells. Measurements using 2P-FLIM with 960 nm excitation are comparable for both diI-C$_{18}$ and A488-IgE (Fig. 4.3), suggesting a negligible cellular autofluorescence contribution under our experimental conditions and minimal excitation wavelength dependence.

The excited state of the lipid analog diI-C$_{18}$, which preferentially partitions into the cross-linked IgE-FcεRI domains, experiences a longer average fluorescence lifetime in cross-linked cells ($\tau_f = 0.8 \pm 0.1$ ns, $p = 2.36 \times 10^{-5}$; Table 4.2), as compared with cells that do not undergo receptor cross-linking ($\tau_f = 0.60 \pm 0.04$ ns; Table 4.2). These average lifetime values closely agree with those obtained by 2P-FLIM of diI-C$_{18}$ labeled cells, with and without IgE-FcεRI cross-linking. In single point experiments, diI-C$_{18}$ fluorescence in cell decays as a triexponential, whether in the presence ($\tau_1 = 0.13 \pm 0.03$ ns [$p = 2.2 \times 10^{-4}$], $\alpha_1 = 0.45 \pm 0.03$ [$p = 1.0 \times 10^{-4}$], $\tau_2 = 0.75 \pm 0.08$ ns [$p = 2.2 \times 10^{-4}$], $\alpha_2 = 0.40 \pm 0.05$ [$p = 0.016$], $\tau_3 = 3.1 \pm 0.4$ ns and $\alpha_3 = 0.15 \pm 0.04$ [$p = 0.017$]) or absence of FcεRI cross-linking ($\tau_1 = 0.08 \pm 0.01$ ns, $\alpha_1 = 0.53 \pm 0.05$,
Figure 4.3. 2P-FLIM of RBL-2H3 cells to examine excitation wavelength dependence fluorescence lifetime. 2P-FLIM images were acquired with $\lambda_{ex} = 960$ nm, as a comparison to the 2P-FLIM images in Fig. 4.2 obtained with $\lambda_{ex} = 760$ nm. Representative 2P-FLIM images of diI-C$_{18}$ (B and D) and A488-IgE (A and C) in the plasma membrane of cells with (C and D) without (A and B) $\alpha$-IgE cross-linking. $n = 7$ (diI-C$_{18}$, – cross-linking), $n = 7$ (diI-C$_{18}$, + cross-linking), $n = 5$ (A488-IgE, – cross-linking), and $n = 8$ (A488-IgE, + cross-linking). Bar, 5 µm.
$\tau_2 = 0.62 \pm 0.05 \text{ ns}, \ \alpha_2 = 0.35 \pm 0.04, \ \tau_3 = 2.9 \pm 0.2 \text{ ns and } \alpha_3 = 0.12 \pm 0.02; \text{ Table } 4.2$). Fluorescence decays were also calculated from parallel and perpendicularly polarized fluorescence decays (following the denominator of Eq. 2.2) and fit without deconvolution, starting a few time channels beyond time zero. As a result, the shortest lifetime components ($\tau_1$) had lower amplitudes and the average lifetimes were consequently higher, while all time constants remained within standard deviation of the values obtained with deconvolution. For example, in $\alpha$-IgE treated cells ($n = 5$), diI-C$_{18}$ had an average lifetime of $1.3 \pm 0.1 \text{ ns } [p = 7.65 \times 10^{-3}]$, with $\tau_1 = 0.09 \pm 0.01 \text{ ns}, \ \alpha_1 = 0.29 \pm 0.06, \ \tau_2 = 0.92 \pm 0.08 \text{ ns } [p = 0.015], \ \alpha_2 = 0.50 \pm 0.03, \ \tau_3 = 3.7 \pm 0.4 \text{ ns } [p = 0.013] \text{ and } \alpha_3 = 0.21 \pm 0.06 [p = 0.017]$. In uncross-linked ($n = 6$), diI-C$_{18}$ again had a lower average lifetime of $0.98 \pm 0.09 \text{ ns}$, with $\tau_1 = 0.07 \pm 0.02 \text{ ns}, \ \alpha_1 = 0.32 \pm 0.07, \ \tau_2 = 0.7 \pm 0.1 \text{ ns}, \ \alpha_2 = 0.46 \pm 0.08, \ \tau_3 = 2.8 \pm 0.5 \text{ ns and } \alpha_3 = 0.22 \pm 0.06$. These complementary single point lifetime measurements enable us to directly correlate the observed excited-state dynamics of the diI-C$_{18}$ lipid and A488-IgE labels with their surrounding environment. As shown in Fig. 4.4A, the diI-C$_{18}$ magic angle fluorescence decays for these two cellular conditions are shaped differently, with diI-C$_{18}$ in cross-linked cells demonstrating a slower lifetime decay (Fig. 4.4A, curve 1) than in uncross-linked cells (Fig. 4.4A, curve 2). This observation suggests that the diI-C$_{18}$ lipid analog localizes to regions of differing membrane structure upon treatment with $\alpha$-IgE and that environmental constraint increases in cross-linking-induced domains. These single point lifetime measurements follow the same trend we observed in the FLIM experiments—
Table 4.2. Single point, time-resolved fluorescence lifetime of diI-C₁₈ and A488-IgE in RBL cells, with and without IgE-FcεRI cross-linking by α-IgE<sup>a</sup>.

<table>
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<th>τ₁/µs</th>
<th>α₁</th>
<th>τ₂/µs</th>
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<td>0.53(5)</td>
<td>0.62(5)</td>
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<td>2.9(2)</td>
<td>0.12(2)</td>
<td>0.60(4)</td>
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<td>0.45(3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.75(8)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.40(5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1(4)</td>
<td>0.15(4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8(1)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>− α-IgE</td>
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<td>0.19(2)</td>
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<td>0.52(2)</td>
<td>3.38(2)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>2.13(3)&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>λ<sub>ex</sub> = 480 nm, T ≈ 20°C. The fitting parameters represent an average over a number of cells (n) with the standard deviation in the last digit shown in parentheses to reflect the cell-to-cell variation of the individual fit parameters. All data shown, except for A488-IgE labeled cells, are from single point decays acquired at the magic angle, using deconvolution with the system response function (FWHM ~60 ps) as in 2P-FLIM experiments (see text). A comparison was made with the fitting parameters of fluorescence decays calculated from parallel and perpendicularly polarized fluorescence decays (see text). <sup>b</sup>p < 0.05, as determined from unpaired, two-tailed Student’s t-tests, indicating a statistically significant difference in the means as compared to cells in the absence of receptor cross-linking. <sup>c</sup>The pH of water is ~5–5.5, and the pH of PBS is 7.4.
Figure 4.4. Time-resolved fluorescence decays of diI-C<sub>18</sub> and A488-IgE in living RBL cells, under different conditions of IgE cross-linking. Representative single point fluorescence lifetime decays for diI-C<sub>18</sub> (A) in the plasma membrane of mast cells in the presence (1) or absence (2) of α-IgE; and A488-IgE (B) on the surface of mast cells with (3) or without (4) cross-linking ($\lambda_{ex} = 480$ nm for both diI-C<sub>18</sub> and A488-IgE). Triexponential diI-C<sub>18</sub> decays were measured at magic angle polarization and biexponential A488-IgE decays were calculated from the measured parallel and perpendicularly polarized fluorescence decays (following the denominator of Eq. 2.2). In the calculated magic angle fluorescence decays, the fitting was started beyond the FWHM of the system response function. All decays were fit following Eq. 2.1, and the fit parameters of time-resolved fluorescence decays are summarized in Table 4.2. Comparison between both lifetime methods (with and without deconvolution) is included in the text where appropriate. With samples having long excited-state lifetimes (i.e., A488), there are no significant differences in the fitting parameters with each method.
that is, lifetime increases for both dil-C18 and A488-IgE upon FcεRI cross-linking (Table 4.2). To validate the cross-linking-induced dil-C18 enhancement and the underlying mechanism of membrane ordering, we compared our cell results with time-resolved dil-C18 fluorescence in GUVs of various phases (F. Ariola, J. Heetderks, P.S. Weiss and A.A. Heikal, unpublished data). In Ld phase (DOPC) GUVs, dil-C18 fluorescence decays as a biexponential with $\tau_1 = 0.87 \pm 0.02$ ns, $\alpha_1 = 0.83 \pm 0.01$, $\tau_2 = 1.5 \pm 0.1$ ns, $\alpha_2 = 0.17 \pm 0.01$, and an estimated average lifetime of $0.97 \pm 0.01$ ns ($n = 5$). For dil-C18 in GUVs of $L_\omega$ composition (DPPC:cholesterol, 0.7:0.3, mol/mol) that is near the 2:1 ratio of phospholipid:cholesterol found in RBL plasma membranes (Gidwani et al., 2001), we measured an average lifetime of $1.22 \pm 0.07$ ns ($n = 5$). These results agree with previous studies by Packard and Wolf (1985), who reported increased dil-C18 average lifetime as lipid order increased, with 0.80 ns for dil-C18 in Ld phase (DOPC), 0.96 ns in egg phosphatidylcholine, 1.13 ns in $L_\beta$ phase (DPPC) and 1.54 ns in a $L_\omega$ composition (DPPC:cholesterol, 0.7:0.3, mol/mol). We also observed longer average lifetime of another head-group labeled phospholipid analog (NBD-DPPE) in $L_\beta$ phase (DPPC) versus Ld phase (POPC) planar lipid bilayers (see Chapter 3). The GUV results, in particular, support our argument that increased dil-C18 fluorescence lifetime in cross-linked mast cell membranes is due to greater lipid order (i.e., $\tau_\mu = 0.60 \pm 0.04$ ns in uncross-linked cells and $0.8 \pm 0.1$ ns in cells cross-linked with $\alpha$-IgE). Furthermore, the lifetime components of dil-C18 in either natural (Table 4.2) or model membranes are longer than the lifetime of free dil-C18 in DMSO, which only decays as a single exponential ($\tau_\mu = 0.61 \pm 0.03$ ns and $0.54 \pm 0.02$ ns from fitting measured and calculated fluorescence decays, respectively). When dil-C18 is intercalated within the membrane, fluorophore conforma-
tional changes become restricted due to steric hindrance and hydrogen bonding of neighboring lipid molecules (Ariola et al., 2006).

Similarly, fluorescence lifetime measurements were also conducted on A488-IgE labeled RBL mast cells for comparison. A488-IgE excited-state fluorescence lifetime increases when cells undergo IgE receptor cross-linking, as compared with uncross-linked conditions. The measured average lifetime for cross-linked A488-IgE-FcεRI is significantly larger ($\tau_{fl} = 1.67 \pm 0.04$ ns, $p = 0.004$; Table 4.2) when compared to monomeric IgE-FcεRI ($\tau_{fl} = 1.5 \pm 0.2$ ns; Table 4.2). A488-IgE fluorescence in cross-linked cells decays as a biexponential ($\tau_1 = 1.10 \pm 0.06$ ns [$p = 0.025$], $\alpha_1 = 0.77 \pm 0.01$ [$p = 6.49 \times 10^{-4}$], $\tau_2 = 3.6 \pm 0.1$ ns and $\alpha_2 = 0.23 \pm 0.01$ [$p = 6.10 \times 10^{-3}$]; Table 4.2), similar to A488-IgE fluorescence in the absence of cross-linking ($\tau_1 = 1.0 \pm 0.1$ ns, $\alpha_1 = 0.81 \pm 0.02$, $\tau_2 = 3.4 \pm 0.3$ ns and $\alpha_2 = 0.19 \pm 0.02$; Table 4.2). The overall profile of the lifetime decays (Fig. 4.4B) and the fitting parameters from single point time-resolved fluorescence measurements support the trend in 2P-FLIM experiments regarding the local diI-C18 and IgE environments in IgE-FcεRI cross-linked or uncross-linked cells. The longer fluorescence decays that occur when α-IgE is present are further substantiated by our time-resolved anisotropy results, as well as by polarized fluorescence depletion experiments ($\mu$s timescale) in which the lifetime of IgE on mast cells increased upon cross-linking (Rahman et al., 1992).

As a comparison, time-resolved fluorescence measurements on the fluorophores of interest were carried out in solution. As previously noted, the excited-state fluorescence of free diI-C18 (in DMSO) decays as a single exponential (Table 4.2) with an
average lifetime of 0.61 ± 0.03 ns. This ultrafast excited-state lifetime is consistent with the low dil-C18 fluorescence quantum yield in solution, and the non-radiative pathway for the excited-state depopulation of dil-C18 is likely to include isomerization (Windengren and Schwille, 2000). The fluorescence of free A488 (in water) decays as a single exponential with an average lifetime of 4.1 ± 0.2 ns (Table 4.2), which is longer than when the dye is covalently attached to IgE and similar in magnitude to the 3.75 ± 0.05 ns obtained by Agronskaia et al. (2003) who analyzed a 50 µM aqueous A488 solution with 2P-TCSPC ($\lambda_{ex} = 800$ nm). The longer excited-state lifetime of A488 in water (Table 4.2), as compared to when it is covalently linked to receptor-bound IgE, indicates either an active non-radiative pathway is available in the cellular environment of A488 or a greater stabilization of A488 excited state by the polar solvent molecules. The average A488-IgE lifetime in PBS (2.13 ± 0.03 ns) is appropriately shorter than free A488 in solution, yet significantly longer than A488-IgE-FcεRI in uncross-linked cells ($p = 0.016$). A clear assignment of the active non-radiative mechanism for A488 upon covalently modifying IgE is rather difficult to define due to the random A488 labeling of IgE. While previously published results of lifetime measurements on fluorescently-labeled, receptor-bound IgE (using comparable techniques) are not available, we hypothesize that the observed A488-IgE lifetime represents an average over a variety of dye environments that vary in both solvent accessibility and possible quenching by protein amino acids, whereas the A488 aqueous solution environment is much more homogeneous. To confirm the structural bases of the dil-C18 and A488-IgE fluorescence lifetime increases upon IgE cross-linking, we carried out time-resolved fluorescence anisotropy measurements.
4.3.3 Dipole-moment distribution and rotational diffusion report increased membrane order upon IgE receptor cross-linking

In previous fluorimetry experiments, Gidwani et al. (2001) measured the steady-state fluorescence anisotropy of plasma membrane blebs and detergent-resistant membranes isolated from RBL cells. In that study, both biomembrane systems were labeled with 2-[3-(diphenylhexatrienyl) propanoyl]-1-hexadecanoyl-sn-glycero-3-phosphocholine (DPH-PC) and revealed sensitivity to cholesterol-dependent membrane ordering. The plasma membrane blebs and lipids extracted from plasma membrane vesicles exhibited enhanced steady-state anisotropy (40% greater) compared to $L_d$ phase (POPC) vesicles and decreased steady-state anisotropy (60% less) compared to $L_d$ phase (DPPC:cholesterol, 0.66:0.33, mol/mol) vesicles (Gidwani et al., 2001). Using SUVs comprised of more simple lipids, we also observed higher steady-state anisotropy of a phospholipid analog (NBD-DPPE) in $L_{\beta}$ phase (DPPC), as compared to $L_d$ phase (POPC) (see Chapter 3). However, these cuvette-based experiments lack dynamics information, as well as spatial resolution, for describing signaling molecules in intact, living cells. To overcome these limitations, we used steady-state fluorescence anisotropy imaging to evaluate the spatial distribution of lipid, and protein, order. In addition, complementary single point time-resolved fluorescence anisotropy measurements were carried out using pulsed 1P-excitation ($\lambda_{ex} = 480$ nm, 4.2 MHz), and simultaneous parallel and perpendicularly polarized emission detection, on singly labeled cells to follow diluted-C$_{18}$ dynamics within the live RBL cell membrane and protein-protein interaction dynamics between A488-IgE-Fc$\varepsilon$RI and $\alpha$-IgE.
We used steady-state fluorescence anisotropy imaging to visualize local diI-C\textsubscript{18} (Figs. 4.1 and 4.5) and A488-IgE (Fig. 4.5) within the cell membrane of live mast cells, treated and untreated with $\alpha$-IgE. Such measurements enable us to further evaluate how the local plasma membrane nanostructure is altered upon IgE-FceRI cross-linking with secondary antibody and to provide a structural basis for the observed fluorescence lifetime increase. The confocal fluorescence images reveal diI-C\textsubscript{18} colocalization (Fig. 4.1E) with A488-IgE (Fig. 4.1D) in the presence of FcεRI cross-linking, without a striking visual difference in the average diI-C\textsubscript{18} anisotropy in cells with (Fig. 4.1F) or without (Fig. 4.1C) cross-linking. Cells with $\alpha$-IgE (e.g., Fig. 4.1F) exhibit the expected patching with a more homogeneous diI-C\textsubscript{18} anisotropy distribution within the IgE receptor domains. In contrast, the diI-C\textsubscript{18} anisotropy images of cells in the absence of $\alpha$-IgE (e.g., Fig. 4.1C) generally display more heterogeneity in fluorescent label dipole-moment orientation throughout the plasma membrane. Higher anisotropy was consistently observed at the domain perimeters, which may be a reflection of increased interfacial tension or barriers between the more ordered domains and the less ordered bulk plasma membrane. In contrast, NBD-DPPE phospholipid analog exhibits clearly higher, homogeneous average anisotropy in $L_b$ phase (DPPC) versus $L_d$ phase (POPC) planar lipid bilayer (see Chapter 3). As described in Chapter 2, to avoid artifacts due to polarization-biased detection sensitivity, we estimated the $G$-factor (0.96) from comparative steady-state anisotropy measurements of several free fluorescent molecules in solution and in viscous media performed in a spectrofluorimeter with polarization capabilities and on our confocal microscope. Based on the selected set of filters and dichroic mirrors, the scattered light contribution to our images is negligible, and because the parallel and
perpendicularly polarized images were recorded simultaneously, potential cell movement effects can also be ruled out.

Similar trends were also observed using 2P-fluorescence polarization anisotropy imaging (Fig. 4.5), which offers the advantages of larger dynamic range (i.e., maximum theoretical $r_0$-value of 0.57, compared with 0.4 for 1P-excitation), an accurately determined $G$-factor, and negligible cellular autofluorescence and scattered light. These single cell anisotropy images indicate heterogeneous local lipid and protein environments under the different IgE-FcɛRI cross-linking states in live RBL cells. The spatial resolution inherent in steady-state anisotropy offers new information that may be averaged out in previous bulk studies on cell blebs or plasma membrane vesicles studied in suspension using spectrofluorimetry (Gidwani et al., 2001). While the diI-C$_{18}$ steady-state anisotropy images provide insight into plasma membrane spatial organization, they unfortunately lack the dynamics information related to membrane nanostructural changes and the effect of IgE-FcɛRI cross-linking on the membrane. To obtain such information, we conducted time-resolved anisotropy studies of diI-C$_{18}$ and A488-IgE-FcɛRI in mast cells, as a function of IgE receptor cross-linking with secondary antibody. Single point fluorescence anisotropy measurements, with picosecond temporal resolution, reveal notably higher restrictions on diI-C$_{18}$ rotational mobility upon cross-linking (Fig. 4.6 and Table 4.3). diI-C$_{18}$ fluorescence anisotropy decays as a single exponential with a residual anisotropy ($r_\infty$) component, indicating a wobbling (or tumbling) motion of the fluorophore around a flexible tether to/within the membrane. While there is substantial
Figure 4.5. 2P-steady-state anisotropy of RBL-2H3 cells for comparison with 1P-steady-state confocal anisotropy imaging. $\lambda_{ex} = 960$ nm for 2P-steady-state anisotropy imaging of A488-IgE and dil-C$_{18}$; whereas $\lambda_{ex} = 488$ nm and 543 nm for 1P-steady-state anisotropy imaging of A488-IgE and dil-C$_{18}$, respectively, as shown in Fig. 4.1. Representative 2P-anisotropy images of dil-C$_{18}$ (B and D) and A488-IgE (A and C) in the plasma membrane of cells that had been cross-linked (C and D) or not (A and B) with $\alpha$-IgE. $n = 10$ (dil-C$_{18}$, – cross-linking), $n = 18$ (dil-C$_{18}$, + cross-linking), $n = 5$ (A488-IgE, – cross-linking), and $n = 5$ (A488-IgE, + cross-linking). Bar, 5 µm.
cell to cell variation, the characteristic diI-C\textsubscript{18} rotational correlation time differs with IgE-FcεRI cross-linking state ($\phi = 0.13 \pm 0.01 \text{ ns } [p = 0.048]$ and $0.10 \pm 0.01 \text{ ns}$ in the presence and absence of $\alpha$-IgE, respectively; Table 4.3), indicating increased membrane nanostructure with IgE cross-linking. Under our experimental conditions, such a fast rotational time constant may not be assigned to free diI-C\textsubscript{18} fluorophores due to the substantial observed residual anisotropy ($0.19 \pm 0.07$ and $0.21 \pm 0.04$ for cells with and without IgE cross-linking, respectively). The overall initial diI-C\textsubscript{18} anisotropy is comparable in the membrane of cells incubated with $\alpha$-IgE ($r_0 = 0.30 \pm 0.06$) and in cells without $\alpha$-IgE ($r_0 = 0.33 \pm 0.02$; Fig. 4.6A and Table 4.3). Our initial anisotropy values for diI-C\textsubscript{18} in RBL cells are somewhat larger than the $\sim 0.25$ obtained for DPH-PC in RBL plasma membrane blebs and detergent-resistant membrane vesicles at room temperature using steady-state spectrofluorimetric anisotropy measurements (Gidwani et al., 2001), which may be due to different membrane curvature of the blebs and vesicles versus RBL cells, to ensemble averaging in the cuvette experiments as compared with the single cell approach reported here, or to using a different anisotropy probe. The degree of orientational constraint, as reflected by the $r_\infty / r_0$ ratio (Kinosita et al., 1982; Kinosita et al., 1977), is also similar for diI-C\textsubscript{18} in the plasma membrane of cross-linked cells as compared to uncross-linked cells ($0.64 \pm 0.02$ and $0.64 \pm 0.09$, respectively; Table 4.3). In contrast to diI-C\textsubscript{18} in mast cells (Table 4.3), free diI-C\textsubscript{18} in DMSO has an initial anisotropy of $0.30 \pm 0.01$ and a rotational correlation time of $0.73 \pm 0.06 \text{ ns}$, consistent with its molecular mass (934 Da) and the viscosity of DMSO (~2 cP). The longer diI-C\textsubscript{18} rotational diffusion upon receptor cross-linking correlates well with the increased probe lifetime in the same cells. These results are further substantiated by previous FRAP
Figure 4.6. Time-resolved fluorescence anisotropy decays of diI-C\textsubscript{18} and A488-IgE in living RBL cells, under different conditions of IgE cross-linking. Representative single point time-resolved anisotropy decays of diI-C\textsubscript{18} (A) in the plasma membrane of mast cells with (1) or without (2) IgE cross-linking and A488-IgE (B) on the surface of cells in the presence (3) or absence (4) of α-IgE ($\lambda_{\text{ex}} = 480$ nm for both diI-C\textsubscript{18} and A488-IgE). Curves were fit, following Eq. 2.2, as a single (diI-C\textsubscript{18} labeled cells) or biexponential (A488-IgE labeled cells), both with an additional residual anisotropy component. Points of the decays at times well beyond the excited-state lifetime of the probe where noise levels were high (3 ns for diI-C\textsubscript{18}) were not included in the fit. Time-resolved fluorescence anisotropy decay fit parameters are summarized in Table 4.3. The observed associated-anisotropy feature (Vishwasrao et al., 2005) for diI-C\textsubscript{18} in uncross-linked cells was not reproducible in all measurements and may be attributed to the low signal-to-noise at times much longer than the excited-state lifetime (see Fig. 4.4).
Table 4.3. Single point, time-resolved fluorescence polarization anisotropy of diI-C₁₈ and A₄₈₈-IgE in RBL cells, with and without IgE-FcεRI cross-linking by α-IgE.

