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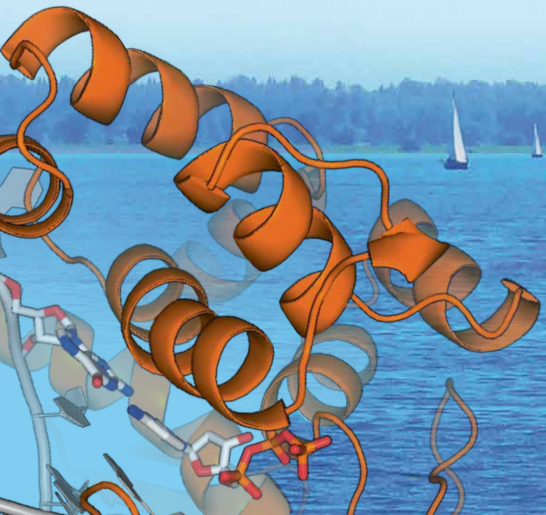

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Dual color subunit counting method using GFP/YFP overlapping spectra

X-ray crystallography, Cryo EM are most used method to determine protein structure. Although efficient, the methods determine protein stoichiometry in vitro. Besides, crystallography requires high density proteins and Cryo-EM uses detergents to solubilize proteins. If we want to study protein structure in its native environment, we need to use optical microscopy. The problem is, the size of proteins are far smaller (~2-5 nm) than the diffraction limit of optical microscope (~20 nm). Hence we cannot determine subunit composition spatially.

Subunit counting method, a robust method, uses photo-bleaching steps of fluorophores to decipher membrane receptor stoichiometry in its native environment [1]. The method counts bleaching steps of fluorophores to count subunits (if all the fluorophores are functional, number of bleaching steps will be directly proportional to the number of subunits present). mEGFP is quite widely used fluorophore in subunit counting as it is photo-stable and its bleaching step counting is easy. The bleaching step count from YFP is also good, it can be excited with the same laser as GFP and it gives emission in different spectral range than GFP but shares overlapping emission spectra. In this research, we established a method to bypass this disadvantage.

We used NMDA receptor, an obligatory dimer of dimer as positive control. We genetically fused YFP with GluN1 and GFP with GluN3A. We expressed them in Xenopus laevis oocytes and after 18-24 hour imaged them using TIRF microscopy in dual view mode. We have used a long pass filter (cutoff wavelength around 520 nm) to divide the emission spectra of GFP and YFP in two different channels.

Hypothesis used to determine a membrane receptor stoichiometry using this method is as follows:

We observed bleaching steps in both channels but with different step heights. This is because a small fraction of GFP emission that is above 520 nm can pass through the filter to be recorded in YFP channel (vice versa for YFP emission). Therefore, the intensity drop of a GFP bleaching step will be higher in GFP channel compared to the YFP channel and vice versa for YFP steps. We can use this difference in step heights to count number of GFP and YFP bleaching steps, which subsequently will be used to determine subunit composition of a receptor.

Here, we successfully reproduced the dimer of dimer stoichiometry of NMDA receptor using this method.

Reference: 1. Ulbrich, M.H., Isacoff, E.Y. (2007). Nature Methods