22

Large-Scale Purification of Latex Bead Phagosomes from Mouse Macrophage Cell Lines and Subsequent Preparation for High-Throughput Quantitative Proteomics

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Summary

Phagocytosis involves the engagement of a diverse array of cell surface receptors whose signals must be integrated on the membrane of the forming phagosomal cup. This method enables the quantitative proteomic analysis of phagosome fractions derived from phagocytes stimulated under two different conditions, thus allowing the complexity of phagosomal signaling to be analyzed in terms of the quantitative changes in phagosomal fraction protein content.

Key Words: Phagosome purification; macrophage; isotope coded affinity tag; liquid chromatography tandem mass spectrometry; sucrose gradient; RAW 264.7.

1. Introduction

The process of phagocytosis enables immune cells to interrogate the environment around them. This interrogation involves a plethora of cell surface pattern recognition receptors, opsonin receptors, and receptors that recognize self ligands such as those presented by apoptotic cells ***(1)***. The integration of these signals occurs on the phagosomal membrane and leads to molecular decisions that determine whether inflammatory or anti-inflamatory mediators will be released, whether antigen presentation will be performed, and what path phagosomal maturation will take ***(2,3)***.

The protocol described here began as a method to purify latex bead–containing phagosomes on sucrose step gradients using the density of latex as a

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way to float phagosomal membranes away from other cellular membranes ***(4)***. Proteomic analysis has been used to identify the protein content of latex bead–isolated phagosome fractions and provides the standard from which to measure new techniques ***(5)***. With the advent of quantitative proteomic techniques, such as the use of cleavable ICAT (isotope coded affinity tag) reagent, the relative abundance of two sister peptides labeled differentially with heavy and light markers (identified by the collision dissociated peptide fingerprint) can be determined from the original ions' peak intensities which differ by 9 mass units ***(6,7)***. In this way the relative abundance of many peptides from a complex mixture of two samples can be determined. The ability to determine how different activation states of the macrophage affect the protein makeup of the phagosome has become a reality.

The protocol to purify latex bead–containing phagosomes from mouse macrophage cell lines was adapted in order to maximize the yield of phagosomal protein from one purification experiment (though multiple repetitions are required to yield one mg of total protein) and enable high-throughput proteomic techniques (which are best performed with mg quantities of protein) to be used as a method to ask questions about how different opsonins, pathogen-associated patterns, or inflammatory mediators might change the protein content of the phagosome. It consists of four major steps: the presentation of IgG opsonized latex beads to macrophages in an efficient and large-scale procedure, the purification of the latex bead phagosomes by isopycnic centrifugation with sucrose step gradients, labeling of the proteins found in the purified fraction with cleavable ICAT reagent, and separation of labeled peptides from this complex mixture by strong cation exchange chromatography and avidin affinity chromatography in order to reduce the complexity of each fraction.

Due to space limitations and the complexity of this process, the method for determination of the relative abundance and identity of sample peptides by LC-MS/MS and the use of software tools designed to enable high-throughput analysis of the data with statistical confidence will not be covered in this protocol. The end result will be purified peptide fractions labeled with heavy and light ICAT reagent that can be analyzed by a variety of mass spectrometry platforms. References will be given for those who are interested in the specific details of the platform used by the authors.

2. Materials

2.1. Latex Bead Preparation

1. Nonpyrogenic Eppendorf tubes.
2. Latex beads (0.82 μm, 5081A), (Duke Scientific Corporation, Palo Alto, CA).
3. Mouse monoclonal anti-Ova (A 6075 Sigma Aldrich, St. Louis, MO).

4. 5X crystalline chicken ovalbumin (32467 Calbiochem) other highly purified sources acceptable.
5. Bead wash buffer (BWB), 25 m*M* 2-(N-morpholino) ethanesulfonic acid (MES) pH 6.1, 0.05% Triton X 100 (TX100).
6. Ova binding buffer (OBB), 25 m*M* MES pH 6.1, 0.1 % w/v ovalbumin, 0.05% TX100, 0.05% NaN_3; filter sterilize with a 0.2-µm low-protein-binding filter; can be stored at 4°C for multiple months.
7. Final resuspension buffer, 25 m*M* MES pH 6.1.
8. Limulus amoebocyte assay (50-647U, Cambrex).
9. High-speed desktop centrifuge for 1.5-mL Eppendorf tubes.
10. Rotator or shaker in a refrigerator.

