A Cell-Free Scintillation Proximity Assay for Studies on Lysosome-to-Phagosome Targeting

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Sci. STKE **2007** (392), pl3. [DOI: 10.1126/stke.3922007pl3]

A Cell-Free Scintillation Proximity Assay for Studies on Lysosome-to-Phagosome Targeting

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Introduction

Phagocytosis is a multistep process that involves the attachment of particles to the cell surface, formation of membrane-bound vacuoles called phagosomes, movement of phagosomes through the cytoplasm, and ultimately the merger of phagosomes with endosomes and lysosomes. The final step of phagolysosome formation is essential for phagosomal acidification, killing of microbial pathogens, and the digestion and presentation of antigens to T lymphocytes. Phagocytosis removes effete tissue material such as apoptotic bodies, photoreceptor outer segment fragments, or collagen fibrils. Moreover, as part of the immune system, phagocytosis is essential for the destruction of invading microorganisms. Opsonization of substrate particles with immunoglobulin G (IgG) facilitates phagocytosis by triggering signaling pathways that lead to the generation of microbicidal oxygen species and that facilitate the engulfment process. Moreover, we recently found that IgG-induced signaling promotes the targeting of lysosomes to phagosomes (*1*).

To study interactions between lysosomes and phagosomes biochemically, we developed a scintillation proximity approach that is illustrated in Fig. 1A. The key components of this assay are phagosomes containing scintillant latex beads and lysosomes labeled with [^{3}H]cholesteryl oleyl ether (Fig. 1B). The probability of a tritium-derived beta particle reaching the scintillant decreases exponentially as a function of distance, such that scintillation is generated only when phagosomes and tritium-labeled lysosomes form a close complex (*2*). Complex formation is facilitated in the presence of adenosine triphosphate (ATP), cytosol from macrophages, and cytosol from rat liver (Fig. 2, A and B). When macrophage cytosol is supplied from cells that have been exposed to IgG-coated particles, complex formation between lysosomes and phagosomes is enhanced (Fig. 2, C and D) (*1*).

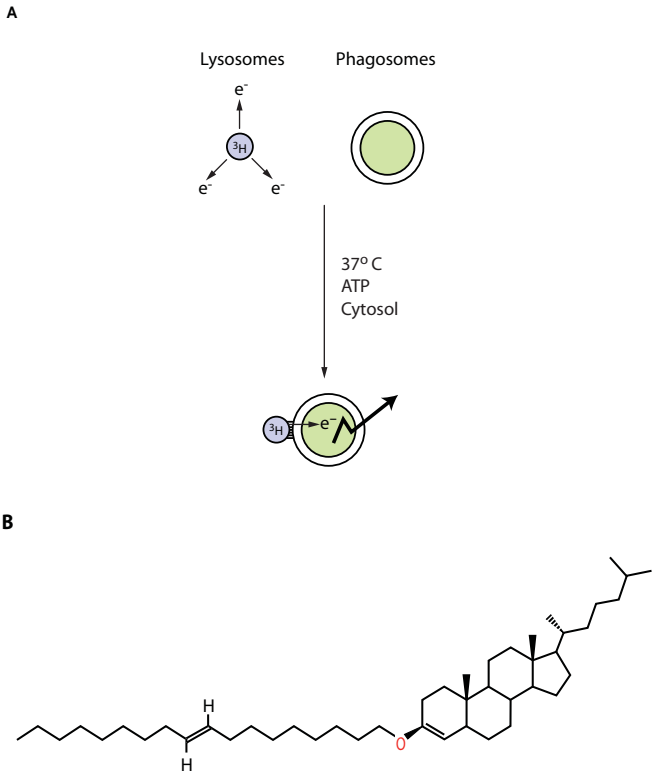

Fig. 1. (**A**) Schematic illustrating the principle of the scintillation proximity assay. (**B**) Structure of cholesteryl oleyl ether (3β[*Z*]-3-[9-octadecenyloxy]-cholest-5-ene), a chemical that accumulates in lysosomes.

Cholesteryl ether is similar to cholesteryl fatty acyl esters that accumulate in lysosomes of patients deficient in acidic cholesteryl ester hydrolase (lysosomal acid lipase) activity (*3*). Because cholesteryl ether is inherently resistant to hydrolysis (*4*), it accumulates specifically in lysosomes (*5*). The specificity of labeling makes it possible to study lysosomes as a component of crude membrane extracts. In addition, cholesteryl ether can be incorporated into anionic liposomes that are avidly engulfed by macrophages (*6*–*8*).

The proximity scintillation assay described below is particularly useful for biochemical studies on the tethering and docking steps of lysosome-to-phagosome targeting and the regulation of this process by IgG. Proximity scintillation assays could in principle be adapted to study any interaction between different vesicle pools, provided that a radiolabeled compound can be selectively incorporated into one vesicle population and scintillant can be incorporated into the other.

Materials

Cell Culture Reagents and Supplies

10-cm cell culture dishes (Greiner Bio-One, #664160)

6-well cell culture dishes (Corning, #3506)

Cocktail of penicillin (10,000 U/ml) and streptomycin sulfate (10,000 μg/ml) (Mediatech Inc., #30-002-CI)

DMEM:F12 medium (a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium; Mediatech Inc., #10-090-CV)

Fetal bovine serum (FBS; Invitrogen, #10438-026)

Mouse J774A.1 cells (ATCC, Manassas, VA; #TIB-67)

Phosphate-buffered saline (PBS; Mediatech Inc., #21-040-CV)

Trypsin EDTA (0.25% trypsin and 2.21 mM EDTA in Hank's buffered salt solution; Mediatech Inc., #25-053-CI)

Antibodies

Human IgG (Sigma, #I4506)

Mouse IgG (Sigma, #I8765)

Microbeads

Polybead® amino microspheres (6-μm monodisperse polystyrene beads with surface-coupled primary amine groups; Polysciences, #19118)

Scintillant polyethyleneimine (PEI)–coated polyvinyltoluene (PVT) beads (average diameter, 5 μm; density, 1.05 g/cm^3; GE Healthcare, #RPNQ0097)

