Live Cell Super-resolution Microscopy Quanitifies an Interaction Between Influenza Hemagglutinin and Phosphatidylinositol 4,5-bisphosphate

Jaqulin N. Wallace
jaqulin.wallace@maine.edu
LIVE CELL SUPER-RESOLUTION MICROSCOPY QUANTIFIES AN INTERACTION BETWEEN INFLUENZA HEMAGGLUTININ AND PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE

By

Jaquin Nicole Wallace

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Dr. Samuel T. Hess, Professor of Physics, Advisor

Dr. Robert W. Meulenberg, Associate Professor of Physics

Dr. MacKenzie R. Stetzer, Associate Professor of Physics
Influenza virus, colloquially known as the flu, is an acute respiratory disease that infects several millions of individuals each year in the U.S. and kills tens of thousands of those infected. Yearly viral vaccines are widely available, however, due to the virus’s high mutation rate, their efficacy varies greatly. Due to the variability in vaccine efficiency against seasonal influenza, and the potential for even more pathogenic versions of influenza to emerge at any time, there is a high demand for a universal treatment option.

Influenza virus hijacks a variety of host cell components in order to replicate. The glycoprotein hemagglutinin (HA), which is found in the envelope of influenza virions and assembles in the plasma membrane (PM) of host cells, is involved in the attachment, entry, and assembly stages of the viral life cycle. To perform its membrane fusion function, HA must cluster at high densities, although the mechanism for HA clustering remains unknown. Previous work has observed an association between the cytosolic protein actin and HA at the PM of cells. Actin has been shown to affect the motion of HA within clusters, but their connection is not understood. Phosphoinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP2), are implicated in actin polymerization, remodeling, and depolymerization and are theorized to be the mediator between the HA-actin connection through their direct interactions with actin.
binding proteins (ABPs). Disruption of factors that induce clustering of HA may lead to novel treatment methods for influenza infection.

To elucidate the mechanism of HA clustering, we used Fluorescence Photoactivation Localization Microscopy (FPALM) to study HA and PIP2 in living cells at the PM. We found that HA and PIP2 colocalize at the PM in living cells and that HA modulates the mobilities of PIP2 molecules. Further analysis of PIP2 and HA revealed a time-dependent correlation in their dynamics, indicating the existence of a direct connection between the molecules. In addition, we found that HA and PIP2 are delivered together to the PM at high frequencies, suggesting that HA is delivered to the PM already clustered. These HA clusters persist long enough at the PM that HA and PIP2 recycling events are observed at similar frequencies. Our observations strengthen the hypothesis that HA and PIP2 interact at the PM and suggest that PIP2 plays a role in HA clustering mechanisms.
DEDICATION

To my mom and dad:

who taught me how to persevere in the face of a challenge,

to fight for the things I want most from this life,

and, most importantly, to love with all my heart.
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# TABLE OF CONTENTS

DEDICATION ............................................................................................................................................... iii

ACKNOWLEDGEMENTS ........................................................................................................................... iv

LIST OF TABLES .......................................................................................................................................... x

LIST OF FIGURES ......................................................................................................................................... xii

LIST OF EQUATIONS .................................................................................................................................. xiv

Chapter

## 1. INTRODUCTION ........................................................................................................................................ 1

1.1 Influenza A Virus Review ......................................................................................................................... 1

1.2 Brief Overview of Influenza A Life Cycle ................................................................................................. 4

1.3 Hemagglutinin (HA) ................................................................................................................................. 5

1.4 Actin ......................................................................................................................................................... 7

1.5 Actin Binding Proteins (ABPs) ............................................................................................................... 8

1.5.1 α-Actinin .............................................................................................................................................. 9

1.5.2 Cofilin 1 ............................................................................................................................................ 9

1.5.3 Myosin 1 .......................................................................................................................................... 11

1.5.4 Tropomyosin 4 .................................................................................................................................. 11

1.6 Phosphatidylinositol 4,5-bisphosphate (PIP2) ....................................................................................... 11

1.6.1 Platforms .......................................................................................................................................... 14

1.6.2 Local Synthesis or Release of PIP2 (LSRP) ..................................................................................... 14

1.6.3 Megapool .......................................................................................................................................... 15

1.6.4 Budding/Recycling ............................................................................................................................ 16

1.6.5 Exocytosis/Delivery .......................................................................................................................... 16

v
1.7 Diffraction Limited Microscopy ................................................................. 17
1.8 Fluorescence Microscopy ................................................................. 17
1.9 Super Resolution Microscopy ................................................................. 18
1.10 Introduction Review ........................................................................... 19

2. MATERIALS AND METHODS ................................................................ 21

2.1 Biological and Sample Preparation Methods ........................................ 21
  2.1.1 Cell Passage .................................................................................. 21
  2.1.2 Plating Procedure ........................................................................ 22
  2.1.3 Transient Transfection of Sample .................................................. 23
  2.1.4 Sample Preparation for Live Cell Imaging ....................................... 24
  2.1.5 Sample Preparation for Fixed Cell Imaging ..................................... 24
  2.1.6 Bead Sample Preparation .............................................................. 24
  2.1.7 BODIPY TMR-PIP2 Labeling ....................................................... 25

2.2 Super Resolution Imaging ...................................................................... 26
  2.2.1 Fluorescence Photoactivation Localization Microscopy Alignment .... 26
  2.2.2 Imaging Procedures ...................................................................... 29
  2.2.3 Imaging Acquisition ...................................................................... 30
  2.2.4 Acquisition of Configuration Images .............................................. 30

2.3 Post-Processing Analysis ...................................................................... 33
  2.3.1 Localization .................................................................................. 33
  2.3.2 Species Separation ........................................................................ 34
  2.3.3 Bleed-Through Correction .............................................................. 36
  2.3.4 Correlation Coefficients ................................................................. 37
    2.3.4.1 Pearson’s Correlation Coefficient ............................................ 37
2.3.4.2 Manders’ Co-localization Coefficients .................................................. 38

2.3.5 Dynamics ........................................................................................................... 39

2.3.5.1 Trajectory Analysis ....................................................................................... 39

2.3.5.2 Mean Squared Displacement ....................................................................... 40

2.3.5.3 Molecular Mobility ......................................................................................... 40

2.3.5.4 Molecular and Lateral Flux ............................................................................ 42

2.3.6 Density-Based Clustering .................................................................................. 44

2.3.7 Image Rendering ............................................................................................... 45

3. RESULTS .................................................................................................................. 46

3.1 Preface .................................................................................................................... 46

3.2 Actin Binding Proteins’ Association with Influenza Hemagglutinin Clusters
in Fixed Cells .............................................................................................................. 46

3.3 Comparison of PIP2 and PH Domain with and without Influenza Hemagglutinin
using Single Species Imaging of Living Cells .............................................................. 52

3.4 Measurement of Colocalization of Influenza Hemagglutinin and PH Domain
in Living Cells ............................................................................................................. 59

3.5 Dynamic Properties of Influenza Hemagglutinin and PH-Domain .................... 62

3.5.1 Measuring Molecular Flux as a Function of Time ........................................... 62

3.5.2 Measuring Lateral Net Flux as a Function of Time ........................................ 73

3.6 Measuring the Frequency of Membrane Models as a Function of Time ............ 76

4. DISCUSSION ............................................................................................................. 82

4.1 Influenza Hemagglutinin Clustering Properties are Affected by High
Concentrations of Actin Binding Proteins ................................................................ 83

4.2 Influenza Hemagglutinin Affects PIP2 Mobility at the Plasma Membrane .......... 86
4.3 Influenza Hemagglutinin Modulates PIP2 Dynamics in High-Density Regions ........... 88

4.4 Live Cell Analysis Supports Spatial Dependence of Influenza Hemagglutinin and PIP2 ................................................................................................................................................................. 89

4.5 Influenza Hemagglutinin and PIP2 Cooperatively Modulate Membrane Organization .................................................................................................................................................................................. 91

4.5.1 Influenza Hemagglutinin and PIP2 Dynamics Fluctuate in Same Time-Dependent Trend .............................................................................................................................................................................................. 92

4.5.2 Analysis Indicates More Likely Membrane Models for Influenza Hemagglutinin and PIP2 Interactions at the PM ................................................................................................................................................................................................. 93

4.5.2.1 Platforms .................................................................................................................................................................................................................................................................................................................. 93

4.5.2.2 Megapool ................................................................................................................................................................................................................................................................................................................. 94

4.5.2.3 Budding/Endocytosis ......................................................................................................................................................................................................................................................................................... 95

4.5.2.4 Exocytosis/Delivery ......................................................................................................................................................................................................................................................................................... 96

4.5.2.5 Local Synthesis or Release of PIP2 (LSRP) ................................................................................................................................................................................................................................. 97

4.6 A Proposed Model for Influenza Hemagglutinin Clustering at the Plasma Membrane .............................................................................................................................................................................................................. 97

5. CONCLUSION .................................................................................................................................................................................................................................................................................................................. 101

5.1 Conclusion .................................................................................................................................................................................................................................................................................................................. 101

5.2 Possible Future Experimental Directions ............................................................................................................................................................................................................................................................................................................. 102

BIBLIOGRAPHY .................................................................................................................................................................................................................................................................................................................. 100

APPENDICES .................................................................................................................................................................................................................................................................................................................. 113

Appendix A. MATLAB Codes.................................................................................................................................................................................................................................................................................................................. 113

A.1. color_1_MobilityvsDensity_plotter_v2 ............................................................................................................................................................................................................................................................................................................. 113

A.2. HA_PIP2_changes_traj_stats_wallace_v42 ........................................................................................................................................................................................................................................................................................................ 115
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.3. HA_PIP2_changes_traj_stats_bargraph_plotter_v7</td>
<td>128</td>
</tr>
<tr>
<td>Appendix B. Statistical Tests Tables</td>
<td>134</td>
</tr>
<tr>
<td>B.1. Statistical Tests from Chapter Three</td>
<td>134</td>
</tr>
<tr>
<td>B.2. P-Values</td>
<td>136</td>
</tr>
<tr>
<td>Appendix C. Equation Derivations</td>
<td>142</td>
</tr>
<tr>
<td>C.1. Diffusion of Molecules</td>
<td>142</td>
</tr>
<tr>
<td>C.2. Radius of Mobility</td>
<td>143</td>
</tr>
<tr>
<td>Appendix D. Additional Experimental Results</td>
<td>144</td>
</tr>
<tr>
<td>BIOGRAPHY OF THE AUTHOR</td>
<td>145</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<p>| Table 2.1 | Biological Supplies and Manufacturer Details .......................................................... 18 |
| Table 2.2 | Sample Preparation for 2-Species Imaging of PH Domain-PAmKate and Dendra2-Hemagglutinin .......................................................... 22 |
| Table 2.3 | Optical Instruments and Manufacturer Details .......................................................... 25 |
| Table 2.4 | Acquisition Information for 2-Species Imaging of PH Domain-PAmKate and Dendra2-Hemagglutinin .......................................................... 28 |
| Table 3.1 | Correlation Coefficients of Actin Binding Proteins with Hemagglutinin ..................... 45 |
| Table 3.2 | Mean Density and Area Values of Hemagglutinin Clusters with Low/High Actin Binding Proteins .......................................................... 48 |
| Table 3.3 | Fitting Parameters for MSD vs Time for PH Domain-Dendra2 Molecules ..................... 53 |
| Table 3.4 | Fitting Parameters for Mobility vs Density of PH Domain-Dendra2 Molecules ............ 55 |
| Table 3.5 | Description of Conditions (Molecular Flux) for Dendra2-Hemagglutinin and PH Domain-PAmKate .......................................................... 60 |
| Table 3.6 | Normalized Conditions (Molecular Flux) Increasing with Time, $\tau$ ....................... 62 |
| Table 3.7 | One-Way ANOVA Significance Testing of Two Time Points for Each Condition (Molecular Flux) .......................................................... 63 |
| Table 3.8 | Fitting Parameters for Time Point Difference for Each Condition (Molecular Flux) ....... 65 |
| Table 3.9 | One-Way ANOVA Significance Testing of Conditions (Molecular Flux) ....................... 68 |
| Table 3.10 | Normalized Conditions (Molecular Flux) with Lateral Flux of Dendra2-Hemagglutinin and PH Domain-PAmKate .......................................................... 71 |
| Table 3.11 | Definition of State Vectors for Hemagglutinin and PIP2 Membrane Models ............... 74 |
| Table 3.12 | Normalized Frequency of Events of Membrane Models for Hemagglutinin and PIP2 .... 76 |
| Table 3.13 | Rates of Membrane Models for Hemagglutinin and PIP2 ....................................... 77 |</p>
<table>
<thead>
<tr>
<th>Table B.1</th>
<th>Ordinary One-Way ANOVA Significance Testing of Rates of Membrane Models for Hemagglutinin and PIP2</th>
<th>134</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table B.2</td>
<td>One-Way ANOVA Significance Testing of Fitting Parameters for Time Point Difference for Each Condition</td>
<td>135</td>
</tr>
<tr>
<td>Table B.3</td>
<td>P-Values for Table 3.3</td>
<td>136</td>
</tr>
<tr>
<td>Table B.4</td>
<td>P-Values for Table 3.4</td>
<td>136</td>
</tr>
<tr>
<td>Table B.5</td>
<td>P-Values for Table 3.7</td>
<td>137</td>
</tr>
<tr>
<td>Table B.6</td>
<td>P-Values for Table B.2</td>
<td>138</td>
</tr>
<tr>
<td>Table B.7</td>
<td>P-Values for Table 3.9</td>
<td>139</td>
</tr>
<tr>
<td>Table B.8</td>
<td>P-Values for Table 3.12</td>
<td>140</td>
</tr>
<tr>
<td>Table B.9</td>
<td>P-Values for Table B.1</td>
<td>140</td>
</tr>
<tr>
<td>Table B.10</td>
<td>P-Values for Table 3.2</td>
<td>141</td>
</tr>
<tr>
<td>Table D.1</td>
<td>Total Number of Events Used to Calculate Variables Listed for Each Time Difference</td>
<td>144</td>
</tr>
<tr>
<td>Table D.2</td>
<td>Average Cell Properties for 2-Color FPALM Data</td>
<td>144</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Influenza A Virion Structure</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Hemagglutinin Structure</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>Actin Binding Protein Structures</td>
<td>10</td>
</tr>
<tr>
<td>1.4</td>
<td>Diagram of Proposed Hemagglutinin and PIP2 Membrane Models</td>
<td>13</td>
</tr>
<tr>
<td>1.5</td>
<td>FPALM Illumination Methods Diagram</td>
<td>17</td>
</tr>
<tr>
<td>2.1</td>
<td>Widefield FPALM Diagram</td>
<td>24</td>
</tr>
<tr>
<td>2.2</td>
<td>Examples of Configuration Images</td>
<td>29</td>
</tr>
<tr>
<td>2.3</td>
<td>FPALM Concept Diagram</td>
<td>32</td>
</tr>
<tr>
<td>2.4</td>
<td>Trajectory Analysis Concept Diagram</td>
<td>38</td>
</tr>
<tr>
<td>3.1</td>
<td>Correlation Coefficients for Actin Binding Proteins with Hemagglutinin</td>
<td>44</td>
</tr>
<tr>
<td>3.2</td>
<td>Mean Density and Area of Hemagglutinin Clusters with Low/High Actin Binding Proteins</td>
<td>46</td>
</tr>
<tr>
<td>3.3</td>
<td>Mean Squared Displacement Curves of GloPIP</td>
<td>50</td>
</tr>
<tr>
<td>3.4</td>
<td>Diffusion Curves of PH Domain-Dendra2 Molecules</td>
<td>52</td>
</tr>
<tr>
<td>3.5</td>
<td>Mobility Curves of PH Domain-Dendra2 Molecules</td>
<td>54</td>
</tr>
<tr>
<td>3.6</td>
<td>Render of Dendra2-Hemagglutinin and PH Domain-PAmKate</td>
<td>57</td>
</tr>
<tr>
<td>3.7</td>
<td>Pearson’s Correlation Coefficient of Dendra2-Hemagglutinin and PH Domain-PAmKate</td>
<td>58</td>
</tr>
<tr>
<td>3.8</td>
<td>Manders’ Co-localization Coefficients of Dendra2-Hemagglutinin and PH Domain-PAmKate</td>
<td>58</td>
</tr>
<tr>
<td>3.9</td>
<td>Normalized Molecular Flux of Dendra2-Hemagglutinin and PH Domain-PAmKate Molecules</td>
<td>61</td>
</tr>
<tr>
<td>3.10</td>
<td>Fitted Curves of Time-Dependent Normalized Molecular Flux Graphs</td>
<td>64</td>
</tr>
</tbody>
</table>
Figure 3.11  τ=10s, τ=30s Normalized Molecular Flux Graphs ............................................. 67
Figure 3.12  Lateral Net Flux and Molecular Flux Graphs for τ=10s and τ=30s ....................... 70
Figure 3.13  Frequency of Membrane Models ........................................................................ 75
Figure 4.1   Proposed Model for Influenza Hemagglutinin Clustering at the Plasma Membrane .... 96
# LIST OF EQUATIONS

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation 1.1</td>
<td>Rayleigh Criterion</td>
<td>14</td>
</tr>
<tr>
<td>Equation 1.2</td>
<td>Localization Precision</td>
<td>15</td>
</tr>
<tr>
<td>Equation 2.1</td>
<td>Gaussian Fit</td>
<td>31</td>
</tr>
<tr>
<td>Equation 2.2</td>
<td>$\alpha$ Ratio</td>
<td>33</td>
</tr>
<tr>
<td>Equation 2.3</td>
<td>Uncorrected Channel A Grid Value</td>
<td>33</td>
</tr>
<tr>
<td>Equation 2.4</td>
<td>Uncorrected Channel B Grid Value</td>
<td>33</td>
</tr>
<tr>
<td>Equation 2.5</td>
<td>Corrected Channel A Grid Value</td>
<td>33</td>
</tr>
<tr>
<td>Equation 2.6</td>
<td>Corrected Channel B Grid Value</td>
<td>34</td>
</tr>
<tr>
<td>Equation 2.7</td>
<td>Pearson’s Correlation Coefficient</td>
<td>35</td>
</tr>
<tr>
<td>Equation 2.8</td>
<td>Manders’ Co-localization Coefficient for Green Channel</td>
<td>35</td>
</tr>
<tr>
<td>Equation 2.9</td>
<td>Manders’ Co-localization Coefficient for Red Channel</td>
<td>35</td>
</tr>
<tr>
<td>Equation 2.10</td>
<td>Distance Between Two Points</td>
<td>37</td>
</tr>
<tr>
<td>Equation 2.11</td>
<td>Mobility of a Molecules</td>
<td>37</td>
</tr>
<tr>
<td>Equation 3.1</td>
<td>Mean Squared Displacement Curve Fit</td>
<td>49</td>
</tr>
<tr>
<td>Equation 3.2</td>
<td>Diffusion Coefficient</td>
<td>49</td>
</tr>
<tr>
<td>Equation 3.3</td>
<td>Radius of Mobility</td>
<td>51</td>
</tr>
<tr>
<td>Equation 3.4</td>
<td>Mobility Curve Fit</td>
<td>55</td>
</tr>
<tr>
<td>Equation 3.5</td>
<td>Time Dependent Curves Fit</td>
<td>63</td>
</tr>
<tr>
<td>Equation 3.6</td>
<td>State Vector</td>
<td>72</td>
</tr>
<tr>
<td>Equation 3.7</td>
<td>Rate per Unit Area $(R_A)$</td>
<td>73</td>
</tr>
<tr>
<td>Equation C.1</td>
<td>Mean Squared Displacement Curve Fit</td>
<td>142</td>
</tr>
<tr>
<td>Equation C.2</td>
<td>Simplified MSD Curve Fit</td>
<td>142</td>
</tr>
<tr>
<td>Equation C.3</td>
<td>Einstein’s Two-Dimensional Diffusion</td>
<td>142</td>
</tr>
<tr>
<td>Equation C.4</td>
<td>Diffusion Coefficient</td>
<td>.................................................................</td>
</tr>
<tr>
<td>Equation C.5</td>
<td>Area of a Circle</td>
<td>........................................................................</td>
</tr>
<tr>
<td>Equation C.6</td>
<td>Radius of Mobility</td>
<td>........................................................................</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Influenza virus is a known public health threat. Colloquially known as the flu, influenza virus infects several millions of people each year and kills tens of thousands of those infected (Center for Disease Control and Prevention, 2020b). A wide variety of individuals are at higher risk for contracting the virus, including the elderly, young children, individuals with pre-existing conditions such as cancer or diabetes, and woman who are pregnant (Center for Disease Control and Prevention, 2020c). A universal treatment option has not been discovered, however, preventative measures are in place, including the creation of yearly vaccines (Center for Disease Control and Prevention, 2020a). Due to its high mutation rate, the efficacy of influenza vaccines varies yearly, resulting in an inexact prevention and protection from the infectious disease. New viral strains are constantly surfacing which in severe cases can lead to nationwide epidemics or world-wide pandemics, such as resulted in 1918 with the Spanish Flu which infected an estimated 500 million people and killed 40 million of those infected (Taubenberger et al., 2001). Due to this ongoing race between the mutations of the virus and the development of new countermeasures, there is a high demand for a universal treatment for influenza virus. Nevertheless, certain crucial mechanisms by which the virus operates remain widely unknown.

1.1 Influenza A Virus Review

The influenza virus, which causes acute respiratory disease, belongs to the Orthomyxoviridae family of viruses, which is itself divided into five genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus, and Isavirus (Mahy & van Regenmortel, 2008). Influenza viruses have segmented, single-stranded, negative-sense ribonucleic acid (RNA) genomes that are translated and replicated in infected host cells. Influenza A virus (IAV) has 8 genome segments that encode 11 proteins: hemagglutinin (HA),
neuraminidase (NA), matrix 1 protein (M1), matrix 2 protein (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2; also known as nuclear export protein, NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase basic protein 1 – F2 (PB1-F2) (Mahy & van Regenmortel, 2008). IAV particles can have a spherical (100nm diameter) or filamentous morphology (between 300nm to several micrometers in length) (Mahy & van Regenmortel, 2008). Typically, influenza virus is named by its HA and NA subtypes, where 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) have been recorded. Of these combinations H1N1, H2N2, H3N2, H5N1, H1N2, and some H7 variations have been identified to infect humans (Belser et al., 2011; Mahy & van Regenmortel, 2008; Wong & Yuen, 2006). The remaining subtype combinations are associated with animals, most abundantly avian species.

IAVs are enveloped particles consisting of a lipid bilayer derived from the host cell, and thought to be enriched in certain lipids (Chen et al., 2005, 2007; Takeda et al., 2003; Wilson et al., 2015). Implanted in the IAV lipid envelope are the two viral glycoproteins, HA and NA, and the M2 ion channel (Mahy & van Regenmortel, 2008; Samji, 2009). Just beneath the lipid envelope lies M1, which forms a meshed matrix surrounding the viral ribonucleoproteins (vRNPs) made up of the viral negative stranded RNA genome (vRNA). These vRNPs are coated with NP and small amounts of NEP. vRNPS are also associated with the heterotrimeric polymerase complex containing PB1, PB2, and PA proteins (Mahy & van Regenmortel, 2008). A visualization of an IAV can be found in figure 1.1.
Figure 1.1: Influenza A Virion Structure. Hemagglutinin, Neuraminidase, and M2 ion channels are in the lipid membrane of the influenza A virion (IAV). Just beneath the lipid membrane, matrix protein 1 forms a meshed matrix. Within the enveloped particle are nuclear export proteins and viral ribonucleoproteins (vRNPs). These vRNPs encode the IAV genome and are associated with polymerase basic proteins 1 and 2 and polymerase acidic protein.
1.2 Brief Overview of Influenza A Life Cycle

To initiate entry, HA binds to sialic acid found on the outer surface of the host cell’s plasma membrane (PM) (Skehel & Wiley, 2000). Receptor-mediated endocytosis of the IAV particle occurs and the virus enters the cell via an endocytic compartment (endosome). Acidification of the endosome releases vRNPs from M1 and causes a conformational change in HA leading to fusion between the virion and endosomal membranes, opening a pore to release the contents of the virion into the cytoplasm (Mahy & van Regenmortel, 2008). Once released from the endosome, the vRNPs are then transported to the nucleus of the host cell where RNA transcription and replication take place (Mahy & van Regenmortel, 2008). A unique mechanism, cap snatching, allows the transcription of vRNAs by cleaving the methylated capped primer at the 5’ end of host cell mRNA molecules (Mahy & van Regenmortel, 2008). This method of stealing methylated caps from the host cell depletes the cells ability to produce its own proteins while simultaneously allowing viral proteins to be translated by host cell ribosomes. Five of the eight vRNP segments encode for one protein each: HA, NA, NP, PB2, and PA. The remaining 3 vRNP segments encode for two proteins each: segment 2 encodes for PB1 and PB1-F2, segment 7 encodes for M1 and M2, and segment 8 encodes for NS1 and NEP (Mahy & van Regenmortel, 2008). Once all 11 proteins are translated by the cell, viral assembly at the PM can occur. HA, NA, and M2 proteins are transported to the PM and cluster at a putative assembly site (Leser & Lamb, 2005). At the same time, vRNPs are released from the nucleus where they are then transported to the assembly site (Takizawa et al., 2016). M1 then multimerizes (M1 linkage to create a matrix) beneath the PM at glycoprotein clustering sites (Ali et al., 2000; Rossman & Lamb, 2011), having attached to the vRNPs either in the cytoplasm or during transport to the PM (Mahy & van Regenmortel, 2008; Nayak et al., 2004) and helps induce membrane curvature of infectious virions (Chlanda et al., 2015). Once assembly has occurred, the virus will bud from the PM. M2 will assist in scission of the virion from the host cell PM. To ensure the virions do not re-infect host cells, NA cleaves sialic acids on the PM of the host cell during the budding process (Mahy & van Regenmortel,
Virions are then free to infect other cells or may be released via respiratory droplets to infect other organisms.

1.3 Hemagglutinin (HA)

The mature glycoprotein, hemagglutinin (HA), is a homotrimer that is found in the IAV envelope and embedded in the plasma membrane (PM) of infected cells (Mahy & van Regenmortel, 2008). HA consists of two subunits: HA1, containing the sialic acid receptor binding site, and HA2, containing the fusion peptide. The precursor to HA, HA0, is cleaved into these subunits by a host cell enzyme, which is required to activate the fusion properties of mature HA (Chaipan et al., 2009). No longer a single chain of amino acids (such in the case of HA0), the HA subunits are linked together by a disulfide bond (Q. Huang et al., 2003). The enzymes required for cleaving are primarily found in or near bronchial cells (Böttcher-Friebertshäuser et al., 2010; Goto & Kawaoka, 1998; Lazarowitz et al., 1973; Su et al., 2018). However, more severe strains of Influenza A virus can be cleaved by other cell types (Chaipan et al., 2009). The HA structure can be broken down into three sections: an ectodomain, a transmembrane domain, and a cytoplasmic tail domain (CTD) (Mahy & van Regenmortel, 2008). The ectodomain is located outside the plasma membrane and is comprised of HA1 and most of HA2. The transmembrane domain spans the plasma membrane and serves as the membrane anchor region. The CTD is a short amino acid chain, typically ten to eleven amino acids long, extending into the cytoplasm close to the plasma membrane. The CTD is anchored to the membrane by three cysteines which had undergone acylation (Veit et al., 1991), the process of binding to an acyl group either a palmitic or stearic acid (Alberts et al., 2015). Mutations of these cysteine sites to different amino acids have caused interruptions in HA association with M1 (Chen et al., 2005; Chlanda et al., 2017), inhibition of viral growth (Chen et al., 2005; Zurcher et al., 1994), or were reverted back to the original cysteine during live infection (Chlanda et al., 2017; Jin et al., 1994).
Figure 1.2: Hemagglutinin Trimer Structure. The glycoprotein, Hemagglutinin (HA), is located in the plasma membrane of IAVs and is most frequently found in trimers. Each HA can be broken into three sections: the ectodomain, which is found on the outside of the IAV; the transmembrane domain, which is section found in the plasma membrane; and the cytoplasmic tail domain, which is a small sequence of amino acids found in the cytosolic region of the IAV known to anchor the HA to the plasma membrane. The structure shown here was extracted from the RCSB database (Lu et al., 2013) but does not show the full transmembrane domain or cytoplasmic tail.
The mechanism for HA clustering at the plasma membrane (PM) of host cells is still unknown; however, it is essential for proper Influenza A virus (IAV) infectivity (Ellens et al., 1990). To properly infect host cells, a minimum number of HA trimers is required; in addition, higher HA density on the virion is correlated with higher fusion efficiency (Ellens et al., 1990). Mutations of the transmembrane region and CTD led to a decrease in HA clustering at the PM which in turn decreased infection rates (Takeda et al., 2003). HA clustering is also important for assembly of viral components at the PM just before the budding process. Clustering of IAV membrane proteins signal for vRNAs to be transported to the PM (Takizawa et al., 2016) and recruitment of M1 for multimerization begins beneath the PM (Rossman & Lamb, 2011). To understand this mechanism further, it is important to understand how cellular components are affected by infection. Viruses necessarily hijack host cell components for replication (Mañes et al., 2003) and some are found in purified flu virus (Shaw et al., 2008). Cytosolic cellular components have been shown to regulate HA at the PM (Gudheti et al., 2013; Simpson-Holley et al., 2002) and are also involved in trafficking HA to the PM (Guerriero et al., 2006; Rozelle et al., 2000).

1.4 Actin

Actin is a ubiquitous protein found in most eukaryotic cells and is involved in many cellular processes, such as cell motility, cell division and cytokinesis, muscle contraction, vesicle and organelle movement, cell signaling, and the establishment and upkeep of cell shape (Alberts et al., 2015). Monomeric actin, sometimes called globular or G-actin, is a cytosolic protein carrying an energy-rich ATP or ADP (Alberts et al., 2015). G-actin will link together head-to-tail to form a right-handed helix called filamentous or F-actin. Actin filaments are formed with all G-actin monomers facing the same direction creating a filament polarity with “barbed” and “pointed” ends (Alberts et al., 2015). The ends specify the direction in which the filament is more likely to further polymerize, with the “barbed” end having a higher affinity for addition of new G-actin monomers, while the “pointed” end has a higher rate of monomer loss.
Actin filaments are regulated by a set of actin binding proteins (ABPs) which assist in the process of actin polymerization and disassembly (Pollard, 2016). Individual actin filaments are very flexible but may be cross-linked together forming bundles (branched actin regions) to increase rigidity (Alberts et al., 2015). It has been theorized that actin rich regions can affect the mobilities of both proteins and lipids in the PM (Kusumi & Sako, 1996; Sako & Kusumi, 1994) and it has been observed to affect membrane-associated proteins by modulating their motion (Heinemann et al., 2013b, 2013a; Sadegh et al., 2017).

Actin plays a series of vital roles in IAV infection, especially during the assembly of viral components at the plasma membrane (PM) (Avalos et al., 1997; Bedi & Ono, 2019; Gudheti et al., 2013; Kumakura et al., 2015; Simpson-Holley et al., 2002). HA has been seen in close vicinity to actin filaments at the PM (50nm to 1μm) and actin affects the motion of HA within clusters (Gudheti et al., 2013; Simpson-Holley et al., 2002). Actin also plays a role in HA assembly at the PM through PI5K-mediated actin comet delivery of Golgi-derived vesicles containing HA0 trimers (Guerriero et al., 2006; Rozelle et al., 2000). This vesicle delivery mechanism involves the rapid assembly of Arp2/3 nucleated actin filaments beneath a Golgi-derived vesicle which transports proteins and lipids to the PM (Alberts et al., 2015). Actin is implicated in both the assembly of HA at the PM and the confinement of HA in clusters once there. For a more in-depth summary of the role of actin, and other cytoskeletal components, in IAV infection see the review by Bedi & Ono (Bedi & Ono, 2019).

1.5 Actin Binding Proteins (ABPs)

Actin is regulated by a group of proteins named actin binding proteins (ABPs) (Pollard, 2016). These proteins are involved in polymerization (addition of monomers to actin filaments), disassembly of filaments (the removal of G-actin from actin filaments), or stabilization of existing filaments. For example, formins are involved in polymerization of actin on the “barbed” end of filaments (Chesarone et al., 2010)
and to create branched actin regions, actin-related protein 2/3 (Arp2/3) will bind to the side of existing filaments to initiate new filament growth at a distinct angle (Mullins et al., 1998; Zigmond, 2004). In this work, four ABPs were chosen to be studied alongside HA, α-actinin, cofilin 1, myosin1, and tropomyosin 4, due to their probable affiliation with HA (figure 1.3).

### 1.5.1 α-Actinin

α-actinin is a rod-shaped protein with one actin binding domain at each end of the rod (Sjöblom et al., 2008). In non-muscle cells, this ABP is known to bundle actin filaments together near the plasma membrane (PM) and can directly bind phosphoinositides in the PM (Burn et al., 1985; Catimel et al., 2008; Fukami et al., 1994). The binding of phosphoinositides regulates actin binding to α-actinin (Fraley et al., 2003; Full et al., 2007; Sjöblom et al., 2008). In muscle cells, α-actinin forms a lattice structure which stabilizes muscle contraction (Sjöblom et al., 2008). While there are no known direct connections between α-actinin and the influenza viral life cycle, HA has been shown to affect phosphoinositide clustering at the PM (Curthoys et al., 2019; Parent, 2020) and α-actinin has a direct binding to those phosphoinositides.

### 1.5.2 Cofilin 1

Cofilin is involved in both disassembly of actin filaments at the “pointed” end (Ichetovkin et al., 2000) and assists with polymerization of actin at the “barbed” end depending on local concentrations (Andrianantoandro & Pollard, 2006; Ichetovkin et al., 2002). Cofilin can sever actin filaments, thus creating free “barbed” ends and potentially triggering new actin polymerization (Bravo-Cordero et al., 2013; Kanellos & Frame, 2016). During actin remodeling near the end of the influenza viral life cycle, cofilin concentrations increase (G. Liu et al., 2014). Knockdown of cofilin concentrations has been shown to reduce IAV yields from host cells (G. Liu et al., 2014) and cofilin has been identified in purified flu virus particles (Shaw et al., 2008).
Figure 1.3: Actin Binding Protein Structures. All four structures of the actin binding proteins (ABPs) studied in this work are visualized here: α-actinin, cofilin 1, myosin 1, and tropomyosin 4. The structures shown here were extracted from the RCSB database: α-actinin (J. Liu et al., 2004), cofilin 1 (Klejnot et al., 2013), myosin 1 (Dominguez et al., 1998), and tropomyosin 4 (Brown et al., 2001).
1.5.3 Myosin 1

Myosin 1 is a monomeric motor protein that binds to F-actin in the presence of ATP (a packet of energy located in G-actin monomers) and moves along these actin filaments (Alberts et al., 2015; Hartman & Spudich, 2012). These myosin motors have been found to move Golgi-derived vesicles to the plasma membrane (PM) of cells (Fath et al., 1994; Montes de Oca et al., 1997). Arp2/3 mediated actin comets, in which myosin 1 is associated with, transport HA0 trimers from the Golgi to the PM in these vesicles (Guerriero et al., 2006; Taunton et al., 2000). This myosin-actin network is important for IAV to recruit M1 and vRNPs to the PM (Kumakura et al., 2015) and may play an important role in HA recruitment to the PM as well.

