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In Vitro Phagosome–Endosome Fusion

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Summary

Phagolysosome biogenesis plays a pivotal role in elimination of foreign particles and pathogens by leukocytes. This process is achieved by multiple cycles of membrane fusion between the phagosome and the endosomal system. In vitro reconstitution of phagosome fusion with endosomes is instrumental in studying this intricate process. Such an assay is also invaluable for the identification of effectors produced by intracellular pathogens that may hamper the pathway. In this chapter we describe a highly sensitive and relatively rapid method to measure fusion between phagosomes and early, as well as late, endosomal compartments. The assay is based on the formation of a stable biotin–streptavidin complex upon fusion between biotinylated–peroxidase loaded endosomes and magnetic streptavidin conjugated bead-containing phagosomes. Fusion is quantified using a fluorescence-based detection method that measures the peroxidase activity associated with the beads.

Key Words: Phagosome; endosomes; macrophages; fusion; cell-free system; magnetic beads.

1. Introduction

Phagolysosome biogenesis, also referred as phagosome maturation, is a highly regulated membrane trafficking process essential for killing and degradation of pathogens, as well as for antigen presentation by professional phagocytes ***(1,2)***. Once engulfed by phagocytosis, microorganisms or inert particles reside in a specialized organelle, the phagosome. The biogenesis of the phagolysosome results from a sequential series of fusion events between the phagosome and the compartments of the endocytic pathway. Typically, the newly formed phagosome undergoes maturation by fusing initially with early endosomes, then with the late endosomes, and finally with lysosomes. The

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use of cell-free assay that reconstitutes fusion of phagosomes with endocytic compartments is a very powerful tool to dissect this extremely complex and dynamic process and to uncover new pathogenic effectors interfering with normal biogenesis.

The first biochemical assay that monitored fusion between phagosomes and early endosomes was developed by Stahl and colleagues ***(3)***. This pioneering work found that the system required cytosol, adenosine triphosphate (ATP), and NEM-sensitive fusion protein (NSF) ***(3)***. However, this procedure did not allow the reconstitution of phagosome fusion with later components of the endocytic pathway. A few years later, Jahraus et al. published a new assay and showed the involvement of Rab5 in fusion of latex bead–containing phagosomes with early endosomes ***(4)***. Although this protocol had the advantage to permit measurement of fusion between phagosomes and late endosomes, the time-consuming step of phagosome isolation and reisolation had prevented the utilization of this assay for extensive studies.

The procedure described in this chapter is a modified and optimized version of the one developed by Jahraus et al. ***(4)***, with the yield of a rapid and sensitive biochemical assay in mind. The method is based on the same principle as the one used in the earliest studies ***(3,4)***, which is the content mixing of phagosomes and endosomes and the formation of a stable complex upon fusion **(Fig. 1)**. The endosomes are loaded with biotinylated peroxidase, and the specific loading of early endosomes and late endosomes is achieved by employing different pulse-chase incubation times. Separately, macrophages are allowed to uptake magnetic streptavidin-conjugated beads and to form phagosomes. The introduction of magnetic beads permits rapid and simple isolation of phagosomes and, therefore, significantly reduces the experimental time. It also allows an increase in the number of conditions tested per set of experiments and facilitates washing steps. The endosome and phagosome populations are mixed in the presence of cytosol and ATP. Fusion between the bead-containing phagosome and endosomes results in formation of a stable biotin–streptavidin complex that can be quantified after membrane permeabilization by measuring peroxidase activity associated with the beads. Fusion is directly proportional to the amount of complexes formed and, thereby, to peroxidase activity. High sensitivity is obtained by using a fluorogenic peroxidase substrate. Another advantage of this procedure is that it does not require prelabeling of the beads or the endosome probe.

