

# 1 A model-free method for measuring dimerization free energies of CLC-ec1 in 2 lipid bilayers

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## 8 9 **ABSTRACT**

10  
11 We previously reported the equilibrium dimerization reaction of the CLC-ec1 Cl<sup>-</sup>/H<sup>+</sup> transporter in 2:1  
12 POPE/POPG membranes (Chadda et al. 2016). This was determined by measuring the probability  
13 distributions of subunit capture into extruded liposomes by single-molecule photobleaching analysis  
14 across a wide range of subunit/lipid mole fraction densities. In this approach, knowledge of the liposome  
15 size distribution is necessary in order to correct the data for random co-capture events and extract the  
16 underlying dimerization reaction. For this we used a previously reported cryo-electron microscopy (cryo-  
17 EM) measured size distribution of 400 nm extruded liposomes made of *E. coli* polar lipids (Walden et al.  
18 2007). While the model and data agreed at low densities, we observed systematic inaccuracies at higher  
19 densities limiting our ability to extract  $F_{Dimer}$  in this range. To address this issue, we measured the 400 nm  
20 extruded 2:1 POPE/POPG liposome size distribution by cryo-EM and found that there is a small, but  
21 significant amount of larger liposomes in the population. Re-analysis of the I201W/I422W ‘WW’  
22 photobleaching data using this distribution shows that the protein is monomeric in the membrane and can  
23 serve as an experimental control. Dimer controls were constructed by glutaraldehyde cross-linking of  
24 C85A/H234C ‘WT’ or introducing R230C/L249C, which forms a spontaneous disulfide bond.  
25 Determination of  $F_{Dimer}$  based on the experimental controls yields improved fits and no change in the  
26 previously reported  $\Delta G^\circ$  values, providing an alternate model-free approach to measuring CLC-ec1  
27 dimerization in membranes.

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29

## 30 **RESULTS**

31

32 Equilibrium association reactions of stable membrane protein complexes in lipid bilayers have been  
33 challenging to measure due to limited protein signals. This has restricted studies to that of weak

34 complexes (Yano & Matsuzaki 2006; Cristian et al. 2011; North et al. 2006; Yano et al. 2015), though  
35 advances into steric-trapping have enabled the study of stable oligomers (Hong et al. 2010; Hong &  
36 Bowie 2011). For an alternate approach, it has been demonstrated that reconstitution of membrane  
37 proteins into liposomes can report on protein stoichiometry at low densities in the membrane (Walden et  
38 al. 2007; Robertson et al. 2010; Stockbridge et al. 2013; Fang et al. 2006). Using this approach and  
39 taking advantage of the sensitivity of single-molecule photobleaching analysis, we were able to measure  
40 CLC-ec1 stoichiometry at various subunit/lipid densities, including extremely dilute conditions where we  
41 observed a shift from dimer to monomer (Chadda et al. 2016; Chadda & Robertson 2016). The fraction  
42 of protein in the dimer state showed a reversible dependency on the membrane density, allowing for the  
43 determination of the dimerization free energy in lipid bilayers.

44  
45 The major challenge in this approach is to measure population stoichiometry accurately across a wide  
46 range of protein densities. In order to observe a complete change in the population from monomers to  
47 dimers, we must examine the stoichiometry across five orders of magnitude, and at densities greater than  
48 the Poisson limit where liposomes are likely to contain more than one protein species. This issue can be  
49 exacerbated in a heterogenous liposome population as larger liposomes tend to act like protein sinks.  
50 This random co-capture of protein subunits has been demonstrated to falsely report on oligomerization for  
51 membrane proteins in detergent micelles (Tanford & Reynolds 1976; Stanley & Fleming 2005), and it  
52 must be corrected for in any reconstituted system. Previously, we used a stochastic simulation of the  
53 Poisson process of subunit reconstitution into a defined liposome population based on the ‘Walden’  
54 distribution of 400 nm extruded vesicles comprised of *E. coli* polar lipids (EPL) (Figure 1A,B) (Walden  
55 et al. 2007). While the data and model agreed at lower densities, it systematically deviated at higher  
56 densities where the data contained fewer single-steps and more multi-step photobleaching events as  
57 compared to the model (Figure 1C,D). We hypothesized that the larger liposomes were under-represented  
58 in the Walden distribution leading to an underestimation of liposomes containing more than 3 steps in the  
59 model. We directly investigated this by measuring the 400 nm extruded 2:1 POPE/POPG liposome size  
60 distribution by cryo-electron microscopy (Figure 1E), which shows that there is a significant proportion  
61 of larger liposomes that is missing in the Walden distribution. The difference is small but the effect is  
62 amplified when considering the fractional surface area (Figure 1F), which dictates the Poisson process of  
63 subunit capture. This underrepresentation of larger liposomes in the Walden distribution could arise due  
64 to size selection during the freezing of cryo-electron microscopy samples, or it could be a result of minor  
65 differences in the lipid composition. EPL is a crude extract with approximately 67% POPE, 20% POPG  
66 and 10% cardiolipin, while our experimental lipid conditions is a synthetic mimic made of 67% POPE  
67 and 23% POPG.

68  
69 With the updated liposome size distribution, we re-examined the I201W/I422W ('WW') CLC-ec1  
70 photobleaching data from Chadda et al. (Chadda et al. 2016). Previously, this construct was found to be  
71 monomeric in detergent by both glutaraldehyde cross-linking, x-ray crystallography and also in 3:1 egg  
72 PC/POPG liposomes reconstituted at  $\chi = 1.5 \times 10^{-5}$  subunits/lipid (1  $\mu\text{g}/\text{mg}$ ) (Robertson et al. 2010). We  
73 also observed differences in the fraction of empty liposomes ( $F_0$ ) by single-molecule co-localization  
74 microscopy for the Cy5 labeled protein and Alexa Fluor 488 labeled liposomes, indicating that the protein  
75 occupancy was consistent with a monomer at high densities (Chadda et al. 2016). However, when we  
76 calculated  $F_{Dimer}$  using the Walden distribution, we observed a weak apparent dimerization reaction that  
77 either indicated an actual formation of dimers or inaccuracies of the modeling at higher densities. With  
78 the new liposome distribution, we find that the experimental data converges with the simulated monomer  
79 probabilities (Figure 1G) and that the apparent dimerization is no longer present (Figure 1H). This,  
80 together with the other evidence presented in previous studies, demonstrates that WW is monomeric  
81 within our experimental range of densities. This observation indicates that WW can serve as a monomeric  
82 control in future experiments.

