Homo-FRET Imaging of CEACAM1 in Living Cells using Total Internal Reflection Fluorescence Polarization Microscopy

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
The Institute of Biomaterials and Biomedical Engineering
University of Toronto

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2012

Abstract

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) undergoes homotypic and heterotypic \textit{cis}- and \textit{trans}- interactions that regulate processes including metabolism, immune response, and tumorigenesis. To better understand and eventually control CEACAM1, we characterized the localization, homotypic \textit{cis}-oligomerization, and regulation of CEACAM1 at the molecular scale using steady-state TIRFPM homo-FRET imaging in living cells. We established the anisotropy sensitivity of our TIRFPM platform using Venus monomers and dimers, which had significantly different anisotropy values. Heterogeneously distributed across the plasma membrane, CEACAM1-4L-EYFP was a mixture of monomers and oligomers, with a slightly more monomeric population at the high intensity regions. In addition, perturbation with ionomycin or $\alpha$-CEA pAb increased CEACAM1 monomers, potentially in a localized manner. Although limited in detecting anisotropy differences between CEACAM1-4L-EYFP and monomeric G432,436L-CEACAM1-4L-EYFP populations, TIRFPM homo-FRET imaging can be a useful tool for studying membrane protein self-association with proper controls and studies that focus on relative anisotropy changes.
Acknowledgments

This body of work was only possible with the mentorship, support, intellectual discourse, and friendship of many amazing people.

I am extremely grateful to Dr. Christopher Yip for his sharp insight, enduring patience, and steadfast guidance, which made my my graduate studies possible.

I would also like to thank Dr. Jonathan Rocheleau and Dr. Scott Gray-Owen for their mentorship, which have been instrumental sources of inspiration for my graduate studies.

Thank you to my Toronto family, who sang and danced with me on those late lab nights, always laughed so loud that the entire floor knew we had arrived, and fed my mind, my spirit, and my stomach. Thank you — you guys know who you are — for preserving my sanity.

Finally, thank you to my home friends and family, who always grounded and supported me.
## Table of Contents

### Contents

Acknowledgments .......................................................................................................................... iii

Table of Contents .......................................................................................................................... iv

List of Abbreviations .................................................................................................................... viii

List of Tables .................................................................................................................................. x

List of Figures ................................................................................................................................ xi

List of Appendices ......................................................................................................................... xiii

Chapter 1 ......................................................................................................................................... 1

1 Introduction ................................................................................................................................ 1

1.1 CEACAM1 ................................................................................................................................ 1

1.1.1 CEACAM1 Structure .............................................................................................. 1

1.1.2 Interactions and Regulation of CEACAM1 ............................................................ 2

1.1.3 CEACAM1 Heterotypic Interactions ....................................................................... 2

1.1.4 CEACAM1 Homotypic Interactions ....................................................................... 4

1.1.5 Regulation of *cis*- and *trans*- Heterotypic and Homotypic Interactions ............... 5

1.2 Fluorescence Imaging Techniques ...................................................................................... 6

1.2.1 Fluorescence Background ....................................................................................... 6

1.2.2 Fluorescence Imaging Techniques to Study Protein Interactions ......................... 8

1.2.3 Hetero-FRET Imaging .......................................................................................... 10

1.2.4 Steady-State Homo-FRET Imaging ...................................................................... 12

1.2.5 Applications of Homo-FRET Imaging ................................................................. 16

1.2.6 Homo-FRET Imaging in Total Internal Reflection Fluorescence Polarization Microscopy ......................................................................................................................... 17

Chapter 2 ....................................................................................................................................... 19
2 Steady-State Homo-FRET TIRFPM Imaging Instrumentation Development and Calibrations ................................................................. 19

2.1 Chapter Summary ........................................................................................................................................................................... 19

2.2 Background ......................................................................................................................................................................................... 19

2.3 Materials and Methods ................................................................................................................................................................. 21

2.3.1 Molecular Cloning ........................................................................................................................................................................ 21

2.3.2 Cell Culture and Transfections ................................................................................................................................................. 21

2.3.3 Immunoblot ................................................................................................................................................................................... 21

2.3.4 TIRFM Instrumentation and Imaging ................................................................................................................................. 22

2.3.5 Homo-FRET Imaging in TIRFPM ........................................................................................................................................... 22

2.3.6 Homo-FRET Imaging in Epifluorescence .................................................................................................................................. 24

2.3.7 Statistical Analysis .................................................................................................................................................................... 24

2.4 Results .............................................................................................................................................................................................. 25

2.4.1 Epifluorescence and TIRFPM Anisotropy Distinguishes Monomeric and Dimeric Venus ....................................................................................................................................................................................... 25

2.4.2 Investigation of Potential Homo-FRET Imaging Artifacts ........................................................................................................ 27

2.5 Discussion ....................................................................................................................................................................................... 29

Chapter 3 .......................................................................................................................................................................................... 30

3 Steady-State Homo-FRET TIRFPM Imaging of EYFP-labeled CEACAM1 ................................................................. 30

3.1 Chapter Summary ............................................................................................................................................................................. 30

3.2 Background ..................................................................................................................................................................................... 30

3.3 Materials and Methods .................................................................................................................................................................. 34

3.3.1 Generation of EYFP-labeled CEACAM1 Mutants ................................................................................................................................. 34

3.3.2 Cell Culture and Transfections ................................................................................................................................................. 34

3.3.3 TIRFM Settings ........................................................................................................................................................................... 34

3.3.4 FITC-Dextran Flow-through Experiment in TIRFM ................................................................................................................................................. 35

3.3.5 Homo-FRET Imaging in TIRFPM ........................................................................................................................................... 35
3.3.6 Time-lapse Homo-FRET Imaging in TIRFPM ................................. 37
3.3.7 Two-color Imaging to determine Anisotropy at Junctions in TIRFPM .... 37
3.4 Results ........................................................................................................... 38
  3.4.1 CEACAM1 has Heterogeneous Distribution across the HeLa Cell Surface .... 38
  3.4.2 Steady-state Anisotropy Images of CEACAM1-4L-EYFP in TIRFPM ........ 39
  3.4.3 Investigation of Possible CEACAM1-4L-EYFP Homo-FRET Imaging Artifacts ................................................................. 41
  3.4.4 Characterization of EYFP-labeled CEACAM1 Mutants ............................ 43
  3.4.5 CEACAM1 $cis$-Homotypic Oligomer Response to Ionomycin ................ 46
  3.4.6 CEACAM1 $cis$- Homotypic Oligomer Response to $\alpha$-CEA pAb ............... 48
  3.4.7 CEACAM1 exists primarily as $cis$-Oligomers at Cell-Cell Contacts ....... 51
3.5 Discussion ....................................................................................................... 54
  3.5.1 Cytoplasmic EYFP Label ........................................................................ 54
  3.5.2 EYFP-labeled CEACAM1 Mutant Constructs ........................................... 55
  3.5.3 CEACAM1 $cis$-Homotypic Oligomerization in Response to Ionomycin .. 58
  3.5.4 CEACAM1-Substrate Contact ................................................................. 58
  3.5.5 CEACAM1 $cis$-Homotypic Oligomerization in Response to $trans$-Ligation by pAb ................................................................. 59
  3.5.6 CEACAM1 $cis$-Homotypic Oligomerization at Cell-Cell Contacts ......... 60
3.6 Conclusion ....................................................................................................... 62
  4 Conclusions and Future Directions ............................................................... 62
  4.1 Chapter Summary ....................................................................................... 62
  4.2 Conclusions and Future Directions ........................................................... 62
    4.2.1 Sensitivity of the TIRFPM System ......................................................... 62
    4.2.2 CEACAM1 $cis$-Homotypic Oligomerization, Structure, and Kinetics .... 64
    4.2.3 The Roles of CEACAM1’s Interactions in Inside-Out and Outside-In Signal Transduction ................................................................. 67
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>Acceptor</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>CEACAM</td>
<td>Carcinoembryonic antigen-related cell adhesion molecule</td>
</tr>
<tr>
<td>CEACAM1-L</td>
<td>CEACAM1-long cytoplasmic tail</td>
</tr>
<tr>
<td>CEACAM1-S</td>
<td>CEACAM1-short cytoplasmic tail</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>$D$</td>
<td>Donor</td>
</tr>
<tr>
<td>$\bar{E}$</td>
<td>Electric field</td>
</tr>
<tr>
<td>$E(\lambda)$</td>
<td>Excitation spectra</td>
</tr>
<tr>
<td>$E_{\text{FRET}}$</td>
<td>Efficiency of energy transfer</td>
</tr>
<tr>
<td>EYFP</td>
<td>Enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>$F(\lambda)$</td>
<td>Fluorescence intensity</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FCCS</td>
<td>Fluorescence cross-correlation spectroscopy</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence lifetime imaging microscopy</td>
</tr>
<tr>
<td>FPM</td>
<td>Fluorescence polarization microscopy</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>$F_{x,y,or z}$</td>
<td>Fluorescence intensity polarized along $x$-, $y$- or $z$- axis, respectively</td>
</tr>
<tr>
<td>$F_{\parallel}$</td>
<td>Fluorescence intensity through the emission polarizer oriented parallel to the excitation polarization</td>
</tr>
<tr>
<td>$F_{\perp}$</td>
<td>Fluorescence intensity through the emission polarizer oriented perpendicular to the excitation polarization</td>
</tr>
<tr>
<td>G</td>
<td>G factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>ICS</td>
<td>Image correlation spectroscopy</td>
</tr>
<tr>
<td>IgC-like</td>
<td>Immunoglobulin-constant-like</td>
</tr>
<tr>
<td>IgV-like</td>
<td>Immunoglobulin-variable-like</td>
</tr>
<tr>
<td>ITIM</td>
<td>Inhibitory tyrosine motif</td>
</tr>
<tr>
<td>$J(\lambda)$</td>
<td>Spectral overlap integral</td>
</tr>
<tr>
<td>$K_a, K_b, K_c$</td>
<td>High NA-correction factors</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>$n$</td>
<td>Refractive index</td>
</tr>
<tr>
<td>N</td>
<td>Noise</td>
</tr>
<tr>
<td>$N$</td>
<td>Number of cells</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>$P$</td>
<td>Probability for excitation</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PALM</td>
<td>Photoactivation localization microscopy</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>$Q$</td>
<td>Quantum yield</td>
</tr>
<tr>
<td>$r$</td>
<td>Fluorescence anisotropy</td>
</tr>
</tbody>
</table>
\( r_c \) High NA-corrected anisotropy
\( r_d \) Intermolecular distance
\( r_{\text{inf}} \) Limiting anisotropy
\( R_0 \) Förster distance at which FRET efficiency is 50%
\( S \) Signal
\( S_0 \) Ground state electronic energy level
\( S_1 \) First excited electronic energy level
\( \text{SDS} \) Sodium dodecyl sulfate
\( \text{SE} \) Standard error
\( \text{SHP} \) Src homology region 2 domain-containing phosphatase
\( \text{TIRFPM} \) Total internal reflection fluorescence polarization microscopy

\( \alpha \) Incident angle
\( \alpha_c \) Critical angle
\( \varepsilon \) Extinction coefficient
\( \theta \) Relative angle between fluorophore dipole moments
\( \Theta \) Angle between \( \vec{E} \) and \( \vec{\mu} \)
\( \kappa^2 \) Dipole orientation factor
\( \lambda \) Wavelength
\( \vec{\mu} \) Dipole moment
\( \sigma \) Half-cone angle of objective
\( \tau \) Fluorescence lifetime
\( \omega \) Rate of energy transfer
List of Tables

Table 2.1: Average $r_e$ of Monomeric and Dimeric Venus ......................................................... 27

Table 3.1: Mean $r_e$ of EYFP-labeled CEACAM1 Mutants ......................................................... 44

Table 3.2. Qualitative Assessment of CEACAM1-4L-EYFP cis- Homotypic Oligomers based on Anisotropy at Cell-Cell Contact Compared to the Rest of the Cell. ........................................ 54

Table 4.1: Summary of Imaging Techniques for Future CEACAM1 Studies............................ 70
List of Figures

Figure 1.1: Schematic of Naturally Occurring CEACAM1 Isoforms. .................................................. 2

Figure 1.2: Jablonski Diagram. ............................................................................................................. 7

Figure 1.3: Overview of Advanced Fluorescence Imaging Techniques. .............................................. 9

Figure 1.4: Overview of Motivations for FRET Approaches. .............................................................. 12

Figure 1.5: Schematic of Homo-transfer during Homo-FRET Imaging ............................................ 14

Figure 1.6: Schematic of TIRFM. .......................................................................................................... 18

Figure 2.1: TIRFPM Platform. ............................................................................................................... 23

Figure 2.2: Immunoblot of HeLa Cell Lysates Transiently Transfected with Monomeric (M) or Dimeric (D) Venus. ............................................................................................................. 25

Figure 2.3: Anisotropy Images of HeLa Cells Transiently Transfected with Soluble Venus Monomer and Venus Dimer ........................................................................................................... 26

Figure 2.4: TIRFPM Anisotropy Imaging Controls of HeLa Cells Transiently Transfected with Soluble Venus Monomer or Venus Dimer .................................................................................. 28

Figure 2.5: Progressive Photobleaching of HeLa Cells Transiently Transfected with Soluble Monomeric Venus or Dimeric Venus using TIRFPM ................................................................. 29

Figure 3.1: Schematic Diagram of EYFP-labeled CEACAM1 Mutants .................................................. 32

Figure 3.2: Representation of Updated Anisotropy Analysis ............................................................... 36

Figure 3.3: Time-lapse TIRFIM Imaging of CEACAM1-4L-EYFP’s Heterogeneous Distribution. ........................................................................................................................................ 38

Figure 3.4: TIRFPM Anisotropy Imaging of HeLa cells Transiently Transfected with CEACAM1-4L-EYFP ....................................................................................................................................... 40
Figure 3.5: TIRFPM Anisotropy Imaging Controls of HeLa Cells Transiently Transfected with CEACAM1-4L-EYFP ............................................................ 42

Figure 3.6: Progressive Photobleaching of HeLa Cells Transiently Transfected with CEACAM1-4L-EYFP in TIRFPM. ............................................................ 42

Figure 3.7: Representative Intensity and TIRFPM Anisotropy Images of EYFP-labeled CEACAM1 Mutants. ........................................................................ 45

Figure 3.8: Baseline Time-lapse Characterization of CEACAM1-4L-EYFP’s cis-Homotypic Oligomerization. ........................................................................ 47

Figure 3.9: Ionomycin Perturbation of CEACAM1 cis-Homotypic Oligomerization. ............. 48

Figure 3.10: FITC-Dextran Exclusion Studies in TIRFM ................................................. 50

Figure 3.11: pAb Perturbation of CEACAM1 cis-Homotypic Oligomerization...................... 52

Figure 3.12: CEACAM1 cis-Homotypic Oligomers at Cell-Cell Contacts.............................. 53
List of Appendices

Appendix 1: $r_c$ vs. Intensity for EYFP-labeled CEACAM1 Mutants................................. 77

Appendix 2: $r_c$ vs. Time for CEACAM1 Perturbation Studies........................................ 78

Appendix 3: Updated ImageJ Macro for CEACAM1 $r_c$ Calculation.................................. 79

Appendix 4: ImageJ Macro for Registration of Time-lapse Intensity and $r_c$ Images ............. 89
Chapter 1

1 Introduction

1.1 CEACAM1

Membrane protein interactions are important for regulating many cellular processes, including solute transport and signal transduction. The membrane protein, carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1) is a member of the CEA protein family known to affect downstream signaling processes ranging from metabolism to immune response through their multifaceted interactions. CEACAM1, in particular, has been linked to both pathogenic and tumorigenic pathways, although its effects vary depending on isoform, heterotypic and homotypic interactions, glycosylation state, and expression levels in different cell types (1).

1.1.1 CEACAM1 Structure

Like other members of the CEA family, CEACAM1 has multiple isoforms, with varying numbers of extracellular domains and cytoplasmic tail lengths (Figure 1.1). CEACAM1 is a transmembrane protein with immunoglobulin-like extracellular domains: up to three membrane-proximal immunoglobulin-constant-like (IgC-like) domains, and an N-terminal immunoglobulin-variable-like (IgV-like) domain containing a β-pleated sheet that is important for mediating trans- homotypic and heterotypic interactions (1). CEACAM1-4L, a naturally expressed CEACAM1 isoform, has 4 extracellular domains and a 71-amino acid long cytoplasmic tail, containing two inhibitory tyrosine motifs (ITIMs) that mediate CEACAM1 interactions with Src family protein tyrosine kinases and Src homology region 2 domain-containing phosphatase -1 and -2 (SHP-1 and SHP-2, respectfully) (Figure 1.1). Another naturally expressed isoform, CEACAM1-4S, has a 10-amino acid long cytoplasmic tail that lacks these ITIMs but maintains
its interactions with calmodulin (Figure 1.1) (1-2). CEACAM1’s numerous isoforms and their respective interactions further contribute to CEACAM1 complexity.

1.1.2 Interactions and Regulation of CEACAM1

CEACAM1 affects many signaling pathways by undergoing numerous cell type-dependent homotypic and heterotypic cis- and trans- interactions through its extracellular, transmembrane, and cytoplasmic domains. How these intracellular and intercellular, or cis- and trans- respectively, interactions affect each other (and ultimately the downstream cell signaling processes) is an important question that has only recently begun to be investigated at the single-cell and molecular level.

1.1.3 CEACAM1 Heterotypic Interactions

CEACAM1-4L’s cytoplasmic tail is responsible for many interactions with SHP-1 (3-4), SHP-2 (3), c-Src tyrosine kinase (4), calmodulin (5-6), and actin (2, 7), which are important for

![Figure 1.1: Schematic of Naturally Occurring CEACAM1 Isoforms.](image)

Predicted domain structure and glycosylation patterns of naturally occurring CEACAM1 isoforms with an amino-terminal IgV-like domain and varying number of membrane-proximal IgC-like domains are shown. Nomenclature number indicates number of extracellular domains, followed by letter indicating cytoplasmic tail length, long (L) or short (S). Reprinted from reference (1), with permission.
regulating downstream signaling. In murine intestinal CT51 cells, CEACAM1-L’s localization to cell-cell contacts was dependent on intact actin and increased upon over-expression of constitutively-activated Rho-like small GTPases like Rac1 and Cdc42, thereby revealing the importance of these proteins for proper CEACAM1 regulation (2). Although CEACAM1-L seemed to associate with actin indirectly in these CT51 rounded cells, in more adherent cells like the MC38 murine colon cancer cells, direct binding between CEACAM1-L as well as CEACAM1-S with G-actin was observed. However, interaction of CEACAM1-L, but not CEACAM1-S, with F-actin appeared to be dependent on cytoplasmic tail tyrosine phosphorylation, indicating that modifications to CEACAM1’s cytoplasmic tail can affect its interactions (2, 7). CEACAM1-S’s interactions with actin, on the other hand, were influenced by its heterotypic interactions with tropomyosin, and tropomyosin’s interactions with either CEACAM1-S or CEACAM1-L were, in turn, affected by the presence of actin or calmodulin (7). While CEACAM1’s cytoplasmic tail interacts with the cytoskeleton, it also initiates different signaling pathways through ITIM-dependent interactions with c-Src, SHP-1, and SHP-2 (1, 4). In addition to its cis-interactions, CEACAM1 also undergoes heterotypic interactions in trans.

Trans-heterotypic interactions with CEACAM1’s extracellular domain dramatically influences proper immune cell function. Trans-ligation of CEACAM1 with Opa proteins expressed on pathogenic Neisseria reduces proper activation of human periphery CD4+ cells (8) and dendritic cells (1), thereby suppressing the normal immune response to these pathogens. In addition, trans-ligation of CEACAM1 with monoclonal anti-CEACAM1 antibody has been shown to inhibit some lymphokine-activated killer activity (9). CEACAM1 can also interact with other members of the CEA family. Trans-interactions between CEACAM1 in natural killer cells and CEACAM5 in target cells inhibit natural killer cell-mediated cytotoxicity; this has serious implications for tumor growth since some melanoma patients have high CEACAM1 expression in their natural killer cells (10). Heterotypic interactions mediated through CEACAM1’s extracellular domain, clearly have important downstream immunological effects.
CEACAM1 not only undergoes numerous *cis-* and *trans-* heterotypic interactions, but also homotypic interactions. Therefore, it is important to understand the relationship between, and the signaling roles of, CEACAM1’s heterotypic and homotypic interactions.

1.1.4 CEACAM1 Homotypic Interactions

Human CEACAM1-4L (6) and rat CEACAM1-L (5) have been shown biochemically to exist as a mixture of monomers and non-covalently associated *cis-*dimers in a cell-type dependent manner. At the cell surface of epithelial NBT II cells and RALA cells, rat CEACAM1-L was found to be a mixture of monomers and *cis-* dimers. Interestingly, although rat CEACAM1-L could undergo *trans-* homotypic interactions in Chinese hamster ovary (CHO) cells, CEACAM1-L *cis-*dimers were not found (5). This illustrated the importance of cell type-dependent regulation of CEACAM1’s *cis-*interactions. Müller et. al. confirmed that CEACAM1-L undergoes *cis-*homotypic binding in rat NBT-II cells using confocal acceptor photobleaching. Furthermore, FRET efficiency vs. acceptor-density curves indicated that CEACAM1-L was a mixture of monomers and *cis-* dimers at free edges but was more *cis-* dimeric/oligomeric at the regions of cell-cell contact (i.e. areas undergoing *trans-*homotypic interactions). In addition, the FRET efficiency of CEACAM1 mutants, with either truncated N-domain or cytoplasmic tail mutants, indicated that CEACAM1’s N-domain and not the cytoplasmic tail can influence CEACAM1 *cis-* dimerization (4). Recently, however, the transmembrane domain GXXG motif has been shown to be the primary motif responsible for CEACAM1’s *cis-* dimerization (11), although clearly the N-domain also plays a role.

Recent studies have demonstrated that some proteins interact with CEACAM1 in a monomer- or *cis-*oligomer- dependent manner (4, 6). These studies began to address how signals are transmitted across the plasma membrane, suggesting that *trans-* homotypic and heterotypic interactions can regulate *cis-* oligomerization, which in turn affects interactions with signaling proteins.
1.1.5 Regulation of cis- and trans- Heterotypic and Homotypic Interactions

Although CEACAM1’s network of interactions is complex, studies are beginning to investigate how trans- homotypic interactions influence cis- homotypic interactions and vise versa. These studies have shown that calmodulin can disrupt CEACAM1’s cis- homotypic interactions (5-6) and that CEACAM1’s trans- homotypic interactions can regulate its cis- homotypic interactions (4).