<table>
<thead>
<tr>
<th></th>
<th>φ₁/ns</th>
<th>β₁</th>
<th>φ₂/ns</th>
<th>β₂</th>
<th>rₜ</th>
<th>r₀</th>
<th>rₜ / r₀</th>
<th>n</th>
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<tr>
<td><strong>diI-C₁₈ labeled cells</strong></td>
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<tr>
<td>– α-IgE</td>
<td>0.10(1)</td>
<td>0.12(3)</td>
<td>–</td>
<td>–</td>
<td>0.21(4)</td>
<td>0.33(2)</td>
<td>0.64(9)</td>
<td>7</td>
</tr>
<tr>
<td>+ α-IgE</td>
<td>0.13(1)ᵇ</td>
<td>0.10(3)</td>
<td>–</td>
<td>–</td>
<td>0.19(7)</td>
<td>0.30(6)</td>
<td>0.64(2)</td>
<td>7</td>
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<tr>
<td><strong>A₄₈₈-IgE labeled cells</strong></td>
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<tr>
<td>– α-IgE</td>
<td>0.13(2)</td>
<td>0.10(3)</td>
<td>1.56(2)</td>
<td>0.07(2)</td>
<td>0.08(3)</td>
<td>0.18(5)</td>
<td>0.45(9)</td>
<td>13</td>
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<tr>
<td>+ α-IgE</td>
<td>0.16(3)ᵇ</td>
<td>0.07(1)</td>
<td>1.83(5)ᵇ</td>
<td>0.07(1)</td>
<td>0.11(4)</td>
<td>0.19(4)</td>
<td>0.59(1)ᵇ</td>
<td>11</td>
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<td><strong>Free markers in solution</strong></td>
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<tr>
<td>A₄₈₈ (waterᵈ)</td>
<td>0.11(6)</td>
<td>0.33(7)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.33(7)</td>
<td>–</td>
<td>2</td>
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<tr>
<td>A₄₈₈-IgE (PBSᵈ)</td>
<td>0.17(2)</td>
<td>0.12(3)</td>
<td>2.01(2)ᵇ</td>
<td>0.06(1)</td>
<td>0.03(1)ᶜ</td>
<td>0.15(1)</td>
<td>0.22(3)ᵇ</td>
<td>4</td>
</tr>
<tr>
<td>diI-C₁₈ (DMSO)</td>
<td>0.73(6)</td>
<td>0.30(1)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.30(1)</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>fluorescein (waterᵈ)</td>
<td>0.12(1)</td>
<td>0.29(9)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.29(9)</td>
<td>–</td>
<td>3</td>
</tr>
</tbody>
</table>

ᵃ λₘₑₓ = 480 nm, T ≈ 20°C. The fitting parameters represent an average over a number of cells (n) with the standard deviation in the last digit shown in parentheses to reflect the cell-to-cell variation of the individual fit parameters. ᶦ p < 0.05, as determined from unpaired, one-tailed Student’s t-tests, indicating a statistically significant difference in the means as compared to cells in the absence of receptor cross-linking. ᵇ The time constant associated with the third exponential term is ~40–100 ns with a pre-exponential factor that is shown in this case as residual anisotropy (rₜ). The uncertainty in this time constant is due to the rather small amplitude and relatively long rotational time as compared with the A₄₈₈-IgE excited-state lifetime. ᵇ The pH of water is ~5–5.5, and the pH of PBS is 7.4.
studies that showed restricted diI-C$_{16}$ lateral diffusion (~30–40% decrease in the mobile fraction) within domains of RBL cells that had been extensively cross-linked in the cold, as compared to cells that had not been cross-linked (Pyenta et al., 2003; Thomas et al., 1994). These time-resolved anisotropy studies provide support for the hypothesis that diI-C$_{18}$ lipid analog redistributes into IgE-FcεRI-containing membrane domains of greater structural order upon cross-linking.

To probe possible changes in protein-protein interactions upon α-IgE binding, similar time-resolved anisotropy measurements were conducted on A488-IgE. In the cell environment, A488-IgE anisotropy decays biexponentially with a residual ($r_\infty$) anisotropy component (Fig. 4.6 and Table 4.3), in IgE receptor cross-linked and uncross-linked cells. The rotational times of A488-IgE bound to the cell surface were slower when IgE-FcεRI was cross-linked with α-IgE ($\phi_1 = 0.16 \pm 0.03$ ns [$p = 0.045$], $\beta_1 = 0.07 \pm 0.01$, $\phi_2 = 1.83 \pm 0.05$ ns [$p = 0.010$], and $\beta_2 = 0.07 \pm 0.01$; Table 4.3), as compared to cells in the absence of cross-linking ($\phi_1 = 0.13 \pm 0.02$ ns, $\beta_1 = 0.10 \pm 0.03$, $\phi_2 = 1.56 \pm 0.02$ ns, and $\beta_2 = 0.07 \pm 0.02$; Table 4.3). In agreement with the observed lifetime increase, the enhanced A488-IgE-FcεRI rotational times support conformational changes of membrane order (i.e., a constraining environment) and the likelihood of the recruitment of other proteins upon α-IgE-IgE-FcεRI cross-linking. The corresponding total initial anisotropies of A488-IgE-FcεRI in cells with ($r_0 = 0.19 \pm 0.04$) and without cross-linking ($r_0 = 0.18 \pm 0.05$) are not statistically different (see Fig. 4.6B for representative decays); however the degrees of orientational constraint are significantly different between the two, with $r_\infty / r_0 = 0.59 \pm 0.01$ ($p = 0.034$) for A488-IgE in cells with IgE cross-linking.
and 0.45 ± 0.09 in cells in the absence of cross-linking (Table 4.3). Because of the short time scale of our time-resolved anisotropy experiments (ps–ns), the anisotropy reported by A488-IgE may reflect rapid tumbling of covalently attached A488 or wagging motions of IgE Fab segments (Holowka et al., 1990) that are somewhat slowed by secondary antibody binding, and not motions of the much larger IgE (~180 kDa). This explanation is further supported by the substantial segmental mobility associated with free A488-IgE rotational diffusion in solution (Table 4.3). A488-IgE (in PBS) anisotropy decays bi-exponentially with a residual anisotropy component ($\phi_1 = 0.17 ± 0.02$ ns, $\beta_1 = 0.12 ± 0.03$, $\phi_2 = 2.01 ± 0.02$ ns [$p = 0.023$], $\beta_2 = 0.06 ± 0.01$, and $r_\infty = 0.03 ± 0.01$; Table 4.3), where the slowest time constant, associated with such a small $r_\infty$ amplitude fraction, is uncertain (~40–100 ns, as compared to the relatively shorter excited-state lifetime, i.e., the rotational diffusion observation time window).

To the best of our knowledge, this is the first time that the ultrafast (ps–ns) excited-state dynamics and rotational diffusion of fluorescently-labeled IgE have been monitored for elucidating the structure-function relationship of IgE receptor cross-linking in intact, live mast cells. Previously, time-resolved fluorescence (Holowka et al., 1990) and phosphorescence measurements (Myers et al., 1992; Song et al., 2002) of IgE-FceRI on mast cells in solution were carried out with nanosecond-microsecond resolution. In one of these studies, the rotational correlation time of IgE-FceRI on both RBL plasma membrane blebs and cells increased substantially for cells that had been treated with $\alpha$-IgE, and the degree of orientational constraint, $r_\infty / r_0$, also increased from 0.515 in uncross-linked cells to 1.00 in $\alpha$-IgE-treated cells (Myers et al., 1992). These results agree well with the trend observed in this report with ~20 ps time resolution (Table 4.3).
In addition, the improved time resolution here enables us to monitor ultrafast segmental mobility of the A488-IgE-FcεRI complex in real time, which is beyond the resolution of previous phosphorescence experiments (Zidovetski et al., 1986). Although the time resolution differences between our techniques and those of Myers et al. (1992) do not permit a true comparison of dynamics and order parameters, the enhanced environmental constraint on A488-IgE segmental mobility under cross-linking conditions agree well with former FRAP studies on Cy3-IgE-FcεRI lateral diffusion (Pyenta et al., 2003). In those studies, a cross-linking-induced reduction in Cy3-IgE-FcεRI lateral mobility was reported in mast cells (~40% decrease in the mobile fraction) as the complex moved into a more ordered environment from the uncross-linked state (Pyenta et al., 2003). Overall, our single point time-resolved anisotropy results for diI-C18 and A488-IgE, together with the lifetime results, suggest that fluorophore dynamics become more restricted and local membrane structure increases upon IgE-FcεRI cross-linking.

4.4 Conclusions

Short-range sampling of the immediate local environment of a fluorescent probe, whether membrane-incorporated or protein-associated, allows for real-time molecular-level interrogation and visualization of specialized cholesterol-rich microdomains and associated signaling proteins, as well as the monitoring of their short-range excited-state fluorescence dynamics. In general, the anisotropy and lifetime changes of both diI-C18 and A488-IgE in individual RBL-2H3 mast cells can be correlated with cross-linking-induced changes in local membrane structure, which are indicative of the degree of
Figure 4.7. Model of A488-IgE and diI-C\textsubscript{18} orientation and location within the plasma membrane in the absence and presence of IgE receptor cross-linking. The state of the plasma membrane in a resting, or uncross-linked, cell (A) is contrasted with the membrane of a cross-linked cell (B). Ordered membrane regions are indicated by locally high levels of cholesterol, sphingomyelin, glycosphingolipid, and glycerophospholipid concentrations. Upon receptor cross-linking, these domains cluster to nucleate a larger, functional domain.
membrane heterogeneity, organization and composition. In this Chapter, we have shown that upon extensive IgE-FcεRI cross-linking, diI-C_{18} undergoes nanoscale environmental changes as it co-redistributes in the plasma membrane to well-defined A488-IgE-FcεRI patches (Fig. 4.7B), resulting in longer lifetimes (Figs. 4.2, 4.3 and 4.4; Table 4.2) and higher anisotropy (Figs. 4.1 and 4.6; Table 4.3), as compared to uncross-linked cells (Fig. 4.7A). We have also shown that, upon cross-linking with α-IgE, A488-IgE environment and dynamics are affected by increased protein-protein interactions, which results in distinguishably longer lifetimes and higher anisotropy as compared with A488-IgE bound to uncross-linked cells. Based on these results, we hypothesize that protein-protein interactions (i.e., IgE-FcεRI cross-linking) recruit essential signaling proteins and lipid molecules into more ordered domains that serve as platforms for signaling. The results presented in this Chapter provide a valuable frame of reference for interpretation of studies on mast cells that are stimulated under more physiological conditions (see Chapter 6), where domains are much smaller in size and exist for shorter periods of time, although the exact spatial and temporal scale of these domains remains to be determined. Moreover, these studies offer an opportunity to follow the spatial and temporal relationships linking plasma membrane nanostructure to the dynamics of FcεRI and other signaling molecules, both in living cells and in model membrane systems.
5. MOLECULE-SPECIFIC TOF-SIMS IMAGING OF MEMBRANE LIPID DISTRIBUTION ASSOCIATED WITH EXTENSIVE IGE RECEPTOR CROSS-LINKING IN RBL MAST CELLS: NON-PHYSIOLOGICAL CONDITIONS

This Chapter summarizes mass spectrometry imaging studies performed in the Winograd lab (Penn State, Chemistry) to determine the chemical composition of the membrane domains formed by extensive cross-linking of mast cells. These studies (to be published) provide a basis for the development of statistical models of ToF-SIMS resolution dependence on ionization efficiency and concentration.

5.1 Introduction

The localization of various molecules in the complex plasma membrane of cells is critical for understanding numerous cellular processes. Cholesterol-rich membrane domains have been hypothesized to play key roles in signaling and trafficking and are the focus of many membrane structure-function studies (Brown and London, 1998; Simons and Toomre, 2000). However, directly observing the spatial arrangement of these domains, and the lipids that comprise them, in vivo with good resolution and obtaining relevant chemical information has remained elusive due to their size and lifetime.

Mass spectrometry has been one of the primary methods used traditionally to reveal the chemical composition of model membrane systems and actual biological samples without exogenous labeling, but has been restricted by its lack of spatial information (Sodhi, 2004). Field et al. (1997) previously demonstrated that ligand-mediated IgE receptor (FceRI) cross-linking on RBL mast cells caused its co-isolation with detergent-resistant membranes and consequent phosphorylation by the tyrosine kinase, Lyn, early in the FceRI signaling pathway. To determine the structural bases for these
interactions, tandem mass spectrometry was subsequently performed on lipid extracts of whole RBL cell lysates, on induced blebs isolated from RBL cells and on detergent-resistant vesicles from unstimulated or stimulated (i.e., cross-linked) RBL cells. These tandem mass spectrometry experiments suggested that detergent-resistant membranes were enriched in polyunsaturated phospholipids when cells were stimulated with antigen (Fridriksson et al., 1999), which may result from the membrane trafficking that occurs upon stimulation (Naal et al., 2003). This result was surprising considering that a variety of model membrane and cellular data imply that detergent resistance correlates with increased ordering resulting from interactions between saturated lipids and cholesterol (the liquid-ordered phase). In addition to the lack of spatial information about lipid distribution, these studies also provided no data about membrane component colocalization with signaling molecules (Fridriksson et al., 1999), and may be artifactual due to the non-ionic detergent extraction (Johansson, 2006).

In contrast to traditional mass spectrometry, ToF-SIMS allows imaging of biological samples on substrates, as discussed in Chapter 2, with high chemical specificity and surface sensitivity and a spatial resolution of ~1 µm or smaller (Johansson, 2006). Because cells do not require homogenization, cell morphology and the context of the chemical species are maintained. Further, primary ion source improvements have permitted larger, well-identifiable mass fragments to be generated, making the mass limit well above \( m/z \) 1000, which is amenable to analysis of many biomolecules, such as lipids (Johansson, 2006). Micron-sized domains, similar to those found with fluorescence microscopy upon lipid-phase specific labeling, have been detected by ToF-SIMS imaging (with a Ga\(^+\) primary ion source) of model phospholipid bilayers formed by
the Langmuir-Blodgett method (e.g., POPC:sphingomyelin: cholesterol, 30:47:23 (mol/mol) (McQuaw et al., 2007) or DPPC:DPPS (1,2-dipalmitoyl-sn-glycero-3-phosphoserine), 4:1 (mol/mol) (Ross et al., 2001)). Model membrane studies such as these provide a frame of reference for interpreting ToF-SIMS images of single cells and subcellular structures by identifying characteristic phospholipid headgroups (e.g., phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS)), sphingomyelin, and cholesterol mass fragments.

Although a variety of biomolecules have been imaged via ToF-SIMS of bacterial, yeast and mammalian cells, membrane lipids are the most commonly studied (Johansson, 2006). For example, dried bacterial cells have been distinguished on the basis of their surface lipids by ToF-SIMS with a ReO$_4^-$ primary ion source (Ingram et al., 2003). Further, ToF-SIMS with a Ga$^+$ ion source has been used to analyze the PC and cholesterol spatial distributions in freeze-dried blood cells adhering to glass (Sjovall et al., 2003). However, sample integrity is best maintained by sample preparation methods such as flash-freezing, freeze-fracture in vacuum or under nitrogen, and analysis of the sample in the frozen-hydrated state (Johansson, 2006). Membrane lipid inhomogeneities of *Tetrahymena*, flash-frozen and freeze-fractured under vacuum, have been compared at fusion sites versus the rest of the membrane during mating using ToF-SIMS with an In$^+$ primary ion source (Ostrowski et al., 2004).

In this Chapter, our goal is to correlate the enhanced lipid ordering that we observe in the plasma membrane of RBL cells upon IgE receptor cross-linking via fluorescence lifetime and polarization anisotropy (see Chapter 4) with the chemical identity of the lipids in these regions. Using ToF-SIMS imaging, we identify the key
lipid species present in the membrane of individual, suspended RBLs extensively cross-linked with α-IgE, or not, and cryogenically preserved via flash freezing and freeze fracturing. ToF-SIMS imaging and analysis was carried out primarily by Paul Piehowski, under the supervision of Professor Nicholas Winograd (Penn State, Chemistry), while we provided the biological system and facilitated data interpretation. (A further description of this work will be discussed in Paul Piehowski’s Dissertation.) Taken together with the studies described in Chapter 4, these ToF-SIMS experiments represent one of the first studies to correlate intact cell membrane structure (i.e., chemical identity) with biological function (i.e., IgE receptor cross-linking) and lend insight into the molecular basis of functional membrane domains on the cell surface.

5.2 Materials and methods

5.2.1 Cell and whole cell lipid extract preparation

Uncross-linked or cross-linked suspended RBL mast cells were prepared as described in Section 2.2.1 and in Davey et al. (2007). The cryogenic portion of the preparation is described in detail in Section 2.6.1 and also by Piehowski et al. (2008). Total cell membrane lipid extracts were prepared from RBLs, similar to Fridriksson et al. (1999), to assess whether mass fragments of interest could be identified by the ToF-SIMS instrumentation. Adherent RBLs (1.1 × 10^8 cells) were harvested with cell harvest buffer (135 mM NaCl, 5 mM KCl, 20 mM HEPES pH 7.6, 1.3 mM EDTA), pelleted by centrifugation (200 × g, 8 min) in BSS and washed two times in BSA/BSS. After the second wash, the cell pellet was resuspended in 5 mL distilled deionized water, centrifuged again, and resuspended in 5 mL methanol. The solution was transferred to an ethanolic-KOH-cleaned Duall ground glass tissue grinder and after 20 strokes, 5 mL chloro-
form was added, and the solution was transferred to an ethanolic-KOH cleaned glass vial. The solution was vortexed and probe sonicated at highest power for 5 min at 45°C and then rocked at room temperature for 2 h. The solution was centrifuged (400 × g, 10 min) and the supernatant transferred to a clean vial. The pellet was re-extracted in 5 mL 1:1 chloroform:methanol (v/v), vortexed and centrifuged again (400 × g, 10 min). Supernatants consisting of the total lipid extracts were combined and stored under nitrogen at −20°C. Total RBL lipid extract was applied to Si shards and allowed to dry prior to ToF-SIMS analysis. (Shards were maintained at liquid nitrogen temperatures (−210°C to −196°C) prior to introduction into the ultrahigh vacuum (5 × 10⁻⁹ – 5 × 10⁻⁸ torr) (Roddy et al., 2003) of the ToF-SIMS spectrometer.)

5.2.2 Mass spectrometry

The mass spectrometry instrumentation, theory and analysis are described in Sections 2.6.2–3. Secondary ion images were acquired in parallel with ToF-SIMS images to monitor cell morphology and integrity. The In⁺ primary ion source uniformly sampled the entire top of a cell (~1% of the surface is destroyed), with the three-dimensional information compressed into a two-dimensional image. ToF-SIMS images were processed with bin = 1 or smoothed in MATLAB, unless otherwise noted. With image binning, each pixel in the original image (with bin = 0) is summed with adjacent pixels, thus decreasing the spatial resolution such that with bin = 1, each pixel is the sum of four pixels from the original image. With image smoothing, pixel size remains the same (i.e., 350 nm, here), but each pixel is displayed as the average of itself and the eight surrounding pixels. Increases in the level of binning are discussed below.
5.3 Results and discussion

5.3.1 ToF-SIMS of RBL total lipid extracts identifies mass fragments of interest to IgE receptor cross-linking

In early ToF-SIMS studies using a C$_{60}^+$ primary ion source, the Winograd lab acquired mass spectra of various membrane lipids from single-constituent lipid bilayer model systems (McQuaw et al., 2005; Roddy et al., 2003; Sostarecz et al., 2004) to identify characteristic ion fragments that were then used to identify the location of these molecules within actual cell membranes (Ostrowski et al., 2007; Parry and Winograd, 2005; Piehowski et al., 2008). However, we wanted to ensure that these ion fragments of interest could be adequately detected from our cells using the current ToF-SIMS method. As shown in Fig. 5.1, ToF-SIMS analysis of total RBL lipid extracts revealed fragments ascribed to the PE ($m/z$ 142) and PC ($m/z$ 184) phospholipid headgroups, the latter of which are found in higher concentrations in the plasma membrane outer leaflet (Fridriksson et al., 1999; Yeagle, 1987). Sphingomyelin ($m/z$ 284) was detected with lower efficiency in lipids extracted from cells than in artificial membranes (McQuaw et al., 2006; McQuaw et al., 2007). Cholesterol was primarily identified via the ion fragment at $m/z$ 147; however it was also detected at $m/z$ 369 (Piehowski et al., 2008), albeit with fewer counts (data not shown). Based on cholesterol-dependent fluorescence colocalization experiments of IgE receptor with various signaling-associated molecules and on our fluorescence lifetime and anisotropy studies (Chapter 4), we hypothesized that cholesterol and sphingomyelin would be enriched in the cell membrane domains with IgE-FceRI when intact RBL cells
Figure 5.1. Mass spectrum of total lipid extract from RBL cells obtained via ToF-SIMS. The characteristic ion fragments for the phosphatidylethanolamine (PE) headgroup, phosphatidylcholine (PC) headgroup, sphingomyelin and cholesterol are indicated at $m/z$ 142, $m/z$ 184, $m/z$ 284 and $m/z$ 147 (see inset), respectively. Cholesterol is also identified at $m/z$ 369 (Piehowski et al., 2008), but with fewer counts than at $m/z$ 147 (data not shown).
were cross-linked with α-IgE. Therefore, ToF-SIMS images of individual, cryogenically preserved, suspended RBLs that were extensively cross-linked with α-IgE, or not, were acquired to determine the spatial distribution of the mass fragments associated with molecules that comprise the RBL cell membrane.

5.3.2 ToF-SIMS reveals cholesterol and membrane lipid distribution in uncross-linked versus cross-linked RBL cells

As an initial effort toward visualizing micron-sized cholesterol-rich domains in individual cells, we performed ToF-SIMS on RBL cells that were extensively cross-linked with α-IgE. Due to sample preparation restrictions, all ToF-SIMS imaging of cells discussed herein was performed using an In⁺ primary ion source, rather than C₆₀⁺. A representative set of ToF-SIMS images is shown in Fig. 5.2. In SIMS, the ion intensity can be affected by sample topography and primary ion current fluctuations. Thus, these images were normalized to a common hydrocarbon fragment derived from membrane lipids (C₅H₉⁺) at m/z 69 (Piehowski et al., 2008) (Fig. 5.2A). Substantial ion yields were obtained for the PC and PE headgroups (Fig. 5.2B and D, respectively), and cholesterol (Fig. 5.2C), while the sample integrity was assessed by mapping C₅H₉⁺ and ions from solution, such as Na⁺, and K⁺ (data not shown). When the very top plane of the cells is imaged via ToF-SIMS, the spatial distribution of PC is rather uniform (Fig. 5.2B) with high count levels, while cholesterol (Fig. 5.2C) and PE (Fig. 5.2D) are more heterogeneously distributed throughout the cell membrane at lower concentrations than PC, suggesting micron-sized domains of these latter membrane components. ToF-SIMS imaging of intact RBL cells, whether cross-linked with α-IgE or not, is novel because
Figure 5.2. Representative ToF-SIMS images of a single, cross-linked RBL cell. The following ion fragments of interest were isolated and used to form each image: hydrocarbon standard fragment, C$_5$H$_9^+$, at $m/z$ 69 (A), PC headgroup, including phosphate at $m/z$ 184 (B), cholesterol at $m/z$ 147 (C), and PE headgroup, including phosphate at $m/z$ 142 (D), all at bin = 1. The color bar represents the signal intensity level for each mass species, so the scales are all different. The background in (A) is attributed to residual hydrocarbon fragments on the analysis surface and is not observed for any of the other fragments of interest. Bar, 5 µm.
these experiments are the first to achieve sub-cellular localization of multiple membrane lipid components in individual, cryogenically prepared mammalian cells.

Relationships between membrane components can be assessed qualitatively by overlaying two images and quantitatively by performing Pearson’s correlation tests with image pairs. For example, when the cholesterol and PE images from Fig. 5.2C and D, respectively, are overlaid, anti-correlation between these two components is implied (data not shown). Statistical analysis substantiates anti-correlation by generating a correlation coefficient \( P \) of \(-0.78\) (with \( p < 1 \times 10^{-4} \)), which indicates that both components are likely not localized to the same pixel, i.e., as one component increases the other tends to decrease. In fact, Yeagle and others have suggested that it is thermodynamically unfavorable for cholesterol to partition with PE in model membranes (Yeagle, 1987) and cell membranes (House et al., 1989).

We next obtained ToF-SIMS images of the membrane of RBL cells that were cross-linked with \( \alpha \)-IgE, or not, to compare the distributions of various lipid species across the cell surface and gain information about changes induced by receptor cross-linking and possible co-redistribution into cholesterol-rich microdomains. Representative images of cross-linked and uncross-linked cells are shown in Fig. 5.3A and B, respectively, with only the cholesterol \((m/z 147)\) distribution highlighted in this particular figure. When images are smoothed identically during analysis, similar micron-sized cholesterol domains appear on both the cross-linked cell membrane and the uncross-linked control. Further, when the distributions of various lipid species of interest, including cholesterol, are determined, there is no substantial change in the standardized intensities,
Figure 5.3. Representative ToF-SIMS images of cross-linked and uncross-linked RBL cells. Images are pseudo-colored to show cholesterol (m/z 147) intensity in an individual cross-linked cell (A) and uncross-linked cell (B). These images highlight the similarity in the cholesterol distributions in cross-linked and uncross-linked cells. Images are smoothed, as described in Section 5.2.2. Bar, 5 µm.
Figure 5.4. Relative concentrations of various ion fragments assigned to RBL cell membrane components of interest. These intensities or relative concentrations are standardized to the hydrocarbon fragment, C$_5$H$_9^+$ ($m/z$ 69). PE ($m/z$ 142), cholesterol ($m/z$ 147) and PC ($m/z$ 184) concentrations are not significantly different between uncross-linked (white bars, $n = 5$) and cross-linked (black bars, $n = 6$) cell samples. These data were extracted from raw, unbinned, unsmoothed ToF-SIMS images.
or relative concentration, of any membrane components upon cross-linking (Fig. 5.4). In other words, under these experimental conditions there is no detectable enrichment in the concentration or localization of PE, cholesterol or PC in the membrane of cells cross-linked with α-IgE. These results are in contrast to our fluorescence lifetime and anisotropy data supporting cholesterol-enrichment in cross-linked mast cells (Chapter 4), as well as other previous fluorescence imaging and biochemical analyses (Field et al., 1997; Sheets et al., 1999a). These results may be attributed to the current limitations of ToF-SIMS imaging, as described below.

