Chapter 7

The Application of Fluorescent Probes for the Analysis of Lipid Dynamics During Phagocytosis

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Abstract

Phagocytosis is the process whereby specialized leukocytes ingest large particles. This is an extremely dynamic and localized process that requires the recruitment to the sites of ingestion of numerous effector proteins, together with extensive lipid remodelling. To investigate such a dynamic series of events in living cells, non-invasive methods are required. The use of fluorescent probes in conjunction with spectroscopic analysis is optimally suited for this purpose. Here we describe a method to express in RAW264.7 murine macrophages genetically encoded probes that allow for the spatio-temporal analysis of lipid distribution and metabolism during phagocytosis of immunoglobulin-opsonized beads. The fluorescence of the probes is best analysed by laser scanning or spinning disc confocal microscopy. While the focus of this chapter is on phagocytic events, this general method can be employed for the analysis of lipid distribution and dynamics during a variety of biological processes in the cell type of the investigator's choice.

Key words: Phagocytosis, macrophage, fluorescent probes, phosphatidylserine, phosphoinositide, confocal fluorescence microscopy.

1. Introduction

Biological membranes are dynamic entities that not only function as physical barriers for cells and organelles but also play important roles in metabolic and cell signalling events. The species of lipids that comprise these membranes dictate cellular processes by recruiting proteins through lipid-specific binding domains, by electrostatic interactions or by serving as substrates to generate important signalling molecules (1, 2). All of these aspects of lipid function are critically involved in phagocytosis.

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Phagocytosis is a complex process by which specialized cell types, such as neutrophils, microglia and macrophages, ingest large (>0.5 μm) particles such as bacteria or apoptotic bodies. Particle engulfment by phagocytosis is essential for the clearance of infection and plays an important role in tissue remodelling (3). The ingested particles are internalized into membrane-bound vacuoles termed "phagosomes" that upon maturation become increasingly acidic and degradative in nature (reviewed in (4)).

Phagosome formation is a receptor-driven process that is triggered by recognition of intrinsic components of the target particle or of serum components – such as complement proteins or immunoglobulins – that coat the particle (5, 6). As a result of receptor engagement, tyrosine kinases are activated, effector proteins are recruited, lipids are redistributed and metabolized, and actin undergoes re-arrangement (5). The localized remodelling of the actin cytoskeleton drives the formation of pseudopods that engulf the phagocytic target. The formed vacuole undergoes extensive changes in order to acquire microbicidal properties, a process known as "maturation". The phagosomal membrane undergoes significant and rapid alterations in lipid composition throughout the stages of phagosome formation and maturation (3). Thus, phosphatidylinositol-4,5-*bis*phosphate ($PI(4,5)P_2$) is initially enriched at the base of the forming phagocytic cup, where it is required to initiate localized actin polymerization to drive pseudopod formation (7, 8). As actin continues to polymerize, pushing the phagocyte membrane around the target, $PI(4,5)P_2$ is maintained at the leading edges of the pseudopod, but is cleared from the base of the phagocytic cup. $PI(4,5)P_2$ clearance is caused in part by its phosphorylation by class-I phosphatidylinositol 3-kinase, generating phosphatidylinositol-3,4,5-*tris*phosphate ($PI(3,4,5)P_3$) (9, 10). Further clearance of $PI(4,5)P_2$ is caused by $PI(3,4,5)P_3$-dependent recruitment of PLCγ, which hydrolyses $PI(4,5)P_2$ to diacylglycerol (DAG) and inositol *tris*phosphate (IP_3) (7, 11). Upon fusion of the pseudopod tips and closure of the phagosome, $PI(3,4,5)P_3$ rapidly disappears from the phagosome membrane. At that stage phosphatidylinositol 3-phosphate (PI(3)P), another phosphoinositide, begins to accumulate (9). PI(3)P persists for several minutes on the limiting membrane of phagosomes, where it is essential for subsequent maturation of the phagosome through endosomal fusion (12). The role of phosphatidylserine (PS) during phagosome formation has not been defined, although this negatively charged lipid is abundant in the plasma membrane and the phagocytic cup (13). After sealing, PS persists on the phagosomal membrane, but whether this lipid is required for maturation is not yet known.

