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Studying Phagocytosis by Live-Cell Scintillation Proximity Assay

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Summary Phagocytosis of microorganisms, senescent cells, apoptotic bodies, and effete tissue material is an important process in host defense and tissue homeostasis. A method is described to measure, in living macrophages, the kinetics of particle engulfment and lysosome/phagosome targeting. Plasma membranes or lysosomes are labeled with tritiated lipids, followed by exposure of cells to scintillant microbeads. Because of the short range of tritium β-particles, geometric factors, and the confinement of lipids to membranes, scintillation can only be elicited by tracer molecules in membranes immediately vicinal to the scintillant. When the plasma membrane is labeled with [^{3}H]cholesterol, a signal is produced on bead–cell contact and engulfment and then reaches steady state within 45 min. When lysosomes are labeled with nonhydrolyzable [^{3}H]cholesterol oleyl ether, scintillation requires intracellular lysosome/phagosome attachment or fusion, and steady state is attained only after several hours. The live-cell scintillation proximity approach is useful for examining the effects of pharmacological and genetic manipulations on particle uptake and on lysosome/phagosome targeting.

Keywords Cholesterol; endocytosis; endosomes; lysosomes; phagosomes; plasma membrane.

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1 Introduction

Phagocytosis involves binding of particles to cell surface receptors, formation of intracellular phagosomes by engulfment, transport of phagosomes to the perinuclear region, and fusion of phagosomes with endo-/lysosomes *(1)*. Studies of the biochemical mechanisms and regulation of these processes often require kinetic measurements with living cells. Conventionally, live-cell experiments use light microscopy to follow the distribution of fluorescently labeled proteins. The approach is characterized by high spatial and temporal resolution, applicability to many different cell types, and the opportunity to study several markers at the same time *(2,3)*. On the downside, analyzing the effects of multiple parameters by live-cell microscopy is time consuming, results are often difficult to quantify, and the approach can be susceptible to selection bias during both analyses and documentation *(4)*.

Here, we describe a complementary approach that is based on scintillation proximity technology. Scintillation proximity assays generally combine scintillant particles with analytes containing weak β-emitters *(5,6)*. Because of the short range of β-radiation emitted by isotopes such as tritium and ^{14}C, only those labeled molecules can produce scintillation that are within about 1 µm of the scintillant *(7)*. The approach has long been used for in vitro measurements of enzyme activity, receptor–ligand binding, and other biochemical interactions. The data are quantitative, and assays are amenable to high-throughput formats *(8)*.

Since commercially available scintillant particles are small enough to be taken up by phagocytes (2.5–5 µm), we recently developed scintillation proximity assays for studies with living macrophages and tritiated lipids *(7,9,10)*. In one approach, the plasma membrane is labeled with [^{3}H]cholesterol to study particle envelopment and uptake *(7,9)*. In a second protocol, lysosomes are specifically labeled with nonhydrolyzable [^{3}H]cholesterol oleyl ether to follow the kinetics of lysosome/phagosome targeting *(10)*.

Cells are grown in glass scintillation vials, and scintillation is measured in standard scintillation counters. Alternatively, cells can be cultured in multiwell plates, and data are collected in a microplate scintillation reader. When a temperature-controlled counting chamber is available, data can be collected continuously with a temporal resolution of up to about 1 min. We have used the live-cell scintillation proximity approach to study (i) the kinetics of particle engulfment *(7,9)*, (ii) lysosomal cholesterol storage *(7)*, and (iii) lysosome/phagosome targeting *(10)*.

To illustrate what types of data can be obtained with this method, we performed the experiment shown in Fig. 1. The solid line shows the results of an experiment designed to analyze the particle engulfment process. A line of immortalized mouse macrophages, J774 cells, was incubated overnight with liposomes containing [^{3}H]cholesterol oleyl ester, which is hydrolyzed to [^{3}H]cholesterol and oleate in lysosomes. Cholesterol partitions preferentially to the plasma membrane but distributes across all cellular organelles (11,12). The labeled cells were then placed on ice, and cultures received suspensions of scintillant yttrium silicate beads. The plate was left on ice for 15 min to allow the particles to settle on top of the cells and then moved