To determine whether micron-sized cholesterol domains were accurately detected in cross-linked and uncross-linked RBLs, a film of pure cholesterol (applied to the Si substrate and analyzed in the same way as RBL total lipid extract) was examined via identical ToF-SIMS methods. Similar cholesterol distributions are seen in an individual cross-linked mast cell image (Fig. 5.5A) and a pure cholesterol film image (Fig. 5.5B), both smoothed to the same degree. Thus, at this resolution (350 nm), regions of high and low intensity that suggest cholesterol domains in cross-linked and uncross-linked cells are artifactual based on counting errors due to low signal, resulting in the observation of heterogeneous distributions of various lipid species even in a pure, homogeneous film. In other words, an image of a uniform surface appears heterogeneous because the pixel intensities follow a normal distribution.

As a consequence of these studies on mast cell membrane lipid distribution in the presence and absence of IgE receptor cross-linking, the Winograd lab has made progress toward developing statistical models to predict the useful lateral resolution of ToF-SIMS
Figure 5.5. Comparison of cholesterol distribution in a cross-linked RBL cell membrane versus a pure cholesterol film as assessed by ToF-SIMS imaging. The cholesterol ion fragment at \( m/z \) 147 was selected to construct these images from total ion images of a cross-linked RBL cell (same cell as shown in Fig. 5.2C, but with image smoothing) (A) and a pure cholesterol film (B). Both samples were smoothed to the same degree, revealing similar cholesterol distributions at this resolution (350 nm), although the signal intensity is higher overall for the pure cholesterol film. Images are smoothed, as described in Materials and methods. Bar, 5 µm.
measurements, and therefore the binning amount appropriate for mass spectrometry image analysis. The molecule being probed, concentration of that molecule, primary ion source and spot size affect not only lateral resolution, but also the detection limit of ToF-SIMS. For a particular lipid species’ ionization efficiency, we can determine the lateral resolution limit (or concentration difference) required to conclude that the species concentration is significantly different between two pixels with 95% confidence (see Section 2.6.3). As the ionization efficiency increases, fewer molecules are required to obtain a z-score of three standard deviations (i.e., ~95% confidence level), thus increasing lateral resolution (Fig. 5.6A) (such that less binning is required for image analysis) for an estimated percent change in lipid species concentration (e.g., 100%). Analogously, with increasing ionization efficiency, the percent change in concentration required to resolve two pixels decreases (Fig. 5.6B) for a given lateral resolution or pixel size (e.g., 1.2 µm). With this model, ToF-SIMS images can be acquired at the highest resolution allowed by the primary ion source spot size and then binned according to the model to resolve concentration differences of the species of interest. For example, a 100% cholesterol concentration change in an uncross-linked mast cell membrane, versus in cross-linking induced membrane domains, is not resolvable at our current 350 nm resolution (Fig. 5.6A). If images are binned 4 × 4, to make the effective pixel size ~1.2 µm, then at best, an ~125% change in cholesterol concentration can be distinguished at the expense of lateral resolution. Cholesterol concentration would have to increase 2-fold or more to be detectable at a 350 nm spatial resolution. The current detection limitations do not allow us to currently distinguish between the subtle changes in lipid concentration that likely occur as a function of extensively cross-linking IgE-FcεRI.
Figure 5.6. Statistical modeling of ToF-SIMS resolution limit dependence on lipid species ionization efficiency and concentration. \( z \)-score is plotted as a function of lateral resolution limit (A) and percent change in lipid species concentration (B) for two representative ionization efficiencies, or lipid species. PC and cholesterol (or PE) ionization efficiencies are represented by squares (higher ionization efficiency) and diamonds (lower ionization efficiency), respectively. Horizontal lines are drawn on each plot to represent three standard deviations, used to calculate the \( z \)-score. For (A), the change in lipid species concentration was held constant at 100% and for (B), the pixel size, or lateral resolution, was held constant at 1.2 \( \mu \text{m} \).
Directly relating the spatial distributions of diI-C18 (our cholesterol-rich membrane probe) and IgE observed with fluorescence microscopy (Chapter 4) to the distribution of cholesterol and other membrane components measured by ToF-SIMS, remains a key concern. Such experiments could potentially determine the chemical composition specifically within these micron-sized, cross-linking induced domains, and would be feasible with the addition of a higher magnification, higher numerical aperture fluorescence microscope objective in the ToF-SIMS spectrometer. Further, there is little possibility of resolving IgE protein (or $\alpha$-IgE), let alone IgE-FcεRI domains, because none of the distinct mass fragments obtained from an IgE solution control were identified in cross-linked or uncross-linked cell images. Tandem mass spectrometry methods must be used to resolve high mass fragments that arise from a large protein, such as IgE (~180 kDa). It would also be interesting to apply ToF-SIMS imaging to the antigen-mediated exocytotic release of histamine-containing granules. In fact, early ToF-SIMS experiments of intact uncross-linked mast cells revealed little or no histamine signal; however histamine concentrated inside the cells was easily detected via through-fracture analysis (data not shown).

5.4 Conclusions

Using ToF-SIMS imaging with 350 nm lateral resolution, we were able to visualize the sub-cellular distribution of multiple key lipid species present in the membrane of individual, cryogenically preserved, intact RBL cells that were extensively cross-linked with $\alpha$-IgE, or uncross-linked. Our results suggest that IgE receptor cross-linking-induced changes in cholesterol distribution, or concentration, in the membrane of mast cells may be very subtle, requiring sensitive analytical methods such as fluorescence
lifetime and polarization anisotropy (Chapter 4). Cell membrane lipid detection is currently limited by ToF-SIMS spatial resolution and sensitivity; however, further resolution improvements are likely to arise through increased use of buckminsterfullerene ion sources and continued improvements in the collection and ionization efficiency, as well as data analysis. In addition, much effort in the Winograd lab has been focused on developing new analysis methods to interpret the complex data sets generated by ToF-SIMS experiments. As ToF-SIMS imaging resolution and sensitivity improves, it may become possible to apply this method to RBL cells stimulated under more physiological conditions (i.e., conditions described in Chapter 6).
6. MULTIVALENT ANTIGEN-MEDIATED IGE RECEPTOR SIGNALING IN RBL MAST CELLS: PHYSIOLOGICAL CONDITIONS

This Chapter describes the application of ultrafast excited-state fluorescence dynamics methods, including lifetime and anisotropy, to probe mast cell membrane structure during physiological IgE receptor signaling, where membrane domains are not optically resolvable. These studies provide evidence for the association of IgE receptor with specialized cholesterol-rich domains to facilitate signaling. The work detailed in this Chapter was published, in part, as Davey et al., “Molecular perspective of antigen-mediated mast cell signaling”, Journal of Biological Chemistry 283:7117–7127 (2008).

6.1 Introduction

Cholesterol-rich plasma membrane domains, also known as rafts, are believed to engage in various cellular functions, such as IgE receptor (FcεRI) signaling in mast cells and basophils (Abramson and Pecht, 2007; Draber and Draberova, 2002; Holowka et al., 2005; Mukherjee and Maxfield, 2004; Rivera and Olivera, 2007). Antigen-mediated cross-linking 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 exocytotic histamine release in the allergic response (Holowka et al., 2005; Sheets et al., 1999a). Due to the inherent complexity of cellular membranes, there has been major interest in understanding the thermodynamics and kinetics regulating membrane domain formation in model systems, such as giant unilamellar vesicles (Bacia et al., 2005; Feigenson and Buboltz, 2001; Veatch et al., 2004) and supported bilayers and monolayers (Dietrich et al., 2001a; Dietrich et al., 2001b; Stottrup et al., 2004), as well as in cells or plasma membrane-derived blebs (Bacia et al., 2004; Dietrich et al., 2002; Ge
and Freed, 1999; Ge et al., 2003; Gidwani et al., 2001; Pyenta et al., 2003; Sengupta et al., 2007b; Sheets et al., 1997; Sinha et al., 2003; Swamy et al., 2006; Thomas et al., 1994). Forming model membranes with physiological proportions of cholesterol and various lipids (e.g., phosphatidylcholine and sphingomyelin), however, has produced conflicting evidence of whether segregated domains actually form at 37°C (Silvius and Nabi, 2006). In addition to imaging such microdomains with optical microscopy (Bacia et al., 2004; Dietrich et al., 2001a; Dietrich et al., 2001b; Feigenson and Buboltz, 2001; Stottrup et al., 2004; Thomas et al., 1994; Veatch et al., 2004), translational diffusion (ms–s) has been the main observable in various SPT (Dietrich et al., 2002; Sheets et al., 1997), FRAP (Dietrich et al., 2001b; Pyenta et al., 2003; Thomas et al., 1994), and FCS studies (Bacia et al., 2004; Bacia et al., 2005; Pyenta et al., 2003).

Many studies of cholesterol-rich membrane domains associated with IgE receptor signaling, including those described in Chapter 4, have mainly focused on RBL mast cells in suspension that are substantially (i.e., non-physiologically) perturbed by cross-linking with a secondary antibody at 4°C (Davey et al., 2007; Holowka et al., 2000; Pyenta et al., 2003; Sheets et al., 1999a; Thomas et al., 1994). In such studies, ordered microdomains within the plasma membrane (labeled with a fluorescent lipid analog such as diI-C₁₈ (Davey et al., 2007)) co-redistribute with patches of cross-linked receptor-bound IgE (labeled, for example, with Alexa Fluor 488 (A488) (Davey et al., 2007)). In Chapter 4, we reexamined existing spatial relationships between microdomains and extensively cross-linked IgE-FcεRI patches at the single cell level by probing the spatio-temporal dynamics (ps–ns) of the molecules that comprise these domains (Davey et al., 2007). Using ultrafast excited-state dynamics and rotational diffusion (Davey et al.,
we observed that plasma membrane lipid order (i.e., packing) or nanostructure is enhanced within cross-linking-induced microdomains, or patches, as evidenced by increased diI-C18 fluorescence lifetime and rotational diffusion. Similar trends in the same dynamic properties were also observed for cross-linked, colocalized A488-IgE-FcεRI. These results supported the hypothesis that lipids are recruited into more ordered domains that serve as IgE-FcεRI signaling platforms. Further, the gross perturbations inherent under these non-physiological cross-linking conditions provided a simple platform on which to test and optimize our fluorescence dynamics imaging assay (Ariola et al., 2006; Davey et al., 2007) for probing molecule-molecule interactions and structural changes relevant to cell signaling.

Nonetheless, under physiological conditions, cholesterol-rich membrane 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 (Jacobson et al., 2007; Owen et al., 2007). 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 (Bacia et al., 2004) and in suspended cells subjected to non-physiological IgE-FcεRI cross-linking (Pyenta et al., 2003; Thomas et al., 1994). Further, electron-spin resonance spectroscopy (Swamy et al., 2006) and FRET (Sengupta et al., 2007b) have been used to investigate suspended cell membrane phase, or order, and lateral distributions, but not within the context of IgE-FcεRI cross-linking and signaling.
In this Chapter, 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 (Ariola et al., 2006; Davey et al., 2007). Our rationale is that membrane-associated molecular events on the ps–ns timescale translate to interrogating structural changes on the spatial order of 100 nm (at 100 ps resolution) (Zewail, 2001), which is below the diffraction limit of traditional optical microscopy. By interrogating plasma membrane fast molecular dynamics and order as mast cells are stimulated with multivalent antigen at room temperature (~20°C), where domains may be transient and smaller than the diffraction limit, we could relate these changes directly to the stimulated FcεRI tyrosine phosphorylation, the earliest biochemical step in the pathway (under identical conditions).

The various experimental controls that will be discussed in this Chapter are outlined in Table 6.1. 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.

6.2 Materials and methods

6.2.1 Cell preparation

Adherent mast cell preparation is described in Section 2.2.2. Cells were dually labeled with diI-C_{18} and A488-IgE for most experiments. For some control experiments, cells were fluorescently labeled with either diI-C_{18} (in the presence of unlabeled IgE for possible cross-linking) or A488-IgE. Other control experiments involved fluorescently labeling cells with diI-C_{12} or Δ^9-diI-C_{18}, omitting multivalent antigen (DNP-BSA) addition, and stimulating cells with 80 nM PMA, a protein kinase C activator that
Table 6.1. Experimental controls performed on variously labeled adherent mast cells at 0, 5, 10, 20 and 30 min time points with respect to treatmenta.

<table>
<thead>
<tr>
<th>fluorescent molecule monitored, treatment</th>
<th>FLIM</th>
<th>pseudo- single point lifetime</th>
<th>single point lifetime</th>
<th>2P- anisotropy imaging</th>
<th>pseudo- single point anisotropy</th>
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<td>dil-C\textsubscript{18}, + DNP-BSA</td>
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<td>X\textsuperscript{b}</td>
<td>X</td>
<td>not shown</td>
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<td>X\textsuperscript{c}</td>
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<tr>
<td>dil-C\textsubscript{18}, – DNP-BSA</td>
<td>X</td>
<td>–</td>
<td>X</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>dil-C\textsubscript{18}, + PMA</td>
<td>X\textsuperscript{b}</td>
<td>–</td>
<td>X\textsuperscript{b}</td>
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<tr>
<td>dil-C\textsubscript{18}, + DNP-BSA, + cyto-D</td>
<td>X\textsuperscript{b}</td>
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<tr>
<td>dil-C\textsubscript{12}, + DNP-BSA</td>
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<tr>
<td>(\Delta)-dil-C\textsubscript{18}, + DNP-BSA</td>
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<td>A488-IgE, + DNP-BSA\textsuperscript{d}</td>
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<tr>
<td>A488-IgE, + dil-C\textsubscript{18}, + DNP-BSA\textsuperscript{d}</td>
<td>X</td>
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<td>–</td>
<td>not shown</td>
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</table>

\(\Delta\)-dil-C\textsubscript{18} refers to the experimental controls that are described in this Chapter. \(\Delta\)-dil-C\textsubscript{18} was often performed in the presence of A488-IgE as a marker for antigen-stimulated IgE receptor clustering (or not). A488-IgE was not detected under the given excitation and emission filter conditions. These experiments were performed without respect to time after antigen stimulation to avoid averaging over dipole moment ruffling and obscuring the interpretation of dil-C\textsubscript{18} rotational dynamics within the plane of the plasma membrane. A488-IgE dynamics were monitored in the presence of dil-C\textsubscript{18} for energy transfer experiments that probe lateral interactions between the two fluorophores.
stimulates actin polymerization and leads to cell ruffling (Xu et al., 1998b), instead of DNP-BSA.

For solution experiments, A488-IgE was diluted in PBS to ~1 µM and diI-C_{18} in DMSO to ~10 µM. Absorption spectra of 10 µM A488 in water, 0.052 µM A488-IgE in PBS, and 10 µM diI-C_{18} 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; Keating lab, Penn State, Chemistry) was used to record the fluorescence emission spectra of 0.1 µM A488 in water, 0.01 µM A488-IgE in PBS, and 2.5 µM diI-C_{18} in DMSO.

6.2.2 In vivo tyrosine phosphorylation assays

The protocol for performing tyrosine phosphorylation assays on adherent, IgE-sensitized RBL cells is noted in Section 2.2.3. Cells were stimulated with DNP-BSA at ~20°C for 0, 5, 10, 20, and 30 min.

6.2.3 Confocal fluorescence microscopy

The experimental setup and data analysis have been described in more detail in Section 2.3 and elsewhere (Ariola et al., 2006; Davey et al., 2007). A488-IgE was imaged using 488 nm argon ion laser excitation and a 525/30 bandpass filter, while diI-C_{18} was imaged with 543 nm HeNe laser excitation 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).
6.2.4 Fluorescence lifetime imaging

2P-FLIM experiments were performed using 960 nm excitation, as described in Sections 2.4.2.1 and 2.4.3, with data acquired at 0, 5, 10, 20 and 30 min after antigen stimulation (or control treatment). Prior to detection, the epi-fluorescence was separated from the excitation laser using a dichroic mirror and filters (690SP for all samples, plus a 600LP to detect only diI-C<sub>18</sub> or plus a 525/50 bandpass to detect only A488-IgE, Chroma). As noted in Section 2.4.2.1, 2P-FLIM images were recorded using 256 × 256 pixels and an 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 timescales of ruffling (s) versus excited-state dynamics (ps–ns). Experiments were performed over several days on different cell preparations.

Complementary 1P-time-resolved fluorescence measurements were conducted using 480 nm excitation to enhance the time resolution and signal-to-noise ratio, as detailed in Sections 2.4.2.2 and 2.4.3. Pseudo-single point, i.e., single-cell averaging, measurements were also carried out in some cases to enhance the signal-to-noise ratio and temporal resolution using 960 nm low repetition rate laser pulses scanned over the entire cell.

To probe lateral plasma membrane heterogeneity associated with IgE-FceRI stimulation, we also carried out time-resolved FRET (Lagerholm et al., 2005; Navratil et al., 2006; Silvius and Nabi, 2006; Suhling et al., 2005). Based on the spectral overlap between the membrane (diI-C<sub>18</sub>) and IgE (A488) labels, we examined the potential of these fluorophores to be a FRET donor-acceptor pair. Energy transfer experiments were
performed on cells labeled with A488-IgE (donor) in the absence or presence of diI-C$_{18}$ (acceptor), as described in Sections 2.4.2.3 and 2.4.3. A 690SP plus a 525/50 bandpass filter (Chroma) were used to isolate the emission of the donor (A488-IgE). Under these filter conditions, no signal was detected from acceptor (diI-C$_{18}$) only labeled cells. Here, the refractive index of the medium, $n$, is 1.4 (for biomolecules in aqueous solution (Lakowicz, 1999)) and the quantum yield of the donor, $Q_d$, is 0.6 (for A488) (Panchuk-Voloshina et al., 1999).

### 6.2.5 Fluorescence polarization anisotropy imaging

Steady-state imaging and time-resolved fluorescence anisotropy measurements were performed using 2P- and 1P-excitation, respectively, with data acquired at 0, 5, 10, 20 and 30 min after antigen stimulation (or control treatment). For steady-state 2P-anisotropy imaging, the parallel (0°) and perpendicular (90°) fluorescence polarizations were recorded simultaneously in two-channel, laser scanning mode (see Sections 2.5.1.3 and 2.5.3). For time-resolved fluorescence anisotropy, 1P-laser pulses were positioned on cell regions of interest, or a single cell was averaged via scanning 2P-FLIM, and the epi-fluorescence polarizations were resolved and detected simultaneously as described earlier (see Sections 2.5.2 and 2.5.3). Both polarization images and time-resolved fluorescence polarization decays were constructed using the SPC830 module, and the $G$-factor (0.74) was calculated using the tail matching approach (Hess et al., 2003; Lakowicz, 1999) to account for the detection efficiency polarization dependence (Lakowicz, 1999).
6.3 Results and discussion

Antigen-stimulated FcεRI phosphorylation 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 (Sheets et al., 1999a). Cholesterol-rich membrane domains have also been shown to protect Lyn from tyrosine phosphatases, thereby enhancing its activity (Young et al., 2003). The first indication IgE-FcεRI association with low-density cholesterol-enriched membrane domains upon cross-linking was provided by sucrose gradient ultracentrifugation experiments on RBL cells lysed with Triton X-100 detergent (Field et al., 1995). Further, methyl-β-cyclodextrin cholesterol depletion was shown to reduce the degree of stimulated FcεRI tyrosine phosphorylation as assessed by immunoblotting (Sheets et al., 1999a). When suspended mast cells are cross-linked with secondary antibody at 4°C, a fluorescent lipid analog, which partitions preferentially into cholesterol-rich regions, colocalizes with fluorescently labeled IgE-FcεRI into micron-sized patches, as observed using conventional optical microscopy (Chapter 4; Davey et al., 2007; Thomas et al., 1994).

However, imaging cholesterol-rich domains in vivo, without substantial non-physiological perturbation (i.e., low temperatures or extensive IgE-FcεRI cross-linking (Chapter 4; Davey et al., 2007; Holowka et al., 2000; Sheets et al., 1999a; Thomas et al., 1994)) has remained a challenge. As a result, little direct information about the formation and chemical and physical nature of these domains within complex biomembranes is known, and their functional role in signaling is poorly understood and speculative at best. Such difficulty in characterizing these domains arises from their perceived small size and
transient nature, as well as the inherent diffraction-limited resolution of optical microscopy.

To circumvent the temporal resolution of translational diffusion-based spectroscopic techniques (µs–s) (Bacia et al., 2004; Dietrich et al., 2002; Pyenta et al., 2003; Sheets et al., 1997; Thomas et al., 1994), we used a fluorescence dynamics assay capable of probing molecular dynamics (fluorescence lifetime and rotational diffusion) on a ps–ns timescale to interrogate antigen-mediated changes in the membrane structure of RBL mast cells. This fluorescence dynamics assay was previously tested on massively cross-linked, suspended mast cells, demonstrating increased fluorescence lifetime and rotational diffusion of diI-C₁₈ and A488-IgE, and thus providing evidence for plasma membrane nanostructural changes within the non-physiologically induced microdomains (Davey et al., 2007).

### 6.3.1 Antigen-mediated changes in cell morphology and IgE distribution are demonstrated by confocal microscopy

We monitored cell morphology, IgE-FcεRI clustering and plasma membrane structural changes 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 adding soluble multivalent antigen to establish an initial baseline (0 min), and then subsequently imaged after the cells were stimulated for 5, 10, 20 and 30 min. Fig. 6.1 shows a representative time series, in which panel A depicts cell morphology while panels B and C show the corresponding diI-C₁₈ and A488-IgE-FcεRI fluorescence images, respectively. (Note that the fluorescence background
Figure 6.1. Confocal microscopy of adherent antigen stimulated mast cells labeled with dil-C$_{18}$ and A488-IgE. Antigen-mediated changes in morphology (A), lipid analog, dil-C$_{18}$, distribution (B), and A488-IgE-FceRI distribution (C) are monitored over 30 min of antigen stimulation at room temperature. No obvious dil-C$_{18}$ colocalization with cross-linked A488-IgE is observed. The crosshairs (and arrows) indicate the focused laser beam position for single point time-resolved fluorescence decay measurements (decays shown in Fig. 6.7, with corresponding data in Table 6.2). Bar, 20 µm.
observed only in Fig. 6.1B is due to diI-C\textsubscript{18} 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 (Holowka et al., 2000; Ortega et al., 1999).

