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EM Analysis of Phagosomes

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Summary

Electron microscopy (EM) is the only technique that can combine sensitive protein-detection methods with detailed information on the substructure of cellular compartments. The purpose of this chapter is to describe some of the methods at the EM level that can be used to analyze the spatial organization of cell organelles with respect to phagosomes or vacuoles in which pathogens are sequestered, characterize the compartment in which pathogens are harbored, ie immature phagosomes, phagolysosomes, autophagosomes, and ER-derived vacuoles, to cite a few, and decipher the molecular mechanisms involved in survival of pathogens within infected host cells.

Key Words: Electron microscopy; enzyme cytochemistry; phagosomes; autophagosomes; pathogens.

1. Introduction

Intracellular pathogens have evolved a wide variety of strategies to manipulate host cell organelles and/or constituents, thus enabling them to find favorable conditions for survival and multiplication (for review *see*, e.g, **refs. *1*** and ***2***). After binding to cell surface receptors, microorganisms and particles are internalized by phagocytosis into membrane-bound compartments called phagosomes. Under normal conditions, the newly formed phagosomes intermingle contents and membrane with the successive compartments of the endocytic pathway (early endosomes, late endosomes, lysosomes) through a complex series of fusion and fission events. As they are processed into phagolysosomes, they undergo gradual modifications by specific addition and removal of membrane constituents. In addition, they become acidified due

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to the vacuolar proton pump ATPase located in the membrane and acquire toxic constituents, including hydrolases, which will ultimately destroy bacteria. One of the major strategies used by endoparasites, but by no means the only one, is to modulate these interactions. A wide variety of situations have been described. Pathogens can (1) use the acidic and hydrolase-rich phagolysosomal environment to survive and multiply or (2) avoid the cytolytic environment of the phagolysosome by preventing phagosome maturation and fusion with lysosomes at different steps of the endocytic pathway. Mycobacteria, for example, do not inhibit phagosome–lysosome fusions directly but rather affect the preceding step of phagosome maturation without which fusion with lysosomes cannot occur (reviewed in **refs. *3*** and ***4***). Other pathogens escape the endocytic pathway. They can either (1) escape from the phagosome, after lysis of the phagosome membrane, and invade the cytoplasm, in which they multiply, (2) exclude, from the phagosome membrane, plasma membrane–derived constituents and/or non-plasma membrane–derived fusion-mediating factors, thereby depriving the phagosome membrane of recognition signals required for fusion with the successive compartments of the endocytic pathway, or even (3) segregate from the endocytic pathway to interact with the endoplasmic reticulum in which they multiply. Whatever the strategy used to modulate or prevent interactions with compartments of the endocytic pathway, it is of the utmost importance since it will profoundly affect drug targeting to the intracellular site of replication of pathogens and also antigen presentation. More recently, it has been shown that under certain conditions, several pathogens can be sequestered within autophagic compartments (***5***; reviewed in **ref. *6***). The fate of bacteria within autophagosomes as well as the significance of these observations remains unclear, but autophagy might have implications for the development of vaccines against such pathogens.

Our understanding of the molecular mechanisms of pathogen survival depends a great deal on the tools and techniques we use to obtain information about the pathogen and the cellular machinery. Electron microscopy methods, especially when combined with molecular biology tools (mutants, knockouts) or with drugs that modify the cellular machinery, are extremely valuable tools to unravel the cellular and molecular mechanisms that enable pathogens to survive and multiply within the host cells. EM is indeed at the highest-resolution limit of a spectrum of complementary morphological techniques. It is the only technique that can combine sensitive protein-detection methods with detailed information on the substructure of intracellular compartments and on the spatial organization of organelles within the cell, especially when the latter are in intimate contact but not necessarily interacting with one another.

Before going into the more complex and sensitive EM protein-detection approaches, it is important to master the landscape in which pathogenic bacteria survive and replicate within their host cell. I have, therefore, chosen to devote this chapter to basic conventional EM and to a number of methods, including enzyme cytochemistry, that are extremely useful in gaining insight into many relevant questions regarding pathogen survival within its host, such as:

1. What is the morphological state of bacteria, i.e., are they intact, altered, or damaged (intact as well as certain altered bacteria are usually live, and damaged ones are dead)?
2. What is the correlation between CFU counts and replication, in particular when CFU counts remain stationary; is it because bacteria have become dormant or because some bacteria are being degraded while others multiply?
3. Do bacteria replicate in a single, increasingly larger, phagosome, or does bacterial division induce separation of the phagosome?
4. Is there any specific interaction between the pathogen and the phagosomal membrane? For example, is a close apposition between the phagosome membrane and the bacterial surface all around the bacterium required for prevention of normal phagosome processing?
5. Do the phagosomes contain vesicular or tubular structures of either bacterial or cellular origin which might be important for bacterial replication?
6. What is the spatial organization of the phagosome with respect to other cellular compartments such as the endocytic organelles, the endoplasmic reticulum, or mitochondria?
7. Do phagosomes intermingle content and membrane with the successive compartments of the endocytic pathway, or do they block phagosome processing at some stage?
8. Do they escape the endocytic pathway and interact with other organelles?
9. Do bacteria lyse the phagosome membrane to reside in the cytoplasm?
10. Do bacteria induce a reorganization of the cytoskeletal network, thereby modifying the interactions with the organelles of the endocytic pathway?

Given the broad spectrum of EM methods and applications, it is not possible to describe here all the possible methods that one can use. The analysis of phagosome membrane composition by quantitative autoradiography (described in **ref. *7***) or by a variety of EM immunolocalization approaches (described in **refs. *8*** and ***9***), as well as more recently developed approaches such as electron tomography for three-dimensional imaging (*see*, e.g., **ref. *10***) will not be discussed here. Although many EM approaches have come within reach of every laboratory that is willing, and able, to invest resources, and although EM equipment has become increasingly user-friendly, consultation and training with experienced researchers who are familiar with the cell and pathogen ultrastructure is invaluable for learning EM methods, especially these more sophisticated ones, and ensuring that data are interpreted accurately.

2. Materials

2.1. Basic Conventional Transmission Electron Microscopy (TEM)

Many EM products are very toxic and even carcinogenic. Handle with care, under fume hood, and follow security measures indicated on product data sheets.

2.1.1. Products

1. Glutaraldehyde, 25% solution in water (EM grade I, Sigma, St. Louis, MO, USA). Store at –20°C. Once thawed, store at 4°C, no longer than 1 mo. Avoid putting in refrigerator containing cell culture products as fumes might affect such products. Very toxic.
2. Osmium tetroxide 4% solution in water (Electron Microscopy Sciences [EMS], Hatfield, PA, USA or Sigma). Store sealed ampullae at 4°C. Once opened, keep remainder in a clean glass vial with a glass stopper. Stable at 4°C for several months. If solution turns brown or black, discard. Fumes will blacken refrigerator: seal vial with parafilm, store in a plastic container also sealed with parafilm. Avoid putting in same refrigerator as cell culture products as fumes will affect such products. Very toxic.
3. Cacodylic acid, sodium salt, trihydrate (Sigma or EMS). Store at room temperature (RT). Toxic: contains arsenate.
4. $CaCl_2$ and $MgCl_2$ (Sigma). Prepare 1*M* solutions. Store at RT.
5. Uranyl acetate (EMS or Merck, White house Station, NJ, USA). Store powder at RT. Radioactive.
6. 5,5'-Diethylbarbituric acid, sodium salt (Fluka, Sigma-Aldrich, Buch, SG, Switzerland) for preparation of Na-veronal buffer. Store powder at RT. Very toxic. Regulations on purchase and delivery are strict in many countries. If unavailable, buy maleic acid (Sigma) for preparation of Na-H-maleate-NaOH buffer instead of Na-veronal buffer.
7. Agarose low melting point, molecular biology grade (Sigma).
8. Molecular sieve dehydrate with indicator for drying solvents (Fluka). Store at RT. Toxic, carcinogenic by inhalation.
9. Ethyl alcohol 96.2% and acetone (Carlo Erba, Rodano, Italy).
10. Spurr resin. The medium-grade TLV low-viscosity resin premix kit, from TAAB, Reading, Berkshire, UK, gives excellent results. Store at RT (*see* **Note 1**). Very toxic.
11. Lead nitrate (Fluka). Store at RT. Very toxic.
12. Other products of common use: sucrose, sodium acetate, trisodic sodium citrate, NaCl, NaOH, HCl 1 *N*.
13. Rubber policeman.
14. Microvettes CB 300 Z (Sartorius, Goettingen, Germany).
15. Microcentrifuge with fixed vertical axis or with free angle rotor.
16. Glass vials (content: 2–3 mL).