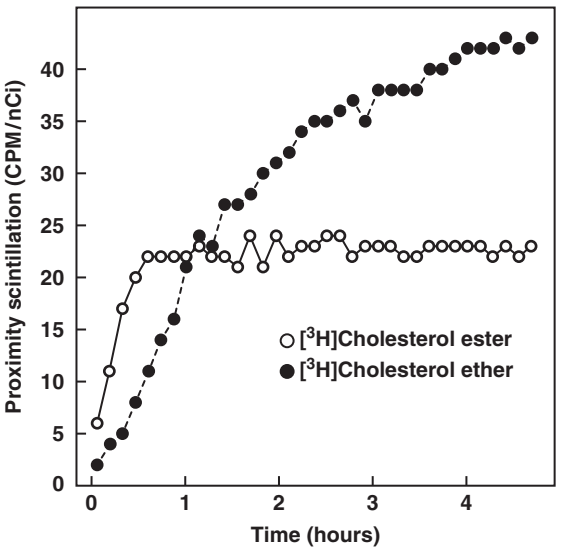

Fig. 1 Live-cell scintillation proximity assays. On d 0, mouse J774 macrophages were set up in a 24-well plate at 5×10^5 cells/well in medium A. After 30 min at 37 °C, the cells were switched to medium A plus liposomes containing [^{3}H]cholesterol oleyl ester (solid line) or [^{3}H]cholesterol oleyl ether (dashed line) (1 µCi per 0.5 mL per well final concentrations). On d 1, the medium was removed, cells were washed two times with PBS, and each well received 0.5 mL of a suspension of 100 mg/mL of scintillant yttrium silicate beads in medium B. The plate was left on ice for 15 min and then moved to a microplate scintillation counter at 33 °C. Scintillation was measured every 8 min for 5 h. The cells were then lysed to determine the total cellular radioactivity by liquid scintillation counting. Data represent proximity scintillation corrected for total cellular radioactivity. The average coefficient of variation in wells containing [^{3}H]cholesterol oleyl ester was 5.3% with a range of 0.4% to 39.1% (n = 3). The average coefficient of variation in wells containing [^{3}H]cholesterol oleyl ether was 9.1% with a range of 0.8% to 37.5% (n = 3).

to a microplate scintillation counter at 33 °C. Scintillation was measured every 8 min. The signal rapidly increased and reached steady state after about 45 min.

Time curves of similar shape were obtained with primary mouse peritoneal macrophages *(7)* and with immortalized mouse HD1A macrophages *(9)*, although the rate of uptake in these cell types was slower. When the scintillant particles are added in the presence of phagocytosis inhibitors, scintillation remains at background levels, indicating that the signal changes can be used as a proxy for the kinetics of engulfment *(7)*. When detergent is added to cultures at the end of experiments, the scintillation is reduced to almost zero, which demonstrates that the signal results from the presence of [^{3}H]cholesterol in particle-proximal membranes (unpublished data).

The values represented by the dashed line in Fig. 1 were obtained with J774 cells that had been labeled overnight with liposomes containing [^{3}H]cholesterol oleyl ether. Endocytosed cholesterol ether is stored specifically in lysosomes *(10,13)*. The cultures were then supplemented with yttrium silicate beads, and scintillation

was measured as above. Compared to [^{3}H]cholesterol-labeled samples, the scintillation signal in [^{3}H]cholesterol ether-labeled cells at early time-points was significantly lower as little cholesterol ether is found at the cell surface *(10)*. As would have been predicted, the rate of signal increase during the first 33 min was slower in [^{3}H]cholesterol ether-labeled cells (16.1 ± 1.5 cpm/nCi/h vs 32.7 ± 4.9 cpm/nCi/h), but scintillation continued to climb for much longer. As the data in Fig. 1 were corrected for total cellular radioactivity, the higher specific proximity scintillation in [^{3}H]cholesterol ether-labeled cells at later time-points can be partly explained by the notion that cholesterol ether accumulates predominantly in lysosomes *(10)*, whereas cholesterol equilibrates across a much broader range of cellular pools *(11)*.

In summary, the live-cell scintillation proximity assay provides a useful experimental tool for quantitative analyses of phagocytosis in living cells.

2 Materials

2.1 Cell Culture

1. J774A.1 macrophages (American Type Culture Collection, Manassas, VA; cat. no. TIB-67).
2. A 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12, Mediatech, Herndon, VA, cat. no. 10-090-CV).
3. DMEM/F12 without phenol red (Mediatech, cat. no. 16-405-CV)
4. Fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA).
5. A cocktail of penicillin and streptomycin sulfate (Mediatech, cat. no. 30-002-CI).
6. Medium A: DMEM/F12, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin.
7. Medium B: Phenol red-free DMEM/F12, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin.
8. Plastic 10-cm cell culture dishes (various manufacturers).
9. Trypsin ethylenediaminetetraacetic acid (EDTA) solution (Mediatech, cat. no. 25-052-CI).
10. Phosphate-buffered saline (PBS) (Mediatech, cat. no. 21-040-CM).
11. Tissue culture grade 1 *M* HEPES-KOH at pH 7.4 (Mediatech, cat. no. 25-060-CI).

