Single Molecule Imaging

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Abstract

Single-molecule imaging is a powerful tool to understand working mechanisms of glycoproteins and glycolipids in cell plasma membranes by overcoming intrinsically stochastic and inhomogeneous properties of biological molecular systems. Here, basic techniques for single-molecule imaging microscopy are described. This method can be carried out by total internal reflection fluorescence microscopy or by oblique illumination microscopy with high-sensitive CCD cameras. The densities of fluorescently labeled membrane proteins and lipids must be low (<2 molecules/μm^2) in cell plasma membranes because high-density expression interferes with tracking of individual fluorescent spots. Therefore, single-molecule imaging in cell membranes requires that conditions be optimized for protein expression, fluorescent labeling, and lipid incorporation. In this chapter, methods for observing and detecting colocalization of

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N. Taniguchi et al. (eds.), *Glycoscience: Biology and Medicine*,
DOI 10.1007/978-4-431-54841-6_122

individual fluorescent spots of proteins or lipids are described. Furthermore, analysis methods for single-molecule imaging, focusing on colocalization of two different fluorescent spots, are explained.

Keywords
Single-molecule imaging • Dual-color observation • mGFP • ACP-tag • Halo7 tag • Colocalization • Dimer

Introduction

Single-molecule fluorescence microscopy is a very useful tool to investigate localization and dynamics of membrane components including glycolipids and glycoproteins and to unravel mechanisms of the molecular interactions. For examples, simultaneous dual-color observation of single molecules of glycosylphosphatidylinositol-anchored proteins (GPI-APs) in cell plasma membranes revealed that all of the GPI-APs were mobile and continually formed transient (~200 ms) homodimers through ectodomain protein interactions, stabilized by the presence of the GPI-anchoring chain and cholesterol (Suzuki et al. 2012). The heterodimers with prolonged lifetimes did not form, suggesting a fundamental role for the specific ectodomain protein interaction. Upon ligation of a GPI-AP, CD59, it formed stable homooligomers which slowly diffused and frequently exhibited temporal confinement in small (~50 nm in diameter) membrane area where the downstream signaling molecules such as PLCγ2 were transiently (~250 ms) recruited and activated (Suzuki et al. 2007a, b). Another example of a notion which was proposed by single-molecule imaging study is compartmentalization of cell plasma membranes by cytoskeletal actin filaments. Single-molecule observation at ultrahigh temporal resolution (~25 μs/frame) showed that phospholipids and membrane proteins diffused rapidly in small membrane area (30 ~ 200 nm), were confined there for short periods (1 ms ~ 100 ms), suddenly hopped to the adjacent compartment, and repeated this diffusional behavior which are called "hop diffusion" (Fujiwara et al. 2002). Thus, single-molecule imaging studies have elucidated mechanisms of molecular events which occur stochastically and transiently in cell plasma membranes. This chapter describes protocols for single-molecule imaging of membrane proteins and lipids in living cell plasma membranes and the data analysis.

Preparation of Cells for Single-Molecule Imaging

Single-molecule imaging in cell membranes requires that conditions be optimized for protein expression, fluorescent labeling, and lipid incorporation. If the density of membrane proteins or lipids in cell membranes is high, the individual spots of the attached fluorophores are indistinguishable, precluding single-molecule imaging. Therefore, observing single molecules in cell membranes requires that the density of fluorescent spots be low (<2.0 spots/μm^2) enough to observe single molecules.

Incorporation of Small Amounts of Fluorescent Lipids to Cell Plasma Membranes

1. Cells are cultured in HAM's F12 medium (Gibco) supplemented with 10 % fetal bovine serum. Cells are sparsely (4×10^3/coverslip) seeded on a coverslip-based dish (35 mmϕ (diameter) with a window of 12 mmϕ, 0.15-mm-thick glass, Iwaki) and grown for 2 days before each experiment.
2. A lipid probe is dissolved in an appropriate solvent. For example, Cy3-dioleoylphosphatidylethanolamine (Cy3-DOPE) (10 μg/ml) is dissolved in methanol.
3. A small volume (typically 1 μl) of a lipid probe solution is placed on the bottom of a glass vial, and a thin film of the lipid probe is prepared.
4. One milliliter of Hank's balanced salt solution (HBSS) buffered with 2 mM Pipes, pH 7.2, is added to the glass vial, and the lipid probe suspension is vigorously vortexed.
5. The lipid probe suspension is further diluted to 1–5 nM with HBSS, and 1 ml of the lipid probe is incubated with cells in a glass base dish for 15 min at room temperature.