83  
84 With a monomeric control in place, we were motivated to identify an experimental dimer control, to  
85 establish a model-free approach to measuring dimerization that does not require prior knowledge of the  
86 liposome size distribution. For this, we turned to covalent cross-linking methods that have already been  
87 well established for CLC-ec1. First, we tried glutaraldehyde that covalently cross-links the dimer state as  
88 demonstrated on SDS-PAGE (Figure 2 – figure supplement 1) (Maduke et al. 1999; Robertson et al.  
89 2010). Glutaraldehyde is a short chain bis-reactive molecule and cross-links proteins via primary amine  
90 groups present on lysines and the N-terminus. CLC-ec1 has 13 native lysine residues (Figure 2A) and  
91 while the dimer is the major cross-linked product, we also observe a small tetramer population and a  
92 small amount of resistant monomer (Figure 2 - supplementary figure 1), highlighting the non-specific  
93 nature of this reaction. Measurement of the photobleaching probability distribution shows that the WT-  
94 Cy5 + glutaraldehyde proteoliposomes follow the 2:1 POPE/POPG dimer model, as well as the saturating  
95 range of the WT-Cy5 photobleaching data (Figure 2B). However, we observe lower values of  $P_1$  and  
96 higher values of  $P_{3+}$ , which agrees with the observation that a small fraction of protein is non-specifically  
97 cross-linked in a higher oligomeric state. This contribution, however, is small and the majority of the  
98 reconstituted protein is dimeric. Upon measurement of functional activity, however, it was found that a  
99 large fraction of the protein is non-functional (Figure 2 – figure supplement 1). Therefore, while the  
100 protein is present in the membrane, in mainly a dimeric form, the background glutaraldehyde  
101 modifications or cross-linking states have a significant effect on transport function. With this in mind, we

102 reserve glutaraldehyde cross-linked WT as a structural dimeric control in the membrane, but not one with  
103 a proper biological fold.

104  
105 For an alternate approach, we investigated disulfide cross-linking across the dimerization interface.  
106 Previously, Nguitrageol & Miller demonstrated that the CLC-ec1 dimer could spontaneously cross-link  
107 via a disulfide bond between R230C and L249C, during expression and/or purification (Nguitrageol &  
108 Miller 2007). We introduced R230C/L249C onto the C85A/H234C ‘WT’ background (Figure 2C) and  
109 found that the protein expressed, purified as a dimer in detergent micelles (Figure 2 – figure supplement  
110 2), and ran as a dimer on SDS-PAGE indicating disulfide formation prior to purification (Figure 2C). The  
111 disulfide bond is not modified by the reducing agent tris(2-carboxyethyl)phosphine (TCEP) included in  
112 the purification, which allows for H234C to remain reactive for Cy5-maleimide labeling at yields  
113 comparable to the WT. The photobleaching probability distributions were measured for 2:1 POPE/POPG  
114 membranes incubated at room temperature for 1-4 days and then extruded using a 400 nm filter (Figure  
115 2D). The data shows that the photobleaching probability distribution of R230C/L249C-Cy5 corresponds  
116 to the ideal dimer simulation based on the updated 2:1 POPE/POPG liposome size distribution, as well as  
117 the saturating range of the WT-Cy5 data. In addition, we measured the chloride transport function of the  
118 R230C/L249C proteoliposomes reconstituted at  $\chi = 1.5 \times 10^{-5}$  subunits/lipid, which showed comparable  
119 function to WT (Figure 2 – figure supplement 2). Therefore, R230C/L249C provides a functionally  
120 competent dimer control for CLC-ec1 dimerization reactions, preserving the native functional fold.

121  
122 With these non-reactive, ‘ideal’ monomer and dimer experimental controls, we re-calculated  $F_{Dimer}$  for the  
123 WT-Cy5 and W-Cy5 photobleaching data. The fits of the equilibrium dimerization isotherm (Figure 3)  
124 are improved using either WT + glutaraldehyde or R230C/L249C data for the dimer state and WW data  
125 defining the monomeric state. The values of  $\Delta G^\circ$  and  $\Delta\Delta G$  are not significantly different compared to our  
126 previous report (Figure 3E), showing that the simulation method is sufficient to estimate  $F_{Dimer}$  but that  
127 the physical controls provide a model-free method of obtaining the same results.

128  
129 This study reports on methodological advances developed for measuring dimerization of CLC-ec1 in 2:1  
130 POPE/POPG lipid bilayers. Using a newly determined liposome size distribution, we increase the  
131 dynamic range of the measurement one order of magnitude up to  $\chi = 3.8 \times 10^{-5}$  subunits/lipid, allowing  
132 for stability measurements of weaker complexes. We also establish experimental monomeric (WW) and  
133 dimeric (WT + glutaraldehyde or R230C/L249C) controls that provide an easy way of correcting the  
134 experimental data for random co-capture without knowledge of the liposome size distribution. This  
135 simplifies measurement of the reaction under changing conditions, for instance temperature and lipid

136 composition, both of which are expected to change the liposome size distribution. The overall agreement  
137 between the statistical modeling approach and experimental controls demonstrates that the subunit capture  
138 and accounting approach is sufficiently rigorous for reporting on membrane protein stoichiometry in lipid  
139 bilayers.

140

## 141 **METHODS**

142 The bulk of the methods used in this study follow that reported in (Chadda et al. 2016). Details of  
143 experiments specific to this study are outlined here.

144

145 **Cross-linking of ‘WT’ C85A/H234C CLC-ec1.** For running on SDS-PAGE, glutaraldehyde (Sigma  
146 Aldrich, St. Louis, MO) was added to 8  $\mu$ M WT in size exclusion buffer (SEB; 150 mM NaCl, 20 mM  
147 MOPS pH 7.5, 5 mM analytical-grade DM, Anatrace, Maumee, IL), for a final concentration of  
148 glutaraldehyde of 0.4% wt/vol (~40 mM). The reaction was allowed to proceed for 8 minutes after which  
149 10x Tris or Glycine buffer was added to quench the reaction. For reconstitution, ‘WT’ protein was  
150 labeled with Cy5-maleimide as described previously, then cross-linked with glutaraldehyde and quenched  
151 as described above, before reconstitution into 2:1 POPE/POPG liposomes (Avanti Polar Lipids,  
152 Alabaster, AL). For the R230C/L249C disulfide cross-linked construct (Nguitragool & Miller 2007),  
153 mutations were added to the C85A/H234C background using a Quickchange II site-directed mutagenesis  
154 kit (Agilent Technologies, Santa Clara, CA). Purification was carried out as described previously (Chadda  
155 et al. 2016), in the presence of 1 mM TCEP until the SEC purification step. Labeling and reconstitution  
156 was carried out as before. For SDS-PAGE, the sample was run on non-reducing gels. For DTT  
157 reduction, 10  $\mu$ M of protein was incubated with 100 mM DTT at 30 °C for 1 hour.

