Investigating the Role of Lipid Anchors in Live Cell Membrane Organization Using Fluorescence Fluctuation Spectroscopy

by

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A dissertation submitted in partial satisfaction of the requirements for the degree of
Doctor of Philosophy
in
Chemistry
in the
Graduate Division
of the
University of California, Berkeley

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Spring 2011
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Abstract

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The cellular plasma membrane is a heterogeneous and dynamic two-dimensional fluid. It has long been considered the necessary boundary between the internal components of a cell and the surrounding environment. The organization of membrane proteins, originally thought to freely and randomly diffuse throughout the plasma membrane, has been recognized as essential to the proper functioning of a cell. Evidence from detergent extraction of membranes, model membranes, and nanometer resolution microscopy techniques requiring cell fixation have supported the idea that the membrane, through lipid-lipid and lipid-protein interactions, plays an active role in membrane protein organization. Advanced fluorescence microscopy and spectroscopy techniques offering subdiffraction resolution have added to the evidence and knowledge of heterogeneity in the membrane lipid bilayer of live cells, but have led to mixed results on the exact role that lipid-lipid and lipid-protein interactions play in lateral membrane organization.

Here we employ pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS) to study the co-diffusion of lipid anchors in live cell membranes. Lipid anchors are lipid moieties often found attached to membrane proteins. They function to tether proteins to membranes, and we ask if they also play a larger role in lateral membrane organization. We use fusions of fluorescent proteins and lipid anchors that participate in only lipid-lipid and lipid-peptide interactions without any direct protein-protein interactions. PIE-FCCS allows us to observe the co-diffusion of two different colored species while completely removing cross-talk due to spectral bleed-through and simultaneously measuring the fluorescence lifetimes to monitor energy transfer. We find a highly specific organizing scheme wherein different anchors partition into distinct domains, and we see no evidence of Förster Resonance Energy Transfer through fluorescence lifetimes indicating the absence of tight labeled-anchor complexes.
I dedicate this work to my husband and our families, for their unwavering love and support.
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Acknowledgments

Foremost, I’d like to acknowledge Hector H. Huang, with whom I shared a large part of the work done during my graduate career. Hector provided lots of inspiration, conversation, and good hard work that ultimately led to results. Dr. Adam W. Smith also significantly contributed to the same work, and was especially helpful in keeping us grounded and in giving advice from the perspective of experience. Il-Hyung Lee wrote the invaluable Matlab scripts that convert photon streams to correlation curves, and Michael Coyle wrote scripts that helped tremendously in streamlining data analysis. Without the contributions of these two, I would still probably be stuck in front of a computer. Eldon Chou did the cloning that allowed us to reach high densities of lipid anchors in live cells, and his work is much appreciated. Wan-Chen Lin was always helpful in optical issues with the microscope, and for much of the beginning of my time in graduate school was a very patient and very thorough teacher. I am lucky that she chose to mentor me in those early days.

I would also like to acknowledge all of the members of the Groves Lab for all that they do to contribute to an environment of scientific collaboration. I am often happily reminded of how much I appreciate the friendly and supportive atmosphere that surrounds every group meeting and qualifying exam practice. It was a pleasure to be able to celebrate scientific and personal successes with so many of you.

My advisor, Professor Jay T. Groves is always encouraging, always grounding, and always optimistic. He has quite a mind for science, and I have learned a lot about being a scientist from him.

There are many people outside of the lab, outside of UC Berkeley, and even outside of science who stood by me, encouraged me, and gave me confidence in my ability to complete this degree. Dr. Chrystal Bruce, one of my undergraduate professors at Erskine College, believed in my ability to do whatever I put my mind to, and often encouraged me to reach farther than I thought I could or should. Friends in my small group and in Veritas graduate fellowship always smiled and told me I could do it, even when they didn’t quite understand what it was that I thought I couldn’t do. My family, including the Curtiss, Cullifer, Van Epps, Fyke, Triffo, Watson, and Hartsel families, have ALL been supportive and encouraging. Finally, I acknowledge my husband, who gave the most meaningful encouragement at the most crucial times and continues to support me in all I try to do.
Chapter 1

Organization of Cell Membranes
1.1 Abstract

Much more than merely a container for cell components, the cellular plasma membranes is a complex, heterogeneous mixture of lipids and proteins. This chapter gives a brief overview of past and current ideas about the organization of the plasma membrane and the direct or indirect role that the membrane plays in the lateral organization of membrane proteins. Evidence from membrane extractions and model membranes, which support lipid-driven organization are discussed as well as more advanced optical techniques that can be used to make direct observations in live cells.

1.2 Cell Membranes

Lipid bilayers consist of two layers of lipids whose hydrophilic headgroups interface the cytosol on the inside of the cell and the aqueous environment surrounding the cell, and their hydrophobic tails face inward and interact with each other. The fluid-mosaic model proposed by Singer and Nicholson in 1972 identified the membrane as a two-dimensional fluid in which proteins matching the width of the membrane float freely. At that time, the cell plasma membrane was thought to be homogeneous with proteins randomly distributed. Scientists quickly started recognizing the important roles played by membranes in cell-cell communication and signaling within a cell. In a review by Engelman, the author contrasts what Singer and Nicholson defined in 1972 to the modern view of cell membranes densely packed with proteins that offer a functional patchiness to the membrane structure. The understanding of the composition, structure, and function of the cell membrane has grown and changed dramatically with the advent of new scientific ideas and techniques. The essential roles the plasma membrane plays in many signaling pathways and other cell processes have become increasingly understood and appreciated. Despite these advances, we are far from having a complete understanding of exactly how the membrane functions, especially with respect to organization and heterogeneity of membrane proteins.

1.3 Diversity of Lipids

There are over 1000 different types of lipids in a typical mammalian cell. Lipids that make up cell membranes are characterized by a head group along with the length and degree of saturation of each of its fatty acid tails. The main lipid components of the eukaryotic plasma membrane include glycerophospholipids, sphingolipids, and cholesterol. Several lipids, such as ceramides and sphingosines, are bioactive, and participate directly in cell signaling and regulating cell processes. Other lipids have a more indirect effect by recruiting or dispersing membrane proteins as their populations change. Still others may do nothing more than maintain the impermeability and fluidity that is necessary for the compartmentalization of cellular components. The diversity of lipids and the evidence for
protein organization and colocalization in membranes leads to numerous investigations into whether lipids play a direct role in the lateral organization of cell membranes.7-10

1.4 Membrane Proteins

A large fraction of the total area of a cell membrane is covered with membrane proteins. Membrane proteins can facilitate cell signaling, ion transport, cell-cell interactions, and other cell functions. They are of such importance that about 30% of genes in eukaryotic cells code for membrane proteins.11 Trans-membrane proteins span the width of the bilayer and have segments compatible with the hydrophobic tails of lipids that make up the inner portion of the lipid bilayer. These and all membrane proteins can interact with each other and intracellular proteins through various chemical interactions. Other methods by which proteins bind to the outer or inner leaflet of a membrane include hydrophobic segments that insert and hold a protein to a membrane, amphiphilic alpha helices that bind to the intermediary region between the lipid headgroup and the fatty acid tails, specific lipid-binding domains, and covalent attachment of lipid moieties, which insert into the membrane. Often, proteins will have a combination of anchoring or binding motifs, and can also be stabilized by charged lipid headgroups.12 This diversity in methods of binding proteins to membranes allows proteins to bind distinct lipid membranes in different organelles and possibly distinct lipid membrane domains.

1.5 Lipid-Anchored Proteins

Lipid-anchored proteins have covalently attached lipid moieties. These moieties are attached during or after translation, or synthesis of the protein inside the cell. While functional in facilitating proper trafficking to specific organelle or plasma membranes, these moieties also keep proteins bound to membranes as depicted in Figure 1.1.13,14 This class of protein is the primary subject of chapter 3, and will be discussed in more detail there.

The many different methods of binding or tethering proteins to membranes can be, and often are, combined to form a library of anchoring motifs. The implications of these different anchors have been of great interest recently. Particularly, because of the ability of lipid anchors to directly interact with other lipids, they are intriguing subjects to use to study lipid driven membrane organization. There is also evidence that the lipid modifications play a crucial role in the cellular function of proteins. For example, H-Ras, N-Ras, and K-Ras give distinct signal outputs despite their close homology. The Ras isoforms differ only in the c-terminal region, which encodes for specific lipid moiety modification. The difference in function has been attributed to localization to distinct domains in the plasma membrane.15
1.6 Membrane Organization

One large-scale example of lipid sorting or lipid domain formation is the differential sorting of lipids between the apical and basolateral membranes of polarized epithelial cells. The two membranes have different compositions of proteins and lipids and serve different functions.\textsuperscript{16} Another example of organized membrane structures are caveolae, which are small (50-100nm) invaginations of cell membranes. Many proteins are found localized in caveolae, and the lipid composition of caveolae is enriched in cholesterol and sphingolipids compared to the surrounding membrane.\textsuperscript{17} Larger scale organization includes patterns such as the immunological synapse seen in lymphocyte T-cells. This bulls-eye pattern of sorted proteins arises when an antigen-presenting cell interfaces with a T-cell and the T-cell becomes activated.\textsuperscript{18}

The finding of compositional heterogeneity in cell membranes has led to many studies of the existence and formation of lipid domains. One of the more popular schools of thought on this includes the existence of lipid “rafts” whose definition has undergone many changes since the coining of the term by Simons et al. in 1997.\textsuperscript{19} Membrane rafts are said to be enriched in sphingolipids and cholesterol, which causes them to be more tightly packed and recruit certain “raft” proteins. Membrane rafts are implicated as organizing principles in cell signaling and trafficking.\textsuperscript{20} In the past decade, many studies have been performed on live, fixed, and extracted cell membranes in an attempt to find evidence of these membrane rafts and elucidate their characteristics. While some surprising and intriguing findings have been made in giant plasma membrane vesicles, there is arguably no live-cell evidence of stable raft domains, as originally defined, in live cells.\textsuperscript{8,21,22} Collectively, membrane researchers have refined the original raft definition, which described stable ordered domains on the micron scale that float in the cell membrane, to instead be highly dynamic, on the nanoscale, and transient.\textsuperscript{9,23} Still, many scientists use the lipid raft hypothesis in terms of a stable binary phase separation to explain phenomenological results of membrane protein colocalization and other membrane studies. While the definition of membrane rafts has become ambiguous enough to be more confusing than clarifying, the evidence needed to better understand the mechanisms of membrane organization continues to grow.
1.7 Domain Formation in Model Membranes

Lipids have interesting phase behavior when reconstituted into giant unilamellar vesicles (GUV’s) and other forms of model membranes.\textsuperscript{24,25} Compositions, though simplified, that mimic the composition of the outer leaflet of cell membranes phase separate into liquid ordered (Lo) and liquid disordered (Ld) phases. The Lo phase is composed of high melting temperature ($T_m$) lipids with saturated fatty acid tails and cholesterol, which fills in the spaces between lipids enhancing order while still allowing relatively fast lipid diffusion characteristic of a liquid phase. The Ld phase is composed of low $T_m$ lipids with unsaturated or branched tails as these take up more volume and do not compress into an ordered configuration. Diffusion in the Ld phase is faster than diffusion in the Lo phase.