Expression of Small Amounts of Membrane Proteins and Fluorescent Labeling

Single molecules of membrane proteins can be observed via fluorescently labeled Fab fragments attached to most of the proteins, but the dye/protein ratio is not necessarily 1:1 in this case, which interferes with the estimation of monomer/dimer ratios of the expressed proteins. Therefore, cells are usually transfected with cDNA of proteins with tags such as monomeric (A206K) green fluorescent protein (mGFP) (Zacharias et al. 2002), Halo7 (Promega) (Keuning et al. 1985), and ACP (New England Biolabs) (Meyer et al. 2006; Rock and Cronan 1979). Halo7-tag and ACP-tag can be labeled with fluorophores at a precise 1:1 mol ratio. Furthermore, neither Halo7 nor ACP forms dimers and therefore does not induce receptor clustering. Notably, however, when these tags are inserted at the extracellular side of receptors, the signal peptides of the receptors sometimes need to be replaced by others for expression of the receptors in cell plasma membranes. Halo7-tag (molecular weight = 33 kDa) is a modified haloalkane dehalogenase designed to bind covalently to synthetic ligands that are suicide substrates. Fluorescently labeled receptors tagged with Halo7 can be labeled at high efficiency (>80 %) (Suzuki et al. 2012). To maintain protein functions, some linkers with lengths between 15 and 21 amino acids should be inserted between Halo7-tag and proteins. Because the fluorophore-conjugated Halo7 ligand is often membrane permeable if the fluorophore molecules are membrane permeable, Halo7-tag at the cytoplasmic site of proteins can also be labeled with membrane-permeable fluorescent ligands. ACP-tag (molecular weight = 8 kDa) is a small protein tag based on ACP from *Escherichia coli*. ACP-tag can be enzymatically conjugated with fluorophores

using the substrates derived from coenzyme A (CoA). Because CoA and fluorescent CoA substrates are membrane impermeable, proteins tagged with ACP at its extracellular surface can be fluorescently labeled.

1. Using LipofectAMINE PLUS (Life Technologies), cells are transfected with cDNAs encoding membrane proteins. For GPI-APs and intracellular signaling molecules, the cDNA sequences are placed in the Epstein-Barr virus-based episomal vector pOsTet15T3 – which carries tetracycline-regulated expression units, a transactivator (rtTA2-M2), and a Tet operator sequence (a Tet-on vector) – and expressed in the cells. For transmembrane proteins such as GPCRs, the cDNA sequences are placed in pcDNA3+ (Invitrogen). The cells that stably expressed the molecules of interest are selected and cloned using 800 μg/ml G418 (final concentration).
2. These cells are sparsely seeded in a glass base dish as mentioned in the part of lipid incorporation. The expression levels of GPI-APs and intracellular signaling molecules are adjusted by finely controlling the concentration of doxycycline added to the cell culture medium. The doxycycline concentrations are optimized by measuring the spot number densities of fluorophores attached to the expressed proteins in cell plasma membranes. Typically, the optimized doxycycline concentrations are 1 ~ 5 ng/ml for GPI-APs.
3. Membrane proteins with Halo7-tag are labeled with fluorescent Halo ligands according to manufacturer instructions (Promega), but for single-molecule imaging, cells are incubated with 50 nM Halo ligand conjugated with fluorophores for 15 min to label the extracellular Halo7 of membrane proteins and for 60–120 min to label cytoplasmic Halo7 (Suzuki et al. 2012). Under these conditions, more than 80 % of the Halo7-tag can be fluorescently labeled.
4. Membrane proteins tagged with ACP ligand are labeled with fluorophores according to manufacturer instructions (New England Biolabs). However, note that for single-molecule imaging of ACP tagged GPI-APs, cells are incubated with 50–1,000 nM ACP ligand conjugated with fluorophores for 30 min (Suzuki et al. 2012). More than 95 % of ACP tagged at the extracellular site of receptors can be labeled with fluorophores (Meyer et al. 2006).

