SUPPLEMENTARY MATERIALS AND METHODS

Buffers

The following buffers were used: Buffer A, 100 mM NaCl, 20 mM HEPES pH 7.5, 0.1% dodecylmaltoside (Anatrace); Buffer B, Buffer A and 2 mM EDTA; Buffer C, Buffer A with 300 μM alprenolol (Sigma) and 2 mM $CaCl_2$; Buffer D, Buffer A with 2 mM CaCl2; Buffer E, Buffer A with 0.01% cholesterolhemisuccinate; Buffer F, Buffer A with no dodecylmaltoside; Buffer G, Buffer F with 1% octylglucoside; Lysis buffer, 10 mM Tris-HCl, pH 7.5, with 1 mM EDTA, 1 μM Alprenolol, 160 μg/ml benzamidine and 2.5 μg/ml leupeptin; Solubilization buffer, 20 mM Tris-HCl, pH 7.5, with 1.0% *n*-dodecyl-maltoside (DDM) (Anatrace), 100 mM NaCl, 160 μg/ml benzamidine, 2.5 μg/ml leupeptin and 1 μM alprenolol. Reconstitution buffer, 20 mM HEPES pH 7.5, 100 mM NaCl.

Expression and purification of β_2-AR from Sf9 Insect Cells

β_2-AR was purified from Sf9 cells using a three-step purification procedure as described previously (Granier et al, 2007). Briefly, cell pellets were lysed in lysis buffer and centrifuged (15 min at 30,000*g*). Lysed cells were resuspended in solubilization buffer and dounced with ~ 30 strokes using a Wheaton tissue grinder (Millville, NJ) and then stirred for 1 h at 4 °C. Solubilized receptor was purified by chromatography using M1 Flag antibody affinity resin (Sigma). The eluate from the M1 anti-Flag column was further purified on an alprenolol-sepharose affinity column and finally through a second M1 Flag antibody affinity resin purification step. Purified detergent-soluble receptor was stored in Buffer E. The concentration of functional, purified receptor was determined as follows: purified receptor was incubated in triplicate in Buffer A with a saturating concentration of [^{3}H]-DHA (10 nM) in a total volume of 100 μL for 1 hr at room temperature. Free [^{3}H]-DHA was separated from bound by passing through a Sephadex G50 Medium column (4 cm x 0.5 cm). Non-specific binding was determined in the presence of 1 μM alprenolol.

Factor Xa , PNGase F Proteolysis and Modification by NHS-PEO_4-Biotin

For proteolysis, 15 pmol of reconstituted samples were subjected to either 1 μl of Factor Xa, or 1 μl of PNGase F or 1 μl of both Factor X and PNGase F for 2 h at room temperature. Proteolysis was stopped by the addition of SDS sample buffer. Samples were ran on 10% SDS-PAGE and transferred onto nitrocellulose membranes. An M1 antibody conjugated to an Alexa-680 fluorophore was used for detection on Odyssey Li-Cor imager. Similarly, for modification of the FLAG epitope, 15 pmol of reconstituted samples were treated with a 20-fold molar excess of NHS-PEO_4-Biotin and incubated on ice for 2 h, per manufacturer recommendation. Excess NHS-PEO_4-Biotin was removed using gel filtration by Sephadex G-50. Samples were then processed and imaged similar to proteolysis samples, with the addition of blotting for the C-terminal 6-Histidine tag using secondary goat-antimouse antibody conjugated to IR-800. Quantification of bands was done using Kodak Molecular Imaging Software, v4.0.3.

Isopycnic Density Centrifugation

Briefly, NBD-labeled phosphocholine (Avanti Polar Lipids, Inc.) was added to the DOPC plus cholesterol hemisuccinate lipid mixture at a final of 0.4% of the total lipid content. Cy5 labeled β_2AR was reconstituted into NBD-containing lipids and subjected to a sucrose gradient. Sucrose solutions (60%, 24%, 18%, 12% and 6%, wt/wt) were prepared in reconstitution buffer. First, 250 μl of reconstituted sample was mixed with 3.0 ml of the 60% sucrose and 2.75 ml of reconstitution buffer containing 0.05% Triton X-100. This 6.0 ml solution was added to the bottom of a 40 ml ultracentrifuge tube, followed by layering with the remaining sucrose solutions. The gradients were centrifuged for 16 hrs at 110,000g (SW28 rotor, Beckman). 600 μl aliquots, beginning with the top of the gradient, were collected and analyzed by spectroscopy. Cy5 labeled β_2AR was monitored by excitation at 649 nm and emission collected from 655-690 nm. Lipids were followed by excitation of NBD at 460 nm with

emission collected from 470-590 nm.

Electron Microscopy of Lipid Vesicles and Calculation of the Density of Receptors per Lipid Vesicles

Samples were submitted to the Stanford University Cell Sciences Imaging Facility. A negative staining protocol was used to obtain EM images of lipid vesicles reconstituted at a lipid-to-receptor ratio of 1,000:1. Using these images, the average diameter of 83 ± 12 nm for the vesicles was obtained. Based on this value, we estimated the density of β_2-ARs in lipid vesicles using the method of Mansoor et al (Mansoor et al, 2006). The following equations were used:

$$S.A._{vesicle} = 4\pi r^2$$

$$S.A._{lipid} = 4\pi r^2$$

$$\frac{\#of\ lipids}{vesicle} = \frac{2\,x\,S.A._{vesicle}}{S.A._{lipid}}$$

$$\frac{\#of\ \beta_2 ARs}{vesicle} = \frac{\#of\ lipids}{vesicle}\,x\,\frac{receptor}{lipid}\,ratio$$

We can assume that the surface area of one DOPC lipid is roughly 70 Å (Mansoor et al, 2006) and that the total surface area for the vesicle, based on a diameter of 83 nm, is about 2,163,146 Å^2. Assuming that the vesicle is comprised of a lipid bilayer, we estimated the number of lipids to be ~58,463 lipids per vesicle. From this, we can estimate the number of β_2-ARs in a lipid vesicle as a function of the number of lipids/vesicle multiplied by the ratio of lipid-to-receptor, or 1,000:1. These calculations resulted in a receptor density of ~50-60 β_2-ARs per lipid vesicle.