While using the same molecules, the results in model membranes are far-removed from what is occurring and what can actually occur in the live cell membrane. This is because of the complexity of live cells. Not only are there many more types of lipids, proteins, and other molecules in a live-cell membrane than there are in model membranes, but a live cell membrane also interacts with and is restricted by the actin cytoskeleton and other intracellular proteins and processes.\textsuperscript{26,27} Large-scale phase separation in live cell membranes has never been observed. However, phase separation in osmotically swollen cell membranes and giant plasma membrane vesicles (GPMV’s), which are extracted directly from live cells, do show large-scale phase separations visible by optical microscopy.\textsuperscript{21,22,28} Recently GPMV’s have been shown to pass through a critical point during temperature scanning.\textsuperscript{9} Despite this evidence and the hypothesis that critical fluctuations, which are small and short-lived, could be the physical explanation behind membrane heterogeneity and domain existence in live cells, no evidence of these critical fluctuations or of a phase separation or phase change has been found in live cells.

![Figure 1.2 Schematic of a Lo/Ld phase separated model membrane.](image)
1.8 Labeling Membrane Domains

The choice of label or probe used to identify membrane domains requires careful consideration. In model membranes, this can be done with either a fluorescently labeled lipid or a lipid probe. These are included in appropriate fractions in lipid compositions prepared before forming model membranes. For live cell membranes, the possible methods depend on whether the protein or molecule of interest is incorporated in the inner or outer leaflet of the cell membrane. If on the inner leaflet, genetically fusing a green fluorescent protein (GFP) to the protein of interest is a minimally invasive and common method of labeling intracellular proteins. Labeling with GFP also ensures that there will be a maximum of one fluorophore per protein. This ratio is important when attempting to perform quantitative measurements and holds for expression of any fluorescent fusion protein. The wide range of emission of fluorescent proteins available makes this approach applicable to multicolor experiments as well. Monomeric versions of these proteins should be used when possible as opposed to the wild-type, which have a small but significant propensity to dimerize, so that no additional clustering is induced. Other methods sometimes used to label internal proteins that involve fixing cells and permeabilizing the membranes in order to allow antibodies or other markers to get inside the cell are likely not suitable for experiments studying membrane organization as they may rearrange or change the properties of membranes.

There are fewer restrictions for labeling proteins or lipids on the outer leaflet of the cell. GFP is often used with the benefits of a one to one labeling ratio and very specific labeling. The disadvantage to using GFP is that it usually requires the cell to make additional protein, and one must be careful to consider consequences of over-expressing proteins. Lipid probes may also be used to study diffusion or colocalization of lipid components. Lipid probes such as Dil do not label or bind to specific membrane components, but rather mimic lipid molecules and insert into the outer leaflet of the cell membrane. Lipid probes can be used to mark liquid ordered or liquid disordered phases in model membranes, and may be supposed to do the same thing if those phases exist in live cell membranes. Antibody labeling is another common option. Antibodies can be used as full antibodies, which have two identical binding sites, or just the fab fragments of antibodies that have only one binding site can be used. Because clustering is often interpreted as an indication of membrane domain existence, using monovalently binding species to label proteins or lipids avoids possible artifacts associated with the induced clustering of certain proteins by antibodies or other multivalently binding proteins. Lipid-binding proteins such as Cholera Toxin Subunit B (CTB) are also frequently used to label lipids and domains, but evidence has shown CTB can induce binding and may falsely show evidence of domains.
1.9 Observing Membrane Organization

Two observable characteristics are commonly used to determine whether a lipid or protein is participating in lateral membrane organization in live cells. These are colocalization with other lipids or proteins, or a decrease in its diffusion or mobility.

Observing colocalization can be done with many different techniques. Fluorescence microscopy is often used because it is highly specific and sensitive, detecting only fluorescently labeled molecules. Traditional optical microscopy can resolve micron-scale organization such as signaling clusters in T-lymphocytes, but membrane organization is often thought to occur on a smaller, nanometer, scale. Super-resolution techniques such as photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) and stimulated emission depletion (STED) can better resolve the positions, and therefore nano-scale colocalization, of molecules.\textsuperscript{34-36} These techniques require long imaging time due to imaging only few fluorophores at a time. Membrane organization has been proposed to be highly dynamic, and the time requirement for imaging with these techniques may limit their utility. Promising improvements have been and are constantly being made to these techniques to make them faster and more applicable to dynamic live cell studies.\textsuperscript{37}

Other methods of observing colocalization include Förster resonance energy transfer (FRET) and fluorescence cross-correlation spectroscopy (FCCS). FRET can detect the colocalization of two fluorophores if the are within their Förster radius of one another, which is typically less than 10nm.\textsuperscript{38} FRET can be calculated from wide-field imaging or through lifetime analysis as the fluorescence lifetime of a donor fluorophore undergoing energy transfer decreases. FCCS can detect dynamic colocalization of two fluorophores at length scales of up to the fluorescence excitation area, usually around the size of a diffraction-limited spot (~250nm).\textsuperscript{39,40} This technique will be discussed in more detail in Chapter 2.

The second observable in membrane organization is the diffusion or mobility of lipids and proteins. Techniques used to monitor mobility of molecules in live cells include single particle tracking (SPT), single molecule tracking (SMT), fluorescence recovery after photobleaching (FRAP), and fluorescence correlation spectroscopy (FCS). SPT and SMT track molecules labeled with gold nanoparticles (SPT) or fluorophores (SMT) by imaging localization.\textsuperscript{41,42} Findings of molecules showing confined diffusion or regions where their diffusion is slowed have been observed and attributed to dense “raft-like” domains or interactions of the plasma membrane with the actin cytoskeleton acting as a barrier. FRAP monitors the rate and fraction of recovery after photobleaching to determine if molecules are barred from normal diffusive behavior by inclusion in domains or any other structure that may alter their diffusion.\textsuperscript{43} FCS finds the diffusion coefficient of labeled molecules by calculating the autocorrelation of thermal fluctuations of a system at equilibrium or steady state.\textsuperscript{44} All of these techniques have been in some way modified or combined with others to become even more powerful tools used to investigate membrane organization in live cells.
Chapter 2

Pulsed-Interleaved Excitation Fluorescence Cross-Correlation Spectroscopy on Membranes
2.1 Abstract

Fluorescence correlation spectroscopy (FCS) is a powerful technique for obtaining characteristic parameters from an unperturbed system. Since its original conception, it has seen many variations keeping it at the forefront of modern single-molecule fluorescence techniques. One such variation is pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS). Discussed here are the techniques of FCS, FCCS and PIE-FCCS in the context of the unique challenges of using these techniques to study two-dimensional model and live cell membranes.

2.2 Introduction

Fluorescence correlation spectroscopy (FCS) is a technique that advantageously utilizes the statistics of thermal fluctuations in a system at equilibrium or steady state to derive characteristics of that system. In the context of this dissertation, the systems of interest are composed of diffusing fluorescently labeled molecules in supported lipid bilayers or live cell membranes. The fluctuations arise from the exchange of diffusing molecules in and out of a tightly focused laser spot, which makes up the excitation area. Diffusion will generally be limited to two-dimensions, and concentrations will be considered as density in some two-dimensional area unless otherwise noted. The most common system characteristics obtained by FCS are diffusion coefficients and concentrations.

Magde et al. originally introduced FCS in 1974.45,46 Powerful for use in chemical kinetic experiments, its first experiments involved biological applications: DNA binding ethidium bromide, and diffusion of lipid dyes in cell membranes. Inherently high sensitivity made up for the complicated experimental setup and hours-long acquisition time for some experimentalists, but it was not until dramatic improvements in photon detectors and the addition of a confocal pinhole to limit the background light that the technique started to become more widespread.47 Aside from observing diffusion and concentration, which have been sufficient for answering questions concerning membrane organization discussed in chapter 3, FCS can also be used to observe kinetics of molecular binding and unbinding by observing changes in diffusion as a small, fluorescently labeled species binds to a larger, slower diffusing species over time. Further, by adding a second fluorophore to the second species and a second excitation source, fluorescence cross-correlation spectroscopy (FCCS) can be used to observe the time dependent change in the amount cross-correlation of species of any size, which indicates dynamic colocalization.48,49 Our use of two-color FCCS has been independent of changes in time, and has revealed organizational characteristics of the cell plasma membrane.
Key equations:

Equation 2.1 shows the autocorrelation function used to calculate the correlation curves in figure 2.1 where $\delta I$ is the difference in intensity at time $t$ from the average intensity, $\tau$ is lag time, and the brackets denote the time-average value.

$$G(\tau) = \frac{\langle \delta I(t)\delta I(t + \tau) \rangle}{\langle I \rangle^2} + 1$$

(2.1)

Equation 2.2 shows the cross-correlation function where $I_r$ and $I_g$ are the intensities from the red and green channels, respectively.

$$G_x(\tau) = \frac{\langle \delta I_r(t)\delta I_g(t + \tau) \rangle}{\langle I_r \rangle\langle I_g \rangle} + 1$$

(2.2)

Equation 2.3 is the two-dimensional diffusion model for autocorrelation curves where $N$ is the average number of fluorophores in the focal spot and $\tau_D$ is the diffusion time or average time a fluorophore spends in the focal spot. The amplitude, $G(0)$ is inversely proportional to $1/N$. When the size of the focal spot is known, concentration can be calculated from this parameter. The diffusion time, $\tau_D$, can be related to the diffusion coefficient, $D$, by equation 2.4.
\[
G(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} + 1
\]  
(2.3)

\[
\tau_D = \frac{\nu^2}{4D}
\]  
(2.4)

### 2.3 Fluorescence Spectroscopy Optical Setup

A Nikon TE2000-E, which utilizes several commercial and home-built system is used to perform the FCS/FCCS experiments discussed here. The Nikon Perfect Focus System (PFS) offers stability in focus crucial to fluorescence fluctuation spectroscopy data acquisition from two-dimensional membranes. Also, a 100% right port crystal allows all emission light to be directed toward the FCS/FCCS detection path as opposed to default crystals which split the light 80%/20% between a port and the eyepiece. The excitation path consists of two lasers that can be combined into a single mode optical fiber to perform single-color FCS or two-color FCCS. The detection path directs the emission through a confocal pinhole then splits the light into two equal parts for FCS or directs the light according to wavelength for two-color FCCS to two single-photon detectors.

