CHAPTER 14

Visualization of Protein Interactions in Living Cells

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Abstract

Ligand binding to cell membrane receptors sets off a series of protein interactions that convey the nuances of ligand identity to the cell interior. The information may be encoded in conformational changes, the interaction kinetics and, in the case of multichain immunoreceptors, by chain rearrangements. The signals may be modulated by dynamic compartmentalization of the cell membrane, cellular architecture, motility, and activation—all of which are difficult to reconstitute for studies of receptor signaling in vitro. In this chapter, we will discuss how protein interactions in general and receptor signaling in particular can be studied in living cells by different fluorescence imaging techniques. Particularly versatile are methods that exploit Förster resonance energy transfer (FRET), which is exquisitely sensitive to the nanometer-range proximity and orientation between fluorophores. Fluorescence correlation microscopy (FCM) can provide complementary information about the stoichiometry and diffusion kinetics of large complexes, while bimolecular fluorescence complementation (BiFC) and other complementation techniques can capture transient interactions. A continuing challenge is extracting from the imaging data the quantitative information that is necessary to verify different models of signal transduction.

Introduction

Recognition of extracellular ligands by cell surface receptors depends on membrane compartmentalization, subcellular organization, and whole cell dynamics. Ligand-engaged or free receptors can interact with numerous proteins that co-inhabit the cell membrane, partition in different membrane domains, as well as being targets for intracellular adaptors, effector enzymes, cytoskeleton terminals, and the recycling machinery. Any of these interactions may modulate the activity of receptor components and all are themselves subject to continuous change according to subcellular localization, cell motility, polarity, state of activation, and the extracellular environment. Not surprisingly, the mechanisms of ligand recognition by different receptors can be difficult to understand based on in vitro studies alone and have to be verified in the milieu of the living cell. Particularly puzzling is the signal transduction by the multichain immunoreceptors that use dedicated chains for ligand binding and a number of noncovalently associated signaling chains to interface with the intracellular effector enzymes. How the information about the quality of binding between the ligand and the extracellular domain is projected by multichain receptors to the cell interior is of great general interest; especially for understanding antigen recognition, cytokine communication, and homeostasis in the immune system and beyond. According to the structural models, binding of a ligand to the extracellular domain induces a range of structural changes that
propagate to the intracellular domains to expose sites for docking of various adaptors and signaling enzymes. The structural changes could be conformational, chain rearrangements, ligand-driven dimerization, or multimerization. In contrast, the kinetic models favor the view that the information is conveyed by the net balance of otherwise unstructured interactions between the receptors and the membrane-resident kinases and phosphatases, which have opposing effects on signaling. To distinguish between the alternative mechanisms requires quantitative characterization of various parameters of protein interactions in living cells. Verifying the structural mechanisms of ligand recognition requires determining distances and orientations between protein domains within and between receptors, while the kinetic models call for determination of affinities, lifetimes, diffusion coefficients, and frequencies of random collisions—all with subcellular resolution in living cells.

The last two decades witnessed significant refinement of fluorescence microscopy techniques that allowed looking non-invasively inside cells and visualizing receptor dynamics in situ. The most powerful approaches harness fluorescence to provide information about protein interactions (Fig. 1). The general strategy is to hyperlink the structural data on a pixel-by-pixel basis to additional parameters of fluorescence that are sensitive to the local environment. The most direct and versatile are imaging modalities based on Förster (fluorescence) resonance energy transfer (FRET), which is sensitive to the proximity and orientation between fluorophores and is amenable to the structural and the kinetic analysis. Complementary information about diffusion and stoichiometry of large protein complexes can be obtained by fluorescence correlation microscopy (FCM), while bimolecular fluorescence complementation (BiFC) and other complementation techniques can be used to determine protein interactions.

**FRET**

FRET microscopy is the most powerful and popular approach to study protein interactions in living cells. Occurring through dipole-dipole resonance between the excited donor fluorophore and a nearby acceptor, FRET allows direct detection of nanometer-range proximity between appropriately labeled proteins as well as conformational changes. FRET can be imaged based on several parameters that are detectable by wide field, confocal, multiphoton, as well as total internal reflection fluorescence microscopy. Being a proximity effect, FRET can be used to detect both the specific complex formation as well as random collisions—both of which may be important for signaling by multichain immunoreceptors. We will focus later on how quantitative FRET imaging can be leveraged to study the underlying mechanisms of protein interactions.

**Bimolecular Fluorescence Complementation**

The BiFC technique is based on nonfluorescent, complementary fragments of fluorescent proteins (FPs) that can refold into a fluorescing product. By genetically attaching the fragments to different proteins, their interactions can be detected based on de novo fluorescence. Due to irreversible refolding, BiFC is not a general approach to monitor the dynamics of protein interactions but it excels as an end-point kinetic assay. Quantitative application of BiFC is possible by multiplexing fragments from different color FPs. That way, the relative efficiency of competing interactions can be evaluated ratiometrically. Recent improvements include new fragments of the Cerulean and Venus FPs that offer faster refolding kinetics and better sensitivity. The BiFC assay can complement FRET to determine and screen for protein interactions.

**Fluorescence Correlation Techniques**

The formation of large protein complexes that exceed the nanometer range of FRET can be studied at the single-molecule level in living cells by FCM. This method uses highly sensitive detectors to detect bursts of fluorescence due to diffusion of single fluorophores through a small observation volume, which can come from a confocal or multiphoton excitation. The diffusion coefficient, which depends on the mass of freely diffusing complexes, can be discerned by applying the autocorrelation function to the fluctuations of fluorescence. Classic FCM is performed under free diffusion conditions to quantify the absolute molecular mass...