1.5.4 Tropomyosin 4

Tropomyosin 4 is a helical coiled protein which binds to F-actin and is implicated in stabilizing these filaments (Alberts et al., 2015). In most cases, binding of tropomyosin 4 to F-actin subunits prevents binding of other ABPs in that region. Tropomyosin 4 has been seen to regulate the interactions of formins with the “barbed” end of F-actin (Gunning et al., 2015; Ujfalusi et al., 2012; Wawro et al., 2007) and is not easily bound to Arp2/3 nucleated actin bundles (Hsiao et al., 2015). Although there is no known association between tropomyosin 4 and the influenza viral life cycle, this ABP is found in purified flu virus (Shaw et al., 2008) indicating that it is at least found in the budding region of IAV.

1.6 Phosphatidylinositol 4,5-bisphosphate (PIP2)

The phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP2), is a cellular lipid found primarily on the inner leaflet of the plasma membrane (PM) and only makes up a small fraction of the phospholipids found on the PM (Alberts et al., 2015). The PIP2 structure is made up of a negatively charged, hydrophilic head group with a phosphate group attached to the 4th and 5th positions. This head group is attached to two fatty acid chains that are hydrophobic and are generally found inside the
cytoplasmic leaflet of the plasma membrane. PIP2 is involved in a number of cellular functions, such as cell motility, adhesion, actin reorganization, vesicle transportation, exocytosis, and endocytosis (Alberts et al., 2015; Balla, 2013; Catimel et al., 2008; McLaughlin et al., 2002). PIP2 functions as a second messenger or by directly interacting with proteins to manage membrane organization. PIP2 is known to recruit, bind, and regulate proteins at a variety of locations in the cell. However, a majority of PIP2 function occurs at the PM (Czech, 2000). An important PIP2 regulator is actin binding proteins, and by extension actin-network organization (Catimel et al., 2008). Of the ABPs mentioned previously, α-actinin, coflin 1, and myosin 1 all have direct interactions with PIP2 (Catimel et al., 2008).

There is evidence of influenza virus exploiting PIP2-dependent signaling pathways (Ehrhardt et al., 2006; Fujioka et al., 2019; Hale et al., 2006; Shin et al., 2007). Some proteins that have been identified in purified flu virus have direct interactions or associations with PIP2 (Catimel et al., 2008; Shaw et al., 2008). PIP2 is modulated by HA clustering in the PM (Curthoys et al., 2019) and its clustering is affected by HA clustering (Curthoys et al., 2019; Parent, 2020).

The involvement of PIP2 in numerous cellular functions identifies it as a primary target for understanding membrane organization (Balla, 2013; McLaughlin et al., 2002), protein-lipid interactions (Won et al., 2006), and viral infection (Curthoys et al., 2019; Favard et al., 2019; Gc et al., 2016; Johnson et al., 2018; Mücksch et al., 2017; Rocha-Perugini et al., 2014). Previous studies have tried to theorize and explain the role of phosphoinositides, such as PIP2, in membrane organization (Anderson & Jacobson, 2002; Kusumi & Sako, 1996; Sako & Kusumi, 1994). However, further observations are needed to test these theories. PIP2 concentrates into clustered regions in the PM to function, and understanding how PIP2 clusters would further elucidate how this small lipid is involved in various cellular activity (Hammond, 2016). Hammond proposed three membrane models to predict the way in which PIP2 concentrates in the PM for use by membrane-associated proteins (figure 1.4); Platforms, Local Synthesis or Releases of
Figure 1.4: Diagram of Proposed Hemagglutinin and PIP2 Membrane Models. Five membrane models explaining the possible ways PIP2 may concentrate at the plasma membrane for use by membrane-associated proteins. The membrane-associated protein we chose to study was hemagglutinin (HA) due to its clustering properties and importance to influenza infection. Each panel depicts a before and after membrane picture for how PIP2 and HA may move based on its corresponding model requirements. 1) Platforms predicts that PIP2 pre-clusters at the membrane and recruits membrane-associated proteins to these regions. 2) Local Synthesis or Release of PIP2 (LSRP) predicts one of two possibilities: PI kinases beneath the clustered proteins in the membrane synthesis PIP2 as needed or PIP2 is released by proteins known to bind PIP2 at the PM (not pictured). 3) Megapool predicts that membrane-associated proteins are pre-clustered in the PM and recruit freely diffusing PIP2 molecules as needed to these regions. 4) Regions of the PM are removed through budding or recycling methods. 5) Both PIP2s and proteins are delivered together pre-clustered in Golgi-derived vesicles to the PM.
1.6.1 Platforms

The Platforms membrane model predicts that domains of lipids are already enriched in the PM (Hammond, 2016). These PIP2 domains act as platforms onto which membrane-associated proteins can congregate. Once the protein recruitment has occurred lipid signaling or protein activation can ensue. There are three constraints on this model: firstly, PIP2 molecules are highly dynamic, with a reported diffusion coefficient of upwards of $1 \, \mu m^2/s$, which can escape clusters easily and would require a corolling factor to maintain clustered regions; secondly, membrane-associated proteins would need a basis to distinguish PIP2 platforms dedicated for them versus another cellular function; and thirdly, PIP2 has a negatively charged head group that would prevent highly concentrated regions to exist without a corolling factor to keep them together. Nevertheless, PIP2 has been observed to cluster in the PM (Curthoys et al., 2019; Golebiewska et al., 2011; Y. Liu et al., 1998; Van Den Bogaart et al., 2011; Wang & Richards, 2012), although some studies have observed the opposite in differing cell types (Ji et al., 2015; Van Rheenen et al., 2005). These constraints can be easily overcome by co-operation with neighboring membrane proteins, such as those containing polybasic domains, which have been observed previously to interact with PIP2 (Van Den Bogaart et al., 2011; Won et al., 2006). It is still unclear whether pre-clustered PIP2 platforms exist consistently in all cell types in which PIP2 signaling is required for cell function and how platforms would be able to persist over longer time scales, although there is evidence for a role played by corolling proteins (Won et al., 2006).

1.6.2 Local Synthesis or Release of PIP2 (LSRP)

The Local Synthesis or Release of PIP2 (LSRP) model postulates that PIP2 is locally acquired or synthesized as needed by membrane-associated proteins, rather than by existing in pre-formed clusters prior to signaling (Hammond, 2016). There are two ways this can occur: by local synthesis of PIP2 by PI
kinases located beneath the PM or by signaling the release of PIP2 bounded by other membrane-associated proteins, such as MARCKS. We have modified the originally published model by Hammond, named ‘selfish’ PIP2 synthesis, to include this second possibility (PIP2 release), as the two are indistinguishable using our experimental technique. This model predicts that the majority of PIP2 would be bound by proteins in the PM, only to be released when signaled; however, this would make labeling of PIP2 nearly impossible by the fluorescently labeled PH domain, which would need to compete against other PIP2-binding proteins in order to find and bind PIP2. Previous studies have successfully used fluorescently labeled PH domain to label PIP2 at the PM which is inconsistent with this prediction (Curthoys et al., 2019; Hammond et al., 2009), except if it only describes a subset of the PIP2 present within the PM. This model also predicts the recruitment of PI kinases to PM regions with clustered proteins (Hammond, 2016). Previous studies have observed PI kinase-enriched regions near sites of PIP2-associated proteins (Di Paolo et al., 2002; Ling et al., 2002; Nakano-Kobayashi et al., 2007). Constraints on this model include the availability of substrates and kinases required for synthesis of PIP2 (such as PI4P and PI5P) and the possible over-production of PIP2 at protein-rich regions with the unwanted effect of activating nearby PIP2-dependent functions. It is unclear how these constraints may affect PIP2 concentrations under this membrane model and further observations are needed.

1.6.3 Megapool

Similar in principle to the Platforms model, the Megapool model requires that regions of clustered membrane-associated proteins recruit freely diffusing PIP2 molecules from the surrounding areas (Hammond, 2016). The PIP2 is not pre-clustered into platforms by a corralling factor and can easily diffuse into regions. This model predicts that the abundance of PIP2 molecules in the PM would be enough to support PIP2-dependent functions. There are no constraints on this model, making it a likely candidate for PIP2. Yet, no evidence of this model currently exists and further observations are needed to favor this model.
1.6.4 Budding/Recycling

To efficiently repurpose proteins and lipids or to expel certain molecules, there are two cellular functions that maintain equilibrium: Budding and Recycling. Budding is the process in which regions of the membrane are “pinched” away from the cell to create vesicles that are expelled from the cell (Alberts et al., 2015). This may happen for a variety of reasons, but a most common purpose is for controlled release of vesicles from the cell surface. Conversely, it is possible for a cell to secrete a protein or other small molecule via exocytosis, which leads to an increase in the area of the plasma membrane. Recycling is the process of “taking in” (i.e. retracting, engulfing) regions of the membrane into the cell (Alberts et al., 2015). This mechanism is important for regulation of protein concentrations in the PM. Both mechanisms require removal of lipids (such as PIP2) and proteins (such as HA) from the PM and therefore make up the fourth membrane model. PIP2 recruitment into clusters at the PM may be mediated for the cell to conserve energy. Both budding of vesicles and recycling of cellular components are exhaustive processes. It is likely that the PM is organized for efficient recovery or expulsion of regions no longer necessary for cellular function.

1.6.5 Exocytosis/Delivery

The model Exocytosis/Delivery predicts that PIP2 is delivered to the PM in protein-rich Golgi-derived vesicles. The Golgi apparatus is an organelle inside the cell that packages proteins into membrane-bound vesicles to be delivered to the membrane (Alberts et al., 2015). As evidence for this model, it has been shown that one of the kinases (PI5K) implicated in production of PIP2 mediates vesicle transport to the PM from the Golgi (Guerriero et al., 2006; Rozelle et al., 2000). It is possible that membrane-associated proteins are delivered pre-clustered to the membrane alongside the necessary PIP2 needed to carry out a cellular function. If this mechanism of lipid-protein association exists, it would help explain the clustering of multiple viral proteins at the PM.
All the biological components mentioned previously associate at the nanoscale. To further understand their interactions and elucidate their membrane structure, or their associations with influenza proteins, there is a need to image these molecules at the nanoscale.

1.7 Diffraction Limited Microscopy

When light passes through a lens, however perfect the lens may be, it is focused to a shape at the lens’ focal length, not to an infinitesimally small point. Focused light from a point source forms a point spread function (PSF) in the shape of an Airy pattern. Individual Airy patterns are resolvable if the distance of separation is greater than or equal to the Rayleigh criterion, described by

\[ R = 0.61 \frac{\lambda}{NA} \]  

where \( R \) is the separation distance between two PSFs to be resolved, \( \lambda \) is the emission wavelength of the PSFs, and \( NA \) is the numerical aperture of the lens (Born & Wolf, 1997). A weakness of light microscopy has been primarily led by this diffraction limit, with lateral resolution on the order of 200 nanometers. Conventional light microscopy is largely limited by its resolution and in most cases cannot distinguish biological structures on the nanoscale level, e.g. virions, proteins, lipids, etc.

1.8 Fluorescence Microscopy

To better observe nanoscale structure, techniques optimizing the use of fluorophores have been invented and improved upon (Pawley, 2006). In 1962, the discovery and isolation of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* paved the way for fluorescence microscopy (Prasher et al., 1992; Prendergast & Mann, 1978; Shimomura et al., 1962). Since its discovery, several other fluorescent proteins have been isolated (Gunewardene et al., 2011; Gurskaya et al., 2006; Lešková et al., 2019). Fluorophores like GFP are structured so that the chromophore is protected within the center of a
tightly built β-barrel (Chalfie & Kain, 2005). Light at the proper excitation wavelength can penetrate this barrel structure and excite the chromophore within. The chromophore will then emit light at a Stokes shifted wavelength (which is longer due to loss of energy) that can be observed and collected through a microscope set-up (Chalfie & Kain, 2005). The attachment, or tagging, of fluorophores to proteins creates a fusion protein which can then be expressed inside cells. Other biological tagging systems are possible through the use of ligand to fluorophore attachment (Middleton & Kellam, 2005) or by using fluorescently tagged antibodies (Sadun et al., 1961), however, these tagging systems are not as precise since their binding may either be non-specific (the possibility of tagging other native proteins or lipids) or transient (will not stay bound together for long periods of time) in nature.

1.9 Super Resolution Microscopy

The invention of super resolution fluorescence microscopy broke the diffraction limit, allowing structures to be resolved down to tens of nanometers providing new observations of cellular molecules (Betzig et al., 2006; S T Hess et al., 2006; Rust et al., 2006). In the work presented here, we used the technique Fluorescence Photoactivation Localization Microscopy (FPALM) which utilizes the properties of photoactivatable or photoswitchable fluorescent proteins to resolve molecules at length scales shorter than the Rayleigh criterion (S T Hess et al., 2006). Photoactivatable proteins remain in a dark state until activated by the appropriate wavelength of light. Photoswitchable proteins are similar but begin in a fluorescent state and undergo changes to their emission wavelengths. These proteins are then intermittently turned on at low frequencies and imaged over time. Resolvable PSFs are localized according to a fitted approximation with a localization precision, \( \sigma_{xy} \), given by

\[
\sigma_{xy}^2 = \frac{r^2 + q^2/12}{N} + \frac{8\pi r^4 b^2}{q^2 N^2}
\]

Equation 1.2
where \( r \) is the standard deviation of the PSF, \( N \) is the total number of photons for a given localization, \( b \) is the background noise in photons per pixel, and \( q \) is the effective pixel size at the sample (Thompson et al., 2002). Equation 1.2 has since been optimized for localization analysis of single-molecule tracking and super-resolution microscopy (Mortensen et al., 2010). Depending on the biological application there are multiple illumination methods available for use in microscopy. Widefield and total internal reflection (TIRF) (figure 1.5) illumination geometries are both used in this work. Widefield illuminates a circular column within the sample (figure 1.5A) while TIRF illuminates a small region (~100nm) just above the coverslip (figure 1.5B) (Stout & Axelrod, 1989). TIRF will primarily illuminate the PM of adherent cells as well as a small region just above the coverslip which is ideal for observing PM proteins or protein interactions with PM components. Widefield is better suited to imaging deeper within the cell but typically induces more background fluorescence from out of focus molecules.

1.10 Introduction Review

Influenza HA is a membrane glycoprotein that plays vital roles during flu infection of host cells (Mahy & van Regemortel, 2008). High HA density at the plasma membrane (PM) is required for flu infectivity (Ellens et al., 1990) but the mechanism for clustering is still unknown. HA’s affiliation with actin (Gudheti et al., 2013; Simpson-Holley et al., 2002) led us to study the possible interaction between HA and the phosphoinositide, PIP2, along with multiple actin binding proteins (ABPs). We find that HA clustering properties are affected by ABP concentrations and that PIP2 mobilities are modulated by the presence of HA. We also studied the possible ways PIP2 may concentrate in the PM using HA as a membrane-associated protein. We observed that PIP2 and HA are more likely to be delivered to the membrane together than to be recruited into clusters once in the PM.
Figure 1.5: FPALM Illumination Methods Diagram. A visualization of a laser exciting a sample in two alternative illumination methods. (A) Widefield microscopy requires the laser to be passing through the central region of the objective lens. The laser will illuminate a circular column, highlighted in red, of the sample. In focus fluorescence will be collected at the focal plane of the objective lens. (B) Total Internal Reflection (TIRF) microscopy (Stout & Axelrod, 1989) requires the laser to be shifted until a critical angle is achieved in the glass coverslip to bounce the laser light back down into the objective lens. A small region above the sample, approximately 100nm of depth above the coverslip, is excited by evanescent waves created by the TIRF, highlighted in red. Panels are not drawn to scale.
CHAPTER 2
MATERIALS AND METHODS

2.1 Biological and Sample Preparation Methods

All sample preparations follow standard protocols for fluorescence labeling. Biological components, such as complete growth media, are prone to auto-fluorescence that can increase background during data acquisition. After labeling, samples should be shielded from stray light so that they are not activated prematurely. A list of all supplies used in this chapter can be found in table 2.1.

2.1.1 Cell Passage

Single species samples were prepared with NIH3T3 mouse fibroblast cells (ATCC) or NIH3T3-HAb2 (HAb2) cells (NIH3T3 mouse fibroblast cells stably expressing hemagglutinin protein (Ellens et al., 1990)). All two-species samples were prepared with NIH3T3 mouse fibroblast cells.

Both NIH3T3 and HAb2 cells were grown in T25 flasks with filter caps (Thermo Fisher Scientific) in a cell incubator and maintained at less than 90% confluency per split cycle. The cell incubator was maintained at 37°C and 5% CO₂ levels. Cells were grown in growth media consisting of Dulbecco’s Modified Eagle’s Medium (DMEM) with high glucose, phenol red, and L-Glutamine (Lonza) which was then supplemented to 10% calf bovine serum (ATCC) and to 2% Penicillin-Streptomycin (antibiotic, GIBCO). When 60-90% confluency was reached, old media was removed, and cells were prewashed with 0.5 milliliter of 0.05% Trypsin-EDTA solution (Thermo Fisher Scientific) prewarmed to 37°C. Cells were then incubated with 1 milliliter of 0.05% Trypsin-EDTA solution for ~5 minutes, or until cells had detached from the surface, at 37°C. After the cells detached from the flask’s bottom, they were resuspended in 4 milliliters of growth media prewarmed to 37°C, carefully breaking up any cell clumps during the pipetting procedure. After counting with a hemocytometer, approximately 100,000 cells were then seeded into a
### Table 2.1: Biological Supplies and Manufacturer Details

A list of all biological supplies and their manufacturers mentioned in biological methods and sample preparation methods.

<table>
<thead>
<tr>
<th>MANUFACTURER</th>
<th>BIOLOGICAL SUPPLY</th>
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<tbody>
<tr>
<td>Alfa Aesar</td>
<td>4% Paraformaldehyde</td>
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<tr>
<td>ATCC</td>
<td>Calf Bovine Serum&lt;br&gt;NIH3T3, mouse fibroblast cells</td>
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<tr>
<td>Echelon BioSciences</td>
<td>BODIPY TMR-Phosphatidylinositol 4,5-biphosphate (GloPIP)</td>
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<td>Gibco</td>
<td>Opti-MEM&lt;br&gt;Penicillin-Streptomycin</td>
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<td>Invitrogen</td>
<td>Tetraspeck Beads, 100-nanometer diameter</td>
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<td>Lonza</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>MatTek Corporation</td>
<td>Petri Dishes, 35-millimeter No 1.5 glass bottom</td>
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<tr>
<td>Omega Bio-Tek</td>
<td>E.Z.N.A. Endo-Free Plasmid DNA Mini Kit II</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>Thermo Fisher Scientific</td>
<td>D-Glucose&lt;br&gt;Lipofectamine 3000 Transfection Kit&lt;br&gt;NanoDrop™ One Microvolume UV-Vis Spectrophotometer&lt;br&gt;Nunc™ Chambered Coverglass, 8-well&lt;br&gt;Poly-L-lysine&lt;br&gt;T25 Flasks with filter caps&lt;br&gt;Trypsin-EDTA</td>
</tr>
</tbody>
</table>

new T25 flask with 5 milliliters of growth media and placed back into the incubator to be split again three days later. If necessary, any remaining cells were then used for sample preparation.

#### 2.1.2 Plating Procedure

Both NIH3T3 and HAb2 cells were plated in a 35-millimeter, #1.5 glass bottom petri dish (MatTek Corporation) at 25,000 cells per milliliter of plating media warmed to 37°C, (approximately 50,000 cells per dish). Plating media consisted of DMEM with high glucose and L-Glutamine (Lonza) which was then supplemented to 10% with calf bovine serum (ATCC). Plating media does not contain phenol red or
antibiotic, unlike growth media, see section 2.1.1. Petri dishes were then incubated for 24 hours before transfection.

### 2.1.3 Transient Transfection of Sample

A variety of fusion proteins were utilized in the work presented here. Two fusion proteins were used to label HA; Hemagglutinin-Dendra2 (Gudheti et al., 2013), where the hemagglutinin is translated first, and Dendra2-Hemagglutinin (plasmid kindly made by Dr. Hang Waters, Zimmerberg Lab, National Institute of Child Health and Human Development), where the Dendra2 is translated first. Fluorescently labeled actin binding proteins were tagged with PAmCherry; α-actinin-PAmCherry (plasmid kindly made by Dr. Vladislav Verkhusha, Albert Einstein College of Medicine), Cofilin 1-PAmCherry (Gudheti et al., 2013), Myosin 1-PAmCherry (plasmid kindly made by Dr. Vladislav Verkhusha, Albert Einstein College of Medicine), and Tropomyosin 4-PAmCherry (plasmid kindly made by Dr. Nikki Curthoys, University of Wollongong, Australia). Two fusion proteins were used to label PIP2 via a protein named phospholipase C-δ1 pleckstrin homology (PH) domain; PH domain-PAmKate (Curthoys et al., 2019) and PH domain-Dendra2 (Curthoys et al., 2019).

All plasmid deoxyribonucleic acid (DNA) was prepared using an E.Z.N.A. Endo-Free Plasmid DNA Mini Kit II (Omega Bio-Tek). DNA was then measured by a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) for recording of concentrations. Typically, a 260/280 ratio is found between 1.7-1.9 and a 260/230 ratio is between 2.0-2.2 to demonstrate a sufficient DNA purity. Concentrations less than 500 \( \text{ng} \mu\text{l} \) may indicate a poor DNA yield and were not used for experiments as a precaution.

After a 24-hour incubation period, plated cells were transfected with plasmid DNA using the manufacturer’s protocol for Lipofectamine 3000 (Thermo Fisher Scientific) with Opti-MEM (Gibco). For fixed cell two species plates, a total of 2-3 micrograms of DNA was added to each plate at a concentration of 1:1 Hemagglutinin-Dendra2 to an actin binding protein (α-Actinin, Cofilin 1, Myosin 1, or Tropomyosin
4) fluorescently labeled with PAmCherry. For live cell two species plates, a total of 2-3 micrograms of DNA was added to each plate at concentrations of 1:1, 1.3:1, and 3:1 Dendra2-Hemagglutinin to PH domain-PAmKate (see table 2.2 for additional details). For single species plates, a total of 2 micrograms of DNA per species was transfected onto a plate; Dendra2-PH Domain was used for single species data presented below (Curthoys et al., 2019). After the desired DNA concentration was added, plates were then wrapped in aluminum foil to prevent unwanted activation from room lights and incubated for 24-hours before imaging.

2.1.4 Sample Preparation for Live Cell Imaging

Just prior to imaging, plates were removed from the incubator and washed 2-3 times with room temperature phosphate-buffered saline (PBS, Sigma-Aldrich). Imaging media, containing PBS and 10 millimolar glucose (Thermo Fisher Scientific), was then added to plates to a depth of 2 millimeters, and cells were then immediately (within 10 minutes) imaged. Samples should remain in plating media and covered until they are needed for imaging to avoid dehydration or potential complications from cell starvation.

2.1.5 Sample Preparation for Fixed Cell Imaging

After a 24-hour incubation period, transiently transfected two-species plates were removed from the incubator to be washed 2-3 times with 1 milliliter of room temperature phosphate-buffered saline (PBS, Sigma-Aldrich) to remove any lingering media. Immediately following, cells were washed with 1 milliliter of 4% paraformaldehyde (PFA, Alfa Aesar) and let stand for 10-20 minutes. Plates were then washed 2-3 times with 1 milliliter of room temperature PBS to remove any lingering PFA. Plates were imaged within a week of fixation and stored at 4°C.

2.1.6 Bead Sample Preparation

A bead sample was required for two species imaging. Preparation begins by adding 200-microliters of ploy-L-lysine (Thermo Fisher Scientific) into each well of an 8-well Nunc™ chambered
coverglass (Thermo Fisher Scientific) and incubated for 2 hours. Each well was then rinsed three times with PBS (Sigma-Aldrich) before the addition of ~100-microliters of tetraspeck beads (Invitrogen) diluted into distilled water, with dilutions ranging from 1:20 to 1:100 beads to water. Each well was then incubated for 2 hours. Wells were then rinsed three times with PBS to ensure all excess material was removed. A few droplets of FluorSave (Millipore), enough to cover the bottom of the well, was added and left to dry for 1-3 hours. Bead samples can be used for approximately 2-3 months after which the sample is suboptimal. Images of beads were acquired and analyzed to allow correlation between the two-color channels according to previously published methods (Gunewardene et al., 2011).

### 2.1.7 BODIPY TMR-PIP2 Labeling

For instructions on methods associated with the labeling of BODIPY TMR-Phosphatidylinositol 4,5-biphosphate (GloPIP, Echelon Biosciences) in NIH3T3 and NIH3T3-HAb2 (HAb2) cells; see Ozark et al. for biological methods instructions (Ozaki et al., 2000) and Curthoys et al. for super-resolution imaging instructions (Curthoys et al., 2019).

<table>
<thead>
<tr>
<th>REPLICATE NUMBER</th>
<th>DATE</th>
<th>PASSAGE NUMBER</th>
<th>PLASMID</th>
<th>DNA AMOUNT</th>
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<td>1</td>
<td>11/12/2019</td>
<td>p-12</td>
<td>PH Domain -PAmKate Dendra2-HA</td>
<td>1.09 μg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.42 μg</td>
</tr>
<tr>
<td>2</td>
<td>11/19/2019</td>
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<td>PH Domain -PAmKate Dendra2-HA</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.42 μg</td>
</tr>
<tr>
<td>3</td>
<td>01/11/2020</td>
<td>p-31</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5 μg</td>
</tr>
<tr>
<td>4</td>
<td>01/13/2020</td>
<td>p-32</td>
<td>PH Domain -PAmKate Dendra2-HA</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5 μg</td>
</tr>
</tbody>
</table>

**Table 2.2:** Sample Preparation for 2-Species Imaging of PH Domain-PAmKate and Dendra2-Hemagglutinin. A list of biological information for sample preparation of 2-species imaging of PH Domain-PAmKate and Dendra2-HA in living cells.
2.2 Super Resolution Imaging

All experiments were conducted using a set-up similar to figure 2.1. All equipment information for the instrumentation used in this set-up can be found in table 2.3. Figure 1.5 depicts two illumination methods for FPALM – widefield and total internal reflection (TIRF). Alignment procedures for both illumination methods are described below.

2.2.1 Fluorescence Photoactivation Localization Microscopy Alignment

The illumination path for a typical fluorescence photoactivation localization microscopy (FPALM) alignment consists of any lasers and microscope required for sample illumination. An activation laser, wavelength of 405 nanometers (CrystaLaser), was aligned co-linearly with a readout laser, wavelength of 558 nanometers (CrystaLaser), to pass through a 250-millimeter focal length lens (ThorLabs) positioned at approximately one focal length from the objective back aperture plane. Laser light was focused through the rear (arc lamp) port of the Olympus IX71 microscope (Olympus) and onto the back focal plane of the objective lens (Olympus), OBJ, after reflecting from a dichroic mirror (Chroma), DM1, and bouncing upwards in the positive z-direction towards the OBJ. For Widefield illumination, it is important that all laser light and fluorescence light in the detection path should pass straight through the center of all lenses to prevent unwanted bending of light (this is not the case for TIRF illumination). Laser light entering the center of the OBJ leads to a widefield FPALM illumination geometry (figure 1.5A). Once passed through the infinity corrected OBJ, laser light excites a sample, S, located at the focal plane of the OBJ. A portion of the fluorescence light was collected by the OBJ (light traveling in the negative z-direction), and then passed through a dichroic mirror (Semrock), DM2, a variety of emission filters (see table 2.3 for instrumentation information), and the microscope tube lens (Olympus), TL, which has a focal length of 180-millimeters. Fluorescence from the sample was focused by the TL, converging at the TL focal plane, FP2, which is conjugate to S, and was magnified by the ratio of TL to objective focal length, or in this case 60x.
Figure 2.1: Widefield FPALM Diagram. Above is the basic layout for a typical Widefield FPALM set-up where all optics are in the same plane (xy-plane) except for the microscope, which is perpendicular (in the z-direction) to this plane. The activation laser and readout laser were controlled using neutral density filters (ND) and a shutter (SH) along each path. Both laser paths were combined (colinear) at a mirror (M2) and reflected to a lens (L1) by M1. Laser light passed into the microscope box where it was then reflected by a dichroic mirror (DM2) upwards to the objective lens (OBJ). The sample (S), located in the focal plane above the OBJ, was then excited by laser light, causing it to emit fluorescence. Fluorescence was collected through the OBJ, passing downwards through DM2 and a filter (F1). Light then passed through the tube lens (TL) and was reflected off M6 to pass into the detection path located to the left of the microscope box. Fluorescence passed through the focal plane of the tube lens, where the sample image has been magnified by the OBJ, and AP2, which was used to restrict the field of view for proper detection by the Electron Multiplying CCD (EMCCD). Passing through a 2x telescope created by L2 and L3, the light (A) continued straight to the EMCCD for single-color detection or (B) was split into two channels by DM3 where red light was passed through to M7, M8, and F3 while green light was reflected to M9 and F2.
## OPTICAL INSTRUMENTS AND MANUFACTURER DETAILS

<table>
<thead>
<tr>
<th>PART NAME</th>
<th>PART DETAILS (IF AVAILABLE)</th>
<th>MANUFACTURER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation Laser</td>
<td>405 nanometers, total power: ~2mW</td>
<td>CrystaLaser</td>
</tr>
<tr>
<td>Aperture</td>
<td></td>
<td>ThorLabs</td>
</tr>
<tr>
<td>Autofluorescent Slides</td>
<td></td>
<td>ChromaLabs</td>
</tr>
<tr>
<td>Dichroic Mirror</td>
<td>(DM1) Z405RDC</td>
<td>(DM1) Chroma</td>
</tr>
<tr>
<td></td>
<td>(DM2) Di01-R405/488/561/635</td>
<td>(DM2) Semrock</td>
</tr>
<tr>
<td></td>
<td>(DM3) FF580-FD101</td>
<td>(DM3) Semrock</td>
</tr>
<tr>
<td>Electron Multiplying CCD</td>
<td>Andor iXon+ DU897</td>
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</tr>
<tr>
<td>Filter</td>
<td>(F1 - 1) 405 Notch Filter</td>
<td>Semrock</td>
</tr>
<tr>
<td></td>
<td>(F1 - 2) 561 Notch Filter x2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F2) 585/40 Band Pass Filter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F3) 630/92 Band Pass Filter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F4) 476/10 Emission Filter</td>
<td></td>
</tr>
<tr>
<td>Immersion Oil</td>
<td>Immersion 518F oil; 12-624-66A</td>
<td>Zeiss</td>
</tr>
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<td>Lens</td>
<td>(L1) 250-millimeter focal length</td>
<td>ThorLabs</td>
</tr>
<tr>
<td></td>
<td>(L2) 200-millimeter focal length</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(L3) 400-millimeter focal length</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(L4) 300-millimeter focal length</td>
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</tr>
<tr>
<td>Mercury Lamp</td>
<td>U-RFL-T</td>
<td>Olympus</td>
</tr>
<tr>
<td>Microscope</td>
<td>Olympus IX71</td>
<td>Olympus</td>
</tr>
<tr>
<td>Neutral Density Filter</td>
<td></td>
<td>ThorLabs</td>
</tr>
<tr>
<td>Objective Lens</td>
<td>PlanApo, 60X oil, NA = 1.45, infinity corrected</td>
<td>Olympus</td>
</tr>
<tr>
<td>Readout Laser</td>
<td>558 nanometers, total power: ~100mW</td>
<td>CrystaLaser</td>
</tr>
<tr>
<td>Shutter</td>
<td></td>
<td>Thor Labs</td>
</tr>
<tr>
<td>Tube Lens</td>
<td>180-millimeter focal length</td>
<td>Olympus</td>
</tr>
</tbody>
</table>

**Table 2.3:** Optical Instruments and Manufacturer Details. A list of all optical instruments used in a FPALM set-up, their part details, and manufacturers. Multiple instruments are labeled according to their appearance in figure 2.1.
To image just above the coverslip of the sample, an approximately 100-200 nm region above the top of the coverslip, an alternative illumination technique (figure 1.5B) was conducted, referred to as total internal reflection microscopy (TIRFm) (Stout & Axelrod, 1989). Alignment only differs from a typical widefield illumination set-up in that the translation stage was shifted until laser light was focused off-center in the back aperture, causing the beam to be emitted at an angle from the objective which is totally internally reflected at the upper surface of the glass coverslip (figure 1.5B) resulting in no laser “spill” out of the sample dish and a nearly equally bright laser spot exiting the back aperture of the microscope.

The detection path, e.g. all optics located after the microscope and up to the electron multiplying charge-coupled device (EMCCD) camera (Andor), for a single species experiment (figure 2.1A) was aligned with an adjustable aperture (ThorLabs), AP2, placed at FP2 to restrict fluorescence from a region of interest at S. A telescope, with magnification 2x, was placed one focal length away from FP2. The EMCCD camera was placed at a second conjugate plane to S and FP2, with total magnification 120 times that of S, located at the focal plane of the second telescope lens, L3 (ThorLabs).

The detection path for a two species experiment has the same alignment as a single species experiment with the exception that light passing through L3 (ThorLabs) was split into two channels. Both channels converge at the EMCCD camera (figure 2.1B) at the second conjugate plane to S and FP2. Filters, F2 and F3 (Semrock), must be perpendicular to the channel (light propagation direction) to reduce channel distortion and to be filtering at their optimal wavelength.

### 2.2.2 Imaging Procedures

Once the proper FPALM alignment was conducted (section 2.2.1), a drop of immersion oil (Zeiss; 12-624-66A) was added to the objective (OBJ), and a sample was placed on the microscope stage, with the sample region of interest roughly centered on the OBJ. Samples were prepared as stated previously in sections 2.1.4 or 2.1.5. Samples should sit approximately 5-10 minutes on the stage before acquisition to reduce temperature dependent z-direction drift. Using mercury lamp (Olympus) light filtered by F4
(see figure 2.1) a transfected cell was found through the microscope eyepiece. A cell that was sitting flat on the glass coverslip, with few neighboring cells, and glowing a faint-moderate green (representing successful transfection of plasmid) was considered optimal for imaging.

2.2.3 Imaging Acquisition

After selecting a cell, fluorescence from single molecules, in the form of point spread functions (PSFs), were acquired using the Andor Solis software for the Andor iXon+ EMCCD camera (see detection path alignment from section 2.2.1). The camera chip was binned to readout only the region of interest (the section of the chip which will primarily collect fluorescence from the sample, S) and the frame rate was adjusted to reach ~60Hz (i.e. 60 frames per second). In some replicates, to reach this optimal frame rate the shift speed of the camera (the speed of the shift register of the camera) was decreased. All other Andor software settings remained the same between replicates, i.e. the electron multiplying (EM) gain was set to 200, cooling temperature was set at -80°C, the camera readout rate was set to 10MHz, etc. All frame rates and shift speed adjustments are recorded in table 2.4. Cells were imaged for 10,000 frames and saved in an uncompressed TIFF format to later be analyzed using MATLAB software.