IgE-Fc\textsubscript{ε}RI phosphorylation has been hypothesized to result from cross-linking-induced coalescence of nanoscopic, transient, thermodynamically unstable cholesterol-rich membrane domains (Sheets et al., 1999b) that are smaller than the diffraction limit of optical microscopy (~\(\lambda/2\)), thereby explaining the absence of clear diI-C\textsubscript{18}-labeled membrane domains (Fig. 6.1B) that colocalize with A488-IgE-Fc\textsubscript{ε}RI (Fig. 6.1C). This physiological observation contrasts with non-physiological studies on RBL cells at 4°C (Davey et al., 2007; Holowka et al., 2000; Pyenta et al., 2003; Sheets et al., 1999a; Thomas et al., 1994), plasma membrane blebs below 20°C (Baumgart et al., 2007) and model systems such as GUVs (Bacia et al., 2004; Bacia et al., 2005; Feigenson and Buboltz, 2001; Veatch et al., 2004), in which micron-sized domains were clearly observed. Although antigen-mediated changes in cell morphology and antigen-induced receptor cross-linking and their associated dynamics can easily be visualized with fluorescence microscopy (Fig. 6.1), putative sub-diffraction membrane nanostructural changes are not readily apparent in conventional imaging. To overcome this limitation, we performed excited-state dynamics experiments and 2P-FRET on diI-C\textsubscript{18} and/or A488-IgE labeled mast cells, which report on the immediate fluorophore environment (\(\leq 10\) nm) (Lakowicz, 1999).
6.3.2 Antigen-mediated IgE receptor cross-linking alters membrane nanostructure as reported by ultrafast excited-state dynamics

We investigated 2P-fluorescence lifetime and time-resolved FRET changes 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 2P-FLIM image acquisition time (~120 s) is on the same timescale as membrane ruffling, the inherent three-dimensional resolution of 2P-excitation and the photoselectivity associated 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 dil-C18 in RBL cells, which were dually labeled with dil-C18 and A488-IgE, were fit to a biexponential and are shown before and after antigen stimulation in Fig. 6.2A. Corresponding A488-IgE confocal images were sequentially recorded after each dil-C18 FLIM image to observe how IgE-FcεRI distribution changes correlate with membrane nanostructural changes, as reported by fluorescence lifetime (Fig. 6.2B). dil-C18 FLIM images reveal morphological changes similar to those observed with confocal microscopy (Fig. 6.1). Based on these images, three apparent dil-C18 populations can be readily identified in the corresponding pixel-lifetime histogram (Fig. 6.2C), with lifetime components centered at ~0.4 ns and ~1.4 ns, and a fraction at ~2.4 ns. The second (~1.4 ns) component undergoes the most notable shift upon antigen stimulation. The effects of membrane structure on dil-C18 isomerization, and thus
Figure 6.2. 2P-FLIM of dil-C\textsubscript{18} reveals nanostructural changes in the mast cell plasma membrane upon antigen stimulation. dil-C\textsubscript{18} 2P-FLIM imaging (A) shows antigen-stimulated changes in membrane nanostructure (binning = 3). Confocal imaging (B) demonstrates A488-IgE-FcεRI distribution with stimulation. When compared with dil-C\textsubscript{18} in the membrane, the background features in the dil-C\textsubscript{18} FLIM images have a distinctly shorter lifetime (~0.3 ns), which can be fit to a single exponential (Davey et al., 2007), confirming that these features are unincorporated free dil-C\textsubscript{18} aggregates on the coverslip surface. Total FLIM image histograms (C), 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 average lifetime distribution changes as a function of antigen stimulation. The most noteworthy average lifetime shift is between 0 and 5 min. Bar, 10 \(\mu\)m.
photophysics, likely result from increased local saturated and mono-unsaturated phospholipid concentrations (Sengupta et al., 2007a), or solvent protection by cross-linked membrane proteins and the underlying actin cytoskeleton (Holowka et al., 2005; Hullin-Matsuda and Kobayashi, 2007). The widths and amplitudes of pixel-lifetime histogram peaks vary with cell morphology and signal-to-noise, thus it is difficult to assign structural significance to such changes.

As a control to test whether phase-specific diI analogs are affected by IgE receptor cross-linking, cells labeled with shorter chain diI-C<sub>12</sub> (Fig. 6.3) or mono-unsaturated diI-C<sub>18</sub> (Δ<sup>9</sup>-diI-C<sub>18</sub>) (Fig. 6.4) and stimulated with antigen exhibited no substantial lifetime shifts over 30 min, as assessed by 2P-FLIM. diI-C<sub>12</sub> and Δ<sup>9</sup>-diI-C<sub>18</sub> preferentially partition into cholesterol-depleted areas of model and cell membranes, whereas diI-C<sub>18</sub> prefers cholesterol-rich regions. As an additional control to determine whether diI-C<sub>18</sub> lifetime depends upon IgE receptor cross-linking specifically, 2P-FLIM of cells labeled with diI-C<sub>18</sub> and not subsequently stimulated (Fig. 6.5) or activated in the absence of antigen with PMA, which bypasses the IgE signaling pathway and activates protein kinase C, also demonstrated no diI-C<sub>18</sub> average lifetime distribution changes (Fig. 6.6). (As with diI-C<sub>18</sub> at 0 min, before DNP-BSA stimulation, the various diI analogs, unstimulated and PMA controls still exhibit a major lifetime population at ~1.4 ns, under the same fitting conditions.) These controls indicate that the changes in diI-C<sub>18</sub> fluorescence lifetime, and thus membrane structure, specifically result from IgE receptor cross-linking via antigen, which leads to stimulation and its subsequent down regulation.

Complementary pseudo-single point time-resolved fluorescence studies (4.2 MHz laser pulses, λ<sub>ex</sub> = 960 nm) on RBL cells labeled with diI-C<sub>18</sub> (Fig. 6.7A; Table 6.2)
Figure 6.3. 2P-FLIM of dil-C₁₂ does not report nanostructural changes in the mast cell plasma membrane as a function of antigen stimulation. Cell morphology and dil-C₁₂ distribution are shown in bright field (A) and confocal (B) images, respectively. dil-C₁₂ 2P-FLIM imaging (C) shows no detectable antigen-stimulated changes in membrane nanostructure (binning = 3). Total FLIM image histograms (D), with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple) time points, clearly demonstrate that there are no notable average lifetime shifts. Bar, 20 µm.
Figure 6.4. 2P-FLIM of mono-unsaturated dil-C_{18} does not report nanostructural changes in the mast cell plasma membrane as a function of antigen stimulation. Cell morphology and Δ^9-diI-C_{18} distribution are shown respectively in bright field (A) and confocal (B) images. Δ^9-diI-C_{18} 2P-FLIM imaging (C) shows no detectable antigen-stimulated changes in membrane nanostructure (binning = 3). Total FLIM image histograms (D), with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple) time points, clearly demonstrate that there are no notable average lifetime shifts. Bar, 20 µm.
Figure 6.5. 2P-FLIM of diI-C$_{18}$ does not reveal nanostructural changes in the mast cell plasma membrane in the absence of DNP-BSA. Cell morphology and dil-C$_{18}$ distribution are shown in bright field (A) and confocal (B) images, respectively. dil-C$_{18}$ 2P-FLIM imaging (C) shows no major membrane structural changes when lifetime is monitored on the usual timescale, but without DNP-BSA addition (binning = 3). Total FLIM image histograms (D), with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple) time points, demonstrate no resolvable average lifetime shifts. Bar, 20 µm.
Figure 6.6. 2P-FLIM of diI-C\textsubscript{18} shows that there are no nanostructural changes in the mast cell plasma membrane when cells are activated with PMA in the absence of antigen. Cell morphology and diI-C\textsubscript{18} distribution are respectively shown in bright field (A) and confocal (B) images. diI-C\textsubscript{18} 2P-FLIM imaging (C) shows no notable membrane structural difference when lifetime is monitored after treatment with PMA, an activator of protein kinase C that stimulates actin polymerization similarly to antigen, yet IgE-FceRI remains monomeric (binning = 3). Total FLIM image histograms (D), with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple) time points, exhibit no distinguishable average lifetime distribution shifts. Bar, 20 µm.
Figure 6.7. Time-resolved fluorescence decays of diI-C$_{18}$ indicate variations in membrane and protein environment with antigen stimulation. diI-C$_{18}$ pseudo-single point time-resolved fluorescence decays (A), 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 single point measurements. Note that specific spatial information is lost due to the binning although overall changes that occur upon antigen stimulation are related. Table 6.2 shows data for several of these pseudo-single point time series, revealing transiently increasing diI-C$_{18}$ average fluorescence lifetime within 5 min of stimulation. Representative single point time-resolved diI-C$_{18}$ fluorescence decays (B) 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 focused laser beam location on the representative dually labeled cell is indicated by the arrow and crosshairs shown in Fig. 6.1. The average fluorescence lifetime transiently increases after 5 min. Statistics for several of these time series are shown in Table 6.2. The average lifetime ($\tau_{fl}$) from single point measurements (Table 6.2 and representative panel B) are plotted as a function of stimula-
tion time (C). The asterisk indicates $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests, or that $\tau_f$ is statistically significantly different from $\tau_f$ prior to antigen stimulation.
Table 6.2. Pseudo-single point and single point, time-resolved fluorescence lifetime of diI-C18 and A488-IgE in adherent mast cells.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$/ns</th>
<th>$\alpha_1$</th>
<th>$\tau_2$/ns</th>
<th>$\alpha_2$</th>
<th>$\tau_3$/ns</th>
<th>$\alpha_3$</th>
<th>$\tau_{fl}$/ns</th>
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<tbody>
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<td><strong>diI-C18</strong></td>
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<td>0 min (pre-stimulation)</td>
<td>0.05(1)$^a$</td>
<td>0.7(1)</td>
<td>0.39(2)</td>
<td>0.2(1)</td>
<td>1.7(2)</td>
<td>0.1(1)</td>
<td>0.26(2)</td>
<td>4</td>
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<tr>
<td>5 min</td>
<td>0.06(1)$^a$</td>
<td>0.6(1)</td>
<td>0.39(4)</td>
<td>0.3(1)</td>
<td>1.66(2)</td>
<td>0.1(1)</td>
<td>0.32(4)$^b$</td>
<td>4</td>
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<tr>
<td>10 min</td>
<td>0.048(7)$^a$</td>
<td>0.7(1)</td>
<td>0.42(7)</td>
<td>0.2(1)</td>
<td>1.85(8)</td>
<td>0.1(1)</td>
<td>0.28(6)</td>
<td>4</td>
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<td>20 min</td>
<td>0.06(1)$^a$</td>
<td>0.6(1)</td>
<td>0.45(3)$^b$</td>
<td>0.3(1)</td>
<td>2.0(2)</td>
<td>0.1(1)</td>
<td>0.36(9)</td>
<td>4</td>
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<td>30 min</td>
<td>0.06(2)$^a$</td>
<td>0.6(1)</td>
<td>0.41(6)</td>
<td>0.3(1)</td>
<td>1.9(4)</td>
<td>0.1(1)</td>
<td>0.38(8)</td>
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<td>0 min (pre-stimulation)</td>
<td>0.4(2)</td>
<td>0.4(1)</td>
<td>1.1(3)</td>
<td>0.5(1)</td>
<td>2.8(4)</td>
<td>0.1(1)</td>
<td>1.1(1)</td>
<td>5</td>
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<td>5 min</td>
<td>0.8(2)$^b$</td>
<td>0.5(1)</td>
<td>1.4(3)</td>
<td>0.4(1)</td>
<td>3.2(6)</td>
<td>0.1(1)</td>
<td>1.25(9)$^b$</td>
<td>5</td>
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<td>10 min</td>
<td>0.7(2)</td>
<td>0.4(1)</td>
<td>1.2(1)</td>
<td>0.5(1)</td>
<td>2.7(3)</td>
<td>0.1(1)</td>
<td>1.2(1)</td>
<td>5</td>
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<tr>
<td>20 min</td>
<td>0.8(2)$^b$</td>
<td>0.6(1)</td>
<td>1.3(3)</td>
<td>0.3(1)</td>
<td>3.0(6)</td>
<td>0.1(1)</td>
<td>1.2(1)</td>
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<td>30 min</td>
<td>0.8(1)$^b$</td>
<td>0.5(1)</td>
<td>1.2(2)</td>
<td>0.4(1)</td>
<td>2.9(9)</td>
<td>0.1(1)</td>
<td>1.16(8)</td>
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<td><strong>A488-IgE in the absence of diI-C18</strong></td>
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<td>0 min (pre-stimulation)</td>
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<td>1.4(4)</td>
<td>0.3(1)</td>
<td>3.50(6)</td>
<td>0.7(1)</td>
<td>2.94(5)</td>
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<td>1.3(2)</td>
<td>0.3(1)</td>
<td>3.3(4)</td>
<td>0.7(1)</td>
<td>2.7(1)$^b$</td>
<td>5</td>
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<td>10 min</td>
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<td>–</td>
<td>1.3(4)</td>
<td>0.3(1)</td>
<td>3.1(3)$^b$</td>
<td>0.7(1)</td>
<td>2.5(2)$^b$</td>
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<td>20 min</td>
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<td>–</td>
<td>0.7(3)$^b$</td>
<td>0.3(1)</td>
<td>3.1(3)$^b$</td>
<td>0.7(1)</td>
<td>2.4(2)$^b$</td>
<td>5</td>
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<td>30 min</td>
<td>–</td>
<td>–</td>
<td>0.9(2)$^b$</td>
<td>0.3(1)</td>
<td>3.0(2)$^b$</td>
<td>0.7(1)</td>
<td>2.4(1)$^b$</td>
<td>5</td>
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<td>Time (min)</td>
<td>0 min (pre-stimulation)</td>
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<td>A488-IgE in presence of diI-C&lt;sub&gt;18&lt;/sub&gt;</td>
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<td>0 min</td>
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<td>0.9(4)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3(1)</td>
<td>3.0(1)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>2.41(7)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>5 min</td>
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<td>0.5(2)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3(1)</td>
<td>2.8(2)</td>
<td>0.7(1)</td>
<td>2.1(1)&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>—</td>
<td>0.7(3)</td>
<td>0.3(1)</td>
<td>2.9(3)</td>
<td>0.7(1)</td>
<td>2.15(1)&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>—</td>
<td>0.5(1)</td>
<td>0.3(1)</td>
<td>2.6(2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7(1)</td>
<td>2.14(8)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>—</td>
<td>0.9(3)</td>
<td>0.3(1)</td>
<td>2.8(1)</td>
<td>0.7(1)</td>
<td>2.19(5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The very short time constant (~60 ps) for diI-C<sub>18</sub> pseudo-single point magic angle decays is due to aggregated free dye on the coverslip being scanned along with the cell. <sup>b</sup><sub>p</sub> < 0.05, as determined from unpaired, two-tailed Student’s <sup>t</sup>-tests. For decays of either diI-C<sub>18</sub> (<i>n</i> = 5 or <i>n</i> = 4), A488-IgE (<i>n</i> = 5) or A488-IgE in the presence of diI-C<sub>18</sub> (<i>n</i> = 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. <sup>c</sup><sub>p</sub> < 0.05, as determined from unpaired, two-tailed Student’s <sup>t</sup>-tests. For A488-IgE decays, this indicates that the mean of the given fit parameter is statistically significantly different in the presence of diI-C<sub>18</sub>, versus in the absence of diI-C<sub>18</sub>, at the same time point of stimulation.
reveal a statistically significant diI-C_{18} average fluorescence lifetime increase 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 6.2). The very short time constant (\(\tau_1 \sim 60\) ps) does not vary considerably with antigen stimulation and is likely due to aggregated, free diI-C_{18} on the coverslip. Note that the pseudo-single point lifetime data shown in Table 6.2 for diI-C_{18} 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-C_{18} labeled cells (Table 6.2 and Fig. 6.7B) were carried out to confirm 2P-FLIM and pseudo-single point experiment results and reveal that diI-C_{18} fluorescence decays triexponentially. Compared with results prior to antigen stimulation \((\tau_\beta = 1.1 \pm 0.1\) ns), diI-C_{18} average fluorescence lifetime increases significantly (as determined by unpaired, two-tailed Student’s \(t\)-tests) within the first 5 min after stimulation, with \(\tau_\beta = 1.25 \pm 0.09\) ns \((p = 0.035; n = 5)\). As signaling continues, \(\tau_\beta\) returns to its original value for the experimental duration (Fig. 6.7C). Further, the single point lifetime data shown in Table 6.2 for diI-C_{18} labeled cells at 0 min \((n = 5)\) agree with additional data from cells that were not subsequently stimulated \((n = 25;\) data not shown).

As further support that the significantly increased diI-C_{18} lifetime at 5 min is distinctively due to IgE receptor cross-linking, parallel 1P-single point lifetime experiments were performed on diI-C_{18} labeled cells not stimulated with antigen (Fig. 6.8) and cells activated with PMA (Fig. 6.9). In contrast to antigen-stimulated cells, cells that are not stimulated with DNP-BSA exhibit little ruffling (Fig. 6.8A, B and C) and no IgE clustering (Fig. 6.8C). Appropriately, PMA-treated cells ruffle due to actin
Figure 6.8. Time-resolved fluorescence decays of diI-C$_{18}$ reveal no increase in average lifetime in the absence of DNP-BSA. The location of the focused laser beam on the dually labeled cell is indicated by the arrow and crosshairs (A-C). Although DNP-BSA addition is omitted, diI-C$_{18}$ single point fluorescence decays (D) are displayed as a function of time (0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple)), and the average fluorescence lifetime is unchanging. Bar, 20 µm.
Figure 6.9. Time-resolved fluorescence decays of diI-C$_{18}$ exhibit no increase in average lifetime when cells are activated with PMA. The location of the focused laser beam on the dually labeled cell is indicated by the arrow and crosshairs in the upper image panels (A-C). diI-C$_{18}$ single point fluorescence decays (D) are displayed as a function of time after PMA activation (0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple)). The average fluorescence lifetime does not change with time. Bar, 20 µm.
polymerization (Fig. 6.9A, B and C), but IgE does not cluster because the IgE receptor signaling pathway is circumvented (Fig. 6.9C). The lifetime components obtained from fitting the decays in Figs. 6.8D and 6.9D are on the order of those for diL-C18 in antigen-stimulated cells (see Table 6.2), and in either case, there is no significant change in the average lifetime (or any of the lifetime components) over the 30 min time period ($n = 5$ for both). For example, at 5 min after PMA treatment, diL-C18 decays triexponentially ($\tau_1 = 0.9 \pm 0.2$ ns, $\alpha_1 = 0.5 \pm 0.1$, $\tau_2 = 1.2 \pm 0.2$ ns, $\alpha_2 = 0.4 \pm 0.1$, $\tau_3 = 2.6 \pm 0.4$ ns, $\alpha_3 = 0.1 \pm 0.1$, with $\tau_\rho = 1.1 \pm 0.2$ ns ($n = 5$)) and the fit parameters are statistically the same ($p > 0.05$) at all other time points monitored (data not shown).

6.3.3 Stimulated tyrosine phosphorylation of IgE receptor supports fluorescence dynamics observations

The functional relevance of the transient diL-C18 lifetime increase was examined by following the time course of tyrosine phosphorylation of cells stimulated under the same conditions as the micro-spectroscopy experiments (1 $\mu$g/mL antigen, $\sim$20°C). The first identifiable biochemical step that occurs upon IgE-FcεRI cross-linking with multivalent antigen is the phosphorylation of the FcεRI $\beta$ and $\gamma$ subunits by the tyrosine kinase Lyn (Sheets et al., 1999a). Because our experiments are performed at room temperature, as compared to 37°C, the signaling rate is reduced. 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 DNP-BSA concentration used in these studies (Holowka et al., 2000; Sheets et al., 1999a). Although ongoing IgE-FcεRI cross-linking continues throughout the experiment,
Figure 6.10. Antigen stimulation kinetics assessed by immunoblotting agree with plasma membrane nanostructural changes. RBL cells are stimulated with antigen under the same conditions as the micro-spectroscopy experiments (1 µg/mL DNP-BSA, ~20°C). The representative anti-phosphotyrosine immunoblot (A) shows stimulated \textit{in vivo} tyrosine phosphorylation, and the band corresponding to the FcεRI β subunit (Sheets et al., 1999a) is indicated. The level of FcεRI β tyrosine phosphorylation for the immunoblot shown in panel A is quantified using densitometry (B).
its phosphorylation decreases with time as the receptors compete for limited amounts of Lyn (Xu et al., 1998a), and as stimulated actin polymerization separates Lyn from IgE-FcεRI as signaling proceeds (Frigeri and Apgar, 1999; Holowka et al., 2000), resulting in downregulation.

Fig. 6.10A shows a representative immunoblot \((n = 4)\) of time-dependent cell stimulation, and Fig. 6.10B provides the corresponding densiometric trace for FcεRI β phosphorylation. Under these conditions, phosphorylation is maximal at 5 min and remains sustained for the experimental duration. The phosphorylation kinetics agree with the kinetics of diI-C\(_{18}\) fluorescence lifetime changes (Fig. 6.7C) and further strengthen the relevance of the lifetime results.

6.3.4 Time-resolved FRET between A488-IgE and diI-C\(_{18}\)-labeled membrane reveals lateral, nanoscale heterogeneity associated with antigen stimulation

To examine the potential interaction between ordered membrane domains (diI-C\(_{18}\)) 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 overcome the spatial limitation of conventional optical microscopy \((\lambda / 2)\) by probing nanoscale (0.1–10 nm) lateral membrane heterogeneities in real time, lending insight into the transient nature of IgE-FcεRI associations with cholesterol-rich nanodomains that may act as signaling platforms. Recently, using steady-state FRET between carbocyanine probes that preferentially partition into the liquid-ordered \((L_o)\) phase based on their alkyl chains, Sengupta et al. demonstrated nanoscale lipid heterogeneity in the RBL cell plasma membrane that was enhanced by non-physiological IgE-FcεRI cross-linking (Sengupta et al., 2007b).
Figure 6.11. Steady-state absorption and emission spectra of A488 and diI-C\textsubscript{18} indicate energy transfer potential. The absorption (1) and emission (2) of A488-IgE (donor) in PBS and the absorption (3) and emission (4) of diI-C\textsubscript{18} (acceptor) in DMSO are shown. Cyanine dyes, other than diI-C\textsubscript{18}, have exhibited negative solvatochromism, or a spectral red-shift in nonpolar solvents (Hubener et al., 2003); thus, we measured the absorption and emission of diI-C\textsubscript{18} in DMSO rather than methanol. The structure of A488 is shown to the left of its spectra, while the structure of diI-C\textsubscript{18}, used to label the plasma membrane, is shown to the right.
Steady-state spectroscopy of A488-IgE and diI-C18 (Fig. 6.11) indicated the potential of these fluorescent labels as a donor and acceptor, respectively, in a FRET pair. Based on the spectral overlap of the donor emission and acceptor absorption, the estimated Förster distance ($R_0$) was calculated as $3.33 \pm 0.04$ nm. Using spectrophotometric resonance energy transfer methods on RBL cell-derived membrane vesicles (i.e., ensemble averaging in a cuvette), Baird and Holowka (1985) determined distances between various IgE regions and the outer cell membrane surface ranging from $\sim 4.5$–$10$ nm. Their findings, in combination with the $R_0$ obtained here ($3.33 \pm 0.04$ nm), suggested that time-resolved energy transfer would be feasible between A488-IgE and diI-C18.

We measured A488-IgE fluorescence lifetime in RBL cells as a function of antigen stimulation, in the presence and absence of diI-C18 (acceptor). Using pseudo-single point measurements, the A488-IgE excited-state fluorescence lifetime was reduced in the presence of diI-C18, versus 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. 6.12A, and the lifetimes ($\tau_i$) and amplitudes ($\alpha_i$) of all decay components are summarized in Table 6.2. In unstimulated cells labeled solely with A488-IgE, $\tau_\beta$ is $2.94 \pm 0.05$ ns ($n = 5$) as compared with $2.41 \pm 0.07$ ns ($p = 2.73 \times 10^{-7}$) in the presence of diI-C18 ($n = 6$).

Using higher time resolution (4 ns timescale), the A488-IgE $\tau_\beta$ reduction in the presence of acceptor is further emphasized (data not shown). Based on the measured
Figure 6.12. Time-resolved fluorescence decays of A488-IgE, in the absence and presence of diI-C18, indicate that the probes interact transiently when mast cells are stimulated with antigen. A488-IgE (donor) pseudo-single point fluorescence (A) decays more rapidly in the presence of diI-C18 (acceptor) (2) as compared to in the absence of acceptor (1). The representative decays were acquired prior to antigen stimulation, however A488-IgE fluorescence decays faster in the presence of diI-C18 at all time points of stimulation (see Table 6.2). The energy transfer efficiency ($E_T$) is plotted as a function of stimulation time (B) to show that the probes experience maximal association 5 min after stimulation, corresponding to the largest energy transfer efficiency increase (from $0.18 \pm 0.01$ before stimulation to $0.22 \pm 0.01$ at 5 min). $E_T$ standard deviation is determined by the error in $\tau_{DA}$ ($n = 6$; Table 6.2) and $\tau_D$ ($n = 5$; Table 6.2). The asterisk indicates $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests, or that $E_T$ is statistically significantly different from $E_T$ prior to antigen stimulation.
Table 6.3. Fluorescence resonance energy transfer parameters obtained from steady-state spectrophotometry of diI-C₁₈ and A488-IgE and pseudo-single point, time-resolved fluorescence lifetime of A488-IgE, in the absence and presence of diI-C₁₈, in adherent mast cells.