Analysis of FRET data

FRET data was analyzed as described previously (Granier et al, 2007). Samples were either excited at the donor wavelength (ExcD; 550 nm) or at the acceptor wavelength (ExcA; 649 nm), resulting in the following scans:

$$\begin{cases} A = & \text{receptor labeled with donor and acceptor, ExcD} \\ A' = & \text{receptor labeled with donor and acceptor, ExcA} \\ B = & \text{receptor labeled with acceptor, ExcD} \\ B' = & \text{receptor labeled with acceptor, ExcA} \\ A_{drug} = & \text{same as } A\text{, with drug, ExcD} \\ A'_{drug} = & \text{same as } A'\text{, with drug, ExcA} \\ S_{buff} = & \text{buffer, ExcD} \\ S'_{buff} = & \text{buffer, ExcA} \\ S_{drug} = & \text{drug alone, in buffer, ExcD} \\ S'_{drug} = & \text{drug alone, in buffer, ExcA} \end{cases}$$

Background fluorescence from either buffer or drug is removed as follows:

$$\begin{cases} C = & A - S_{buff} \\ C' = & A' - S'_{buff} \\ D = & A_{drug} - S_{drug} \\ D' = & A'_{drug} - S'_{drug} \\ E = & B - S_{buff} \\ E' = & B' - S_{buff} \end{cases}$$

In spectrum E, the only signal is from direct excitation of the acceptor at ExcD. In spectra C and D, the signal from direct excitation of the acceptor is mixed with donor and FRET signals. However, the contribution from direct excitation of the acceptor in spectrum *C* and *D* is proportional to spectrum *E*, with a scaling factor that depends on the amount of acceptor fluorophores in the different samples. Importantly, the amount of acceptor is directly proportional to the intensities in spectra *C′*, *D′* and *E′*, where the donor and the FRET signals do not contribute at all. Thus, the contribution from direct excitation of acceptor in spectra C and D can be subtracted as follows:

$$\begin{cases} \mathrm{F} = C - E \times \dfrac{C'_{\max}}{E'_{\max}} \\ \mathrm{G} = D - E \times \dfrac{D'_{\max}}{E'_{\max}} \end{cases}$$

where "max" is defined as the intensity at the acceptor emission peak. The wavelength used to determine this value is 670 nm.

Thus, the final corrected spectrum is:

$$H = G - \left(E \times \frac{G_{\max}}{E_{\max}} \right)$$

Finally, spectra are normalized to keep the area under the curve constant, which also removed the contribution from any drug-induced fluorescent intensity change of the donor. The proximity ratio is then:

$$\text{Proximity Ratio} = \frac{I_A}{I_A + I_D}$$

where I_D and I_A are the intensities of the donor and acceptor peaks, respectively. The drug response is calculated as the change in proximity ratio between spectrum *F* and spectrum *H*.

FRET efficiency values are calculated using a modified version of the proximity ratio equation:

$$\text{FRET Efficiency} = \frac{I_A}{I_A + \gamma I_D}$$

The correction factor γ is defined as:

$$\gamma = \frac{\eta_A \Phi_A}{\eta_D \Phi_D}$$

where η_D and η_A are the collection efficiencies of donor and acceptor signals, and Φ_A and Φ_D are the fluorescence quantum yields of the donor and acceptor, respectively. We assume η_A/η_D to be 1 to obtain the following equation:

$$\text{FRET Efficiency} = \frac{I_A}{I_A + (\Phi_A / \Phi_D) I_D}$$

Φ_A and Φ_D were assumed to be 0.24 and 0.20 for Cy3 and Cy5, respectively (Malicka et al. 2002).

Anisotropy of bound Cy3 and Cy5

The anisotropy was determined according to the equation:

$$r = \frac{I// - GI\perp}{I// + 2GI\perp}$$

where I// and I⊥ are the fluorescence intensities measured for parallel and perpendicular components relative to the polarized excitation and G the collection efficiencies of parallel and perpendicular signals. We assumed the collection efficiencies of parallel and perpendicular signals to be the same (G = 1) to obtain:

$$r = \frac{I// - I\perp}{I// + 2I\perp}$$

Intensities were acquired at the maximum emission wavelength of each fluorophore over a 60 sec time period and subsequently averaged. Samples were excited at the wavelengths described above. For determining any potential drug-induced changes in the anisotropy, samples were mixed and incubated for 20 min at room temperature with saturating concentrations of drug.

FRET Saturation and Mathematical Modeling

FRET saturation experiments were set-up similar to BRET saturation experiments performed previously (Mercier et al, 2002); (Harikumar et al, 2007); (James et al, 2006). Briefly, β_2AR labeled samples were reconstituted at the following donor-to-acceptor ratios: 4:1, 2:1, 1:1, 1:2, 1:4, 1:6 and 1:10. Importantly, the total receptor concentration for these experiments remained unchanged. A mathematical model proposed previously predicts the relationship between FRET efficiency and donor-to-acceptor ratio, describing the number of GPCR protomers in a multimeric assembly (Harding et al, 2009; Harikumar et al, 2008; James et al, 2006; Mercier et al, 2002; Veatch & Stryer, 1977). The equation used to describe dimers and oligomers is:

$$\text{maximal FRET} = \frac{[(a+d)^n - a^n - d^n]}{[(a+d)^n - a^n - d^n + nd^n]} \quad (1)$$

where a is the number of Cy5-β_2AR acceptor molecules, d is the number of Cy3-β_2AR donor molecules and n is the number of protomers in the complex. Data for all three constructs were combined after maximal FRET was calculated, giving an analysis of the receptor/lipid bilayer system as a whole.

Factor Xa , PNGase F Proteolysis and Modification by NHS-PEO$_4$-Biotin

Reconstituted β_2AR samples were subjected to Factor Xa and PNGase F (New England BioLabs) proteolysis or NHS-PEO$_4$-Biotin to determine the orientation of receptors in lipid vesicles. Factor Xa cleaves at the third intracellular loop of the β_2AR, while PNGase F deglycosylates asparagine-linked oligosaccharides. NHS-PEO$_4$-Biotin, which covalently modifies primary amines, is predicted to disrupt antigen recognition of the FLAG sequence on the N-terminus of the receptor by M1 antibody (Sigma, St Louis).