2.2.4 Acquisition of Configuration Images

Readout and activation lasers were controlled manually by neutral density filter wheels, NDs (figure 2.1). Laser powers were measured using a power meter (ThorLabs) and approximate intensity values were ~15mW for the 558-nanometer laser and ~1.0-100µW for the 405-nanometer laser. Laser beam profiles were imaged using autofluorescent slides (Chroma) in widefield illumination (figure 2.2A and figure2.2B).

A bead sample was imaged with fluorescence split into two channels in the detection path (see Gunewardene et al., 2011, and figure 2.1 for details; section 2.1.1 for alignment specifics). A bead sample was illuminated with the readout laser to emit fluorescence and approximately 100 frames were acquired with the EMCCD camera using Andor Solis software. The bead sample was imaged after the camera chip
was binned to properly correlate the two channels (see section 2.2.3 for details regarding camera settings and figure 2.2D for a single example of a bead sample image).

In addition to a bead sample, an image of a scale with defined distance markings was required for proper configuration of acquired data (Figure 2.2C).

<p>| ACQUISITION INFORMATION FOR 2-SPECIES IMAGING OF PH DOMAIN-PAMKATE AND DENDRA2-HA |
|-------------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>REPPLICATE NUMBER</th>
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<th>SHIFT SPEED</th>
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<td>3.3 μs</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>4</td>
<td>01/15/2020</td>
<td>59.63 Hz</td>
<td>1.7 μs</td>
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**Table 2.4:** Acquisition Information for 2-Species Imaging of PH Domain-PAmKate and Dendra2-Hemagglutinin. List of camera settings for 2-color imaging of PH Domain-PAmKate and Dendra2-HA in living cells.
Figure 2.2: Examples of Configuration Images. Light is split by a dichroic mirror (FF580-FDi01, Semrock) at 595 nanometer light in the detection path of the FPALM set-up, see figure 2.1, resulting in two channels at the EMCCD camera chip. The left channel is identified as the red channel (primary acquisition of PAmKate) and the right channel is identified as the green channel (primary acquisition of Dendra2, technically wavelengths that would be seen as orange). (A) A beam profile image of the activation laser (405 nanometers, CrystaLaser) taken in widefield illumination. (B) A beam profile image of the readout laser (558 nanometers, CrystaLaser) taken in widefield illumination. (C) An example of a scale image with a width (marked in white) of 20 micrometers taken in widefield illumination. (D) An example of a bead sample image taken in TIRF illumination. See section 2.2.4 for configuration acquisition procedures.
2.3 Post-Processing Analysis

All post-processing analysis was done using MATLAB, a programming language and IDE developed by MathWorks. Any novel codes written, or co-written, by the author are found in the appendix of this work.

2.3.1 Localization

After the acquisition of data (typically saved as an uncompressed TIFF stack), localizations of point spread functions (PSFs) were obtained. The method used for localization of PSFs is consistent between one species and two species data, with a few exceptions detailed here.

To begin, a conversion factor, q, was obtained from the scale image which designates a pixel to micron conversion factor for the camera (q is equal to the distance at the sample occupied by a single pixel within the image). This was done manually using Fiji (Fiji is just ImageJ) software (Schindelin et al., 2012). If data contains two or more species, a correlation matrix was obtained by analyzing a bead sample TIFF stack using “correlate2color.m”, which uses image registration to correlate two manually selected channels (green and red). The resulting correlation matrix is an affine transformation matrix containing translation, scale, shear, and rotation, which maps the green channel to the red channel. A correlation file was saved, “corr_file.m”, containing both the correlation matrix and the pre-selected coordinates of the two channels.

Analysis of TIFF stacks for localizations was conducted using “M_Batch_Adv_1Cv2.m” for one species data and “M_Batch_Adv_2C2Tv2.m” for two or more species data. Simply, each code is a wrapper which contains the background subtraction, thresholding, localization mapping, concatenation, and tolerancing analysis needed to output an analyzed mat file for each cell. Background subtraction was done using a temporal median filtering method (TMF) (Hoogendoorn et al., 2014), where a defined frame window (~100 frames) was centered on a selected frame. The median photon value per pixel was found from all frames within the frame window and then subtracted from the selected frame. The frame
window was then shifted by one frame within the stack (i.e. by one frame in time) and repeated until all frames have been background subtracted successfully. In order to identify localizations, a threshold limit was chosen in which possible PSFs were selected with photon values greater than the threshold limit. In two or more species data, a threshold value was selected for each channel independently. Localizations are then “grabbed” by extracting a seven by seven box centered on a pixel with a photon value above the threshold limit. A Gaussian approximation of the PSF was fitted to each “grabbed” box using a non-linear fitting algorithm. Fitted parameters outputted from the algorithm are the x and y coordinates, the Gaussian magnitude, the Gaussian radius, an offset factor and their associated errors. The fitted Gaussian is given by

\[ I(x, y) = I_o e^{-\frac{[(x-x_o)^2+(y-y_o)^2]}{\sigma^2}} + C \]

where \( I(x, y) \) represents the pixel intensity at any given x, y coordinate, \( I_o \) represents the peak intensity of the Gaussian, \( x_o \) and \( y_o \) represent the center coordinates of the Gaussian, \( \sigma \) represents the radius of the Gaussian, and \( C \) represents an offset from zero. If uncompressed TIFF stacks acquired for each cell were split into two files due to file size limits, concatenation was done to combine all data from split files into a single file. Localized data then underwent tolerancing, which is the process of removing poorly fitted PSFs. Toleranced data includes only PSFs that fall within user-defined ranges for the fit parameters of a PSF (which must be chosen carefully to ensure the poor fits are excluded, while maximizing the number of PSFs with good fits that are included). A visual representation of the localization method explained above is found in figure 2.3.

2.3.2 Species Separation

For two or more species data, an alpha ratio was calculated for each localized PSF. This ratio, \( \alpha \), is the intensity ratio composed of pixel intensity sums from the grabs from each of the two channels for a given PSF written as
Figure 2.3: FPALM Concept Diagram. This visual representation for the concept of FPALM uses a known image shown in A. A random subset of molecules was activated and readout in each frame. These molecules are then localized and recorded. The localized point spread functions (PSFs) were simulated as a Gaussian with a radius of 50 nanometers. A) The original image of a cartoon elephant as seen by the simulation. B) A diffraction limited version of the simulated image. C) An example of a FPALM rendered image of the simulated image at frame 1000 with n=5553 molecules. D-F) The left column is a recording of localized PSFs in raw frames 1 (D), 50 (E), and 500 (F) respectively with green boxes around individual molecules which can be resolved and red boxes around those that are within the Rayleigh criteria which are therefore not resolvable; multiemitter algorithms must be used to analyze such PSFs (F. Huang et al., 2011). The right column shows the high-resolution render of the simulated image at that respective frame and the number of molecules localized from all previous frames. This simulation was run using “FPALMConceptSim.m” courtesy of Dr. Matthew T. Parent.
Equation 2.2
\[ \alpha = \frac{I_R}{I_R + I_G} \]

where \( I_R \) represents the intensity in the red channel of a given PSF, and \( I_G \) represents the intensity in the green channel of the same given PSF (Gunwardene et al., 2011). Within each cell, alpha ratios for tolerated data were visually graphed using a histogram format using the MATLAB code “nrat_plot_batch_v2.m”. This graph will hopefully contain two peaks, the positions of which are used for separation of the two species, and to minimize bleed-through between channels (Kim et al., 2013). Based on those peaks, \( \alpha \) ranges for each species were chosen and used for further analysis. Alpha ranges for each species varied slightly from cell to cell, however, were typically 0.0 to 0.60 for green channel (Dendra2) and 0.61 to 1.0 for the red channel (PAmKate).

2.3.3 Bleed-Through Correction

Two-color super-resolution imaging can be subject to bleed-through of the two species (Kim et al., 2013). This bleed-through occurs when a species’ spectra overlap into another channel resulting in misidentification of localizations. This misidentification can be corrected for by using a bleed-through correction method. Two-color data was binned into grids with density grid pixel width of 80 nanometers by their predetermined \( \alpha \) ratios (section 2.3.2). The two species were labeled A and B for simplicity. The uncorrected grid values, \( n_A^{\text{meas}} \) and \( n_B^{\text{meas}} \), are represented by equations 2.3 and 2.4,

\[ n_A^{\text{meas}} = n_A^{\text{corr}} - k_{AB} n_A^{\text{corr}} + k_{BA} n_B^{\text{corr}} \]  
Equation 2.3

\[ n_B^{\text{meas}} = n_B^{\text{corr}} - k_{BA} n_B^{\text{corr}} + k_{AB} n_A^{\text{corr}} \]  
Equation 2.4

which can then be solved to obtain the corrected grid values, \( n_A^{\text{corr}} \) and \( n_B^{\text{corr}} \), in equations 2.5 and 2.6,

\[ n_A^{\text{corr}} = \frac{n_A^{\text{meas}} - k_{BA} n_A^{\text{meas}} - k_{BA} n_B^{\text{meas}}}{1 - k_{AB} - k_{BA}} \]  
Equation 2.5
\[ n_{B}^{corr} = \frac{n_{B}^{meas} - k_{AB}n_{A}^{meas} - k_{BA}n_{B}^{meas}}{1 - k_{AB} - k_{BA}} \]

Equation 2.6

where \( k_{AB} \) represents the bleed-through rate of species A into the channel of species B and \( k_{BA} \) represents the bleed-through rate of species B into the channel of species A (Kim et al., 2013). These bleed-through rates were found using the MATLAB codes “bleedthrustimate.m”. One color samples of each species were imaged and analyzed in the same manner as their two-color counterparts (using the same FPALM two-color set-up and on the same day of acquisition). Using the \( \alpha \) ratios previously identified (section 2.3.2), the rate of bleed-through for each species can be determined by dividing the number of molecules misidentified (molecules with \( \alpha \) ratios outside the accepted limits) into the total number of localized molecules for a given species.

### 2.3.4 Correlation Coefficients

In order to quantify the overlap of two species (the relative frequency of two species being found in the same pixel together), two specific correlation coefficients were determined, Pearson’s correlation coefficient (Pearson, 1901) and Manders’ co-localization coefficients (Manders et al., 1992). Data with two (or more) species were split by alpha ranges corresponding to the peak of that species within the alpha histogram (section 2.3.2). All two-color localization data was binned into a pixel grid with pixels that had a side length of prescribed size (density grid pixel width), and whose pixel value (intensity) was equal to the number of localizations contained within the area of each given pixel. Images were then masked manually to highlight only the illuminated area which contained a cell. The masked areas were then used to calculate both coefficients. The coefficients testing was done using the MATLAB code, “Manders_Pearson_Batch_MP.m”.

#### 2.3.4.1 Pearson’s Correlation Coefficient

Pearson’s correlation coefficient (PCC) is a standard marker for describing the correlation between two patterns, in this case the correlation between two species’ spatial distributions inside a cell.
This coefficient measures the similarity between two distributions with no dependence on the average intensity of the two channels. The PCC, \( r \), for an image is given by

\[
r = \frac{\sum_i (R_i - \bar{R})(G_i - \bar{G})}{\sqrt{\sum_i (R_i - \bar{R})^2 \sum_i (G_i - \bar{G})^2}}
\]

where, \( R_i \) represents the red intensity for a pixel \( i \), \( \bar{R} \) represents the average intensity of all masked pixels in the red channel, \( G_i \) represents the green intensity for a pixel \( i \), and \( \bar{G} \) represents the average intensity of all masked pixels in the green channel (Pearson, 1901). A PCC value can fall between the range -1 and 1, where -1 is an anti-correlation of the two channels and 1 is a perfect correlation of the two channels.

### 2.3.4.2 Manders’ Co-localization Coefficients

To measure the degree of colocalization of two species, Manders’ co-localization coefficients (MCC) were calculated to measure the degree to which one species was located with a second. These coefficients circumvent the drawbacks of the PCC when the fluorescence of the two species differs greatly, since the MCC is not dependent on products of fluctuations from the mean. Two coefficients are defined, \( M_G \) for the green channel (species with greener fluorescence) and \( M_R \) for the red channel (species with redder fluorescence). The MCC in the green channel, \( M_G \), for an image is given by

\[
M_G = \frac{\sum_i G_{i,\text{coloc}}}{\sum_i G_i}
\]

where \( G_{i,\text{coloc}} \) represents the green intensity in pixel \( i \) with a non-zero red intensity and \( G_i \) represents the green intensity in pixel \( i \) (Manders et al., 1992). While the MCC in the red channel, \( M_R \), for an image is given by

\[
M_R = \frac{\sum_i R_{i,\text{coloc}}}{\sum_i R_i}
\]

where \( R_{i,\text{coloc}} \) represents the red intensity in pixel \( i \) with a non-zero green intensity and \( R_i \) represents the red intensity in pixel \( i \) (Manders et al., 1992). An MCC value can range from 0 to 1, where 0 signifies no colocalization of the two channels and 1 signifies perfect colocalization of the two channels.
2.3.5 Dynamics

2.3.5.1 Trajectory Analysis

In order to properly measure the dynamic properties of a species, molecular trajectories were determined using a single-molecule tracking algorithm (Manley et al., 2008; Schütz et al., 1997). Batch trajectory analysis of one species data was conducted using “SM_ID_dist_batch_auto_TG1_ajn3.m”. Data with two (or more) species were split by alpha ranges corresponding to that species peak (section 2.3.2) and trajectory analysis was then conducted on each species separately (this process is identical to that of the one species analysis) then combined into a single MATLAB file using a batch code named “Two_Color_Trajectories_Batch.m”. To find the trajectories of localized molecules, a circle of prescribed radius \( r_1 \) equal to the expected maximum distance a molecule could travel between two camera frames \( \bar{x}_{HA} = 150 \text{nm}; \bar{x}_{PH \text{ domain}} = 300 \text{nm} \) was drawn around all molecules in a select frame \( f_i \). Only a single molecule can be found inside \( r_1 \) within the same frame \( f_i \) for that molecule to be considered by the algorithm; this check is used to prevent confusion between molecules and to avoid overcounting. The same circle \( r_1 \) with identical coordinates was then drawn in the following frame \( f_{i+1} \). If a single localized molecule was again found within the same circle, the algorithm records the x-y coordinates of both localizations (from \( f_i \) and \( f_{i+1} \)) and connects them as part of a trajectory, then repeats this process for all remaining molecules and frames in the data set. In addition to the aforementioned check, two additional checks were made to prevent misidentification of molecules. Firstly, a second, larger circle with twice the radius length \( r_2 = 2r_1 \) was drawn in addition to the initial circle \( r_1 \) in both frames \( f_i; f_{i+1} \). If a molecule was found in the second radius \( r_2 \) drawn in the initial frame \( f_i \), neither molecule was considered by the algorithm as it is difficult to distinguish them in the following frame \( f_{i+1} \). Secondly, there can be no other molecule within \( r_2 \) of the selected molecules position in the second frame \( f_{i+1} \). All trajectory lengths (the number of molecules in each trajectory) and their corresponding
molecular positions were recorded. A visual representation of the trajectory analysis can be seen in figure 2.4.

2.3.5.2 Mean Squared Displacement

Capturing how molecules move over differing time frames requires the calculation of the mean squared displacement (MSD) between molecules. The MSD between two localizations of the same molecule, such as in the $i^{th}$ and $(i + 1)^{th}$ frames within a selected trajectory, was found according to

$$MSD = \bar{x}^2 = (x - x_o)^2 + (y - y_o)^2$$  \hspace{2cm} \text{Equation 2.10}$$

where $x_o$ and $y_o$ represent the coordinates for the $i^{th}$ localization and $x$ and $y$ represent the coordinates for the $(i + 1)^{th}$ localization. All MSDs for this step size ($t_{frame}$) were averaged. The MSD was then found between the next localization in the trajectory length $(i + 2)^{th}$ and the initial localization ($i^{th}$). All MSDs for this new step size ($2t_{frame}$) were averaged. The process was repeated until there were no more localizations (time steps) within a trajectory length. All MSD vs time values were averaged by cell and graphed. This analysis was conducted using the MATLAB code “einstein.m”.

2.3.5.3 Molecular Mobility

Determination of molecular mobility as a function of the local localization density requires the calculation of molecular mobilities ($\mu$) within a subregion of the cell. The mobility of a molecule ($\mu$) is the molecule’s mean squared displacement (MSD) divided by the time elapsed between position measurements ($t_{frame}$).

$$\mu = \frac{MSD}{t_{frame}}$$  \hspace{2cm} \text{Equation 2.11}$$

The location of the $i^{th}$ molecule used to calculate the MSD was calculated within a grid with pixel width of 80 nanometers. A second grid, the density plot, was created with the same dimensions and pixel width,
Figure 2.4: Trajectory Analysis Concept Diagram. Concept for trajectory analysis of FPALM localization data in live cells. Molecules fluorescing for multiple frames can be tracked using a single-molecule tracking algorithm (Manley et al., 2008; Schütz et al., 1997) as long as the density of molecules in each frame is low. To visually represent this tracking method, a multitude of molecules were simulated with random positions and movements. Rows A and B show a single molecule that was simulated in three frames (frames 1-3) with random movement. Row A) The first of the three frames, labeled frame 1 and frame 2 in the diagram, with a simulated molecule including the trajectory path it followed. Row B) The second and third frames, labeled frame 2 and frame 3 in the diagram, records the second step in the molecule’s trajectory. The molecule’s full trajectory path is shown in the final panel of row B. Row C) Five molecules were randomly simulated in three frames (frames 1-3) and their trajectory paths are traced in the final panel. This simulation was run using “FPALMTrajSim_v3.m” courtesy of Dr. Matthew T. Parent.
within which the numbers of localizations within each pixel were calculated. All localized molecules in the cell were binned into the density plot by increasing each pixel value by one if a molecule would fall within that region. All molecular mobilities corresponding to a location in the MSD grid were then spatially overlaid on the density plot. Mobilities were then separated by density values and averaged together for a given cell. All molecular mobility vs density values were averaged by cell and graphed. This analysis was conducted using the MATLAB code “color1_MobilityvsDensity_plotter_v2.m”.

2.3.5.4 Molecular and Lateral Flux

To study the flux properties of two species as a function of time, the molecular flux and lateral flux of both species was calculated using the code named “HA_PIP2_changes_traj_stats_wallace_v42.m”. To begin, two frame stacks named “pre” and “post” were selected, each with an equal length of time, \( \tau \). Time \( \tau \) cannot be larger than half the time needed to acquire a 10,000-frame stack. These stacks must be consecutive in nature, leaving no gaps between groups of frames. Data was then separated into species (red and green data) by their predetermined alpha values (section 2.3.2). Red data was binned into grids for both “pre” and “post” stacks with density grid pixel width of 80 nanometers. Each pixel represents the number of red localizations found in that region of interest for the selected section of frames. The “post” grid for the red data was then subtracted from the “pre” grid resulting in values which are potentially positive, negative, or zero; the sign of the resulting value is then either +1, -1, or 0. A value of +1 represents an increase in localizations between the “pre” and “post” grids, a value of -1 represents a decrease in localizations, and 0 represents no change between the two grids where each flux is within a predetermined threshold (a difference of 3 localizations is required for an increase or decrease to occur). This process was then repeated for the green data resulting in two change grids (one for each colored species). The red and green grids were then compared (each pixel region has two values, one for the change in red and one for the change in green) to record the frequency of the nine distinct conditions (all permutations of red and green changes: +G+R, +G-R, +G=R, etc.) and normalized by the total frequency
of events found between the two grids. The nine normalized distinct conditions are referred to as “molecular flux” in this text. Note that the “green” and “red” channels typically correspond in this work to Dendra2 and PAmKate, respectively.

To then study the lateral flux (net lateral motion) of a species (i.e. red or green) in regions associated with the nine normalized conditions (molecular flux), a grid of the same size was created. Pixels in this grid contain information regarding the net lateral flux of the species (for a given species, defined as the number of trajectory steps crossing into a given pixel minus the number of trajectory steps crossing out of that pixel). A pixel has either a net increase due to lateral movement (+1), a net decrease due to lateral movement (-1), or no net lateral flux (0) within a predetermined threshold (a minimum of two trajectory steps inward or outward are required to count toward a net change). The regions (pixels) with lateral flux (red or green) were then compared to the regions where molecules were changing in number (“molecular flux regions”) where each pixel region in the lateral flux grid was mapped directly to each pixel in the molecular flux grid. This comparison was used to record the frequencies of twenty-seven distinct conditions (all permutations of net lateral flux (3 total) combined with the observed molecular flux (9 total)) and then were normalized by the total frequency of events. This was done separately for red and green data, producing information about the lateral movement of each species in regions where both species were found (as stated above, grid pixels were required to contain at least 3 localizations for each species to be considered for analysis).

We then determined that certain kinds of events (for example, a combination of a net lateral motion of one species combined with an overall change in both species) would be expected to result from the occurrence of certain models of phosphoinositide membrane organization (Hammond, 2016; figure 1.1). For each model being considered, a state vector describing the changes in each species’ molecular flux and the lateral flux was defined. To calculate the observed frequency of a given type of event (model) occurring, the molecular flux, red lateral net flux, and green lateral net flux grids (each grid has identical
density grid pixel widths) were searched for regions matching the state vector for a given model, and the number of occurrences were recorded. These frequencies were then normalized by the total number of events for all five models.

“Pre” and “post” frames were both shifted by τ (if a frame stack equivalent to a time τ was available) and this process was repeated (finding molecular flux, lateral net flux, frequency of models, and model rates) to allow as much of the full dataset to be used as possible. All shifted values were averaged for a given cell and then all stored variables were averaged together by cell.

2.3.6 Density-Based Clustering

To accurately identify clusters from localization data, density-based clustering analysis was conducted using the MATLAB code “grid_plot_greenvsred_clusterID_Batch_v18.m” and further plotting analysis was conducted using “plot_HA_density_vs_X_dataall_v6.m”. Localization data with two or more species were split by alpha ranges corresponding to that species peak (section 2.3.2) and binned into grids with prescribed density grid pixel width (~10nm), one for each species’ corresponding channel (red or green). Starting with a grid containing green data, green localizations were individually convolved with a circle with a radius approximately equal to localization precision (~30-50nm). A mask was then drawn along the outermost convolved green localizations to map the outside of the sampled cell and to calculate the cell area by adding all pixel areas found within the mask of green localizations. Using this mask, the green density within each cell (the average density of green molecules per unit area) was then determined by dividing the number of localized molecules found within the mask by the cell area. To accurately identify clusters, the convolved green localization grid was then thresholded by 3-4 times the average green density for that cell. All pixels found above that threshold remain for green cluster identification and were saved in a grid of equal size. A MATLAB function “bwconncomp” was used to identify green clusters from the thresholded grid by finding all “linked” (i.e. contiguous) pixels. Green cluster density and areas were then calculated using a MATLAB function “regionprops”. The process was then repeated
for the red species. All density values were normalized by the average density in the given cell and denoted as “relative to average” in this text.

Using the grid where identified green clusters were defined, red localizations were overlaid to find the density of red localizations found within a green cluster and vice versa. Low and high concentrations of the red species within green clusters was identified according to the average red density for the cell where “low concentrations” were defined as less than the red average density (between zero and 1 times) and high concentrations were greater than three times the red average density (between 3 times and the largest concentration of red). Green cluster densities were then plotted against green cluster areas according to high or low concentrations of red localizations.

2.3.7 Image Rendering

Localized molecules for each species were rendered using “DeltaGauss2colorBatch.m”. Localizations were then plotted using intensity-weighted gaussians of prescribed size ($\sigma = 20\, nm$) for all molecules. Molecules localized in the “green channel” were rendered in green and molecules localized in the “red channel” were rendered in magenta. Overlap in molecules, signifying colocalization, was represented in light grey or white. A scale bar of one micron was also included.
CHAPTER 3

RESULTS

3.1 Preface

All significance testing was done using GraphPad Prism 8.3.1 software. Significance p-value cut-offs were indicated as follows, with increasing significance, $p \geq 0.05$ (ns = not significant), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). The p-value specifies the probability that the test cannot reject the null hypothesis, that the two groups come from the same underlying distribution. Exact p-values can be found in the appendix. All nonlinear fits (other than localization) were done using Microcal™ Origin 6.0 software. Parameter errors denote the 95% confidence interval and were computed using the standard deviation of the experimental data.

3.2 Actin Binding Proteins’ Association with Influenza Hemagglutinin Clusters in Fixed Cells

Multiple experiments were conducted to measure the association of actin binding proteins (ABPs), α-Actinin-PAmCherry, Cofilin 1-PAmCherry, Myosin 1-PAmCherry, and Tropomyosin 4-PAmCherry, with Hemagglutinin-Dendra2 (HA-Dendra2) in fixed NIH3T3 cells. Correlation coefficient analysis of these ABPs-PAmCherry with HA-Dendra2 (figure 3.1B, table 3.1) shows a positive correlation using Pearson’s correlation coefficient (PCC) (Pearson, 1901). PCC values can range from -1 to 1 where a negative coefficient is an anti-correlation between two species and the more positive the PCC is, the more spatially correlated. Among the four ABPs, Tropomyosin 4-PAmCherry has the highest correlation with HA-Dendra2 (figure 3.1B). The ABPs and HA also show a high colocalization using Manders’ co-localization coefficient (MCC) (Manders et al., 1992). MCC values can range from 0 to 1, where values represent the fraction of one species found with the other. All four MCC values for $M_G$ (the fraction of HA-Dendra2 with...
Figure 3.1: Correlation Coefficients for Actin Binding Proteins with Hemagglutinin. Actin binding proteins (ABPs), α-actinin (blue), coflin 1 (purple), myosin 1 (green), and tropomyosin 4 (yellow), are colocalized with hemagglutinin (HA) at the plasma membrane, with TM4 holding the strongest correlation. All four ABPs are fluorescently labeled with PAmCherry and imaged in fixed NIH3T3 cells along with HA-Dendra2 using the widefield illumination method. Number of cells for each ABP is as follows: α-actinin, n=37 cells; coflin 1, n=28 cells; myosin 1, n=13 cells; and tropomyosin 4, n=8 cells. All coefficients testing was conducted with density grid pixel width of 80-nanometers and are bleed-through corrected (section 2.3.3). A) Manders’ co-localization coefficients (MCC) (Manders et al., 1992) of all four ABPs-PAmCherry with HA-Dendra2 was conducted and plotted with $M_G$ against $M_R$ where the green channel represents HA-Dendra2 and the red channel represents an ABP attached to PAmCherry. B) A bar chart showing Pearson’s correlation coefficient (PCC) (Pearson, 1901) of all four ABPs-PAmCherry with HA-Dendra2. Error bars represent standard error of the mean.
Table 3.1: Correlation Coefficients of Actin Binding Proteins with Hemagglutinin. Calculated values for Pearson’s correlation coefficient (PCC) and Manders’ co-localization coefficients (MCC) between Hemagglutinin-Dendra2 and four actin binding proteins (ABPs), α-actinin, cofilin 1, myosin 1, and tropomyosin 4, fluorescently labeled with PAmCherry. PCC values are represented by $r$ and MCC values are represented by $M_G$ and $M_R$ which corresponds to the green and red channel respectively. Error is standard error of the mean.

<table>
<thead>
<tr>
<th>ACTIN BINDING PROTEINS</th>
<th>NUMBER OF REPlicates</th>
<th>NUMBER OF CELLS</th>
<th>PEARSON’S CORRELATION COEFFICIENT</th>
<th>MANDERS’ CO-LOCALIZATION COEFFICIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ACTININ</td>
<td>2</td>
<td>37</td>
<td>$0.100 \pm 0.003$</td>
<td>$0.6950 \pm 0.0056$ $0.6390 \pm 0.0045$</td>
</tr>
<tr>
<td>COFILIN 1</td>
<td>3</td>
<td>28</td>
<td>$0.1094 \pm 0.0049$</td>
<td>$0.67 \pm 0.01$ $0.54 \pm 0.01$</td>
</tr>
<tr>
<td>MYOSIN 1</td>
<td>1</td>
<td>13</td>
<td>$0.16 \pm 0.01$</td>
<td>$0.602 \pm 0.009$ $0.692 \pm 0.016$</td>
</tr>
<tr>
<td>TROPOMYOSIN 4</td>
<td>1</td>
<td>8</td>
<td>$0.196 \pm 0.013$</td>
<td>$0.759 \pm 0.024$ $0.797 \pm 0.014$</td>
</tr>
</tbody>
</table>

To better understand how hemagglutinin (HA) cluster properties may change when colocalized with high concentrations of an actin binding protein, HA-Dendra2 clusters were identified with four ABPs, α-Actinin-PAmCherry, Cofilin 1-PAmCherry, Myosin 1-PAmCherry, and Tropomyosin 4-PAmCherry. HA-Dendra2 cluster densities were plotted versus HA-Dendra2 cluster areas (figure 3.2) for low and high concentrations for each ABP-PAmCherry (see section 2.3.6 for more details on analysis). The distribution of clusters varies for all eight scenarios presented in figure 3.2. HA-Dendra2 average cluster areas increased significantly (****, p<0.0001) between low and high concentrations of ABPs-PAmCherry, with
Figure 3.2: Mean Density and Area of Hemagglutinin Clusters with Low/High Actin Binding Proteins. HA cluster density and area change when in the presence of high concentrations of actin binding proteins, α-actinin (blue), coflin 1 (purple), myosin 1 (green), and tropomyosin 4 (yellow). All four ABPs are fluorescently labeled with PAmCherry and imaged in fixed NIH3T3 cells along with HA-Dendra2 using the widefield illumination method. Data was bleed-through corrected (section 2.3.3). Number of cells for each ABP is as follows: α-actinin, n=37 cells; coflin 1, n=28 cells; myosin 1, n=13 cells; and tropomyosin 4, n=8 cells. Cluster analysis was conducted using a density grid pixel width of 10-nanometers. HA-Dendra2 clusters were distributed between low (left-most column) and high (right-most column) concentrations of A) α-Actinin-PAmCherry, B) Cofilin 1-PAmCherry, C) Myosin 1-PAmCherry, and D) Tropomyosin 4-PAmCherry. Error bars represent standard error of the mean.
### Table 3.2: Mean Density and Area Values of Hemagglutinin Clusters with Low/High Actin Binding Proteins

Calculated values for mean density and mean area of Hemagglutinin-Dendra2 clusters with low and high concentrations of four actin binding proteins (ABPs), α-actinin, cofilin 1, myosin 1, and tropomyosin 4, fluorescently labeled with PAmCherry. Number of clusters used for analysis is also included. A Kolmogorov-Smirnov test was found between low and high concentrations for each ABP’s density and area. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), and p<0.0001 (****). Significance testing was generated using GraphPad Prism 8.3.1. software. Error is standard error of the mean.

<table>
<thead>
<tr>
<th>Actin Binding Proteins</th>
<th>Concentration of Actin Binding Proteins</th>
<th>Number of Clusters</th>
<th>Mean Density (Relative to Average)</th>
<th>Mean Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Actinin</td>
<td>low</td>
<td>464</td>
<td>5.6865 ± 0.0026</td>
<td>0.0381 ± 0.00013</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>649</td>
<td>7.362 ± 0.005</td>
<td>0.0925 ± 0.0024</td>
</tr>
<tr>
<td><strong>Significance Between Concentrations</strong></td>
<td>****</td>
<td>****</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cofilin 1</td>
<td>low</td>
<td>174</td>
<td>6.873 ± 0.016</td>
<td>0.0601 ± 0.0006</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>396</td>
<td>9.289 ± 0.014</td>
<td>0.1085 ± 0.0003</td>
</tr>
<tr>
<td><strong>Significance Between Concentrations</strong></td>
<td>****</td>
<td>****</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myosin 1</td>
<td>low</td>
<td>195</td>
<td>6.793 ± 0.007</td>
<td>0.0368 ± 0.0002</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>380</td>
<td>7.153 ± 0.007</td>
<td>0.1066 ± 0.0005</td>
</tr>
<tr>
<td><strong>Significance Between Concentrations</strong></td>
<td>ns</td>
<td>****</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropomyosin 4</td>
<td>low</td>
<td>84</td>
<td>6.433 ± 0.029</td>
<td>0.0184 ± 0.00017</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>404</td>
<td>6.3473 ± 0.0048</td>
<td>0.0478 ± 0.00014</td>
</tr>
<tr>
<td><strong>Significance Between Concentrations</strong></td>
<td>ns</td>
<td>****</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The largest increase occurring in Myosin 1-PAmCherry (Table 3.2). Higher concentrations of α-Actinin-PAmCherry and Cofilin 1-PAmCherry significantly (****, p<0.0001) increased the average density of HA-Dendra2 clusters with Cofilin 1-PAmCherry having the largest effect on HA-Dendra2 clusters. There were small changes in HA cluster densities in Myosin 1-PAmCherry and Tropomyosin 4-PAmCherry, but these changes were not significant using a Kolmogorov-Smirnov test.
3.3 Comparison of PIP2 and PH Domain with and without Influenza Hemagglutinin using Single Species Imaging of Living Cells

An analysis was conducted in NIH3T3 and NIH3T3-HAb2 (HAb2) cells to measure the mean squared displacement (MSD) of phosphatidylinositol 4,5-bisphosphate (PIP2) molecules fluorescently tagged with BODIPY TMR (section 2.1.7) inside and outside clusters (Curthoys et al., 2019). It was hypothesized that visualization of GloPIP molecules within hemagglutinin expressing cells (HAb2 cells) would exhibit altered confinement compared to molecules within non HA-expressing cells (NIH3T3 cells). Following the analysis methods of Curthoys et al. 2019, the mean squared displacement (MSD) of all trajectories inside and outside of clusters was calculated for NIH3T3 and HAb2 data (figure 3.3) and curve fitted to

\[ MSD = MSD_p \left(1 - e^{-\frac{t}{\tau}}\right) \]  \hspace{1cm} \text{Equation 3.1}

where \( MSD_p \) represents the plateau MSD in \( \mu m^2 \), \( t \) represents time in seconds, and \( \tau \) represents the time constant in seconds. All points in the HAb2 curves are found below their NIH3T3 counterparts for inside and outside clusters, i.e. the red curve is always below the blue curve and the orange curve is always below the green curve (figure 3.3). GloPIP molecules found inside clusters had MSDs much lower than outside clusters (figure 3.3B). Inside clusters, the plateau MSD value for the NIH3T3 curve was reported as \( MSD_{p,NIH3T3} = (0.030 \pm 0.002) \mu m^2 \) and \( MSD_{p,HAb2} = (0.0213 \pm 0.0013) \mu m^2 \) was reported for the HAb2 curve (Curthoys et al., 2019). Assuming two-dimensional diffusion, equation 3.1 can be simplified to find the diffusion coefficient, \( D \), given by

\[ D = \frac{MSD_p}{4\tau} \]  \hspace{1cm} \text{Equation 3.2}

where \( MSD_p \) represents the plateau MSD in \( \mu m^2 \), and \( \tau \) represents the time constant in seconds. Using
Figure 3.3: Mean Squared Displacement Curves of GloPIP. Enhanced confinement of BODIPY TMR-phosphatidylinositol 4,5-bisphosphate (GloPIP) molecules inside clusters in NIH3T3-HAb2 (HAb2) cells. The mean squared displacement (MSD) of all trajectories inside and outside of clusters was calculated for each cell, n=22 cells for HAb2 and n=25 cells for NIH3T3. Error bars represent the standard error of the mean (SEM). (A) A view of all MSD curves where blue represents GloPIP trajectories outside clusters in NIH3T3 cells and red represents trajectories outside clusters in HAb2 cells. All data was fit to \( MSD = MSD_p \left( 1 - e^{-t/\tau} \right) \) where \( MSD_p \) represents the plateau value for the curve and \( \tau \) represents the time constant. (B) An enlarged view of the MSDs for trajectories inside clusters where green represents NIH3T3 cells and orange represents HAb2 cells. (This figure was adapted from the manuscript, Curthoys et al., 2019).
equation 3.2, diffusion rates outside of clusters were calculated, \( D_{HAb2, outside} = (1.76 \pm 0.09) \frac{\mu m^2}{s} \) and \( D_{NIH3T3, outside} = (2.70 \pm 0.37) \frac{\mu m^2}{s} \), and diffusion rates inside of clusters were calculated, \( D_{HAb2, inside} = (0.61 \pm 0.04) \frac{\mu m^2}{s} \) and \( D_{NIH3T3, inside} = (0.61 \pm 0.22) \frac{\mu m^2}{s} \) (Curthoys et al., 2019).