17. Flat embedding molds, single tapered ends, in clear silicone. Measures: 14 mm(L) × 5 mm (W) 4 mm(D) (from EMS). Gelatin or plastic capsules of different shapes and sizes can also be used (see with local EM facility).
18. 60°C incubator.

2.1.2. Preparation of Buffers, Dehydrating Agents, Resins, Stains for EM Processing

1. Na-cacodylate buffer 0.1 *M*, pH 7.2 + 5 m*M* $CaCl_2$ and 5 m*M* $MgCl_2$ +/– 0.1 *M* sucrose: Dissolve, e.g., 5.35 g Na-cacodylic acid in 220 mL distilled water, adjust pH to 7.2 with HCl 1 *N*, complete to 250 mL with distilled water. Add 1.25 mL $CaCl_2$ 1 *M* and 1.25 mL $MgCl_2$ 1 *M*. When necessary, add 8.5 g sucrose to maintain osmolarity. Store at 4°C. Stable for several months but beware of molds. Very toxic.
2. Na-veronal buffer, pH 6.0. (a) Solution A: dissolve 1.94 g of sodium acetate, 2.94 g of 5,5´- diethylbarbituric acid, sodium salt, and 2.8 g NaCl in 100 mL distilled water. Store at 4°C until further use. Stable for several months. (b) Na-veronal buffer: Mix 20 mL of solution A, 28 mL of HCl 0.1 *N* and 56 mL of distilled water. Adjust pH to 6.0 with a few drops of 10% acetic acid if necessary. Add 2.5 mL of $CaCl_2$ 1 *M*. Stable at 4°C for 2–3 mo. If crystals form, warm up at 37°C before use.
3. 0.05 *M* Na-H-Maleate-NaOH buffer, pH 6.0. Dissolve 0.58 g maleic acid and 0.2 g NaOH in 20 mL distilled water, adjust pH to 6.0 with NaOH, complete to 100 mL with distilled water. Store at 4°C.
4. Uranyl acetate 1% in Na-veronal buffer or in Na-H-maleate-NaOH buffer. Dissolve uranyl acetate in buffer, e.g., 250 mg in 25 mL, in a brown glass bottle (because light sensitive) and store at 4°C. Stable for 1 mo.
5. Agar 2% solution in Na-cacodylate buffer devoid of sucrose.
6. Graded series of ethanol and acetone, i.e., 25, 50, 75, 90 and 100% solutions. The 100% solution must be completely dried as follows: add the commercial solution (~96%) to a glass bottle (with a glass stopper) containing molecular sieve, mix vigorously, and let settle for at least 24 h before use. Avoid mixing before use. Stable at RT for several months.
7. Spurr resin: Follow instructions, i.e., add all three components in same vial, mix vigorously, let settle for an hour to remove bubbles, use immediately afterward or store in 5- or 10-mL syringes (use only Norm-ject devoid of black rubber stopper inside the syringe). Add caps to tip to avoid contact with air and store at –20°C. Once thawed, use content of syringe on the same day; do not refreeze.
8. Uranyl acetate 1% in distilled water for staining of thin sections. Should be available in EM facility. Otherwise, dissolve 200 mg uranyl acetate in 20 mL of distilled water. Store in 10-mL Norm-ject syringes with a 0.22-µm filter at the tip of the syringe. Light sensitive, cover syringe with aluminum foil. Stable for several months at 4°C.
9. Lead citrate for staining of thin sections. Should be available in EM facility. Otherwise, prepare the following solutions only in boiled distilled water. Solution A: lead nitrate 1 *M*, i.e., 3.3 g in 10 mL water. Solution B: trisodic sodium citrate 1 *M*, i.e., 3.57 g in 10 mL water. Solution C: NaOH 1 *N*, i.e., 1 g in 25 mL water.

To 16 mL of boiled distilled water, add 3 mL of solution B and mix gently with hand. Add 2 mL of solution A and mix gently with hand. A milky precipitate will form. Add, dropwise, 4 mL of solution C while mixing gently with hand. The precipitate must disappear completely. If not, discard and start over. Dispatch in 2- or 5-mL syringes with a 0.22-µm filter at the tip of the syringe. Stable for several months at 4°C. Discard if nonspecific fine precipitates are observed on the thin sections under the EM.

2.2. Acquisition of the Newly Endocytosed Content Marker, Horseradish Peroxidase (HRP), by Preexisting Phagosomes

In addition to all the EM products listed in **Subheading 2.1.**:

1. Peroxidase from horseradish type II, RZ ~2.0 (Sigma). Store at –20°C.
2. 3,3´-Diaminobenzidine tetrahydrochloride (DAB-HCl) from EMS. Store tablets at RT. Carcinogenic.
3. H_2O_2 30% solution, reagent ACS (EMS). Store at 4°C. Once opened, stable for 2 mo at 4°C.
4. Tris-HCl 0.1 *M*, pH 7.6. Store at 4°C, stable for several months.
5. Prepare a 10 mg/mL solution of DAB in 0.1M Tris-HCl, pH 7.6. Filter the solution and store small aliquots at –20°C. Stable for about 6 mo. Once thawed, do not refreeze an aliquot.

2.3. Acquisition of the Content Marker Bovine Serum Albumin Coupled to Gold Particles (BSA-Au) Chased to Lysosomes Prior to Phagocytic Uptake

In addition to all the EM products listed in **Subheading 2.1.**:

1. BSA coupled to gold particles (BSA-G10). Store at 4°C. Stable for several months (*see* **Note 2** for preparation).

2.4. Enzyme Cytochemistry: Staining for Acid Phosphatase

In addition to all the EM products listed in **Subheading 2.1.**:

1. Ammonium chloride 1 *M*. Stable at 4°C for several months.
2. Na-acetate, trihydrate, reagent ACS (EMS). Store at RT.
3. Acetic acid 10% solution. Store at RT. Stable for several months.
4. Glycerol 2-phosphate, disodium salt hydrate (Sigma). Store powder at RT.
5. Ammonium sulfide 20% solution in water (Sigma). Store at RT.
6. Na-fluoride (Sigma). Prepare a 10 m*M* solution in water. Store at 4°C.
7. Water for gradient elution for high-performance liquid chromatography (HPLC) (Fluka).

2.5. Enzyme Cytochemistry: Staining for Glucose 6-Phosphatase

In addition to all the EM products listed in **Subheading 2.1.**:

1. Water for gradient elution for HPLC (Fluka).
2. D-Glucose-6-phosphate, disodium salt dehydrate (Sigma). Store at –20°C.
3. Piperazine-*N*,*N*´-bis(2-ethanesulfonate) (PIPES) (Sigma). Store powder at RT.
4. Maleic acid (EMS). Store powder at RT.
5. Tris base, purissimo.

2.6. Assessment of Phagosome Acidity: DAMP Treatment and Immunolocalization

In addition to all products from **Subheading 2.1.** (except for osmium tetroxide and Spurr resin):

1. DAMP (3-(2,4-dinitroanilino)-3´-amino-*N*-methyldipropylamine) from Oxford Biomedical Research (Oxford, MI).
2. Phosphate-buffered saline (PBS).
3. Bovine serum (do not heat inactivate).
4. Rabbit anti-dinitrophenol antibodies.
5. Protein A coupled to gold particles 10 nm in diameter (PAO-G10) (*see* **Note 2** for preparation.
6. Resin: LRWhite. Purchase kit from EMS. Store kit at 4°C. Stable for several months.

