Video Article

"Phagosome Closure Assay" to Visualize Phagosome Formation in Three Dimensions Using Total Internal Reflection Fluorescent Microscopy (TIRFM)

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Abstract

Phagocytosis is a mechanism used by specialized cells to internalize and eliminate microorganisms or cellular debris. It relies on profound rearrangements of the actin cytoskeleton that is the driving force for plasma membrane extension around the particle. In addition, efficient engulfment of large material relies on focal exocytosis of intracellular compartments. This process is highly dynamic and numerous molecular players have been described to have a role during phagocytic cup formation. The precise regulation in time and space of all of these molecules, however, remains elusive. In addition, the last step of phagosome closure has been very difficult to observe because inhibition by RNA interference or dominant negative mutants often results in stalled phagocytic cup formation.

We have set up a dedicated experimental approach using total internal reflection fluorescence microscopy (TIRFM) combined with epifluorescence to monitor step by step the extension of pseudopods and their tips in a phagosome growing around a particle loosely bound to a coverslip. This method allows us to observe, with high resolution the very tips of the pseudopods and their fusion during closure of the phagosome in living cells for two different fluorescently tagged proteins at the same time.

Video Link

The video component of this article can be found at http://www.jove.com/video/54470/

Introduction

Phagocytosis is a major cell function that starts with the recognition and binding of material to surface receptors, which then leads to the internalization and degradation of the ingested material. While single-celled eukaryotes such as the mold *Dictyostelium discoideum* and amoebae use phagocytosis for feeding on bacteria, higher organisms have evolved with professional cells. Macrophages or dendritic cells are the first line of defense against pathogens in various tissues and organs, and are crucial to activate the adaptive immune system through antigen presentation and cytokine production [1-4]. Under certain circumstances phagocytosis can be performed by non-professional phagocytic cells, *e.g.,* endothelial and epithelial cells. This process is important to maintain homeostasis during development and in adulthood for normal tissue turnover and remodeling. Finally, specialized phagocytes such as Sertoli cells in the testis or retinal pigment epithelial cells are extremely potent phagocytes [5].

The formation of a phagosome where degradation of microorganisms or cellular debris occurs starts with the clustering of phagocytic receptors on the surface of the phagocytic cell. Downstream signaling events following clustering of opsonic receptors such as Fc receptors (FcR) or complement receptors (CRs) have been well characterized. However, there are also numerous non-opsonic receptors including Toll-like receptors (TLRs), lectins, mannose receptors and scavenger receptors. These receptors recognize determinants on the particle surface such as mannose or fucose residues, phosphatidylserine, and lipopolysaccharides [1,6-9].

Pathogen or cell debris recognition involves binding to and clustering of several types of phagocytic receptor, which then lead to intense and transient actin remodeling. In parallel, focal exocytosis of intracellular compartments contributes to the release of membrane tension and is important for efficient phagocytosis of large particles. The signaling events leading to actin polymerization and membrane deformation were dissected in experimental models that triggered a single phagocytic receptor. During FcR-mediated phagocytosis, there is intense actin polymerization that is regulated by small GTPases (Rac, Cdc42). Among their downstream effectors, the Wiskott-Aldrich syndrome protein (WASP) leads to activation of the Actin-Related Protein 2/3 complex (Arp2/3) that nucleates actin filaments [1,2,4,10]. Local production of phosphatidylinositol-4, 5-bisphosphate ($PI(4,5)P_2$) is crucial for initial actin polymerization that drives pseudopod formation. Its conversion to $PI(3,4,5)P_3$ is required for pseudopod extension and phagosome closure [11]. Several pathways contribute to the disappearance of $PI(4,5)P_2$. Firstly detachment of phosphatidylinositol phosphate kinases (PIPKIs) from the phagosome arrests $PI(4,5)P_2$ synthesis. Secondly, it can be phosphorylated and consumed by class I PI3K kinases (PI3K) and converted in $PI(3,4,5)P_3$ [12]. A role for phosphatases and phospholipases has also been implied in $PI(4,5)P_2$ hydrolysis and F-actin removal during phagocytosis in mammalian cells and in *Dictyostelium*[13,14]. The phospholipase C (PLC)δ hydrolyzes $PI(4,5)P_2$ into diacylglycerol and inositol-1,4,5-*tris*phosphate. The $PI(4,5)P_2$ and $PI(3,4,5)P_3$ phosphatase OCRL (oculocerebrorenal syndrome of Lowe) has also been implicated in phagosome formation. Precise local formation of F-actin and its

