Protocol

Isolation and immunofluorescence staining of *Aspergillus fumigatus* conidia-containing phagolysosomes

The analysis of phagolysosomes within professional phagocytic cells is facilitated by their isolation. Here, we optimized a protocol for the isolation of intact phagolysosomes from macrophages infected with the spores of *Aspergillus fumigatus*. Purified phagolysosomes allow improved immunostaining, e.g., of phagolysosomal membrane proteins, or proteome analysis.

**HIGHLIGHTS**

- Improved protocol to isolate intact phagolysosomes from macrophages
- Optimized cell separation allows phagolysosome analyses without cell background
- Isolated phagolysosomes are usable for immunofluorescence and protein analyses
Protocol

Isolation and immunofluorescence staining of Aspergillus fumigatus conidia-containing phagolysosomes

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SUMMARY
The analysis of phagolysosomes within professional phagocytic cells is facilitated by their isolation. Here, we optimized a protocol for the isolation of intact phagolysosomes from macrophages infected with the spores of Aspergillus fumigatus. Purified phagolysosomes allow improved immunostaining, e.g., of phagolysosomal membrane proteins, or proteome analysis.
For complete details on the use and execution of this protocol, please refer to Schmidt et al. (2020).

BEFORE YOU BEGIN
The protocol is optimized from the published work of Kyrmizi et al. (2018).

1. Phagocytic cells should be maintained according to the accepted protocols. Their viability should be higher than 90%, and confluency in flasks or wells should be around 80%.
2. For efficient uptake by phagocytic cells, fungal spores should be freshly harvested from mycelia.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-vATPase V1 antibody | Abcam   | ab73404    |
| DyLight633          | Invitrogen | #35562    |
| Chemicals           |        |            |
| ATP                 | Sigma-Aldrich | A7699-1G  |
| Biocoll             | Merck   | L6113      |
| Bovine serum albumin| Sigma-Aldrich | A3294-50G |
| Mounting medium     | Vector Laboratories | H-1000-10 |
| Protease inhibitor  | cOmplete, Roche | S892791001 |

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MATERIALS AND EQUIPMENT
For cultivation of RAW264.7 macrophages, the listed DMEM medium is recommended.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Experimental models: cell lines | RAW264.7 | ATCC Cat#TIB-71 |
| Experimental models: organisms/strains | Aspergillus fumigatus strain ATCC 46645 pksP | Jena Microbial Resource Collection (JMRC), HKI Jena (Langfelder et al., 1998) |
| Software | BioRender | BioRender 2021 https://biorender.com/ |
| | Zeiss ZEN software 2.3 | Zeiss https://www.zeiss.de/ |

Reagent Vendor/ manufacturer Final concentration Amount

| Reagent | Vendor/ manufacturer | Final concentration | Amount |
|---------|----------------------|----------------------|--------|
| DMEM medium (#11960044, high glucose) | Gibco | 89% | 178 mL |
| Fetal calf serum | GE Healthcare | 10% | 20 mL |
| Ultraglutamine | Gibco | 1% | 2 mL |
| Gentamicin sulfate 50 mg/mL | Gibco | 27.5 μg/mL | 110 μL |
| Total | | | 200 mL |

STEP-BY-STEP METHOD DETAILS
Preparation of *A. fumigatus* conidia and macrophage cell line (RAW264.7)
For the isolation of phagolysosomes from macrophages seeding of a defined amount of macrophages in well plates and cultivation of *A. fumigatus* conidia until sporulation is necessary and described below.

© Timing: 10 min

1. Revival of *A. fumigatus* conidia from cryopreservation
   a. Use an inoculation loop to spread conidia on a petri dish with *Aspergillus Minimal Medium* agar (Pontecorvo et al., 1953)
   b. Incubate conidia-plated agar plate for 5 days at 37°C

© Timing: 30 min plus 3 days

2. Seed macrophage cell line
   a. Preheat cell culture medium (see “Materials and equipment”), which should be stored at 4°C, to 37°C in a water bath
   b. Prepare 2 × 25 cm² cell culture flasks with 3 × 10⁵ macrophages (revived from cryopreserved cell line) and 7 mL culture medium each
   c. Incubate flasks for 3 days at 37°C and 5% (v/v) CO₂