158

159 **Cryo-electron microscopy measurements of liposome size distributions.** Liposomes were  
160 freeze/thawed seven times, incubated at room temperature, and then extruded through a 400 nm  
161 nucleopore filter (GE Life Sciences, Chicago, IL) 21 times prior to sample freezing. For freezing, 3  $\mu$ L of  
162 the undiluted sample was loaded onto glow-discharged Lacey carbon support films (Electron Microscope  
163 Sciences, Hatfield, PA), blotted and plunged into liquid ethane using a Vitrobot System (FEI, Hillsboro,  
164 OR). Images were collected at 300 kV on a JEOL 3200 fs microscope (JEOL, Tokyo, Japan) with a Gatan  
165 K2 Summit direct electron detector camera (GATAN, Pleasanton, CA). Magnifications of 15K and 30K  
166 were used. Liposome sizes were analyzed in Fiji & ImageJ (Schneider et al. 2012; Schindelin et al.  
167 2012), and all liposomes were included in counting, even those on the carbon outside of the vitreous ice.

168

169 ***F<sub>Dimer</sub>* calculator.** A MATLAB app was created to calculate the fraction of dimer using the various  
170 models and experimental controls, and the sum of  $R^2$  analysis. All of the models and experimental  
171 control data are implemented in the code. For the modeling,  $P_{Cys} = 0.72$ ,  $P_{non-specific} = 0.14$  was used, and a  
172 bias of 4 for size exclusion of the dimer model, and  $A_{lipid} = 0.6 \text{ nm}^2$  as described in (Chadda et al. 2016;  
173 Chadda & Robertson 2016). The app file is available for download as a source file in the supplementary  
174 information. MATLAB 2016b or higher is required.

175

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## 182 REFERENCES

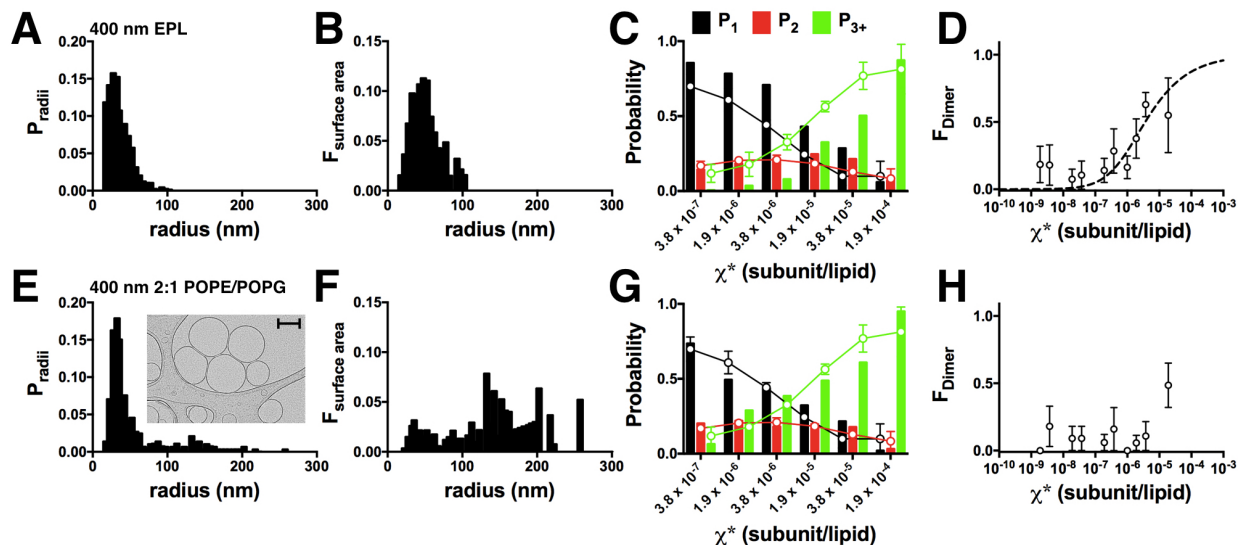
- 183 Chadda, R. & Robertson, J.L., 2016. Measuring Membrane Protein Dimerization Equilibrium in Lipid  
184 Bilayers by Single-Molecule Fluorescence Microscopy. *Methods in enzymology*, 581, pp.53–82.
- 185 Chadda, R. et al., 2016. The dimerization equilibrium of a ClC Cl(-)/H(+) antiporter in lipid bilayers.  
186 *eLife*, 5, p.e17438.
- 187 Cristian, L., Lear, J.D. & DeGrado, W.F., 2011. Use of thiol-disulfide equilibria to measure the energetics  
188 of assembly of transmembrane helices in phospholipid bilayers. *Proceedings of the National*  
189 *Academy of Sciences of the United States of America*, 100(25), pp.14772–14777.
- 190 Fang, Y., Kolmakova-Partensky, L. & Miller, C., 2006. A Bacterial Arginine-Agmatine Exchange  
191 Transporter Involved in Extreme Acid Resistance. *Journal of Biological Chemistry*, 282(1), pp.176–  
192 182.
- 193 Hong, H. & Bowie, J.U., 2011. Dramatic destabilization of transmembrane helix interactions by features  
194 of natural membrane environments. *Journal of the American Chemical Society*, 133(29), pp.11389–  
195 11398.
- 196 Hong, H. et al., 2010. Method to measure strong protein-protein interactions in lipid bilayers using a  
197 steric trap. *Proc Natl Acad Sci USA*, 107(46), pp.19802–19807.
- 198 Maduke, M., Pheasant, D.J. & Miller, C., 1999. High-level expression, functional reconstitution, and  
199 quaternary structure of a prokaryotic ClC-type chloride channel. *The Journal of general physiology*,  
200 114(5), pp.713–722.
- 201 Nguitragool, W. & Miller, C., 2007. ClC Cl /H+ transporters constrained by covalent cross-linking.  
202 *Proceedings of the National Academy of Sciences*, 104(52), pp.20659–20665.
- 203 North, B. et al., 2006. Characterization of a Membrane Protein Folding Motif, the Ser Zipper, Using  
204 Designed Peptides. *Journal of molecular biology*, 359(4), pp.930–939.

