FÖRSTER RESONANCE ENERGY TRANSFER IN LIPID BILAYERS:  
THEORY AND APPLICATIONS FOR EXAMINING PHASE BEHAVIOR

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
Frederick Andrew Heberle
January 2011
© 2011 Frederick Andrew Heberle
The spatial organization of lipids and proteins in biological membranes is of considerable interest. Multiple lines of evidence support the existence of specialized lipid microdomains or "membrane rafts", that are involved in processes as diverse as protein sorting, vesicular transport, viral entry and exit from cells, and signaling. The chemical complexity of cell membranes, and the small size and fleeting nature of rafts, pose serious challenges to experimentalists who seek to understand their thermodynamic origins.

Using high-resolution FRET and ESR measurements, we have examined phase behavior, order parameters, and the partitioning behavior of fluorescent probes in three ternary lipid mixtures that are models for the outer leaflet of mammalian plasma membranes. In two of these mixtures, we observe a region of coexisting fluid phases characterized by small (nanometer scale) phase domains. We developed a FRET model suitable for characterizing the size of these small domains, and were able to recover domain sizes and probe partition coefficients with good accuracy in a global analysis of simulated FRET data. These studies confirm that many of the critical properties of membrane rafts can be reproduced in lipid-only mixtures. Systematic study of these nanodomain mixtures will provide insight into the thermodynamic origins of membrane rafts.
BIOGRAPHICAL SKETCH

Fred Heberle (who is known back home as Andy) was born and raised in southeastern Montana, in the foothills of the Rocky Mountains, and was extremely fortunate to have two loving parents who supported his interests. His childhood was spent playing under a big sky: on the banks of the Yellowstone River, on hard dirt ball fields, in the back yard of the white house at 14th and River. In the frigid winter months, he damaged furniture swinging baseball bats indoors, making his parents long for the spring.

Fred grew up with a dream to play center field for the Atlanta Braves. While waiting for the scouts to call, he passed time reading textbooks. Some of this knowledge managed to stick, and he graduated at the top of his class of 55 students at Forsyth High School in 1995. Fred earned a B.A. in chemistry from Cornell University in 2003, and in the final semester of his senior year began a research project with Dr. Gerald Feigenson, using FRET to examine phase behavior in lipid bilayers. That project turned into a two-year appointment as a lab technician, and ultimately into this doctoral dissertation.

In his life, Fred has called four places home: Forsyth, MT; Missoula, MT; Seattle, WA; and Ithaca, NY. He continues to chase his dream of living a calendar year without seeing snow.
To Mom and Dad
ACKNOWLEDGMENTS

I owe an enormous debt to my advisor and mentor Dr. Gerald Feigenson, who provided me with an opportunity to do fascinating science. Not only has he shaped the way I conduct research and think about scientific problems, he is also a world-class motivator. As a graduate student, this was an invaluable resource: The ups and downs of research can be difficult to endure, but I could always count on Jerry to get me back on track and in the right frame of mind after a failed experiment (of which there were many). His door is always open.

The "big data sets" discussed in Chapter 2 were truly a massive undertaking and could not have happened without the help of my labmates Jing Wu, Shih Lin Goh, and Robin Petruzielo. They have my extreme gratitude for sacrificing a few months of their lives for my thesis work. Many current and former Feigenson lab members have contributed to this work either directly or with stimulating conversations: Dr. Adam Hammond, Dr. Jeffrey Buboltz, Dr. Juyang Huang, Dr. Thalia Mills, Dr. Jiang Zhao, Nelson Morales, Tatyana Konyakhina, Jon Amazon, David Ackerman, David Stringer, Grace Huang, and Greg Costanza. I've benefitted greatly from discussions and interactions with Drs. Gilman Toombes, Andrew Smith, Qi Wang, Sarah Veatch, Nozomi Ando, and Lovy Pradeep. I thank the immortal Dr. Richard Feynman for inspiration.

I thank Drs. Jack Freed, Boris Dzikovski, and Mingtao Ge of ACERT for assisting with ESR experiments, providing their spectral simulation software, and getting us started with the simulations.

Dr. John Nagle and Dr. Stephanie Tristram-Nagle gave me an opportunity to participate in x-ray experiments conducted both in their home lab at Carnegie Mellon University and at CHESS, under the guidance of Dr. Thalia Mills. Though that work did not end up in my thesis, I learned a great deal (and had a great deal of fun).
Many thanks to my committee members Dr. Barbara Baird and Dr. Holger Sondermann for a critical reading of the thesis.

I thank the following sources for supporting this work: NIH Molecular Biophysics Training Grant 1-T32-GM08267; and research awards NIH R01 GM077198 and NSF MCB 0842839 to G.W. Feigenson.

Along my path, I've been extremely fortunate to have the friendship and support of some wonderful people: Paul and Mary Kunst, Grayson Kunst, Caroline Kunst, Dillon Kunst, Eric Watson, John Forsythe, Kay Wright, Cindy Larsen, Ira Gresens, Karen Seward, Cathy Byron, Brett Kraut, Dwayne Albertson, Sheila Haselhuhn, Stace Sisco, Farhad Dhabhar, Vipul Kothari, Matt LePere, Dr. Nate Morehouse, Dr. Michael Weingarten, Rob and Jessica Costanzo, Greg Allen, Will Ko, Dave Yarmchuck, Cheryl MacKenzie, Jessica Radbord, Madeline Galac, the Galac family (for brightening up a particularly lonely Easter weekend), Frank Petruzielo, Dr. David Lee, Laura Byrnes, Kylan Szeto, Sarah Shelby, and Dr. Petya Krasteva. I owe much to my "Ithaca parents" Albert and Cindy Smith, and their son Mike Smith.

Finally, my love and thanks to my family. My sister, Susie Sanders, has been an endless source of encouragement. My father and best friend, Fred Heberle, made great sacrifices to allow me to pursue this career. And to my mother and biggest supporter, Gay Heberle, who saw me off on this journey but wasn't able to see the end: I love you and miss you.
TABLE OF CONTENTS

BIographiesketch .......................................................................................... iii
Dedication ........................................................................................................ iv
Acknowledgments .......................................................................................... v
Table of Contents ............................................................................................ vii
List of Figures .................................................................................................. xi
List of Tables ................................................................................................... xii
List of Abbreviations ....................................................................................... xiii

Chapter 1  Introduction ....................................................................................... 1
  1.1 Overview .................................................................................................. 1
  1.2 Lipid bilayer phases ............................................................................... 2
  1.3 Plasma membrane rafts ......................................................................... 4
    1.3.1 The membrane raft hypothesis ....................................................... 4
    1.3.2 Relationship between rafts and fluid bilayer phases ..................... 5
    1.3.3 Nanodomains in ternary mixtures ............................................... 8
    1.3.4 Methods for measuring nanodomain size .................................... 10
  1.4 FRET in membranes ............................................................................ 10
    1.4.1 The FRET mechanism ................................................................. 10
    1.4.2 FRET for random, planar arrays of donor and acceptor ............. 12
    1.4.3 FRET in macroscopically phase separated mixtures .................. 13
    1.4.4 Measuring domain size with FRET ............................................ 15
  1.5 Thesis outline ......................................................................................... 16
REFERENCES ................................................................................................. 17

Chapter 2  Comparison of three ternary lipid bilayer mixtures: FRET and ESR reveal nanodomains ......................................................................................... 26
  2.1 Abstract ................................................................................................. 26
  2.2 Introduction ............................................................................................. 27
  2.3 Materials and Methods ......................................................................... 29
    2.3.1 Materials ......................................................................................... 29
    2.3.2 FRET sample preparation .............................................................. 29
    2.3.3 FRET data analysis ....................................................................... 30
    2.3.4 ESR sample preparation ................................................................. 31
    2.3.5 ESR data analysis ......................................................................... 31
  2.4 Results ................................................................................................... 32
    2.4.1 Regions of enhanced (reduced) FRET efficiency correspond to phase-coexistence regions .......................................................................................... 32
    2.4.2 FRET surfaces in DSPC/DOPC/chol .............................................. 35
      2.4.2.1 BoDIPY-PC to Fast-DiI: Enhanced FRET ................................. 35
      2.4.2.2 DHE to BoDIPY-PC: Reduced FRET ..................................... 36
      2.4.2.3 Probe partitioning in DSPC/DOPC/chol ................................. 37
    2.4.3 FRET surfaces in DSPC/POPC/chol and DSPC/SOPC/chol .......... 40
Chapter 5  Applications of quantitative FRET in bilayers II: Determination of domain size ................................................................. 113
5.1 Abstract .................................................................................. 113
5.2 Introduction ............................................................................. 113
5.3 Materials and Methods ............................................................. 115
  5.3.1 Monte Carlo simulations of energy transfer efficiency .......... 115
    5.3.1.1 Bilayer parameters ...................................................... 115
    5.3.1.2 Probe parameters ...................................................... 116
    5.3.1.3 Simulation details ...................................................... 117
  5.3.2 Nonlinear least-squares fitting of simulated data .................. 119
    5.3.2.1 Domain surface coverage function ............................. 119
    5.3.2.2 Nonlinear least-squares fitting of E data ..................... 120
5.4 Results ..................................................................................... 121
  5.4.1 Simulations of transfer efficiency ....................................... 121
  5.4.2 Simulated data fit to the FP-FRET model ....................... 128
    5.4.3 Simulated data fit to the FP-FRET model: departure from assumptions .. 133
      5.4.3.1 Distribution of transverse probe locations ................ 135
      5.4.3.2 Uncoupling of phase domains ............................... 135
      5.4.3.3 Variation in domain size across the tieline ............. 135
5.5 Discussion ................................................................................. 140
  5.5.1 Choice of fixed parameters ............................................... 140
  5.5.2 Single-trajectory vs global data analysis ......................... 142
  5.5.3 Assumptions of the FP-FRET model ................................ 144
      5.5.3.1 Probe height distribution ..................................... 144
      5.5.3.2 Cross-leaflet domain coupling ............................. 145
      5.5.3.3 Variation in domain size ..................................... 147
      5.5.3.4 Additional considerations .................................. 148
  5.5.4 Computational considerations ......................................... 153
5.6 Conclusions ............................................................................. 154
REFERENCES ............................................................................ 156

Chapter 6  Conclusions and Future Directions .................................. 162
6.1 Summary of findings and conclusions .................................... 162
  6.1.1 Phase studies in ternary lipid mixtures ............................. 162
  6.1.2 The FP-FRET model ....................................................... 163
6.2 Future directions ...................................................................... 164
  6.2.1 Ternary systems ............................................................. 164
  6.2.2 Four-component systems ............................................. 165
  6.2.3 Improving the FP-FRET model ..................................... 165
  6.2.4 Molecular Dynamics ................................................... 166
  6.2.5 Computational efficiency .............................................. 167
REFERENCES ............................................................................ 168
LIST OF FIGURES

Figure 2.1 Two types of FRET patterns depend................................................................. 33
Figure 2.2 SAE surfaces in DSPC/DOPC/chol ................................................................. 34
Figure 2.3 Lipid and probe $K_p$ in the Ld + Lo tieline field ............................................ 38
Figure 2.4 SAE surfaces in DSPC/POPC/chol and DSPC/SOPC/chol ............................ 39
Figure 2.5 FRET data on an Ld + Lo tieline trajectory .................................................. 43
Figure 2.6 ESR reveals similarities in phase properties ..................................................... 44
Figure 2.7 Phase diagrams for systems in this study ......................................................... 49
Figure 3.1 Transformation of sample coordinates to the Gibbs triangle ......................... 66
Figure 3.2 A visual guide to the tieline field parameterization .......................................... 69
Figure 3.3 Tieline generating functions for DSPC/DOPC/chol ......................................... 70
Figure 3.4 Percolation map for the Ld + Lo region of DSPC/DOPC/chol ......................... 72
Figure 3.5 GUVs near the critical point in DSPC/DOPC/chol ........................................... 73
Figure 3.6 Monte Carlo simulated FRET data ................................................................. 81
Figure 3.7 The uncertainty of a FRET signal ................................................................. 82
Figure 4.1 Snapshots of the binary hard disk fluid ......................................................... 97
Figure 4.2 Domain surface coverage is related to the RDF ............................................. 98
Figure 4.3 Geometric considerations for calculating domain surface .............................. 102
Figure 5.1 The average acceptor density seen by donors ................................................. 122
Figure 5.2 Comparison of three models for predicting FRET ........................................ 125
Figure 5.3 Simulated FRET data reveal the effect of domain radius ............................... 126
Figure 5.4 Simulated FRET data reveal the effect of domain radius ............................... 127
Figure 5.5 A distribution of probe transverse locations .................................................. 137
Figure 5.6 Cross-leaflet uncoupling of phase domains .................................................. 138
Figure 5.7 Variation in domain size along a tieline ......................................................... 139
Figure 5.8 FRET orientation factors are properly described ........................................ 149
Figure A.1 Typical 9.6 GHz ESR spectra for 16-PC ...................................................... 171
Figure A.2 Best-fit NLSL spectra for six- and three-parameter fits ................................... 174
Figure A.3 Composition dependence of selected parameters ........................................ 175
LIST OF TABLES

Table 2.1  Best-fit parameter values for FRET data in DSPC/DOPC/chol ............... 40
Table 2.2  Best-fit phase boundaries and 16-PC partition coefficients ............... 46
Table 3.1  Fixed parameters for the Ld + Lo tieline field .............................. 68
Table 3.2  Simulation parameters for MC data ............................................... 80
Table 4.1  Parameters used in energy transfer simulations ................................ 107
Table 5.1  Parameters used in energy transfer simulations ............................. 123
Table 5.2  Best-fit parameters from $R_{\text{sim}}=10$ nm data sets .................... 129
Table 5.3  Best-fit parameters from global fits .............................................. 130
Table 5.4  Correlation matrices for single-trajectory fits ............................... 131
Table 5.5  Correlation matrices for global fits ............................................... 134
Table 5.6  Best-fit parameters for simulations with relaxed assumptions .......... 136
Table A.1  Model parameters used in nonlinear least-squares fitting .............. 172
Table A.2  A typical correlation matrix showing strong correlations .......... 172
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-PC</td>
<td>1-palmitoyl-2(16-doxyl stearoyl) phosphatidylcholine</td>
</tr>
<tr>
<td>A</td>
<td>FRET acceptor</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>BoDIPY-PC</td>
<td>2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>BSM</td>
<td>brain sphingomyelin</td>
</tr>
<tr>
<td>C12:0-DiI</td>
<td>1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>C18:0-DiO</td>
<td>3,3'-dioctadecyloxycarbocyanine perchlorate</td>
</tr>
<tr>
<td>CAS</td>
<td>computer algebra system</td>
</tr>
<tr>
<td>Chol</td>
<td>cholesterol</td>
</tr>
<tr>
<td>D</td>
<td>FRET donor</td>
</tr>
<tr>
<td>DAPC</td>
<td>1,2-diarachidoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DHE</td>
<td>ergosta-5,7,9(11),22-tetraen-3β-ol</td>
</tr>
<tr>
<td>DLPC</td>
<td>1,2-dilauroyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPhPC</td>
<td>1,2-diphytanoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DSPC</td>
<td>1,2-distearoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>E</td>
<td>FRET efficiency</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>Fast-DiI</td>
<td>1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>FP-FRET</td>
<td>finite phase separation FRET</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>FS</td>
<td>Fung and Stryer</td>
</tr>
<tr>
<td>GPMV</td>
<td>giant plasma membrane vesicle</td>
</tr>
<tr>
<td>GUV</td>
<td>giant unilamellar vesicle</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>MC</td>
<td>Monte Carlo</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>MLV</td>
<td>multilamellar vesicle</td>
</tr>
<tr>
<td>MOMD</td>
<td>microscopic order macroscopic disorder</td>
</tr>
<tr>
<td>NBD</td>
<td>7-nitro-2-1,3-benzoxadiazol-4-yl</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PSM</td>
<td>N-palmitoyl-D-erythro-sphingosylphosphorylcholine</td>
</tr>
<tr>
<td>RDF</td>
<td>radial distribution function</td>
</tr>
<tr>
<td>REE</td>
<td>region of enhanced efficiency</td>
</tr>
<tr>
<td>Rhod</td>
<td>rhodamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RHS</td>
<td>right hand side</td>
</tr>
<tr>
<td>RRE</td>
<td>region of reduced efficiency</td>
</tr>
<tr>
<td>RSE</td>
<td>rapid solvent exchange</td>
</tr>
<tr>
<td>SAE</td>
<td>stimulated acceptor emission</td>
</tr>
<tr>
<td>SOPC</td>
<td>1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>SP-FRET</td>
<td>steady-state probe-partitioning FRET</td>
</tr>
<tr>
<td>SSM</td>
<td>N-stearoyl-D-erythro-sphingosylphosphorylcholine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>WAXS</td>
<td>wide angle x-ray scattering</td>
</tr>
<tr>
<td>WH</td>
<td>Wolber and Hudson</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

1.1 Overview

This work seeks to expand the usefulness of Förster resonance energy transfer (FRET) as a means of characterizing phase behavior in model membranes. FRET is indeed a remarkably useful biophysical tool, capable of providing nanometer-scale spatial information while being relatively easy and inexpensive to use. Since the first quantitative models for membrane FRET appeared in the literature 30 years ago, FRET has been successfully used to examine lipid mixing in bilayers. During this time, evidence for highly nonrandom mixing of lipids and proteins in cell membranes has accumulated (obtained with a variety of methods, including FRET), and models for membrane function have evolved to include these observations, culminating in the membrane raft hypothesis. The basic idea of the raft hypothesis is simply stated: The preferential association of certain lipids (particularly sphingomyelin and cholesterol) can effectively compartmentalize the membrane into relatively ordered and disordered regions, providing the cell with a mechanism for controlling the spatial distribution of its components, and hence membrane chemistry. Belying its conceptual simplicity, the membrane raft hypothesis has generated significant controversy. This is in part due to the fleeting nature of rafts, and the great challenge this poses to experimentalists who wish to determine their size and lifetime distributions in the resting cell.

In this chapter we provide background information for the work presented in chapters 2-5: an introduction to lipid bilayer phases and plasma membrane rafts, and an overview of FRET and its applications in membranes, particularly for examining phase behavior and domain size. We end with a statement of the specific problems to be addressed in this work.
1.2 Lipid bilayer phases

Lipids are amphiphilic molecules, composed of a polar headgroup and nonpolar tail. In mixtures with water, lipids form a wide variety of structures depending primarily on the relative size of the headgroup and tail: the most important of these structures in biology is the bilayer. The bilayer is a lamellar (L) structure that can exist in several phases, which differ in chain conformational and positional order. Conformational order is expressed as the ratio of trans to gauche conformers in the hydrocarbon chains: lipids with a high degree of conformational order possess chains that are fully extended, with carbon segments mostly in the trans conformation. Conformational order is related to the segmental order parameter, which can be determined experimentally with ESR, NMR, fluorescence polarization, or WAXS. Positional order refers to the two-dimensional spatial correlation among the chains: bilayers with a high degree of positional order have a well-defined inter-chain spacing, with spatial correlations that persist over long distances. Lateral positional order can be examined with x-ray scattering in oriented samples, and is usually correlated with the rate of translational lipid diffusion.

The three lamellar phases that will be discussed in this thesis are differentiated by order. The liquid disordered (Ld) phase is characterized by low conformational and positional order, in contrast to the solid ordered (L\(\beta\)) phase, in which both conformational and positional order are high. Pure phospholipid and sphingolipid species can exist in either state, with a cooperative phase transition occurring at a well-defined temperature known as the melting transition temperature \(T_M\). For the purposes of the present discussion, it is convenient to categorize these lipids as either low-\(T_M\) or high-\(T_M\) relative to room temperature. For lipids with fully saturated chains, \(T_M\) increases with chain length due to an increase in Van der Waals interactions: for the phosphatidylcholines, the division between low- and high-\(T_M\) occurs at 14 carbons
with DMPC ($T_M=23^\circ$C). Lipids with one or multiple unsaturations in either or both chains are categorically low-$T_M$ lipids. Binary mixtures of lipids with distinctly different transition temperatures exhibit gel/fluid coexistence at intermediate temperatures. The classical method for studying these transitions is differential scanning calorimetry, though the positions of phase boundaries have been determined with many other types of measurements including fluorescence spectroscopy and microscopy, FRET, and ESR.

A third lamellar phase, the liquid ordered or Lo phase, is only found in lipid mixtures containing substantial amounts of sterol. Cholesterol is the most abundant lipid in mammalian plasma membrane. It is also an amphiphile, composed of a rigid, hydrophobic steroid tail and a polar hydroxyl headgroup that orients the molecule in the bilayer (in a PC bilayer, the hydroxyl is located at the level of the carbonyl oxygen). The energetics of cholesterol in bilayers is strongly influenced by the small size of the hydroxyl, relative to the bulky steroid ring: cholesterol's headgroup cannot effectively shield its hydrocarbon shoulders from water. Driven by the hydrophobic effect, neighboring lipids reconfigure their chains and headgroups to accommodate cholesterol, protecting the hydrocarbon from water like an umbrella (1).

The umbrella effect provides a useful framework for understanding the behavior of mixtures of PC and cholesterol. Starting from an Ld phase PC bilayer, cholesterol increases the conformational order, as disordered chains assume an extended conformation to create room under the headgroup for cholesterol. In a gel phase bilayer, where the chains are already mostly trans, cholesterol disrupts the long-range positional order due to the strong energetic penalty for multiple cholesterol contacts. The result in either case is a remarkable state of matter: a fluid phase, lacking in long-range positional order and characterized by fast diffusion, but possessing high
chain conformational order. In effect, cholesterol decouples conformational order from positional order (2).

1.3 Plasma membrane rafts

1.3.1 The membrane raft hypothesis

Lipid rafts were first proposed as a mechanism to explain the distinctly different lipid and protein compositions of the apical and basolateral membranes of polarized epithelial cells, each of which is supplied material from the trans Golgi network (3). It was postulated that the lipid and protein sorting was driven by favorable interactions between sphingolipids and cholesterol give rise to lipid microdomains in trans Golgi membranes. The hypothesis was partly based on structural considerations of the lipids: the sphingosine base can act as both hydrogen bond donor and acceptor, unlike the glycerol backbone of glycerophospholipids. The lipid domains (later termed “rafts”) could also target proteins based on some affinity for the chemical and physical properties of the raft, and furthermore the affinity could be regulated by chemical modifications such as ligand binding, oligomerization, acylation, or deacylation of the protein(s) (4). Much work was done over the ensuing decade to catalogue the targeting ability (expressed as a partition coefficient between raft and nonraft domains) of these and other modifications for a variety of proteins, in a variety of cell types (5,6).

It was soon recognized that rafts should not be unique to the trans Golgi membranes. Plasma membrane is similarly rich in sphingolipid and cholesterol (7)—perhaps raft-like phenomena were functioning there as well. Indeed, membranes evolved to effect a three-dimensional compartmentalization necessary for life's chemical reactions, and it was (and is) appealing to consider that a two-dimensional compartmentalization may have co-evolved to regulate processes within the plane of
the membrane itself. As biochemical studies were identifying the chemical sequences necessary for domain targeting, biophysical studies were employed to measure raft size. The earliest estimates for raft size in resting cells were on the order of hundreds of nanometers (8); subsequent work, utilizing a variety of techniques, have yielded size estimates that converged on 10s of nanometers (9-11). These observations raise the possibility that both the regulated partitioning of membrane components and the regulated size of membrane domains might act in concert to control the chemical reactions of the membrane, by modulating the spatial concentrations (surface densities) of enzymes and their substrates. Such regulation was shown to be important for cell signaling (6,12,13), the pathogenesis of bacteria (14) and viruses (4), and vesicular transport (15). An excellent review of the myriad raft functions can be found in (5).

1.3.2 Relationship between rafts and fluid bilayer phases

As biochemists set their sights on understanding the physical properties of membrane rafts, parallel developments in the field of membrane biophysics were shedding light on the nature of the Lo phase. Using a combination of IR spectroscopy and fluorescence quenching, Silvius and Lefleur showed that a ternary mixture of a low-$T_M$ PC, high-$T_M$ PC, and cholesterol exhibited complex phase behavior, including a large region of coexisting Ld + Lo phases (16). Shortly afterward, the discovery that coexisting phases could be directly visualized in giant unilamellar vesicles (GUVs) using fluorescent lipids as a contrast agent opened the floodgates for investigations of similar mixtures (17). It was soon observed that a ternary composition mimicking the lipid content of the apical membrane gave rise to micron-sized, fluid domains (18). GUV microscopy was turned toward examining the composition-dependent phase behavior, and partial phase diagrams were obtained for several ternary outer leaflet models (19-21). Phase regions undetectable by
fluorescence microscopy were also discovered, leading to the realization that multiple techniques spanning distance scales from nanometers to microns were necessary for solving ternary lipid phase diagrams (22). Relatively complete phase diagrams have now been obtained for the ternary systems: DPPC/DLPC/chol (23), PSM/POPC/chol (24), BSM/POPC/chol (25), DPPC/DOPC/chol (26), DPPC/DPhPC/chol (27), DSPC/DOPC/chol (28), PSM/DOPC/chol (21), and SSM/DOPC/chol (29).

The abundance of experimental data has led to theoretical work attempting to understand the physical mechanisms responsible for fluid-fluid phase coexistence. Elliot et. al were able to reproduce a fluid-fluid coexistence region using mean field theory, but this outcome relied on phase coexistence in one of binary PC/chol systems (30,31); this theory cannot explain the existence of closed-loop miscibility gaps in for example DPPC/DPhPC/chol (27). A phenomenological model from the same group reproduced the closed loop Ld + Lo region through a differential ordering effect of cholesterol on the saturated and unsaturated lipid (32). Simulation work has also yielded insights into the interactions responsible for fluid-fluid miscibility gaps. Monte Carlo simulations have shown how the set of three unlike pairwise interaction energies between two phospholipids and cholesterol can generate the basic shape of the Ld + Lo coexistence region of DSPC/DOPC/chol, and that an additional line tension-like energetic term can refine the boundary to closely match experimental observations (J. Huang, unpublished). Atomistic MD simulations are unable to reach timescales necessary to see phase separation, but recent progress has been made with coarse grained simulations, which can extend the available timescales into the 10s of microseconds (33,34). Starting from an initially random mixture, bilayers composed of DPPC/DOPC/cholesterol separated into Ld + Lo phases in planar and spherical bilayers, with cholesterol enriched in the Lo phase as expected (35).
The obvious connections between raft properties and the liquid ordered phase has led to the point where the two terms have become essentially synonymous, with raft phenomena in general viewed as a manifestation of coexisting fluid phases (36,37). As studies on the partitioning of putative raft components in the Ld + Lo coexistence regions of ternary model systems have accumulated, this viewpoint has been subjected to some scrutiny. In many cases, proteins that partition into the raft phase in cells (usually determined by detergent resistance assays) prefer the liquid disordered phase in model systems (38). The extent to which detergent-resistant membrane fractions faithfully report the partitioning of membrane components in the unperturbed cell has been called into question (39,40). In giant plasma membrane vesicles (GPMVs) blebbled from cells, lipid-anchored proteins faithfully reproduce their raft affinities, while transmembrane proteins do not, being almost completely excluded from Lo phase (41,42). In contrast, other plasma membrane vesicle preparation techniques (i.e. "membrane spheres") show preferential raft partitioning for some transmembrane peptides (43). Further studies showed that order in the GPMV Lo phase was greater than in membrane spheres (44), possibly owing to cross-linking artifacts associated with the GPMV prep, which may explain some of the partitioning discrepancies.

Aside from partitioning behaviors of proteins, the connection has been questioned from other standpoints as well. Munro noted that if the outer leaflet PM composition were to be mapped to a ternary phase diagram, the most probable phase state would be a uniform Lo mixture (12). This possibility was supported by a finding that in three cell lines the majority of the plasma membrane exhibited order comparable to that of Lo phases in model membranes (45). Feigenson and others have pointed out that ternary phase diagrams demonstrate there is no "prototype" Lo phase, and that the composition and properties of raft domains in cells might vary
considerably both in time and space (28). Recently, Lingwood and Simons have argued that the terms Lo and Ld should not be applied to cells, and should instead be reserved for lipid systems where order parameters can be accurately measured (46).

1.3.3 Nanodomains in ternary mixtures

Buboltz and Feigenson were the first to report "nanoscopic" domains, in the ternary mixture DPPC/DLPC/chol (23). Confocal fluorescence microscopy of GUVs showed coexisting gel and fluid phases at compositions along the binary DPPC/DLPC axis that persisted with the addition of cholesterol, ending abruptly at 16 mole % cholesterol; above this concentration, GUVs appeared uniform. FRET measurements over the entire composition space confirmed the microscopy results for low cholesterol but revealed that the experimental signature of phase separation—a pronounced decrease in energy transfer efficiency due to the segregation of donor and acceptor between phase domains—continued up to 25 mole % cholesterol. The flatness of the microscopy boundary at 16 mole % cholesterol provided support for the interpretation of FRET results in terms of first-order phase coexistence: together with the phase rule, this linear feature suggested the existence of a three-phase region, which in turn implied an adjoining region of Ld + Lo phases.

Using spectroscopic techniques with nanometer spatial sensitivity, rich phase behavior including a three phase region and coexisting Ld + Lo phases was also observed in PSM/POPC/cholesterol (24) and BSM/POPC/cholesterol (25). The discovery of nanoscopic phase behavior in the latter systems was significant, because these lipids are generally thought to comprise the most biologically relevant ternary system, in terms of lipid abundance in the mammalian plasma membrane. That Ld + Lo compositions in these mixtures should so closely resemble the prevailing picture of membrane rafts, both in terms of phase properties and size scale, attracted considerable attention.
The explosion of interest in ternary phase diagrams has yielded valuable information, summarized in recent reviews (47-49). An intriguing result of ternary model studies is that the structure of the low-\(T_M\) lipid seems to determine fluid domain size. Highly disordered lipids like DOPC (with unsaturations in each acyl chain) and diphytanoyl-PC (with methyl branches down the length of the chains) form micron-sized fluid domains with a variety of high- \(T_M\) lipids and cholesterol. Less perturbing lipids like POPC and SOPC (with one saturated and one unsaturated chain) and DLPC (with fully saturated, 12-carbon chains) do not exhibit micron-sized domains, though heterogeneity is detected with spectroscopic techniques. Feigenson has proposed a classification scheme for ternary phase diagrams based on the GUV results: Type II diagrams have a region of "macroscopic" \(L_d + L_o\) coexistence in addition to \(L_d + \lambda\beta\) and three phase (\(L_d + L_o + \lambda\beta\)) regions, while Type I diagrams only show a region of macroscopic \(L_d + \lambda\beta\) (22). In general, low-\(T_M\) lipids that give rise to Type II diagrams are biologically rare, especially in the plasma membrane of animal cells. In contrast, the low-\(T_M\) lipids of Type I systems are relatively abundant (50).

An interpretation of Type I systems in terms of first-order phase separation is controversial. The unfavorable line tension at domain boundaries is minimized by domain coalescence, and in the absence of competing interactions, the equilibrium state is a single (large) domain. A variety of interactions has been proposed to fill this role, including domain curvature and electrostatic repulsion (51), though both theory and experiment are lacking. Alternative explanations for nanodomains include non-ideal mixing, microemulsions, and 2D Ising-like critical phenomena (52). Recently, Ising-like critical fluctuations have been observed in DPPC/DPhPC/chol (53), DPPC/DOPC/chol (26), and GPMVs (54), lending support to this viewpoint. Regardless of the mechanism at work, it is clear that the lipid solvent of the membrane
can exhibit heterogeneity on an enormous range of size scales, and in the absence of proteins.

1.3.4 Methods for measuring nanodomain size

Only a few techniques are capable of delivering size measurements of small membrane domains, including FRET (10,55), AFM (56), electron microscopy (57,58), and the newly-emerging super-resolution techniques (11,59). FRET offers several advantages over other methods. FRET can be measured in free-standing bilayers, in contrast to nanoscale imaging techniques that often require a rigid bilayer support that may influence the phase behavior (60,61). Steady-state FRET can be measured with inexpensive equipment found in many labs, and a variety of suitable fluorescent probes are commercially available.

FRET also suffers from drawbacks. Unlike direct visualization techniques, modeling domain size with FRET requires information about the coexisting phases, including their compositions, molecular areas, and thicknesses. The fluorescent probes must be well-characterized, particularly with respect to their transverse location in the bilayer. Finally and most famously is the problem of the orientation factor $\kappa^2$. The distribution of $\kappa^2$ in a membrane has not been experimentally determined for any probe pair, and the assumption of an isotropic distribution may not be valid in the highly anisotropic membrane environment.

1.4 FRET in membranes

1.4.1 The FRET mechanism

Förster resonance energy transfer or FRET is a valuable biophysical tool for acquiring information at molecular length scales. FRET results from a weak dipole-dipole coupling between electric oscillators (a donor and an acceptor) in the near field: it occurs over distances that are greater than molecular contact, but much shorter than
the excitation or emission wavelengths of the molecules involved. Because the ultimate quenching of the donor excitation energy does not involve molecular collision, FRET is considered to be a long-range interaction. Also, as its name implies, FRET does not involve emission of a photon by the donor: no propagating electromagnetic waves are present in the near field of an oscillating dipole (62), though a "virtual" photon is often invoked to describe the phenomenon.