![Diagram of excitation and detection paths on home-built FCS/FCCS setup.](image)

Figure 2.2: Schematic of excitation and detection paths on home-built FCS/FCCS setup.
In more detail as shown in figure 1, a Kr/Ar mixed gas laser (Stabilite 2018-RM, Newport Corp., Irvine, CA) provides excitation at 488nm, 568nm, or 647nm by manually tuning the laser to the desired wavelength. A pulsed diode laser (PicoQuant, LDH-P-C-485, Series 1010767) provides excitation at 479nm. Previously, both lasers were split by 90%/10% beam splitters, and the 90% path went to one single mode fiber intended for Total Internal Reflection Fluorescence (TIRF) excitation, while the 10% went to the single mode fiber intended for FCS/FCCS. This was done using an Ar gas laser in place of the diode laser, and allowed quick switching between TIRF and FCS excitation modes. Recently, the beam splitter in the 479nm path was removed allowing 100% of its power to go toward the FCS dedicated fiber, leaving the possibility for only one-color TIRF, but increasing the power as was needed for FCS excitation.

After splitting by the 90%/10% splitter the two laser paths are combined by a 500nm long pass beam combiner configured so that the 568nm or 647nm path passes through while the 479nm reflects, and both are directed into the fiber launchers. The terminating end of one fiber couples to a beam expander for FCS/FCCS excitation, while the other couples to a set of lenses that expand and then focus with a long focal length (the distance to the back of the objective) the beam for TIRF excitation.

A dichroic holder custom built by the machine shop in the College of Chemistry at UC Berkeley holds custom dichroic mirrors (Chroma Technology Corp., Rockingham, VT) that reflect only the two laser lines in use while allowing all other wavelengths to pass through. The custom dichroic holder was built to hold a dichroic between the filter wheel and the Perfect Focus System so that the excitation light does not enter through the side port of the microscope and does not pass through the filter wheel before excitation.

For FCS/FCCS a collimated beam reaches the back aperture of a Nikon 100x TIRF objective with a 1.49 NA. The objective focuses the excitation light to a nearly diffraction limited spot on the fluorescent sample. After fluorescence emission passes back through the objective, a notch filter that blocks 568nm and 647nm and a 488nm long pass filter sit in a filter cube in the filter wheel to block any reflected or scattered laser light.

The emitted light is then entirely directed out of the side port of the microscope body and through a 50μm pinhole. A focusing lens further focuses the beam and either a 50/50 beam splitter directs the fluorescence emission equally to two avalanche photodiodes (APD) (SPCM-AQRH-16, Perkin&Elmer, Canada) for FCS or a 580nm long pass beam combiner splits the light for FCCS so that the emission from each fluorophore is directed to one or the other APD. Filters are appropriately placed for the specific application. For example, if performing FCS on a GFP labeled molecule, a 488nm long pass and a 520nm short pass filter are used to block any shorter or longer wavelengths of stray light before the beam is split. For FCCS, the 488nm long pass filter remains, but the 520nm short pass filter is placed directly in front of APD B while a 615/75 or 625/45 band pass filter is placed directly in front of APD A.
2.4 Data Acquisition

The disadvantage of highly sensitive experiments like FCS is the possibility of sources of error causing artifactual results. The possible artifacts are increased when measuring fluorescence fluctuations of a membrane or any nearly two-dimensional sample as changes in focus lead to relatively large changes in excitation area. With care in experimental setup and techniques, these artifacts can be completely avoided.

2.4.1 Alignment

Proper alignment of the excitation and emission paths is crucially important before any fluorescence fluctuation bilayer experiment. A supported-lipid bilayer serves as a great sample to use for alignment because it is stable, fluid, and essentially two-dimensional. Typically a 99.992% unlabeled to 0.008% labeled ratio of lipids (TR-DHPE, BD-DHPE, or 0.008% of both) give a good stable fluorescence signal at a wide range of excitation powers. First, the beam must be adjusted so that it is perpendicularly incident to the mounted sample. If the beam is off perpendicular, any small change in focus, or variation in focus from sample to sample, will result in a change in excitation area due to the skew of the beam. Briefly, the excitation beam can be viewed live by directing fluorescence emission to a mounted camera. The appropriate excitation path (FCS) is chosen by putting the “flip mirror” seen in figure 1 up, so that it is blocking the TIRF beam and reflecting the FCS beam into the objective path. The beam should be adjusted so that it is centered on a previously saved target region in imaging acquisition software, and as the focus is moved up and down, no uneven side-to-side or up and down distortion of the beam shape should be observed. If too little light is detected by the camera when the focus is out of plane of the bilayer, the laser power can be increased until the beam appears round and in the same position as when it is focused on the bilayer. Every time the camera is moved or the detection path needs large adjustments, a new FCS target region needs to be saved in acquisition software. This target should be used to make sure the incident FCS beam is in the position that coincides with the position of the pinhole before every set of FCS or FCCS experiments and makes alignment of the detection path much easier.

Aligning the pinhole and APD’s requires care and patience. To start, once the FCS spot is properly aligned in the excitation path, with the APD’s enclosed in a light protecting box and the room lights off, the APD’s should be turned on. The focus should be optimized for maximum detected photon counts in APD A, and the PFS should be set to maintain that focus or any other means of avoiding focus drift should be done. The focus, and the positions of the pinhole and APD A, or the APD directly in line with the emission beam, should be adjusted, in that order, to give the maximum intensity counts detected. This should be repeated first in APD A until a maximum is reached. Only after the pinhole and APD A are maximized should APD B be adjusted to suit the beam path. It is always necessary to check the alignment of the detection path, and is most important to do after changing the dichroic or beam splitter that splits the emitted fluorescence between the two APD’s as each mirror slightly changes the angle of the beam toward APD B. Anything that
causes a drift in intensity while aligning the bilayer, such as slow bleaching, fluctuating laser power, or stage/focus drift, should be avoided at all costs as a change in intensity for any reason other than a change in pinhole, focus, or APD position makes alignment extremely difficult. Methods to avoid these problems are discussed below. If the pinhole and APD’s are not properly aligned, FCS measurements will not reflect real conditions of the sample.

2.4.2 Focus

Because membranes and supported bilayers are essentially two-dimensional, they are more susceptible to problems caused by focus drift or improper focus than three-dimensional solution measurements. Focus can be optimized by maximizing photon counts detected given that more fluorophores exist within or bound to the membrane than in the solution above the membrane. Alternatively, proper focus can be found by taking a z-scan, that is to take FCS curves at several z-positions, of the alignment bilayer. The z-position that corresponds to the lowest value of N (highest G(0) amplitude, see equation 1) is the most accurate. To minimize the effects of focus drift, shorter measurements can be taken and focus checked frequently, however any focus drift limits the accuracy and precision of measurements. Figure 2.3 shows a set of curves acquired consecutively with drifting focus. The changing amplitude reflects the change in the number of fluorophores within the effective excitation area, which increases as the focus drifts away from the waist of the focused beam. Nikon’s Perfect Focus System does a superb job of maintaining constant focus. Designed for maintaining focus for long-term time-lapse imaging, the PFS also works well for maintaining precise focus over short times on systems that otherwise suffer from focus drift. In addition, this system allows much finer control of the focus than the microscope’s default fine focus knob. It is important to note that it is possible for the optimal focus for the FCS detection path to differ from that of the optimal focus for the eyepiece or mounted cameras. This is true on our setup, and care should be taken to readjust and optimize focus for FCS after acquiring image data.
2.4.3 Excitation Power

For FCS, FCCS, or any other fluorescence fluctuation experiment that relies on the fluctuations from the average fluorescence intensity, the molecular brightness, or counts per second per molecule, is the most important parameter when it comes to statistical accuracy. Therefore, using an excitation power that maximally excites fluorophores without, or with minimal, photobleaching is crucial. Laser power necessary for proper excitation also depends on the identity of the fluorophore. Fluorescent proteins, for example, require much less power for excitation and will bleach at lower power than most synthetic dyes, and synthetic dyes usually do not give good enough signal to noise at the same excitation power used to excite GFP. Performing a power scan by taking data at a wide range of excitation powers should be done when using a new fluorophore or using a new optical setup. The appropriate range of excitation power is located by finding the linear region of a plot of detected intensity versus excitation power. A plateau in this curve indicates bleaching of fluorophores.

Usually, power is measured before the excitation beam is passed through the objective lens. As a cautionary note, if a change in the components or alignment of the excitation path causes a change in the excitation spot size, this changes the distribution and effective excitation power that reaches the fluorophores. Power scans should be repeated if such changes in alignment are made.

Obviously using too much power will lead to photobleaching of fluorophores, which will lead to artificially low concentrations and faster diffusion times. When photobleaching occurs, consecutive curves will be inconsistent, and they will not fit a two-dimensional diffusion model. FCS curves of bleaching fluorophores will show a long lag time decay appearing as a drop in the baseline of the decay curve as can be seen in figure 2.4. Using too little power can also have detrimental effects on the observed concentrations. Less discussed and perhaps less universal, is the consequence that when exciting at such low power that the noise is similar in size to the fluctuations caused by diffusing fluorophores, the observed N from the amplitude of an autocorrelation curve will overestimate the

Figure 2.3: Changing amplitudes due to focus drift. These are autocorrelation curves of diffusing labeled lipids in a supported lipid bilayer. The changing amplitudes of these curves, acquired consecutively, are a result of focus drift, which causes a change in the excitation area.
concentration. The power scans shown in Figure 2.5 demonstrate the importance determining proper excitation power by systematically performing a power scan over a wide range of power and looking at multiple parameters. An initial scan (Figure 2.5 A) led to the interpretation that GFP was bleaching as power increased from 0.2μW to 1.0μW while a later scan (Figure 2.5 B) shows that this was an artificial inflation of N due to noise. While N is not the most important parameter to consider when performing a power scan, it is important to note the effect of using too little power.