Understanding the cell biology of phagocytosis or any other dynamic cellular process is not a trivial task. Some of the lipids that contribute to phagosome formation exist only transiently and

the entire phagocytic process occurs locally and rapidly. Conventional methods for lipid analysis, such as thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC), are not optimal for investigation of such a dynamic process, as they lack the spatial and temporal resolution necessary to monitor localized and rapid events, and generally require large number of cells and use of radioisotopes. To overcome these hurdles, it is most convenient to employ fluorescent probes to detect and track the fate of specific lipids in living cells using dynamic detection methods such as confocal fluorescence microscopy. These lipid-binding probes or "biosensors" require the expression of chimeric constructs consisting of a fluorescent protein, such as green fluorescent protein (GFP), fused to a specific lipid-binding domain (**Fig. 7.1**). There exist several variants of GFP as well as variants of a red fluorescent protein (RFP) that possess unique spectral properties that can be used in combination for a variety of imaging applications (reviewed in (14)). These can be coupled to domains or motifs isolated from several proteins that bind defined species of lipids, such as PS or $PI(4,5)P_2$ (**Table 7.1**). There are several advantages to using fluorescent biosensors: they are relatively non-invasive and enable analysis of dynamic events in living cells. In addition, and importantly, they can be genetically encoded and therefore introduced into cells by transfection of plasmid DNA, by transduction with viruses or by microinjection of the cDNA encoding the probe.

Herein we describe a general method for the use of lipid-binding fluorescent probes in conjunction with confocal microscopy for the dynamic, non-invasive analysis of lipids during phagocytosis. By no means is this protocol limited to the

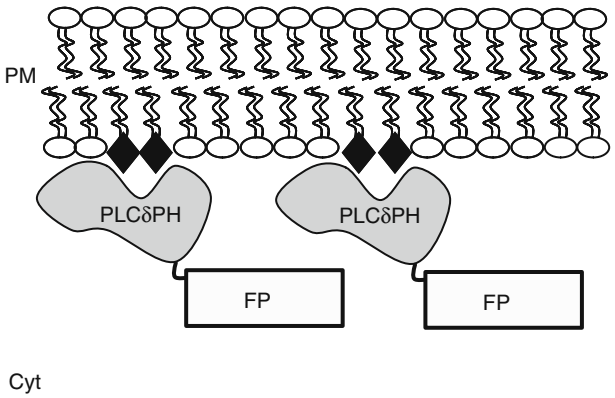

Fig. 7.1. Strategy for lipid detection using fluorescent fusion protein probes. Cells are transfected with plasmids that express a fusion protein comprised of a lipid-binding domain (e.g. PLCδPH) and a fluorescent protein (FP). The lipid-binding domain interacts reversibly with its endogenous ligand (e.g. $PI(4,5)P_2$ for PLCδPH), resulting in accumulation of the probe and increased fluorescence signal that can be detected spectroscopically.

Table 7.1
Lipid-binding probes that can be used in live cell imaging

Lipid-binding domain	Ligand	Protein source	References
FYVE	PI(3)P	EEA1 Hrs	(15) (15)
PX		$P40^{Phox}$	(16)
Pleckstrin homology (PH)	PI(4)P	OSH2 FAPP-1	(17) (18)
PH Tubby domain	$PI(4,5)P_2$	PLCδ Tubby	(19) (20)
PH	$PI(3,4)P_2$	TAPP1	(21)
PH	$PI(3,4)P_2/PI(3,4,5)P_3$	AKT PDK1 CRAC	(22) (23) (24)
PH	PI(3,4,5)P3	Btk GRP1 ARNO	(25) (26) (27)
C2 Annexin	PS	Lactadherin Annexin V	(28) (29)
C1	DAG	PKCγ1	(30)
BH3 CLBD	Cardiolipin	Bid MTCK1b	(31) (32)
SBD	Sphingolipids	Gp120, PrP, β-amyloid peptide	(33)
D4	Cholesterol	Perfringolysin θ toxin	(34)

study of phagocytosis and the same general procedure can be used to analyse many other processes in phagocytes and other cells. A fundamental component of the method described here is the transfection of plasmid DNA into macrophages. Primary macrophages are often refractory to transfection procedures and therefore we often use the murine macrophage cell line RAW264.7 for analysis of phagocytic events. Described below is a general protocol for the analysis of dynamic lipid behaviour during phagocytosis of IgG-coated polystyrene beads.

