# 21

# Monitoring Time-Dependent Maturation Changes in Purified Phagosomes from *Dictyostelium discoideum*

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## Summary

The amoeba *Dictyostelium discoideum* is an established model to study phagocytosis. The sequence of events leading to the internalization and degradation of a particle is conserved in *D. discoideum* compared to metazoan cells. As its small haploid genome has been sequenced, it is now amenable to genome-wide analysis including organelle proteomics. Therefore, we adapted to *Dictyostelium* the classical protocol to purify phagosomes formed by ingestion of latex beads particles. The pulse-chase protocol detailed here gives easy access to pure, intact, and synchronized phagosomes from representative stages of the entire process of phagosome maturation. Recently, this protocol was used to generate individual temporal profiles of proteins and lipids during phagosome maturation generating a proteomic fingerprint of six maturation stages ***(1)***. In addition, immunolabeling of phagosomes on a coverslip was developed to visualize and quantitate antigen distribution at the level of individual phagosomes.

**Key Words:** Phagosomes, organelle purification, phagocytosis, *Dictyostelium*.

## 1. Introduction

The social amoeba *Dictyostelium discoideum* is a recognized eukaryotic model for professional immune phagocytes ***(2)***. The range of ingested particles includes latex beads, live and dead cells of the same species, yeast, and bacteria. It is used to study host–pathogen interactions with bacteria such as *Legionella pneumophilia* ***(3)***, *Pseudomonas aeruginosa* ***(4)***, and *Mycobacterium marinum* ***(5,6)***. The sequence of events leading to the internalization

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and degradation of a particle is conserved in *D. discoideum* compared to metazoan. This includes particle recognition and signaling, recruitment of the actin cytoskeleton leading to the formation of pseudopods, and the intense vesicular trafficking involving the delivery of digestive enzymes and membrane retrieval ***(2,7)***. Two peculiarities of *D. discoideum* are the difficulty to identify phagocytic receptors ***(8–10)*** and constitutive exocytosis of undigested remnants. However, phagosome/endosome exocytosis is also used by dendritic cells to present antigens on their surface. Additionally, exocytosis of latex beads can be triggered by appropriate secretagogues in macrophages ***(11)***. Compared to macrophage-like cell lines, the use of *D. discoideum* as a phagocytic model has several advantages. The rate of phagocytosis is up to 20-fold higher. The time scale of phagosome maturation from uptake to exocytosis does not exceed 3 h. Its small haploid genome (33 Mbp, 12,000 predicted genes) is now sequenced and thoroughly annotated, allowing rapid targeted mutagenesis and large-scale analysis by random insertion of plasmid sequences, microarrays, and proteomics ***(1,8,12)***.

The protocol presented here has been used notably to generate individual temporal profiles of proteins and lipids during phagosome maturation ***(1, Brügger et al., in preparation)***. The method is based on the purification of low-density latex bead–containing phagosomes via their flotation in sucrose step gradients. The method was originally introduced by Wetzel and Korn for *Acanthamoeba* and adapted successfully to macrophages by Desjardins and collaborators ***(13,14)***. The latter protocol has been further refined and adapted into a pulse-chase protocol that covers the entire maturation process. A large number of pure, intact, and synchronized phagosomes are isolated at six different times spanning the entire maturation process of 3 h. Synchronicity is ensured by a preincubation in the cold of cells and beads at high concentration. Cells are broken with a ball homogenizer for optimal breakage while keeping the phagosomal membrane intact. Phagosome purity is significantly improved by a brief incubation of the cell lysate with a physiological concentration of ATP, which releases the rigor mortis actin–myosin interactions, thereby avoiding co-purification of enmeshed organelles ***(15)***. This protocol has been fine-tuned to yield equal amounts of phagosomes at each time point. As it has been shown that phagocytosis was sensitive to particle size ***(16)***, shape ***(17)***, and nature ***(18)***, small adjustments were introduced to isolate phagosome formed with different bead sizes (0.5, 0.8, 3.0 μm).

The quantitation of the number of purified phagosomes is precisely performed by measuring light scattering in the fraction collected from the sucrose gradient. An average of $4 \times 10^{10}$ phagosomes per time point can be isolated. Phagosome concentration is normalized according to absorbance at 600 nm ***(15)***. Protein quantitation indicates that the total amount of protein

per phagosome does not vary significantly throughout maturation (less than 15%) and is approximately 12 µg/$10^9$ phagosomes (≈450 µg total) ***(1)***. This is further demonstrated by the equal loading of total proteins on 1D gels after normalization ***(1,7,15)***.