**Figure 2.4:** Correlation curves from a photobleaching sample. The downward curves at the long lag times of these correlation curves indicate photobleaching of the fluorophore, YFP in this case. These curves were acquired consecutively, and the noisier curves correspond to later time points.

**Figure 2.5:** N vs. Laser Power
(A) Power scan showing decreasing N from 0.2 to 1.0uW. This plot was incorrectly interpreted as photobleaching of GFP and mCherry as power increased. (B) Scanning the power over a wider range together with other data (not shown) indicates that N is artificially inflated at low power settings due to noise, and photobleaching does not occur until well above 2uW of power.
2.5 Pulsed Interleaved Excitation Fluorescence Cross-Correlation Spectroscopy

2.5.1 Spectral Bleed Through Leads to False Positive Cross-Correlation

Spectral bleed-through occurs when photons emitted from one fluorophore bleed into the detection pathway intended for a different fluorophore due to long emission profile tails. In FCCS, this leads to artificially high cross-correlation because a single fluorophore, detected by both detectors, will appear as two fluorophores with identical dynamics and lead to a higher cross-correlation signal. PIE-FCCS corrects this problem by allowing complete removal of all photons that possibly originate from spectral bleed-through before reconstructing the intensity time traces. The following description of PIE-FCCS is given in the context of experiments similar to those in chapter 3 on the setup described above.

While EGFP and mCherry are good, photostable biological fluorescent probes, their broad emission spectra lead to spectral bleed-through. In the optical detection pathway described above, the emission from EGFP and mCherry, or a green and a red fluorophore, are separated by a dichroic beam splitter allowing longer, red, wavelengths to pass through to APD A while reflecting shorter, green, wavelengths to APD B. In addition to the dichroic, long pass and short pass filters are used for the red and green paths, respectively. However, because EGFP has such a long emission tail, a small but significant percentage of photons emitted from EGFP are of low enough energy to pass straight to the red APD A. At the same time, photons from the same protein will go toward the green APD B. This problem cannot be ignored as it always leads to false positive cross-correlation.

A few conditions amplify this problem of artificial cross-correlation due to bleed-through. One of them is the condition where the green or shorter wavelength fluorophore is much brighter, i.e. has a greater molecular brightness, than the red fluorophore. This happens to be true for EGFP and mCherry. In this case, even a balanced ratio of green to red fluorophores leads to significant bleed-through.

2.5.2 Pulsed-Interleaved Excitation

Pulsed-Interleaved Excitation FCCS (PIE-FCCS) is a guaranteed method of removing false-positive cross-correlation due to spectral bleed-through. Briefly, PIE-FCCS pulses one laser at a time such that the arrival times of photons in each of two detection channels are associated with a pulse from a single excitation source. The pulses are interleaved in short enough periods to allow millisecond-scale dynamics to be observed while still allowing fluorescence to relax between each pulse. Photon signals are acquired in time-tagged time resolved (TTTR) format, which records each photon with an arrival time or time relative to a periodic pulse, and a total time or a time that records in which period the photon arrived so that a continuous intensity trace can be constructed. Sorting the photons by their arrival time (Figure 2.6), one can select only the photons in each channel corresponding to the correct excitation source, and remove all other photons before reconstructing the total
time intensity trace. This can result in a large decrease in the photon counts included for the red fluorophore if it is excited by the blue laser source. These photons will be removed because they are indistinguishable from photons resulting from bleed-through.

2.5.3 PIE-FCCS Optics and Electronics

The optical setup is slightly modified from that of FCS/FCCS for PIE-FCCS. Aside from acquisition electronics, the only thing needed in addition to what is used for continuous FCCS is a way to pulse the cw Kr/Ar mixed gas laser. In our system, we added a pockels cell (ConOptics, Inc, Model 25D Driver, Model 350-160 KD*P Series), which is an electro-optic modulator in order to pulse our second excitation color. The pockels cell is aligned so that it blocks light when it is off or receiving no voltage, but can be tuned with its bias voltage to let cw light through when experiments require it.

The electronic needs to perform PIE-FCCS are more extensive. The most important piece of equipment is the TCSPC card. Our system uses a TimeHarp200 (PicoQuant, Germany), which has 36-picosecond arrival time resolution and acquires data in a TTTR format. The arrival time can be used to construct lifetime histograms or perform any time-gated fluorescence fluctuation technique, and the total time is necessary techniques that utilize changes or fluctuations in fluorescence intensity with time.

Figure 2.6: PIE-FCCS optical and electronic schematic.

O – Objective
DM – Dichroic Mirror
NF – Notch Filter
CP – Confocal Pinhole
PDL – pulsed diode laser
EOM – Electro-optic modulator (pockels cell)
2.5.4 Interleaving, Not Overlapping, Pulses

There are a few options for achieving the appropriate timing for PIE-FCCS. Following are the settings we most frequently use for taking PIE-FCCS data at 10MHz. The pulse generator (Quantum Composers, 9530 Series) provides the master clock for the PIE system. The pockels cell driver is connected through Channel A of the pulse generator. The Channel A source is set to the internal oscillator at $T_0$ of 100ns. The pulse width of the Channel A pulse is 18ns corresponding to a 9ns rise and fall time characteristic of the pockels cell model. The pulse generator also supplies a clock through a reference out to the PDL oscillator module. This module, when set to external trigger, sends the clock on to the 479nm diode laser as a trigger. The delay of the pulse from the Kr/Ar laser can be arbitrarily moved by adding time to the delay option on the channel A menu. This should be done while viewing the live histogram (~.7 sec, osc.) on the TimeHarp 200 acquisition software and should allow the photons received from the diode laser pulse to decay to zero before the next pulse appears. The exact arrival time of the PDL pulse excitation cannot be changed through the pulse generator interface. The arrival time of fluorescence signal from the PDL pulse is changed through the length of BNC cable from the APD to the router. This only changes the timing of the arrival of the signal to the TCSPC card so that it falls in an appropriate detection window for signal acquisition. It does not change the timing of the actual pulse. Although the pulse from the Kr/Ar laser through the pockels cell can be arbitrarily delayed, the length of cable from both APD’s to the router must be dictated by the PDL pulse signal arrival and be kept the same for both APD’s in order to keep the path length of red and green emission the same from sample to detection. If this is not done, it is possible to have both pulses occurring simultaneously while detecting them separately, but this will not allow proper gating and removal of bleed-through photons. Usually signal from the blue pulse can be seen in both channels due to bleed-through and direct excitation of red fluorophore with the blue laser, and this allows confirmation that the pathlengths are the same when the pulse is simultaneous in both channels.

![Figure 2.7: Arrival times from the 479nm pulse at about 10ns appear in both channel B (top) and channel A (bottom) due to spectral bleed-through and direct excitation of the red fluorophore with 479nm. Arrival times from the 568nm pulse appear later (~60ns) and only in channel A, as expected. The shapes of the peaks are reflective of the picosecond time scale of the 479nm pulsed diode laser and the 18ns pulse of the EOM.](image)
2.5.6 Physical and Practical Limits of PIE-FCCS

There are a number of components necessary in the acquisition of TCSPC data that impose limits on length of data acquisition, maximum intensity of a sample, and even diffusion coefficients of fluorescent molecules. Some of these can be overcome by advanced techniques or upgraded hardware.

The first and most obvious limiting pieces of hardware are the APD’s. As single photon detectors, one must use caution and ensure that the APD’s are guarded when powered on from too much light by never allowing the dark box to be open while the lights are on and by always ensuring that filters are in place to block any reflected laser light. However, there is much less danger than one would think of “blowing out” the APD’s with a bright sample. In truth, the SPCM-AQRH-16 model, which is the model of both APD A and B on Scope 3, can detect 29 MCPS before saturation (according to information from the manufacturer), though this should never be approached in an experiment for many reasons. Of real concern is the loss in linearity of output with increasing counts per second (cps). These APD’s remain linear into the high 100’s of kCPS, but require a correction factor of 1.03 at 1Mcps, and the correction factor continues to rise from there. This means that a fraction of photons arriving at the APD’s are not detected and recorded. While taking data at intensities in this high range is possible, if good data can be acquired at lower intensity, preferably with fewer fluorophores, it should be. Perhaps for FCS/FCCS data acquired using a hardware online correlator, the limits of which are not discussed here, the nonlinearity of APD’s at high intensities may be the limiting factor. TCSPC and TTTR data, however, has stricter limits imposed by other hardware and software components.

Limits of the TCSPC card include the large amount of data in a TTTR file, and a relatively long dead time (<350ns according to PicoQuant) that directly affects the amplitude of cross-correlation curves from FCCS. The large TTTR files impose practical limits on the total amount of data acquired in terms of length of acquisition and overall intensity of sample. Both of these lead to higher total numbers of photons. Calculating the auto- and cross-correlation curves of any amount of data is time-intensive and computationally expensive. Using SymphoTime (SymphoTime 5.1.3, PicoQuant, Berlin, Germany) to calculate the auto- and cross-correlation curves may take less time per curve, but it cannot be automated and requires a person to sit and click for many hours. The algorithm used by the SymphoTime software is not apparent, and often leads to very noisy tails for two-dimensional or slow three-dimensional diffusion. A custom Matlab (The MathWorks, Inc.) script used to convert, gate, and calculate correlation curves, can run for days in Matlab to convert data for one day’s experiments, but requires user input only at the beginning and is transparent in its algorithm. Data file size limitations make acquiring TTTR data for longer than a few minutes, especially with a bright (>100 kCPS) sample, impractical, but not impossible.
2.5.7 Effects of the TCSPC Dead Time

Another limitation to the maximum count rate is the dead time of the TCSPC card, which may be up to a few hundred nanoseconds. If working with a sample with reasonable intensity (~1-100kCPS) and acquiring FCS data, this dead time has little to no effect on experimental results. However, it has a dramatic impact on experimental results from FCCS experiments when samples start to get moderately bright. As the intensity of non-correlated samples increases due to increased numbers of fluorophores or increased excitation power, the amplitude of the cross-correlation curve decreases from 0 to negative values. The cross-correlation amplitude of a completely correlated sample also noticeably decreases with increased intensity. Although two detectors are used, the same TCSPC card processes signals from both detectors. This means that once one photon is detected, the next cannot be detected for another ~350ns or the length of the dead time. As the samples get brighter, the TCSPC card is busier and unable to detect photons for a higher percentage of time. When two species are correlated they are likely to emit photons close together in time, but the TCSPC card will only process one of the two. This leads to anti-correlation (negative correlation values) at short lag times as seen in Figure 2.8. This artifact may be increased because of the difference in molecular brightness of EGFP and mCherry, which are the fluorophores used in Chapter 3. EGFP is almost twice as bright as mCherry, which means it gives off twice as many photons. The TCSPC card is then twice as likely to process a signal from a photon from GFP than from mCherry.