3. Methods

3.1. Basic Conventional TEM (see *Figs. 1 and 2)*

Cells must be fixed, dehydrated, and embedded in resin prior to sectioning and observation under the TEM. Important: Do not let cells dry up at any stage during the entire processing of samples. Illustrations of this method can be found in **refs. *7***, ***11***, and ***12***.

1. Fixative 1 (F1): Prepare a 2.5% solution of glutaraldehyde in 0.1*M* Na-cacodylate buffer, pH 7.2 containing 5 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, and 0.1 *M* sucrose. Prepare fresh; can be kept on ice for half a day but warm up to RT before use.
2. Remove medium from cells. If necessary, wash cells with medium without serum. Do not use PBS for washes as this can introduce tiny calcium or magnesium phosphate precipitates during the fixation steps.
3. Add F1 immediately to culture dish. Fix for 1 h at RT under fume hood (*see* **Note 3**).
4. Remove fixative, wash twice (2 × 15 min) with above buffer at RT (s*ee* **Note 4**).
5. Fixative 2 (F2): Prepare a 1% solution of osmium tetroxide in sucrose-free 0.1 *M* Na-cacodylate buffer, pH 7.2, containing 5 m*M* $CaCl_2$ and 5 m*M* $MgCl_2$. Sucrose is not necessary at this or following steps. Prepare fresh and use immediately.

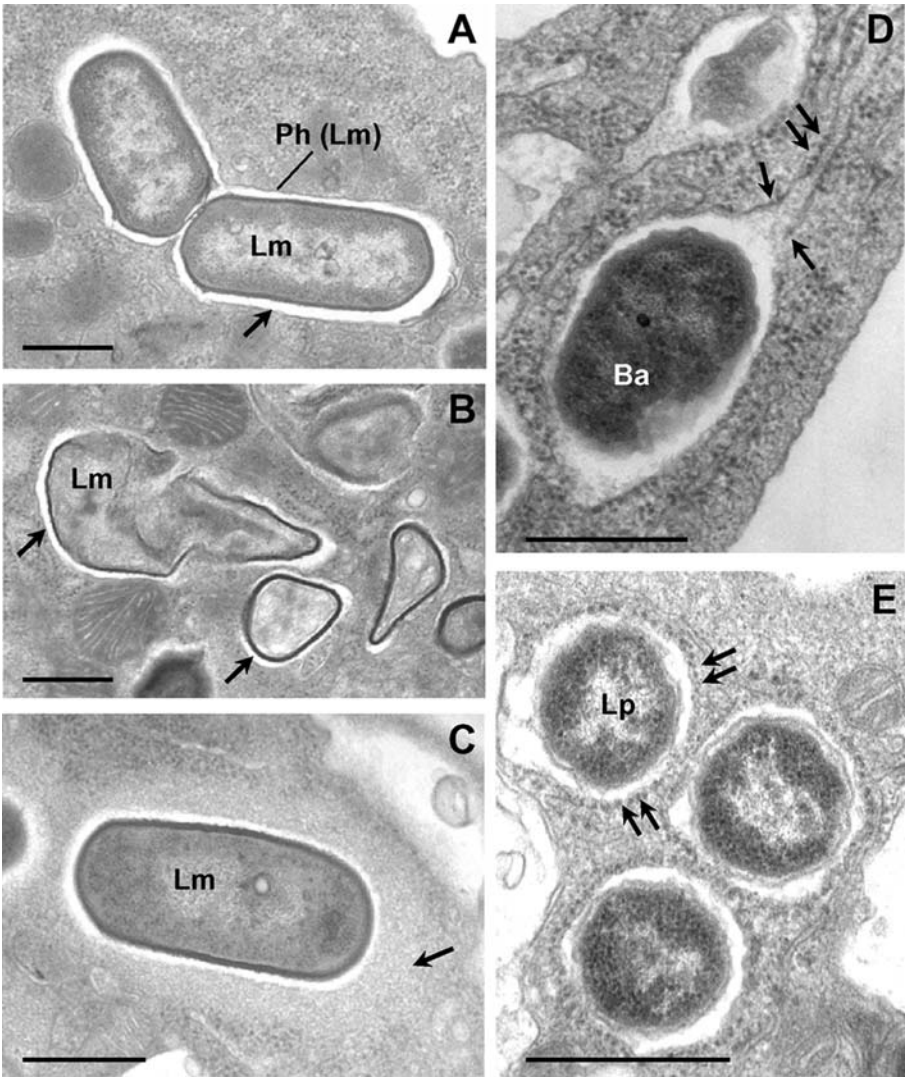

Fig. 1. Illustration of how one can use basic conventional microscopy **(Subheading 3.1.)** to determine morphological appearance of bacteria and identify the compartment in which they survive/replicate. **(A, B, C)**: Bone marrow–derived macrophages were infected with *Listeria monocytogenes* (Lm), **(D)** *Brucella abortus* (Ba) or **(E)** *Legionella pneumophila* (Lp), fixed and processed for conventional TEM. These thin sections show whether bacteria are morphologically intact or degraded and give indications on the compartment in which they are enclosed. In **(A)** Lm is morphologically intact and therefore viable and is in a membrane-bound (arrow) phagosome; in **(B)** all the Lm are enclosed in membrane-bound (arrow) phagosomes and are degraded, as shown by their distorted shape; in **(C)** Lm is intact.

6. Remove buffer, add F2. Fix for 1 h at RT under fume hood.
7. Wash cells three times rapidly to remove remaining osmium. Add 1-1.5mL of buffer in the culture dish (*see* **Note 5**).
8. Scrape cells off gently from culture dishes with a rubber policeman and put sample in a conical 1.5-mL Eppendorf tube (*see* **Note 6**).
9. Spin down samples in a microcentrifuge (10,000 rpm, 3 min).
10. Prepare a water bath at 42°C. Heat 2% agar. Once liquefied, put in water bath. Prepare a microvette containing about 0.3 mL agar.
11. Take samples in turn, remove supernatant, put Eppendorf tube in water bath, add about 0.3 mL agar to Eppendorf, mix cells and agar, and transfer to a microvette. Centrifuge immediately at 10,000 rpm for 2 min. **Step 10** must be done rapidly in order to avoid solidification of agar before the cells have concentrated in the cap at the bottom of the microvette. Put microvette in ice, to harden the agar, for about 5 min.
12. Prepare a glass vial (2–3 mL content) with 1 mL of distilled water for each sample. Water will serve to wash samples briefly before putting them in Na-veronal buffer.
13. Remove microvette from ice. With a razor blade, cut along one side of the bottom cap, then remove the cap gently. Gently push through the agar with a match over a glass slide. The sample, easily recognizable by its black color due to osmium fixation, will come out first, then the agar. Keep 3–4 mm of agar to protect the sample during further manipulations, and cut off the remainder. Put the sample in the glass vial. Do not leave samples in water for more than 5 min.
14. Remove water. Wash samples for 2 min with 1 mL of Na-veronal buffer (or Na-H-maleate-NaOH buffer).
15. Remove buffer, add 1 mL of 1% uranyl acetate prepared in Na-veronal buffer (or Na-H-maleate-NaOH buffer). Incubate cells for 1 h at RT. This step serves to improve fixation of membrane phospholipids.
16. Dehydrate samples at RT in a graded series of ethanol or acetone (ethanol is preferable when samples are embedded in Spurr resin), i.e., 25, 50, 75, and 90% followed by three successive baths in 100% dried on molecular sieve, 10 min per bath.

Fig. 1. The bacterium has completely lysed the phagosome membrane and resides in the cytoplasm where it is surrounded by a thick actin filament meshwork (arrow); in **(D)** the Ba-containing vacuole (BCV) has fused with the endoplasmic reticulum (arrows). The latter is easily identifiable by the presence of ribosomes (double arrows); in **(E)** the Lp-containing vacuole is decorated with ribosomes (double arrows), thereby indicating that the LCV membrane has acquired ER characteristics. Bar = 0.5 μm. Figures **A, B, C)** reproduced with kind permission from ASM Press; **D)** with permission from the Rockefeller University Press and **E)** with kind permission from Blackwell Publishing Ltd, as indicated in **Note 17**.