## 2. Materials

### 2.1. Buffers and Equipment

1. Soerensen buffer (SB): 15 m*M* $KH_2PO_4$, 2 m*M* $Na_2HPO_4$, pH 6.0.
2. Soerensen/Sorbitol buffer (SSB): Soerensen buffer containing 120 m*M* sorbitol.
3. HESES: 20 m*M* hydroxyethyl piperazine ethane sulfonate (HEPES)-KOH, pH 7.2, 0.25 *M* sucrose.
4. Homogenization buffer (HB): HESES, 2X Complete ethylenediamine tetraacetic acid (EDTA)-free (protease inhibitor cocktail; Roche, Hertfordshire, UK).
5. Membrane buffer: 20 m*M* HEPES-KOH, pH 7.2, 20 m*M* KCl, 2.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), 20 m*M* NaCl.
6. Storage buffer: 25 m*M* HEPES-KOH, pH 7.2, 1.5 m*M* Mg-acetate, 1 m*M* $NaHCO_3$, 1 µ*M* $CaCl_2$, 25 m*M* KCl, 1 m*M* ATP, 1 m*M* DTT, 1X Complete EDTA-free, 100 m*M* sucrose.
7. Ball homogenizer (Isobiotec, Heidelberg, Germany, barrel 8.000 mm, ball diameter 7.990 mm, resulting in an annular void clearance of 5 µm).
8. Piperazine-*N*,*N*´–bis(2-ethanesulfonate) (PIPES) buffer: 20 m*M* PIPES, pH 6.0.
9. Saturated picric acid solution. Dissolve 3 g of solid picric acid in 1 L of double-distilled water, warm up to 80°C overnight. Cool down to room temperature and adjust the pH to 6.0. Store at 4°C.
10. Picric acid/PFA fixative. Mix 0.4 g of paraformaldehyde, 7 mL of double distilled water, 10 mL of PIPES buffer. Microwave in brief pulses until it dissolves, then cool immediately to room temperature (on ice). Add 3 mL of a saturated picric acid solution.
11. Phosphate-buffered saline (PBS) buffer: 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* $Na_2HPO_4$, 1.8 m*M* $KH_2PO_4$, pH 7.4.

### 2.2. Cell Culture

*Dictyostelium discoideum* cells of wild-type strain Ax2 are grown axenically in HL5c medium (ForMedium Ltd, Norwich, UK) in shaking culture (at 180 rpm) at 22°C to a density of $5 \times 10^6$ cells/mL.

### 2.3. Preparation of Latex Beads

Always prepare fresh. 2 × 2 mL of 0.8-µm latex beads suspension (Sigma, St. Louis, MO) are spun down in an Eppendorf tube (10,000*g*, 5 min). The beads are then washed twice in SSB, pH 8, to remove the detergent and sodium azide

contained as preservatives in the suspension supplied by the manufacturer, and finally resuspended in 2 mL of SSB, pH 8, and kept on ice. This appears critical, as insufficient washing blocks phagocytosis. Before use, the bead suspension is sonicated for 5 min in a bath sonicator. For use of other bead sizes *see* **Note 1**.

### 2.4. Preparation of Sucrose Step Gradients

Prepare sucrose step gradients in disposable centrifuge tubes (polyallomer tubes 25 × 89 mm, Beckman) by layering the following sucrose solutions on top of each other: 4 mL of 60%, 12 mL of 35%, 12 mL of 25% sucrose in 20 m*M* HEPES-KOH pH 7.2. The gradients can be stored up to overnight avoiding vibrations. The last layer composed of 4 mL of 10% sucrose in 20 m*M* HEPES-KOH pH 7.2 is added after the phagosome samples have been loaded to obtain an undisturbed 25%/10% interface and to balance the tubes exactly.

### 2.5. Preparation of Poly-L-Lysine–Coated Cover Slips

Coverslips (12 mm diameter, No. 1, Assistant, Germany) are coated with a 1 mg/mL poly-L-lysine hydrobromide (MW (vis) 93,800, Sigma) solution for 1 h. The coverslips are then dipped once in double-distilled water and left to dry.

