doi:10.1038/nature12000

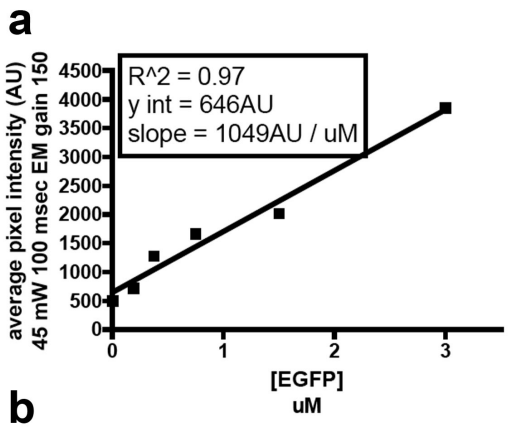
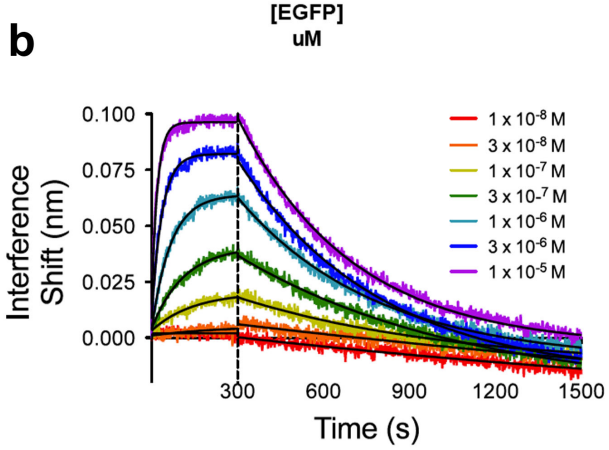
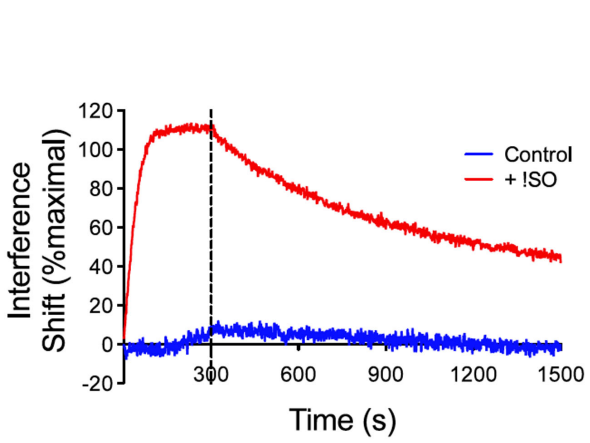
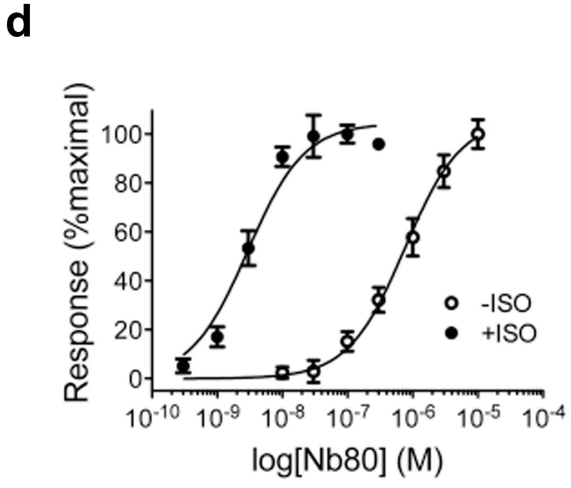
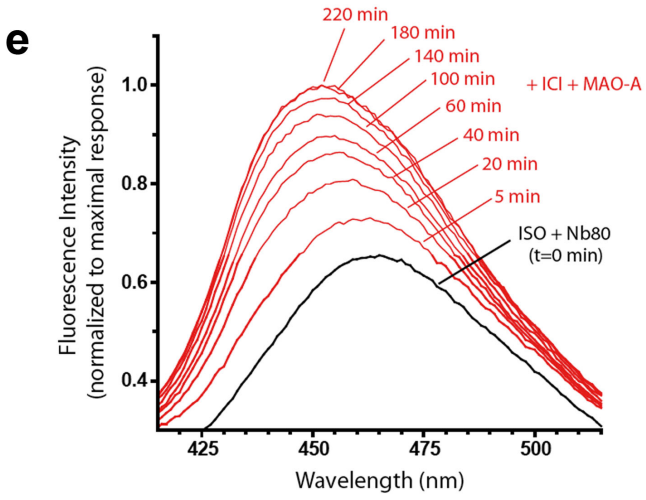