2.2 Liposomes

1. Egg yolk phosphatidylcholine (Sigma, St. Louis, MO; cat. no. 61751) is dissolved at 10 m*M* in a mixture of 95% chloroform and 5% methanol.
2. Bovine brain phosphatidylserine (Sigma, cat. no. 79406) is dissolved at 10 m*M* in chloroform/methanol (95:5).

3. Dicetyl phosphate (Sigma, D-2631) is dissolved at 4 m*M* in chloroform/methanol (95:5).
4. 1 mCi/mL (1 Ci = 37 GBq) [^{3}H]cholesterol oleyl ester (GE Healthcare, Piscataway, NJ; cat. no. TRK886).
5. 1 mCi/ml [^{3}H]cholesterol oleyl ether (GE Healthcare, cat. no. TRK888).
6. Water bath sonicator.

2.3 Scintillation Proximity Assay

1. Topcount NXT microplate scintillation counter equipped with two photomultipliers (Perkin Elmer, Wellesley, MA).
2. Yttrium silicate scintillating beads (GE Healthcare, cat. no. RPNQ0013).
3. Opaque white 24-well plates (Perkin Elmer, cat. no. 6005168).
4. Adhesive transparent plate covers (Perkin Elmer, cat. no. 6005198).
5. Beckman LS 6000 IC scintillation counter.
6. Liquid scintillation cocktail.
7. 20-mL scintillation vials.
8. Nonidet P-40 (Sigma, cat. no. NP40).
9. Bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Rockford, IL).

3 Methods

3.1 Preparation of Liposomes

1. In a glass screw-cap tube, combine 20 µL of 10 m*M* phosphatidylcholine, 20 µL of 10 m*M* phosphatidylserine, 50 µl of 4 m*M* dicetyl phosphate, and 80 µL of 1 mCi/mL [^{3}H]cholesterol oleyl ester (to label the plasma membrane) or [^{3}H]cholesterol oleyl ether (to label lysosomes).
2. A slow current of nitrogen gas is directed into the tube through a 1-mL plastic pipet. Nitrogen-facilitated evaporation of the solvents should be performed in a fume hood. After evaporation of the solvents, the lipids will form a thin but visible film on the wall of the glass tube.
3. The lipid film is supplemented with 200 µL PBS, vigorously vortexed for 30 s, and sonicated for 2 min in a water bath sonicator. Vortexing and sonication are repeated two times. This will yield a liposome suspension containing 400 µCi/mL of tritiated lipid.
4. Suspensions can be stored at 4 °C in the dark for up to 4 wk but are sonicated again for 1 min before each additional use.

3.2 Culture of J774 Macrophages

1. J774 cells are grown in 10-cm dishes at 37 °C in an atmosphere of 8–9% CO_2 in medium A.
2. J774 cells adhere very strongly to the surface of culture dishes and are most easily trypsinated when grown to complete confluency.
3. Cultures should not be grown continuously for more than 3 mo before replacement with a fresh batch from frozen stocks.
4. J774 cultures should occasionally be tested for contamination with mycobacteria.

3.3 Proximity Scintillation Assay

1. On the evening of d 0, J774 cells are set up in 0.5 mL medium A in an opaque 24-well plate at a density of 5×10^5 cells per well (*see* **Note 1**).
2. Note that the white opaque microplates cited above exhibit phosphorescence after exposure to light. To reduce background during live scintillation counting, the plates should be stored in the dark and during experiments should be handled under low-light conditions (for an alternative culture vessel, *see* **Note 2**).
3. After 30 min at 37 °C, the cells have attached, and the medium is replaced with 0.5 mL per well of medium A containing 5 µL liposomes per milliliter medium. The resulting final concentration of tritiated lipids is 1 µCi per well per 0.5 mL medium (*see* **Note 3**).
4. On the morning of d 1, the medium is removed, cells are washed twice with PBS to remove the radioactive liposomes, and each well receives 0.5 mL fresh medium B.
5. The cells are incubated for another hour at 37 °C to allow for uptake of surface-bound liposomes and to chase all [^{3}H]cholesterol ether into lysosomes or to complete hydrolysis of [^{3}H]cholesterol ester and equilibration of [^{3}H]cholesterol.
6. The plate is then placed on ice, the medium is removed, and each well receives 0.5 mL of an ice-cold suspension of 100 mg/mL yttrium silicate beads in medium B plus 50 m*M* HEPES-KOH. To ensure that each well receives the same amount of yttrium silicate, the suspension needs to be vortexed repeatedly while pipetting the 0.5-mL aliquots. At this point, the cells are kept in phenol red-free medium B to later prevent quenching of the scintillation signal. HEPES is added to help buffer the pH in the absence of CO_2 (*see* **Note 4**).
7. The plate is left on ice for 15 min to let the scintillant particles settle on top of the cell monolayer. This step helps synchronize the subsequent phagocytosis process. The plate should be protected from light to reduce background that might result from phosphorescence of the plate material.
8. The dish is then sealed with an adhesive plastic foil to reduce evaporation and to avoid contamination of the instrument during the subsequent counting phase.
9. After the yttrium silicate beads have settled, the plate is transferred to a Topcount NXT multiwell plate scintillation reader, and continuous scintillation