The FRET process requires a population of excited state donors: for biophysical applications, these are almost always fluorophores. Depopulation of the donor excited state via FRET will occur at some rate in the presence of a population of ground-state energy acceptors, which are also typically fluorescent molecules. Just as a molecule in the far field can "accept" a photon whose energy matches that of an electronic transition, that same molecule can accept energy in the near field of an oscillating dipole (the donor), and with the same requirement of matching energies. The precise details of this frequency matching were worked out by Förster and are expressed mathematically as a spectral overlap integral (63):

$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \int_0^\infty F_D(\lambda)d\lambda \quad 1.1$$

where $F_D$ is the (dimensionless) corrected fluorescence emission spectrum of the donor, and $\varepsilon_A$ is the absorption spectrum of the acceptor (in units of $M^{-1}cm^{-1}$).

The basis of FRET as a "molecular ruler" is its strong dependence on the distance between donor and acceptor transition dipoles. The rate of energy transfer between a donor and acceptor separated by a distance $r$ is:

$$k_T(r) = \frac{9000 \ln 10 Q_D \kappa^2}{128\pi^3 N_A n^4 \tau_D r^6} = \frac{1}{\tau_D} \left(\frac{R_0}{r}\right)^6 \quad 1.2$$
where $Q_D$ and $\tau_D$ are the donor quantum yield and fluorescence lifetime in the absence of acceptor, $n$ is the index of refraction of the medium, $N_A$ is Avogadro's number, and $\kappa^2$ describes the relative orientation of the donor and acceptor transition dipoles.

The right hand side of Equation 1.2 combines the terms in a convenient way, such that the donor-acceptor separation distance at which half of the excited state donors are quenched via FRET is expressed as the value $R_0$ (the Förster distance). Förster distances are specific to a given donor and acceptor (expressed by the dependence on donor quantum yield and lifetime, and probe spectral properties), and in a given dielectric environment (expressed by the refractive index). Though often considered to be constant, in most physical systems $R_0$ is properly described by a distribution, due to the distribution of possible orientations that can be assumed by the donor and acceptor transition dipoles (i.e., the distribution of $\kappa^2$). In cases where the molecules exhibit rapid and isotropic rotational diffusion, $\kappa^2 = 2/3$ can be used as a fixed constant in the calculation of $R_0$ (63,64).

Typical values of $R_0$ range from 1-10 nm, which sets an approximate sensitivity range of 1-20 nm for FRET distance measurements using donor and acceptor separated by a fixed distance. We will see that for an ensemble of freely-diffusing fluorophores, the upper limit of sensitivity can be extended to nearly 100 nm. FRET can be measured in the steady-state either by observing the relative quenching of the donor (i.e. fluorescence intensity in the presence and absence of acceptor, which gives a direct measurement of transfer efficiency), or by observing the enhanced fluorescence emission of the acceptor.

### 1.4.2 FRET for random, planar arrays of donor and acceptor

(N.B.: this section is intended to provide an overview of theoretical and experimental FRET developments that are most relevant to this thesis. Comprehensive reviews of FRET applications in membranes can be found in (65,66)).
A quantitative description of FRET for an ensemble of freely diffusing lipid fluorophores in a membrane is challenging: the equations must account for a distribution of donor-acceptor distances, rather than a single fixed distance. In the late 70s, three groups published solutions to the problem, one of which (67) was more restrictive in its assumptions and will not be discussed here. A comparison of the theories of Fung and Stryer (FS) and Wolber and Hudson (WH) is found in Chapter 2, but the main results are summarized here (68,69). Both treatments showed that FRET efficiency for a monolayer depended on essentially three parameters: the surface density of acceptor, the distance of closest approach between donor and acceptor (taken to be the sum of their Van der Waals radii), and \( R_0 \). FS also derived equations for \( E \) in planar and spherical bilayers, and proposed an experimental test to examine the extent of non-random mixing in a membrane: \( E \) is measured as a function of acceptor concentration and fit to the model to determine an apparent \( R_0 \) which, if different from \( R_0 \) calculated from the spectral overlap integral, would indicate non-random mixing of donor and acceptor.

1.4.3 FRET in macroscopically phase separated mixtures

In the mid-90s, Pederson showed that FRET between freely-diffusing lipid probes could be used to examine phase transitions in membranes (70). In that study, a decrease in FRET efficiency between NBD-PE and Rhod-PE was observed at the gel/fluid transition of pure DPPC vesicles. The authors reproduced these results with Monte Carlo simulations, in which the donor and acceptor were assigned different affinities for the gel and fluid phase domains. In the vicinity of the phase transition temperature, large clusters of gel and fluid domains are present; the segregation of donor and acceptor increased the average distance between these lipids relative to their random distribution in bilayers far from the transition temperature, and resulted in decreased transfer efficiency.
Buboltz and Feigenson applied this principle in their study of DPPC/DLPC/cholesterol (23), using the acronyms REE and RRE to describe the relatively enhanced or reduced FRET efficiency observed in phase coexistence regions due to non-uniform probe partitioning. A phenomenological model was used to recover probe partition coefficients from the FRET profiles with the lever rule. It was also demonstrated that phase boundaries could be accurately determined with FRET, as compositions where transfer efficiency changed abruptly.

Buboltz later put these ideas on a solid quantitative basis with the development of a Stern-Volmer quenching model for stimulated acceptor emission (SAE) data in single phase mixtures (71). With a set of additional assumptions (including, importantly, that domain size \( \gg R_0 \)), he provided a quantitative basis for SAE measurements along a tieline, in terms of the donor and acceptor partition coefficients and the lever rule; the resulting model was called “steady-state probe-partitioning FRET” or SP-FRET (72). The good agreement between experimental data and the SP-FRET model was demonstrated in gel/fluid mixtures of DPPC/DLPC at 25°C. SP-FRET was also measured in the ternary system DPPC/DOPC/cholesterol as a function of temperature (73). While no quantitative analysis was presented, this important study demonstrated that REE and RRE lineshapes observed in phase-separated binary mixtures had two-dimensional counterparts (surface features) in three component mixtures: probe separation resulted in a "valley" of reduced efficiency in the Ld + Lo and Ld + L\(\beta\) regions. Above the melting transition temperature of the high-\(T_M\) lipid DPPC, FRET efficiency was essentially uniform across composition space, consistent with random mixing of the probes.

The derivation of SP-FRET in (72) follows a standard approach for describing signals in phase-separated systems. In many cases, the signal measured in a bulk sample can be modeled as arising from two distinct, non-interacting populations of
probe: the observed signal is simply the sum of signals arising from each population. This approach does not strictly apply to a FRET measurement, because donors in one phase and near a domain boundary may be quenched by acceptors in the other phase. One of the explicit assumptions of the SP-FRET model is that the total amount of domain interface is small, such that this population of donors—those that are near enough to the phase boundary to be able to transfer energy to acceptors in either phase—is negligible. This assumption poses an obvious problem for the study of nanodomains.

1.4.4 Measuring domain size with FRET

After publication of a phase diagram showing a region of coexisting Ld + Lo in the ternary mixture PSM/POPC/cholesterol, the Prieto lab established a protocol for estimating domain sizes using FRET (74,75). Importantly, their model does not explicitly account for small domains. The idea is relatively simple: for a given acceptor partition coefficient, the acceptor distribution in the vicinity of a donor appears increasingly more like a random probe distribution as the size of phase domains decreases toward $R_0$. The practical corollary is that donor fluorescence decay curves, when fit to a model for FRET that assumes infinitely large phase domains, will return best-fit values of acceptor $K_p$ that are smaller than their true values. By comparing $K_p$ values measured from donor decay curves to values measured with techniques insensitive to domain size (like fluorescence quantum yield or anisotropy), the presence of small domains could be inferred. Domain sizes can be estimated (but not recovered) with this method. Using this method, domain sizes in the Ld + Lo region of PSM/POPC/chol were found to vary within the coexistence region, ranging from small (<20 nm diameter) Lo domains near the Ld boundary, to large (75-100 nm diameter) Ld domains near the Lo boundary (76).
Towles and Dan developed a model that explicitly accounted for domain size, starting from a picture of the bilayer as monodisperse disks of one phase randomly distributed in the second, continuous phase (77). They imagined two populations of donors: those located in domains, and those located in the surround phase, for which the apparent domain surface coverage as a function of increasing distance would differ. They recognized that this radial domain surface density function was related to the pair correlation function for non-interacting, two-dimensional disks, though they did not attempt to derive the relationship. Rather, they used Monte Carlo techniques to simulate a domain surface coverage function, from which they could calculate distance-dependent acceptor densities for the two donor pools. Their model was successful in recovering domain size from Monte Carlo simulations of donor decay functions. In a subsequent paper, the same group attempted to recover domain size from experimental data, but used an entirely different model, based on a different set of assumptions that is valid only in a highly limited subset of parameter space (78,79).

1.5 Thesis outline

This research presented in this thesis aims to extend the usefulness of FRET for studying phase behavior in membranes. In Chapter 2, we apply FRET and ESR to three ternary mixtures to determine Ld + Lo boundaries and order parameters, and we identify an Ld + Lo tieline in DSPC/POPC/cholesterol that is suitable for examining nanoscopic phase domains. In Chapter 3, we outline a procedure for extracting tieline and probe partitioning information from FRET measurements in a ternary Ld + Lo coexistence region, which is applied to the Ld + Lo region of DSPC/DOPC/cholesterol. We develop a model to extract domain size information from FRET measurements in Chapter 4, and then assess the performance of the model with simulated data in Chapter 5. We end with conclusions and future directions.
REFERENCES


Chapter 2
Comparison of three ternary lipid bilayer mixtures: FRET and ESR reveal nanodomains*

2.1 Abstract

Phase diagrams of ternary lipid mixtures containing cholesterol have provided valuable insight into cell membrane behaviors, especially by describing regions of coexisting liquid-disordered (Ld) and liquid-ordered (Lo) phases. Fluorescence microscopy imaging of giant unilamellar vesicles (GUVs) has greatly assisted the determination of phase behavior in these systems. However, the requirement for optically resolved Ld + Lo domains can lead to the incorrect inference that in lipid-only mixtures, Ld + Lo domain coexistence generally shows macroscopic domains. Here we show this inference is incorrect for the low melting temperature phosphatidylcholines abundant in mammalian plasma membranes. By use of high compositional resolution FRET measurements, together with ESR data and spectral simulation, we find that ternary mixtures of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol together with either 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (SOPC), do indeed have regions of Ld + Lo coexistence. However, phase domains are much smaller than the optical resolution limit, likely on the order of the Förster distance for energy transfer ($R_0$, ~ 2-8 nm).

2.2 Introduction

What is the connection between the membrane properties of living cells, and chemically simplified model bilayer mixtures? While the physical chemical behaviors of even simplified lipid bilayer mixtures are not fully understood, complex membranes of living cells have provided important clues to the underlying physical properties of bilayers. The functional lipid raft, a compositionally distinct membrane domain, is now thought to play a role in normal cell functions including signaling, membrane transport and protein sorting, and virus pathogenesis (1). These characteristics of biological membranes suggest highly nonuniform mixing of membrane components. But what are the sizes, shapes, lifetimes, connectivities, and partitioning behaviors of membrane heterogeneities? Are proteins required for raft formation? Such descriptions are important, as many functions ascribed to rafts require the existence of domains large enough to accommodate several proteins, and stable for at least the time required for proteins to find each other and interact (2). Measurements of raft size in resting cells have resulted in estimates from a few to hundreds of nanometers (3), underscoring the difficulty of teasing apart mechanisms that mediate domain size in an experimental system as complex, dynamic, and variable as the plasma membrane (PM).

Model lipid bilayers offer a measure of simplification to the problem. Model bilayers can be chemically well-defined and systematically studied within the powerful framework of equilibrium thermodynamics. Indeed, model studies have figured prominently in the development of the raft hypothesis by providing a picture of PM domains as coexisting liquid-disordered (Ld) and liquid-ordered (Lo) phases. The minimal requirement for liquid phase coexistence in model systems, met by all animal cell PM, is a ternary mixture of cholesterol, low-, and high-melting temperature ($T_M$) lipids (4). Furthermore, the low-$T_M$ lipid seems to be an important
factor in controlling liquid phase domain size. The well-studied low-$T_M$ lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), though rare in mammalian PM, exhibit micron-sized domains in ternary mixtures (5, 6). In contrast, biologically-abundant low-$T_M$ lipids including POPC and SOPC do not (7-9), though methods sensitive to submicron length scales consistently indicate liquid phase heterogeneity in POPC- and SOPC-containing ternary mixtures (7,8,10,11). Driven by these reports, a growing theoretical literature seeks to explain submicron domains in lipid-only bilayers in terms of composition-dependent membrane properties like line tension and bending stiffness (12,13). Theoretical treatments have indeed outpaced experiments, largely due to the difficulty of obtaining reliable data at length scales below the optical resolution limit (3). Composition-dependent data are particularly valuable, as composition is the primary mechanism by which a cell can alter membrane phase behavior. These data are also challenging to acquire: Precise control of membrane composition is laborious, and the effect of small, systematic changes in membrane composition on domain properties remains largely unexplored.

FRET between diffusing lipid fluorophores (SP-FRET, 14) is sensitive to membrane domains larger than $R_0$ (typically 2–8 nm). To address the effect of bilayer composition on domain size, we measured SP-FRET over the entire composition space of the ternary systems DSPC/DOPC/chol, DSPC/POPC/chol, and DSPC/SOPC/chol. The first of these mixtures, well-established as exhibiting first-order Ld + Lo phase coexistence with clearly-defined phase boundaries, provides a standard for comparison (6). We report that the latter two systems show FRET and ESR behavior remarkably similar to that seen in DSPC/DOPC/chol. This behavior is described by probe partitioning between phases and the lever rule (14), even at compositions where GUV images appear uniform. Apparently, nanoscopic liquid
domains in these systems behave closely enough to genuine phases that they may be usefully described by the tools of equilibrium thermodynamics.

2.3 Materials and Methods

2.3.1 Materials

Phospholipids were from Avanti Polar Lipids, Inc (Alabaster, AL), cholesterol from Nu Chek Prep (Elysian, MN). Fluorescent dyes 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BoDIPY-PC) and 1,1’-dilinoleyl-3,3’,3”,3”-tetramethylindocarbocyanine perchlorate (Fast-DiI) were from Invitrogen (Carlsbad, CA). Ergosta-5,7,9(11),22-tetraen-3β-ol (DHE) was from Sigma-Aldrich (St. Louis, MO). Spin-labeled lipid 1-palmitoyl-2-(16-doxyl stearoyl) phosphatidylcholine (16-PC) was a gift from Boris Dzikovski of the National Biomedical Center for Advanced ESR Technology (ACERT) at Cornell University. Purity of > 99% was confirmed by thin layer chromatography (TLC) on washed, activated Adsorbosil TLC plates (Alltech, Deerfield, IL), developed with chloroform/methanol/water (C/M/W) 65/24/4 for phospholipids, 65/35/4 for 16-PC, C/M 9/1 for Fast-DiI, and petroleum ether/diethyl ether/chloroform 7/3/3 for cholesterol. Solvents were HPLC grade. Concentration of phospholipid stocks was determined to < 1% by inorganic phosphate assay, and of fluorescent dye stocks by absorption spectroscopy using an HP 8452A spectrophotometer (Hewlett-Packard, Palo Alto, CA). Cholesterol stocks were prepared by standard gravimetric methods to ~ 0.2%.

2.3.2 FRET sample preparation

Chloroform mixtures of lipids in 2% compositional increments were prepared in glass culture tubes using a syringe and repeating dispenser (Hamilton USA, Reno, NV). Samples received a fixed volume of a combined chloroform stock of fluorescent
probes to achieve probe/lipid mole ratios of ~ 1/200 (DHE), 1/1500 (BoDIPY-PC), and 1/3000 (Fast-DiI). Multilamellar vesicles (MLVs) were prepared from these mixtures using rapid solvent exchange (RSE, 15) as previously described (6). After measurement, randomly selected samples were analyzed by TLC and showed no evidence of breakdown.

Fluorescence data were collected at 22°C with a Hitachi F7000 spectrofluorimeter (Hitachi High Technologies America, Schaumburg, IL). A 100 µL sample aliquot was diluted into 1.90 mL RSE buffer (200 mM KCl, 5 mM PIPES, 1 mM EDTA) gently stirring in a cuvette. Intensity (2.5 nm bandpass for excitation and emission slits, 10 s integration time) was measured in six channels (excitation/emission λ, nm): DHE fluorescence (327/393); BoDIPY-PC stimulated emission (327/517); BoDIPY-PC fluorescence (509/517); Fast-DiI stimulated emission (509/565); Fast-DiI fluorescence (549/565); and vesicle scattering (420/420). Signal in the stimulated acceptor emission (SAE) channels contains non-FRET contributions from donor (D) and acceptor (A) emission through their direct excitation pathways, as well as excitation light scattered by the vesicle suspension. Control samples were used to correct for these contributions: full details are provided in Chapter 3.

2.3.3 FRET data analysis

Following (14) and (16), SAE in a 2D tieline field is modeled by:

\[ F(u, S_{L0}, \kappa_D, \kappa_A, \varphi) = \]

\[ f_{ld}(u, \varphi) + S_{L0} \left[ f_{lo}(u, \varphi) K(u, \kappa_D) K(u, \kappa_A) - f_{ld}(u, \varphi) \right] \]

\[ 1 + (K(u, \kappa_D) - 1)S_{L0} \]

\[ K(u, \kappa) = 10^{\kappa_0 u + (\kappa_1 - \kappa_0)u^2} \]

\[ f(t, \varphi) = \varphi_0 + (\varphi_1, \varphi_2) \cdot B(t) \]
where \( u \) is the tieline coordinate, \( S_{Lo} \) is the Lo phase mole fraction, \( K \) are functions describing D and A partition coefficient variation within the tieline field, \( f_{ld} \) and \( f_{Lo} \) are functions describing SAE variation in compositions along the phase boundaries, and \( B(t) \) is the Ld + Lo boundary. Equations 2.2 and 2.3 were chosen to vary smoothly and allow a range of reasonable behaviors with a minimal number of fitting parameters (\( \kappa \) and \( \varphi \)). Data in the 2-phase region were fit by optimizing \( \kappa^D \), \( \kappa^A \), and \( \varphi \). Analysis was performed with Mathematica 7.0.1 (Wolfram Research, Champaign, IL). Full details of the analysis are provided in Chapter 3.

2.3.4 ESR sample preparation

MLV samples were prepared by hydrating lipid films. Lipids and probe (16-PC) were dispensed into glass culture tubes with a Hamilton syringe. Samples contained \(~2000 \) nmoles total lipid with 0.2 or 0.3 mol % 16-PC. Samples were dried to a thin film by rotary evaporation at \(~60^\circ C\) and placed under vacuum for 12-24 h to remove residual solvent. The dry film was hydrated at \( 60^\circ C \) with 400 \( \mu L \) prewarmed RSE buffer, immediately followed by vortexing and five freeze/thaw cycles between liquid nitrogen and \( 60^\circ C \) water. Samples were sealed under Ar and placed in a \( 60^\circ C \) water bath, cooled at 2°C/h to ambient temperature, and incubated for \( > 24 \) h. Prior to measurement, samples were pelleted and transferred to 1.5-1.8 x 100 mm glass capillaries.

2.3.5 ESR data analysis

Dynamic parameters for 16-PC in the slow-motional regime were obtained from nonlinear least-squares fits of simulations (17) based on the stochastic Liouville equation (18). Briefly, spectra are modeled as arising from Brownian diffusion of the nitroxide in an ordered liquid. The diffusion rates are contained in an axially-symmetric diffusion tensor \( R \), with principal components \( R_\perp \) and \( R_\parallel \) representing diffusion rates perpendicular and parallel to the bilayer normal. In an anisotropic
membrane environment, the motion of the nitroxide also depends on its orientation with respect to the bilayer normal. The tendency of the probe to orient in the bilayer is expressed as a restoring potential $U(\Omega)$ defined relative to the local director, and modeled as an expansion of generalized spherical harmonics $D_{MK}^I(\Omega)$ keeping only the lowest-order ($M = 0$) terms:

$$\frac{-U(\Omega)}{kT} = c_0^2D_{00}^2(\Omega) + c_2^2[D_{02}^2(\Omega) + D_{0-2}^2(\Omega)]$$  \hspace{1cm} (2.4)

where $\Omega \equiv (0, \theta, \phi)$. Preferential alignment of the molecular $z$-axis with the bilayer normal can then be expressed by an order parameter:

$$S_0 \equiv \langle D_{00}^2 \rangle = \langle 1/2 (3 \cos^2 \theta - 1) \rangle$$  \hspace{1cm} (2.5)

A vesicle suspension is essentially an isotropic distribution of such locally-ordered regions; the microscopic order with macroscopic disorder (MOMD) model accounts for this by integrating the spectral lineshape over the director tilt angle $\psi$ (17). MOMD was incorporated in the simulation as a $\sin \psi$-weighted averaging of spectra calculated at 23 discrete values of $\psi$. Details of the analysis are found in Appendix A.

2.4 Results

2.4.1 Regions of enhanced (reduced) FRET efficiency correspond to phase-coexistence regions

Figure 2.1 models a particularly useful FRET experiment for examining composition-dependent phase behavior in lipid bilayers: a set of samples is prepared along a tieline, with each sample receiving a fixed amount of D and A probe. Shown is the family of FRET curves generated by Equation 3.8 as $K^A$ varies with fixed $K^D$, revealing two general lineshapes. When D and A prefer the same phase, FRET increases abruptly at the phase boundary where the favored phase first appears.
Figure 2.1 Two types of FRET patterns depend on probe partitioning: enhanced FRET efficiency when probes colocalize in the coexistence region (REE); and reduced FRET efficiency when probes separate (RRE). Equation 3.8 plotted for $K^D$ of 0.33 (favoring the Ld phase) and various $K^A$. 

\[ K^A = 0.05 \]

\[ K^D = 0.33 \]
Figure 2.2 SAE (stimulated acceptor emission) surfaces in DSPC/DOPC/chol show regions of enhanced or reduced FRET efficiency corresponding to phase coexistence regions. Contour plots A and B from 1116 data points, corresponding to 2 mol % sampling of the ternary composition space. Data were smoothed by averaging nearest-neighbor values. The relatively lowest values are blue, and the relatively highest values are red as shown by the scale bar. (A) BoDIPY-PC to Fast-Dil FRET: Donor and acceptor colocalization in Ld phase domains results in enhanced FRET, most pronounced near the ordered phase boundary (arrow 1). (B) DHE to BoDIPY-PC FRET: Donor and acceptor segregation between ordered and disordered phases results in reduced FRET. Symbols and arrows refer to surface features mentioned in the text. (C) and (D), predicted surfaces for the Ld + Lo region corresponding to a best-fit of data in panels A and B (respectively) to Equations 2.1-3. Critical point (star) and tieline field used to model the data are shown.
Relative to a straight line joining the endpoint values, FRET is enhanced at all phase-separated compositions. When D and A prefer different phases, the opposite effect is seen: FRET decreases abruptly at each phase boundary and is reduced at all compositions along the tieline. These two characteristic lineshapes are termed REE or RRE for “region of enhanced (or reduced) efficiency". Although FRET varies with composition even in the absence of phase coexistence, changes within a single phase are expected to be gradual and small relative to those induced by phase separation.

Analogous to the one-dimensional tieline, the FRET surface for a 2D tieline field with smoothly varying $K^p$, described by Equations 2.1-3, exhibits a characteristic 3D peak of enhanced efficiency if D and A colocalize, and a 3D valley of reduced efficiency if D and A separate in the coexistence region. We chose two FRET pairs to generate both behaviors: a cholesterol analog (DHE) that partitions into Lo phase, paired with the Ld-preferring probe BoDIPY-PC; and BoDIPY-PC paired with a second Ld-preferring probe, Fast-DiI. Consistent with expectations, clear and interpretable patterns are observed: FRET between DHE (D) and BoDIPY-PC (A) is reduced in DSPC/DOPC/chol phase-coexistence regions relative to the surrounding single-phase regions (see Figure 2.2 B), while FRET between BoDIPY-PC (D) and Fast-DiI (A) is enhanced (see Figure 2.2 A). We now describe key features of these surfaces.

2.4.2 FRET surfaces in DSPC/DOPC/chol

2.4.2.1 BoDIPY-PC to Fast-Dil: Enhanced FRET

Both probes prefer Ld phase, yielding REE peaks in compositional regions where Ld (a) coexists with an ordered phase; and (b) is the minor component. We note these qualitative features of the FRET surface in Figure 2 A:

1. A ridge of enhanced FRET (gray curved arrow 1). Most of the composition space below $\chi_{\text{chol}} = 0.4$ separates into coexisting Ld and ordered phase (Lo,
Lβ, or both). Tie-lines have small positive slope indicating slightly increased concentration of cholesterol, up to ~ 2.5-fold, in Lo. The ridge of enhanced FRET results from increased concentration of D and A in the diminishing Ld phase, and concomitant decrease in average D-A separation.

2. The upper boundary of the liquid/liquid region at $\chi_{CHOL} \sim 0.4$, manifest as a sharp increase in FRET (e.g., from point D to B).

3. The relative magnitude of FRET in the gel/liquid and liquid/liquid regions differs, with the REE peak in the latter (point B) ~ 15% greater than in the former (point A). This difference in FRET intensity could result from the geometry and small size of gel/liquid phase domains at high $\chi_{DSPC}$.

4. FRET efficiency is enhanced in single-phase compositions near the critical point. The path through composition space marked by gray curved arrow 2 follows a *continuous* phase change from Ld to Lo; a modest rise and fall in FRET intensity occurs in the single phase vicinity of the critical point (point C). Thus, even in the absence of a sharp transition, molecular interactions giving rise to Ld and Lo coexistence at lower $\chi_{CHOL}$ cause compositional fluctuations sensed by the probes in this single-phase region.

5. Along the DOPC/chol binary axis, modest changes in FRET are consistent with complete miscibility.

6. Along the binary DSPC/chol axis, FRET decreases from a high value at the DSPC vertex to a low value near $\chi_{CHOL} = 0.27$, then remains nearly constant up to high $\chi_{CHOL}$. The gradual change in FRET is consistent with the absence of any first-order phase transition along this axis (19).

2.4.2.2 DHE to BoDIPY-PC: Reduced FRET

Like cholesterol, DHE partitions modestly into ordered phases. The FRET surface between DHE and BoDIPY-PC in the Lβ + Lo and Ld + Lo regions is
dominated by valleys of reduced efficiency due to probe separation (Figure 2 B, regions near points E and B). We further note the following:

1. The upper Ld + Lo boundary is seen as an abrupt decrease in FRET efficiency upon entering the 2-phase region (e.g., from point D to B).

2. FRET in the Ld + Lβ region at low cholesterol (region near point A) is modestly enhanced relative to single-phase Ld, suggesting that DHE (or cholesterol) prefers Ld over Lβ'. The same result was found for DHE in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) at 20°C and DPPC/DOPC at 25°C (14,20), and likely reflects an energetic penalty for creating defects in a tilted Lβ lattice. As cholesterol is added to DSPC/DOPC mixtures (e.g., from point A to B), a decrease in FRET occurs as DHE preference shifts from Ld to Lβ. The change in DHE partitioning might relate to the transformation of the gel from a tilted Lβ' to an untilted Lβ phase that more easily accommodates sterols (19).

3. Modest changes in FRET along the DOPC/chol binary axis are consistent with uniform mixing.

2.4.2.3 Probe partitioning in DSPC/DOPC/chol

FRET data in the Ld + Lo region were modeled with Equations 2.1-3 to recover probe $K^p$, using a fixed tieline field. The phase boundary was taken from (6) with slight modification. The critical point and tieline slopes were constrained with phase percolation data and direct observation of critical fluctuations in GUVs (see Figures 3.4 and 3.5). Best-fit surfaces are shown in Figure 2.2, C and D, and the recovered parameters are listed in Table 2.1.

Equation 2.2 describes probe partitioning between coexisting phases: for a particular tieline (i.e., a particular value of $u$), the best-fit parameters $\kappa_0$ and $\kappa_1$ give the partition coefficient $K^p$. Figure 2.3 plots $K^p$ for DHE, BoDIPY-PC, and Fast-DiL
Figure 2.3 Lipid and probe $K^P$ in the Ld + Lo tieline field of DSPC/DOPC/chol. Each value of $u$ represents a different tieline, beginning at the critical point ($u = 0$) and ending at the Ld + Lo segment of the three-phase triangle ($u = 1$). DHE (dotted), BoDIPY-PC (dashed), and Fast-DiI (dot-dash) $K^P$ are calculated from Equation 2.2 and the respective best-fit values of $\kappa_0$ and $\kappa_1$ listed in Table 1. Lipid $K^P$ (solid gray lines) are calculated from tieline endpoints.
Figure 2.4 SAE surfaces in DSPC/POPC/chol and DSPC/SOPC chol show RRE and REE. Contour plots A and B each from 1116 data points, corresponding to 2% sampling of the ternary composition space. Data were smoothed by averaging nearest-neighbor values. BoDIPY-PC to Fast-DiI FRET in DSPC/POPC/chol (A) and DSPC/SOPC/chol (B). As in Figure 1, colocalization of these probes in Ld phase domains results in enhanced FRET efficiency at phase-separated compositions. DHE to BoDIPY-PC FRET in DSPC/POPC/chol (C) and DSPC/SOPC/chol (D). Separation of these probes between ordered and disordered phases results in reduced FRET efficiency. Symbols and arrows refer to surface features mentioned in the text.
using their respective best-fit values of $\kappa_0$ and $\kappa_1$ found in Table 2.1, across the Ld + Lo tieline field. For comparison, $K^P$ of lipid components calculated from tieline endpoint compositions are also shown in Figure 2.3. The structural analogs DHE and cholesterol partition similarly to each other, weakly favoring the Lo phase. Both BoDIPY-PC and Fast-DiI strongly prefer the Ld phase; like DOPC, these probes have structural motifs that disrupt ordered lipid lattices.

<table>
<thead>
<tr>
<th>$\kappa_0^{DHE}$</th>
<th>$\kappa_1^{DHE}$</th>
<th>$\kappa_0^{BoDIPY}$</th>
<th>$\kappa_1^{BoDIPY}$</th>
<th>$\kappa_0^{DiI}$</th>
<th>$\kappa_1^{DiI}$</th>
<th>$\phi_0^{RE}$</th>
<th>$\phi_1^{RE}$</th>
<th>$\phi_2^{RE}$</th>
<th>$\phi_3^{RE}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.45(1)</td>
<td>0.1(4)</td>
<td>-1.0(2)</td>
<td>-1.0(6)</td>
<td>-0.8(2)</td>
<td>-1.0(6)</td>
<td>3.3(4)</td>
<td>-2(1)</td>
<td>8(3)</td>
<td>1.51(4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5(3)</td>
</tr>
</tbody>
</table>

### 2.4.3 FRET surfaces in DSPC/POPC/chol and DSPC/SOPC/chol

FRET was examined for DSPC/POPC/chol and DSPC/SOPC/chol under sample preparation and measurement conditions essentially identical to those used for DSPC/DOPC/chol. As with DSPC/DOPC/chol, these mixtures exhibit coexisting gel/liquid at low cholesterol concentration when GUVs are examined with fluorescence microscopy (9). Unlike DSPC/DOPC/chol, GUVs with compositions above the gel/liquid region appear uniform under the microscope. Nevertheless FRET features, shown in the last section to arise from probe-partitioning and the lever rule, exist above the gel/liquid region in these systems as described below.

#### 2.4.3.1 BoDIPY-PC to Fast-DiI

1. A ridge of enhanced FRET is marked by gray curved arrow 1 in Figure 2.4, A and B, analogous to arrow 1 in Figure 2.2 A. A peak of enhanced FRET in DSPC/POPC/chol (point B) corresponds to point B in the Ld + Lo region of DSPC/DOPC/chol (see Figure 2.2 A), the result of probe colocalization in Ld domains. In contrast, the ridge in DSPC/SOPC/chol (see Figure 2.4 B arrow 1)
contains no local peak near B, but instead runs continuously into the larger REE peak of the gel/liquid region (point A). The relative FRET enhancement in the Ld + Lo region of these systems is smaller than for the DOPC-containing system, indicating that phase domains are comparable in size to $R_0$. The absence of a FRET peak at point B in DSPC/SOPC/chol might indicate a further reduction in domain size in the SOPC system compared with the POPC system.

2. The upper Ld + Lo boundary is marked by an increase in FRET efficiency upon entering the 2-phase region, shown clearly on the path between points D and B.

2.4.3.2 DHE to BoDIPY-PC

1. The upper Ld + Lo boundary shows up in a similar way in all three mixtures. There is a remarkable similarity of Figure 2.4, C and D to Figure 2.2 B: A phase boundary appears as a relatively abrupt change in FRET at $\chi_{CHOL} \sim 0.30$ (e.g., between points D and B in Figure 2.4, C and D). This upper boundary extends from the binary DSPC/chol axis to at least $\chi_{DSPC} = 0.2$ and therefore must include parts of both the Lo + Lβ and Ld + Lo boundaries. It is clearly distinct from the upper boundary of macroscopic gel/liquid coexistence observed in GUV experiments, which does not extend above $\chi_{CHOL} = 0.18$ in either system (9).

2. FRET efficiency in Ld + Lo is reduced relative to that in the surrounding 1-phase regions, but to a lesser extent than in the DOPC-containing mixture. This effect is expected when phase domain size is comparable to $R_0$. 

41
2.4.4 Summary of FRET surfaces for the three mixtures

1. Overall FRET patterns, both RRE and REE, are the same for the three mixtures, reflecting similarity of their phase behavior over all composition space.

2. Cholesterol interacts more favorably with POPC and SOPC than with DOPC. 40 mol % cholesterol must be added to DSPC/DOPC mixtures to achieve miscibility, compared to 30 mol % for DSPC/POPC or DSPC/SOPC.