Crosslinking in lipid vesicles

Briefly, 15 pmol of Cy5-β_2AR reconstituted samples were treated with a final concentration of 1 mM *Bis*(NHS)PEO$_5$ (Pierce) on ice for the times indicated. When ligands were present, receptors were incubated with ligands prior to treatment with crosslinker for 30 minutes at room temperature and then crosslinker was added on ice. The crosslinking reaction was stopped by the addition of 20 mM final Tris, followed by the addition of SDS sample buffer. Samples were resolved on 10% SDS-PAGE and Cy5 fluorescence was imaged using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Quantification of bands was done using Kodak Molecular Imaging Software, v4.0.3.

In vitro reconstitution of β_2AR into rHDL

Wild type human apoA-I was purified from human serum as described in detail in Whorton *et al*.(Whorton et al, 2007). Monobromobimane labeled β_2AR was reconstituted into rHDL particles as previously described (Yao et al, 2009).

[^{3}H]-DHA Saturation and Competition Binding

Saturation and competition binding assays were performed using [^{3}H]-dihydroalprenolol (DHA) on reconstituted β_2AR. Concentrations of [^{3}H]-DHA varied for saturation binding; 1 nM was used for competition binding. Competing ligands were added at the indicated concentrations and the assays were carried out for 60 min at room temperature with shaking at 230 rpm. All data points were obtained in duplicates and experiments were repeated three times. Saturation binding and competition binding data were fit to theoretical saturation and one-site competition binding models using Prism (GraphPad Software, San Diego, CA).

[^{35}S]-GTPγS Binding

Purified β_2AR and tethered-Gαs protein (Tet-Gαs) (Lee et al, 1999) were mixed in a molar ratio of 1:5 and reconstituted as described previously (Swaminath et al, 2005). Briefly, reconstituted receptor (100 nM final concentration) and Tet-Gαs were resuspended in 500 μl of cold binding buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$ and 1 mM EDTA) supplemented with 0.05% (w/v) bovine serum albumin, 0.4 nM [^{35}S]-GTPγS, GDP (1 μM) with or without β_2AR ligands. Incubations were performed for 30 min at 25°C with shaking at 230 rpm. Non-specific binding was determined in the presence of 100 μM GTPγS and was always less than 0.2% of total binding. Bound [^{35}S]-GTPγS was separated from free [^{35}S]-GTPγS by filtration through glass fiber filters followed by three washes with 3 ml of cold binding buffer. Filter-bound radioactivity was determined by liquid scintillation counting.

Purification of Gs heterotrimer and Tet-Gs

Gs heterotrimer ($G\alpha s_s$, his6-β_1, γ_2) was expressed in Sf9 cells and purified as previously described (Kozasa & Gilman, 1995). Tethered-Gs (Tet-Gs) consists of the amino terminus and first transmembrane segment of the β_2AR linked to the carboxyl terminus of the β_2AR and the amino terminus of the alpha subunit of Gs, as previously described (Lee et al, 1999). Tet-Gs was purified by a single Flag antibody affinity chromatography step in the presence of GDP and AlF to remove insect cell beta/gamma subunits as previously described (Swaminath et al, 2005).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Orientation of reconstituted β_2AR assessed by bimane quenching

Purified receptors were labeled at the base of the sixth transmembrane domain with stoichiometric amounts of monobromobimane (mBBr) and reconstituted. (**A**) Reconstituted samples in reconstitution buffer (black) or in reconstitution buffer containing 1 mM L-tryptophan (red). (**B**) Reconstituted samples solubilized in reconstitution buffer containing 0.2% DDM (black) or the same buffer containing 1 mM L-tryptophan. Bimane fluorescence was followed by excitation at 370 nm and emission collected from 439-550 nm. Data are representative of three independent experiments.

Figure S2: Distribution of β_2ARs in lipid vesicles reconstituted at a lipid-to-receptor ratio of 10,000:1.

To determine the distribution of β_2ARs in lipid vesicles, sucrose density gradients of samples containing 0.4% NBD-phosphocholine and Cy5-β_2ARs reconstituted at a lipid-to-receptor ratio of 10,000:1 were performed as described in Materials and Methods. Detection of lipid fractions was performed by following NBD fluorescence (λ_{ex} = 460 nm) and receptor fractions by following Cy5 fluorescence (λ_{ex} = 649 nm).

Figure S3: Saturation binding of reconstituted β_2ARs

The Kd of radiolabeled [^{3}H]-dihydroalprenolol was determined for Δ5-T66C (**A**), Δ5-A265C (**B**), Δ5-R333C (**C**) and wild-type (**D**) by saturation binding. Concentrations of [^{3}H]-DHA were varied as shown. Data represent the mean ± S.E.M. of three independent experiments performed in triplicate.

Figure S4: Time-course of ICI-induced FRET changes

The time-course of FRET changes upon ICI treatment (1 μM) for H8/H8 samples was measured. Data represent the mean ± S.E.M. of three independent experiments performed

Figure S5: Anisotropy of Cy3 or Cy5-labeled β_2ARs

Anisotropy measurements in the presence of isoproterenol (**A**) or ICI 118,551 (**B**) were performed as described in Supplemental Materials and Methods.

Figure S6: Crosslinking of β_2ARs in liposomes in the presence of agonist, antagonist or inverse agonist

(**A**) Crosslinking of reconstituted Cy5-labeled β_2AR samples in the presence or absence of saturating amounts of isoproterenol, alprenolol or ICI 118,551 was carried-out as described in Materials and Methods. Data are representative of at least three independent experiments. (**B**) Quantification of monomeric band from crosslinking experiments in the presence or absence of saturating amounts of ligands. Data represent the mean ± S.E.M. of three independent experiments performed. * ($P < 0.05$).

Figure S7: ICI-mediated resistance of β_2AR oligomers to detergent dissolution

FRET between H8/H8 interactions was monitored after supplementing assay buffer with DDM to a final concentration of 0.2%. Samples were either pre-incubated at room temperature for 30 min with saturating

amounts of ICI (1 μM) or in buffer alone, all without DDM. After the addition of 0.2% final DDM, samples were mixed immediately and placed in a cuvette for measurements up to 15 min. *Inset* shows an expanded view of the time-course up to 120 sec. Data represent the mean ± S.E.M. of three independent experiments performed.

Figure S8: Effect of oligomerization on agonist-induced conformational changes in TM6. β_2AR was labeled on C265 at the cytoplasmic end of TM6 with monobromobimane (mBBr-β_2AR) and reconstituted into either phospholipids vesicles (**A**), or recombinant HDL particles (**B**) under conditions that produce only monomers (Whorton et al, 2007). The agonist isoproterenol (10 μM) induced similar changes in intensity and λ_{MAX} in both reconstitution conditions.