2.2. RAW 264.7 Cell Preparation and Bead Presentation

1. 125- or 250-mL Erlenmeyer flasks heat treated at 180°C for 4 h to destroy endotoxin.
2. RPMI 1640 with L-glutamine (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Ogden, UT) and penicillin/streptomycin (full medium).
3. Full medium with 25 m*M* hydroxyethyl piperazine ethane sulfonate (HEPES) (from 1 *M* HEPES buffer stock).
4. RAW 264.7 cells (ATCC).
5. 15-cm Petri dishes, non-tissue culture treated, sterile, nonpyrogenic.
7. 15-, 50-, and 250-mL conical cell culture centrifuge tubes.
6. Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS), Cellgro.com.
7. DPBS, 1.5 m*M* ethylenediaminetetraacetic acid (EDTA), filter sterilize.
8. DPBS, 120 m*M* sorbitol, filter sterilize.
9. Refrigerated benchtop centrifuge for cell culture applications.
10. 37°C shaking incubator.

2.3. Cell Disruption

1. Nitrogen decompression bomb (4639, Parr Instrument Company).
2. N_2 gas tank.
3. Homogenization buffer (HB) 20 m*M* HEPES pH 7.4 (use KOH to titrate pH), 0.25 *M* sucrose, filter sterilize, and store at 4°C.
3. Homogenization buffer with protease inhibitors and Ca (HB + PI + Ca) 20 m*M* HEPES pH 7.4, 0.25 *M* sucrose, 1x protease inhibitors cocktail (Complete Mini EDTA free, 11 836 170 001, Roche); dissolve one tablet in 10 mL of buffer, 0.2 m*M* $CaCl_2$, add protease inhibitors on day of use, and store on ice or at 4°C.
4. 15% sucrose w/v in 20 m*M* HEPES pH 7.4; *see* **Subheading 2.4.** for instructions on sucrose solutions.

2.4. Phagosome Purification by Sucrose Step Gradient

1. Beckman Ultra-Clear 1 × 3.5 in centrifuge tubes (344058).
2. Sucrose, molecular biology grade.
3. 20 m*M* HEPES pH 7.4 (use KOH to titrate pH), filter sterilize and store at 4°C.
4. 62% sucrose w/v, 20 m*M* HEPES pH 7.4 (use KOH to titrate pH), filter sterilize and store at 4°C.
5. Using the 62% sucrose and 20 m*M* HEPES solutions above, make enough 40, 35, 25, 15, and 10% sucrose solutions to perform the experiment and equilibrate to 4°C.
6. Beckman L8-55M ultracentrifuge.
7. Beckman SW-28 swinging bucket rotor.
8. ICAT labeling buffer: 200 m*M* Tris-HCl pH 8.3, 6 *M* urea, 5 m*M* EDTA, 0.1% SDS; make fresh on day of use.

2.5. Assessment of Phagosome Purity

1. Antibodies: anti-Lamp1 (1D4B rat monoclonal antibody (mAB), University of Iowa) anti-rab5 (BD, mouse mAB, 610282), p62 nuclear porin (mAB414, mouse mAB, Covance), GM130 (BD, 610822, mouse mAB), anti-mouse IgG horseradish peroxidase (HRP, Amersham), anti-rat IgG HRP (Pierce).
2. Mini gel sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) apparatus and electroblotting apparatus.
3. 4x SDS-PAGE loading buffer: 0.25*M* Tris-HCl pH 6.8, 8% SDS, 30% glycerol, 0.02% bromophenol blue; add 10% β-mercaptoethanol before use.
4. Acrylamide: Bis 37.5:1.
5. Ammonium persulfate, make a fresh10% w/v solution in ddH_2O.
6. Upper-Tris buffer: 0.5 *M* Tris-HCl pH 6.8, 0.3% SDS.
7. Lower-Tris buffer: 1.5 *M* Tris-HCl pH 8.8, 0.4% SDS.
8. Towbin buffer: 25 m*M* Tris, 192 m*M* glycine, 20% v/v methanol.
9. Methanol.
10. Polyvinylidene fluoride transfer membrane (PVDF).
11. 3mm filter paper.
12. Tris-buffered saline with Tween 20 (TBS-T): 10 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 0.05% Tween 20 w/v.
13. TBS-T with 5% nonfat dry milk w/v.
14. TBS-T with 5% bovine serum albumin (BSA) w/v.
15. ECL plus detection reagent (Amersham).
16. Kodak Biomax XAR film.
17. Silver Stain Plus kit (BioRad).