<table>
<thead>
<tr>
<th></th>
<th>$E^a_T$</th>
<th>$R$/nm</th>
<th>$k_{\text{FRET}}$/ns⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>0.18(1)</td>
<td>4.3(1)</td>
<td>0.07(2)</td>
</tr>
<tr>
<td>5 min</td>
<td>0.22(1)ᵇ</td>
<td>4.1(1)</td>
<td>0.10(2)</td>
</tr>
<tr>
<td>10 min</td>
<td>0.14(4)</td>
<td>4.5(4)</td>
<td>0.06(5)</td>
</tr>
<tr>
<td>20 min</td>
<td>0.11(4)ᵇ</td>
<td>4.7(5)</td>
<td>0.04(5)</td>
</tr>
<tr>
<td>30 min</td>
<td>0.09(1)ᵇ</td>
<td>4.9(1)ᵇ</td>
<td>0.03(1)ᵇ</td>
</tr>
</tbody>
</table>

ᵃ The donor (A488-IgE) fluorescence lifetime in the presence of acceptor (diI-C₁₈) ($\tau_{DA}$) and the donor fluorescence lifetime in the absence of acceptor ($\tau_D$), used in the energy transfer efficiency ($E_T$) calculation, were taken from the Table 6.2 columns beginning with $2.41 \pm 0.07$ ns and $2.94 \pm 0.05$ ns, respectively. $R_0$ was calculated as $3.33 \pm 0.04$ nm and used to determine $R$, as described in Section 2.4.3. ᵇ $p < 0.05$ was determined from unpaired, two-tailed Student’s $t$ tests, indicating that the mean is statistically significant different from the corresponding mean prior to antigen stimulation.
donor (A488-IgE) fluorescence lifetime reduction in resting cells (i.e., prior to stimulation) in the presence or absence of acceptor (diI-C18), the estimated $E_T$, $R$, and $k_{FRET}$ are $0.18 \pm 0.01$, $4.3 \pm 0.1$ nm, and $0.07 \pm 0.02$ ns$^{-1}$, respectively (Table 6.3). Mathematical modeling studies of FRET data and electron microscopy images from cell membranes imply that functional membrane domains are $\sim$5–20 nm in diameter (Shaw, 2006), which is on the approximate order of the donor-acceptor separation distance obtained prior to stimulation (i.e., $R = 4.3 \pm 0.1$ nm).

Following antigen stimulation, the donor (A488-IgE) $\tau_\beta$ reduction is significant in the presence of diI-C18, going from $2.7 \pm 0.1$ ns to $2.1 \pm 0.1$ ns ($p = 8.88 \times 10^{-4}$) at 5 min of stimulation, and from $2.5 \pm 0.2$ ns to $2.15 \pm 0.01$ ns ($p = 5.93 \times 10^{-3}$) at 10 min (Table 6.2). By 20 min, the A488-IgE average lifetime in the presence of diI-C18 is no longer statistically significantly different from that with A488-IgE alone. Interestingly, $\tau_\beta$ decreases significantly between 0 min and 5 min, both for cells labeled only with A488-IgE ($2.94 \pm 0.05$ ns at 0 min and $2.7 \pm 0.1$ ns [$p = 5.52 \times 10^{-3}$] at 5 min) and for those dually labeled with A488-IgE and diI-C18 ($2.41 \pm 0.07$ ns at 0 min and $2.1 \pm 0.1$ ns [$p = 3.16 \times 10^{-3}$] at 5 min) (Table 6.2). This trend of decreasing $\tau_\beta$ 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 A488-IgE 2P-FLIM images are fit under moderate binning conditions to improve histogram signal-to-noise and better resolve lifetime components, the pixel image histograms reveal decreased $\tau_\beta$ by 5 min and at all subsequent time points relative to 0 min (Fig. 6.13). (At low binning, there were no resolvable, significant A488-IgE $\tau_\beta$ shifts with antigen
Figure 6.13. 2P-FLIM of A488-IgE suggests protein environmental changes during antigen-mediated stimulation. A488-IgE 2P-FLIM images (A) and the corresponding histograms (B), with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red) and 30 min (purple) time points, are shown to demonstrate that the A488-IgE average lifetime components change slightly as stimulation proceeds. To enhance the histogram signal-to-noise and better resolve lifetime components, the A488-IgE 2P-FLIM images were fit with binning = 5. Bar, 20 µm.
stimulation; data not shown.) The major $\tau_\text{p}$ peak at $\sim3.0$ ns (Fig. 6.13) further agrees with $\tau_\text{p}$ acquired via pseudo-single point measurements (Table 6.2).

The modest antigen-mediated changes in average A488-IgE fluorescence lifetime observed with 2P-FLIM can be attributed to the fact that DNP-BSA binding to IgE-FcεRI follows a transient hapten exposure model (Holowka et al., 2007; Xu et al., 1998a), in which the antigen and antibody interact at fast on/off rates. A488-IgE populations that are variously bound by DNP-BSA are suggested by the multi-Gaussian pixel-lifetime histogram fits from total FLIM images (Fig. 6.13). At various DNP-BSA stimulation time points, the A488-IgE average lifetimes obtained with 2P-FLIM (Fig. 6.13) are longer than what we observed in suspended cells that were extensively cross-linked with a secondary antibody ($\tau_\text{p} = 0.9$ ns and 1.35 ns (Davey et al., 2007)). 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 cross-linked suspended cells (Davey et al., 2007). Notably, using either 2P-FLIM or single point lifetime methods, the A488-IgE average fluorescence lifetime ($\tau_\text{p}$) decreases within the first 5 min of antigen stimulation, in direct contrast with the transiently increasing diI-C₁₈ lifetime, and may be explained by increased environmental disorder due to presenting multivalent antigen to IgE-FcεRI.
When we calculate $E_T$ for dually labeled cells as a function of stimulation time (Fig. 6.12B), we find that the FRET efficiency increases from $0.18 \pm 0.01$ prior to stimulation to $0.22 \pm 0.01$ after 5 min of stimulation, followed by a gradual decrease with $0.14 \pm 0.04$ at 10 min, $0.11 \pm 0.04$ at 20 min and $0.09 \pm 0.01$ at 30 min (see Table 6.3 and Fig. 6.12B). These data show that A488-IgE and diI-C$_{18}$ experience maximal association 5 min after stimulation begins, as indicated by the significant ($p = 8.05 \times 10^{-3}$) energy transfer efficiency increase and also by the A488-IgE rotational correlation time decrease in the presence of diI-C$_{18}$ between 0 min and 5 min (see Fig. 6.14; Table 6.4). The time of maximal FRET agrees with maximal FcεRI tyrosine phosphorylation 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. (2000), the ultrafast time resolution of our FRET studies provides an advantage for resolving sub-diffraction association of various molecules (e.g., IgE-FcεRI) with cholesterol-rich domains (Varma and Mayor, 1998).

Relative to 0 min, the reduced FRET efficiency ($E_T$) at 20 min post-stimulation (Table 6.3) indicates reduced lateral interactions between IgE-FcεRI and diI-C$_{18}$-labeled membranes and may be related to the hypothesis that actin microfilaments help to separate FcεRI from ordered membrane domains (Frigeri and Apgar, 1999; Holowka et al., 2000), resulting in signaling downregulation. Antigen-induced IgE-FcεRI cross-linking at 37°C results in endocytosis of ~50% of receptor complexes, with a half-time of 5 min (Furuichi et al., 1984; Xu et al., 1998b), so it is unlikely that the reduced FRET observed here at 20 min is due to IgE-FcεRI internalization, even with its much slower
room temperature kinetics. Moreover, if IgE-FcεRI is internalized, reduced FRET suggests that diI-containing domains are not co-internalized with IgE receptor, and we are currently investigating this possibility. The FRET results, together with the diI-C\textsubscript{18} lifetime results, support the hypothesis that cross-linked IgE-FcεRI transiently translocates into membrane regions with greater nanostructural order to facilitate signaling; however, our results do not rule out the possibility that receptor engagement may recruit functional lipid nanodomains (Shaw, 2006).

From the acceptor perspective of FRET, we would expect diI-C\textsubscript{18} fluorescence to increase at a rate of $k_{\text{FRET}}$ and decay with a time constant corresponding to the diI-C\textsubscript{18} excited-state lifetime(s) (Lakowicz, 1999). To directly measure $k_{\text{FRET}}$, we measured the diI-C\textsubscript{18} fluorescence decay 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 system response FWHM) without a rise, in either the presence ($n = 8$; data not shown) or absence ($n = 5$; Table 6.2) of donor. These results may be attributed to the presence of a large acceptor population thereby causing the emission from directly-excited diI-C\textsubscript{18} to surpass the emission due to FRET. Another possibility is that $k_{\text{FRET}}$ is much faster than our temporal resolution. In our FRET studies, we assume that the donor and acceptor dipole moments are fully randomized and thus an orientation parameter ($\kappa^2$) 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.
6.3.5 Antigen-mediated variations in membrane and protein order are probed by fluorescence polarization anisotropy

To assess further whether antigen-induced IgE-FceRI cross-linking 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 as a function of stimulation time (0, 10, 20 and 30 min) reveal that diI-C₁₈ averaged initial anisotropy ($r_0$), or dipole-moment distribution, does not change as a result of stimulation or the accompanying morphological changes ($n = 7$, data not shown). Perhaps this is not surprising considering that there are no optically resolvable membrane domains. Similarly, there was little difference in A488-IgE averaged $r_0$ over the entire cell, although individual A488-IgE-FceRI puncta that form as a result of ongoing cross-linking do have higher initial anisotropies than uncross-linked A488-IgE-FceRI ($n = 9$, 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, probe motions, which could act as a fluorescence depolarization mechanism. Cell morphological changes, 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, and these may be reflected in the pixel-to-pixel anisotropy variations that we observed during cell stimulation. We are currently developing image processing algorithms to further quantify these possible variations.

Rotational diffusion of molecular dipoles directly probes fast conformational changes and possible restricting effects of the surrounding environment. Further, these
Table 6.4. Pseudo-single point and single point, time-resolved fluorescence polarization anisotropy of diI-C$_{18}$ and A488-IgE in adherent mast cells.

<table>
<thead>
<tr>
<th></th>
<th>$\phi_t$/ns</th>
<th>$\beta_t$</th>
<th>$r_\infty$</th>
<th>$r_0$</th>
<th>$r_\infty/r_0$</th>
<th>$n$</th>
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</thead>
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<tr>
<td><strong>diI-C$_{18}$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single point:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 min (pre-stimulation)</td>
<td>0.65(9)</td>
<td>0.04(2)</td>
<td>0.18(6)</td>
<td>0.22(5)</td>
<td>0.8(1)</td>
<td>8</td>
</tr>
<tr>
<td><strong>A488-IgE</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Pseudo-single point:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min (pre-stimulation)</td>
<td>0.6(1)</td>
<td>0.17(3)</td>
<td>0.11(1)</td>
<td>0.28(2)</td>
<td>0.40(7)</td>
<td>7</td>
</tr>
<tr>
<td>5 min</td>
<td>0.6(2)</td>
<td>0.14(2)</td>
<td>0.12(2)</td>
<td>0.26(1)</td>
<td>0.46(9)</td>
<td>4</td>
</tr>
<tr>
<td>10 min</td>
<td>0.6(2)</td>
<td>0.17(4)</td>
<td>0.11(2)</td>
<td>0.28(2)</td>
<td>0.4(1)</td>
<td>4</td>
</tr>
<tr>
<td>20 min</td>
<td>0.7(3)</td>
<td>0.17(3)</td>
<td>0.11(2)</td>
<td>0.28(4)</td>
<td>0.40(4)</td>
<td>4</td>
</tr>
<tr>
<td>30 min</td>
<td>0.5(2)</td>
<td>0.16(4)</td>
<td>0.14(4)</td>
<td>0.30(3)</td>
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<tr>
<td><strong>A488-IgE in presence of diI-C$_{18}$</strong></td>
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<tr>
<td>Pseudo-single point:</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 min (pre-stimulation)</td>
<td>0.8(2)$^a$</td>
<td>0.18(3)</td>
<td>0.13(3)</td>
<td>0.31(5)</td>
<td>0.4(1)</td>
<td>7</td>
</tr>
<tr>
<td>5 min</td>
<td>0.61(1)$^b$</td>
<td>0.19(1)$^a$</td>
<td>0.11(1)</td>
<td>0.30(1)$^a$</td>
<td>0.36(4)</td>
<td>3</td>
</tr>
<tr>
<td>10 min</td>
<td>0.6(1)</td>
<td>0.1(1)</td>
<td>0.13(1)</td>
<td>0.2(1)</td>
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<td>20 min</td>
<td>0.50(8)</td>
<td>0.15(1)</td>
<td>0.15(1)</td>
<td>0.30(1)</td>
<td>0.50(1)$^{ab}$</td>
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<td>30 min</td>
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<td>0.17(4)</td>
<td>0.12(3)</td>
<td>0.29(6)</td>
<td>0.43(1)</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$ $p < 0.05$, 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-C$_{18}$, as compared to in the absence of diI-C$_{18}$, at the same time point of stimulation.

$^b$ $p < 0.05$, 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.
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-C$_{18}$ labeled cells (Table 6.4) 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 interpretation of diI-C$_{18}$ 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-C$_{18}$ decays as a single exponential ($\phi_1 = 0.65 \pm 0.09$ ns, $\beta_1 = 0.04 \pm 0.02$), with an additional residual anisotropy component (Table 6.4). The fast rotational correlation time reflects rapid depolarizations within our observation timescale, suggesting temporal dipole-moment randomization of diI-C$_{18}$ as a FRET acceptor. The degree of orientational constraint ($r_\infty / r_0 = 0.8 \pm 0.1$) is high because diI-C$_{18}$ is intercalated in the membrane.

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 (Davey et al., 2007), suggesting that lipid in adherent cell membranes may have an intrinsically higher packing order than in spherical, suspended cell membranes. Collectively, these diI-C$_{18}$ 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 (2004). It is possible that the longer diI-C$_{18}$ fluorescence and rotational dynamics observed in stimulated adherent cells, as compared to cross-linked suspended
cells (Davey et al., 2007), result from greater restriction due to diI-C$_{18}$ confinement into many nanoscopic domains with higher line tension (London, 2005) than the large micro-domains of suspended cells.

The fast A488-IgE rotational correlation time at all time points of stimulation (Table 6.4), in combination with that of diI-C$_{18}$, validates the randomization assumption in our FRET calculations (i.e., $\kappa^2 = 2/3$). Prior to antigen stimulation, A488-IgE 2P-pseudo-single point anisotropy decays are somewhat slower in the presence of diI-C$_{18}$ (Fig. 6.14). Upon stimulation, the rotational correlation time of A488-IgE in the presence of diI-C$_{18}$ decreases by 5 min (as determined by unpaired, two-tailed Student’s $t$-tests), indicating enhanced donor-acceptor association (Sharma et al., 2004) (Fig. 6.14). 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-C$_{18}$ in the membrane. Single point anisotropy decays of free fluorophores in solution (data not shown) generated fitting parameters that agree with Davey et al. (2007), verifying the consistency in our experimental setup.
Figure 6.14. Time-resolved fluorescence polarization anisotropy decays of A488-IgE in the absence and presence of diI-C_{18} demonstrate FRET sensitivity. 2P-pseudo-single point anisotropy decays prior to antigen stimulation (A) indicate that A488-IgE (donor) exhibits slower decays, and thus longer rotational correlation times in the presence of diI-C_{18} (acceptor) (1), versus in the absence (2) of diI-C_{18}. In the presence of diI-C_{18} (B), A488-IgE anisotropy decays faster by 5 min of antigen stimulation (4), as compared to 0 min (3), indicating increased donor-acceptor association upon stimulation.
6.4 Conclusions

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-FcεRI cross-linking and reorganization that occur on the seconds timescale (Fig. 6.1). Despite the lack of visible domain formation during the antigen stimulation course or colocalization between diI and the IgE receptor, diI-C18 fluorescence lifetime imaging reveals plasma membrane nanostructural changes as a function of antigen-stimulation time (Figs. 6.2 and 6.7; Table 6.2). The appropriate controls demonstrate that a $L_o$-specific diI analog is required for reporting antigen stimulation-dependent lifetime changes (Figs. 6.3 and 6.4) and that diI-C18 lifetime depends specifically on IgE receptor cross-linking via multivalent antigen (Figs. 6.5, 6.6, 6.8 and 6.9). The observed diI-C18 lifetime changes also correlate with the kinetics of the earliest biochemical step in the IgE receptor signaling pathway—that is, stimulated FcεRI tyrosine phosphorylation as assessed by immunoblotting (Fig. 6.10). These results suggest IgE-FcεRI stimulation-dependent membrane nanostructural changes at early times of the signaling process, which is further supported by the transient increase in FRET between A488-IgE-FcεRI and diI-C18 within 0.1–10 nm spatial resolution (Fig. 6.12; Table 6.3). The results presented in this Chapter suggest that IgE-FcεRI is recruited into transient, ordered membrane domains that serve as IgE-FcεRI signaling platforms. Furthermore, these studies offer an experimental approach that is applicable to other signaling pathways, including the related T cell and B cell immunoreceptors that depend upon localized membrane ordering.
7. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this Dissertation, I have described studies of the structure, dynamics and composition of lipid membranes and their role in immunoreceptor signaling. Membrane structure and dynamics were assessed using a novel ultrafast fluorescence dynamics imaging assay that includes fluorescence lifetime and polarization anisotropy imaging, as well as time-resolved FRET. The membrane composition of RBL mast cells was also interrogated using ToF-SIMS imaging.

The sensitivity of ultrafast excited-state fluorescence dynamics methods to phase was initially tested using SUVs and supported planar lipid bilayers composed of simple lipid compositions and labeled with a fluorescent phospholipid analog. Fluorescence lifetime, steady-state structural order (anisotropy), and rotational dynamics of the lipid probe reported the membrane phase, with longer lifetime, higher order and longer overall rotational correlation time in the gel phase as compared to the fluid, or liquid-disordered, phase. Bilayer data agreed with values and trends described in the literature (Araiso and Koyama, 1995; Gidwani et al., 2001) for SUVs analyzed via traditional spectrofluorimetric lifetime and anisotropy experiments, with the ultrafast fluorescence dynamics methods offering the advantage of location-specific spatio-temporal dynamics (Chapter 3).

Information gained from model membrane studies was subsequently related to the structure and formation dynamics of cholesterol-enriched plasma membrane domains in individual, live RBL-2H3 mast cells, and thus to their biological function in IgE receptor signaling. Cells were extensively cross-linked with α-IgE at 4°C to form micron-sized domains of fluorescently labeled, cross-linked IgE (A488-IgE-FcεRI) and the membrane-
incorporated, \( L_o \) preferring, probe diI-C\(_{18} \). diI-C\(_{18} \) exhibited an environmental change as it co-redistributed in the plasma membrane to the cross-linked A488-IgE-Fc\( \varepsilon \)RI domains, resulting in longer lifetime and higher anisotropy as compared to uncross-linked cells. The increase in diI-C\(_{18} \) lifetime is attributed to the fluorophore having fewer conformational degrees of freedom to relaxation from the excited state, due to a more restrictive, or ordered, environment. With cross-linking, the environment and dynamics of A488-IgE were also affected by increased protein-protein interactions, causing longer lifetime and higher anisotropy as compared to uncross-linked cells. These findings support the hypothesis that, upon IgE receptor cross-linking cholesterol-rich membrane domains serve as platforms for signaling, recruiting signaling proteins and lipids (Chapter 4).

In collaboration with the Winograd group (Penn State, Chemistry), ToF-SIMS imaging was used in an attempt to correlate the enhanced lipid ordering observed in the RBL cell plasma membrane upon IgE receptor cross-linking via fluorescence lifetime and polarization anisotropy with the lipid chemical identity and distribution in these regions. The sub-cellular distribution of several key lipids in individual extensively cross-linked and uncross-linked mammalian cells was observed. Unfortunately, current limitations in ToF-SIMS imaging preclude further detection of the subtle changes in lipid composition and distribution that occurs as a result of receptor crosslinking (Chapter 5).

Results on non-physiologically cross-linked mast cells provided a valuable context for interpreting ultrafast fluorescence dynamics studies on mast cells stimulated with antigen under more physiological conditions (~20\(^\circ\)C), where membrane domains are transient and smaller than the diffraction limit. Although no domains were visible under
these conditions, diI-C<sub>18</sub> fluorescence lifetime imaging revealed dynamic plasma membrane nanostructural changes as a function of antigen-stimulation time. diI-C<sub>18</sub> lifetime changes were specifically attributed to a liquid-ordered preferring diI analog and to IgE receptor cross-linking via multivalent antigen. These diI-C<sub>18</sub> lifetime changes were further correlated with stimulated IgE receptor phosphorylation kinetics as assessed by immunoblotting. Stimulation-dependent membrane nanostructure changes at early times of the signaling process were further supported by transiently increasing FRET between A488-IgE-FceRI and diI-C<sub>18</sub>. These results suggest that cross-linked IgE-FceRI is recruited transiently into ordered membrane domains that serve as signaling platforms. This work represents the first application of ultrafast dynamics methods to assess localized membrane ordering and membrane-protein relationships during physiological cell signaling (Chapter 6).

Taken together, this research has demonstrated that fluorescence lifetime and rotational mobility of a molecule are exquisitely sensitive to the immediate membrane environment, even when domain formation is not optically resolvable. Thus, fluorescence lifetime and anisotropy methods offer an opportunity to follow the real-time, molecular-level, spatio-temporal dynamics of local heterogeneities or cholesterol-rich domains found in biological membranes and to relate these properties to the distribution and dynamics of various signaling proteins, such as the IgE receptor.

Along with the studies described here, several other projects not included in this Dissertation have been pursued. In collaboration with the Williams lab (Penn State, Chemistry) and my former colleagues, Minjoung Kyoung and Christal Lee, I worked on developing monovalent quantum dot/oligonucleotide conjugates for hybridization to
complementary oligonucleotide/lipid conjugates incorporated into planar lipid bilayers to ultimately assess lipid dynamics; yet proof of monovalency was not achieved using various chemical separations methods due to effects of conjugate charge and size. In another project, to determine the role of actin polymerization in kinetic regulation of early IgE receptor signaling steps, I probed GFP-labeled actin (GFP-actin) molecular dynamics during antigen-mediated signaling (~20°C), in the presence and absence of actin polymerization, using our ultrafast fluorescence dynamics assay (see Appendix A). Time of increased GFP-actin average lifetime seemingly corresponded to time of increased diI-C18 average lifetime and IgE receptor phosphorylation (discussed in Chapter 6), suggesting that actin polymerization facilitates ordered membrane domain recruitment to IgE receptor complexes. However, due to varying degrees of polymerization at later time points, such measurements of this particular actin probe were only sensitive at the onset of actin polymerization. Initially in collaboration with Minjoung Kyoung, and then independently, I added FCCS to our multi-modal microscope with conventional FCS capabilities (Appendix B). Using specific calibration samples and specialized optics and hardware, I co-aligned two, independent laser beams for dual-color excitation, and optimized the detection pathway. I also worked with photon counting histogram (PCH) analysis of FCS data, which differentiates similarly diffusing fluorescent species by molecular brightness, establishing FCS data acquisition conditions, analysis software operation and data interpretation (Appendix C).

In future research, the fluorescence lifetime, anisotropy and FRET techniques described in this Dissertation can be used to study the dynamics and interactions of other proteins, such as Lyn, LAT, phospholipase C and PKC, as IgE receptor signaling pro-
ceeds. With the development of an appropriate FcεRI fluorescent construct, the role of actin polymerization in regulating FcεRI/Lyn interactions during IgE receptor signaling can be assessed. These methods may also be used to probe the role of membrane domains in the antigen-mediated exocytosis of histamine-containing granules. As ToF-SIMS resolution and sensitivity continue to improve and new data analysis methods are developed, this technique will provide chemical information regarding the role of membrane domains in signaling under physiological conditions that is highly complementary to excited-state fluorescence dynamics studies. FCCS may also be used to examine interactions between different fluorescent IgE receptor signaling protein constructs, e.g., IgE-FcεRI/LAT, IgE-FcεRI/PLCγ and LAT/PLCγ, or membrane/protein interactions, e.g., diI-C₁₈ labeled membrane with a fluorescent LAT construct. Finally, the oligomerization of FcεRI or other signaling molecules can be monitored via PCH as signaling proceeds.
REFERENCES


APPENDIX A

GFP-actin dynamics in antigen-mediated RBL mast cell signaling

Stimulated actin polymerization results from cross-linking of IgE-FcεRI and manifests itself as membrane ruffling. This actin polymerization is thought to regulate FcεRI tyrosine phosphorylation kinetics by controlling the time or extent of interactions between Lyn and the cross-linked receptor (Holowka et al., 2000; Sheets et al., 1999b), and is hypothesized to organize the cell membrane (and FcεRIs) for more efficient signaling responses (Frankel et al., 2006). When RBL cells are stimulated with multivalent antigen (DNP-BSA) at 37°C in the presence of cytochalasin D, an inhibitor of stimulated actin polymerization, the transient IgE-FcεRI phosphorylation peak is extended from 5 min (in the absence of cytochalasin D) to 10 min, thus providing evidence of the down regulatory role of actin polymerization in IgE receptor signaling, prior to Syk phosphorylation (Holowka et al., 2000). Fluorescence cross-correlation spectroscopy experiments confirm the increased associations between Lyn and antigen cross-linked FcεRI upon cytochalasin D treatment at ~20°C (Larson et al., 2005). Other studies show that treating human basophils with latrunculin A, which sequesters G-actin released from F-actin filaments and prevents it from repolymerizing, also increases downstream IgE receptor signaling events, such as histamine release and intracellular calcium signaling (Vilarino and MacGlashan, 2004). Further, upon cross-linking mast cells with DNP-BSA at 22°C in the presence of cytochalasin D, F-actin colocalizes with IgE-FcεRI and raft markers such as Thy-1 and GD1b; however, the molecular bases for the underlying structural organization remain to be defined (Holowka et al., 2005; Holowka et al., 2000).