## 3. Methods

### 3.1. Phagosome Isolation After Pulse-Chase Feeding of Latex Beads

1. Cells from an overnight culture are counted with a hemocytometer, and $8 \times 10^9$ cells are centrifuged at 500*g* for 5 min. Subsequent steps are performed at 4°C or on ice when possible, except indicated otherwise.
2. Resuspend the cells in 50 mL SSB pH 8 and pool into a 50-mL Falcon tube.
3. Wash cells by spinning them down at 500*g* for 5 min.
4. Gently resuspend cell pellet in 20 mL of ice cold SSB pH 8.0. If desired, biotinylate the cell surface (for **steps 4-6**, *see* **Note 2**).
5. Add the bead suspension to the cells. Mix by inverting the tube.
6. Incubate for 15 min on ice.
7. Our typical setup is based on six time points (*see* **Note 3**):

   - **P1**: 5 min pulse
   - **P2**: 15 min pulse
   - **P3**: 15 min pulse/15 min chase
   - **P4**: 15 min pulse/45 min chase
   - **P5**: 15 min pulse/1 h 45 min chase
   - **P6**: 15 min pulse/2 h 45 min chase

Pour the bead/cell mixture into 100 mL of HL5c kept at 22°C in a 250-mL flask. Starting with the latest time point P6, pipet 5 mL of the cell/beads suspension for P6 to P3, 6 mL for P2, and 7 mL for P1 (*see* **Fig. 1** and **Note 4**).

8. Incubate at 22°C while shaking at 120 rpm for the appropriate period of time.
9. At the chosen time point, in order to stop phagocytosis, pour the 100-mL sample into 330 mL of ice-cold SSB prealiquoted in centrifugation bottles (500 mL, 69 × 160 mm) kept on ice (*see* **Note 5**).
10. Centrifuge cells for 8 min at 2000 g (Beckman rotor JLA-10.500).
11. Resuspend the cell pellet in 50 mL of ice-cold HESES and centrifuge again for 5 min at 500*g*.
12. For time point P3 to P6, resuspend the cells in 5 mL of ice cold HL5c and pour into 100 mL of HL5c kept at 22°C. Incubate at 22°C while shaking at 120 g for the appropriate chase period. Proceed then from **step 9** to **step 11** to stop phagocytosis.
13. Following **step 11**, repeat the HESES washing step twice for all time points to thoroughly wash away uningested latex beads. Repeat the HESES wash once more if 3-µm beads are used.

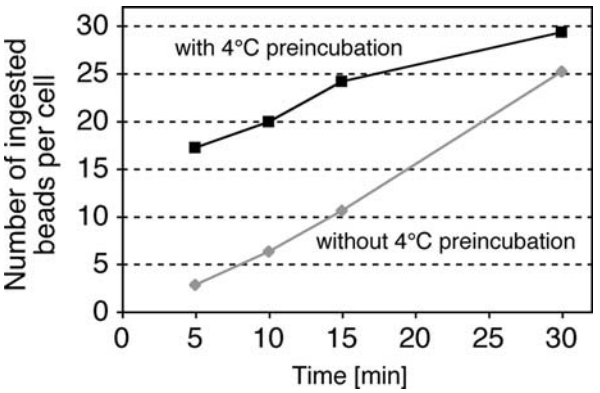


Fig. 1. Effect of beads adsorption on phagocytic uptake as measured by FACS. Cells were either preincubated (black curve) or not (grey curve) at 4°C with fluorescent beads (carboxylated Fluoresbrite YG microspheres, 1.0 µm diameter, Polysciences Inc.) for 15 min before starting the uptake at 22°C. Preadsorption of the latex beads onto cells in the cold enables an efficient and synchronous uptake during the pulse period, resulting in a more homogeneous population of isolated phagosomes. In comparison, the initial uptake without preincubation is reduced and more than 20 min are needed to get the same number of beads inside the cells as with preincubation in the cold. Uptake was stopped by plunging the bead/cell suspension in an azide-containing buffer. As cells round up and contract due to azide treatment, subsequent centrifugation detaches uningested beads from the cell surface ***(1)***. Therefore, the only beads monitored by FACS have been internalized.

