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Measuring phagocytosis of Aspergillus fumigatus conidia by human leukocytes using flow cytometry

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Corresponding Author:	Susann Hartung, Ph.D. Leibniz Institute for Natural Product Research and Infection Biology Jena, GERMANY
Corresponding Author's Institution:	Leibniz Institute for Natural Product Research and Infection Biology
Corresponding Author E-Mail:	Susann.Hartung@leibniz-hki.de
Order of Authors:	Susann Hartung, Ph.D.
	Christopher Rauh
	Sarah Böttcher
	Mai Thi Ngoc Hoang
	Susanne Jahreis
	Silke Rummler
	Andreas Hochhaus
	Marie von Lilienfeld-Toal
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TITLE:

Measuring Phagocytosis of *Aspergillus fumigatus* Conidia by Human Leukocytes Using Flow Cytometry

AUTHORS AND AFFILIATIONS:

Susann Hartung[1,2], Christopher Rauh[2], Sarah Böttcher[1], Mai Thi Ngoc Hoang[1,2], Susanne Jahreis[1,2], Silke Rummler[3], Andreas Hochhaus[2], Marie von Lilienfeld-Toal[1,2]

[1]Infections in Hematology and Oncology, Leibniz Institute for Infection Biology and Natural Product Research, Jena, Germany
[2]Department for Hematology and Medical Oncology, Jena University Hospital, Jena, Germany
[3]Institute for Transfusion Medicine, Jena University Hospital, Jena, Germany

Email addresses of co-authors:

Christopher Rauh	christopher.rauh@uni-jena.de
Sarah Böttcher	sarah.boettcher@leibniz-hki.de
Mai T.N. Hoang	mai.hoang@leibniz-hki.de
Susanne Jahreis	susanne.jahreis@leibniz-hki.de
Silke Rummler	silke.rummler@med.uni-jena.de
Andreas Hochhaus	andreas.hochhaus@med.uni-jena.de
Marie von Lilienfeld-Toal	Marie.von_Lilienfeld-Toal@med.uni-jena.de

Corresponding author:

Susann Hartung susann.hartung@leibniz-hki.de

KEYWORDS:

phagocytosis, flow cytometry, *Aspergillus fumigatus*, conidia, FITC, anti-FITC antibody, aspergillosis

SUMMARY:

This protocol provides a fast and reliable method to quantitatively measure phagocytosis of *Aspergillus fumigatus* conidia by human primary phagocytes using flow cytometry and to discriminate phagocytosis of conidia from mere adhesion to leukocytes.

ABSTRACT:

Invasive pulmonary infection by the mold *Aspergillus fumigatus* poses a great threat to immunocompromised patients. Inhaled fungal conidia (spores) are cleared from the human lung alveoli by being phagocytosed by innate monocytes and/or neutrophils. This protocol offers a fast and reliable measurement of phagocytosis by flow cytometry using fluorescein isothiocyanate (FITC)-labeled conidia for co-incubation with human leukocytes and subsequent counterstaining with an anti-FITC antibody to allow discrimination of internalized and cell-adherent conidia. Major advantages of this protocol are its rapidness, the possibility to combine the assay with cytometric analysis of other cell markers of interest, the simultaneous analysis of monocytes and neutrophils from a single sample and its applicability to other cell wall-bearing

fungi or bacteria. Determination of percentages of phagocytosing leukocytes provides a means to microbiologists for evaluating virulence of a pathogen or for comparing pathogen wildtypes and mutants as well as to immunologists for investigating human leukocyte capabilities to combat pathogens.