Biochemical studies showed that treating cells with ionomycin activated calmodulin, which then separated CEACAM1 cis-oligomers into monomers (5-6). Furthermore, HeLa cells transiently transfected with CEACAM1 had increased cellular aggregation (and hence trans-binding) upon treatment with ionomycin, suggesting that cis- monomers were responsible for trans- interactions (6). While cis- homotypic oligomerization may play a role in mediating trans-homotypic interactions, recent studies have also begun investigating how trans- interactions may regulate cis- interactions. Trans- ligation of CEACAM1-specific antibody with rat CEACAM1-L altered the monomer and cis- oligomer equilibrium, which in turn affected binding of SHP-2 and c-Src, but not SHP-1 (4). Cis- oligomerization, therefore, plays an important role in CEACAM1-mediated cell signaling and needs to be further investigated.

While confocal acceptor photobleaching analysis of FRET at free edges compared to cell contacts for different combinations of rat CEACAM1 mutants is informative about the regulation of interactions, it is still unclear how the human CEACAM1 monomer-oligomer equilibrium is spatially and dynamically regulated. Therefore, in order to better characterize the spatio-dynamic organization of CEACAM1 oligomers at the cell surface, we implemented homo-FRET imaging of living cells on a total internal reflection fluorescence polarization microscopy (TIRFPM) system.
1.2 Fluorescence Imaging Techniques

Since protein interactions affect many biological processes, it is important to understand their mechanisms and how they can be regulated. Recent advances in the quantification and sensitivity of fluorescence imaging has made fluorescence microscopy a powerful tool for investigating the structural details — such as molecular distances, orientations, or oligomeric states— and kinetic parameters — such as lifetime, diffusion coefficients, and binding affinities— of proteins in a highly-resolved spatial and temporal manner (12). Furthermore, fluorescence imaging approaches are advantageous for studying dynamic or transient interactions that may otherwise not be detected through more traditional biochemical methods, like X-ray crystallography, NMR spectroscopy, or co-immunoprecipitation.

1.2.1 Fluorescence Background

Fluorescence can be described by the Jablonski diagram, which describes different paths a molecule can take to occupy other electronic states. For example, an excited fluorophore can relax back to its ground state electronic energy level ($S_0$), through fluorescence, intersystem crossing (energy transfer) or quenching (Figure 1.2). For fluorescence, the emitted photon has a lower energy than the excitation photon, which results in the Stokes shift (red-shifted emission wavelengths relative to excitation wavelengths). This emission typically occurs from the first excited state ($S_1$), where fluorescence can compete with faster non-radiative processes (13).

Fluorophores can be described by several characteristics that ultimately influence the emission properties of the fluorophore, and therefore influence the possibility of detecting the fluorophore. The extinction coefficient, $\varepsilon$, describes how well a fluorophore absorbs light. Quantum yield, $Q$, on the other hand, is the ratio of emitted photons relative to photons absorbed by the fluorophore, and therefore describes how efficiently absorbed light is converted to emitted light. These properties, including fluorescence lifetime, $\tau$, are characteristic of specific fluorophores, but can also be influenced by the environment.
Fluorophores are able to fluoresce due to their typically aromatic structures, which have a dipole moment, \( \mu \), associated with them. When the dipole moment is aligned with the excitation electric field \( E \) there is a high probability for excitation, \( P \), according to:

\[
P = |\mu \cdot E|^2 = \cos^2 \theta \tag{1}
\]

in which \( \theta \) is the angle between the \( \mu \) and \( E \) vectors. Therefore, the more aligned the excitation electric field is with the dipole moment of the fluorophore, the greater the probability of excitation. This photo-selective excitation property for exciting oriented fluorophores is an important property for homo-FRET imaging, described later.

Green fluorescent protein (GFP) is particularly popular for studying proteins because it enables fluorescence imaging with high label specificity in living cells. GFP has a 27 kDa \( \beta \)-can structure composed of 11 \( \beta \)-strands and is capped by \( \alpha \)-helix components at either end. These \( \alpha \)-helices protect and rigidly constrain the Ser65-Tyr66-Gly67 fluorophore on the central \( \alpha \)-helix (15-18). After maturation through cyclization and oxidation, the GFP fluorophore is able to fluoresce. GFP is an especially useful molecule because it can be encoded into a protein of interest for highly specific labeling while typically maintaining normal protein function.
Furthermore, labeling proteins with spectrally-distinct GFP variants (like the yellow variants, enhanced yellow fluorescent protein (EYFP) and Venus) can be useful for investigating the interactions or relationships between proteins (15, 19-20). While fluorescence images alone can be used to suggest colocalization between labeled proteins, more advanced imaging techniques are required to identify molecular interactions.

### 1.2.2 Fluorescence Imaging Techniques to Study Protein Interactions

Several advanced fluorescence imaging and analyses techniques are becoming popular for characterizing proteins and their interactions (Figure 1.3). Some techniques focus on rigorous analysis to extract information from the images. For example, fluorescence correlation spectroscopy (FCS) and image correlation spectroscopy (ICS) auto-correlate signal fluctuations caused by diffusing fluorophores in a sub-femtoliter volume to determine labeled protein dynamics, concentration and/or interactions (Figure 1.3 C). Also analyzing signal fluctuation, molecular number and brightness can be calculated from the intensity and variance of a time-stack to determine the brightness and number of molecules (21). These methods are not dependent on molecular distance, as is the case for FRET discussed later, but can be sensitive to imaging artifacts and require relatively involved analysis algorithms.

While some techniques use post-image processing to characterize proteins, others implement advancements in fluorescence tools like fluorescent probes and optical instrumentation. Super-resolution microscopy, like photoactivation localization microscopy (PALM), breaks the diffraction-limited resolution barrier by stochastically activating a subset of photoactivatable or photo-reversible fluorophores, followed by exciting and localizing the fluorophores to achieve ~20 nm lateral resolution (Figure 1.3 A) (22). Furthermore, by combining these super-resolution techniques with super-registration techniques, high spatiotemporal interactions between labeled proteins can be imaged, although this requires sophisticated analysis (Figure 1.3 G) (21). While studies are beginning to apply PALM to study living cells (23) or to characterize organelle-scale interactions in fixed cells (24), PALM requires optical expertise for implementation and is still limited in its use for investigating dynamic interactions in living cells.

Other techniques use fluorescence emission characteristics to investigate molecular interactions. For example, bimolecular fluorescence complementation (BiFC) uses the onset of fluorescence
as an indication of protein interaction (Figure 1.3 F). When two non-fluorescent segments of a fluorescent protein are close enough, they can regenerate the native fluorescent protein. However, since this interaction is typically irreversible, BiFC is limited to studying very specific questions about the onset of protein interaction and subsequent trafficking (21). Förster resonance energy transfer (FRET), like BiFC, characterizes molecular-scale protein interactions at distances much smaller than the diffraction limit. FRET, unlike BiFC, is used to determine molecular interactions based on changes in fluorescence emission properties that result from energy transfer between closely interacting donor and acceptor fluorophores (Figure 1.3 E). FRET, however, is sensitive to optical as well as biological artifacts, and therefore requires the use of proper controls.

Figure 1.3 Overview of Advanced Fluorescence Imaging Techniques.
Schematic demonstration of advanced imaging techniques are shown, including (A) super-resolution microscopy such as PALM, (B) multi-photon microscopy, (C) fluorescence fluctuation spectroscopy, (D) FRAP or FLIP, (E) FRET, (F) BiFC, and (G) Super-registration microscopy. Reprinted from reference (21), with permission.
Although many techniques are emerging to study molecular interactions, we use one ideal for investigating the dynamic homotypic interactions of CEACAM1: homo-FRET imaging, an alternate FRET strategy to the more typical hetero-FRET imaging.

### 1.2.3 Hetero-FRET Imaging

FRET is a powerful technique that, depending on the experimental design, can be used to characterize protein interaction, orientation, and/or conformational changes. Hetero-FRET uses spectrally distinct donor and acceptor fluorophores to gauge molecular interactions. When the donor and acceptor fluorophores are within 1-10 nm apart, energy can be transferred non-radiatively from the excited donor to the nearby acceptor. Essentially, the donor fluorophore’s excited state couples with the acceptor such that the acceptor is able to occupy its dipolar-excited state. To relax back to the ground state, the acceptor then emits light with its characteristic energy level(s) (and emission wavelength(s)) (12-13). The efficiency of energy transfer between the donor and acceptor fluorophores, $E_{\text{FRET}}$, can be calculated by:

$$E_{\text{FRET}} = \frac{1}{1 + \left(\frac{r_d}{R_0}\right)^6}$$  \hspace{1cm} (2)

where $R_0$ is the Förster distance at which FRET efficiency is 50%, and $r_d$ is the intermolecular distance. As shown in Equation (2), FRET efficiency is inversely related to the sixth power of $r_d$, such that the larger the distance between the fluorophores, the lower the FRET efficiency in a $r_d^6$-dependent manner (12, 25).

$R_0$ can be determined for different fluorophore pairs and is a function of the dipole orientation factor ($\kappa^2$), donor’s quantum yield ($Q_D$), acceptor’s extinction coefficient ($\varepsilon_A$), and the fluorophores’ spectral overlap integral ($J(\lambda)$):

$$R_0 = [2.8 \times 10^{17} \cdot \kappa^2 \cdot Q_D \cdot \varepsilon_A \cdot J(\lambda)]^{1/6} \text{ nm}$$  \hspace{1cm} (3)

$$J(\lambda) = \int F_D(\lambda) \cdot E_A(\lambda) \cdot \lambda^4 d\lambda$$  \hspace{1cm} (4)

where $F_D(\lambda)$ is the normalized donor fluorescence and $E_A(\lambda)$ is the acceptor excitation spectra (12). Assuming a freely rotating fluorophore, $\kappa^2$ is typically 2/3, which is the value integrated
over all angles. An ideal FRET pair would consist of a donor with high quantum yield and large spectral overlap for the donor and acceptor (12). For these reasons, sensitivity for detecting $E_{\text{FRET}}$ decreases in cases of perpendicular donor and acceptor dipole orientation ($\kappa^2 \to 0$) or with large distances ($r_{d} \to 0$). These features of FRET can, however, be used to investigate interactions and conformational changes of proteins of interest (Figure 1.4) (12).

Hetero-FRET typically results in donor fluorescence quenching, increased sensitized emission, decreased donor lifetime, and fluorescence emission depolarization (13, 25). These characteristics can be measured to indicate the extent of energy transfer (and therefore determine interactions or conformational changes) by measuring sensitized emission or acceptor photobleaching using conventional microscopy, donor lifetime using fluorescence lifetime imaging microscopy (FLIM), or emission polarization using fluorescence polarization microscopy (FPM) (25-26). (For a more comprehensive list of FRET strategies and motivations, see Figure 1.4) Since hetero-FRET results in the sensitized emission of the acceptor, conventional microscopy can measure donor and acceptor fluorescence intensity to determine the extent of FRET. Alternately, since FRET decreases donor fluorescence intensity, donor fluorescence can also be used to measure FRET efficiency, although this is typically limited to fixed samples due to the need for proper image registration after long photobleaching times. Both techniques require additional controls for bleed-through and cross-excitation. Time-domain and frequency-domain FLIM generate more definitive FRET data; these techniques can, for example, sensibly determine donor lifetime, $\tau_D$, which is reduced during FRET. FLIM-FRET, however, may be sensitive to environmental changes and photobleaching, and is more costly due to the need for specialized instrumentation (27). Since FRET results in the depolarized emission of slowly rotating fluorescent probes, fluorescence polarization anisotropy (discussed in Section 1.2.4), can be used to assess depolarization as an indicator of FRET. For more rigorous detection of energy transfer, fluorescence anisotropy can be coupled with sensitized emission or FLIM, or used to study homo-FRET between identical fluorophores, which is ideal for studying homotypic oligomerization.

Practical issues arise with the use of hetero-FRET, including the need for numerous control samples and acquisition of numerous images to assess the presence of cross-excitation or bleed-through artifacts (12). Since we are interested in studying CEACAM1’s homotypic interactions, these additional hetero-FRET complications are avoided by using homo-FRET.
1.2.4 Steady-State Homo-FRET Imaging

While hetero-FRET has emerged as a popular tool for investigating molecular interactions, homo-FRET is an ideal approach for studying homotypic interactions between like fluorophores. Homo-transfer does not alter the spectral or fluorescence lifetime properties of the identical fluorophores, so changes in fluorescence polarization anisotropy are instead used to detect homo-

Figure 1.4: Overview of Motivations for FRET Approaches.
S/N↑ indicates this technique is ideal for high fluorescence signal (S) and therefore low noise (N), and S/N↓ indicates this technique is ideal for low fluorescence signals. Reprinted from reference (27), with permission.
FRET, assuming that the fluorophore has parallel absorption and emission dipole moments, a small Stokes shift, negligible rotation during the fluorescence lifetime, and random fluorophore orientations (17, 28).

In homo-FRET imaging, polarized light is used to photoselectively excite a subset of fluorophores with dipole moments aligned with the excitation polarization, as described by Equation (1). These fluorophores then emit light parallel to the excitation polarization if they are fairly dispersed (Figure 1.5 A). Polarized emission therefore typically describes monomers. However, if the photoselected fluorophore is in close proximity to another identical fluorophore (less than 1.6X $R_0$) (17)) then homo-transfer can occur between the two fluorophores. Assuming a flexible linker between the protein of interest and the fluorophore, the acceptor can assume orientations different than the photoselectively excited donor and therefore emits depolarized light relative to the excitation polarization (Figure 1.5 B) (28). As a result, depolarized fluorescence emission, relative to the excitation source, is indicative of dimers and oligomers, whereas polarized fluorescence emission is indicative of monomers (29).

Applying homo-FRET imaging to conventional microscopy requires the use of a polarized excitation light source and two emission polarizers, one oriented parallel and one oriented perpendicular to the excitation polarization. Fluorescence intensity collected through the parallel emission polarizer ($F_\parallel$) and through the perpendicular emission polarizer ($F_\perp$) is then used to calculate fluorescence polarization anisotropy, $r$, which is corrected by the instrument polarization bias correction factor, $G$:

$$r = \frac{F_\parallel - GF_\perp}{F_\parallel + 2GF_\perp} \quad (5)$$

In this case, anisotropy is normalized against the total intensity shown in the denominator; the multiplication factor of 2 for $F_\perp$ is used for a randomly oriented sample that emits light symmetrically along the two axes perpendicular to the excitation polarization axis (30-31). Therefore, as shown by Equation (5), a higher anisotropy value is indicative of a more monomeric population, since the polarized light emitted by the monomers will primarily be
Figure 1.5: Schematic of Homo-transfer during Homo-FRET Imaging.

(A) Polarized excitation (blue) of randomly oriented fluorophores. Absorption dipole moments (gray arrows) are photoselectively excited when aligned with polarized excitation. Since the emission dipole moments (black arrows) are nearly parallel with absorption dipole moment, these dispersed fluorophores will emit light (red) parallel to excitation polarization. (B) When the photoselectively excited donor fluorophore is in close proximity to an acceptor fluorophore, homo-FRET (green) can occur. As a result of the acceptor fluorophore’s different orientation, the acceptor fluorophore, emits depolarized fluorescence, which can then be measured as an indication of homo-FRET. Reprinted from reference (27), with permission.

collected by the $F_{\parallel}$ channel. Lower anisotropy is indicative of a more oligomeric population, since the oligomers’ more depolarized light will primarily be collected by the $F_{\perp}$ channel.

The G factor is calculated using polarized images of an isotropic dye:

\[
G = \frac{F_{yy} \times F_{xy}}{F_{yx} \times F_{xx}} \quad (6)
\]

Here we use the microscope convention with the $z$-axis as the optical axis, $y$-axis as the axis parallel to the excitation polarization, and the $x$-axis as the axis perpendicular to the excitation.
polarization (Figure 2.1). In Equation (6), the first subscript refers to the excitation polarization alignment with either the $y$- or $x$- axes, and the second subscript refers to the axis alignment of the emission polarizer (29, 32-33). Equation (5) applies primarily to spectroscopic measurements and must be corrected for the use of a high numerical aperture (NA) objective, described by:

$$NA = n \sin \sigma$$  \hspace{1cm} (7)

where $n$ is the refractive index of the medium, and $\sigma$ is the half-cone angle of the objective. High NA objectives collect light at steep angles, resulting in “bleed-through” of light from the different axes into the traditional $F_\parallel$ and $F_\perp$ channels, which are $F_{xy}$ and $F_{yx}$ for the low NA limits. To correct for this “bleed-through,” the $F_\parallel$ and $F_\perp$ channels are re-written to ascribe intensity to the respective polarization components along the different imaging axes (31).

$$F_\parallel = K_a F_z + K_b F_x + K_c F_y$$  \hspace{1cm} (8)

$$GF_\perp = K_a F_z + K_c F_x + K_b F_y$$  \hspace{1cm} (9)

where the high NA-correction factors ($K_a$, $K_b$, and $K_c$) are:

$$K_a = \left(\frac{1}{6}\right) (2 - 3 \cos \sigma + \cos^3 \sigma)(1 - \cos \sigma)^{-1}$$  \hspace{1cm} (10)

$$K_b = \left(\frac{1}{24}\right) (1 - 3 \cos \sigma + 3 \cos^2 \sigma - \cos^3 \sigma)(1 - \cos \sigma)^{-1}$$  \hspace{1cm} (11)

$$K_c = \left(\frac{1}{8}\right) (5 - 3 \cos \sigma - \cos^2 \sigma - \cos^3 \sigma)(1 - \cos \sigma)^{-1}$$  \hspace{1cm} (12)

With smaller NA and therefore as $\sigma \to 0$, $K_c \to 1$ while $K_a$ and $K_b \to 0$ (31). Therefore, for a randomly oriented fluorophore where $F_x = F_z$, the high NA-corrected anisotropy, $r_c$, can be expressed as:

$$r_c = \frac{(K_a + K_b + K_c)(F_\parallel - GF_\perp)}{(K_a - 2K_b + K_c)F_\parallel + (-K_a - K_b + 2K_c)GF_\perp}$$  \hspace{1cm} (13)
1.2.5 Applications of Homo-FRET Imaging

As with hetero-FRET imaging, time-resolved measurements lead to more unambiguous homo-FRET results. Time-resolved fluorescence anisotropy can be used to determine details such as the homo-FRET transfer rate, dynamically-averaged Förster distance, and the range of intermolecular distance(s). These properties can be calculated from the range of $\kappa^2$, the averaged $\langle \kappa^2 \rangle$, the limiting anisotropy ($r_{\text{inf}}$), relaxation time, and/or the fit of the anisotropy decay curve (17). Furthermore, time-resolved anisotropy combined with other techniques, like spectrally-resolved microscopy (34), can provide even more homo-FRET details. Time-resolved fluorescence anisotropy can be used to determine intermolecular fluorophore distances (35-36), relative protein orientations, protein flexibility (18), fractional monomer and oligomer composition (28), and oligomer enumeration (17, 28, 37). Although extremely informative, time-resolved anisotropy requires specialized instrumentation, which limits its accessibility to the broader research community (38). Therefore in these studies, we use the more accessible steady-state homo-FRET imaging.

Steady-state homo-FRET imaging has been applied to several types of microscopy, including epifluorescence (39-40) and confocal microscopy using one-photon excitation (32, 41-43) or two-photon excitation (28). Furthermore, steady-state homo-FRET imaging has become an increasingly popular technique for investigating homotypic interactions (32, 43), oligomer enumeration (28, 38, 44), protein orientation (41, 45-46), plasma membrane heterogeneity and lipid confinement (40), and has been used in high-content screens (33).

To access more detailed information about CEACAM1’s oligomerization and organization at the cell surface, we implemented homo-FRET imaging on TIRFPM platform, as first described previously (47).
1.2.6 Homo-FRET Imaging in Total Internal Reflection Fluorescence Polarization Microscopy

Reducing out-of-focus fluorescence at the cover slip, TIRFPM is advantageous for imaging processes at the cell-substrate interface with high axial resolution. When incident light travels through a medium with high refractive index \(n_3\) to one with lower refractive index \(n_1\) at an incident angle \(\alpha\) at or above the critical angle \(\alpha_c\), the incident light is totally internally reflected (TIR) as opposed to refracted (Figure 1.6 A). This generates an electromagnetic field that exponentially decays away from the surface, and is therefore useful for exciting fluorophores within 100 nm of the cover slip surface (48-49). The polarization of the incident light affects the evanescent field’s polarization. \(s\)-polarized light generates an evanescent field polarized normal to the plane of incidence (the plane containing both the incident and reflected light paths) (Figure 1.6 C), whereas \(p\)-polarized light creates an evanescent field with polarization that “cart wheels” along the plane of incidence (Figure 1.6 B) (49).

These polarization effects make TIRFPM ideal for some polarized fluorescence imaging applications. TIRFPM has been used to study processes such as plasma membrane topographical changes by photoselectively exciting fluorophores with the distinct \(s\)-polarized versus \(p\)-polarized evanescent wave polarization components (50). Here, to investigate membrane protein \(cis\)- homotypic oligomerization and spatial distribution across the plasma membrane, we apply homo-FRET imaging to our TIRFPM platform. While previous work established the platform and initial anisotropy calculation macro (47), it was critical that we corrected the image acquisition procedure and analysis, used more rigorous controls, and processed results more quantitatively to ensure proper assessment of anisotropy. With an ultimate aim of understanding how CEACAM1 is able to regulate numerous important downstream processes, we applied homo-FRET imaging to TIRFPM and were able to characterize some factors that regulate CEACAM1’s localization and homotypic \(cis\)- oligomerization.
Figure 1.6: Schematic of TIRFM.

(A) Schematic of TIR evanescent field generated when \( n_3 > n_1 \) and \( \alpha > \alpha_c \). Note that the intermediate layer is not necessary for TIR illumination, although the intermediate layer can be useful for other applications like surface plasmon microscopy, discussed in reference (51). The dashed oval is magnified to show the effects of (B) \( p \)-polarized incident light and (C) \( s \)-polarized incident light on the evanescent field polarization for the distance of one wavelength. Adapted from reference (51), with permission.
Chapter 2

2 Steady-State Homo-FRET TIRFPM Imaging
Instrumentation Development and Calibrations

2.1 Chapter Summary

TIRFPM anisotropy was able to differentiate between pure monomeric and pure dimeric populations. Monomeric Venus had a significantly higher anisotropy than dimeric Venus in both epifluorescence (0.252 and 0.222, respectively) and TIRFPM (0.189 and 0.140, respectively), with an average anisotropy difference of 0.030 and 0.049, respectively. Although there was some photobleaching during image acquisition, it did not affect the TIRFPM’s sensitivity to monomers compared to dimers. Furthermore, we confirmed that the Venus dimer’s lower anisotropy was caused by homo-transfer between like fluorophores and not concentration-induced depolarization. Therefore, TIRFPM homo-FRET imaging is a promising technique for studying biological systems with varying oligomer states, especially for membrane proteins like CEACAM1.