3. The magnitudes of FRET variations across all compositions are greatest in the DOPC-containing mixtures: the maximum FRET enhancement in the Ld + Lo region is greatest in DSPC/DOPC/chol, smaller when DOPC is replaced by POPC, and smaller still with replacement by SOPC. These changes can be explained by reduced probe $K^p$, reduced phase domain size, or both.

2.4.5 ESR spectroscopy

Figure 2.5 shows interpolated FRET data along an Ld + Lo tieline near the three phase region, corresponding to the dashed lines in Figures 2.2 and 2.4. The patterns of enhanced and reduced FRET efficiency predicted by equation 3.8 are apparent, although precise phase boundaries are in some cases difficult to determine. ESR was used to further examine the physical properties of mixtures along this sample trajectory. Experimental spectra were simulated to extract order and dynamic parameters. Plots of typical spectra are shown in Figure 2.8, and a discussion of simulation parameters are found in Appendix A.

Figure 2.6 A shows the order parameter $S_0$ for 16-PC as a function of mixture composition, assuming a single environment for the probe. For samples near the binary DSPC/chol axis $S_0 \sim 0.25$, typical of 16-PC in an Lo phase (21). Chain order decreases as low-$T_m$ lipid is incorporated into the bilayer and eventually falls to values ($S_0 \sim 0.05$) typical of 16-PC in an Ld phase. A sharp drop in $S_0$ occurs in each
Figure 2.5 FRET data on an Ld + Lo tieline trajectory. FRET values are interpolated from raw surface data, along a trajectory corresponding to the dashed line in Figures 2.1 and 2.3. This trajectory also corresponds to the ESR data shown in Figure 2.6. (A) Donor BoDIPY-PC and acceptor Fast-DiI both partition into Ld phase, resulting in enhanced FRET in the Ld + Lo phase coexistence region of DSPC/DOPC/cholesterol (black diamonds), DSPC/POPC/cholesterol (blue triangles), and DSPC/SOPC/cholesterol (red circles). (B) Donor DHE partitions into Lo phase, resulting in reduced FRET to the acceptor BoDIPY-PC in the Ld + Lo region (symbols as in panel A). In favorable cases the FRET signal changes abruptly at the onset of a phase transition, allowing determination of phase boundaries by eye. The apparent Lo phase boundaries in these FRET trajectories are in good agreement with ESR data of Figure 2.4, with the exception of DSPC/SOPC/cholesterol: in addition to a change of slope in the enhanced FRET data at the expected phase boundary ($\chi_{DSPC} = 0.5$), another abrupt change is observed in both data sets at $\chi_{DSPC} = 0.6$. It is possible that the sample trajectory crosses the boundary of Ld + Lβ phase coexistence, which is not precisely determined in this system.
Figure 2.6 ESR reveals similarities in phase properties of mixtures forming macroscopic and nanoscopic phases. Compositional trajectories run in the approximate direction of Ld + Lo tie lines (see Figure 2.2 A, dashed line) and differ only in the identity of the low-TM lipid. (A) Composition-dependent order parameters obtained from ESR spectral simulations in DSPC/DOPC/chol (diamonds), DSPC/POPC/chol (triangles), and DSPC/SOPC/chol (circles). (B) Fraction of 16-PC spin probe in the Lo phase determined by spectral subtraction using Equation 2.6 (symbols as in panel A). Predicted fractions from Equation 2.7 shown as lines for DSPC/DOPC/chol (solid), DSPC/POPC/chol (dashed), and DSPC/SOPC/chol (dotted), with best-fit parameters listed in Table 2.2.
ternary mixture, consistent with redistribution of 16-PC between two phase environments. Though the recovered value of $S_0$ represents an average of values in the coexisting phases, it does not simply reflect the relative amounts of the two phases present. Because the first-derivative signal varies inversely with the square of the resonance linewidth, we speculate that the narrow disordered component is dominating the fit.

To quantify the distribution of 16-PC between Ld and Lo, we modeled each spectrum as a weighted superposition of Ld- and Lo-like spectra, using the binary-axis (endpoint) compositions as basis spectra. The best-fit weights $(w_{Lo}, w_{Ld})$ directly yield the fraction of 16-PC in the Lo phase:

$$f_{16PC}^{Lo} = \frac{w_{Lo}}{w_{Lo} + w_{Ld}}$$  \hspace{1cm} 2.6

Figure 2.6 B plots the 16-PC fraction in the Lo phase determined at each composition using Equation 2.6. Assuming the sample trajectory is collinear with a tieline and the probe does not partition preferentially to the interface between domains, the probe fraction in the Lo phase can be expressed as a function of the phase boundaries $(\chi^{Ld}, \chi^{Lo})$ and the $K^P$ of 16-PC:

$$f_{16PC}^{Lo}(\chi; \chi^{Ld}, \chi^{Lo}, K^{16PC}) = \begin{cases} 
0 & \chi \leq \chi^{Ld} \\
\frac{K^{16PC}}{K^{16PC} + \frac{\chi^{Lo} - \chi}{\chi - \chi^{Ld}}} & \chi^{Ld} < \chi < \chi^{Lo} \\
1 & \chi \geq \chi^{Lo}
\end{cases}$$  \hspace{1cm} 2.7

Both phase boundaries and $K^{16PC}$ were varied in the fit. Model predictions are shown as lines in Figure 2.6 B, with recovered parameters listed in Table 2.2. 16-PC partition between Ld and Lo is close to unity, similar to values seen for coexisting gel/liquid of DPPC/DLPC/chol (22). The recovered Lo phase boundaries coincide with the abrupt drop in $S_0$ shown in Figure 2.6 A. The recovered Ld phase boundary for
DSPC/DOPC/chol is considerably lower in DSPC than values determined by FRET and confocal fluorescence microscopy, most likely due to the paucity of data points near this boundary.

Table 2.2 Best-fit phase boundaries and 16-PC partition coefficients with standard deviations, modeled by Equation 2.7.

<table>
<thead>
<tr>
<th>System</th>
<th>( \chi^{Ld}_{DSPC} )</th>
<th>( \chi^{Lo}_{DSPC} )</th>
<th>( K^{16PC} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC/DOPC/chol</td>
<td>0.00(2)</td>
<td>0.640(3)</td>
<td>0.80(6)</td>
</tr>
<tr>
<td>DSPC/POPC/chol</td>
<td>0.06(1)</td>
<td>0.500(5)</td>
<td>1.05(8)</td>
</tr>
<tr>
<td>DSPC/SOPC/chol</td>
<td>0.05(1)</td>
<td>0.480(5)</td>
<td>1.3(1)</td>
</tr>
</tbody>
</table>

The location of the phase boundary at high \( \chi_{DSPC} \) reveals quantitative differences in molecular interactions: the Lo phase accommodates significantly more monounsaturated lipid, evidence of a more favorable interaction with DSPC for these lipids compared to DOPC. Only 8 mol % DOPC is required to precipitate an Ld phase along this trajectory. In contrast, ~25 mol % POPC or SOPC is required for phase separation. Up to the point of phase separation, the Lo phase maintains a nearly constant order parameter of 0.25 and diffusion coefficient of \( 9 \times 10^7 \) s\(^{-1} \) (data not shown), indicating only minor changes in bilayer properties as a function of composition within the Lo region.

Physical properties of the Ld phase exhibit greater dependence on lipid structure, apparent in Figure 2.6A. The Ld phase at low DSPC becomes progressively ordered in the series DOPC < POPC < SOPC. For compositions on the binary axis consisting of the low-\( T_M \) lipid with \( \chi_{CHOL} = 0.09 \), \( S_0 \) increases 75% upon saturation of the sn-1 chain (equivalent to replacing DOPC with SOPC). In contrast, shortening the sn-1 chain by two carbons (i.e., replacing SOPC with POPC) increases fluidity, evidenced by a 10% decrease in \( S_0 \). This trend continues with addition of DSPC and persists until the disappearance of Ld phase, at which point \( S_0 \) is the same for the three
mixtures. Figure 2.6 B shows that these differences in order are also reflected in 16-PC partitioning between Ld and Lo. As $\Delta S_0$ between the coexisting environments decreases in the series DOPC > POPC > SOPC, there is less tendency for the bulky spin probe to be driven out of the ordered phase, and the 16-PC concentration in Lo increases.

2.5 Discussion

2.5.1 Motivation and experimental design

Systems studied here are simple models for the mammalian plasma membrane outer leaflet: ternary mixtures containing cholesterol and the high-$T_M$ lipid DSPC, with a series of three low-$T_M$ lipids DOPC, POPC, and SOPC. Despite the structural similarity of the low-$T_M$ lipids, exchanging DOPC for either POPC or SOPC results in dramatically different mixing behavior at biologically relevant cholesterol concentrations (10-40 mol %), as revealed by fluorescence microscopy of GUVs. The micron-sized liquid phase domains in DSPC/DOPC/chol are not observed at any composition in DSPC/SOPC/chol or DSPC/POPC/chol (4,9). POPC is an important lipid for model studies due to its biological abundance and has been chosen as the representative low-$T_M$ lipid in several recent studies of ternary mixtures (7,8,11). Each of these studies used a sphingomyelin (SM) as the high-$T_M$ lipid, employed methods sensitive to small length scales, and reported a region of Ld + Lo phase coexistence. In contrast, a FRET study comparing DPPC/DOPC/chol with DPPC/POPC/chol reported Ld + Lo coexistence in the former system, but not in the latter (23). Together, these studies reveal that even small structural differences in both the high- and low-$T_M$ components can dramatically affect phase coexistence and/or domain size. We add to these reports a comparison of three ternary mixtures at high-compositional resolution using methods that are sensitive to small (> 2 nm) heterogeneities.
The high throughput of our RSE sample preparation technique enabled us to evenly sample the ternary composition space at 2% resolution. For each mixture, we examined ~1100 samples and several hundred control samples. We previously discovered that systematic errors occur in large data sets when samples are prepared and measured in a well-defined order (e.g., low to high DSPC, or low to high cholesterol), due primarily to a small, gradual change in the concentration of chloroform solutions of lipid and probe. In this study, data were collected and measured in random order to minimize any systematic distortion of surface features. The trade-off inherent in this approach is a greater overall noise level. FRET surfaces are consequently less useful for establishing *precise* phase boundaries than targeted, smaller-scale experiments (i.e. short linear sample trajectories that cross a phase boundary). Their value lies in establishing the overall pattern of phase behavior of a mixture.

2.5.2 Comparison of phase behavior in three ternary systems

Our basis mixture for investigating influence of the low-$T_M$ lipid on liquid domain size is DSPC/DOPC/chol. We begin with a discussion of phase behavior in this system. The complete phase diagram for DSPC/DOPC/chol is shown in Figure 2.7. For illustrative purposes, we consider the phase behavior of a hypothetical sample with equal mole fractions of DOPC and DSPC and continuously increasing cholesterol concentration. In the absence of cholesterol, DOPC is practically insoluble in the pure DSPC gel (Lβ'), and the sample is composed of roughly equal mole fractions of Ld and Lβ' phase with compositions $\chi_{DSPC} = 0.1$ and 1, respectively (6). As cholesterol is added, its distribution between the coexisting phases initially favors Ld, as evidenced by enhanced FRET between DHE and the Ld-preferring BoDIPY-PC at low $\chi_{CHOL}$ (see Figure 2.2 B). Upon further addition of cholesterol, DSPC chain tilt is abolished, and both cholesterol and DOPC solubility increase in the gel phase (now Lβ). As
Figure 2.7 Phase diagrams for systems in this study: DSPC/DOPC/chol (solid lines), DSPC/POPC/chol (dashed), and DSPC/SOPC/chol (dotted). Solidus boundary extensions are not well-determined in the POPC- and SOPC-containing mixtures.
cholesterol distribution shifts to favor the Lβ phase, FRET efficiency between DHE and BoDIPY-PC decreases. Cholesterol eventually reaches a saturating concentration of $\chi_{CHOL} \sim 0.16$ in the Lβ phase and a third, Lo phase with higher cholesterol content forms. Along this 1:1 DSPC/DOPC trajectory, the three phases coexist over a small composition range, with Lo effectively “replacing” Lβ as cholesterol concentration increases from 12 to 18 mol % (the fraction of Ld phase is roughly constant along this path). Further addition of cholesterol beyond $\chi_{CHOL} \sim 0.18$ has a differential effect on chain order in the coexisting phases, as seen in the ESR trajectories. Order increases in the Ld phase as DOPC chains are forced into a smaller cross-sectional area to shield cholesterol from contact with water in the headgroup region, while order in the Lo phase is essentially unchanged. Eventually the difference in chain order is too small to sustain phase separation: above $\chi_{CHOL} = 0.4$ the bilayer exists as a single phase with small compositional fluctuations.

Upon replacing DOPC with either POPC or SOPC, the same phase coexistence regions are observed with FRET, but phase boundaries have shifted in a manner consistent with increased acyl chain order in POPC- or SOPC-rich Ld phases (see Figure 2.7). POPC and SOPC are significantly more soluble in the Lβ and Lo phases than is DOPC, composing > 25 mol % of these phases at maximum solubility, before precipitation of the Ld phase. However, as in the DOPC-containing mixtures, cholesterol is soluble in gel up to $\chi_{CHOL} \sim 0.16$. Both POPC and SOPC mixtures are more ordered than DOPC mixtures at all cholesterol concentrations. Consequently, Ld + Lo phase separation is abolished at lower cholesterol concentrations, with FRET data indicating an upper phase boundary at $\chi_{CHOL} \sim 0.3$.

Quantitative analysis of dye partitioning in DSPC/DOPC/chol (see Figure 2.3) confirms that the fluorescent sterol DHE is a faithful reporter of cholesterol distribution (24). Similarly, the distribution of BoDIPY-PC and Fast-DiI between Ld
and Lo reflects the distribution of DOPC between these phases. Maximum partitioning occurs at the 3-phase triangle just before precipitation of the Lβ phase, where physical differences (e.g., order) between Ld and Lo phases are greatest. Consequently the FRET peak between BoDIPY-PC and Fast-DiI is located on this tieline (see Figure 2.2 A). The central FRET peak in DSPC/POPC/chol (see Figure 2.4 A, point B) similarly must be located at a composition very near the Ld + Lo leg of the 3-phase triangle. Furthermore, GUV and FRET data (not shown) narrowly constrain the location of the Ld vertex of the 3-phase triangle, and together these two points define the approximate direction of the first Ld + Lo tieline.

2.5.3 Comparison of DSPC/POPC/chol with other POPC-containing ternary systems

Phosphatidylcholines are among the most abundant lipids in mammalian PM. Approximately 50 mol % of the outer leaflet lipid is PC, with POPC and SOPC typically being the predominant species (25). Because of its biological relevance, POPC was used as the low-\(T_m\) lipid in much of the pioneering work on ternary phase behavior. Our study invites comparison with these systems, particularly those studied using techniques sensitive to nanometer-scale heterogeneities.

De Almeida et al. used fluorescence anisotropy and lifetime-weighted quantum yield to obtain a phase diagram for PSM/POPC/chol (7). Halling et al. reported similar results with the same lipid system and experimental techniques, though they also employed probes sensitive to the onset of gel phase and thereby recorded the Ld + Lo segment of the 3-phase triangle to greater precision (11). Both reported similar phase behavior at 23°C: three 2-phase binary systems extending into the ternary composition space and joined by a 3-phase region. Pokorny et al. published a similar phase diagram for BSM/POPC/chol, with the notable difference that the Ld + Lo region terminated in a critical point near the binary POPC/chol axis (8).
The reported phase behavior for the POPC/chol axis (where the diagrams are directly comparable) deserves further comment. De Almeida and Halling show a region of Ld + Lo coexistence between $\chi_{\text{CHOL}} = 0.12-0.44$, whereas Pokorny, in agreement with our study, shows complete miscibility of the components to at least $\chi_{\text{CHOL}} = 0.4$. This discrepancy has important consequences for the estimated uncertainty of a proposed tieline in (7), as de Almeida's analysis relied on the limiting tieline slopes at the 3-phase triangle and binary POPC/chol axis. Spatial sensitivity of the experiments is the most likely explanation for conflicting results. SP-FRET detects changes in D and A spatial distributions on length scales greater than a few times $R_0$, and formation of lipid clusters smaller than a few nm cannot be observed above the baseline FRET signal of a random probe distribution. In contrast, fluorescence anisotropy and quantum yield are sensitive to the immediate environment of the fluorescent molecule: composition differences in the nearest-neighbor shell surrounding a probe can yield distinct signals, and hence small clusters of lipids can appear as distinct “nano-environments” even for ordinary nonideal mixing. An important distinction must be drawn between coexisting phase domains and these very local compositional fluctuations that are present in any nonideal mixture, as only the former are constrained by the lever rule for first-order phase separation. FRET is therefore an important tool for distinguishing these cases: the sixth-power distance dependence effectively acts as a spatial filter by averaging out the effects of short-range compositional fluctuations.

2.5.4 Limitations of SP-FRET for establishing phase behavior

As noted in the previous section, diminished sensitivity of SP-FRET to the smallest domains (i.e. those comparable in size to $R_0$) may limit its usefulness in establishing phase coexistence in some mixtures. Variation in the domain size distribution within a phase coexistence region can confound the precise determination
of phase boundaries, particularly if parts of the phase-coexistence region near one or both phase boundaries exhibit small domains: the "apparent" coexistence region would be smaller than the true coexistence region. In the extreme case that an entire coexistence region exhibits domains smaller than $R_0$, it might be indistinguishable from surrounding one-phase regions in the SP-FRET surface; the probability of this artifact is minimized by using probes with small $R_0$ (14). The D/A pairs used in this study have $R_0$ of ~ 2.5 nm (DHE/BoDIPY-PC) and 6.5 nm (BoDIPY-PC/Fast-Dil) as calculated from spectral overlap integrals. The close correspondence of SP-FRET surface features of the two probe pairs (particularly in the Ld + Lo region) suggests that liquid domain sizes are no smaller than ~ 5 nm in the mixtures studied.

2.5.5 Phase-like behavior of nanodomains

It is becoming increasingly clear that sub-optical lateral organization is a general phenomenon that occurs in many binary and ternary mixtures (26,27). However, considerable debate continues as to whether nanoscopic domains constitute first-order phase separation. Line tension at domain boundaries always favors coalescence of small domains into a single large domain at equilibrium. Thermodynamic stability of small domains thus would require both small line tension together with a free energy contribution opposing domain coalescence. Line tension is reduced by the presence of line-active molecules, a role that has been suggested for cholesterol (28,29), asymmetric saturated/monounsaturated lipids like POPC and SOPC (30), and fluorescent impurities (31). Theoretical work has related line tension to hydrophobic mismatch at domain boundaries (32), and a recent AFM/fluorescence microscopy study demonstrated that a decreasing height mismatch between Ld and Lo domains resulted in stable arrays of increasingly smaller domains (33). In GUV systems containing POPC, cholesterol, and a saturated PC of varying length, liquid domains were not observed at 1/1/1 composition for DPPC and DSPC (with 16- and
18-carbon chains, respectively), but were visible with 20-carbon DAPC (4). Further exploration is needed to understand the molecular origin of line tension and its dependence on lipid composition and temperature. Ultimately, no special mechanism is required to explain vanishingly small line tensions in phase-separated mixtures, as line tension must approach zero near a critical point. This has been demonstrated experimentally in ternary systems both by varying temperature at a fixed composition toward an upper miscibility critical point (34) and by varying composition at fixed temperature toward a consolute point (35).

Even though small line tensions are expected and observed in phase-separated compositions near critical points, the thermodynamic stability of small phase domains requires a competing energy term favoring dispersed domains. For unsupported bilayers like those studied here, curvature has been proposed to compete with line tension (13,36). Another possibility is that within the Lo phase, the competition of individual cholesterol molecules for “solvating PC” neighbors is frustrated as the phase domain grows (37). Much work remains to explain the stability of small domains.

2.6 Conclusions

We previously reported the absence of visible liquid domains in DSPC/POPC/chol and DSPC/SOPC/chol (9). Using methods with submicron sensitivity, we have found that in fact these mixtures exhibit similar phase behavior to DSPC/DOPC/chol, including liquid phase coexistence. We have shown that FRET and ESR data in DSPC/POPC/chol and DSPC/SOPC/chol are consistent with first-order phase transitions: changes in signal with composition are consistent with phase boundaries and the lever rule. Our data also suggest the location of an Ld + Lo tieline in DSPC/POPC/chol. As an experimental system, this tieline offers several advantages
to the study of raft properties: the compositions of coexisting phases are well-defined, the phase fractions are constrained by the lever rule, and the domain sizes are comparable to those suggested for biological rafts. We expect that further study of this tieline will provide more realistic data for lipid and protein partitioning between “raft-like” Ld and Lo domains, and valuable insight into the physical mechanisms that limit domain size in biological membranes.
REFERENCES


Chapter 3
Applications of quantitative FRET in bilayers I: Modeling a tieline field

3.1 Abstract

Measurements of FRET between freely diffusing lipid probes in a bilayer mixture can potentially yield a wealth of quantitative information, including phase boundaries, critical points, and probe partition coefficients. We have developed a model for extracting this information from measurements of stimulated acceptor emission, which we used in Chapter 2 to determine probe partition coefficients in DSPC/DOPC/cholesterol. Here, we elaborate on the mathematical details of the model, and discuss a procedure for estimating uncertainty in high compositional resolution FRET measurements.

3.2 Introduction

In the previous chapter, we reported our investigation of phase behavior in three ternary lipid mixtures. Our evidence for the existence of submicron domains in two of these mixtures is based partly on data presented in Figures 2.2 and 2.4. The six contour plots in these figures are a compact representation of FRET surfaces derived from measurements of ~3300 MLV samples.

The seemingly complex patterns in the contour plots arise from two simple physical principles. First, FRET efficiency depends on donor-acceptor separation distance: for an ensemble of donors and acceptors like that of our MLV samples, steady-state FRET is determined by the time-averaged distribution of donor-acceptor separation distances. Second, if coexisting phases are present in the sample, carefully chosen probe molecules will display a preference, described mathematically by the
partition coefficient. To a first-order approximation, patterns in the FRET surfaces are qualitatively explained by these two principles. Any change in the phase state of the bilayer will change the spatial distribution of donors and acceptors via partitioning, which in turn changes the distribution of separation distances. If donor and acceptor prefer the same phase, the average separation distance decreases, and FRET increases; if they prefer different phases, the opposite effect is observed. In either case, these changes are relative to the baseline FRET signal of a random distribution of donors and acceptors, which is the first-order approximation for a single-phase mixture.

With these principles in mind, much can be learned about the phase behavior of a mixture by simple inspection of the FRET surfaces. However, the data potentially contain a wealth of quantitative information, including the location of phase boundaries and the critical point, the directions of tielines, and the partition coefficients of probe molecules. Previously, Buboltz developed a quantitative model for FRET in phase-separated mixtures and applied it to experimental data on an individual tieline of phase coexistence (1). Here, we develop a mathematical procedure to simultaneously fit FRET data in an entire two-phase region consisting of an infinite set of non-intersecting tielines. Our method is based on a procedure reported by Smith and Freed for modeling ESR data (2). The basic idea is to define the two-phase region, or “tieline field”, through a set of functions with a limited number of parameters. The functions are carefully chosen to satisfy certain physical constraints, including non-intersection of tielines. The tieline field functions must specify the coexisting compositions (i.e., the tieline) for every composition contained in the field. The fraction of each phase is then obtained from the lever rule.

With tieline field functions and their associated parameters in place, the FRET model can be restated in terms of the tieline field parameters, and experimental data can be fit with a nonlinear least squares routine. In the case of
DSPC/DOPC/cholesterol, we were able to constrain the Ld + Lo phase boundaries, critical point, and tieline slopes using CFM data, leaving only the probe partition coefficients and boundary FRET values as adjustable parameters.

This chapter provides a detailed description of the procedure used to model FRET data surfaces in DSPC/DOPC/cholesterol. Section 3.3.1 gives an overview of corrections applied to raw fluorescence measurements. Section 3.3.2 describes the linear algebra used to transform ternary sample compositions into the Gibbs triangle to simplify analysis. The SP-FRET model for a tieline is introduced in Section 3.3.3. The mathematical basis of the tieline field parameterization is stated in Section 3.3.4 with an emphasis on graphical representation. Section 3.3.5 provides details of experiments used to constrain key tieline field parameters. Section 3.3.6 combines the tieline field parameterization and SP-FRET model, yielding a set of equations suitable for modeling a FRET surface. Finally, Section 3.3.7 discusses a mathematical procedure for estimating measurement uncertainty in the tieline field.

3.3 Discussion

3.3.1 Data processing: Corrections to the raw FRET signal

Energy transfer requires overlap between donor emission and acceptor excitation frequencies. This requirement nearly always ensures the existence of (a) an illumination frequency that efficiently excites the donor with minimal direct excitation of the acceptor, and (b) an observation frequency at which emission arises primarily from the acceptor with minimal contribution from the donor. This is the basis of the inherently low signal-to-background of stimulated acceptor emission (SAE), making it an extremely useful FRET metric at low probe concentrations. In this discussion, we refer to a data channel (excitation/emission λ) that satisfies these criteria as an SAE channel.
Even under optimal conditions, signal measured in an SAE channel will contain non-FRET contributions including fluorescence emission from both donor and acceptor through the direct excitation pathway, as well as scattering of direct excitation light by the vesicle suspension. Signal originating from these pathways can be taken into account with an additional measurement in a reference channel at which the signal arises entirely from one pathway (3). The intensity in the reference channel is proportional to the intensity in the SAE channel, and the constant of proportionality is determined by measuring the intensities in the reference and SAE channels in an appropriate control sample:

\[ \delta_{SAE} = \frac{F_{SAE}}{F_D} \quad (\text{sample contains lipid + donor}) \]  

\[ \alpha_{SAE} = \frac{F_{SAE}}{F_A} \quad (\text{sample contains lipid + acceptor}) \]  

\[ (\sigma_D, \sigma_{SAE}, \sigma_A) = \left( \frac{F_D}{F_S}, \frac{F_{SAE}}{F_S}, \frac{F_A}{F_S} \right) \quad (\text{sample contains only lipid}) \]  

where \( F_{SAE} \) is the intensity in the SAE channel and \( F_D, F_A, \) and \( F_S \) are the intensities in the donor, acceptor, and scattering reference channels, respectively. In a sample containing both donor and acceptor, FRET is calculated as:

\[ F = F_{SAE} (1 - \sigma_{SAE}) - \delta_{SAE} F_D (1 - \sigma_D) - \alpha_{SAE} F_A (1 - \sigma_A) \]  

(Note the correction for scattering in the donor and acceptor reference channels.) To account for any composition-dependence of the donor and acceptor fluorescence surfaces, we prepared donor- and acceptor-only samples over the entire ternary space at 10% compositional resolution (a total of 56 compositions for each of the three probes), calculated \( \alpha_{SAE} \) and \( \delta_{SAE} \) at these compositions, and interpolated at 2% resolution.
3.3.2 The Gibbs triangle and transformation of coordinates

The Gibbs triangle offers a useful representation of ternary mixtures. Ternary mole fractions can be used directly as Cartesian coordinates for plotting data (e.g., phase boundaries) on an equilateral triangle. A drawback to this approach is that it places the inherently 2D triangle in a 3D space, adding unnecessary complexity for plotting, modeling, or otherwise manipulating data. A convenient transformation places the Gibbs triangle in the plane $z = 0$, with the lower-left and lower-right vertices positioned at $(0, 0, 0)$ and $(1, 0, 0)$, respectively. This is accomplished by a geometric contraction, rotation, and translation of the mole fraction coordinates, operations that can be combined into a single affine transformation matrix $A$:

$$A = \begin{bmatrix}
\frac{1}{2} & 0 & -\frac{1}{2} & 1 \\
0 & 1 & -\frac{1}{2} & 2 \\
\frac{1}{2\sqrt{3}} & \frac{1}{\sqrt{3}} & -\frac{2\sqrt{3}}{3} & \frac{2\sqrt{3}}{3} \\
\frac{1}{\sqrt{6}} & \frac{1}{\sqrt{6}} & -\sqrt{6} & -\sqrt{6} \\
0 & 0 & 0 & 1
\end{bmatrix} \tag{3.5}$$

Affine transformations preserve collinearity and ratios of distances, and thus preserve the lever rule for phase-separated mixtures. An affine transformation matrix operates on a homogeneous coordinate vector (formed by appending the homogeneous component $w = 1$ to the end of the composition vector $\chi$), and generates a new homogeneous coordinate vector:

$$A \begin{bmatrix} \chi_{DSPC} \\ \chi_{CHOL} \\ \chi_{DOPC} \\ 1 \end{bmatrix} = A \begin{bmatrix} \psi_x \\ \psi_y \\ 0 \\ 1 \end{bmatrix} = \begin{bmatrix} \psi \\ 0 \\ 1 \end{bmatrix} \tag{3.6}$$

The first two entries in the transformed vector are the new coordinates $\psi = (\psi_x, \psi_y)$. The transformation is undone by applying the inverse of $A$ to the homogeneous coordinate vector formed from $\psi$.


The transformations of Equations 3.6 and 3.7 are shown in Figure 3.1.

### 3.3.3 A model for energy transfer along a tieline: SP-FRET

Buboltz developed a model for composition-dependent SAE in phase-separated lipid bilayers (termed SP-FRET) and demonstrated its application to the well-characterized gel/liquid binary system DPPC/DLPC at 25°C (1). For the case of coexisting Ld and Lo phases, the observed steady-state SAE signal $F$ is given by:

$$ F = \frac{f_{Ld} + S_{Lo}(f_{Lo}K^1K^2 - f_{Ld})}{[1 + (K^1 - 1)S_{Lo}][1 + (K^2 - 1)S_{Lo}]} $$

where the independent variable $S_{Lo}$ is the mole fraction of Lo phase, $f_{Ld}$ and $f_{Lo}$ are the observed $F$ at the Ld and Lo phase boundaries, and $K^1$ and $K^2$ are the donor and acceptor partition coefficients ($K^i \equiv \chi_{Lo}/\chi_{Ld}$). Equation 3.8 was derived by assuming dilute probe concentration, moderate excitation intensity, and infinitely large phase domains (for practical purposes, domain radius > 10 $R_0$). We note that Equation 3.8 makes no explicit distinction between donor and acceptor partition coefficients because the equation is symmetric with respect to these variables, a consequence of the linear behavior of SAE with both donor and acceptor concentration in the dilute limit. Figure 2.1 plots the family of curves generated by Equation 3.8, demonstrating the two general lineshapes of reduced and enhanced FRET efficiency.

Equation 3.8 is straightforward to implement when data are constrained to a single tieline (e.g., in a binary mixture). This constraint is lifted in a ternary mixture, where each sample may lie on a different tieline with independent $K^i$, $f_{Lo}$, and $f_{Ld}$. Modeling ternary data therefore requires imposing constraints to limit the number of adjustable parameters; these constraints should include functions that identify the...
Figure 3.1 Transformation of sample coordinates to the Gibbs triangle. When used directly as Cartesian coordinates, mole fraction coordinates $\chi$ lie on an equilateral triangle in 3-space (black triangle). The affine transformation matrix $A$ operates on $\chi$ to generate coordinates $\psi = (\psi_x, \psi_y, 0)$ that lie on a Gibbs triangle in the $z = 0$ plane (blue triangle); the $z$-coordinate is then dropped, yielding 2D coordinates that simplify plotting and modeling data. Mole fractions are easily recovered by applying $A^{-1}$ to $\psi$. 
coexisting compositions at each phase-separated composition. This is accomplished with the “tieline field parameterization” described in the next section.

3.3.4 The tieline field parameterization

The description of a 2-phase coexistence region as a ruled surface was introduced by Smith and Freed, and we have mostly retained the mathematical structure found in that work (2). The exception is our introduction of a smooth Bézier curve to represent the phase boundary, which we find offers an improvement in computational efficiency over the chord-length boundary parameterization of the original work. We have also changed some notation for clarity. The tieline field parameterization is stated mathematically by the following equations:

\[ B(t) = \sum_{i=0}^{n} \binom{n}{i} (1 - t)^{n-i} t^i p_i \] 3.9

\[ t_{Lo}(u) = t_c + (1 - t_c)u \] 3.10

\[ t_{Ld}(u) = t_c - \frac{t_c u}{u + c(1 - u)} \] 3.11

\[ \psi_{Ld} = \psi_{Ld}(u) = B(t_{Ld}(u)) \] 3.12

\[ \psi_{Lo} = \psi_{Lo}(u) = B(t_{Lo}(u)) \] 3.13

\[ \psi_{Ld}\psi_{Lo} = \psi_{Lo} - \psi_{Ld} \] 3.14

\[ \psi_l(u, S_{Lo}) = \psi_{Ld} + S_{Lo} \psi_{Ld}\psi_{Lo} \] 3.15

Equation 3.9 parameterizes the phase boundary as a Bézier curve of degree \( n \). We found that a degree 5 curve was able to closely reproduce the Ld + Lo boundary in DSPC/DOPC/chol with control points listed in Table 3.1.
Table 3.1 Fixed parameters for the Ld + Lo tieline field in DSPC/DOPC/chol.