Image	F	bkd	est [EGFP]
Nb80-EGFP 'medium' cell #1	524	506	17 nM
Nb80-EGFP 'medium' cell #2	519	507	11 nM
Nb80-EGFP 'medium' cell #3	527	506	20 nM
Nb80-EGFP 'bright' cell	533	507	25 nM



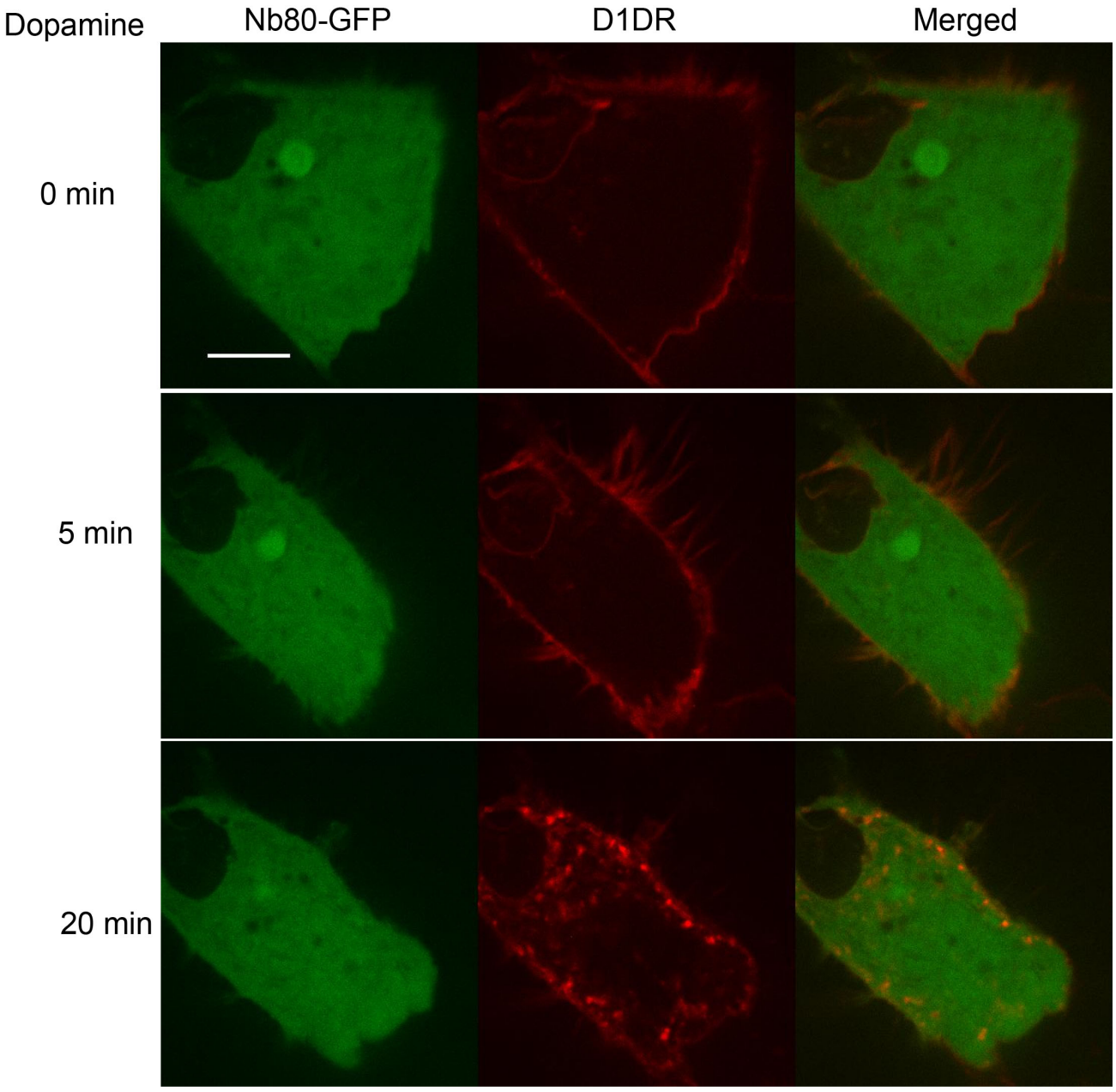




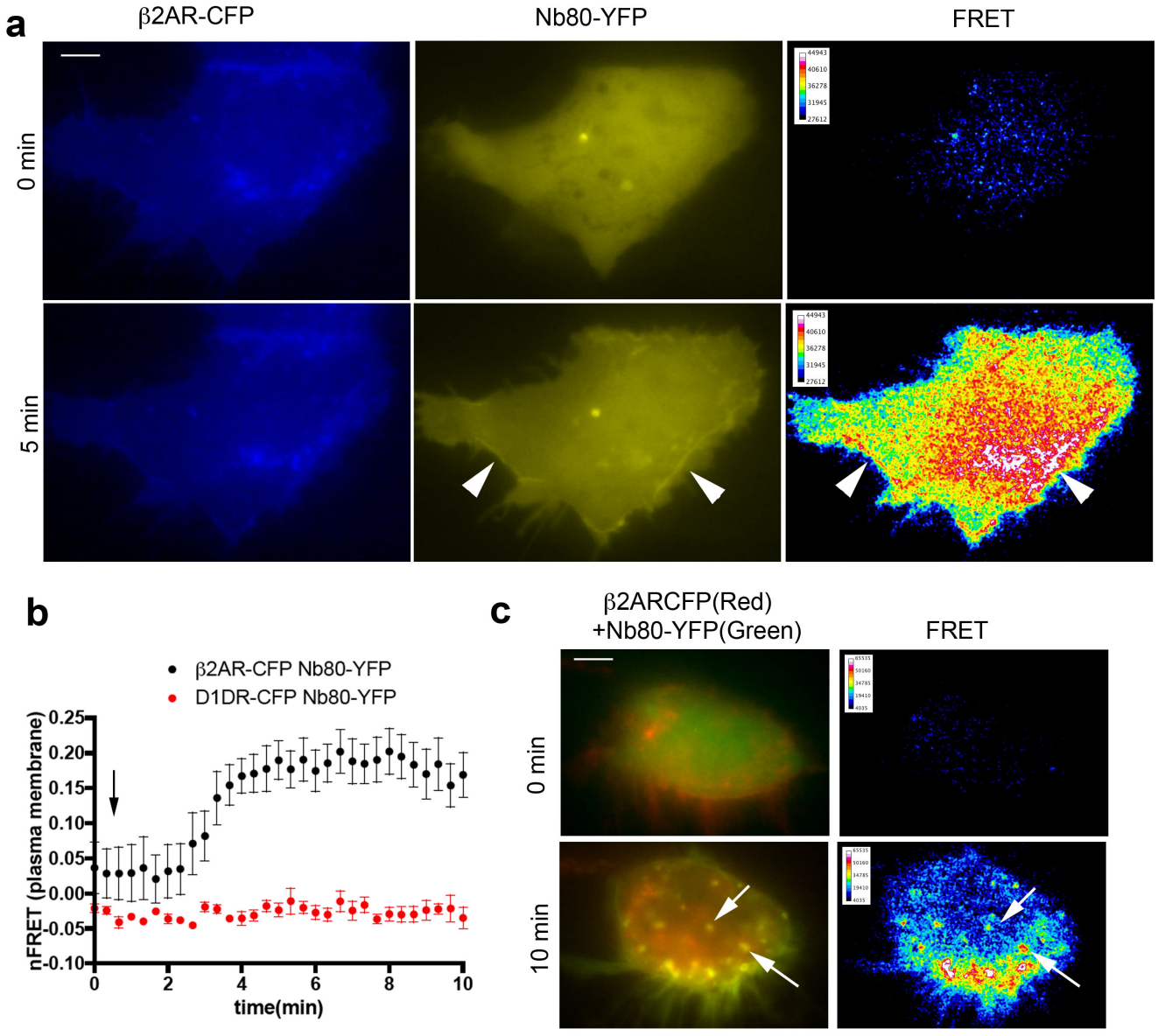


Supplementary figure 1- Nb80-GFP is expressed at a concentration suitable to function as a conformational biosensor. a) Graph shows a standard plot of purified EGFP (dissolved in Hank's balanced salt solution) fluorescence intensity imaged by confocal sectioning of droplets in vitro; line represents linear least-squares fit. Box shows estimation of cytoplasmic Nb80-GFP fluorescence intensities in confocal sections through the cytoplasm, from individual cells expressing Nb80-GFP in the range used in the present analysis, imaged in the absence of agonist, and with background for each determined from a blank region of the same image. Chemical concentration of Nb80-GFP in the cytoplasm was estimated by calibration of background-subtracted cytoplasmic fluorescence intensity for each example cell to the in vitro standard plot. A similar