2.6. Protein Labeling with Isotope Coded Affinity Tag

1. BCA protein assay (Pierce).
2. Tris(2-carboxyethyl)phosphine (TCEP).

3. Cleavable ICAT reagent (Applied Biosystems).
4. Dithiothreitol (DTT).
5. 50 m*M* Tris-HCl, pH 8.3.
6. Sequencing Grade Modified Trypsin (V5113, Promega).
7. 0.2-μm low-protein-binding syringe filter.

2.7. Cation Exchange Chromatography and Avidin Affinity Chromatography

1. High-performance liquid chromatography (HPLC) system with ability to make linear gradients and track UV absorbance at 214 nm.
2. Two-mL sample loop for HPLC system.
3. Polysulfoethyl A column, 2.1 mm × 200 mm, 5-μm particles, 300 Å pore size (PolyLC, Inc.).
4. Strong cation exchange buffer A (SCX buffer A), 25% acetonitrile (volatile, flammable, hazardous), 5 m*M* KH_2PO_4, pH 3.0.
5. SCX buffer B, 25% acetonitrile, 5 m*M* KH_2PO_4, pH 3.0, 350 m*M* KCl.
6. 5% Phosphoric acid.
7. 100 m*M* Phosphate buffer pH 10.0.
8. 2x PBS pH 7.2.
9. ICAT avidin affinity buffer pack (4326740, Applied Biosystems) good for 50 purifications.
10. One-mL glass Hamilton syringe with a blunt needle.

3. Methods

3.1. Latex Bead Preparation

1. Prepare enough latex beads for a 1:50 dilution with the resuspended RAW 264.7 cells. (*see* **Subheading 3.2.** to have RAW 264.7 cells prepared for the day of the experiment).
2. Add 750 μL of beads/1.5 mL Eppendorf tube. Centrifuge at 10,000*g* for 2 min, remove supernatant and resuspend with 1 mL of BWB, repeat centrifugation step, remove supernatant, and resuspend with OBB and rock or rotate overnight at 4°C (shorter incubation times are not sufficient). Alhough inclusion of surfactant in the buffers helps to reduce bead compaction upon centrifugation, rapid resuspension of the beads is best performed by digging with the pipet tip while pipetting up and down.
3. Wash two times with BWB with centrifuge at maximum *g* force for 5 min.
4. Resuspend the beads in BWB with a 1:1000 dilution of anti-ova antibody and rock or rotate at room temperature for 30 min (*see* **Notes 1** and **2**).
5. Wash once with BWB and resuspend with 25 m*M* MES pH 6.1. Perform final wash after the cells are prepared for incubation with beads and equilibrated to 37°C in a shaking incubator.