<table>
<thead>
<tr>
<th>$P_0$</th>
<th>$P_1$</th>
<th>$P_2$</th>
<th>$P_3$</th>
<th>$P_4$</th>
<th>$P_5$</th>
<th>$t_c$</th>
<th>$c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.155, 0.074)</td>
<td>(0.176, 0.265)</td>
<td>(0.3, 0.38)</td>
<td>(0.624, 0.365)</td>
<td>(0.646, 0.391)</td>
<td>(0.764, 0.224)</td>
<td>0.506</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 3.2 A plots the control points and the Bézier curve $B(t)$ traced out by Equation 3.9 as the Bézier parameter $t$ varies from 0 to 1. The value of $t$ corresponding to the critical point ($t_c = 0.506$, marked by the star in Figure 3.2 B) was determined by first locating the critical composition experimentally and then numerically solving for the closest point on $B(t)$ (see Section 3.3.5). The critical point divides $B(t)$ into two curves or “directrices” corresponding to the Ld and Lo phase boundaries and defined in terms of their Bézier parameter ($t$) domains:

$$Ld \text{ directrix} = B(t) \quad t \in [0, t_c]$$

$$Lo \text{ directrix} = B(t) \quad t \in [t_c, 1]$$

Given two directrices, a tieline field is defined through functions that specify how points on the directrices are joined to form rulings. We first introduce a new parameter $u \in [0, 1]$ that identifies a particular tieline, with $u = 0$ corresponding to the infinitesimal tieline at the critical point, and $u = 1$ corresponding to the last tieline (the Ld + Lo segment of the three-phase triangle). We then define two tieline generating functions that map $u$ to $t$ in the domains of the Lo and Ld directrices. The Lo endpoint function, Equation 3.10, is arbitrarily defined by a linear mapping (black curve in Figure 3.3). The Ld endpoint function, Equation 3.11, allows for variability in tieline slope, and must be a strictly decreasing function of $u$ due to the physical constraint of non-overlapping tielines. Smith introduced a monotonic function with a single parameter $c \in (0, \infty)$ that generates a family of curves shown as red lines in Figure 3.3. We used a linear mapping of $u$ to $t_{Ld}$ (corresponding to the constraint $c=1$,
Figure 3.2 A visual guide to the tieline field parameterization. (A) A set of Bézier control points \( \{P_i\} \) generate a smooth representation \( B(t) \) of the Ld + Lo phase boundary in DSPC/DOPC/chol (for values, see Table 3.1). (B) The critical point (star), defined by a particular value of the Bézier parameter \( t = t_c \), divides \( B(t) \) into two directrices: \( 0 \leq t \leq t_c \) (the Ld phase boundary) and \( t_c \leq t \leq 1 \) (the Lo phase boundary). Tielines are generated with Equations 3.10-11, which specify unique values of \( t \) on each directrix for an input parameter \( u \in [0, 1] \). Shown is the particular tieline corresponding to \( u = 0.6 \). (C) The tieline field of DSPC/DOPC/chol. (D) Vector representation of the composition \( \psi_i \) defined by tieline field coordinates \( (u, S_{Lo}) = (0.6, 0.5) \).
Figure 3.3 Tieline generating functions for DSPC/DOPC/chol. The functions $t_{Lo}(u)$ (black curve) and $t_{Ld}(u)$ (family of red curves) determine the tieline endpoints. For each function $t_c = 0.506$, the experimentally determined value of the critical point (see section 3.3.5). Tieline slopes were fixed by setting $c = 1$, which selects a particular curve for $t_{Ld}(u)$ (bold red line). Values of $t_{Lo}$ and $t_{Ld}$ for the $u = 0.6$ tieline are shown (dashed lines).
the bold red line in Figure 3.3), as suggested by the symmetric appearance of the phase boundary and phase percolation map (see Section 3.3.5).

The Cartesian endpoints $\psi_{L0}$ and $\psi_{Ld}$ of a given tieline $u$ are found by inserting the Bézier parameters $t_{L0}(u)$ and $t_{Ld}(u)$ into the boundary equation $B(t)$. Figure 3.2 $B$ demonstrates this for the particular tieline $u = 0.6$, and Figure 3.2 $C$ shows a larger set of tielines. Any point in the 2-phase region is now uniquely specified by its tieline parameter $u$ and the Lo phase fraction $S_{Lo}$ given by the lever rule: for a point $\psi_i$ on the tieline, $S_{Lo}$ is equal to the ratio of its distance from the Ld endpoint to the total tieline length. These relationships are stated in vector form by Equations 3.12-15, and shown for the $u = 0.6$ tieline in Figure 3.2 $D$.

3.3.5 Constraining the tieline field with GUV data

We previously determined the phase diagram for DSPC/DOPC/chol (4). This work included a relatively complete examination of the Ld + Lo region with confocal fluorescence microscopy. However, no critical phenomena were examined, and the location of the room temperature critical composition $\chi_c$ was not reported. Since that publication, much work has been done to establish the experimental signatures of critically fluctuating membranes, including the visual appearance of critically fluctuating giant vesicles (5,6). In light of these new developments, we reexamined the Ld + Lo region in an attempt to locate $\chi_c$.

We first narrowed the possible location of $\chi_c$ by reexamining GUV data from (4), noting the percolating phase for compositions in the Ld + Lo region (Figure 3.4). The locus of compositions for which the percolating phase changes from Ld to Lo comprises the percolation threshold curve and terminates at the critical point. From Figure 3.4, the percolation threshold curve can be approximated by a line joining the compositions $(\chi_{DSPC}, \chi_{CHOL}, \chi_{DOPC}) = (0.4, 0.18, 0.42)$ and $(0.26, 0.39, 0.35)$; the latter composition was taken to be $\chi_c$. We note that phase percolation was recently
Figure 3.4 Percolation map for the Ld + Lo region of DSPC/DOPC/chol determined from fluorescence micrographs of GUVs. Samples composed of mostly Ld phase exhibit disconnected Lo phase domains in a continuous Ld background (light gray circles). As the fraction of Lo phase increases the pattern eventually reverses (dark gray circles). The percolation threshold curve divides the two regions and must terminate at the critical point (star). Both types of connectivity are observed at some compositions (half circles), an indication of proximity to the percolation threshold.
Figure 3.5 GUVs near the critical point in DSPC/DOPC/chol, imaged with C12:0-DiI at a probe/lipid ratio of 1/5000. Fluctuating irregularly shaped domain boundaries are consistent with proximity to a critical point. All GUVs have nominal composition \((\chi_{DSPC}, \chi_{CHOL}, \chi_{DOPC}) = (0.27, 0.37, 0.36)\). Scale bar = 10 microns.
used to determine the 22°C critical point in egg-sphingomyelin/DOPC/chol, at a composition remarkably close to that in DSPC/DOPC/chol: $(\chi_{ESM}, \chi_{CHOL}, \chi_{DOPC}) = (0.26, 0.40, 0.34)$ (7). Several groups have reported room-temperature critical points for the Ld + Lo boundary near the 1:1 low-$T_M$/high-$T_M$ composition (6,8,9). Figure 3.5 shows typical GUVs prepared at compositions near $\chi_c$. Consistent with previous reports (6,8), fluctuating irregularly shaped phase boundaries indicative of critical behavior were observed in these vesicles.

Having determined $\chi_c$, the critical Bézier parameter $t_c$ was fixed by minimizing the distance between $B(t)$ and $\chi_c$ in the Gibbs triangle space:

$$
\begin{bmatrix}
\psi_x \\
\psi_y \\
0 \\
1
\end{bmatrix}_c = A
\begin{bmatrix}
\chi_{DSPC} \\
\chi_{CHOL} \\
\chi_{DOPC}
\end{bmatrix}_c
$$

3.18

$$
t_c = \min_t \|B(t) - (\psi_x, \psi_y)_c\| 3.19$$

The symmetric appearance of the percolation threshold map and the location of $\chi_c$ near the center of the boundary curve suggest a symmetric tieline field and provide justification for constraining the value of the tieline slope parameter $c$ of Equation 3.11 at unity. As $c$ deviates from unity, tieline endpoints “bunch” together near the Ld or Lo vertex of the three-phase triangle. In this scenario, the composition of one phase would necessarily change rapidly as $\chi_c$ is approached from inside the 2-phase region, giving the phase diagram an asymmetric fan-like appearance. With $c \approx 1$ tieline fanning is minimal.

### 3.3.6 Data fitting

Considering the 2-phase tieline FRET model Equation 3.8, $S_{Lo}$ and $u$ are defined at every phase-separated composition by Equations 3.9-15. The remaining parameters in Equation 3.8 ($K^1, K^2, f_{ld}, f_{lo}$) are physically constrained to be constant.
along a tieline and therefore can be expressed as functions of \( u \) and a set of additional fitting parameters:

\[
K = K(u, \kappa) \quad 3.20
\]
\[
f = f(u, \varphi) \quad 3.21
\]

Beyond the physical constraint that the partition coefficients are unity at the critical point (i.e., \( K(0, \kappa) = 1 \)), the functional forms of Equations 3.20-21 are arbitrary. They are chosen using the criteria that they vary smoothly with \( u \) and can describe a range of reasonable behaviors with a minimal number of additional fitting parameters (\( \kappa \) and \( \varphi \)). Combining Equations 3.8, 3.20, and 3.21 gives the final expression for FRET in a 2-phase tieline field:

\[
F(u, S_{Lo}, \kappa^D, \kappa^A, \varphi) = \frac{f_{La}(u, \varphi) + S_{Lo}[f_{La}(u, \varphi)K(u, \kappa^D)K(u, \kappa^A) - f_{La}(u, \varphi)]}{[1 + (K(u, \kappa^D) - 1)S_{Lo}][1 + (K(u, \kappa^A) - 1)S_{Lo}]} \quad 3.22
\]

The partition coefficient function \( K \) is specified as:

\[
K(u, \kappa) = 10^{\kappa_0u + (\kappa_1 - \kappa_0)u^2} \quad 3.23
\]

and boundary FRET is modeled as the intersection of a plane with the open right cylinder surface of \( B(t) \):

\[
f(t, \varphi) = \varphi_0 + (\varphi_1, \varphi_2) \cdot B(t) \quad 3.24
\]

Data in the 2-phase region are then fit by optimizing the donor and acceptor partition coefficient parameters \( \kappa^D \) and \( \kappa^A \), and boundary FRET parameters \( \varphi \). The data coordinates \((\psi_x, \psi_y)\) are converted to \((u, S_{Lo})\) coordinates by numerically solving Equations 3.9-15:

\[
(\psi_x, \psi_y, F)_i \xrightarrow{(t_e,c)} (u, S_{Lo}, F)_i \quad 3.25
\]

The weighted chi-square merit function
\[ \chi^2 = \sum_{i=1}^{N} w_i [F_i - F((u, S_{Lo})_i; \kappa^D, \kappa^A, \varphi)]^2 \]

is minimized over \( \kappa^D, \kappa^A \), and \( \varphi \) using the Levenberg-Marquardt algorithm, as implemented by the `NonlinearModelFit` function in Mathematica 7.0.1 (Champaign, IL). The weights \( w_i \) are taken as the inverse of the estimated variance of \( F_i \), calculated from a propagation of experimental error described in Section 3.9. The calculated weights depend on the best-fit parameter set, and therefore the minimization is implemented as an iterative reweighting, terminating with convergence of \( \chi^2 \) to within 0.1% (typically achieved in 3-5 iterations). The two FRET surfaces modeled have common parameters \( \kappa^{BoDIPY} \), so the data are analyzed globally by minimizing the sum of their weighted \( \chi^2 \).

3.3.7 Modeling measurement uncertainty in a tieline field

Proper modeling of experimental data requires knowledge of the error associated with each measurement. The true uncertainty of a given measurement is the standard deviation of the distribution from which that measurement is drawn. This uncertainty is the proper normalization factor for its residual term in the chi-square merit function. Fitting with true measurement uncertainties provides an unbiased best estimate of the adjustable model parameters, an objective means of assessing how well the model describes the data, and a means of combining data sets with shared parameters but different measurement uncertainties into a global analysis (10).

In the most favorable cases, measurement errors are either known in advance or can be easily determined (e.g., with sample replicates). When errors cannot be easily determined, they are often omitted from the chi-square function under the assumption of uniform sample error. When this assumption is justified, the chi-square minimum still provides an unbiased estimate of model parameters, although the goodness-of-fit cannot be independently or objectively assessed.
The least favorable case occurs when measurement errors are not known in advance, are not uniform, and cannot be efficiently determined. The large FRET data sets in this study consist of ~1400 independently prepared samples of differing compositions, with data obtained over several months. To achieve high compositional-resolution, we sacrificed sample replicates and therefore an experimentally determined estimate of uncertainty. Furthermore, we cannot justifiably assume uniform sample error, as will become clear in the following discussion.

Despite these limitations, there is clearly quantitative information to be gleaned from the data. Our approach here is to use our analytical model for FRET, Equation 3.8, to derive the measurement uncertainty: if we know the sources of random error that creeps into sample preparation and measurement, we can propagate known (or estimated) error magnitudes into an expected uncertainty. The large number of data points allows us to compare the expected uncertainty to the fit residuals to evaluate our estimates of measurement error.

3.3.7.1 Deriving an expression for random measurement error of FRET samples

We imagine a binary system of lipids A and B, with a 2-phase coexistence region that meets the assumptions of the SP-FRET model (i.e., dilute probe concentrations, large phase domains, and no interfacial probe partitioning). We rearrange Equation 3.8, factoring out the \( f_{Ld} \) term and replacing it with the variable \( I \) (to represent fluorescence intensity), and using the lever rule to recast the mole fraction of Lo phase \( (S_{Lo}) \) in terms of absolute lipid compositions:

\[
F = F(I, K^1, K^2, \rho, \chi_{Ld}, \chi_{Lo}, \chi_B)
\]

\[
= I \left( \frac{1 + \frac{\chi_B - \chi_{Ld}}{\chi_{Lo} - \chi_{Ld}} (\rho K^1 K^2 - 1)}{1 + (K^1 - 1) \frac{\chi_B - \chi_{Ld}}{\chi_{Lo} - \chi_{Ld}} (1 + (K^2 - 1) \frac{\chi_B - \chi_{Ld}}{\chi_{Lo} - \chi_{Ld}})} \right) \quad 3.27
\]
where $\chi_B$ is the sample composition (in terms of the mole fraction of component B), $\chi_{Ld}$ and $\chi_{Lo}$ are the coexisting phase compositions, and $\rho$ is the ratio of observed FRET in the coexisting phases. The term in parenthesis is a generalized SP-FRET curve that depends only on the physics of the system (probe partition coefficients, mole fraction of Lo phase, and ratio of intrinsic FRET in the coexisting phases). The coefficient $I$ scales the general curve and depends on details of the experiment, including the particular probes and probe concentrations chosen, sample concentration in the cuvette, and the details and settings of the fluorimeter used to measure the samples.

Using partial derivatives, the error associated with each parameter in Equation 3.27 propagates as:

$$
\sigma_I^2 = \left( \frac{\partial F}{\partial I} \right)^2 \sigma_I^2 + \left( \frac{\partial F}{\partial K^1} \right)^2 \sigma_{K^1}^2 + \left( \frac{\partial F}{\partial K^2} \right)^2 \sigma_{K^2}^2 + \left( \frac{\partial F}{\partial \rho} \right)^2 \sigma_{\rho}^2 \\
+ \left( \frac{\partial F}{\partial \chi_{Ld}} \right)^2 \sigma_{\chi_{Ld}}^2 + \left( \frac{\partial F}{\partial \chi_{Lo}} \right)^2 \sigma_{\chi_{Lo}}^2 + \left( \frac{\partial F}{\partial \chi_B} \right)^2 \sigma_{\chi_B}^2
$$

assuming no correlation among the variables. This expression is simplified by considering the relative contributions of the various terms. Error in $I$ and $\chi_B$ (the fluorescence intensity and sample composition, respectively) arises primarily from random errors in fluid handling: the lipid and probe stock solutions that must be prepared, assayed for concentration, and combined to create the sample mixture; the volume of buffer added to create the MLV suspension; evaporative loss of buffer during RSE sample preparation as the sample is exposed to vacuum; transfer of an MLV sample aliquot to a cuvette; etc. In contrast, the parameters $K^1, K^2, \rho, \chi_{Ld}$ and $\chi_{Lo}$ are physical constants for a given probe pair and tieline, and as such their uncertainty is tightly constrained by our ability to control sample temperature and
pressure (which is quite good in an ambient laboratory setting). We therefore assume that error in $K^1, K^2, \rho, \chi_{Ld}$ and $\chi_{Lo}$ is negligible compared to error in $I$ and $\chi_B$ and drop their associated terms from Equation 3.28. This leads to a first-order approximation of the standard deviation of a FRET measurement:

$$\sigma_F(I, K^1, K^2, \rho, \chi_{Ld}, \chi_{Lo}, \chi_B, \sigma_I, \sigma_{\chi_B}) = \sqrt{\left(\frac{\partial F}{\partial I}\right)^2 \sigma_I^2 + \left(\frac{\partial F}{\partial \chi_B}\right)^2 \sigma_{\chi_B}^2} \quad 3.29$$

(N.B. The choice to omit the correlation term from Equation 3.28 can now be explained: because $K^1, K^2, \rho, \chi_{Ld}$ and $\chi_{Lo}$ are constant for a given tieline, their errors are certainly correlated; however, just as the individual errors are driven to zero by good thermal control, so too is the correlation term).

Finally, for a given nominal composition $\chi_B$, the expected value of $\sigma_F$ is found by averaging Equation 3.29 over a compositional distribution of width $\sigma_{\chi_B}$:

$$\langle \sigma_F \rangle = \int_{-\infty}^{\infty} dx \frac{\sigma_F}{\sqrt{2\pi}\sigma_{\chi_B}} \exp\left(-\frac{(x - \chi_B)^2}{2\sigma_{\chi_B}^2}\right) \quad 3.30$$

Equation 3.30 gives the value of $\sigma_F$ to which a set of sample replicates prepared at nominal composition $\chi_B$ will converge, given the assumptions of the derivation. It does not have a closed form solution, but is easily evaluated using CAS software.

### 3.3.7.2 Composition-dependence of measurement uncertainty: Simulation vs. model

To evaluate the validity of Equation 3.30, raw data was simulated at 2% compositional resolution for a hypothetical binary system with model parameters listed in Table 3.2. At each nominal composition $\chi_B$, random (Monte Carlo) values of $I$ and $\chi_B$ were drawn from normal distributions with standard deviations $\sigma_I$ and $\sigma_{\chi_B}$. Equation 3.27 was evaluated using the Monte Carlo values, and the resulting value of $F$ was paired with the nominal value of $\chi_B$ to form a Monte Carlo data point. Figure
3.6 shows Equation 3.27 (solid line) and a typical Monte Carlo data set (open circles) constructed with parameters listed in Table 3.2. Figure 3.7 shows the standard deviation calculated at each composition from $10^5$ simulated data sets (open circles) along with the prediction of Equation 3.30 (solid line), demonstrating good agreement between simulation and model.

Table 3.2 Simulation parameters for MC data used to evaluate Equation 3.30.

<table>
<thead>
<tr>
<th>Trajectory</th>
<th>$l$</th>
<th>$\sigma_I$</th>
<th>$\sigma_{\chi_B}$</th>
<th>$\chi_{ld}$</th>
<th>$\chi_{lo}$</th>
<th>$\rho$</th>
<th>$K^1$</th>
<th>$K^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRE</td>
<td>10</td>
<td>0.5</td>
<td>0.02</td>
<td>0.2</td>
<td>0.8</td>
<td>1.5</td>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td>REE</td>
<td>10</td>
<td>0.1</td>
<td>0.02</td>
<td>0.2</td>
<td>0.8</td>
<td>1</td>
<td>0.1</td>
<td>0.167</td>
</tr>
</tbody>
</table>

Figure 3.7 clearly shows that the assumption of uniform variance is not appropriate for composition-dependent FRET data. Consider a set of sample replicates with Ld-preferring probes (i.e., Figure 3.6 A), prepared at nominal compositions corresponding to the phase boundaries. Near the phase boundary at $\chi_B = 0.2$, small random differences in the composition of replicates have little effect on the observed signal. Some of the samples contain a small amount of Lo phase, but because the probes strongly prefer the Ld phase there will be little sample-to-sample variation in the distribution of D-A distances, and hence little variance in the observed signal. In contrast, small differences in composition near the Lo boundary at $\chi_B = 0.8$ result in dramatic differences in the spatial distribution of D and A, and correspondingly large changes in observed signal. Indeed, for the conditions listed in Table 3.2, there is a > 25-fold difference in expected error near the Lo phase boundary compared to the Ld phase boundary. The result for the RRE probe pair (Figure 3.7 B) also shows significant changes in expected error across the phase-coexistence region: an ~ 4-fold difference between the lowest and highest $\sigma_F$, with local peaks in error occurring near the phase boundaries.
Figure 3.6 Monte Carlo simulated FRET data for a hypothetical binary A/B system with a phase-coexistence region between $\chi_B = 0.2$ and $\chi_B = 0.8$. (A) REE resulting from donor/acceptor colocalization in the B-rich phase. (B) RRE resulting from donor/acceptor separation. The model FRET curves described by Equation 3.27 (solid lines) were calculated using parameters listed in Table 3.2. Noisy data (open circles) were randomly generated, as described in the text.
Figure 3.7 The uncertainty of a FRET signal exhibits composition dependence. The standard deviation predicted by Equation 3.30 (solid lines) for the FRET curves shown in Figure 3.6, along with the standard deviation calculated from $10^5$ Monte Carlo data sets. Uncertainty in FRET measurements exhibits a strong dependence on composition in the phase-coexistence region. This uncertainty must be taken into account when fitting data to a model.
3.3.7.3 Composition-dependent error in a tieline field

Equation 3.30 gives the expected standard deviation of a FRET measurement for a binary (1D) tieline. Extending this result to a 2D tieline field is conceptually simple but nontrivial to implement in practice. Unlike Equation 3.8, Equation 3.22 (the appropriate starting point for a derivation of uncertainty for the 2D case) does not have a closed form in terms of lipid composition. Consequently, we cannot evaluate the partial derivative with respect to composition, and so we cannot derive an expression for $\sigma_F$ in terms of a compositional uncertainty $\sigma_X$.

We can, however, use the 1D case as an approximation to the 2D case by considering each composition to lie on a pseudo-binary tieline, and using the equations derived in the previous section to calculate $\sigma_F$. The fitting routine is then implemented as an iterative reweighting, as follows:

1. Using uniform weights (i.e., $w_i = 1$), minimize Equation 3.26 to find the best-fit parameter set $a_0$.
2. Set $\sigma_X$ to 0.02 (i.e., the estimated 2% compositional uncertainty of our sample preparation), and choose starting values of $\sigma_I$ for the RRE and REE surfaces (which will in general be different).
3. Evaluate $\langle \sigma_{F,i} \rangle$ from Equations 3.27, 3.29, and 3.30 using $a_0, \sigma_X, \sigma_I^{\text{REE}}$ and $\sigma_I^{\text{RRE}}$.
4. Calculate the weights $w_i = 1/\langle \sigma_{F,i} \rangle^2$.
5. Using the new weights $w_i$, minimize Equation 3.26 to find the best-fit parameter set $a_1$.
6. Iterate steps 3-5 (using the best-fit parameter set $a_j$ from the previous iteration in step 3) until some convergence criterion is met (e.g., parameter values change by less than 1 part in 1000).
The derivation of uncertainty assumes that the data are well-described by the model. In this case the reduced chi-square should be approximately equal to unity.
REFERENCES


4.1 Abstract

Multicomponent lipid mixtures exhibit complex phase behavior, including coexistence of nanoscopic fluid phases in ternary mixtures mimicking the composition of the outer leaflet of mammalian plasma membrane. The physical mechanisms responsible for the small size of phase domains is unknown, due in part to the difficulty of determining the size and lifetime distributions of small, fleeting domains. Steady-state FRET provides information about the spatial distribution of lipid fluorophores in a membrane, and with an appropriate model can be used to determine the size of phase domains. Starting from a radial distribution function for a binary hard disk fluid, we develop a domain size-dependent model for stimulated acceptor emission. We compare the results of the model to two similar, recently published models.

4.2 Introduction

Förster resonance energy transfer (FRET) is an immensely useful tool for probing molecular length scales. As its name implies, the physical mechanism for FRET is a through-space interaction of electric oscillators in the near field: it occurs at distances greater than molecular contact, but much smaller than the wavelength of visible light. Resonance between electric dipoles in an excited-state donor molecule and ground-state acceptor molecule (which for the purposes of this work are fluorophores) results in simultaneous donor quenching and acceptor excitation, without emission of a donor photon. The accessibility of FRET to experimentalists is
largely due to Förster, who correctly described the now well-known dependence of FRET efficiency on donor-acceptor separation distance, and who provided a valuable set of equations for quantifying FRET in many physically realistic scenarios (1). It is for this reason that the phenomenon, which was first observed well before Förster's contributions, today bears his name.

Because of its inherent distance dependence, FRET between freely diffusing membrane fluorophores can provide insight into the mixing behavior of lipids. This was recognized as early as 30 years ago, when three (nearly simultaneous) extensions to Förster's theory provided a quantitative description of FRET for a random, planar array of fluorophores (2-4). With the recent emergence of the lipid raft hypothesis, these models have been revisited in the context of coexisting membrane phases (5,6). Indeed, FRET is a particularly attractive tool for studying lateral organization in biological membranes because it can be employed in biologically relevant free-standing bilayers, in contrast to many other methods for probing small length scales that require a rigid bilayer support. The technology required to do FRET experiments is inexpensive and ubiquitous, and a wide variety of suitable probes are commercially available; consequently the literature for membrane FRET experiments is rapidly expanding.

As evidence mounts that the size of plasma membrane rafts may play an important biological role, the quantitative use of membrane FRET as a "molecular ruler" for phase domains has been explored (7,8). The exquisite sensitivity of FRET to precise details of molecular geometry demands precision in the analysis. Care must be taken to ensure that the geometric picture on which the model is based—that is, the spatial distribution of donors and acceptors—is an accurate description of the system. To this end, several groups have recently proposed modifications to the original models for membrane FRET to better account for energy transfer near domain
boundaries, where the approximations of the early models are not valid (9,10). Such considerations are critically important for an accurate determination of domain size in the 2-50 nm range, a range that overlaps with many experimental and theoretical estimates of domain size in both cells and model membrane systems (11-13).

This chapter provides a brief overview and comparison of the two most influential early models for membrane FRET, those of Wolber and Hudson (WH), and Fung and Stryer (FS) (3,4). An extension of the FS model to multibilayer systems is then proposed, and its application to the characterization of lamellarity in liposome preparations is discussed. We then address a crucial update to the model: a quantitative description of energy transfer efficiency for probes that partition between phase domains of arbitrary size. Included is a discussion of the recent work of Towles et al. and Brown et al. to extend the WH and FS models (respectively) to account for small domains, and the deficiencies in the latter model that motivated this work (9,10).

4.3 Methods

4.3.1 Monte Carlo simulations of transfer efficiency

Refer to Chapter 5 for details of these simulations.

4.4 Results and Discussion

This section is organized as follows:

1. We describe and compare two early models for membrane FRET, upon which nearly all subsequent work is based.

2. We propose and develop an extension to FS for the case of small domains: the Finite Phase-separation FRET (FP-FRET) model.

3. The FP-FRET model is compared to two existing models for FRET efficiency derived for the case of small domains.
4.4.1 Comparison of two influential models for membrane FRET

The theory of either Wolber and Hudson (WH) or Fung and Stryer (FS), published nearly simultaneously in the late 1970s, serves as the starting point for most subsequent treatments of membrane FRET (3,4). A survey of the literature reveals that the WH equations are by far the most commonly used, although a discussion of what differences exist between these models (if any) is difficult to find. The popularity of WH may be due to the greater generality of their presentation, including an easily computed approximation to the exact solution. In contrast, the FS model requires numerical integration, which was far more computationally expensive at the time that work was published than it is today. As the FS model serves as the basis for the theory in the rest of this chapter, an explanation of this choice is in order.

By way of comparison, both derivations explicitly employed the following assumptions:

1. The concentration of excited-state donors is much less than the concentration of acceptors (the limit of moderate excitation intensity).
2. Donors and acceptors are randomly distributed in the membrane.
3. There is no change in the distribution of donor-acceptor distances (i.e., no lateral diffusion) on the timescale of fluorescence emission. In real membranes these timescales are separated by at least two orders of magnitude, lending validity to this assumption.
4. The Förster distance $R_0$, which quantifies the spatial extent of the FRET interaction for a particular donor-acceptor pair, is not a function of distance between a donor and acceptor.
5. There is no time-dependence to $R_0$ on the timescale of fluorescence decay (i.e., either the static or dynamic averaging limit holds). In practice, assumptions 4
and 5 allow all donor-acceptor interactions to be described by a single value of $R_0$.

In either model, the relative donor quenching due to FRET and the transfer efficiency are given by the following equations:

$$q_r = \int_0^\infty e^{-t/\tau_0} e^{-n_A S(t)} dt$$  \hspace{1cm}  \text{(4.1)}$$

$$E = 1 - q_r$$  \hspace{1cm}  \text{(4.2)}$$

where $n_A$ is the acceptor concentration (a surface density given in number of acceptors per unit area), and $S(t)$ is the so-called “energy transfer term”. For FS, the energy transfer term has the form:

$$S_{FS}(t) = \int_{R_e}^\infty 2\pi r [1 - e^{-(t/\tau_0)(R_0/r)^6}] dr$$  \hspace{1cm}  \text{(4.3)}$$

while for WH, it is:

$$S_{WH}(t) = \pi R_0^2 (t/\tau_0)^{1/3} \gamma[2/3, (t/\tau_0)(R_0/R_e)^6] + \pi R_e^2 [1 - e^{-\left(\frac{t}{\tau_0}\right)(R_0/R_e)^6}]$$  \hspace{1cm}  \text{(4.4)}$$

where $\gamma$ is the incomplete gamma function given by:

$$\gamma(x, y) = \int_0^y z^{x-1} e^{-z} dz$$  \hspace{1cm}  \text{(4.5)}$$

Both models ultimately reveal dependence of donor quenching on three parameters: the surface density of acceptors $n_A$, the Förster distance $R_0$, and the distance of closest approach between a donor and acceptor $R_e$. Neither model has a closed form algebraic solution, so that the equations must be solved by numerical integration.
Given the identical assumptions of the two models and the similar forms of the equations, what then are the differences between the WH and FS models? First, the WH equations have a very simple power series expansion when the distance of closest approach between donor and acceptor is much less than $R_0$ (mathematically, when $R_e \sim 0$). The resulting infinite sum converges rapidly, making a precise computation of $q_r$ extremely fast and efficient. However, in real membrane systems, this approximation is rarely if ever valid: the closest-contact distance for donors and acceptors in the same leaflet is approximately the sum of the respective molecular radii (often not much smaller than $R_0$), and the closest-contact distance for acceptors in the opposing leaflet is often greater than $R_0$. An additional exponential term in Equation 4.1 is required to account for this contribution to donor quenching. In the more physically realistic geometries with nonzero $R_e$, both the WH and FS equations must be integrated numerically. As a second and more important difference, WH provided an approximate expression for donor quenching (with a stated accuracy of < 1%) in the form of a simple sum of two exponentials, and included a table of best-fit values for the pre-exponential and exponential fitting parameters over a range of acceptor concentrations. Although the double exponential form is orders of magnitude faster to compute than a numerical integral, either can now be calculated in a fraction of a second on any laptop computer.

4.4.2 Finite Phase-separation FRET (FP-FRET): A model for FRET in the presence of small domains

It is now clear that many lipid mixtures containing cholesterol, from the simple three-component model systems to the immensely complex plasma membrane, exhibit non-random mixing of lipid components over a wide range of size scales. In the case of unstimulated plasma membrane, while much evidence supports the presence of lateral domains (11-13), direct observation by conventional fluorescence microscopy
has failed to detect large-scale phase separation. The thermodynamic nature of these lateral heterogeneities is the subject of much debate, and reliable measurements of size- and timescales of domains are critically important. FRET between freely-diffusing membrane probes is sensitive to domain sizes in the range of ~ 2-20 nm, given an appropriate model for the spatial arrangement of donors and acceptors.

Defining a one-size-fits-all geometry to model lateral domains in even "simple" three-component membranes is itself problematic, let alone for modeling rafts in the vastly more variable and chemically heterogeneous plasma membrane. A simple geometric model should be considered a first-order approximation for small phase domains. Nevertheless, the derivation outlined here can serve as a starting point for investigating more complicated geometries.

The following thermodynamic and geometric picture is used in the derivation:

1. Domains result from first-order phase separation of two liquids.
2. The donor and acceptor molecules are non-interacting, and distribute between the phases according to a well-defined partition coefficient.
3. At a given composition of interest, one of the liquid phases exists as circular domains dispersed in the other, surrounding liquid phase.
4. The circular phase domains are monodisperse with a radius $R$.
5. The domains are non-interacting: there are no special forces that cause domains to attract or repel, resulting in a random domain distribution.

It is worth stating explicitly the practical implications of these assumptions. By assumption 1, the total area fraction of domains can be determined mathematically from the lever rule and the average molecular areas of the pure phases, while assumption 2 allows us to treat the probes as being randomly distributed within the phase domains. Importantly, the model parameters resulting from assumptions 1 and 2 (phase fractions, molecular areas, and partition coefficients) can be determined by
independent experiments. Assumptions 3 and 4 simplify the mathematics and minimize the number of fitting parameters (though it should be noted that a distribution of domain sizes can be accommodated with a fairly straightforward modification of the equations derived in this section). Assumption 5 ensures that the circular domains are randomly distributed within the surrounding phase. Together, assumptions 3-5 enable the introduction of an important concept in statistical mechanics—the pair correlation function—to model the spatial distribution of domains. As a final note, the derivation that follows is for energy transfer from donors to acceptors in the same plane. Using the results of the previous section and the assumption of cross-leaflet domain coupling, the equations in this section are easily modified to account for non-equivalent donor-acceptor planes and cross-leaflet energy transfer. This situation is explicitly addressed in Chapter 5.