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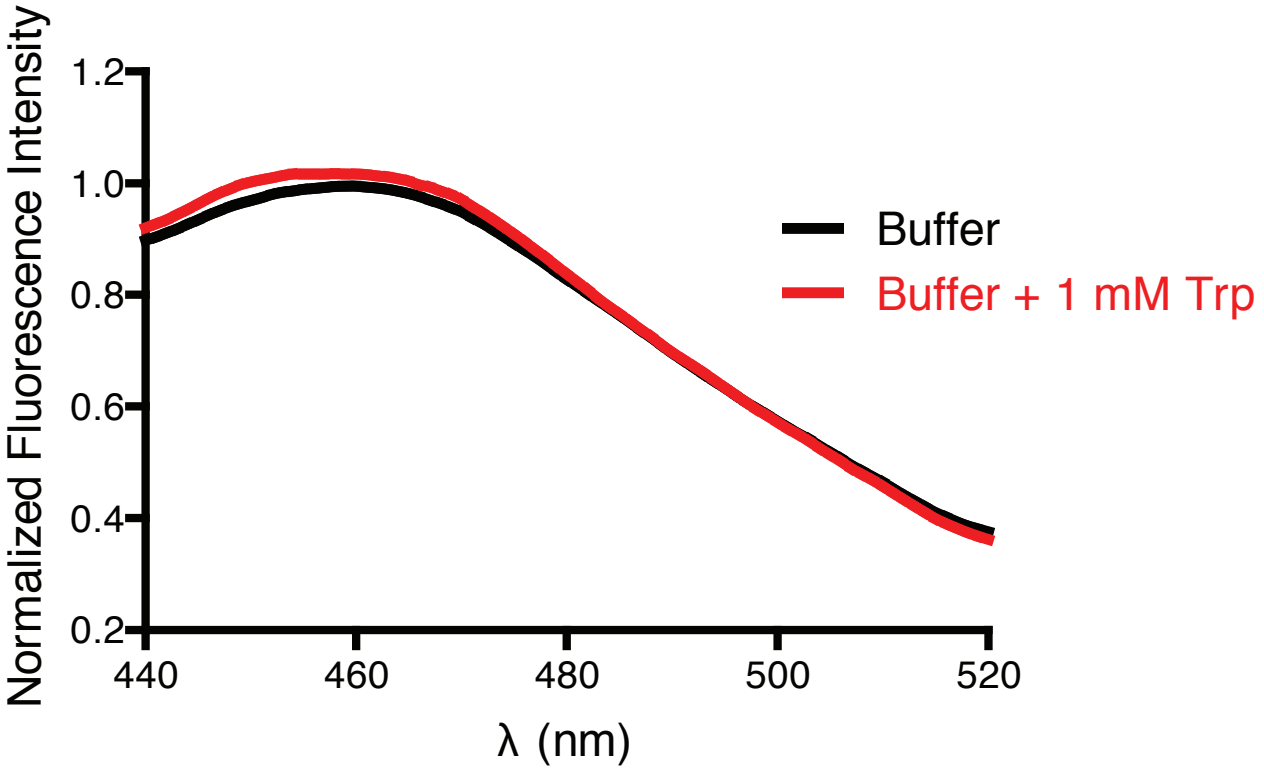
Supplementary Table I. Moles of fluorophore per mol of receptor and donor to acceptor ratio for the single-reactive cysteine receptors.[a]

	Fluorophore Labels per Receptor	
	Cy3	**Cy5**
T66C	0.73 ± 0.2	0.68 ± 0.2
A265C	0.64 ± 0.1	0.63 ± 0.2
R333C	0.80 ± 0.2	0.74 ± 0.2

[a] Stoichiometry of labeling was determined as described in Materials and Methods. Data represent the mean ± S.E.M. of at least three independent experiments.

