Chapter 6

Genetically Encoded Fluorescent Biosensors for Live Cell Imaging of Lipid Dynamics

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Abstract

Fluorescence imaging provides a powerful technique to visualize spatiotemporal dynamics of biomolecules in living cells, if fluorescent biosensors for the relevant biomolecules become available. Here I describe a fluorescent biosensor for a lipid second messenger, phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P_3). The biosensor overcomes limitations of existing methods for the lipid analysis and allows us to pinpoint that the PI(3,4,5)P_3 concentrations are increasing and/or decreasing not only at the plasma membrane but also at organelle membranes, such as the Golgi apparatus membranes and endoplasmic reticulum membranes. The present biosensor has also been shown to be applicable to a variety of lipid second messengers, including diacylglycerol and phosphatidylinositol 3,4-bisphosphate.

Key words Fluorescent biosensors, Fluorescence resonance energy transfer (FRET), Live cell imaging, Lipid second messengers, Phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P_3), Subcellular dynamics

1 Introduction

PI(3,4,5)P_3 is a lipid second messenger that regulates diverse cellular functions, including cell proliferation and apoptosis, and has a role in the progression of diabetes and cancer (Fig. 1a) [1–3]. PI(3,4,5)P_3 is generated by phosphoinositide 3-kinase (PI3K) [4]. When generated at cellular membranes, PI(3,4,5)P_3 recruits and activates its binding proteins, including Akt, PDK1, Btk, and GRP1, to regulate various cellular functions. To analyze the PI(3,4,5)P_3 levels, labeling cells with [^{32}P]orthophosphate has been widely used. However, because millions of cells must be smashed and analyzed to obtain sufficient radiochemical signals this method has several limitations with respect to its spatial and temporal resolution. Recently, several PI(3,4,5)P_3 biosensors have been constructed by fusing *Aequorea victoria* green fluorescent protein (GFP) to a PI(3,4,5)P_3-binding domain derived from Btk, GRP1, ARNO, or Akt. These sensors, which translocate from the

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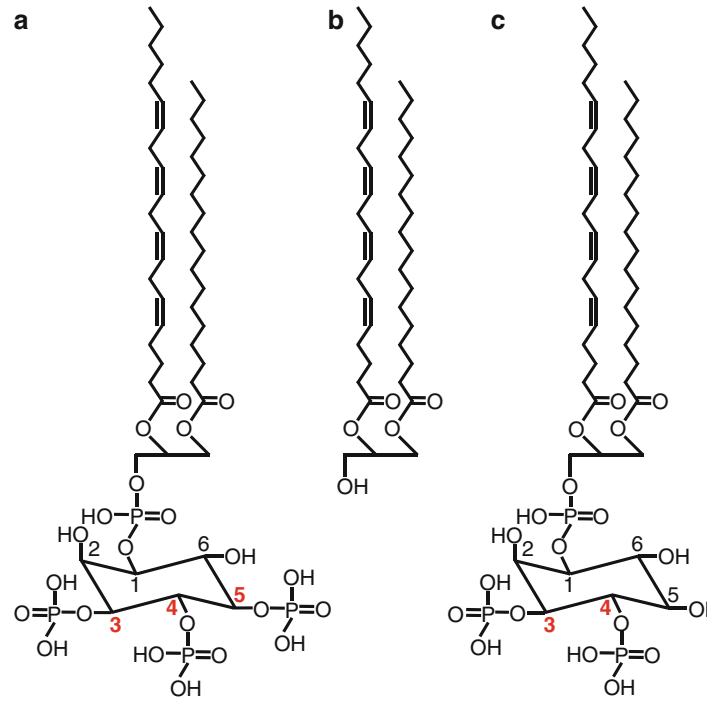

Fig. 1 Structure of lipid second messengers. (**a**) Phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$). (**b**) Diacylglycerol (DAG). (**c**) Phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$)

cytosol to the membrane following $PI(3,4,5)P_3$ generation, have been used to visualize changes in the $PI(3,4,5)P_3$ levels in the cellular membrane[5–8]. However, several factors frequently observed during fluorescence imaging experiments, such as membrane ruffles and changes in the cell shape, affect the fluorescence intensity change in the region of interest in a $PI(3,4,5)P_3$-independent manner, causing serious artifacts. Moreover, it is difficult with these types of fluorescent fusion proteins to distinguish to which membranes the fusion proteins are translocated in the cell.