14. Resuspend the cell pellet (approximately 1 mL) in 2 mL of HB-containing a protease-inhibitor cocktail (Complete EDTA-free, Roche) at 2X concentration, resulting in a 1X final concentration.
15. Homogenize cells by eight passages through a ball homogenizer. (*see* **Fig. 2** and **Note 6**).
16. Adjust the final concentrations of ATP to 10 m*M*, of $MgCl_2$ to 10 m*M* and of sucrose to about 45–50% from freshly made stocks (0.1 *M* ATP in 40% sucrose, 20 m*M* HEPES-KOH buffered to pH 7.2; 1 *M* $MgCl_2$; 71.4% sucrose in 20 m*M* HEPES-KOH pH 7.2) (*see* **Note 7**).
17. Mix gently for 15 min using an overhead tumbler or a wheel.
18. Load the density-adjusted homogenate between the 60% and 35% layer of the sucrose step gradients using a syringe and a needle (100 mm × 1.5 mm), and overlay with 4 mL of 10% sucrose solution (for **steps 18** and **19**, *see* **Note 8**).
19. Centrifuge gradients at 28,000 g (Beckman rotor SW 28, 100,000*g* avg), 4°C for 2 h 30 min.
20. Collect the interphase between 10% and 25% with a Pasteur pipet and dilute with membrane buffer to a final volume of 15 mL, mix by inverting the tube (for **steps 20**–**23**, *see* **Note 9**).
21. Take a 50-µL aliquot to measure scattering at 600 nm to calculate the number of phagosomes in the sample. A standard curve can be made with serial dilutions

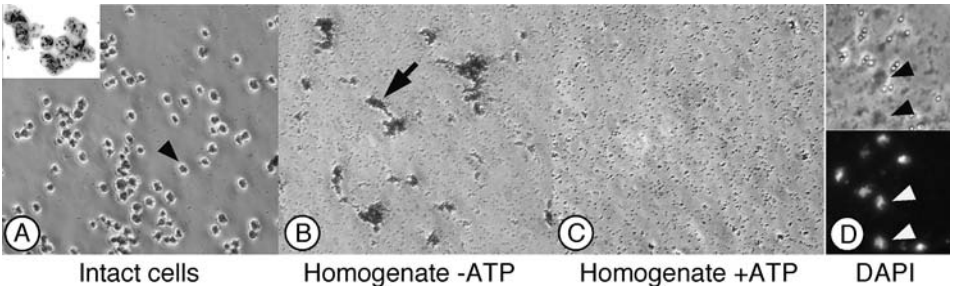


Fig. 2. Optimal cell breakage and effect of ATP incubation. Phase contrast pictures of cells before **(A)** and after (**B, C, D** upper panel) six to eight passages through a ball homogenizer. The cell suspension and homogenate were dropped on a glass slide and covered with a cover slip. Pictures were taken with an Axiophot 2 and a 20x objective. Optimal cell breakage is obtained when 95% of the cells have been lysed and have lost their refractile appearance (compare **A** to **B/C**). The contrast-inverted picture reveals the presence of beads inside the cells (**A**, small panel). ATP treatment in the cold releases phagosomes from enmeshed organelles. Aggregates present in **B** are absent from **C** (arrows). The intactness of the nuclei (arrowheads) is also essential to avoid aggregation of the organelles around free chromatin. As illustrated here by DAPI staining of a homogenate **(D)**, ball homogenization preserves the nuclei from breakage.

of the beads, generating a linear correlation between scattering and bead concentration in a range between $2 \times 10^7$ and $2 \times 10^8$ beads/mL ***(15)***.

22. The 14.95 mL of phagosome suspension are further diluted with membrane buffer up to 37 mL and pelleted in the SW 28 again for 50 min at 28,000 g (100,000*g* avg).
23. Resuspend the pellet in storage buffer. Adjust the amount of buffer according to the scattering measurements so as to reach the same concentration of phagosomes in all samples.
24. Snap-freeze aliquots in liquid nitrogen and store at –80°C until further use. Samples are routinely analysed by SDS-PAGE (*see* **Fig. 3**).

### *3.2. Immunofluorescence on Purified Phagosomes*

Purified phagosomes are taken from **step 21**. The amount of phagosomes to be used per coverslip is determined as follow: $300/OD_{600} = n$, where $n$ is the volume in µL of phagosome suspension per cover slip (*see* **step 21** for $OD_{600}$ measurements). Adjust the volume up to 0.5 mL per cover slip with HESES buffer. Place the coverslips in a 24-well plate and add 0.5 mL of the phagosome suspension per well. Centrifuge 7 min at 1500 g in a clinical centrifuge with multiwell plate swinging buckets. Wash the poly-L-lysine–coated coverslips