3.2. RAW 264.7 Cell Preparation and Bead Presentation

1. Expand RAW 264.7 cells to yield the number of cells required for the experiment. Use 5-6 × 10^8 cells/sucrose gradient. To obtain this number of cells requires 10 15-cm Petri dishes grown to high density (*see* **Note 3**). An SW-28 rotor has six buckets, and thus six gradients can be run per rotor, this requires 60 confluent 15-cm dishes be ready on the day of the experiment. We seed the cells from confluent, healthy (not a lot of floating cells) plates at 1:5 into 12 plates and allow them to grow for 3 d, then expand 1:5 into 60 plates and allow the cells to grow for 2 d prior to the experiment, thus optimizing the density and health of the cells.
2. Remove growth medium and wash the cells **gently** with 10 mL of DPBS. Remove DPBS and add 10 mL DPBS with 1.5 m*M* EDTA. Allow the cells to incubate at room temperature while all plates are washed and filled with DPBS, 1.5 m*M* EDTA. Remove cells by vigorously pipetting up and down with an electric pipet aid.
3. Collect the cells by centrifugation at 250*g* (average) for 10 min and resuspend once in DPBS. Collect the cells by centrifugation again and resuspend in prewarmed (37°C) full growth medium with 25 m*M* HEPES at 1 × 10^7 cells/mL. Place cells in a sterile, heat treated 250-mL Erlenmeyer flask, shake at 250 rpm in a 37° C incubator.
4. Present prepared beads as a 1:50 dilution (1mL of prepared beads for 50 mL of prepared cells) into the suspended cells and replace flask into the 37°C shaking incubator. Incubate with shaking for the desired period of time (*see* **Note 4**).

3.3. Cell Disruption

1. Collect cells by centrifugation at 250*g* and wash with cold (4°C) DPBS with 120 m*M* sorbitol. The sorbitol increases the density of the solution and helps to remove uninternalized beads, but is not necessary. Repeat until supernatant appears clear, usually two to three washes.
2. Wash one time with HB. Resuspend half of the cells in 15 mL HB + PI + Ca and keep on ice. Repeat for the other half. We often performed the experiment where 30 plates were treated with interferon (IFN)-γ for 18 h and the others were unstimulated. If all 60 plates are used as a control or experimental, then break the cells in two separate 15-mL aliquots. Conditions can be worked out for different volumes and numbers of cells.
3. Load resuspended cells into a prechilled cell disruption bomb. Follow the manufacturer's procedures and pressurize the bomb with N_2 gas to 400 psi (*see* **Note 5**). Allow the bomb to incubate on ice for 10 min. Slowly release the pressurized contents of the bomb by turning the release valve counterclockwise until the cell lysate begins to run through the release tube into a 50-mL conical tube. Carefully, watch the released volume as pressurized gas will follow the last contents of the bomb and can blow the cell lysate out of the 50-mL conical tube. This can be avoided by releasing the lysate slowly and holding the angle of the

release tube such that released gas will not directly blow down into the tube but against a side wall.

The pressure and time of incubation in the bomb should be determined empirically by visual inspection of the cell lysate under a tissue culture microscope. Stain the homogenate with Trypan blue and note the number of unbroken cells and the state of the nuclei in a counting chamber. The optimal pressure will disrupt more than 98%of the cells, but all the nuclei will appear intact.

4. Layer the cell homogenate over 15% sucrose (10 mL) in a 50-mL conical tube and centrifuge at 525*g* (avg) for 30 min at 4°C without the brake. The nuclei can also be spun down without layering over sucrose, but the pellet is often difficult to see.
5. Remove the supernatant, which contains the latex bead phagosomes. Save an aliquot (100 µL) of the supernatant for Western blot and/or silver stain analysis. This is the postnuclear supernatant (PNS).

3.4. Phagosome Purification by Sucrose Step Gradient

1. Preequilibrate all solutions on ice and perform the rest of the procedure on ice.
2. Mix the phagosome sample with 62% sucrose to give a final (c) of 40% sucrose.
3. Layer sequentially in a Beckman Ultra Clear SW-28 rotor tube: 1 mL 62% sucrose, 8–10 mL phagosome sample at 40% sucrose, 8 mL 35% sucrose, 12 mL 25% sucrose, 7 mL 10% sucrose. The final volume is 38 mL; the tube will hold 40 mL.
4. Assure that the tubes are balanced and centrifuge in a swinging bucket rotor (Beckman SW-28) at 100,000*g* (rmax) for 1 h.
5. Carefully remove the tubes from the rotor and collect the phagosome fraction from the 10–25% sucrose interface using a transfer pipet. The bead layer will be clearly visible.
6. Mix the collected fraction with 20 m*M* HEPES pH 7.4 to 38 mL and centrifuge again in an SW-28 rotor at 100,000*g* (rmax) for 30 min. Fractions may be pooled, but the final concentration of sucrose must be 10% or less or the beads will not pellet. Much of the 25% layer often gets aspirated with the beads.
7. Carefully remove the tubes from the rotor and aspirate the supernatant completely.
8. Resuspend the pellet with an appropriate volume of ICAT labeling buffer, or 2x SDS-PAGE buffer, if the phagosome fraction will only be used for Western blot analysis.