Single-Molecule Imaging

Single-molecule fluorescence imaging is performed using an objective lens-type TIRF microscope (Tokunaga et al. 2008) constructed on an inverted microscope. Figure 1 shows a schematic diagram of the TIRF microscope which is built to perform single- or dual-color single-molecule imaging. An Olympus IX-70 microscope is used as the base with a modified mirror turret to allow side entry of the excitation laser beams to the microscope. A laser beam attenuated with neutral density filters is expanded by two lenses (L1, f = 15 mm; L2, f = 150 mm for 488-nm excitation; or L1, f = 20 mm; L2, f = 80 mm for 594-nm excitation), focused at the back focal plane of the objective lens with an L3 lens (f = 350 mm),

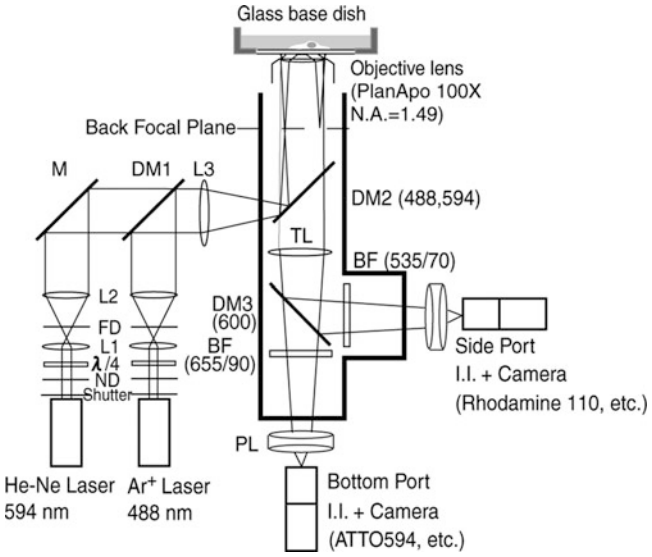

Fig. 1 Microscope setup for simultaneous, dual-color imaging of two fluorescent single molecules of different species. For the observations of other dyes, the microscope is also equipped with 543- and 642-nm lasers. The excitation arm consists of the following optical components: *BP* band-pass filter, *DM* dichroic mirror, *FD* field diaphragm, *L1* and *L2* working as a 10x beam expander for 488-nm excitation or as a 4x beam expander for 594-nm excitation; *L3* focusing lens, *M* mirror, *ND* neutral density filter, *r/4* quarter-wave plate, *S* electronic shutter. The two-color fluorescence emission signal is split by a dichroic mirror (DM3) and detected by two cameras at the side and bottom ports. *BF* barrier filter, *I.I.* image intensifier, *PL* projection lens (2X), *TL* tube lens (1x or 2x)

and steered onto the microscope at the edge of an objective lens with a high numerical aperture (PlanApo 100x; numerical aperture = 1.49; Olympus). A single- or dual-color dichroic mirror (Chroma Technology) is also used.

For single-molecule observation of fluorescent probes in the basal side of cell membranes, the laser beams are totally internally reflected at the coverslip-medium interface, and an evanescent field (1/e penetration depth = ~100 nm) is formed on the surface of the coverslip. The basal cell membranes are locally illuminated with this evanescent field. The emission from the fluorophore is collected by the objective lens and passes the single- or dual-color excitation path dichroic mirror (DM2). For single-molecule observation at the apical side of membranes, oblique illumination is useful. For dual-color observation, the emission is split into two imaging systems by an observation path dichroic mirror (DM3).

The image in each arm is projected onto the photocathode of the image intensifier with a two-stage microchannel plate (C8600-03; Hamamatsu Photonics), which is lens coupled to the camera. Hamamatsu electron bombardment charge-coupled cameras (C7190-23) are used. The cameras on the two detecting arms are synchronized frame by frame by coupling the sync out of one camera to the trigger of the second (gen-locked). Observations are performed only for individual

fluorescent spots located within the central region (20 μm in diameter) of the illuminated area, in which nonuniformities appear to be small. The camera images are stored on a digital videotape (8PDV-184ME; Sony) for postexperiment spatial synchronization.