We first note that the FS (or WH) equations are easily modified for the "infinite phase separation" condition—that is, for the case where \( R \) (domain radius) approaches infinity. (In practice, the “infinite phase separation” case is indistinguishable from a domain diameter greater than \( \sim 40 \) times the Förster distance, see Chapter 5). Here, the idea is to simply treat the coexisting phase domains in the sample as if they could be separated by some mechanical means, and their quenching measured separately. One only needs to know the fraction of total donor and acceptor found in each phase; FRET is then independently calculated for each donor pool using the FS equations, and summed:

\[
E = f_D^d E^d + f_D^s E^s
\]

where \( E^d \) and \( E^s \) are the phase-specific transfer efficiencies calculated from Eqs 4.1-3. The fraction of total donors in each phase is calculated by mass balance, from the mole fraction of domain phase \( \chi^d \) (determined from the lever rule) and the partition coefficient of the donor \( K_P^d \):

\[
\frac{f_D^d}{f_D^s} = \frac{\chi^d}{1 - \chi^d}
\]

\[
\chi^d = \frac{V^d}{V} = \frac{N^d}{N}
\]

\[
f_D^d = \frac{N^d}{N}
\]

\[
f_D^s = \frac{N^s}{N}
\]

\[
E = \frac{f_D^d E^d + f_D^s E^s}{f_D^d + f_D^s}
\]
where $K_D^d \in (0, \infty)$ is defined so that values greater than unity indicate preference for the domain phase. It should be noted that the absolute donor concentration does not enter into these equations: transfer efficiency is independent of absolute donor concentration in the limit of low excited state donor concentration (i.e., excited state donors are not competing for acceptors). Additionally, the phase-specific acceptor surface density must be used in Equation 4.1, and is similarly calculated by mass balance:

$$n_A^d = K_A^d \chi_A/[a^d (1 - \chi^d + \chi^d K_A^d)]$$

$$n_A^s = \chi_A/[a^s (1 - \chi^d + \chi^d K_A^s)]$$

$$n_A^\infty = \chi_A/[a^d \chi^d + a^s (1 - \chi^d)]$$

where $n_A^d$ and $n_A^s$ are the respective acceptor surface densities in the domain and surround phases, $a^d$ and $a^s$ are the respective molecular areas of the domain and surround phases, $\chi_A$ is the total acceptor mole fraction, and $K_A^d$ is the acceptor partition coefficient with values >1 indicating preference for the domain phase. The bulk acceptor surface density (Equation 4.11) is the area-weighted average acceptor surface density, and can be thought of as the acceptor density of a thin shell at an infinite distance from any particular donor in the bilayer. It is included here for completeness.

Why do the FS or WH models fail when domains are small? After all, we have explicitly assumed that the properties of the two coexisting phases are no different from the case of “macroscopic” phase separation: the lever rule still holds, and probes still partition between phases with a well-defined $K_p$. To answer this question, we
must first recognize that the population of donors located within domains really consists of two sub-populations: donors that are near domain boundaries “see” an acceptor environment that is different from donors located well inside the domain. What is meant by “near” a boundary? The inverse sixth-power distance dependence of FRET ensures that less than 1% of energy transfer occurs to an acceptor located further than 2.2 $R_0$ from a given donor (for the largest common $R_0$, this is about 18 nm). By this criterion, any donor within 20 nm of a domain boundary has a non-negligible contribution to quenching from acceptors outside the domain. This is in fact true even in the limit of infinite phase separation: the difference is that for micron-sized domains, these problematic donors—those located within 20 nm or so of a domain boundary—make up a negligible fraction of the total donor pool. As domains get smaller and domain perimeter increases, the problematic donors become the majority, and so we must find a way to quantify their local acceptor environment.

The key to a solution is a subtle point implicit in Equations 4.9-10: the ensemble-averaged acceptor concentration in the neighborhood of a donor located far from a phase boundary does not depend on distance from the donor. This is not the case for donors located near a phase boundary. If we can mathematically account for the distance-dependence in local acceptor concentration (in other words, determine the correct $r$-dependence for Equations 4.9-10, given the assumptions of the model), we can simply insert those functions into the existing FS machinery:

$$S(t) = \int_{R_e}^{\infty} \langle n_A(r) \rangle 2\pi r \left[ 1 - e^{-(t/\tau_0)(R_0/r)^6} \right] dr$$

4.12

The rest of this derivation focuses on arriving at the ensemble-averaged acceptor density functions $\langle n^{d}_A(r) \rangle$ and $\langle n^{s}_A(r) \rangle$. We imagine observing a donor located within a domain over time, occasionally pausing to measure the distances to
Figure 4.1 Snapshots of the binary hard disk fluid. All particles (domains) are identical and non-interacting, so the only parameter is the disk packing fraction $f$ (for these studies, equal to the domain area fraction). Shown are snapshots for packing fraction 0.2 (panel A) and 0.5 (panel B).
Figure 4.2 Domain surface coverage is related to the radial distribution function of the binary hard disk fluid. Shown are intermediate functions from the derivation of the ensemble domain surface coverage. (A) The RDF for disk packing fraction 0.5 shown in Figure 4.1 B: $g(r'; 0.5)$. (B) The local number density function corresponding to the RDF in panel A: $\gamma(r'; 0.5)$. The local number density function is simply the RDF rescaled by the disk number density of the system, $N = f/\pi$. (C) The domain surface coverage function relative to the center of a reference domain. (D) The ensemble averaged domain surface coverage function, derived by averaging the function in panel C over the disk.
all acceptors in its vicinity. Over long periods of observation, a normalized histogram of these distance measurements will converge on \( n_A^d(r) \), the ensemble-averaged acceptor surface density function for donors *inside* domains. If instead we choose to observe a donor located *outside* a domain (in the *surround* phase), we will arrive at the function \( n_A^s(r) \) (although I will show that through mass balance, we can obtain this function from \( n_A^d(r) \) for free).

We approach the problem by first ignoring the probe molecules, and considering only the domains: from a given reference point within a domain, what is the probability that some randomly chosen point at a distance \( r \) will also be located inside a domain? The problem, stated in this way, closely resembles a classical problem in statistical mechanics with a very large literature, the two-dimensional fluid. The simplest 2D fluid—monodisperse, non-interacting particles, also known as the "hard-disk fluid"—is characterized by a single parameter, the disk packing fraction \( f \). For our purposes the disk packing fraction is equivalent to the domain area fraction, defined as:

\[
f = a^d \chi^d / [a^d \chi^d + a^s (1 - \chi^d)]
\]

Figure 4.1 shows Monte Carlo snapshots of a hard disk fluid for two packing fractions, \( f = 0.2 \) (panel A) and \( f = 0.5 \) (panel B). Of central importance to theoretical treatments of the hard disk fluid is the pair correlation function \( g(r; f) \), also referred to as the radial distribution function or RDF (14). The RDF for unit-radius disks with packing fraction \( f = 0.5 \) is shown in Figure 4.2 A. The RDF describes the relative probability of finding another disk center some distance \( r \) away from the center of a reference disk. Characteristics of the RDF for a hard-disk fluid are:

1. It is zero between 0 and \( 2R \), a consequence of the hard-core repulsion (disks cannot overlap).
2. It is normalized to the number density of disks in the system, so that at large distances \( g(r) \) approaches 1.

3. There are oscillations in probability (about 1) at short distances that increase in magnitude and decay length as the disk packing fraction increases. The peaks occur approximately at integer multiples of the disk diameter, and reflect "shells" of nearest-neighbor disks surrounding the reference disk centered at 0.

   It is interesting to note that the short-range oscillations in \( g(r) \) are an apparent attraction that is purely statistical in nature, as there is no explicit interaction between disks except a hard-core repulsion. They are the consequence of local hexagonal order that arises at higher number densities, as disks are packed ever more tightly into the system.

   As it turns out, there is no analytical solution for \( g(r) \) in even-numbered dimensions (15): the function must be obtained either by Monte Carlo simulation (as was done for the curve in Figure 4.2 A, see Chapter 5 for simulation details) or through an approximate expression. With this in mind, we begin with the RDF as given, for a particular packing fraction \( f \) and scaled distance \( r' = r/R \), where \( R \) is the domain radius. We will use the function notation \( g(r'; f) \) where the independent variable (here, \( r' \)) is separated from any fixed parameters by a semicolon. This notation serves as a reminder that \( g(r) \), or any function derived from it, is specific to some packing fraction \( f \). The number density for unit-radius domains is \( f/\pi \), and the local number density \( \gamma(r'; f) \) is defined as:

\[
\gamma(r'; f) = \left( f/\pi \right) g(r'; f)
\]

\( \gamma(r') \) for \( f = 0.5 \) is shown in Figure 4.2 B. \( \gamma(r') \) is useful because it gives the expected number of domain centers \( N \) found in an annulus of width \( dr' \), located at a distance \( r' \) from the center of the reference domain:
We now return to the question that prompted our diversion into statistical mechanics: What is the probability that a randomly chosen point at a distance \( r' \) from the reference domain center will itself be located inside a domain? This probability is equal to the domain surface coverage at \( r' \), which we will call \( \sigma^d \), and to answer the question we must know how many domains we expect to observe at \( r' \). Equation 4.15 tells us the number of domain centers we will see at \( r' \), but these are not the only domains contributing to the surface coverage at \( r' \): in fact, any domains located between \( r' - 1 \) and \( r' + 1 \) will contribute to the average. Figure 4.3 shows how a unit-radius domain near \( r' \) will contribute to surface coverage of a thin shell at \( r' \), through an angle \( 2\theta \). The infinitesimal domain surface coverage at \( r' \) contributed by a domain centered at \( r' + x \) is proportional to the area of the annular segment of width \( dr' \) between \( -\theta \) and \( +\theta \), and to the expected number of domain centers at \( r' + x \) (given by Equation 4.15):

\[
d\sigma^d \propto 2r'\theta(r', x)dr' 2\pi(r' + x)\gamma(r' + x; f)dx
\]

\[
\theta(r, x) = \cos^{-1}\left[\frac{2r^2 + 2rx + x^2 - 1}{2r(x + r)}\right]
\]

Integrating Equation 4.16 for all contributing values of \( x \), and normalizing to the total area of the annulus located at \( r' \) (that is, \( 2\pi r'dr' \)) gives:

\[
\sigma^d(r'; f) = \begin{cases} 
1, & 0 \leq r' < 1 \\
2 \int_{-1}^{1} (r' + x)\gamma(r' + x; f) \theta(r', x)dx, & r' \geq 1
\end{cases}
\]

Equation 4.18 gives the probability of finding a domain at a distance \( r' \) from the center of some reference domain. The equation is shown for packing fraction \( f = 0.5 \) in Figure 4.2 C. A more useful function gives the probability of finding a
Figure 4.3 Geometric considerations for calculating domain surface coverage. All unit radius domains between $r - 1$ and $r + 1$ contribute to the domain surface coverage at $r$. The domain surface coverage at $r$ can be thought of as the average domain density in a thin shell located at distance $r$ from the center of a reference domain (located at the origin). The RDF gives the number of disk centers found at $r$, but these are not the only disks contributing to the domain surface coverage at $r$. Shown is a disk centered at $r' < r$, which contributes the shaded area to the domain surface coverage at $r$. Equations 4.16-18 account for the contributions of all disks located between $r - 1$ and $r + 1$. 
domain at a distance $r'$ from a *randomly chosen point* within the reference domain. This is the ensemble-averaged domain surface coverage $\langle \sigma^d(r'; f) \rangle$, valid for all donors inside domains in the long-time average. It is found by averaging Equation 4.18 over all positions within the reference domain:

$$\langle \sigma^d(r'; f) \rangle = \frac{1}{\pi} \int_0^1 \rho \; d\rho \int_0^{2\pi} \sigma^d(\sqrt{r^2 + \rho^2 + 2r\rho \cos \varphi}; f) \; d\varphi$$  

Equation 4.19 is shown for packing fraction $f = 0.5$ in Figure 4.2 D. We can now express the acceptor density near an average donor located inside a domain as:

$$\langle n_A^d(r; f, R) \rangle = n_A^d(\langle \sigma^d(Rr'; f) \rangle) + n_A^s(1 - \langle \sigma^d(Rr'; f) \rangle)$$  

where we have reintroduced the absolute distance $r = Rr'$. This is the first of the two sought-after equations, the distance-dependent counterpart of Equation 4.9. The second is obtained by mass balance: rearranging Equation 4.9 and replacing $n_A^d$ with Equation 4.20, we arrive at an expression for a distance-dependent "apparent" partition coefficient of the acceptor into the domain phase:

$$K_A^{d,app}(r; f, R) = \frac{a^d(1 - \chi^d)}{\chi_A/\langle n_A^d(r; f, R) \rangle - \chi^d a^d}$$  

Inserting this expression into Equation 4.10 gives the distance-dependent counterpart to Equation 4.20, valid for donors located in the surround phase:

$$\langle n_A^s(r; f, R) \rangle = \chi_A/[a^s(1 - \chi^d + \chi^d K_A^{d,app}(r; f, R))]$$  

Equations 4.20 and 4.22 give the dependence of acceptor surface density on distance from an average donor in the two donor pools: those located in domains, and those located in the surround. They are inserted directly into the integrand of the FS energy transfer integral (Equation 4.3) to complete the small domain model, taking the place of what had previously been a constant local acceptor density (no dependence on $r$). It is worth emphasizing the manipulations of Equations 4.21-22, which generate a
necessary function essentially for free (i.e., this function does not need to be simulated independently).

Figure 4.4 A shows Equation 4.20 (red curve) and Equation 4.22 (blue curve) for \( f = 0.4 \), and for (arbitrarily chosen) values of acceptor partition coefficient \( (K_A^d = 10) \), mole fraction \( (\chi_A = 0.002) \), and phase molecular areas \( (a^d = 0.675, a^s = 0.45) \). Several interesting features are evident in these curves. At the closest distances, the "local vicinity" of a given donor must be either inside a domain for \( \langle n^d_A(r) \rangle \), or in the surround for \( \langle n^s_A(r) \rangle \): consequently the limiting values of these curves at \( r = 0 \) are simply the domain or surround acceptor densities defined by Equations 4.9-10. At the other extreme \( r = \infty \), the acceptor density is equal to the bulk density defined by Equation 4.11: regardless of where a particular donor is located (in a domain or in the surround), at far enough distances the acceptor environment must look like the bulk average. At short distances the curves oscillate about the bulk acceptor density, which reflects the locally hexagonal ordering of randomly packed domains. Fig 4.4 B shows a histogram of the acceptor surface density for the domain (red circles) and surround (blue circles) phases, determined by Monte Carlo simulations (see Chapter 5 for simulation details). These simulations demonstrate the validity of Equations 4.20 and 4.22.

### 4.4.3 Comparison to existing models

Similar FRET models have recently been published elsewhere (9,10). In the model of Towles and Dan (TD), the WH equations were modified with a directly simulated domain surface coverage function similar to Equation 4.19, and the resulting model was evaluated with simulated data (9). An advantage of the present work is that the domain surface coverage function is derived through mathematical manipulation of the pair correlation function for hard disks \( g(r) \), a more general approach that allows for the use of existing functional forms for \( g(r) \). The theoretical literature for
Figure 4.4 The average acceptor density seen by donors in a nanodomain system. The two donor pools (those located in domains, and those located in the surrounding phase) see different acceptor densities in the ensemble average. Shown are the acceptor surface densities for domain area fraction 0.4, acceptor mole fraction 0.002, and acceptor partition coefficient of 10 (favoring the domain phase). (A) Acceptor surface density seen by donors in domains (red) and surround (blue) predicted by Equation 4.20 and 4.22. (B) The same functions shown with MC simulated data, demonstrating the validity of the acceptor density functions.
two-dimensional fluids is extensive, and many well-characterized approximations to \( g(r) \) exist (15). In principle, any functional form for \( g(r) \) can be used, including those derived for polydisperse (16) or interacting disks (17). Indeed, a long-range interaction between fluid phase domains has been suggested as a potential mechanism for stabilizing small domains (18): using the approach outlined above, the effect of such an interaction on the spatial distribution of probes can be accounted for via \( g(r) \).

A second model, published by the same research group, was derived from the FS equations. In contrast to the TD model (which was only tested against simulated data), the Brown model was used to extract domain size for experimentally obtained FRET data in several model bilayer mixtures (DMPC/cholesterol, DPPC/DOPC/cholesterol, and DPPC/POPC/cholesterol) (10,19). For this reason, it deserves special scrutiny. In addition to the largely geometric assumptions found in the TD and FP-FRET models, several additional (and critically important) simplifications were made:

1. For donors located inside domains, the quenching contribution to acceptors within the same domain is neglected. Effectively, this limits analysis to experimental systems where donors and acceptors partition into opposite phases, and where acceptor partition is exceptionally strong.
2. Donors inside domains were assumed to reside on a ring at \( 2/3 \ R \) (the mass-weighted "average" radial position of an ensemble of randomly distributed donors).
3. The acceptor surface density was assumed to be constant (and equal to its surround-phase density, viz. Equation 4.18) throughout the membrane.

The result is a highly specialized model that is valid only in a small and poorly defined subset of the overall parameter space: when the FRET probes partition into
different phases, and acceptor partition is strong, and domain area fraction is very small (the infinitely dilute domain limit, realized only near tieline endpoints).

Figure 4.5 compares predictions of the FP-FRET (solid line), TD (dashed line), and Brown (dotted line) models for two sets of parameters chosen to mimic experimental data on an Ld + Lo tieline (Table 4.1).

Table 4.1 Parameters used in energy transfer simulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>REE</th>
<th>RRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_{Ld}$</td>
<td>Average molecular area of Ld phase (nm$^2$)</td>
<td>0.675</td>
<td></td>
</tr>
<tr>
<td>$a_{Lo}$</td>
<td>Average molecular area of Lo phase (nm$^2$)</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>$\chi_{perc}$</td>
<td>Phase percolation threshold</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>$\chi_A$</td>
<td>Mole fraction acceptor</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>$\tau_0$</td>
<td>Donor fluorescence lifetime (ns)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$\varphi$</td>
<td>Relative donor quantum yield</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>donor, acceptor radius (nm)</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>$d$</td>
<td>donor, acceptor transverse location (nm)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>$R_0$</td>
<td>Förster distance (nm)</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>$K_D$</td>
<td>donor partition coefficient</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>$K_A$</td>
<td>acceptor partition coefficient</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Figure 4.5 A shows tieline E for strong donor and acceptor partition into the Ld phase, while Figure 4.5 B shows the case of strong donor preference for Lo and strong acceptor preference for Ld. Also shown in both plots are data generated from Monte Carlo simulations using the same parameter sets (circles, see Chapter 5 for simulation details). The predictions of the FP-FRET and TD models are similar for the length of the tieline, with small differences in E (\(< \sim 1\%\)) seen at some compositions. The differences may be due to differences in the domain surface coverage functions. In both cases, surface coverage data were simulated at discrete values of $f$ and $r$, and an integrable function suitable for use in the model was generated from the data (see
Figure 4.5 Comparison of three models for predicting energy transfer efficiency in bilayers composed of nanoscopic domains. Monte Carlo simulated data (open circles) were generated by constructing random snapshots of domain configurations and randomly placing donors and acceptors, subject to the assumptions of the model. Predictions of the FP-FRET (solid), Towles and Dan (dashed), and Brown (dotted) models. A, tieline E profiles for strong donor and acceptor partition into the Ld phase. B, tieline E profiles for the case of strong donor partition into Lo, and strong acceptor partition into Ld.
Chapter 5). The number of data points in both the $f$ and $r$ dimensions is considerably higher in the present work, which should produce more accurate results. Even still, the observed differences in the predictions of the two models are small relative to the accuracy of the domain size analysis reported in Chapter 5.

The limitations of the Brown model are evident in Figure 4.5. As Brown et al. note, their model is valid only for opposite probe partitioning, and only in the regime of small domain packing fractions (i.e., small domain area fractions): these restrictions correspond to regions near the tieline endpoints in Figure 4.5 B. Despite this significant caveat, the model was used to estimate domain sizes over the entire Ld + Lo coexistence region of two ternary mixtures. Figure 4.5 demonstrates that $E$ predicted from the Brown model deviates significantly from simulated values along the length of the tieline, suggesting that the region of validity is perhaps more narrow than the authors appreciated.

### 4.5 Conclusions

We have derived a model for transfer efficiency between freely diffusing membrane probes in phase-separated bilayers that is valid in the regime of finite-sized phase domains. The FP-FRET model yields predictions that are similar to those of a recently published model derived under similar assumptions, and which closely agree with $E$ data generated with Monte Carlo simulations over the entire range of domain packing fractions. The small differences between these models are unlikely to result in substantial differences in recovered domain size. In contrast, a third model (Brown) was shown to deviate significantly from the simulated data, due to a highly restrictive set of assumptions. Consequently, domain sizes recovered from experimental data in the Ld + Lo coexistence regions of DPPC/DOPC/chol and DPPC/POPC/chol are
likely to contain significant errors. These results graphically illustrate the importance
of geometric considerations in models for transfer efficiency in membranes.
REFERENCES


Chapter 5
Applications of quantitative FRET in bilayers II: Determination of domain size

5.1 Abstract

A model (Finite Phase-separation FRET, or FP-FRET) has recently been developed by us to describe FRET efficiency (E) between freely-diffusing membrane probes in phase-separated bilayers, that in principle applies to any system where phase domain dimensions are larger than $\sim R_0$. We used Monte Carlo techniques to simulate E for a range of probe partitioning behaviors and domain sizes, and then fit the simulated data to the FP-FRET model to recover simulation parameters. We found that FP-FRET can determine domain size to within 5% of simulated values for domain diameters up to $\sim 5 R_0$, and to within 15% for diameters up to $\sim 20 R_0$. We also investigated the performance of the model in cases where specific model assumptions are not valid.

5.2 Introduction

There is evidence that the controlled size of plasma membrane rafts is an important aspect of their functionality (1). Conceptually, a raft describes the compartmentalizing of membrane components, based on the differential physical and chemical properties of the raft and its surrounding "sea". The extent to which a given component (for example, a transmembrane protein) is compartmentalized can be expressed by its partition coefficient between the raft and non-raft domains (if there are only two types of domain). Binding to regions of a membrane, and rates of chemical reactions in a membrane can conceivably be controlled not only by the lipid composition of a raft, but also by its size.
In resting cells, rafts cannot be visualized by conventional light microscopy, a fact that sets an approximate upper limit of 200 nm on raft spatial dimensions. Though precise measurements on nanometer scales are experimentally challenging, estimates for raft dimensions are converging on the order of 2-20 nm (2). After stimulation (for example by an addition of an external cross-linker), an increase in raft size is frequently observed. In the most extreme cases, micron-sized domains can be directly visualized with fluorescence microscopy.

In parallel with cell studies, observations of nanometer-scale heterogeneity in 3-component lipid mixtures have stimulated the interest of researchers. A lively debate exists as to the thermodynamic origins of small domains, with some evidence pointing toward genuine first-order phase separation (3), and some evidence supporting Ising-like critical phenomena (4,5). The dependence of domain size on lipid composition and temperature may yield important insights into the thermodynamic nature of these domains. Furthermore, there is an obvious connection between observations of rafts in cells and small domains in model membranes, with a growing consensus that the physical origin of raft phenomena is closely related to lipid phase separation.

Only a few techniques are capable of delivering size measurements of small membrane domains, including FRET (6,7), AFM (8), and the newly-emerging super-resolution techniques (9,10). Uniquely among these techniques, FRET between freely-diffusing lipid probes does not require a bilayer support, which can introduce strong interactions (11,12). This is an especially important consideration for nanodomains, where even small perturbations might tip the delicate balance of interaction energies and cause significant artifacts in size measurements.

In Chapter 4, we developed a model (FP-FRET) to predict energy transfer efficiency in a bilayer composed of nanoscopic phase domains. Using simulated data, we report on the performance of the FP-FRET model for predicting the size of phase
domains, and show that a global analysis of multiple donor-acceptor pairs can yield accurate estimates for domain diameters up to 20 times the Förster distance. We also investigate the reliability of recovered parameters when various physical assumptions of the model are not strictly met.

5.3 Materials and Methods

5.3.1 Monte Carlo simulations of energy transfer efficiency

All simulation and data analysis was performed with Mathematica 7.0.1 (Wolfram Research, Champaign, IL). Energy transfer simulations for donor and acceptor fluorophores in phase-separated bilayers were performed by constructing random snapshots of probe configurations subject to the considerations described below.

5.3.1.1 Bilayer parameters

Bilayer structural parameters were based on an experimentally determined Ld + Lo tieline in DSPC/POPC/cholesterol at 23°C (3). The average molecular area for each phase was determined by assigning areas to the individual lipid components in each phase, and computing the mole-fraction weighted sum of these values. For both Ld and Lo phases, the cholesterol area was set to 0.285 nm² (13,14), and the area of DSPC was set to its value of 0.475 nm² in the tilted gel phase (15). Little information is available about areas in POPC/cholesterol, so we made use of the experimental observation that DOPC and POPC are very similar structurally (16). We used a value of 0.631 nm² for POPC, reflecting the decrease in DOPC area that occurs with the addition of 10 mol% cholesterol (17). Hydrocarbon thicknesses for Ld and Lo phases of 27.1 Å and 37.1 Å (respectively) were taken from POPC and DSPC values (15,16), with 2.2 Å added to the POPC thickness to account for the cholesterol content of the Ld phase (17). No offset was applied to the Lo phase thickness, as it has been shown
that the addition of cholesterol to saturated gel-phase lipids has little effect on bilayer thickness. The percolation threshold has not been determined for DSPC/POPC/cholesterol, but the threshold in similar systems appears to be approximately half-way along the tieline, so we used a value of $\chi_{Lo} = 0.5$ (See Figure 3.4).

### 5.3.1.2 Probe parameters

Probe parameters were based on commercially available fluorescent lipid analogs: DHE donor to DiO acceptor, and DiO donor to DiI acceptor. Donor fluorescence lifetimes were set to 1.0 ns for both donors in both phases, and relative donor fluorescence quantum yields in the Ld and Lo phases were set to 1.0. Förster distances in each phase were set to 2.5 nm for DHE to DiO and 5.7 nm for DiO to DiO (18). Acceptor mole fractions were 0.005 (DiO) and 0.001 (DiI).

The transverse positions of chromophore planes were referenced to electron density profiles of POPC and DSPC bilayers, with adjustments made for the effects of cholesterol on bilayer thickness (15-17). The DiI and DiO chromophores were taken to reside 6.3 Å below the phosphate peak (19). The chromophore position of DHE was estimated from a molecular model to be 5-8 Å below the hydroxyl, which is approximately the same transverse location as the DiI and DiO chromophores; for convenience, the same value was used.

Probe radii are required to determine the distance of closest approach between donor and acceptor. Cholesterol radius was taken to be $\sqrt{0.37 \text{ nm}^2/\pi} = 0.34 \text{ nm}$. Average DiI radius was calculated from MD simulations of DiI in fluid DPPC bilayers: briefly, the diameter (defined as the distance across the chromophore) was calculated for each of $10^4$ frames in a 100 ns movie and averaged.
5.3.1.3 Simulation details

Energy transfer simulations were performed at 51 evenly-spaced compositions along the tieline. At each composition, Monte Carlo bilayer snapshots were constructed by randomly placing non-overlapping round domains of specified radius in a square simulation box with periodic boundary conditions. The dimensions of the box were chosen based on the acceptor mole fraction so that ~1000 acceptors would be found in the box, and subject to the constraint of an integer number of domains. Two leaflets with different acceptor configurations were then constructed from each domain configuration, corresponding to the physical constraint of cross-leaflet coupling between phase domains. The average number of acceptors in the domain and surround phases was calculated from box dimensions, total acceptor mole fraction, and acceptor partition coefficients; for each leaflet snapshot, the actual number of acceptors to be placed in domains and surround was drawn from a Poisson distribution using the average values. Trial acceptor coordinates were generated and tested for location in a domain and non-overlap with existing acceptors: this process was repeated until the specified number of acceptors in each leaflet was placed.

The steady-state transfer efficiency observed in an ensemble sample is an average of transfer efficiency from donors in each phase, weighted by the relative numbers of donors in those phases. (N.B.: If $E$ is calculated from steady-state donor emission, the weight includes the relative quantum efficiency of the donor in each phase; for simplicity, we assumed equal quantum efficiencies in this study). For each bilayer snapshot, the ensemble $E$ was determined by averaging $E$ calculated for individually placed donors. The total number of donors to be averaged from the domain and surround phases was calculated from the total number of donors ($N_D$, typically $10^4$), the partition coefficient of donor into the domain phase, and the mole fraction of domain phase:
Trial donor coordinates were generated randomly and tested for both non-overlap with existing acceptors and inclusion in a domain, rejecting trial coordinates as needed to achieve the correct domain and surround counts. For each donor $D_j$, the set of distances $\{r\}$ to all acceptors $A_k$ within a cutoff radius $20 R_0$ (including acceptors in both leaflets) was determined. Following (20), fluorescence decay for the $j^{th}$ donor in the $i^{th}$ phase was calculated from $\{r\}$ and $R_0$:

$$\rho_{i,j,DA}(t) = e^{-t/\tau_i} \prod_{k=1}^{N_A} e^{-t/\tau_i(R_{0,ij}/r_{jk})^6}$$ \hspace{1cm} (5.3)

The average decay for the set of $N_D$ donors in the presence of acceptors is given by:

$$i_{DA}(t) = 1/N_D \sum_i \sum_{j=1}^{N_D} \rho_{i,j,DA}(t)$$ \hspace{1cm} (5.4)

The decay function for donors in the absence of acceptors is:

$$i_D(t) = 1/N_D \sum_i N_{D,i} e^{-t/\tau_i}$$ \hspace{1cm} (5.5)

and the ensemble transfer efficiency is:

$$E = 1 - \int_0^\infty i_{DA}(t)dt / \int_0^\infty i_D(t)dt$$ \hspace{1cm} (5.6)

The above process was iterated and the results averaged until the desired confidence for $E$ was achieved.

Additional simulations were performed in which various assumptions of the FP-FRET model were relaxed; the specific details of these simulations are discussed in the Results section.
5.3.2 Nonlinear least-squares fitting of simulated data

5.3.2.1 Domain surface coverage function

The ensemble-averaged domain surface coverage function \( \langle \sigma^d(r', f) \rangle \) (see Equation 4.27) was obtained from Monte Carlo simulations. Briefly, a domain snapshot with periodic boundary conditions was generated for unit-radius disks at a given packing fraction \( f \) as described in the previous section. The domain surface coverage function, which gives the domain density as a function of distance from an "average" point within a domain, was obtained by iterating the following procedure:

1. A random disk center \((x, y)_l\) was chosen from the set of disk centers in the snapshot.
2. A random point on the disk, \( p_l = (x_l + \sqrt{r} \cos \theta, y_l + \sqrt{r} \sin \theta) \), was chosen by drawing random values from the uniform distributions \( r \in [0,1] \) and \( \theta \in [0,2\pi) \).
3. An additional \( 10^4 \) random points \( \{q\} \) were generated as in step 2, with the value of \( r \) drawn from the uniform distribution \( r \in [0,c^2] \), where \( c \) is a predetermined cutoff radius. The cutoff is chosen by balancing computational efficiency with useful information: as \( r' \) increases, \( \langle \sigma^d(r', f) \rangle \) oscillates about \( f \) with decaying amplitude, and at some distance the amplitude of the oscillations are no longer meaningful in the calculation of transfer efficiency, which decays as \( (R_0/r)^6 \) (see Figure 4.5 B). We determined that for the largest value of \( f \) simulated (0.65), this cutoff occurs at \( r' < 14 \): for all simulated packing fractions, \( c \) was therefore set to 14.
4. The set of distances \( \{r\}_{l,q} \) from the reference point \( p_l \) to each \( q_j \) was calculated.
5. The points \( \{q\} \) were tested for inclusion in a domain to generate a subset \( \{d\} \), with a corresponding subset of distances \( \{r\}_{l,d} \)
This process was repeated for \(~10^3\) random disk points \(p_l\); a new domain snapshot was then generated, and the entire process repeated. In this manner, a histogram of domain surface coverage was built up. The histogram bin width was set to 0.06 to give a total of 234 bins between \(r' = 0\) and \(r' = 14.04\). The \(\sigma^d\) value for each bin was calculated from the ratio of points found in domains (the binned values of \(\{r\}_d\)) to total points (the binned \(\{r\}_q\)), and the \(r'\) value was taken to be the midpoint of the bin. The simulation was terminated when the desired confidence for each bin was achieved (typically, between \(3\times10^5\) and \(6\times10^5\) total counts for each bin in \(\{d\}\)). The histogram was then converted to a set of 234 data points \((f, r', \sigma^d)\) and smoothed with a Savitsky-Golay filter. A total of 65 packing fractions between 0.01 and 0.65 were simulated, to generate a combined 2D data set of 15,210 points. This data set constitutes a discrete approximation to the full ensemble-averaged domain surface coverage function, \(\langle \sigma^d(r', f) \rangle\); a continuous, integrable functional representation of \(\langle \sigma^d(r', f) \rangle\) was generated with a 1st-order (linear) interpolation using Mathematica's built-in Interpolation function.

5.3.2.2 Nonlinear least-squares fitting of E data

Simulated E data were fit to the FP-FRET model using a custom Levenberg-Marquardt algorithm written in Mathematica. The partial derivatives of the model with respect to the variable parameters (i.e., the Jacobian matrix \(J\)) were calculated with a forward-difference approximation (21). All numerical integrals were performed using Mathematica's built-in NIntegrate function with the integration method set to "Automatic", and the integration accuracy set to 10 digits of accuracy. Function calls to the model were performed in parallel on a 128-kernel Mathematica cluster, using Mathematica's built-in parallelization functions.