- 205 Robertson, J.L., Kolmakova-Partensky, L. & Miller, C., 2010. Design, function and structure of a  
206 monomeric ClC transporter. *Nature*, 468(7325), pp.844–847.
- 207 Schindelin, J. et al., 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods*,  
208 9(7), pp.676–682.
- 209 Schneider, C.A., Rasband, W.S. & Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image  
210 analysis. *Nature Methods*, 9(7), pp.671–675.
- 211 Stanley, A.M. & Fleming, K.G., 2005. The transmembrane domains of ErbB receptors do not dimerize  
212 strongly in micelles. *Journal of molecular biology*, 347(4), pp.759–772.
- 213 Stockbridge, R.B. et al., 2013. A family of fluoride-specific ion channels with dual-topology architecture.  
214 *eLife*, 2.
- 215 Tanford, C. & Reynolds, J.A., 1976. Characterization of membrane proteins in detergent solutions.  
216 *Biochimica et biophysica acta*, 457(2), pp.133–170.
- 217 Walden, M. et al., 2007. Uncoupling and turnover in a Cl<sup>-</sup>/H<sup>+</sup> exchange transporter. *The Journal of*  
218 *general physiology*, 129(4), pp.317–329.
- 219 Yano, Y. & Matsuzaki, K., 2006. Measurement of Thermodynamic Parameters for Hydrophobic  
220 Mismatch 1: Self-Association of a Transmembrane Helix †. *Biochemistry*, 45(10), pp.3370–3378.
- 221 Yano, Y. et al., 2015. Cholesterol-induced lipophobic interaction between transmembrane helices using  
222 ensemble and single-molecule fluorescence resonance energy transfer. *Biochemistry*, 54(6), pp.1371–  
223 1379.
- 224

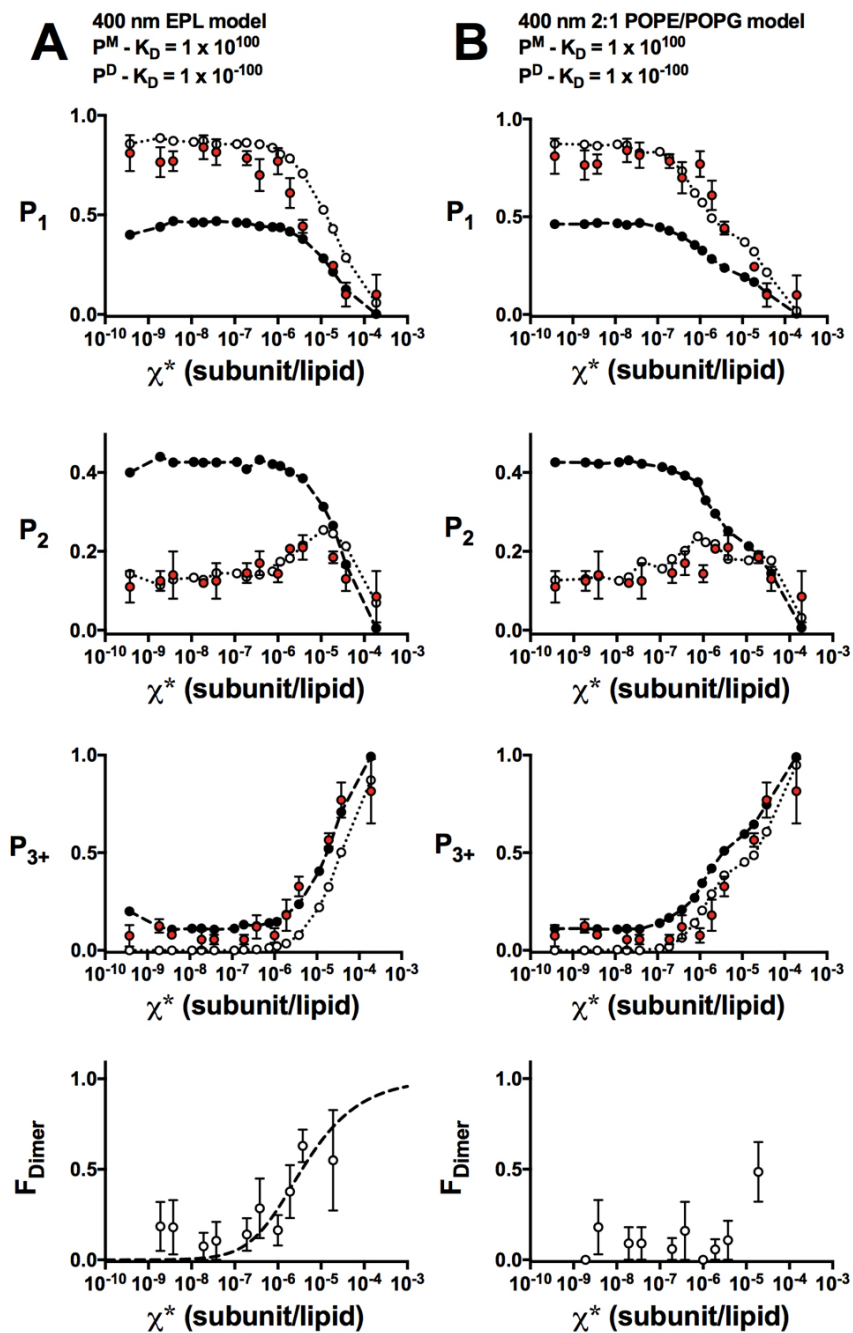
**Table 1. Summary of free energy values for CLC-ec1 dimerization in 2:1 POPE/POPG lipid bilayers.** Free energies are calculated as  $\Delta G^\circ = -RT \ln(K_{eq}/\chi^\circ)$  where  $\chi^\circ$  is the standard state of 1 subunit/lipid, R is the gas constant  $1.99 \times 10^{-3} \text{ kcal mole}^{-1} \text{ K}^{-1}$ , and T is 298 K (25 °C), where  $K_{eq}$  is determined by fitting to the equilibrium dimerization isotherm. Data represent best-fit  $\pm$  error of fit ( $R^2$ ).

Monomer/dimer data	$\Delta G^\circ_{WT}$ (kcal/mole)	$\Delta G^\circ_W$ (kcal/mole)
P <sup>M</sup> : 400 nm EPL, $K_D = 1 \times 10^{100}$ P <sup>D</sup> : 400 nm EPL, $K_D = 1 \times 10^{-100}$	-11.1 $\pm$ 0.1 (0.91)	-9.4 $\pm$ 0.2 (0.87)
P <sup>M</sup> : 400 nm 2:1 POPE/POPG, $K_D = 1 \times 10^{100}$ P <sup>D</sup> : 400 nm 2:1 POPE/POPG, $K_D = 1 \times 10^{-100}$	-10.8 $\pm$ 0.2 (0.80)	-8.7 $\pm$ 0.2 (0.71)
P <sup>M</sup> : WW P <sup>D</sup> : WT + glutaraldehyde	-10.3 $\pm$ 0.1 (0.88)	-8.4 $\pm$ 0.2 (0.78)
P <sup>M</sup> : WW P <sup>D</sup> : R230C/L249C	-10.8 $\pm$ 0.1 (0.89)	-8.8 $\pm$ 0.2 (0.80)