Supplementary Figure 1

A



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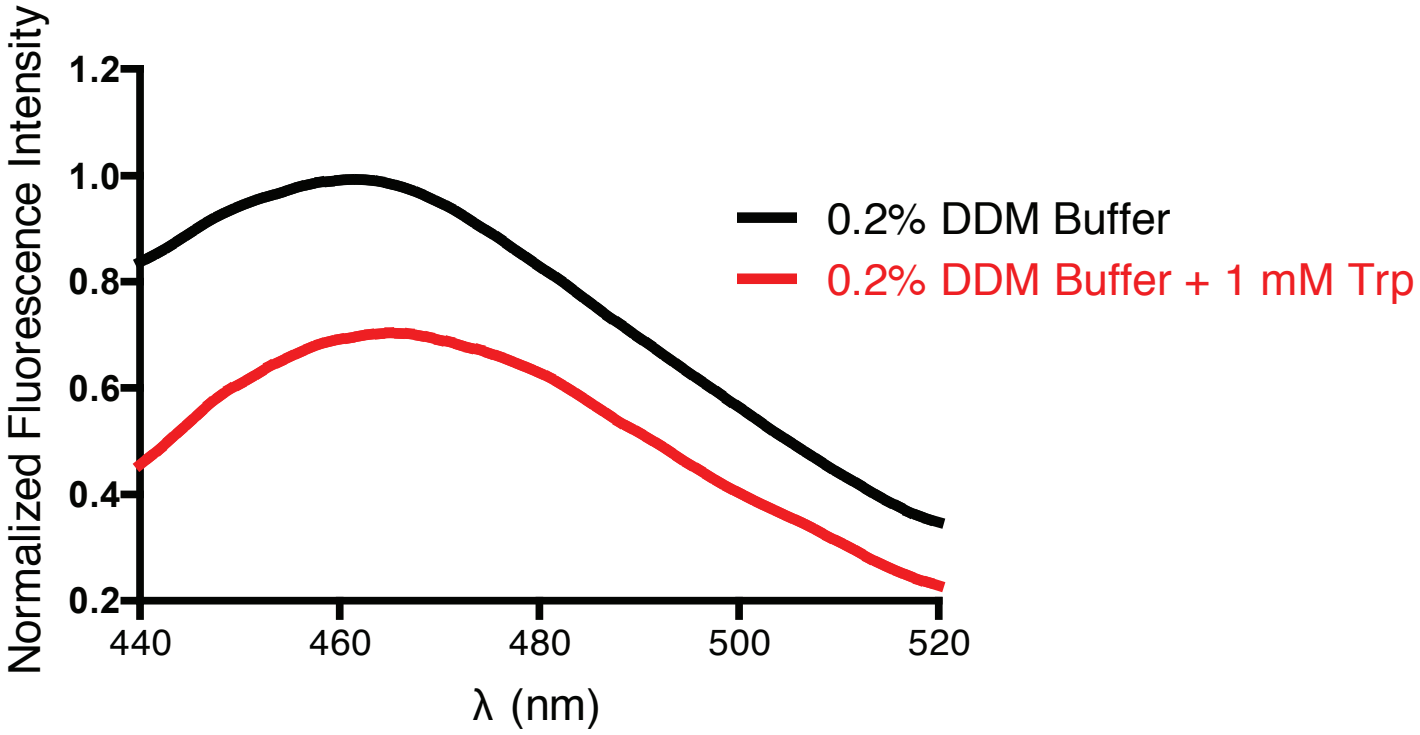
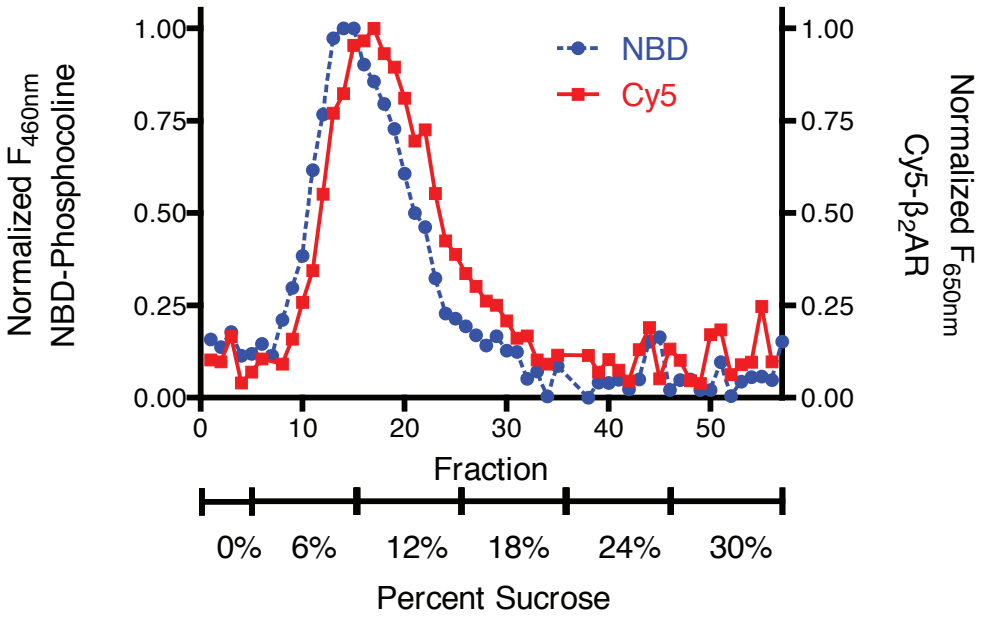


Supplementary Figure 2



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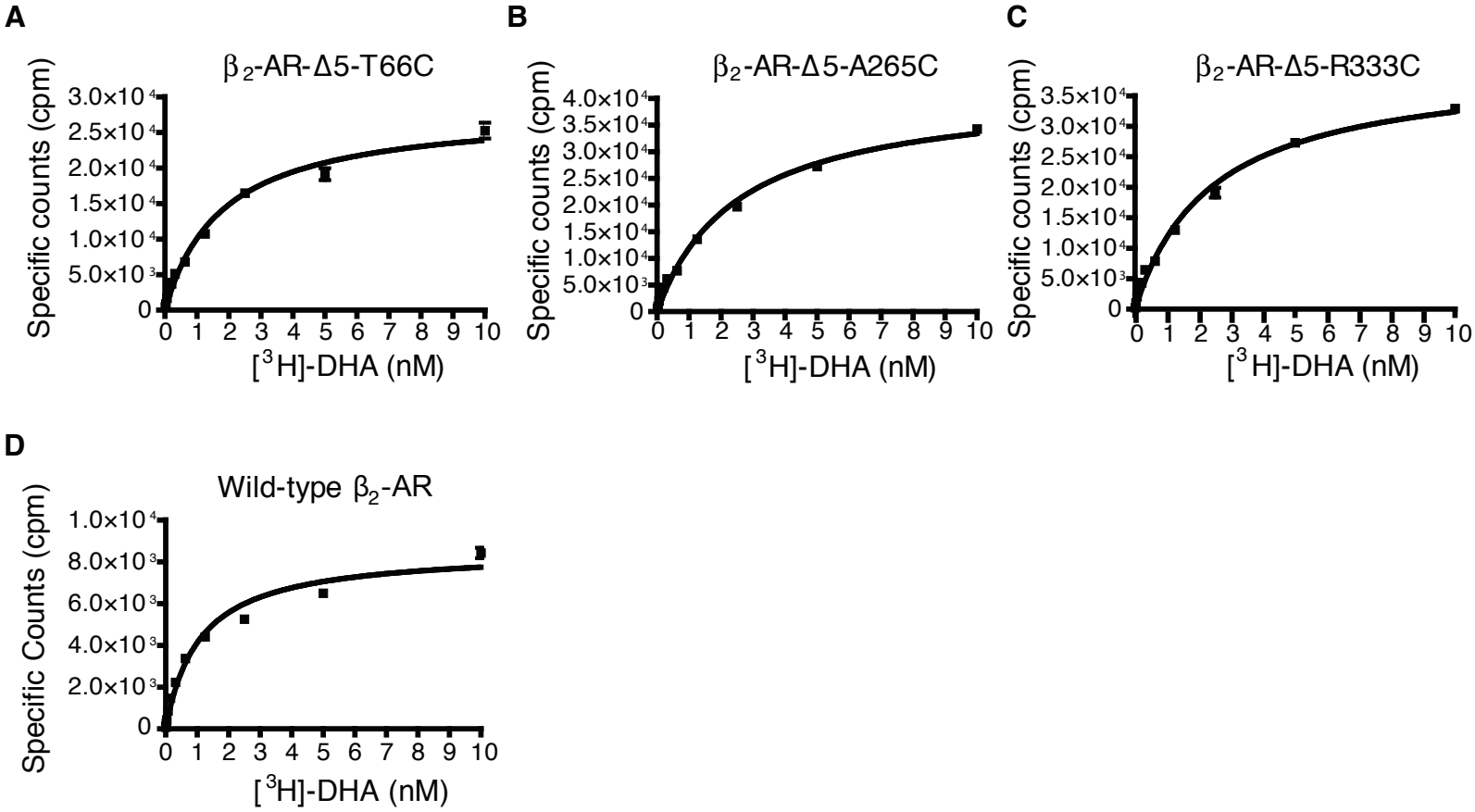