This assay can be used to directly test the effect of pathogenic factors on different steps of phagolysosome biogenesis and to examine the involvement of host proteins and of signaling lipids in phagosome maturation. In combination with in vivo studies, this assay was key in demonstrating the role of Rab14 in the fusion of phagosomes with early endosomes, as well as examining the

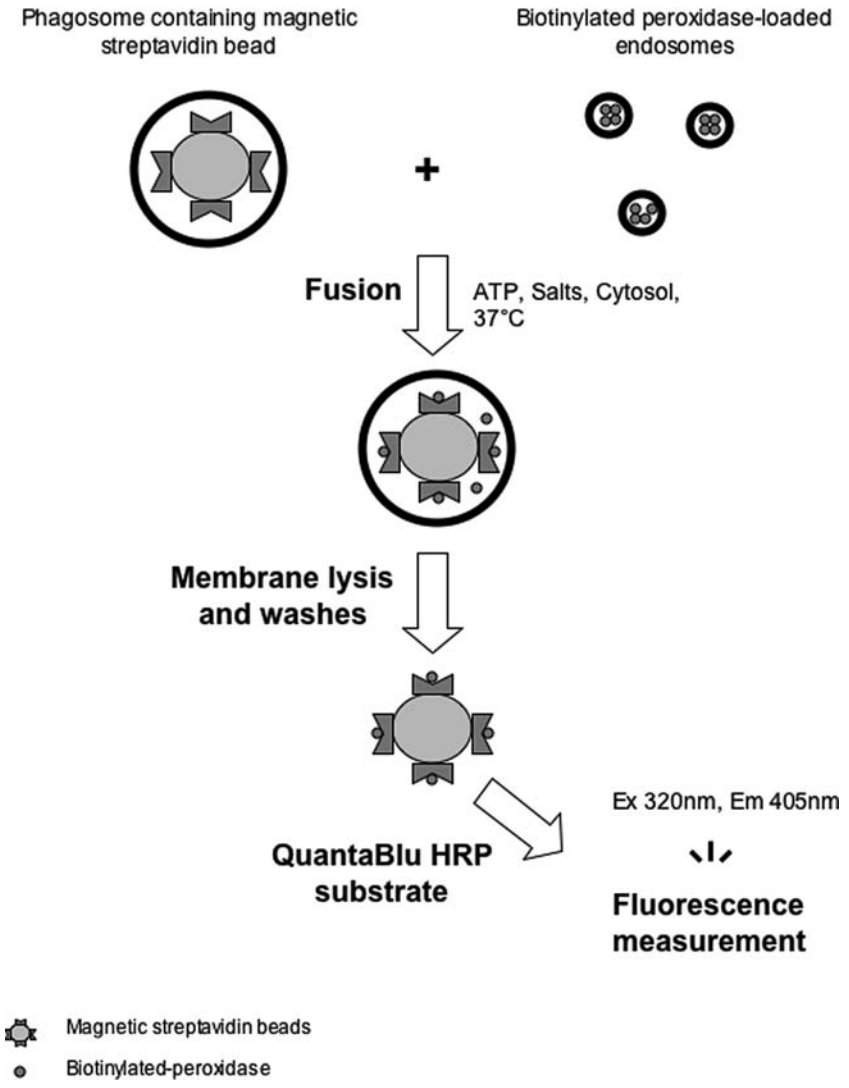

Fig. 1. Outline of the method measuring in vitro fusion between phagosomes and endosomes. Isolated phagosomes containing magnetic streptavidin beads are incubated with endosomes loaded with biotinylated peroxidase in presence of ATP-regenerating system, salts, and cytosol for 1 h at 37°C. Then, organellar membranes are lysed and magnetic beads are washed several times. Peroxidase activity associated with the beads is measured by using a fluorogenic substrate.

action of SapM, an *M. tuberculosis* PI3P phosphatase, and phosphatidylinositol mannoside on phagolysosome biogenesis ***(5–7)***.

2. Materials

2.1. Cell Culture, Transfection

1. Macrophage culture: J774A.1 (ATCC TIB-67) and RAW264.7 (ATCC TIB-71) cells are grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone, Ogden, UT) and 4 m*M* L-glutamine (BioWhittaker).
2. 293T cell medium: DMEM supplemented with 10% FBS, 4 m*M* L-glutamine, penicillin 100 units/mL and streptomycin 100 µg/mL (Invitrogen, Carlsbad, CA).
3. 293T cell transfection: Calcium phosphate transfection kit (#K2780-01, Invitrogen).