2. Materials

2.1. Cell Culture

1. RAW264.7 murine macrophage cell line (ATCC®, Manassas, VA).
2. 1× Dulbecco's modified Eagle's medium (DMEM) (WISENT, Mississauga, ON, Canada), supplemented with

5% (v/v) heat-inactivated fetal bovine serum (HI-FBS) (WISENT).

3. 1× solution RPMI 1640 buffered with 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (WISENT), referred to hereafter as HPMI.
4. Sterile 1× phosphate-buffered saline (PBS).
5. 0.05% trypsin solution containing 0.53 mM ethylenediaminetetra-acetic acid (EDTA) and sodium bicarbonate (WISENT).
6. T-25 or T-75 tissue culture flasks (Sarstedt, Montreal, QC, Canada).
7. Tissue culture dishes with 6 or 12 wells (Becton Dickinson, Mississauga, ON, Canada).
8. Round glass cover-slips (18 mm diameter for 12-well dishes or 25 mm for 6-well dishes) (Fisher Scientific, Ottawa, ON, Canada).
9. Humidified tissue culture incubator at 37°C with 5% CO_2.

2.2. Transfection Reagents

1. Fugene HD transfection reagent (Roche Applied Science, Mississauga, ON, Canada).
2. Serum-free DMEM (WISENT).

2.3. Plasmid Isolation

1. Transformed bacterial stock (usually an *Escherichia coli* strain that was used during plasmid construction) in glycerol (*see* **Note 1**).
2. Sterile Luria-Bertani (LB) bacteriological medium (Tryptone, 10 g/L, NaCl 5 g/L and yeast extract 5 g/L) (BioShop Canada, Burlington, ON, Canada).
3. Antibiotic stocks for plasmid selection (ampicillin 100 mg/mL, kanamycin 40 mg/mL, chloramphenicol 30 mg/mL) (Sigma-Aldrich, Canada).
4. High Speed plasmid Maxi kit (QIAGEN Inc., Mississauga, ON, Canada).

2.4. Phagocytosis Assays

1. Inert polystyrene beads (3.87 μm diameter) with 2% (v/v) divinylbenzene (Bangs Laboratories, Fishers, IN,).
2. Lyophilized human IgG (Sigma-Aldrich), re-suspended in sterile PBS at a concentration of 50 mg/mL.

2.5. Microscopy

1. A spinning-disk confocal microscope equipped with at least a 63× magnifying objective, a light source and filter set that is appropriate for the fluorescent protein of choice and an objective heater. The system in our laboratory consists of a Zeiss Axiovert 200 M inverted microscope

(Carl Zeiss Canada, Toronto, ON, Canada) equipped with diode-pumped solid-state laser (Spectral Applied Research, Richmond Hill, ON, Canada) providing lines 440, 491, 561, 638 and 655 nm, a motorized X-Y stage (API, WA) and an piezo focus drive (Quorum Technologies, Guelph, ON, Canada). Images on this system are captured using a back-thinned EM-CCD camera (Hamamatsu) controlled by the software Volocity version 4.1.1 (Improvision Inc., Waltham, MA).

2. Live-cell imaging chamber for 18 or 25 mm cover-slips (Invitrogen Canada Inc., Burlington, ON, Canada).
3. Digital temperature regulator with a heated P insert (PeCon, Germany).
4. Images are analysed using analysis software such as Volocity or Image J (http://rsb.info.nih.gov/ij /).