© Timing: 1 h plus 1 day

3. Harvest conidia 5 days after mycelial growth and sporulation on agar plates
   a. Slowly add 10 mL 0.9% (w/v) NaCl, 0.01% (v/v) Tween 20 solution to the petri dish
   b. Use a T-formed spatula to scrap the conidia
   c. Measure concentration of conidia by using a 1:100 dilution and counting with a Thoma chamber or another counting method

4. Harvest adherent macrophages with a confluency of 80%–90%
a. Pre-warm culture medium to 37°C in water bath
b. Remove the old medium from macrophages' culture flask by aspiration
c. Add 3 mL of fresh pre-warmed medium
d. Use a cell scraper to scrape the macrophages from the base of the culture flask
e. Collect cell suspension in a 15 mL Falcon tube
f. Measure cell concentration by Thoma chamber or another counting system

5. Seed cells for infection with A. fumigatus conidia
   a. Add 5 mL medium + 4 x 10⁶ macrophages to each well of a 4-well plate (21.8 cm²/well)
   b. Incubate cells 24 h at 37°C and 5% (v/v) CO₂

**Infection of macrophages with A. fumigatus conidia**

Macrophages are incubated with a defined amount of conidia to allow phagocytosis and formation of a conidia-containing phagolysosome. The implementation of infection is explained in the following.

**Timing:** 3 h

6. Replace culture medium of the 4-well plate containing macrophages
   a. Remove the old medium
   b. Add 3 mL pre-warmed fresh medium to the cells

7. Phagocytosis of A. fumigatus conidia by macrophages
   a. Infect macrophages using an MOI of 5; incubate for 30 min at 4°C to synchronize phagocytosis
   b. Allow phagocytosis for 2 h at 37°C and 5% (v/v) CO₂

**Isolation of conidia-containing phagolysosomes**

Formed phagolysosomes are isolated by the described cell lysis and Biocoll-separating process.

**Timing:** 3 h

8. Isolation of infected cells from plate
   a. Pre-cool a tabletop centrifuge to 4°C
   b. Remove old medium of the 4 wells of the infected macrophages by aspiration
   c. Wash cells by adding 3 mL PBS per well and remove PBS
   d. Harvest cells with cell scraper in 1.5 mL PBS per well
   e. From each well, transfer cell suspension to a separate 1.5 mL Eppendorf tube
   f. Centrifuge at 200 x g for 5 min at 4°C

9. Homogenization of cells
   a. Discard supernatant
   b. Resuspend cell pellet in 1 mL homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4) containing protease inhibitors
   c. Lyse cells on ice by passing the cell suspensions 20 times per pellet through a 26G needle on a 1 mL syringe

**CRITICAL:** Slowly pass the cell suspension through a needle to avoid air bubbles in the tube. Passing times should not fall below 20 times to improve cell lysis.

10. Check cell breakage
    a. Add 5 μL trypan blue to 5 μL cell suspension
    b. Microscopic observation shows blue staining, which indicates disrupted cell membranes

11. Release of actin/myosin interaction
    a. Incubate each cell homogenate with 300 μL of 10 mM ATP for 15 min with rotation at 4°C

12. Separation of phagolysosomes from cell debris
a. Split the 1 mL homogenate into portions of 500 μL; carefully overlay each 500 μL on top of 500 μL Biocoll (1.077 g/mL) in a reaction tube by using a 200 μL tip

△ CRITICAL: A small pipette tip avoids too fast layering of the homogenate onto Biocoll and improves separating process.

b. Centrifuge at 600 × g for 20 min at 4°C
c. Discard supernatant
d. Wash pelleted conidia-containing phagolysosomes twice with ice-cold 500 μL PBS with centrifugation in-between for 5 min, 600 × g, at 4°C
e. Resuspend pellet in 150 μL ice-cold PBS and collect all samples in one tube
f. Seed 20 μL suspension + 1 mL PBS or count phagolysosomes and place a certain amount on a coverslip in a 24-well plate and store at 4°C (possible for max. 1 day) to allow the phagolysosomes to attach onto the coverslip. Alternatively, isolated phagolysosomes can be lysed for Western Blot analysis.

**Immunofluorescence staining of conidia-containing phagolysosomes**

© Timing: 6 h

Membrane-associated targets of isolated phagolysosomes can be stained with specific antibodies using immunofluorescence technology as described below.