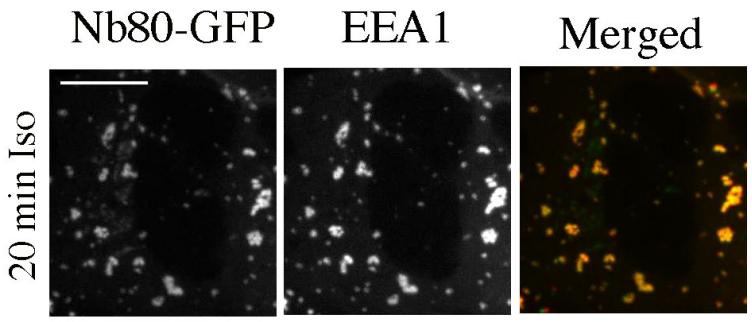
intensity range was selected for analysis of Nb37-GFP distribution in individual cells, supporting an estimated cytoplasmic concentration for both biosensors of ~20 nM. **b)** Representative data showing the typical association and dissociation responses for Nb80 binding to β_2AR reconstituted into biotinylated rHDL particles when measured in the absence of isoproterenol (in color) along with single-phase exponential curve fits (in black). **c)** Time scan of 30 nM Nb80 binding in the absence (blue) or presence of 100μM isoproterenol (red). **d)** Maximum association responses at each Nb80 concentration in the absence (open circles) or presence of 100μM isoproterenol (ISO, closed circles), normalized to the overall maximum response produced and expressed as a percent of maximum. Data are from three experiments each and are plotted as mean ± SEM. K_D values estimated for unliganded and isoproterenol-bound β_2AR are 7.6±1.4 x10^{-7} M and 2.9±0.5 x10^{-9} M, respectively. The association and dissociation assays suggest that Nb80 binds to isoproterenol-bound β_2AR with k_{on} and k_{off} values of 1.1x10^6 ± 4.0 x10^4 $M^{-1}s^{-1}$ and 1.4 x10^{-3} ± 5 x10^{-5} s^{-1}, respectively, in generally good agreement with equilibrium binding affinity estimated from the maximal response data. **e)** Alternate estimate of Nb80 dissociation kinetics by spectral shift of bimane fluorescence measured for mBB-β_2AR reconstituted into rHDL particles. Sequential spectra are shown after pre-binding isoproterenol and Nb80 followed by addition of the inverse agonist ICI-118551 and monoamine oxidase to minimize agonist re-binding.