depolymerization is tightly regulated in space and time and we have shown that recruitment of intracellular compartments is important to deliver locally the OCRL phosphatase, thus contributing to local actin depolymerization at the base of the phagocytic cup [13,15]. For this, we used the experimental set up described here.

The mechanism and molecular players required for phagosome closure and membrane scission remain poorly defined because of the difficulties in visualizing and monitoring the site of phagosome closure. Until recently, phagocytosis was observed on fixed or living cells that internalize particles on their dorsal face or on their sides, making the timely visualization of the site of phagosome closure difficult. In addition, fixing methods could cause retraction of membranes and bias the results on pseudopodia extension and closure. By contrast, the assay that we have set up and describe here allows us to visualize pseudopod extension and the closure step of phagocytosis in living cells [13], based on total internal reflection microscopy (TIRFM) [16]. This optical technique uses an evanescent wave to excite fluorophores in a thin area at the interface between a transparent solid (coverslip) and a liquid (cell culture medium). The thickness of the excitation depth is around 100 nm from the solid surface, allowing visualization of molecular events close to the plasma membrane. TIRFM allows a high signal-to-background ratio, and limits the out-of-focus fluorescence collected and the cytotoxicity due to illumination of cells.

Taking advantage of TIRFM, we developed the "phagosome closure assay" in which coverslips are activated with poly-lysine and then coated with IgG-opsonized red blood cells (IgG-SRBCs). Macrophages expressing transiently fluorescently tagged proteins of interest are then allowed to engulf the IgG-SRBCs. While cells detach the target particles that are non-covalently bound to the glass surface, the tips of the pseudopods can be observed and recorded in the TIRF mode. TIRF acquisitions are combined with acquisitions in the epifluorescence mode, after shifting the stage 3 µm above, which allows the visualization of the base of the phagocytic cup. Addition of pharmacological drugs such as those inhibiting actin or dynamin during the process is also possible to further dissect the process at the molecular level.

The protocol described in detail here is for a RAW264.7 murine macrophage cell line and opsonized particles, but virtually, it can be adapted to any other phagocytic cell and with other targets such as beads. This method will allow better characterization of the regulation in time and space of the molecular players involved in pseudopod extension and phagosome closure during various phagocytic processes.

Protocol

Note: The plasmid used Lifeact-mCherry is a kind gift of Dr. Guillaume Montagnac, Institut Curie, Paris, generated after [17].

1. Cells and Transfection

Note: RAW264.7 macrophages are grown to sub confluency in complete medium (RPMI (Roswell Park Memorial Institute) 1640 medium, 10 mM HEPES, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 2 mM L-Glutamine and 10% FCS (Fetal Calf Serum)) in a 100 mm plate. They are transfected with plasmids encoding fluorescently tagged proteins by electroporation. Routinely approximately 5 - 6 x 10^6 cells are transfected with 20 µg or 10 µg of plasmid for each transfection or co-transfection respectively. Note that other means of transfection based on electroporation or lipofection can be used as alternative approaches.