2.2. Phagosome, Endosomes, and Cytosol Preparation

1. Streptavidin magnetic beads: BioMag® binding sreptavidin (Polysciences, Warrington, PA).
2. Peroxidase–biotinamidocaproyl conjugate (Biot-HRP, Sigma, St. Louis, MO).
3. Internalization medium: 150 m*M* NaCl, 20 m*M* hydroxyethyl piperazine ethane sulfonate (HEPES) pH 7.4, 6.5 m*M* glucose, 1 mg/mL bovine serum albumin (BSA).
4. BioMag Multi-32 Microcentrifuge Tube Separator (Polysciences).
5. Protease inhibitors. Prepare the following stock solutions: 10 mg/mL N_a-Tosyl-L-lysine chloromethyl ketone (TLCK, Sigma), 1.79 mg/mL E64 (in DMSO, Sigma), 1 m*M* pepstatin (in DMSO, Sigma), 10 mg/mL leupeptin (Sigma).
6. Homogenization buffer. For cytosol: 250 m*M* sucrose, 3 m*M* imidazole pH 7.2 (HB1). For endosomes: 250 m*M* sucrose, 20 m*M* HEPES/KOH pH 7.2, 0.5 m*M* ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*´,*N*´-tetraacetic acid (EGTA) (HB2). For phagosomes: 250 m*M* sucrose, 3 m*M* imidazole pH 7.2, 0.5 m*M* EGTA (HB3). Add to each homogenization buffer 1/1000 of protease inhibitors.
7. Phosphate-buffered saline (PBS) and PBS with 5 mg/mL BSA.
8. Syringe apparatus for homogenization: two 22-gauge needles, two plastic syringes (3 mL), one tubing (5 inches, Pharmed AYX42605). Fit 22-gauge needle to each syringe and connect syringes with 5 inches tubing **(Fig. 2A)**.

2.3. Fusion Reaction

1. Biotinylated-insulin: Insulin-biotinamidocaproyl labeled (Sigma) is dissolved at 5 mg/mL in distilled water, aliquoted, and stored at –20°C.
2. ATP-regenerating system: Prepare MgATP solution (42 mM, adjust to pH 7 with KOH, Sigma), creatine phosphate solution (336 mM, Sigma), and creatine

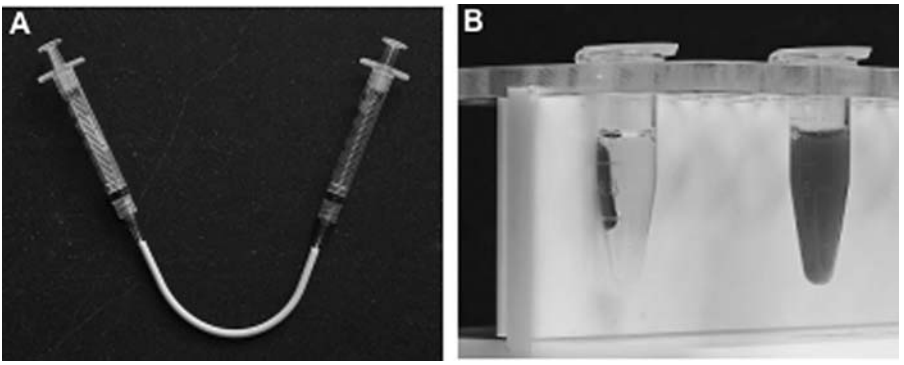

Fig. 2. **(A)** Syringe apparatus for cell homogenization. Two 3-mL syringes fitted with 22-gauge needle are connected with 5 in. tubing. **(B)** Isolation of phagosomes containing magnetic beads. After phagocytosis of magnetic beads, macrophages are homogenized and phagosomes are isolated by placing the cell extract in a multi-32 microcentrifuge tube separator for 15 min. Before separation (right tube); after separation (left tube).

phosphokinase solution in 50% glycerol (1180 units/mL, Sigma). Each solution is aliquoted and stored at –20°C. Just before starting the fusion reaction, prepare ATP-regenerating system by mixing 1/1/1 ATP, creatine phosphate, and phosphocreatine kinase (in that order).

3. ATP-depleting system: Prepare apyrase solution in distilled water (278 units/mL), aliquot and store at –20°C. Prepare glucose solution (278 mM) and store at 4°C. Just before starting the fusion reaction, prepare ATP-depleting system by mixing 1/1 apyrase and glucose solutions.
4. Fusion buffer: 190 m*M* HEPES/KOH pH 7.2, 10 m*M* dithiothreitol (DTT), 15 m*M*$MgCl_2$, 500 m*M* KCl, 3 m*M* EGTA, 750 m*M* sucrose.