To overcome these limitations of existing methods, we have developed a fluorescent biosensor for $PI(3,4,5)P_3$ based on fluorescence resonance energy transfer (FRET) (Fig. 2) [9]. The biosensor, named Fllip, is composed of two different-colored mutants of GFP and a $PI(3,4,5)P_3$-binding domain. The $PI(3,4,5)P_3$ level was observed by dual-emission ratio imaging, thereby allowing stable observation of $PI(3,4,5)P_3$ without suffering from the artifacts described above. Fllip is further connected with the membrane localization sequence (MLS) for the localized analysis of the $PI(3,4,5)P_3$ concentration in subcellular membranes. Fllip has allowed us to visualize the spatiotemporal regulation of the $PI(3,4,5)P_3$ generation in single living cells.

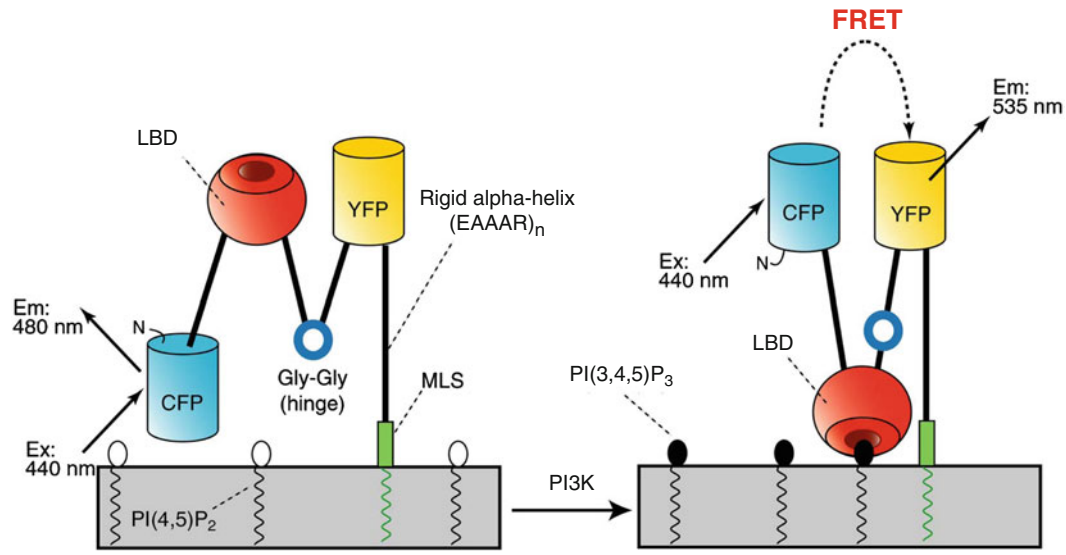

Fig. 2 Principle of the present fluorescent biosensor, Fllip. Abbreviations: *CFP* cyan fluorescent protein, *YFP* yellow fluorescent protein, *LBD* lipid binding domain, *MLS* membrane localization domain, *PI(4,5)P_2* phosphatidylinositol 4,5-bisphosphate, *PI(3,4,5)P_3* phosphatidylinositol 3,4,5-triphosphate, *PI3K* phosphatidylinositol 3-kinase, *Ex* excitation, *Em* emission, *FRET* fluorescence resonance energy transfer

2 Materials

2.1 Materials for Cell Culture and Transfection

1. Chinese hamster ovary cells expressing platelet-derived growth factor receptor (CHO-PDGFR cells).
2. Ham's F-12 medium.
3. Fetal bovine serum.
4. Penicillin and Streptomycin.
5. Trypsin–EDTA.
6. PBS tablet.
7. LipofectAMINE 2000.
8. OptiMEM.
9. Glass-bottomed dish.

2.2 Materials for Live Cell Imaging

1. Hanks balanced salt solution.
2. Excitation filter for CFP (440AF21) (Omega Optical, Inc., Brattleboro, VT, USA).
3. Dichroic mirror (455DRLP) (Omega Optical, Inc., Brattleboro, VT, USA).
4. Emission filter for CFP (480AF30) (Omega Optical, Inc., Brattleboro, VT, USA).

5. Emission filter for YFP (535AF26) (Omega Optical, Inc., Brattleboro, VT, USA).
6. Platelet-derived growth factor (PDGF).