1. Scrape the cells with a cell lifter and resuspend them in 10 ml of culture medium by pipetting up and down several times.
2. Centrifuge the cell suspension 300 x g, 5 min at RT in swinging angle rotor.
3. Preheat 10 ml of complete medium supplemented with 10 µg/ml of gentamicin at 37 °C.
4. Following centrifugation, discard the supernatant and resuspend the pellet in 3 ml of "washing buffer A" from the electroporation kit.
5. Centrifuge the cell suspension 300 x g, 5 min at room temperature in swinging angle rotor.
6. In the meantime prepare the DNA mix at room temperature: 120 µl 2x Buffer B, 20 µg of plasmid DNA coding for the protein of interest, *q.s.p.* 240 µl H_2O.
7. Following centrifugation, discard the entire supernatant.
8. Resuspend the cell pellet in the DNA mix and transfer into 4 mm electroporation cuvettes.
9. Incubate at RT for 3 min.
10. Electroporate at 250 V, 900 µF.
11. Immediately resuspend the cells in the pre-warmed (37 °C) complete medium supplemented with gentamicin and plate them in a 100 mm dish.
12. Incubate the transfected cells overnight at 37 °C, 5% CO_2.
13. The next morning, replace the complete medium with 10 ml of serum-free microscopy medium (RPMI 1640 without phenol red, 10 mM HEPES, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 2 mM L-Glutamine).

2. Opsonization of Red Blood Cells

Note: As a model of particle target for macrophages, sheep red blood cells (SRBCs) are used. Usually, around 7 x 10^6 SRBCs per 35 mm glass bottom dish is used.

1. Wash the SRBCs with 100 µl of 1x PBS (phosphate buffered saline)/0.1% BSA (bovine serum albumin) and centrifuge (600 x g, 4 min).
2. Following centrifugation, discard the supernatant and resuspend the SRBCs in 100 µl of 1x PBS/0.1% BSA and centrifuge (600 x g, 4 min).
3. Following centrifugation, discard the supernatant and resuspend the SRBCs in 1x PBS/0.1% BSA with rabbit IgG anti-SRBCs at sub-agglutinating concentration. Use 500 µl of solution containing antibody/5 µl of SRBCs.
   Note: The sub-agglutinating concentration of antibodies corresponds to the lowest concentration that did not induce agglutination detected as formation of a network. In general, the sub-agglutinating concentration is 8.2 µg/ml.
   1. Determine the concentration of IgG anti-SRBCs required to opsonize the SRBCs by a hemagglutination test. Serially dilute the IgG anti-SRBCs (stock at 13.1 mg/ml) between 1/50 - 1/25,600 in a microplate. Add 2 x 10^6 SRBCs in each well. Incubate the plate in the dark at RT for several hr.

4. Incubate at RT for 30 mins with slow rotation.
5. After two washes in 1x PBS/0.1% BSA as described above, resuspend the IgG-opsonized SRBCs (IgG-SRBCs) in pre-warmed serum-free microscopy medium (2 ml/dish).

3. Poly-lysine Coating of Coverslips

1. In the meantime, treat 35 mm glass bottom dishes with 2 ml of 0.01% poly-L-lysine for 30 min at room temperature.
2. Wash the dishes two times with 2 ml of 1x PBS.

4. Non-covalent Fixation of SRBCs on Glass Bottom Dishes

1. Pour 2 ml of SRBCs suspension per 35 mm glass bottom dish.
2. Centrifuge with a swinging rotor at 500 x g during 2 min onto 35 mm glass bottom dishes.
3. Remove the supernatant and wash once with 2 ml of 1x PBS/10% BSA.
4. Incubate the particles for 30 min with 2 ml of 1x PBS/10% BSA per dish.
5. Wash the dishes three times with 2 ml of 1x PBS.
6. Replace 1x PBS with 2 ml of pre-warmed (37 °C) serum-free microscopy medium.

5. Phagocytosis Visualized by TIRFM

1. **Microscope**
   Note: TIRFM was performed using a microscope equipped with an oil-immersion objective (N 100X, NA1.49), a heating chamber with CO_2, and two single photon detection cameras EMCCD (Electron Multiplying Charge Coupled Device) coupled with a 1.5X lens.
   1. The day before the TIRF microscope session, turn on the heating chamber at 37 ºC to allow a homogeneous heating of the microscope stage.