3.5. Assessment of Phagosome Purity

The easiest method of assessing phagosome fraction purity is by Western blot analysis. The phagosome fractions should be highly enriched with lamp-1, cathepsin D, and rab proteins such as Rab7 ***(8)***. Determinations of which antibodies to use must be made based on the nature of the fraction (a very short 5-min pulse with latex beads will not result in a phagosome fraction

enriched with lamp-1 or cathepsin D, but should be enriched with rab5). In our project, we were interested in IFN-γ–induced proteins that might be localized to phagosomal membranes. One candidate was Lrg-47 ***(9)***, so we used an antibody to Lrg-47 to demonstrate enrichment in our phagosome fraction from INF-γ treated cells (*see* **Fig. 1**). Other data had suggested that Lrg-47 was localized to Golgi membranes in macrophages, so we demonstrated the absence of contamination with Golgi membranes by using antibodies against Golgi membrane markers such as GM130 ***(10)***. The nature of the questions being asked will dictate which proteins one should be concerned about when assessing purity. Western blot analysis is also a great way to confirm that protein hits by mass spectrometry are truly enriched in the phagosome fraction.

1. Pour a 10% polyacrylamide mini gel with a stacking gel using the discontinuous buffer system of Laemmli ***(11)***.
2. Prepare the samples that have not been resuspended in SDS-PAGE buffer by mixing the samples with 4x SDS-PAGE buffer (including 10% β-mercaptoethanol) to yield 1x SDS-PAGE buffer in the final sample. Boil the samples for 5 min before loading on to the gel.
3. Load 5–10 µg of protein from the PNS fraction and the phagosome fraction and run the gel until the desired molecular weight markers are near the bottom of the gel.
4. Generate Western blots by electoblotting the gel to PVDF membrane (must be wet with methanol) using Towbin buffer in a mini-gel electroblotter for 1 h at 100 V constant voltage ***(12)***.
5. Block the membrane with 5% milk in TBS-T for 1 h.

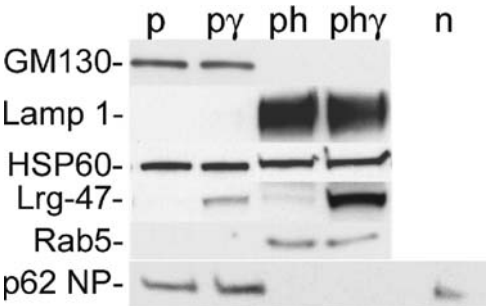

Fig. 1. Assessment of purified phagosome fraction purity. 5 µg of protein (pns) from cells stimulated or not with 30 units/mL IFN-γ purified latex bead phagosome fractions after a 1-h pulse, and nuclear fractions from those cells were separated by SDS-PAGE and Western blotted. Lanes: pns (p), phagosome fraction (ph), nuclear fraction (n); fractions from IFN-γ–treated cells are denoted by a γ symbol. Western blots were decorated with abs as described in **Subheading 2.5**.

6. Wash one time with TBS-T.
7. Add primary antibody in TBS-T with 5% BSA and incubate overnight with agitation at 4°C.
8. Wash the blot with TBS-T three times for 5 min.
9. Add the secondary antibody in TBS-T with 5% milk and incubate at room temperature with shaking for 1 h.
10. Wash the blot 3 × 5 min and leave in TBS-T to keep wet.
11. Incubate the blot for 5 min with ECL plus detection reagent according to the manufacturer's protocol.
12. Expose the blot to Kodak Biomax XAR film for various times and develop the film with an automated film processor.
13. Alternatively, silver stains can be performed with the gel after SDS-PAGE using the BioRad Silver Stain Plus kit according to the manufacturer's protocol. When performing silver stains, it is imperative to use clean glass plates in order to reduce background staining.