Chemicals

[1α,2α(n)-^{3}H]cholesteryl oleyl ether (1 mCi/ml, GE Healthcare, #TRK888)

25% glutaraldehyde solution (Sigma, #G6257)

ATP (Roche, #519987)

Bovine serum albumin (BSA; Sigma)

Coomassie Plus protein assay reagent (Pierce, #1856210)

Creatine kinase (Roche, #127566)

Creatine phosphate (Roche, #621714)

Dicetylphosphate (Sigma, #D2631)

Dioleylphosphatidylcholine (Avanti Polar Lipids, #850375C)

Dioleylphosphatidylserine (Avanti Polar Lipids, #840035C)

Dithiothreitol (DTT; Sigma)

EDTA (Sigma)

Guanosine triphosphate (GTP; Roche, #414581)

HCl

Hepes, free acid

KCl

KOH pellets

NaOH pellets

Protease inhibitor cocktail [200×; containing 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin hydrochloride, *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), leupeptin hemisulfate, and pepstatin A; Sigma #P8340]

Sucrose

Animals

Male Charles River CD® IGS (Sprague-Dawley–derived) rats (retired breeders, Charles River Laboratories)

Equipment

37°C humidified, 5% CO_2 incubator (Hereaus model Heracell or equivalent)

Baby Dieffenbach clamp (Harvard Apparatus, #523241)

Barnstead/Thermolyne Labquake rotator

BD Insyte Autoguard shielded I.V. catheters (1.1 mm × 25 mm; BD Biosciences, #381433)

Benchtop centrifuge (Sorvall Biofuge Fresco or equivalent)

CO_2 tank with attached general purpose regulator (for killing rats)

Dissecting board pins (Richard-Allan Scientific, #DB3000)

Hoefer SG15 gradient maker (GE Healthcare)

I.V. bag (Braun, #L8000 or equivalent)

Latex tubing (inner diameter 1/16 inch; VWR, #62996-350)

Neoprene cork dissection board (40.6 cm × 30.5 cm × 2.5 cm; Mopec Inc., #BC005)

Nitrogen tank with attached general-purpose pressure regulator (Airgas, #120-2-4F-4F)

Note: The tank should be placed next to a chemical hood certified for tritium use.

Peristaltic pump (Bio-Rad Econo Pump or equivalent)

Polyallomer ultracentrifuge tubes (14 × 89 mm; Beckman, #331372)

Polypropylene storage tank (GE Healthcare model SE 100 plate washer)

Polytron homogenizer

Preparative ultracentrifuge (Beckman Coulter model Optima L-90K or equivalent)

Scintillation counter (Beckman LS 6000 IC or equivalent)

Stainless steel dissecting drain tray (63.5 cm × 45.72 cm × 2.54 cm; Mopec Inc., #BC010)

Sterile hood (Baker Company model Sterilgard III Advance or equivalent)

Syringe needles (22G1 gauge, BD Biosciences, #305155)

Syringes (1 ml, BD Biosciences, #309602)

Tabletop centrifuge (Sorvall Legend RT or equivalent, equipped with swinging bucket rotor and adapters for 15-ml and 50-ml tubes)

Tissue culture microscope (Olympus model CK40 or equivalent)

Ultracentrifuge swinging bucket rotor (Beckman Coulter SW41 Ti)

Recipes

Recipe 1: Buffer A

Reagent	*Volume of Stock Solution*	*Final Concentration*
Hepes-KOH (1 M, pH 7.3)	8 ml	40 mM
KCl (2 M)	10 ml	100 mM
$MgCl_2$ (1 M)	0.6 ml	3 mM
EGTA (200 mM, pH 7.3)	0.5 ml	0.5 mM

Dilute the reagents in 150 ml of ultrapure H_2O (ddH_2O, double-distilled or double-deionized) and adjust the pH to 7.3 with 10 M KOH. Then adjust the final volume to 200 ml with ddH_2O. Before use, aliquot an amount (typically, we use 5 ml, which is sufficient for one gradient) required for a single experiment, add 0.001 volume of 1 M DTT (final concentration, 1 mM) plus 0.005 volumes of 200× protease inhibitor cocktail (final concentration, 1×), and store on ice.

Recipe 2: Buffer B

Add 30 g of sucrose to 100 ml of Buffer A (Recipe 1) to yield 30% (w/v) sucrose. Before use, aliquot an amount required for a single experiment, add 0.001 volume of 1 M DTT plus 0.005 volumes of 200× protease inhibitor cocktail, and store on ice.

Recipe 3: Buffer C

Reagent	*Volume of Stock Solution*	*Final Concentration*
Hepes	47.7 g	400 mM
KCl	37.25 g	1 M
$MgCl_2$	3.045 g	30 mM
EGTA	0.951 g	5 mM

Dissolve the reagents in 400 ml of ddH_2O. Adjust the pH to 7.3 with 10 M KOH and then adjust the final volume to 500 ml.

Recipe 4: Homogenization Buffer

Reagent	*Volume of Stock Solution*	*Final Concentration*
Hepes-KOH (1 M)	5 ml	10 mM
KCl (2 M)	2.5 ml	10 mM
$MgCl_2$ (1 M)	0.75 ml	1.5 mM

Dilute stock solutions in 400 ml of ddH_2O. Adjust pH to 7.3 with 10 M KOH; then adjust the final volume to 500 ml with ddH_2O. Before use, aliquot an amount required for a single experiment, add 0.001 volume of 1 M DTT (final concentration, 1 mM) plus 0.005 volumes of 200× protease inhibitor cocktail (final concentration, 1×), and store on ice.

Recipe 5: Reaction Buffer

Add 8.5 g of sucrose to 100 ml of Buffer A (Recipe 1) to yield a final concentration of 250 mM sucrose. Before use, aliquot an amount required for a single procedure, add 0.001 volume of 1 M DTT plus 0.005 volumes of 200× protease inhibitor cocktail, and store on ice.