3 Methods

3.1 Design and Construction of the Biosensor

1. *General design*. To construct the present biosensor, named Fllip, the pleckstrin homology (PH) domain, which is derived from GRP1 [6] and selectively binds with PI(3,4,5)P_3, is sandwiched between cyan (CFP) and yellow (YFP) variants of *Aequorea victoria* GFP [10–12] (Fig. 2).
2. *The molecular switch*. The PH domain and the fluorescent proteins are connected with rigid α-helical linkers, consisting of repeated EAAAR sequences [13] (Fig. 2). Within one of the rigid linkers, a single di-glycine motif is introduced as a hinge. When PI(3,4,5)P_3 is produced at the membrane upon activation of phosphatidylinositol 3-kinase (PI3K), the PH domain of Fllip binds with PI(3,4,5)P_3 (Fig. 2) and induces a significant conformational change through the flexible di-glycine motif introduced in the rigid α-helical linker. The flip-flop type of conformational change exhibited by Fllip results in intramolecular FRET from CFP to YFP, allowing detection of the PI(3,4,5)P_3 dynamics at the cellular membrane.
3. *Membrane targeting*. Fllip can be tethered to the membrane by fusing it with an MLS via the rigid α-helical linker (Fig. 2). Three different sequences, termed MLS_{pm}, MLS_{em}, and MLS_{mit}, have been tested. These sequences tether Fllip to the plasma membrane (pm), endomembranes (em), and mitochondrial outer membranes (mit), respectively (Fig. 3). The endomembranes include the Golgi apparatus membranes and the endoplasmic reticulum membranes. The amino acid sequence of MLS_{pm} (QGCMGLPCVVM) is derived from the CAAX box motif of N-Ras [14]. The N-terminal and C-terminal cysteines are palmitoylated and farnesylated, respectively. MLS_{em} (QGSMGLPCVVM) is derived from a mutated CAAX box motif of N-Ras, in which the original N-terminal cysteine is substituted with a serine not to be palmitoylated [14]. The amino acid sequence of MLS_{mit} (FNRWFLTGMTVAGVVLLGSLFSRK) is derived from the C-terminal sequence of Bcl-xL [15]. Fllip biosensors connected with MLS_{pm}, MLS_{em}, and MLS_{mit} are named Fllip-pm, Fllip-em, and Fllip-mit, respectively.