2.2 Background

Steady-state homo-FRET imaging is commonly applied to epifluorescence microscopy, and one-photon and two-photon excitation confocal microscopy (28, 32, 39-40, 52-53). Recently, homo-FRET imaging has been briefly performed in a TIR configuration (54). Offering unique access to study membrane protein self-association with high axial resolution, our TIRFPM homo-FRET imaging platform was used to study CEACAM1’s monomer and cis-homotypic oligomer equilibrium. As this application on TIRFPM is not well established, it was first necessary to characterize the homo-FRET imaging system. Previous studies have determined the anisotropy sensitivity of their fluorescence polarization microscopes using fluorescent protein monomers, dimers, and occasionally larger order oligomers (39, 42, 52), inducible GFP oligomers attached
to ligand binding-induced dimerization domains (28), and/or through the use of deliberate progressive photobleaching (38, 44).

Fluorescent protein members of the GFP family are well-suited for homo-FRET imaging for several reasons. Like the other fluorescent proteins, EYFP has a small 14 nm Stoke’s shift that enables energy transfer between the two identical fluorescent proteins. Fluorescent proteins also have well-aligned absorption and emission dipole moments and therefore negligible fluorescence depolarization due to dipole moment rotation. This simplifies time-resolved homo-FRET calculations and facilitates enumeration of fluorescent protein oligomer state (16-17, 55). In addition, fluorescent proteins, like EYFP, have rotational correlation times (23.4 ns) that are much longer than their fluorescent lifetimes (2.6 ns) (17). Therefore, fluorescence depolarization can be attributed to homo-transfer between the fluorescent proteins, rather than rotational depolarization.

In addition to the use of fluorescent protein oligomer controls, controlled photobleaching by sequential image acquisition can be used to confirm the occurrence of homo-transfer. Fluorescent protein monomers are progressively photobleached without altering the anisotropy, compared to oligomers, in which random fluorescent molecule(s) in an oligomer can be photobleached. The photobleached member of the oligomer can no longer undergo homo-FRET, so the anisotropy increases with photobleaching. Therefore, the anisotropy vs. intensity curve provides details about the oligomeric state of the protein.

Here we use the Venus monomer and tandem Venus dimer constructs to establish the sensitivity and functionality of the TIRFPM homo-FRET imaging platform for studying CEACAM1, discussed in Chapter 3.
2.3 Materials and Methods

2.3.1 Molecular Cloning

In collaboration with the Rocheleau group (University of Toronto), we generated a dimer construct from two monomeric Venus constructs (A206K mutant) linked by 10 amino acids, using an approach described by Rizzo et. al. (20, 56-57). More specifically, the tandem construct was made by ligating the Venus C1 vector’s Nhel-BglII fragment into the Venus N3 vector’s Nhel-BamHI digested site.

2.3.2 Cell Culture and Transfections

HeLa cells were grown in RPMI 1640 media (Invitrogen Life Technologies; ON, Canada) with 10% FBS and 4mM GlutaMAX (Invitrogen) at 37 °C and 5% CO₂. For imaging, cells were trypsinized and plated onto untreated 35 mm WillcoWells cover slip-bottom dishes (WillCo Wells; Amsterdam, Netherlands). After 24 hours, cells were transfected using FuGene6 (Roche; PQ, Canada), according to the manufacturer’s protocol, in Opti-MEM media (Invitrogen). 18-24 hours after transfection, cells were imaged in RPMI 1640 media supplemented with HEPES but without phenol red or sodium bicarbonate (Wisent Inc., Canada).

2.3.3 Immunoblot

Total cell lysates of HeLa cells transiently transfected with monomeric or dimeric Venus were resolved under non-reducing conditions on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel probed using anti-GFP antibody (Santa Cruz Biotechnology, Inc.; CA, USA). The gel was then incubated in goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, Inc.; PA, USA) and developed using Western Lightning Plus enzymatic chemiluminescence kit (Perkin-Elmer; ON, Canada).
2.3.4 TIRFM Instrumentation and Imaging

A home-built TIRFPM system, described previously (47), was used for all TIRFPM imaging, including homo-FRET imaging. Briefly, the TIRFPM instrument was built on an inverted microscope (Olympus Canada; ON, Canada) and controlled using µManager (47, 58) (Version 1.3.48, http://www.micro-manager.org). TIRFPM images were acquired on an Evolve 512 EMCCD camera (Photometrics; AZ, USA) using a PLAN-APO 60X 1.45 NA oil-immersion objective (Olympus). Venus images were acquired using 473 nm laser excitation, a 505 nm cut-on wavelength dichroic mirror, and a 500-550 nm band-pass interference filter. Image analysis was performed using the ImageJ software (59) (version 1.43j, http://rsbweb.nih.gov/ij/) and the specified plug-ins. Intensity images in the figures are \( F_\parallel \) channel intensity images, unless otherwise indicated, that were treated with a non-linear gamma filter \( f(i) = \left(\frac{i}{255}\right)^\gamma \times 255, i = 8 \) bit pixel intensity; \( \gamma = 0.7 \) for better simultaneous visualization of high intensity and low intensity regions. The gamma filter was used specifically for figures and was only applied after anisotropy image processing.

2.3.5 Homo-FRET Imaging in TIRFPM

The TIRFPM platform was previously adapted for fluorescence polarization microscopy, including homo-FRET imaging (47). Briefly, the excitation polarization was oriented such that it photoselectively excites fluorophores aligned along the \( y \)-axis (Figure 2.1), and the emission polarizers were oriented either parallel or perpendicular to the excitation polarization, along the \( y \)-axis or \( x \)-axis, respectively (Figure 2.1). The excitation polarization was refined through a Glan-Taylor linear polarizer followed by a half-wave plate that was used to achieve \( s \)-polarized incident light in TIRF. This generated a TIRF evanescent field polarized along the \( y \)-axis (Figure 2.1). By translating the mirror that directs the excitation light into the microscope base (Object 3, Figure 2.1) along the \( x \)-axis, the TIRF angle could be adjusted for epifluorescence or TIRF imaging. \( F_\parallel \) and \( F_\perp \) emission polarizers were positioned in an additional filter turret placed in the microscope’s infinity space (Object 5, Figure 2.1).
To ensure proper homo-FRET imaging, the polarization quality and orientation of the excitation laser along the visible optical path and at the sample stage were assessed using a flat polarizer and power meter. Emission polarizers were previously installed using a procedure described by Dix (47, 60) and checked against an LCD monitor that emits linearly polarized light (47, 60). The $G$ factor (473 nm) was consistently 0.93 for isotropic solutions of fluorescein isothiocyanate (FITC) using the 20X 0.4 NA objective and 60X 1.45 NA oil-immersion objective in epifluorescence. Equation (6), was used for the $G$ factor calculation as opposed to $G = \frac{F_{yy}}{F_{yx}}$ because it gave the most consistent $G$ factor measurement for several exposure times and dilute concentrations of FITC (29).

![Figure 2.1. TIRFPM Platform.](image)

(1) 473 nm excitation laser directed through (2) Glan-Taylor linear polarizer followed by a half-wave plate for rotating excitation polarization to generate TIRF evanescent field polarized along $y$-axis. (3) TIRF mirror translated along $x$-axis to shift from epifluorescence to TIRF imaging at (4) sample plane. Sample emission is collected through emission polarizers parallel, $F_\parallel$ (along $y$-axis), and perpendicular, $F_\perp$ (along $x$-axis), to excitation polarization positioned in (5) additional filter turret before (6) EMCCD image acquisition. $z$-axis is the optical axis of microscope. Dotted line indicates laser path behind visible optics; Glan-Taylor linear polarizer and half-wave plate are also behind visible optics.
Anisotropy image sets were collected sequentially through the $F_\parallel$ then the $F_\perp$ emission polarizers for a 1000 ms exposure time per image. The image sets were then run through an updated version of the lab’s ImageJ pixel-wise anisotropy calculation macro (similar to Appendix 3, but without dual auto-thresholding) (47). To acquire anisotropy statistics, background subtraction, automatic thresholding, and ROI selection were updated from the original anisotropy calculation macro (similar to Figure 3.2).

2.3.6 Homo-FRET Imaging in Epifluorescence

Homo-FRET imaging in epifluorescence was acquired similar to Section 2.3.5, except the TIRF angle was adjusted to achieve epifluorescence. Due to a high excitation laser power compared to the TIRF evanescent field, epifluorescence images were acquired using 200-500 ms exposure time per image.

Progressive photobleaching of the Venus constructs was performed in epifluorescence (using a 20X 0.4NA objective) because the TIRF evanescent field was not effective for photobleaching Venus constructs, likely due to the low intensity of the evanescent TIRF field and/or the rapid recovery of soluble Venus into the TIRFM field.

2.3.7 Statistical Analysis

Two-tailed, unpaired Student T-tests were used to determine significant differences between mean anisotropy values of the indicated cell populations using Graphpad Prism (GraphPad Prism, GraphPad Software, Inc.; CA, USA).
2.4 Results

2.4.1 Epifluorescence and TIRFPM Anisotropy Distinguishes Monomeric and Dimeric Venus

Immunoblot analysis showed the monomeric Venus band at ~27 kDa and the dimeric Venus band at ~55 kDa, which was consistent with the reported fluorescent protein molecular weight for the monomer and that expected for the dimer (Figure 2.2) (17). These data are therefore consistent with the expected protein expression.

Epifluorescence homo-FRET imaging of transiently transfected HeLa cells demonstrated that the mean anisotropy of soluble monomeric Venus (0.252) was significantly higher (p<0.0001) than that of the soluble dimeric Venus (0.222), indicating that the TIRFPM platform could properly distinguish monomers from dimers with an anisotropy difference comparable to that reported by Squire et. al. (0.038) (Figure 2.3 A-B; Table 2.1) (39). Although the epifluorescence absolute anisotropy values were lower than those typically reported for monomeric fluorescent proteins (0.29), others have also reported different anisotropy values (0.22, monomeric GFP) (42, 52).

TIRFPM anisotropy of monomeric Venus (0.189) was significantly higher (p<0.0001) than that of the dimeric Venus (0.140) (Figure 2.3 C-D; Table 2.1). TIRFPM and epifluorescence anisotropy differences between monomeric and dimeric Venus were comparable, proving our TIRFPM system can be successfully adapted for homo-FRET imaging. TIRFPM absolute anisotropy, however, were lower than those in epifluorescence, which are likely due to the

![Figure 2.2: Immunoblot of HeLa Cell Lysates Transiently Transfected with Monomeric (M) or Dimeric (D) Venus.](image-url)
methods’ inherent sampling differences (54). These results were comparable to the TIRFPM anisotropy recently reported for GFP (~0.18), which also confirmed the observation that TIRFPM anisotropy values were lower than those generally reported for monomeric fluorescent proteins in epifluorescence (54).

Figure 2.3: Anisotropy Images of HeLa Cells Transiently Transfected with Soluble Venus Monomer and Venus Dimer.

(A-B) Representative epifluorescence and (C-D) TIRFPM image sets (\(F_\parallel\), \(F_\perp\), \(r_c\) from left to right) and histogram of the indicated ROI are shown for (A,C) Venus monomers and (B,D) dimers. Raw histograms (light gray) are overlaid with least squares Gaussian fits (black line); mean \(r_c \pm \text{SD}\) are shown next to histogram peak. \(F_\parallel\) and \(F_\perp\) fluorescence images were treated with gamma filter function (\(\gamma=0.7\)) after image processing to facilitate simultaneous visualization of high intensity and low intensity features. Brightness and contrast settings are equal for these images. Inset is 9 µm X 9 µm.
Table 2.1: Average $r_c$ of Monomeric and Dimeric Venus

<table>
<thead>
<tr>
<th>Construct</th>
<th>Acquisition</th>
<th>$N$</th>
<th>Anisotropy, $r_c$ ( ^\ast )</th>
<th>Ratio ( ^\dagger )</th>
<th>Difference ( ^\ddagger )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomeric Venus</td>
<td>Epifluorescence (60X)</td>
<td>27</td>
<td>0.252 ± 0.006</td>
<td>1.13</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>TIRFPM</td>
<td>109</td>
<td>0.189 ± 0.002</td>
<td>1.36</td>
<td>0.050</td>
</tr>
<tr>
<td>Dimeric Venus</td>
<td>Epifluorescence (60X)</td>
<td>29</td>
<td>0.222 ± 0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TIRFPM</td>
<td>119</td>
<td>0.140 ± 0.002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Average anisotropy ± SE of $N$ number of cells
† Mean value of measurements determined on a per cell basis
‡ Ratio = $<r_{\text{monomer}}>$ / $<r_{\text{dimer}}>$
§ Difference = $<r_{\text{monomer}}>$ - $<r_{\text{dimer}}>$

Average epifluorescence and TIRFPM anisotropy (± SE) for $N$ number of cells transiently transfected with indicated constructs. Mean per cell $r_c$ were measured for central 9 µm x 9 µm ROI within the TIRFPM field of view. Average $r_c$ for a population of cells transfected with monomeric Venus was significantly higher (p<0.0001) than average $r_c$ for a population of cells transfected with dimeric Venus in both epifluorescence and TIRFPM.

2.4.2 Investigation of Potential Homo-FRET Imaging Artifacts

Several control experiments were performed to further assess the presence of possible artifacts, including concentration-induced depolarization or photobleaching during image acquisition, and to confirm detection of homo-transfer using progressive photobleaching.

Comparison of anisotropy vs. intensity for a representative subset of cells showed no intensity-dependent anisotropy trend, suggesting that the anisotropy was not subject to concentration-induced depolarization (Figure 2.4 A). Concentration-induced depolarization would cause crowding-induced homo-transfer, such that anisotropy would decrease with increasing intensity. Since the anisotropy image set ($F_\parallel, F_\perp$) used a long exposure time, for comparability to that required for imaging EYFP-labeled CEACAM1 in Chapter 3, this could lead to photobleaching during image acquisition. Theoretically, Venus could become photobleached during the first acquired image, resulting in a relatively lower intensity for the second acquired image. Photobleaching could therefore affect the calculated anisotropy. While absolute anisotropy was dependent on the acquisition order ($F_\parallel$ then $F_\perp$, compared to $F_\perp$ then $F_\parallel$) suggesting that
Figure 2.4: TIRFPM Anisotropy Imaging Controls of HeLa Cells Transiently Transfected with Soluble Venus Monomer or Venus Dimer.

(A) $r_c$ vs. intensity used to investigate intensity/concentration dependence of anisotropy for subset of cells. Each data point corresponds to the mean $r_c$ at the central 9 µm X 9 µm area of an individual cell. (B) Image set acquired through emission polarizers in the following orders: (left) either $F_{\parallel}$ channel first, then $F_{\perp}$ channel second or (right) $F_{\perp}$ channel first, then $F_{\parallel}$ channel second. Average $r_c$ for subset of cells is shown above each bar.

Photobleaching did occur, the relative anisotropy difference between the Venus monomer and dimer (~0.05) was independent of acquisition order (Figure 2.4 B). Therefore, although this long exposure time affected the absolute anisotropy, photobleaching between acquired images did not alter the TIRFPM platform’s ability to distinguish monomers and dimers. Consequently, to maintain consistency, we acquired all anisotropy image sets in the same order ($F_{\parallel}$ then $F_{\perp}$).

The anisotropy of dimeric Venus increased with progressive photobleaching (gray, Figure 2.5), approaching a value indicative of monomers (dotted line, Figure 2.5) whereas the anisotropy of the monomeric Venus did not change with progressive photobleaching (black, Figure 2.5). Furthermore, since monomeric Venus anisotropy did not increase with photobleaching, the soluble Venus constructs were not subject to concentration-induced depolarization. Thus, the dimeric Venus’ lower absolute anisotropy was indicative of homo-transfer and not imaging or concentration artifacts. Photobleaching of the Venus constructs using TIRFM was not effective, likely due to the attenuated laser power of the evanescent TIRF field compared to epifluorescence.
Figure 2.5: Progressive Photobleaching of HeLa Cells Transiently Transfected with Soluble Monomeric Venus or Dimeric Venus using TIRFPM.
Photobleaching curves of monomeric Venus (black, N = 5 cells) and dimeric Venus (gray, N = 3 cells) in epifluorescence using a 20X 0.4 NA objective are shown. The dashed line represents the average anisotropy of monomeric Venus.

2.5 Discussion

The epifluorescence FPM and TIRFPM imaging strategies were able to use anisotropy to distinguish soluble homogeneous monomers from dimers. Although the average anisotropy in epifluorescence was lower than those typically reported for monomeric fluorescent proteins (0.29), reported anisotropy values are not always consistent (42, 52). Our lower anisotropy value was unlikely due to the G factor measurement because G factor measurements were consistently 0.93, nor due to concentration-induced depolarization because we did not observe an intensity-dependent anisotropy trend. Furthermore, transient transfection of 4-fold lower Venus plasmid concentrations did not alter the anisotropy values (data not shown). Irrespective of our lower reported anisotropy values, monomeric Venus had a significantly higher anisotropy than dimeric Venus in both epifluorescence and TIRFPM, with an average anisotropy difference of 0.030 and 0.050, respectively.

Although there was slight photobleaching during image acquisition, it did not alter TIRFPM’s sensitivity to distinguish Venus monomer and dimer anisotropy. We have shown the ability to resolve populations of monomers and dimers using the TIRFPM homo-FRET imaging platform, making it a powerful tool for studying homotypic oligomerization in biological systems.
Chapter 3

3 Steady-State Homo-FRET TIRFPM Imaging of EYFP-labeled CEACAM1

3.1 Chapter Summary

TIRFPM imaging revealed that CEACAM1-4L-EYFP, CEACAM1-4S-EYFP, CEACAM1-4Δcyto-EYFP and G432,436L-CEACAM1-4L-EYFP were concentrated into high intensity features, previously characterized as ezrin-rich regions for CEACAM1-4L-EYFP (47), and were also more diffusely distributed across the rest of the membrane. CEACAM1-4L-EYFP existed as a mixture of monomers and cis-oligomers across both the high intensity and low intensity regions with a slightly higher amount of monomers in the high intensity regions. Upon perturbation with ionomycin and pAb, CEACAM1 became more monomeric, potentially in a localized manner. Together, these studies indicated the importance of intracellular and extracellular interactions in disrupting CEACAM1’s cis-homotypic oligomers. While TIRFPM anisotropy was sensitive to real-time changes in CEACAM1 oligomerization, it was insensitive to static differences between CEACAM1-4L-EYFP, which had been shown biochemically to exist as a mixture of monomers and oligomers (6), and the monomeric mutant G432,436L-CEACAM1-4L-EYFP. Therefore, TIRFPM homo-FRET imaging studies can be effective for probing membrane protein oligomerization, provided they assess relative shifts in anisotropy, such as time-course studies or progressive photobleaching experiments, rather than solely relying on absolute anisotropy values.

3.2 Background

CEACAM1 undergoes a complex network of heterotypic and homotypic cis- and trans-interactions that affect cell growth, apoptosis, immune cell function, and metabolism in a cell type-dependent manner (1). Several CEACAM1-4L interactions seem to be regulated by CEACAM1-4L’s cis- monomer-dimer equilibrium. Rat CEACAM1-4L and CEACAM1-4S, and
more recently, human CEACAM1-4L transfected in HeLa cells, have been biochemically characterized as a mixture of monomers and \textit{cis}-dimers at the cell surface (5-6), although the possibility of larger order oligomers has not been eliminated. These are some of the few studies beginning to identify motifs responsible for CEACAM1’s \textit{cis}-oligomerization and to investigate intracellular and extracellular interactions responsible for regulating \textit{cis}-oligomerization.

The Gray-Owen group (University of Toronto) generated several fluorescently-labeled CEACAM1 constructs with mutations at the cytoplasmic, transmembrane, or extracellular domains to investigate how CEACAM1’s domains regulate its interactions (Figure 3.1). CEACAM1-4L cytoplasmic tail mutants — CEACAM1-4Δcyto-EYFP with no cytoplasmic tail and CEACAM1-4S-EYFP that can be found naturally with a shorter, 10 amino acid, cytoplasmic tail — may give insight into cytoplasmic tail-mediated interactions with calmodulin, SHP-1 and SHP-2, tyrosine kinases, and/or actin. Although not as frequently studied, CEACAM1’s transmembrane domain contains a GXXG motif that was recently shown to play an important role in CEACAM1’s \textit{cis}-homotypic oligomerization. Mutating this motif in CEACAM1-4L generated a \textit{cis}-monomeric mutant G432,436L-CEACAM1-4L-EYFP, that could still undergo \textit{trans}-interactions. A monomeric mutant of CEACAM1-S using the same mutation was also recently confirmed by Lawson et. al. (11). At the extracellular domain, CEACAM1-4L’s \textit{trans}-interactions are predominantly mediated through the N-terminal IgV-like domain. Two point mutations in this domain generated the RQ43,44SL-CEACAM1-4L-EYFP mutant that no longer undergoes \textit{trans}-homotypic interactions but can still undergo \textit{cis}-homotypic interactions (61). In this study, we used these mutants to investigate the motifs’ roles in \textit{cis}-homotypic oligomerization and heterotypic interactions.

CEACAM1-4L’s cytoplasmic tail and extracellular domains have been shown to interact with proteins that may influence CEACAM1-4L’s \textit{cis}-oligomerization, which in turn transmits signals across the plasma membrane (4, 6, 61). CEACAM1-4L and CEACAM1-4S monomer and \textit{cis}-dimer equilibrium are controlled by interactions with, for example, calmodulin. Biochemical assays demonstrated that incubating cells with ionomycin, a calcium ionophore, activated calmodulin, which then bound to a cytoplasmic tail motif present in CEACAM1’s membrane-proximal 10 amino acids (1, 62). \textit{Ca}^{2+}-activated calmodulin binding to CEACAM1 then disrupted CEACAM1 \textit{cis}-oligomers into monomers (5-6, 62). The ionomycin-induced monomers have been shown to increase cell aggregation, mediated through \textit{trans}-homotypic...
interactions, indicating that cis- homotypic interactions can affect trans- interactions (Gray-Owen group, personal communication).