To help determine the role of stimulated actin polymerization in membrane nanostructural changes during IgE receptor signaling, we performed fluorescence lifetime studies of diI-C18 (1P-single point lifetime and 2P-FLIM as described in Chapter 6, data not shown) in the membrane of cells stimulated with antigen in the presence of cytochalasin D. With actin polymerization inhibited via cytochalasin D, diI-C18 fluorescence decays biexponentially and its lifetime does not change substantially with stimulation time (data not shown). These observations contrast those in Chapter 6, i.e., that diI-C18 fluorescence decays triexponentially with significantly higher lifetime by 5 min after antigen stimulation, and suggest that actin polymerization is required for membrane structure to change dynamically during signaling.

Because diI-C18 lifetime does not change with cell activation via PMA treatment (see Figs. 6.6 and 6.9), which permits stimulated actin polymerization while bypassing the IgE receptor signaling pathway, membrane nanostructural changes are seemingly not a function of actin polymerization alone. Further, if diI-C18 lifetime changes result directly from IgE receptor phosphorylation, we would expect diI-C18 lifetime to be sustained beyond 5 min when actin polymerization is inhibited via cytochalasin D, agreeing with sustained phosphorylation (Holowka et al., 2000); yet we observe no diI-C18 lifetime changes under these conditions (data not shown). Thus, these studies suggest that actin polymerization may play a key role in increasing local concentrations of
saturated and mono-unsaturated lipids (Sengupta et al., 2007a) or in initiating better association or closer proximity between cholesterol-rich membrane areas and FcɛRI, resulting in diI-C18 lifetime changes during antigen-induced signaling. Such roles for actin polymerization may be even more emphasized at lower temperatures, i.e., ~20°C as compared to 37°C.

Here, to complement the membrane dynamics studies performed as a function of actin polymerization (data not shown), we examine actin molecular dynamics during antigen-mediated IgE receptor signaling at ~20°C by the same fluorescence dynamics assay (Chapter 6; Ariola et al., 2006; Davey et al., 2007). Using fluorescence lifetime and polarization anisotropy imaging, we probe the molecular dynamics of GFP-labeled actin (GFP-actin) polymerization at the single cell level as mast cells are stimulated with DNP-BSA at ~20°C, in the absence and presence of cytochalasin D. As actin polymerizes, GFP-actin packs closer together, seemingly becoming more dynamically restricted. By examining GFP-actin conformational and environmental changes, we aim to determine how actin polymerization may regulate the kinetics of early IgE receptor signaling steps.

Materials and methods

Cell preparation

RBL-2H3 mast cells were resuspended in phenol-red free medium (minimal essential medium (MEM) without phenol red, with Earle’s salts and without L-glutamine, 20% (v/v) fetal bovine serum (FBS), 50 µg/mL gentamicin sulfate, 4 mM L-glutamine) at 2.0 × 10^5 cells/mL and plated onto glass-bottomed 35-mm Petri dishes two days prior, as previously described (Hess et al., 2003). The day before the experiment, cells were transfected using GenePorter (Gene Therapy Systems) following the manufacturer’s protocol, except that 100 nM phorbol 12,13-dibutyrate (Sigma) in Optimem (Gibco) was added to the cells immediately before DNA/GenePorter incubation to increase transfection yield (Hess et al., 2003). 2.5 µg of GFP-actin (0.18 mg/mL) (or 2.5 µg GFP-N1 (0.54 mg/mL)) were used per dish. After 3–5 h incubation with the DNA/GenePorter complexes, the cells were washed with phenol-red free medium, sensitized overnight with 1 µg of unlabeled α-DNP IgE per 1 mL of cell suspension, and further handled as described in Section 2.2.2. The day of the experiment, some control dishes were labeled in Tyrodes with a 100× dilution of 1 mg/mL diI in DMSO stock. Otherwise, cells were singly labeled with the GFP construct, with a 1:1 ratio of GFP:monomeric actin, for example. Dishes were stimulated with DNP-BSA at 1 µg/mL final concentration. As an additional control, stimulated F-actin polymerization was inhibited by treating some dishes with 2 µM cytochalasin D (MP Biomedicals, Inc.) in BSA/BSS for 10 min at ~20°C prior to DNP-BSA (supplemented with cytochalasin D at 2 µM final concentration) stimulation (Holowka et al., 2000). For parallel solution experiments, fluorescein and GFP (Benkovic lab, Penn State, Chemistry) were diluted in PBS (pH 7.4) to ~1 µM and ~10 µM, respectively. All experiments described below were carried out at ~20°C.
Confocal fluorescence microscopy

The experimental setup was detailed in Section 2.3. Briefly, GFP-actin (and GFP-N1) were imaged using 488 nm argon ion laser excitation and a 525/30 bandpass filter, while dIl-C18 was imaged with 543 nm HeNe laser excitation and a 605/70 bandpass filter. DIC imaging (488 nm excitation and a 525/30 emission filter) was used to monitor cell viability before and after all experiments to ensure that no photodamage had occurred, and unlabeled RBL cells showed negligible autofluorescence (data not shown).

Fluorescence lifetime imaging

2P-FLIM images were acquired at 0, 5, 10, 20 and 30 min after antigen stimulation (or control treatment) after 940 nm excitation, per 2.4.2.1 and 2.4.3. Prior to detection, the epi-fluorescence was passed through a dichroic mirror and filters (690SP plus a 525/35 bandpass to detect only GFP, Chroma). 2P-FLIM images were recorded using $256 \times 256$ pixels, with 120 s acquisition time. Fluorescein and GFP solutions in PBS (pH 7.4) were used as experimental standards.

2P-pseudo-single point measurements were conducted at high temporal resolution and signal-to-noise, using 940 nm low repetition rate laser pulses scanned over the entire cell (see Sections 2.4.2.2 and 2.4.3). Here, 2P-fluorescence intensity decays were acquired at the magic angle or calculated from the parallel and perpendicular intensity decays of 2P-pseudo-single point anisotropy measurements (see below). The fit parameters (single exponential) agree for both methods and the fits of decays that were not deconvoluted were started after 60 ps.

Time-resolved FRET experiments were performed on cells labeled with GFP-actin (donor) in the absence or presence of dIl-C18 (acceptor), analogous to studies described in Sections 2.4.2.3 and 2.4.3. A 690SP plus a 525/35 bandpass filter (Chroma) isolated GFP-actin emission. Under these filter conditions used for monitoring GFP-actin dynamics, no signal was detected from dIl-C18 only labeled cells. Here, $\eta = 1.4$ (for biomolecules in aqueous solution (Lakowicz, 1999)) and $Q_D = 0.6$ (for GFP) (Tsien, 1998).

Fluorescence polarization anisotropy imaging

2P-pseudo-single point time-resolved fluorescence anisotropy measurements were carried out at 0, 5, 10, 20 and 30 min after antigen stimulation (or control treatment) using 940 nm excitation (see Sections 2.5.2 and 2.5.3) and the same filter conditions used for fluorescence lifetime experiments. Time-resolved fluorescence polarization decays were constructed using the SPC830 module, and the $G$-factor (1.22) was calculated using the tail matching approach (Hess et al., 2003; Lakowicz, 1999). Fits were started after 60 ps, to avoid the instrumental response function, because deconvolution could not be performed.
Results and discussion

Actin polymerization causes antigen-stimulation dependent membrane ruffling

Changes in cell morphology and GFP-actin distribution were monitored with confocal microscopy as a function of antigen stimulation to provide a basis for actin dynamics imaging studies. A representative 30 min time series of DIC and GFP-actin confocal fluorescence images is shown in Fig. A.1. The 0 min time point was acquired prior to adding multivalent antigen (DNP-BSA) at ~20°C. Subsequently, stimulated actin polymerization induces cell ruffling (Fig. A.1A), with an onset that varies dish-to-dish from ~10 min to ~20 min, and causes GFP-actin movement to the cell periphery and accumulation in actin plaques (Fig. A.1B) over time. Brighter actin areas formed by ~10–20 min, which is likely a reflection of the degree of actin polymerization resulting from stimulation. Under our confocal microscopy and cell stimulation conditions (~20°C, 1 µg/mL DNP-BSA), individual GFP-actin filaments are not resolved among the mixture of actin monomers and filaments. To ensure that GFP-actin was properly localized, cells transfected with GFP-N1 to yield cytosolic GFP were also imaged with confocal microscopy (data not shown). In contrast to GFP-actin, GFP-N1 did not move to the cell periphery and enrich in cell ruffles, nor did it form puncta resembling actin plaques. When cells were also labeled with the membrane probe diI-C18, it appeared to colocalize with GFP-actin in plaques near the coverslip surface (data not shown). A model of stimulated actin polymerization as a result of IgE receptor cross-linking is depicted in Fig. A.2. Actin is in its monomeric form (G-actin) before IgE receptor cross-linking (Fig. A.2A) and polymerizes to form actin filaments (F-actin) upon cross-linking (Fig. A.2B). To relate GFP-actin distribution and localization to actin polymerization behavior in IgE receptor signaling, we performed excited-state dynamics experiments of GFP-actin in antigen-stimulated mast cells for which polymerization was either allowed to occur normally, or inhibited.

Ultrafast excited-state dynamics of GFP-actin reflect the onset of antigen-induced actin polymerization

We used 2P-FLIM to follow the timescale of antigen stimulated actin polymerization and obtain a spatial representation of GFP-actin fluorescence lifetime changes. The excited-state dynamics, and therefore the environment and molecular conformation, of GFP-actin undergo subtle changes with antigen stimulation, as highlighted in Fig. A.3A and C. Cells exhibited normal variations in the degree of ruffling and GFP-actin brightness (Fig. A.3B). FLIM images of both control solutions and GFP-actin transfected cells were best fit to a single exponential in SPCImage. The total image histograms for GFP in solution were fit to a single Gaussian distribution, with an ~3 ns average lifetime (data not shown), while histograms for GFP-actin in cells were fit to a bi-Gaussian, with a major lifetime population at ~2.7 ns and a minor component at ~3.2 ns (Fig. A.3C). For all dishes examined (n = 6), the GFP-actin average lifetime distribution shifted slightly at 5 min. We expected that as actin polymerized during antigen stimulation, the GFP-actin lifetime would continue to increase. By 20 and 30 min, however, the trends in GFP-actin lifetime are not always consistent in that the lifetime does
Figure A.1. Confocal microscopy of adherent antigen stimulated mast cells labeled with GFP-actin. Antigen-mediated changes in cell morphology (A) and GFP-actin distribution (B) are monitored over 30 min at ~20°C. Cell ruffling is due to stimulated actin polymerization and with time, GFP-actin moves to the cell periphery and accumulates in punctae, or actin plaques. Bar = 20 µm.
Figure A.2. Model of stimulated actin polymerization as a result of IgE receptor cross-linking. In the absence of cross-linking (A), actin is unpolymerized and in the monomeric, or G-actin, form. With IgE receptor cross-linking (B), G-actin polymerizes to form actin filaments as indicated. Individual G-actin monomers are depicted by the circles that comprise the actin filaments. The plasma membrane is shown as labeled with diI-C$_{18}$, which preferentially partitions into cholesterol-enriched membrane areas upon cross-linking. Stimulated actin polymerization separates cholesterol-rich domains from cross-linked IgE-Fc$\varepsilon$RI after signal initiation (Holowka et al., 2000).
Figure A.3. 2P-FLIM of GFP-actin demonstrates excited-state dynamics changes upon antigen stimulation. 2P-FLIM imaging (A) demonstrates subtle changes in the average fluorescence lifetime, and therefore environment and molecular conformation, of GFP-actin as a function of antigen stimulation time (binning = 5). Corresponding GFP-actin confocal images (B) indicate changing cell morphology and actin localization. Total FLIM image histograms (C), with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red), and 30 min (purple) time points, represent overall average lifetime distribution changes. Bar = 20 μm.
not always continue to increase. This may be observed because by ~20 min, the cells must contain a mixture of actin at varying polymerization stages, perhaps complicating interpretation of the later time points.

Complementary 2P-pseudo-single point time-resolved fluorescence experiments were performed on GFP-actin transfected cells to determine whether subtle GFP-actin lifetime changes could be discerned using higher temporal resolution methods. Time resolved fluorescence decays of GFP-actin in cells stimulated with multivalent antigen were fit to a single exponential and are sensitive to GFP environment as indicated by the slower GFP decay in PBS (Fig. A.4A). GFP-actin (see Table A.1) has a shorter lifetime than GFP in solution \( \tau_p = 2.7 \pm 0.2 \) ns due to the decreased GFP quantum yield upon association with protein (Hess et al., 2003). The average fluorescence lifetime, obtained from fitting several fluorescence decays \((n = 12)\) for each time point of antigen stimulation, shows a statistically significant increase at 5 min relative to 0 min \((p = 0.014)\), which was maintained at 10 min \((p = 9.77 \times 10^{-4})\), 20 min \((p = 5.88 \times 10^{-3})\) and 30 min \((p = 0.019)\) (Fig. A.4B). Average fluorescence lifetime \(\tau_p\) data for GFP-actin are also shown in Table A.1 and the trend of increasing average lifetime at 5 min agrees with 2P-FLIM results; however the average lifetimes obtained from single point methods are shorter due to the faster time resolution. When actin polymerization is inhibited by stimulating cells with cytochalasin D-supplemented antigen, there is no substantial change in GFP-actin average fluorescence lifetime as a function of stimulation time (Fig. A.4B and Table A.1), indicating that GFP-actin lifetime changes are actin polymerization-specific.

Hess et al. (2003) previously observed that cytoplasmic GFP and LynB-GFP (tethered to the inner plasma membrane leaflet) had approximately the same \(\tau_p\), despite the GFP probe having clearly different environments. Our comparative GFP-N1 and GFP-actin measurements reveal the same tendency (Table A.1), and our average lifetime values for GFP-N1 and GFP-actin are on the order of those obtained for GFP-N1 and LynB-GFP by Hess et al (2003). However, GFP-N1 average fluorescence lifetime does not change substantially with antigen stimulation time (Table A.1). Both studies demonstrate that GFP appears to have a lifetime that is insensitive to viscosity differences between the cytoplasm and a protein environment. Due to the insensitivity of GFP lifetime to viscosity and the varying degrees of GFP-actin polymerization at later time points of antigen stimulation, our excited-state dynamics measurements are only sensitive to the onset of actin polymerization. The GFP-actin average lifetime increase at 5 min, that corresponds to diI-C\textsubscript{18} average lifetime increase and IgE-Fc\textepsilon RI phosphorylation peak (Chapter 6), initially suggests that the onset of actin polymerization stabilizes ordered membrane domain recruitment to Fc\textepsilon RI complexes. To further investigate the role of stimulated actin polymerization inception in stabilizing ordered membrane domain recruitment to Fc\textepsilon RI complexes during IgE receptor signaling, we performed time-resolved FRET experiments between GFP-actin and diI-C\textsubscript{18} in the mast cell plasma membrane.
Figure A.4. Time-resolved fluorescence decays are sensitive to GFP environment and indicate polymerization-specific changes in GFP-actin lifetime. 2P-pseudo-single point time-resolved fluorescence decays (A) of GFP in PBS (brown, upper curve) and GFP-actin in cells stimulated with multivalent antigen for 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red), and 30 min (purple) show that GFP fluorescence decays more rapidly when it is associated with actin. The average lifetime obtained from several fluorescence decays is plotted (B) with respect to time of antigen stimulation (open circles, $n = 12$) and time of antigen stimulation in the presence of cytochalasin D (closed circles, $n = 12$), which inhibits stimulated actin polymerization. The asterisk indicates $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests, or that the average lifetime is statistically significantly different from the average lifetime prior to antigen stimulation.
Table A.1. 2P-pseudo-single point, time-resolved fluorescence lifetime of GFP-actin, and GFP-N1, in adherent mast cells stimulated with antigen in the absence and presence of cytochalasin D.

<table>
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<th>GFP-actin cells ( (n = 12) )</th>
<th>GFP-actin cells, + cytochalasin D ( (n = 12) )</th>
<th>GFP-N1 cells ( (n = 12) )</th>
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</thead>
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<tr>
<td>0 min (pre-stimulation)</td>
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<td>2.54(7)</td>
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</tbody>
</table>

<sup>a</sup> Solution controls of GFP and fluorescein in PBS gave \( \tau_f = 2.7 \pm 0.2 \text{ ns} \) and \( 3.99 \pm 0.07 \text{ ns} \), respectively.  
<sup>b</sup> \( p < 0.05 \), as determined from unpaired, two-tailed Student’s \( t \)-tests with the 0 min time point.
Time-resolved FRET between GFP-actin and dil-C18-labeled membrane is sensitive to the onset of antigen-mediated actin polymerization

To study possible interactions between GFP-actin and ordered membrane domains (dil-C18) during IgE receptor stimulation, we also conducted time-resolved FRET measurements on GFP-actin in dually labeled cells. Based on the spectral overlap of GFP emission and dil-C18 absorption, the estimated Förster distance ($R_0$) for energy transfer is 3.16 ± 0.03 nm. In the presence of dil-C18 (acceptor), GFP-actin (donor) fluorescence decays faster (Fig. A.5A), significantly reducing GFP-actin average fluorescence lifetime from 2.47 ± 0.02 ns to 2.36 ± 0.04 ns ($p = 1.19 \times 10^{-3}$) as determined by 2P-pseudo-single point fluorescence dynamics prior to antigen stimulation. At every time point after antigen stimulation, GFP-actin $\tau$ is significantly reduced in the presence of dil-C18 due to energy transfer, going from 2.51 ± 0.01 ns to 2.34 ± 0.03 ns ($p = 2.07 \times 10^{-5}$) at 5 min, 2.54 ± 0.02 ns to 2.37 ± 0.03 ns ($p = 4.44 \times 10^{-5}$) at 10 min, 2.52 ± 0.01 ns to 2.40 ± 0.05 ns ($p = 5.53 \times 10^{-5}$) at 20 min, and 2.51 ± 0.01 ns to 2.41 ± 0.02 ns ($p = 5.02 \times 10^{-6}$) at 30 min.

GFP-actin $\tau$ in the absence (Table A.1) and presence of dil-C18 was used to calculate the energy transfer efficiency ($E_T$) between the two probes with respect to antigen stimulation time, as described in Chapter 2. The only significant $E_T$ change is an increase from 0.04 ± 0.02 at 0 min to 0.07 ± 0.01 at 5 min ($p = 0.026$) (Fig. A.5B and Table A.2), which may be attributed to the onset of stimulated actin polymerization that in turn initiates maximal association between GFP-actin and the membrane ($R = 4.9 ± 0.1$ nm; $p = 0.018$). Localization of dil-C18 in the plasma membrane outer leaflet and GFP-actin on the cytoplasmic membrane side likely explains the low energy transfer efficiency and rate ($k_{FRET}$) at all time points (Table A.2). Owing to such inefficient energy transfer, it is not possible to assert the role of actin polymerization in ordered membrane domain recruitment during IgE receptor signaling. Because GFP was only shown to be sensitive to the onset of GFP-actin polymerization, and not the degree of polymerization, we assessed the possibility of homo-FRET due to polymerization, and thus GFP-actin aggregation, using time-resolved fluorescence polarization anisotropy methods.

Rotational diffusion reports GFP environment and GFP-actin polymerization

To further evaluate GFP sensitivity to its environment and GFP-actin polymerization state, we carried out 2P-pseudo-single point time-resolved fluorescence polarization anisotropy measurements of GFP-actin in cells stimulated with antigen in the absence or presence of cytochalasin D, as an inhibitor of stimulated actin polymerization. Fig. A.6A shows representative anisotropy decays of free GFP in PBS, cytoplasmic GFP (GFP-N1) and GFP-actin, in order of fastest to slowest anisotropy decay, or greatest to least rotational mobility. Free GFP in solution decays as a single exponential, while GFP-N1 and GFP-actin decay as a bi-exponential due to the presence of a fast decay
Figure A.5. Time-resolved fluorescence decays of GFP-actin, in the absence and presence of diI-C_{18}, reveal that the probes associate transiently upon antigen-activated actin polymerization. 2P-pseudo-single point fluorescence of the donor, GFP-actin, (A) decays more quickly in the presence of the acceptor, diI-C_{18}, (2) versus in the absence of acceptor (1). Although these representative decays were acquired prior to antigen addition (0 min), the donor fluorescence decays more rapidly in the presence of acceptor at all time points after antigen stimulation. Energy transfer efficiency ($E_T$) is plotted with respect to antigen stimulation time (B), with $n = 8$ at each time point. The asterisk denotes $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests, indicating that $E_T$ is statistically significantly different from $E_T$ prior to antigen stimulation.
Table A.2. Fluorescence resonance energy transfer parameters obtained from steady-state spectrophotometry of GFP-actin and diI-C_{18} and 2P-pseudo-single point, time-resolved fluorescence lifetime of GFP-actin, in the absence and presence of diI-C_{18}, in antigen-stimulated adherent mast cells.

<table>
<thead>
<tr>
<th>Time</th>
<th>$E_T$</th>
<th>$R$/nm</th>
<th>$k_{FRET}$/ns$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>0.04(2)</td>
<td>5.3(2)</td>
<td>0.019(9)</td>
</tr>
<tr>
<td>5 min</td>
<td>0.07(1)$^b$</td>
<td>4.9(1)$^b$</td>
<td>0.029(5)</td>
</tr>
<tr>
<td>10 min</td>
<td>0.07(2)</td>
<td>4.9(2)</td>
<td>0.029(9)</td>
</tr>
<tr>
<td>20 min</td>
<td>0.05(2)</td>
<td>5.2(3)</td>
<td>0.020(9)</td>
</tr>
<tr>
<td>30 min</td>
<td>0.04(1)</td>
<td>5.3(2)</td>
<td>0.017(4)</td>
</tr>
</tbody>
</table>

$^a$ The donor (GFP-actin) fluorescence lifetime in the absence ($\tau_D$) (Table A.1; $n = 12$) and presence of acceptor (diI-C_{18}) ($\tau_{DA}$) ($n = 8$) at each time point of antigen stimulation were used to calculate energy transfer efficiency ($E_T$). $R_0$ was calculated as 3.16 ± 0.03 nm and used to determine $R$, as described in Chapter 2. $^b p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests, indicating that the mean is statistically significant different from the corresponding mean at 0 min.
component (see Table A.3). The slow ($\phi_1$) and fast ($\phi_2$) rotational correlation time components, their corresponding fractions ($\beta_1$ and $\beta_2$) and the initial anisotropies ($r_0$), or sums of the fractions, are shown in Table A.3. The slow rotational component can be attributed to overall GFP molecular rotation, which is directly proportional to environmental viscosity and molecular weight; whereas the fast component reflects fast wobbling of the GFP chromophore that occurs when GFP is tethered to a protein. For example, for GFP-actin, $\phi_2$ likely reports rapid wobbling due to the flexibility of the ~5 amino acid linker between GFP and the actin monomer C-terminus. GFP-N1 may exhibit a $\phi_2$ component due to the crowded cellular environment, because it is not tethered to protein. GFP-actin initial anisotropy ($r_0$) is somewhat higher (~0.48) than that of cytosolic GFP or GFP in solution (~0.45) due to the higher degree of GFP order when it is associated with actin.