In most cases the variable parameters were donor \(K_P\), acceptor \(K_R\), and domain size \(R\); these variables were searched in logarithmic space. In linear space, \(K_P \in (0,1]\)
and $K_p \in [1, \infty)$ represent the same range of partitioning behavior, but into opposite phases; with logarithmic scaling, these ranges become $(-\infty, 0]$ and $[0, \infty)$. Logarithmic scaling therefore produces variables that are symmetric about 0 in terms of their partitioning strength, and which have the appropriate ranges for an unconstrained nonlinear fit $(-\infty, \infty)$. The domain size $R$ has a similar logarithmic effect on $E$, and is also naturally constrained to positive values. We found that this simple conversion to logarithmic space dramatically improved the convergence properties of the fitting routine. Confidence limits were computed from the covariance matrix at the solution in logarithmic space, and then converted back to linear space. All parameter values and confidence intervals in the text are reported in linear space.

5.4 Results

5.4.1 Simulations of transfer efficiency

Table 5.1 lists the bilayer and probe parameters used as input in Monte Carlo simulations of tieline energy transfer efficiency ($E$), and Figure 5.1 shows the system schematically. The simulated bilayer system represents an experimentally established Ld + Lo tieline in DSPC/POPC/cholesterol (3): properties of the Ld and Lo phases including average molecular areas and thicknesses were set based on the tieline endpoint compositions as described in Materials and Methods. Two donor-acceptor pairs with different $R_0$ were simulated: donor DHE to acceptor DiO ($R_0 = 2.5$ nm); and donor C18:2-DiO to acceptor DiI ($R_0 = 5.7$). The partition coefficient of the cholesterol analog DHE was set to 2.5, the approximate value of cholesterol $K_p$ calculated from cholesterol concentration at the tieline endpoints. C18:2-DiO $K_p$ was set to 0.1, based on values determined for the structurally similar probe C18:2-DiI in the DSPC/DOPC/chol Ld + Lo region (3).
Figure 5.1 Bilayer structural parameters used in nanodomain FRET simulations. Parameters are based on the Ld and Lo compositions of a tie line in DSPC/POPC/chol as described in Materials and Methods. Composition-dependent parameters include the molecular area of the phases $a$, and the mean probe transverse location $d$, measured relative to the bilayer midplane. The full list of simulation parameters is found in Table 5.1.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Ld value</th>
<th>Lo value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\chi_{DSPC}$</td>
<td>Mole fraction DSPC</td>
<td>0.11</td>
<td>0.52</td>
</tr>
<tr>
<td>$\chi_{POPC}$</td>
<td>Mole fraction POPC</td>
<td>0.8</td>
<td>0.26</td>
</tr>
<tr>
<td>$\chi_{CHOL}$</td>
<td>Mole fraction cholesterol</td>
<td>0.09</td>
<td>0.22</td>
</tr>
<tr>
<td>$a$</td>
<td>Average molecular area ($\text{nm}^2$)</td>
<td>0.583</td>
<td>0.45</td>
</tr>
<tr>
<td>$\chi^{\text{perc}}_{Lo}$</td>
<td>Percolation threshold</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>$R$</td>
<td>Domain radius</td>
<td>2, 5, 10, 20, 40, $\infty$</td>
<td></td>
</tr>
<tr>
<td>$R_{DHE-DIO}^0$</td>
<td>DHE to DiO Förster distance (nm)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>$R_{DiO-DII}^0$</td>
<td>DiO to DiI Förster distance (nm)</td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>$\tau_0$</td>
<td>Donor fluorescence lifetime (ns)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>$\varphi$</td>
<td>Relative donor quantum yield</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$\tau_{DHE}$</td>
<td>DHE chromophore radius (nm)</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>$\tau_{DiO}$</td>
<td>DiO chromophore radius (nm)</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>$\tau_{DiI}$</td>
<td>DiI chromophore radius (nm)</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>$d$</td>
<td>Transverse chromophore plane (nm)</td>
<td>1.35</td>
<td>1.75</td>
</tr>
<tr>
<td>$K_p^{DHE}$</td>
<td>DHE partition coefficient</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>$K_p^{DiO}$</td>
<td>DiO partition coefficient</td>
<td>0.1, 0.25, 4</td>
<td></td>
</tr>
<tr>
<td>$K_p^{DiI}$</td>
<td>DiI partition coefficient</td>
<td>0.1, 0.25, 1, 4</td>
<td></td>
</tr>
<tr>
<td>$\chi_{DiO}$</td>
<td>Mole fraction DiO acceptor</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>$\chi_{DiI}$</td>
<td>Mole fraction DiI acceptor</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.2 plots simulated E data along the tieline with fluid domain radius fixed at 10 nm. Figure 5.2 A shows data for DHE efficiency to a set of DiO acceptors with different partitioning behavior, ranging from strong partition into Ld phase ($K_p =0.1$, lower curve) to strong partition into Lo phase ($K_p =4$, upper curve). Figure 5.2 B shows a corresponding set of trajectories for the DiO to DiI pair: in this case, the upper and lower curves are for DiI $K_p =0.1$ and 4, respectively. The data in each panel of Figure 5.2 can be thought of as an experiment in which a single donor species is paired with a carbocyanine acceptor series with identical chromophore but different alkyl chains.

The trajectories in Figure 5.2 reveal qualitative behavior similar to observations of stimulated acceptor emission (SAE) in tieline trajectories (3,22). In particular, regions of enhanced or reduced FRET efficiency (REE and RRE) are seen, depending on the relative partitioning of donor and acceptor. The REE and RRE are a direct result of dramatic changes in the distribution of donor-acceptor distances that occur in the phase-coexistence region: they are observed only when both donor and acceptor have sufficiently strong $K_p$. The necessity of this condition is demonstrated in Figure 5.2, for the trajectories with acceptor $K_p =1$ (green circles). In this case, despite the presence of coexisting phases, the spatial distribution of acceptors remains completely random at all compositions and no REE or RRE is observed.

Figures 5.3-4 show the effect of phase domain size on the E profile. For each probe pair, simulated E is shown for the cases of maximum REE and RRE (i.e., the upper and lower trajectories in each panel of Figure 5.2), and for a range of domain radii. Increasing the domain size has an effect that is qualitatively similar to increasing the strength of probe $K_p$: as phase domains grow, the peak height (or valley depth) increases rapidly at first, ultimately approaching a limiting value (the so-called "infinite phase-separation" limit). For both probe pairs, it is difficult to distinguish by
Figure 5.2 Simulated FRET data reveal the effect of acceptor $K_p$. (A) Simulated $E$ for DHE donor ($K_p = 2.5$) to DiO acceptor with $K_p = 0.1$ (black circles), 0.25 (red circles), 1 (green circles), and 4 (blue circles). Regions of enhanced efficiency (REE, blue circles) are observed when donor and acceptor prefer the same phase, and regions of reduced efficiency (RRE, red and black circles) are observed for opposite donor/acceptor partitioning. No change in $E$ (relative to a straight line joining the tieline endpoints) is observed for uniform acceptor $K_p$ (green circles). (B) Data for DiO donor ($K_p = 0.1$) to DiI acceptor with varying $K_p$ (color coding as in panel A). The RRE and REE are reversed relative to panel A due to the opposite phase preference of the donor. Fits of individual trajectories to the FP-FRET model are shown as dashed lines, and global fits are shown as solid lines.
Figure 5.3 Simulated FRET data reveal the effect of domain radius $R$. Data for DHE donor ($K_P = 2.5$) to DiO acceptor with $K_P = 0.1$ (bottom 6 sets of circles) and 4 (top 6 sets of circles), for $R = 2$ nm (red), 5 nm (orange), 10 nm (yellow), 20 nm (green) and 40 nm (blue). Data predicted for the "infinite phase separation" limit, corresponding to the maximum possible domain size at each composition in a 100 nm diameter vesicle (purple). Fits of individual trajectories to the FP-FRET model are shown as dashed lines, global fits as solid lines.
Figure 5.4 Simulated FRET data reveal the effect of domain radius \( R \). Data for DiO donor \( (K_R = 0.1) \) to DiI acceptor with \( K_R = 0.1 \) (top 6 sets of circles) and 4 (bottom 6 sets of circles), for \( R = 2 \) nm (red), 5 nm (orange), 10 nm (yellow), 20 nm (green) and 40 nm (blue). Data predicted for the "infinite phase separation" limit, corresponding to the maximum possible domain size at each composition in a 100 nm diameter vesicle (purple). Fits of individual trajectories to the FP-FRET model are shown as dashed lines, global fits as solid lines.
eye the 40 nm (blue) and "infinite" radius (purple) data in the REE curves (though somewhat better separation is achieved in the RRE curves), demonstrating the sensitivity limits of the experiment. Finally, we note the appearance of the percolation threshold at $\chi_{lo} = 0.5$ as a widow's peak in the E profile for smaller domain sizes. This effect, which is caused by a discontinuity in the spatial distribution of probes when the continuous phase switches from Ld to Lo, disappears in the infinite-phase separation limit.

5.4.2 Simulated data fit to the FP-FRET model

Simulated data were fit to the FP-FRET model derived in Chapter 4, as described in Materials and Methods. Curves in Figures 5.2-4 show the recovered E profiles. In all cases, the unknown parameters were assumed to be the probe partition coefficients and the domain size R. Within this framework, two fitting schemes were used. In the first, each trajectory was fit independently of the others by varying donor and acceptor $K_p$ and $R$: the best-fit E profile for this fitting scheme is shown as a dashed line in the figures. In the second scheme, three trajectories for each donor/acceptor pair were fit globally, with a total of 5 adjustable parameters: a single donor $K_p$, three acceptor $K_p$'s, and $R$ (for global fitting, the trajectory with acceptor $K_p = 1$ was omitted). Predicted E profiles of the global fit are shown as solid lines in the figures. In all cases, both fitting schemes yield predicted curves that match the simulated data remarkably well, as judged both by eye and by reduced chi-square (see Table 5.2). The minor exception is for DHE/DiO REE data in the regime of Ld domains (the right-hand portion of the upper curves in Figure 5.2 A and Figure 5.3): a small, systematic disagreement is present here, with the model predicting lower values than are observed in the simulated data.

Table 5.2 lists the best-fit parameters and 95% confidence intervals corresponding to the data in Figure 5.2. Several trends are apparent in this data. First,
Table 5.2 Best-fit parameters from $R_{sim}=10$ nm data sets fit to the FP-FRET model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sim. Val.</th>
<th>Traj. 1</th>
<th>Traj. 2</th>
<th>Traj. 3</th>
<th>Traj. 4</th>
<th>Global</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHE to DiO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R$</td>
<td>10</td>
<td>10 (2)</td>
<td>13 (6)</td>
<td>14 (9)</td>
<td>7 (4)</td>
<td>10.0 (9)</td>
</tr>
<tr>
<td>$K_P^{DHE}$</td>
<td>2.5</td>
<td>2.3 (1)</td>
<td>2.3 (2)</td>
<td>3 (2)</td>
<td>2 (5)</td>
<td>2.42 (7)</td>
</tr>
<tr>
<td>$K_P^{DiO,1}$</td>
<td>0.1</td>
<td>0.10 (1)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.100 (6)</td>
</tr>
<tr>
<td>$K_P^{DiO,2}$</td>
<td>0.25</td>
<td>--</td>
<td>0.27 (3)</td>
<td>--</td>
<td>--</td>
<td>0.26 (1)</td>
</tr>
<tr>
<td>$K_P^{DiO,3}$</td>
<td>4</td>
<td>--</td>
<td>--</td>
<td>3 (3)</td>
<td>--</td>
<td>6 (1)</td>
</tr>
<tr>
<td>$K_P^{DiO,4}$</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2 (7)</td>
<td>--</td>
</tr>
<tr>
<td>$\chi^2_{red}$</td>
<td>--</td>
<td>0.716</td>
<td>0.924</td>
<td>1.592</td>
<td>1.727</td>
<td>1.117</td>
</tr>
<tr>
<td><strong>DiO to DiI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R$</td>
<td>10</td>
<td>9.7 (5)</td>
<td>9 (1)</td>
<td>10.4 (5)</td>
<td>10 (10)</td>
<td>9.9 (2)</td>
</tr>
<tr>
<td>$K_P^{DiO}$</td>
<td>0.1</td>
<td>0.102 (5)</td>
<td>0.11 (2)</td>
<td>0.107 (6)</td>
<td>0.15 (9)</td>
<td>0.101 (3)</td>
</tr>
<tr>
<td>$K_P^{DiI,1}$</td>
<td>0.1</td>
<td>0.10 (2)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.107 (7)</td>
</tr>
<tr>
<td>$K_P^{DiI,2}$</td>
<td>0.25</td>
<td>--</td>
<td>0.23 (5)</td>
<td>--</td>
<td>--</td>
<td>0.249 (7)</td>
</tr>
<tr>
<td>$K_P^{DiI,3}$</td>
<td>4</td>
<td>--</td>
<td>--</td>
<td>4.2 (2)</td>
<td>--</td>
<td>4.4 (2)</td>
</tr>
<tr>
<td>$K_P^{DiI,4}$</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1.0 (1)</td>
<td>--</td>
</tr>
<tr>
<td>$\chi^2_{red}$</td>
<td>0.956</td>
<td>0.859</td>
<td>1.443</td>
<td>1.112</td>
<td>1.081</td>
<td></td>
</tr>
</tbody>
</table>
### Table 5.3 Best-fit parameters from global fits to the FP-FRET model.

#### DHE to DiO

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( R_{\text{sim}} = 2 )</th>
<th>( R_{\text{sim}} = 5 )</th>
<th>( R_{\text{sim}} = 10 )</th>
<th>( R_{\text{sim}} = 20 )</th>
<th>( R_{\text{sim}} = 40 )</th>
<th>( R_{\text{sim}} = \infty )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R )</td>
<td>2.3 (1)</td>
<td>5.4 (3)</td>
<td>10.0 (9)</td>
<td>23 (4)</td>
<td>50 (20)</td>
<td>800 (--)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DHE}} )</td>
<td>1.93 (8)</td>
<td>2.35 (9)</td>
<td>2.42 (7)</td>
<td>2.42 (6)</td>
<td>2.43 (5)</td>
<td>2.40 (4)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DiO,1}} )</td>
<td>0.13 (3)</td>
<td>0.107 (8)</td>
<td>0.100 (6)</td>
<td>0.104 (5)</td>
<td>0.106 (5)</td>
<td>0.112 (5)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DiO,2}} )</td>
<td>0.38 (3)</td>
<td>0.28 (1)</td>
<td>0.26 (1)</td>
<td>0.265 (9)</td>
<td>0.266 (8)</td>
<td>0.272 (8)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DiO,3}} )</td>
<td>11.23 (6)</td>
<td>6 (1)</td>
<td>6 (1)</td>
<td>5.9 (8)</td>
<td>6.0 (8)</td>
<td>6.3 (8)</td>
</tr>
<tr>
<td>( \chi_{\text{red}}^2 )</td>
<td>1.230</td>
<td>1.072</td>
<td>1.117</td>
<td>1.042</td>
<td>1.299</td>
<td>1.052</td>
</tr>
</tbody>
</table>

#### DiO to DiI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( R_{\text{sim}} = 2 )</th>
<th>( R_{\text{sim}} = 5 )</th>
<th>( R_{\text{sim}} = 10 )</th>
<th>( R_{\text{sim}} = 20 )</th>
<th>( R_{\text{sim}} = 40 )</th>
<th>( R_{\text{sim}} = \infty )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R )</td>
<td>2.00 (3)</td>
<td>5.07 (8)</td>
<td>9.9 (2)</td>
<td>20.1 (9)</td>
<td>42 (4)</td>
<td>90 (20)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DiO}} )</td>
<td>0.05 (3)</td>
<td>0.108 (6)</td>
<td>0.101 (3)</td>
<td>0.102 (2)</td>
<td>0.101 (2)</td>
<td>0.105 (1)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DiI,1}} )</td>
<td>0.16 (6)</td>
<td>0.10 (1)</td>
<td>0.107 (7)</td>
<td>0.101 (6)</td>
<td>0.099 (6)</td>
<td>0.098 (6)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DiI,2}} )</td>
<td>0.33 (8)</td>
<td>0.24 (1)</td>
<td>0.249 (7)</td>
<td>0.245 (6)</td>
<td>0.247 (5)</td>
<td>0.249 (5)</td>
</tr>
<tr>
<td>( K_{p}^{\text{DiI,3}} )</td>
<td>3.7 (7)</td>
<td>4.5 (3)</td>
<td>4.4 (2)</td>
<td>4.2 (1)</td>
<td>4.0 (1)</td>
<td>3.83 (9)</td>
</tr>
<tr>
<td>( \chi_{\text{red}}^2 )</td>
<td>1.429</td>
<td>0.884</td>
<td>1.081</td>
<td>1.493</td>
<td>1.455</td>
<td>1.473</td>
</tr>
</tbody>
</table>

130
Table 5.4 Correlation matrices for single-trajectory fits of DiO to DiI energy transfer, $R_{stim}=10$ nm data. Bold entries indicate strong correlations ($|r_{ij}|>0.9$).

<table>
<thead>
<tr>
<th>Trajectory 1</th>
<th>$K_P^{DiO}$</th>
<th>$K_P^{DiI,1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$</td>
<td>-0.706</td>
<td>0.882</td>
</tr>
<tr>
<td>$K_P^{DiO}$</td>
<td>0.934</td>
<td></td>
</tr>
<tr>
<td>Trajectory 2</td>
<td>$K_P^{DiO}$</td>
<td>$K_P^{DiI,2}$</td>
</tr>
<tr>
<td>$R$</td>
<td>-0.871</td>
<td>0.945</td>
</tr>
<tr>
<td>$K_P^{DiO}$</td>
<td>0.979</td>
<td></td>
</tr>
<tr>
<td>Trajectory 3</td>
<td>$K_P^{DiO}$</td>
<td>$K_P^{DiI,3}$</td>
</tr>
<tr>
<td>$R$</td>
<td>0.711</td>
<td>-0.813</td>
</tr>
<tr>
<td>$K_P^{DiO}$</td>
<td>-0.288</td>
<td></td>
</tr>
<tr>
<td>Trajectory 4</td>
<td>$K_P^{DiO}$</td>
<td>$K_P^{DiI,4}$</td>
</tr>
<tr>
<td>$R$</td>
<td>-0.390</td>
<td>-0.946</td>
</tr>
<tr>
<td>$K_P^{DiO}$</td>
<td>0.645</td>
<td></td>
</tr>
</tbody>
</table>
it is clear that for the single-trajectory fits, the best result for both probe pairs is obtained with trajectory 1: both the recovered domain size $R$ and probe $K_p$ are in good agreement with the simulation values, a result that is observed for the other domain sizes as well (data not shown). It may be significant that of the four trajectories, acceptor $K_p$ strength (defined as $|\log K_p|$) is strongest for trajectory 1. Trajectories 2 and 3 (in which the acceptor partitions to different phases but with identical strength) perform worse than trajectory 1 for DHE/DiO. Trajectories 1-3 perform similarly for DiO/DiI, which may reflect the comparatively better S/N of these data. For both probe pairs, trajectory 4 performs poorly as judged by the parameter confidence intervals: uniform acceptor $K_p$ essentially collapses the effects of $R$ and donor $K_p$ onto a single, straight-line trajectory, drastically reducing the information content of the data. For both probe pairs, the most accurate results are obtained by global fitting of trajectories 1-3 (the final column in Table 5.2).

The results of global fitting of tieline E data simulated at 6 domain sizes are shown in Table 5.3. For both probe pairs, the agreement between recovered values of $R$ and simulation values is good at smaller domain sizes, with accuracy decreasing as domain sizes increases. Still, recovered $R$ are within 15% of the simulation value for domain radius up to at least $10R_0$. The trend for probe $K_p$ is reversed: the least accurate values occur at the smallest $R$, with continuous improvement as $R$ increases. Both of these observations are consistent with the fact that in the infinite domain size limit, all information regarding domain size is lost, and the curves are entirely controlled by donor and acceptor $K_p$. We note that the true (simulation) value of $R$ is in every case but two contained in the 95% confidence interval for recovered $R$ calculated from the parameter covariance matrix, which is a reasonable outcome for a sample of 12 fits.
For both probe pairs, the global fit produces better results than any of the individual trajectory fits: in general, recovered values are closer to the simulation values, and confidence intervals are smaller. It is instructive to examine the parameter correlation coefficients $r_{ij}$ obtained from the off-diagonal elements of the covariance matrix at the best-fit solution. Table 5.4 lists $r_{ij}$ for the single-trajectory fits listed in the lower half of Table 5.2, and corresponding to the DiO/DiI data shown in Figure 5.2 B. Strong correlations ($|r_{ij}| > 0.9$) are observed between pairs of parameters for all but trajectory 3. Parameters will exhibit strong correlations when their effect on the data is similar, and we have seen in Figures 5.2-4 that increasing the partition coefficients and increasing the domain size have at least superficially similar effects. It can be difficult to separate these effects in the absence of a sufficient number of data points, or when S/N is poor. In short, large $r_{ij}$ are an indication that the data do not contain enough information to support all of the variable parameters.

By combining multiple data sets in a global analysis, subtle differences in parameter effects are more likely to be distinguished. Table 5.5 lists the correlation matrices for DiO/DiI energy transfer for all domain sizes: the matrix for the $R_{sim} = 10$ nm data corresponds to the matrices in Table 5.4. The pairwise parameter correlations for $R_{sim} = 10$ are in nearly every case reduced by the global analysis, with all $r_{ij}$ less than 0.8 in magnitude. Similar trends are seen at other domain sizes.

5.4.3 Simulated data fit to the FP-FRET model: departure from assumptions

To this point, the simulation parameters have exactly followed the assumptions that were used to derive the FP-FRET model. It is important to assess how the model fares when the underlying assumptions are not strictly met. Testing every case is beyond the scope of these studies, but we can address some of the most likely scenarios. Wherever literature data can be found to suggest how a real bilayer might depart from the assumptions, we have tried to incorporate that into the simulations.
Table 5.5 Correlation matrices for global fits of DiO to DiI energy transfer. Bold entries indicate strong correlations (\( |\gamma| > 0.9 \)).

<table>
<thead>
<tr>
<th>( R_{\text{sim}} )</th>
<th>( K_{p}^{\text{DiO}} )</th>
<th>( K_{p}^{\text{DiI,1}} )</th>
<th>( K_{p}^{\text{DiI,2}} )</th>
<th>( K_{p}^{\text{DiI,3}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_{\text{sim}} = 2 )</td>
<td>( K_{p}^{\text{DiO}} )</td>
<td>0.167</td>
<td>0.160</td>
<td>-0.225</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,1}} )</td>
<td>-0.97</td>
<td>-0.913</td>
<td>0.139</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,2}} )</td>
<td>0.901</td>
<td>-0.162</td>
<td>-0.153</td>
</tr>
<tr>
<td>( R_{\text{sim}} = 5 )</td>
<td>( K_{p}^{\text{DiO}} )</td>
<td>0.449</td>
<td>0.498</td>
<td>-0.717</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,1}} )</td>
<td>-0.880</td>
<td>-0.780</td>
<td>0.441</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,2}} )</td>
<td>0.843</td>
<td>-0.637</td>
<td>-0.633</td>
</tr>
<tr>
<td>( R_{\text{sim}} = 10 )</td>
<td>( K_{p}^{\text{DiO}} )</td>
<td>0.485</td>
<td>0.529</td>
<td>-0.799</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,1}} )</td>
<td>-0.795</td>
<td>-0.670</td>
<td>0.361</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,2}} )</td>
<td>0.750</td>
<td>-0.631</td>
<td>-0.626</td>
</tr>
<tr>
<td>( R_{\text{sim}} = 20 )</td>
<td>( K_{p}^{\text{DiO}} )</td>
<td>0.503</td>
<td>0.537</td>
<td>-0.842</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,1}} )</td>
<td>-0.717</td>
<td>-0.597</td>
<td>0.294</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,2}} )</td>
<td>0.679</td>
<td>-0.613</td>
<td>-0.608</td>
</tr>
<tr>
<td>( R_{\text{sim}} = 40 )</td>
<td>( K_{p}^{\text{DiO}} )</td>
<td>0.507</td>
<td>0.534</td>
<td>-0.861</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,1}} )</td>
<td>-0.660</td>
<td>-0.550</td>
<td>0.245</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,2}} )</td>
<td>0.634</td>
<td>-0.598</td>
<td>-0.594</td>
</tr>
<tr>
<td>( R_{\text{sim}} = \infty )</td>
<td>( K_{p}^{\text{DiO}} )</td>
<td>0.522</td>
<td>0.543</td>
<td>-0.875</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,1}} )</td>
<td>-0.610</td>
<td>-0.514</td>
<td>0.210</td>
</tr>
<tr>
<td></td>
<td>( K_{p}^{\text{DiI,2}} )</td>
<td>0.608</td>
<td>-0.597</td>
<td>-0.594</td>
</tr>
</tbody>
</table>
5.4.3.1 Distribution of transverse probe locations

Figure 5.5 shows simulated E data in which the transverse height for each donor and acceptor was drawn from a normal distribution centered at 0.35 nm (see Table 5.1) and with a standard deviation of 0.25 nm. For comparison, data for the case of a single transverse height (i.e., Figure 5.2) are reproduced in a lighter shade. Clearly, the effect of a small distribution of probe heights is within the noise of the data, indicating that the FP-FRET model can be applied in this case without modification. Best-fit parameter values for global fitting are listed in the third column of Table 5.6, and are, within error limits, identical to the case of a single probe height.

5.4.3.2 Uncoupling of phase domains

Figure 5.6 shows simulated data for the case of uncoupled phase domains: that is, the case where the lateral positions of domains in the upper and lower leaflets are completely uncorrelated. Here, clear distortions are observed: for both the RRE and REE and relative to the case of coupled domains (reproduced in a lighter shade), transfer efficiency moves in the direction of random probe mixing. In principle this could manifest in the recovered parameters as either a reduction in probe partitioning strength or domain size (or some combination of the two): for the two data sets examined, both effects are observed (column 4 in Table 5.6). It is likely that in the case of uncoupled domains, fitting to a model that assumes domain coupling will lead to an apparent domain size that is smaller than the true value.

5.4.3.3 Variation in domain size across the tieline

Experimental evidence suggests that domain size may vary in Ld + Lo coexistence regions (23). Figure 5.7 shows data that were simulated with a linear variation in domain radius, ranging from 5 to 20 nm across the tieline (domain size is shown on the upper x-axis scale). For clarity, only two of the simulated trajectories are shown for each probe pair, those corresponding to the maximum and minimum
Table 5.6 Best-fit parameters recovered from global fits of data sets with relaxed assumptions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sim. Val.</th>
<th>Probe z dist.</th>
<th>Uncoupling</th>
<th>Variable R 1</th>
<th>Variable R 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>10</td>
<td>10 (1)</td>
<td>8.8 (8)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>R1</td>
<td>5</td>
<td>--</td>
<td>--</td>
<td>18 (2)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>R2</td>
<td>20</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>19 (5)</td>
</tr>
<tr>
<td>$K_p^{DHE}$</td>
<td>2.5</td>
<td>2.46 (8)</td>
<td>2.34 (5)</td>
<td>2.08 (7)</td>
<td>2.6 (3)</td>
</tr>
<tr>
<td>$K_p^{DIO,1}$</td>
<td>0.1</td>
<td>0.099 (6)</td>
<td>0.125 (8)</td>
<td>0.096 (5)</td>
<td>0.105 (5)</td>
</tr>
<tr>
<td>$K_p^{DIO,2}$</td>
<td>0.25</td>
<td>0.26 (1)</td>
<td>0.29 (1)</td>
<td>0.25 (1)</td>
<td>0.27 (1)</td>
</tr>
<tr>
<td>$K_p^{DIO,3}$</td>
<td>4</td>
<td>5.1 (6)</td>
<td>7 (1)</td>
<td>6.8 (5)</td>
<td>5 (1)</td>
</tr>
<tr>
<td>$\langle R_0 \rangle$</td>
<td>2.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$\chi^2_{red}$</td>
<td>--</td>
<td>0.743</td>
<td>1.267</td>
<td>2.8523</td>
<td>1.050</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sim. Val.</th>
<th>Probe z dist.</th>
<th>Uncoupling</th>
<th>Variable R 1</th>
<th>Variable R 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>10</td>
<td>9.9 (2)</td>
<td>5.9 (1)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>R1</td>
<td>5</td>
<td>--</td>
<td>--</td>
<td>17.7 (8)</td>
<td>5.4 (6)</td>
</tr>
<tr>
<td>R2</td>
<td>20</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>18.8 (8)</td>
</tr>
<tr>
<td>$K_p^{DIO}$</td>
<td>0.1</td>
<td>0.101 (3)</td>
<td>0.120 (6)</td>
<td>0.079 (2)</td>
<td>0.102 (2)</td>
</tr>
<tr>
<td>$K_p^{DII,1}$</td>
<td>0.1</td>
<td>0.111 (7)</td>
<td>0.14 (1)</td>
<td>0.167 (7)</td>
<td>0.105 (8)</td>
</tr>
<tr>
<td>$K_p^{DII,2}$</td>
<td>0.25</td>
<td>0.254 (7)</td>
<td>0.28 (1)</td>
<td>0.298 (7)</td>
<td>0.247 (8)</td>
</tr>
<tr>
<td>$K_p^{DII,3}$</td>
<td>4</td>
<td>4.41 (2)</td>
<td>3.8 (2)</td>
<td>3.04 (9)</td>
<td>4.3 (2)</td>
</tr>
<tr>
<td>$\langle R_0 \rangle$</td>
<td>5.7</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$\chi^2_{red}$</td>
<td>1.389</td>
<td>2.559</td>
<td>4.424</td>
<td>1.785</td>
<td></td>
</tr>
</tbody>
</table>

\[136\]
Figure 5.5 A distribution of probe transverse locations has a minor effect on FRET efficiency, compared to the assumption of a single location. Simulated FRET data for DHE to DiO (A) and DiO to DiI (B), with transverse probe locations drawn from a distribution as described in the text. Colors and symbols as in Figure 5.2. For comparison, Figure 5.2 data are shown in a lighter shade. Global fits to the FP-FRET model are shown as solid lines.
Figure 5.6 Cross-leaflet uncoupling of phase domains has a large effect on FRET. Simulated FRET data for DHE to DiO (A) and DiO to DiI (B), corresponding to uncoupling of domains across leaflets as described in the text. Colors and symbols as in Figure 5.2. For comparison, Figure 5.2 data are shown in a lighter shade. Global fits to the FP-FRET model are shown as solid lines.
Figure 5.7 Variation in domain size along a tieline affects FRET. Data for DHE to DiO (A) and DiO to DiI (B), simulated using a composition-dependent domain size shown on the upper x-axis. Colors and symbols as in Figure 5.2. The shaded area marks the region bounded by predicted E values for $R = 5$ and 20 nm, for the given acceptor $K_p$. Global fits to the unmodified FP-FRET model are shown as dashed lines, and global fits to a modified FP-FRET model accounting for a linear variation in domain size are shown as solid lines.
acceptor partition strength. Also shown is the range of E bounded by curves predicted for 5 and 20 nm domains. For both FRET pairs, simulated data lie closer to the 20 nm curve for most of the length of the tieline, owing to the steep increase in E that occurs at the smallest domain sizes (see Figures 5.3-4). The dashed line shows the results of fitting the simulated data to the unmodified FP-FRET model: that is, a single value of domain size $R$ was varied along with the probe $K_p$ to achieve the best fit to the data. Consistent with a visual inspection of the data, the best-fit domain radius for each probe pair is closer to the maximum domain size of 20 nm. In both cases, reduced chi-square (shown in Table 5.6, column 5) is significantly larger than for any fits discussed to this point, a good indication that the model fails to capture an important aspect of the underlying probe distribution.

It is trivial to account for smooth variation in domain size in the model. We modified the FP-FRET model to account for linear variation, which added one adjustable parameter: the best-fit to this model is shown as the solid lines in Figure 5.7. Column 6 in Table 5.6 shows that the recovered domain size parameters (the endpoint values) are in good agreement with the simulation values. As expected, a significant improvement in reduced chi-square is also observed.

**5.5 Discussion**

**5.5.1 Choice of fixed parameters**

The primary goal of this report is to assess the performance of the FP-FRET model and its nonlinear least-squares implementation: given data generated with known parameters, can those parameters be accurately recovered? We chose to simulate data with snapshots of discrete, non-overlapping, randomly placed donors and acceptors, and subject to the thermodynamic constraints of the phase-separated system. This approach allows us to independently evaluate some of the more subtle
assumptions of the model. The FP-FRET model is, after all, a continuous representation of an inherently discrete system: acceptors are treated as a continuous number density with spatial variation that depends on domain size, partitioning strength, and distance from a donor. It is reasonable that such a continuous description would break down for very small domains, consisting of several tens of molecules, and for which the discrete nature of a lipid lattice may strongly influence energy transfer.

For a model built on the assumption of first-order phase separation, it is essential to work on a known tieline, and the established Ld + Lo tieline in the ternary system DSPC/POPC/cholesterol is a convenient choice (3). Molecular areas and bilayer thicknesses can be estimated from the abundant x-ray and neutron scattering data and molecular dynamics simulations available for these lipids (15-17). We emphasize that the applicability of the domain size analysis described in this report relies on the availability of such data.