The best way to avoid the anti-correlation due to the dead time would be to incorporate a second TCSPC card into the system. However, these cards are quite expensive. A cheaper way to avoid this problem is to use samples that are less bright with fewer fluorophores. Anything around ~100kCPS or less would be preferable. The only problem here is if the experimental system’s cross-correlation has a dependence on fluorophore or labeled molecule concentration. Even when this is the case, careful experimentation and use of good controls can yield interpretable results, as discussed in Chapter 3.

**Figure 2.8:** Negative cross-correlation due to TCSPC dead time.
Auto- and cross-correlation curves of a supported lipid bilayer containing Bopdipy 510/500 and Texas Red labeled lipids. At high concentrations of lipids, high count rates increase the anti-correlation effect that the dead time of the TCSPC card has on cross-correlation.
Chapter 3

Specific Lipid-Anchor Organization in Cell Membranes is Revealed by PIE-FCCS
3.1 Abstract

Many proteins are anchored to cellular membranes through various combinations of covalently attached lipid moieties. These modifications, along with polybasic regions of the proteins, are known to be essential for proper cellular localization. It has further been suggested that the anchors themselves may determine the lateral organization of lipid-anchored proteins in the plasma membrane. However, there is as yet no consensus on the spatial targeting characteristics of the various anchor types in living cells. In this study, we examine the dynamic colocalization of lipid anchored fluorescent proteins using pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS) and fluorescence lifetime analysis. Specifically, we look at the colocalization of anchors from LCK (myristoyl, palmitoyl, palmitoyl), RhoA (geranylgeranyl), and K-Ras (farnesyl) proteins. The results reveal varying degrees of homogeneous colocalization of RhoA and LCK anchor types with no cross correlation between different anchors observed in Jurkat cell membranes. Fluorescence lifetime information compiled from the same data streams reveals essentially no Förster resonance energy transfer (FRET). Taken together, these observations suggest the lipid anchored fluorescent proteins are co-clustering with other native proteins or lipids in the membrane in an anchor specific manner.

3.2 Introduction

The cell membrane, primarily a two dimensional fluid lipid bilayer, is densely packed with a variety of proteins and conjugated polysaccharides that are laterally fluid in the plane of the membrane. Lipid anchors are commonly found on membrane proteins, and are known to facilitate trafficking to intracellular locations as well as regulate interactions between certain proteins. They also provide physical interactions necessary to tether many proteins to cellular membranes. Lipid moieties that are attached as lipid anchors include isoprenyl groups, such as farnesyl and geranylgeranyl, and saturated fatty acids such as palmitoyl and myristoyl. Lipid-anchored proteins are often found to have multiple lipid modifications and/or basic amino acids that aid in stabilizing the protein-membrane interaction. Ras proteins are an example of a family of small GTPases that differ only in their hypervariable C-termini and lipid modification, and as a result are distributed amongst different organelle membranes and display differential sorting behavior in the plasma membrane. Swapping the anchors in other natively anchored proteins has been shown to disrupt protein function even when proper subcellular localization is maintained. These results suggest that anchors play a role in the differential lateral sorting of lipid-anchored proteins in live cell membranes.

Here, we study the organization of the anchor for the Lymphocyte Cell Kinase (LCK), an immediate downstream activator of T cell receptor activation during the immune response, the anchor for the small GTPase, RhoA, and the anchor for a member of the Ras oncogenic superfamily, K-Ras. Our study simply considers whether interactions between lipid anchors and membranes are capable of driving organization and whether different
anchors are capable of differential sorting in the membrane. In order to study the lateral membrane partitioning behavior of lipid anchors, absent of any short-range protein-protein interactions with high temporal and spatial resolution, we express truncated forms of our proteins of interest fused to either a red fluorescent protein, mCherry, or a green fluorescent protein, GFP, in live cells for use with pulsed interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS). These truncated proteins contain only the anchor domain, which is the lipidation motif and the polybasic region of the peptide (see Fig. 3.1). We employ PIE-FCCS to measure the degree to which these anchors colocalize in live cell membranes. By expressing a green anchored protein and red anchored protein in each cell, we can do a pairwise comparison of our lipid anchors to look at homogeneous and heterogeneous colocalization.

Evidence from detergent resistant membranes suggests that palmitoylation strongly biases the partitioning of proteins into tightly packed domains, however, these assays require permeabilization of the cell membrane which disrupts the native organization and cannot be regarded as representative of the structure of the cell membrane. Model membrane systems also predict that lipid anchors will sort into domains based on their chemical structure. Giant unilamellar vesicles (GUV’s) show that lipids and lipid anchors sort into liquid ordered (Lo) domains, consisting of ordered, tightly packed saturated lipid tails and cholesterol, and liquid disordered (Ld) domains, consisting of loosely packed unsaturated lipid tails. However, a simplified model membrane system does not reflect the complexity in the mixture of lipids and proteins from a cell membrane. A recent study in giant plasma membrane vesicles (GPMVs), which are taken directly from cell membranes, shows inconsistent partitioning of lipid anchors between vesicles. The anchor of LCK, consisting of three saturated fatty acids partitioned into the Lo domain in some GPMV’s, into the Ld domain in others, and still in others it resided equally in both. While GPMV’s maintain a similar membrane composition to live cells, they are removed from cytoskeletal interactions and active maintenance of the membrane composition, which may critically change lipid phase behavior. Finally, studies of lipid anchors in live cells further confuse the issue. Different Förster resonance energy transfer (FRET) studies have shown both clustering and non-clustering behavior of lipid-anchored fluorescent proteins in live cell membranes. On the other hand, studies using single particle tracking (SPT) and fluorescence recovery after photobleaching (FRAP), both of which observe diffusive behavior, found no evidence of anchors partitioning into stable domains.

The general consensus taken from these results is that there is no clear model of how anchors organize in the membrane. FCCS shows a definite correlation for two co-diffusing species regardless of their separation distance or orientation as long as they are both within the excitation area (~0.1 \( \text{um}^2 \)). PIE-FCCS provides cross-talk free cross-correlation and lifetime measurements from the same data stream allowing us to compare the amount of energy transfer (FRET) with the amount of cross-correlation.

We see varying levels of homogeneous colocalization in membranes of Jurkat cells transfected with either RhoA anchors or LCK anchors, but we see no heterogeneous colocalization between two different anchors. This finding suggests a minimum of two distinct domains, specific to each anchor, existing in a background of various other
membrane components. This pattern was not consistent in the membrane of COS 7 cells, in which no colocalization was observed. In addition, we also notice no significant difference between the fluorescent lifetimes of GFP in different anchored pairs, regardless of the amount of correlation observed. This suggests that clusters are greater than ~10 nm and include other native membrane components. Our results suggest that membrane organization of lipid anchors is anchor specific as well as membrane specific.

3.3 Materials and Methods

3.3.1 Cloning

Construction of truncated lipid anchor-fluorescent protein fusion genes: Constructs of GFP-kRas-CT, mCherry-kRas-CT, mCherry-mGFP-kRas-CT in pN1 vector with a strong CMV$_{IE}$ promoter were given as gifts from Dr. Nick Endres and Dr. John Kuriyan (UC Berkeley). Retroviral plasmids containing LCK-NT-mCherry, LCK-NT-GFP, mCherry-RhoA-CT, GFP-RhoA-CT were given as gifts from Dr. Björn Lillemoier and Dr. Mark Davis (Stanford). These genes were subcloned into the pN1 vector between the NcoI/NotI restriction sites. Polymerase chain reaction (PCR) primers and sequences of the genes can be found in the Supplementary Information. All oligonucleotides were synthesized by Elim Bioscience (Fremont, CA) and sequenced by Elim Bioscience or the University of California Berkeley core DNA Sequencing Facility (Berkeley, CA).

Construction of His-tagged fluorescent protein (FP-His12): Genes were cloned into the Ncol/XhoI restriction sites in the multiple cloning region downstream of a T7 promoter in the vector pET-28b(+) (Novagen). Genes for mCherry and mGFP were amplified by PCR and cloned into the NcoI/HindIII sites of pET-28b(+)-His12. mCherry-mGFP-His12 was constructed sequentially by first cloning mCherry into the Ncol/BamHI sites of pET28b(+) with an oligo cassette encoding a 12 X His-tag downstream of the fluorescent protein to generate pET-28b(+)mCherry1-His12. mGFP was then inserted into the BamHI/HindIII sites of pET-28b(+)mCherry1-His12 to produce pET-28b(+)mCherry1-mGFP2-His12. All cloning was accomplished in E. coli XL1-Blue strain (Stratagene).

3.3.2 Protein Expression and Purification

FP-His12 Protein was expressed in E. coli BL21 Star (DE3) strain (Invitrogen). Expression was induced during log phase growth with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma), in 1 L suspension of Luria-Bertani bacterial media (Sigma) at 37°C for 3-5 hours. Cells were lysed by a freeze-thaw cycle, conventional treatment with 1 mg/mL lysozyme (Sigma) for 1 hour at 4°C in lysis buffer (40mM Tris pH 7.4, 275mM NaCl, 20mM
Imidazole, 2% Protease Inhibitor Cocktail for His tag (Sigma)) and then by probe sonicator (Sonics & Materials Inc., VCX750). Samples were on ice during pulse sonication (5s ON/ 9s OFF, 150 seconds, amplitude = 35%, with a 3 mm stepped microtip). Lysate was clarified by addition of, and incubation with, nuclease (100 ng/mL RNaseA (Roche) and 25 ng/mL DNaseI (Roche)) and high-speed centrifugation (6,000 rcf) for 45min at 4˚C then filtered through a 0.45 µm syringe filter. His-tagged proteins were purified by Immobilized Nickel Affinity Chromatography in a 1 mL His-Trap column on an AKTA Explorer (GE Life Sciences) and by Gel Filtration chromatography on a Superdex-100 HR Size Exclusion Column (GE Life Sciences) in Phosphate Buffered Saline, pH 7.4 (PBS, Gibco, Cellgro) and 20% glycerol (EMD). Purified proteins were concentrated with Amicon centrifugal filters and flash frozen in liquid nitrogen in aliquots and stored at -80˚C.