Recipe 6: ATP Regenerating System (8×)

Reagent	*Volume of Stock Solution*	*Final Concentration*
ATP (sodium salt)	654.4 mg	8 mM
GTP (sodium salt)	202.8 mg	2 mM
Creatine phosphate	52.3 mg	40 mM
Creatine kinase	1.0 mg	20 μg/ml

Dissolve reagents in 50 ml of Reaction Buffer (Recipe 5). Verify that the pH is 7.3 and, if necessary, adjust with 1 N NaOH. (Aliquots are snap-frozen on liquid nitrogen and stored at –80°C until use.)

Recipe 7: Growth Medium A

Working in a sterile hood, add 5 ml of 100× antibiotic cocktail [containing penicillin (10,000 U/ml) and streptomycin (10 mg/ml)] to 500 ml of DMEM:F12.

Recipe 8: Growth Medium B

Add FBS to Growth Medium A (Recipe 7) to yield 10% (v/v) FBS. Sterilize by passing through a 0.22-μm filter and store at 4°C for up to 1 month.

Recipe 9: 5% BSA Solution

Dissolve 0.5 g of BSA in 10 ml of ddH_2O, then sterilize by passing the solution through a 0.22-μm filter under a cell culture hood. Store at 4°C.

Recipe 10: Growth Medium C

Add 4 ml of 5% BSA Solution (Recipe 9) to 96 ml of Growth Medium A (Recipe 7) to yield 0.2% (w/v) BSA. Prepare fresh for each experiment.

Recipe 11: Phospholipid Stock Solutions

Dioleylphosphatidylcholine Stock

Dissolve dioleylphosphatidylcholine (20 mg/ml) in a mixture of 95% chloroform and 5% methanol and store at –20°C.

Dioleylphosphatidylserine Stock

Dissolve dioleylphosphatidylserine (10 mg/ml) in a mixture of 95% chloroform and 5% methanol and store at –20°C.

Dicetyl Phosphate Stock

Dissolve dicetyl phosphate (8 mM) in a mixture of 95% chloroform and 5% methanol and store at –20°C.

Recipe 12: Liposome Buffer

Reagent	*Volume of Stock Solution*	*Final Concentration*
PBS (10×)	1 ml	1×
Glucose (1 M)	0.3 ml	0.3 M
ddH_2O	8.7 ml	

Prepare fresh for each preparation.

Recipe 13: Liposomes

Reagent	*Volume of Stock Solution*	*Final Concentration*
Dioleylphosphatidylcholine (20 mg/ml, Recipe 11)	37.5 μl	1 mM
Dioleylphosphatidylserine (10 mg/ml, Recipe 11)	80 μl	1 mM
Dicetyl phosphate (8 mM, Recipe 11)	25 μl	0.2 mM
[^{3}H]cholesteryl oleyl ether (1 mCi/ml)	250 μl	250 μCi/ml

Combine all reagents in a 1.5-ml microcentrifuge tube and then dry the solvent under a stream of nitrogen. The lipids will form a thin but visible film on the wall of the tube. Add 1 ml Liposome Buffer (Recipe 12). Vortex for 2 min and sonicate for 2 min; repeat four times. Store at 4°C. Repeat vortex and sonication steps (2 min each) once before each use.

Note: The liposomes are radioactive, and proper protection must be used. Any plasticware that comes into contact with the liposomes must be discarded in accordance with institutional policies regarding radioactive waste.

Recipe 14: 1 M Glycine Solution

Dissolve 75.1 g of glycine in 1 liter of ddH_2O.

Instructions

Procedure 1: Coating Amine Beads with Antibodies

This process couples amine beads (Polybead® amino microspheres) to IgG or BSA. Confirm that the IgG-coated beads are effectively coupled by testing the supernatant for protein before and after cross-linking. These beads may be stored at 4°C for up to 6 weeks and are used for the generation of conditioned low-salt cytosol (Procedure 7) (Fig. 2, C and D).

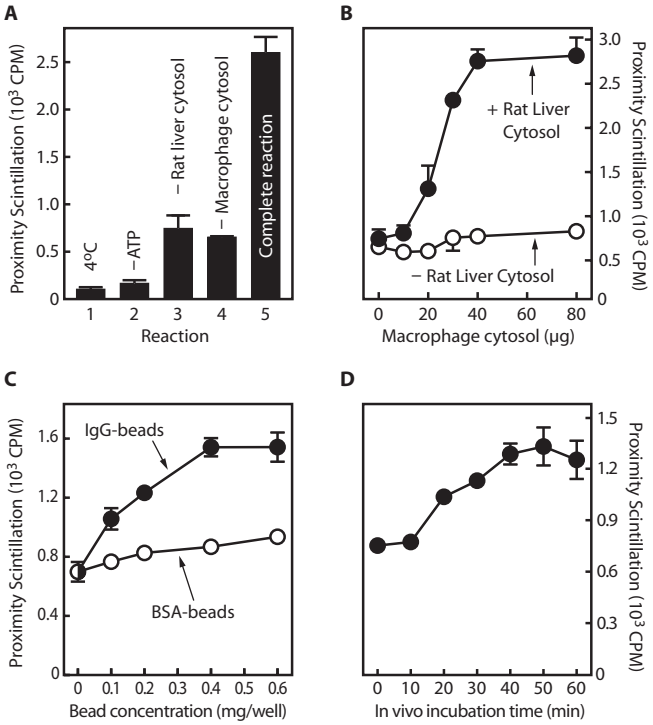

Fig. 2. Measurements of lysosome-to-phagosome targeting in vitro. Lysosome-to-phagosome targeting was measured with the cell-free scintillation proximity assay. (**A**) Complete reactions contained [^{3}H]cholesteryl ether–labeled lysosomes, J774 cell phagosomes containing scintillant latex beads with or without cytosol from J774 cells, rat liver, and an ATP-regenerating system. After 1 hour of incubation, proximity scintillation was measured and is expressed in counts per minute (CPM). (**B**) Reactions were as in (A) except that macrophage cytosol concentrations were varied in the absence (open circles) and presence (solid circles) of rat liver cytosol (6 mg/ml). (**C**) Reactions were as in (A) except that low-salt cytosol was from J774 macrophages exposed to the indicated concentrations of 6-μm BSA-coated beads (open circles) or IgG-coated beads (solid circles) in medium C for 1 hour. (**D**) Low-salt cytosol was from J774 cells exposed to 6-μm IgG-coated beads (0.2 mg/ml) in medium C for the indicated time. Background scintillation counts in reactions performed without macrophage cytosol were 446 CPM (C) and 195 ± 41 CPM (D). Error bars are SD (n = 3). [Taken from figures 2 and 3 in (*1*)]