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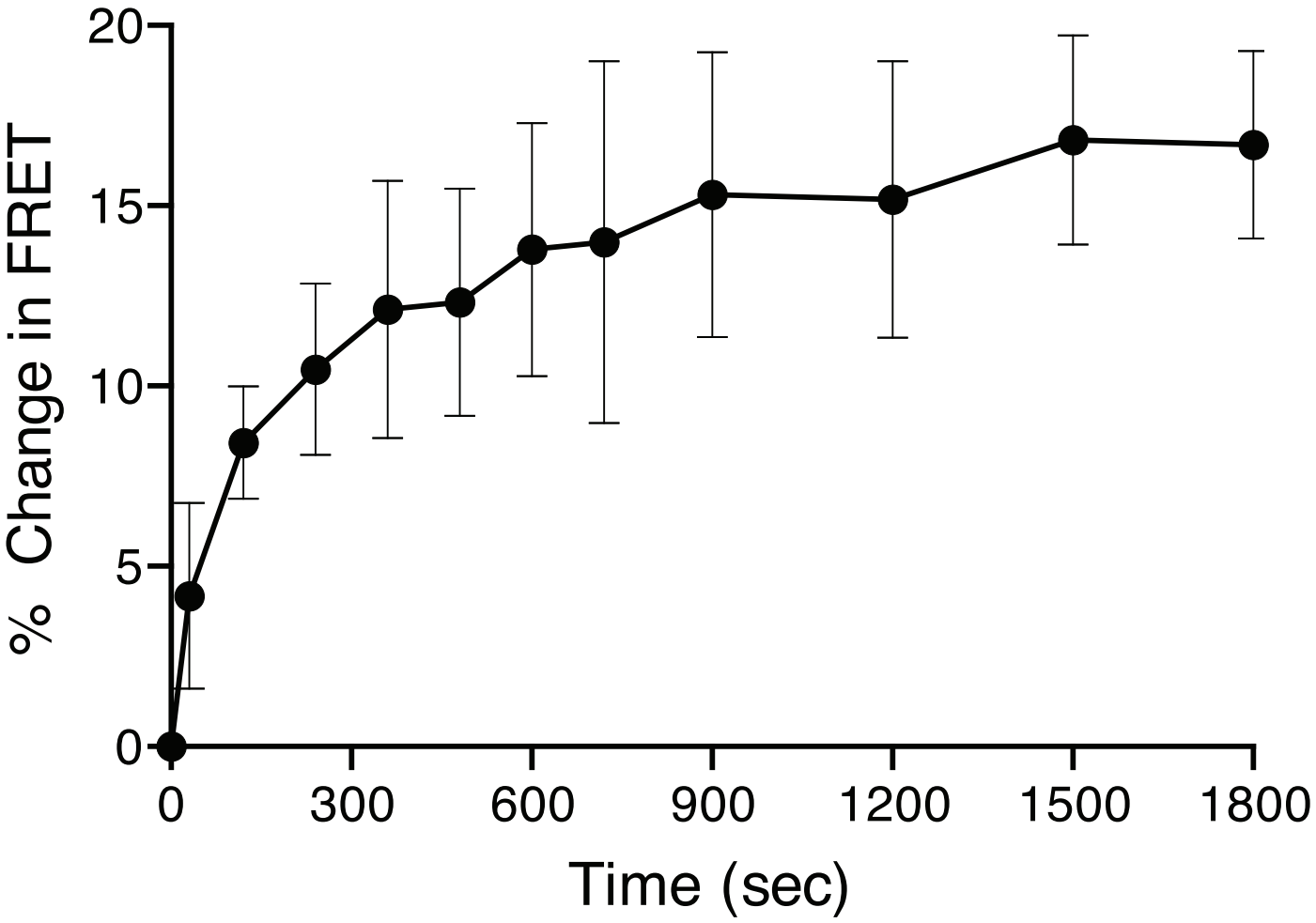

Supplementary Figure 5

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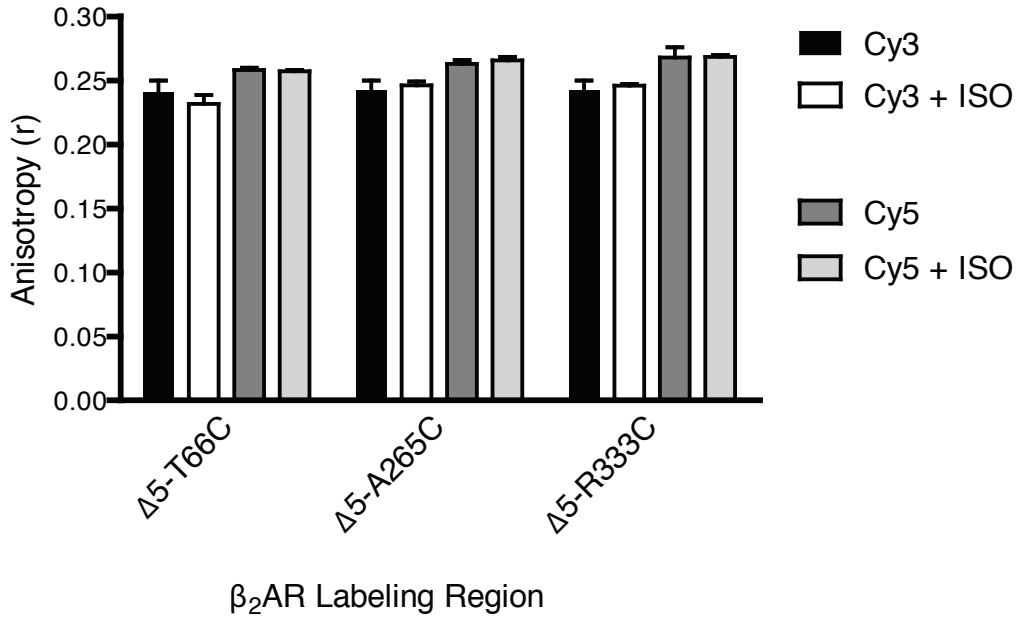