Significance differences were observed comparing the diffusion of GloPIP2 molecules inside and outside of clusters for both cell types, NIH3T3 cells (****, p<0.0001) and HAb2 cells (**, p<0.01) using an ordinary one-way ANOVA test. Inside clusters, GloPIP molecules moved within a mean radius of mobility, \( r_{mobility} \), defined as

\[
  r_{mobility} = \sqrt{\frac{MSD_p}{\pi}}
\]

\textit{Equation 3.3}

where \( MSD_p \) represents the plateau MSD in \( \mu m^2 \). For GloPIP molecules inside clusters, \( r_{mobility} = (0.098 \pm 0.005) \mu m \) in NIH3T3 cells and \( r_{mobility} = (0.082 \pm 0.004) \mu m \) in HAb2 cells.

To compare to the above GloPIP data, an analysis of PH domain-Dendra2 trajectories was conducted to measure the MSD in NIH3T3 and HAb2 cells. In HAb2 cells, the PH domain-Dendra2 diffusion curve was below that of the curve for NIH3T3 cells at all time points (figure 3.4). To quantify this observation, the data sets were compared using two statistical significance tests, the paired t-test and Kolmogorov-Smirnov (K-S) test. The MSD curves for NIH3T3 vs HAb2 cells were significantly different using a paired t-test (**, p<0.01), however, yielded no significance (ns, p≥0.05) when using a K-S test. The data was fitted to equation 3.1 and recorded in table 3.3. Another indication that the HAb2 curve is below the NIH3T3 curve is in the \( MSD_p \) values, \( MSD_{p, NIH3T3} = (0.244 \pm 0.011) \mu m^2 \) and \( MSD_{p, HAb2} = (0.182 \pm 0.005) \mu m^2 \), and \( \tau \) values, \( \tau_{NIH3T3} = (0.360 \pm 0.018) s \) and \( \tau_{HAb2} = (0.275 \pm 0.010) s \), of which the HAb2 curve had both a smaller plateau and \( \tau \) value. Using equation 3.2, yielded the following diffusion coefficient for PH domain-Dendra2 molecules in NIH3T3 cells, \( D_{NIH3T3} = (0.169 \pm 0.011) \frac{\mu m^2}{s} \).
Figure 3.4: Diffusion Curves of PH Domain-Dendra2 Molecules. PH Domain-Dendra2 molecules exhibit altered confinement in the presence of hemagglutinin. The mean squared displacement (MSD) of each trajectory was calculated for each time point and averaged across all cells of each type, n=10 cells for NIH3T3 data and n=10 cells for HAb2 data. The data was fit to \( MSD = MSD_p \left( 1 - e^{-t/\tau} \right) \) where \( MSD_p \) represents the plateau MSD and \( b \) represents the inverse time constant. MSD curves have two-star (**, p<0.01) significance using a paired t-test, however, are not significant using a K-S test. Significance testing \( p \)-values are accompanied by a significance star rating ranging as follows: \( p \leq 0.05 \) (ns), \( p < 0.05 \) (*), \( p < 0.01 \) (**), \( p < 0.001 \) (***) , and \( p < 0.0001 \) (****). Significance testing was generated using GraphPad Prism 8.3.1 software. Error bars are standard error of the mean.
FITTING PARAMETERS FOR MSD VS TIME FOR PH DOMAIN-DENDRA2 MOLECULES

<table>
<thead>
<tr>
<th></th>
<th>NIH3T3</th>
<th>HAb2</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSD$_p$ ($\mu$m$^2$)</td>
<td>0.244</td>
<td>0.182</td>
<td>0.005</td>
</tr>
<tr>
<td>$\tau$ (s)</td>
<td>0.360</td>
<td>0.275</td>
<td>0.010</td>
</tr>
<tr>
<td>$X^2$</td>
<td>0.0055</td>
<td>0.0062</td>
<td></td>
</tr>
<tr>
<td>R$^2$</td>
<td>0.9991</td>
<td>0.9993</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.3:** Fitting Parameters for MSD vs Time for PH Domain-Dendra2 Molecules. The fitting parameters for MSD curves in NIH3T3 cells and HAb2 cells (n=10 cells, each). The data shown in figure 3.4 was fit to $MSD = MSD_p \left(1 - e^{-t/\tau}\right)$ where MSD$_p$ represents the plateau MSD and b represents the inverse time constant. Significance testing p-values are accompanied by a significance star rating ranging as follows: $p \leq 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). Significance testing was generated using GraphPad Prism 8.3.1. software. Fits were calculated using the standard deviation of the data. Fits were done using Microcal™ Origin 6.0 software.

and in HAb2 cells, $D_{HAb2} = (0.165 \pm 0.008) \frac{\mu m^2}{s}$. Using equation 3.3, the following mean radius of mobilities were obtained $r_{mobility} = (0.279 \pm 0.006) \mu m$ for NIH3T3 cells and $r_{mobility} = (0.241 \pm 0.003) \mu m$ for HAb2 cells. The fitting parameters for the MSD curves are significantly different (****, $p<0.0001$) comparing NIH3T3 data and HAb2 data using both the multiple t-test and unpaired t-test (table 3.3).

To better understand how the PH Domain-Dendra2 molecules behave in the presence of HA, we analyzed PH Domain-Dendra2 mobilities (units $\frac{\mu m^2}{s}$) as a function of PH-Dendra2 density (figure 3.5). In figure 3.5, the HAb2 data curve was below the NIH3T3 curve at all densities. To confirm this observation, the data sets were compared and tested for significance by two tests, paired t-test and K-S test, the same as was done for the MSD curves. The mobility curves showed four- star (****, $p<0.0001$) significance
Figure 3.5: Mobility Curves of PH Domain-Dendra2 Molecules. PH domain-Dendra2 mobilities are altered when in the presence of hemagglutinin. The mobilities ($\mu_m$) of each trajectory was calculated for a given density and averaged across all cells of each type at a density grid pixel width of 80 nanometers – n=10 cells for NIH3T3 and n=10 cells for HAb2. These curves were fitted to $\mu = \mu_0 + A e^{-\rho \alpha}$ where $\mu_0$ represents the asymptotic plateau value, $\mu_0 + A$ represents the free diffusion of a molecule, $\rho$ represents the density, and $\alpha$ represents the decay rate of the density. Mobility curves have four-star (****, $p<0.0001$) significance using a paired t-test and the same significance using a K-S test. Significance testing $p$-values are accompanied by a significance star rating ranging as follows: $p \leq 0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****). Significance testing was generated using GraphPad Prism 8.3.1. software. Error bars are standard error of the mean.
Fitting Parameters for Mobility vs Density of PH Domain-Dendra2 Molecules

<table>
<thead>
<tr>
<th></th>
<th>NIH3T3</th>
<th></th>
<th></th>
<th>HAb2</th>
<th></th>
<th></th>
<th>MULTIPLE T-TEST</th>
<th>UNPAIRED T-TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VALUE</td>
<td>ERROR</td>
<td>VALUE</td>
<td>ERROR</td>
<td>VALUE</td>
<td>ERROR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>μ₀ (μm²/s)</td>
<td>0.49</td>
<td>0.04</td>
<td>−0.0016</td>
<td>0.095</td>
<td>0.095</td>
<td>0.0016</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td>A (μm²/s)</td>
<td>0.22</td>
<td>0.03</td>
<td>0.71</td>
<td>0.09</td>
<td>0.71</td>
<td>0.09</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td>μ₀ + A (μm²/s)</td>
<td>0.71</td>
<td>0.04</td>
<td>0.71</td>
<td>0.13</td>
<td>0.71</td>
<td>0.13</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>α (molecules/μm²)</td>
<td>1971</td>
<td>577</td>
<td>3528</td>
<td>717</td>
<td>3528</td>
<td>717</td>
<td>ns</td>
<td>****</td>
</tr>
<tr>
<td>X²</td>
<td>1.6581</td>
<td>−</td>
<td>2.7461</td>
<td>−</td>
<td>2.7461</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>R²</td>
<td>0.8525</td>
<td>−</td>
<td>0.9486</td>
<td>−</td>
<td>0.9486</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 3.4: Fitting Parameters for Mobility vs Density of PH Domain-Dendra2 Molecules. The fitting parameters for mobility curves in NIH3T3 cells and HAb2 cells (n=10 cells, each). The data shown in figure 3.5 was fit to \( \mu = \mu_0 + A e^{-\rho} \) where \( \mu_0 \) represents the asymptotic plateau value, \( \mu_0 + A \) represents the free diffusion of a molecule, \( \rho \) represents the density, and \( \alpha \) represents the decay rate of the density. Significance testing p-values are accompanied by a significance star rating ranging as follows: \( p \leq 0.05 \) (ns), \( p < 0.05 \) (*), \( p < 0.01 \) (**), \( p < 0.001 \) (***), and \( p < 0.0001 \) (****). Significance testing was generated using GraphPad Prism 8.3.1. software. Fits were calculated using the standard deviation of the data. Fits were done using Microcal™ Origin 6.0 software.

using a paired t-test and the same significance level using a K-S test. The mobilities of PH domain-Dendra2 molecules in NIH3T3 cells was compared to those mobilities in HAb2 cells. These mobilities, \( \mu \), were fitted to

\[
\mu = \mu_0 + A e^{-\rho/\alpha}
\]

Equation 3.4

where \( \mu_0 \) represents the asymptotic plateau value in \( (\mu m^2/s) \), \( \mu_0 + A \) represents the free diffusion of a molecule in \( (\mu m^2/s) \), \( \rho \) represents the density in molecules per \( \mu m^2 \), and \( \alpha \) represents the decay rate of the density in molecules per \( \mu m^2 \). Both curves’ y-intercepts were within error of each other, \( (\mu_0 + \)
\[ A_{NIH3T3} = (0.709 \pm 0.043) \mu m^2/s \text{ and } (\mu_0 + A)_{HAb2} = (0.708 \pm 0.130) \mu m^2/s \]

where \( \mu_0 + A \) represents the free diffusion of a molecule at a density of zero, and were not significantly (ns, p≤0.05) different using a multiple t-test method. In contrast, the curves decayed to significantly different plateau values,

\[ \mu_{0, NIH3T3} = (0.489 \pm 0.0329) \mu m^2/s \text{ and } \mu_{0, HAb2} = (-0.0016 \pm 0.095) \mu m^2/s, \]

which represent the mobility each curve asymptotically approaches at large densities. The fitting parameters had a four-star (****, p<0.0001) significance using multiple t-tests (table 3.4) except for their decay rates, which had no significance using this test, while all three fit parameters had four-star (****, p<0.0001) significance ratings using an unpaired t-test method.

3.4 Measurement of Colocalization of Influenza Hemagglutinin and PH Domain in Living Cells

Following the results found with PH domain-Dendra2, we performed simultaneous imaging of both HA and PH domain together inside cells. Using TIRF illumination, three biological replicates of two-species live-cell FPALM were successfully completed in a total of 27 cells. Following localization analysis, each cell was inspected for the expression level of each species. Figure 3.6 shows image rendering of each species, (panel A) Dendra2-Hemagglutinin in green and (panel B) PH domain-PAmKate in magenta, in a NIH3T3 cell. Panels C and D show a merge of the two channels with white areas signifying colocalization between the two species.

To measure how well the two species, HA and PH domain, spatially correlate together inside NIH3T3 cells, a measurement of their Pearson’s correlation coefficient (PCC) was done (Pearson, 1901). A positive PCC measures correlation between two species while a negative PCC measures anti-correlation. Figure 3.7A shows a distribution of 27 cells each with a density grid pixel width (pixel width for a grid masking the cell) of 80 nanometers and their PCC values between Dendra2-HA and PH domain-PAmKate. This distribution is overall positive with a mean PCC of \( PCC_{mean} = (0.267 \pm 0.023) \). Additionally (figure
Figure 3.6: Render of Dendra2-Hemagglutinin and PH Domain-PAmKate. Hemagglutinin coclusters with PH domain in the plasma membrane of NIH3T3 cells. Two color (species) rendering of Dendra2-Hemagglutinin and PH domain-PAmKate of the basal surface of an NIH3T3 cell imaged with the TIRF illumination method using intensity-weighted Gaussians of prescribed size (20 nanometers) for all localizations. (A) Green rendering of all Dendra2-Hemagglutinin localizations in an NIH3T3 cell. (B) Magenta rendering of all PH-PAmKate localizations in an NIH3T3 cell. (C) A merged render of both species’ localizations in an NIH3T3 cell. White represents colocalization between the two species. (D) Close-up view of weighted render which includes yellow arrows to identify colocalization regions. Scale bars represent 1 micrometer.
Figure 3.7: Pearson’s Correlation Coefficient of Dendra2-Hemagglutinin and PH Domain-PAmKate. Mean Pearson’s correlation coefficient (PCC) of Dendra2-Hemagglutinin and PH domain-PAmKate is overall positive in NIH3T3 cells. (A) PCCs were calculated for n=27 cells at a density grid pixel width of 80 nanometers. The mean PCC is 0.267 ± 0.023, which is shown as a solid vertical black line. (B) Grid widths range from 50 – 150 nanometers and PCCs are calculated for n=27 cells. Error bars represent standard error of the mean.
by increasing the density grid pixel width there is a positive increase in the mean PCC for Dendra2-HA and PH domain-PAmKate. Live cell PCC values shown here were larger than previously reported in fixed cells (Curthoys et al., 2019).

A second metric of colocalization between the two species (HA and PH domain in NIH3T3 cells) is the Manders’ colocalization coefficient (MCC) (Manders et al., 1992). There are two MCC values, one for the green channel and another for the red channel, which relate how much of one species is located with the second species relative to the total for the first species. An MCC of 1 is perfectly colocalized (i.e. all of that species is found with the other) while an MCC of zero is not colocalized. Figure 3.8A measures both MCCs for n=27 cells with a density grid pixel width of 80 nanometers. For this data, Dendra2-HA is the green channel and PH domain-PAmKate is the red channel (plotted using magenta, figure 3.6). The mean MCC for Dendra2-HA was measured as $MCC_G = 0.77 \pm 0.05$ and the mean MCC for PH domain-PAmKate was measured as $MCC_R = 0.58 \pm 0.05$. In figure 3.8B, there is a bi-modal distribution of MCCs with a small group found at much lower values. Most of the lower group are from the same replicate. When the density grid pixel width was increased (figure 3.8B), the MCCs increase positively towards a value of 1. This trend was also seen with the PCC values stated previously.

3.5 Dynamic Properties of Influenza Hemagglutinin and PH-Domain

3.5.1 Measuring Molecular Flux as a Function of Time

Measuring the dynamics of both species relative to each other can reveal how the two species interact over various timescales, represented by $\tau$, the time delay between the segments of the dataset used for “pre” and “post”. To do this, each cell (n=27 cells) was divided into a grid with density grid pixel width of 80 nanometers and divided temporally into consecutive stacks of frames, each of total duration $\tau$. Each grid pixel was assigned a category representative of the condition met in that pixel. A condition
Figure 3.8: Manders’ Co-localization Coefficients of Dendra2-Hemagglutinin and PH Domain-PAmKate. Dendra2-Hemagglutinin Manders’ colocalization coefficient (MCC) is larger than PH Domain-PAmKate MCC suggesting that HA is more likely to be found colocalized with PH domain, than PH domain is with HA, on average. (A) Plotted distribution is of n=27 cells for a density grid pixel width of 80 nanometers. The mean MCC for Dendra2-HA is 0.771 ± 0.046, which is shown as a solid horizontal green line, and for PH Domain-PAmKate is 0.581 ± 0.053, which is shown as a solid vertical red line. (B) Each grid pixel width is averaged over 27 cells with a range from 50-150 nanometers. Error bars represent standard error of the mean.
is defined by the increases and decreases in molecular numbers over time (green representing Dendra2-HA; red representing PAmKate-PH). Within a grid pixel where each species is found, either species’ molecular flux can increase, decrease, or remain the same encoded by the symbols (+), (-), and (=) respectively (table 3.5). If both species were not found in a given grid pixel, it was ignored. Each pixel which measured a given condition (molecular flux) was counted as an event. Each number of events was then normalized by the total number of events for that cell, yielding a normalized frequency of events, P(t). Normalized P(t) values were then averaged by cell, and their standard error of the mean was calculated.

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>DENDRA2-HA MOLECULAR FLUX (HA)</th>
<th>PH DOMAIN-PAMKATE MOLECULAR FLUX (PIP2)</th>
<th>DYNAMIC?</th>
</tr>
</thead>
<tbody>
<tr>
<td>+HA =PIP2</td>
<td>increase</td>
<td>remains the same</td>
<td>partially</td>
</tr>
<tr>
<td>-HA =PIP2</td>
<td>decrease</td>
<td>remains the same</td>
<td>partially</td>
</tr>
<tr>
<td>+HA +PIP2</td>
<td>increase</td>
<td>increase</td>
<td>yes</td>
</tr>
<tr>
<td>-HA +PIP2</td>
<td>decrease</td>
<td>increase</td>
<td>yes</td>
</tr>
<tr>
<td>+HA -PIP2</td>
<td>increase</td>
<td>decrease</td>
<td>yes</td>
</tr>
<tr>
<td>-HA -PIP2</td>
<td>decrease</td>
<td>decrease</td>
<td>yes</td>
</tr>
<tr>
<td>=HA =PIP2</td>
<td>remains the same</td>
<td>remains the same</td>
<td>no</td>
</tr>
<tr>
<td>=HA +PIP2</td>
<td>remains the same</td>
<td>increase</td>
<td>partially</td>
</tr>
<tr>
<td>=HA -PIP2</td>
<td>remains the same</td>
<td>decrease</td>
<td>partially</td>
</tr>
</tbody>
</table>

Table 3.5: Description of Conditions (Molecular Flux) for Dendra2-Hemaglutinin and PH Domain-PamKate. An overview of defined conditions in terms of Dendra2-HA and PH domain-PamKate flux. Conditions are also defined by their dynamics – dynamic, partially dynamic, and no dynamics.
### Table 3.6: Normalized Conditions (Molecular Flux) Increasing with Time, \( \tau \)

<table>
<thead>
<tr>
<th>( \tau ) (s)</th>
<th>+HA+PIP2</th>
<th>-HA+PIP2</th>
<th>+HA-PIP2</th>
<th>-HA-PIP2</th>
<th>+HA+PIP2</th>
<th>-HA-PIP2</th>
<th>=HA+PIP2</th>
<th>=HA-PIP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6.8 ± 1.6</td>
<td>7.1 ± 1.7</td>
<td>20.50 ± 1.08</td>
<td>18.7 ± 0.9</td>
<td>18.2 ± 0.9</td>
<td>23.07 ± 1.29</td>
<td>1.58 ± 0.59</td>
<td>1.93 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>9.04 ± 1.81</td>
<td>9.4 ± 1.8</td>
<td>16.32 ± 1.49</td>
<td>15.6 ± 1.5</td>
<td>15.2 ± 1.5</td>
<td>19.06 ± 1.93</td>
<td>9.74 ± 3.69</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>20</td>
<td>11.8 ± 2.2</td>
<td>12.79 ± 2.29</td>
<td>13.57 ± 1.59</td>
<td>13.5 ± 1.7</td>
<td>12.46 ± 1.57</td>
<td>16.52 ± 2.16</td>
<td>14.33 ± 4.58</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>25</td>
<td>13.02 ± 2.09</td>
<td>14.3 ± 2.3</td>
<td>11.7 ± 1.5</td>
<td>12.4 ± 1.8</td>
<td>10.8 ± 1.5</td>
<td>14.8 ± 2.2</td>
<td>17.79 ± 5.05</td>
<td>2.56 ± 0.56</td>
</tr>
<tr>
<td>30</td>
<td>13.38 ± 2.24</td>
<td>15.31 ± 2.56</td>
<td>10.05 ± 1.5</td>
<td>11.02 ± 1.83</td>
<td>9.3 ± 1.6</td>
<td>13.3 ± 2.4</td>
<td>22.9 ± 5.9</td>
<td>2.4 ± 0.5</td>
</tr>
</tbody>
</table>

A histogram styled bar graph with frequency of each condition \( P(\tau) \), averaged by cell (n=27 cells), was plotted as a function of increasing time delay \( \tau \) (figure 3.9, table 3.6). Columns with dynamics, i.e. +HA+PIP2, +HA-PIP2, -HA+PIP2, and -HA-PIP2, decreased in \( P(\tau) \) with increasing \( \tau \). It was more likely to see the two species change together in the same direction than to see them change oppositely. Concurrently, the percentage of constant PH domain-PAmKate (labeled PIP2 in figure 3.9) with dynamic Dendra2-HA (+HA, -HA) increased with increasing \( \tau \). Conditions (molecular flux) with constant dynamic PH domain-PAmKate (+PIP2, -PIP2) neither increase nor decrease in \( P(\tau) \) with increasing \( \tau \). The three conditions in which PH domain-PAmKate was kept constant, =HA=PIP2, +HA=PIP2, and -HA=PIP2, are all increased in \( P(\tau) \) as a function of \( \tau \).

Table 3.7 represents a one-way ANOVA significance testing of two time points for each condition represented in figure 3.9. Significant differences between values at different \( \tau \), all compared against the \( \tau=10 \) s time point, are only found in the most dynamic columns (+HA+PIP2, +HA-PIP2, -HA+PIP2, and -HA-PIP2) and the constant (=HA=PIP2) column. There is no significant difference between event frequencies.
Figure 3.9: Normalized Molecular Flux of Dendra2-Hemagglutinin and PH Domain-PAmKate Molecules. The majority of dynamics of colocalized HA and PH domain is found on short time scales. Normalized conditions (molecular flux) for Dendra2-Hemagglutinin and PH domain-PAmKate (labeled PIP2) molecules averaged by cell (n=27 cells) for various time differences (τ), for a density grid pixel width of 80 nanometers. Each bar within a column is described as follows: τ=10 s (dark blue), τ=15 s (light blue), τ=20 s (green), τ=25 s (orange), and τ=30 s (yellow). The y-axis is the normalized percentage, P(τ), of a given event by the total frequency of events for a given cell and then averaged across all cells. Along the x-axis, a + represents an increase in molecules, a – represents a decrease in molecules, and an = represents no change (remains the same) in molecules. Error bars represent standard error of the mean.
### Table 3.7: One-Way ANOVA Significance Testing of Two Time Points for Each Condition (Molecular Flux)

Significant decrease in dynamic hemagglutinin and PIP2 normalized conditions with increase in time differences, $\tau$. Comparison of $\tau=10$ s, to time points, $\tau=15$ s, $20$ s, $25$ s, and $30$ s respectively using a one-way ANOVA significance test for normalized conditions of HA and PIP2 molecules in NIH3T3 cells (n=27 cells). Significance testing $p$-values are accompanied by a significance star rating ranging as follows: $p \leq 0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****). Significance testing was generated using GraphPad Prism 8.3.1. software.

<table>
<thead>
<tr>
<th></th>
<th>10s vs 15s</th>
<th>10s vs 20s</th>
<th>10s vs 25s</th>
<th>10s vs 30s</th>
</tr>
</thead>
<tbody>
<tr>
<td>$+HA = PIP2$</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>$-HA = PIP2$</td>
<td>ns</td>
<td>ns</td>
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</tr>
<tr>
<td>$+HA + PIP2$</td>
<td>ns</td>
<td>**</td>
<td>***</td>
<td>****</td>
</tr>
<tr>
<td>$-HA + PIP2$</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>$+HA - PIP2$</td>
<td>ns</td>
<td>*</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>$-HA - PIP2$</td>
<td>ns</td>
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<tr>
<td>$=HA + PIP2$</td>
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<td>ns</td>
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<tr>
<td>$=HA - PIP2$</td>
<td>ns</td>
<td>ns</td>
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</tbody>
</table>

P($\tau$) as a function of $\tau$ for conditions with constant PIP2 and dynamic Dendra2-HA (+HA, -HA) or with constant Dendra2-HA and dynamic PH domain-PAmKate (+PIP2, -PIP2). The largest significant difference is between time points $\tau=10$ s and $\tau=30$ s for $+HA+PIP2$ with a four-star (****, $p<0.0001$) significance rating. All other time combinations yield no significance except for $\tau=15$ s to $\tau=30$ s with a one-star (*, $p<0.05$) significance.

A striking trend was observed in the normalized P($\tau$) (figure 3.9) with increasing $\tau$ for all nine conditions (figure 3.10) – exponential growth and decay. Time dependent curves, P($\tau$), (figure 3.9, table 3.8) are exponentially related when fitted to
\[ P(\tau) = P_{plateau} + A e^{-k\tau} \quad \text{Equation 3.5} \]

where \( P(t) \) represents the normalized percentage, \( P_{plateau} \) represents the asymptotic plateau normalized percentage value, \( P_{plateau} + A \) represents the y-intercept, and \( k \) represents the inverse time constant in inverse seconds. Panels A – I have y-axis limits from 0 – 35% while panels J and K are enlarged views of panels H and I, respectively, with y-axis limits from 0 – 4% to emphasis the exponential nature of the fitted curves. The conditions, +HA=PIP2, -HA=PIP2, =HA=PIP2, and =HA-PIP2 (panels A,B,G-I), are growing as a time-dependent exponential buildup (i.e. \( 1-e^{-t/\tau} \)), while, the conditions, +HA+PIP2, +HA-PIP2, -HA+PIP2, and -HA-PIP2 (panels C-F), are decaying exponentially with time. The decaying curves are all conditions where each species is dynamic (changing substantially with time) and were fitted with positive A values signifying exponential decay. The remaining curves all contain at least one species that is observed to be constant (partial and no dynamics conditions) and were fitted with negative A values, signifying exponential buildup. Dynamic conditions have positive y-intercepts, while the remaining curves (partial and no dynamics conditions) have y-intercepts of approximately zero (± error). Fits of conditions =HA+PIP2 and =HA-PIP2 were forced through the origin, using Microcal™ Origin 6.0 software, resulting in a zero y-intercept value. None of the decay rate values, \( k \), are significantly different from each other using a Brown-Forsythe ANOVA test.

It was shown in figure 3.9 that dynamic conditions dominate on a short time scale, \( \tau=10 \) s, in comparison to longer time scales, \( \tau=30 \) s. In order to take a closer look at these time scales, all conditions (molecular flux) for both \( \tau=10 \) s and \( \tau=30 \) s are presented in figure 3.11. For \( \tau=10 \) s, \(~80\% \) \( (P_{dynamics}(\tau) = 80.191 \pm 4.372) \) of all counted events are found in columns +HA+PIP2, +HA-PIP2, -HA +PIP2, and -HA -PIP2. In contrast, \(~17\% \) \( (P_{partial}(\tau) = 17.546 \pm 3.871) \) of all counted events are found in the opposite four columns where at least one species is observed to be constant (partial dynamics) and less than 3%
Figure 3.10: Fitted Curves of Time-Dependent Normalized Molecular Flux Graphs. Time dependence of normalized conditions are exponentially related. (A-I) Fitted graphs for each condition (molecular flux) are fit to the equation, $P(\tau) = P_{plateau} + Ae^{-k\tau}$, where $P(\tau)$ represents the normalized percentage, $P_{plateau}$ represents the asymptotic plateau percentage value, $P_{plateau} + A$ represents the y-intercept, and $k$ represents the inverse time constant. Each box is labeled with its corresponding condition from figure 3.9 where a + represents an increase in molecules, a - represents a decrease in molecules, and an = represents no change in molecules (table 3.5). Y-axis ranges from 0-35%. (J, K) An enlarged view of panels H and I respectively with y-axis ranges from 0.0-4.0%. Error bars represent the standard error of the mean.
Fitting Parameters for Time Point Difference for Each Condition (Molecular Flux)

<table>
<thead>
<tr>
<th></th>
<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>+HA +PIP2</th>
<th>-HA +PIP2</th>
<th>+HA -PIP2</th>
<th>-HA -PIP2</th>
<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>+HA +PIP2</th>
<th>-HA +PIP2</th>
<th>+HA -PIP2</th>
<th>-HA -PIP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_{\text{plateau}} )</td>
<td>16.6 ± 3.6</td>
<td>21 ± 8</td>
<td>6.8 ± 0.8</td>
<td>8 ± 1</td>
<td>4 ± 1</td>
<td>10 ± 1</td>
<td>30 ± 10</td>
<td>2.6 ± 0.2</td>
<td>2.36 ± 0.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>-18.73 ± 2.05</td>
<td>-23.35 ± 4.38</td>
<td>27.4 ± 0.7</td>
<td>19 ± 1</td>
<td>23.1 ± 0.4</td>
<td>25± 3.5</td>
<td>-50.7 ± 3.5</td>
<td>-2.6 ± 0.2</td>
<td>-2.36 ± 0.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P_{\text{plateau}} + A )</td>
<td>-2.09 ± 4.18</td>
<td>-1.8 ± 9.3</td>
<td>34.15 ± 1.05</td>
<td>27.75 ± 1.69</td>
<td>27.75 ± 1.09</td>
<td>35.55 ± 1.47</td>
<td>-20.08 ± 10.58</td>
<td>0.0 ± 0.3</td>
<td>0.0 ± 0.24</td>
<td></td>
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</tr>
<tr>
<td>( k \left( \frac{1}{s} \right) )</td>
<td>0.062 ± 0.038</td>
<td>0.046 ± 0.038</td>
<td>0.070 ± 0.007</td>
<td>0.064 ± 0.015</td>
<td>0.054 ± 0.006</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.03</td>
<td>0.14 ± 0.04</td>
<td>0.19 ± 0.07</td>
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<tr>
<td>X^2</td>
<td>0.0035</td>
<td>0.0048</td>
<td>0.0046</td>
<td>0.0593</td>
<td>0.0164</td>
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<td>0.0040</td>
<td>0.0095</td>
<td>0.0103</td>
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<td>R^2</td>
<td>0.9805</td>
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<td>0.9994</td>
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<td>0.9984</td>
<td>0.9925</td>
<td>0.7043</td>
<td>0.4636</td>
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</tbody>
</table>

Table 3.8: Fitting Parameters for Time Point Difference for Each Condition (Molecular Flux). Fitting parameters for normalized conditions with increasing time differences, \( \tau \). The data shown in figure 3.8 were fitted to \( P(\tau) = P_{\text{plateau}} + A e^{-k\tau} \) where \( P(\tau) \) represents the normalized percentage, \( P_{\text{plateau}} \) represents the asymptotic plateau normalized percentage value, \( P_{\text{plateau}} + A \) represents the \( y \)-intercept, and \( k \) represents the inverse time constant in inverse seconds. Fits were calculated using the standard deviation of the data. Fits were done using Microcal™ Origin 6.0 software.

\( (P_{\text{no dynamics}}(\tau) = 2.264 ± 0.893) \) of the events show both species as constant (figure 3.11A). In contrast, when looking at the 30 second time difference, less than 44\% \( (P_{\text{dynamics}}(\tau) = 43.721 ± 7.401) \) of all counted events are found in dynamic columns (+HA+PIP2, +HA-PIP2, -HA+PIP2, and -HA-PIP2), while \( \approx 33\% \) \( (P_{\text{partial}}(\tau) = 33.285 ± 5.749) \) of all counted events are found in the opposite four columns with partial dynamics, and \( \approx 23\% \) \( (P_{\text{no dynamics}}(\tau) = 22.994 ± 5.893) \) of the events are found where both species are constant (figure 3.11B). Between \( \tau=10 \) s and \( \tau=30 \) s, there is a decrease in dynamics, an increase in partial dynamics, and a significant increase in the non-dynamic column (=HA=PIP2) all with increasing time scales.
Figure 3.11: $\tau=10$ s, $\tau=30$ s Normalized Molecular Flux Graphs. Dynamic conditions between HA and PH domain dominate at short time scales ($\tau=10$ s) while partial dynamics increase over long time scales ($\tau=30$ s). Normalized conditions for Dendra2-Hemagglutinin and PH domain-PAmKate (labeled PIP2) molecules averaged by cell ($n=27$ cells) for (A) $\tau=10$ s and (B) $\tau=30$ s for a density grid pixel width of 80 nanometers. The y-axis is the normalized percentage of a given event by the total frequency of events for a given cell and then averaged across all cells. Along the x-axis, a “+” represents an increase in molecules, a “–” represents a decrease in molecules, and an “=” represents no change (remains the same) in molecules. Error bars represent standard error of the mean.
### Table 3.9: One-Way ANOVA Significance Testing of Conditions (Molecular Flux)

Comparison of one-way ANOVA significance testing of normalized conditions (molecular flux) for Dendra2-Hemagglutinin and PH domain-PAmKate molecules in NIH3T3 cells (n=27 cells) for two time points, 10 seconds and 30 seconds. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***) , and p<0.0001 (****). Significance testing was performed using GraphPad Prism 8.3.1. software.

#### \( \tau = 10 \text{ seconds} \)

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>+HA=PIP2</th>
<th>-HA=PIP2</th>
<th>+HA=PIP2</th>
<th>-HA=PIP2</th>
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<td>+HA+PIP2</td>
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#### \( \tau = 30 \text{ seconds} \)

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<tr>
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Table 3.9: One-Way ANOVA Significance Testing of Conditions (Molecular Flux). Comparison of one-way ANOVA significance testing of normalized conditions (molecular flux) for Dendra2-Hemagglutinin and PH domain-PAmKate molecules in NIH3T3 cells (n=27 cells) for two time points, 10 seconds and 30 seconds. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***) , and p<0.0001 (****). Significance testing was performed using GraphPad Prism 8.3.1. software.
To understand the differences between conditions (molecular flux) for both the $\tau=10$ s and $\tau=30$ s time points, a one-way ANOVA significance test was used for all combinations (table 3.9). For $\tau=10$ s, the dynamic columns, $+HA+PIP2$, $+HA-PIP2$, $-HA+PIP2$, and $-HA-PIP2$, are significantly different (****, $p<0.0001$) from other (less dynamic) conditions, but have no significant differences (ns, $p \leq 0.05$) when compared to each other. In comparison to $\tau=30$ s, columns $=HA+PIP2$ and $=HA-PIP2$ show some significant differences (ranging from *, $p<0.05$, to $$$*, p<0.001$, values) compared to all other conditions except for $=HA=PIP2$. The dynamic conditions show significant differences when compared to the less dynamic conditions at $\tau=10$ s, but much lower levels of significance compared to the less dynamic conditions at the $\tau=30$ s time point.