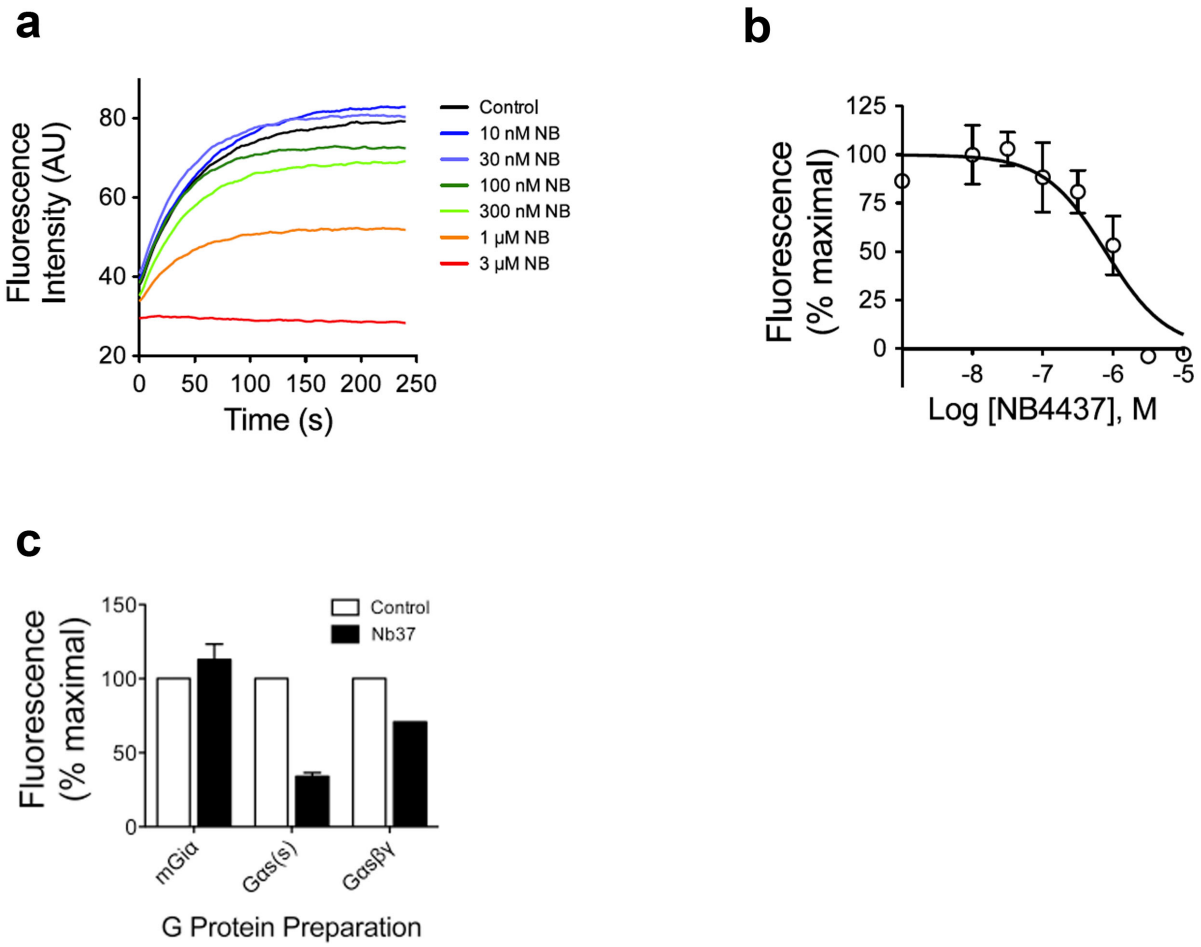
Supplementary figure 2- D1 Dopamine receptor does not recruit Nb80-GFP to the PM or endosomes. Representative images of cell coexpressing Nb80-GFP (green) and D1DR (red) at the indicated time (left) after 10μM dopamine addition. Scale bar=10 μm.



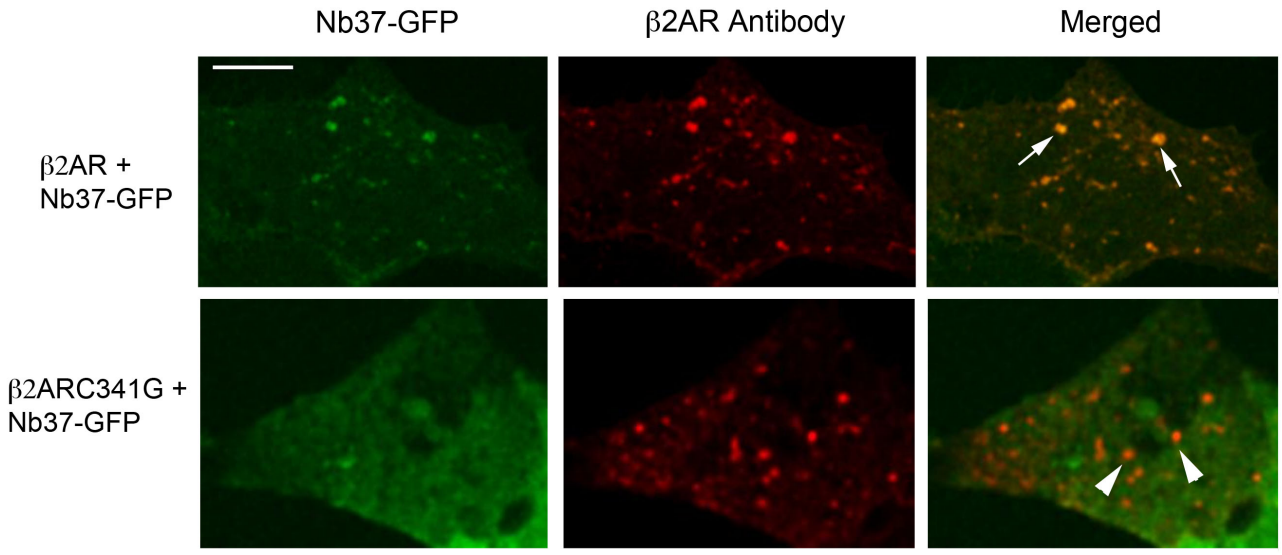
Supplementary figure 3- FRET analysis of biosensor interaction with β2AR in intact cells. a) Representative image of β2AR-CFP, Nb80-YFP and a pseudo-color image of pixel-by-pixel calculated FRET prior to (top row) and 5 min (bottom row) after 10 μM isoproterenol addition to the culture medium (arrowheads indicate representative regions of the peripheral PM; it is not possible in these wide field images to resolve PM from cytoplasm in more central regions). **b)** Time course of nFRET determined from integrated intensity measurements of an ROI drawn around a peripheral region of the PM for β2AR-CFP/NB80-YFP nFRET after addition of 10 μM isoproterenol (black line, arrow indicates time of agonist addition) and for D1DR-CFP/NB80-YFP (red line) after addition of 10 μM dopamine; n=10 cells, 3 experiments. **c)** Representative image of merged β2AR-CFP, Nb80-YFP and a pseudo-color image of calculated FRET prior to (top row) and 10 min (bottom row) after 10 μM isoproterenol addition (arrows indicate representative endosomes), in which FRET signal is evident also at endosomes. Scale bar=5 μm.



