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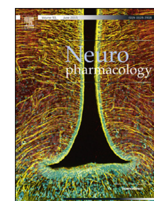

Invited review

Tracking individual membrane proteins and their biochemistry: The power of direct observation

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ABSTRACT

The advent of single molecule fluorescence microscopy has allowed experimental molecular biophysics and biochemistry to transcend traditional ensemble measurements, where the behavior of individual proteins could not be precisely sampled. The recent explosion in popularity of new super-resolution and super-localization techniques coupled with technical advances in optical designs and fast highly sensitive cameras with single photon sensitivity and millisecond time resolution have made it possible to track key motions, reactions, and interactions of individual proteins with high temporal resolution and spatial resolution well beyond the diffraction limit. Within the purview of membrane proteins and ligand gated ion channels (LGICs), these outstanding advances in single molecule microscopy allow for the direct observation of discrete biochemical states and their fluctuation dynamics. Such observations are fundamentally important for understanding molecular-level mechanisms governing these systems. Examples reviewed here include the effects of allostery on the stoichiometry of ligand binding in the presence of fluorescent ligands; the observation of subdomain partitioning of membrane proteins due to microenvironment effects; and the use of single particle tracking experiments to elucidate characteristics of membrane protein diffusion and the direct measurement of thermodynamic properties, which govern the free energy landscape of protein dimerization. The review of such characteristic topics represents a snapshot of efforts to push the boundaries of fluorescence microscopy of membrane proteins to the absolute limit.

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1. Introduction

1.1. The power of direct observation and the promise of single molecule experiments

Experimental biology is a discipline that relies on direct observation and ample measurements to impart statistical relevance. New advances in single molecule fluorescence imaging promise to bring that experimental approach to the single protein level by allowing for the direct observation of (1) the way proteins move and interact with one another, (2) the binding and unbinding of small molecules, (3) the stochastic kinetic rates that arise from different conformational manifolds, (4) the distribution of discrete states in small ensembles of individually tracked proteins, and (5) individual chemical reactions arising from discrete protein states. This suite of direct observations can be used to describe the statistical mechanical mechanisms of how proteins function, complete with fluctuation dynamics describing the kinetic rates between discrete states. (Lu et al., 1998; Rollins et al., 2014; Schuler et al., 2002; Yang et al., 2003).

Membrane proteins are of particular interest in neuroscience and can be more easily studied by single molecule fluorescence techniques than proteins that diffuse freely in all three dimensions.(Harms et al., 2003; Owen et al., 2012; Wang et al., 2014) Discussed in this article are a few examples of membrane bound proteins that act either as ion channels or enzymes, all of which can

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be described as complex molecular machines with moving parts and two-dimensional lateral motions. A stochastic model can be used to describe the operation of molecular machines, which links allosteric motions and chemical reactions to changes in free energy. (Elber and Karplus, 1987; Jülicher and Bruinsma, 1998; Keller and Bustamante, 2014; Kurzynski and Chelminiak, 2014; Lazaridis and Karplus, 1999; Tsai, 2008) Such descriptions include the following phenomena: the closing of ligand binding clefts, (McCammon et al., 1976) rotations of subdomains, (De Groot et al., 1998; Noji et al., 1997) twisting of subunits, binding and unbinding of ligands, (Ghanouni et al., 2001; Lau and Roux, 2007; Ma et al., 2000; Ravindranathan et al., 2005) the metabolism of substrates (enzymes), (Rout et al., 2014) and the movement of proteins through lipid domains. (Wang et al., 2014) Each machine has a small number of key conformational degrees of freedom linked to these motions and chemical reactions, which result in the observable functional states (discrete states) of the protein. (Cooper, 1976; Kim, 2002a, 2002b; Thomas, 1996; Weiss, 2000) These motions and states are controlled by an underlying potential energy surface. (Best and Yng-Gwei, 2005) This surface is a function of the key conformational coordinates and interactions between the protein and the lipid membrane, all of which gives the free energy of each important functional state within specific microenvironments (domains). (Chou, 2001; Maddox, 2002) The local energy minima on this surface represent the discrete biochemical states that can, in principle, be observed at the single protein level. Hence, experimentally measured free energy differences between biochemical states tell us about the contours of the potential energy surface and the mechanisms that drive their properties. New single molecule experiments that allow for the direct observation of the functioning and interactions of membrane bound proteins (e.g. receptors, ion channels, membrane bound enzymes, etc.) will no doubt lead to paradigm shifts in our understanding of how such molecular machines work. (Yudowski et al., 2007).