3.5.2 Measuring Lateral Net Flux as a Function of Time

Understanding the lateral net flux of both species overlapping with the conditions mentioned previously (table 3.5) required mapping individual trajectory steps over density grid boxes, with grid width of 80 nanometers, that contain a condition (molecular flux). The trajectory movement was defined as into a box representing net inward lateral flux, out of a box representing net outward lateral flux, or contained within a box designating no net lateral flux. Each channel’s lateral net flux of each density grid box was thresholded (two or more trajectories were required for a flux to be calculated) and then overlapped with conditions, $P(\tau)$, (whether HA and/or PIP2 changed during the same time window). This process was repeated for two-time differences, $\tau=10$ s and $\tau=30$ s (figure 3.12). Figure 3.12 shows trends between the normalized $P(\tau)$ of Dendra2-Hemagglutinin and PH domain-PAmKate lateral net flux for two time points, $\tau=10$ s and $\tau=30$ s, overlapping with previously explained conditions (table 3.5). These normalizations are averaged by cell ($n=27$ cells) and calculated for a density grid pixel width of 80 nanometers. In figure 3.12, panel A shows the regions with inward lateral net flux, panel B shows the regions with outward lateral net flux, and panel C shows regions of no normalized lateral net flux (the
Figure 3.12: Lateral Net Flux and Molecular Flux Graphs for $\tau=10$ s and $\tau=30$ s. Lateral net flux of PIP2 and HA trend similarly with a majority of events resulting in zero lateral net flux. Conditions (molecular flux) for Dendra2-Hemagglutinin and PH domain-PAmKate (labeled PIP2) are overlapped with lateral net flux (trajectory steps into, out of, or within a density grid box and thresholded) for each species. Normalization is done for each species for all three lateral flux types (inward, outward, and zero) by total frequency of events for a given cell and then averaged across all cells ($n=27$ cells) for a density grid pixel width of 80 nanometers. This is repeated for two-time differences, $\tau=10$ s and $\tau=30$ s. Panels are defined as (A) inward lateral net flux, (B) outward lateral net flux, and (C) zero, or no, lateral net flux. Bar colors are defined: purple is PH domain-PAmKate lateral net flux at $\tau=10$ s; blue is PH domain-PAmKate lateral net flux at $\tau=30$ s; orange is Dendra2-Hemagglutinin lateral net flux at $\tau=10$ s; and yellow is Dendra2-Hemagglutinin lateral net flux at $\tau=30$ s. The $y$-axis limits of panels A and B are 0-1.2%, and panel C is 0-30%. Calculated values can be found in table 3.8. Error bars represent standard error of the mean.
### Normalized Conditions (Molecular Flux) with Lateral Flux of Dendra2-Hemagglutinin and PH Domain-PamKate

#### \( \tau = 10 \text{ seconds} \)

<table>
<thead>
<tr>
<th></th>
<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>TOTAL %</th>
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</thead>
<tbody>
<tr>
<td>PIP2 + FLUX</td>
<td>0.224 ± 0.038</td>
<td>0.208 ± 0.038</td>
<td>0.32 ± 0.04</td>
<td>0.29 ± 0.04</td>
<td>0.338 ± 0.069</td>
<td>0.42 ± 0.05</td>
<td>0.046 ± 0.015</td>
<td>0.049 ± 0.008</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>PIP2 – FLUX</td>
<td>0.19 ± 0.03</td>
<td>0.20 ± 0.03</td>
<td>0.333 ± 0.057</td>
<td>0.346 ± 0.048</td>
<td>0.255 ± 0.036</td>
<td>0.39 ± 0.07</td>
<td>0.047 ± 0.014</td>
<td>0.048 ± 0.009</td>
<td>0.044 ± 0.007</td>
</tr>
<tr>
<td>NO PIP2 FLUX</td>
<td>7.2 ± 1.4</td>
<td>7.4 ± 1.5</td>
<td>18.93 ± 1.03</td>
<td>18.25 ± 1.07</td>
<td>17.5 ± 1.1</td>
<td>21.5 ± 1.3</td>
<td>1.6 ± 0.5</td>
<td>1.9 ± 0.3</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>HA + FLUX</td>
<td>0.076 ± 0.025</td>
<td>0.09 ± 0.03</td>
<td>0.138 ± 0.025</td>
<td>0.114 ± 0.023</td>
<td>0.09 ± 0.02</td>
<td>0.14 ± 0.03</td>
<td>0.035 ± 0.013</td>
<td>0.046 ± 0.012</td>
<td>0.038 ± 0.009</td>
</tr>
<tr>
<td>HA – FLUX</td>
<td>0.07 ± 0.02</td>
<td>0.084 ± 0.027</td>
<td>0.177 ± 0.038</td>
<td>0.128 ± 0.046</td>
<td>0.094 ± 0.019</td>
<td>0.19 ± 0.04</td>
<td>0.039 ± 0.015</td>
<td>0.043 ± 0.009</td>
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</tr>
<tr>
<td>NO HA FLUX</td>
<td>6.86 ± 1.58</td>
<td>6.95 ± 1.69</td>
<td>19.4 ± 1.1</td>
<td>17.66 ± 1.28</td>
<td>16.3 ± 0.9</td>
<td>21.66 ± 0.34</td>
<td>2.0 ± 0.6</td>
<td>2.6 ± 0.4</td>
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#### \( \tau = 30 \text{ seconds} \)

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<tr>
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<th>+HA =PIP2</th>
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<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>+HA =PIP2</th>
<th>-HA =PIP2</th>
<th>TOTAL %</th>
</tr>
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<tbody>
<tr>
<td>PIP2 + FLUX</td>
<td>0.5 ± 0.1</td>
<td>0.53 ± 0.13</td>
<td>0.36 ± 0.07</td>
<td>0.37 ± 0.07</td>
<td>0.31 ± 0.05</td>
<td>0.417 ± 0.069</td>
<td>0.8 ± 0.2</td>
<td>0.22 ± 0.04</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>PIP2 – FLUX</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.31 ± 0.05</td>
<td>0.36 ± 0.07</td>
<td>0.31 ± 0.05</td>
<td>0.391 ± 0.068</td>
<td>0.8 ± 0.2</td>
<td>0.20 ± 0.04</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>NO PIP2 FLUX</td>
<td>11.4 ± 1.8</td>
<td>13.24 ± 2.03</td>
<td>9.8 ± 1.5</td>
<td>10.0 ± 1.6</td>
<td>10.49 ± 2.12</td>
<td>12.14 ± 2.07</td>
<td>20.3 ± 5.2</td>
<td>27.4 ± 5.7</td>
<td>25.9 ± 5.6</td>
</tr>
<tr>
<td>HA + FLUX</td>
<td>0.29 ± 0.06</td>
<td>0.29 ± 0.058</td>
<td>0.101 ± 0.019</td>
<td>0.089 ± 0.025</td>
<td>0.070 ± 0.013</td>
<td>0.10 ± 0.01</td>
<td>0.4 ± 0.04</td>
<td>0.059 ± 0.015</td>
<td>0.060 ± 0.016</td>
</tr>
<tr>
<td>HA – FLUX</td>
<td>0.299 ± 0.059</td>
<td>0.302 ± 0.056</td>
<td>0.100 ± 0.023</td>
<td>0.11 ± 0.04</td>
<td>0.14 ± 0.09</td>
<td>0.083 ± 0.016</td>
<td>0.4 ± 0.1</td>
<td>0.051 ± 0.012</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>NO HA FLUX</td>
<td>13.97 ± 2.03</td>
<td>15.27 ± 2.39</td>
<td>10.2 ± 1.6</td>
<td>10.2 ± 1.7</td>
<td>10.1 ± 1.9</td>
<td>12.77 ± 2.28</td>
<td>19.9 ± 4.8</td>
<td>2.5 ± 0.4</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

**Table 3.10:** Normalized Conditions (Molecular Flux) with Lateral Flux of Dendra2-Hemagglutinin and PH Domain-PamKate. Calculate frequencies (percentages of total events) of all normalized conditions (molecular flux) with lateral net flux of Dendra2-Hemagglutinin and PH domain-PamKate for time differences, \( \tau = 10 \text{ s} \) and \( \tau = 30 \text{ s} \). Normalization is done for each species for all three lateral flux types (inward, outward, and zero) by the total frequency of events for a given cell, averaged by cell (n=27 cells) with standard error of the mean, with a density grid pixel width of 80 nanometers. The final column shows the summation of all conditions with standard error of the mean.
average net flux for a pixel was zero). The y-axis for panels A and B ranges from 0.0-1.2% while panel C ranges from 0-30%, suggesting it is much more likely that an average 80nm by 80nm membrane zone (pixel) has an overall zero lateral net flux. All normalized P(τ) are written in table 3.10 with the final column showing the total percentage for a given lateral net flux. Zones with net lateral flux comprise a small fraction of total events, consistent with what is shown in figure 3.12. PH domain-PAmKate and Dendra2-Hemagglutinin inward lateral net flux (figure 3.12A) exhibit similar trends between the τ=10 s and τ=30 s time points (both species increase or decrease simultaneously with time) except for +HA+PIP2, -HA+PIP2, -HA-PIP2, =HA+PIP2, and =HA-PIP2 conditions. This trend of similar behavior increases when looking at outward lateral net flux (figure 3.12B) except for =HA+PIP2 and =HA-PIP2 conditions. For no lateral net flux (figure 3.12C), all conditions follow the same trend between the τ=10 s and τ=30 s time points.

### 3.6 Measuring the Frequency of Membrane Models as a Function of Time

Each membrane model (section 1.6) is defined by a particular state vector describing the molecular count and lateral movement for a given pixel with density grid pixel width of 80 nanometers. A state vector is defined for each model tested as

$$
\langle d\text{PIP2} \mid d\text{HA} \mid \text{PIP2 flux} \mid \text{HA flux} \rangle
$$

where the first element, $d\text{PIP2}$, is the flux difference in PH domain-PAmKate molecules, the second element, $d\text{HA}$, is the flux difference in Dendra2-HA molecules, the third element, PIP2 flux, is the lateral net flux of PH domain-PAmKate molecules, and the fourth element, HA flux, is the lateral net flux of Dendra2-HA molecules for a given density grid pixel. Each element may be defined as 1 representing an increase or inward motion, 0 representing no difference or motion, or -1 representing a decrease or outward motion. Each model’s state vector is defined in table 3.11, and a model may be defined with multiple state vectors as in the case of Exocytosis/Delivery model. All events of a given model are
summed, normalized, and averaged by cell (n=27 cells). These normalized events for five-time differences, $\tau$, are shown in figure 3.13 and displayed in table 3.12. The models Budding/Endocytosis and Exocytosis/Delivery have more than ~75% of the events (see seventh row in table 3.12) while the remaining membrane models, Platform, Local Synthesis or Release of PIP2 (LSRP), and Megapool, do not exceed ~25% of the events (see fourth row in table 3.12). A t-test between both totals mentioned above was performed (see final row in table 3.12) confirming the differences as two-star (**, $p<0.01$) or higher. A dependence on increasing time difference, $\tau$, is shown in the Platform, LSRP, and Megapool models. The Budding/Exocytosis model has a decreasing trend with increasing time while the trend for the Exocytosis/Delivery model is on average decreasing with increasing time.

To study how often a model will occur as a function of time delay $\tau$, the rate of events for each model was calculated. Here, rates per unit area ($R_A$) are defined as the average number of events found in each time difference divided by both the total time elapsed and the sampled cell area. Because the two subsets of frames are each $\tau$ long and adjoin one another, the total elapsed time is equal to twice the time difference, $\tau$ (section 2.3.5.4). The $R_A$ values for a given membrane model are then defined as

$$R_A = \frac{n}{2\tau A_{\text{sampled}}}$$

where $n$ is the average number of events for a time difference found in a given cell, $\tau$ is the time difference in seconds, and $A_{\text{sampled}}$ is the cell area that has been sampled in $\mu m^2$. Table 3.13 shows the $R_A$ values for all five models for five-time differences, $\tau$. The table has units ($\frac{\text{# events}}{s \, \mu m^2}$) and all values are multiplied by $10^{-3}$. Over time differences, $\tau$, rates vary minimally which is confirmed with an ordinary one-way ANOVA significance test which reports that all values are not significant (ns, $p \leq 0.05$) from each other in a row. The rates for Budding/Endocytosis and Exocytosis/Delivery are always larger than other models at every time interval (significance testing of models against each other can be found in table B.1).
Table 3.11: Definition of State Vectors for Hemagglutinin and PIP2 Membrane Models. Membrane models and their defined state vector. It is noted that a model may possess two or more state vectors, as seen in Exocytosis/Delivery.
Two membrane models, Budding/Endocytosis and Exocytosis/Delivery, are, on average, found more abundantly in NIH3T3 cells. Each membrane model is accompanied by a state vector defined in table 3.9. Models are normalized by the total number of events for a given cell and then averaged over all cells (n=27 cells) for a density grid pixel width of 80 nanometers. This is then repeated for all time differences, $\tau=10$ s, $\tau=15$ s, $\tau=20$ s, $\tau=25$ s, and $\tau=30$ s. Error bars represent standard error of the mean.

**Figure 3.13**: Frequency of Membrane Models. Two membrane models, Budding/Endocytosis and Exocytosis/Delivery, are, on average, found more abundantly in NIH3T3 cells. Each membrane model is accompanied by a state vector defined in table 3.9. Models are normalized by the total number of events for a given cell and then averaged over all cells (n=27 cells) for a density grid pixel width of 80 nanometers. This is then repeated for all time differences, $\tau=10$ s, $\tau=15$ s, $\tau=20$ s, $\tau=25$ s, and $\tau=30$ s. Error bars represent standard error of the mean.
### Normalized Frequency of Events of Membrane Models for HA and PIP2

<table>
<thead>
<tr>
<th></th>
<th>$\tau = 10s$</th>
<th>$\tau = 15s$</th>
<th>$\tau = 20s$</th>
<th>$\tau = 25s$</th>
<th>$\tau = 30s$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLATFORM</strong></td>
<td>0.04 ± 0.04</td>
<td>0.5 ± 0.2</td>
<td>1.4 ± 0.6</td>
<td>1.78 ± 0.68</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td><strong>LSRP</strong></td>
<td>5.7 ± 1.5</td>
<td>13.19 ± 3.57</td>
<td>14.7 ± 3.7</td>
<td>17.0 ± 3.8</td>
<td>21 ± 5</td>
</tr>
<tr>
<td><strong>MEGAPOOL</strong></td>
<td>0.15 ± 0.09</td>
<td>0.38 ± 0.18</td>
<td>0.71 ± 0.24</td>
<td>0.89 ± 0.26</td>
<td>1.36 ± 0.36</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>5.9 ± 1.5</td>
<td>14.07 ± 3.58</td>
<td>16.8 ± 3.8</td>
<td>19.7 ± 3.9</td>
<td>24 ± 5</td>
</tr>
<tr>
<td><strong>BUDDING/ENDOCYTOSIS</strong></td>
<td>52.4 ± 5.8</td>
<td>46.8 ± 4.4</td>
<td>42.8 ± 4.2</td>
<td>40.7 ± 4.6</td>
<td>38.6 ± 4.3</td>
</tr>
<tr>
<td><strong>EXOCYTOSIS/DELIVERY</strong></td>
<td>41.7 ± 5.5</td>
<td>39 ± 4</td>
<td>40 ± 4</td>
<td>39.7 ± 3.6</td>
<td>37.07 ± 3.56</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>94 ± 8</td>
<td>86 ± 6</td>
<td>83.2 ± 5.8</td>
<td>80.3 ± 5.8</td>
<td>75.7 ± 5.5</td>
</tr>
</tbody>
</table>

**Significance Between Totals:**
- ***
- **
- **
- **
- **

**Table 3.12:** Normalized Frequency of Events of Membrane Models for Hemagglutinin and PIP2. Normalization of the frequency of events for five membrane models, Platform, Local Synthesis or Release of PIP2 (LSRP), Megapool, Budding/Exocytosis, and Exocytosis/Delivery. Three membrane models, Platform, LSRP, and Megapool, total percentages are presented in row four while the remaining two membrane models, Budding/Endocytosis and Exocytosis/Delivery, total percentages are in seventh row. The final row shows t-test significance between rows four and seven previously mentioned. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), and p<0.0001 (****). Significance testing was generated using GraphPad Prism 8.3.1. software. All values include the standard error of the mean.
### Rates of Membrane Models for HA and PIP2

<table>
<thead>
<tr>
<th>Model</th>
<th>( \tau = 10s )</th>
<th>( \tau = 15s )</th>
<th>( \tau = 20s )</th>
<th>( \tau = 25s )</th>
<th>( \tau = 30s )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platform</strong></td>
<td>0.03 ± 0.03</td>
<td>0.154 ± 0.057</td>
<td>0.39 ± 0.13</td>
<td>0.53 ± 0.19</td>
<td>0.55 ± 0.18</td>
</tr>
<tr>
<td><strong>LSRP</strong></td>
<td>2.6 ± 0.8</td>
<td>4.2 ± 1.1</td>
<td>4.6 ± 1.3</td>
<td>5.6 ± 1.7</td>
<td>6.42 ± 2.14</td>
</tr>
<tr>
<td><strong>MEGAPOOL</strong></td>
<td>0.07 ± 0.04</td>
<td>0.125 ± 0.005</td>
<td>0.23 ± 0.09</td>
<td>0.28 ± 0.09</td>
<td>0.40 ± 0.14</td>
</tr>
<tr>
<td><strong>Budding/Endocytosis</strong></td>
<td>14.2 ± 2.9</td>
<td>14 ± 3</td>
<td>11.72 ± 2.18</td>
<td>9.7 ± 1.7</td>
<td>8.4 ± 1.6</td>
</tr>
<tr>
<td><strong>Exocytosis/Delivery</strong></td>
<td>11.7 ± 2.4</td>
<td>11.4 ± 2.5</td>
<td>11.33 ± 2.16</td>
<td>9.8 ± 1.8</td>
<td>9.2 ± 1.8</td>
</tr>
</tbody>
</table>

**Table 3.13**: Rates of Membrane Models for Hemagglutinin and PIP2. Lists of rates for each membrane model (Platform, Local Synthesis or Release of PIP2 (LSRP), Megapool, Budding/Exocytosis and Exocytosis/Delivery) in units \((\frac{\text{events/s}}{\mu m^2}) \times 10^{-3}\). Rates are calculated using \( R_A = \frac{n}{2 \tau A_{sampled}} \), where \( n \) is the average number of events for a time difference found in a given cell, \( \tau \) is the time difference in seconds, and \( A_{sampled} \) is the cell area that has been sampled in \( \mu m^2 \). Error is standard error of the mean.
Influenza virus, colloquially known as the flu, is a public health threat which causes thousands of deaths in the United States each year (Center for Disease Control and Prevention, 2020b). Due to its high mutation rate, a universal treatment option has not been created; however, each year a flu vaccine is produced which has a variable effectiveness (Center for Disease Control and Prevention, 2020a) resulting in inexact prevention and protection from the infectious disease. The flu, and other enveloped viruses such as Ebola, HIV, and Coronaviruses, continue to infect humans by exploiting host cell membrane organization (Flint et al., 2003). An improved understanding of these processes is needed.

Entry of influenza virions (virus particles) into the host cell depends on membrane fusion of hemagglutinin (HA), a protein located on the viral envelope (Flint et al., 2003). This HA-dependent membrane fusion relies on high density of HA trimer clustering (Ellens et al., 1990; Takeda et al., 2003), however, the mechanism for this clustering is unknown. To further understand influenza virus exploitation of host cells, there is a need for insight into the mechanism of HA trimer clustering on the plasma membrane (PM).

The actin cytoskeleton network has been linked to the influenza viral lifecycle, both during internalization of the virus (Sun & Whittaker, 2007) and through budding of virions (Roberts & Compans, 1998; Simpson-Holley et al., 2002). HA and actin have been shown to colocalize at the PM (Gudheti et al., 2013; Simpson-Holley et al., 2002) and disruption of actin comets resulted in virtually no observations of HA at the PM (Rozelle et al., 2000). It can be stated that actin is essential to viral infection. Yet, a direct interaction between HA and actin has yet to be observed. We hypothesize that the cytoplasmic tail domain (CTD) of HA monomers is interacting with phosphoinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP2), to modulate actin assembly at the PM through actin binding proteins (ABPs).
Mutations of the CTD of HA have shown changes in infectivity of influenza virus (Jin et al., 1994; Lazarovits & Roth, 1988; Simpson & Lamb, 1992) and HA clustering properties (Parent, 2020), suggesting the importance of the HA CTD to influenza infection. The CTD of HA has three conserved cysteine (an amino acid) residues that serve as acylation sites (Veit et al., 1991) for a palmitic or stearic acid (Mahy & van Regenmortel, 2008). The phosphoinositide, PIP2, includes two acyl chains and a charged head group (Alberts et al., 2015). We hypothesize that PIP2 is the link between HA and actin (Curthoys et al., 2019) since PIP2 is known to interact with a plethora of actin binding proteins (ABPs) (Alberts et al., 2015; Catimel et al., 2008), and a number of these same ABPs are found in purified influenza virus (Shaw et al., 2008). To better understand the role actin has in influenza infection, and by indirect association PIP2, we began by studying four ABPs, α-actinin, cofilin 1 (cofilin), myosin 1 (myosin), and tropomyosin 4 (TM4), and HA in fixed NIH3T3 cells.

4.1 Influenza Hemagglutinin Clustering Properties are Affected by High Concentrations of Actin Binding Proteins

Phosphoinositides, such as PIP2, are known to interact with actin binding proteins (Alberts et al., 2015; Catimel et al., 2008) which have been hypothesized to be the link between actin and HA (Curthoys et al., 2019). Four actin binding proteins (ABPs), α-actinin, cofilin, myosin, and TM4, were studied in this work to better understand their effect on HA clustering. We see positive correlation between all four ABPs and HA (figure 3.1) with the strongest correlation between HA and TM4.

Tropomyosin 4 (TM4) is an ABP primarily associated with stabilization of actin filaments by binding several actin subunits along filamentous actin and preventing interactions with other ABPs (Alberts et al., 2015). TM4 can also regulate the interaction between formins, actin binding proteins whose main function is nucleation of filamentous actin which occurs primarily at the plasma membrane (PM) (Alberts et al., 2015), and the barbed end (growing end) of actin filaments (Gunning et al., 2015; Ujfalusi et al.,
TM4 has previously been identified in purified Influenza virions (Shaw et al., 2008). We observed a significant increase in HA cluster areas with higher concentrations of TM4 but no significant difference between HA cluster densities between high and low concentrations of TM4 (figure 3.1D). It has been shown previously that TM4 cannot easily bind to Arp2/3 (an actin binding protein whose main function is to nucleate branched actin) nucleated actin bundles (Hsiao et al., 2015), though a high concentration of TM4 may indirectly indicate formin-nucleated actin filaments. These formin-nucleated actin filaments have been observed to be more flexible (Bugyi et al., 2006; Papp et al., 2006; Ujfalusi et al., 2012) implying a less stable structure for HA clusters resulting in wider cluster areas (figure 3.1D).

Observations of formins and Arp2/3 with HA in cells has yet to be done, however, based on our observations thus far we would expect to see small, densely packed HA clusters to be associated with Arp2/3 and formins associated with spread-out or “leaking” HA clusters.

Of the four ABPs we measured, Cofilin has the lowest colocalization coefficient with HA (figure 3.1B), nonetheless, the two molecules are positively correlated. Cofilin is known to depolymerize older actin filaments to enhance filament assembly and turnover (Alberts et al., 2015) and has been identified in purified influenza virions (Shaw et al., 2008). With higher concentrations of Cofilin, we see a significant increase in both density and area of HA clusters (figure 3.1B). Near the end of the Influenza virus cycle, influenza infection enhances actin remodeling and increases Cofilin concentrations (G. Liu et al., 2014). Since Cofilin is needed for actin turnover, it is possible that the increase in Cofilin is directly related to an increase in actin filaments with increased HA density. HA clustering alters organization of actin filaments (Gudheti et al., 2013) which may be mediated by Cofilin concentrations.

Myosin is a monomeric myosin motor protein that moves along actin filaments (Alberts et al., 2015) and has been implicated in translating Golgi-derived vesicles to and from the PM (Fath et al., 1994; Montes de Oca et al., 1997). We observed that Myosin in high concentrations significantly increases HA cluster areas while not significantly changing their cluster densities (figure 3.1C). Arp2/3 mediated actin
comets, with which Myosin is associated, have been suggested as transporters of HA from the Golgi to the PM (Guerriero et al., 2006; Taunton et al., 2000). The actin-myosin network has been isolated as playing an important role in viral assembly at the PM (Kumakura et al., 2015). However, Myosin has not been identified in purified influenza virus (Shaw et al., 2008) suggesting Myosin is implicated in early assembly of HA to the PM but may be recycled before influenza budding. Increased Myosin concentrations under HA clusters may represent an increase in vesicle movement along actin filaments for HA delivery, or more simply, the HA area is directly proportional to the Myosin concentration, as Myosin is required to push HA to the PM. Quantifying how much and what kind of actin is near these regions would help elicit more details about the association between HA and Myosin, as well as observations of their dynamics.

To indirectly understand how HA may interact with PIP2, we studied the possible affect α-Actinin has on HA clustering properties (figure 3.1A). In non-muscle cells (such as NIH3T3 cells), α-Actinin binds actin filament bundles to the PM (Alberts et al., 2015) and can directly bind PIP2 molecules (Burn et al., 1985; Catimel et al., 2008; Fukami et al., 1994). α-Actinin binding to PIP2 molecules is a regulatory pathway that determines the extent of actin filament formation; observations show PIP2 inhibits α-Actinin binding to actin filaments (Fraley et al., 2003; Full et al., 2007; Sjöblom et al., 2008). In this work, we see that higher concentrations of α-Actinin significantly increase density and area of HA clusters (figure 3.1A). We theorize that α-Actinin is directly binding a palmitic acid (PIP2) on the CTD of HA. However, it is unknown how this interaction may affect actin filament bundling beneath the PM. We theorize that this interaction occurs directly following the insertion of HA molecules into the plasma membrane and is no longer necessary just before viral budding. Future observations of α-Actinin and mutated HA where the cysteines have been replaced (HA-MAY) are needed to further explore this theory.

Of the four ABPs we studied, Cofilin and TM4 have been identified in purified influenza virus (Shaw et al., 2008). However, all four ABPs are found in HA clusters (figure 3.1) strengthening previously published observations of a relationship between HA and actin (Gudheti et al., 2013). It has been
hypothesized that PIP2 has a major role in HA clustering (Curthoys et al., 2019) which has connections to a wide variety of ABP functions (Alberts et al., 2015; Catimel et al., 2008). Of the ABPs studied in this work, TM4 has not been identified to be associated with the PIP2 interactome (Catimel et al., 2008). Nevertheless, Myosin (Hokanson & Ostap, 2006), Cofilin (Zhao et al., 2010), and α-Actinin (Fukami et al., 1994) are associated with the PIP2 interactome. HA and PIP2 have been shown to colocalize at the PM and large PIP2 concentrations increase the area of HA clusters (Curthoys et al., 2019). We observed that each ABP affected the clustering of HA in either density and/or area (figure 3.1), each with their own possible effect on HA assembly at the PM. Although the study of ABPs is important to understand the relationship between HA and actin, it does not elucidate the mechanism of HA and PIP2 interactions. Expanding on the observations published in Curthoys et al. (2019), we observed fluorescently labeled PIP2 in living cells with and without HA to study the affect HA may have on the mobility of PIP2 molecules.

4.2 Influenza Hemagglutinin Affects PIP2 Mobility at the Plasma Membrane

When two molecules interact, their mobilities are modulated by the presence of the interacting partner. To determine whether the presence of HA modulates the mobility of PIP2, we needed a method to visualize the dynamics of HA and PIP2 in living cells. Previously published work has successfully demonstrated the ability to view PIP2 using super-resolution microscopy techniques (Curthoys et al., 2019; Ji et al., 2015). PIP2 can be observed at the plasma membrane (PM) using a Bodipy TMR organic dye attached to PIP2 (GloPIP) (Curthoys et al., 2019; Golebiewska et al., 2008) or with a pleckstrin homology (PH) domain from phospholipase C delta attached to a photo-switchable fluorescent protein (PH domain-Dendra2) (Curthoys et al., 2019; Hammond et al., 2009). We performed both methods of labeling of PIP2 molecules in living NIH3T3 and HAβ2 cells, measuring molecular dynamics using previously published trajectory analyses (Gudheti et al., 2013; Manley et al., 2008).
Measurement of their mean squared displacements (MSD) as a function of time revealed that both GloPIP (figure 3.3) and PH domain-Dendra2 (figure 3.4) exhibit confinement in NIH3T3 and HAb2 cells. These curves were used to calculate diffusion coefficients for both labels in NIH3T3 cells and in HAb2 cells. There are some subtle differences between calculated diffusion coefficients presented here and with the literature. GloPIP diffusion \( D_{\text{GloPIP}} = 1.13 \ \mu m^2/s \) in this work (Curthoys et al., 2019) is larger than published previously \( D_{\text{GloPIP}} = (0.8 \pm 0.2) \ \mu m^2/s \) for NIH3T3 cells (Golebiewska et al., 2008), however, the temperatures at which each experiment was conducted were 37°C and 25°C, respectively. It is known that lipid mobility does increase as a function of temperature (Cicuta et al., 2007; Filippov et al., 2004; Korlach et al., 1999; Wu et al., 1977) so the variation was expected. PH domain-Dendra2 diffusion \( D_{\text{PH}} = (0.165 \pm 0.011) \ \mu m^2/s \) in NIH3T3 cells in this work is an order of magnitude lower than PH domain-GFP diffusion \( D_{\text{PH}} = (1.24 \pm 0.14) \ \mu m^2/s \) in HEK cells (human embryonic kidney cells) (Hammond et al., 2009), with both experiments conducted at room temperature. The observed differences compared to HEK cells can thus be attributed to differing cell type.

Both PIP2 labeling methods, GloPIP (figure 3.3) and PH domain-Dendra2 (figure 3.4), exhibit enhanced confinement of PIP2 molecules in (HA-expressing) HAb2 cells in comparison to (non-HA-expressing) NIH3T3 cells. This result indicates a possible connection with HA at the PM, as the rate of diffusion for both labeling methods decreased in HAb2 cells in comparison to NIH3T3 cells (figure 3.3, figure 3.4). Both labeling methods of PIP2 show a decrease in their mean radius of mobility when in HAb2 cells compared to NIH3T3 cells (see results) indicating that the presence of HA in the PM limits the movement of PIP2 molecules. We also see a steep decrease in PH domain labeled PIP2 mobilities as we increase PIP2 density when in HAb2 cells (figure 3.5). These high-density regions, which will normally decrease PIP2 mobilities in NIH3T3 cells (figure 3.5), are significantly decreasing PIP2 mobilities in HAb2
cells. This may imply that HA is contributing to the confinement of PIP2 in high-density regions, as has been seen previously (Curthoys et al., 2019).

4.3 Influenza Hemagglutinin Modulates PIP2 Dynamics in High-Density Regions

High-density regions in the plasma membrane (PM) of cells are directly associated with clustering and unique PM organizations are potentially useful for producing infectious influenza virions (Flint et al., 2003). The study of influenza virions has revealed that HA density is important to viral budding (Ellens et al., 1990) while phosphoinositides have been shown to compartmentalize for proper signaling (Y. Liu et al., 1998). The reason for HA clustering is still widely unknown, still, HA has been seen colocalized with actin filaments (Gudheti et al., 2013) and PIP2 clusters (Curthoys et al., 2019). The presence of a link between HA and actin filaments indicates the potential utilization of a cellular signaling pathway by the virus to cluster vital components at the PM.

To understand how HA in the PM may affect PIP2 movements, we studied PIP2 (fluorescently labeled PH domain) mobilities, in living NIH3T3 and HAb2 cells. We find that PIP2 mobilities in both cell types outside of high-density regions (at densities approaching zero) approach the same value and are not significantly different from each other (figure 3.5). These highly mobile molecules ($\mu = 0.709 \pm 0.043 \frac{\mu m^2}{s}$) outside of high-density regions indicate that PIP2 can be found outside of clustered domains and are not obviously affected by HA. The finding that PIP2 can be found outside of clusters is supported by previously published work using antibody labeled PIP2 (Wang & Richards, 2012).

Quantification of the membrane regions containing a high density of PIP2 shows a significant difference between PIP2 mobilities in NIH3T3 and HAb2 cells (figure 3.5). Mobility values in HAb2 cells are always lower than in NIH3T3 cells and there is a significant difference between these distributions (figure 3.5). After curve fitting the dependence of mobility vs density for the two cell types, we saw significant differences between parameter values indicating some HA dependence on PIP2 mobilities in
all density regions. HA decreases the mobility of PIP2 molecules at any given density. This suggests that these PIP2 clusters are more crowded when HA is in the PM. This could be due to a manipulation of signaling pathways by HA in the PM (Fujioka et al., 2013; Haidari et al., 2011) or an overall change in the distribution of cellular components, such as actin (Gudheti et al., 2013). It was shown in Gudheti et al. (2013) that HA mobilities decreased with increasing actin density, suggesting that actin-associated structures could be restricting the movement of HA in the PM (Gudheti et al., 2013). We predict that as the density of PIP2 increases, it is highly likely that actin and HA are there as well. The mobility of PIP2 molecules in the densest regions, represented as $\mu_0$, drops to zero within error in HAb2 cells, indicating a density above which molecules are effectively immobile (figure 3.5). However, the error in the free diffusion parameter is large and further testing is needed for verification of this observation. Visualization of these low mobility molecules could not be addressed with our GloPIP data as low mobilities were removed due to the dye sticking to the coverslip (Curthoys et al., 2019).

Super-resolution microscopy in fixed cells has previously revealed that HA and the phosphoinositide, PIP2, colocalize and potentially interact at the PM (Curthoys et al., 2019). It was also shown that PIP2 diffusion is altered by the presence of HA in live cells (Curthoys et al., 2019, figure 3.4). In the work presented next, we explore how HA and PIP2 interact in a time-dependent manner using super-resolution microscopy of living cells, revealing additional evidence for their possible interaction.