1. Pipette 100 μl of amine beads (25 mg/ml) into 15-ml centrifuge tubes.
2. Pellet the beads in a tabletop centrifuge by spinning for 15 min at 4000 RPM (3600*g*).
3. Remove the supernatant and resuspend the beads in 10 ml of PBS.
4. Wash the beads three times with 10 ml of PBS; centrifuge for 15 min at 3600*g* after each wash.
5. After the final wash, resuspend the beads in 6 ml of PBS and add, drop by drop, 4 ml of 25% glutaraldehyde (final concentration, 10%) while swirling the solution.
6. Incubate on a rotator for 2 hours at room temperature.
7. Wash the beads three times with 10 ml of PBS; centrifuge for 15 min at 3600*g* after each wash.
8. After the final wash step, resuspend the beads in 10 ml of PBS and add 10 μl of IgG (10 mg/ml) or BSA (10 mg/ml).
9. Incubate at room temperature on a rotator overnight.
10. Centrifuge for 15 min at 3600*g* and determine the protein concentration in the supernatant to calculate the percent cross-linking of the antibodies to the beads.

    Note: 80% of the protein is typically cross-linked.
11. Wash the beads four times with 10 ml of PBS, pelleting by centrifugation for 15 min at 4000 RPM after each wash.
12. After the final wash step, remove the supernatant, resuspend the pellet in 1 ml of PBS, and add 50 μl of 1 M glycine (Recipe 14) to quench remaining cross-linkers.
13. Wash the beads once more with 1 ml of PBS, resuspend in 1 ml of sterile PBS, and store the suspension at 4°C. Vortex before use.

Procedure 2: Preparation of Rat Liver Cytosol

For each preparation, we routinely process four male rats. The animals are euthanized by CO_2 inhalation according to institutional guidelines. To minimize quenching of the scintillation signal by rat liver cytosol (which is colored), livers should be thoroughly perfused and then rinsed. Once the tissue is excised, work quickly and keep the material at 4°C to prevent degradation.

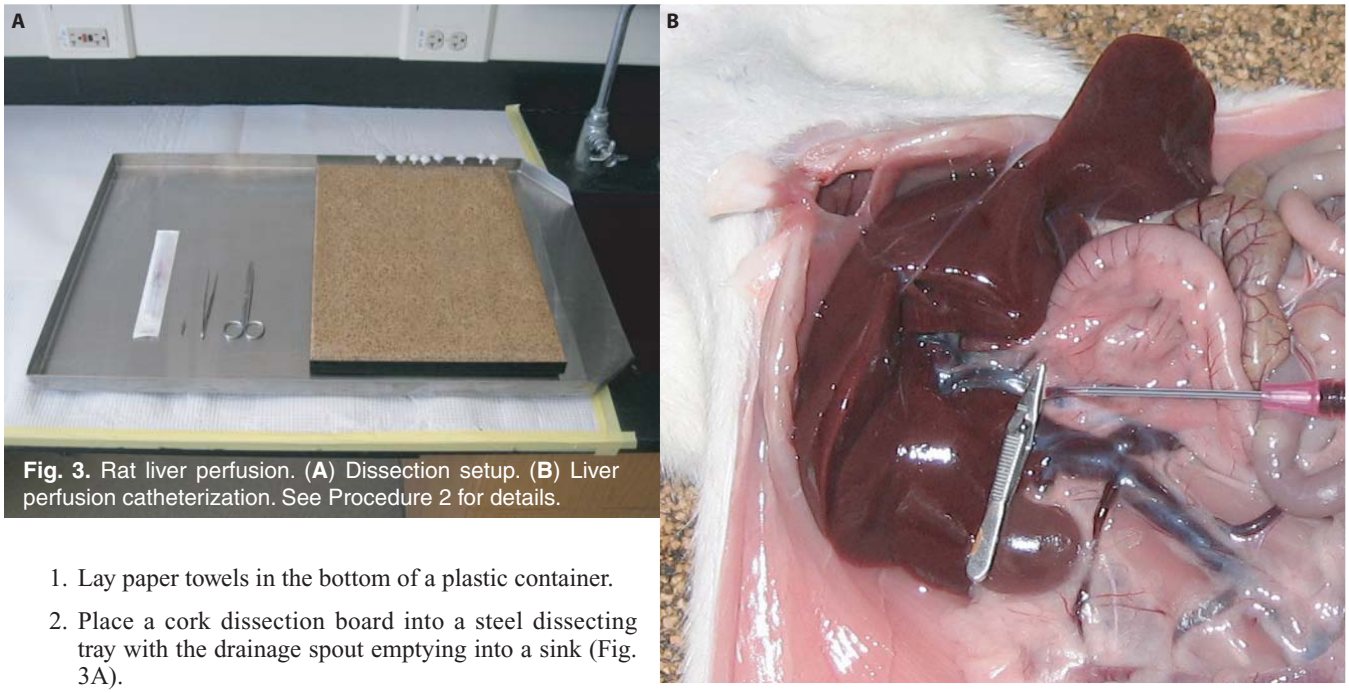

Fig. 3. Rat liver perfusion. (**A**) Dissection setup. (**B**) Liver perfusion catheterization. See Procedure 2 for details.