3. Methods

3.1. Cell Culture

1. RAW264.7 macrophages are routinely cultured in 10 mL of DMEM supplemented with HI-FBS (5% v/v) at 37°C and 5% CO_2 in a T-25 tissue culture flask. If several transfections are to be performed it may be useful to culture the macrophages in a T-75 flask, which will yield a greater number of cells that can subsequently be seeded onto cover-slips. When the macrophages reach ~70–80% confluence they are ready to be trypsinized and can be used to seed tissue culture dishes (12- or 6-well dishes) containing sterile glass cover-slips (*see* **Note 2**).

3.2. Transfection of Cells

3.2.1. Day 1

1. Prior to trypsinizing the macrophages, add to a 12-well tissue culture dish sterile 18-mm glass cover-slips (one per well) using sterile tweezers. To each well containing a cover-slip add 1 mL of pre-warmed (37°C) DMEM plus HI-FBS. If using a 6-well dish, 2 mL of medium should be added to each well containing a 25-mm cover-slip.
2. The RAW264.7 macrophages in the T-25 flask that have reached 70–80% confluence are washed once with 8 mL of sterile PBS pre-warmed to 37°C. The PBS is then carefully aspirated.
3. Add 1 mL of pre-warmed (37°C) 0.05% trypsin/0.53 mM EDTA to the T-25 flask (for T-75 flasks add up to 2.5 mL trypsin/EDTA). Gently rotate the flask to ensure that the trypsin solution is dispersed evenly over the bottom of the flask and incubate for 1–5 min while observing for detachment of the cells (*see* **Note 3**).

4. When the majority of the RAW cells appear to be detached, gently add 8 mL of pre-warmed DMEM plus HI-FBS to wash the cells off the bottom of the flask. The addition of medium containing FBS inactivates the trypsin so that further proteolysis does not occur. Carefully pipette up and down to disperse any clumped cells. This will also ensure that any weakly adherent macrophages are detached from the bottom of the flask (*see* **Note 4**).
5. To the 12-well dish containing pre-warmed DMEM with HI-FBS add 1–4 drops of the macrophage suspension from step 4. For a 6-well dish, up to six drops of the macrophage suspension may be necessary to achieve the desired confluence. The exact number of macrophages per drop is influenced by the efficiency of trypsinization and the initial confluence of the cell culture. While it is not necessary to precisely enumerate the RAW cells in the suspension, after an overnight incubation (18 h) the adherent cells should be approximately 50% confluent (*see* **Note 5**).
6. Incubate the dish containing cover-slips seeded with RAW cells overnight in a tissue culture incubator at 37°C with 5% CO_2.
7. For plasmid isolation, 500 mL of sterile LB containing the appropriate antibiotic is inoculated with bacteria from a glycerol stock carrying the desired plasmid. The bacterial culture is then incubated overnight at 37°C, unless specific growth conditions are indicated. Note that this culture should be started 1 day prior to splitting the RAW cells. The overnight bacterial culture is lysed and the plasmid DNA isolated by directly following the QIAGEN instruction manual for the High Speed Maxi Prep kit. Typically 0.75–1 μg/μL of plasmid DNA is obtained. Once the plasmid is isolated it can be stored and used repeatedly for transfections.

3.2.2. Day 2

8. Remove Fugene HD and serum-free DMEM from the 4°C refrigerator and warm up to room temperature.
9. Transfections are performed as described in Roche's protocol that is supplied with the reagent. Typically, 2 μg of plasmid DNA and 3 μL of Fugene HD are used per well for transfections. Importantly, the ratio of DNA to transfection reagent should be determined for each construct and batch of RAW cells, as some variability can occur. When two plasmids are being co-transfected, the combined amount of DNA added to each well should not exceed what is used for only one plasmid (i.e. 1 μg of each plasmid).
10. Add 100 μL of the transfection solution prepared in the previous step drop-wise to each tissue culture well and

incubate the plate (12- or 6-well) for 16–24 h at 37°C with 5% CO_2.

3.3. Bead Opsonization

1. This is to be performed the day that phagocytosis will be examined. Dilute 50 μL of the suspension of 3.87 μm polystyrene beads into 200 μL of sterile PBS (*see* **Note 6**).
2. To the bead suspension from step 1 add 16 μL of human IgG reconstituted in sterile PBS. Mix the bead suspension continuously for 1–2 h at room temperature.
3. Wash the IgG-opsonized beads three times with 1 mL of sterile PBS and re-suspend in a final volume of 800 μL.