### 4.4 Live Cell Analysis Supports Spatial Dependence of Influenza Hemagglutinin and PIP2

Studying PIP2 and influenza HA simultaneously can be used to better understand their interdependence in the PM. It was shown in fixed cells that HA and PIP2 colocalize in the plasma membrane of NIH3T3 cells using both diffraction limited and super-resolution microscopy techniques (Curthoys et al., 2019). To further study their interaction, we imaged HA-Dendra2 and the PLCδ-PH domain (a PIP2 marker) tagged with PAmKate in living NIH3T3 cells (figure 3.6) and quantified their spatial
dependence (figure 3.7, figure 3.8) using previously published measures of colocalization (Manders et al., 1992; Pearson, 1901). Using HA-Dendra2 and PH domain-PAmKate, Curthoys et al. (2019) quantified a Pearson’s correlation coefficient (Pearson, 1901) of approximately $0.20 \pm 0.04$ at a pixel width of 100-nanometers in fixed NIH3T3 cells, while this work calculated a PCC of $0.267 \pm 0.023$ at a pixel width of 80-nanometers in living NIH3T3 cells (figure 3.7). This work finds a positive correlation coefficient which supports the previously reported result that HA and PIP2 share spatial dependence in the PM (Curthoys et al., 2019). PIP2 is known to be sequestered in the PM by other membrane-associated proteins other than HA (Catimel et al., 2008; Gambhir et al., 2004; McLaughlin & Murray, 2005; Rauch et al., 2002) explaining a positive but modest PCC between PIP2 and HA (figure 3.7). Although positive in nature, the two PCC values are only moderate in magnitude despite visible overlap between the two species when rendering an image (figure 3.7). There are a few possible reasons this could be the case, however they are subtle (Dunn et al., 2011). Choosing a masked area for calculating a PCC is crucial for calculating an appropriate quantitative number as background pixels (regions of the cell not labeled, or any extracellular regions included in the mask) will add a positive correlation, inadvertently raising the average PCC (Dunn et al., 2011). Cell to cell variability in expression levels between the two fluorescent probes can average out correlation resulting in a lower PCC value than seen in any individually calculated cell (Dunn et al., 2011). These shortcomings of the coefficient (Dunn et al., 2011) could possibly explain the low, positive values.

To garner a better understand of any existing correlation between the two probes (HA-Dendra2 and PH domain-PAmKate), a second coefficient was calculated in this work, Manders’ co-localization coefficient or MCC (Manders et al., 1992). We calculate an MCC of $0.77 \pm 0.05$ for HA and $0.58 \pm 0.05$ for PIP2 with a pixel width of 80-nanometers (figure 3.8). A higher MCC value for HA indicates it is more likely for a HA molecule to be located near a PIP2 molecule. The lower PIP2 coefficient makes intuitive sense when considering the wide range of pathways and interaction partners with which PIP2 has been
associated (Czech, 2000; McLaughlin et al., 2002; Raucher et al., 2000). It is expected that the cell still requires those pathways to exist while also producing viral proteins, such as HA (and in this study the cells were not infected, but rather only expressing HA from the virus).

4.5 Influenza Hemagglutinin and PIP2 Cooperatively Modulate Membrane Organization

The phosphoinositide PIP2 is a cellular lipid found primarily on the inner leaflet of the plasma membrane (PM) (Alberts et al., 2015) and is involved in a number of cellular functions such as cell motility, adhesion, exocytosis, and endocytosis (Alberts et al., 2015; Catimel et al., 2008; McLaughlin et al., 2002). This involvement in numerous cellular functions identifies PIP2 as a primary target for understanding membrane organization (McLaughlin et al., 2002), protein-lipid interactions (Won et al., 2006), and viral infection (Curthoys et al., 2019; Favard et al., 2019; Gc et al., 2016; Johnson et al., 2018; Mücksch et al., 2017; Rocha-Perugini et al., 2014). PIP2 is known to recruit, bind, and regulate proteins at a variety of locations in the cell, but the majority of PIP2 function occurs at the PM (Czech, 2000). There is evidence of influenza virus exploiting PIP2 signaling pathways (Ehrhardt et al., 2006; Fujioka et al., 2019; Hale et al., 2006; Shin et al., 2007) and we hypothesize that PIP2 plays a major role in HA clustering (Curthoys et al., 2019). We found that the presence of HA altered PIP2 mobilities (figure 3.5), suggesting that HA and PIP2 may share a common signaling pathway. If PIP2 does play a major role in HA organization, an analysis of these molecules’ dynamics should detect potential membrane models proposed for lipid regulation of proteins (Hammond, 2016).

Viruses necessarily exploit cellular functions to properly replicate. The study of these exploitations has led to a better understanding of cellular function in general (Flint et al., 2003). Aspects of the mechanism for organization of PIP2 in the PM are still poorly understood (spatial and temporal dependence for clustering and/or synthesis), however, a variety of hypotheses have been postulated (Hammond, 2016). To better understand this mechanism, we studied the simultaneous interplay between
HA and PIP2 dynamics in living cells. HA has been used previously as a surrogate protein for observing lipid membrane models (Takeda et al., 2003) and is delivered to the PM by PIP2-dependent actin comets (Guerriero et al., 2006). In this work, we observe the frequency at which each membrane model (figure 1.1) occurs to better understand how HA may be exploiting a PIP2-dependent pathway.

4.5.1 Influenza Hemagglutinin and PIP2 Dynamics Fluctuate in Same Time-Dependent Trend

We have previously shown colocalization between HA and PIP2 in fixed cell super-resolution observations (Curthoys et al., 2019). The work presented here observed HA and PIP2 at the PM in living cells (see results). We first analyzed HA and PIP2’s dynamical fluctuations in a manner similar to fluorescent cross-correlation spectroscopy (FCCS) (Eigen & Rigler, 1994). FCCS is primarily used to measure interactions between two fluorescently labeled molecules in living cells and in vitro (Eigen & Rigler, 1994). However, FCCS is constrained by slow diffusion rates leading to unwanted photobleaching of molecules (Eigen & Rigler, 1994). Our method allows nanoscale resolution observations of slower moving molecules on a longer time scale (see methods).

The influenza lifecycle requires the dynamic transport of HA from the cell’s Golgi to the PM through PIP2-mediated actin comets (Guerriero et al., 2006). Observing the dynamics of HA and PIP2 at the PM has the potential for unlocking a correlation not previously seen. We studied the molecular flux properties of influenza HA and PIP2 at a variety of time points to visualize the probabilistic frequency of certain events (figure 3.9, table 3.6). Observations of these events at short time points (figure 3.10A) indicate a high affinity for dynamic flux while longer time points (figure 3.10B) show a substantial increase in constant levels of each protein. We also observed that all flux events over time could be fitted to the same exponential function (figure 3.11) with similar time constants (table 3.8). This dependence on timescale ($\tau$) shows that the dynamics of both species are coupled, suggesting an interaction between HA and PIP2.
To better understand how these dynamic events associate with lateral movement of molecules, we studied the lateral net flux of both PIP2 and HA independently in regions associated with these events (figure 3.12). We found that many events exhibited no lateral net flux of either species; therefore, events in this category (figure 3.12C) follow the same trends as seen in figure 3.9. The probabilistic frequencies of dynamic events with inward (figure 3.12A) and outward (figure 3.12B) lateral net flux stay the same at differing time points, which is different from all other columns (figure 3.12). This indicates the possibility that dynamic columns with lateral net flux are stable over longer time scales than regions with constant levels of one or both species. The stability of this flux over time may imply a different process is occurring when the two molecules are moving laterally, which is keeping them correlated for longer time scales. The difference between these processes cannot be discerned using this analysis and requires another experimental approach.

4.5.2 Analysis Indicates More Likely Membrane Models for Influenza Hemagglutinin and PIP2 Interactions at the PM

Hammond et al. (2016) proposed three membrane models for PIP2 membrane organization, based upon previously published experimental work (Hammond, 2016). In this work, we are suggesting two additional membrane models (figure 1.2) that could potentially explain how PIP2 organizes in the PM to interact with HA (figure 3.6). We observed the frequency at which each of these models occurs for a variety of time points. The five models in question are discussed as follows, Platforms, Megapool, Budding/Endocytosis, Exocytosis/Delivery, and Local Synthesis or Release of PIP2 (LSRP).

4.5.2.1 Platforms

The Platforms membrane model is defined as the recruitment of membrane-associated proteins to pre-clustered PIP2 domains at the PM (Hammond, 2016). Super-resolution microscopy has confirmed the presence of PIP2 domains in the PM (Curthoys et al., 2019) which strengthens the possibility for this model. It has also been widely hypothesized that HA clustering occurs at the PM (Gudheti et al., 2013;
Samuel T. Hess et al., 2007; Takeda et al., 2003). The criteria for this model include a constant PIP2 molecular flux with increasing HA molecular flux and no PIP2 lateral flux with inward HA lateral flux (figure 3.13). Our observations show ~2% of events are contributed to Platforms. Due to its low occurrence (figure 3.13), Platforms is not a probable model for HA recruitment into clusters. The low number of events for the Platform model suggests that HA clustering does not occur (to a large extent) from recruitment of HA into PIP2 domains. Although this model seems not to apply to HA recruitment or clustering, it still has the potential to explain other (native) PM-associated protein clustering and, therefore, can only be ruled out for HA using our work.

4.5.2.2 Megapool

The Megapool membrane model requires that free PIP2 molecules within the PM can be induced to cluster by a protein which attracts them (Hammond, 2016). Due to its low event rate, ~2% of the time (figure 3.13), this model is also inconsistent with our observations for HA and PIP2. We observe that PIP2 domains exist in the PM “naturally” (without HA present). These PIP2 domains may be clustered due to associations with other membrane-associated proteins, indicating that the presence of HA is not required for PIP2 clustering. Despite a few examples showing unclustered PIP2 in the PM (Ji et al., 2015), our findings, and those of many others (McLaughlin & Murray, 2005), show a highly clustered distribution of most of the PIP2 in the PM with very little unclustered PIP2. It has been suggested previously that PIP2 clustering may be cell-type dependent (Kwiatkowska, 2010); thus our findings that PIP2 is highly clustered at the PM may not hold in all cell types. Presumably, when labeling PIP2 using a PH domain, some PH domains would be able to bind free PIP2 and highlight unclustered PIP2 at the PM. However, we do not see this to a large degree in our cell type. This is evidence against the Megapool model to explain PIP2 and membrane-associated protein interactions, which would predict a substantial pool of unclustered free PIP2. An alternative to freely diffusing PIP2 specifies that MARCKS (a membrane-associated protein known to sequester PIP2 molecules) may bind free PIP2 molecules (McLaughlin et al., 2002) and release
these molecules when needed. This alternative, however, would be difficult to distinguish from the LSRP model using our method and cannot be tested in this work (thus we group Local Synthesis and Release together, see below). At the very least for HA clustering, the Megapool model does not describe our observations of HA and PIP2 dynamics at the PM.

**4.5.2.3 Budding/Endocytosis**

The Budding/Endocytosis model includes budding of a vesicle from the PM (i.e. removal of the PM outward from the cellular surface) as well as the recycling of portions of the PM through endocytosis (i.e. retraction of the PM into a vesicle inside the cell) (figure 3.13). Due to our imaging geometry (TIRF illumination at the bottom of the cell), we cannot distinguish plasma membrane budding from endocytosis. Our criteria for this model require that there be no lateral net flux of either species and that their molecular fluxes be decreasing with time. Due to this, we are unable to distinguish between these two possibilities (i.e. budding from recycling) as both events would meet the same criteria. We do observe a high probability of this model occurring (figure 3.13). PIP2 has been identified to play a role in PM endocytosis (He et al., 2017; Koch & Holt, 2012), and we predict that PIP2 is associated with HA during its extraction from the PM, either through recycling or budding events. Future observations of fluorescently labeled PIP2 and HA during viral budding could strengthen this finding.

We observe that this model occurs, on average, at the same rate as the model, Exocytosis/Delivery (figure 3.13). Although we cannot distinguish between budding and recycling, the two events necessarily remove portions of the membrane. A cell cannot sustain only removal of its plasma membrane and would therefore need delivery of components as well, which potentially explains the similarity of the frequency of the model Exocytosis/Delivery (addition of molecules into the membrane) to the frequency of Endocytosis/Budding.

Endocytic recycling of the PM occurs with both fast (1-5 minutes) and slow (10-20 minutes) rates which are highly dependent on the associated PM-based processes (Alberts et al., 2015; Hao & Maxfield,
2000; Mayor et al., 1993). In this work, we calculate a rate at which the model Budding/Endocytosis occurs at the plasma membrane to be \( R_A = (14.2 \pm 2.9) \times 10^{-3} \frac{\text{# events}}{s \cdot \mu m^2} \) (table 3.13). Correspondingly, a Budding/Endocytosis event occurs every \( 70 \pm 14 \text{ s} = 1.17 \pm 0.24 \text{ min} \), which is consistent with average fast recycling events at the PM (Hao & Maxfield, 2000; Mayor et al., 1993).

4.5.2.4 Exocytosis/Delivery

Exocytosis is the process of delivering vesicles to the plasma membrane via fusion from the Golgi (Alberts et al., 2015). This model occurs at a similar probability as that of Budding/Endocytosis. As mentioned in Budding/Endocytosis, these two models should be balanced at the plasma membrane to maintain PM area and cell membrane function. We find that the probabilities of the two events are similar (figure 3.13) and that their rates are comparable (table 3.13). Previous work proposed that the process of HA clustering was happening at the plasma membrane (Samuel T. Hess et al., 2007; Takeda et al., 2003). The Platforms and Megapool models would have been consistent with this proposal; however, we observe that the Exocytosis/Delivery model occurs with a much higher probability (figure 3.13). The definition of the Exocytosis/Delivery model as simultaneous arrival of HA and PIP2 together at the PM and the high frequency of this model together imply that the clustering of HA is occurring in intracellular compartments such as secretory vesicles, the ER, or the Golgi, prior to insertion into the PM. Not only is HA clustered before arriving at the PM, it is accompanied by PIP2 molecules which are also pre-clustered. We predict that HA and PIP2 are packaged within the Golgi to be delivered together to the PM in the same clusters.

We also observe that Exocytosis/Delivery is not \( \tau \)-dependent (table 3.12, figure 3.13). We would predict that the number of events from \( \tau = 10 \text{ s} \) to \( \tau = 30 \text{ s} \) would increase by three-fold, as with \( \tau = 30 \text{ s} \) there would be three times as much time for delivery events to occur in comparison to \( \tau = 10 \text{ s} \). However, we see that the number of events is relatively constant. If the number of zones (regions associated with this model) used for Exocytosis/Delivery increases with time, we would expect to see the number of events to increase with \( \tau \). We, however, observe a steady number of events as \( \tau \) increases implying that the number
of zones used for Exocytosis/Delivery may not be increasing with time, but is rather staying constant. This may suggest that the same zones along the PM are being re-used for Exocytosis/Delivery events over long periods of time. Future work, for example using an endocytosis inhibitor, is needed to further test this possibility.

4.5.2.5 Local Synthesis or Release of PIP2 (LSRP)

Lastly, the model LSRP, which was originally named Local Synthesis in Hammond et al. (2016), indicates the synthesis of PIP2 at a localized region or the release of PIP2 molecules at clustered domains of membrane-associated proteins (Hammond, 2016). This model requires that there be no lateral flux of either species within a designated region, and that the PIP2 molecular flux is increasing with constant HA molecular flux. As mentioned previously when discussing the Megapool model, it is difficult to distinguish this PIP2 molecular increase arising from direct synthesis of PIP2 underneath the PM from the release of free PIP2 by sequestering proteins, such as MARCKS. In order to distinguish between the two events, additional observations of phosphoinositide kinases (enzymes required for synthesis) and/or MARCKS (sequestering protein) are needed alongside PIP2 and a membrane-associated protein. The low occurrence, ~15% of events (figure 3.13), for this model suggests it is not the dominant mode of HA and PIP2 interactions at the PM. However, other membrane-associated proteins could utilize this model for recruitment of PIP2 molecules to a greater or lesser degree.

4.6 A Proposed Model for Influenza Hemagglutinin Clustering at the Plasma Membrane

Observations of HA at the PM co-localized with phosphoinositides (Curthoys et al., 2019, figure 3.7) and actin binding proteins (figure 3.1, figure 3.2), in addition to previously discovered associations with actin (Gudheti et al., 2013), led us to propose an integrated model for HA clustering (figure 4.1). We have observed that HA affects the motion of PIP2 molecules at the PM (figure 3.4, figure 3.5) and that HA and PIP2 are associated in a time-dependent manner (figure 3.9). PIP2 and HA are most frequently
delivered together to the PM and persist in clusters together long enough to be removed through either recycling methods or budding (figure 3.13). We observed that actin binding protein concentrations affect the clustering properties of HA, strengthening the theory that actin plays a vital role in HA clustering (figure 3.2).

Our observations lead us to suggest that HA clustering may be occurring before insertion into the PM (figure 4.1A). Phosphatidylinositol-4-phosphate 5-kinase (PI5K)-mediated actin comets deliver vesicles containing HA0 (the precursor to HA) trimers to the PM from the Golgi (Guerriero et al., 2006). It has been observed that HA0 trimerizes before entering the PM (Constance S. Copeland et al., 1988). These vesicles contain phosphatidylinositol 4-phosphate (PI(4)P) molecules (Guerriero et al., 2006) which PI5K phosphorylates (by addition of a phosphate group to the phosphoinositide head group at position 5) to produce PIP2 molecules (Alberts et al., 2015). We observed that HA and PIP2 arrive together at the PM at a high frequency (figure 3.13). If PI(4)P is located near HA trimers within the vesicle (figure 4.1A), their phosphorylation by PI5K at the PM after vesicle insertion would explain our observations of PIP2 being delivered at the same time as HA (figure 3.13). We propose that HA clustering is mediated by phosphoinositides before arrival at the PM.

After insertion into the PM, HA trimer clustering persists due to actin, ABPs, and phosphoinositides (figure 4.1B). HA trimers cluster at the PM (Chen et al., 2005; Gudheti et al., 2013; Samuel T. Hess et al., 2007; Takeda et al., 2003) and colocalize with PIP2 molecules (Curthoys et al., 2019, figure 3.7). HA and PIP2 colocalize at the PM long enough to be removed together via natural cellular processes (figure 3.13). This implies that PIP2 plays a major role in HA remaining clustered long enough for 1) the delivery of other viral components, 2) for viral budding to begin, or 3) cellular recycling. We theorize that the link between HA and actin is through their mutual associations with PIP2. HA dynamics and cluster morphologies are both mediated by actin rich membrane regions of the PM (e.g. HA is found colocalized with actin) (Gudheti et al., 2013; Simpson-Holley et al., 2002). PIP2 plays a vital role in actin
polymerization, reorganization, and depolymerization through actin binding proteins (ABPs) (Alberts et al., 2015; Catimel et al., 2008) and we observed some level of HA colocalization with the four ABPs examined: α-actinin, coflin 1, myosin 1, and tropomyosin 4 (figure 3.1). Differing concentrations of the ABPs affect HA clustering properties (figure 3.2), implying they may play a vital role to HA clustering at the PM.

It has been theorized that actin filaments compartmentalize regions of the plasma membrane and potentially restrict motion of membrane-associated proteins and lipids (Kusumi & Sako, 1996; Sako & Kusumi, 1994). This model predicts that actin would function as a “fence” creating compartments between 40 and 300nm in size (Kusumi et al., 2012) allowing membrane-associated proteins and lipids to freely diffuse within these compartments, however, requiring the molecules to “hop” the “fence” to move between adjacent compartments, potentially slowing diffusion at the boundaries (Fujiwara et al., 2002; Kusumi et al., 2012). It has been observed that actin compartments, or “fences”, can section membrane-associated proteins into smaller domains and modulate their motion (Heinemann et al., 2013b, 2013a; Sadegh et al., 2017). We theorize that actin is anchored to the PM through its interaction with PIP2 and ABPs which may be the mechanism holding HA clusters together, limiting diffusion, and preventing the clusters from falling apart over time (figure 4.1B), while being distinct from a fence in that the HA is colocalized with the actin-rich regions rather than being surrounded by them.
Figure 4.1: Proposed Model for Influenza Hemagglutinin Clustering at the Plasma Membrane. A proposed model for HA clustering at the PM. (A) PISK-mediated actin comets deliver a vesicle containing HA0 trimers and PI(4)P to the PM. (B) PI(4)P is then converted into PIP2 molecules at the PM near HA trimers and delivered to the PM together. These HA trimers co-localize with PIP2, α-actinin, tropomyosin 4, myosin 1, coflin 1, and actin filaments. 1) PIP2 is primarily found in the inner leaflet of the plasma membrane (McLaughlin et al., 2002; McLaughlin & Murray, 2005). 2) PI(4)P is found on the outer leaflet of vesicles transported via PISK-mediated actin comets (Guerriero et al., 2006; Ueno et al., 2011). 3) HA0, the precursor to HA, trimerizes before insertion into the plasma membrane (C. S. Copeland et al., 1986; Constance S. Copeland et al., 1988). 4) PISK-mediated actin comets deliver vesicles containing HA trimers to the plasma membrane (Guerriero et al., 2006). 5) HA trimers cluster at the plasma membrane (Chen et al., 2005; Gudheti et al., 2013; Samuel T. Hess et al., 2007; Takeda et al., 2003). 6) PIP2 colocalizes with HA at the plasma membrane (Curthoys et al., 2019, figure 3.7). 7) HA may interact with PIP2 via the CTD (Parent, 2020). 8) α-actinin colocalizes with HA (figure 3.1). 9) Myosin 1 colocalizes with HA (figure 3.1). 10) Tropomyosin 4 colocalizes with HA and binds to actin filaments (Alberts et al., 2015, figure 3.1). 11) Actin colocalizes with HA and modulate HA mobility (Gudheti et al., 2013, Simpson-Holley et al., 2002). 12) Cofilin 1 colocalizes with HA (Gudheti et al., 2013, figure 3.1).
5.1 Conclusion

Influenza hemagglutinin (HA) is found in nanoscale clusters at the plasma membrane (PM) (Samuel T. Hess et al., 2007). These clusters have been shown to colocalize with actin (Gudheti et al., 2013; Simpson-Holley et al., 2002) and we observed that HA colocalizes with four actin binding proteins (ABPs), α-actinin, coflin 1, myosin 1, and tropomyosin 4 (figure 3.1). Higher concentrations of these ABPs showed altered HA cluster properties (figure 3.2) implying they may have a connection to HA clustering at the PM. Of the four ABPs we studied, three are directly connected to the PIP2 interactome (Catimel et al., 2008): α-actinin (Fukami et al., 1994), coflin 1 (Zhao et al., 2010), and myosin 1 (Hokanson & Ostap, 2006), while two of the ABPs are found in purified flu virus, coflin 1 and tropomyosin 4 (Shaw et al., 2008). PIP2 is found to colocalize with HA (Curthoys et al., 2019, figure 3.7) as well as its dynamics being modulated by HA (Curthoys et al., 2019, figure 3.3, figure 3.4, figure 3.5) implying a direct connection between the two molecules. Further analysis of PIP2 and HA molecules in living cells revealed a time-dependent trend among their molecular flux (figure 3.9) reinforcing the theory that there exists a direct connection between the two.

PIP2 is found primarily in the inner leaflet of the plasma membrane and is used in a variety of cellular functions (Alberts et al., 2015; Catimel et al., 2008; McLaughlin et al., 2002). PIP2 has been used as a primary target for understanding membrane organization (McLaughlin et al., 2002), protein-lipid interactions (Won et al., 2006), and viral infection (Curthoys et al., 2019; Favard et al., 2019; Gc et al., 2016; Johnson et al., 2018; Mücksch et al., 2017; Rocha-Perugini et al., 2014). How PIP2 concentrates at the PM to fulfill its cellular role is widely unknown but membrane models have been postulated (Hammond, 2016). Due to their physiology, viruses exploit cellular functions to replicate and the study of
this exploitation has led to a better understanding of cell membrane organization (Flint et al., 2003). In this work, we looked at how HA, a membrane-associated protein, potentially affects PIP2 and quantified the likelihood of each possible membrane model (figure 3.13). Our analysis found that PIP2 and HA are delivered together to the PM a high percentage of the time and that these clusters persist long enough to be removed from the PM together (figure 3.13). We propose a model for how HA clusters at the PM based on our findings and previously discovered associations. HA is delivered to the PM via PI5K-mediated actin comets alongside PIP2. These HA clusters contain actin filaments, which reduces molecular lateral diffusion, allowing clusters to persist for long time periods. We observed actin binding proteins beneath HA clusters. These ABPs mediate actin filaments and we theorize that this mediation is involved in the persistence of HA clusters.

5.2 Possible Future Experimental Directions

To better understand the mechanisms behind Influenza HA clustering properties, more information regarding its effect on and/or by cellular phosphoinositides and cellular proteins is needed. Our work observed the enhanced confinement of PIP2 molecules in HAb2 cells versus NIH3T3 cells using two labeling methods (figure 3.3, figure 3.4). Can HA diffusion also be confined when in the presence of PIP2 molecules? A possible experiment to test this theory would be to quantify the confinement of HA molecules in clusters with and without PIP2 present, and vice versa for PIP2 clusters with and without HA, using a two-color super-resolution technique in living cells. In this work, we tested colocalization of HA with four actin binding proteins (ABPS), α-actinin, cofilin 1, myosin 1, and tropomyosin 4. Are there other ABPs that colocalize with HA? A variety of other ABPs are found in purified flu virus (Shaw et al., 2008) and some worthy candidates to look at are profilin, tropomyosin 1, and tropomyosin 3. ABPs not found in purified flu virus can also be imaged to better understand the end of the viral life cycle. ABPs which may colocalize with HA but are not found in virus particles may be important for HA delivery via actin
comets or play a vital role in HA clusters persisting at the PM. To quantify these possibilities, imaging of HA clusters with ABPS in living cells using super-resolution techniques may be the next step.

HA clusters at the PM with actin rich membrane regions (Gudheti et al., 2013; Simpson-Holley et al., 2002) and is found colocalized with PIP2 (Curthoys et al., 2019, figure 3.7). Is HA clustering associated with both molecules simultaneously or are these findings independent? To better understand this narrative, studying HA, PIP2, and actin in living cells via a 3-color super-resolution experiment would view all three molecules at the PM simultaneously. By studying living cells, and collecting dynamic information about all three molecules, it is possible to quantify confinement of HA and PIP2 near actin rich regions.
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APPENDIX A:

MATLAB CODES

A.1. color1_MobilityvsDensity_plotter_v2

```matlab
% Mobility vs Density plotter: for 1-color data only
% [JW] 2020

% User options:
boxsize   = 0.080;       % in um
framerate = 50.63;       % in Hz
timeperframe = 1/framerate;  % time per frame in seconds.
binwidth  = 1;          % width of binning for density

% Load Files in Folder:
% HAb2:
folder = '';
fileslist = dir(fullfile(folder,'*traj.mat'));

% Run function:
[densityh,averagemobh,err_averagemobh,mobilitiesh,standarddevh,mobilities_factorh] =
color1_MobilityvsDensity_v6(boxsize,timeperframe,binwidth,fileslist,folder);
denlimith = densityh<6000;

% Plotting HAb2:
figure
errorbar(densityh(denlimith),averagemobh(denlimith),err_averagemobh(denlimith),'bo')
hold on

% Load Files in Folder:
% NIH3T3:
folder = '';
fileslist = dir(fullfile(folder,'*traj.mat'));

% Run function:
[densityn,averagemobn,err_averagemobn,mobilitiesn,standarddevn,mobilities_factorn] =
color1_MobilityvsDensity_v6(boxsize,timeperframe,binwidth,fileslist,folder);
denlimitn = densityn<6000;

% Plotting NIH3T3:
```
errorbar(densityn(denlimitn),averagemobn(denlimitn),err_averagemobn(denlimitn ),'.','Color',[0.8500 0.3250 0.0980],'LineWidth',1,'MarkerSize',10)
xlabel({'Density of PIP2 Molecules','(PIP2 Molecules per \mum^2)'});
ylabel({'Mobility of PIP2 Molecules','(\mum^2 per Second)'});
% % title('Mobility of Molecules vs. Density of Molecules','FontSize',16)
legend({'HAb2','NIH3T3'})
ylim([0 1])
set(gca,'YTick',[0:0.1:1]);
grid on

% Plotting fitted functions:
% (Values taken from fitting program)
% NIH3T3:
mob_n0 = 0.48874;
A_n = 0.22006;
alpha_n = 1972.13421;
fitted_n = mob_n0 + A_n*exp(-densityn(denlimitn)./alpha_n);
% HAb2:
mob_h0 = -0.00164;
A_h = 0.70984;
alpha_h = 3527.72044;
fitted_h = mob_h0 + A_h*exp(-densityh(denlimith)./alpha_h);
%Plot:
plot(densityh(denlimith),fitted_h,'--','Color',[0 0.4470 0.7410],'LineWidth',1)
plot(densityn(denlimitn),fitted_n,'--','Color',[0.8500 0.3250 0.0980],'LineWidth',1)
hold off
legend({'HAb2','NIH3T3','HAb2 curve fit','NIH3T3 curve fit'})
clear all;

% User Variables:
time_change  = 20; % Time chunk in seconds
endtimeloop  = 6; % Total time changes (i.e. which chunk of frames are you interested in?)
f0           = 1; % Starting frame
interlaced   = 1; % interlaced == 0 if you want A vs B, C vs D THIS HALF'S FINAL ENDTIMELOOP*
check        = 0; % Will display checks for each cell in data set.
min_delta    = 3; % Minimum change in HA or PIP2 grid plot to count as "different"
change_thresh= 2; % Minimum influx / outflux (# particles)
dx           = 0.080; % Bin width in micrometers
frames_per_second = 60; % Frame rate taken from data
startfilenum = 1; % Starting file number
endfilenum   = 27; % Ending file number
inpath = ''; % File path
infile = '*combined.mat';

% CODE STARTS HERE:
disp('Batching:')

% File input continued:
inpathsearch = strcat(inpath,infile);
filelist = dir(inpathsearch);
nfiles = length(filelist);

% Timeloop:

% Normalize Grids:

% normalize_grid = normalize_grids_to_np
% % Normal grids to number of points multiplied by 30,000(?)
% % Time per frame:
time_per_frame = 1/frames_per_second; % Time per frame

% TIMELOOP FRAMES: 

if interlaced == 1
    for u = 1:endtimeloop
        % PREFRAME
        preframe(u,1) = floor(f0+df*(u-1)+(u-1));
        preframe(u,2) = floor(f0+df*u+(u-1));
        % POSTFRAME
        postframe(u,1) = floor(f0+df*u+u);
    end
end
postframe(u,2) = floor(f0+df*(u+l)+u);
end
last_frame = postframe(endtimeloop,2);
disp(['Last Frame: ',num2str(last_frame)])
end

if interlaced == 0
    for u = 0:floor(endtimeloop/2)-1
        v = 2*u+1;
        % PREFRAME
        preframe(u+1,1) = floor(f0+df*(v-1)+(v-1));
        preframe(u+1,2) = floor(f0+df*v+(v-1));
        % POSTFRAME
        postframe(u+1,1) = floor(f0+df*v+v);
        postframe(u+1,2) = floor(f0+df*(v+1)+v);
    end
    last_frame = postframe(floor(endtimeloop/2),2);
disp(['Last Frame: ',num2str(last_frame)])
endtimeloop = floor(endtimeloop/2);
end

%% FILE LOOP: -------------------------------------------------------------
for fileloop = startfilenum:endfilenum
    % LOAD IN FILES: -------------------------------------------------------------
    infile = strcat(inpath,filelist(fileloop).name);
    load(infile);
    disp('...')
    disp([num2str(fileloop),'/',num2str(endfilenum)])
    % Matrices needed for <TIMELOOP> iterations:
    num_change_all_total = [];
    percent_change_all_timeloop = [];
    % Creating a mask
    gw2 = ceil(max([max(Gdata.xf_all),max(Gdata.yf_all),max(Rdata.xf_all),max(Rdata.yf_all)]).*Gdata.q)+.01;
    % Maximum width
    xbins2 = (0:dx:gw2)+0.5*dx;  % x-axis length
    ybins2 = (0:dx:gw2)+0.5*dx;  % y-axis length
    mask = zeros(numel(ybins2),numel(xbins2));

    % TIME LOOP: -------------------------------------------------------------
    for timeloop = 1:endtimeloop
        disp(['Timeloop: ',num2str(timeloop),'/',num2str(endtimeloop)])

        % BINNING FOR CELL MATRICES: -------------------------------------------------------------
        % NEVER GO TO 10 NANOMETERS.
        gw = ceil(max([max(Gdata.xf_all),max(Gdata.yf_all),max(Rdata.xf_all),max(Rdata.yf_all)]).*Gdata.q)+.01;  % Maximum width
        xbins = (0:dx:gw)+0.5*dx;  % x-axis length
        ybins = (0:dx:gw)+0.5*dx;  % y-axis length
        edges = cell(2,1);  % Cell matrix
        edges{1} = xbins;
        edges{2} = ybins;

        % PRE -------------------------------------------------------------
        % Indices of pre framed localizations

120
ind_green_pre = find(Gdata.framenum_all>=preframe(timeloop,1) &
Gdata.framenum_all<=preframe(timeloop,2));  % GREEN
ind_red_pre = find(Rdata.framenum_all>=preframe(timeloop,1) &
Rdata.framenum_all<=preframe(timeloop,2));  % RED

% x, y coordinates of GREEN
x1i = Gdata.xf_all(ind_green_pre)*Gdata.q;  % GREEN x coordinates
y1i = Gdata.yf_all(ind_green_pre)*Gdata.q;  % GREEN y coordinates

% x, y coordinates of RED
x2i = Rdata.xf_all(ind_red_pre)*Gdata.q;  % RED x coordinates
y2i = Rdata.yf_all(ind_red_pre)*Gdata.q;  % RED y coordinates

% Pre Grid Plot
% Matrices with Pre Coordinates
X1i = [x1i y1i];  % GREEN axis length
X2i = [x2i y2i];  % RED axis length

% Plot of Pre data
grid_green_pre = hist3(X1i,edges);  % GREEN
grid_red_pre = hist3(X2i,edges);  % RED
% Normalization of grids to points
if normalize_grids_to_np == 1
    grid_green_pre = grid_green_pre*30000/length(ind_green_pre);
    grid_red_pre = grid_red_pre*30000/length(ind_red_pre);
end

% Thresholded Pre data
green_thresh_pre = 20*mean(grid_green_pre(:));
red_thresh_pre = 20*mean(grid_red_pre(:));
grid_green_pre_thresh = double(grid_green_pre>green_thresh_pre);  % FINAL GREEN
grid_red_pre_thresh = double(grid_red_pre>red_thresh_pre);  % FINAL RED

% Merging Pre Color Plots
grid_size = size(grid_green_pre);
grid_merge_pre = zeros(grid_size(1),grid_size(2),3);
grid_merge_pre(:,:,1) = grid_red_pre_thresh';  % RRED
grid_merge_pre(:,:,2) = grid_green_pre_thresh';  % GREEN
grid_merge_pre(:,:,3) = grid_red_pre_thresh';  % BOTH

% POST ---------------------------------------------------------------
% indices of post framed localizations
ind_green_post = find(Gdata.framenum_all>=postframe(timeloop,1) &
Gdata.framenum_all<=postframe(timeloop,2));  % Indices of green data in frame range
ind_red_post = find(Rdata.framenum_all>=postframe(timeloop,1) &
Rdata.framenum_all<=postframe(timeloop,2));  % Indices of red data in frame range