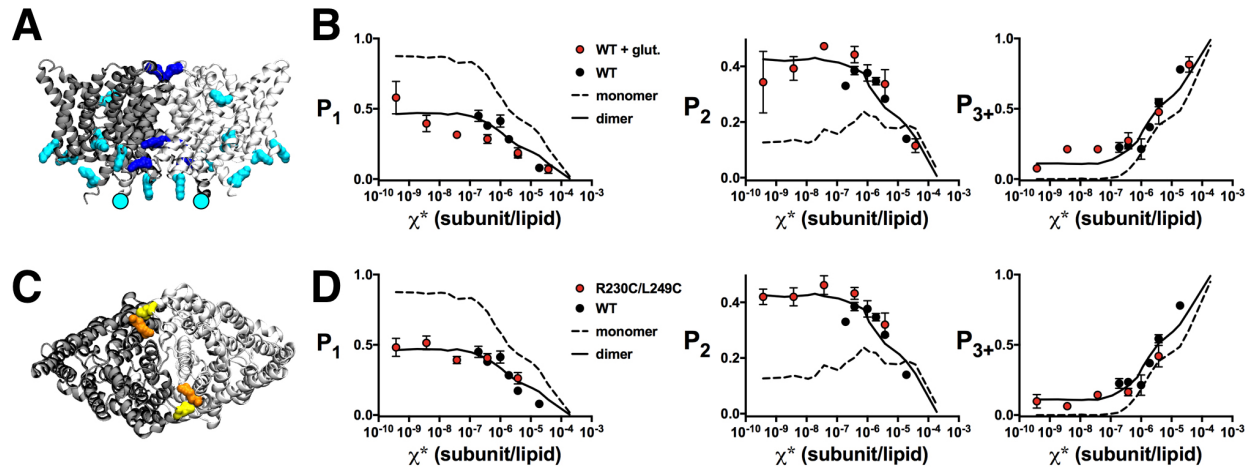




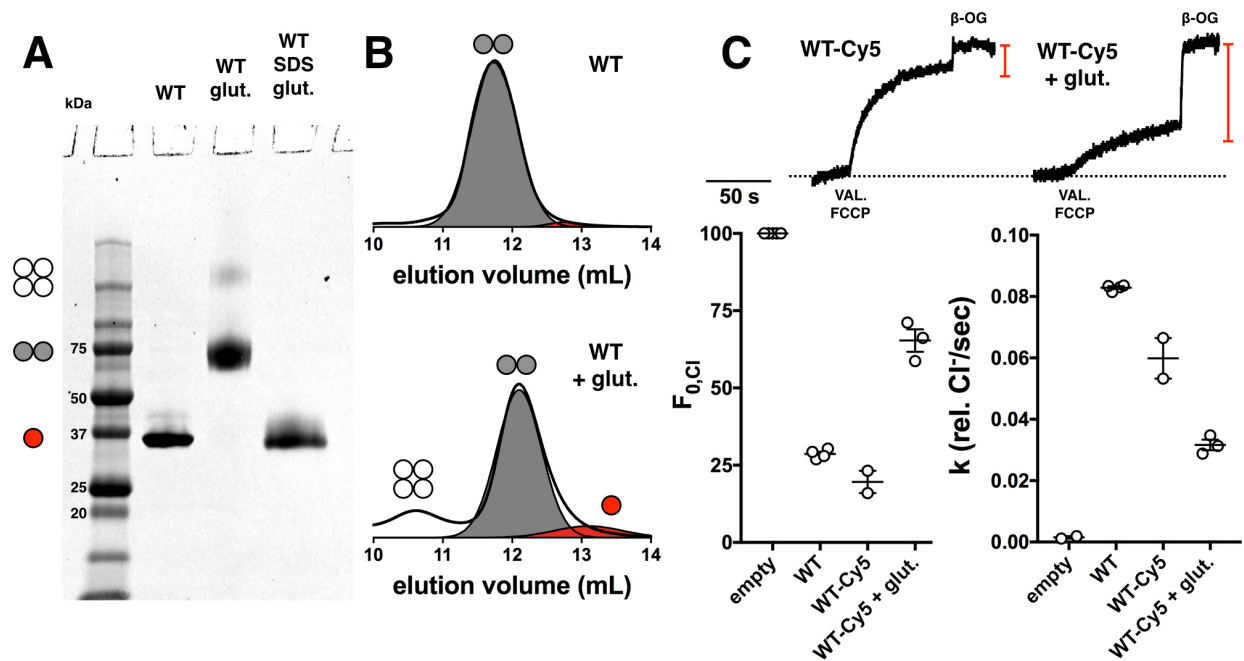
**Figure 1. The 400 nm 2:1 POPE/POPG liposome size distribution indicates WW-Cy5 is monomeric.** (A) 400 nm EPL liposome size distribution reported in Walden et al. (Walden et al., 2007). (B) Fractional surface area distribution. (C) Photobleaching distribution of model (bars) and experimental WW data (circles-lines). (D)  $F_{Dimer}$  values for WW-Cy5 determined using the Walden distribution, reported in Chadda et al. (Chadda et al., 2016). (E) The 400 nm 2:1 POPE/POPG liposome size distribution (mean, n=2). Inset shows a cryo-electron microscopy image of the liposomes (raw image attached as source data), scale bar - 200 nm. (F) Fractional surface area distribution ( $F_{surface\ area}$ ). (G) Photobleaching distribution of model (bars) and experimental WW data (circles-lines). (H) Re-calculated  $F_{Dimer}$  values for WW-Cy5 from the updated 2:1 POPE/POPG distribution, demonstrating that WW-Cy5 is monomeric across the dynamic range of the experiment. Data represented as mean  $\pm$  SEM, n=2-3.