2. **Critical Angle Determination**
   Note: ImageJ Color Profiler software is used to process TIRF image streams.
   1. Place a 35 mm glass bottom dish containing opsonized SRBC with serum-free microscopy medium under the microscope.
   2. Scrape the cells and resuspend them in the medium before adding them (100 - 500 µl) in the dish.
   3. Use the "Live Acquisition" software to control the microscope and perform excitation with a 491 nm and/or a 561 nm laser to identify cells expressing fluorescently tagged proteins.
   4. Identify a cell that expresses the fluorescently tagged proteins.
   5. Place it in the middle of the field and acquire 500 images at one excitation wavelength, with different angles starting from 0º up to 5º, with an increment of 0.01º (**Figure 2A**). The angles are automatically changed by the microscope system.
   6. Determine the critical angle allowing the incident light to be totally reflected at the glass/ medium interface and generate the evanescent wave. Using ImageJ Color Profiler software, open the image sequence by clicking on "File", "Open" and select the file.
   7. Select a region of interest (ROI), with the rectangular tool, in the cell with a uniform fluorescence (**Figure 2B yellow 1**).
   8. Plot the "Z axis profile" mean fluorescence intensity measured in the ROI with function of the angles on the x axis by clicking on "Image" tab, "Stacks" in the drop down menu and "Plot Z-axis Profile" (**Figure 2B red 2**).
      Note: The critical angle is the angle leading to the maximum fluorescence before a sharp decrease in fluorescence.
   9. Use any value of angle on the x-axis superior to the critical angle during the microscopy session to obtain a TIRF signal as example 2.00 (**Figure 2C**).

3. **Acquisitions**
   1. Using Live Acquisition software, set up the parameters of acquisition with the module “Protocol Editor” (**Figure 3A**).
      1. Create a protocol with a “loop” comprising “Multi Channels” acquisition to acquire fluorescent signal from proteins of interest in the TIRF region. Enter the TIRF angle (for example 2.00), the exposure time (for example 50 msec) and the laser intensity (for example 50%) **(Figure 3B 1**).
      2. Introduce a “Z move” of the objective 3 µm above the TIRF region to obtain signal acquisition in epifluorescence with the “Multi Channels “tool. Enter an angle below the critical angle (as example 1.00), the time of exposition (50 msec) and the laser intensity (50%) (**Figure 3B, 2 and 3**).
      3. Next add a "z move" of the objective 3 µm below, to return in the TIRF region (**Figure 3B 4**). Add a "snapshot" of the cell in bright light LED (Light-Emitting Diode) (**Figure 3B 5**).

   2. Find a cell of interest with a moderate level of fluorescently tagged protein expression that initiates phagocytosis of SRBC by extending plasma membrane around the particle.
   3. Place it in the middle of the field.
   4. Start streaming acquisition of 500 - 1,000 frames. In the “loop count” tab, enter “750 frames” (**Figure 3B 1**).
      Note: ImageJ Color Profiler software is used to process TIRF image streams.
   5. Open the sequence images in Image J software by clicking “File”, “Open” and choosing the file.
   6. Separate the channels by clicking on the tab “Image “, “Hyperstacks” drop down menu and on “Stack to hyperstack” function. Complete the appeared window: Order: xyctz; Channel (c): number of channels (ex: “2” if you have two fluorochromes); Slices (z): number of slices in z axis (ex: “1” if there is no movement in the z- axis); Frames (t): number of images divided per number of channels; Display mode: Grayscale.
      Note: Two separate image sequences are generated, corresponding to the two different channels.

Representative Results

The experimental system described in this manuscript is schematically represented in **Figure 1**. Transfected RAW264.7 macrophages expressing the proteins of interest fused to a fluorescent tag are placed into contact with IgG-opsonized sheep red blood cells (SRBCs) that were non-covalently fixed on the coverslip. The macrophages can detach the SRBC from the coverslip to engulf it. The TIRF microscope used allows concomitant acquisition of signals from the TIRF area corresponding to the tips of the pseudopods and signals in the epifluorescence mode after a Z shift 3 µm above.