1. Lay paper towels in the bottom of a plastic container.
2. Place a cork dissection board into a steel dissecting tray with the drainage spout emptying into a sink (Fig. 3A).
3. Hang an I.V. bag with 1 liter of 0.9 M NaCl ~50 cm above the work area and attach to 1/16-inch latex tubing with terminal Luer-Lock adaptor.
4. Place dissection board pins, blunt operating scissors, a baby Dieffenbach clamp, I.V. catheters, and a squirt bottle with 70% ethanol near the work area.
5. For each liver, prepare one 50-ml tube containing 15 ml of Reaction Buffer (Recipe 5) on ice.
6. Euthanize a rat by CO_2 inhalation in the plastic container.
7. Affix the animal to the dissection board, ventral side up.
8. Sterilize the abdomen with 70% (v/v) ethanol and open the abdominal cavity with blunt scissors.
9. Insert a shielded I.V. catheter into the portal vein, remove the needle from the catheter, and fix the catheter in place with a baby Dieffenbach clamp (Fig. 3B).
10. Connect the 0.9 M NaCl solution to the catheter with latex tubing and a Luer-Lock adaptor.
11. Start perfusion by cutting the vena cava and continue until the perfusate becomes clear and the color of the liver has turned gray (~10 min).
12. Excise the liver and cut it into small pieces (~0.5 cm in diameter).
13. Briefly rinse the tissue pieces three times in 10 to 20 ml of PBS and then transfer the entire liver from one rat into a 50-ml tube containing 15 ml of ice-cold Reaction Buffer (Recipe 5).
14. Homogenize the tissue in a Polytron homogenizer for ~10 s at room temperature at maximum speed and then place the tube on ice.
15. Repeat the entire procedure for each rat.
16. Centrifuge the homogenates for 30 min at 3600*g* in a tabletop centrifuge at 4°C.
17. Using a gel-loading tip attached to a vacuum trap, aspirate the foamy top layer.
18. Pool the remaining supernatant, leaving behind the viscous bottom fraction, and transfer the supernatant into six 14 × 89 mm centrifuge tubes.

19. Centrifuge the supernatant for 90 min at 40,000 RPM (~198,000*g*) at 4°C in a SW40Ti rotor.

    Note: A larger rotor may be used, if available.

20. If necessary, carefully aspirate the whitish top layer.

21. Transfer the remaining supernatant to fresh 14 × 89 mm centrifuge tubes, and centrifuge for 4 to 6 hours at 198,000*g* at 4°C.

22. If necessary, carefully aspirate the whitish top layer.

23. Remove the remaining supernatant with a 10-ml pipette and transfer it to a 50-ml tube on ice.

    Note: If several rats were processed, the supernatants may be pooled at this point.

24. Dilute 5 µl of the cytosol in 1 ml of Reaction Buffer (Recipe 5) and determine the protein concentration.

25. Aliquot 0.5 to 1 ml of cytosol into 1.5-ml microcentrifuge tubes on ice, snap-freeze on liquid nitrogen, and store at –80°C.

26. Before experiments, place tubes in a 37°C water bath just long enough for the cytosol to thaw, vortexing every 15 to 30 s. Then keep the tube on ice until use.

Procedure 3: Preparation of Scintillant Phagosomes

Phagosomes are formed by culturing macrophages in the presence of scintillant PEI-PVT beads. The cells are then harvested and the phagosomes are separated from other organelles by ultracentrifugation through a 30% to 0% sucrose gradient. The phagosomes containing the scintillant beads collect in a discrete white band in the gradient (Fig. 4A). No homogenization step is necessary before centrifugation (Fig. 4B). Phagosomes obtained from one 10-cm dish of J774 cells provide enough material for about 24 in vitro reactions.