The fluorescent probes chosen should also be carefully considered. The FP-FRET model assumes a single transverse location for each chromophore (which can be different in each phase). In a real membrane, the position of the chromophore will fluctuate in time, so that the single fixed value in the model represents the mean of a distribution. The accuracy of E predicted by the model might therefore be expected to depend both on the width of the distribution of chromophore distances, and on the sensitivity of E to changes in chromophore transverse position (which will in turn depend on \( R_0 \) and bilayer thickness). In any case, the best probes will be those that have relatively stable transverse locations in the bilayer. With the exception of bilayers having a high concentration of polyunsaturated chains, cholesterol is anchored in the membrane with its long axis nearly coincident to the bilayer normal, and its polar hydroxyl group at the level of the carbonyl oxygen (24), making DHE (a structural
analog of cholesterol) a good candidate for transverse stability. A recent MD study of C18:0-DiI in fluid DPPC bilayers found a relatively narrow and approximately normal vertical distribution of the DiI chromophore, with an average location 6.3 Å below the DPPC phosphate and a standard deviation of ~ 2.5 Å (19). The narrow distribution was attributed to the delocalized positive charge on the conjugated-π system: when the charge was artificially eliminated, the chromophore position exhibited significantly greater transverse fluctuations. The positively charged DiO chromophore differs from DiI by a single heteroatomic substitution; it is reasonable to assume that if the positive charge is indeed responsible for stabilizing the chromophore position just below the interface, DiO will behave similarly to DiI. Finally, and by way of motivating the next section, we emphasize that the effectiveness of a global analysis of multiple data sets is related to their interconnectedness: they must share some common adjustable parameters. The DiI and DiO fluorophores are particularly attractive in this respect, as both are commercially available with a variety of different alkyl chains that exhibit markedly different partitioning behavior between Ld and Lo phases (25). By pairing a carbocyanine acceptor series with a single donor (like the hypothetical experiments in this report), we can achieve distinctly different E profiles, all linked by a common donor $K_p$ and $R_0$.

5.5.2 Single-trajectory vs global data analysis

The most important FP-FRET parameters are those that control the spatial distribution of probes in the membrane: these are the domain size, and the donor and acceptor partition coefficient. $K_p$ between the coexisting phases can be independently measured with a variety of techniques, including fluorescence intensity or anisotropy (26,27). The fluorescence signal arises from the very local environment of a probe molecule, and as such offers the advantage that it does not depend on domain size. There are however some potential drawbacks to these measurements. It can be
difficult to achieve good signal-to-noise at the low probe concentrations desired for studies of phase behavior. A related and much more serious problem concerns the difference in signal observed in the two phases $\Delta S$. It can easily be shown that for real data with a finite S/N, the confidence limits on the best-fit $K_p$ diverge rapidly as $\Delta S$ approaches the S/N ratio. As discussed in the previous section, the number of probes for which reliable information about transverse location is available is already quite limited; clearly, we are in a weak position if we must also rely on a large difference in quantum yield or anisotropy in the coexisting phases for this relatively small set of probes. Rather, we would like to identify controllable experimental conditions—for example, the number of independent trajectories, and the quantity of data points in a trajectory—that allow us to recover probe $K_p$ from the E data itself.

To this end, we performed two types of fits on the simulated data trajectories, in each case with no assumed knowledge of probe $K_p$ or domain size. First, each 51-sample trajectory was fit as an independent experiment. We then analyzed three trajectories (each with the same donor $K_p$, but different acceptor $K_p$) simultaneously in a global fit. A global fit essentially uses all of the information contained in multiple data sets that share one or more parameters. A single goodness-of-fit criterion is established by summing the squared residuals for all of the data points (28). An essential aspect of global analysis is that the relative error for each data point is known or can be estimated: the appropriate weight for each residual term is the relative error, and different data sets will in general have different errors.

A rather surprising result of this study is that for even a single 51-point trajectory with a single probe pair, in most cases both the probe $K_p$ and the domain size can be reasonably estimated (see Tables 5.2-3). The accuracy depends on the strength of probe partitioning (with larger $K_p$ leading to more accurate values), $R_0$ for the probe pair (with smaller $R_0$ showing reduced accuracy at large domain sizes), and
of course S/N. The clear exception is for uniform acceptor $K_p$, in which case the trajectory contains virtually no information. As expected, a better fit (as judged by the relative difference between simulated and recovered values, and the confidence interval of the recovered parameters) is achieved in every case with a global analysis. Certainly, some of the improvement can be attributed to the larger ratio of independent data points to fit parameters: the single-trajectory analysis includes 51 data points and 3 adjustable parameters, with the global analysis adding 102 data points and only two adjustable parameters. However, some of the improvement is the result of reduced parameter correlations. Figures 5.2-4 demonstrate that either increasing the domain size or increasing the probe partition coefficients has similar effects on the E lineshape. This can potentially cause strong correlations in these parameters (see Table 5.2), which increases their confidence intervals. When we add another trajectory with the same donor but a different acceptor, the effect is to weaken the correlations among all of the parameters (Table 5.3). The effects of domain size and probe partitioning are essentially decoupled, leading to improved accuracy in the recovered parameters.

5.5.3 Assumptions of the FP-FRET model

We tested the performance of the FP-FRET model for a variety of simulated data sets in which a particular assumption of the model was relaxed. We now discuss the results of these simulations.

5.4.3.1 Probe height distribution.

We used a distribution of probe transverse positions that was based on MD simulations of DiI in DPPC bilayers (19). In that study, DiI transverse position was observed to fluctuate with a standard deviation of $\sim 2.5 \, \text{Å}$. As shown in Figure 5.5, no significant effect was observed in the simulated data due to the distribution, and we conclude that relatively narrow distributions need not be explicitly accounted for by the model in order to obtain reliable domain size measurements. It is likely that wider,
or highly asymmetric distributions may show more significant deviations than were observed in our simulations. Furthermore, an incorrect estimate of the mean position may lead to larger errors. Mean fluorophore depth can in many cases be measured by addition of an external (29) or membrane incorporated (30) fluorescence quencher. MD studies of membrane fluorophores have begun to appear in the literature (19,31-36), and such studies will undoubtedly be an important tool for characterizing vertical distributions for use in bilayer energy transfer studies, particularly for cases where experimental data cannot be easily obtained.

5.5.3.2 Cross-leaflet domain coupling

Experimentally, cross-leaflet coupling of phase domains in macroscopically-separated bilayers is always observed: we are aware of no published exceptions. A variety of mechanisms has been put forward to explain these observations, including chain interdigitation, cholesterol flip-flop, and electrostatic coupling (37). Collins has recently proposed that surface tension at the bilayer midplane not only greatly reduces the probability of domain uncoupling, but makes even small overhang of domains very unlikely except near a critical point, where the interfacial energy vanishes (38). Using coarse-grained MD simulations of DPPC/DOPC/chol, Risselada and Marrink found evidence for the existence of this surface tension and estimated its magnitude, concluding that domain overhang > 20 nm² is effectively suppressed (39). A recent review concluded that the largest contributor to the midplane interfacial energy is chain interdigitation (37). Considering the prevailing viewpoint, we are relatively comfortable with the assumption of coupled leaflets. Still some uncertainty remains, particularly for nanoscopic domains, where direct verification of coupling in free-standing bilayers is impossible.

When data arising from an uncoupled system are fit to the unmodified FP-FRET model, the recovered domain size is always smaller than the true value (Table
5.6). Fitting to an inappropriate model in this case also decreases the quality of the fit, evidenced by the larger reduced chi-square: changes in E due to uncoupling are qualitatively different from the effects of domain size or probe partitioning, and the effect cannot be easily absorbed into the existing parameters. Figure 5.6 shows that the effect is exacerbated by increased acceptor partitioning strength, and by increased $R_0$. These results are easy to understand. The inverse-sixth power dependence of E on donor-acceptor distance dramatically reduces the frequency of quenching events at distances larger than even a few nm, which is the typical distance between probes in opposing leaflets. In general, the only cross-leaflet acceptors that contribute in a meaningful way to donor quenching are those that are almost directly opposite the donor. When domains are coupled, the time-averaged acceptor density immediately above a given donor is enhanced or reduced (depending on the direction of acceptor partitioning) relative to a random acceptor distribution. Uncoupling of the leaflets destroys this correlation, and the time-averaged value of acceptor density opposite the donor is now the same as it would be for randomly distributed acceptors. Figure 5.6 reveals that uncoupling is more important for long $R_0$ probe pairs, where a larger fraction of the total donor quenching occurs to acceptors in the opposite leaflet. For small $R_0$ pairs and large transverse probe distances, the effect is expected to be negligible. In either case, it is trivial to account for this effect in the model by using the average acceptor surface density in the term for cross-leaflet energy transfer (the S integral).

The simulated data reveal that uncoupling is clearly a differential effect with respect to $R_0$, more significant for larger than for smaller values. Furthermore, compared to distortions in E due to the failure of other model assumptions, this differential effect seems to be unique. This suggests an experimental test for uncoupling of small domains. Data are collected with a variety of probe pairs covering
a range of $R_0$ and fit as usual to the coupled domain FP-FRET model. If the recovered domain size decreases with increasing $R_0$, the data should be refit to an uncoupled model: if a consistent value of $R$ is obtained, uncoupling of domain leaflets may be responsible for the discrepancies in the former case.

Finally, we note that theoretical calculations raise an intriguing third possibility regarding domain coupling: that below a certain critical radius, estimated to be $\sim 2$ nm, the most energetically favorable configuration is the anti-registration of Ld and Lo domains (37). Such a scenario would seem to require nearly equal area fractions of Ld and Lo phases to significantly affect model predictions, and as such may be unimportant except near the percolation threshold.

### 5.5.3.3 Variation in domain size

There is no theoretical work of which we are aware to relate the size (or size distribution) of nanodomains to lipid composition along a tieline. Experimentally, the dependence of domain size on mixture composition has been estimated in only a handful of cases (23,40,41). Though domain size measurements in those studies are at best crude, the evidence suggests that domain size may vary across the Ld + Lo region in some mixtures. Using FRET, de Almeida et al. estimated that Lo phase domains near the Ld boundary were $< 20$ nm in diameter, while Ld domains near the Lo boundary were $\sim 75-100$ in diameter (23). These estimates seem large based on a comparison of FRET profiles obtained in the Ld + Lo region of DSPC/DOPC/chol and DSPC/POPC/chol (3). For simulating the effects of variable domain size, a smaller variation of 5-20 nm (domain radius) was used. Our results indicate that such a linear variation in domain size can be successfully incorporated into the model (Figure 5.7 and Table 5.6). Furthermore, the inappropriateness of a single-radius model was easily diagnosed by the fit statistics. A good strategy might be to in all cases fit to a model with varying domain size, and then use the fit statistics to evaluate the results. Similar
values and strong cross-correlation between the endpoint domain size parameters would indicate that the variation in domain size across the tieline is negligible.

5.5.3.4 Additional considerations

It is beyond the scope of this report to investigate every possible way in which assumptions of the FP-FRET model may fail to be met in real membranes. Still, some unaddressed and potentially significant complications merit a brief discussion here.

First, we must consider the issue of the FRET orientation factor $\kappa^2$. $\kappa^2$ is related to the relative orientations of the donor and acceptor transition dipoles, shown in Figure 5.8 A, as:

$$\kappa^2 = (\cos \theta_T - 3 \cos \theta_D \cos \theta_A)^2$$  \hspace{1cm} 5.7

and is related to the Förster distance through the relationship:

$$R_0^6 = \frac{9000 \ln 10 \frac{Q_D}{\kappa^2}}{128\pi^5 N_A \eta^4} = C \kappa^2$$  \hspace{1cm} 5.8

(42). In Equation 5.8, the photophysical parameters (which are constant for a given probe pair in a given environment) have been folded into a single constant $C$. Much print has been devoted to the problem of the uncertainty associated with $\kappa^2$. This uncertainty arises from two sources: a lack of knowledge of the probability distribution of $\kappa^2$ (which is related to the range of allowed motions of the probes), and a lack of knowledge of the rate at which the transition dipoles sample their allowed orientations (which is related to the rate of rotational diffusion of the chromophores).

In many cases, it is assumed that all orientations of the transition dipoles are allowed (the isotropic assumption), and that the entire range of orientations is sampled during the transfer time (the dynamic averaging assumption). The isotropic distribution of $\kappa^2$ has been exactly calculated (43), and is shown in Figure 5.8 B: the expectation value of $\kappa^2$ on this distribution is $\langle \kappa^2 \rangle = 2/3$. When both the isotropic and dynamic averaging assumptions hold, it is valid to use the average value of $\kappa^2 = 2/3$ (and hence,
**Figure 5.8** FRET orientation factors are properly described by a distribution. (A) A donor excitation transition dipole D and acceptor emission transition dipole A separated by a distance R, showing the geometric considerations for calculating $\kappa^2$ with Equation 5.7 (from Dale et al., 1979). (B) The isotropic distribution of $\kappa^2$ resulting from complete orientational freedom of both donor and acceptor.
a single value of $R_0$) to describe all probe pairs (43). We will now consider these two sources of uncertainty in the context of our experiments.

First is the possibility of a restricted range of motional freedom of the lipid fluorophore. Fluorophores attached via a flexible linker to the headgroup may more or less meet the approximation of an isotropic distribution of orientations, but fluorophores located within the highly asymmetric bilayer environment, such as those considered here, almost certainly do not. A common model for rotational diffusion of lipids in membranes is wobble-in-a-cone, where the long axis of the molecule is allowed to sample an axially symmetric subset of orientations about a mean orientation (44). The fact of restricted motion does not itself invalidate the use of a single value of $R_0$ to describe all probe pairs: a single value can be used whenever the entire range of allowed motions (e.g., a restricted conical distribution) is sampled during the transfer time. Such a case is by definition within the dynamic averaging regime, though the distribution of $\kappa^2$ may well be unknown. For cases where the desired information is the unknown D-A separation distance (or distance distribution), this fact is of little comfort, as there are now two unknowns ($R$ and $\langle \kappa^2 \rangle$) in the transfer efficiency equation. However, this is not the case for the model presented here: consider that in the single-phase compositions at the tieline endpoints, where there are no phase domains and hence no probe partitioning, the assumption of a random probe distribution has precisely accounted for all donor-acceptor separation distances (at least, to the extent that the bilayer structural parameters and probe transverse locations are known, and we have in this work assumed that those parameters are fixed by independent experiments). If we relax the assumption that $\langle \kappa^2 \rangle = 2/3$, we now have one unknown parameter ($R_0$, via its relationship to $\kappa^2$). Stated another way, we can account for the effects of restricted motion simply by allowing $R_0$ to vary in the fit. An alternative (and conceptually equivalent) procedure
is to measure E vs acceptor concentration in the pure phases and fit the data to Equations 4.1-3 to recover $R_0$ for use in the model (41). It is worth reemphasizing that this is only strictly valid in the dynamic averaging regime.

The second source of uncertainty—the averaging regime (dynamic vs. static)—seems to be a source of confusion, and is less frequently discussed in the literature. The static averaging limit holds when the motion of the transition dipoles is much slower than the timescale of energy transfer (that is, the timescale of fluorescence decay). In this case, each D-A pair has a different value of $\kappa^2$, and the average transfer efficiency of an ensemble is the average of $E$ for all of the individual pairs, each with its own $R_0$ determined by Equation 5.8 (45). It can be shown that, given the same underlying distribution of orientation factors, the value of $E$ obtained in the static averaging limit is always less than the value obtained in the dynamic averaging limit (45). This is an intuitive result, because in the dynamic regime every D-A pair must sample all orientations during the transfer time, including those most favorable for energy transfer (43).

Rotational diffusion for fluorescent lipids in membranes can be examined with fluorescence anisotropy. The analysis typically reports two rotational correlation times: a fast component that is ascribed to rotational motions of the chromophore, and a slow component that is ascribed to the whole lipid. Experimentally determined values of the fast component for dialkyl-DiI probes in fluid phases range from 0.18-1.6 ns (46,47). Gullapalli has reported a similar value of 0.99 ns obtained from MD simulations of C18:0-DiI (19). These values are comparable to the fluorescence lifetimes of these probes, an indication that the dynamic averaging regime may not be strictly valid for DiO to DiI energy transfer. Dale and Eisinger have noted that the intermediate regime is almost impossible to model analytically (45), though they have also claimed that it can in many cases be reasonably approximated by the dynamic
averaging limit (43). Though modeling the intermediate regime may prove extremely
difficult, it will be important to at least assess the effects of probe rotational motions
on E with MC and MD simulations.

A second issue for consideration is that of domain shape, which in this study is
assumed to be perfectly round. For bilayers exhibiting macroscopic phase coexistence,
round fluid domains are always observed away from the critical point, though it is
possible that the very low line tensions inherent in nanodomain systems will result in
different shapes. It has been shown that low line tensions in the presence of competing
interactions can cause phase-separated systems to undergo a shape instability; in
certain interaction energy regimes, this can result in elongated domains (48). Such
patterns have been observed in Ld + Lo phase coexistence regions (49, J. Wu
unpublished). Transfer efficiency arising from striped domains may be difficult to
model analytically, though in such cases E can still be easily simulated with Monte
Carlo techniques.

Another, related issue is a potential distribution of domain sizes. Here,
experimental evidence is completely lacking, though some theoretical work suggests
that nanodomains may have a very narrow size distribution (50). Towles and Dan have
addressed the effects of domain polydispersity on transfer efficiency measurements,
concluding that as the degree of polydispersity increases, the recovered domain size
decreases, owing to a biasing effect of increased sensitivity of FRET at smaller
domain sizes (51).

Finally, we note the possibility that the domains are not randomly dispersed in
the membrane as assumed by the model, but rather arranged in some ordered array.
For example, hexagonal arrays of Ld + Lo domains are occasionally observed in
DOPC-containing mixtures (49). In such cases, it may be possible to use an expression
for the 2D RDF that explicitly accounts for domain interactions. The domain surface coverage is then derived from this RDF as described in Chapter 4.

Apart from geometrical considerations, a larger issue is whether or not a model of first-order phase separation is valid for the case of nanoscopic domains. Several explanations for the observation of nanodomains have been developed both theoretically and experimentally, including highly nonideal mixing (52), microemulsions (53-55), and 2D critical phenomena (4,5). This and other relevant work has been summarized in recent reviews (2,56). On the other hand, much theoretical work has shown that systems undergoing first-order phase separation can also exhibit nanometer-scale domains due to spatial modulation arising from competing energetic considerations (48). The interaction driving domains to coalesce is the domain edge energy (related to the line tension), and several potential competing interactions have been identified, including lipid intrinsic curvature and electrostatic repulsion.

Regardless of the underlying mechanism of domain formation, the FP-FRET model remains a simplistic representation of a real membrane, just as every model is, by definition, a simplification of physical reality. It is perhaps best to think of the FP-FRET model as a convenient way of approximating the spatial distribution of donor-acceptor distances that will be found whenever lipids have a strong tendency to cluster. Like any model, its success or failure should be judged based on its ability to reproduce experimental data, its predictive power, and the consistency of domain sizes it reports compared with measurements from other techniques.

5.5.4 Computational considerations

We conclude with a brief discussion of the computational considerations of the FP-FRET model. The equations of the FP-FRET model do not have simple, analytical derivatives with respect to the model parameters, yet these derivatives are required in
the Levenberg-Marquardt algorithm for nonlinear least-squares fitting, in the calculation of the Jacobian matrix. The standard approach in such cases is to use a finite-difference approximation of the derivatives to estimate the Jacobian. Finite-difference methods are computationally expensive: for the forward-difference method used in this work, each iteration of the fitting routine results in \( N(M + 1) \) function calls, where \( N \) is the number of data points, and \( M \) is the number of variable parameters. The global analyses in this work varied 5 parameters to fit 153 data points, or a total of 918 function calls per iteration. The fits shown in Table 5.6 required an average of 3111 function calls: this corresponds to just over 3 iterations per fit, demonstrating the efficiency of the Levenberg-Marquardt algorithm.

Still, the sheer number of function calls poses a problem. The average computation time of a function call in the global analyses was 16 seconds (on a 2.34 GHz processor). If the function calls are performed serially, a typical analysis would take ~ 14 hours to complete. By performing the function calls in parallel, this time can be reduced to ~ 3.5 hours on a quad-core machine—a long wait to be sure, but at least within the realm of acceptability. We were fortunate to have access to a 128-node Mathematica cluster, and we found that our parallel implementation of the fitting routine scaled with an average efficiency of 83%. Operating with the full cluster, a typical simulation was completed in ~ 8 minutes.

5.6 Conclusions

We have shown that the FP-FRET model can provide accurate estimates of membrane domain sizes up to 20 times the Förster distance. Conservatively, with suitable fluorophores it should be possible to measure domain sizes up to 40 nm with better than 10% accuracy, and up to 100 nm with better than 20% accuracy. Though the model relies on good information about the phase separated system (i.e. the
compositions and molecular areas of the coexisting phases), it is robust to some less certain but physically realistic scenarios, such as a distribution of transverse probe distances and variation in domain size across the tieline. Furthermore, we demonstrated that by simply varying $R_0$ in the fit, recovered parameter values are accurate in the face of a distribution of probe orientation factors, despite the fact that such a distribution is not explicitly accounted for by the model. For these reasons, the FP-FRET model should prove valuable for determining domain sizes in nanodomain-forming bilayer systems.
REFERENCES


Chapter 6
Conclusions and Future Directions

6.1 Summary of findings and conclusions

6.1.1 Phase studies in ternary lipid mixtures

The use of FRET for examining phase transitions and phase coexistence regions in lipid mixtures is well-established (1-8). Using high compositional-resolution FRET and ESR measurements, we found similar overall phase behavior for ternary mixtures containing DSPC, cholesterol, and one of a series of structurally similar low-TM lipids: DOPC, POPC, or SOPC. The extent of Ld + Lo phase separation is reduced in the POPC and SOPC systems, compared to the DOPC system: the upper boundary decreased in cholesterol content from 0.4 to 0.3 mole %, and the right-hand (Lo) boundary decreased in DSPC content from 0.64 mole % in the DOPC-containing system to 0.50 and 0.48 mole % in the POPC and SOPC systems respectively. GUV phase-region diagrams were previously determined for these systems (6,9). DSPC/DOPC/cholesterol is a Type II GUV system, exhibiting a wide compositional region of Ld + Lo phase coexistence with micron-sized domains visible with CFM, in addition to Ld + Lb and three-phase coexistence regions (6). In contrast, DSPC/POPC/chol and DSPC/SOPC/chol are Type I GUV systems: only the Ld + Lb region at low cholesterol shows visible phase domains, with no fluid-fluid coexistence evident at any composition (9). Taken together, these findings suggest that the Ld + Lo region of the POPC- and SOPC-containing systems is composed of nanoscopic phase domains with dimensions significantly smaller than the optical resolution limit of ~ 200 nm.

ESR measurements confirmed the presence of a sharp Lo phase boundary in the three mixtures consistent with FRET measurements. Differences in order were
observed in the Ld phases as a function of the low-TM lipid, with order increasing in the series DOPC < POPC < SOPC. In contrast, Lo phases for the three mixtures exhibited similar order. These findings suggest that the extent of the Ld + Lo miscibility gap is controlled by the difference in order between the coexisting phases. ESR spectra were also quantitatively consistent with partitioning of the spin probe between Ld and Lo environments, suggesting that tieline slopes in the three mixtures are substantially similar.

We analyzed FRET data in the Ld + Lo region of DSPC/DOPC/chol using the tieline field method of Smith and Freed (10). While the data did not support an independent determination of the critical point and tieline slopes, we were able to determine partition coefficients for the three fluorescent lipid analogs. We found that DHE and cholesterol partitioning are identical to within error, lending support to the use of DHE as a quantitative reporter of cholesterol. BoDIPY-PC and Fast-DiI prefer Ld phase, with partitioning strength increasing with decreasing cholesterol content: strongest partitioning occurs at the three phase triangle, just before precipitation of the gel phase.

6.1.2 The FP-FRET model

We developed a model for quantitative FRET studies suitable for circular phase domains of any size. The FP-FRET equations derive directly from a pair correlation function for two-dimensional disks, which allows for a straightforward modification of the model to account for domain-domain interactions. We showed that this model can be used to extract domain sizes and probe partition coefficients from simulated data with good accuracy. Importantly, we showed that global analysis of large data sets containing multiple probe pairs improves accuracy and extends the range of spatial sensitivity: domain diameters up to 20 \( R_0 \) were determined to within 15% of their true value. With suitable probe pairs, the regime of domain diameters
amenable to this analysis is ~2-120 nm. We also tested the performance of the model against simulated data sets for which certain model assumptions were relaxed. We found that the model is relatively insensitive to a distribution of probe transverse locations, but can detect uncoupling of phase domains. We also found that variation in domain size along a tieline can be accounted for with a simple modification to the fitting algorithm.

6.2 Future directions

Using simulated data, we have shown that the FP-FRET model is in principle capable of extracting domain size information from lipid systems with well-defined phase behavior. The physical picture underlying the model is one of first-order phase separations: the geometric considerations are simplistic, though consistent with observations of macroscopic fluid domains in Type II systems. Whether or not this picture is suitable for describing the fluid domains of Type I systems is an open question.

6.2.1 Ternary systems

We and others have stressed the importance of conducting domain size measurements on a well-defined tieline (11,12). We have determined an Ld + Lo tieline in DSPC/DOPC/chol and DSPC/POPC/chol to reasonable certainty for proceeding with this analysis. The DOPC system is a good control, as domains in this system are known to be in the infinite-phase limit of FP-FRET sensitivity.

Chapter 5 is a guide for conducting the domain size experiments. Either probe pair (DHE/DiO or DiO/DiI) should provide usable data, though higher acceptor concentrations are required in the former case due to its inherently lower S/N: the small $R_0$ results in less transfer efficiency at a given acceptor concentration, and DHE has a relatively low extinction coefficient, resulting in smaller donor fluorescence
signals. Before conducting the experiments, it is advisable to determine $R_0$ from an acceptor concentration series at each of the tieline endpoints for comparison with values recovered in the fit.

6.2.2 Four-component systems

The existence of micron-sized fluid phase domains in DSPC/DOPC/chol, and nanometer-sized phase domains in DSPC/POPC/chol, implies that a transition between these size regimes occurs in the four-component mixture DSPC/DOPC/POPC/chol. Recent work in the Feigenson lab has explored this transition with GUV microscopy and FRET. Stimulated acceptor emission trajectories in the approximate direction of Ld + Lo tielines are informative: as DOPC is replaced with POPC, an REE peak is observed to decrease gradually, evidence of a smooth transition between size regimes (J. Wu, unpublished). It may be possible to recover domain sizes from E trajectories in the four-component system, though the analysis will require additional assumptions about tielines in this system. If successful, these experiments will provide valuable information that can be used to inform theoretical treatments of nanodomains.

6.2.3 Improving the FP-FRET model

The model assumes the existence of well-defined partition coefficients for the probes. This implies that the lipid composition at domain phase boundaries behaves like a step function between the Ld and Lo compositions. The concept of partitioning is murky at phase boundaries, where a finite transition zone must exist over which the lipid composition changes from that of the Ld phase to that of the Lo phase. The transition zone can be safely ignored in the case of infinite-phase separation where its area is negligible compared to the area of the bulk phase domains. However, the transition zone must certainly contain a few shells of lipid: depending on its width, it may be important to account for its effects at small domain sizes. MD simulations of
DPPC/DOPC/chol found a transition zone of ~ 5nm over which the average composition changed nearly linearly from Ld to Lo (13).

What are the practical consequences of a transition zone, in terms of modeling FRET data? Clearly, the apparent partitioning strength of the probes will be reduced to an extent that depends upon the width of the transition zone. The consequences of this effect are to some extent mitigated when probe partition coefficients are allowed to vary in the fit. Nevertheless, it seems likely that the existence of boundary transition zones will reduce sensitivity of FP-FRET at the smallest domain sizes. It should be relatively straightforward to explore this effect with MC simulations like those conducted in Chapter 5. Depending on the results of these simulations, it may be necessary to modify the model to account for the existence of transition zones.

Broader questions about the thermodynamic nature of small domains cannot be addressed with a single model. Ising-like critical phenomena have been reported both in model membranes (14,15) and in plasma membrane vesicles (15), and a model for FRET in critically fluctuating membranes has been explored (S. Veatch, personal communication). The relative abilities of these models to describe experimental data may shed light on the nature of membrane rafts.

6.2.4 Molecular Dynamics

We have stressed the importance of a realistic geometric picture for accurately modeling FRET. Over the course of this work, it became clear that MD simulations have a unique ability to provide estimates of some parameters necessary for simulating FRET data, particularly the location and dynamics of fluorescent probes in the bilayer. We can achieve a better understanding of our lipid/probe model systems by conducting MD simulations in parallel with FRET studies. MD simulations will allow us to characterize the distribution of fluorophore transverse locations and the distribution of FRET orientation factors. Comparing the $\kappa^2$ distributions over short
and long time scales will allow us to assess our assumption of dynamic averaging of the transition dipoles. These studies should be conducted in both the Ld and Lo phases.

6.2.5 Computational efficiency

The FP-FRET model equations contain integrals that must be solved numerically. This is a computationally expensive task: for calculating a single value of \( E \) from a fixed set of parameters, we found an average evaluation time of 10-20 s with our Mathematica implementation on a modern processor. For fitting large data sets, typically several thousand function calls are made. We achieved reasonable analysis times by performing these function calls in parallel on a 128 kernel computing cluster.

The FP-FRET analysis will be more generally useful if it can be done on a desktop computer. Mathematica 8.0, released in November 2010, contains built-in support for performing parallel computation on a graphics processing unit (GPU), commonly known as a video or graphics card. Whereas a typical desktop processor contains 4 computing cores, modern GPUs contain 100s of cores: a current top-of-the-line NVIDIA Fermi video card has 480 processing cores, and retails for ~$500. We have shown that the FP-FRET analysis scales well, achieving 83% efficiency on a 128 kernel cluster. We expect that a GPU implementation will achieve similar scaling, and result in even faster analyses due to the larger number of available cores.
REFERENCES


APPENDIX A
Modeling ESR Spectra

Chiang et al. studied the composition-dependence of the spin labeled lipid 16-PC in the ternary lipid system DPPC/DLPC/cholesterol at 24°C, extracting dynamic and order parameters from nonlinear least-squares fitting of experimental spectra to a slow-motional regime model (1). While only small variations in these parameters were observed within a given phase, significant differences between ordered and disordered phases were found. In particular, Ld and Lo phases exhibit substantial differences in the $S_0$ order parameter, which measures the extent of alignment between the molecular axis and the local bilayer director. The effects of these differences are readily observed in the spectra of Figure A.1. Our ESR studies were designed to probe Ld + Lo phase coexistence in similar ternary mixtures, using molecular order as a contrast mechanism.

Following Chiang et al., experimental ESR spectra were fit using the NLSL program available from the Freed lab, with model parameters listed in Table A.1. These include magnetic and structural parameters for 16-PC in bilayers, as well as integer parameters required by the model. An excellent discussion of the nonlinear least-squares analysis of slow-motion ESR spectra using the NLSL program can be found elsewhere (2).

We modified the fitting procedure of Chiang et al. as follows. Initially, we varied six model parameters as described in (1). These adjustable parameters can be classified as three pairs of related variables (Table A.1): $c_0^2$ and $c_2^2$, the first two coefficients of the orienting potential; $\tilde{R}$ and $N$, the average rotational rate constant and rotational anisotropy; and $\Delta^{(0)}$ and $\Delta^{(2)}$, which account for Gaussian
Figure A.1 Typical 9.5 GHz ESR spectra for 16-PC in Ld and Lo phases. (A) 16-PC in an Ld-phase with composition $(\chi_{DSPC}, \chi_{CHOL}, \chi_{DOPC}) = (0, 0.09, 0.91)$. (B) 16-PC in an Lo-phase with composition $(\chi_{DSPC}, \chi_{CHOL}, \chi_{DOPC}) = (0.7, 0.3, 0)$. All CW spectra were obtained on a Bruker Instruments EMX ESR spectrometer operating at 9.3 GHz at ambient temperature (~ 22°C). Typical instrument settings were: center field = 3320 G, sweep width = 100 G, microwave power = 2 mW, modulation frequency = 1 G, time constant = conversion time = 81.92 s, resolution = 1024 points. 20 scans were averaged for each sample.
inhomogeneous spectral broadening. We discovered strong correlations (typically, $r_{ij} > 0.9$) between each of these pairs of variables at the solution (Table A.2).

**Table A.1** Model parameters used in nonlinear least-squares fitting of ESR spectra.

<table>
<thead>
<tr>
<th>Parameter symbol</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_{xx}$, $g_{yy}$, $g_{zz}$</td>
<td>2.0085, 2.0058, 2.0024</td>
<td>Principal components of the g tensor</td>
</tr>
<tr>
<td>$A_|$, $A_\perp$</td>
<td>33.4, 5.0</td>
<td>Principal components of the axially symmetric A (hyperfine) tensor (G)</td>
</tr>
<tr>
<td>$\alpha_D, \beta_D, \gamma_D$</td>
<td>0, 0, 0</td>
<td>Diffusion tilt angles (°)</td>
</tr>
<tr>
<td>$L_{emx}, L_{omx}, K_{mx}, M_{mx}, P_{mx}$</td>
<td>8, 7, 4, 4, 2</td>
<td>Basis set truncation indices</td>
</tr>
<tr>
<td>$n_{MOMD}$</td>
<td>23</td>
<td>Number of orientations in MOMD model</td>
</tr>
<tr>
<td>$N \equiv R_|/R_\perp$</td>
<td>10*</td>
<td>Rotational anisotropy</td>
</tr>
<tr>
<td>$\bar{R} \equiv \sqrt[3]{R_|^2 R_\perp}$</td>
<td>Variable</td>
<td>Average rotational diffusion rate (s$^{-1}$)</td>
</tr>
<tr>
<td>$c_0^0, c_2^2$</td>
<td>Variable, 0*</td>
<td>Coefficients for orienting potential</td>
</tr>
<tr>
<td>$\Delta^{(0)}, \Delta^{(2)}$</td>
<td>Variable, 0*</td>
<td>Gaussian inhomogeneous broadening (p-p width, G)</td>
</tr>
</tbody>
</table>

* variable in Chiang et al., but fixed in this study

**Table A.2** A typical correlation matrix showing strong correlations between related pairs of adjustable parameters (bold values).