counting is started. Nuclide settings of the instrument control software are as follows: glass scintillator; low energy range; high-sensitivity efficiency mode; region A, 0–50; region B, 0–256. Wells are read for 30 s at a time. The instrument may be connected to a circulating water bath to control the temperature in the counting chamber (*see* **Note 5**).

10. After the final reading, the seal is removed, cells are washed three times with PBS, and the wash buffer is tested for contamination with radioactivity by liquid scintillation counting to control for washing efficiency.
11. After the final wash, the plates are sealed once more, and proximity scintillation is measured again to control for loss of cells during the washing procedure.
12. Finally, the cells are lysed by the addition of PBS containing 0.5% NP-40. To facilitate cell lysis, the plate is subjected to one freeze–thaw cycle, and lysates are transferred to microcentrifuge tubes. Particulate material containing the YSi beads and nuclei are spun down for 30 s at 16,000 *g* in a microcentrifuge. A 20-µL aliquot of the supernatant is removed to determine total incorporated radioactivity by liquid scintillation counting, and a second 20-µL aliquot is used to determine the protein concentration with BCA protein assay reagent.

3.4 Data Processing and Statistics

1. All conditions should be analyzed at least in triplicate wells.
2. Data generated by the Topcount NXT software can be loaded into Microsoft Excel for further analyses.
3. Scintillation counts should be corrected for total cellular radioactivity determined after proximity scintillation readings. In addition, the resulting values can be corrected for protein concentrations.
4. The data from triplicate wells can be graphed directly to illustrate the degree of scatter. Alternatively, for each round of counting, both time and scintillation counts are averaged and plotted with the standard deviation. Since the standard deviation tends to scale with the scintillation counts *(7)*, statistical dispersion is best analyzed by calculating, for each graph point, the coefficient of variation (i.e., the quotient of the standard deviation divided by the mean); for a given trace, the overall dispersion may then be summarized by averaging all coefficients of variation and indicating their range (see legend to Fig. 1).

4 Notes

1. In addition to immortalized mouse J774 cells, we have successfully tested primary mouse peritoneal macrophages *(7)* and immortalized mouse HD1A macrophages *(9)*.

2. Instead of using microplates, cells can be grown in standard glass scintillation vials that have previously been washed and autoclaved. Macrophages grow well on glass. Proximity scintillation readings are then done in a regular liquid scintillation counter using the tritium settings. This setup works well for single measurements, the vials are inexpensive, and the necessary equipment is available in most laboratory environments.
3. Scintillation readings with empty wells gave background values of about 50 cpm, whereas the protocol described above should produce readings between 500 and 2000 cpm (Fig. 1). From time to time, we found that experiments resulted in unusually low counts, making data difficult to interpret. In most cases, these problems could be solved either by preparing a new batch of liposomes or by thawing a fresh vial of cells.
4. In the experiments described above, cells were made radioactive first and then supplied with scintillant beads. The reverse approach is also possible. When studying the effects of pharmacological or genetic manipulations on phagosome maturation, it may be important to rule out effects on the uptake process. To analyze the effects of actin and calmodulin inhibitors on lysosome/phagosome targeting, we have added scintillant beads first and then supplied the cells with [^{3}H]cholesterol ether-containing liposomes plus or minus the inhibitors *(10)*. To verify that the inhibitors did not interfere with endocytosis of liposomes, a parallel set of wells was labeled with [^{3}H]cholesterol ester and treated identically *(10)*.
5. We initially did all proximity scintillation readings with the Topcount NXT microplate reader attached to a circulating water bath. However, liquid occasionally leaked into the instrument, necessitating extensive repairs. The problem was solved when it became apparent that the temperature in the counting chamber will reach 33 °C even in the absence of external control with the instrument at room temperature.

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