Before antigen stimulation, the slow rotational correlation time component, $\phi_1$, of GFP in solution is ~2.5 times faster than cytosolic GFP and ~4.5 times faster than GFP-actin (Table A.3), which is analogous to the trends observed by Hess et al. (2003) for GFP solution versus cytosolic GFP and LynB-GFP. GFP-actin $\phi_1$ also appears to be sensitive to actin polymerization activation, as demonstrated in Fig. A.6B by a statistically significant $\phi_1$ increase 5 min after initiation of antigen stimulation ($p = 0.029$). There are no substantial GFP-actin $\phi_1$ changes when cells are stimulated in the presence of cytochalasin D to inhibit actin polymerization. GFP-actin molecular weight, and thus $\phi_1$, should seemingly increase as a function of polymerization; however with a significant $\phi_1$ increase only at 5 min, mixed polymerization levels may conceal polymerization-dependent changes in overall rotational dynamics, as with the excited-state dynamics discussed above. Further, Fig. A.6C suggests that GFP-actin $\phi_2$ may report on the actin polymerization peak at ~20 min, as substantiated by a statistically significantly reduced $\phi_2$ at 20 min ($p = 6.09 \times 10^{-3}$) and 30 min ($p = 0.027$) after antigen stimulation, only in the absence of cytochalasin D. A peak in actin polymerization could cause enough restriction of the GFP molecules tethered to actin, resulting in confined segmental mobility and faster wobbling motions of GFP-actin. Further, this peak in actin polymerization should correspond to the peak in FcεRI phosphorylation downregulation; however at ~20°C, we did not observe clear signaling downregulation, but rather a leveling off (Chapter 6). These polarization anisotropy studies did not provide any evidence of anticipated GFP-actin homo-FRET, i.e., decreased $\phi_1$ or $\phi_2$ (Sharma et al., 2004), due to stimulated polymerization.
Figure A.6. Time-resolved fluorescence polarization anisotropy decays are sensitive to GFP environment, surrounding viscosity and molecular weight, and indicate polymerization-specific changes in GFP-actin rotational correlation times. 2P-pseudo-single point time-resolved anisotropy decays (A) of GFP-actin curve (1) and cytoplasmic GFP-N1 curve (2) in cells prior to antigen stimulation (i.e., 0 min), and GFP in PBS curve (3). Decays of GFP in solution were fit to a single exponential, and GFP-N1 and GFP-actin to a bi-exponential. The average longer rotational correlation time component ($\phi_1$) obtained from several anisotropy decays is plotted (B) with respect to time of antigen stimulation (open circles, $n = 6$) and time of antigen stimulation in the presence of cytochalasin D (closed circles, $n = 6$). The asterisk indicates $p < 0.05$, as determined from unpaired, two-tailed Student’s t-tests, or that $\phi_1$ is statistically significantly different from $\phi_1$ prior to antigen stimulation and applies to the open circles. The average shorter rotational correlation time component ($\phi_2$) is plotted (C) in the same manner as the data shown in (B), and the asterisk applies to both the open and closed circles.
Table A.3. 2P-pseudo-single point, time-resolved fluorescence polarization anisotropy of GFP-actin, and GFP-N1, in adherent mast cells stimulated with antigen in the absence and presence of cytochalasin D.

<table>
<thead>
<tr>
<th></th>
<th>$\phi_1$/ns</th>
<th>$\beta_1$</th>
<th>$\phi_2$/ns</th>
<th>$\beta_2$</th>
<th>$r_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFP-actin cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(n = 6)$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min (pre-stimulation)</td>
<td>40(4)</td>
<td>0.44(2)</td>
<td>0.6(1)</td>
<td>0.04(1)</td>
<td>0.480(8)</td>
</tr>
<tr>
<td>5 min</td>
<td>48(1)$^a$</td>
<td>0.44(2)</td>
<td>0.7(3)</td>
<td>0.04(2)</td>
<td>0.481(8)</td>
</tr>
<tr>
<td>10 min</td>
<td>42(8)</td>
<td>0.44(2)</td>
<td>0.6(1)</td>
<td>0.04(2)</td>
<td>0.476(3)</td>
</tr>
<tr>
<td>20 min</td>
<td>41(4)</td>
<td>0.45(1)</td>
<td>0.3(1)$^a$</td>
<td>0.03(1)</td>
<td>0.481(6)</td>
</tr>
<tr>
<td>30 min</td>
<td>47(9)</td>
<td>0.4(1)</td>
<td>0.43(4)$^a$</td>
<td>0.03(1)</td>
<td>0.44(9)</td>
</tr>
<tr>
<td><strong>GFP-actin cells, + cytochalasin D</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 min (pre-stimulation)</td>
<td>54(4)$^b$</td>
<td>0.45(2)</td>
<td>0.7(1)</td>
<td>0.04(1)</td>
<td>0.48(2)</td>
</tr>
<tr>
<td>5 min</td>
<td>53(5)</td>
<td>0.44(1)</td>
<td>0.7(5)</td>
<td>0.05(2)</td>
<td>0.48(2)</td>
</tr>
<tr>
<td>10 min</td>
<td>57(4)</td>
<td>0.44(2)</td>
<td>0.8(1)</td>
<td>0.04(2)</td>
<td>0.48(1)</td>
</tr>
<tr>
<td>20 min</td>
<td>49(15)</td>
<td>0.45(2)</td>
<td>0.9(1)$^b$</td>
<td>0.03(1)</td>
<td>0.48(2)</td>
</tr>
<tr>
<td>30 min</td>
<td>48(13)</td>
<td>0.45(1)</td>
<td>0.7(1)$^b$</td>
<td>0.04(1)</td>
<td>0.49(2)</td>
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<tr>
<td><strong>GFP-N1 cells</strong></td>
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<tr>
<td>$(n = 6)$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min (pre-stimulation)</td>
<td>26(4)$^c$</td>
<td>0.39(5)</td>
<td>2(2)</td>
<td>0.06(4)</td>
<td>0.45(2)$^c$</td>
</tr>
<tr>
<td>5 min</td>
<td>31(1)$^c$</td>
<td>0.35(6)</td>
<td>4(2)$^c$</td>
<td>0.10(5)</td>
<td>0.45(3)$^c$</td>
</tr>
<tr>
<td>10 min</td>
<td>29(3)$^c$</td>
<td>0.39(4)</td>
<td>2(1)</td>
<td>0.06(3)</td>
<td>0.46(3)</td>
</tr>
<tr>
<td>20 min</td>
<td>28(5)$^c$</td>
<td>0.40(6)</td>
<td>1(1)</td>
<td>0.05(4)</td>
<td>0.45(2)$^c$</td>
</tr>
<tr>
<td>30 min</td>
<td>32(1)$^c$</td>
<td>0.39(4)</td>
<td>2(1)$^c$</td>
<td>0.06(2)</td>
<td>0.45(2)</td>
</tr>
<tr>
<td><strong>Solutions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>11(2)</td>
<td>0.45(2)</td>
<td>0.45(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fluorescein</td>
<td>0.151(9)</td>
<td>0.253(9)</td>
<td>0.253(9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests with the 0 min time point.  $^b$ $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests between GFP-actin and GFP-actin, + cytochalasin D, at the same time point.  $^c$ $p < 0.05$, as determined from unpaired, two-tailed Student’s $t$-tests between GFP-actin and GFP-N1, at the same time point.
Conclusions

We used fluorescence lifetime and polarization anisotropy imaging of GFP-actin to probe the molecular dynamics of stimulated actin polymerization at the single cell level and determine how actin polymerization may regulate the kinetics of early IgE receptor signaling steps. GFP-actin distribution and localization (Fig. A.1) were related to subtle antigen-mediated changes in its environment and molecular conformation, and therefore excited-state dynamics (Figs. A.3 and A.4; Table A.1), over the course of antigen stimulation. As GFP-actin polymerizes, it exhibits varying degrees of polymerization, and therefore varying degrees of packing and molecular weight that affect molecular dynamics. Because of the various GFP-actin polymerization states at later time points of antigen stimulation, our excited-state dynamics measurements are only sensitive to the onset of actin polymerization. The statistically significant GFP-actin average lifetime increase at 5 min corresponds to the diIC_{18} average lifetime increase and IgE-FcεRI phosphorylation peak, discussed in Chapter 6, and suggests that the beginning of actin polymerization facilitates ordered membrane domain recruitment to FcεRI complexes. GFP-actin time-resolved FRET measurements demonstrated that initiating stimulated actin polymerization results in maximal association between GFP-actin and diIC_{18}-labeled cholesterol-enriched cell membrane (Fig. A.5; Table A.2).

Although GFP lifetime is insensitive to viscosity differences between the cytoplasm and a protein environment, GFP rotational diffusion was sensitive to environmental viscosity, protein binding, and molecular weight (Fig. A.6; Table A.3). The significant increase in the long rotational correlation time component of GFP-actin at 5 min agrees with GFP-actin dynamics restriction reported by fluorescence lifetime methods at the same time point, whereas the actin polymerization peak is reflected by a reduced short rotational correlation time component of GFP-actin. Mixed actin polymerization levels, and therefore various GFP-actin molecular weights, may also conceal polymerization-dependent changes in overall rotational dynamics, as with the excited-state dynamics discussed above.
APPENDIX B

Protocol for fluorescence cross-correlation spectroscopy (FCCS)

As noted by Kyoung (2008; 2007), fluorescence correlation spectroscopy (FCS) is one of the major biophysical techniques used to assess molecular dynamics and kinetic properties of low concentration samples by measuring small, naturally-occurring thermal fluctuations, and therefore fluorescence fluctuations, through a fL detection volume (Haustein and Schwille, 2007; Hwang and Wohland, 2007). Fluorescence cross-correlation spectroscopy (FCCS) is an extension of FCS that allows excitation of two spectrally distinct fluorophores and the simultaneous detection of two different emission colors in separate detector channels. The signals of the two channels are time correlated with each other to obtain information only about particles with both fluorophores (Weidenmann et al., 2002).

This protocol describes calibration sample preparation, specialized optics and modifications to the conventional fluorescence correlation spectroscopy setup, and excitation and emission pathway alignment procedures. Also presented is a brief discussion of representative data obtained in calibrating the FCCS setup detection pathway and overall alignment.

Calibration sample preparation

1. Apply a thin, ~1 inch long strip of double sticky tape to each of the long sides of a 3” × 1” glass slide, allowing space between the strips for addition of sample later, and top the glass slide with a 22 mm × 22 mm glass coverslip to make a sandwich.

2. Make the following solutions and add ~50 µL to individual sandwich sample chambers before sealing with nail lacquer. Conditions of excitation and detection are also noted.

   a. **6 nM aqueous Cy5 for λ<sub>ex</sub> = 633 nm and PMT A detection.** Expected lateral diffusion coefficient, D, is ~2.5 × 10<sup>-6</sup> cm<sup>2</sup>/s (Windengren and Schwille, 2000).

   b. **10 nM aqueous rhodamine green for λ<sub>ex</sub> = 488 nm and PMT B detection.** Expected D is ~2.8 × 10<sup>-6</sup> cm<sup>2</sup>/s (Heikal et al., 2000).

   c. **10 nM aqueous Alexa Fluor 488 for λ<sub>ex</sub> = 488 nm and PMT B detection.**

   d. **10 nM A488-IgE in PBS for λ<sub>ex</sub> = 488 nm and PMT B detection.**

   e. **10 nM A488-IgE-A647 in PBS for λ<sub>ex</sub> = 633 nm and PMT A detection.**

   f. **10 nM A488-IgE-A647 in PBS for λ<sub>ex</sub> = 488 nm and PMT B detection.**
g. **10 nM A488-IgE-A647 in PBS** for $\lambda_{\text{ex}} = 633 \text{ nm} + 488 \text{ nm}$, with PMT A + PMT B detection.

IgE is first conjugated to Alexa Fluor 488 (A488-IgE) and then to Alexa Fluor 647 via commercially available protein labeling kits (Invitrogen), both of which label the monomeric IgE at exposed primary amines. (The dye/IgE ratio was 4.0 and 4.5 for Alexa Fluor 488 and 647, respectively, as determined by absorption spectroscopy.)

To minimize protein sample sticking, coverslip and slide surfaces are treated with 10 mg/mL BSA in PBS (pH 7.4) for at least 30 min in a room temperature humidified chamber. Without this treatment, autocorrelation is not attainable.

**Specialized optics and modifications implemented for FCCS (see Fig. B.1)**

A beam expander ($5 \times$) is placed in front of the HeNe lasers (BE2) so that the red HeNe (633 nm) focal spot size and $z$-position can be controlled independently from the Ar laser (488 nm), which passes through a $3 \times$ beam expander (BE1). As described below, each of the beam expanders is optimized for the best possible signal level in the autocorrelation curves of both channels and the focal volumes of each laser line are consequently made similar during this adjustment.

A z-594sp-bcm dichroic mirror (DM1) is inserted early in the excitation pathway to transmit Ar laser (488 nm) and reflect red HeNe laser (633 nm) lines. A z488/633rpc and z488/633m dichroic/emission filter combination is mounted in an objective turret filter cube (position #6).

To enhance red emitter signal, the IR filter is removed from the emission split of the multi-image module. A custom-built, dichroic emission filter cube (DM2) holder with attached $x$, $y$, $z$-positioners for the optical fiber adaptors is added to one side of the emission split arm. This filter cube holder allows splitting of the emission to two separate PMT detectors. In addition to adjusting the two beam expanders, the optimal $z$-alignment is set by moving the dichroic emission filter cube (DM2) holder position along the emission split arm.

Two emission filters (HQ680/60 for PMT A and HQ525/30 for PMT B) are added to the dichroic emission filter cube (DM2, a 555dclp, which transmits emission greater than 555 nm to PMT A and reflects less than 555 nm to PMT B). These filters eliminate false correlation in both channels due to signal leakage to the wrong PMTs and optimize cross-correlation.
Figure B.1. Microscope system for FCCS. As described briefly in Chapter 2 (Fig. 2.3) this multimodal system is based on a Nikon TE2000U inverted microscope capable of microscopy and spectroscopy applications (Sheets Lab, Penn State, Chemistry). The excitation and detection pathways are detailed in (A) and (B), respectively. Both Ar laser and HeNe laser lines converge at DM1, after respective size adjustment with BE1 and BE2 (A). Ar laser power is controlled by adjusting ND1 and/or ND2, whereas HeNe laser power is controlled with ND2 (A). Mirrors that flip down, and are not directly involved in this FCCS setup, are shown in white. M7 and M8 are periscope mirrors that direct the beams up to the microscope back aperture (A) and (B). After sample excitation, a dichroic mirror directs the emission to an emission path split (B, shaded area) where the emission can be imaged via CCD (as in Fig. 2.3) or detected via PMT(s). On the PMT detection side, emission passes through a custom-built emission cube holder (DM2), which
typically contains a dichroic mirror cube with emission filters for each PMT but can also accommodate a 50/50 beamsplitter for detection alignment purposes (B). DM2 is linked to each PMT detector by an optical fiber that can be adjusted in x, y, and z-position. A: aperture; BE: beam expander, BS: beam splitter, DM: dichroic mirror, FW: filter wheel, M: mirror, ND: neutral density filters, OBJ: objective. Figure is partially adapted from Kyoung et al (2007).
Alignment procedure

Excitation pathway alignment:
Follow the alignment and startup electronics procedures outlined by Kyoung (2008, see Appendix E, Protocol for fluorescence correlation spectroscopy).

For the alignment procedure, A, BE, DM, M, and ND refer to aperture, beam expander, dichroic mirror, mirror and neutral density filters, respectively. These optics are shown schematically in Fig. B.1.

General startup:
1. Start up the red HeNe laser and Ar ion laser (see Appendix A of Kyoung (2008)).
2. Turn on the LEP module and the PCs.
3. Turn on the shutter controllers for both lasers.

Red HeNe laser:
1. Make sure that M14 is up to direct the red HeNe beam toward the HeNe shutter, which is opened by switching the controller from n.c. to n.o.

2. BE2 should be inserted in the beam path after the HeNe shutter and M13 flipped up to direct the beam to DM1, which will reflect the 633 nm laser line (M3, M4, M5 and M6 should be flipped down to not obstruct the beam path).

3. Alternate adjusting M13 to direct the beam through the center of A1 (aperture after M3) and adjusting DM1 to direct the beam through the center of A2 (aperture after M6). Repeat until the beam passes through the center of both apertures.

Ar ion laser:
1. The laser head aperture should be 8. Laser output power should be ~250 mW and ND ~3.2 should be used. To achieve ND = 3.2, use 1.6 on ND1 and 1.6 on ND2, for example.

2. Make sure M1 is flipped up to direct the Ar laser beam through 488 color filter and BE1, toward M2 and DM1, which will transmit the 488 nm laser line. Open the Ar laser shutter by switching the controller from n.c. to n.o. BE1 should be turned such that the yellow tape on the moving portion of BE1 is aligned with the green line on the stationary portion of BE1. If the yellow tape is lost, the position is ~8 mm away from the permanent white spot on the moving part of BE1, in the direction of reduced beam expansion, or an increased focal spot size at the sample plane.

3. Alternate adjusting M1 to direct the beam through the center of A1 and adjusting M2 to direct the beam through the center of A2. Repeat until the beam passes through the center of both apertures.
Both laser lines:
1. Close both laser shutters temporarily.

2. Place the 60×, 1.2 NA water immersion objective in the path and put the red Chroma slide on the sample stage. Position filter cube #6 under the objective.

3. Remove the Wollaston prism under the objective and the analyzer from the emission pathway.

4. Open the Ar laser shutter to observe a small focal spot in the eyepiece.

5. Adjust M7 and M8 alternately (smaller periscope mirrors) to move the focus near the center of the eyepiece field of view (via M8) and eliminate leaning of the beam through the working distance of the objective.

6. Close the Ar shutter and open the HeNe shutter and repeat the previous step, going back and forth between the two laser excitations. Usually, if the red HeNe beam is centered and not leaning, the Ar beam is also centered, but slightly leaning, which will be corrected in a subsequent step.

7. Using the CCD to view the excitation, move the knobs of M2 slightly, to bring the focused Ar laser spot to the same $x$, $y$ position as the focused red HeNe spot. Use the focus at the higher $z$-focus position of the red Chroma slide.

8. Alternately adjust M1 and M2, while watching the CCD image, to eliminate the Ar laser spot leaning as much as possible and to repeatedly bring the Ar laser spot back to the position of the focused red HeNe spot.

**Emission pathway alignment:**
1. Adjust ND1 and ND2 that the red HeNe laser passes through ND ~1.6 and the Ar ion laser through a total of ND ~3.2 to reduce the power of each beam, and thus the power of the emission reaching the PMTs.

2. Open the emission path split by turning the beamsplitter (BS, Fig. B.1) dial to 2.

3. Ensure that the microscope is on eyepiece view, turn off the room light and turn on PMT A.

4. In the Flex02_12dc software, the photon counts should be ~0.02 kHz.

5. To monitor the photon counts at either or each PMT, RT intensity mode should be used and the microscope must be on detector view (5). If the photon counts are higher than ~1000 kHz, the laser intensities must be temporarily reduced with ND on ND1 or ND2.
6. To optimize the fine $x,y$-alignment of the two beams, remove any emission filter cubes from the holder and use the PMT A fiber as an alignment standard. Using the red Chroma slide, ND = 1.6 and the red HeNe laser, align the PMT A fiber by peaking the $x$, $y$, and $z$ knobs. Peaking, or maximizing the photon counts, is done by turning $x$, $y$, and $z$ separately, and going back and forth. Turn off the HeNe shutter, increase ND to 5.6 and use Ar laser excitation on the red Chroma slide. You will align the green emission to the PMT A fiber by peaking the knobs of M2 early in the Ar laser beam path to optimize the photon counts.

7. Insert the 50/50 beamsplitter mirror into the dichroic emission filter cube holder attached to the emission split. Using the red Chroma slide, ND = 1.6 and red HeNe laser, align the PMT A fiber by peaking its $x$, $y$ and $z$ knobs. Turn off the HeNe shutter and PMT A. Increase ND to 5.6, turn on PMT B and use Ar laser excitation on the red Chroma slide. Align the PMT B fiber by peaking its $x$, $y$ and $z$ knobs.

8. To ensure that the PMTs are functioning properly and that the alignment is optimized, standards, such as Cy5 and rhodamine green, will be tested using the 50/50 beamsplitter detection scheme. The first six controls listed above are typically measured using both PMTs simultaneously. (ND = 1.6 and ND = 3.2 are typically used for red HeNe and Ar laser excitation, respectively.)

9. When you are ready to acquire data, click RT intensity again and S to set the data acquisition parameters, including duration (60s is typical), autosave and multiplerun (10 runs are typical) when using 50-µm optical fibers for detection. Before each sample run, re-optimize the PMT fibers in $x$, $y$ and $z$, as these can drift slightly over time. The idea is that the autocorrelation from each channel, and the pseudo-cross-correlation averaged from both channels should all yield the same diffusion time ($\tau_d$) and number of molecules ($N$), within standard error, upon fitting.

10. Insert the FCCS dichroic mirror into the dichroic emission filter cube holder attached to the emission split and repeat the previous step on the seven controls mentioned above. This time, the same excitation wavelengths as previously will be used for each sample, with only a single PMT used for detection (except in the case of the seventh control, which is dually-excited A488-IgE-A647, i.e., the real cross-correlation calibration sample, which should yield the same diffusion time ($\tau_d$) and number of molecules ($N$), within standard error, as the autocorrelation from each channel upon fitting).
11. Cross-correlation can be calibrated using the A488-IgE-A647 sample, however quad mode must be used for data acquisition. In this case, both channels are measured at once, making five data columns after the time column (1st data column).

1st window: A×A–white trace, autocorrelation, 2nd data column
   A×B–green trace, cross-correlation, 4th data column
2nd window: B×B–white trace, autocorrelation, 3rd data column
   B×A–green trace, cross-correlation, 5th data column

A 6th data column can be calculated as the averaged cross-correlation signal [(A×B)+(B×A)/2] in Excel (Microsoft).

**General shutdown:**
1. Turn off the PMTs.
2. Turn off the HeNe and Ar ion lasers according to Appendix A of Kyoung (2008).
3. Turn off laser shutters, the LEP module and the computer.

**FCCS calibration**

When the above alignment procedure is followed, 50-µm optical fibers are used for detection, and autocorrelation curves are fit to 1-component 3D-diffusion in IgorPro (Wavemetrics), the diffusion times of Cy5, rhodamine green, and Alexa Fluor 488 (A488) diffusion times are expected to be ~70 µs, ~60 µs, and ~0.1 ms, respectively. For A488 conjugated to IgE (A488-IgE), the diffusion is 2-component, with \( \tau_d \) values of ~0.1 ms and ~1 ms. Analogously, when A488-IgE-A647 is excited only with the Ar laser (or excited with both lasers and detected in PMT B after the dichroic emission filter), diffusion is 2-component with \( \tau_d \) ~0.1 ms and ~1 ms, because the additional fluorescent label does not drastically change the protein diffusion properties. The shorter component can be attributed to free A488 in the sample, as the diffusion time is similar to that of free A488 in solution. If only red HeNe excitation is used on A488-IgE-A647 (or if it is excited with both lasers and detected in PMT A after the dichroic emission filter), diffusion is again 2-component, but with \( \tau_d \) ~10 µs and ~1 ms. In this case, the diffusion time of the shorter component is too fast to be free Alexa Fluor 647, yet it is consistently observed for A488-IgE-A647 under red HeNe excitation. The diffusion of the real cross-correlation sample (i.e., dually-excited A488-IgE-A647) is 1-component with \( \tau_d \sim 1 \) ms. Any free dyes present and detected in each channel are not associated with one another, are therefore uncorrelated and do not show up in the cross-correlation curve. Such an observation is proof of the lack of crosstalk between channels (Bacia and Schwille, 2003).

**Detection pathway calibration:**

Provided that the alignment is correct, using the 50/50 beamsplitter mirror in the emission pathway for samples (a)–(f) above, \( \tau_d \) and \( N \) should be the same in both PMTs, and the pseudo-cross-correlation from both PMTs should yield a diffusion time that is within deviation of the auto-correlation diffusion times. To calibrate our FCCS setup detection pathway, we followed the alignment procedure previously outlined and mea-
sured the lateral diffusion of two standard dyes in solution, i.e., Cy5 and rhodamine green, separately using 633 nm red HeNe laser and 488 nm Ar laser excitation, respectively. Fluorescence fluctuations of each dye solution were simultaneously detected in PMT A and PMT B after a 50/50 beamsplitter mirror in the emission pathway; therefore these measurements do not provide true cross-correlation data, but rather serve to verify the alignment. The averaged lateral diffusion parameters (τ_d and N) for each dye solution were obtained from the autocorrelation and pseudo-cross-correlation curves and are shown in Table B.1 as a function of optical fiber size. These data were averaged from curves obtained from multiple slides, at multiple spots per slide, and at multiple z-foci per spot, for a single experimental day, or alignment procedure. The averaged τ_d and N data in Table B.1 are also plotted with respect to optical fiber size in Fig. B.2A and C (for Cy5), and in Fig. B.3A and C (for rhodamine green). For a given fiber size, the τ_d values (or N values) obtained from PMT A and PMT B autocorrelation curves, and from the calculated pseudo-cross-correlation curve, are within standard deviation. This is visually indicated by overlapping error bars in Fig. B.2A and C (for Cy5), and in Fig. B.3A and C (for rhodamine green). Fig. B.2B and D, and Fig. B.3B and D show the scatter of the individual data points used to calculate the averaged lateral diffusion parameters for Cy5 and rhodamine green, respectively. For either of the dyes, there is little scatter in the τ_d and N data for each optical fiber size tested. As optical fiber size is increased, τ_d and N increase linearly in each measured channel. Linear regression was applied to each of the plots in Figs. B.1A and C, and B.2A and C using IgorPro (Wavemetrics) and the results are summarized in Table B.2. The slopes agree for each parameter, for both Cy5 and rhodamine green, and the goodness of the fits are indicated by R^2 ~1.00.
Table B.1. Cy5 and rhodamine green lateral diffusion in aqueous solution obtained from autocorrelation and pseudo-cross-correlation curves measured by FCCS via a 50/50 beamsplitter, as a function of optical fiber size.