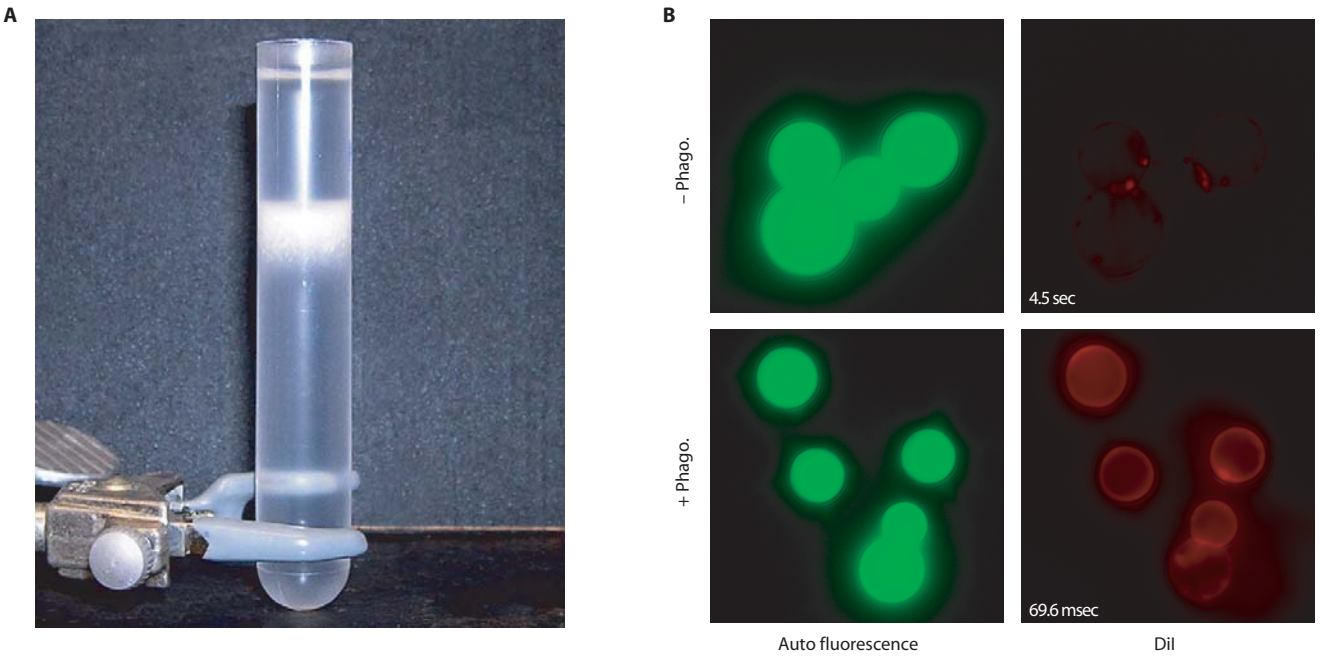

Fig. 4. Isolation and characterization of scintillant bead–containing phagosomes. (**A**) The scintillant bead–containing phagosomes float as an opaque whitish band near the top of the sucrose gradient. (**B**) Vybrant DiI (1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate, Invitrogen #V-22885) labeling of the fraction containing scintillant beads. Upper panels: J774 cells were harvested and mixed with beads just before centrifugation. Lower panels: Beads were added to cultured J774 cells 2 hours before harvest, as described in Procedure 3. The results show that isolated phagosomes are surrounded by membrane and that the preparations are not contaminated with intact cells. The scintillant beads are autofluorescent in the blue channel and were false-colored in green (left panels). DiI fluorescence (red channel) is shown in red (right panels; exposure times are indicated).

1. One day before using the cells, plate J774 mouse macrophages at 70 to 90% confluency in a 10-cm dish in 10 ml of Growth Medium B (Recipe 8) and incubate at 37°C, 5% CO_2 overnight.
2. On the day of the experiment, place the rotor into the ultracentrifuge and switch on the instrument at least 1 hour before starting the experiment to ensure that the chamber and rotor are equilibrated to 4°C by the time of centrifugation.
3. Aspirate the medium from the culture dish, add 25 mg of scintillant PEI-PVT beads in 4 ml of Growth Medium B (Recipe 8), and incubate for 60 min at 37°C, 5% CO_2.
4. Wash the cells three times with 10 ml of PBS, aspirating after each wash.
5. Add 10 ml of Growth Medium B and incubate for 60 min at 37°C, 5% CO_2.
6. Wash the cells three times with 10 ml of PBS, aspirating after each wash.
7. Wash the cells once with 1 ml of Buffer B (Recipe 2), aspirating after the wash.
8. Scrape the cells into 1 ml of Buffer B with a rubber policeman.
9. Transfer the cell suspension to a 14 × 89 mm ultracentrifuge tube and overlay with a 30% to 0% sucrose gradient in Buffer A (Recipe 1).

   Note: Prepare a 30% to 0% sucrose gradient in Buffer A using a 15-ml gradient maker containing 5 ml of Buffer B in the mixing chamber and 5 ml of Buffer A in the reservoir chamber. Add a stir bar to the mixing chamber, place the gradient maker on a magnetic stirrer, and transfer the solution to the centrifuge tube by opening the stopcock and pumping the sucrose solution with a peristaltic pump. For more details about preparation of gradients, refer to a GE Healthcare manual for Hoefer gradient makers.
10. Centrifuge the cells at 40,000 RPM (~198,000*g*) in a SW41Ti rotor for 45 min at 4°C.
11. Collect the phagosomes (white band) with a 1-ml adjustable air-displacement pipette (Gilson Pipetman® or equivalent).
12. To estimate the bead concentration, dilute a 20-µl aliquot of the phagosome suspension in 1 ml of Reaction Buffer (Recipe 5) and measure the OD_{600} in a spectrophotometer. PVT bead suspensions of known concentration are used for calibration.

Procedure 4: Preparation of Postnuclear Supernatants Containing ^{3}H-Labeled Lysosomes

The postnuclear supernatant is a crude mixture of cytosol and cellular membranes. However, density gradient centrifugation after labeling of cells with [^{3}H]cholesteryl ether–containing liposomes revealed that the distribution of the radiolabel overlaps with that of the lysosomal fraction (*5*). Postnuclear supernatant may thus be used directly for in vitro reactions or [^{3}H]cholesteryl ether–containing membranes can be separated from the cytosol by further centrifugation, as described in Procedure 5 below. Alternatively, membrane fractions enriched for labeled lysosomes can be purified by sucrose density gradient centrifugation as described in (*5*).

If the membranes are separated from the cytosol component of the postnuclear supernatant, in vitro reactions must be supplemented with both isotonic cytosol (see Procedure 2) and low-salt cytosol (see Procedures 6 and 7) (Fig. 2, A and B).

Postnuclear supernatants should be centrifuged and lysosomes obtained from the pellet when experiments are designed to compare the activity in different batches of low-salt cytosol (Fig. 2, B to D).

1. Culture one 10-cm dish of J774 mouse macrophages in Growth Medium B (Recipe 8) at 37°C, 5% CO_2 until confluent.
2. Replace medium with 4 ml of Growth Medium B plus 0.1 ml of [^{3}H]cholesteryl oleyl ether–containing liposomes (Recipe 13), and culture for 16 hours at 37°C, 5% CO_2.

   Note: Starting now, gloves should be worn and all solutions and plasticware that contact the cells must be discarded in accordance with institutional policies regarding radioactive waste.
3. Wash the cells three times with 10 ml of PBS, aspirating after each wash.
4. Add 10 ml of Growth Medium B and incubate for 1 hour at 37°C, 5% CO_2 to chase the label into lysosomes.

   Note: To compare the interactions of phagosomes with early endosomes, late endosomes, and lysosomes, a load-and-chase protocol may be used (4). For this procedure, add [^{3}H]cholesteryl ether–containing liposomes to macrophages at 15°C, which causes the tracer to accumulate in early endosomes. Wash to remove unincorporated liposomes and harvest immediately (early endosomes) or incubate the cells for different periods at 37°C to chase [^{3}H]cholesteryl ether into late endosomes and lysosomes.
5. Wash the cells three times with 10 ml of PBS, aspirating after each wash.

6. Rinse the cells with 1 ml of Homogenization Buffer (Recipe 4), removing by aspiration.
7. Scrape the cells into 1 ml of Homogenization Buffer and transfer the suspension to a 1.5-ml microcentrifuge tube.
8. Pass the suspension eight times through a 22-gauge needle (bent two times with pliers), immediately add 100 µl of Buffer C (Recipe 3), and mix by pipetting.