% x, y coordinates of Green
x1f = Gdata.xf_all(ind_green_post)*Gdata.q;  % HA x values
y1f = Gdata.yf_all(ind_green_post)*Gdata.q;  % HA y values

% x, y coordinates of Red
x2f = Rdata.xf_all(ind_red_post)*Gdata.q;  % PIP2 x values
y2f = Rdata.yf_all(ind_red_post)*Gdata.q;  % PIP2 y values

% Post Grid Plot
% Matrices with Post Coordinates
X1f = [x1f y1f];  % GREEN axis length
X2f = [x2f y2f];  % RED axis length

% Plot of Pre data
grid_green_post = hist3(X1f,edges);  % GREEN
grid_red_post = hist3(X2f,edges);  % RED
% Normalization of grids to points
if normalize_grids_to_np == 1
    grid_green_post = grid_green_post*30000/length(ind_green_post);
    grid_red_post   = grid_red_post*30000/length(ind_red_post);
end

% Thresholded Post data
green_thresh_post = 20*mean(grid_green_post(:));
red_thresh_post   = 20*mean(grid_red_post(:));
green_post_threshold = double(grid_green_post>green_thresh_post); %
FINAL
grid_red_post_threshold = double(grid_red_post>red_thresh_post); %
FINAL

% Merging Post Color Plots
grid_merge_post = zeros(grid_size(1),grid_size(2),3); % grid_size is
above in PRE section.
grid_merge_post(:,:,1) = grid_red_post_threshold'; % RED
grid_merge_post(:,:,2) = grid_green_post_threshold'; % GREEN
grid_merge_post(:,:,3) = grid_red_post_threshold'; % BOTH

%% CHANGE IN PRE AND POST (POST MINUS PRE) ------------------------
% Finding zeroed date in both PRE and POST Grid Plot
grid_green_bothzero = double(grid_green_pre==0 & grid_green_post==0);
% When green grid is zero
grid_red_bothzero   = double(grid_red_pre==0 & grid_red_post==0);
% When red grid is zero
% Difference in GREEN pre and post
diff_grid_green = double((grid_green_post-
grid_green_pre)>=min_delta)-double((grid_green_pre-
grid_green_post)>=min_delta)+2*grid_green_bothzero;
% Difference in RED pre and post
diff_grid_red   = double((grid_red_post-
grid_red_pre)>=min_delta)-
double((grid_red_pre-
grid_red_post)>=min_delta)+2*grid_red_bothzero;

%% CONDITION MATRICES BELOW: --------------------------------------
% Increased GREEN at Constant RED
up_green_constant_red   = double(diff_grid_green==1 &
diff_grid_red==0);
% Decreased GREEN at Constant RED
down_green_constant_red = double(diff_grid_green==-1 &
diff_grid_red==0);
% Increased GREEN at Increased RED
up_green_up_red   = double(diff_grid_green==1 & diff_grid_red==1);
% Decreased GREEN at Increased RED
down_green_up_red   = double(diff_grid_green==-1 & diff_grid_red==1);
% Increased GREEN at Decreased RED
up_green_down_red   = double(diff_grid_green==1 & diff_grid_red==-1);
% Decreased GREEN at Decreased RED
down_green_down_red = double(diff_grid_green==-1 & diff_grid_red==-1);
% Increased RED at Increased GREEN
up_red_up_green   = double(diff_grid_green==1 & diff_grid_red==1);
% Decreased RED at Increased GREEN
down_red_up_green   = double(diff_grid_green==1 & diff_grid_red==-1);
% Increased RED at Constant GREEN
up_red_constant_green= double(diff_grid_green==0 & diff_grid_red==1);
% Decreased RED at constant GREEN
down_red_constant_green = double(diff_grid_green == 0 & diff_grid_red == -1);

% Constant RED at constant GREEN
constant_red_constant_green = double(diff_grid_green == 0 & diff_grid_red == 0);

% No data is where GREEN and RED are not together.
no_data = double(grid_green_bothzero | grid_red_bothzero);
% Is data is where GREEN and RED are together.
is_data = 1 - no_data;
is_data_tot_num = size(find(is_data == 1),1); % Total number of boxes where two species are present.
mask = mask | is_data;

% Include all changes above in single matrix named "change_all" with below parameters:
% 1 == +GREEN =RED
% 2 == -GREEN =RED
% 3 == +GREEN +RED
% 4 == -GREEN +RED
% 5 == +GREEN -RED
% 6 == -GREEN -RED
% 7 == =GREEN =RED
% 8 == =GREEN +RED
% 9 == =GREEN -RED
% 10 == NO DATA
change_all = up_green_constant_red + 2 * down_green_constant_red + ...
    3 * up_green_up_red + 4 * down_green_up_red + 5 * up_green_down_red + ...
    6 * down_green_down_red + 7 * constant_red_constant_green + ...
    8 * up_red_constant_green + 9 * down_red_constant_green + 10 * no_data;

% Check that the above changes are independent of each other:
if check == 1
    % Check1 will equal zero if the changes are found in independent boxes.
    check1 = up_green_constant_red & down_green_constant_red &
    up_green_up_red & down_green_up_red & up_green_down_red &
    constant_red_constant_green;
    if isempty(find(check1 == 1))
        disp('change_all Check.')
    else
        disp('change_all Fail.')
    end
    if is_data == (is_data & change_all)
        disp('is_data Check.')
    else
        disp('is_data Fail.')
    end
end

% NUMBER OF EVENTS IN EACH CONDITION FROM CHANGE_ALL: 
num_change_all(1,1) = length(find(change_all == 1)); % 1 == +GREEN =RED
num_change_all(1,2) = length(find(change_all == 2)); % 2 == -GREEN =RED
num_change_all(1,3) = length(find(change_all == 3)); % 3 == +GREEN +RED
num_change_all(1,4) = length(find(change_all == 4)); % 4 == -GREEN +RED
num_change_all(1,5) = length(find(change_all == 5)); % 5 == +GREEN -RED
num_change_all(1,6) = length(find(change_all == 6)); % 6 == -GREEN -RED
num_change_all(1,7) = length(find(change_all == 7)); % 7 == =GREEN =RED
num_change_all(1,8) = length(find(change_all==8)); % 8 == =GREEN +RED
num_change_all(1,9) = length(find(change_all==9)); % 9 == =GREEN -RED
% Concatinate as <TIMELOOP> iterates:
num_change_all_total = vertcat(num_change_all_total,num_change_all);

%% NORMALIZATION (PERCENT) OF EVENTS FROM CHANGE_ALL: ----------
% total number of change_all events in a cell for <TIMELOOP> ==
total_num_change_all

total_num_change_all_timeloop(1,timeloop) = sum(num_change_all);
% Normalization of change_all conditions for <TIMELOOP>
percent_change_all(1,:) = (num_change_all(1,:)/total_num_change_all_timeloop(1,timeloop)).*100;
% Delete zero rows If necessary:
logi1=isnan(percent_change_all);
percent_change_all(logi1(:,1)==1,:)=[ ];
% Concatinate as <TIMELOOP> iterates
percent_change_all_timeloop = vertcat(percent_change_all_timeloop,percent_change_all);

%% FINDING GAINED AND LOST MOLECULES FOR LATERAL RED DATA: -------
% This will render o,x from trajectories during pre & post
traj_minframe = preframe(timeloop,1);
traj_maxframe = postframe(timeloop,2);
q = Rdata.q; % q is the conversion from pixels to microns
% Creating grid plots for gained RED and lost RED:
size_traj_red = size(Rdata.trajectories); % Finds size of red trajectories
ntraj_red = size_traj_red(1); % Length of how many trajectories are in cell
grid_width_pixels = ceil(gw/dx); % Number of pixels in binned data
gain_grid_red = zeros(grid_width_pixels,grid_width_pixels); % Plot in pixels for gained RED
loss_grid_red = zeros(grid_width_pixels,grid_width_pixels); % Plot in pixels for lost RED

Grid in pixels where there is RED trajectory data stored.
% Plotting trajectories in grid plots for RED molecules:
for i = 1:ntraj_red % i cycles through RED trajectories
ind_ith_traj = Rdata.trajectories(i,:); % Index of the ith trajectory
    ind_first_zero = find(ind_ith_traj==0,1,'first'); % Find where the first zero in the trajectory is located
    if length(ind_first_zero)>0
        ind_ith_traj = ind_ith_traj(1:(ind_first_zero-1)); % Removes zeroes from the end of the trajectory
    end
    if Rdata.framenum_all(ind_ith_traj(1))>traj_minframe && Rdata.framenum_all(ind_ith_traj(1))<traj_maxframe
        xpi = q*Rdata.xf_all(ind_ith_traj); % x coordinates of trajectory
        ypi = q*Rdata.yf_all(ind_ith_traj); % y coordinates of trajectory
        xpi_int = floor(xpi/dx)+1; % Bin x coordinates
        ypi_int = floor(ypi/dx)+1; % Bin y coordinates
        dxpi_int = abs(diff(xpi_int)); % find difference between x coordinates

124
\[ dy_{\text{int}} = \text{abs}(\text{diff}(y_{\text{int}})); \] % find difference between y coordinates
\[ dx_{\text{yint}} = dx_{\text{yint}} + dy_{\text{int}}; \] % Add xy coordinates
\[ \text{ind \_ changed \_ box} = \text{find}(dx_{\text{yint}}>0)+1; \] % Index of molecules that changed boxes
\[ \text{traj \_ red \_ grid \_ data}(x_{\text{int}},y_{\text{int}}) = \text{traj \_ red \_ grid \_ data}(x_{\text{int}},y_{\text{int}})+1; \] % Increments by 1 when molecule from trajectory is found in grid.
\[ \text{ind \_ changed \_ box} = \text{find}(dx_{\text{yint}}>0)+1; \] % Index of molecules that changed boxes
\[ \text{traj \_ red \_ grid \_ data}(x_{\text{int}},y_{\text{int}}) = \text{traj \_ red \_ grid \_ data}(x_{\text{int}},y_{\text{int}})+1; \] % Increments by 1 when molecule from trajectory is found in grid.
\[ \text{gain \_ grid \_ red}(x_{\text{int}}(\text{ind \_ changed \_ box}(j)),y_{\text{int}}(\text{ind \_ changed \_ box}(j))) = \text{gain \_ grid \_ red}(x_{\text{int}}(\text{ind \_ changed \_ box}(j)),y_{\text{int}}(\text{ind \_ changed \_ box}(j)))+1; \]
\[ \text{loss \_ grid \_ red}(x_{\text{int}}(\text{ind \_ changed \_ box}(j)-1),y_{\text{int}}(\text{ind \_ changed \_ box}(j)-1)) = \text{loss \_ grid \_ red}(x_{\text{int}}(\text{ind \_ changed \_ box}(j)-1),y_{\text{int}}(\text{ind \_ changed \_ box}(j)-1))+1; \]
\[ \text{end} \]
\[ \text{end} \]
\[ \text{end} \]
\[ \% FINDING GAINED AND LOST MOLECULES FOR LATERAL GREEN DATA: ------ \]
\[ \% Creating grid plots for gained GREEN and lost GREEN: \]
\[ \text{size \_ traj \_ green} = \text{size}(\text{Gdata.trajectories}); \] % Find size of GREEN trajectories
\[ \text{ntraj \_ green} = \text{size \_ traj \_ green}(1); \] % Lenght of how many trajectories are in cell
\[ \text{gain \_ grid \_ green} = \text{zeros}(\text{grid \_ width \_ pixels},\text{grid \_ width \_ pixels}); \] % Plot in pixels for gained GREEN
\[ \text{loss \_ grid \_ green} = \text{zeros}(\text{grid \_ width \_ pixels},\text{grid \_ width \_ pixels}); \] % Plot in pixels in lost GREEN
\[ \text{traj \_ green \_ grid \_ data} = \text{zeros}(\text{grid \_ width \_ pixels},\text{grid \_ width \_ pixels}); \] % Grid in pixels where there is GREEN trajectory data stored.
\[ \% Plotting trajectories in grid plots for GREEN molecules: \]
\[ \% i cycles through GREEN trajectories \]
\[ \text{ind \_ ith \_ traj} = \text{Gdata.trajectories}(i,:); \]
\[ \text{ind \_ first \_ zero} = \text{find}(\text{ind \_ ith \_ traj}==0,1,'first'); \]
\[ \% If length(ind \_ first \_ zero)>0 \]
\[ \text{ind \_ ith \_ traj} = \text{ind \_ ith \_ traj}(1:(\text{ind \_ first \_ zero}-1)); \]
\[ \% If Gdata.framenum \_ all(ind \_ ith \_ traj(1))>\text{traj \_ minframe} \&\& \]
\[ \text{Gdata.framenum \_ all(ind \_ ith \_ traj(1))<\text{traj \_ maxframe} \]
\[ \text{xpi} = q*\text{Gdata.xf \_ all(ind \_ ith \_ traj)}; \]
\[ \text{ypi} = q*\text{Gdata.yf \_ all(ind \_ ith \_ traj)}; \]
\[ \text{xpi \_ int} = \text{ceil}(x_{\text{pi}}/dx); \]
\[ \text{ypi \_ int} = \text{ceil}(y_{\text{pi}}/dx); \]
\[ \text{dxpi} = \text{abs}(\text{diff}(x_{\text{pi}})); \]
\[ \text{dypi} = \text{abs}(\text{diff}(y_{\text{pi}})); \]
\[ \text{dxpi} = \text{dxpi} + dy_{\text{int}}; \]
\[ \text{ind \_ changed \_ box} = \text{find}(dx_{\text{yint}}>0)+1; \]
\[ \text{traj \_ green \_ grid \_ data}(x_{\text{int}},y_{\text{int}}) = \text{traj \_ green \_ grid \_ data}(x_{\text{int}},y_{\text{int}})+1; \] % Increments by 1 when molecule from trajectory is found in grid.
\[ \% If Gdata.framenum \_ all(ind \_ ith \_ traj(1))>\text{traj \_ minframe} \&\& \]
\[ \text{Gdata.framenum \_ all(ind \_ ith \_ traj(1))<\text{traj \_ maxframe} \]
\[ \text{gain \_ grid \_ green}(x_{\text{int}}(\text{ind \_ changed \_ box}(j)),y_{\text{int}}(\text{ind \_ changed \_ box}(j))) = \text{gain \_ grid \_ green}(x_{\text{int}}(\text{ind \_ changed \_ box}(j)),y_{\text{int}}(\text{ind \_ changed \_ box}(j)))+1; \]
loss_grid_green(xpi_int(ind_changed_box(j)-1),ypi_int(ind_changed_box(j)-1)) =
loss_grid_green(xpi_int(ind_changed_box(j)-1),ypi_int(ind_changed_box(j)-1))+1;
    end
    end
    end

%% NET GRID PLOTS RED AND GREEN: --------------------------------------
% Matrices with lateral RED and GREEN flux data
net_gain_grid_red   = gain_grid_red - loss_grid_red;
net_gain_grid_green = gain_grid_green - loss_grid_green;
% Matrices containing net RED and GREEN flux
flux_red_net   = zeros(grid_width_pixels,grid_width_pixels);
flux_green_net = zeros(grid_width_pixels,grid_width_pixels);
% NET RED DATA AT THRESHOLD:
% NET GAIN
ind_net_gain_red = find(net_gain_grid_red>=change_thresh);
flux_red_net(ind_net_gain_red) = 1;
% NET LOSS
ind_net_loss_red = find(net_gain_grid_red<=(-change_thresh));
flux_red_net(ind_net_loss_red) = 2;
% DATA & NO FLUX
ind_nofluxbutdata_red = find(net_gain_grid_red<change_thresh &
net_gain_grid_red>(-change_thresh) & traj_red_grid_data>0);
flux_red_net(ind_nofluxbutdata_red) = 3;
% NET GREEN DATA AT THRESHOLD:
% NET GAIN
ind_net_gain_green = find(net_gain_grid_green>=change_thresh);
flux_green_net(ind_net_gain_green) = 1;
% NET LOSS
ind_net_loss_green = find(net_gain_grid_green<=(-change_thresh));
flux_green_net(ind_net_loss_green) = 2;
% DATA & NO FLUX
ind_nofluxbutdata_green = find(net_gain_grid_green<change_thresh &
net_gain_grid_green>(-change_thresh) & traj_green_grid_data>0);
flux_green_net(ind_nofluxbutdata_green) = 3;

%% NUMBER OF TOTAL EVENTS OF LATERAL FLUX AND MOLECULAR CONDITIONS: 
for j = 1:9 % Cycles through conditions
    for i = 1:3 % Cycles through flux data
        % WHERE i=1 IS POSITIVE FLUX + CHANGE_ALL
        % WHERE i=2 IS NEGATIVE FLUX + CHANGE_ALL
        % WHERE i=3 IS NO FLUX + CHANGE_ALL
        % RED LATERAL FLUX AND CHANGE ALL CONDITIONS
        num_red_flux_change_all(i,j) =
        length(find(double(change_all==j & flux_red_net==i) ==1));
        % GREEN LATERAL FLUX AND CHANGE ALL CONDITIONS
        num_green_flux_change_all(i,j) =
        length(find(double(change_all==j & flux_green_net==i) ==1));
    end
end
% ALL RED LATERAL events for given cell with timeloop steps
num_red_flux_change_all_t(:,:,timeloop) = num_red_flux_change_all;
% ALL GREEN LATERAL events for given cell with timeloop steps
num_green_flux_change_all_t(:,:,timeloop) = num_green_flux_change_all;

% % NORMALIZATION RED LATERAL FLUX & CHANGE_ALL: -------------------------
% total number of RED lateral flux & change_all events in a cell for
<TIMELOOP> == total_num_red_flux_change_all
    total_num_red_flux_change_all(timeloop) = sum(num_red_flux_change_all(:));
% Normalization of RED lateral flux & change_all conditions for
<TIMELOOP>
    for h = 1:size(num_red_flux_change_all,2)
        for g = 1:size(num_red_flux_change_all,1)
            percent_red_flux_change_all(g,h) = (num_red_flux_change_all(g,h)./total_num_red_flux_change_all(timeloop)).*100;
        end
    end
% Delete zero rows if necessary:
    logi2=isnan(percent_red_flux_change_all);
    percent_red_flux_change_all(logi2(:,1)==1,:)=[ ];
% Concatination of normalization of lateral RED flux & change_all as
<TIMELOOP> iterates:
    if ~isempty(percent_red_flux_change_all)
        percent_red_flux_change_all_total(:,:,timeloop) = percent_red_flux_change_all;
    end

% % NORMALIZATION GREEN LATERAL FLUX & CHANGE_ALL: -----------------------
% total number of GREEN lateral flux & change_all events in a cell
for <TIMELOOP> == total_num_green_flux_change_all
    total_num_green_flux_change_all(timeloop) = sum(num_green_flux_change_all(:));
% Normalization of GREEN lateral flux & change_all conditions for
<TIMELOOP>
    for h = 1:size(num_green_flux_change_all,2)
        for g = 1:size(num_green_flux_change_all,1)
            percent_green_flux_change_all(g,h) = (num_green_flux_change_all(g,h)./total_num_green_flux_change_all(timeloop)).*100;
        end
    end
% Delete zero rows if necessary:
    logi2=isnan(percent_green_flux_change_all);
    percent_green_flux_change_all(logi2(:,1)==1,:)=[];
% Concatination of normalization of lateral GREEN flux & change_all
as <TIMELOOP> iterates:
    if ~isempty(percent_green_flux_change_all)
        percent_green_flux_change_all_total(:,:,timeloop) = percent_green_flux_change_all;
    end

% % FREQUENCY OF MODELS: --------------------------------------------------
for freq = 1:timeloop
    % PLATFORMS = change_all==1, flux_red_net==3, flux_green_net==1
    frequencies(freq,1) = length(find(double(change_all==1 & flux_red_net==3 & flux_green_net==1)));
% LOCAL SYNTHESIS = change_all==8, flux_red_net==3, flux_green_net==3
frequencies(freq,2) = length(find(double(change_all==8 & flux_red_net==3 & flux_green_net==3) == 1));
% MEGAPOOL = change_all==8, flux_red_net==1, flux_green_net==3,
frequencies(freq,3) = length(find(double(change_all==8 & flux_red_net==1 & flux_green_net==3) == 1));
% ENDO/EXO FROM PM = change_all==6, flux_red_net==3,
frequencies(freq,4) = length(find(double(change_all==6 & flux_red_net==3 & flux_green_net==3) == 1));
% EXO FROM GOLGI = change_all==3, flux_red_net==3, flux_green_net==3
\ OR \ change_all==3, flux_red_net==2, flux_green_net==2
frequencies(freq,5) = length(find(double(change_all==3 & flux_red_net==3 & flux_green_net==3) == 1)) + length(find(double(change_all==3 & flux_red_net==2 & flux_green_net==2) == 1));
end
end % END OF FOR LOOP LABELED: <TIMELOOP>

%% FREQUENCY OF MODELS FOR ALL CELLS: -------------------------------
% Sum frequencies in <TIMELOOP> direction:
frequencies = sum(frequencies,1);
% Total frequencies for normalization:
total_frequencies = sum(frequencies(:));
% Normalization of Frequencies:
percent_frequencies = frequencies./total_frequencies*100;

%% RATES OF EVENTS FOR FREQUENCY OF MODELS: ----------------------------
% Rates are calculated by averaging the # of events by the # of
% timeloops in your system and then dividing by the box area
multiplied
% by the number of boxes data can be stored in and time of each loop.
number_bins(fileloop) = sum(mask(:));
for i = 1:5
  frequency_rates(1,i) = frequencies(i)./(end timeloop*time_change*2*dx.^2*number_bins(fileloop));
end

%% MATRICES CONTAINING <FILELOOP> INFORMATION: -----------------------------
% CHANGE ALL CONDITIONS:
% Total number of change all events for each <FILELOOP>
total_num_change_all_timeloop = sum(total_num_change_all_timeloop(:,:));
total_num_change_all_cell(fileloop,:) =
total_num_change_all_timeloop;
% Each change all's number of events for each <FILELOOP>
num_change_all_total = sum(num_change_all_total,1);
num_change_all_total_cell(fileloop,:) = num_change_all_total;
% Normalization of change all events for each <FILELOOP>
percent_change_all_timeloop = mean(percent_change_all_timeloop,1);
percent_change_all_cell(fileloop,:) = percent_change_all_timeloop;
% FREQUENCIES:
% Each model's number of events for each <FILELOOP>
frequencies_cell(fileloop,:) = frequencies;

% Number of all events for all models for each <FILELOOP>
total_frequencies_cell(fileloop) = total_frequencies;

% All normalized frequencies (5 models) for each <FILELOOP>
percent_frequencies_cell(fileloop,:) = percent_frequencies;

% Frequency rates for each <FILELOOP>
frequency_rates_cell(fileloop,:) = frequency_rates;

% RED LATERAL FLUX AND CHANGE ALL CONDITIONS:
% Total number of events for RED lateral flux and change all conditions for each <FILELOOP>
total_num_red_flux_change_all = sum(total_num_red_flux_change_all(:));
total_num_red_flux_change_all_cell(fileloop) = total_num_red_flux_change_all;
% Each lateral RED flux and change all conditions' number of events for each <FILELOOP>
num_red_flux_change_all_total = sum(num_red_flux_change_all_t,3);
num_red_flux_change_all_cell(:,:,fileloop) = num_red_flux_change_all_total;
% Normalization of lateral RED flux and change all conditions for each <FILELOOP>
percent_red_flux_change_all_total = mean(percent_red_flux_change_all_total,3);
percent_red_flux_change_all_cell(:,:,fileloop) = percent_red_flux_change_all_total;

% GREEN LATERAL FLUX AND CHANGE ALL CONDITIONS:
% Total number of events for GREEN lateral flux and change all conditions for each <FILELOOP>
total_num_green_flux_change_all = sum(total_num_green_flux_change_all(:));
total_num_green_flux_change_all_cell(fileloop) = total_num_green_flux_change_all;
% Each lateral GREEN flux and change all conditions' number of events for each <FILELOOP>
num_green_flux_change_all_total = sum(num_green_flux_change_all_t,3);
num_green_flux_change_all_cell(:,:,fileloop) = num_green Flux_change_all_total;
% Normalization of lateral GREEN flux and change all conditions for each <FILELOOP>
percent_green_flux_change_all_total = mean(percent_green_flux_change_all_total,3);
percent_green_flux_change_all_cell(:,:,fileloop) = percent_green_flux_change_all_total;

end % END OF FOR LOOP LABELED: <FILELOOP>

disp('...')
disp('Averaging...')

%% AVERAGE FOR EACH CELL: -----------------------------------------------
% TOTAL AREA OF CELLS:
% Average area of cells, std, and standard error

cell_area = number_bins.*dx.^2;
ave_area = mean(cell_area,2);
ave_area_std = std(cell_area,0,2);
ave_area_err = std(cell_area,0,2)./sqrt(size(cell_area,2));

% CHANGE ALL CONDITIONS:
% Final total number of change all events
totnum_ca = sum(total_num_change_all_cell(:));
% Average normalized change all conditions, std, and standard error
pct_ca = mean(percent_change_all_cell,1);
pct_ca_std = std(percent_change_all_cell,0,1);
pct_ca_err = std(percent_change_all_cell,0,1)./sqrt(size(percent_change_all_cell,1));

% FREQUENCIES:
% Final total number of frequency events
totnum_freq = sum(total_frequencies_cell(:));
% Average normalized frequency of events, std, and standard error
% Delete zero rows if necessary:
logi3=isnan(percent_frequencies_cell);
percent_frequencies_cell(logi3(:,1)==1,:)=[];
pct_freq = mean(percent_frequencies_cell,1);
pct_freq_std = std(percent_frequencies_cell,0,1);
pct_freq_err = std(percent_frequencies_cell,0,1)./sqrt(size(percent_frequencies_cell,1));

% Average rates for frequency of events, std, and standard error
rate_freq = mean(frequency_rates_cell,1);
rate_freq_std = std(frequency_rates_cell,0,1);
rate_freq_err = std(frequency_rates_cell,0,1)./sqrt(size(frequency_rates_cell,1));

% RED LATERAL FLUX AND CHANGE ALL CONDITIONS:
% Final total number of RFCA events
totnum_rfca = sum(total_num_red_flux_change_all_cell(:));
% Average normalized lateral RFCA, std, and standard error
pct_rfca = mean(percent_red_flux_change_all_cell,3);
pct_rfca_std = std(percent_red_flux_change_all_cell,0,3);
pct_rfca_err = std(percent_red_flux_change_all_cell,0,3)./sqrt(size(percent_red_flux_change_all_cell,3));

% GREEN LATERAL FLUX AND CHANGE ALL CONDITIONS:
% Final total number of GFCA events
totnum_gfca = sum(total_num_green_flux_change_all_cell(:));
% Average normalized lateral GFCA, std, and standard error
pct_gfca = mean(percent_green_flux_change_all_cell,3);
pct_gfca_std = std(percent_green_flux_change_all_cell,0,3);
pct_gfca_err = std(percent_green_flux_change_all_cell,0,3)./sqrt(size(percent_green_flux_change_all_cell,3));

disp('...')
disp('Code completed.')

savevars = {'frames_per_second','time_change','endtimeloop','min_delta',... 'change_thresh','dx','preframe','postframe','normalize_grids_to_np',... 'startfilenum','endfilenum','df','dx','last_frame',... 'total_num_change_all_cell','num_change_all_total_cell','frequencies_cell',... 'total_frequencies_cell','total_num_red_flux_change_all_cell','...
'num_red_flux_change_all_cell', 'total_num_green_flux_change_all_cell', ...
'num_green_flux_change_all_cell', 'pct_ca', 'pct_ca_std', 'pct_ca_err', ...
'pct_freq', 'pct_freq_std', 'pct_freq_err', 'rate_freq', 'rate_freq_std', ...
'rate_freq_err', 'pct_rfca', 'pct_rfca_std', 'pct_rfca_err', 'pct_gfca', ...

'pct_gfca_std', 'pct_gfca_err', 'totnum_ca', 'cell_area', 'ave_area', 'ave_area_std', ...
'ave_area_err', 'totnum_freq', 'totnum_rfca', 'totnum_gfca');
save([inpath, 'HA_PIP2_changes_traj_stats_time-', num2str(time_change), ...
's_delta-', num2str(min_delta), '_thresh-', num2str(change_thresh), ...
'_interlaced-', num2str(interlaced), '_v6'], savevars{:})
disp('...')
disp('Saved.')
A.3. HA_PIP2_change_traj_stats_bargraph_plotter_v7

% --------------------------------- % Flux Code BAR GRAPH PLOTTER - Version 7 % [JW] 2020 Spring % --------------------------------- % GREEN = Hemagglutinin % RED = PH Domain [PIP2] % --------------------------------- close all; clear all;
% --------------------------------- % User Variables:
% == 1 if yes
% == 0 if no
figure1 = 0;
figure2 = 0;
figure3 = 0;
figure4 = 0;
figure5 = 0;
% LOAD IN DATA:
[file,path]=uigetfile('','multiselect','on');
% --------------------------------- % CODE STARTS HERE: %-----------------------------------
ca        = []; ca_std    = []; ca_err    = [];
freq      = []; freq_std  = []; freq_err  = [];
gfca      = []; gfca_std  = []; gfca_err  = [];
rfca      = []; rfca_std  = []; rfca_err  = [];
rates     = []; rates_std = []; rates_err = [];
for i=1:numel(file)
  load([path,file{i}])
  % CELL AREA:
  area(i)     = ave_area;
  area_err(i) = ave_area_err;
  area_std(i) = ave_area_std;
  % CHANGE ALL CONDITIONS:
  ca     = vertcat(ca,pct_ca);
  ca_std = vertcat(ca_std,pct_ca_std);
  ca_err = vertcat(ca_err,pct_ca_err);
  % FREQUENCY:
  freq   = vertcat(freq,pct_freq);
  freq_std= vertcat(freq_std,pct_freq_std);
  freq_err= vertcat(freq_err,pct_freq_err);
  % LATERAL GREEN FLUX AND CHANGE ALL CONDITIONS:
  gfca   = cat(3,gfca,pct_gfca);
  gfca_std= cat(3,gfca_std,pct_gfca_std);
  gfca_err= cat(3,gfca_err,pct_gfca_err);
  % LATERAL RED FLUX AND CHANGE ALL CONDITIONS:
  rfca   = cat(3,rfca,pct_rfca);
  rfca_std= cat(3,rfca_std,pct_rfca_std);
  rfca_err= cat(3,rfca_err,pct_rfca_err);
  % RATES OF MODELS:
  rates  = vertcat(rates,rate_freq);
  rates_std= vertcat(rates_std,rate_freq_std);
  rates_err= vertcat(rates_err,rate_freq_err);
end
%% BAR GRAPH ORGANIZATION: --------------------------------------------
%% RED FLUX_CHANGE_ALL AVERAGED BY CELL, COMBINED 10S & 30S DATA FOR PLOTTING:
% RED INFLUX:
rfca_influx(1,:) = rfca(1,:,1);
rfca_influx(2,:) = rfca(1,:,5);
rfca_influx_err(1,:) = rfca_err(1,:,1);
rfca_influx_err(2,:) = rfca_err(1,:,5);
% RED OUTFLUX:
rfca_outflux(1,:) = rfca(2,:,1);
rfca_outflux(2,:) = rfca(2,:,5);
rfca_outflux_err(1,:) = rfca_err(2,:,1);
rfca_outflux_err(2,:) = rfca_err(2,:,5);
% RED NET ZERO FLUX:
rfca_netzero(1,:) = rfca(3,:,1);
rfca_netzero(2,:) = rfca(3,:,5);
rfca_netzero_err(1,:) = rfca_err(3,:,1);
rfca_netzero_err(2,:) = rfca_err(3,:,5);
%% GREEN FLUX_CHANGE_ALL AVERAGED BY CELL, COMBINED 10S & 30S DATA FOR PLOTTING:
% GREEN INFLUX:
gfca_influx(1,:) = gfca(1,:,1); % 10 seconds
gfca_influx(2,:) = gfca(1,:,5); % 30 seconds
gfca_influx_err(1,:) = gfca_err(1,:,1); % 10 seconds
gfca_influx_err(2,:) = gfca_err(1,:,5); % 30 seconds
% GREEN OUTFLUX:
gfca_outflux(1,:) = gfca(2,:,1); % 10 seconds
gfca_outflux(2,:) = gfca(2,:,5); % 30 seconds
gfca_outflux_err(1,:) = gfca_err(2,:,1); % 10 seconds
gfca_outflux_err(2,:) = gfca_err(2,:,5); % 30 seconds
% GREEN NET ZERO FLUX:
gfca_netzero(1,:) = gfca(3,:,1); % 10 seconds
gfca_netzero(2,:) = gfca(3,:,5); % 30 seconds
gfca_netzero_err(1,:) = gfca_err(3,:,1); % 10 seconds
gfca_netzero_err(2,:) = gfca_err(3,:,5); % 30 seconds
%% COMBINED RFCA AND GFCA DATA ALL AVERAGED BY CELL, COMBINED 10S & 30S DATA FOR PLOTTING:
% INFLUX:
c0_fca_influx = [rfca_influx; gfca_influx];
c0_fca_influx_err = [rfca_influx_err; gfca_influx_err];
% OUTFLUX:
c0_fca_outflux = [rfca_netzero; gfca_netzero];
c0_fca_outflux_err = [rfca_netzero_err; gfca_netzero_err];
% NET ZERO FLUX:
c0_fca_netzero = [rfca_outflux; gfca_outflux];
c0_fca_netzero_err = [rfca_outflux_err; gfca_outflux_err];

%% BAR GRAPH PLOTS: --------------------------------------------------
%% ERROR BAR WIDTHS:

% For 5 hist bar charts with 9 conditions
width(1,:) = [1:9]-dx*2;
width(2,:) = [1:9]-dx;
width(3,:) = [1:9];
width(4,:) = [1:9]+dx;
width(5,:) = [1:9]+dx*2;
% For 2 hist bar charts with 9 conditions:
width(6,:) = [1:9]-dx*0.95;
width(7,:) = [1:9]+dx*0.95;
% For 4 hist bar charts with 9 conditions:
width(8,:) = [1:9]-dx*1.75;
width(9,:) = [1:9]-dx*0.56;
width(10,:) = [1:9]+dx*0.56;
width(11,:) = [1:9]+dx*1.75;
% For 5 bared hist chart with 5 conditions:
width_5(1,:) = [1:5]-dx*2;
width_5(2,:) = [1:5]-dx;
width_5(3,:) = [1:5];
width_5(4,:) = [1:5]+dx;
width_5(5,:) = [1:5]+dx*2;