3.4. Synchronized Phagocytosis Assay and Image Acquisition

1. Turn on the digital heater to warm objective (63× or 100×) and the P insert. These must be at 37°C prior to acquiring images of cells undergoing phagocytosis. At this time ensure that the spinning disk confocal microscope and the light source are turned on. It is also useful at this time to set up the experimental acquisition parameters for the experiment using the Volocity software.
2. Prior to initiating the phagocytosis assay, warm an aliquot of HPMI to 37°C.
3. Remove a cover-slip from one well containing transfected cells and add to a new 12- or 6-well tissue culture dish after having been incubated for at least 16 h at 37°C with 5% CO_2. Place the plate with the remaining transfectants back into the incubator.
4. Wash the cover-slip twice with cold (4°C) 1× PBS (*see* **Note 7**).
5. To this well add 1 mL of cold (4°C) HPMI and keep plate on ice (*see* **Note 8**).
6. Next, add 15 μL of the IgG-opsonized bead suspension to this well and transfer the chilled tissue culture dish with the beads to a centrifuge with a plate rotor that has been cooled to 4°C. Centrifuge the plate for 1 min at 250×*g* to sediment the beads onto the transfected RAW cells, to synchronize phagocytic events.
7. Transfer cover-slip from step 6 to a pre-chilled live-cell imaging chamber and keep on ice. Add 500 μl of fresh 4°C HPMI to the chamber.
8. Rapidly transfer the live-cell imaging chamber to the spinning-disk confocal microscope and focus on a single cell. The focal plane should be such that you acquire images through the middle portion of the cell. It is also possible to acquire Z-stacks for the cell of interest; however, this may lead to photobleaching over time. A fluorescence image and

a differential interference contrast (DIC) image should be captured for each acquisition (*see* **Note 9**).

9. Once the cell of interest is in focus, the cold HPMI should be aspirated and fresh, warm (37°C) HPMI should be carefully added to the chamber. With the addition of warmed medium phagocytosis will proceed rapidly and imaging should commence immediately. Setting up the acquisition protocol as a time-lapse experiment will enable multiple images of the same cell to be acquired at set intervals during the phagocytic process (*see* **Note 10**). Alternatively, images can be acquired manually at pre-determined time points, though this is slightly more laborious.

3.5. Fluorescence Measurements

The detection of plasmalemma and internal membrane components can be made using a variety of plasmid-encoded fluorescent probes. Furthermore, analysis of the dynamic behaviour of many different lipid components can be monitored separately or simultaneously by microscopy. Proper analysis of the acquired images is of utmost importance so that misinterpretation of data is avoided. Qualitative conclusions can be drawn from careful visual analysis of acquired images; however, only gross changes in the distribution of a cellular probe can be described with any certainty. To strengthen the data it is best to perform quantitative analysis of all fluorescence images (*see* **Note 11**).

Quantitative analysis of fluorescence images is usually carried out by measuring the mean fluorescence intensity per pixel of a particular region of the cell called the region of interest (ROI). Fluorescence intensity can be affected by a number or variables, including probe expression levels, the brightness of the light source used to excite the probe, the exposure time during the acquisition of the image, and photobleaching of the fluorophore over time. For these reasons it is necessary to standardize fluorescence intensity measurements for each cell being analysed. To achieve this, a region of the cell that is predicted not to undergo changes during the experiment should be selected as an internal reference for fluorescence intensity. In our case, during phagocytosis it is useful to compare fluorescence of the ROI to a remote region of the plasma membrane where phagocytic events have not occurred and no particles are bound (i.e. the bulk plasma membrane). The manner in which fluorescence intensities are normalized depends on the properties of the probe being used. In instances where there is readily detectable cytosolic fluorescence, as can be seen in **Fig. 7.2** using the PH-PLCδ/eGFP probe, it is best to correct the measured fluorescent intensities by subtracting the cytosolic fluorescence intensity. To allow for comparison between cells and experiments, fluorescent intensities can