It is essential to determine the critical angle for total internal reflection fluorescence as described in **Figure 2.** This ensures a clean TIRF signal from a 100 nm region below the plasma membrane. **Figure 3** represents the development of acquisition parameters through a module called "Protocol Editor" included in the Live Acquisition software. This module allows users to create and manage workflows before they are sent to the microscope, which will execute the process. At the end of the acquisition, the user collects a TIFF stream.

Figure 4 shows, a representative live-cell TIRFM movie of a "phagosome closure assay" in RAW264.7 macrophages transfected with the plasmid (*e.g.*, Lifeact-mCherry, a kind gift of Dr. Guillaume Montagnac, Institut Curie, Paris, generated after [17]) to follow actin polymerization. Macrophages transiently expressing plasmid were allowed to engulf IgG-SRBCs bound to the coverslip. As the tips of the pseudopods were apposed to the glass coverslip around one SRBC, an F-actin ring was detected in the TIRF area (top panel) that progressively narrowed until it closed. In parallel, the blurry F-actin signal detected by epifluorescence after shifting the stage 3 µm above (bottom panel) corresponded to depolymerization at the base of the phagocytic cup. After 3 min of acquisition, the SRBC was totally internalized, as confirmed with transmitted light (panel on the right).

Therefore, this method can be used to properly distinguish the molecular events that take place at the very ends of the pseudopods and the molecular events occurring at the base of the phagocytic cup.

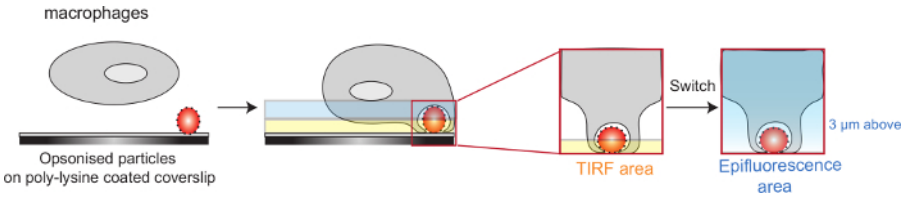

Figure 1. Schematic Representation of the "Phagosome Closure Assay" Analyzed by TIRFM. The phagosome closure assay is performed using macrophages transiently expressing one or two fluorescently tagged-protein. Macrophages are deposited on IgG-opsonized SRBCs non-covalently fixed on poly-lysine coated coverslips. Images are recorded in TIRF mode to detect the site of phagosome closure and in epifluorescence mode to detect the base of the phagocytic cup. Please click here to view a larger version of this figure.