To investigate the extracellular interactions, Müller et. al. used confocal acceptor photobleaching hetero-FRET to show that rat CEACAM1 trans- homotypic interactions at cell-cell contacts induced the formation of cis- oligomers, as compared to the free surface of the cell where CEACAM1 existed as a mixture of monomers, dimers, and larger order oligomers (4, 63). These cis- oligomers, in turn, preferentially interacted with SHP-1 and SHP-2, but not c-Src. Trans-interactions with mAb were also shown to affect rat CEACAM1 cis-oligomerization and recruit c-Src and SHP-2 in a phosphorylation-dependent manner (4). These studies indicate the

![Figure 3.1: Schematic Diagram of EYFP-labeled CEACAM1 Mutants.](image)

Shown are EYFP-labeled CEACAM1 mutants used in this study, including (A) CEACAM1-4L-EYFP with an EYFP attached to the 71 amino acid-long cytoplasmic tail, (B) CEACAM1-4S-EYFP with an EYFP attached to the 10 amino acid-long cytoplasmic tail, (C) CEACAM1-4Δcyto-EYFP with no cytoplasmic tail (D) RQ43,44SL-CEACAM1-4L-EYFP with N-terminal domain mutations and has an EYFP attached to the 71 amino acid-long cytoplasmic tail, and (E) G432,436L-CEACAM1-4L-EYFP with transmembrane domain mutations and has an EYFP attached to the 71 amino acid-long cytoplasmic tail. For all the constructs, the extracellular domain consists of 3 membrane proximal IgC-like domains and an N-terminal IgV-like domain. For simplification purposes, EYFP barrels are shown in same orientation with a vertical dipole moment (black line), although we assumed that the cytoplasmic tail is flexible, allowing EYFP to adopt multiple orientations. Adapted from original figure, courtesy of Prerna Patel.
importance of trans-interactions in initiating signaling cascades that are dependent on the regulation of the CEACAM1-4L and CEACAM1-4S phosphorylation, and CEACAM1 monomer and cis-oligomer equilibrium.

Intra- and extra-cellular interactions are clearly important for regulating CEACAM1’s cis-oligomers and subsequently transmitting signals across the cell membrane, but these studies do not address how the membrane localization, organization, and dynamics of CEACAM1 may together influence these interactions. Therefore, previous work in the Yip group began characterizing human CEACAM1-4L-EYFP in TIRFPM, and revealed that transiently transfected CEACAM1-4L-EYFP was heterogeneously distributed across the surface of HeLa cells, with high concentrations of CEACAM1-4L-EYFP in ezrin-rich regions and low concentrations of CEACAM1-4L-EYFP across the rest of the plasma membrane. Using acceptor photobleaching FRET and sensitized emission FRET in TIRF, it was also suggested that the high intensity, ezrin-rich regions were monomeric compared to the low intensity regions (47). Early attempts to implement homo-FRET, a FRET strategy better suited for studying homotypic interactions, in TIRFPM were unsuccessful. Therefore, while the platform and initial anisotropy calculation macro were previously created (47), it was still necessary to correct image acquisition procedure and analysis, to develop more relevant controls (Chapter 2), and to use more high-throughput, statistical analysis.

Therefore, here we show that homo-FRET imaging in TIRFPM can be used to investigate the real-time localization and regulation of CEACAM1-EYFP cis- oligomers in response to intracellular and extracellular interactions. This work establishes the baseline for future studies into how the regulation of CEACAM1 monomers and cis- homotypic oligomers can affect the interactions with, and recruitment of, other proteins or lipids to ultimately influence inside-out and outside-in signaling across the cell membrane.
3.3 Materials and Methods

3.3.1 Generation of EYFP-labeled CEACAM1 Mutants

The Gray-Owen group (University of Toronto) attached EYFP or mCherry to the C-terminal end of CEACAM1 mutants’ cytoplasmic tails to create the following labeled-CEACAM1 constructs: CEACAM1-4L-EYFP, CEACAM1-4L-mCherry, CEACAM1-4S-EYFP, CEACAM1-Δcyto-EYFP, G432,436L-CEACAM1-4L-EYFP, and RQ43,44SL-CEACAM1-4L-EYFP. Here, we assumed CEACAM1-4L has a flexible cytoplasmic tail, discussed further in Discussion Section 3.5.1. For details on generating the aforementioned chimeric EYFP-labeled CEACAM1, see H. Lee’s thesis (6). Lifeact-RFP (for labeling F-actin) was a kind gift from S. Grinstein (University of Toronto).

3.3.2 Cell Culture and Transfections

Methods described previously in Section 2.3.2.

3.3.3 TIRFM Settings

Intensity images were acquired using our TIRFPM platform, described previously in Methods Section 2.3.4. Cells transfected with EYFP-labeled CEACAM1 or incubated with FITC-dextran were imaged using 473 nm laser excitation, a 505 nm cut-on wavelength dichroic mirror, and a 500-550 nm band-pass interference filter. Red fluorophores were imaged using 532 nm laser excitation, a 565 nm cut-on wavelength dichroic mirror, and a 565-605 nm band-pass interference filter. Figures show F∥ channel intensity images, and where indicated the intensity images were treated with a non-linear gamma filter \( f(i) = (i/255)^\gamma \times 255 \), \( i = 8 \) bit pixel intensity; \( \gamma = 0.7 \) for better simultaneous visualization of the high and low intensity regions. The gamma filter was only applied after anisotropy image processing.
3.3.4 FITC-Dextran Flow-through Experiment in TIRFM

HeLa cells were transiently transfected with CEACAM1-4L-mCherry, soluble mCherry, or Lifeact-RFP (that binds to F-actin). Twenty-four hours after transfection, cells were incubated with final concentration 100µg/mL FITC-dextran (molecular weight 10,000) for 15 minutes at room temperature prior to imaging (64-65). Images were acquired using TIRFM settings previously described in Methods Section 3.3.3. For better visualization of colocalization in the merged channel, the intensity of the FITC-dextran channel was inverted such that intensity is inversely correlated to the distance from the untreated glass cover slip. Bright regions correspond to areas in closest proximity to the cover slip and dark regions correspond to areas further away.

3.3.5 Homo-FRET Imaging in TIRFPM

Anisotropy image analysis and statistical significance calculations were similar to Methods Section 2.3.5. An anisotropy image set was collected sequentially through the $F_\parallel$ then the $F_\perp$ emission polarizers for 2 s exposure time per image, resulting in a total acquisition time that was faster than the observed μm-scale motions of the CEACAM1-4L-EYFP high intensity regions (Figure 3.3). Instead of using a single intensity threshold to mask the cell boundary as was done for Venus constructs with homogenous intensity, two ImageJ auto-threshold algorithms, MaxEntropy and Triangle, were used as masks for CEACAM1-EYFP’s high intensity and low intensity regions, respectively: http://pacific.mpi-cbg.de/wiki/index.php/Auto_Threshold (Appendix 3). These automatic threshold algorithms best matched visual thresholding of the high intensity and low intensity regions (Figure 3.2).
Figure 3.2: Representation of Updated Anisotropy Analysis.
(1) The ImageJ macro subtracts the image background, (2) removes any saturated pixels, and (3) calculates \( r_c \) per pixel [using original macro calculations (47)]. (4a) ImageJ’s MaxEntropy auto-threshold and (4b) Triangle auto-threshold (after subtraction of the MaxEntropy auto-threshold mask) are used as masks for CEACAM1-EYFP’s high intensity and low intensity regions, respectively. A representative 9\( \mu \)m X 9\( \mu \)m ROI is selected at the center of the TIRFPM field, and the mean anisotropy is calculated for the ROI. Further statistical analysis is performed in MS Excel and Graphpad Prism.
3.3.6 Time-lapse Homo-FRET Imaging in TIRFPM

Time-lapse homo-FRET imaging experiments were started at negative time points to establish baseline anisotropy fluctuations before adding the indicated soluble factor at t = 0 minutes. Cells were imaged at room temperature in RPMI 1640 media supplemented with HEPES (Wisent Inc., Canada) but without phenol red or sodium bicarbonate. For ionomycin experiments, ionomycin (1 µM), MgCl₂ (0.5 mM) and CaCl₂ (1mM) were added at t = 0 minutes. For pAb experiments, filtered pAb (20 µg/mL) or isotype (20 µg/mL) resuspended in PBS were added at t = 0 minutes. For each experiment, anisotropy image sets of 4-5 cells were acquired every 10 to 15 minutes. Since imaging multiple cells for each time point led to some lateral translation between sequential images, an ImageJ macro for registering and analyzing the images was written using the ImageJ “TurboReg” plugin (Appendix 4) (66). For some cells, photobleaching occurred over this time-course, which can artificially increase anisotropy, as shown previously (Figure 2.5). Therefore, two-tailed T-tests were used to determine the significance of Pearson’s correlation for anisotropy vs. intensity using GraphPad Prism. Traces with p<0.05 and a negative correlation between anisotropy and intensity were eliminated from the data set.

3.3.7 Two-color Imaging to determine Anisotropy at Junctions in TIRFPM

HeLa cells transiently transfected with either CEACAM1-4L-EYFP or CEACAM1-4L-mCherry were trypsinized, resuspended together, spun down to remove residual trypsin, and then plated for 9-12 hours at 37°C at 5% CO₂ prior to imaging. Images were acquired using TIRFPM settings previously described in Methods Section 3.3.3 and 3.3.5. These settings resulted in CEACAM1-4L-EYFP’s bleedthrough into the mCherry channel, but no bleedthrough of CEACAM1-4L-mCherry into the EYFP channel. Therefore, the mCherry channel was used to visualize cell-cell contacts between CEACAM1-4L-EYFP cells (also visible in EYFP channel) and CEACAM1-4L-mCherry cells (only visible in mCherry channel) without affecting CEACAM1-4L-EYFP anisotropy calculations.
3.4 Results

3.4.1 CEACAM1 has Heterogeneous Distribution across the HeLa Cell Surface

As previously reported by the Yip group, CEACAM1-4L-EYFP had a heterogeneous distribution across the plasma membrane of HeLa cells, with CEACAM1-4L-EYFP localized to high intensity regions as well as diffusely distributed across the rest of the plasma membrane (Figure 3.3) (47). The high intensity regions, previously identified as ezrin-rich regions (47), typically ranged from 0.5 µm to 5 µm in size and slowly changed shape in the seconds time frame (arrowheads, Figure 3.3). From the intensity images, it was unclear how these structures affected the organization of CEACAM1’s monomer- cis- oligomer equilibrium across the cell surface, so we investigated cis- self-associations use homo-FRET imaging in TIRFPM.

Figure 3.3: Time-lapse TIRFM Imaging of CEACAM1-4L-EYFP’s Heterogeneous Distribution.
Arrowheads indicate high intensity regions that have changing shape and/or size over the time period. Images were treated with gamma filter function (γ=0.7) to facilitate simultaneous visualization of high intensity and low intensity regions. Insets are 9 µm X 9 µm.
3.4.2 Steady-state Anisotropy Images of CEACAM1-4L-EYFP in TIRFPM

Anisotropy images of CEACAM1-4L-EYFP were more heterogeneous compared to those of soluble Venus, which were homogenously monomeric or dimeric (Figure 3.4). This suggested that CEACAM1-4L-EYFP was a mixture of monomers and homotypic oligomers across the cell membrane, which is consistent with immunoblots of CEACAM1-4L (6). Furthermore, when thresholds were applied to the high intensity and low intensity regions, it was visually apparent that both regions had heterogeneous oligomer composition. However, in some CEACAM1-4L-EYFP cells, the high intensity regions had a higher anisotropy than that of the low intensity regions (Figure 3.4 B-C). Yet, in other cells, the high intensity regions had a lower anisotropy (data not shown). As many factors have been shown to influence the cis- homotypic oligomerization of CEACAM1, more rigorous and controlled studies are required to determine the causes for these cell-to-cell variations, which may include phosphorylation (4), glycosylation, or regulation of other proteins that may be interacting with CEACAM1. What is clear, however, is these regions exist as an equilibrium of monomers and cis- homotypic oligomers.

Characterization of CEACAM1-4L-EYFP cell populations suggested that both the high intensity and low intensity regions had fairly oligomeric CEACAM1-4L-EYFP populations, although high intensity regions on average tended to have a higher anisotropy than the rest of the plasma membrane (Table 3.1). Since the anisotropy difference was small, more rigorous studies are needed to confirm that the high intensity region’s higher anisotropy is actually indicative of more monomeric CEACAM1-4L-EYFP. However, we also observed that 71% of the CEACAM1-4L-EYFP cells showed \( \frac{r_{\text{high intensity region}}}{r_{\text{low intensity region}}} > 1 \), suggesting that for the majority of the cells, the high intensity regions were more monomeric than the low intensity regions (Figure 3.4 B-C). This also meant that 29% of the CEACAM1-4L-EYFP cell population had \( \frac{r_{\text{high intensity region}}}{r_{\text{low intensity region}}} < 1 \), suggesting that the high intensity regions of these cells were more oligomeric. It should be noted that we used \( \frac{r_{\text{high intensity region}}}{r_{\text{low intensity region}}} = 1 \) as a theoretical value to indicate a cell with equal amounts of CEACAM1 monomers and cis-oligomers in the high intensity and low intensity regions. This value should be determined experimentally, ideally calibrated against biologically monomeric and dimeric CEACAM1 constructs that should show homogeneous oligomerization across the cell surface (regardless of intensity distribution).
To exclude imaging artifacts as the cause of the variations in anisotropy, control experiments were performed on CEACAM1-4L-EYFP by TIRPFM.

Figure 3.4 TIRFPM Anisotropy Imaging of HeLa cells Transiently Transfected with CEACAM1-4L-EYFP.

TIRFPM intensity image, anisotropy image and histogram of indicated ROI are shown (from left to right) for cell (A) without thresholding, (B) high intensity thresholding, and (C) low intensity thresholding. Raw histograms (light gray) are overlaid with least squares Gaussian fit (black line); mean $r_c \pm$ SD are shown next to histogram peak. Intensity images were treated with gamma filter function ($\gamma=0.7$) after image processing to facilitate simultaneous visualization of high intensity and low intensity features. Brightness and contrast settings are equal for these images. Inset is 9 µm X 9 µm.
3.4.3 Investigation of Possible CEACAM1-4L-EYFP Homo-FRET Imaging Artifacts

Since CEACAM1-4L-EYFP is a membrane protein and not soluble like the Venus proteins, TIRFPM imaging of CEACAM1-4L-EYFP was also assessed for imaging artifacts, including concentration-induced depolarization or photobleaching during image acquisition, and for confirmation of homo-transfer between fluorophores using progressive photobleaching.

CEACAM1-4L-EYFP, like soluble Venus monomer and dimer, showed no intensity-dependent anisotropy (Figure 3.5 A). Therefore, CEACAM1-4L-EYFP was also not subject to concentration-induced depolarization. As was also the case for monomeric and dimeric Venus, there was some photobleaching during the anisotropy image set acquisition (Figure 3.5 B). However, the difference in anisotropy between the high intensity and low intensity regions for a subset of cells was consistent regardless of image acquisition order, confirming that the effect of photobleaching was the same for the high intensity and low intensity regions. Anisotropy image set acquisition order (F\parallel, F\perp) was consistent throughout this study. It should also be mentioned that photobleaching caused a greater effect on CEACAM1-4L-EYFP anisotropy than Venus, likely due to the difference in protein concentration or photobleaching recovery (Figure 2.4 B, Figure 3.5 B). This indicated that Venus anisotropy was not an ideal calibration tool for directly enumerating CEACAM1-4L-EYFP oligomers on this platform. Therefore, we used anisotropy as an indication of the relative monomer and homotypic oligomer equilibrium rather than as an absolute ruler.

CEACAM1-4L-EYFP’s high intensity and low intensity regions both increased slightly with photobleaching, which was the trend also shown with the soluble Venus dimer (Figure 3.6). This confirmed the presence of CEACAM1-4L-EYFP cis- homotypic oligomers in both regions, as demonstrated from static steady-state anisotropy images. However, unlike the photobleaching curves of soluble Venus, the curves of the CEACAM1 high intensity and low intensity regions had varying mean initial anisotropy as well as varying slopes, which might be expected for a mixed population of monomers and oligomers. Cells with lower initial
Figure 3.5. TIRFPM Anisotropy Imaging Controls of HeLa Cells Transiently Transfected with CEACAM1-4L-EYFP.
(A) $r_c$ vs. intensity used to investigate intensity/concentration dependence of anisotropy. Each data point corresponds to the mean anisotropy at the central 9 µm X 9 µm area of an individual cell. (B) The anisotropy image set was acquired through emission polarizers in the following orders: (*left*) either F$_\parallel$ channel first, then F$_\perp$ channel second or (*right*) F$_\perp$ channel first, then F$_\parallel$ channel second. Mean $r_c$ for the subset of cells is shown above each bar.

Anisotropy, which would be more oligomeric, qualitatively had a larger increase in anisotropy upon photobleaching compared to cells with a higher initial anisotropy, which would be more monomeric. Furthermore, while the low intensity regions were inherently subject to more background noise than high intensity regions, we showed that these low intensity regions were composed of homotypic oligomers since the low intensity anisotropy was also sensitive to progressive photobleaching. Thus, the detection of homo-transfer in CEACAM1-4L-EYFP’s high intensity and low intensity regions was not the result of imaging artifacts.

Figure 3.6: Progressive Photobleaching of HeLa Cells Transiently Transfected with CEACAM1-4L-EYFP in TIRFPM.
Photobleaching curves of CEACAM1-4L-EYFP for high intensity regions (*black*, N = 5 cells) and dimeric Venus (*gray*, N = 3 cells) in epifluorescence using 20X 0.4 NA objective are shown.
3.4.4 Characterization of EYFP-labeled CEACAM1 Mutants

CEACAM1-4L-EYFP, CEACAM1-4S-EYFP, CEACAM1-4Δcyto-EYFP, and G432,436L-CEACAM1-4L-EYFP had similar distributions into high intensity and low intensity regions across the cell membrane (Figure 3.7 A-D). Compared to the other mutants, RQ43,44SL-CEACAM1-4L-EYFP had fewer high intensity regions and also less distinct difference in intensity between the high intensity regions and low intensity regions (Figure 3.7 E). This demonstrated that the N-terminal domain, and not the cytoplasmic tail nor cis-oligomerization, affected the distribution of CEACAM1 into these ezrin-rich regions. Currently it is unclear why a mutation in the extracellular domain would greatly affect CEACAM1 distribution, although Müller et. al. and Klaile et. al. have illustrated the importance of the N-terminal domain on CEACAM1 interactions (4, 63).

Like CEACAM1-4L-EYFP, the intensity-independent anisotropy of all the CEACAM1 mutants was more heterogeneous than that of the pure Venus monomers and dimers, which was expected for proteins that exist as a mixture of monomers and oligomers (Figure 3.7; Appendix 1). Comparison of the cytoplasmic tail mutants showed $r_{\text{CEACAM1-4L-EYFP}} < r_{\text{CEACAM1-4S-EYFP}} < r_{\text{CEACAM1-4Δcyto-EYFP}}$ for both high and low intensity regions (Figure 3.7; Table 3.1). Since CEACAM1’s decreasing cytoplasmic tail length may lead to a more rigid connection to EYFP, the anisotropy of the shorter cytoplasmic tail constructs may be more indicative of segmental orientation or distance (end of the cytoplasmic tail vs. closer to the transmembrane region) rather than oligomerization. This interpretation is supported by immunoblot studies that do not show any cytoplasmic tail-dependent regulation of CEACAM1’s monomer-oligomer equilibrium (6). If this is the case, then the aforementioned cytoplasmic tail-dependent anisotropy trend may be indicative of more structured high intensity regions as compared to low intensity regions, which may be expected if these regions are associated with ezrin and possible supramolecular structures.

RQ43,44SL-CEACAM1-4L-EYFP’s absolute anisotropy was comparable to that of CEACAM1-4L-EYFP for both the high intensity and low intensity regions (Figure 3.7 E; Table 3.1). From this data alone, it is not clear whether R43 and Q44 use homotypic or heterotypic interactions to recruit CEACAM1 to the high intensity regions. The N-terminal domain’s ability to mediate cis– homotypic interactions (4, 63) combined with our observation of an increased homogeneity
of RQ43,44SL-CEACAM1-4L-EYFP’s anisotropy compared to CEACAM1-4L-EYFP’s anisotropy for both the high intensity and low intensity regions (data not shown), suggests that the recruitment of CEACAM1 to the high intensity regions may be dependent on either R43- and Q44- mediated CEACAM1 cis-homotypic interactions. However, since we did not observe a change in CEACAM1’s intensity distribution for cis- monomer G432,436L-CEACAM1-4L-EYFP, nor a large difference in anisotropy between RQ43,44SL-CEACAM1-4L-EYFP’s high intensity and low intensity regions, nor a large difference between RQ43,44SL-CEACAM1-4L-EYFP and CEACAM1-4L-EYFP, it seems more probable that CEACAM1’s recruitment to the high intensity regions is dependent on R43- and Q44- mediated cis- heterotypic, and not homotypic, interactions.

Although we expected the monomeric mutant, G432,436L-CEACAM1-4L-EYFP, to have a homogeneously higher anisotropy (more similar to the Venus monomer) than CEACAM1-4L-EYFP, comparison of CEACAM1-4L-EYFP and G432,436L-CEACAM1-4L-EYFP did not show a difference in anisotropy. This suggested that the TIRFPM platform was limited in its ability to detect static differences between proteins at an equilibrium of monomers and oligomers, as compared to pure monomers and oligomers, which can be differentiated by TIRFPM anisotropy. However, applying progressive photobleaching to homo-FRET imaging may be more sensitive to the monomer-oligomer equilibrium differences between the CEACAM1 mutants.

Table 3.1: Mean $r_c$ of EYFP-labeled CEACAM1 Mutants

<table>
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<tr>
<th>Construct</th>
<th>N</th>
<th>Anisotropy, $r_c$</th>
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<td></td>
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<td>High Intensity ROI</td>
<td>Low Intensity ROI</td>
<td>$rac{r_{high intensity region}}{r_{low intensity region}}$</td>
</tr>
<tr>
<td>CEACAM1-4L-EYFP</td>
<td>122</td>
<td>0.161 ± 0.003</td>
<td>0.151 ± 0.002</td>
<td>1.07 ± 0.01</td>
</tr>
<tr>
<td>CEACAM1-4S-EYFP</td>
<td>32</td>
<td>0.171 ± 0.004</td>
<td>0.154 ± 0.004</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>CEACAM1-4Δcyto-EYFP</td>
<td>27</td>
<td>0.186 ± 0.005</td>
<td>0.166 ± 0.004</td>
<td>1.13 ± 0.02</td>
</tr>
<tr>
<td>G432,436L-CEACAM1-4L-EYFP</td>
<td>85</td>
<td>0.160 ± 0.003</td>
<td>0.149 ± 0.002</td>
<td>1.07 ± 0.01</td>
</tr>
<tr>
<td>RQ43,44SL-CEACAM1-4L-EYFP</td>
<td>18</td>
<td>0.164 ± 0.005</td>
<td>0.153 ± 0.005</td>
<td>1.08 ± 0.02</td>
</tr>
</tbody>
</table>

* Average anisotropy ± SE of $N$ number of cells
† Average value of measurements determined on a per cell basis
‡ Ratio = $\frac{r_{high intensity region}}{r_{low intensity region}}$
§ Difference = $r_{high intensity region} - r_{low intensity region}$

Average TIRFPM anisotropy (± SE) for $N$ number of cells transiently transfected with indicated constructs. Mean per cell $r_c$ were measured for central 9 µm x 9 µm ROI within the TIRFPM field of view.
Figure 3.7: Representative Intensity and TIRFPM Anisotropy Images of EYFP-labeled CEACAM1 Mutants.