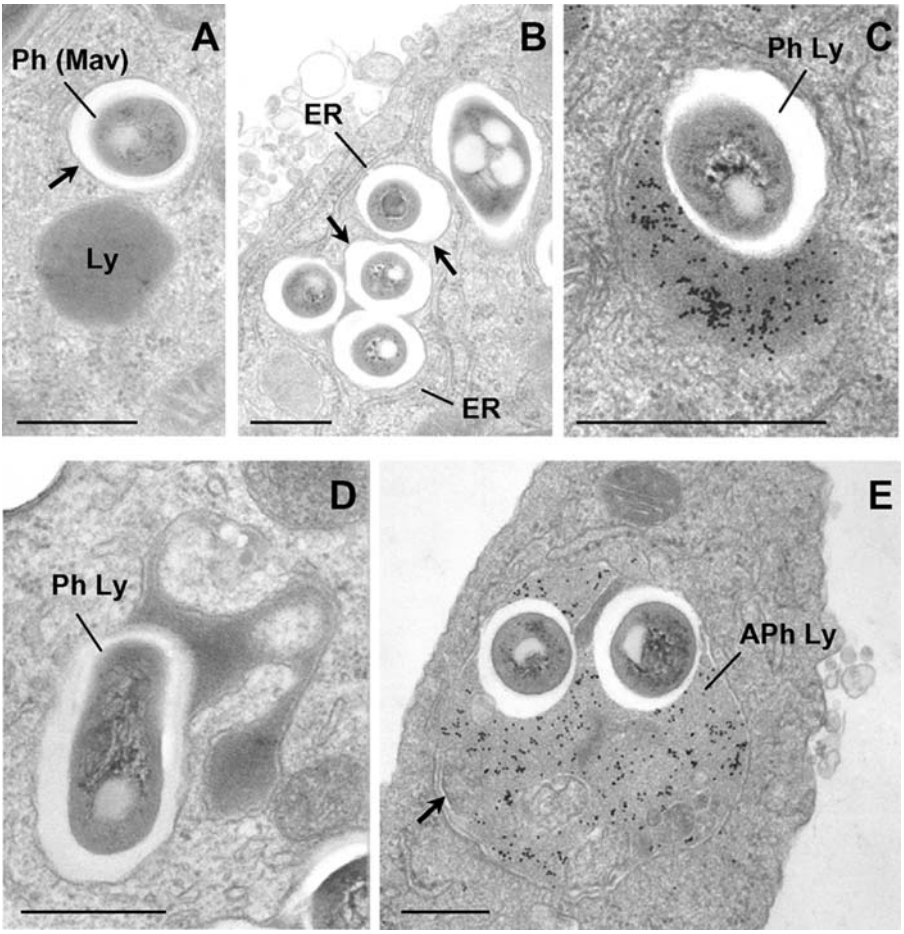

Fig. 2. Illustration of how one can use basic conventional microscopy (**Subheading 3.1.**) to identify cell components involved in phagosome processing. Bone marrow–derived macrophages were infected with *Mycobacterium avium*. At day 7 postinfection, cells were treated for 0–6 h with 5 m*M* methyl-β-cyclodextrin (CD), which leads to a gradual depletion of cholesterol in cellular membranes, including phagosomes. (**A**) Thin section of a control cell, no CD. The phagosome membrane is smooth and closely apposed to the surface of *M. avium* all around (arrow). Such phagosomes remain immature and, therefore, do not fuse with surrounding lysosomes (Ly). (**B**) After a 30-min treatment with CD, the phagosome membrane starts to become loose (arrows) and undulates around the bacterial surface. Notice the close proximity of the endoplasmic reticulum (ER). (**C**) Cells were loaded for 30 min with bovine serum albumin coupled to 10-nm-diameter gold particles (BSA-Au10) as described in **Subheading 3.3.**, followed by a 2-h chase to label lysosomes and then treated with CD for 2 h. Phagosomes contain BSA-Au, thereby indicating that they have matured and fused with Ly. (**D**) After a 3-h

17. Embedding in Spurr resin at RT: Prepare 25, 50 and 75% solutions of Spurr resin (1 mL per sample). The solutions must be prepared with the 100% dried solution of the dehydrating agent used for dehydration, i.e., either ethanol or acetone. Incubate the samples in the successive baths of resin for 30 min each. Then incubate in a first bath of pure resin for 30 min and a second bath for 2 h.
18. Put pure resin in molds. Add sample. (Do not forget to identify it! Write with a pencil on a small piece of paper, with number towards manipulator.) Add resin to fill mold.
19. Polymerize in a 60°C incubator for 24 h.
20. Section samples (70- to 75-nm-thick sections) at EM facility.
21. Staining of thin sections. Usual case: (a) Deposit grids on a drop of 1% uranyl acetate prepared in distilled water, incubate for 6 min, wash rapidly on five successive drops of distilled water, dry on filter paper; (b) then deposit grids on Reynold's lead citrate for 2.5 min, wash rapidly on five successive drops of distilled water. Dry on filter paper.

3.2. Acquisition of HRP by Preexisting Phagosomes
(*see** *Fig. 3A, B, D, E, G)

HRP has been widely used by endocytologists as a content marker because it is very sensitive to staining by cytochemical methods even in standard fixation conditions, and the reaction product forms a dense insoluble deposit that is easily visualized under the EM. The great advantage of this marker is that early endosomes can be distinguished from prelysosomes and lysosomes in terms of two parameters ***(7)***. First, they differ in their cytochemical staining pattern after HRP uptake. In early endosomes, the HRP reaction product only lines the inner face of the membrane (**Fig. 3A**; *see also* **refs.** ***7*** and ***11***). In prelysosomes and lysosomes, the entire lumen is filled with HRP reaction product (**Fig. 3B**; *see also* **refs.** ***7*** and ***11***). Second, early endosomes acquire newly internalized HRP immediately, whereas lysosomes display the marker only after a characteristic lag of 5–10 mins in macrophages and up to 15–30 min in other cells. The first parameter is used to observe whether phagosomes fuse

Fig. 2. treatment with CD, *M. avium*–containing phagolysosomes (PhLy) become deformed. (**E**) After a 4- to 6-h treatment, cytoplasmic organelles, including other phagosomes, have been engulfed during the formation of an autophagolysosome (APhLy), which also contains gold particles accumulated in Ly prior to cholesterol depletion. In this autophagosome one can still see remnants of the inner membrane (arrow). Notice that bacteria are morphologically intact and surrounded by their electron translucent zone which is part of the cell wall. Bar = 0.5 μm. Reproduced with kind permission from Blackwell Publishing Ltd as indicated in **Note 17**.

with early endosomes or with lysosomes (*see* **Fig. 3D,E,G**), and the second is used to classify phagosomes as resembling either early endosomes or lysosomes (*see*, e.g., **refs. *7*** and ***11*** for illustration of method; *see also* **Note 7** for other possible markers).

1. Prepare a concentrated solution of HRP in culture medium (RPMI or DMEM).
2. Dilute HRP in complete medium. Prepare the solution fresh, warm up to 37°C, and use immediately (*see* **Note 8** for appropriate concentration).