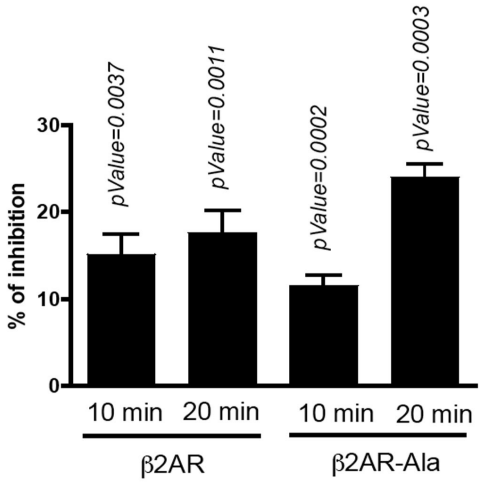
Supplementary figure 4- Nb80-GFP colocalized with early endosomal marker. Colocalization of Nb80-GFP with EEA1 in cells fixed after 20 min isoproterenol exposure; n=7 cells. Scale bar=5 μm.



Supplementary figure 5- Inhibition of bodipy-GTPγS-FL binding by Nb37. The effect of Nb37 on GTPγS binding to purified G proteins was measured using 100 nM bodipy-GTPγS-FL as a fluorescent probe. **a)** Representative time scan of bodipy-GTPγS-FL binding to Gαs in the presence of varying concentrations of Nb37. **b)** Concentration dependence of Nb37 binding to Gαs. The fluorescence was measured at 240s following the addition of G protein at the concentrations indicated. Fluorescence intensity was plotted as a percent of control (no nanobody). The data were fitted by non-linear regression using Prism 5 (GraphPad, San Diego, CA) and are derived from two independent experiments. IC50 for Nb37 inhibition of bodipy-GTPγS-FL is approximately 7.9 ± 2.4x10-7 M. **c)** Maximal inhibition data showing that Nb37 inhibits bodipy-GTPγS-FL to Gαs and the Gαsβγ heterotrimer but not Gαi.



Supplementary figure 6- Nb37-GFP is not recruited by the Gs coupling-defective mutant β2AR. Representative images of a cell co-expressing Nb37-GFP (green) and β2AR (red) showing extensive colocalization between the two fluorophores in isoproterenol-treated cells (top row, Pearson's coefficient = 0.710; n= 340 endosomes). Representative images of a cell co-expressing Nb37-GFP (green) and the G protein coupling-defective mutant β2ARC341G showing greatly reduced colocalization (bottom row, Pearson's coefficient = 0.294; n= 555 endosomes). Cells were fixed after 20 min incubation in the presence of 10μM isoproterenol. Arrows in merge indicate examples of endosomes colocalized with Nb37-GFP, arrowheads indicate examples of receptor-containing endosomes without detectable biosensor colocalization. Scale bar=10 μm.



Supplementary figure 7- The Dyngo-dependent component of initial cellular cAMP accumulation requires β2AR endocytosis but not recycling. Inhibition of whole cell cAMP response produced by 15 min pre-incubation with 30μM Dyngo4a, assessed using luminometry assay 10 and 20 min after subsequent addition of 10 μM isoproterenol to the culture medium. Left set of bars represents results from cells expressing wild type β2AR. Right set of bars represents results from cells expressing the recycling-defective mutant β2AR-Ala (mean ± SEM, n=10 experiments; P values determined at each time point above the corresponding bar).