Intensity and anisotropy images of (A) CEACAM1-4L-EYFP, (B) CEACAM1-4S-EYFP, (C) CEACAM1-4Δcyto-EYFP, (D) G432,436L-CEACAM1-4L-EYFP, and (E) RQ43,44SL-CEACAM1-4L-EYFP, masked into high intensity and low intensity regions. Intensity images were treated with gamma filter function ($\gamma=0.7$) after image processing to facilitate simultaneous visualization of high intensity and low intensity features. Brightness and contrast settings are equal for these images. Inset is 9 µm X 9 µm.
3.4.5 CEACAM1 cis-Homotypic Oligomer Response to Ionomycin

Although the TIRFPM platform was not optimal for detecting anisotropy differences between static images of CEACAM1 mutants, we suspected it might still be able to detect relative changes in anisotropy upon perturbation with factors that influence CEACAM1’s cis-homotypic interactions. Therefore, we investigated the effects of ionomycin on CEACAM1. This established a baseline for anisotropy changes upon cytoplasmic tail-mediated separation of cis-dimers that can be useful for investigating how monomers affect CEACAM1’s trans-interactions or heterotypic interactions at the plasma membrane.

CEACAM1-4L-EYFP anisotropy measured in the high intensity and low intensity regions showed little change over time after incubation with additional media at t = 0 minutes, indicating that CEACAM1-4L-EYFP was stable over the 2 hour imaging time frame used for the perturbation studies (Figure 3.8 A-B). Occasionally, cells exhibited a photobleaching-induced increase in anisotropy during the observed timeframe. Thus, the cells that had significant, negative correlations between their anisotropy and intensity (p<0.05) were disregarded (Figure 3.8 C).

When ionomycin (1 µM) was added to the CEACAM1-4L-EYFP cells, the anisotropy noticeably increased for the high intensity regions in comparison to CEACAM1-4L-EYFP cells perturbed by the loading vehicle (DMSO) or CEACAM1-4Δcyto-EYFP cells incubated with ionomycin (1 µM) (circles, Figure 3.9 A-B; Appendix 2 A). Since we did not observe this effect in CEACAM1-4Δcyto-EYFP cells, the ionomycin-induced anisotropy increase for CEACAM1-4L-EYFP’s high intensity regions was likely due to activated calmodulin binding to and disrupting CEACAM1’s cis- homotypic oligomers, as has been shown to occur biochemically over a similar timeframe (5-6). The limited response in the low intensity regions could have been due to instrumental insensitivity in detecting small changes in anisotropy, or due to the localization of activated-calmodulin to CEACAM1’s high intensity regions. Interestingly, time-course trends of average anisotropy for several cells suggested that CEACAM1-4L-EYFP’s low intensity
regions, like its high intensity regions, may also become more monomeric in response to ionomycin (Appendix 2 A). Further investigation is needed for clarification.

Figure 3.8: Baseline Time-lapse Characterization of CEACAM1-4L-EYFP’s cis-Homotypic Oligomerization.
(A) Additional media added to CEACAM1-4L-EYFP cell at t = 0 minutes. Images are shown at indicated time points. Intensity images were treated with gamma filter function (γ=0.7) after image processing to facilitate simultaneous visualization of high intensity and low intensity features. Brightness and contrast settings are equal for these images. Inset is 9 µm X 9 µm. (B) Raw histograms corresponding to the indicated ROIs at t = -30 minutes (light gray) and t = 60 minutes (pink line), were overlaid with least squares Gaussian fits at t = -30 minutes (black line) and t = 60 minutes (red line). (C) r_c vs. intensity traces for representative cells are shown: the cell displayed in A with no significant correlation between intensity and anisotropy (pink line) and a photobleaching cell showing significant negative correlation between anisotropy and intensity over the timeframe (black line). Photobleached cells were eliminated from the study.
Figure 3.9: Ionomycin Perturbation of CEACAM1 cis- Homotypic Oligomerization. (A) CEACAM1 response to activated calmodulin assessed using ionomycin (1µM) added to CEACAM1-4L-EYFP cells, DMSO vehicle added to CEACAM1-4L-EYFP cells, or ionomycin (1µM) added to CEACAM1-4Δcyto-EYFP at t = 0 minutes. Images are shown at indicated time points. Intensity images were treated with gamma filter function (γ=0.7) after image processing to facilitate simultaneous visualization of high intensity and low intensity features. Brightness and contrast settings are equal for these images. Inset is 9 µm X 9 µm. (B) Raw histograms corresponding to the indicated ROIs at t = -35 minutes (light gray) and t = 80 minutes (pink line), were overlaid with least squares Gaussian fits at t = -35 minutes (black line) and t = 80 minutes (red line).

3.4.6 CEACAM1 cis- Homotypic Oligomer Response to α-CEA pAb

CEACAM1 not only undergoes interactions that are mediated by its cytoplasmic tail but also interactions mediated by its extracellular domains. Perturbation with different α-CEACAM1 mAb has been shown to influence rat CEACAM1-4L and CEACAM1-4S cis- dimerization, which subsequently affected interactions with downstream signaling proteins (4). Here, we perturbed human CEACAM1 with polyclonal α-CEA antibody (Dako) to further investigate how the extracellular domain may regulate cis-dimerization and downstream signaling in a localized manner.
Unlike ionomycin, which increased intracellular calcium levels through the formation of membrane pores, pAb needed to physically access the extracellular domain of CEACAM1-4L-EYFP in order to induce clustering and potentially influence downstream signaling. We used FITC-dextran flow-through experiments to assess the accessibility of CEACAM1-4L-EYFP’s extracellular domain to pAb. Since larger distances between the cell and the cover slip create larger volumes for FITC-dextran to occupy, high FITC intensity typically indicates a large distance between the cell and cover slip. However, here the FITC channel intensity image was inverted for easier visualization of colocalization, such that high intensity now indicates a small distance between the cell and cover slip.

FITC-dextran flow-through experiments revealed that CEACAM1-4L-EYFP’s high intensity structures were not adhered to the untreated glass cover slip, which was expected from the mobility of the high intensity structures (Figure 3.10). Soluble mCherry and FITC-dextran were colocalized (yellow in the merged channel), demonstrating that the closer the cell membrane was to the surface, the larger the cell volume occupied by mCherry molecules within the exponentially decaying TIRF evanescent field (arrowhead, Figure 3.10 A). High concentrations of RFP-labeled F-actin also colocalized with areas in close proximity to the glass cover slip (yellow in the merged channel), suggesting that actin was present at tight contacts, most likely adhesions to the glass surface (arrowhead, Figure 3.10 B). Some F-actin did not colocalize with the FITC channel (arrow, Figure 3.10 B), indicating that some F-actin was not adhered to the glass cover slip. This actin distribution is consistent with the cytoskeleton’s numerous roles, many of which are not exclusively related to cellular adhesion (67). The CEACAM1-4L-mCherry’s high intensity regions (arrow, Figure 3.10 C) did not colocalize as well as soluble mCherry and F-actin to regions in closest contact with the cover slip (arrowhead, Figure 3.10 C), indicating that the high intensity regions were not in tight contact with the cover slip. Since CEACAM1-4L-EYFP’s high intensity regions were not tightly adhered to the glass cover slip, they were potentially accessible to external soluble factors, such as α-CEA antibody.
Figure 3.10: FITC-Dextran Exclusion Studies in TIRFM.
The red channel (red) shows HeLa cells transiently transfected with (A) soluble mCherry, (B) Lifeact-RFP (for labeling F-actin), or (C) mCherry-CEACAM1 WT. The red channel images were merged with the FITC-dextran channel images (green) to show correlation between protein position and cell surface proximity to the untreated glass cover slip. FITC-dextran channel images were thresholded to the excitation laser illumination area using ImageJ Huang auto-threshold plugin. The intensity LUT was then inverted, such that bright regions now indicate close proximity to a glass cover slip (typically indicative of adhesions to the substrate), and dark regions correspond to distances far from the glass cover slip surface. Arrows indicate high concentrations of expressed protein (in red fluorophore channel); arrowheads indicate regions of close proximity to the glass cover slip. Images are shown with auto-brightness and contrast to show comparable intensities in the merged channel.

α-CEA pAb was able to access the extracellular domains of EYFP-labeled CEACAM1 constructs and caused μm-scale clustering. pAb clustering was qualitatively visualized by the appearance of circular clustered features and increasing intensity in the low intensity regions, apparent at 80 minutes with internalization becoming apparent at 140 minutes (Figure 3.11 A). When pAb was added to CEACAM1-4L-EYFP cells, high intensity and low intensity regions showed an increase in anisotropy compared to the isotype control (circles, Figure 3.11). By binding multiple epitopes on different CEACAM1s, the pAb may have stabilized CEACAM1-
4L-EYFP monomeric conformation or clump between the CEACAM1-4L-EYFP monomers, thereby separating them at the molecular level enough to reduce the occurrence of homo-transfer.

Although the anisotropy of G432,436L-CEACAM1-4L-EYFP cells incubated with pAb did not appear different from that of cells incubated with isotype, both showed an increase in anisotropy after the addition of pAb (Figure 3.11; Appendix 2 B). This may have been due to unstable G432,436L-CEACAM1-4L-EYFP during the imaging time period. Already difficult to discern from static images of CEACAM1-4L-EYFP, G432,436L-CEACAM1-4L-EYFP anisotropy has to be characterized further before drawing definitive conclusions.

3.4.7 CEACAM1 exists primarily as cis-Oligomers at Cell-Cell Contacts

CEACAM1-4L-EYFP cells resuspended with CEACAM1-4L-mCherry cells formed cell-cell contacts that had characteristically high concentrations of CEACAM1, as expected from other studies (2, 4). Therefore, cell-cell contacts were identified as regions of high intensity between CEACAM1-4L-EYFP cells (visible in EYFP and mCherry channels due to bleed-through) and CEACAM1-4L-mCherry cells (visible only in mCherry channel). Anisotropy was calculated for CEACAM1-4L-EYFP to determine the effect of CEACAM1-4L-mCherry trans-homotypic interactions with CEACAM1-4L-EYFP on cis-homotypic oligomerization (Figure 3.12). Note that the CEACAM1-4L-mCherry anisotropy image was only background noise (Figure 3.12 A). Cell-cell contacts (ROI 1, arrowhead, Figure 3.12 C) typically had lower anisotropy than the rest of the cell (ROI 2, Figure 3.12 C), indicating cell-cell contacts were enriched in cis-homotypic oligomers relative to the rest of the cell (Table 3.2).
Figure 3.11: pAb Perturbation of CEACAM1 cis- Homotypic Oligomerization.
Figure 3.11 (Cont.): pAb Perturbation of CEACAM1 cis- Homotypic Oligomerization.

(A) CEACAM1 response to external clustering using pAb (20 µg/mL) added to CEACAM1-4L-EYFP cells, isotype (20 µg/mL) added to CEACAM1-4L-EYFP cells, pAb (20 µg/mL) added to G432,436L-CEACAM1-4L-EYFP cells, and isotype (20 µg/mL) added to G432,436L-CEACAM1-4L-EYFP cells at t = 0 minutes. Images are shown at indicated time points. Intensity images were treated with gamma filter function (γ=0.7) after image processing to facilitate simultaneous visualization of high intensity and low intensity features. Brightness and contrast settings are equal for these images. Inset is 9 µm X 9 µm. (B) Raw histograms corresponding to the indicated ROIs at t = -30 minutes (light gray), t = 80 minutes (light blue line), and t = 130 minutes (pink line), were overlaid with least squares Gaussian fits at t = -30 minutes (black line), t = 80 minutes (blue line), and t = 130 minutes (red line).

Figure 3.12: CEACAM1 cis-Homotypic Oligomers at Cell-Cell Contacts.

(A) CEACAM1-4L-mCherry cell, only visible in the mCherry channel, (B) CEACAM1-4L-EYFP cell, visible in both the EYFP and mCherry channel, and (C) cell-cell contact between CEACAM1-4L-mCherry cell (top of image) and CEACAM1-4L-EYFP cell (bottom of image) are shown. ROI 1 shows the cell-cell contact, and ROI 2 shows the rest of the cell, not in contact with other cells. The arrow indicates the boundary between cells. Intensity images were treated with gamma filter function (γ=0.7) after image processing to facilitate simultaneous visualization of high intensity and low intensity features. Brightness and contrast settings are equal for these images.
Table 3.2. Qualitative Assessment of CEACAM1-4L-EYFP cis- Homotypic Oligomers based on Anisotropy at Cell-Cell Contact Compared to the Rest of the Cell.

<table>
<thead>
<tr>
<th>r_{cell-cell} relative to r_{rest of cell}</th>
<th>N</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>More monomeric</td>
<td>1</td>
<td>8.3</td>
</tr>
<tr>
<td>More oligomeric</td>
<td>10</td>
<td>83.3</td>
</tr>
<tr>
<td>Mixture (monomers, oligomers)</td>
<td>1</td>
<td>8.3</td>
</tr>
</tbody>
</table>

3.5 Discussion

3.5.1 Cytoplasmic EYFP Label

We assumed that CEACAM1-4L-EYFP’s cytoplasmic tail was flexible, since studies have reported that the location of the fluorescent protein can result in a rigid fluorescent label or flexible linker (32). Using time-resolved fluorescence anisotropy, Hink et. al. showed that depending on GFP-label location, anisotropy relaxation curves can indicate segmental protein motion rather than entire protein rotation (18). Without access to time-resolved fluorescence anisotropy data or the CEACAM1 cytoplasmic tail structure, it was difficult to unambiguously determine the flexibility of CEACAM1’s cytoplasmic tail. However, it appeared that in comparison with the CEACAM1 mutants with shorter cytoplasmic tails, CEACAM1-4L-EYFP was more flexible; anisotropy increased with decreasing cytoplasmic tail length for both high intensity and low intensity regions, although immunoblots did not show this same trend (6). This implicated that the observed anisotropy trends may be reflective of cytoplasmic tail rigidity rather than oligomerization (further discussed in Discussion Section 3.5.2). For these reasons, we assumed that EYFP had a relatively flexible connection to the CEACAM1-4L-EYFP. This might enable the long cytoplasmic tail to sample many orientations and emit depolarized light upon homo-transfer (28, 53), or to reach distances close enough to undergo homo-transfer that are not accessible by shorter cytoplasmic tails. In the future, cytoplasmic tail flexibility using steady-state anisotropy could be assessed by labeling CEACAM1 with EYFP at different locations and with a small peptide linker.
3.5.2 EYFP-labeled CEACAM1 Mutant Constructs

CEACAM1-4L-EYFP was a mixture of monomers and oligomers in both its high intensity and low intensity regions, with some cells appearing more monomeric and others more oligomeric at the high intensity, ezrin-rich regions. On average, CEACAM1-4L-EYFP’s high intensity regions had a slightly higher anisotropy than the low intensity regions; this finding correlates with our group’s previous hetero-FRET TIRFM images suggesting that monomers were localized to these high intensity regions (47). CEACAM1-4L-EYFP’s more monomeric distribution in the high intensity regions may be reflective of localized protein interactions with CEACAM1 monomers. The hetero-FRET images, however, showed more discrete separation of monomers and oligomers compared to TIRFPM homo-FRET images that showed CEACAM1 as a more heterogeneous mixture of monomers and cis-oligomers with a rather small difference in average anisotropy between high and low intensity regions. These findings are in agreement with confocal hetero-FRET investigation of rat CEACAM1 showing CEACAM1 as a mixture of monomers, dimers and oligomers at the free cell edge (4), although here we have begun characterizing the spatial distribution of this mixture across the cell membrane and at points of cell-cell contact. Since rat CEACAM1 monomer-dimer equilibrium is sensitive to clone (5), expression levels (5), and phosphorylation (4), studies investigating these factors would more rigorously determine the high intensity structures’ role(s) in cis-oligomerization and causes for their variability.

To further assess the sensitivity of steady-state TIRFPM anisotropy to CEACAM1 homotypic oligomerization and to investigate the functional roles of CEACAM1’s domains, several chimeric EYFP-tagged CEACAM1 were studied. Untagged CEACAM1-4L, CEACAM1-4S, CEACAM1-4Δcyto, RQ43,44SL-CEACAM1-4L showed monomeric and dimeric immunoblot bands, although larger order oligomers have not been excluded (6). The RQ43,44SL-CEACAM1-4L mutant, unlike the others, did not undergo trans- homotypic interactions (6). The transmembrane momoneric mutant, G432,436L-CEACAM1-4L, ran as a single monomeric band (Gray-Owen group, personal communication).

Although CEACAM1-4S-EYFP, CEACAM1-4Δcyto-EYFP, and G432,436L-CEACAM1-4L-EYFP mutants had intensity distributions resembling that of CEACAM1-4L-EYFP, RQ43,44SL-
CEACAM1-4L-EYFP had fewer and less discrete high intensity regions. These characteristics of RQ43,44SL-CEACAM1-4L-EYFP were visible on the basal and apical side of the cell using confocal (data not shown). This suggested that the N-terminal domain, and not the cytoplasmic tail nor cis-homotypic oligomerization, regulates CEACAM1’s localization to these high intensity, ezrin-rich regions (47). Furthermore, although this N-terminal domain mutant was designed for studying CEACAM1’s intercellular interactions, it clearly also affects intracellular regulation of CEACAM1. Müller et. al. and Klaile et. al. have also reported the importance of the N-terminal domain in facilitating trans- and cis- homotypic interactions (4, 63), indicating the ability for the N-terminal domain to regulate cis- interactions. Since these changes are unlikely due to CEACAM1’s cis- homotypic interactions (specifically those mediated by the GXXG transmembrane motif), it is possible that CEACAM1’s R43 and Q44 undergo heterotypic cis- interactions that concentrate CEACAM1 to these regions, which in turn recruits ezrin.

In spite of the cytoplasmic tail mutants’ similar intensity distributions, the trend $r_{\text{CEACAM1-4L-EYFP}} < r_{\text{CEACAM1-4S-EYFP}} < r_{\text{CEACAM1-4Δcyto-EYFP}}$ was observed for both the high and low intensity regions. Since a shorter cytoplasmic tail might act as a more rigid EYFP linker to CEACAM1, depolarization as a consequence of homo-transfer may be less apparent (28). Alternately, the EYFP rigidly attached to the shorter cytoplasmic tails may not be able to undergo homo-transfer if CEACAM1’s transmembrane domains are separated at distances >10 nm. Either way, anisotropy would be higher for a shorter linker (in this case the cytoplasmic tail), which was consistent with our observations. Although the trend of increasing anisotropy with decreasing cytoplasmic tail length could have been a consequence of cytoplasmic tail-dependent differences in oligomer regulation, it was unlikely since immunoblot studies showed that the cytoplasmic tail length did not greatly affect monomer – cis-dimer equilibrium (Gray-Owen group, personal correspondence) (6). Therefore, this trend may reflect fluorescent protein restraint or rigidity rather than oligomerization. Furthermore, since these constructs still concentrated to the high intensity regions with similar appearances, we suspected this anisotropy difference was not a result of cytoplasmic tail-dependent regulation. More thorough characterization of the high intensity regions’ composition(s) is needed, however, to confirm that the nature of these high intensity regions is similar (or not) for the different mutants.

Although RQ43,44SL-CEACAM1-4L-EYFP had a different cell surface distribution than CEACAM1-4L-EYFP, RQ43,44SL-CEACAM1-4L-EYFP’s and CEACAM1-4L-EYFP’s
average anisotropy values were similar, for both their high intensity and low intensity regions. Across the cell surface, RQ43,44SL-CEACAM1-4L-EYFP, like CEACAM1-4L-EYFP, also showed more heterogeneous anisotropy than the Venus constructs, although it showed less heterogeneous anisotropy than the other CEACAM1 mutants (Figure 3.7; data not shown). Without the use of a dimeric CEACAM1 control or progressive photobleaching homo-FRET studies, it is unclear if RQ43,44SL-CEACAM1-4L-EYFP’s more homogeneous anisotropy, compared to the other CEACAM1 constructs, is an indication of more homogeneous oligomers (or monomers) that is simply undetected by absolute anisotropy. However, RQ43,44SL-CEACAM1-4L-EYFP’s and CEACAM1-4L-EYFP’s anisotropy similarities, conservation of RQ43,44SL-CEACAM1-4L-EYFP monomers and cis-dimers at the cell surface according to immunoblots (6), and cis-monomeric G432,436L-CEACAM1-4L-EYFP’s and CEACAM1-4L-EYFP’s similar intensity distributions, suggested that the recruitment of CEACAM1 to the high intensity regions was regulated by R43- and Q44-dependent heterotypic, and not homotypic, interactions. Studies are ongoing to determine the mechanism and significance of this mutation.

While TIRFPM anisotropy was useful for distinguishing monomeric Venus from dimeric Venus, this technique was unable to differentiate between CEACAM1-4L-EYFP and biochemically monomeric G432,436L-CEACAM1-4L-EYFP. Since G432,436L-CEACAM1-4L-EYFP’s high intensity and low intensity regions showed similar anisotropy values to CEACAM1-4L-EYFP, even though low intensity regions were much less concentrated than the high intensity regions, our inability to detect G432,436L-CEACAM1-4L-EYFP monomerization was not likely due to concentration-induced depolarization. Therefore, this data suggested that the absolute anisotropy values obtained on our TIRFPM platform were unable to detect static differences in CEACAM1’s monomer-oligomer equilibrium. Whether this absolute anisotropy insensitivity to G432,436L-CEACAM1-4L-EYFP monomers is due to a small CEACAM1 anisotropy range compared to that of the pure Venus constructs or due to the segmental freedom of the cytoplasmic tail that may not be reflective of the monomerization (or oligomerization) of the entire protein, cannot be resolved without a dimeric CEACAM1 construct or a CEACAM1 labeled with EYFP at different positions. However, progressive photobleaching curves of $r_c$ vs. intensity may be more sensitive to shifts in the monomer-oligomer equilibrium for the different CEACAM1 mutants, and detect differences specifically between CEACAM1-4L-EYFP and G432,436L-CEACAM1-4L-EYFP.
3.5.3 CEACAM1 cis-Homotypic Oligomerization in Response to Ionomycin

Although steady-state anisotropy differences between static mutants were not well-resolved, we were able to detect relative changes in anisotropy by perturbing CEACAM1. Ionomycin, a calcium ionophore, increases free calcium in the cytoplasm that activates calmodulin, which can then separate CEACAM1-4L cis-dimers but not CEACAM1-Δcyto dimers that lack the calmodulin-binding motif (6). Ionomycin increased anisotropy slightly for CEACAM1-4L-EYFP high intensity regions relative to CEACAM1-4L-EYFP incubated with DMSO and CEACAM1-4L-Δcyto-EYFP incubated with ionomycin. This correlated with immunoblot studies (6) showing that calmodulin-dependent interactions with CEACAM1’s cytoplasmic tail regulate CEACAM1’s cis-homotypic oligomerization (since we do not see this effect with the mutant lacking the cytoplasmic tail.)