3.6. Protein Labeling with Isotope Coded Affinity Tag

Cleavable ICAT reagent is a thiol reactive molecule that is manufactured in heavy ($9 \times C^{13}$) and light ($9 \times C^{12}$) forms with an acid-cleavable biotin group *(7)*. The ICAT buffer denatures the proteins in the sample, which are then reduced, giving the ICAT reagent access to reduced cysteine residues. This enables two samples—in our case, one collected from IFN-γ–stimulated cells and one collected from unstimulated cells—to be labeled in separate reactions with heavy and light reagents and then be combined and processed for mass spectrometry analysis.

1. Determine the concentration of protein in your samples with a BCA or Bradford assay. Follow the manufacturer's instructions and dilute the BSA standard with ICAT buffer to make a standard curve and assure there are no inhibitory components in the buffer. This protocol works very well with 1 mg of total protein, 0.5 mg from each sample. Less protein will not yield as good a result, and much less will not yield any reasonable result by this protocol. More protein may be labeled, but the column referred to for cation exchange in this protocol can only bind up to 5 mg of protein and runs optimally with 1 mg of total protein.
2. Reduce the samples by adding TCEP to a final concentraton of 5 m*M*. TCEP in H_2O is very acidic, but the amount of Tris in the ICAT buffer used in this protocol is high in order to account for this step.
3. Check the sample pH; it must remain between 8.0 and 8.5 for ICAT labeling to proceed. Adjust the pH with ammonium bicarbonate if necessary. The sample pH can be checked by spotting a small amount (1 μL) on pH paper with an appropriate range.
4. Incubate the samples for 30 min at 37°C.

5. Remove 4 µg of protein from each sample to use as a control for labeling efficiency and digestion efficacy by silver stain.
6. Estimate the moles of cysteine residues in each sample. Assumptions: average protein 50 kDa, average of six cysteines per protein. Formula: *x* g protein/ 50,000 g/mol = mol protein × 6 = mol of cyteine residues.
7. Determine the amount of ICAT reagent in nmol that will be necessary to achieve a 1.2 m*M* concentration in the samples. Determine how many nmol of ICAT reagent are necessary to achieve a twofold molar excess of ICAT reagent to cysteine residues. Use the greater amount. Each tube contains 175 nmol of ICAT reagent. Try to label both samples under very similar conditions. Use the same amount of protein; try to use the same volume for the reaction.
8. Determine the number of tubes required to label the samples, round up to the nearest full tube. First, spin the tubes in a desktop centrifuge to collect the reagent powder in the bottom. Add the reduced sample to the tube, vortex, spin, and add the sample to any additional tubes necessary to achieve the predetermined molar ratio. Label one fraction (control) with light ICAT reagent and the other fraction (stimulus) with heavy ICAT reagent.
9. Incubate the tubes for 2 h at 37°C with agitation in the dark.
10. Stop the reaction by adding a 10-fold molar excess of DTT to the reaction tube, vortex, and incubate at room temperature for 5 min.
11. Remove 4 µg of protein from each sample to use as a control for labeling efficiency and digestion efficacy by silver stain.
12. Combine the light and heavy samples and dilute the combined sample sixfold with 50 m*M* Tris-HCl pH 8.3; check the final pH to ensure it is at 8.3.
13. Add trypsin 1:50 w/w with respect to µg of protein and let the sample digest overnight at 37°C with agitation.
14. Remove 4 µg of protein from the combined sample to use as a control for labeling efficiency and digestion efficacy by silver stain.
15. Confirm by SDS-PAGE and silver stain (*see* **Subheading 3.5.**) that the sample has been labeled and digested, if the digestion is incomplete, return the sample to 37°C incubation. When the digestion is complete, continue with the next step.
16. Centrifuge the sample in a desktop refrigerated centrifuge at 2500*g* (rmax) for 1.5 h, carefully remove the supernatant, and filter out the remaining latex beads with a 0.2-µm low-protein-binding syringe filter.

3.7. Cation Exchange Chromatography and Avidin Affinity Chromatography

1. Prepare the cation exchange column as per the manufacturer's instructions.
2. Dilute the sample 1:1 with SCX buffer A and verify that the sample pH is less than or equal to 3.0. If the pH is not less than or equal to 3.0, titrate with 5% phosphoric acid to achieve the appropriate pH.