INTRODUCTION:

Invasive pulmonary aspergillosis is a great threat to immunocompromised patients as treatment options are limited and only successful upon early diagnosis, which leads to high mortality rates[1]. Infectious agents are conidia (spores) of the mold *Aspergillus fumigatus* that are ubiquitous to most habitats[2]. Conidia are inhaled, pass through the airways and can finally enter the lung alveoli. In immunocompetent humans, these conidia are cleared by innate immune cells such as monocytes or macrophages and neutrophil granulocytes, which take up (phagocytose) and digest the pathogens[3]. Phagocytosis is important for microbiologists and immunologists likewise when interested in host-pathogen interactions. Confrontation assays, such as co-incubation of leukocytes and conidia, often include labeling of the spores by fluorescein or its derivative fluorescein isothiocyanate (FITC). Using a microscope, it is straightforward to identify internalized fluorescent conidia and to determine attached/adherent conidia, although this approach is cumbersome and realistically restricted to a few hundreds of cells[4]. However, in flow cytometry which easily allows the analysis of hundreds of thousands of cells within minutes, differential staining of phagocytosed and adherent conidia is vital. Therefore, many protocols rely on Trypan Blue to quench FITC-fluorescence from adherent conidia[5-8]. Another approach is exploiting fluorescence resonance energy transfer of ethidium bromide and FITC to emit red instead of green fluorescence from adherent conidia[9-11]. If specific antibodies are available, as is the case for some bacteria, cell-bound particles can be directly stained[12,13].

Here, we present a protocol to quickly and quantitatively assess phagocytosis of FITC-labeled *A. fumigatus* conidia by human leukocytes along with attachment of spores to cells and lack of interaction by employing an allophycocyanin (APC)-coupled anti-FITC antibody. The method also allows for the simultaneous flow cytometric analysis of further cell markers that can be employed for separate analysis of phagocytosis by monocytes and neutrophils from the same sample.

The protocol can be applied for characterization of fungal strains (e.g., several species of *Aspergillus* and other molds from the genus *Mucorales* presented here) and their mutants[14] and immunological research on phagocytes, such as leukocytes from immunocompromised individuals.

PROTOCOL:

This protocol includes the use of human buffy coats obtained from the Institute for Transfusion Medicine, Jena University Hospital and fresh venous blood drawn from patients, both after written informed consent of the donors in accordance to ethics committee approval 4357-03/15.

1. Preparation of *Aspergillus fumigatus* conidia

1.1. Grow *A. fumigatus* on 1.5% malt agar Petri dishes for 5 days at 37 °C without CO_2.

CAUTION: *A. fumigatus* is a biosafety level 2 microorganism and must be handled in an appropriate facility using a biosafety cabinet and wearing lab coat, gloves and a filter mask.

NOTE: The plate should be covered completely with a green-greyish layer of conidia. White cultures do not sporulate. Composition of malt agar: 4% (w/v) malt extract, yeast extract 0.4% (w/v), agar 1.5% (w/v), Aqua dest.

1.2. Harvesting conidia

1.2.1. Place a paper towel wet with disinfectant into the biosafety cabinet and put the plate on top to prevent overdistribution of volatile conidia.

1.2.2. Add 10 mL of phosphate buffered saline (PBS) + 0.01% detergent on top of the fungus, use a Drigalski spatula to spread the liquid over the plate and rub off the dark colored conidia. Be careful not to remove the white mycelium.

1.2.3. Put the conidia suspension to a 50 mL tube using a 30 µm cell strainer to remove any residual mycelium.

1.2.4. Repeat steps 1.2.2 and 1.2.3 and collect in the same 50 mL tube.

1.2.5. Spin for 5 min at 2,600 x *g* at room temperature.

1.2.6. Remove the supernatant and resuspend in 20 mL of sterile Aqua dest.

1.2.7. Determine conidia concentration with a Thoma counting chamber.

NOTE: The protocol can be paused here and the conidia suspension stored for up to 1 month at 4 °C with the lid screwed tightly.

1.3. FITC labeling

1.3.1. Prepare a 0.1 mM solution of FITC powder in sterile 0.1 M Na_2CO_3 (dissolved in PBS).

CAUTION: FITC powder is hazardous and should be handled with gloves, goggles and a filter mask. Collect waste according to local regulations.

NOTE: Omit artificial light during this and the following steps involving conidia.