1.2. Technical overview

Our current understanding of membrane proteins comes primarily from two types of information: structural information, which is mainly derived from x-ray crystallography and dynamical information from ensemble kinetic experiments. Though structural information describes how proteins are built and could, in principle at least, be used to predict dynamical information, direct measurement of kinetics is more practical. Connecting kinetic and thermodynamic properties to structure is a major goal in single molecule biophysics and great effort has been made to push techniques to higher spatial resolution (structure) (Rust et al., 2006) and ever faster time resolution (kinetics). (English et al., 2006; Turunen et al., 2014).

With regard to improvements in spatial resolution in particular, there are several well-established super-resolution single molecule fluorescence imaging techniques capable of resolving fluorescent emitters separated on the order of tens of nanometers (i.e PALM, (Betzig et al., 2006) FPALM, (Hess et al., 2006) STORM, (Rust et al., 2006) dSTORM, (Heilemann et al., 2008) STED(Hell and Wichmann, 1994; Klar and Stefan, 1999), etc). The reader is referred to some of the many good reviews on the subject. (Coltharp et al., 2014; Henriques et al., 2011; Orrit et al., 2014; Patterson et al., 2010; Tonnesen and Nagerl, 2013) The term 'super-resolution' has evolved to mean that the image generated by the experiment has higher resolution than the diffraction limit of the microscope. Many super-resolution techniques use a form of stochastic reconstruction to build images with resolution accuracies that are ~10–100 times better than conventional fluorescence microscopy. All stochastic super-resolution techniques are based on the same two-step process: super-localization (Thompson et al., 2002) followed by reconstruction. Super-localization forms the basis of single protein tracking and with the advent of fast highly sensitive EMCCD cameras and new fluorescent probe designs it is now possible to construct time-lapse super-resolution images on the ms time scale. This makes it possible to track membrane protein motion and biochemistry with spatial resolution well beyond the diffraction limit.

The diffraction limit, d, of a conventional fluorescence microscope in the x,y-plane is given by Abbe's formula: $d = \lambda/2NA$ where λ is the wavelength of the fluorescence and NA is the numerical aperture of the microscope objective. (Abbe, 1873) Using Abbe's formula, is can be shown that the typical diffraction limit is ~200 nm when using modern high NA objectives (1.4–1.45) and fluorophores like Cy3. As a reference, most small organic fluorophores are ~1 nm, commercially available quantum dots range from ~10 to 20 nm (LifeTechnologies™), and fluorescent proteins are ~ 3–4 nm (GFP). (Yang et al., 1996) Because of their small size, all will give rise to a diffraction limited spot with a full width at half maximum (FWHM) of ~200 nm (the diffraction limit). But since the emitted photons are a sampling of a fluorophore's true position, the location of the emitter can be determined with low nanometer precision by determining the peak of this distribution. The peak of the distribution is determined by fitting it to a point spread function (PSF; an Airy function or 2D-Guassian) and the localization precision of the fluorophore (in the x,y-plane) is estimated from the Thompson–Larsen–Webb equation below:

$$\sigma_{xy} = \left[\left(\frac{s_i^2}{N}\right) + \frac{a^2}{12N} + \frac{8\pi s_i^4 b}{a^2 N^2}\right]^{\frac{1}{2}}$$

where σ_{xy} is the standard error of the mean (SEM) from a single point emitter, s_i is the standard deviation of the Gaussian distribution that equals $(2.2)^{-1}$ of the PSF width, a is the pixel size, b is the background, and N is the number of photons collected. (Thompson et al., 2002) In general, this type of analysis is called super-localization and the reconstruction of an image based on super-localization data produces a super-resolution image. The ability to carry out a time sequence of super localization measurements or super-resolution images is the essential step needed to carry out single protein tracking experiments used to determine elementary kinetic rates associated with discrete mechanistic states.

1.3. Super resolution imaging and tracking of individual membrane proteins

Most stochastic super-resolution techniques like STORM, dSTORM, PALM, FPALM, and their many variants are primarily used to image densely packed biological structures including: mitochondria,(Shim et al., 2012) other organelles, synapses, (Dani et al., 2010) large membrane 'patches,' viruses, and whole cells with a lateral resolution of ~20 nm and an axial resolution of ~50 nm. (Juette et al., 2008; Nickerson et al., 2014) These techniques involve the use of large numbers of photo-switchable or photoactivatable fluorescent probe molecules (STORM) or fluorescence proteins (PALM) to specifically label a protein structure(s). This provides contrast to the fluorescent image and gives fundamental details about its biochemical make-up. The fluorophores are then activated with a laser pulse to stochastically 'turn on'—make fluorescent—a small spatially separated subset of these probes, which are excited by a second laser, and appear as diffraction limited spots on a camera. This process is repeated many times storing each image separately. After data collection is complete, the super-localization

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