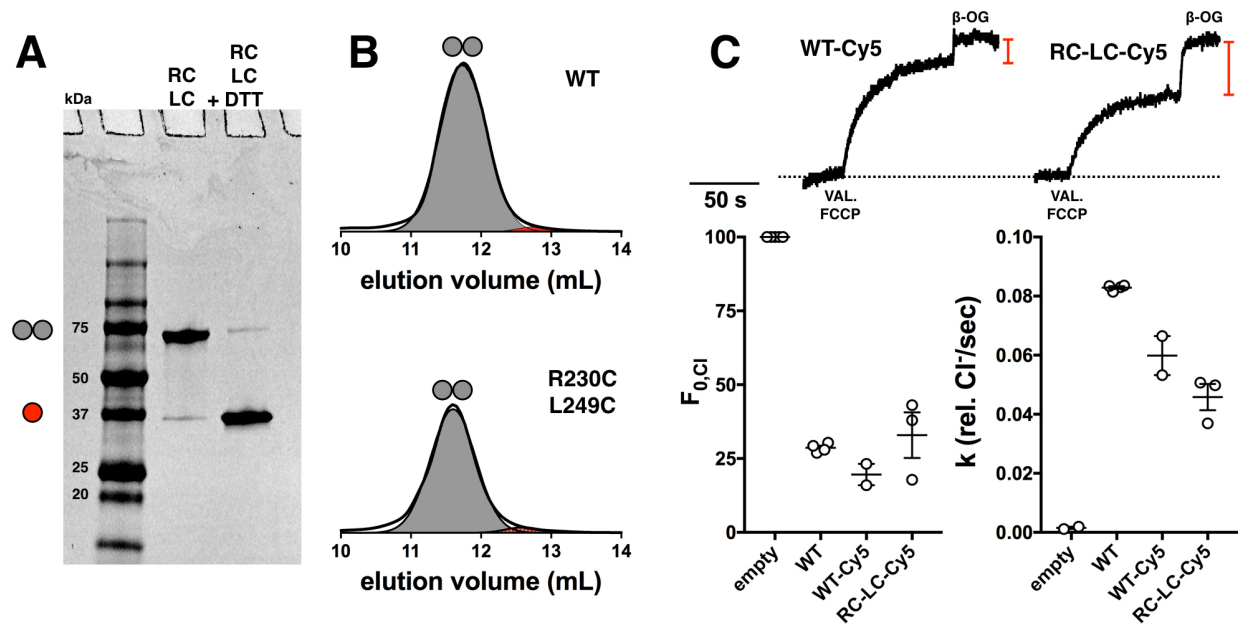
**Figure 1 - figure supplement 1.  $F_{\text{Dimer}}$  analysis of WW-Cy5 in 2:1 POPE/POPG membranes.** Photobleaching probabilities ( $P_1$ ,  $P_2$ ,  $P_{3+}$ ) and  $F_{\text{Dimer}}$  analysis (bottom row) of WW-Cy5 (red) along with monomer (white) and dimer (black) probabilities. Monomer and dimer probabilities determined by (A) modeling using the 400 nm EPL Walden liposome size distribution,  $\Delta G^\circ = -7.4 \pm 0.2$  kcal/mole,  $R^2 = 0.30$  (B) modeling using the 400 nm 2:1 POPE/POPG liposome size distribution, no fit shown. Model parameters set as:  $P_{\text{Cy5}} = 0.72$ ,  $P_{\text{non-specific}} = 0.14$ , bias = 4 and  $A_{\text{lipid}} = 0.6$ . Data represent mean  $\pm$  SEM,  $n=2-3$ .



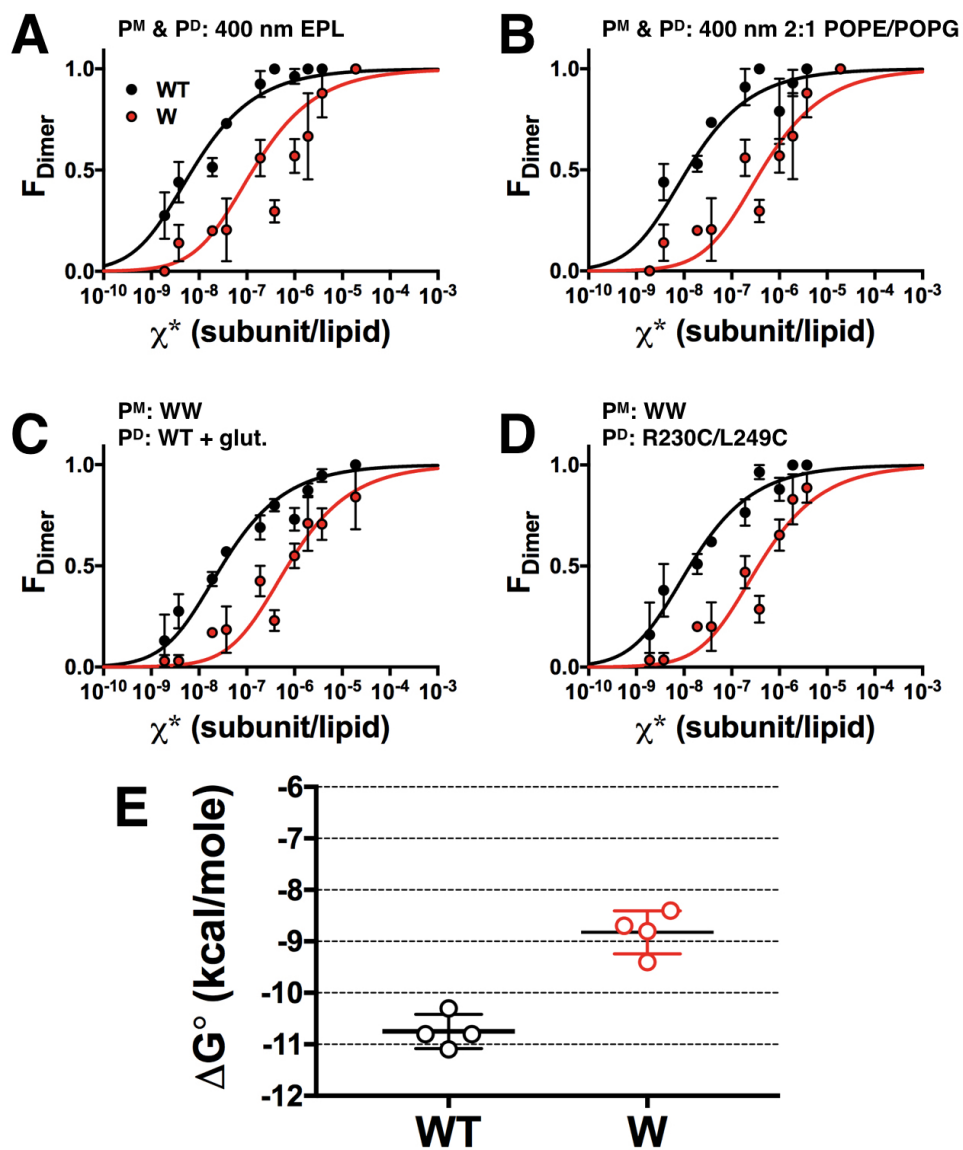
**Figure 2. Photobleaching analysis of cross-linked CLC dimers.** (A) Side view of CLC-ec1 homodimer, with the two subunits colored light and dark grey. Residues with primary amine groups that are potential glutaraldehyde cross-linking sites are highlighted. Lysines are turquoise with those within 5 Å of another lysine colored dark blue. The N-terminus is highlighted as a circle. (B) Photobleaching data of WT-Cy5 + glutaraldehyde (red) compared to the saturating range of WT-Cy5 (black) and monomer/dimer models using the 2:1 POPE/POPG liposome size distribution. Data are represented as mean  $\pm$  SEM,  $n = 4-6$ ,  $P_{Cy5} = 0.70 \pm 0.04$ . (C) Top view of the CLC-ec1 homodimer showing R230C (orange) and L249C (yellow) positions. (D) Photobleaching data of R230C/L249C-Cy5 (red) compared to the saturating range of WT-Cy5 (black) and monomer/dimer models using the 2:1 POPE/POPG liposome size distribution. Data are represented as mean  $\pm$  SEM,  $n=5$ ,  $P_{Cy5} = 0.72 \pm 0.02$ . Model parameters set as:  $P_{Cy5} = 0.72$ ,  $P_{non-specific} = 0.14$ ,  $bias = 4$  and  $A_{lipid}=0.6$ .