1.3.2. Resuspend 1 x 10^8 (or less) conidia in 5 mL of FITC solution in a 15 mL tube. Incubate for 20 min at 37 °C in a rotator.

1.3.3. For the negative control, resuspend conidia in 0.1 M Na_2CO_3 (without FITC) in a 15 mL tube. Incubate for 20 min at 37 °C in a rotator.

NOTE: When calculating the amount of conidia to be stained, take into account a loss of up to 70% during staining, swelling and all necessary washing steps. If no rotator is available, the suspension should be shaken three times during incubation.

1.3.4. For washing, add 10 mL of PBS + 0.01% detergent to the suspension and spin for 5 min at 2,600 x *g* at room temperature.

1.3.5. Remove supernatant and repeat washing twice with 10 mL of PBS + 0.01% detergent.

1.4. Swelling of conidia (may be omitted if not desired)

1.4.1. Resuspend FITC labeled conidia in 5 mL of Roswell Park Memorial Institute (RPMI) medium + 10% Fetal Calf Serum (FCS) and incubate in a rotator at 37 °C for the desired time (e.g., 2 h, 4 h).

NOTE: If no rotator is available, the suspension should be shaken at least every 20 min during incubation.

1.4.2. Add 10 mL of PBS + 0.01% detergent to the suspension and spin for 5 min at 2,600 x *g* at room temperature.

1.4.3. Remove the supernatant and wash twice with 10 mL of PBS + 0.01% detergent.

1.4.4. Filter through a 30 µm cell strainer to remove large clumps of conidia.

1.5. Fixing of conidia (may be omitted if not desired)

1.5.1. Resuspend FITC labeled and swollen conidia in 1 mL of formaldehyde and incubate for 1 h at room temperature.

1.5.2. Add 10 mL of PBS + 0.01% detergent to the suspension and spin for 5 min at 2,600 x *g* at room temperature.

1.5.3. Remove the supernatant and wash twice with 10 mL of PBS + 0.01% detergent.

NOTE: The protocol can be paused here and the conidia stored in PBS in the dark (tube wrapped up in aluminum foil) for up to 1 week at 4 °C.

2. Preparation of human primary leukocytes

2.1. Put 5 mL of buffy coat in a 50 mL tube. Alternatively, use fresh human peripheral venous blood drawn into ethylenediaminetetraacetic acid (EDTA) monovettes (10 mL per 50 mL tube).

CAUTION: Human blood may transmit viruses such as Human Immunodeficiency Virus and Hepatitis B Virus. Handle only after being vaccinated against Hepatitis B. Handle in a biosafety cabinet wearing lab coat and gloves.

2.2. Fill up the tube with Erythrocyte Lysis (EL) Buffer, invert three times and incubate for 5-8 min horizontally until the milky appearance of the mixture turns clear.

NOTE: Occasionally, the blood may take longer to lyse. Go by the appearance. It is not unusual that two tubes of the same blood take different times to lyse. Composition of EL buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA

2.3. Spin for 10 min at 300 x *g* at room temperature. Discard the supernatant.

2.4. Resuspend the pellet in 1 mL of EL Buffer by pipetting. Then add another 24 mL of EL buffer, and invert several times.

2.5. Spin for 5 min at 300 x *g* at room temperature.

2.6. Discard the supernatant and resuspend cells in 1 mL of RPMI + 10% FCS.

2.7. Determine the cell concentration with a Neubauer counting chamber.

3. Phagocytosis assay

3.1. Incubate 2 x 10^6 leukocytes and 4 x 10^6 FITC-labeled conidia (multiplicity of infection = 2) in 1.5 mL of RPMI + 10% FCS in a 12-well cell culture plate. As controls, include cells only (no conidia) and cells + unlabeled conidia.

3.2. Place in a humidified CO_2 incubator at 37 °C and incubate for the desired period of time (e.g., 0.5 h, 2 h or 4 h).

3.3. After incubation, harvest cells with a cell scraper and put in a 15 mL tube.

3.4. Spin for 5 min at 300 x *g* at room temperature.

3.5. Collect supernatant for cytokine analysis or discard if not wanted. Resuspend each sample in 100 µL of PBS + 2 mM EDTA.