2.4. Permeabilization and Washing Buffers

1. Permeabilization buffer: 1% Triton X100, 0.1% (w/v) sodium dodecyl sulfate (SDS), 50 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 1 mg/mL heparin, 100 µg/mL biotinylated insulin.
2. Washing buffer: 1% (v/v) TritonX100, 0.1% (w/v) SDS, 50 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* ethylenediamine tetraacetic acid (EDTA), 0.5% fish skin gelatin (preheated 15 min, 100°C) (Sigma).

2.5. Peroxidase Activity

1. Fluorescent substrate: QuantaBlu fluorogenic peroxidase substrate (Pierce, Rockford, IL).
2. 96-well black plates (Nunc).
3. Fluorescence plate reader (Gemini, Molecular Devices, Sunnyvale, CA).

3. Methods

3.1. 293T Transfection

1. 293T cells are seeded at 1×10^6 per 100-mm tissue culture dish. Prepare 20 dishes.
2. 293T cells are transfected with calcium phosphate tranfection kit, according to the manufacturer's protocol (http://www.invitrogen.com).
3. Transfections are incubated for 48 h.
4. Wash cells with PBS and prepare cytosol as described in **Subheading 3.2.** (resuspend cells in 1 mL of HB1 prior to homogenization) (*see* **Note 1**).

3.2. Cytosol Preparation

1. Grow macrophages in T175 flasks (24 flasks) to 80% confluency (*see* **Note 2**).
2. Wash cells twice with cold PBS.
3. Scrape cells in 12.5 mL cold PBS.
4. Pool cells, wash them once with cold PBS and once with cold HB1.
5. Resuspend cells in 2 mL HB1.
6. Homogenize cells by passing them, 10–20 times, through a syringe apparatus (*see* **Note 3**). The homogenization is carried out until about 90% of cells are broken without major breakage of the nucleus, as monitored by light microscopy.
7. Centrifuge the homogenate at 3000*g* for 20 min at 4°C.
8. Centrifuge the supernatant at 100,000*g* for 1 h at 4°C in 1.5 mL polyallomer microcentrifuge tube (Beckman, Palo Alto, CA).
9. Cytosol is obtained by collecting the supernatant.
10. Make 50-µl aliquots, freeze rapidly in ethanol–dry ice and store at –80°C, up to 1 yr. The protein concentration of the cytosol should be 6 mg/mL minimum.
11. The day of the fusion reaction: thaw aliquots in 37°C water bath, adjust the protein concentration to 6 mg/mL with HB1, and keep them on ice.

3.3. Endosome Preparation

The preparation of endosome-enriched fractions has been adapted from Mayorga et al. ***(3)***.

1. Grow macrophages in T175 flasks (24 flasks) to 80% confluency.
2. Wash cells twice with cold PBS.
3. Scrape cells in 12.5 mL cold PBS.
4. Pool cells, wash them once with cold PBS and once with internalization medium.
5. Cells are resuspended in 3 mL of internalization medium containing 1.7 mg/mL biot-HRP.
6. For loading of early endosomes: incubate cells for 5 min at 37°C, 5% CO_2. Wash cells twice with cold PBS with 5 mg/mL BSA and twice with cold PBS. For loading of late endosomes: incubate cells for 20 min at 37°C, 5% CO_2.

Wash cells twice with cold PBS with 5 mg/mL BSA and once with cold PBS. Resuspend cells with 12.5 mL warm media and incubate for 40 min at 37°C, 5% CO_2. Wash cells once with cold PBS.

7. Cells are washed once with HB2.
8. Cells are resuspended in 7.5 mL of HB2.
9. Homogenize cells by passing them, 5–10 times, through a syringe apparatus (2.5 mL of cell suspension per apparatus).
10. Pool the homogenates.
11. Pellet unbroken cells and nuclei at 200*g* for 6 min. Take the supernatant and repeat this step until no more pellet is observed. The last supernatant is the postnuclear supernatant (PNS). Make aliquots (approximately 400 µL), freeze in liquid nitrogen, and store at –80°C up to 1 yr.
12. The day of the fusion reaction: thaw 3 aliquots of PNS in a 37°C water bath.
13. Pool the aliquots and dilute up to 5.5 mL with HB2.
14. Transfer the suspension to Swti55 ultra-clear centrifuge tube (Beckman, Palo Alto, CA).
15. For early endosomes: centrifuge 1 min, 20,000*g* at 4°C. Then, centrifuge the supernatant 5 min, 23,000*g* at 4°C. Resuspend the pellet with 90 µL of cold HB2 (enough for 15 fusion reactions).
16. For late endosomes: centrifuge 1 min, 16,000*g* at 4°C. Then, centrifuge the supernatant 5 min, 23,000*g* at 4°C. Resuspend the pellet with 90 µL of cold HB2.
17. Keep the endosomes on ice.