3.3.3 Supported Lipid Bilayer Formation and Protein Binding

Supported bilayers for empirical mapping of correlated states were made as previously described. 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 (Δ9-Cis) DOPC) and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (18:1 Ni-NTA DGS) were purchased from Avanti Polar Lipids and stored at -20˚C. 2 mol% and 10 mol% Ni-NTA DGS (with 98 mol% and 90 mol% DOPC, respectively) small unilamellar vesicles (SUVs) were prepared by sonication according to alternate protocol 1 in Lin et al. Glass coverslip membrane supports (#1 Fisherbrand 25 mm round coverglass) are cleaned of organic contaminants by 10 min submersion in highly oxidizing Piranha etch solution (3:1 H₂SO₄:H₂O₂) thereby increasing the hydrophilicity of the support. 15 µl SUVs are mixed 1:1 with 2X Tris-Buffered Saline, pH 7.4 (TBS, Cellgro), and deposited on a clean, dry coverglass. Vesicles fuse to form a fluid supported bilayer on the coverglass. Coverslip and supported membranes are enclosed in a metal imaging chamber and the bilayer must remain hydrated in order to maintain fluidity. The water is exchanged for 100 mM NiCl₂, 2X TBS solution and incubated for 5min in order to charge the Ni-NTA DGS. The solution is washed with filtered H₂O and then exchanged with 5 mL of 1X PBS. 2, 6, 10 mol% Ni-NTA DGS bilayers were incubated with ~3, 6, or 9 nM FP-His12 proteins in PBS for ~ 30-40 min, after which all unbound proteins are washed away by exchanging the solution with 10 mL of PBS.

3.3.4 Cell Culture/Transfection/Sample Preparation

Mammalian cell cultures were obtained from Dr. Ann Fischer in the University of California Berkeley Tissue Culture Facility. Jurkat T cells were cultured in RPMI1640 medium (Gibco) supplemented with 1 mM sodium pyruvate (Cellgro), 100 µg/mL Penicillin/Streptomycin (Cellgro), and 10% Fetal Bovine Serum (FBS, Atlanta Biologicals). Cells were passaged every two or three days by seeding ~ 10⁶ cells in 5mL media in a T-25 cell culture flask and were disposed of after ~ 15 passages. COS 7 cells were cultured in Dulbecco’s Modification Eagle’s Medium (4.5 g/L glucose DMEM, Cellgro) supplemented with 1 mM sodium.
pyruvate, 2 mM L-glutamine, and 10% FBS and passaged 1:20 at ~95% confluency and disposed of after 20 passages.

Cells were transiently transfected either 1 or 2 days before the experiment. COS 7 cells are seeded at a density of 250,000 cells/9.6 cm² well in a 6 well culture plate in 2.5 mL reduced serum Opti-MEMI (Invitrogen) the day before transfection, while 10⁶ Jurkat cells in 2.5 mL Jurkat media are seeded in each well with 2.5 mL of culturing media on the same day as transfection. For transfection, 2.5 µg plasmid DNA is added to 250 µl Opti-MEMI, then 10 µl Lipofectamine 2000 transfection reagent (Invitrogen) is added to this mixture and incubated at room temperature for 30 min. This is then added to cells in 6 well culture plates and incubated at 37° C, 5% CO₂ for ~12-36 hours before the FCCS experiment.

In order to image Jurkat cells, cell culture media was exchanged in 2 x 5 mL PBS, pH 7.4 prewarmed to 37° C, by centrifuge (5 min, 250 rcf) and resuspended in 500 µl Hepes Buffered Saline Solution (pH 7.2) prewarmed to 37° C and deposited on poly-l-lysine coated #1 coverglass (cleaned as before, and with 0.01% poly-l-lysine (PLL, Sigma) solution deposited on coverglass surface for 30 min, then aspirated) enclosed in a metal imaging chamber. Cells are allowed at least 15 min in the incubator in order to settle and adhere to the P-L-L coated coverslips. COS 7 cells are washed with 2 mL prewarmed PBS, pH 7.4 and then lifted from the surface by 1 mL CellStripper™ (Cellgro) for 5-10 min, then neutralized with 500 µl un-supplemented DMEM. Cells are centrifuged (5-10 min, 250 rcf) and the solution is aspirated. Remaining cells are resuspended in 500 µl un-supplemented DMEM, and added to P-L-L coated coverslips in imaging chambers and allowed at least 15 min in the incubator to adhere to the coverslips.

3.3.5 PIE-FCCS

FCCS measurements of lipid-anchored proteins in live cells were taken on a customized microscope setup. A Kr/Ar mixed gas laser (Stabilite 2018-RM, Newport Corp., Irvine, CA) provides a wavelength of 568 nm while a pulsed diode laser (LDH-P-C-485, PicoQuant, Berlin, Germany) provides a 479 nm wavelength at frequencies from 2.5 MHz to 40 MHz. For FCCS, the 568 nm and 479 nm lines are combined and coupled into a single mode optical fiber. The beams are decoupled, expanded, and directed via a custom polychroic mirror (Chroma Technology Corp., Rockingham, VT) into the optical path of the microscope (TE2000E, Nikon Corp., Tokyo, Japan). A 100X TIRF oil objective, NA 1.49 (Nikon Corp., Tokyo, Japan), focuses down the excitation beam. The fluorescence is collected through the same objective and passed through a custom notch filter (Semrock, Rochester, NY) to remove any reflected laser light. The emitted light is then passed through a 50 µm confocal pinhole (Thorlabs, Newton, NJ) to limit collection of out-of-plane fluorescence. A 580 nm long pass beamsplitter then splits and directs the emitted light toward two avalanche photodiodes (SPCM-AQRH-16, Perkin&Elmer, Canada). Optical filters 550 nm short pass and 645/75 nm band pass (Chroma Technology Corp., Rockingham, VT) for the green and red channels, respectively, further select for fluorescently emitted light of the proper wavelengths. A time-correlated single photon-counting (TCSPC) card (PicoQuant, TimeHarp 200, Berlin, Germany) collects signal from the APD’s through a router (PRT 400,
TTL SPAD router, PicoQuant, Berlin, Germany) in the form of time-resolved data, which is used for fitting fluorescence lifetimes. The power of each laser was measured before entering the optical path of the microscope and was kept between 0.9 μW and 1.5 μW. Measurements were taken with the lasers pulsing at 10 MHz. The cw Kr/Ar beam is pulsed by passing through a pulsing electro-optic modulator (EOM). The pulsing of the EOM and the diode laser is controlled and synchronized by a pulse generator (Quantum Composers, 9530 Series). A delay of about 50 ns is set between the diode pulse and the EOM to ensure that the fluorescence completely decays between excitation pulses.

Cells were selected by looking at the epi-fluorescence with filter cubes suitable for GFP and mCherry, individually. A suitable cell would have similar intensity in the both channels. When taking FCCS measurements, we avoided areas of the cell from which we could see background from proteins inserted into membranes of organelles or intracellular vesicles. The bottom membrane of the cell was brought into focus and the PFS (The Perfect Focus System, Nikon Corp., Tokyo, Japan) maintained that focus throughout FCCS measurements. Three to five 15-second measurements were taken from each of three to five spots on a single cell, and three to five cells were observed per sample. The cell samples were kept at ambient temperature and atmosphere during data acquisition for no more than 1.5 hours.

### 3.3.6 Lifetime Acquisition

Fluorescence data for lifetime analysis was acquired either simultaneously with FCCS data, or was acquired from separate samples (e.g. for cells expressing GFP-LCK only). Lifetime histograms were constructed from 15 to 120 second traces, and were tail-fit with SymphoTime software (SymphoTime 5.1.3, PicoQuant, Berlin, Germany).

### 3.3.7 Data Analysis

PIE-FCCS data were time-gated as illustrated in Figure 3.2 B to remove photons possibly resulting from spectral bleed-through using custom Matlab (The MathWorks, Inc) programs. The auto- and cross- correlation curves were calculated from the reconstructed intensity traces without the gated photons using a multiple-tau algorithm as discussed by Wohland et al.\(^7\) Auto- and cross-correlation curves from a single spot were averaged before fitting. Curves that were calculated from intensity traces with large and irregular intensity fluctuations or resulting curves showing long and irregular decays indicative of large intensity fluctuations were discarded, as these irregularities cause artificially high amplitudes in the correlation curves and are usually the result of membrane fluctuations or diffusion of intracellular vesicles into the excitation area. Auto- and cross-correlation curves were fit by finding the average of the earliest ten points in order to get an accurate \(G(0)\) value.
3.4 Results

3.4.1 Live Cells Express Lipid-Anchor ﬁred Fluorescent Proteins

Here we transiently transfect Jurkat and COS7 cells with pairs of lipid anchored green- and red-ﬂuorescent protein constructs. Transient transfections allow us to observe a broad distribution of densities of lipid anchored ﬂuorescent proteins in a population of cells. As seen in Figure 3.1, transfected cells imaged by epi-ﬂuorescence show a homogeneous distribution of ﬂuorescent proteins on the plasma membrane. The lipid anchor ﬂuorescent protein fusions described here are similar to those used in previous studies.22,62,64,65,72 Because the native protein is not included in these chimeras, the localization and diffusion of the ﬂuorescent lipid anchors are unaffected by any direct protein-protein interactions. Instead, the movement and colocalization of the ﬂuorescent lipid anchors is a direct consequence of interactions between the lipid anchor and native components of the plasma membrane, including possible interactions between the basic residues of the peptide and lipid headgroups.22,73

Figure 3.1. Epifluorescence of Jurkat cells expressing anchored ﬂuorescent proteins shows homogeneous distribution in the plasma membrane. Green, red epifluorescent, and reﬂection interference contrast microscopy (RICM) images of Jurkat cells expressing (A) GFP-RhoA-CT and mCherry-RhoA-CT and (B) LCK-NT-GFP and LCK-NT-mCherry. Anchored ﬂuorescent proteins are localized to the plasma membrane, and bright masses are due to intracellular organelles. RICM shows cell membranes are well adhered to P-L-L coated coverslips. Images are false-colored and the intensity scaled to show homogeneous distribution of anchored ﬂuorescent proteins in the plasma membrane. The scale bar is 10 µm.