It was surprising that the increase in anisotropy was isolated to CEACAM1-4L-EYFP high intensity regions and not to low intensity regions, suggesting either a localized effect of calmodulin or TIRFM anisotropy’s insensitivity to oligomer changes in the low intensity regions due to low signal. It is also possible that changes in oligomerization in the high intensity regions, as a result of calmodulin binding, might outweigh changes in the more diffuse plasma membrane regions, thus explaining the discrepancies between the immunoblots (showing complete conversion to monomers upon ionomycin treatment) and homo-FRET trends reported here. On the other hand, averaged anisotropy values showed an increase in the anisotropy of CEACAM1-4L-EYFP’s low intensity regions upon perturbation with ionomycin, indicating the occurrence of some cell-to-cell variability in ionomycin response (Appendix 2 A). Therefore, hetero-FRET investigations into labeled-calmodulin’s localization at the cell membrane and interactions with CEACAM1-4L-EYFP, after perturbation with ionomycin, can resolve some of the aforementioned discrepancies.

3.5.4 CEACAM1-Substrate Contact

FITC-dextran exclusion studies indicated that CEACAM1-4L-mCherry’s high intensity, ezrin-rich regions were not localized to areas in tight contact with the untreated glass cover slip, unlike soluble mCherry and F-actin. This and subsequent experiments showed the extracellular
domains of CEACAM1-4L-EYFP visible at the basal membrane were accessible to extracellular perturbations, like α-CEA antibody.

3.5.5 CEACAM1 cis-Homotypic Oligomerization in Response to trans-Ligation by pAb

α-CEA pAb was able to access and cluster CEACAM1-4L-EYFP, as was observed by the increasing intensity in the low intensity regions as well as increased grainy and circular microclustered appearance across the cell membrane. CEACAM1-4L-EYFP incubated with pAb had larger anisotropy increase in high intensity regions and low intensity regions compared to incubation with isotype, suggesting that antibody-binding separated CEACAM1-4L-EYFP dimers (Figure 3.11; Appendix 2 B). Müller et. al. previously showed different α-CEACAM1 mAb could cause either an increase or decrease in FRET efficiency, postulating that mAb stabilized different rat CEACAM1 conformations (4), confirmed by Klaile et. al. (63). Since we used a pAb as opposed to mAb, pAb possibly stabilized monomeric conformations or separated CEACAM1-4L-EYFP by clumping between CEACAM1’s extracellular domains to reach all the epitopes.

Although G432,436L-CEACAM1-4L-EYFP also had an increasing anisotropy trend with the addition of pAb, which was unexpected for a monomeric protein, the anisotropy trend was similar to that observed with the isotype (Figure 3.11; Appendix 2 B). It is unclear why the anisotropy increased for G432,436L-CEACAM1-4L-EYFP with pAb as well as isotype, but may reflect a low stability of G432,436L-CEACAM1-4L-EYFP over the imaging time frame that was not caught by static images. The anisotropy trend is unlikely an imaging artifact because CEACAM1-4L-EYFP with the isotype did not show the same trend (Appendix 2 B). These observations can be assessed by characterizing unperturbed G432,436L-CEACAM1-4L-EYFP over the same time period.

While CEACAM1-4L-EYFP cis- homotypic oligomer was responsive to pAb perturbation, it is unclear how the increase in CEACAM1 monomers may influence downstream signaling. Therefore it would be ideal to study pAb-induced changes on signaling molecule recruitment and interactions with the cytoskeleton.
3.5.6 CEACAM1 cis-Homotypic Oligomerization at Cell-Cell Contacts

Although it is well-established that CEACAM1’s trans-homotypic interactions mediate cell-cell adhesion, researchers have only recently begun investigating the role that CEACAM1’s trans-homotypic oligomers may have on cis-oligomerization and vise versa. Here we report an increase in CEACAM1-4L-EYFP cis-homotypic oligomers at cell-cell contacts. However, since CEACAM1-4L-EYFP is highly concentrated at these cell-cell contacts, additional controls, such as progressive photobleaching or dilution of CEACAM1-4L-EYFP, should be performed to address the possibility of concentration-induced depolarization. These results, though, are in agreement with Klaile et. al. and Müller et. al. studies showing that rat CEACAM1 trans-homotypic interactions promote CEACAM1 cis-oligomerization (4, 63). In contrast, the Gray-Owen group used cell aggregation studies to show an increasing rate of growth and size of monomeric G432,436L-CEACAM1-4L cell aggregates compared to CEACAM1-4L cell aggregates, demonstrating that the CEACAM1-4L monomer is responsible for forming the cell-cell trans-interactions (Gray-Owen group, personal communication). However, different cells also showed a decrease in cell aggregations upon treatment of CEACAM1-4L with ionomycin, suggesting that other cell regulation-dependent factors may influence the ability to form trans-interactions (6).

By assessing different steps during cell-cell contact formation, these studies together suggest that monomers in cis trigger CEACAM1 trans-homotypic interactions. However, since there was some variability in the outcomes, other factors may also influence the effectiveness of cis-mediated trans-interactions. Furthermore, Müller et. al. showed that the perturbation of cells with mAb that reduced cis-homotypic oligomers increased cell adhesion to rat CEACAM1-Fc coated dishes, whereas mAb that increased cis-homotypic oligomers decreased cell adhesion to rat CEACAM1-Fc coated dishes (4). This difference in trans-homotypic adhesion was attributed to different conformational stabilizations of the N-terminal domain (4). In light of the Gray-Owen group’s studies, the formation of trans-homotypic interactions may have been regulated by the stabilization of CEACAM1 cis-monomers, which then affected the N-terminal domain conformation and trans-binding.
After initial CEACAM1 cis-monomer-mediated trans-interactions, CEACAM1 may undergo structural rearrangement(s) that favors or necessitates cis-homotypic oligomerization for maintaining trans-interactions. This is suggested by our homo-FRET studies showing cis-homotypic oligomers at cell contacts since our biological model - transiently transfected CEACAM1-4L-EYFP HeLa cells – was nearly identical to that used for the Gray-Owen group’s cell aggregation studies, except the homo-FRET studies, were performed 12 hours after cell resuspension. This would allow many hours for cell contact formation and stabilization, whereas cell aggregation studies assessed early formation of aggregation. Furthermore, Müller et. al. also found cell-cell contacts to contain more rat CEACAM1 cis-homotypic oligomers, when imaged 24-48 hours after transfection.

This model can be addressed by time-course anisotropy study of the formation and development of cell-cell contacts using appropriate CEACAM1 mutants and more rigorous imaging controls (like progressive photobleaching) to eliminate the possibility of concentration-depolarization effect at these CEACAM1-rich cell contacts. In addition, these homo-FRET studies would also require extension of the current CEACAM1 mutant library, which is primarily EYFP-tagged, in order to image contacts between EYFP-labeled CEACAM1 and mCherry-labeled CEACAM1 constructs. Finally, since TIRFPM only accesses the side-view of cell-cell contacts, confocal homo-FRET would be a more suitable platform for imaging CEACAM1 localization and oligomerization during cell-cell contact formation.
Chapter 4

4 Conclusions and Future Directions

4.1 Chapter Summary

Here we present a study of the localization and regulation of CEACAM1’s *cis*- homotypic oligomerization using TIRFPM homo-FRET imaging. The results revealed that the perturbation of *cis*-homotypic oligomerization can be monitored by real-time imaging, providing impetus for future investigations into how CEACAM1’s *cis*- homotypic oligomerization can influence CEACAM1’s inside-out and outside-in signaling pathways that ultimately affect tumorigenesis, metabolism, and immune function. Future studies can focus on applying other advanced imaging techniques to study CEACAM1’s multifaceted homotypic and heterotypic interactions with CEACAM1 ligands such as calmodulin, actin, SHP-1 and SHP-2, on the TIRFPM system. These imaging approaches can then be applied to study interactions of other membrane proteins.

4.2 Conclusions and Future Directions

4.2.1 Sensitivity of the TIRFPM System

We applied TIRFPM homo-FRET imaging to study the regulation of CEACAM1’s *cis*- homotypic oligomerization. Although the TIRFPM absolute anisotropy was lower than the epifluorescence anisotropy, it could still resolve differences between pure soluble Venus monomers and dimers. In application, however, the TIRFPM anisotropy was unable to distinguish CEACAM1-4L-EYFP from the monomeric CEACAM1-4L mutant G432,436L-CEACAM1-4L-EYFP, suggesting that the TIRFPM absolute anisotropy value may not be well-suited for determining static differences between membrane proteins existing in a monomer-oligomer equilibrium. However, this is still ambiguous without a dimeric CEACAM1 control. Since the Venus monomer and dimer are not ideal constructs for establishing CEACAM1’s anisotropy range, generation of a constitutive or inducible CEACAM1-4L-EYFP dimer would unambiguously establish the lower anisotropy threshold for CEACAM1’s monomer-oligomer equilibrium. In fact, the fusion of GFP to one or two FK506-binding protein domains has already been confirmed to undergo either ligand binding-induced dimerization or
oligomerization, respectively, using homo-FRET imaging (28). This would serve as a useful model and tool for studying CEACAM1-4L-EYFP’s cis-dimer function and for rigorously characterizing the TIRFPM homo-FRET imaging’s suitability for studying membrane protein interactions (Table 4.1).

In addition to biological controls, additional homo-FRET imaging strategies can be useful for more comprehensive and conclusive studies about CEACAM1’s homotypic oligomerization. Ideally, time-resolved anisotropy would more definitively determine molecular details such as the homo-FRET transfer rate, dynamically-averaged Förster radius, and the range of intermolecular distance(s). However, when that is not an option, steady-state homo-FRET imaging can be used to more quantitatively enumerate homotypic oligomers through progressive photobleaching or controlled oligomerization (Table 4.1) (28, 38, 44). This would enable the determination of monomers, dimers, and higher order oligomers from trends in anisotropy rather than the absolute anisotropy, which was not useful in distinguishing monomeric G432,436L-CEACAM1-4L-EYFP from the mixed monomeric and oligomeric CEACAM1-4L-EYFP. Differentiating dimers and larger order oligomers may also be biologically relevant. For example, another adhesion molecule, platelet endothelial cell adhesion molecule-1 (PECAM-1), regulates trans-interactions through shifts in its cis-dimer and cis-oligomer equilibrium (68). Currently, it is unclear if changes specifically in the oligomer size may also influence CEACAM1’s trans-interactions.

Several strategies have been developed to use steady-state anisotropy to enumerate oligomer size. Fits for anisotropy vs. photobleaching (or fractional fluorescence labeling) curves using mathematical models developed by Yeow et. al. can be used to determine monomers, dimers, tetramers, and larger order oligomers (38). In contrast, Bader et. al. used monomer, dimer, and oligomer GFP controls to calibrate relative oligomer composition within clusters of GFP-labeled proteins, like EGFR. Although not as sensitive for enumerating N-mers as the mathematical models (38, 44), this technique can be quite accurate if using time-resolved anisotropy to determine $r_{inr}$. It was also shown that the typical $r_{inr} = 0$, assumed for some mathematical models, may not be accurate especially for labeled membrane proteins (28, 44). Either of these methods can be applied to our anisotropy image processing for a more quantitative assessment of
CEACAM1 cis-oligomers. However, as we have already observed issues with TIRFPM sensitivity to differences between mutants, this quantification should be done in comparison to more well-established imaging platforms, like confocal homo-FRET, and ideally with proper controls, like the dimeric CEACAM1 or other known monomeric and dimeric membrane proteins (since soluble Venus was not easily photobleached in TIRFPM). Ideally, these anisotropy quantification studies would also be compared with alternate fluorescence analysis techniques used for studying oligomerization, such as numbers and brightness or FCS (Table 4.1). Since the read-out for these complementary techniques are not dependent on fluorescence depolarization, these techniques would also illuminate whether anisotropy of the EYFP attached to CEACAM1-4L’s cytoplasmic tail is affected by cytoplasmic tail orientation changes between the high intensity and low intensity regions.

4.2.2 CEACAM1 cis-Homotypic Oligomerization, Structure, and Kinetics

While homotypic oligomerization plays an important role in mediating signals across the plasma membrane, it is unlikely to be the only factor. Conformational changes and kinetics often affect signal transduction for other adhesion proteins, like integrins (69). Therefore, it is also necessary to consider CEACAM1’s structure and dynamics and how they interface with oligomerization. Investigations into CEACAM1’s heterotypic interactions, conformational changes, kinetics, and sub-diffraction-limited distribution at the high intensity and low intensity regions may offer insight into the nature and purpose of CEACAM1’s distribution in HeLa cells.

Currently, the role of CEACAM1’s high intensity regions in HeLa cells is not entirely clear. CEACAM1-4L-EYFP, CEACAM1-4S-EYFP, CEACAM1-4Δcyto-EYFP, and G432,436L-CEACAM1-4L-EYFP had similar distributions across the cell membrane, with µm-sized high intensity regions and more diffuse distribution across the rest of the plasma membrane. These structures were previously determined to be ezrin-rich for CEACAM1-4L-EYFP (47), though characterization of other components in these high intensity regions (such as lipids, integrins, and/or other CEACAMs) would clarify whether the composition of the high intensity regions is conserved for all the mutants. Since only the RQ43,44SL-CEACAM1-4L-EYFP, and not the cytoplasmic tail mutants nor cis- oligomers, showed fewer high intensity regions and less discrete high intensity regions than the other constructs, CEACAM1’s localization to the high
intensity regions appeared to be dependent on the interactions of the N-terminal extracellular domain’s R43 and Q44.

CEACAM1’s recruitment to the high intensity regions through interactions mediated by its N-terminal extracellular domain appeared to be dependent on heterotypic rather than homotypic interactions because (1) RQ43,44SL-CEACAM1-4L-EYFP and CEACAM1-4L-EYFP had distinct intensity distributions but comparable anisotropy values; (2) cis-monomeric G432,436L-CEACAM1-4L-EYFP and CEACAM1-4L-EYFP had similar intensity distributions; and (3) immunoblot detection of RQ43,44SL-CEACAM1-4L-EYFP showed presence of monomers and cis- homotypic dimers. Due to the TIRFPM’s insensitivity to detect monomeric G432,436L-CEACAM1-4L-EYFP using absolute anisotropy, additional controls (like progressive photobleaching) should be used to confirm both RQ43,44SL-CEACAM1-4L-EYFP’s and G432,436L-CEACAM1-4L-EYFP’s monomer – cis- homotypic oligomer equilibrium (Table 4.1).

We suspect that R43’s and Q44’s recruitment of CEACAM1 depends on cis- heterotypic interactions with other membrane proteins or lipids. Therefore, systematic studies of membrane protein or lipid composition using hetero-FRET or colocalization studies with actin, labeled lipids, or other labeled-adhesion proteins, including integrins and other CEACAMs, can be used to investigate members of the supramolecular structure responsible for organizing the high intensity regions. These studies may be further complicated by the ability of CEACAM1’s extracellular domain to adopt numerous conformations, enabling it to mediate both cis- and trans- homotypic interactions (63). This is also likely to occur in the case of any heterotypic interactions, such that CEACAM1 may undergo conformational changes that expose or hide its N-terminal extracellular domain’s interacting face(s). Single molecule hetero-FRET could be useful for characterizing transient conformational changes of dual-labeled CEACAM1 constructs. Ideally, these experiments would be performed on a library of CEACAM1 constructs, with a fluorescent donor and acceptor attached to different positions on each construct. Depending on E_{FRET} (and calculated r_d) changes in response to different interactions or perturbations, the conformational changes of CEACAM1’s domains could then be mapped (Table 4.1). Combined, these studies may generate a more comprehensive model of
CEACAM1’s homotypic and heterotypic interactions at the cell membrane and may illuminate any signaling roles of the high intensity regions.

Homo-FRET imaging can also be used to study membrane protein orientation using fluorophores rigidly linked to the protein of interest; this may be useful for studying CEACAM1’s structural and conformational changes in the context of the cell membrane. Interestingly, decreasing CEACAM1’s cytoplasmic tail length correlated with increased anisotropy; this trend seemed consistent with an increasingly rigid EYFP linker to a protein. The rigidity of the CEACAM1 mutants’ cytoplasmic tails can be more directly assessed by mapping anisotropy as a function of position/angle on a circular bleb (32, 45-46). Position-independent anisotropy would suggest a flexible linker, whereas position-dependent anisotropy would suggest a rigid linker. Although distinct regulation of the cytoplasmic-tail CEACAM1 mutants may result in the mutants’ different anisotropy values, this discrepancy can be resolved by determining the anisotropy of a chimeric CEACAM1-4L with an EYFP inserted between the transmembrane domain and the cytoplasmic tail. We suspect the anisotropy of this CEACAM1-EYFP-4L construct would be similar to that of CEACAM1-4Δcyto-EYFP since CEACAM1-4Δcyto-EYFP, in spite of its nonexistent cytoplasmic tail, still showed similar distribution patterns to CEACAM1-4L-EYFP. However, studies of the protein and lipid composition in these high intensity regions are necessary to confirm whether the high intensity regions are conserved for the different CEACAM1 constructs. From these preliminary results, anisotropy of the shorter cytoplasmic tail-labeled CEACAM1 constructs (or a CEACAM1-EYFP-4L construct) may act as a reporter of CEACAM1 segmental orientation or distance, rather than just oligomerization (Table 4.1) (46). Therefore, this application of homo-FRET imaging would enable investigation of protein orientation or distance relative to the plasma membrane and importantly, how different protein interactions transmit signals through conformational changes in CEACAM1.

To better understand the monomer – cis-oligomer heterogeneity in the high intensity and low intensity regions for all the CEACAM1 mutants, additional imaging approaches can be used. For example, by combining brightness studies with fluorescence recovery after photobleaching (FRAP) (which assesses diffusion and mobility of a labeled protein or lipid of interest by the fluorescence signal recovery in an intentionally photobleached ROI), Mandl et. al. studied membrane protein oligomerization and dynamics in highly concentrated areas at the single
molecule level (70). After photobleaching a 3 µm X 3 µm ROI, labeled protein clusters diffusing back into the photobleached ROI were analyzed using numbers and brightness to simultaneously quantify oligomer size and rate of diffusion. FRAP can also be combined with homo-FRET, as illustrated by Roberti et. al. (71); this would be useful to simultaneously assess single molecule-level dynamics, directionality, and heterogeneity of CEACAM1 oligomerization for both high intensity and low intensity regions (Table 4.1). However, for TIRFPM studies in particular, it would be important to first assess the effects that such low signal may have on anisotropy. By then adding, depleting, or blocking different potential CEACAM1 ligands, we could use this technique to determine the importance of these high intensity regions in mediating signaling pathways. A complementary approach for studying oligomerization and kinetics, FCS could be used to determine CEACAM1 cluster size and possible directional migration of CEACAM1 sub-clusters within these high intensity regions. For two-color experiments, fluorescence cross-correlation spectroscopy (FCCS) could then also be used to assess co-migration of CEACAM1 and other proteins or lipids. In addition, super-resolution imaging strategies, like PALM, can be used to assess nm-scale localization of CEACAM1 within the µm-scale high density clusters (Table 4.1). Other members of the Yip group are currently implementing many of the aforementioned studies.

4.2.3 The Roles of CEACAM1’s Interactions in Inside-Out and Outside-In Signal Transduction

While static steady-state TIRFPM anisotropy images were unable to resolve differences between the distinct CEACAM1 mutants, it was capable of monitoring changes in anisotropy induced by intracellular and extracellular interactions. Incubation with ionomycin induced a shift towards more monomeric CEACAM1-4L-EYFP for the high intensity regions compared to cells incubated with DMSO. Therefore, as previously reported, CEACAM1’s monomer-oligomer equilibrium can be controlled through intracellular interactions, perhaps in a localized manner (5-6). We also report the disruption of CEACAM1 cis-oligomers by α-CEA pAb’s trans-ligation of CEACAM1. Additional studies with mAb may also offer insight into Ab regulation of CEACAM1 by locking specific epitopes on the extracellular domain thereby controlling oligomerization, as was also shown by Müller et. al. (4). While this suggests that extracellular
factors are also capable of influencing CEACAM1 cis-oligomerization, it is unclear how this trans- ligation may influence downstream signaling. Müller et. al. showed trans-ligation with different mAbs could either increase or decrease cis-oligomerization of rat CEACAM1-L, which in turn led to differential recruitment of c-src, SHP-1, and SHP-2 in an ITIM-dependent manner (4). Thus cis-homotypic oligomerization of CEACAM1 can transmit signals through the plasma membrane to regulate intracellular downstream signaling. Therefore, future studies should investigate whether downstream pathways, including recruitment of signaling molecules (calmodulin, c-src, SHP-1, SHP-2, actin) are localized to particular regions or influenced by changes in CEACAM1 cis-homotypic oligomerization. We have already demonstrated that TIRFPM anisotropy was sensitive to real-time changes in EYFP-labeled CEACAM1 oligomerization, so coupling these studies with hetero-FRET or colocalization studies of labeled signaling molecules may illuminate cis-oligomerization-dependent recruitment of signaling proteins in a spatially- and dynamically- resolved fashion across the cell membrane (Table 4.1).

The CEACAM1 field has recently begun focusing on molecular-scale studies of CEACAM1’s cis- and trans- interactions. Aggregation studies of cells exhibiting monomeric CEACAM1 at the cell surface, either untreated G432,436L-CEACAM1-4L or CEACAM1-4L treated with ionomycin, showed increased aggregation compared to untreated CEACAM1-4L cells (Gray-Owen lab, personal communication). Therefore, it seems CEACAM1 monomers are responsible for initiating trans- homotypic interactions. In addition, Müller et. al. previously showed that the N-terminal domain is important for mediating both cis- and trans- interactions (4). Results from these CEACAM1 studies suggested that CEACAM1’s regulation of its cis- and trans- interactions may be similar to that of other immunoreceptors, shown to use homotypic and/or heterotypic interactions in cis to reduce the number of receptors free to undergo trans- interactions (72). Our homo-FRET studies, in contrast to the cell aggregation studies, were performed several hours after the formation of cell-cell contacts, suggesting that the cis-homotypic oligomers may form as a result of conformational changes mediated by the trans-homotypic interactions during the stabilization of cell-cell contacts. This is supported by Klaile et. al. studies, showing different extracellular domain conformations that can be adopted by CEACAM1 monomers, cis- oligomers, and trans- oligomers (63). Homo-FRET studies were consistent with acceptor photobleaching FRET showing that rat CEACAM1 had higher FRET
efficiency at cell-cell contacts compared to the free edges (4). These studies were also done at a later time point, 24-48 hours after transfection, and therefore many hours after cell contact formation.