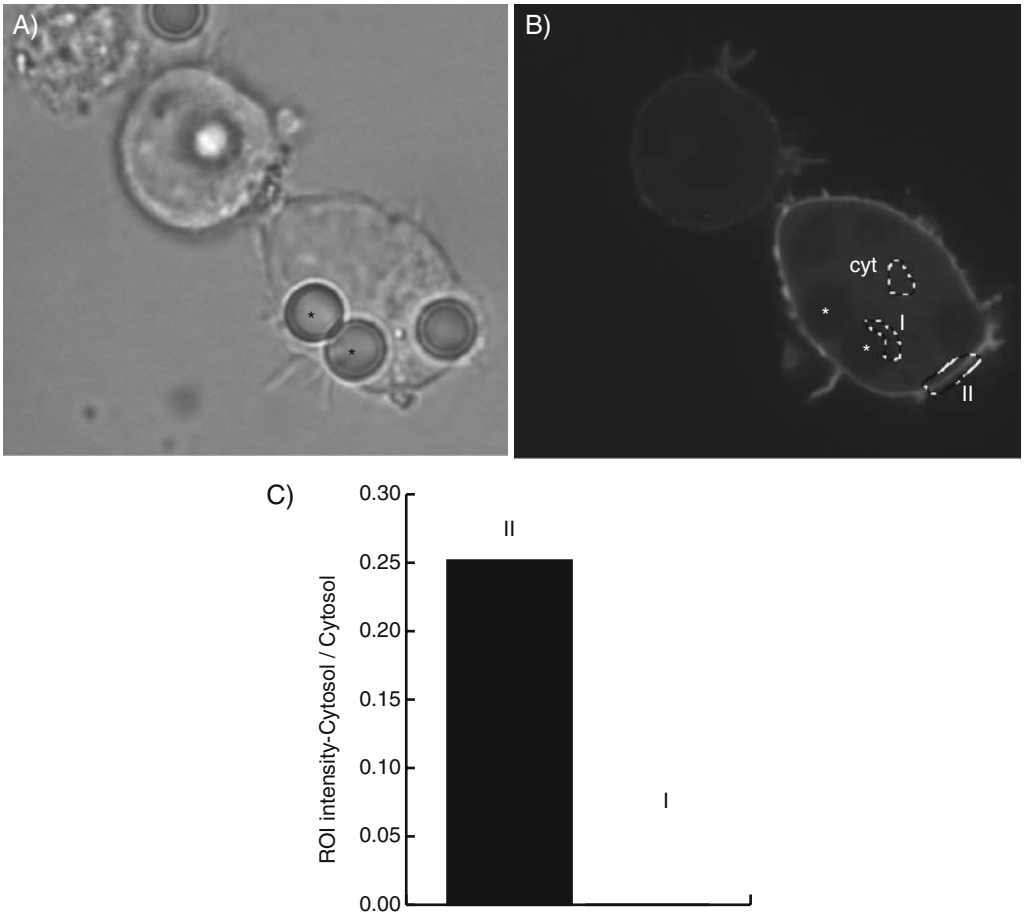

Fig. 7.2. Distribution of $PI(4,5)P_2$ in RAW264.7 cells having internalized IgG-opsonized beads. (**a)** Differential interference contrast (DIC) image of RAW macrophages that have internalized 3.87-μm polystyrene beads. (**b)** Confocal image of the fluorescence of the RAW cells from panel A, which had been transfected with a plasmid expressing the PH domain of PLCδ fused to eGFP. Accumulation of the probe at the plasma membrane indicates that $PI(4,5)P_2$ is abundant in this compartment of the cell. The regions of interest (ROI) used in the analysis of this image are shown and labelled as follows: I, phagosome membrane region; II, plasma membrane region; cyt, cytosolic region. (**c)** The normalized mean fluorescence intensity per pixel of the plasma (II) and phagosomal (I) membranes.

be normalized by dividing the corrected ROI intensities by the measured cytosolic emission.