3.4. Phagosome Preparation

1. Grow macrophages in T175 flasks (8 flasks) for 2 d to 80% confluency.
2. Remove media, add in each flask 12.5 mL of internalization medium containing 2.3% (v/v) of magnetic beads (37°C), and incubate cells for 30 min at 37°C, 5% CO_2.
3. For late phagosomes only: remove medium, wash three times with warm DMEM, 10% FBS, and incubate cells with DMEM, 10% FBS for 10 min at 37°C, 5% CO_2.
4. Wash cells three times with cold PBS.
5. Scrape cells in 12.5 mL cold PBS.
6. Pellet cells (centrifugation 10 min at 200*g*) and wash once with cold HB3.
7. Resuspend cells with 5 mL cold HB3.
8. Homogenize cells and prepare PNS as described in **Subheading 3.3**.
9. Isolate magnetic bead containing phagosomes using the microcentrifuge tube separator, 15 min at 4°C **(Fig. 2B)**.
10. Remove the supernatant and resuspend beads with 900 µL of cold HB3.
11. Keep the phagosomes on ice.

This amount of phagosomes allows 15 fusion reactions.

3.5. Fusion Reaction

1. Place 15 Eppendorf tubes (five conditions in triplicate) on ice.
2. Add to each Eppendorf tubes the following in order: 50 µL of phagosome suspension, 4.2 µL of biotinylated-insulin (*see* **Note 4**), 50 µL of cytosol, 5 µL of endosome suspension, 15 µL of fusion buffer.
3. Add for one condition 10.8 µL of ATP-depleting system and the other conditions 10.8 µL of ATP-regenerating system.
4. Add 15 µL of compound to be tested (inhibitors, effectors) or buffer used for solubilization (control).
5. The fusion reaction is initiated by placing the eppendorf tubes at 37°C for 1 h on a rocking table.

3.6. Permeabilization and Washes

1. Stop the reaction by placing the tubes on ice.
2. Add 650 µL of HB2 in each tube.
3. Isolate the magnetic beads with separator for 15 min at 4°C.
4. Resuspend the beads in permeabilization buffer (900 µL).
5. Incubate 30 min on ice.
6. Isolate beads.
7. Wash beads two times with washing buffer and three times with PBS.
8. Resuspend beads in 50 µL PBS.

3.7. Peroxidase Activity Associated with Beads

1. Mix 9/1 (v/v) QuantaBlu substrate and QuantaBlu stable peroxide solution, keep on ice.
2. Add 150 µL of QuantaBlu mix to each 50 µL of bead suspension, vortex, and incubate for 30 min at 37°C.
3. Stop reaction by putting tube on ice and by adding 150 µL of Stop solution.
4. Vortex and spin down beads for 5 min at 12,000*g* (microcentrifuge).
5. Transfer 250 µL of supernatant to 96-well plate.
6. Read relative fluorescence unit (RFU) at 405 nm by exciting at 320 nm using a fluorescence plate reader.

3.8. Data Analysis

Fusion efficiency is determined by subtracting RFU of fusion reaction with ATP-depleting system to RFU of reaction with ATP-regenerating system.

4. Notes

1. Because they are highly transfectable, 293T cells are used instead of macrophages to prepare cytosol containing large amount of ectopically expressed protein. To

allow fusion 293T cytosol is mixed with cytosol prepared from macrophages (1/5, v/v).
2. Cytosol is depleted of specific protein by transfecting RAW264.7 cells for 24 h with siRNA (*see* Chapter 26 for protocol). Use scrambled siRNA as a control.
3. To avoid the formation of bubbles during homogenization, the needles and the tubing are preloaded with homogenization buffer.
4. Biotinylated insulin is added to the fusion reaction to prevent formation of complexes that could result from damaged organelles.

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