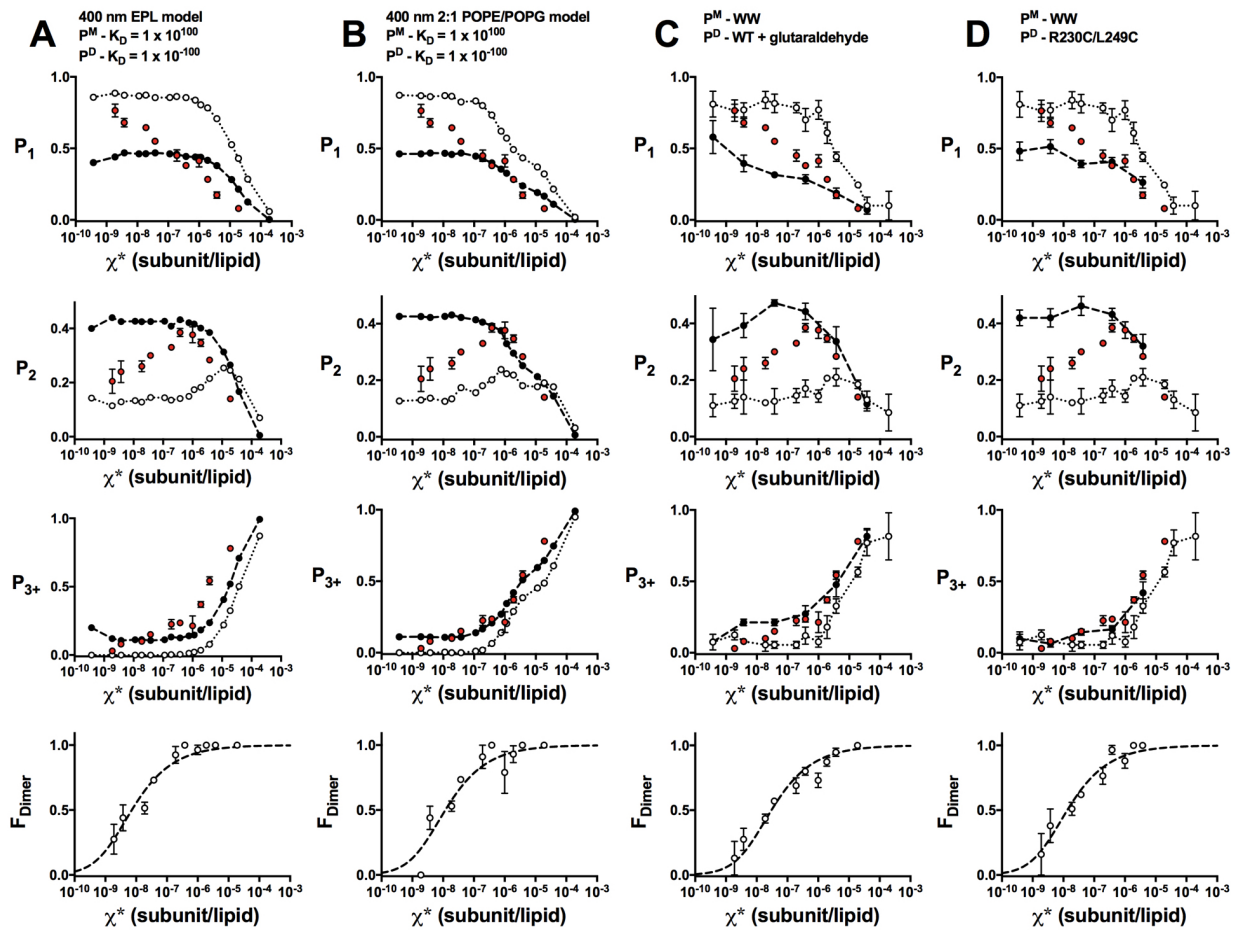
**Figure 2 - figure supplement 1. Glutaraldehyde cross-linking of WT-Cy5.** (A) SDS-PAGE of WT, WT + glutaraldehyde, WT + 2% SDS followed by addition of glutaraldehyde. Red circle – monomer, grey circles – dimer, white circles - tetramer. (B) Size exclusion chromatography profiles of WT and glutaraldehyde cross-linked WT. (C) Functional Cl<sup>-</sup> transport of WT-Cy5 and WT-Cy5 + glutaraldehyde, reconstituted at 1 μg/mg ( $\chi = 1.5 \times 10^{-5}$  subunits/lipid). Data represented as mean ± SEM. The fractional volume of empty liposomes ( $F_{0,Cl}$ , red bar) and transport rate ( $k$ ) shows a significant reduction in CLC function in the presence of glutaraldehyde ( $p < 0.0001$  &  $p = 0.009$  respectively).