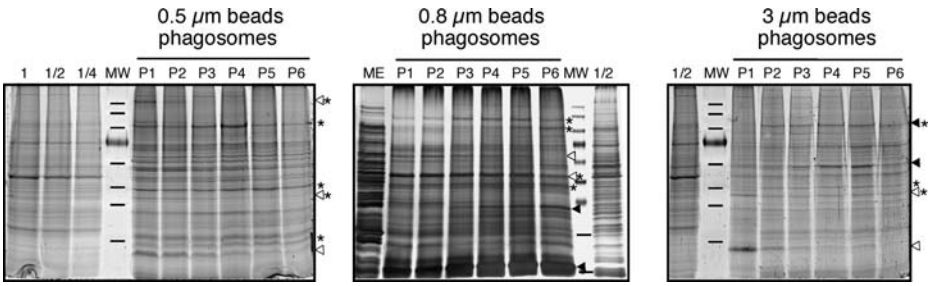


Fig. 3. Characterization of the phagosomal fractions. The fractions obtained after a pulse-chase feeding experiment with latex beads of, respectively, 0.5, 0.8, and 3.0 µm in diameter have been separated on a 10% SDS-PAGE. The gels containing the 0.5- and 3.0-µm samples are Sypro Ruby stained (BioRad, according to the manufacturer). The gel containing the 0.8-µm samples is silver stained (GE Healthcare, according to the manufacturer). Total membrane extracts (ME) and/or total cell extract at different dilutions (1 [$0.5 \times 10^6$ cells/lane], ½, ¼) have been loaded beside the phagososme fractions. The maturation process can be monitored through the appearance/disappearance (open/closed arrows) of bands. Comparison with total cell lysate, total membrane extract shows the unique band pattern of the phagosome fractions. Differences in composition and temporal profiles (asterisks) are visible between phagosome samples obtained by ingestion of beads of different sizes.

by dipping them once into SSB and place them back in a 24-well plate. The phagosomes are fixed for 30 min at room temperature with a PFA/picric acid solution, washed once with PBS and placed for 30 min with 100 m*M* glycine in PBS in order to quench the free aldehyde groups, and blocked for 1 h in 2% FCS in PBS. The coverslips are then processed as for standard immunofluorescence (*see* **Fig. 4**). Alternatively, a rapid freezing fixation procedure in methanol at –85°C can be used ***(19)***.

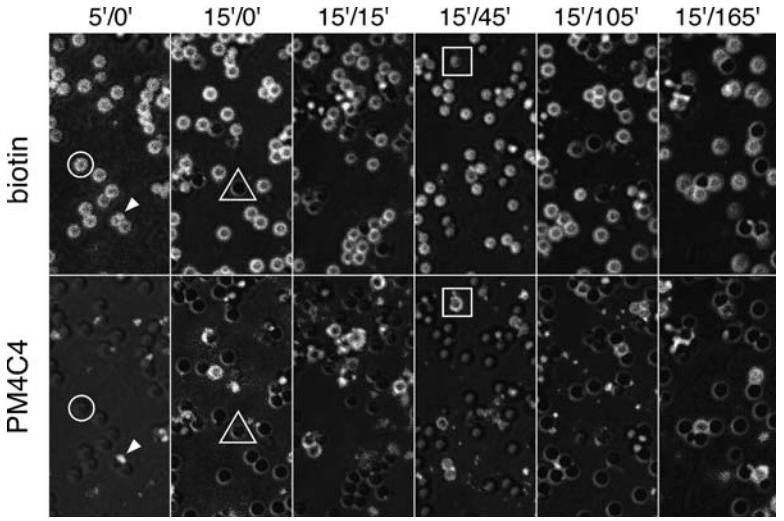


Fig. 4. Visualization of membrane trafficking at the level of single phagosomes. Plasma membrane proteins were biotinylated prior to latex bead uptake and phagosome purification (*see* **Note 1**). Phagosomes isolated at the indicated time points of a pulse/chase feeding experiment were centrifuged on poly-L-lysine–coated coverslips and processed for immunofluorescence. Biotinylated membrane proteins were revealed by streptavidin-TRITC (upper row) and PM4C4 recognized by a monoclonal antibody ***(20)*** followed by a goat–anti-mouse Alexa488-coupled IgG. Shortly after bead ingestion (5' and 15'), the vast majority of phagosomes contain biotinylated plasma membrane proteins, but during maturation (from 15'/15' to 15'/165'), while this marker is retrieved to the surface, it is replaced by nonbiotinylated PM4C4 from later endosomes. Interestingly, the transition from one to the other marker appears not to be gradual but abrupt, reflecting the very intense trafficking to and from maturing phagosomes ***(1)***. Arrowhead: a patch of PM4C4 on a biotin-positive phagosome; circle: a biotin-positive, PM4C4-negative phagosome; triangle: an unstained bead; square: a PM4C4-positive, biotin-negative phagosome. Images were taken with a Leica SP2 confocal microscope. The fluorescence and phase signals were superimposed to allow easier visualization of all beads.