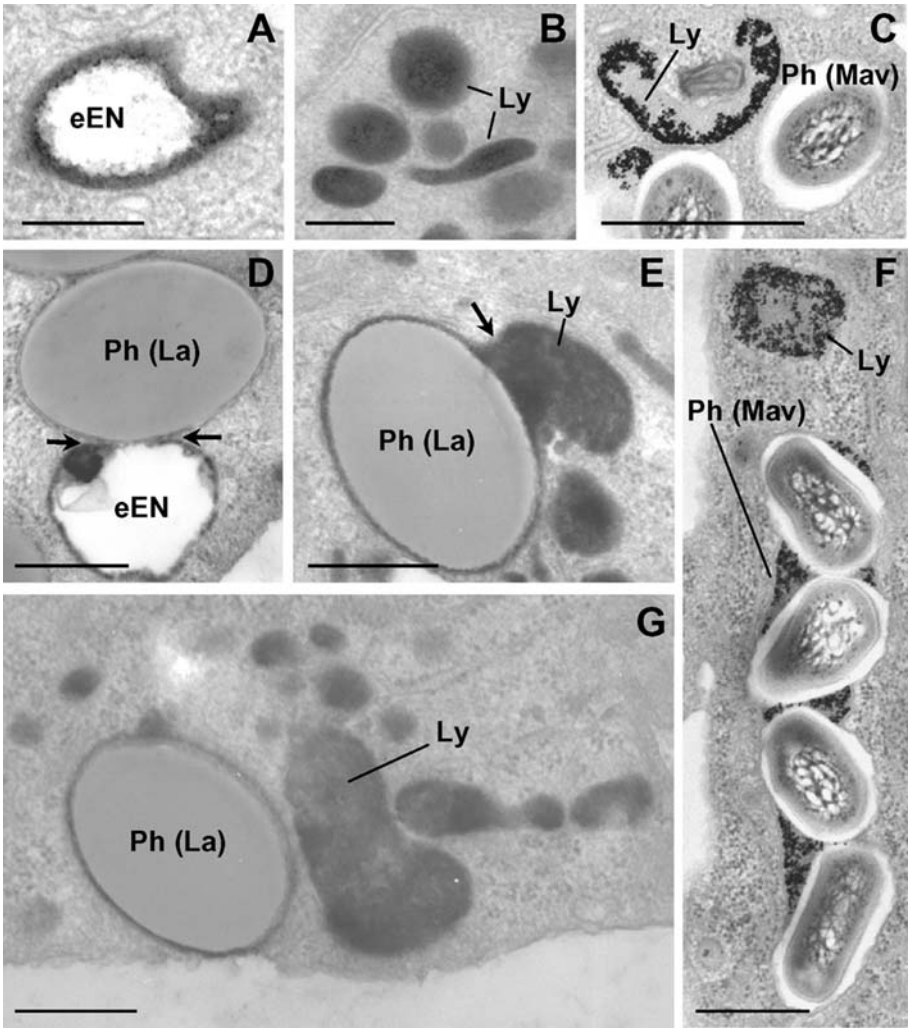

Fig. 3. (*Continued*)

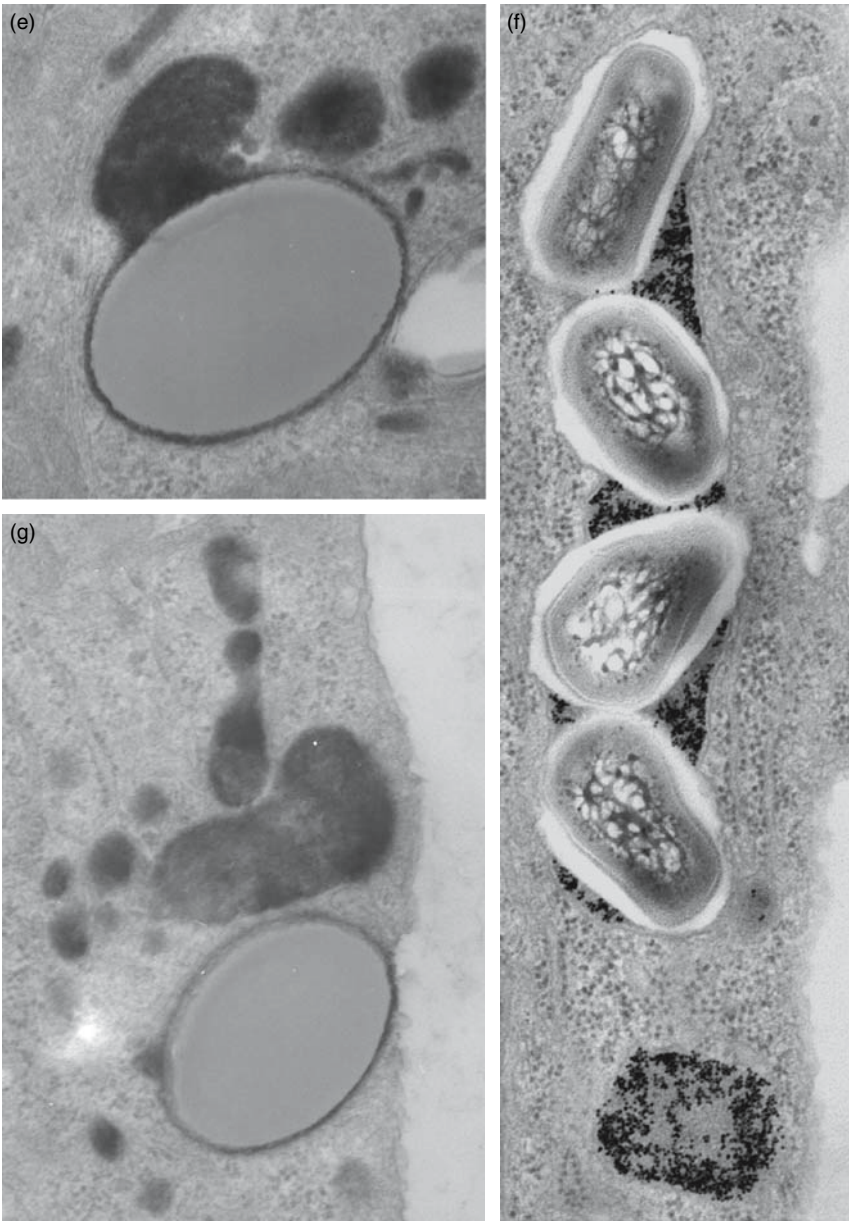

Fig. 3. (*Continued*)

3. At selected time points after exposure to HRP, between 0 and 60 min (or more), proceed to fixation with 2.5% glutaraldehyde, followed by washes with cacodylate buffer, as described in **steps 1–4** in **Subheading 3.1**.
4. Wash cells 3 × 1 min with Na-cacodylate buffer devoid of sucrose and then 3 × 1 min with Tris-HCl 0.1M. Warm buffers to RT before use.
5. During the washes, prepare the cytochemical reaction medium: for 10 mL of medium, add 1 mL of DAB 10 mg/mL to 9 mL of 0.1 *M* Tris-HCl buffer, pH 7.6. Then add 100 µL of a 1% solution of H_2O_2 (final concentration 0.01%). The 1% solution of H_2O_2 must be prepared immediately prior to use. This medium is light sensitive, proceed swiftly to next step.
6. Remove last washing medium, add reaction medium to cells, and incubate for 15 min at RT in the dark. Caution: DAB is carcinogenic and must be handled with care. After incubation, inactivate DAB with H_2O_2 before discarding.
7. Wash three times (3 × 1 min) with Tris-HCl buffer, then three times (3 × 1 min) with Na-cacodylate buffer devoid of sucrose.
8. Process samples for EM from **Subheading 3.1.**, **steps 5–21**.

Fig. 3. Illustration of how one can use **Subheadings 3.2. (A, B, D, E, G)** and **3.3. (C, F)** to analyze phagosome processing. **(A, B)** Morphological appearance of early endosomes (eEN) and lysosomes (Ly) stained with HRP. Bone marrow–derived mouse macrophages were given HRP (25 µg/mL) for 30 min, fixed, and stained for the endocytic content marker, HRP. In eEN **(A)**, the reaction product only lines the inner face of the membrane, whereas in Ly **(B)**, it entirely fills the lumen. **(D, E, G)** Fusion of phagosomes with eEN or Ly stained with HRP. Macrophages were given different types of latex beads. At 2 h after phagocytic uptake, cells were given HRP for 60 min as an endocytic content marker, then fixed and stained for HRP. The 1-µm-diameter hydrophobic bead–containing phagosomes (Ph(La)) fuse with eEN (arrows) **(D)** but not with Ly **(G)**. The 1-µm-diameter hydrophilic bead–containing phagosomes (Ph(La)) have matured and fuse with Ly (arrow) **(E)**. **(C, F)** Acquisition of lysosomal marker by phagosomes. Macrophages were incubated for 30 min with BSA tagged with 10-nm-diameter gold particles (BSA-Au10), washed and incubated for 2 h in medium devoid of BSA-AU10 to chase the marker to lysosomes (Ly). Cells were then infected with *M. avium* for 4 h. Phagosomes containing a single bacterium and for which the phagosome membrane is closely apposed to the bacterial surface do not fuse with Ly **(C)** , whereas those containing several bacteria and for which the phagosome membrane is, therefore, not closely apposed to the bacterial surface all around mature and fuse with Ly **(F)**. Bar = 0.5 µm. **A, B, E, G)** reproduced with kind permission of ASM Press, **C)** with kind permission from Blackwell Science Ltd, **D)** with kind permission from Elsevier and **F)** with kind permission from Springer Science and Business media as indicated in **Note 17**.