It should be noted that when HeLa cells stably transfected with human CEACAM1 were treated with ionomycin, thereby shifting cis-homotypic oligomers to monomers, cell aggregation was reduced, suggesting that in this assay, dimers were responsible for trans- homotypic binding (6). Therefore, depending on cellular regulation or possibly transient changes in cis- homotypic oligomerization, the effect of monomers versus cis- oligomers on trans- interactions may vary. This is not unexpected, as CEACAM1 regulation is affected by many factors, including homotypic and heterotypic interactions, cell type, concentration (5), glycosylation, and phosphorylation kinetics (4). Therefore, systematic studies assessing these factors on CEACAM1 oligomerization may give insight into the variability of our studies. Furthermore, real-time characterization of CEACAM1’s anisotropy, localization, and trafficking during cell contact formation and stabilization would give important insight into the regulation of CEACAM1 trans-homotypic interactions (Table 4.1). These studies of trans-interactions can be combined with perturbations with, for example ionomycin (5-6), pervanadate (for sustained phosphorylation) (4, 7), or other intracellular regulators, to illuminate additional methods for preventing, controlling, or even disrupting CEACAM1 trans- interactions. Furthermore, since the cell-cell contacts were wide and densely populated by CEACAM1, better lateral resolution through PALM imaging may be useful for identifying sub-structural organization of CEACAM1 at cell-cell contacts.

If CEACAM1’s extracellular domain can influence cis- homotypic interactions, it may also affect cis- heterotypic interactions with other membranes proteins and/or lipids, especially because we see strong localization of CEACAM1 into high intensity regions in an intact N-terminal extracellular domain-dependent manner. In addition to cell-cell studies, studies on patterned surfaces can be used to specifically control trans-interactions and to map the effects of CEACAM1’s trans- interactions on its oligomerization, conformation, and downstream signaling. These studies would be ideal in TIRFPM where membrane processes are readily imaged with high axial resolution.
Our studies have revealed that TIRFPM anisotropy can readily distinguish between pure monomeric and dimeric protein populations. However, we did observe limitations of the TIRFPM platform in detecting differences between a mixed monomeric and oligomeric CEACAM1-4L-EYFP population and a biochemically-characterized monomeric CEACAM1 population, indicating the TIRFPM platform’s limitations in detecting potentially small changes in CEACAM1-4L-EYFP’s monomer-oligomer equilibrium. We also showed that CEACAM1 concentrates at ezrin-rich regions (47) in an N-terminal extracellular domain-dependent manner, but exists as a mixture of monomers and oligomers across the cell surface, with potentially more monomers in the ezrin-rich regions. This work provides a basis for more comprehensive studies aimed at understanding CEACAM1’s complex network of cis- and trans- heterotypic and homotypic interactions that likely transmits signals across the plasma membrane through changes in CEACAM1’s cis- homotypic and heterotypic oligomerization, conformation, and/or kinetics in cell type-, lipid-, phosphorylation- and glycosylation- dependent manner.

Table 4.1: Summary of Imaging Techniques for Future CEACAM1 Studies.

<table>
<thead>
<tr>
<th>Research Topics</th>
<th>Proposed Imaging Techniques</th>
<th>Additional Tools*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intracellular characteristics (cis)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homotypic oligomerization</td>
<td>Homo-FRET (progressive photobleaching), FCS, numbers and brightness, FRAP / homo-FRET, FRAP / numbers and brightness, BiFC</td>
<td>Dimeric CEACAM1, EYFP-CEACAM1-4L, GFP fragment-labeled CEACAM1s, Mathematical modeling, Analysis software</td>
</tr>
<tr>
<td>Heterotypic interactions</td>
<td>Hetero-FRET, colocalization, FCCS</td>
<td>Labeled membrane proteins, Labeled lipids, Analysis software</td>
</tr>
<tr>
<td>Kinetics</td>
<td>FRAP, FCS, single particle tracking</td>
<td>Analysis software</td>
</tr>
<tr>
<td>Conformational changes</td>
<td>Homo-FRET, single molecule FRET</td>
<td>CEACAM1-EYFP-4L, Dual-labeled CEACAM1</td>
</tr>
<tr>
<td>Localization</td>
<td>PALM, STORM</td>
<td>Photoactivatable GFP-mCherry-CEACAM1, Analysis software</td>
</tr>
<tr>
<td><strong>Intercellular characteristics (trans)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homotypic oligomerization</td>
<td>Homo-FRET, Micro-patternning</td>
<td>Micro-patternning stamps, Labeled proteins, EYFP-CEACAM1-4L</td>
</tr>
<tr>
<td>Heterotypic interactions</td>
<td>Hetero-FRET, FCCS, Micro-patterning</td>
<td>Labeled proteins, EYFP-CEACAM1-4L</td>
</tr>
<tr>
<td>Localization</td>
<td>PALM, STORM</td>
<td>Photoactivatable GFP-mCherry-CEACAM1, Analysis software</td>
</tr>
</tbody>
</table>

* Tools include the constructs used in this study (not shown in the table).
References


Appendix 1: $r_c$ vs. Intensity for EYFP-labeled CEACAM1 Mutants.

$r_c$ vs. intensity plots are shown for (A) CEACAM1-4L-EYFP, (B) CEACAM1-4S-EYFP, (C) CEACAM1-4Δcyto-EYFP, (D) G432,436L-CEACAM1-4L-EYFP, and (E) RQ43,44SL-CEACAM1-4L-EYFP. Each point is the mean $r_c$ of a single cell’s high intensity region (filled) or low intensity region (hollow).
Appendix 2: $r_c$ vs. Time for CEACAM1 Perturbation Studies.

Time traces show average anisotropy upon (A) ionomycin (1 µM) or DMSO control perturbation and (B) α-CEA pAb (20 µg/mL) or isotype control (20 µg/mL) perturbation of CEACAM1-4L-EYFP or G432,436-CEACAM1-4L-EYFP for high intensity regions and low intensity regions. Soluble factors added at t = 0 minutes. Error bars show standard error.
Appendix 3: Updated ImageJ Macro for CEACAM1 $r_c$ Calculation

// FPM_AnisotropyCorrected_TIRF_473nm ImageJ macro
// Jocelyn Lo, John Oreopoulos
// 10/01/11
// Version 12

// This macro calculates and saves in the selected directory the corrected
// fluorescence polarization microscopy (FPM) anisotropy image with 532 nm excitation wavelength.
// See Axelrod 1989 and Piston 2008 for more details on the theory of FPM as well as the corrections due to high NA.

// JOHN'S VERSIONS:
// Version 13: The intensity image Fpar is now used to generate a threshold mask that removes background regions.
// Version 14: An image histogram of the calculated anisotropy image is performed and saved.
// Version 15: "State1" and "State2" CEACAM1-YFP structures are isolated and analyzed using image histograms.
// This version is specially designed for CEACAM1 analysis.
// Version 16: Bimodal state ROI analysis saved as a separate macro. Specification of excitation wavelength in macro
// Version 17: The denominator of the corrected anisotropy equation is saved as the corrected total intensity (see references cited
// in comments above).
// Version 17c: The calibration bar and scale are put in a separate saved image.
// Version 17: "Close all"

// JOCELYN'S VERSIONS:
// Version 4: Background subtraction
// diffuse intensity selection using "Li thresholding".
// Remove unnecessary steps, but leaving text in: measuring anisotropy values and intensity values for images; generating r
// vs. I for entire cell;
// Version 7: CLEAN UP, remove text of unnecessary steps
// Version 8: SELECT ROI, take measurements, save images. Ideal for systems that are homogeneous, such as YFP-soluble cells
// Make translation #s variables
// Version 9: Select high intensity ROI's, then low intensity ROI's
// Rework Step 9, ROI selection because need it to pull out low I and high I images, not entire anisotropy image
// Version 6-8 use IsoData to threshold for high intensity ROI, but actually MaxEntropy or Renyi Entropy algorithms
// better suited. See JRL-002-192
// Saves combined images (no threshold, high I threshold, low I threshold); 5/5/11
// Version 10: Tests out value of Gaussian blur: want to see if improves threshold selection. Want to see if smoothes STD for r
// values
// Version 11: Bypasses Version 10 (Gaussian blur); V11 is essentially corrected version 9. Correct for saturated pixels in Fpar,
// by selecting and cutting saturated pixels. But appears to affect fidelity of Li auto-thresholding.
// Therefore changed low threshold to "Huang" instead of "Li".
// Version 12: To remove saturated pixels, instead of creating and deleting a selection (which sometimes deletes entire
// image if no saturated pixels),
// Create mask (1-65534) and multiply against Fpar image to remove saturated (65535 pixels). Confirmed
// function by comparing to
// manual selection of saturated pixels, and that this addition did not affect Fpar average intensity values.
// This version may work with Triangle thresholding (vs. Li, which worked previously with Version9).
// Confirmed with JRL-002-187 by comparing auto-thresholding to manual thresholding.
// Checked against Version 9 in JRL-002-191; looks good!
// Version 12 (9/19/11) : Shift corrected between Fpar and Fperp to -2,3
// Create stack, then background subtract
// Generate 0 to 0.65 anisotropy images
// BGSub5: Fpar-> Fperp shift
// STEP: Combined with mode generator for anisotropy and corrected total intensity
// STEP: BGSub5: Combined with anisotropy and corrected total intensity mode measurements
(FPM_PlotAnisotropyVsTotalIntensity_532nm_V2_for4.txt)

setBatchMode(true); //batch mode on
// 1. MEASURED MICROSCOPE-SPECIFIC PARAMETERS
var Version = 12.93

var Lambda = 473 //nm
var NA=1.45;
var nOIL=1.516;
var sig=asin(NA/nOIL); // objective half-angle of acceptance in radians

// Axelrod's high NA correction coefficients
// NOTE: Axelrod's coordinate convention is used
var Ka=(1/3)*(2-3*cos(sig)+pow(cos(sig),3));
var Kb=(1/12)*(1-3*cos(sig)+3*pow(cos(sig),2)-pow(cos(sig),3));
var Kc=(1/4)*(5-3*cos(sig)-pow(cos(sig),2)-pow(cos(sig),3));

// Polarization bias correction factor
// (measured ratio of Fpar/Fperp for isotropic solution of TRITC in EtOH using low NA objective lens)
var g=0.93;

// Translation from Fpar to Fperp (where a is x direction, and b is y direction);
//var a = -1;
//var b = 3;

// 2. BEGIN IMAGE PROCESSING
// Open the parallel and perpendicular emission polarization fluorescence images
// Note that MicroManager's time-lapse acquisition convention of labeling images is used
path = getDirectory("");
open(path+"img_000000000_Close all_000.tif") // NS image or Up/Down or Vertical
rename("Fparfirst");
run("32-bit");

//Remove saturated pixels
selectWindow("Fparfirst");
setThreshold(1, 65534);
run("Create Mask", "");
selectWindow("mask");
run("Divide...", "value=255");
imageCalculator("Multiply create 32-bit", "Fparfirst", "mask");
selectWindow("Result of Fparfirst");
rename("Fpar");

selectWindow("Fparfirst");
run("Close","";
selectWindow("mask");
run("Close","";

open(path+"img_000000001_Close all_000.tif") // EW image or Left/Right or Horizontal
rename("Fperp");
run("32-bit");
var a = -1;
var b = 3;
//run("Translate...", "x=a y=b");
run("Translate...", "x=-1 y=3");

// The pixel shift correction in the above line has been
// predetermined from a calibration of the microscope. This correction will
// need to be determined again should the microscope emission filter wheel
// ever be modified or if the microscope itself is
// moved to another location in the lab

// 3. RUN BACKGROUND SUBTRACTION
selectWindow("Fpar");
run("Concatenate ", "title=[Stack] image_1=[Fpar] image_2=[Fperp] image_3=[-- None --]");
makeRectangle(0,412,100,100);  // Looked through 30 images to identify region where no fluorescence
run("BG Subtraction from ROI", "");
run("Stack to Images", "");

selectWindow("Stack-0001");
save(path + "Fpar.tif");
rename("Fpar");

selectWindow("Stack-0002");
save(path + "Fperp.tif");
rename("Fperp");

// 4. BEGIN CORRECTED ANISOTOPY IMAGE CALCULATION
// NOTE: These image calculations are done using the corrected anisotropy calculations shown in John Oreopoulos thesis, 2009.
// (ie has K^2 and K^3 values, which are result of not simplifying the equation at a certain step in the calculations. However, get
same values as with the more simplified corrected anisotropy equation, because ratio'd out)
selectWindow("Fpar");
run("Duplicate...", "title=[FparDuplicate]");
selectWindow("Fpar");
run("Duplicate...", "title=[FparDuplicate2]");
selectWindow("Fpar");
run("Duplicate...", "title=[FparDuplicate3]");
selectWindow("Fpar");
var V1 = Ka+Kc;
run("Multiply...", "value="+V1");
selectWindow("Fperp");
var V2 = (Ka+Kb)*g
run("Multiply...", "value="+V2");

imageCalculator("Subtract create 32-bit", "Fpar","Fperp");
//run("Image Calculator...", "image1=Fpar operation=Subtract image2=Fperp create 32-bit");
selectWindow("Result of Fpar");
rename("CHI");
var V3 = Kc*(Ka+Kc)-Kb*(Ka+Kb)
run("Divide...", "value="+V3");
selectWindow("CHI");
run("Duplicate...", "title=[CHIDuplicate]");

selectWindow("FparDuplicate");
var V4 = 1/(Ka+Kb)
run("Multiply...", "value="+V4");
selectWindow("FparDuplicate2");
var V5 = 2/(Ka+Kb)
run("Multiply...", "value="+V5");

selectWindow("CHI");
var V6 = 1+Kc/(Ka+Kb)
run("Multiply...", "value="+V6");
selectWindow("CHIDuplicate");
var V7 = 1-2*Kc/(Ka+Kb)
run("Multiply...", "value="+V7");

imageCalculator("Subtract create 32-bit", "CHI","FparDuplicate");
//run("Image Calculator...", "image1=CHI operation=Subtract image2=FparDuplicate create 32-bit");
selectWindow("Result of CHI");
rename("AnisotropyCorrectedNumerator");
imageCalculator("Add create 32-bit", "CHIDuplicate","FparDuplicate2");
//run("Image Calculator...", "image1=CHIDuplicate operation=Subtract image2=FparDuplicate2 create 32-bit");
selectWindow("Result of CHIDuplicate");
rename("AnisotropyCorrectedDenominator");

run("Duplicate...", "title=[CorrectedTotalIntensity]");
save(path + "CorrectedTotalIntensity" + ".tif");

imageCalculator("Divide create 32-bit", "AnisotropyCorrectedNumerator","AnisotropyCorrectedDenominator");
//run("Image Calculator...", "image1=AnisotropyCorrectedNumerator operation=Divide image2=AnisotropyCorrectedDenominator create 32-bit");

// 5. REMOVE BACKGROUND FROM ANISOTROPY IMAGES
selectWindow("Result of AnisotropyCorrectedNumerator");
save(path + "AnisotropyCorrected_"+Lambda+"nm"+ Version + ".tif");

selectWindow("FparDuplicate3");
rename("BackgroundMask"); // Create Background mask
//run("16-bit");
run("Threshold..."); // open Threshold tool
resetMinAndMax();
setAutoThreshold("Triangle dark");
run("Create Mask", "");
save(path + "Triangle dark_mask.tif");
rename("mask");
run("Divide...", "value=255");
//run("32-bit");
open(path+"AnisotropyCorrected_"+Lambda+"nm"+ Version + ".tif")
rename("AnisotropyCorrected");
imageCalculator("Multiply create 32-bit", "AnisotropyCorrected", "mask");

// Resave anisotropy image
selectWindow("Result of AnisotropyCorrected");
setThreshold(0.00001, 0.65);
run("NaN Background");
run("Red Hot");
setMinAndMax(0.000, 0.4000); // Adjust display range
save(path + "AnisotropyCorrected_"+Lambda+"nm"+ Version + ".tif");
save(path + "AnisotropyCorrected_"+Lambda+"nm"+ Version + ".RedHot.jpg");

run("Close All", ");
// End background removal

// 6. BEGIN FORMATTING IMAGES OF ENTIRE CELL

// Begin histogram analysis
var nBins = 50
var BinMin = -0.05
var BinMax = 0.40

open(path + "AnisotropyCorrected_"+Lambda+"nm"+ Version + ".tif");
resetThreshold();
run("Histogram", ",bins=n+Nbins", ",x_min=x_min", ",x_max=x_max", ",BinMin=", ",BinMax=", ",y_max=Auto");
save(path + "Histogram of "+"AnisotropyCorrected_"+Lambda+"nm"+ Version + ".tif");
selectWindow("AnisotropyCorrected_"+Lambda+"nm"+ Version + ".tif");
run("Clear Results");
var row = 0;
getHistogram(Anisotropy, Counts, nBins, BinMin, BinMax);
for (i=0; i<nBins; i++) {
    setResult("Anisotropy", row, Anisotropy[i]);
    setResult("Counts", row, Counts[i]);
    row++;
updateResults();
saveAs("Measurements", path + "Histogram of " + "AnisotropyCorrected_" + Lambda + "nm_V" + Version + ".xls");
selectWindow("Results");
run("Close All", "");
// End histogram analysis

// Add calibration bars for anisotropy images
open(path + "AnisotropyCorrected_" + Lambda + "nm_V" + Version + ".tif");
setMinAndMax(0.000, 0.4000); // Adjust display range
run("royal"); // Ensure that the "royal" LUT is installed with ImageJ before using this macro
save(path + "AnisotropyCorrected_" + Lambda + "nm_V" + Version + ".jpg");
selectWindow("AnisotropyCorrected_" + Lambda + "nm_V" + Version + ".tif");
run("Set Scale...", "distance=1 known=.178 pixel=1 unit=um");
run("Scale Bar...", "width=20 height=3 font=14 color=White background=none location=\[Lower Right\] bold");
run("Calibration Bar...", "location=\[Upper Right\] fill=White label=Black number=5 decimal=2 font=12 zoom=1");
save(path + "AnisotropyCorrected_" + Lambda + "nm_V" + Version + "Calibration Scale Bar" + ".jpg");
run("Close All", "");

// 7. THRESHOLD FOR HIGH INTENSITY ROI'S
open(path + "Fpar.tif");
run("Duplicate...", "title=\[HighIMask\]");
run("Threshold..."); // Open Threshold tool
setAutoThreshold("MaxEntropy dark");
run("Create Mask", ");
run("Divide...", "value=255");
save(path + "ThreshROI_HighI_Mask_" + Lambda + "nm_V" + Version + ".tif");
// Run(32-bit)

open(path + "AnisotropyCorrected_" + Lambda + "nm_V" + Version + ".tif");
run("Duplicate...", "\[AnisotropyCorrected\]");
imageCalculator("Multiply create 32-bit", "AnisotropyCorrected", "ThreshROI_HighI_Mask_" + Lambda + "nm_V" + Version + ".tif");

// Resave anisotropy image
selectWindow("Result of AnisotropyCorrected");
setThreshold(0.00001, 0.6000); 
run("NaN Background");
run("Red Hot"); // Ensure that the "Red Hot" LUT is installed with ImageJ before using this macro
setMinAndMax(-0.0500, 0.6000); // Adjust display range

// Get thresholding value
open(path + "CorrectedTotalIntensity.tif");
imageCalculator("Multiply create 32-bit", "CorrectedTotalIntensity.tif", "ThreshROI_HighI_Mask_" + Lambda + "nm_V" + Version + ".tif");
setThreshold(0.00001, 100000);
run("NaN Background");
getMinAndMax(min, max);
print(min, max);
// Run("Measure");
save(path + "ThreshROI_HighI_CorrectedTotalIntensity_" + Lambda + "nm_V" + Version + ", min + ".tif");
selectWindow("Result of AnisotropyCorrected");
save(path + "ThreshROI_HighI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + ".tif"); //run("Measure");

open(path + "Fpar.tif");
imageCalculator("Multiply create 32-bit", "Fpar.tif", "ThreshROI_HighI_Mask_" + Lambda+"nm_V" + Version + ".tif");
setThreshold(0.00001, 65530);
run("NaN Background"); //run("Measure");
save(path + "ThreshROI_HighI_Fpar_"+Lambda+"nm_V" + Version + ".tif");

open(path + "Fperp.tif");
imageCalculator("Multiply create 32-bit", "Fperp.tif", "ThreshROI_HighI_Mask_" + Lambda+"nm_V" + Version + ".tif");
setThreshold(0.00001, 65330);
run("NaN Background"); //run("Measure");
save(path + "ThreshROI_HighI_Fperp_"+Lambda+"nm_V" + Version + ".tif");

//saveAs( "Measurements", path + "ThreshROI_HighI_AvgMeasurements_V" + Version + ".xls" );
//selectWindow( "Results" );
//run("Close");
run("Close All"; "");

// 8. SELECT LOW INTENSITY ROI MEASUREMENTS
//BEGIN SELECTION OF COMPLEMENTARY LOW I REGIONS

// Make low intensity region mask, which complements high intensity region mask just made above
open(path + "ThreshROI_HighI_Mask_" + Lambda+"nm_V" + Version + ".tif");
setThreshold(1, 2); //Selects high intensity mask regions
run("Create Selection", "");
open(path + "AnisotropyCorrected_"+Lambda+"nm_V" + Version + ".tif");
run("Restore Selection", ""); // High Intensity mask marks the high intensity boundary for Low Intensity image. The anisotropy
value border/periphery of the a cell, previously determined, marks the lower boundary of low intensity ROI.
run("Cut", ""); // Clears out ROI that were selected as "high intensity" regions previously, thereby selecting low intensity regions
setThreshold(0.0001, 0.70);
run("NaN Background"); // Makes background values "NaN"
selectWindow("AnisotropyCorrected_"+Lambda+"nm_V" + Version + ".tif");
run("Select None", "");
run("Duplicate...", "title=[BackgroundMask]" );
setThreshold(0.00001, 0.70);
run("Create Mask", "");
run("Divide...", "value=255");
save(path + "ThreshROI_LowI_Mask_" + Lambda+"nm_V" + Version + ".tif");

// Start saving and measuring low I region values
selectWindow("AnisotropyCorrected_"+Lambda+"nm_V" + Version + ".tif");
//run("Measure");
save(path + "ThreshROI_LowI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + ".tif");

open(path + "CorrectedTotalIntensity.tif");
imageCalculator("Multiply create 32-bit", "CorrectedTotalIntensity.tif", "ThreshROI_LowI_Mask_" + Lambda+"nm_V" + Version + ".tif");
setThreshold(0.00001, 100000);
run("NaN Background"); //run("Measure");
save(path + "ThreshROI_LowI_CorrectedTotalIntensity_"+Lambda+"nm_V" + Version + ".tif");
open(path + "Fpar.tif");
imageCalculator("Multiply create 32-bit", "Fpar.tif", "ThreshROI_LowI_Mask_" + Lambda+"nm_V" + Version + ".tif");
setThreshold(0.00001, 65330);
run("NaN Background");
//run("Measure");
save(path + "ThreshROI_LowI_Fpar_"+Lambda+"nm_V" + Version + "." + min + ".tif");

open(path + "Fperp.tif");
imageCalculator("Multiply create 32-bit", "Fperp.tif", "ThreshROI_LowI_Mask_" + Lambda+"nm_V" + Version + ".tif");
setThreshold(0.00001, 65330);
run("NaN Background");
run("Measure");
save(path + "ThreshROI_LowI_Fperp_"+Lambda+"nm_V" + Version + "." + min + ".tif");