Data Analysis

This section describes analysis method for single-molecule imaging, focusing on colocalization of two different fluorescent spots. Fluorescent spots in the image are identified using a custom computer program, which takes the cross-correlation of the observed image using a reference two-dimensional Gaussian function with a full width of 200 nm (Fujiwara et al. 2002). In addition to identifying fluorescent spots, this method generates the local peaks in the correlation image, and thus determines whether an observed spot represents one unresolvable spot or two resolvable spots. In this manner, fluorescence intensities of individual spots can be determined (Fig. 2).

Because ACP- or Halo7-tagged protein molecules can be labeled with dyes at a 1:1 D/P ratio and high efficiency, the absolute number of molecules in a single colocalized spot can be determined by measuring the fluorescence intensities of individual spots of ACP- or Halo7-tagged proteins.

After each individual spot in the image is identified, the fluorescence intensities of the identified spots are determined, yielding the histograms. The histograms are fitted by the sum of log-normal functions and provide the spot fractions of monomers, dimers, and oligomers via comparison with the histogram for the fluorescence intensities of individual spots of monomer reference molecules (Suzuki et al. 2012). To determine the fluorescence intensity distribution of monomers, the individual spot intensities of ACP- or Halo7-tagged proteins linked to the TM domains of non-raft molecule low-density lipoprotein receptors (ACP-TM or Halo-TM) are measured.

In dual-color simultaneous single-molecule observation that uses two fluorophores – such as pairs of mGFP and tetramethylrhodamine or ATTO488 and ATTO594 – the two full images synchronously obtained in different colors are spatially corrected and overlaid (Koyama-Honda et al. 2005). In dual-color simultaneous single-molecule observation, the distance between the two molecules can be measured directly from the coordinates (x and y positions) of each molecule (as in photoactivated localization and stochastic optical reconstruction microscopies), which can be determined independently in each image in different colors (Koyama-Honda et al. 2005; Suzuki et al. 2007b, 2012). Even when pairs of different colored molecules are known to be truly associated, the probability of scoring the two molecules as associated is limited by the localization accuracies of each molecule and the accuracies of superimposing the two images. Based on a method developed previously (Koyama-Honda et al. 2005; Suzuki et al. 2007b) and the accuracies determined herein, it was found that for truly associated molecules, the probability of scoring the two molecules as associated increases to 99 % using

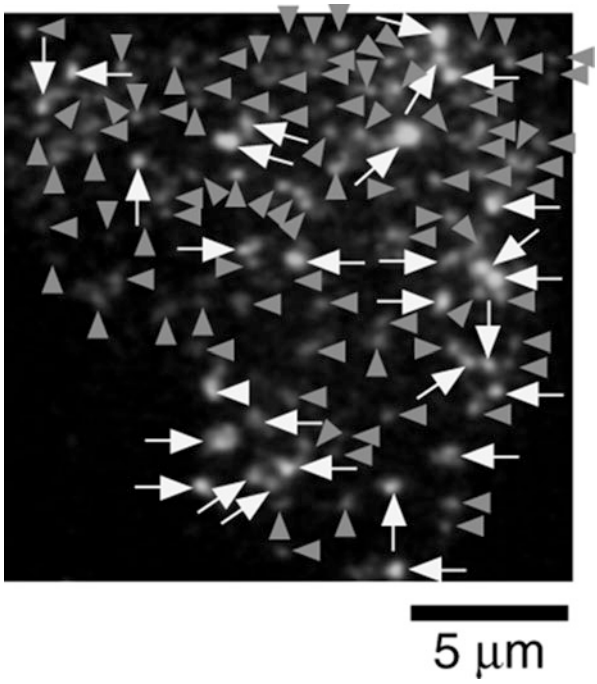

Fig. 2 Identification of individual spots. Typical single-frame total internal reflection image of single ACP-CD59 molecules labeled with Dy547 expressed in the CHO-K1 cell membrane. Individual spots are identified by taking the cross-correlation of the observed image with a reference image of a single-fluorescent molecule spot. *Arrowheads* and *arrows* indicate the spots with monomeric and dimeric intensities, respectively

the criterion that the molecules lie within 240 nm of each other. Therefore, this criterion is used as the definition of colocalization in simultaneous two-color single-molecule observations. The distance of 240 nm coincided with the definition of colocalization in single-color experiments. Given this coincidence, the colocalization of two fluorescent molecules is defined as the event in which the two fluorescent spots representing these molecules become localized within 240 nm of each other.

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