3.3. Acquisition of BSA-Au Chased to Lysosomes Prior to Phagocytic Uptake (see Figs. 2C and 3C,F)

A widely used method to determine whether phagosomes have been processed into phagolysosomes or not involves chasing an endocytic content marker to lysosomes prior to phagocytosis and then analyzing acquisition of the marker, by phagosomes, at selected intervals during or after phagocytic uptake. The most frequently used marker, at present, is BSA-Au. This method is illustrated in **Figs. 2C and 3 C,F** to illustrate no fusion or fusion with lysosomes. (*See also* **refs. *11–13*** for illustration of method; *see* **Note 9** for other possible markers.)

1. Sixteen hours prior to labeling, dialyze BSA-Au against serum-free culture medium (DMEM or RPMI) to remove azide.
2. Dilute BSA-Au in serum-free medium in order to have an OD of 3–5. Make fresh and use immediately.
3. Remove medium from cells, wash twice with serum-free medium and add BSA-Au solution. Incubate cells for 30 min (60 min if uptake is low) at 37°C.
4. Wash cells three times rapidly with serum-free medium.
5. Remove last wash, add complete medium, and incubate for a further 2 h to chase marker to lysosomes (*see* **Note 10**).
6. Process samples as in **Subheading 3.1., steps 1–21**.

3.4. Enzyme Cytochemistry: Staining for Acid Phosphatase (see Fig. 4A)

To determine whether phagosomes have been processed into phagolysosomes, it is possible to stain cells for the presence of lysosomal enzymes. Enzymes are not electron dense and are, therefore, not visible in electron microscopy unless enzyme cytochemistry methods are applied. Such methods do not visualize the enzyme itself but the product of the enzymatic reaction. The fact that the latter must be electron dense to be visualized has limited the number of enzymes that can be localized. Acid phosphatase (AcPase), aryl sulfatase, and trimetaphosphatase are enzymes of choice, because the phosphate liberated during the enzymatic reaction in the presence of substrate will react with the lead citrate used as a capture agent to form insoluble and electron-dense lead phosphate precipitates. These precipitates remain in the organelles containing the enzyme provided that the ultrastructure is well preserved (*see* **ref. *14*** for illustration of method).

1. All products for the reaction medium must be prepared with boiled distilled water (boiled water for gradient elution for HPLC [Fluka] is even better) (*see also* **Note 11**).
2. Prepare Na-acetate buffer 0.05 *M*, pH 5.0. Stable at 4°C for 5–7 d.

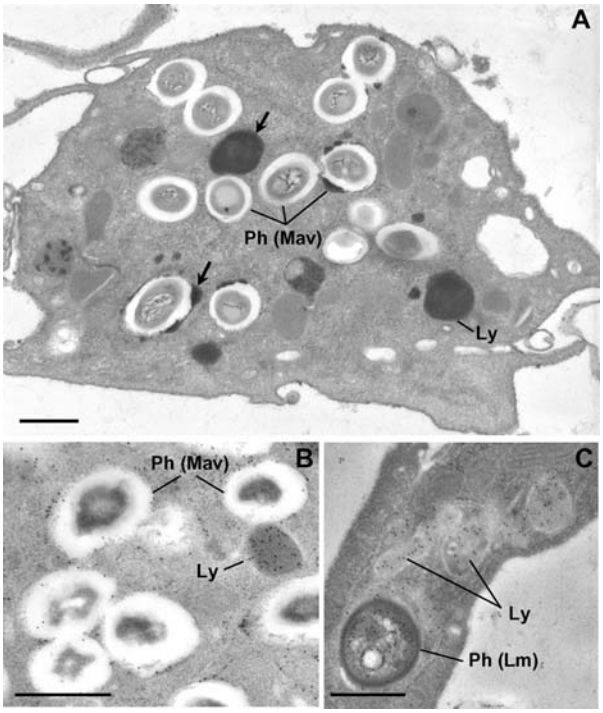

Fig. 4. Illustration of how one can use acid phosphatase (AcPase) cytochemistry **(Subheading 3.4.)** to characterize phagosomes and **Subheading 3.6.** to assess phagosome acidification. **(A)** Bone marrow–derived mouse macrophages were infected with *M. avium*. Seven days later, cells were fixed, stained for AcPase, and processed for EM. Lysosomes (Ly) were strongly labeled as shown by the dark deposits (arrow) in the entire lumen of the Ly, but most *M. avium*–containing phagosomes (Ph (Mav)) were not stained. Only a few of them displayed small deposits (arrow). **(B, C)** Macrophages were infected either for 4 h with *M. avium* as in **(B)** or for 45 min with *Listeria monocytogenes* (Lm) as in **(C)**, washed and reincubated in fresh medium. One hour later, cells were exposed to DAMP (60 m*M*, 30 min), fixed and embedded in LRWhite. The probe was localized by the postembedding method described in **Subheading 3.6**. **(B)** Many gold particles were observed in Ly, but not in Ph(M av), thereby indicating that the latter were poorly acidic. (**C**) A high abundance of gold particles was observed in both Ly and Ph(Lm), thereby indicating that the latter are acidic. Bar = 0.5 μm. **A, C)** Reprinted with kind permission from ASM Press, **(B)** with kind permission from Elsevier as indicated in **Note 17**.

3. Prepare a solution of lead nitrate 6% (60 mg in 1 mL of freshly boiled water) immediately before preparing the reaction medium. Toxic.
4. Cytochemical reaction medium: add 30 mg of glycerol 2-phosphate, disodium salt hydrate to 11 mL of 0.05 *M* Na-acetate buffer. Mix, adjust pH to 5.0

with 10% acetic acid (one or two drops usually sufficient). Then add dropwise, under vigourous stirring, 200 µL of the lead nitrate solution. If mixture becomes cloudy, discard and start over. Incubate reaction medium at 37°C for at least 2 h before adding to cells (overnight is often better). If the medium is still slightly cloudy, one can try to filter it on a 0.22-µm filter.

5. Prepare a 1.25% solution of glutaraldehyde in 0.1 *M* Na-cacodylate buffer, pH 7.2, containing 5 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, and 0.1 *M* sucrose. Prepare fresh, keep on ice, stable for half a day.
6. At time points of interest, remove medium from cells. If necessary, wash cells with serum-free medium. Do not use PBS for washes as this can introduce tiny calcium or magnesium phosphate precipitates during the fixation and incubation steps.
7. Add cold fixative immediately to culture dish. Fix for 1 h at 4°C (*see* **Note 12**).
8. Remove fixative, wash twice (2 × 15 min) at RT with above buffer containing 50 m*M* NH_4Cl, twice (2 × 15 min) at RT with same buffer devoid of NH_4Cl, and twice (2 × 5 min) with Na-acetate buffer prewarmed to 37°C.
9. Remove buffer, add prewarmed reaction medium and incubate cells at 37°C for 30 min. Controls: (a) incubate cells in substrate-free reaction medium or (b) in complete reaction medium containing 10 m*M* sodium fluoride.
10. Wash cells twice (2 × 2 min) with Na-acetate buffer and twice (2 × 2 min) with sucrose-free Na-cacodylate buffer.
11. Remove last wash and process cells for conventional electron microscopy as indicated in **Subheading 3.1., steps 5–21** (*see* **Note 13**).
12. Interpretation: *see* **Note 14**.

*3.5. Enzyme Cytochemistry: Staining for G6Pase (***see** *Fig. 5)*

G6Pase is a specific marker of the endoplasmic reticulum (ER) compartment. In addition to basic conventional EM, staining for G6Pase can, therefore, be used to determine whether the ER interacts with and transfers its contents to phagosomes, and whether phagosomes acquire ER characteristics (*see* **refs. *15–17***). The method described here was adapted from Griffiths et al. ***(18)***.

