The stoichiometry and number of subunits comprising membrane proteins can be addressed using single-molecule fluorescence imaging. Recombinantly-expressed subunits fused genetically to a fluorescent protein are subjected to a fluorescence intensity recording. The number of fluorescently labelled subunits within a single oligomer can then be determined by counting the photobleaching steps observed as each fluorescent label loses permanently its fluorescence by photochemical destruction (*Ulbrich and Isacoff, 2007, Nature Methods*). Although this technique is powerful, drawbacks arise concerning the analysis, which is time consuming and could be biased by the user. The counting process is usually done manually, since our brain has a good ability for step detection. However, imperfections of the traces caused by background fluorescence and fluorescence blinking could be interpreted differently from one user to another. To address these issues of objectivity and productivity, we have developed a fully automated algorithm for the accurate and impartial analysis of photobleaching step counting data. After automatically selecting a spot to analyze, this analysis program extracts the step-like behaviour from a noisy trace by "Progressive Idealization and Filtering" (PIF). The quality of the trace and the fit are also determined in order to reject the traces which are not showing clear step-like events. Prior analysing real data with the automated routine, simulations were made to evaluate its step detection accuracy and limitations. Using PIF, we analyzed the composition of a homomeric kainate-subtype ionotropic glutamate receptor, GluK2 recombinantly expressed in HEK293T cells. GluK2 was fused with a modified version of the superfolder GFP (*Pedelacq et al., 2006, Nature Biotechnology*) and we confirmed its known tetrameric architecture in mammalian cells.

579-Pos Board B365
Wavelet Shrinkage to Resolve Single Molecule FRET Structural Landscape of the Isolated Ligand Binding Domain of the AMPA Receptor
Christy F. Landes.
Rice University, Houston, TX, USA.
Single molecule fluorescence resonance energy transfer (smFRET) spectroscopy has provided significant advances in our understanding of the relationship between structure and function in biological systems. Currently, simplifications must be made for experimental systems, data analysis, and theoretical modeling because biomolecules often exhibit mechanistic or conformational heterogeneity. For example, it is often necessary to treat biomolecular processes as transitions between two well-define states (e.g. folded vs. unfolded) despite clear experimental evidence or theoretical predictions to the contrary. The conformations explored by the agonist binding domain of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor represent such a system. The distribution of conformations in the raw data was so wide it was not possible to extract conformational details. By employing a model-free data analysis technique called wavelet shrinkage, it was determined that each protein form comprised multi-state, sequential equilibria. The results illustrate that the extent of activation is dependent not on a rigid closed cleft, but instead on the probability that a given subunit will occupy a closed cleft conformation, which in turn is determined by the range of states that the protein explores. Also, the results emphasize both the need for and the utility of advanced data processing techniques to quantify structure and dynamics in heterogeneous systems.

580-Pos Board B366
Phosphoinositide Signaling Regulates the Surface Localization of the δ2 Ionotropic Glutamate Receptor
Vasileios I. Petrou[1,2], Diomedes E. Logothetis[2].
[1]Mount Sinai School of Medicine, New York, NY, USA, [2]Virginia Commonwealth University School of Medicine, Richmond, VA, USA.
The δ2 glutamate receptor (GluD2) is a member of the ionotropic glutamate receptor family. Since GluD2 remains an orphan receptor, it has not been shown to conduct ions. It is highly expressed in the parallel fiber-Purkinje cell (PF-PC) synapse and its role in cerebellar physiology is increasingly appreciated. GluD2 has been linked to the induction of cerebellar long-term depression (LTD) and presynaptic terminal differentiation. A single point mutation in the second transmembrane domain (A654T), named Lurcher (GluD2[Lc]), confers constitutive activity to the receptor.
We have used the Lurcher mutant as a model to examine whether phosphoinositides regulate GluD2. We show that decreasing PIP_2 levels in the membrane, through Gq-coupled receptor activation or pretreatment with a PI4K inhibitor, potentiates GluD2[Lc] currents. Conversely, increasing PIP_2 levels by co-expressing PIP5K leads to decreased GluD2[Lc] currents. On the other hand, utilizing PI3K inhibitors wortmannin or LY294002, and presumably decreasing PIP_3 levels, reduces GluD2[Lc] currents, while co-expression of PI3K leads to potentiation of GluD2[Lc] currents.
A chemiluminescence-based assay that quantifies surface localization of the GluD2 and GluD2[Lc] receptors, showed that manipulations of the membrane phosphoinositide levels evoke changes in the cell surface localization of both wild-type and mutant receptors. These changes in surface localization of the receptor correspond to the effects we have observed by monitoring GluD2[Lc] currents, suggesting that current measurements from this mutant receptor serve as a good reporter for the localization of the wild-type δ2 receptor. These results are consistent with the interpretation that increased PIP_2 levels decrease, while increased PIP_3 levels increase localization of the receptor at the cell surface. Signals that affect the levels of these phosphoinositides simultaneously are likely to regulate the surface localization of GluD2 based on the net change of the two opposing effects.