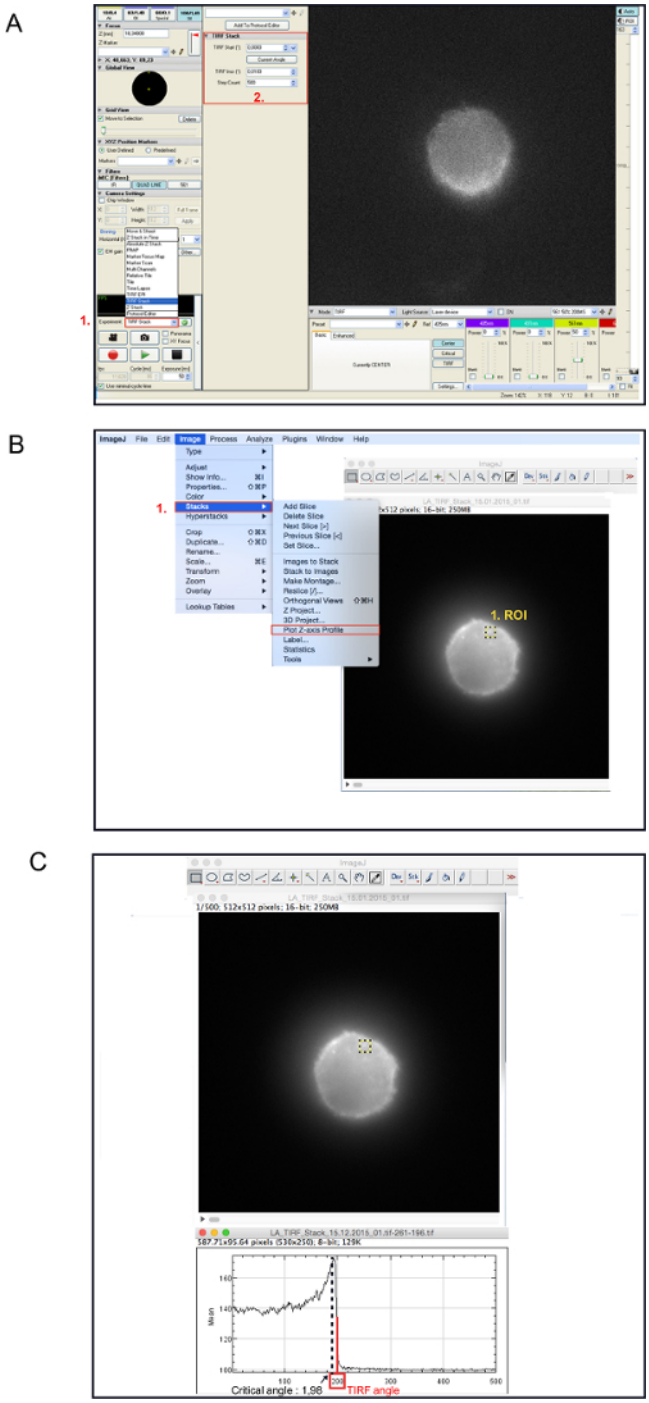

Figure 2: Determination of the Critical Angle for TIRFM. (A) Using "Live acquisition", a cell expressing fluorescently tagged proteins is placed in the middle of the field (region 1 in red). With the TIRF Stack option, images were acquired at one excitation wavelength, with different angles starting from 0° up to 5°, with an increment of 0.01° (region 2 in red). **(B)** The sequence of images is opened using ImageJ Color Profiler software and the mean fluorescence intensity of a region of interest (ROI) is plotted with function of the angles on the x axis using "Stacks" and "PlotZ axis profile" (region 1 in red). **(C)** The x position of the peak of fluorescence on the plot corresponds to the critical angle: 1.98° (black dotted line). Any value after this angle can be used. As an example, 2.00° can be chosen (red line). Please click here to view a larger version of this figure.

Figure 3. Workflow Process using Live Acquisition Module: "Protocol Editor". (A) In the "Protocol Editor" window, a workflow canvas is created. **(B)** This protocol comprises a "Loop" of actions that the microscope will repeat the number of times decided by the user. As an example: 750 (1). One loop included: "Multi Channels" acquisition with laser excitation of fluorescent proteins of interest in TIRF mode. As example: Laser 491 nm intensity 50% -TIRF angle 2.00 (2); "Z move" of the objective 3 µm (3); "Multi Channels" acquisition in epifluorescence mode (4); "Z move" of the objective back to the TIRF region (5) and a "Snapshot" in transmitted light (6). Please click here to view a larger version of this figure.

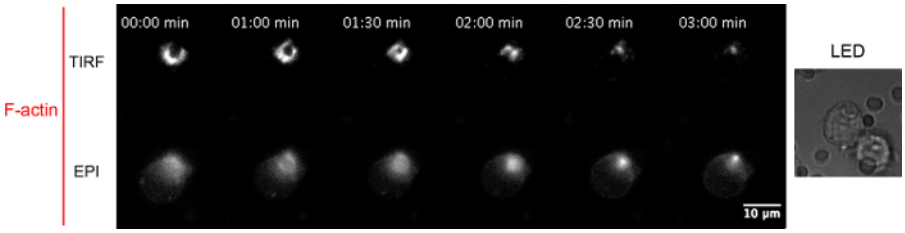

Figure 4. F-actin is Accumulated as a Point at the site of Phagosome Closure. Phagosome closure assay was performed using RAW264.7 macrophages transiently expressing the plasmid Lifeact-mCherry (a kind gift of Dr. Guillaume Montagnac, Institut Curie, Paris, generated after [17]). The plasmid signal was acquired in TIRF area (top) and in epifluorescence mode (bottom). The red arrowhead indicates the actin accumulation in the TIRF region. Transmitted light image at 3 min is presented, indicating an internalized SRBC. Scale bar, 10 µm. Please click here to view a larger version of this figure.