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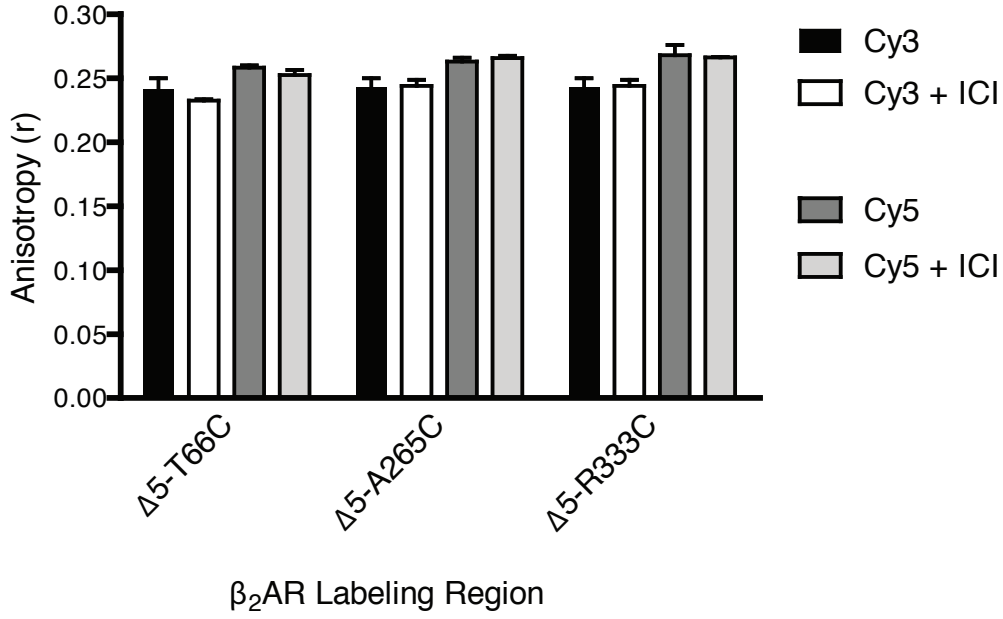

Supplementary Figure 6

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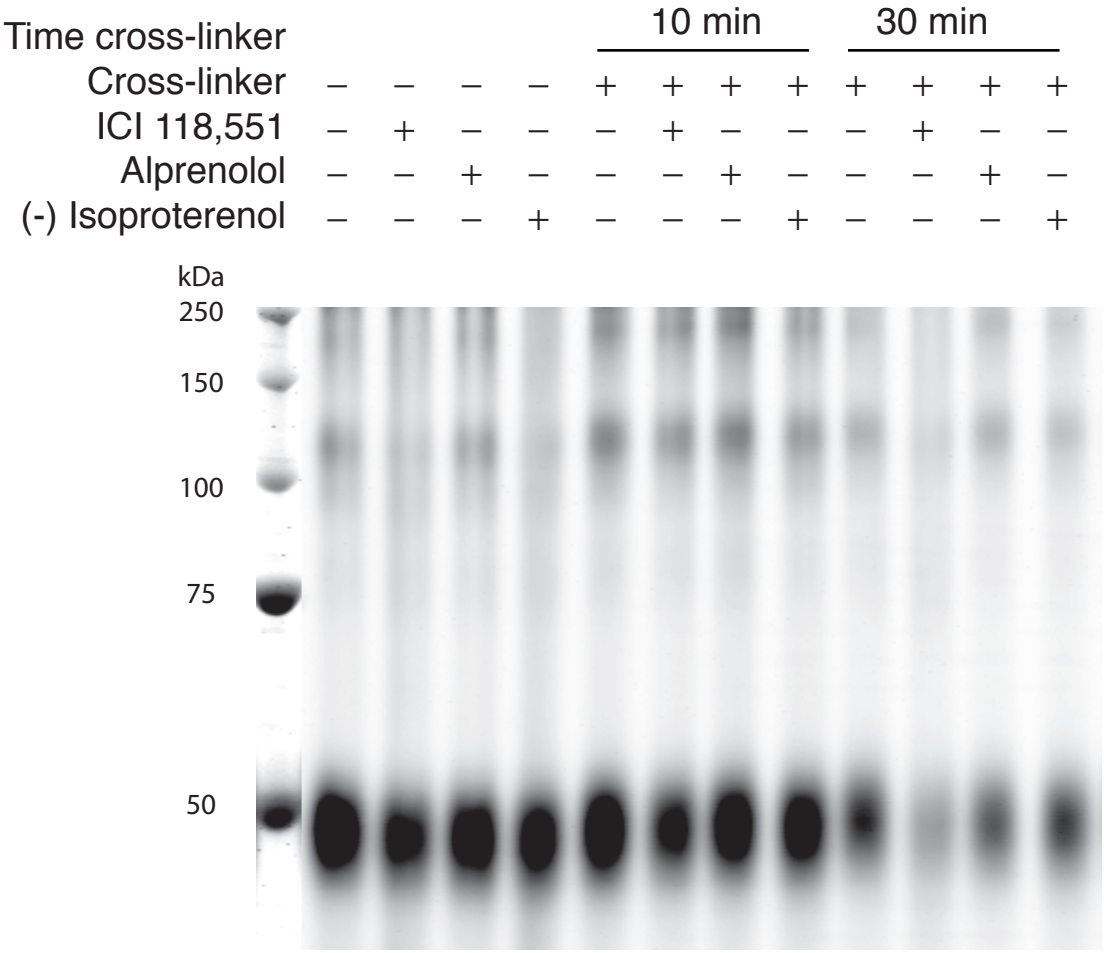