581-Pos Board B367
Purification and In Vitro Functional Analysis of Glutamate Receptor
Sun Joo Lee, Nazzareno D'Avanzo, Colin G. Nichols, James E. Huettner.
Washington University, St. Louis, MO, USA.
Ionotropic glutamate receptors are predominantly localized to neuronal synapses and are principal mediators of excitatory neurotransmission in the brain. Receptor function is regulated by multiple factors including ligands, ions, pH, posttranslational modification, subunit composition, RNA editing, and elements of the lipid environment. Analysis of channel function in native membranes has provided much quantitative information on these various regulatory features, however, investigation of lipid regulation is seriously hampered by an inability to control membrane composition in living cells. Although an inhibitory effect of free polyunsaturated fatty acids on GluK2(R) activation has been demonstrated, the specific bulk lipid requirements for normal channel operation, as well as susceptibility to fatty acid modulation, are unknown. To address this, we aim to purify rat kainate receptor 2 (GluK2), to reconstitute the purified protein, and to assess channel function under different defined lipid environments. Using Saccharomyces cerevisiae as an expression host, we have been able to express wild type GluK2 with either Gln or Arg at the editing site, and with different tags. In addition, we have expressed modified forms of GluK2 including an amino terminal domain (ATD) deletion mutant, and a double point mutation (Y590C/L572C) that allows disulfide crosslinking between ligand binding domain (LBD) dimers. We have succeeded in purifying wild type GluK2(R) through Flag affinity and subsequent size exclusion chromatography after solubilizing the protein using the detergent Foscholin-14 (F14). A major band at the expected monomer size of ~100kDa and several bands at lower molecular weights are resolved on 1D SDS PAGE and are confirmed to be GluK2 through mass spectrometry (MS) analysis. MS analysis also reveals that a fraction of the heterologously expressed protein in yeast maintains a signal sequence at the N-terminus and is phosphorylated on a presumed extracellular residue, indicating misoriented topology.

582-Pos Board B368
Activation of Ionotropic Glutamate Receptors using Tethered Photoswitchable Ligands
Andreas Reiner, Ehud Y. Isacoff.
University of California Berkeley, Berkeley, CA, USA.
Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels, which mediate and modulate excitatory neurotransmission in the central nervous system. iGluRs are tetramers that are formed by identical or homologous subunits, but little is known about how ligand binding at individual subunits contributes to gating. Here we address this question using photoswitchable ligands that are tethered to specifically introduced cysteine residues. The ligand, termed MAG, consists of a cysteine-reactive maleimide group, an azobenzene photoswitch, and a glutamate analogue as head group. After coupling to an attachment site close to the binding cleft, light of specific wavelengths can be used to switch MAG between its cis and trans form, which allows to control ligand binding and unbinding with high temporal precision. We combined photoswitching with voltage-clamp recordings to measure the effect of partial receptor occupation on the extent and time course of activation and desensitization in GluK2, a member of the kainate receptor family. Extending these experiments to heteromeric complexes and complexes of defined subunit stochiometry will provide further information on how individual subunits contribute to the activation, deactivation and desensitization of this important class of signaling molecules.

583-Pos Board B369
N-Terminal Domain of NMDA Receptors as Studied by LRET
Rita E. Sirrieh, Vasanthi Jayaraman.
UT-Houston, Houston, TX, USA.
NMDA Receptors, one of the three main classes of glutamate receptors that mediate excitatory transmission, are tetramers composed of glycine- binding (GluN1) subunits and glutamate- binding (GluN2) subunits. Allosteric