## 4. Notes

1. For phagocytosis of different bead sizes, we used approximately the same bead surface–to–cell surface ratio (360 $\mu m^2$ of bead/cell). But to compensate for the low uptake of 3.0-µm beads, we decided to double the offered bead surface. Therefore, the calculated volumes of the supplied bead suspensions are 2.5 mL of 0.5-µm beads ($14.62 \times 10^{11}$ beads/mL, LB-5, Sigma), 4 mL of 0.8-µm beads ($3.57 \times 10^{11}$ beads/mL, LB-8, Sigma), 10 mL of 3.0-µm beads ($0.20 \times 10^{11}$ beads/mL, LB-30, Sigma). As bead size increases, the offered bead surface does not increase linearly. Therefore, the used bead to cell ratios are, respectively, 460 beads/cell (0.5 µm), 180 beads/cell (0.8 µm), and 25 beads/cell (3.0 µm).
2. The preincubation step is performed to maximize the number of latex beads adsorbed onto the cells. If desired, it is also possible to concomitantly biotinylate plasma membrane proteins *(7)*, using 30 mg of Imuno-Pure NHS-LC-Biotin from Pierce (Rockford, IL). The high pH is thus necessary for efficient biotinylation, but the cells should not be kept for extended periods of time at this pH.
3. This pulse-chase time course has been shown to cover the entire phagosome maturation process ***(1,7)***. The time points of 5 and 15 min are extremely dynamic and cover the first signaling, cytoskeleton, and membrane trafficking phases. The 5-min sample is processed and homogenized before the 15-min chase is stopped, whereas the other four pellets are processed for the chase period as described in **step 12**. SDS-PAGE and Western blot analysis show the enrichment of the fractions in phagosomal markers and highlight the progression of the maturation process and the unique identity of phagosomes at each stage (*see* **Fig. 3**).
4. To start phagocytosis, a 20-fold excess (100 mL) of HL5c at 22°C is added to the sample, instantaneously raising the temperature and thus generating a sharp synchronous wave of uptake (*see* **Fig. 1**). The uneven distribution of the cell/beads suspension (5, 6, or 7 mL) ensures an equal yield for all the time points.
5. To stop the phagocytic process, the 100-mL cell suspension is poured into 3.3 volumes of ice-cold SSB and immediately centrifuged. The use of 120 m*M* sorbitol is crucial to increase bead flotation and nevertheless not perturb cell pelleting. These cycles of resuspension/centrifugation are sufficient to eliminate the vast majority of uningested beads.
6. Homogenization using a ball homogenizer results in homogeneous cell breakage together with preservation of organelle integrity. The osmolarity of the buffer, the void clearance, and the number of passages is optimized to yield about 95% cell breakage (*see* **Fig. 2**). Higher ratios of cell breakage only result in increased nuclear lysis and contamination of cytoplasm with chromatin.
7. Use of a physiological concentration of ATP for a few minutes in the cold avoids artefactual formation of a rigor mortis meshwork of actin and myosins that was shown to entrap contaminating organelles *(7)*. Omission of ATP has visible consequences as it prevents fast and clean flotation of the phagosomes and promotes co-fractionation of an actin–myosin II meshwork (as judged by Coomassie staining of the resulting fractions after SDS-PAGE). This can only partially be compensated by longer centrifugation.

8. If the centrifugation time is reduced to below 2 h 30 min, banding is not as sharp, and phagosome yield is about 20–30% lower. The rotor has to stop without brake to avoid vibrations. Alternatively, centrifugation can be performed overnight.
9. This dilution is necessary to decrease the sucrose concentration and thus decrease the buoyancy of the latex beads phagosomes. We determined that scattering was caused only by the latex beads and not contaminating particles and/or organelles by treating the latex beads phagosome sample with SDS and pelleting. The resulting "clean" latex beads yielded the same scattering values as when residing inside phagosomes.

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