   Note: The yield is improved when the needle is bent two times at 90° angles. If the needle is rinsed several times with water immediately after use, it may be used repeatedly. If the concentrations of protein and radioactivity in postnuclear supernatants suddenly drop, prepare a new needle or increase the number of strokes. Cell lysis may be verified by inspecting a few microliters under a microscope.
9. Centrifuge the lysate for 5 min at 1000*g*.
10. Transfer the supernatant to a fresh microcentrifuge tube and designate as "postnuclear supernatant."
11. Mix a 10-µl aliquot of postnuclear supernatant with 20 ml of liquid scintillation cocktail and measure the radioactivity.
12. Determine the protein concentration with Coomassie Plus protein assay reagent.

    Note: A good preparation yields about 2 mg of protein and 1.5 µCi of radioactivity per milliliter of postnuclear supernatant.

Procedure 5: Preparation of Membrane Fractions Containing ^{3}H-Labeled Lysosomes

This step is optional, but is necessary to study lysosome-phagosome targeting in the absence of low-salt macrophage cytosol (Fig. 2, A and B) or to measure the effect of supplying low-salt cytosol at different concentrations (Fig. 2B), from different cell types (*1*), or from cells conditioned by exposure to IgG beads (see Procedures 6 and 7) (Fig. 2, C and D).

1. Centrifuge the postnuclear supernatant from Procedure 4, step 10, at 16,000*g* for 5 min at 4°C.
2. Remove the supernatant and discard.
3. Resuspend the pellet in 1 ml of Reaction Buffer (Recipe 5).
4. To determine the radioactivity in the membrane fraction, add 10 µl to 20 ml of liquid scintillation cocktail and analyze in a scintillation counter.
5. Determine the protein concentration with Coomassie Plus protein assay reagent.

   Note: Membrane fractions containing ^{3}H-labeled lysosomes should be prepared as freshly as possible; storage on ice for more than 2 hours reduces the in vitro activity.

Procedure 6: Preparation of Low-Salt Cytosol

Low-salt cytosol is required only if the lysosomes are purified from the postnuclear supernatant (see comments under Procedure 5). Cells are cultured in 6-well dishes, as the cells from one confluent well of a 6-well dish will yield enough cytosol for one in vitro reaction.

1. Plate J774 cells at 80 to 90% confluency in 6-well dishes and culture overnight in Growth Medium B (Recipe 8) at 37°C, 5% CO_2.

   Note: One confluent 10-cm dish of J774 cells is sufficient to set up three 6-well dishes.
2. Wash the cells three times with 1 ml of PBS, aspirating after each wash.
3. Rinse the cells with 1 ml of Homogenization Buffer (Recipe 4) and aspirate.
4. Scrape the cells into 1 ml of Homogenization Buffer and transfer the suspensions to 1.5-ml microcentrifuge tubes.
5. Pass the suspension eight times through a 22-gauge needle (bent two times with pliers).
6. Add 100 µl of Buffer C (Recipe 3) and vortex.
7. Centrifuge the lysate for 5 min at 1000*g* at 4°C.
8. Transfer the supernatant to a fresh microcentrifuge tube and centrifuge for 30 min at 16,000*g* at 4°C.
9. Collect the supernatant and designate as "low-salt cytosol."
10. Determine the protein concentration with Coomassie Plus protein assay reagent.

Procedure 7: Preparation of Conditioned Low-Salt Cytosol

The following procedure can be used to study how extracellular signals, inhibitors, and agonists affect the activity of cytosolic targeting factors (*1*). The procedure is described using IgG-coated beads as an example.

1. Plate J774 cells at 80 to 90% confluency in three 6-well dishes in Growth Medium B (Recipe 8) at 37°C, 5% CO_2.

   Note 1: One confluent 10-cm dish of J774 cells is sufficient to set up three 6-well dishes.

   Note 2: When setting up the cells, consider that one well of J774 cells yields enough cytosol for one in vitro reaction and that each condition should be analyzed in triplicate reactions.

2. Culture cells overnight.
3. Wash the cells once with 1 ml of PBS, aspirating after the wash.
4. Switch cells to Growth Medium C (Recipe 10), and incubate for 1 hour at 37°C, 5% CO_2.

   Note: This step is used to reduce background activity from serum IgG.

5. Add IgG-coated latex beads at 0 to 1 mg per well (see Procedure 1) in 1 ml of Growth Medium C.

   Note: See Fig. 2C for an example of how the cytosolic activity varies with the bead concentration.

6. Incubate for up to 2 hours at 37°C, 5% CO_2.

   Note: See Fig. 2D for an example of how the cytosolic activity in IgG-conditioned cells increases over time.

7. Follow steps 2 through 10 in Procedure 6, designating these samples as "conditioned low-salt cytosol."

Procedure 8: Scintillation Proximity Assay

Each experiment should include control reactions in which individual reaction components (ATP, rat liver cytosol, macrophage cytosol) are omitted or in which samples are incubated on ice (Fig. 2A). Because the coloration of rat liver cytosol reduces the scintillation signal, when performing control reactions in which rat cytosol is omitted during the 37°C incubation period, ice-cold rat liver cytosol should be added to the control sample just before reading to correct for quenching.

Scintillation Proximity Assay with Postnuclear Supernatant

1a. Set up reactions in clear microcentrifuge tubes on ice containing the following:

50 µl of an 8× ATP Regenerating System (Recipe 6)

2.4 mg of rat liver cytosol (Procedure 2)

0.75 mg of scintillant phagosomes (Procedure 3)

Postnuclear supernatant containing [^{3}H]cholesteryl ether–labeled lysosomes (containing ~80 µg of protein and ~40 nCi of tritium; Procedure 4)

Reaction Buffer (Recipe 5) to a final volume of 0.4 ml

Scintillation Proximity Assay with Membrane Fractions Containing Labeled Lysosomes

1b. Set up reactions in clear microcentrifuge tubes on ice containing the following:

50 µl of an 8× ATP Regenerating System (Recipe 6)

2.4 mg of rat liver cytosol (Procedure 2)

0.75 mg of scintillant phagosomes (Procedure 3)

~40 nCi of a cytosol-free membrane fraction containing [^{3}H]cholesteryl ether–labeled lysosomes (Procedure 5)

~40 µg of low-salt cytosol (Procedure 6 or 7)

Reaction Buffer (Recipe 5) to a final volume of 0.4 ml

2. Incubate reactions 37°C for up to 2 hours.
3. Place the tubes into 20-ml glass scintillation vials and measure the scintillation in a scintillation counter.