3.4.2 Relative Correlation is Measured by PIE-FCCS

Using PIE-FCCS we detect dynamic colocalization of GFP and mCherry lipid anchored constructs in live cells. This technique requires no ﬁxation of cells or extraction of cell membranes, and measurements do not perturb the native organization of the membrane.
We are unrestricted by the small, <10nm, separation distance necessary for FRET as well as the >200nm resolution of traditional optical microscopy. The observation of cross-correlation reports dynamic colocalization and requires no a priori knowledge of the spatial length of organization.

Pulsed-interleaved excitation and proper data analysis ensure that we are seeing no artificial cross-correlation due to spectral bleed-through from the broad emission spectrum of GFP. The time-tagged time-resolved (TTR) data acquisition format records photons with their arrival time and time tag and allows us to acquire data for FCS, FCCS, and fluorescence lifetimes simultaneously.

Autocorrelation of fluctuating fluorescent signals (Figure 3.2 C and E) resulting from movement of fluorophores in and out of the excitation area is calculated by the normalized autocorrelation function in equation 1.

\[
G(\tau) = \frac{\langle \delta I(t)\delta I(t + \tau) \rangle}{\langle I \rangle^2} + 1
\]

(3.1)

Where \( \delta I(t) \) is the fluctuation in fluorescence intensity at time \( t \), and \( \tau \) is the lag time. Fluorescence cross-correlation is expressed similarly in equation 2, and gives the correlation between fluctuations from two different fluorescent signals.

\[
G_x(\tau) = \frac{\langle \delta I_x(t)\delta I_x(t + \tau) \rangle}{\langle I_x \rangle^2} + 1
\]

(3.2)

Figure 3.2 D and F show two representative examples of auto- and cross-correlation curves from live cell membranes transfected with GFP- and mCherry-RhoA-CT (D) and LCK-NT-GFP and -mCherry (F).

While the \( G(\tau) \) intercepts, \( G_r(0) \) and \( G_g(0) \), of the auto-correlation curves of red and green species are inversely proportional to the concentration of fluorophores in the excitation spot, \( G_x(0) \) of the cross-correlation curve is directly proportional to the amount of dynamic colocalization or concentration of species with both red and green fluorophores. Here, we report a measure of cross-correlation in terms of \( F_{cross} \) as defined in equation 3.

\[
F_{cross} = \frac{G_x(0)}{\min\{G_r(0), G_g(0)\}}
\]

(3.3)
To better represent the amount of cross-correlation present in our transfected cells, we empirically map $F_{cross}$ with physical standards on supported lipid bilayers as illustrated in Figure 3.3 A. mCherry-His12, mGFP-His12 (top) and mCherry-mGFP-His12 (bottom) proteins are deposited on bilayers containing DOPC and 2%-10% Ni-NTA DGS. mCherry-His12 and mGFP-His12 diffuse freely and independently of one another on the same supported bilayer allowing us to measure uncorrelated diffusion by PIE-FCCS. Diffusion of mCherry-mGFP-His12 proteins on supported bilayers correspond to entirely correlated diffusion of red and green fluorescent proteins. Remarkably, increased density and, therefore, increased intensity of both fused and independent fluorophores results in decreased and even negative $F_{cross}$ values as seen in Figure 3.3 B. This decrease is linear with respect to the total intensity from both detection channels and is due to the dead time from the TCSPC acquisition card.\(^{51}\)

The TCSPC card has a longer dead time (~350ns) than more conventional correlation cards or detection electronics.\(^{74}\) Photons detected during a “dead time,” or when the TCSPC is processing a signal, are not recorded leading to an anticorrelation between GFP and mCherry at short lag times.\(^{51}\) In our control bilayer measurements in Figure 3.3, the effect becomes more dramatic as the intensity of the samples increases because the acquisition card is “dead” for a larger percentage of time. We have worked around this by empirically mapping our correlated and uncorrelated boundaries (Figure 3.3 B), and as we observe cells with total intensities between 0 and 500 kCPS, the $F_{cross}$ values fall between the negative and positive empirical boundaries of cross-correlation. These empirical values in the bilayer samples allow us to rescale our data to relative correlation values between 0 and 1 as shown in Figure 3.4.

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**Figure 3.2.** PIE-FCCS acquisition generates cross-talk free data. (A) Schematic of our PIE-FCCS microscope setup. (B) Arrival time (time-resolved) histogram of APD A (red) and APD B (green). Photons with arrival times within the diagonal lined boxes are removed before auto- and cross-correlation curves are calculated. (C) Intensity traces from APD A (red) and APD B (green) resulting from detected fluorescence from a bilayer sample with mCherry-mGFP-His12 exhibiting correlated diffusion. (D) Auto- (red and green) and cross-correlation curves (blue) calculated from the intensity traces in (C). (E) Intensity traces from a bilayer sample with mCherry-His12 and mGFP-His12 exhibiting uncorrelated diffusion. (F) Auto- and cross-correlation curves calculated from traces in (E).
Figure 3.3. Empirical Mapping of Correlated States. (A) Schematic of mCherry-mGFP-His12 diffusing on a Ni-NTA DGS containing supported lipid bilayer representing the correlated state (top) and mCherry-His12 and mGFP-His12 diffusing independently on a supported lipid bilayer representing the uncorrelated state (bottom). (B) Scatter plot of $F_{\text{cross}}$ versus intensity of correlated mCherry-mGFP-His12 (▲) and uncorrelated mCherry-His12 and mGFP-His12 (♦). Increased intensity comes from increased surface density of His-tagged fluorescent proteins. Decreasing cross-correlation with respect to intensity is due to TCSPC card dead time and is fit to a linear trend. (C-D) GFP-RhoA-CT/mCherry-RhoA-CT cross-correlation (x) and LCK-NT-GFP/LCK-NT-mCherry cross correlation (+) with respect to increasing intensity in Jurkat cells. Blue and magenta lines represent the linear fits of the empirically mapped cross-correlation states from (B). Error bars represent the standard deviation of $G_0$ at each spot.
3.4.3 Relative Correlation is Dependent on Density

A significant amount of cross-correlation is observed in Jurkat cells transfected with mCherry-RhoA-CT and GFP-RhoA-CT. This cross-correlation is a direct sign of membrane-mediated dynamic colocalization of RhoA anchors. Relative cross-correlation increases with increasing expression and intensity of anchored fluorescent proteins (Figure 3.4 row 2 column 2, data from 4 experiments, 11 cells). At low intensities, the relative correlation is not distinct from uncorrelated bilayer samples, but as we look at brighter cells, the relative correlation increases and approaches the value obtained from the positive correlation control bilayer samples. To translate intensity to an actual density of lipid anchored fluorescent proteins as shown in Figure 3.6 B and C, we divide the total intensity from each channel by the molecular brightness of mGFP-His12 and mCherry-His12 as obtained from the supported bilayer samples to determine the average number of individual fluorescent protein molecules in the excitation spot. The molecular brightness of mGFP-His12 and mCherry-His12 is obtained by fitting the auto-correlation curves with the 2d diffusional model in equation 4. According to this model, the intercept of the function at \( \tau = 0 \), \( G(0) \) of the autocorrelation function is inversely proportional to the number, \( N \), of diffusing species in the excitation spot. We then divide the total intensity from each channel by the \( N \) obtained from fitting.

\[
G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} + 1
\]

(3.4)

Once we find the number of fluorophores in each spot based on intensity, we divide by the area of the excitation spot to get \( \rho \). The excitation area is calibrated by fitting the auto-correlation curve for fluorophores of known diffusion constants and is \( \sim 0.1 \mu m^2 \).

3.4.4 Relative Correlation is Anchor Specific

Examination of a different anchor pair, LCK-NT-mCherry and LCK-NT-GFP, shows less of a trend (Figure 3.4 row 1 column 1, data from 7 experiments, 28 cells). The relative cross-correlation values increase more slowly and are more spread across values from 0 to 1 at high densities.

In order to see if the different anchor types can partition into the same diffusing domains, we consider the pairwise relative correlation of LCK-NT-mCherry/GFP-RhoA-CT and mCherry-RhoA-CT/LCK-NT-GFP (Figure 3.4 row 2 column 1 and row 1 column 2, data from 5 experiments, 16 cells). In both cases, we see no cross-correlation regardless of density.

We also measure the relative correlation of the C-terminal farnesylated anchor of the Ras family kinase, K-Ras, which is chemically similar to the geranylgeranyl anchor of RhoA (see also SI 2). Cherry-K-Ras-CT/GFP-K-Ras-CT FCCS measurements do not reveal significant cross-correlation within our window of observation (Figure 3.4 row 3 column 3,
data from 4 experiments, 13 cells). Similarly, the pairwise measurements of kRas-CT anchor with RhoA-CT anchor also do not exhibit any cross-correlation (Figure 3.4 row 3 column 2 and row 2 column 3, data from 3 experiments, 8 cells).

The lack of cross-correlation in the pairing of two different anchors tells us two things. First, the monotonic increase in RhoA anchor correlation, and in the increase in LCK correlation is not an effect of having increased anchor density in the membrane, but are behaviors specific to those anchors. The second is that these anchors organize orthogonally. RhoA anchors and LCK anchors do not partition into the same clusters.