Movie 1: F-actin is Accumulated in the Tips of the Pseudopods and at the Site of Phagosome Closure, while it is Cleared from the Base of the Phagocytic Cup. Phagosome closure assay was performed using RAW264.7 macrophages transiently expressing the plasmid. Plasmid imaging in TIRF area (top) and in epifluorescence mode (bottom) using the TIRFM was performed alternatively every 50 msec during 3 min at 37 °C. Please click here to view this video.

Discussion

The experimental protocol described here proposes an unprecedented method to follow in real time and in living cells, with high-resolution, the formation of a phagosome and in particular its closure. Several technical aspects have to be discussed. Firstly, the assay is very sensitive to temperature. It is very important to check that the heating chamber is at 37 °C and that all media, devices or cells are kept within the chamber to avoid temperature changes that could impair the efficiency of phagocytosis. We noticed that subtle temperature changes were sufficient to prevent phagosome formation. Secondly, the protocol described here allows a precise determination of the TIRF angle that will be used throughout the experiment. We believe that this constitutes a substantial advantage when performing TIRF experiments, allowing us to be confident when concluding about events near the plasma membrane. Thirdly, when working with transiently transfected cells, it is important to choose cells with moderate levels of expression of the proteins of interest. With the revolution of the genome-editing technology, it will be feasible to work with cells expressing proteins at native levels. Thus, quantitative fluorescence microscopy will be possible to explore the dynamics of major functions in living cells.

With this assay, it is now possible to analyze precisely in time and space the different players involved in pseudopod extension and fusion. The forced orientation of the target to engulf allows better monitoring of extending pseudopods and the closure site, compared with phagosome formation imaging using a confocal microscope and a different experimental set up. The only caveat is the low output. Not all cells chosen on the basis of their fluorescence will phagocytose during the acquisition time. A potential improvement would be to have a patterned surface coated with target particles to increase the number of concomitant acquisitions. Using this method, we have dissected the spatio-temporal organization of F-actin dynamics and recruitment of recycling (VAMP3-positive) vesicles during phagosome formation [13]. We observed recruitment of F-actin at the very tips of closing pseudopods, but no VAMP3-positive vesicles in this closing zone of the phagocytic cup. VAMP3 was only detected as a plasmalemmal diffuse signal. In contrast, VAMP3-positive vesicles were detected at the base of the forming phagosome, as visualized in the epifluorescence images. These results showed that F-actin polymerization occurs in the extending pseudopods and in their tips, while there is depolymerization at the base where the vesicular traffic is concentrated. This spatio-temporal dynamic of F-actin polymerization was proposed in seminal work reviewed in [2] and recently also confirmed in [12].

Using the same experimental model, we recently showed that dynamin-2, which mediates the scission of endocytic vesicles, was recruited early and concomitantly with actin during phagosome formation and pseudopod extension. Dynamin-2 then accumulates at the site of phagosome scission. Acute treatment of cells with pharmacological inhibitors clearly showed that dynamin-2 plays a crucial role in phagosome scission [18]. The unique possibility to monitor with high precision the closure step in phagosome formation allowed us to conclude the possible role of dynamin-2 in phagosome scission.

The phagosome closure assay can be in principle adapted to any other phagocytic cell (dendritic cells, neutrophils), provided that they express fluorescently tagged proteins of interest. In addition, the method can be extended to other target particles like beads or bacteria. The size and shape of the different targets could also be varied to get further insight into the mechanism of phagosome formation and in particular the forces involved in phagosome closure. Following the localization of different molecular players in living phagocytosing cells using the phagosome

closure assay described here opens avenues for better definition of the molecular mechanisms and machineries important for phagosome formation and closure.

Disclosures

The authors have nothing to disclose.

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