//saveAs("Measurements", path + "ThreshROI_LowI_AvgMeasurements" + "." + min + ".xls");
selectWindow("Results");
run("Close");
run("Close All", "");

// 9. SELECT ROI AND MEASURE VALUES OF HIGH I AND LOW I REGIONS

open(path + "CorrectedTotalIntensity.tif");

setBatchMode(false); // batch mode off

msgtitle = "Select ROI";
msg = "Select ROI, using selection box, then click "OK".";
waitForUser(msgtitle, msg);

setBatchMode(true); // batch mode on

// Images of entire cell
selectWindow("CorrectedTotalIntensity.tif");
run("Measure");
getSelectionBounds(xdir, ydir, width, height);
print(xdir, ydir, width, height);
open(path + "AnisotropyCorrected_"+Lambda+"nm_V" + Version + ".tif");
run("Restore Selection", "");
run("Measure");
open(path + "Fpar.tif");
run("Restore Selection", "");
run("Measure");
open(path + "Fperp.tif");
run("Restore Selection", ""); // Fperp already saved as a shifted -1, 3 image, so not need to shift it
run("Measure");

// Images of high I regions of cell
open(path + "ThreshROI_HighI_CorrectedTotalIntensity_"+Lambda+"nm_V" + Version + "." + min + ".tif");
run("Restore Selection", "");
run("Measure");
open(path + "ThreshROI_HighI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + "." + min + ".tif");
run("Restore Selection", "");
run("Measure");
open(path + "ThreshROI_HighI_Fpar_"+Lambda+"nm_V" + Version + "." + min + ".tif"); run("Restore Selection", ""); run("Measure");

open(path + "ThreshROI_HighI_Fperp_"+Lambda+"nm_V" + Version + "." + min + ".tif"); run("Restore Selection", ""); run("Measure");

// Images of low I regions of cell
open(path + "ThreshROI_LowI_CorrectedTotalIntensity_"+Lambda+"nm_V" + Version + "." + min + ".tif"); run("Restore Selection", ""); run("Measure");

open(path + "ThreshROI_LowI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + "." + min + ".tif"); run("Restore Selection", ""); run("Measure");

open(path + "ThreshROI_LowI_Fpar_"+Lambda+"nm_V" + Version + "." + min + ".tif"); run("Restore Selection", ""); run("Measure");

open(path + "ThreshROI_LowI_Fperp_"+Lambda+"nm_V" + Version + "." + min + ".tif"); run("Restore Selection", ""); run("Measure");

// Save cropped images as stack
run("Images to Stack", "name=Stack title=[] use"); run("Restore Selection", ""); run("Crop", ""); save(path + "ROIStack_"+Lambda+"nm_V" + Version + "." + xdir + "+ydir +" + width + "+height + ".tif");

// Save Measurements
saveAs("Measurements", path + "ROI_AvgMeasurements_"+Lambda+"nm_V" + Version + "." + xdir + "+ydir +" + width + "+height + ".xls"); selectWindow("Results"); run("Close");

//Save combined images (no thresholding, high I threshold, low I threshold)
open(path + "AnisotropyCorrected_"+Lambda+"nm_V" + Version + ".tif"); rename("a");
open(path + "ThreshROI_HighI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + "." + min + ".tif"); rename("b");
open(path + "ThreshROI_HighI_Fpar_"+Lambda+"nm_V" + Version + "." + min + ".tif"); rename("c");

run("Combine...", "stack1=[a] stack2=b"); run("Combine...", "stack1=[Combined Stacks] stack2=c");
save(path + "Combined_r_V" + Version + ".tif");

open(path + "Fpar.tif"); rename("d");
open(path + "ThreshROI_HighI_Fpar_"+Lambda+"nm_V" + Version + "." + min + ".tif"); rename("e");
open(path + "ThreshROI_HighI_Fperp_"+Lambda+"nm_V" + Version + "." + min + ".tif"); rename("f");

run("Combine...", "stack1=[d] stack2=e"); run("Combine...", "stack1=[Combined Stacks] stack2=f");
save(path + "Combined_Fpar_V" + Version + ".tif");

run("Close All", "");
// HIGH INTENSITY ROI: GENERATES PIXEL-BY-PIXEL ANISOTROPY vs. INTENSITY PLOTS

open(path + "ThreshROI_HighI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + "." + min + ".tif");
run("Restore Selection", "");
run("Crop", "");

openpath + "ThreshROI_HighI_CorrectedTotalIntensity_"+Lambda+"nm_V" + Version + "." + min + ".tif");
run("Restore Selection", "");
run("Crop", "");

// Begin table creation.

selectWindow("ThreshROI_HighI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + "." + min + ".tif");
for (y=0; y<getHeight; y++) {
    for (x=0; x<getWidth; x++) {
        row = x+y*getWidth;
        setResult("Anisotropy", row, getPixel(x,y));
    }
}

selectWindow("ThreshROI_HighI_CorrectedTotalIntensity_"+Lambda+"nm_V" + Version + "." + min + ".tif");
for (y=0; y<getHeight; y++) {
    for (x=0; x<getWidth; x++) {
        row = x+y*getWidth;
        setResult("Intensity", row, getPixel(x,y));
    }
}

// End table creation.

updateResults();
saveAs("Measurements", path + "ThreshROI_HighI_AnisotropyVsTotalIntensity_" + Lambda+"nm_V" + Version + "." + xdir + "x_" + ydir + "y_" + width + "w_" + height + "h_" + min + ".xls");
saveAs("Measurements", path + "ThreshROI_HighI_AnisotropyVsTotalIntensity_" + Lambda+"nm_V" + Version + "." + xdir + "x_" + ydir + "y_" + width + "w_" + height + "h_" + min + ".txt");
selectWindow("Results");
run("Close");

// LOW INTENSITY ROI: GENERATES PIXEL-BY-PIXEL ANISOTROPY vs. INTENSITY PLOTS

open(path + "ThreshROI_LowI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + "." + min + ".tif");
run("Restore Selection", "");
run("Crop", "");

openpath + "ThreshROI_LowI_CorrectedTotalIntensity_"+Lambda+"nm_V" + Version + "." + min + ".tif");
run("Restore Selection", "");
run("Crop", "");

// Begin table creation.

selectWindow("ThreshROI_LowI_AnisotropyCorrected_"+Lambda+"nm_V" + Version + "." + min + ".tif");
for (y=0; y<getHeight; y++) {
    for (x=0; x<getWidth; x++) {
        row = x+y*getWidth;
        setResult("Anisotropy", row, getPixel(x,y));
    }
}

selectWindow("ThreshROI_LowI_CorrectedTotalIntensity_"+Lambda+"nm_V" + Version + "." + min + ".tif");
for (y=0; y<getHeight; y++) {
    for (x=0; x<getWidth; x++) {
        row = x+y*getWidth;
        setResult("Intensity", row, getPixel(x,y));
    }
}

// End table creation.

updateResults();
saveAs("Measurements", path + "ThreshROI_LowI_AnisotropyVsTotalIntensity_" + Lambda+"nm_V" + Version + "_" + xdir + "x_" + ydir + "y_" + width + "w_" + height + "h_" + min + "_xls");
saveAs("Measurements", path + "ThreshROI_LowI_AnisotropyVsTotalIntensity_" + Lambda+"nm_V" + Version + "_" + xdir + "x_" + ydir + "y_" + width + "w_" + height + "h_" + min + ".txt");
selectWindow("Results");
run("Close");

run("Close All", "");
setBatchMode(false); //exit batch mode

open(path + "Combined_r_V" + Version + ".tif");
run("Red Hot");

// End of macro
Appendix 4: ImageJ Macro for Registration of Time-lapse Intensity and $r_c$ Images

// TimelapseImaging_Registration Macro
// By Jocelyn Lo

// This macro registers images taken over several time points because shifts in lateral positioning naturally occur when moving around the stage and trying to get back to the same coordinates.
// Registers images relative to image taken at last time point.

// This macro is to be used after
// 1) anisotropy image processing using macro, "FPM_AnisotropyCorrected_TIRF_473nm_V12_0.93gfactor.txt".
// 2) making Fpar and anisotropy ($r$) image stacks. This is also mentioned in the first prompt.

// V1
// V2 Replace Dialog with waitForUser
// V3 Replace for loop with do, while
// V4 Run TurboReg once to get rid of last image and second to last image of original stack.
// Then run do, while loop because there will not be another instance in which you'll need to get rid of two images.
// If left all images in same loop, would get error for that reason.
// V5 Scratch V4, that doesn't make sense. Just need to rename the "alone" images to "Fpar - #" in order to get rid of those images for proper number (i) count.
// Add prompt for initial requirements to begin macro
// V8 Do, while if, else
// V9 Open aligned stack as a sequence to re-save as stack
// V10 Threshold Fpar for high I
// V11 Threshold Fpar for high I and low I
// V12 Include thresholding, etc. on reference image (last image in stack)
// Generate stacks of no thresholding, high ROI, low ROI --> Generate combined stack
// V13 Try adapting for processing Anisotropy ($r$) images, and measuring avg values in define/d ROI
// V14 Adapted for Fpar and $r$ images
// V15 Get avg value, STD, etc. for entire stack, rather than just first image
// V16 Move if, else (for running through entire aligned image stack) to different position

// NECESSARY CHANGES:
// 1) change Fpar to $r$
// 2) Thresholding, not need to go through making mask again
// 3) Measure ROI - certain places, will have to open up Fpar images for measuring both
// Use "combined" stacks to make measurements. Simply adjust ROI by x=512,
to get high I and low I avg values
// 4) Correct image numbering/naming, because as is limits macro to register only up to 9

Version = 16

// 1. PROMPT: REQUIREMENTS BEFORE RUNNING MACRO
Dialog.create("TurboReg Macro");
Dialog.addMessage("Click okay when: 
A) You have already created stacks, FparStack.tif and rStack.tif
B) You have closed all windows open in ImageJ 
C) You have downloaded TurboReg into the ImageJ Plugin folder
If you have not already done this, click cancel and start again");
Dialog.show();

// 2. OPEN FILES
path =getDirectory("";
open(path + "FparStack.tif");
open(path + "rStack.tif");
run("Combine...", "stack1=FparStack.tif stack2=rStack.tif"); // Will allow simultaneous processing Fpar and r images of same time point
save(path + "Combined_Fpar.tif");
rename("Fpar");

run("Stack to Images", "");
//selectWindow("Fpar");
//rename("Fpar-0");

// 3. REGISTER IMAGES FROM FPARSTACK

do {

    if (nImages>1) { // Registers images until there are no more images in the stack to register, ie "if (nImages>1)"
        // Then proceeds to "else" to create aligned stack, etc.
        i=nImages;
        // print(j);
        k = i-1;

        run("TurboReg ", "");

        waitForUser("A) Remember to save alignment profile. B) Put Fpar-n with largest n as Target, and Fpar-(n-1) as Source. Then click okay when TurboReg is finished running and has created an aligned image.");

        //setBatchMode(true);

        selectWindow("Fpar-000" + k);
        run("Close","");
        selectWindow("Fpar-000" + i);
        run("Close","");

        selectWindow("Output");
        save(path + "Fpar_000" + k + ".tif");
        selectWindow("Fpar_000" + k + ".tif");
        run("Stack to Images", "");
        selectWindow("Mask");
        run("Close","");
        selectWindow("Data");
        save(path + "Fpar_V" + Version + ".tif");

        // Creates high I mask
        selectWindow("Fpar_V" + Version + ".tif");
        run("Create Mask", "");
        run("Divide...", "value=255");

        selectWindow("mask");
        makeRectangle(0, 0, 512, 512);
        run("Crop", "");
        run("Duplicate...", "title=[mask-1]");
        run("Combine...", "stack1=mask stack2=mask-1"); // Doubling mask for both Fpar and r images

    } else {
        // Proceeds to "else" if there is only one image in the stack
        // Ends loop if nImages=1
    }

}
selectWindow("Combined Stacks");
save(path + "HighIMask_V" + Version + ".000" + k + ".tif");

// Creates Fpar high I image
imageCalculator("Multiply create 32-bit", "Fpar_V" + Version + ".000" + k + ".alone.tif",
"HighIMask_V" + Version + ".000" + k + ".tif");
setThreshold(0.00001, 65530);
runtime("NaN Background");
//runtime("Measure");
save(path + "HighI_Fpar_V" + Version + ".000" + k + ".tif");

// Closes Fpar high I images
selectWindow("HighIMask");
runtime("Close");
selectWindow("HighI_Fpar_V" + Version + ".000" + k + ".tif");
runtime("Close");

// Creates low I mask
selectWindow("Fpar_V" + Version + ".000" + k + ".alone.tif");
runtime("Duplicate...", "title=[LowIMask]"");
runtime("Threshold...");     // opens Threshold tool
resetThreshold();
setAutoThreshold("Triangle dark");
runtime("Create Mask", "");
runtime("Divide...", "value=255");

selectWindow("mask");
makeRectangle(0, 0, 512, 512);
runtime("Crop");
runtime("Duplicate...", "title=[mask-1]"");
runtime("Combine...", "stack1=mask stack2=mask-1");     // Doubling mask for both Fpar and r images
rename("Cellfootprint_mask");

selectWindow("HighIMask_V" + Version + ".000" + k + ".tif");
setThreshold(1.2);
selectWindow("Cellfootprint_mask");
runtime("Create Selection", "");
runtime("Restore Selection", "");     // High Intensity mask marks the high
intensity boundary on low Intensity image. The mask of "entire cell footprint" marks the lower intensity boundary.
runtime("Cut", "");     // Clears out ROI that were
selected as "high intensity" regions previously, thereby selecting only low intensity regions
//setThreshold(0.0001, 0.70);     ---Not need because using mask, which already has background at
0?
//runtime("NaN Background");     // Makes background values "NaN"     ---Not need because using mask,
which already has background at 0?

selectWindow("Cellfootprint_mask");
runtime("Select None", "");
save(path + "LowlMask_V" + Version + ".000" + k + ".tif");

// Creates Fpar low I image
imageCalculator("Multiply create 32-bit", "Fpar_V" + Version + ".000" + k + ".alone.tif",
"LowlMask_V" + Version + ".000" + k + ".tif");
setThreshold(0.00001, 65530);
runtime("NaN Background");
//runtime("Measure");
save(path + "Lowl_Fpar_V" + Version + ".000" + k + ".tif");

// Closes Fpar low I images
selectWindow("LowlMask");
runtime("Close");
selectWindow("HighIMask_V" + Version + ".000" + k + ".tif");
run("Close");
selectWindow("LowIMask_V" + Version + ".000" + k + ".tif");
run("Close");
selectWindow("LowI_Fpar_V" + Version + ".000" + k + ".tif");
run("Close");
selectWindow("Fpar_V" + Version + ".000" + k + ".tif");
rename("Fpar-000" + k);       // Need to rename image to close this window later on, which is important for proper nImage count
print(nImages);

} else {         // If no more images in stack to align, macro proceeds to this line, at which point starts to create aligned stack.
run("Close All", "");

// Thresholding and renaming of reference image (last image in stack) because it is skipped in the loop
open(path + "Combined_Fpar.tif");
rename("Fpar");
run("Stack to Images", "");
k=nImages;
print(nImages);
print(k);
selectWindow("Fpar-000" + k);
save(path + "Fpar_V" + Version + ".000" + k + ".tif"); // This name allows the image to be opened with rest of aligned stack later on in macro
run("Close All", "");
open(path + "Fpar_V" + Version + ".000" + k + ".tif");

// Creates high I mask
selectWindow("Fpar_V" + Version + ".000" + k + ".tif");
run("Duplicate..., "title=[HighIMask]";
run("Threshold...");       // opens Threshold tool
selectWindow("HighIMask");
resetThreshold();
setAutoThreshold("MaxEntropy dark");       //Makes mask only for intensity image
because r<<I values
run("Create Mask", "");
run("Divide..., "value=255";

selectWindow("mask");
makerectangle(0, 0, 512, 512);
run("Crop", "");
run("Duplicate..., "title=[mask-1]";
run("Combine..., "stack1=mask stack2=mask-1");   // Doubling mask for both Fpar and r images
selectWindow("Combined Stacks");
save(path + "HighIMask_V" + Version + ".000" + k + ".tif");

// Creates Fpar high I image
imageCalculator("Multiply create 32-bit", "Fpar_V" + Version + ".000" + k + ".alone.tif", "HighIMask_V" + Version + ".000" + k + ".tif");
setThreshold(0.00001, 65530);
run("NaN Background");       //run("Measure");
save(path + "HighI_Fpar_V" + Version + "_000" + k + ".tif");

// Closes Fpar high I images
selectWindow("HighIMask");
run("Close");
selectWindow("HighI_Fpar_V" + Version + "_000" + k + ".tif");
run("Close");

// Creates low I mask
selectWindow("Fpar_V" + Version + "._000" + k + ".alone.tif");
run("Duplicate...", "title=[LowIMask]");
run("Threshold..."); // opens Threshold tool
selectWindow("LowIMask");
resetThreshold();
setAutoThreshold("Triangle dark");
run("Create Mask");
run("Divide...", "value=255");
selectWindow("mask");
makeRectangle(0, 0, 512, 512);
run("Crop");
run("Duplicate...", "title=[mask-1]");
run("Combine...", "stack1=mask stack2=mask-1"); // Doubling mask for both Fpar and r images
rename("Cellfootprint_mask");
selectWindow("HighIMask_V" + Version + "_000" + k + ".tif");
setThreshold(1, 2); //Selects high intensity mask regions
run("Create Selection");
selectWindow("Cellfootprint_mask");
run("Restore Selection"); // High Intensity mask marks the high intensity boundary on low Intensity image. The mask of "entire cell footprint" marks the lower intensity boundary.
run("Cut"); // Clears out ROI that were selected as "high intensity" regions previously, thereby selecting only low intensity regions
//setThreshold(0.0001, 0.70); ----Not need because using mask, which already has background at 0?
//run("NaN Background"); // Makes background values "NaN" ----Not need because using mask, which already has background at 0?
selectWindow("Cellfootprint_mask");
run("Select None");
save(path + "LowIMask_V" + Version + "_000" + k + ".tif");

// Creates Fpar low I image
imageCalculator("Multiply create 32-bit", "Fpar_V" + Version + "_000" + k + ".alone.tif", "LowI_Fpar_V" + Version + "_000" + k + ".tif");
setThreshold(0.0001, 65530);
run("NaN Background");
//run("Measure");
save(path + "LowI_Fpar_V" + Version + "_000" + k + ".tif");

// Closes Fpar low I images
selectWindow("LowIMask");
run("Close");
selectWindow("HighIMask_V" + Version + "_000" + k + ".tif");
run("Close");
selectWindow("LowIMask_V" + Version + "_000" + k + ".tif");
run("Close");
selectWindow("LowI_Fpar_V" + Version + "_000" + k + ".tif");
run("Close");

//Save stacks
save(path + "FparStack_V" + Version + ".tif");

save(path + "HighI_FparStack_V" + Version + ".tif");

save(path + "LowI_FparStack_V" + Version + ".tif");

run("Close All","");

// Generate stacks
open(path + "FparStack_V" + Version + ".tif");
open(path + "HighI_FparStack_V" + Version + ".tif");
open(path + "LowI_FparStack_V" + Version + ".tif");

//Selects ROIs for Fpar images
selectWindow("FparStack_V" + Version + ".tif");

msgtitle = "Select ROI"
msg = "Select ROI, using selection box, then click "OK".");
waitforUser(msgtitle, msg);

getSelectionBounds(xdir, ydir, width, height);

do {
    //Loop to collect avg value of all time points
    makeRectangle(xdir, ydir, width, height);
    run("Measure");
    makeRectangle(xdir + 512, ydir, width, height); //Selects corresponding r boundary for timepoint, t
    run("Measure");
    if (getSliceNumber() < nSlices) {
        // ie as long as current slice is not the last slice, continue moving through stack and measuring avg intensities.
        // Otherwise proceed to "else" at which point move on to next stack and measure values.
        run("Next Slice [>]", ");
    } else {
        //When finish measuring avg value from Fpar stack, move on to Low I stack
        selectWindow("FparStack_V" + Version + ".tif");
        run("Close");
        selectWindow("LowI_FparStack_V" + Version + ".tif");
        do {
            //Loop to collect avg value of all time points
            makeRectangle(xdir, ydir, width, height);
            run("Measure");
            makeRectangle(xdir + 512, ydir, width, height); //Selects corresponding r boundary for timepoint, t
            run("Measure");
        } while (getSliceNumber() < nSlices);
    }
}
if (getSliceNumber() < nSlices) { // as long as current slice is not the last slice, continue moving through stack and measuring avg intensities.
    // Otherwise proceed to "else" at which point move on to next stack and measure values.
    run("Next Slice [>]", "");
} else { //When finish measuring avg value from LowI stack, move on to HighI stack
    selectWindow("LowI_FparStack_V" + Version + "_aligned.tif");
    run("Close");
    selectWindow("HighI_FparStack_V" + Version + "_aligned.tif");
    do {
        //Loop to collect avg value of all time points
        makeRectangle(xdir, ydir, width, height);
        run("Measure");
        // Measures Fpar intensity for timepoint, t
        makeRectangle(xdir + 512, ydir, width, height); //Selects corresponding r boundary for timepoint, t
        run("Measure");
        if (getSliceNumber() < nSlices) {
            // as long as current slice is not the last slice, continue moving through stack and measuring avg intensities.
            // Otherwise proceed to "else" at which point move on to next stack and measure values.
            run("Next Slice [>]", "");
        } else {
            //When finish measuring avg value from Fpar stack, move on to High I stack
            selectWindow("HighI_FparStack_V" + Version + "_aligned.tif");
            run("Close");
            saveAs("Measurements", path + 
                    "AvgMeasurements_V" + Version + "_" + xdir + "," + ydir + "," + width + "," + height + ".xls");
            run("Close All", "");
            print("Program is done!");
        }
    } while (getSliceNumber() <= nSlices);
}

} while (getSliceNumber() <= nSlices);
i = 0; // For some reason, if not update j=nImages at this point, when reach ")while", reads i > 0 (ie continues running loop), which should not be the case after running "Close All".

} while (i>1);
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