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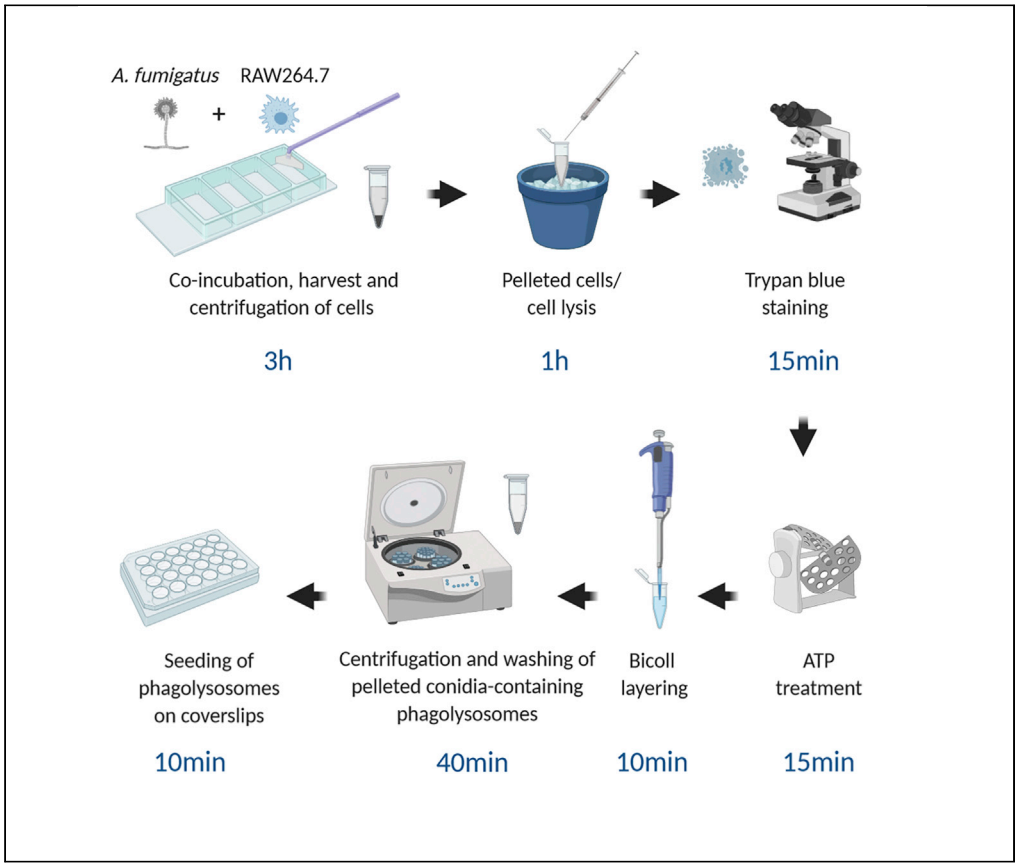
Protocol

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.

Marie Goldmann, Franziska Schmidt, Irene Kyrmizi, Georgios Chamilos, Axel A. Brakhage

axel.brakhage@leibniz-hki.de

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

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

Marie Goldmann,[1,4] Franziska Schmidt,[1,2,4] Irene Kyrmizi,[3,4] Georgios Chamilos,[3,4] and Axel A. Brakhage[1,2,4,5,6,*]

[1]Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute (HKI), 07745 Jena, Germany

[2]Department of Microbiology and Molecular Biology, Institute of Microbiology, Friedrich Schiller University Jena, 07745 Jena, Germany

[3]Department of Medicine, University of Crete, and Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete 71300, Greece

[4]The authors contributed equally

[5]Technical contact

[6]Lead contact

*Correspondence: axel.brakhage@leibniz-hki.de
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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 V_1 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	5892791001

(*Continued on next page*)

Continued		
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/

MATERIALS AND EQUIPMENT

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

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 cm^2 cell culture flasks with 3 × 10^5 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) CO_2

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 × 10^6 macrophages to each well of a 4-well plate (21,8 cm^2/well)
   b. Incubate cells 24 h at 37°C and 5% (v/v) CO_2

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) CO_2

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 × *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 V_1 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

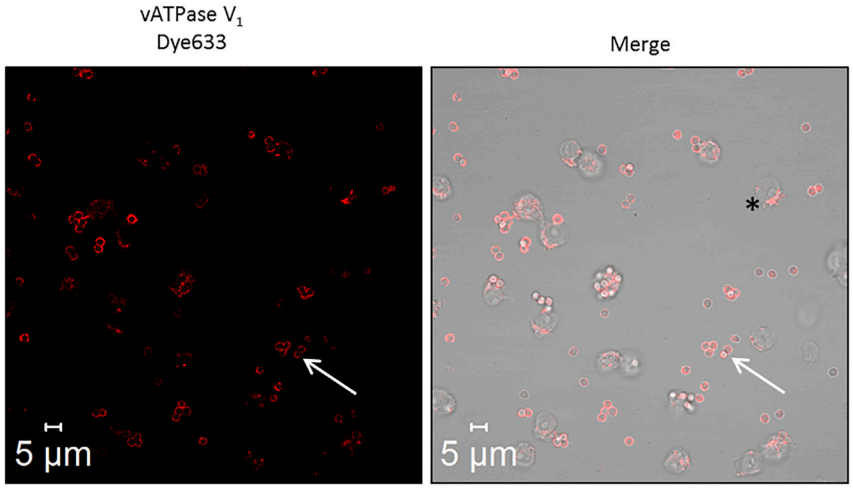

Figure 1. Immunofluorescence staining of vATPase V_1 subunit on isolated phagolysosomes of RAW264.7 macrophages infected with *A. fumigatus pksP* conidia

Anti-vATPase V_1 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.

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.

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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