% CHANGE_ALL AVERAGED BY CELL: ------------------------------------------
if figure1 == 1;
    figure(1)
    bar(ca,'hist')
    hold on
    errorbar(width(1,:),ca(1,:),ca_err(1,:),'LineStyle','none','Color',[0 0 0])
    errorbar(width(2,:),ca(2,:),ca_err(2,:),'LineStyle','none','Color',[0 0 0])
    errorbar(width(3,:),ca(3,:),ca_err(3,:),'LineStyle','none','Color',[0 0 0])
    errorbar(width(4,:),ca(4,:),ca_err(4,:),'LineStyle','none','Color',[0 0 0])
    errorbar(width(5,:),ca(5,:),ca_err(5,:),'LineStyle','none','Color',[0 0 0])
    hold off
    title({"PERCENTAGE_CHANGE_ALL, AVERAGED BY CELL'; 'n=27'})
    grid on
    legend('10 seconds','15 seconds','20 seconds','25 seconds','30 seconds')
    ylim([0 35])
    ylabel('PERCENTAGE')
    xticklabels({'+HA =PIP2','-HA =PIP2','+HA +PIP2','-HA +PIP2','+HA -PIP2','-HA -PIP2','=HA =PIP2','=HA +PIP2','=HA -PIP2'});
end

% PERCENT_FREQUENCIES AVERAGED BY CELL: ----------------------------------
if figure2 == 1;
    figure(2)
    bar(freq,'hist')
    hold on
    errorbar(width_5(1,:),freq(1,:),freq_err(1,:),'LineStyle','none','Color',[0 0 0])
    errorbar(width_5(2,:),freq(2,:),freq_err(2,:),'LineStyle','none','Color',[0 0 0])
    errorbar(width_5(3,:),freq(3,:),freq_err(3,:),'LineStyle','none','Color',[0 0 0])
    errorbar(width_5(4,:),freq(4,:),freq_err(4,:),'LineStyle','none','Color',[0 0 0])
errorbar(width_5(5,:),freq(5,:),freq_err(5,:),'LineStyle','none','Color',[0 0 0])
hold off
title({'PERCENTAGE FREQUENCIES, AVERAGED BY CELL';'n=27'})
grid on
legend('10 seconds','15 seconds','20 seconds','25 seconds','30 seconds')
ylabel('PERCENTAGE')
xticklabels({'PLATFORM','SYNTHESIS','MEGAPOOL','ENDO/EXO','DELIVERY'})
end

% CHANGE_ALL AVERAGED BY CELL FOR 10 SECONDS ONLY: ------------------------
if figure3 == 1;
    figure(3)
    bar(ca(1,:),',w')
    hold on
    errorbar(width(3,:),ca(1,:),ca_err(1,:),',LineStyle','none','Color',[0 0 0])
    hold off
    title({'PERCENTAGE Changes for 10 seconds, AVERAGED BY CELL';'n=27'})
    grid on
    legend('10 seconds')
    ylim([0 35])
    ylabel('PERCENTAGE')
    xticklabels({'+HA =PIP2','-HA =PIP2','+HA +PIP2','-HA +PIP2','+HA -PIP2','-HA -PIP2','=HA =PIP2','=HA +PIP2','=HA -PIP2'});
end

% CHANGE_ALL AVERAGED BY CELL FOR 30 SECONDS ONLY: ------------------------
if figure4 == 1;
    figure(4)
    bar(ca(5,:),',w')
    hold on
    errorbar(width(3,:),ca(5,:),ca_err(5,:),',LineStyle','none','Color',[0 0 0])
    hold off
    title({'PERCENTAGE Changes for 30 seconds, AVERAGED BY CELL';'n=27'})
    grid on
    legend('30 seconds')
    ylim([0 35])
    ylabel('PERCENTAGE')
    xticklabels({'+HA =PIP2','-HA =PIP2','+HA +PIP2','-HA +PIP2','+HA -PIP2','-HA -PIP2','=HA =PIP2','=HA +PIP2','=HA -PIP2'});
end

% COMBINED_FLUX_CHANGE ALL AVERAGED BY CELL BOTH 10, 30 SECOND: -----------
if figure5 == 1;
    figure(5)
    subplot(3,1,1)
    bar(co_fca_influx')
    hold on
    errorbar(width(8,:),co_fca_influx(1,:),co_fca_influx_err(1,:),',LineStyle','none','Color',[0 0 0])
    errorbar(width(9,:),co_fca_influx(2,:),co_fca_influx_err(2,:),',LineStyle','none','Color',[0 0 0])
    errorbar(width(10,:),co_fca_influx(3,:),co_fca_influx_err(3,:),',LineStyle','none','Color',[0 0 0])
errorbar(width(11,:),co_fca_influx(4,:),co_fca_influx_err(4,:),'LineStyle','none','Color',[0 0 0])
hold off
title('LATERAL NET INFLUX')
legend('PIP2 tau = 10 seconds','PIP2 tau = 30 seconds','HA tau = 10 seconds','HA tau = 30 seconds')
grid on
ylim([0 1.2])
yticks([0:0.2:1.2])
ylabel('PERCENTAGE')
xticklabels({'+HA =PIP2','-HA =PIP2','+HA +PIP2','-HA +PIP2','+HA -PIP2','-HA -PIP2','=HA =PIP2','=HA +PIP2','=HA -PIP2'});

subplot(3,1,2)
bar(co_fca_netzero')
hold on
errorbar(width(8,:),co_fca_netzero(1,:),co_fca_netzero_err(1,:),'LineStyle','none','Color',[0 0 0])
errorbar(width(9,:),co_fca_netzero(2,:),co_fca_netzero_err(2,:),'LineStyle','none','Color',[0 0 0])
errorbar(width(10,:),co_fca_netzero(3,:),co_fca_netzero_err(3,:),'LineStyle','none','Color',[0 0 0])
errorbar(width(11,:),co_fca_netzero(4,:),co_fca_netzero_err(4,:),'LineStyle','none','Color',[0 0 0])
hold off
title('LATERAL NET OUTFLUX')
grid on
ylabel('PERCENTAGE')
ylim([0 1.2])
yticks([0:0.2:1.2])
xticklabels({'+HA =PIP2','=HA =PIP2','=HA +PIP2','=HA +PIP2','=HA -PIP2','=HA -PIP2','=HA +PIP2','=HA +PIP2','=HA -PIP2'});

subplot(3,1,3)
bar(co_fca_outflux')
hold on
errorbar(width(8,:),co_fca_outflux(1,:),co_fca_outflux_err(1,:),'LineStyle','none','Color',[0 0 0])
errorbar(width(9,:),co_fca_outflux(2,:),co_fca_outflux_err(2,:),'LineStyle','none','Color',[0 0 0])
errorbar(width(10,:),co_fca_outflux(3,:),co_fca_outflux_err(3,:),'LineStyle','none','Color',[0 0 0])
errorbar(width(11,:),co_fca_outflux(4,:),co_fca_outflux_err(4,:),'LineStyle','none','Color',[0 0 0])
hold off
title('NETZERO FLUX & DATA')
grid on
yticks([0:5:30])
ylabel('PERCENTAGE')
xlabellabels(['+HA =PIP2', '-HA =PIP2', '+HA +PIP2', '-HA +PIP2', '+HA -
           PIP2', '-HA -PIP2', '=HA =PIP2', '=HA +PIP2', '=HA -PIP2']);
end
APPENDIX B:

STATISTICAL TESTS TABLES

B.1. Statistical Tests from Chapter Three

Statistical tests for analyzed data found in chapter 3. These tables do not appear in the order they are referenced in the text of chapter 3. P-values for each table can be found in section B.2.

<table>
<thead>
<tr>
<th>MODEL VS. MODEL</th>
<th>$\tau = 10s$</th>
<th>$\tau = 15s$</th>
<th>$\tau = 20s$</th>
<th>$\tau = 25s$</th>
<th>$\tau = 30s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLATFORM VS. LSRP</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>PLATFORM VS. MEGAPOOL</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PLATFORM VS. BUDDING/ENDOCYTOSIS</td>
<td>****</td>
<td>****</td>
<td>****</td>
<td>****</td>
<td>**</td>
</tr>
<tr>
<td>PLATFORM VS. EXOCYTOSIS/DELIVERY</td>
<td>****</td>
<td>***</td>
<td>****</td>
<td>****</td>
<td>***</td>
</tr>
<tr>
<td>LSRP VS. MEGAPOOL</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>LSRP VS. BUDDING/ENDOCYTOSIS</td>
<td>****</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>LSRP VS. EXOCYTOSIS/DELIVERY</td>
<td>**</td>
<td>*</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>MEGAPOOL VS. BUDDING/ENDOCYTOSIS</td>
<td>****</td>
<td>****</td>
<td>****</td>
<td>****</td>
<td>**</td>
</tr>
<tr>
<td>MEGAPOOL VS. EXOCYTOSIS/DELIVERY</td>
<td>****</td>
<td>***</td>
<td>****</td>
<td>****</td>
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<td>BUDDING/ENDOCYTOSIS VS. EXOCYTOSIS/DELIVERY</td>
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</table>

Table B.1: Ordinary One-Way ANOVA Significance Testing of Rates of Membrane Models for Hemagglutinin and PIP2. Ordinary one-way ANOVA significance testing for five membrane models to explain HA and PIP2 interactions at the PM. Significance testing p-values are accompanied by a significance star rating ranging as follows: $p \leq 0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) , and $p<0.0001$ (****). Significance testing was generated using GraphPad Prism 8.3.1. software.
<table>
<thead>
<tr>
<th>CONDITION</th>
<th>PCTp</th>
<th>A</th>
<th>PCTp + A</th>
<th>k (1/8)</th>
<th>CONDITION</th>
<th>PCTp</th>
<th>A</th>
<th>PCTp + A</th>
<th>k (1/8)</th>
</tr>
</thead>
<tbody>
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<td>ns</td>
<td>ns</td>
<td>+HA =PIP2 vs. =HA =PIP2</td>
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<td>ns</td>
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<td>ns</td>
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<tr>
<td>+HA =PIP2 vs. +HA =PIP2</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>+HA =PIP2 vs. =HA =PIP2</td>
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<td>****</td>
<td>***</td>
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<tr>
<td>+HA =PIP2 vs. -HA =PIP2</td>
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<td>*</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<td>ns</td>
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<td>ns</td>
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<tr>
<td>+HA =PIP2 vs. +HA =PIP2</td>
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<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>-HA =PIP2 vs. =HA =PIP2</td>
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<td>-HA =PIP2 vs. =HA =PIP2</td>
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<td>+HA =PIP2 vs. -HA =PIP2</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>+HA =PIP2 vs. =HA =PIP2</td>
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<td>*</td>
<td>ns</td>
<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>-HA =PIP2 vs. +HA =PIP2</td>
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<td>ns</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>-HA =PIP2 vs. -HA =PIP2</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>-HA =PIP2 vs. +HA =PIP2</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<td>-HA =PIP2 vs. =HA =PIP2</td>
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<td>-HA =PIP2 vs. -HA =PIP2</td>
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<td>-HA =PIP2 vs. +HA =PIP2</td>
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<td>ns</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>-HA =PIP2 vs. =HA =PIP2</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<td>-HA =PIP2 vs. -HA =PIP2</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>-HA =PIP2 vs. +HA =PIP2</td>
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<td>*</td>
<td>ns</td>
<td>ns</td>
<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>-HA =PIP2 vs. =HA =PIP2</td>
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<td>ns</td>
<td>ns</td>
<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
<td>-HA =PIP2 vs. -HA =PIP2</td>
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<td>*</td>
<td>ns</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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</tr>
<tr>
<td>-HA =PIP2 vs. +HA =PIP2</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
<td>+HA =PIP2 vs. =HA =PIP2</td>
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<td>ns</td>
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<td>ns</td>
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<tr>
<td>-HA =PIP2 vs. =HA =PIP2</td>
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<td>ns</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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<tr>
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<tr>
<td>-HA =PIP2 vs. +HA =PIP2</td>
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<td>+HA =PIP2 vs. =HA =PIP2</td>
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</tbody>
</table>
**Table B.2:** One-Way ANOVA Significance Testing of Fitting Parameters for Time Point Difference for Each Condition. Comparisons of all nine conditions using a one-way ANOVA significance test. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***) , and p<0.0001 (****). Significance testing was generated using GraphPad Prism 8.3.1. software.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time Point Difference</th>
<th>p-value</th>
<th>Significance Star Rating</th>
</tr>
</thead>
<tbody>
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<td>Condition 1</td>
<td>0.012</td>
<td>0.034</td>
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</tr>
<tr>
<td>Condition 2</td>
<td>0.002</td>
<td>0.0001</td>
<td>****</td>
</tr>
<tr>
<td>Condition 3</td>
<td>0.005</td>
<td>0.003</td>
<td>**</td>
</tr>
<tr>
<td>Condition 4</td>
<td>0.008</td>
<td>0.001</td>
<td>***</td>
</tr>
<tr>
<td>Condition 5</td>
<td>0.009</td>
<td>0.002</td>
<td>***</td>
</tr>
<tr>
<td>Condition 6</td>
<td>0.010</td>
<td>0.003</td>
<td>**</td>
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<tr>
<td>Condition 7</td>
<td>0.011</td>
<td>0.004</td>
<td>*</td>
</tr>
<tr>
<td>Condition 8</td>
<td>0.012</td>
<td>0.005</td>
<td>*</td>
</tr>
<tr>
<td>Condition 9</td>
<td>0.013</td>
<td>0.006</td>
<td>*</td>
</tr>
</tbody>
</table>
B.2. P-Values

Statistical p-values for analyzed data found in tables in chapter 3 and appendix A. Table captions specific the location in this thesis of the sister table. Tables in this section do not appear in the order they are referenced in the text.

### FITTING PARAMETERS FOR MSD VS TIME FOR PH DOMAIN-DENDRA2 MOLECULES

<table>
<thead>
<tr>
<th></th>
<th>MULTIPLE T-TEST</th>
<th>UNPAIRED T-TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSD_p (µm²)</td>
<td>p&lt;0.000001</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>τ (s)</td>
<td>p&lt;0.000001</td>
<td>p&lt;0.0001</td>
</tr>
</tbody>
</table>

**Table B.3:** P-Values for Table 3.3. P-values for significance testing found in table 3.3. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (**), and p<0.0001 (****). Significance testing was generated using GraphPad Prism 8.3.1. software.

### FITTING PARAMETERS FOR MOBILITY VS DENSITY OF PH DOMAIN-DENDRA2 MOLECULES

<table>
<thead>
<tr>
<th></th>
<th>MULTIPLE T-TEST</th>
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</tr>
</thead>
<tbody>
<tr>
<td>µ₀ (µm²/s)</td>
<td>p=0.000006</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>A (µm²/s)</td>
<td>p=0.000002</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>µ₀ + A (µm²/s)</td>
<td>p=0.994194</td>
<td>p=0.9652</td>
</tr>
<tr>
<td>α (molecules/µm²)</td>
<td>p=0.095191</td>
<td>p&lt;0.0001</td>
</tr>
</tbody>
</table>

**Table B.4:** P-Values for Table 3.4. P-values for significance testing found in table 3.4. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (**), and p<0.0001 (****). Significance testing was generated using GraphPad Prism 8.3.1. software.
**ONE-WAY ANOVA SIGNIFICANCE TESTING OF TWO TIME POINTS FOR EACH CONDITION (MOLECULAR FLUX)**

\[ \tau_1 \text{ vs } \tau_2 \]

<table>
<thead>
<tr>
<th>Condition</th>
<th>10s vs 15s</th>
<th>10s vs 20s</th>
<th>10s vs 25s</th>
<th>10s vs 30s</th>
</tr>
</thead>
<tbody>
<tr>
<td>+HA =PIP2</td>
<td>p=0.9898</td>
<td>p=0.4806</td>
<td>p=0.1770</td>
<td>p=0.1672</td>
</tr>
<tr>
<td>-HA =PIP2</td>
<td>p=0.9855</td>
<td>p=0.3188</td>
<td>p=0.1140</td>
<td>p=0.0826</td>
</tr>
<tr>
<td>+HA +PIP2</td>
<td>p=0.2477</td>
<td>p=0.0089</td>
<td>p=0.0002</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>-HA +PIP2</td>
<td>p=0.6213</td>
<td>p=0.1456</td>
<td>p=0.0492</td>
<td>p=0.0071</td>
</tr>
<tr>
<td>+HA -PIP2</td>
<td>p=0.6186</td>
<td>p=0.0347</td>
<td>p=0.0025</td>
<td>p=0.0004</td>
</tr>
<tr>
<td>-HA -PIP2</td>
<td>p=0.6147</td>
<td>p=0.1425</td>
<td>p=0.0284</td>
<td>p=0.0107</td>
</tr>
<tr>
<td>=HA =PIP2</td>
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<td>p=0.1425</td>
<td>p=0.0490</td>
<td>p=0.0167</td>
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<tr>
<td>=HA +PIP2</td>
<td>p=0.8931</td>
<td>p=0.9599</td>
<td>p=0.9745</td>
<td>p=0.9888</td>
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<tr>
<td>=HA -PIP2</td>
<td>p=0.9522</td>
<td>p=0.9972</td>
<td>p=0.9978</td>
<td>p&gt;0.9999</td>
</tr>
</tbody>
</table>

**Table B.5:** P-Values for Table 3.7. P-values for significance testing found in table 3.7. Significance testing p-values are accompanied by a significance star rating ranging as follows: \( p \leq 0.05 \) (ns), \( p<0.05 \) (*), \( p<0.01 \) (**), \( p<0.001 \) (***) , and \( p<0.0001 \) (****). Significance testing was generated using GraphPad Prism 8.3.1. software.
### Table B.6: P-Values for Table B.2

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<tr>
<th>CONDITION</th>
<th>PCT$_p$</th>
<th>A</th>
<th>PCT$_p$ + A</th>
<th>k (1/2)</th>
<th>CONDITION</th>
<th>PCT$_p$</th>
<th>A</th>
<th>PCT$_p$ + A</th>
<th>k (1/2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+HA =PIP2 vs. -HA =PIP2</td>
<td>p &gt; 0.9999</td>
<td>p &gt; 0.9999</td>
<td>p &gt; 0.9999</td>
<td>p &gt; 0.9999</td>
<td>+HA +PIP2 vs. -HA +PIP2</td>
<td>p = 0.5961</td>
<td>p = 0.0020</td>
<td>p = 0.1239</td>
<td>p &gt; 0.9999</td>
</tr>
<tr>
<td>+HA =PIP2 vs. +HA +PIP2</td>
<td>p = 0.5067</td>
<td>p = 0.7489</td>
<td>p = 0.0321</td>
<td>p &gt; 0.9999</td>
<td>+HA +PIP2 vs. -HA +PIP2</td>
<td>p = 0.1233</td>
<td>p &lt; 0.0001</td>
<td>p = 0.0007</td>
<td>p = 0.8320</td>
</tr>
<tr>
<td>+HA =PIP2 vs. -HA +PIP2</td>
<td>p = 0.6833</td>
<td>p = 0.8735</td>
<td>p = 0.0332</td>
<td>p &gt; 0.9999</td>
<td>+HA +PIP2 vs. -HA -PIP2</td>
<td>p = 0.1010</td>
<td>p = 0.0003</td>
<td>p = 0.0007</td>
<td>p = 0.8290</td>
</tr>
<tr>
<td>+HA =PIP2 vs. +HA -PIP2</td>
<td>p = 0.3703</td>
<td>p = 0.8184</td>
<td>p = 0.0553</td>
<td>p &gt; 0.9999</td>
<td>-HA +PIP2 vs. -HA -PIP2</td>
<td>p = 0.6726</td>
<td>p = 0.2856</td>
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<td>p &gt; 0.9999</td>
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<tr>
<td>+HA =PIP2 vs. -HA -PIP2</td>
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<td>p = 0.7844</td>
<td>p = 0.0134</td>
<td>p &gt; 0.9999</td>
<td>-HA +PIP2 vs. -HA -PIP2</td>
<td>p = 0.9929</td>
<td>p = 0.1196</td>
<td>p = 0.1911</td>
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</tr>
<tr>
<td>+HA =PIP2 vs. =HA +PIP2</td>
<td>p = 0.9677</td>
<td>p = 0.9498</td>
<td>p = 0.9027</td>
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<td>-HA +PIP2 vs. =HA +PIP2</td>
<td>p = 0.6584</td>
<td>p = 0.0029</td>
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<td>+HA =PIP2 vs. =HA -PIP2</td>
<td>p = 0.2453</td>
<td>p = 0.9999</td>
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<td>p = 0.9534</td>
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<td>p = 0.0021</td>
<td>p = 0.0048</td>
<td>p = 0.8248</td>
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<td>p = 0.9999</td>
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<td>p = 0.8750</td>
<td>-HA +PIP2 vs. =HA -PIP2</td>
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<td>p = 0.0047</td>
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<td>p = 0.8208</td>
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<td>p &gt; 0.9999</td>
<td>+HA -PIP2 vs. -HA -PIP2</td>
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<td>p = 0.7954</td>
<td>p = 0.0882</td>
<td>p = 0.9651</td>
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<td>-HA =PIP2 vs. -HA +PIP2</td>
<td>p = 0.8904</td>
<td>p = 0.0226</td>
<td>p = 0.3768</td>
<td>p &gt; 0.9999</td>
<td>+HA -PIP2 vs. =HA +PIP2</td>
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<td>p = 0.0023</td>
<td>p = 0.1691</td>
<td>p &gt; 0.9999</td>
</tr>
<tr>
<td>-HA =PIP2 vs. -HA -PIP2</td>
<td>p = 0.7297</td>
<td>p = 0.0168</td>
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<td>p &gt; 0.9999</td>
<td>+HA -PIP2 vs. =HA -PIP2</td>
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<td>p = 0.0001</td>
<td>p = 0.0014</td>
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</tr>
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<td>-HA =PIP2 vs. =HA +PIP2</td>
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<td>p = 0.0158</td>
<td>p = 0.2276</td>
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<td>+HA -PIP2 vs. =HA -PIP2</td>
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<td>p = 0.0001</td>
<td>p = 0.0013</td>
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<td>p = 0.0481</td>
<td>p = 0.9815</td>
<td>p &gt; 0.9999</td>
<td>-HA -PIP2 vs. =HA -PIP2</td>
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<td>p = 0.0004</td>
<td>p = 0.1175</td>
<td>p &gt; 0.9999</td>
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<td>p &gt; 0.9999</td>
<td>p = 0.8674</td>
<td>-HA -PIP2 vs. =HA -PIP2</td>
<td>p = 0.0449</td>
<td>p = 0.0013</td>
<td>p = 0.0016</td>
<td>p = 0.8394</td>
</tr>
<tr>
<td>-HA +PIP2 vs. -HA +PIP2</td>
<td>p = 0.6167</td>
<td>p = 0.1450</td>
<td>p &gt; 0.9999</td>
<td>p = 0.8050</td>
<td>-HA -PIP2 vs. =HA -PIP2</td>
<td>p = 0.0398</td>
<td>p = 0.0013</td>
<td>p = 0.0015</td>
<td>p = 0.8317</td>
</tr>
<tr>
<td>+HA +PIP2 vs. -HA +PIP2</td>
<td>p = 0.9979</td>
<td>p = 0.0207</td>
<td>p = 0.2771</td>
<td>p &gt; 0.9999</td>
<td>+HA =PIP2 vs. =HA +PIP2</td>
<td>p = 0.4609</td>
<td>p = 0.0079</td>
<td>p = 0.7780</td>
<td>p = 0.8874</td>
</tr>
<tr>
<td>+HA +PIP2 vs. =HA -PIP2</td>
<td>p = 0.9114</td>
<td>p = 0.0513</td>
<td>p = 0.0915</td>
<td>p = 0.8668</td>
<td>+HA =PIP2 vs. +HA -PIP2</td>
<td>p = 0.4529</td>
<td>p = 0.0078</td>
<td>p = 0.7779</td>
<td>p = 0.8352</td>
</tr>
<tr>
<td>+HA +PIP2 vs. -HA -PIP2</td>
<td>p = 0.3854</td>
<td>p = 0.8862</td>
<td>p &gt; 0.9999</td>
<td>p &gt; 0.9999</td>
<td>+HA +PIP2 vs. =HA +PIP2</td>
<td>p = 0.9966</td>
<td>p = 0.9966</td>
<td>p &gt; 0.9999</td>
<td>p &gt; 0.9999</td>
</tr>
</tbody>
</table>

Significance testing 
p-values are accompanied by a significance star rating ranging as follows: p<0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), and p<0.0001 (****). Significance testing was generated using GraphPad Prism 8.3.1. software.
**Table B.7**: P-Values for Table 3.9. P-values for significance testing found in table 3.9. Significance testing p-values are accompanied by a significance star rating ranging as follows: $p \leq 0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****). Significance testing was generated using GraphPad Prism 8.3.1 software.
Table B.8: P-Values for Table 3.12. P-values for significance testing found in table 3.12. Significance testing p-values are accompanied by a significance star rating ranging as follows: p≤0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***) and p<0.0001 (****). Significance testing was generated using GraphPad Prism 8.3.1. software.
## Ordinary One-Way ANOVA Significance Testing of Rates of Membrane Models for HA and PIP2

### Table B.9: P-Values for Table B.1.

<table>
<thead>
<tr>
<th>MODEL VS. MODEL</th>
<th>( \tau = 10s )</th>
<th>( \tau = 15s )</th>
<th>( \tau = 20s )</th>
<th>( \tau = 25s )</th>
<th>( \tau = 30s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLATFORM VS. LOCAL FREE PIP2</td>
<td>p=0.8286</td>
<td>p=0.5150</td>
<td>p=0.2742</td>
<td>p=0.0653</td>
<td>p=0.0375</td>
</tr>
<tr>
<td>PLATFORM VS. MEGAPOOL</td>
<td>p&gt;0.9999</td>
<td>p&gt;0.9999</td>
<td>p&gt;0.9999</td>
<td>p&gt;0.9999</td>
<td>p&gt;0.9999</td>
</tr>
<tr>
<td>PLATFORM VS. BUDDING/ENDOCYTOSIS</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p=0.0017</td>
</tr>
<tr>
<td>PLATFORM VS. EXOCYTOSIS/DELIVERY</td>
<td>p&lt;0.0001</td>
<td>p=0.0002</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p=0.0004</td>
</tr>
<tr>
<td>LOCAL FREE PIP2 VS. MEGAPOOL</td>
<td>p=0.8366</td>
<td>p=0.5078</td>
<td>p=0.2391</td>
<td>p=0.0465</td>
<td>p=0.0306</td>
</tr>
<tr>
<td>LOCAL FREE PIP2 VS. BUDDING/ENDOCYTOSIS</td>
<td>p&lt;0.0001</td>
<td>p=0.0019</td>
<td>p=0.0085</td>
<td>p=0.2043</td>
<td>p=0.8685</td>
</tr>
<tr>
<td>LOCAL FREE PIP2 VS. EXOCYTOSIS/DELIVERY</td>
<td>p=0.0025</td>
<td>p=0.0451</td>
<td>p=0.0151</td>
<td>p=0.1841</td>
<td>p=0.6536</td>
</tr>
<tr>
<td>MEGAPOOL VS. BUDDING/ENDOCYTOSIS</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p=0.0013</td>
</tr>
<tr>
<td>MEGAPOOL VS. EXOCYTOSIS/DELIVERY</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0002</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p=0.0003</td>
</tr>
<tr>
<td>BUDDING/ENDOCYTOSIS VS. EXOCYTOSIS/DELIVERY</td>
<td>p=0.8424</td>
<td>p=0.8489</td>
<td>p=0.9997</td>
<td>p&gt;0.9999</td>
<td>p=0.9950</td>
</tr>
</tbody>
</table>

Significance testing p-values are accompanied by a significance star rating ranging as follows: \( p \leq 0.05 \) (ns), \( p < 0.05 \) (*), \( p < 0.01 \) (**), \( p < 0.001 \) (***) and \( p < 0.0001 \) (****). Significance testing was generated using GraphPad Prism 8.3.1. software.
<table>
<thead>
<tr>
<th>ACTIN BINDING PROTEINS</th>
<th>CONCENTRATION OF ACTION BINDING PROTEINS</th>
<th>NUMBER OF CLUSTERS</th>
<th>MEAN DENSITY (Relative to Average)</th>
<th>MEAN AREA ($\mu$m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ACTININ</td>
<td>low</td>
<td>464</td>
<td>$5.6865 \pm 0.0026$</td>
<td>$0.0381 \pm 0.00013$</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>649</td>
<td>$7.362 \pm 0.005$</td>
<td>$0.0925 \pm 0.0024$</td>
</tr>
<tr>
<td><strong>SIGNIFICANCE BETWEEN CONCENTRATIONS</strong></td>
<td></td>
<td></td>
<td>$p&lt;0.0001$</td>
<td>$p&lt;0.0001$</td>
</tr>
<tr>
<td>COFILIN 1</td>
<td>low</td>
<td>174</td>
<td>$6.873 \pm 0.016$</td>
<td>$0.0601 \pm 0.0006$</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>396</td>
<td>$9.289 \pm 0.014$</td>
<td>$0.1085 \pm 0.0003$</td>
</tr>
<tr>
<td><strong>SIGNIFICANCE BETWEEN CONCENTRATIONS</strong></td>
<td></td>
<td></td>
<td>$p&lt;0.0001$</td>
<td>$p&lt;0.0001$</td>
</tr>
<tr>
<td>MYOSIN 1</td>
<td>low</td>
<td>195</td>
<td>$6.793 \pm 0.007$</td>
<td>$0.0368 \pm 0.0002$</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>380</td>
<td>$7.153 \pm 0.007$</td>
<td>$0.1066 \pm 0.0005$</td>
</tr>
<tr>
<td><strong>SIGNIFICANCE BETWEEN CONCENTRATIONS</strong></td>
<td></td>
<td></td>
<td>$p=0.2222$</td>
<td>$p&lt;0.0001$</td>
</tr>
<tr>
<td>TROPOMYOSIN 4</td>
<td>low</td>
<td>84</td>
<td>$6.433 \pm 0.029$</td>
<td>$0.0184 \pm 0.00017$</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>404</td>
<td>$6.3473 \pm 0.0048$</td>
<td>$0.0478 \pm 0.00014$</td>
</tr>
<tr>
<td><strong>SIGNIFICANCE BETWEEN CONCENTRATIONS</strong></td>
<td></td>
<td></td>
<td>$p=0.5795$</td>
<td>$p&lt;0.0001$</td>
</tr>
</tbody>
</table>

Table B.10: P-Values for Table 3.2. P-values for significance testing found in Table 3.2. Significance testing p-values are accompanied by a significance star rating ranging as follows: $p \leq 0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***, and $p<0.0001$ (****). Significance testing was generated using GraphPad Prism 8.3.1. software.
APPENDIX C:

EQUATION DERIVATIONS

C.1. Diffusion of Molecules

The following equation for the mean squared displacement (MSD) of molecules as a function of time was computed using Microcal™ Origin 6.0 software and explored in detail in chapter 3,

\[ MSD = MSD_p \left( 1 - e^{-\frac{t}{\tau}} \right) \quad \text{Equation C.1} \]

where \( MSD_p \) represents the plateau MSD in \( \mu m^2 \), \( t \) represents time in seconds, and \( \tau \) represents the time constant in seconds. Substitution of the exponential function in equation 3.1 with its Maclaurin series equivalent yields

\[ MSD = MSD_p \left( 1 - \left( 1 + \frac{-t}{\tau} \right) \right) \]

which simplifies to equation 3.2.

\[ MSD = MSD_p \left( \frac{t}{\tau} \right) \quad \text{Equation C.2} \]

It is a good assumption that molecules in the plasma membrane (PM) of cells can only undergo two-dimensional diffusion, which is given by

\[ MSD = 4Dt \quad \text{Equation C.3} \]

where \( D \) is the diffusion coefficient for a given molecule, \( MSD \) represents the mean squared displacement, and \( t \) represents the time. Equating equations 3.2 and 3.3 and solving for the diffusion of molecules is

\[ D = \frac{MSD_p}{4\tau} \quad \text{Equation C.4} \]

where \( MSD_p \) represents the plateau MSD in \( \mu m^2 \), and \( \tau \) represents the time constant in seconds.
C.2. Radius of Mobility

The mean radius of mobility of a molecule in a confined environment (inside a cluster) can move in a circle with area given by

\[ A = \pi r_{\text{mobility}}^2 \quad \text{Equation C.5} \]

where \( r_{\text{mobility}} \) represents the mean radius of mobility. This area (in \( \mu m^2 \)) can be directly related to the mean square displacement a molecule may travel inside a cluster (\( MSD_p \) from equation 3.1, C.1). Solving for the mean radius of mobility then yields

\[ r_{\text{mobility}} = \sqrt{\frac{MSD_p}{\pi}} \quad \text{Equation C.6} \]

where \( MSD_p \) represents the plateau MSD in \( \mu m^2 \).
APPENDIX D:

ADDITIONAL EXPERIMENTAL RESULTS

Total events used to calculate information in chapter 3 can be found in table D.1. Average cell properties used can be found in table D.2.

<table>
<thead>
<tr>
<th>Total Number of Events Used to Calculate Variables Listed for Each Time Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>τ = 10s</td>
</tr>
<tr>
<td>MOLECULAR FLUX</td>
</tr>
<tr>
<td>LATERAL RED FLUX</td>
</tr>
<tr>
<td>LATERAL GREEN FLUX</td>
</tr>
<tr>
<td>FREQUENCY OF MODELS</td>
</tr>
</tbody>
</table>

Table D.1: Total Number of Events Used to Calculate Variables Listed for Each Time Difference. All events used to calculate molecular flux (figure 3.7), lateral red flux (figure 3.10), lateral green flux (figure 3.10), and the frequency of models (figure 3.11) for each time difference, τ, for n=27 cells with density grid pixel width of 80 nanometers.

<table>
<thead>
<tr>
<th>Average Cell Properties for 2-Color FPALM Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Replicates</td>
</tr>
<tr>
<td>Total Cells</td>
</tr>
<tr>
<td>Average Sampled Cell Area</td>
</tr>
</tbody>
</table>
| Average Total Number of Localizations | 81,658 ± 12,284
| Red Channel | 81,658 ± 12,284
| Green Channel | 42,501 ± 7,999 |
| Average Total Number of Trajectories | 10,062 ± 8,468
| Red Channel | 10,062 ± 8,468
| Green Channel | 2,878 ± 524 |

Table D.2: Average Cell Properties for 2-Color FPALM Data. List of average cell properties used for analysis of two-species FPALM data.
BIOGRAPHY OF THE AUTHOR

Jaqulin Wallace was born in New York on August 7, 1994 to John and Nikki Wallace. She was raised in Woodbourne, New York, alongside her sister Frances, and graduated from Tri-Valley Secondary School in 2012 with a regent’s diploma. She then attended Alfred University for four years, graduating in 2016 with a Bachelor of Arts degree in physics and astronomy with concentrations in general physics and astrophysics. She then moved to Maine, where she entered the physics graduate program at the University of Maine in the fall of 2016. Her time in academia has awarded her the Natasha Goldowski Renner Prize in Physics in the spring of 2015 from Alfred University and University of Maine’s College of Liberal Arts and Sciences Graduate Student Excellence in Teaching Award for 2019. During the academic year of 2018-2019, Jaqulin held the office of secretary for the University of Maine’s Graduate Student Government. Jaqulin will be starting a post-baccalaureate position at the National Institute of Health in January of 2021.

Jaqulin is a candidate for the Master of Science degree in physics from the University of Maine in December 2020.