1. All buffers as well as the reaction medium must be prepared with boiled bidistilled water Water for gradient elution for HPLC (Fluka) is even better. Boil water immediately before preparation of products (*see* **Note 11**).
2. Prepare PIPES 0.1 *M*, pH 7.0: Dissolve 3.02 g of PIPES in 75 mL of boiled water. Solution is milky (do not worry!). Adjust the pH to 7.0 with NaOH 1 *N* (about 15 mL). The solution clears up. Complete to 100 mL with boiled water. Split into two 50-mL aliquots, add either 2.5 or 5 g of sucrose to obtain PIPES with 5 or 10% sucrose, respectively. Store at 4°C. Use within a week (but better to prepare the day before).
3. Prepare Tris-maleate 0.08 *M*, pH 6.5: Add 0.485 g Tris base and 0.465 g maleic acid to 40 mL of boiled water, Adjust pH to 6.5 with NaOH 1 *N* (about 1–2 mL)

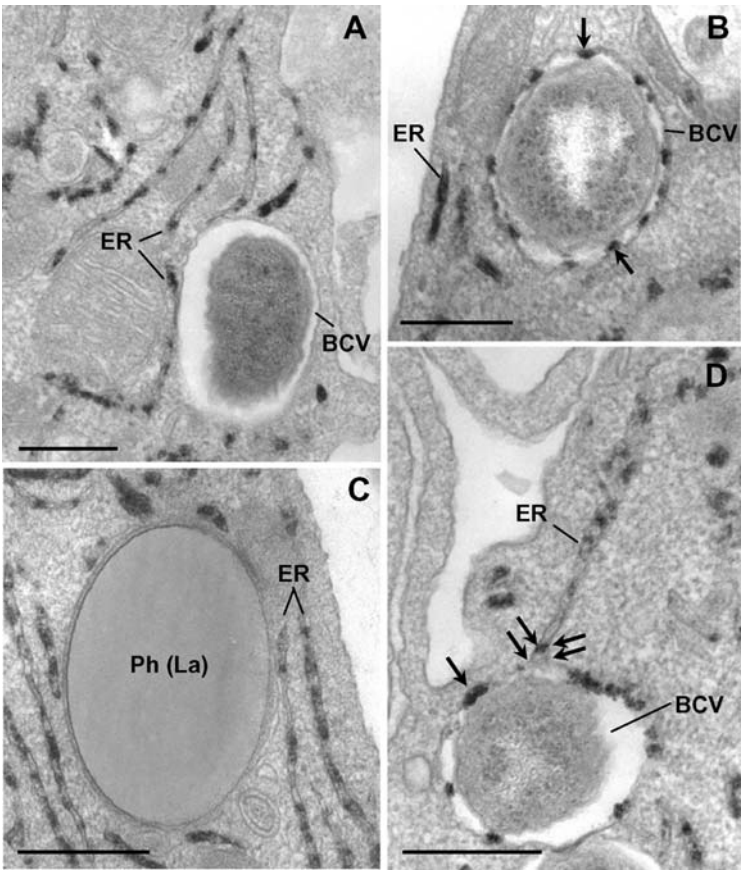

Fig. 5. Illustration of glucose-6-phosphastase (G6Pase) cytochemistry (**Subheading 3.5.**). Bone marrow–derived mouse macrophages were infected with *Brucella abortus* wild-type strain 2308 for various times or given 1-μm-diameter hydrophilic latex beads for 30 min. Samples were processed for detection of G6Pase according to **Subheading 3.5**. Dense deposits in the endoplasmic reticulum (ER) correspond to the reaction product. (**A**) Representative G6Pase-negative *Brucella*-containing vacuole (BCV) at 4 h postinfection. (**C**) Representative G6Pase-negative latex bead-containing phagosome (Ph(La)) processed at 30 min after phagocytic uptake. Notice in both cases that phagosomes are negative, although they are in intimate contact with several G6Pase-positive ER compartments. (**B, D**) Representative G6Pase-positive BCVs at 24 h postinfection as shown by the presence of dense deposits (arrow) within the BCV. (**D**) shows a BCV fusing with the ER (double arrows). Bar = 0.5μm. (**A, B, D**) Reproduced with kind permission from The Rockefeller University Press, as indicated in **Note 17**.

and complete to 50 mL with water. Store at 4°C. Use within a week (but better to prepare the day before).

4. Prepare a 6% solution of lead nitrate: Dissolve 60 mg lead nitrate in 1 mL of freshly boiled water. Prepare fresh and use immediately in reaction medium.
5. Prepare cytochemical reaction medium: Dissolve 95 mg of glucose-6-phosphate, disodium salt in 10 mL of Tris-maleate 0.08 *M*, pH 6.5, adjust pH to 6.5 with a few drops of HCl 0.1 *N*. Add, dropwise, 160 µL of the 6% lead nitrate solution under vigourous stirring. The incubation medium is often slightly milky. It is, therefore, preferable to filter it through a 0.22-µm filter. Stable at RT for 2 h.
6. Prepare fixatives: (a) F1: 1.25% glutaraldehyde in 0.1 *M* PIPES, pH 7.0, containing 5% sucrose; (b) F2: 1.25% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, containing 0.1 *M* sucrose, 5 m*M* $CaCl_2$, and 5 m*M* $MgCl_2$. Keep on ice.
7. Remove medium, add F1, and fix cells for 30 min on ice.
8. Remove F1 and wash cells 3 × 3 min at RT with 0.1 *M* PIPES, pH 7.0, containing 10% sucrose, followed by 1 × 30 s at RT with 0.08 *M* Tris-maleate buffer, pH 6.5.
9. Remove last wash, add cytochemical medium, incubate cells for 2 h at 37°C in a CO_2-free incubator. Possible controls: incubate cells (a) in substrate-free reaction medium or (b) in complete reaction medium containing 10 m*M* sodium fluoride.
10. Remove cytochemical medium, wash cells 3 × 2 min at RT with 0.08 *M* Tris-maleate buffer and 3 × 2 min at RT with 0.1 *M* cacodylate buffer, pH 7.2, containing 0.1 *M* sucrose, 5 m*M* $CaCl_2$, and 5 m*M* $MgCl_2$.
11. Remove last wash, add F2, and fix cells for 1 h at 4°C.
12. Remove F2 and process cells for conventional electron microscopy as indicated in **Subheading 3.1., steps 4–21**.
13. Interpretation: *see* **Note 15**.

*3.6. Assessment of Phagosome Acidity: DAMP Treatment and Immunolocalization (***see** *Fig. 4B,C)*

DAMP (3-(2,4-dinitroanilino)-3‘amino-*N*-methyldipropylamine) is a probe that has been widely used to study phagosome acidification at the TEM level ***(19)***. This weak base accumulates by diffusion within acidic compartments. Once protonated, it can no longer diffuse out. During chemical fixation with aldehydes, it becomes covalently linked to proteins, which allows it to be retained in acidic organelles during processing of samples for EM. DAMP is then localized with an appropriate postembedding immunolabeling method on thin sections of cells embedded in LRWhite resin as described below (*see* **Note 16**).

1. Prepare fixatives: F1: 1.25% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, containing 0.1 *M* sucrose, 5 m*M* $CaCl_2$, and 5 m*M* $MgCl_2$; F2: paraformaldehyde 1% in same buffer. Keep on ice.