1. Using the image analysis software such as Volocity select an experimental ROI that encompasses the membrane that has engaged an IgG-opsonized bead. For the earliest time points imaged it is necessary to compare the region of fluorescence selected with the DIC overlaid in order to ensure that a region with a bead engaged is being analysed.

2. Measure the fluorescence intensity (mean fluorescence intensity per pixel) for the experimental ROI.
3. Define a control ROI on the region of the cell that is not expected to undergo changes and measure its fluorescence intensity.
4. Define a cytosolic ROI and measure the emission intensity.
5. Subtract from the experimental ROI and the control ROI the cytosolic fluorescence, which will also account for background fluorescence.
6. Using the corrected fluorescence intensities for the experimental and control regions of interest determine the ratio of fluorescence intensity of ROI/cytosolic ROI (i.e. phagosomal membrane-cytosol/cytosol). The ratios obtained for several different cells between experimental conditions can now be compared.

Alternatively, when binding of the fluorescent probe within the cell is almost exclusively to membranes, measured fluorescence intensities can be normalized by dividing background-corrected ROI emission intensities by the emission intensity of the bulk plasma membrane.

4. Notes

1. Freeze pure cultures of each *E. coli* strain transformed with a plasmid that is transfected into mammalian cells. This should be done using sterile LB medium containing 25% (v/v) glycerol and then stored at –80°C. These glycerol stocks serve as a continuous source of plasmid DNA that can be easily isolated by inoculating bacterial culture medium. Some laboratories transform competent *E. coli* each time a plasmid has to be isolated, but this increases the time and effort required for plasmid preparation.
2. RAW cells that have been passaged excessively ($>$ than $\approx$35 passages) should not be used, as the cells may become senescent and behave differently from early passage cells.
3. Trypsinization of RAW cells is not always efficient, as these cells can adhere strongly to the tissue culture flask. It is important not to overtrypsinize the cells by incubating for excessive amounts of time because the cells may become activated. If trypsinizing for 5 min at 37°C does not cause detachment of the vast majority of cells, the adherent cells can be mechanically dislodged by gently scraping the bottom of the dish with a sterile tissue culture scraper.

4. When scraping or pipetting to disperse any clumped cells after trypsinization it is important not to handle them too roughly. RAW cells can become activated by mechanical stimulation. It is almost impossible to avoid some activation, but this can be kept to a minimum.
5. While the Roche protocol for use of Fugene HD recommends transfecting cells that have grown to 80% confluence, cells grown to 50% confluence are better suited for phagocytic assays. Overcrowding of RAW cells makes imaging single cells difficult, can cause rounding of the macrophages and can alter their ability to perform phagocytosis.
6. When using polystyrene beads as an opsonin, it is important to use beads containing DVB. In our hands opsonization of polystyrene beads without the DVB additive is not as efficient. As an alternative to inert beads, red blood cells (i.e. sheep red blood cells) can be used and opsonized with anti-red cell specific antibodies.
7. To prevent untimely phagocytic events the cells are chilled to 4°C. This temperature halts phagocytosis until the cells are warmed again to temperatures above ~16°C.
8. HEPES-buffered medium is suitable for incubations where the RAW cells are not kept under CO_2. In the absence of HEPES buffering and CO_2, the pH of the tissue culture medium containing bicarbonate will change progressively, affecting cellular processes that are pH-dependent.
9. There are several advantages to using a spinning-disk confocal microscope over a laser scanning confocal microscope (LSCM) for live cell imaging. First, because the laser beam passes through pinholes in the spinning Nipkow disk the entire specimen is excited simultaneously, making extremely rapid image acquisition possible. Additionally, the low-intensity beams used by the Nipkow disk reduce phototoxicity and photobleaching, favouring time-lapse imaging of live cells. One disadvantage of the spinning disk confocal system is that it is not appropriate for experiments where photoactivation or photobleaching are required.
10. For time-lapse imaging of synchronized phagocytosis we typically capture one frame every 5–10 s for 5–8 min.
11. The quantitative analysis described above can be performed using several different imaging software programs such as Volocity (Improvision) or Image J (which is available free of charge from the National Institutes of Health at http://rsbweb.nih.gov/ij/).

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