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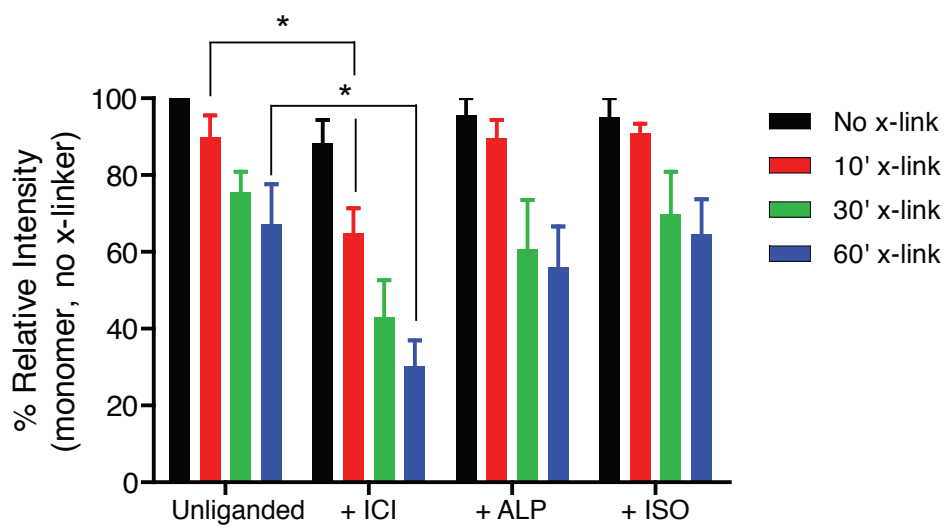
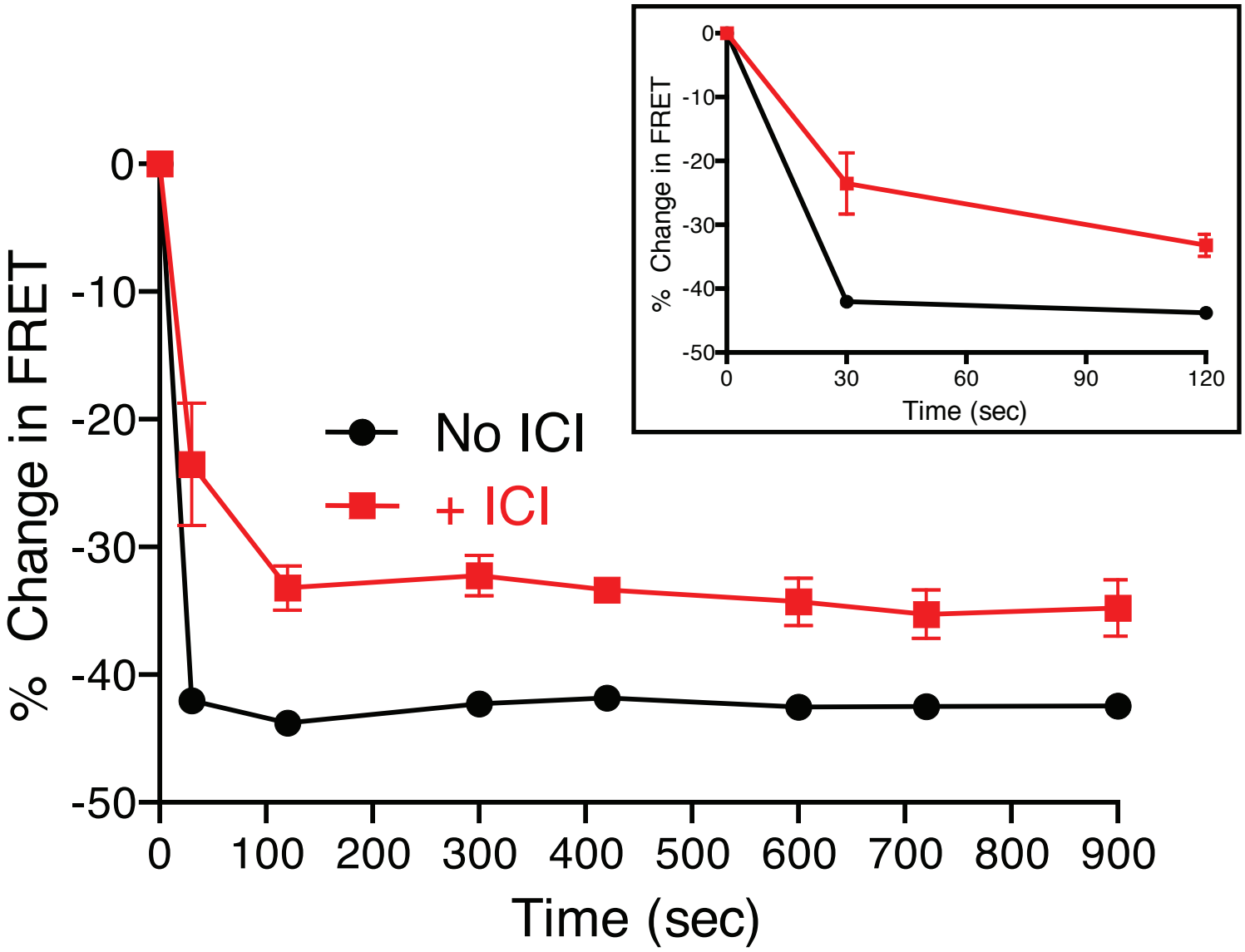


Supplementary Figure 7



Supplementary Figure 8

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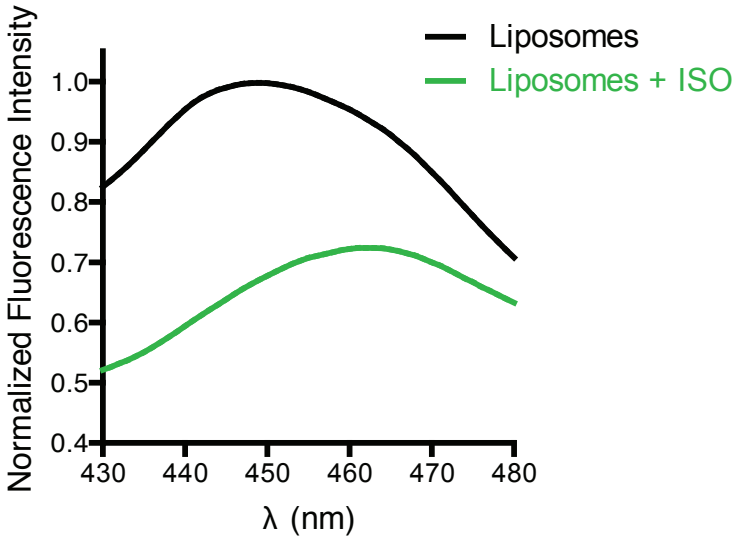

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