Troubleshooting

This assay depends on lysosomes and phagosomes remaining intact; therefore, the quality of the buffers is critical. All buffers and solutions should be prepared with components of the highest grade available, and it is very important to verify the pH of all solutions, even after diluting Tris and HEPES buffers from concentrated, pH-adjusted stocks. Glassware, plasticware, and stir bars used for preparation and storage of all solutions should be thoroughly rinsed several times with ddH_2O to remove any potential traces of detergent. If the assay suddenly stops working, it is advisable to prepare fresh batches of all solutions.

If the readings under control conditions are unusually high, reactions stop responding to conditioning with IgG, or both, the problem can sometimes be solved by reducing the concentration of phagosomes, rat liver cytosol, or postnuclear supernatant in the scintillation proximity assay.

Related Techniques

The scintillation proximity approach has also been used to study phagocytosis in living cells (*2*, *5*, *9*). When cells are labeled with [^{3}H]cholesterol, which localizes predominantly to the plasma membrane, scintillation is initiated upon particle envelopment, allowing kinetic measurements of the engulfment process. Alternatively, labeling cells with [^{3}H]cholesteryl oleyl ether leads to scintillation once phagosomes fuse with lysosomes intracellularly. If cells are analyzed in a temperature-controlled microplate scintillation counter, phagolysosome formation can be followed in real time.

Other reconstitution assays for lysosome-to-phagosome fusion have been developed for permeabilized cells (*10*) or entirely cell-free systems (*11*, *12*). Fusion is commonly inferred from content mixing between lysosomes and phagosomes, and assays often exploit the affinity of biotin and avidin to detect the delivery of lysosomal horseradish peroxidase (HRP) to phagosomes containing appropriately modified particles. In one approach, lysosomes are loaded with avidin-HRP and phagosomes are formed from biotinylated beads (*10*). Similarly, Griffiths and colleagues have loaded lysosomes with biotin-HRP and phagosomes with avidin-conjugated latex beads (*11*). Both assays require ATP and supplementation of reactions with concentrated cytosol.

A different strategy was used by Peyron *et al*., who prepared lysosomes with membranes that had been loaded with two lipid-anchored fluorescent dyes, rhodamine-phosphatidylethanolamine and NBD-phosphatidylethanolamine, at concentrations where the fluorescence is quenched (*12*). Upon incubation with target phagosomes in vitro, dilution of the dyes caused dequenching and up to a 25% increase in the fluorescence signal. Dequenching required cytosol but no additional ATP or GTP. The activity in cytosol from human NB4 neutrophils was shown to be higher after incubation of cells with protein kinase C (PKC) agonist phorbol-12-myristate-13-acetate (PMA) plus calcium ionophore or after exposure to serum-opsonized zymosan. Whether opsonization was necessary for this effect, and what serum component was responsible, was not reported.

Notes and Remarks

We recommend performing pilot experiments designed to optimize concentrations of radiolabeled lysosomes, phagosomes, cytosol, and salt.

This in vitro lysosome-to-phagosome targeting assay requires an ATP-regenerating system, cytosol from rat liver, cytosol from unlabeled macrophages, and a membrane fraction containing [^{3}H]cholesteryl ether–labeled lysosomes. Scintillation signals are about 30 times background (Fig. 2A). The signal is substantially lower in samples lacking an ATP-regenerating system, macrophage cytosol, or rat liver cytosol or when complete reactions are incubated at 4°C instead of 37°C (Fig. 2A). Why these reactions require supplementation with rat liver cytosol is currently not known. Macrophage cytosol alone is insufficient at concentrations up to 0.2 mg/ml (Fig. 2B), but has not yet been assayed at higher concentrations. Fractionation of rat liver cytosol led to the purification of actin and of an actin cross-linking factor consisting of IQGAP, Cdc42, and calmodulin (*5*); possibly, actin or the IQGAP complex are limiting in macrophage cytosol at the concentrations that were used. Alternatively, the low salt concentration in the buffer used for macrophage homogenization might interfere with the integrity or function of the IQGAP complex.

The enhancement of the cytosolic activity by exposure to IgG-coated beads, here shown for mouse J774 macrophages (Fig. 2, C and D), has also been observed in immortalized mouse RAW 264.7 macrophages and primary mouse peritoneal macrophages, as well as in primary human monocytes and human U-937 promyelocytic leukemia cells (*1*). IgG signaling proceeds through a

pathway involving PKC, and the effects of IgG beads can be mimicked by addition of the PKC agonist PMA directly to reactions in vitro or to the medium of macrophages before extraction of cytosol (*1*, *12*).

Because the readout of the current assay requires only proximity of the interacting vesicles, the scintillation signal alone does not reveal whether membranes have tethered, docked, or fused. However, when lysosomes and phagosomes are allowed to attach and are then treated with alkali, which disrupts protein-protein interactions, the scintillation signal returns to background levels (*1*, *5*). Because alkali leaves bilayers intact and does not interfere with the localization of cholesteryl ether (*5*), we conclude that the conditions of the in vitro assay allow for tethering and docking, but not for fusion. When lysosomes and phagosomes are allowed to dock and are then exposed to Ca^{2+}, the scintillation becomes resistant to alkali treatment (*5*), but whether this effect is due to fusion remains to be verified.

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Citation: V. Trivedi, S. C. Zhang, W. Stockinger, A. Nohturfft, A cell-free scintillation proximity assay for studies on lysosome-to-phagosome targeting. *Sci. STKE* **2007**, pl3 (2007).