<table>
<thead>
<tr>
<th>$\Delta^{(0)}$</th>
<th>$\Delta^{(2)}$</th>
<th>$\bar{R}$</th>
<th>$N$</th>
<th>$c_0^2$</th>
<th>$c_2^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta^{(0)}$</td>
<td>1</td>
<td>-0.9211</td>
<td>-0.1413</td>
<td>-0.1021</td>
<td>-0.7223</td>
</tr>
<tr>
<td>$\Delta^{(2)}$</td>
<td>1</td>
<td>0.2778</td>
<td>0.1454</td>
<td>0.7418</td>
<td>-0.6189</td>
</tr>
<tr>
<td>$\bar{R}$</td>
<td>1</td>
<td>0.9602</td>
<td>-0.4112</td>
<td>0.5776</td>
<td></td>
</tr>
<tr>
<td>$N$</td>
<td>1</td>
<td>-0.5424</td>
<td>0.6674</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$c_0^2$</td>
<td>1</td>
<td>-0.9623</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$c_2^2$</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The existence of strong correlations is an indication that the experimental data do not support independent determination of the correlated parameters, and suggests that the parameter search space should be reduced. We therefore fixed the rotational anisotropy ($N$) at 10 (i.e., a physically reasonable 10-fold difference between parallel
and perpendicular rotational diffusion rates), and set the lower-order terms for the orienting potential and spectral broadening \((c_2^2 \text{ and } \Delta^{(2)})\) to 0. For all simulation results quoted in the main text, only three parameters were varied: \(c_0^2, \bar{R}, \text{and } \Delta^{(0)}\).

Figure A.2 shows the best-fit simulated spectra obtained by varying either three or six parameters. Both parameter sets provide a reasonable fit to the experimental spectra (though the larger number of adjustable parameters will always produce a better fit). Nevertheless, systematic deviations between experimental and simulated spectra (for either set) indicate that the model does not fully capture probe dynamics in these systems. The composition-dependence of \(S_0\) and \(R_\perp\) for an Ld + Lo tieline in DSPC/DOPC/chol is shown in Figure A.3, for both three and six parameter fits. In both cases, \(S_0\) is greatest in the Lo phase \((\chi_{DSPC} > 0.64)\) and decreases rapidly at the onset of phase-coexistence \((\chi_{DSPC} = 0.64)\). The physical interpretation is straightforward: for compositions with coexisting Ld and Lo phases, the best-fit value represents an “average” for 16-PC distributed between two environments with markedly different chain segmental order. The magnitude of \(S_0\) clearly depends on which parameters are varied, an effect of strong parameter correlations. Importantly, the trend in \(S_0\) (including the location of the break-point) is the same for both sets. This is not the case for \(R_\perp\) (Figure A.3 B). With the larger adjustable parameter set, a steep decrease in \(R_\perp\) occurs with the onset of Ld phase, concomitant with the decrease in \(S_0\), while \(R_\perp\) for the three-parameter fit remains unchanged. As the fraction of Ld phase increases, \(R_\perp\) eventually increases for both sets, reaching a final value in pure Ld that is approximately twice as large as the Lo value. The initial decrease seen in the larger parameter set is clearly unphysical: the appearance of a small fraction of Ld phase with a faster rate of off-axis diffusion should result in a larger observed value for \(R_\perp\).
Figure A.2 Best-fit NLSL spectra for six- and three-parameter fits. The experimental Ld (A) and Lo (B) spectra from Figure A.1 are shown (gray lines), along with the best-fit NLSL spectra obtained by varying six (green line) or three (blue line) parameters.
Figure A.3 Composition dependence of selected parameters for six- and three-parameter fits. (A) For both six (green circles) and three (blue triangles) parameter fits, the order parameter $S_0$ exhibits a sharp decrease at $\chi_{DSPC} = 0.64$, corresponding to a phase transition. The magnitude of $S_0$ depends on the number of varied parameters, but the trend does not. (B) The best-fit value of $R_\perp$ exhibits an unphysical decrease at the onset of Ld phase for the six-parameter fit (green circles), due to strong correlations with the other fit parameters. The correlations are greatly reduced in the three-parameter fit (blue triangles).
Figure A.3 demonstrates that MOMD simulations should be interpreted with caution at low ESR frequencies. Lou et al. showed that, for 16-PC in DPPC and DPPC/chol bilayers, the dynamic conditions required for a rigorous interpretation of fitting parameters in terms of internal acyl-chain motions are not met at 9.5 GHz: the rates of slower, overall tumbling motions of the lipid are not so small as to be in the rigid limit, and so affect the spectrum (3). These slower motions are accounted for in some ill-defined way by the free parameters, and the final best-fit values therefore represent an averaging of internal (i.e., near the chain end) and overall motions. Nevertheless, the trend in $S_0$ as the temperature was raised through DPPC’s melting transition was identical at 9.5 GHz and 250 GHz, the latter a frequency at which the slow overall motions are frozen out. At both frequencies the melting transition at ~40°C was observed as a sharp decrease in $S_0$. Based on this result, we have confidence that the break-points seen in $S_0$ correspond to the redistribution of 16-PC between high- and low-order environments, even though the magnitude cannot be rigorously interpreted at 9.5 GHz.
REFERENCES


APPENDIX B
A modification of the FP-FRET model to account for different bilayer thicknesses in the two phases

In this Appendix we extend the FP-FRET model developed in Chapter 4 for a monolayer to include the following, more physically realistic assumptions:

1. Within a monolayer, donor and acceptor are located at fixed (though potentially different) distances from the bilayer midplane.
2. In the bilayer, the phase behavior of the two monolayers is coupled: Domains are always in registration across the bilayer.

We start with an expression for transfer efficiency as a weighted sum of $E$ from two donor pools, those in the domain phase and those in the surround phase:

$$E = f_d^d E^d + f_d^s E^s = f_d^d (1 - q_r^d) + f_d^s (1 - q_r^s) \quad \text{B.1}$$

In a bilayer the quenching term is given by

$$q_r = \int_0^\infty e^{-t/\tau_0} e^{-S_{same}(t)} e^{-S_{diff}(t)} dt \quad \text{B.2}$$

where the terms $S_{same}$ and $S_{diff}$ account for energy transfer from donors to acceptors in the same or opposing leaflets, respectively. In favorable cases the transverse location of the donor and acceptor fluorophore (that is, their position in the direction of the bilayer normal) has a narrow distribution and can be approximated by the mean. In these cases, a general form of $S(t)$ is:

$$S_{same}(t) = \int_a \langle n_A(r) \rangle 2\pi r \left[ 1 - e^{-(t/\tau_0)(r_0/\sqrt{|d-a|^2+r^2})^6} \right] dr \quad \text{B.3}$$
where \(d\) and \(a\) are the positions of the donor and acceptor planes with respect to bilayer midplane (see Figure 5.1), and \(\langle n_A(r) \rangle\) is the appropriate acceptor surface density function.

Equations B.3-4 account for two different acceptor pools seen by a given donor: those in the same leaflet, and those in the opposite leaflet. If the transverse location of donor and acceptor planes is different in the coexisting phases, the number of acceptor pools doubles to four. As an example, consider the case where small domains of Lo phase exist in a continuous Ld phase. If the positions of donor planes in the two phases are given by \(d^d\) and \(d^s\), and that of the acceptor planes by \(a^d\) and \(a^s\), then donors in domains can transfer energy to acceptors at relative positions \(|d^d - a^d|\), \(|d^d - a^s|\), \(|d^d + a^d|\), and \(|d^d + a^s|\). The relative population of these pools is related to the fraction of acceptors found in domains \(f_A^d(r)\), given by

\[
\langle f_A^d(r) \rangle = \langle \chi^d(r) \rangle K_A^d / [1 + \langle \chi^d(r) \rangle (K_A^d - 1)]
\]

where \(\langle \chi^d(r) \rangle\) is the mole fraction of domain phase:

\[
\langle \chi^d(r) \rangle = \langle \sigma^d(r) \rangle / a^d / [(\langle \sigma^d(r) \rangle / a^d + (1 - \langle \sigma^d(r) \rangle) / a^s] \]

and \(\langle \sigma^d(r) \rangle\) is the domain surface coverage, found by rearranging Equation 4.20:

\[
\langle \sigma^d(r) \rangle = (\langle n_A^d(r) \rangle - n_A^s) / (n_A^d - n_A^s)
\]

With these definitions,
The ensemble-averaged functions $\langle \sigma^d(r) \rangle$, $\langle \chi^d(r) \rangle$, $\langle n_A(r) \rangle$, and $\langle f_A^d(r) \rangle$ are different for the two donor pools (those in domains, and those in the surround) as discussed in Chapter 4. Finally, it should be emphasized that all of the ensemble-averaged functions are derived from a single radial distribution function $g(r; f)$. 

\begin{align*}
S_{\text{same}}(t) &= \int_a^\infty \langle f_A^d(r) \rangle \langle n_A(r) \rangle 2\pi rdr \left[ 1 - e^{-\left(\frac{t}{\tau_0}\right) \left( R_0/\sqrt{|d^d - a^d|^2 + r^2} \right)^6} \right] dr \\
&+ \int_a^\infty (1 - \langle f_A^d(r) \rangle) \langle n_A(r) \rangle 2\pi rdr \left[ 1 - e^{-\left(\frac{t}{\tau_0}\right) \left( R_0/\sqrt{|d^d - a^d|^2 + r^2} \right)^6} \right] dr \tag{B.8}
\end{align*}

\begin{align*}
S_{\text{diff}}(t) &= \int_a^\infty \langle f_A^d(r) \rangle \langle n_A(r) \rangle 2\pi rdr \left[ 1 - e^{-\left(\frac{t}{\tau_0}\right) \left( R_0/\sqrt{|d^d + a^d|^2 + r^2} \right)^6} \right] dr \\
&+ \int_a^\infty (1 - \langle f_A^d(r) \rangle) \langle n_A(r) \rangle 2\pi rdr \left[ 1 - e^{-\left(\frac{t}{\tau_0}\right) \left( R_0/\sqrt{|d^d + a^d|^2 + r^2} \right)^6} \right] dr \tag{B.9}
\end{align*}
APPENDIX C
A user's guide to the FP-FRET analysis program

C.1 Introduction

This Appendix provides an outline for conducting a domain size analysis with the FP-FRET model. Chapter 5 provides a detailed discussion of the FP-FRET analysis applied to simulated data, but necessarily omits details of running the code. The goal of this section is to provide those important details. The organization is as follows:

1. Experimental considerations
2. Organization of data files
3. Single trajectory analysis
4. Multiple trajectory analysis ("global fitting")

A working copy of Mathematica 7.0 (or higher) is required to run the code (though knowledge of the Mathematica programming language is not required). As discussed in Chapter 5, the code is optimized for a parallel Mathematica environment. The Feigenson lab currently has two such resources. The first is a multiple-platform Mathematica cluster consisting of 24 compute kernels, located on various computers in the Feigenson lab, and linked via Wolfram gridMathematica. The second is a 128 kernel grid installed on nodes 1 through 8 of Feigenson lab's LIPID computing cluster, housed in Rhodes Hall and serviced by Cornell Center for Advanced Computing. This resource can be accessed remotely via secure shell (ssh) network protocol. In this guide, we will only discuss the use of the second (faster) resource.
C.2 Experimental considerations

A discussion of experimental considerations is found in Chapter 5, and will only be summarized here. First, the analysis assumes that data are on a known tieline: for each composition, a value for the mole fraction of the Lo phase (calculated from the lever rule) must be supplied in addition to the observed value of FRET efficiency. Values for molecular areas of the coexisting phases and the percolation threshold must be known. The following probe parameters must be known or estimated: transverse bilayer location, donor lifetime, donor quantum yield, donor and acceptor chromophore radius, and total acceptor mole fraction. The results of Chapter 5 indicate that at a minimum, the following parameters can be uniquely determined from large data sets (containing > 50 data points): donor and acceptor $K_p$, domain radius, and $R_0$. It is possible that additional parameters can be uniquely determined, though care must be taken to ensure the integrity of the result (i.e., parameter correlations should be examined).

When collecting data, is it advisable to use RSE samples to avoid artifactual precipitation of bilayer components, and to extrude the RSE preparation to generate unilamellar vesicles (cross-bilayer energy transfer is not accounted for in this version of the program). The FP-FRET equations are derived for transfer efficiency ($E$), which is typically calculated from donor quenching in the presence and absence of acceptor; parallel samples should therefore be prepared at each composition. It is further advised to prepare and measure sample replicates so that a standard deviation can be calculated for each data point.

C.3 Organization of data files and code

At this point in the discussion, it is assumed that the user has collected one or multiple data trajectories along a tieline of phase coexistence. It is also assumed that
the user has an account on the Feigenson lab LIPID computing cluster (lipid.cac.cornell.edu), and has an open terminal session on the head node. Execute the following steps to prepare data files and code for the analysis:

1. Create a working directory, e.g. home/fs03/yourNetID/working/

2. Upload the following files to the working directory (these files are found in the feigensonlab MBG shared folder, in the directory software/fpfret, or can be requested from the author): fpfret.txt; rdf.dat; paramsTemplate.txt; paramsGTemplate.txt; script.sh

3. Prepare tab delineated data files for each experimental trajectory using the following 4-column format: mole fraction acceptor \t mole fraction Lo phase \t observed transfer efficiency \t measurement standard deviation \n. (Do not include column headers. The \t and \n notation stands for tab and newline, respectively. An example data file is found in Appendix D). Name the files with a .dat extension and upload them to the working directory.

C4. Single trajectory analysis

The fpfret program (which comprises the files fpfret.txt and rdf.dat) requires a parameter file containing the following information: which model parameters to hold and which to vary; fixed or initial values for the parameters; the names of data files and log files generated during the fit; the requested number of computational kernels; and other information used by Mathematica. The simplest approach to setting up the analysis is to copy the parameter template file (paramsTemplate.txt, or paramsGTemplate.txt for a global analysis), and then rename and modify it as described presently.

The following step-by-step guide assumes the existence of a data file (myData.dat) and a copied parameter template file (myParams.txt). The parameter file
contains executable Mathematica code, as well as some unexecuted Mathematica comments, which are set apart by leading "(*" and trailing ")" characters:

(* This is an example comment; Mathematica will not execute any text between the asterisks. *)

Preparing a parameter file for a new data set involves modifying just a few values in the parameter template file; the bulk of this file will remain unchanged. For the following example, the complete parameter file is found in Appendix E.

### C.4.1 Computational kernels

Specify the number of computational kernels to be used by the fitting routine. This is done by "uncommenting" either section 1A or 1B (i.e., deleting the leading "(*" and trailing ")" characters from the block of text). For single trajectory fits, option 1B is recommended with 16 kernels; in this case, the initial lines of the parameter file should appear as follows:

(* Step 1: ONE (and only one) of the following 2 blocks of code is required to launch computation kernels. ONE of the blocks should be uncommented, and the other should be commented out. *)

(* 1A: if > 16 nodes are desired, uncomment the following options and run the code from the command line on the head node *)

(* <<ClusterIntegration`; numKernels=16*8; LaunchKernels[PBS["localhost","QueueName"->"math",numKernels]]; *)

(* 1B: if <= 16 nodes are desired, uncomment the following options and run the code with a submission script *)

numKernels=16;
LaunchKernels[numKernels];

For global fits of several trajectories, option 1A is recommended with the number of kernels set to 16*n, where n is the number of available nodes in the math queue (nodes 1-8, each of which has a licensed copy of Mathematica). Note that the values 16*8 and 128 are both interpreted as 128 by Mathematica; the former notation is useful, as the number of processes (16) and the number of compute nodes (8) are
explicitly stated. In general, all 16 processes of a given node should be requested; on
the other hand, the number of requested nodes will frequently differ, for example
when other users are running jobs on some of the math nodes.

There is an important difference in the way options 1A and 1B are executed on
the cluster. For option 1B, a submission script is used to send the job to a single
compute node in the math queue: all parallel function evaluations are performed on the
16 available kernels on this single node. For option 1A, the code is executed from the
command line on the head node. A local copy of Mathematica is then opened on the
head node, and multiple computational kernels are subsequently opened on nodes 1-8
(as specified by the variable numKernels in myParams.txt). Job submission is covered
in Sections C.4.8 and C.5.6.

C.4.2 File names

Specify the names of the data file, parameter file, and log file:

dataFiles={
    "myData.dat"
};
parameterFile="myParams.txt"; (* name of this parameter file*)
logFileName="myData1.log"; (* name of the log file *)

C.4.3 Probe parameters

Each unique probe in the system is identified by a lowercase letter. For a single
trajectory as in this example, we will use "a" to identify the donor and "b" to identify
the acceptor. Note that in a multiple trajectory analysis, probe "b" may be an acceptor
in one trajectory and a donor in another trajectory (for example).

For each probe, the partition coefficient and chromophore radius are specified
in the list probeData:

probeData={
    {"kalo",Log[2.5],1},("ra",0.35,0), (* probe "a" KP and radius *)
    {"kblo",Log[0.1],1},("rb",0.67,0) (* probe "b" KP and radius *)
};
In this example, probe "a" has 2.5-fold partition into Lo phase, and a radius of 0.35 nm; probe "b" has 0.1-fold partition into Ld phase (i.e., 10-fold partition into Ld phase) and a radius of 0.67 nm. (Note that for the nonlinear least-squares routine, partition coefficients are converted to a base $e$ log; a linear-space $K_P$ can either be entered as the argument of Mathematica's Log function as shown above, or the numerical logarithmic value can be directly specified as the second entry in the $K_P$ list. The results are reported in base $e$ logarithmic form in the log file.) The third entry in the $K_P$ and radius lists tells Mathematica whether the parameter value should be held fixed (0) or varied (1) during the fit; in this example, both partition coefficients will be varied, and both radii will be fixed (recommended).

C.4.4 Probe pairings

Identify the donor and acceptor for each data trajectory using the lowercase letters from the previous step. The list dataSetProbeDefs must contain one entry for each data file in the list dataFiles (see Step 2), and in the same order as the file list. Each entry in dataSetProbeDefs first lists the donor and then the acceptor for a given trajectory. For our example single trajectory analysis with donor probe "a" and acceptor probe "b", we have:

```plaintext
dataSetProbeDefs={
    {"a", "b"}
};
```

C.4.5 Energy transfer parameters

Specify the Förster distance for each probe pair, and whether the value should be fixed (0) or varied (1) in the fit. In the following example, $R_0$ is set to 2.5 nm and held fixed:

```plaintext
r0Data={
    {"R0ab", 2.5, 0}
};
```
C.4.6 Bilayer structural parameters

The list `bilayerData` contains bilayer structural parameters, including molecular areas of phases, percolation threshold, transverse locations of probes, and domain radius. Typically these parameters will be estimated from literature data and held fixed, with the exception of the domain radius. For our example, we have:

```plaintext
bilayerData={
    {"ald",0.583,0}, (* molecular area of Ld phase in nm2 *)
    {"alo",0.45,0}, (* molecular area of Lo phase in nm2 *)
    {"perc",0.5,0}, (* percolation threshold along tieline *)
    {"hald",1.35,0}, (* acceptor transverse location in Ld phase bilayer, in nm *)
    {"hdld",1.35,0}, (* donor transverse location in Ld phase bilayer, in nm *)
    {"halo",1.75,0}, (* acceptor transverse location in Lo phase bilayer, in nm *)
    {"hdlo",1.75,0}, (* donor transverse location in Lo phase bilayer, in nm *)
    {"R",N[Log[10]],1} (* domain radius in nm *)
};
```

Note that in this example, an initial guess of 10 nm is supplied for the domain radius, which is converted to logarithmic space.

C.4.7 Additional parameters

The final block of code specifies some parameters for the fitting routine (a convergence criterion, the maximum number of iterations before termination, and a distance cutoff for calculating transfer efficiency in the local vicinity of the donor), as well as some Mathematica-specific parameters. The values listed in the template file are appropriate for these simulations and should not be modified.

C.4.8 Submitting the job

The final step is to run the job. For a single-trajectory fit using option 1B (see Step 1), this is done with a submission script. The working directory contains a template submission script (script.sh), which is reproduced in Appendix F. In general, only the parameter file name needs updating:

```plaintext
/usr/local/bin/math -run "<<myParams.txt;<<fpfret.txt"
```
If desired, a unique name for the job can also be specified (this is the name that will show up in the job queue on the cluster; note that multiple jobs can have the same name):

```bash
#PBS -N myJobName
```

To submit the job from the working directory, execute the following command at the prompt:

```bash
qsub script.sh
```

Job progress can be monitored online at http://lipid.cac.cornell.edu/ganglia/.

When the job has completed, two files will appear in the working directory: a log file containing fit information and statistics; and a file containing the original and best-fit data points (named by appending "_bestFit.dat" to the data file name).

### C.5 Multiple trajectory analysis ("global fitting")

The parameter file for global fitting is similar to that for single-trajectory fitting, with a few important differences in Steps 1-5. In addition, job submission is handled differently when multiple nodes are requested (recommended for global analysis). The following example assumes the existence of three data trajectories that share a common donor (probe "a"), but have different acceptors (probes "b", "c", and "d"). Copy `paramsGTemplate.txt`, rename it `myParamsG.txt`, and modify it as follows (N.B.: the complete multiple-trajectory parameter file is reproduced in Appendix G).

#### C.5.1 Computational kernels

Specify the number of computational kernels to be used by the fitting routine by uncommenting either section 1A or 1B (option 1A is recommended for global analysis):

```bash
(* Step 1: ONE (and only one) of the following 2 blocks of code is required to launch computation kernels. ONE of the blocks should be uncommented, and the other should be commented out. *)

(* 1A: if > 16 nodes are desired, uncomment the following options and run the code from the command line on the head node *)
```
<<ClusterIntegration';
numKernels=16*8;
LaunchKernels[PBS["localhost","QueueName"->"math",numKernels];

(* 1B: if <= 16 nodes are desired, uncomment the following options
and run the code with a submission script *)

(* numKernels=16;
LaunchKernels[numKernels]; *)

In this example, it is assumed that all 8 math nodes are available for use.

C.5.2 File names

Specify the names of the data files, parameter file, and log file:

dataFiles={
"myData1.dat",
"myData2.dat",
"myData3.dat"
};
parameterFile="myParamsG.txt"; (* name of this parameter file*)
logFileName="myDataG1.log"; (* name of the log file *)

C.5.3 Probe parameters

Specify the partition coefficient and chromophore radius for each unique probe
in the data sets:

probeData=
{"kalo",Log[2.5],1},{"ra",0.35,0}, (* probe "a" KP and radius *)
{"kblo",Log[0.1],1},{"rb",0.67,0}, (* probe "b" KP and radius *)
{"kclo",Log[0.25],1},{"rc",0.67,0}, (* probe "c" KP and radius *)
{"kdlo",Log[4],1},{"rd",0.67,0} (* probe "d" KP and radius *)
};

In this example, the probe partition coefficients will be varied, and the radii
will be held fixed.

C.5.4 Probe pairings

Identify the donor and acceptor for each data trajectory using the lowercase
letters from the previous step:

dataSetProbeDefs=
{"a","b"},
{"a","c"},
{"a","d"}
In this example, a single donor (probe "a") is paired with different acceptors (probes "b", "c", and "d").

C.5.5 Energy transfer parameters

Specify the Förster distance for each probe pair, and whether the value should be fixed (0) or varied (1) in the fit. In the following example, the same value of $R_0$ is used for each probe pair (2.5 nm) and held fixed:

```plaintext
r0Data={
    "R0ab", 2.5, 0,
    "R0ac", 2.5, 0,
    "R0ad", 2.5, 0
};
```

C.5.6 Submitting the job

From the working directory, execute the following command at the prompt:

```plaintext
nohup math -noprompt -run "<<myParamsG.txt;<<fpfret.txt" > output &
```

When the job has completed, several files will appear in the working directory: a log file containing fit information and statistics; and a set of files (one for each trajectory) containing the original and best-fit data points (named by appending "_bestFit_global.dat" to the data file name).
APPENDIX D
FP-FRET analysis: Example data file

The following is an example data file for the FP-FRET analysis described in Chapter 5 and Appendix C. The file must contain four columns with no column headers: mole fraction acceptor \t mole fraction Lo phase \t observed transfer efficiency \t measurement standard deviation \n. The \t and \n notation stands for tab and newline, respectively.

0.005 0.0 0.200 0.003
0.005 0.05 0.189 0.003
0.005 0.1 0.179 0.003
0.005 0.15 0.172 0.003
0.005 0.2 0.167 0.003
0.005 0.25 0.162 0.003
0.005 0.3 0.157 0.003
0.005 0.35 0.155 0.003
0.005 0.4 0.151 0.003
0.005 0.45 0.150 0.003
0.005 0.5 0.148 0.003
0.005 0.55 0.151 0.003
0.005 0.6 0.148 0.003
0.005 0.65 0.146 0.003
0.005 0.7 0.143 0.003
0.005 0.75 0.146 0.003
0.005 0.8 0.143 0.003
0.005 0.85 0.149 0.003
0.005 0.9 0.162 0.003
0.005 0.95 0.179 0.003
0.005 1.0 0.223 0.003
APPENDIX E
FP-FRET analysis: Example parameter file for single-trajectory analysis

(* Step 1: ONE (and only one) of the following 2 blocks of code is required to launch computation kernels. ONE of the blocks should be uncommented, and the other should be commented out. *)

(* 1A: if > 16 nodes are desired, uncomment the following options and run the code from the command line on the head node *)

(* <<ClusterIntegration'; numKernels=16*8; LaunchKernels[PBS["localhost","QueueName"->"math",numKernels]; *)

(* 1B: if <= 16 nodes are desired, uncomment the following options and run the code with a submission script *)

numKernels=16;
LaunchKernels[numKernels];

(* Step 2: Specify FILE NAMES *)

dataFiles={
"myData.dat"
};
parameterFile="myParams.txt"; (* name of this parameter file*)
logFileName="myData1.log"; (* name of the log file *)

(* Steps 3 through 6: specify model parameters. Parameter lists have three elements: {"stringName",realValue,intVary}, where realValue represents a fixed (or initial) value for the parameter, and intVary MUST be either 0 or 1 (if 0, the parameter will remain fixed during the fitting, if 1 it will be adjusted). *)

(* Step 3: Specify PROBE PARAMETERS: there must be one entry for each probe in the system. probe kp and radius must be lettered sequentially, i.e. "kalo", "ra", "kblo", "rb", "kclo", "rc", etc... Note that the value of KP is itself specified in linear space inside a Log function call; values > 1 indicate preference for the Lo phase, and values between 0 and 1 indicate preference for the Ld phase. *)

probeData={
{"kalo",Log[2.5],1},{"ra",0.35,0}, (* probe "a" KP and radius *)
{"kblo",Log[0.1],1},{"rb",0.67,0} (* probe "b" KP and radius *)
};

(* Step 4: Specify PROBE PAIRINGS: there must be one entry for each data file. Defines the donor and acceptor for each data file (in the order they are listed above). Contains two characters identifying
donor and acceptor (in that order) *)

dataSetProbeDefs=
{"a","b"}
);

(* Step 5: Specify ENERGY TRANSFER PARAMETERS: there must be one
entry per data set, corresponding to the probe letters of the energy
transfer pair for that data set *)

r0Data=
{"R0ab",2.5,0}
);

(* Step 6: Specify BILAYER STRUCTURAL PARAMETERS: change the last two
entries in each list to specify the fixed (or initial) value of the
parameter, and whether it should be fixed (0) or varied (1) in the
fit *)

bilayerData=
{"ald",0.583,0}, (* molecular area of Ld phase in nm2 *)
{"alo",0.45,0}, (* molecular area of Lo phase in nm2 *)
{"perc",0.5,0}, (* percolation threshold along tieline *)
{"hald",1.35,0}, (* acceptor transverse location in Ld phase bilayer
in nm *)
{"hdld",1.35,0}, (* donor transverse location in Ld phase bilayer in
nm *)
{"halo",1.75,0}, (* acceptor transverse location in Lo phase bilayer
in nm *)
{"hdlo",1.75,0}, (* donor transverse location in Lo phase bilayer in
nm *)
{"R",N[Log[10]],1} (* domain radius in nm *)
);

(* Step 7: Specify additional parameters *)

ptol=0.001; (* parameter convergence criterion *)
maxIterations=20; (* maximum iterations *)
precision=10; (* numerical precision *)
distanceCutoff=20*R0; (* only acceptors within a cutoff radius of a
given donor will be counted *)
integrationAccuracyGoal=5; (* Mathematica parameter *)
integrationMethod=Automatic; (* Mathematica parameter *)
parallelMethod="FinestGrained"; (* Mathematica parameter *)
APPENDIX F
FP-FRET analysis: Example submission script for single-trajectory analysis

#!/bin/sh
#PBS -N myJobName
#PBS -A gwf3_0001
#PBS -v np=16
#PBS -l nodes=1:ppn=16
#PBS -q math
#PBS -j oe
#PBS -S /bin/bash

cd "$PBS_O_WORKDIR"

/usr/local/bin/math -run "<<myParams.txt;<<fpfret.txt"

cp $PBS_O_WORKDIR/* $PWD
APPENDIX G

FP-FRET analysis: Example parameter file for multiple-trajectory (global) analysis

(* Step 1: ONE (and only one) of the following 2 blocks of code is *
required to launch computation kernels. ONE of the blocks should be *
uncommented, and the other should be commented out. *)

(* 1A: if > 16 nodes are desired, uncomment the following options and *
run the code from the command line on the head node *)

<<ClusterIntegration`;
numKernels=16*8;
LaunchKernels[PBS("localhost","QueueName"->"math",numKernels);

(* 1B: if <= 16 nodes are desired, uncomment the following options *
and run the code with a submission script *)

(* numKernels=16;
LaunchKernels[numKernels]; *)

(* Step 2: Specify FILE NAMES *)

dataFiles={
"myData1.dat",
"myData2.dat",
"myData3.dat"
};
parameterFileName="myParamsG.txt"; (* name of this parameter file*)
logFileName="myDataG1.log"; (* name of the log file *)

(* Steps 3 through 6: specify model parameters. Parameter lists have *
three elements: {"stringName",realValue,intVary}, where realValue *
represents a fixed (or initial) value for the parameter, and intVary *
MUST be either 0 or 1 (if 0, the parameter will remain fixed during *
the fitting, if 1 it will be adjusted). *)

(* Step 3: Specify PROBE PARAMETERS: there must be one entry for each *
probe in the system. probe kp and radius must be lettered sequentially, ie. "kalo", "ra", "kblo", "rb", "kclo", "rc", etc... *
Note that the value of KP is itself specified in linear space inside *
a Log function call; values > 1 indicate preference for the Lo phase, *
and values between 0 and 1 indicate preference for the Ld phase. *)

probeData={
{"kalo",Log[2.5],1},{"ra",0.35,0}, (* probe "a" KP and radius *)
{"kblo",Log[0.1],1},{"rb",0.67,0}, (* probe "b" KP and radius *)
{"kclo",Log[0.25],1},{"rc",0.67,0}, (* probe "c" KP and radius *)
{"kdlo",Log[4],1},{"rd",0.67,0} (* probe "d" KP and radius *)
};
(* Step 4: Specify PROBE PAIRINGS: there must be one entry for each
data file. Defines the donor and acceptor for each data file (in the
order they are listed above). Contains two characters identifying
donor and acceptor (in that order) *)

dataSetProbeDefs=
{  {"a","b"},  
  {"a","c"},  
  {"a","d"}  
};

(* Step 5: Specify ENERGY TRANSFER PARAMETERS: there must be one
entry per data set, corresponding to the probe letters of the energy
transfer pair for that data set *)

r0Data=
{  "{R0ab},2.5,0",  
  "{R0ac},2.5,0",  
  "{R0ad},2.5,0"  
};

(* Step 6: Specify BILAYER STRUCTURAL PARAMETERS: change the last two
entries in each list to specify the fixed (or initial) value of the
parameter, and whether it should be fixed (0) or varied (1) in the
fit *)

bilayerData=
{  "{ald},0.583,0", (* molecular area of Ld phase in nm2 *)  
  "{alo},0.45,0", (* molecular area of Lo phase in nm2 *)  
  "{perc},0.5,0", (* percolation threshold along tieline *)  
  "{hald},1.35,0", (* acceptor transverse location in Ld phase bilayer
  in nm *)  
  "{hdlid},1.35,0", (* donor transverse location in Ld phase bilayer in
  nm *)  
  "{halo},1.75,0", (* acceptor transverse location in Lo phase bilayer
  in nm *)  
  "{hdlo},1.75,0", (* donor transverse location in Lo phase bilayer in
  nm *)  
  "{R",N[Log[10]],1} (* domain radius in nm *)  
};

(* Step 7: Specify additional parameters *)

ptol=0.001; (* parameter convergence criterion *)
maxIterations=20; (* maximum iterations *)
precision=10; (* numerical precision *)
distanceCutoff=20*R0; (* only acceptors within a cutoff radius of a
given donor will be counted *)
integrationAccuracyGoal=5; (* Mathematica parameter *)
integrationMethod=Automatic; (* Mathematica parameter *)
parallelMethod="FinestGrained"; (* Mathematica parameter *)