<table>
<thead>
<tr>
<th>Optical Fiber Size</th>
<th>25-µm optical fibers</th>
<th>50-µm optical fibers</th>
<th>100-µm optical fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>τ_d/µs</td>
<td>N</td>
<td>τ_d/µs</td>
</tr>
<tr>
<td><strong>Cy5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMT A autocorrelation</td>
<td>53(3)</td>
<td>4.6(4)</td>
<td>78(2)</td>
</tr>
<tr>
<td>PMT B autocorrelation</td>
<td>52(4)</td>
<td>4.9(6)</td>
<td>72(4)</td>
</tr>
<tr>
<td>pseudo-cross-correlation</td>
<td>55(2)</td>
<td>5.0(4)</td>
<td>76(3)</td>
</tr>
<tr>
<td><strong>Rhodamine green</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMT A autocorrelation</td>
<td>46(4)</td>
<td>108(13)</td>
<td>74(8)</td>
</tr>
<tr>
<td>PMT B autocorrelation</td>
<td>39(7)</td>
<td>102(18)</td>
<td>66(5)</td>
</tr>
<tr>
<td>pseudo-cross-correlation</td>
<td>53(8)</td>
<td>121(16)</td>
<td>73(7)</td>
</tr>
</tbody>
</table>

Cy5 was excited with 633 nm red HeNe laser, while rhodamine green was excited via 488 nm Ar laser. In both cases, detection was after a 50/50 beamsplitter mirror in the emission pathway, rather than the dichroic emission filter used for actual cross-correlation measurements. The average diffusion times (τ_d) and number of molecules (N) shown in Table B.1 were obtained from 2 slides, at 2 spots per slide, and at 2 z-foci positions per spot (n = 8, total for each dye at each optical fiber size), for a single alignment procedure. The negligible amount of scatter in the data for each dye at each optical fiber size is indicated by the plots in Fig. B.2B and D (for Cy5) and Fig. B.3B and D (for rhodamine green).
Figure B.2. Cy5 diffusion time and number of molecules in aqueous solution obtained from autocorrelation and pseudo-cross-correlation curves measured by FCCS via a 50/50 beamsplitter, as a function of optical fiber size. The open circles, closed circles and open squares represent autocorrelation data from PMT A and PMT B, and cross-correlation data, respectively. The average diffusion times ($\tau_d$) (A) and number of molecules ($N$) (C) obtained from FCCS ($\lambda_{ex} = 633$ nm) of a 6 nM Cy5 solution are also shown with error in Table B.1. All of the $\tau_d$ (B) and $N$ (D) data points used to determine the averages (in A and C) are provided to show the degree of scatter in the data.
Figure B.3. Rhodamine green diffusion time and number of molecules in aqueous solution obtained from autocorrelation and pseudo-cross-correlation curves measured by FCCS via a 50/50 beamsplitter, as a function of optical fiber size. The open circles, closed circles and open squares represent autocorrelation data from PMT A and PMT B, and cross-correlation data, respectively. The average diffusion times ($\tau_d$) (A) and number of molecules ($N$) (C) obtained from FCCS ($\lambda_{ex} = 488$ nm) of a 10 nM rhodamine green solution are also shown with error in Table B.1. All of the $\tau_d$ (B) and $N$ (D) data points used to determine the averages (in A and C) are provided to show the degree of scatter in the data.
Table B.2. Linear regression of averaged Cy5 and rhodamine green diffusion times in aqueous solution and average number of fluorescent molecules in the detection volume, as a function of optical fiber size.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_d$</th>
<th>$N$</th>
<th>slope</th>
<th>$R^2$</th>
<th>slope</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cy5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMT A autocorrelation</td>
<td>0.73(9)$^a$</td>
<td>0.98$^a$</td>
<td>0.30(3)$^b$</td>
<td>0.99$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMT B autocorrelation</td>
<td>0.81(1)$^a$</td>
<td>1.00$^a$</td>
<td>0.35(6)$^b$</td>
<td>0.97$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pseudo-cross-correlation</td>
<td>0.75(3)$^a$</td>
<td>1.00$^a$</td>
<td>0.33(4)$^b$</td>
<td>0.98$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rhodamine green</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMT A autocorrelation</td>
<td>0.86(9)$^c$</td>
<td>0.99$^c$</td>
<td>4.6(8)$^d$</td>
<td>0.97$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMT B autocorrelation</td>
<td>0.84(8)$^c$</td>
<td>0.99$^c$</td>
<td>3.9(8)$^d$</td>
<td>0.96$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pseudo-cross-correlation</td>
<td>0.73(7)$^c$</td>
<td>1.00$^c$</td>
<td>4.1(7)$^d$</td>
<td>0.97$^d$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Linear regression performed on data in Fig. B.2A, $^b$ Fig. B.2C, $^c$ Fig. B.3A, and $^d$ Fig. B.3C.
Overall alignment validation:

When the FCCS dichroic emission filter cube is used in the detection pathway instead, the diffusion times should be appropriately on the order of the values obtained using the 50/50 beamsplitter. If the beams are not similar in terms of focal volume and not optimally aligned, unexpected results will plague one of the detection channels; for example, the diffusion time and $N$ of one channel will be larger than in the other channel. In addition, $\tau_d$ and $N$ can be greater than expected if the detection volumes are imperfectly overlapping (Bacia and Schwille, 2003; Weidenmann et al., 2002).

To validate the overall alignment of the FCCS setup, a representative set of autocorrelation and cross-correlation curves obtained from A488-IgE-A647 is shown in Fig. B.4. Autocorrelation and cross-correlation curves were fit to 2-component and 1-component 3D-diffusion, respectively, according to (Kyoung et al., 2007; Vats et al., 2008)

$$G(\tau) = N^{-1} \sum_{i} f_i \left[ (1 + (\tau/\tau_{d i}))^{-1}(1 + (1/\omega^2)(\tau/\tau_{d i}))^{-0.5} \right], (m = 1 \text{ or } 2), \quad (B.1)$$

where $G(\tau)$ is the normalized autocorrelation function, $N$ is the average number of fluorescent molecules in the detection volume, $\omega$ is the axial-to-lateral dimension ratio of the detection volume, $\tau$ is the time interval, and $\tau_{d i}$ is the characteristic diffusion time for each fraction ($\sum f_i = 1$). Although the diffusion times from each curve are within standard deviation, the number of molecules ($N$) is different for each channel. The discrepancy in $N$ of each autocorrelation curve likely results from free dyes in the A488-IgE-A647 solution, with an apparently greater concentration of A647 (lower amplitude, but higher $N$) detected in PMT A. Veldhuis et al. (2006) observed a similar discrepancy between number of molecules in each channel for their dually-labeled DNA oligomer FCCS calibration sample and calculated correction factors for each channel (see below) to account for this discrepancy. By making the Ar laser beam slightly smaller at the sample plane, it is possible to have nearly identical $N$ values in PMT A and PMT B for A488-IgE-A647; however, $\tau_d$ of the cross-correlation curve then becomes ~2×’s greater than expected.
Figure B.4. A488-IgE-A647 autocorrelation and cross-correlation curves obtained by FCCS. 10 nM A488-IgE-A647 in 1× PBS (pH 7.4) was dually excited with 488 nm Ar laser and 633 nm HeNe laser, and simultaneously detected in PMT A and PMT B using the filter conditions noted above. The acquisition time was 60 s per run and each curve is the average of 10 runs. The open circles, closed circles and open squares represent autocorrelation data from PMT A and PMT B, and cross-correlation data, respectively. The major $\tau_d$ is the same for each curve, within standard deviation. The autocorrelation curves in PMT A and PMT B were fit to 2-component 3D-diffusion, with $\tau_{d1} = 6 \pm 1 \mu$s, $\tau_{d2} = 0.8 \pm 0.1$ ms, $N = 8.6$, and $\tau_{d1} = 0.12 \pm 0.03$ ms, $\tau_{d2} = 0.8 \pm 0.1$ ms, $N = 5.1$, respectively. The cross-correlation curve was fit to 1-component 3D-diffusion, with $\tau_{d1} = 0.9 \pm 0.1$ ms and $N = 16.5$. 
If the amplitude obtained from one detection channel is lower than the other, the cross-correlation amplitude, even for 100% association, can only reach the amplitude of the autocorrelation curve with the lower amplitude (Bacia and Schwille, 2003). Even so, at best, the cross-correlation obtained with A488-IgE-A647 has almost half the amplitude of (or ~2×'s greater than) the autocorrelation from PMT A. Because the two observation volumes will never overlap perfectly, the cross-correlation efficiency must be evaluated to correct for the spatial misalignment. Following the equations outlined by Veldhuis et al. (2006) (see below), we determined that there is ~5 nM free dye detected in PMT A (the red channel) and ~0.9 nM detected in PMT B (the green channel), that N for the cross-correlation should be ~12, rather than 16.5 (see Fig. B.2), and therefore that the estimated cross-correlation efficiency of our setup is ~75%.

Specifically, to estimate cross-correlation efficiency we first assumed that there are no homodimers or free dyes in the A488-IgE-A647 sample and calculated a theoretical N for each channel. Then, the N values determined from fitting the autocorrelation curves were used to calculate correction factors for each channel that were in turn used to determine the theoretical N for cross-correlation. The efficiency of cross-correlation was determined by comparing this theoretical N with the actual N obtained via fitting (Veldhuis et al., 2006).

Assuming no homodimers or free dye, the amplitudes \( G(0) \) of the individual autocorrelation curves of A488-IgE-A647 are given by:

\[
PMT A: \quad G_r(0) = \left[ \frac{(C_{g,r})}{V_r(C_{g,r})^2} \right],
\]

\[
PMT B: \quad G_g(0) = \left[ \frac{(C_{g,r})}{V_g(C_{g,r})^2} \right],
\]

according to Veldhuis et al (2006) and are inversely proportional to \( N \). The concentration of the A488-IgE-A647 heterodimer, \( C_{g,r} \), is 10 nM. The effective observation volumes of each channel, \( V_r \) and \( V_g \), are determined as follows (Veldhuis et al., 2006):

\[
PMT A: \quad V_r = \pi^{3/2} \omega_0^3 \omega_r,
\]

\[
PMT B: \quad V_g = \pi^{3/2} \omega_0^3 \omega_g.
\]

\( \omega_0 \) is the structure parameter that is calculated for each channel following \( D = \omega_0^2 / 4 \tau_d \), where \( \tau_d \) is obtained by fitting the autocorrelation of a 6 nM aqueous Cy5 solution measured in PMT A \((D = 2.5 \times 10^{-6} \text{ cm}^2/\text{s})\) or a 10 nM aqueous rhodamine green solution measured in PMT B \((D = 2.8 \times 10^{-6} \text{ cm}^2/\text{s})\) to 1-component 3D-diffusion (Eq. B.1). \( \omega \) is the axial-to-lateral dimension ratio of each detection volume (Eq. B.1) that is also determined via fitting Cy5 or rhodamine green autocorrelation curves. Using
Eqs. B.2–B.5, we calculated theoretical $N$ values for A488-IgE-A647 in PMT A ($N = 1/G_r(0) = 5.9$) and PMT B ($N = 1/G_g(0) = 4.7$).

The $N$ values determined from fitting A488-IgE-A647 autocorrelation curves measured by PMT A ($N = 1/G_r(0) = 8.6$) and PMT B ($N = 1/G_g(0) = 5.1$) (Fig. B.4) were used in the following equations to determine the correction factors that can be applied to the cross-correlation data (Veldhuis et al., 2006):

PMT A: 
$$G_r(0) = \left[\frac{(C_{g,r}) + (C_{r,r})}{V_r(C_{g,r} + C_{r,r})^2}\right],$$  \hspace{1cm} (B.6)

PMT B: 
$$G_g(0) = \left[\frac{(C_{g,r}) + (C_{g,g})}{V_g(C_{g,r} + C_{g,g})^2}\right],$$  \hspace{1cm} (B.7)

where $C_{r,r}$ and $C_{g,g}$ were calculated as $\sim 5 \text{ nM}$ and $\sim 0.9 \text{ nM}$, respectively, using the previously determined values for all other parameters (Eqs. B.2–B.5). Due to the higher concentration of free dye in PMT A ($\sim 5 \text{ nM A647}$) as compared to PMT B ($\sim 0.9 \text{ nM A488}$), there is a greater discrepancy in PMT A between the theoretically determined $N$ versus the $N$ determined via fitting.

Using the correction factors $C_{r,r}$ and $C_{g,g}$, the theoretical $N$ value for the cross-correlation is determined according to (Veldhuis et al., 2006):

Cross: 
$$G_{g,r}(0) = \left[\frac{(C_{g,r})}{V_{g,r}}(C_{g,r} + 2C_{r,r})(C_{g,r} + 2C_{g,g})\right],$$  \hspace{1cm} (B.8)

where $C_{g,r}$, $C_{r,r}$, and $C_{g,g}$ are as previously noted and $V_{g,r}$ is calculated by:

Cross: 
$$V_{g,r} = \pi^{3/2} \omega_{g,r}^{3/2} \omega_{g,r},$$  \hspace{1cm} (B.9)

$\omega_{g,r}$ and $\omega_{g,g}$ are equal for this setup (i.e., $0.26 \text{ µm}$), so the same value is used for $\omega_{g,r}$.

$\omega_{g,r}$ is the average of $\omega_r$ and $\omega_g$ (i.e., 10 and 8, respectively). Following Eqs. B.8 and B.9, $N = 1/G_{g,r}(0) = \sim 12$, which is $\sim 75\%$ of the $N = 16.5$ that was determined from fitting (Fig. B.4).
Protocol for photon counting histogram (PCH) analysis of FCS data

PCH analysis is used to differentiate species with similar diffusion times by differences in molecular brightness (Thompson et al., 2002). Brightness levels also provide information about the degree of clustering or binding in a system (Thompson et al., 2002). During acquisition of fluorescence fluctuation data (i.e., FCS), a probability histogram of photon counts within the fL detection volume is generated and may be analyzed using Poisson statistics to determine molecular brightness or concentration. Molecular brightness is determined by the average number of photons detected per sampling time, per molecule and is proportional to the product of the quantum yield, extinction coefficient, sampling time and detection efficiency for a particular fluorescent species (Chen et al., 1999). Thus, a brightness distribution may be due to different fluorescent species, or due to concentration changes, or aggregation, of a single fluorescent species with defined brightness (Chen et al., 2002; Sanchez et al., 2001).

In FCS, the excitation volume is not uniformly illuminated such that the fluorescence intensity, as well as the detection efficiency, is different at various locations within the detection volume and follows a 3D-Gaussian point spread function (Chen et al., 1999) in our setup (Fig. B.1). All of the photon count histograms for a particle at these different positions are described by a Poisson distribution; thus, there is a low probability of detecting a large number of particles within a fL detection volume. When this photon counting histogram (PCH) distribution is fit, the average number of fluorescent particles ($N$) and their brightness ($\varepsilon$) is obtained, as well as the number of species with different brightness. For a given brightness, as sample concentration or degree of aggregation increases, the PCH distribution broadens and the statistics become more Poissonian (Chen et al., 1999). PCH statistics also become more Poissonian as brightness decreases.

Considerations for FCS data acquisition

During FCS, sufficient counts per time bin must be obtained to fit the acquired histogram data using PCH analysis. If the Flex02-12dc version (D:\090308_Flex02_for PCH_no norm\Flex02-12dc) of the FCS software is used, this can be achieved with 1–10 nM sample concentration and moderate laser power (i.e., 250 mW of output power from the Ar laser with ND = 2.2–3.2, i.e., ~0.1–1 mW at the sample plane). While FCS data is acquired, the photon histogram can be observed in the histogram window. It is usually best to increase the sample time in the FCS software (under Settings, Photon History Sample Time, change the sample multiplier) from a 1 µs default, until a Gaussian curve, or part of a curve, is observed in the histogram window. Increasing the sample time increases the number of time bins on the histogram x-axis, and increases the Gaussian nature of the distribution.
NOTE: An older version of the FCS software, Flex02_12dc
(D:\082806_add_Flex02c\Flex02_12dc), will automatically normalize all of the photon
counts to one, such that the photon histogram cannot be properly fit with PCH analysis.

To optimize the data acquisition conditions for PCH analysis, it is advisable to
test first the simplest components of a mixture to be analyzed, under various sample
conzentraions, laser powers and sample time conditions. This is because, for a given
brightness ratio between species in a mixture, optimal concentrations exist for each
species at which there is maximal deviation from a single-species PCH distribution
(Müller et al., 2000). Müller et al. (2000) provides thorough discussion of the com-
promises that must be made between the various conditions noted above. The next sec-
tion describes how to determine the goodness of a PCH fit under these conditions. FCS
fitting should be performed first (using IgorPro) to determine the expected N and number
of diffusing species, if applicable, to understand what PCH fit to apply to the data.

PCH analysis procedure

1. The histogram data obtained during FCS measurements is located at the bottom of the
.sin file. Save the left column of data as a .txt file, adding the number of rows to be
analyzed as the first number in the column. It is not necessary to include the many zeros
at the end of this column in the .txt file. (The number at the top of the second column of
data indicates the total number of counts that comprise the first column).

2. PCH analysis is performed using Globals for Spectroscopy software developed at the
Laboratory for Fluorescence Dynamics at the University of Illinois at Urbana-
Champaign. Open the program, go to the file menu and select a default answer file such
as my.ans (C:\Program Files\Globals\my.ans, for example).

3. Open the data file to be analyzed by double-clicking on the Filename line grid and
choosing the .txt file of interest. Enter “0” in the Rec box and “P” in the Type box for
PCH analysis. (Double-clicking on the Rec box will show the contents of the .txt file.)

4. Click on the Select model button on the right side and choose Fluctuation spectro-
scopy, which brings up several options for PCH analysis. After a model is chosen, the
number of species, or specific type of model, can be selected. (For our setup, a 3D-
Gaussian distribution is appropriate, but the number of species will vary depending on the
sample.)

5. Click on the Values/Linking button and enter estimates or initial values for each of the
fit variables associated with the particular model. (This is where N, obtained from fitting
the FCS autocorrelation curves, can be useful.) The Fraction and Background are usually
fixed to 1 and 0, respectively, as designated by “F” next to these fields.
6. Click on the Minimization button to engage the fitting process. The goodness of fit is indicated by the reduced chi square \((\tilde{\chi}^2)\) and residuals (Chen et al., 1999). A reasonable chi square value is \(\sim 1\). An unfavorable chi square is typically obtained when the tail end of the distribution is either sub-Poissonian or super-Poissonian (a super-Poissonian distribution is usually observed at lower sample concentrations), or if the wrong number of species is chosen for fitting.

7. Click on the Report button to display the resulting PCH fit. A summary of the fit parameter values can be obtained from the graph-displaying window as well.

Fig. C.1 provides example PCH fits of A488 (1-species with a 3D-Gaussian distribution) and A488-IgE (2-species with a 3D-Gaussian distribution) in PBS. Histograms are plotted as the detection frequency of a given number of photon counts. (Fit residuals are indicated above the histograms.) FCS data acquisition and PCH fitting conditions are noted in the figure caption. In A488-IgE FCS analysis (see Eq. B.1), two diffusion components were determined, the shorter of which is on the order of free A488 diffusion time (\(-100\ \mu s\)). To further confirm that the secondary diffusion component was due to free dye, PCH analysis was performed. The photon count distribution for an open system is determined by the average of the individual probability functions for \(N\) fluorescent particles diffusing in and out of the detection volume, \(p^{(N)}(h;V_p,\varepsilon)\), weighted by their Poissonian probability of observing \(N\) particles, \(p_h(N)\), according to Chen et al. (1999):

\[
\prod(h;\bar{N}_{PSF},\varepsilon) = \sum_{N=0}^{\infty} p^{(N)}(h;V_{PSF},\varepsilon)p_h(N),
\]  

where the volume of the 3D-Gaussian point spread function, \(V_{PSF}\), is related to the average number of molecules inside that volume, \(\bar{N}_{PSF}\), and the particle concentration, \(c\), by Avogadro’s number, \(N_A\) (\(\bar{N}_{PSF} = cV_{PSF}N_A\)). As a result, \(\bar{N}_{PSF}\) should be equivalent to \(N\) determined FCS fitting of autocorrelation data via Eq. B.1. The average number of photon counts, \(\langle h \rangle\), for an open system can be directly obtained from Eq. C.1 by summing the products of brightness per molecule, \(\varepsilon\), for each species and \(N\) for each species (Chen et al., 1999). One of the two A488-IgE PCH components corresponds to the brightness obtained for A488 under the same concentration and sample time conditions (see Fig. C.1 caption). The second A488-IgE PCH component has higher brightness than the free A488 component, because there are multiple A488 molecules conjugated to each IgE molecule (the average dye/protein ratio is \(\sim 5\), as determined by absorption spectroscopy), and a lower \(N\) due to the random degree of IgE labeling (Müller et al., 2000) (see Fig. C.1 caption). As the sample time is increased, the number of time bins, as well as the molecular brightness (\(\varepsilon\)) reported, increases.
Figure C.1. PCH fitting of A488 and A488-IgE histogram data obtained via FCS at two different sample times. All data shown were acquired using 250 mW of output power from an Ar laser, with ND = 3.2, and a 60 s data acquisition period. Autocorrelation of A488 in PBS (pH 7.4) was fit to 1-component, 3D-diffusion, while A488-IgE in PBS was fit to 2-component, 3D-diffusion (not shown). For PCH, A488 was best fit as 1-species with a 3D-Gaussian distribution, whereas A488-IgE was best fit as 2-species with a 3D-Gaussian distribution. Under 20 µs sample time, 10 nM A488 in PBS (A) yielded $\varepsilon = 0.07$ and $N = 10$, with $\tilde{\chi}^2 = 0.8$, and 10 nM A488-IgE in PBS (B) gave $\varepsilon_1 = 0.06$, $N_1 = 13$, $\varepsilon_2 = 0.31$ and $N_2 = 0.75$, with $\tilde{\chi}^2 = 0.96$. Under 200 µs sample time, 10 nM A488 in PBS (C) yielded $\varepsilon = 0.46$ and $N = 16$, with $\tilde{\chi}^2 = 1.0$, and 10 nM A488-IgE in PBS (D) gave $\varepsilon_1 = 0.59$, $N_1 = 15$, $\varepsilon_2 = 3.9$ and $N_2 = 0.39$, with $\tilde{\chi}^2 = 1.3$. **NOTE:** Molecular brightness can be expressed in terms of photon counts per second per molecule (cpsm) upon dividing $\varepsilon$ by the sample time (in s).
Angel Davey was born on November 18, 1980 in Lewistown, Pennsylvania. She attended high school in Pennsylvania and later in West Virginia where she became interested in science and mathematics. Angel began her academic career at West Virginia University in Morgantown, West Virginia as a chemical engineering major. After one year, she transferred to Shepherd University in Shepherdstown, West Virginia where she graduated *summa cum laude* with a BS in chemistry and a minor in mathematics in May 2003. While at Shepherd, Angel obtained research experience by investigating Vitamin D3 photochemistry with Professor Dan DiLella in the Department of Chemistry and examining the properties of archaeological ceramic adhesives with Dr. Judith Bischoff at the National Park Service, Department of Conservation in Harpers Ferry, West Virginia. Attracted to interdisciplinary research, Angel then attended graduate school at The Pennsylvania State University where she studied the role of the cell membrane in allergic signaling using biophysical approaches, under the supervision of Professor Erin D. Sheets in the Department of Chemistry. As a graduate student, she obtained extensive experience as a teaching assistant and supervising teaching assistant for various general chemistry courses. Angel also had the opportunity to present her research at several scientific meetings, with support from travel awards sponsored by the department, the Biophysical Society and the American Society for Cell Biology. The department offered her further support in the form of a Braddock Graduate Fellowship, Dalaiian Graduate Research Fellowship Award and Dan H. Waugh Memorial Teaching Award. Upon defending her dissertation in December 2008, Angel will continue to pursue biological research with a postdoctoral intramural research training award in the laboratory of Dr. Susan K. Pierce at the National Institute of Allergy and Infectious Diseases in Rockville, Maryland, working in the area of B-cell receptor signaling and memory.