2. At selected intervals postinfection, remove cell culture medium and incubate cells for 30 min at 37°C with 100 μ*M* DAMP diluted in prewarmed complete medium.
3. Wash cells three times (rapidly) with medium (DMEM or RPMI) containing 10% serum (same serum as for cell culturing).
4. Remove last wash, add F1 (warmed to RT), and fix cells for 1 h at RT.
5. Remove F1, add F2, and store sample at 4°C. Cells can be kept at 4°C in F2 for 1 wk.
6. Wash fixed cells twice (2 × 15 min) at RT with complete Na-cacodylate buffer containing 50 m*M* NH_4Cl and three times (3 × 5 min) at RT with the same buffer devoid of NH_4Cl.
7. Process samples as indicated in **Subheading 3.1., steps 8–14**.
8. Put samples on ice. Remove buffer, add 1 mL of 0.5% uranyl acetate prepared in Na-veronal buffer (or Na-H-maleate-NaOH buffer). Incubate for 1 h on ice.
9. Dehydrate samples at RT in 50% ethanol (2 × 15 min) followed by 70% ethanol (3 × 10 min).
10. Embed in LRWhite: three successive baths of 20 min in pure resin.
11. Put pure resin in gelatin capsules. Add sample (do not forget to identify it! Write with a pencil on a small piece of transparent paper, with number towards manipulator). Add resin to fill capsule. Put cap on the capsule to avoid contact with air.
12. Polymerize in a 37°C incubator for 3 d.
13. Sectioning (in EM facility): Pick up sections on formvar and carbon-coated nickel grids and put grids on PBS containing 0.5% bovine serum.
14. For immunolabelling, prepare PBS containing 0.5, 5, and 10% fetal bovine serum (FBS). Do not heat inactivate the serum prior to use.
15. Incubate the grids (face with thin sections against the drops of reagents) on the following antibodies or reagents: preincubate sections for 10 min on PBS containing 10% FBS to block nonspecific sites, and then sequentially incubate for 60 min at RT with rabbit antidinitrophenol (anti-DNP) antibodies and for 30 min with protein A coupled to 10-nm-diameter gold particles (PA-Au10). Antibodies and conjugate are diluted in PBS containing 5% FBS. Sections are washed five times rapidly with PBS containing 0.5% FBS between the incubations and after treatment with the conjugate. After three washes with PBS and distilled water, dry the grids of filter paper. As a control, incubate grids on non specific antibody followed by PA-Au10 or on PA-Au10 only (*see* **Note 16** for estimating intraphagosomal pH).
16. Staining of thin sections: Deposit grids on a drop of 1% uranyl acetate prepared in distilled water, incubate for 1 min, wash rapidly on five successive drops of distilled water, dry on filter paper; then deposit grids on Reynold's lead citrate for 30 s, wash rapidly on five successive drops of distilled water. Dry on filter paper.

4. Notes

1. I suggest Spurr resin because of its low viscosity, which allows for embedding of samples containing difficult to embed bacteria, such as mycobacteria. It is easy to prepare and it polymerizes within 24 h at 60°C. Other resins, such as Epon, are widely used and available as kits from EMS.
2. Preparation of BSA-G10 or PAO-G10. The method has been described at length by J.W. Slot and H. J. Geuze ***(20)***, who introduced the immunogold labeling procedure.
3. Cells can also be fixed overnight but at 4°C instead of RT. Proceed to **step 4** the next morning.
4. Cells can also be washed overnight at 4°C instead of 2 × 15 min at RT. Proceed to **step 5** the next morning.
5. At this stage, one can add buffer to cells and store at 4°C for up to 2 wk. This is practical if one is doing a kinetic study and wishes to process all EM samples at the same time.
6. Cell scraping: This is a critical step. It is important to scrape cells only after they have been fully fixed with both glutaraldehyde and osmium. Otherwise, cells will be damaged during the scraping process.
7. Endocytic content markers added after phagocytic uptake: Other markers can be used, provided that they can be tagged with electron dense probes. One possibility is to use biotinylated ligands (dextran or albumin) as content markers and then exploit the biotin–streptavidin interaction, with streptavidin coupled to HRP or to gold particles, to localize the ligands intracellularly. One can also tag ligands with gold particles (*see also* **Subheading 3.3.**). However, with all these ligands, it is not possible to morphologically distinguish early endosomes from late compartments, as with HRP.
8. Certain primary macrophage cultures, such as mouse bone marrow–derived, are rich in mannose receptors at their surface. In this case, prepare a 1 mg/mL solution and use it at 25 µg/mL (40-fold dilution). HRP will then be essentially internalized via the mannose receptor ***(21)***. If cells have no or very few mannose receptors, one must add more HRP, which will be endocytosed by fluid phase endocytosis. Usually one adds 1 mg/mL—up to 6 mg /mL in certain cell types. It is advisable to try different concentrations of marker and different incubation times in the presence of marker to define the optimal conditions and to use control particles for which it is known whether they fuse with early endosomes only or early endosomes and then lysosomes ***(7,11)***.
9. Other markers: Ferritin, thorotrast, or ligands tagged with electron-dense probes as indicated in **Note 7**.
10. Incubation and chase conditions with BSA-Au: As indicated in **Note 8**, it is advisable to define the optimal working conditions. To obtain the most reliable data, the marker must be internalized by the cell in sufficient amounts so that it will label the entire lysosomal compartment when chased after uptake. It might, therefore, be necessary to expose cells to the marker for 60–120 instead

of 30 min. However, the marker must not be too densely packed within the lysosome, or it might form a sort of rigid gel or network. In such conditions contents would not be transferred to phagosomes upon fusion with lysosomes, and this would be misinterpreted as a "no-fusion" event. Finally, it is important to keep the chase period short because lysosomal contents are recycled out of the lysosomal compartment via small recycling vesicles, and this will eventually lead to excretion of the marker. In macrophages, about 50% of the label is secreted within a 20-h chase period.

11. For enzyme cytochemistry, use only disposable plastic, no reusable glassware or pipets.
12. Enzyme susceptibility to fixatives: The cells must be fixed before the enzymatic reaction occurs. A compromise must, therefore, be reached between preservation of ultrastructure and of enzymatic activity. This is why I use a lower concentration of glutaraldehyde and fix cells in the cold for detection of AcPase (and other phosphatases). Most enzymes are inactivated by chemical fixatives, and below a threshold concentration they will no longer be detected. When studying the acquisition of other lysosomal enzymes by phagosomes, it is advisable to try different fixation conditions and include controls such as phagocytosis of particles that do not prevent phagosome maturation and fusion with lysosomes, and, therefore, acquisition of hydrolases by phagosomes (*see*, e.g., **refs. *13*** and ***14***).
13. One can check under the light microscope whether one was successful with this method. Grow cells on cover slips; proceed through **steps 1–9**. Wash cells once with distilled water, incubate the cover slips for 1 min over a drop of 1% ammonium sulfide (under fume hood), wash cover slips in two successive drops of distilled water. Mount on glass slide. Under the light microscope, the reaction product appears as dark brown deposits in lysosomes (and eventually phagosomes). Material treated with ammonium sulfide cannot be processed for EM.
14. Interpretation: First, one must keep in mind that hydrolases such as AcPase, which are concentrated in lysosomes, are also present in small amounts in early compartments and can, therefore, be acquired by phagosomes via multiple fusion events with early endosomes. The amount of enzyme transferred to phagosomes in such conditions, is, however, usually below the threshold level of detection. Second, the hydrolysis of the substrate can also be catalyzed by other phosphatases, including glucose-6-phosphatase, an ER- and nuclear membrane-specific marker, or alkaline phosphatase, which is enriched in the plasma membrane. Their contribution should, however, be minor if the reaction medium is of good quality. Small or large nonspecific deposits can also occur if the reaction medium is not of good quality. It is advisable to consult a specialist to avoid misleading and wrong conclusions!
15. The hydrolysis of the substrate can also be catalyzed by other phosphatases, including acid phosphatase, which is enriched in lysosomes and phagolysosomes, or alkaline phosphatase, which is enriched in the plasma membrane. If the

cytochemical reaction is done properly, their contribution should, however, be minor. Small or large nonspecific deposits can also occur if the reaction medium is not of good quality. It is advisable to consult a specialist to avoid misleading and wrong conclusions!

16. An important advantage of this method is that the number of gold particles per phagosome can be converted to values for intraphagosomal acidification by the method described by Orci et al. ***(22)*** provided that the phagosome contains sufficient amounts of protein for all the protonated DAMP molecules to be retained in the phagosome after fixation. (For examples of assessment of intraphagosomal pH, *see* **refs. *7*, *12*,** and ***13***.)
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