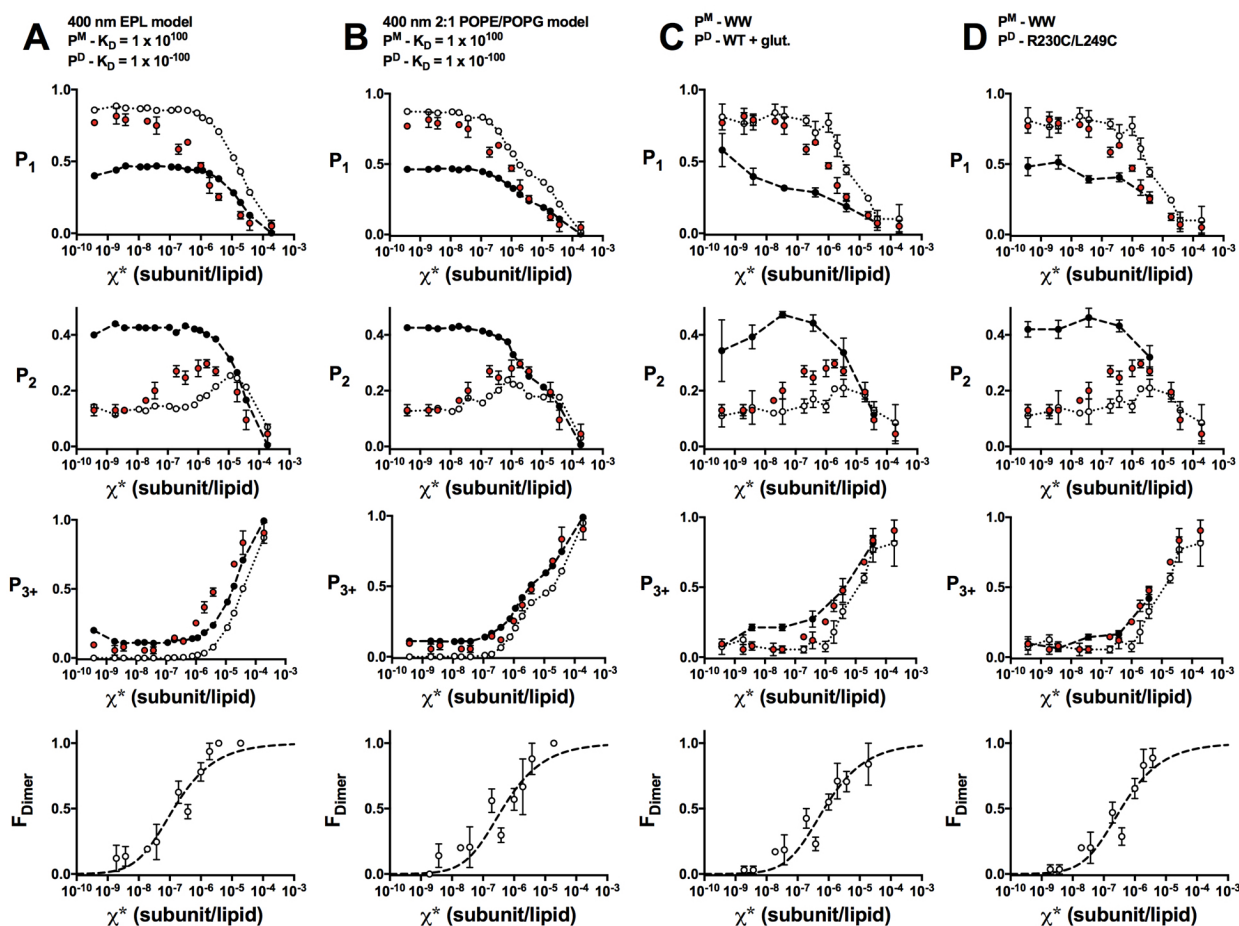
**Figure 2 - figure supplement 2. Disulfide cross-linking of CLC-ec1.** (A) SDS-PAGE of R230C/L249C and R230C/L249C treated with DTT. Red circle – monomer, grey circles – dimer. (B) Size exclusion chromatography profiles of WT and R230C/L249C. (C) Functional Cl<sup>-</sup> transport of WT-Cy5 and R230C/L249C-Cy5 (RC-LC-Cy5), reconstituted at 1  $\mu$ g/mg,  $\chi = 1.5 \times 10^{-5}$  subunits/lipid. Data represented as mean  $\pm$  SEM. The fractional volume of empty liposomes ( $F_{0,Cl}$ , red bar) and Cl<sup>-</sup> transport rate (k) show no significant change between WT-Cy5 and RC-LC-Cy5 samples.



**Figure 3. Model-free measurements of WT-Cy5 and W-Cy5 dimerization free energies in 2:1 POPE/POPG lipid bilayers.**  $F_{\text{Dimer}}$  analysis of WT-Cy5 (black) and W-Cy5 (red). Monomer and dimer probabilities determined by (A) modeling using the 400 nm EPL Walden liposome size distribution (model parameters set as:  $P_{\text{Cy5}} = 0.72$ ,  $P_{\text{non-specific}} = 0.14$ , bias = 4 and  $A_{\text{lipid}}=0.6$ ), (B) modeling the 400 nm 2:1 POPE/POPG liposome size distribution, (C) WW-Cy5 for monomer and glutaraldehyde cross-linked WT-Cy5 for dimer, (D) WW-Cy5 for monomer and R230C/L249C-Cy5 for dimer. Data represent mean  $\pm$  SEM,  $n=2-3$ . (E) Comparison of  $\Delta G^\circ$  values for all monomer/dimer models and controls, mean  $\pm$  SD. Values are reported in Table 1, and figure supplements 1 & 2.



**Figure 3 - figure supplement 1.  $F_{\text{Dimer}}$  analysis of WT-Cy5 in 2:1 POPE/POPG membranes.** Photobleaching probabilities ( $P_1$ ,  $P_2$ ,  $P_{3+}$ ) and  $F_{\text{Dimer}}$  analysis (bottom row) of WT-Cy5 (red) along with monomer (white) and dimer (black) probabilities. Monomer and dimer probabilities determined by (A) modeling using the 400 nm EPL Walden liposome size distribution (model parameters set as:  $P_{\text{Cy5}} = 0.72$ ,  $P_{\text{non-specific}} = 0.14$ , bias = 4 and  $A_{\text{lipid}} = 0.6$ ),  $\Delta G^\circ = -11.1 \pm 0.1$  kcal/mole,  $R^2 = 0.91$  (B) modeling using the 400 nm 2:1 POPE/POPG liposome size distribution,  $\Delta G^\circ = -10.8 \pm 0.2$  kcal/mole,  $R^2 = 0.80$  (C) WW-Cy5 for monomer and glutaraldehyde cross-linked WT-Cy5 for dimer,  $\Delta G^\circ = -10.3 \pm 0.1$  kcal/mole,  $R^2 = 0.88$ , and (D) WW-Cy5 for monomer and R230C/L249C-Cy5 for dimer,  $\Delta G^\circ = -10.8 \pm 0.1$  kcal/mole,  $R^2 = 0.89$ . Data represent mean  $\pm$  SEM.



**Figure 3 - figure supplement 2.  $F_{Dimer}$  analysis of W-Cy5 in 2:1 POPE/POPG membranes.** Photobleaching probabilities ( $P_1$ ,  $P_2$ ,  $P_{3+}$ ) and  $F_{Dimer}$  analysis (bottom row) of W-Cy5 (red) along with monomer (white) and dimer (black) probabilities. Monomer and dimer probabilities determined by (A) modeling using the 400 nm EPL Walden liposome size distribution (model parameters set as:  $P_{Cy5} = 0.72$ ,  $P_{non-specific} = 0.14$ , bias = 4 and  $A_{lipid} = 0.6$ ),  $\Delta G^\circ = -9.4 \pm 0.2$  kcal/mole,  $R^2 = 0.87$  (B) modeling using the 400 nm 2:1 POPE/POPG liposome size distribution,  $\Delta G^\circ = -8.7 \pm 0.2$  kcal/mole,  $R^2 = 0.71$  (C) WW-Cy5 for monomer and glutaraldehyde cross-linked WT-Cy5 for dimer,  $\Delta G^\circ = -8.4 \pm 0.2$  kcal/mole,  $R^2 = 0.78$ , and (D) WW-Cy5 for monomer and R230C/L249C-Cy5 for dimer,  $\Delta G^\circ = -8.8 \pm 0.2$  kcal/mole,  $R^2 = 0.80$ . Data represent mean  $\pm$  SEM.