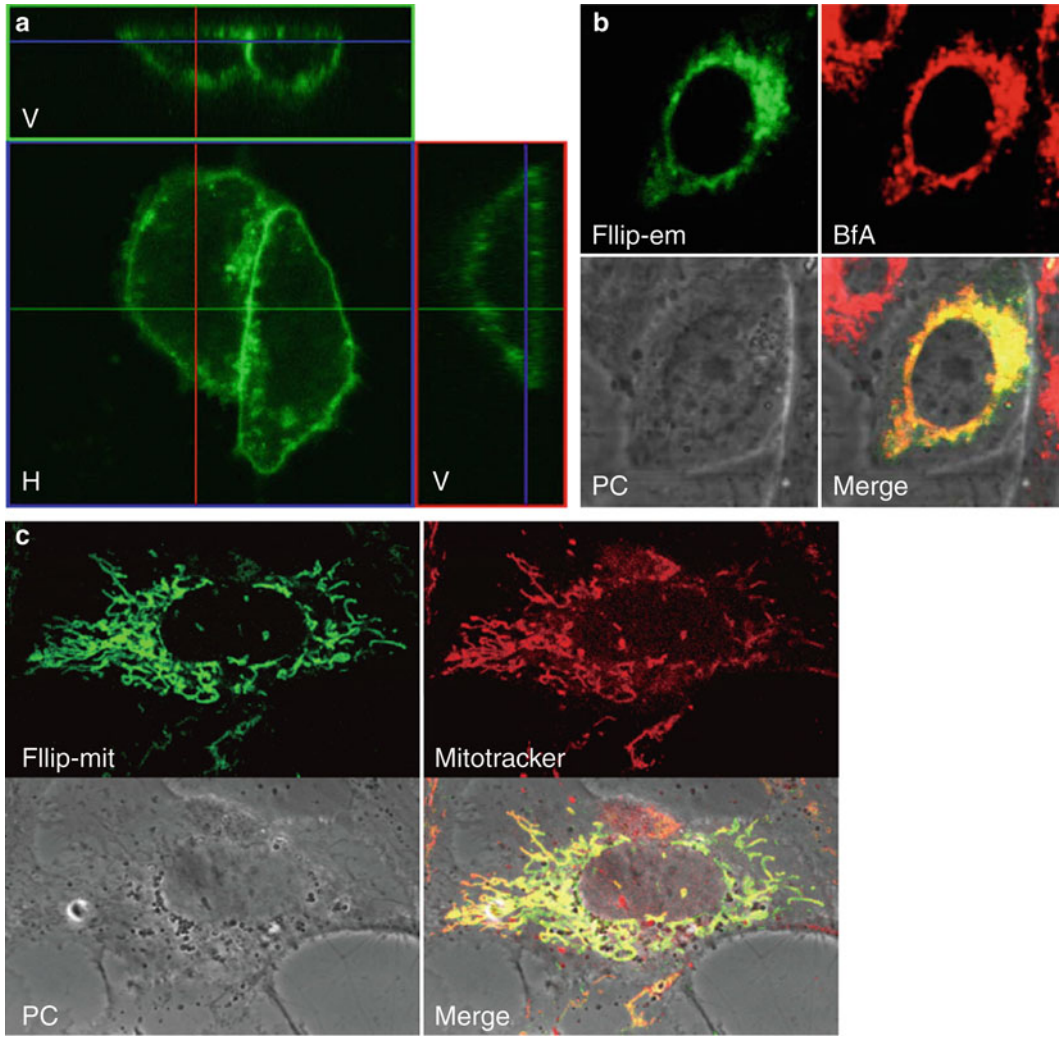

Fig. 3 Subcellular localization of Fllip-pm (**a**), Fllip-em (**b**), and Fllip-mit (**c**). *V* vertical sections, *H* horizontal section, *BfA* BODIPY-brefeldin A, *PC* phase contrast images

3.2 Cell Culture and Transfection

1. Plate CHO-PDGFR cells on 35-diameter glass-bottomed dishes with Ham's F-12 medium supplemented with 10 % fetal calf serum.
2. Incubate the cells at 37 °C in 5 % CO_2 until the cells are 50–80 % confluent.
3. Transfect the cells with 0.8 μg per dish of cDNA encoding Fllip using LipofectAMINE 2000 according to the manufacturer's instructions.
4. The transfection mixture may be replaced with fresh 1.5 mL of Ham's F-12 medium supplemented with 10 % fetal calf serum after 5–12 h of transfection, if desired.

5. Incubate the cells at 37 °C in 5 % CO_2 for 1–3 days after transfection (*see* **Note 1**).
6. Replace the medium with a serum-free Ham's F-12 medium supplemented with 0.2 % bovine serum albumin and incubate the cells for 2–6 h for serum starvation (*see* **Note 2**).
7. Wash with 1 mL of Hank's balanced salt solution (HBSS) and add 1 mL of HBSS for fluorescence imaging of PI(3,4,5)P_3.

3.3 Fluorescence Imaging

1. Place the cells on the stage of an inverted microscope equipped with a cooled CCD camera (*see* **Note 3**).
2. Image acquisition and processing are controlled by a PC connected to the CCD camera and a filter wheel using the MetaFluor software. A shutter for excitation in front of the xenon lamp is also controlled by the PC. Excitation light from a 75-mW xenon lamp is passed through a 440 ± 10.5 nm band-pass filter for CFP excitation (440AF21). The light is reflected onto the cells using a dichroic mirror (455DRLP). The emitted light is collected with a 40× or 63× objective and passed through a 480 ± 15 nm band-pass filter (480AF30) for CFP emission and a 535 ± 13 nm band-pass filter (535AF26) for YFP emission.
3. Define several factors for image acquisition, such as excitation power, time of exposure to the light, image acquisition interval, and binning (*see* **Note 4**).
4. By browsing the cells on the dish, choose moderately bright cells, in which Fllip is well localized in the subcellular membranes of interest, for example, the plasma membrane and endomembranes (Golgi membranes and ER membranes) in the case of Fllip-pm and Fllip-em, respectively (*see* **Note 5**).
5. Draw a region of interest within the cell on the field of view of the CCD camera (*see* **Note 6**).
6. Start to acquire images every 2–10 s with the 440 ± 10.5 nm excitation. The software MetaFluor records fluorescence intensities of the CFP and YFP emissions of Fllip, together with their emission ratios in the selected regions of the cells over time.
7. When the emission ratio of CFP to YFP reaches a steady state, add ligands, such as PDGF, to the dish to stimulate the cells and monitor the PI(3,4,5)P_3 levels through the change in the emission ratio of Fllip (Fig. 4).
8. After finishing all the experiments, wipe the residual immersion oil out of the objective.