13. Fixation of phagolysosomes
   a. Slowly remove PBS from each well
   b. Add 350 μL methanol/acetone (80%/20% (v/v)) or 3.5% (v/v) formaldehyde/PBS or 4% PFA for 15 min RT (depending on the target)
c. Wash three times with 350 μL PBS

14. Blocking and staining
   a. Remove PBS
   b. Add 350 μL 2% (w/v) BSA/PBS for 1 h
   c. Remove blocking solution
   d. Add 300 μL primary antibody diluted in blocking solutions as recommended for 1.5 h
e. Wash three times with PBS for 30 s
   f. Remove PBS
g. Add 300 μL secondary antibody diluted in blocking solutions as recommended for 1 h
   h. Wash three times with PBS for 30 s
   i. Mount coverslips on a glass slide with mounting medium

**EXPECTED OUTCOMES**

For each condition, you should prepare 2 wells of a 4-well plate to isolate a sufficient number of phagolysosomes for immunofluorescence. If ~20 μL of phagolysosome suspension are applied to a glass slide of a 24-well plate, it is possible to analyze 7–8 wells per condition in total. An example of an immunofluorescence staining of the vATPase V₁ subunit on isolated phagolysosomes of RAW264.7 macrophages infected with *A. fumigatus pksP* conidia is shown in Figure 1.

The protocol can also be used for isolation of phagolysosomes from other cell lines and primary cells. Its suitability was confirmed for J774A.1 macrophage cell line and primary bone marrow-derived monocytes/macrophages from C57BL/6 mice.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Samples were visualized using a Zeiss LSM 780 confocal microscope and images were processed with the Zeiss ZEN software. Phagolysosomes with a positive signal for a specific target were
counted and related to non-stained phagolysosomes. One hundred conidia-containing phagolysosomes per biological replicate were evaluated. The values represent the mean ± SD of three biological replicates. Additionally, immunofluorescence intensities could also be evaluated bioinformatically (Schmidt et al., 2020).

LIMITATIONS
The protocol leads to highly purified phagolysosomes; however, some cell debris or intact macrophages may remain in the suspension. A distinction of phagolysosomes from cell debris or intact cells is feasible by microscopy. A quantification of exclusively phagolysosomal proteins by Western blot proved to be more difficult.

TROUBLESHOOTING
Problem 1
The phagolysosomal suspension remains intact cells.

Potential solution
If effective isolation is uncertain, verification of damaged cells via trypan blue staining is necessary. Additional passing steps through a needle are recommended to further reduce the number of intact cells in the sample. Additionally, a careful overlaying improves phase separation between Biocoll and lysate. The use of a yellow tip for a pipetman facilitates the overlaying and improves the separation of phagolysosomes in the pellet and intact cells in the Biocoll-supernatant.

Problem 2
Phagolysosomes are not efficiently stained.

Potential solution
Successful staining of phagolysosomes with antibodies often depends on the fixation reagent, e.g., formaldehyde or methanol/acetone. If the immunofluorescence signal is weak or non-specific, other fixation methods or antibody concentrations can be assessed. For formaldehyde fixation, an addition of 0.3 M glycine to the blocking solution blocks free aldehyde groups and prevents non-specific binding.

Figure 1. Immunofluorescence staining of vATPase V₁ subunit on isolated phagolysosomes of RAW264.7 macrophages infected with A. fumigatus pksP conidia
Anti-vATPase V₁ antibody (Abcam, #ab73404) was used as primary antibody in a 1:500 dilution. Goat-anti-rabbit DyLight633 (Invitrogen, #35562) was used as secondary antibody in a 1:200 dilution. Arrows indicate isolated phagolysosomes. * indicates intact macrophage.
Problem 3
Isolation of phagolysosomes only allows immunofluorescence of target structures on the outer membrane of phagolysosomes.

Potential solution
If your target of interest is expected on the inner phagolysosomal membrane, a permeabilization step with 1% (v/v) Triton X-100/PBS is recommended.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Axel A. Brakhage (axel.brakhage@leibniz-hki.de).

Materials availability
Any material generated in this study is available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
M.G., F.S., and I.K. conducted experiments and analyzed data; A.A.B. and G.C. designed the research and analyzed data; M.G., F.S., I.K., G.C., and A.A.B. wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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