![Graph](image)

**Figure 4.** Comparison of relative cross-correlation of different membrane anchors in Jurkat cells. Normalizing cross-correlation to the empirically mapped correlated (1, blue) and uncorrelated (0, red) states for (top row, left to right) LCK-NT-GFP/LCK-NT-mCherry (28 cells) (♦), LCK-NT-GFP/mCherry-RhoA-CT (5 cells) (●), (Middle Row, Left to right) GFP-RhoA-CT/LCK-NT-mCherry (11 cells) (▲), GFP-RhoA-CT/mCherry-RhoA-CT (11 cells) (♦), and GFP-RhoA-CT/mCherry-kRas-CT (3 cells) (▲), (bottom row, left to right) GFP-kRas-CT/mCherry-RhoA-CT (5 cells) (♦), GFP-kRas-CT/mCherry-kRas-CT (13 cells) (■) in Jurkat T cells. Error bars represent the normalized standard deviation of G(0) for each spot.
3.4.5 Relative Correlation is Cell-Specific

Finally, we compare the cross-correlation of the RhoA anchors and the LCK anchors in the membranes of epithelial COS 7 cells to our results in Jurkat lymphocytes, and notice that anchors do not organize in the same manner. In COS7 cells, we no longer observe clustering in the RhoA anchors (data from 3 experiments, 6 cells), nor do we see it in the LCK anchors (data from 3 experiments, 10 cells). These cross-correlation results tell us that the complexity of membrane organization is specific for different cell types, and that membranes may organize differently based on membrane composition or cell function.

3.4.6 Fluorescence Lifetimes Show No Energy Transfer

In order to examine local spatial organization, we measure the lifetime of the GFP-anchored proteins in our cross-correlation experiments as shown in Figure 3.5. Shortened GFP lifetimes would be an indication of FRET between two anchors. There is a slight decreasing trend in lifetimes of all samples, including cells transfected with only a GFP-anchor and cells transfected with fused mCherry/GFP, as intensity increases, which can be attributed to the dead time effect of the TCSPC card. All cells transfected with a pair of GFP and mCherry anchors show GFP lifetimes similar to cells expressing only LCK-NT-GFP or GFP-RhoA-CT indicating no energy transfer due to FRET between mCherry and GFP. The exception is the fusion, mCherry-mGFP-KRas-CT, which shows strong evidence of FRET due to the short distance between the two fluorophores.

FCCS of our anchored fluorescent proteins gives us one picture of membrane organization, while comparisons of GFP lifetimes add greater detail to this picture. By pairing our co-diffusion studies with a look at very close molecular interactions, we begin to have an idea of the spatial dimensions of the organization. A lack of appreciable FRET in our cross-correlating RhoA anchors indicates that they are partitioning into clusters that are probably larger than ~10 nm in diameter. We do not see energy transfer, even at higher densities, when there is a greater chance of having a cluster multiply labeled with red and green fluorophores.

It is worth noting that FRET experiments alone would not detect RhoA anchor colocalization in this density range. This emphasizes the importance of using FRET alongside techniques that investigate the diffusive behavior of the species involved or are not limited to interactions within ~10nm.
3.5 Discussion

We see from our results, that a density threshold must be overcome before significant dynamic colocalization is detected. This tells us that the anchors partition into a pool of distinct, preexisting clusters present in the membrane. If anchors clustered strongly with one another or partitioned into large domains, we would expect to see significant cross-correlation at much lower densities. To put it another way, a finite number of clusters in the membrane is responsible for frustrating anchor cross-correlation until higher labeled anchor densities are reached. Remarkably, we see that both RhoA anchors and LCK anchors will distribute into clusters and that these clusters are distinct from another.

We describe this trend of increasing cross-correlation with increasing intensity by a probability distribution of observing at least one red and one green anchor probe present in a cluster on the membrane. We start by noting that the labeled anchors are distributed randomly among the clusters, so the probability of one cluster having $N$ probes follows the Poisson distribution, where $\langle N \rangle = \rho / n$ with $\rho$ as the number of probes per $\mu m^2$, and $n$ as the number of clusters per $\mu m^2$:

$$p_{\text{cluster}}(N; \langle N \rangle) = \frac{\langle N \rangle^N e^{-\langle N \rangle}}{N!}$$  \hspace{1cm} (3.5)

By choosing a probe at random, the probability distribution for $N$ other labeled anchors in the same cluster is scaled by the number of labeled anchors in the cluster.
\[ p_{\text{probe}}(N; \langle N \rangle) \propto N \langle N \rangle^N e^{-\langle N \rangle} \frac{1}{N!} \] (3.6)

The normalized probability distribution for \( N \) for the cluster in which the chosen probe resides follow.

\[ p_{\text{probe}}(N; \langle N \rangle) = N \langle N \rangle^N e^{-\langle N \rangle} \frac{1}{N!} \] (3.7)

We now consider that in order to see cross-correlation, at least one of the other probes in the cluster must be the opposite color of the chosen probe. This probability, that at least one of the \((N-1)\) probes is of the opposite color is given by,

\[ p_{\text{cross}}(N) = 1 - \left( \frac{1}{2} \right)^{(N-1)} \] (3.8)

Finally, the total probability that any given probe is in a cluster with at least one other probe of the other color is given by summing up all of the possible ways this can happen:

\[ P_{\text{total}} = \sum_{N=0}^{\infty} \frac{N}{\langle N \rangle} \frac{N}{N!} e^{-\langle N \rangle} \left( 1 - \left( \frac{1}{2} \right)^{(N-1)} \right) \] (3.9)

In effect, \( P_{\text{total}} \) is the probability of observing cross-correlation at a particular probe density in the membrane, which is the same as our measure of relative correlation.

In Figure 3.6, we have converted intensity to density using the molecular brightness of GFP and mCherry from control bilayers, and fit the RhoA clustering data to the probability distribution shown in equation 3.9 to find the average number of clusters where \( \langle N \rangle = \rho / n \). Fitting the RhoA anchor data, we find a minimum of 1640 clusters per square micron \((R^2 = 0.593)\). This result represents a minimum value because the intensity of brighter samples is probably under-detected due to the dead time of the TCSPC card, and the actual density may be greater.

We are limited by our method from seeing higher membrane densities, but in the case of LCK-NT-mCherry/LCK-NT-GFP cross-correlation, we catch a glimpse of the beginning of an increasing trend similar to that which we see in the case of the RhoA anchor.
The best fit of our probabilistic model to the LCK cross-correlation values returns a cluster density of \( \sim 4821 \) clusters per square micron (\( R^2 = 0.2358 \)), on average. These results show that partitioning into large domains is not the dominating behavior of LCK anchors. Instead, the clusters they partition into are very numerous and probably small. This is in stark contrast to reports that the full-length LCK proteins colocalize into large T cell receptor signaling microdomains during T cell activation.\(^{64,75-77}\) Previous results suggest that the LCK anchor will sort into clusters regardless of T cell activation.\(^{62,78,79}\)

Based on our observations, we present a more complex picture than is predicted by general models of membrane organization. Although much research has advanced the understanding of membrane organization beyond stable micron-scale phase-separated lipid domains known as lipid rafts, liquid-ordered and liquid-disordered phase separation is still often referenced as the primary mechanism of sorting of membrane components.\(^{20,72,80,81}\) According to this lipid raft model, anchored proteins with saturated
acyl chains, such as the LCK anchor, are expected to cluster into raft domains, while isoprenylated molecules like the RhoA anchor would be excluded to the surrounding disordered phase with no apparent clustering.\textsuperscript{62,82-84} Here we show the existence of two distinct, non-overlapping domains that LCK anchors and RhoA anchors recognize in a background of perhaps many other protein clusters in the membrane of Jurkat cells. This complexity extends to the differential sorting between the RhoA and K-Ras anchors due to their subtle differences. Our observations, and many previous studies, demonstrate that the complexity from the interactions between all membrane components in addition to interactions with the cytoskeleton and intracellular proteins cannot be fully described by an organizational scheme driven by binary phase separation.\textsuperscript{54,64}

It is also important not to conflate the observations made in different membrane systems. The models of membrane organization derived from GPMVs and model membranes, which are by default at equilibrium, do not predict the behavior seen in living cells maintained at a non-equilibrium steady state. A number of intracellular processes such as cytoskeletal interactions and endocytosis dynamically affect the composition and tension of the plasma membrane. Furthermore, different living cells can exhibit different organizational behaviors, as we see of the RhoA anchors in COS 7 cells, with respect to the anchors in Jurkat cells.

PIE-FCCS has allowed us to see a small window of what must be a complex and highly specific organizational scheme of the live cell membrane. The intensity requirements and limits of PIE-FCCS do not allow us to extend our measurements to more dense samples, but we clearly see that interactions between anchors and the membrane do cause differential sorting of anchors, and that simple binary phase separation does not explain the orthogonal nature of this differential sorting.

### 3.6 Acknowledgements

The authors would like to acknowledge Il-Hyung Lee and Michael Coyle for contributions of Matlab analysis scripts. The authors would also like to thank Ann Fischer from UC Berkeley Tissue Culture Facility for obtained mammalian cell cultures, Dr. Nick Endres and Dr. John Kuriyan for K-Ras anchor containing plasmids, and Dr. Björn Lillemeyer and Dr. Mark Davis for access to LCK and RhoA anchor containing plasmids. Finally, the authors would like to thank Howard Hughes Medical Institute and the Department of Energy for funding and support.
3.7 Supporting Information

SI Figure 1. Epifluorescence of Jurkat cells expressing K-Ras anchor fluorescent fusion protein shows homogeneous distribution in the plasma membrane. Green, red epifluorescent, and reflection interference contrast microscopy (RICM) images of Jurkat cells expressing (A) GFP-K-Ras-CT and mCherry-K-Ras-CT. Images are false-colored and the intensity scaled to show homogeneous distribution of anchored fluorescent proteins in the plasma membrane. The scale bar is 10 µm.

SI Figure 2. Epifluorescence of COS 7 cells and comparison of relative cross-correlation of different membrane anchors in COS 7 cells. Green, red epifluorescent, and reflection interference contrast microscopy (RICM) images of COS 7 cells expressing (A) GFP-RhoA-CT and mCherry-RhoA-CT and (B) NT-LCK-GFP and NT-LCK-mCherry. Images are false-colored and the intensity scaled to show homogeneous distribution of anchored fluorescent proteins in the plasma membrane. The scale bar is 10 µm. (C-D) Normalizing cross-correlation to the empirically mapped correlated (1, blue) and uncorrelated (0, red) states for (C) GFP-RhoA-CT/mCherry-RhoA-CT (6 cells) (▲) and (D) LCK-NT-GFP/LCK-NT-mCherry (10 cells) (♦) in COS 7 cells. Error bars represent the normalized standard deviation of G(0) for each spot.
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