3.4 Customizing the Biosensor

1. It should be mentioned that the modular design of Fllip allows it to be easily adapted to detect a variety of other lipid second messengers. Fllip has two key components: the MLS and the lipid binding domain (LBD). These two components can be tailored to detect the lipid-of-interest.

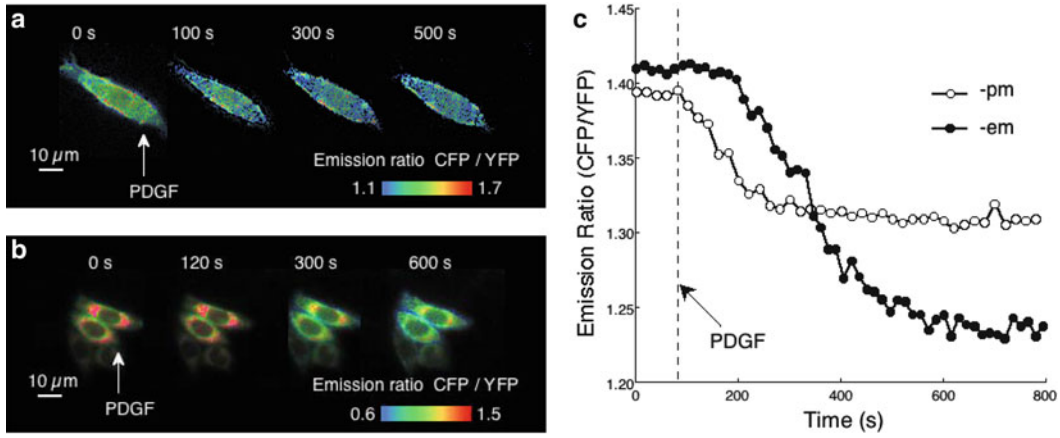

Fig. 4 (**a**, **b**) Pseudocolor images of the CFP/YFP emission ratio before (0 s) and 100, 300, or 500 s after addition of platelet-derived growth factor (PDGF). Results were obtained with CHO-PDGFR cells expressing Fllip-pm (**a**) or Fllip-em (**b**). (**c**) Time course of the CFP/YFP emission ratio of Fllip-pm and Fllip-em before and after stimulation with PDGF

Table 1
Changing the selectivity of the present fluorescent biosensor

Lipids	Lipid binding domains (LBDs)	References
$PI(3,4,5)P_3$	Pleckstrin homology domain derived from Grp1	[9]
DAG	Cysteine rich domain derived from PKC β	[16]
$PI(3,4)P_2$	Pleckstrin homology domain derived from TAPP1	[17]

2. By connecting each specific MLS, Fllip can be directed to various subcellular membranes, such as the plasma membrane and organelle membranes, including membranes of the Golgi apparatus, endoplasmic reticulum, mitochondrion, and the nucleus.
3. Lipid second messengers, such as diacylglycerol (DAG) [16] (Fig. 1b) and phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) [17] (Fig. 1c) can be detected through use of appropriate LBDs, instead of the $PI(3,4,5)P_3$-binding domain, as performed previously (Table 1).

4 Notes

1. Incubation at 28 °C often improves subcellular localizations of Fllip-pm and Fllip-em in the plasma membrane and endomembranes, respectively.

2. This experimental step may not be necessary, depending upon cell types used for imaging.
3. Cells are viable and healthy for at least 60 min at ambient conditions. A CO_2 chamber and a temperature controller are required for long-term experiments.
4. If photobleaching of fluorescent proteins, in particular YFP, is observed, use an ND filter in the range of 0.1–10 % to reduce excitation light intensity and/or decrease exposure time. Also, binning can sum the signal from multiple pixels on the CCD camera so that less light is required while keeping a good signal-to-noise ratio.
5. Avoid cells that are too bright in order to reproducibly obtain quantitative FRET signals. Also avoid cells that are too dim, because these cells often exhibit noisy images.
6. The intensity in the region should be more than five times the background intensity.

Acknowledgments

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