3. Wash the column with 5 mL of SCX buffer A at 0.2 mL/min.
4. Using a 2-mL sample loop, load 2 mL of sample sequentially and wash with SCX buffer A until the UV baseline returns to zero, approximately 3 mL. Continue to load the entire sample.
5. Run a linear gradient to 25% SCX buffer B for 25 min and collect 1-min fractions.
6. Run a linear gradient to 100% SCX buffer B for 50 min and collect 1-min fractions.
7. Wash the column with 8 mL of SCX buffer B.
8. Wash the column with 8 mL of SCX buffer A.
9. Prepare the column for storage as per the manufacturer's recommendations.
10. Neutralize the fractions which contain peptides as determined by the 214-nm trace with 0.4 volumes of 100 m*M* $NaPO_4$ pH 10.0. Add 1 volume of 2x PBS pH 7.2.
11. Perform Avidin affinity chromatography and acid cleavage of the ICAT reagent according to the manufacturer's instructions for each neutralized fraction.
12. The fractions are now ready to be analyzed by a mass spectrometry platform. We further fractionate the avidin purified fractions by reverse phase chromatography in line with MS/MS using an LCQ-Deca ion-trap mass spectrometer (Thermofinnigan) ***(13)***. The recorded data are searched against a database of mouse proteins maintained in house by SEQUEST software ***(14)***. The results are then submitted to a suite of analysis programs that provide a probability that the search results are correct at the peptide level (PeptideProphet) and determine a combined probability score and a best matched list of proteins found in the sample (ProteinProphet) ***(15–17)***. Information about the relative abundance of heavy- and light-labeled peptides is determined by XPRESS software ***(18)***, and averages are generated for all peptides determined to belong to a protein by ProteinProphet. The final combined results from two separate labeling experiments of one latex bead phagosome purified sample obtained using IFN-γas a differential stimulus are shown in **Table 1**.

Table 1
Combined Results from LC-MS/MS Analysis of Two Labeling Experiments (each 1 mg protein total)

	Total peptides	Total proteins	Differential proteins
Number	7932	582	11
p-Value/range	0.05[a]	0.5[b]	(1.7–16.7)[c]

[a] Peptide *p*-values ≥ 0.05, determined by PeptideProphet.
[b] Protein *p*-values ≥ 0.5, determined by ProteinProphet.
[c] Range for average ratio of heavy to light peptides identified in 11 proteins, determined by XPRESS.

4. Notes

1. RAW 264.7 cells are very sensitive to lipopolysaccharide (LPS) and other bacterial contaminants. All reagents should be screened for LPS by use of the Limulus Amoebocyte Lysate (LAL) assay. All glassware must be baked in order to destroy LPS. Antibodies are often highly contaminated with LPS. The antibody used in this step showed no detectable LPS by LAL, but a rabbit anti-ovalbumin antibody was highly contaminated and required clean-up with LPS-removing chromatography beads (Acticlean Etox, Sterogene Bioseparations, Inc.).
2. Confirmation that the beads are IgG opsonized can be performed by immunofluorescence with a fluorescent secondary to the opsonizing antibody. IgG opsonization increases the efficiency of bead uptake by RAW264.7 cells and also elicits an oxidative burst when the cells are prestimulated with IFN-γas measured by a luminol-based assay ***(19)***.
3. RAW 264.7 cells stick tenaciously to many tissue culture–treated flasks. The use of nontreated Petri dishes allows for rapid cell removal and reduced space requirements in an incubator, but one must be careful not to lose cells during the DPBS wash step.
4. We often load the beads for 1 h to maximize the number of internalized beads, but any pulse-chase strategy can be performed. The RAW 264.7 cells are resilient to culture in shaking suspension, but we have not performed chase periods beyond 4 h in shaking suspension. During the chase period, we dilute the cells to 5×10^6/mL in full medium with 25 m*M* HEPES.
5. Other methods of cell disruption can be used, such as Dounce homogenization, but the composition of the HB would have to be changed. We have found the nitrogen decompression method to be extremely reproducible.

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