4. Antibody staining

4.1. For each sample, prepare 100 µL of antibody mix, including APC anti-FITC antibody, according to **Table 1**.

4.2. Put a 100 µL sample into one well of a 96-well V-bottom plate. Add 150 µL of PBS + 2 mM EDTA for washing.

4.3. For color compensation, place 1 x 10^6 cells for each color in further wells of the 96-well V bottom plate. Include a well of cells that is left unstained. Add 150 µL of PBS + 2 mM EDTA for washing.

4.4. Cover plate with an adhesive foil.

4.5. Spin for 5 min at 300 x *g* at room temperature. Remove the foil.

4.6. Discard the supernatant by quickly and forcefully inverting the plate only once over the sink or a disposable paper towel.

NOTE: Do not repeat or knock the plate on a paper until dry as this will result in a massive loss of cells from the plate.

4.7. Resuspend cells in the 100 µL antibody mix, and mix well by pipetting.

4.8. For color compensation, resuspend the respective cells in 100 µL of PBS + 2 mM EDTA and add a single antibody to each well at the same amount used in the antibody mix.

4.9. Cover with an adhesive foil and incubate for 20 min at room temperature in the dark.

4.10. Remove the foil. Add 150 µL of PBS + 2 mM EDTA to each well for washing. Cover with an adhesive foil.

4.11. Spin for 5 min at 300 x *g* at room temperature. Remove the foil. Discard the supernatant by quickly and forcefully inverting the plate over the sink or a disposable paper towel.

4.12. Resuspend in 200 µL of PBS + 2 mM EDTA and transfer cells from each well to a separate round bottom tube. Make sure there are no cell clusters in the suspension. Remove clusters otherwise.

NOTE: Every cluster that is large enough for the eye to see is large enough to potentially clog the cytometer.

5. Flow cytometry

5.1. Start the flow cytometer and let it warm up. Start the acquisition software.

5.2. Create a new experiment and setup and label the samples.

5.3. Set up parameters (FSC 250, SSC 250) and detectors for fluorophores FITC, APC, BUV395, V500, PerCP-Cy5.5.

5.4. Compensation setup

5.4.1. Open compensation setup.

5.4.2. Indicate individual colors.

5.4.3. Using the control cells left unstained or with individual stainings, set the PMT detector voltages to include all events within the scale.

5.4.4. Record at least 10,000 events of each control.

5.4.5. Use the compensation setup to calculate the spillover of fluorophores and apply to the experiment's cytometer settings.

5.5. Recording sample data

5.5.1. Display FSC and SSC in the acquisition software and set gate around leukocytes.

5.5.2. Based on the leukocyte gate, display dot plot SSC/CD45 and gate for CD45+ cells to separate from conidia.

5.5.3. Display CD45+ cells in a dot plot CD14/CD66b and gate monocytes (CD14+) and neutrophils (CD66b+) separately.

5.5.4. Display neutrophils in a dot plot anti-FITC/FITC.

5.5.5. Using the sample with unlabeled conidia, set quadrants for anti-FITC and FITC signals, allowing a maximum of 1% of cells in the respective quadrants.

5.5.6. Repeat steps 5.5.4 and 5.5.5 for monocyte gate.

5.5.7. Record all samples with at least 20,000 events in the leukocyte gate.

REPRESENTATIVE RESULTS:

In measuring phagocytosis of *A. fumigatus* conidia by human phagocytic cells, discrimination between genuine internalization and mere attachment of conidia to the cells is an obstacle, especially when it comes to high-throughput methods such as flow cytometry. In order to overcome this hurdle, we present a fast and reliable protocol based on the staining of conidia

with the fluorescent dye FITC prior to co-incubation of cells and conidia, followed by a counterstaining with an APC-labeled anti-FITC antibody after incubation (**Figure 1A**). As shown in **Figure 1B**, FITC-labeled conidia are phagocytosed by human monocytes and neutrophils that provide a green signal to the cells. These conidia are inaccessible for the anti-FITC antibody and, hence, cannot bind the antibody and the cells appear APC negative (FITC+, APC-). Non-interacting cells do not acquire a green signal from FITC-labeled conidia and remain FITC-, APC-. A few cells appear FITC-, APC+. Since the anti-FITC APC antibody should not be able to bind cells without FITC-labeled conidia, these events are considered staining artifacts. FITC-labeled conidia, which are attached to the cells but not internalized, render cells also positive for FITC but also provide a target for the anti-FITC antibody that makes these cells double positive for FITC and APC (FITC+, APC+). When analyzed microscopically, this population contained up to 20% of cells with attached conidia only in our experiments.

Using the antibodies described in this protocol and following the gating strategy in **Figure 1D**, a general gating of human leukocytes by FSC and SSC characteristics is followed by a separation of leukocytes and free conidia by the pan-leukocyte marker CD45. Especially when using swollen conidia and/or long incubation times, conidia can reach almost cell size at the time of flow cytometry and hence bias analysis. Since human primary monocytes and neutrophils take up conidia differently, this protocol allows to separately analyze these cell population based on staining with the well-established cell lineage markers CD14 for monocytes and CD66b for neutrophils. Gating for phagocytosing and adherent cell populations is done based on control samples with unlabeled conidia that carry neither a FITC nor an anti-FITC APC signal. When APC and FITC are plotted against each other, quadrants are set in such a manner that a maximum of 1% cells are allowed in the gates of interest.

The percentage of human primary phagocytes internalizing conidia can be highly variable among blood donors but also depends on experimental factors such as incubation time and swelling state of conidia. Spore internalization can be detected after 0.5 h of co-incubation already and increases with time (**Figure 2A**). Pre-swollen conidia are taken up easier than resting (not swollen) spores even at short incubation times (**Figure 2B**). If conidia are fixed with formaldehyde, phagocytosis is diminished compared to native conidia (**Figure 2C**).

FIGURE AND TABLE LEGENDS:

Table 1: Antibody mix for flow cytometry. Amounts of each reagent are given as microliters per sample (1 x 10^6 cells) to be analyzed.

Figure 1: Setup and analysis of flow cytometric phagocytosis assay. (**A**) Scheme of the protocol including conidia and cell preparation and counterstaining. (**B**) Phagocytosis is analyzed by plotting of flow cytometric data of FITC-labeled conidia against anti-FITC APC counterstaining. Resulting populations indicate percentages of non-interacting leukocytes (FITC-, APC-), leukocytes with adherent conidia (FITC+, APC+) and phagocytosing leukocytes (FITC+, APC-) as well as staining artifacts (FITC-, APC+). (**C**) Double positive cell populations from 3 different experiments with 3 different donors (two of them performed in duplicates, one performed single) were microscopically counted for internalized and attached only conidia. (**D**)

Representative gating strategy of flow cytometric data to detect leukocytes (CD45), identify monocytes (CD14) and neutrophils (CD66b) and determine interacting populations (FITC, anti-FITC). This figure has been modified from Hartung et al., Cytometry A, 95: 3, p. 332-338 (2019)[14].

Figure 2: Phagocytosis of conidia by human primary phagocytes depends on several conditions. (**A**) Percentage of cells internalizing resting conidia increased with co-incubation time. (**B**) Phagocytosis increased with conidial swelling time when co-incubated for 0.5 h. (**C**) Native conidia were better phagocytosed than fixed conidia. Data were obtained from 10 different donors (**A,B**) or 5 different donors (**C**) in 10 (**A,B**) or 5 (**C**) independent experiments. Error bars indicate SD. This figure has been modified from Hartung et al., Cytometry A, 95: 3, p. 332-338 (2019)[14].

Figure 3: Analysis of phagocytosis is a means to assess functionality of human primary phagocytes and characterize clinically relevant molds. (**A**) Exemplary comparison of monocytes from a healthy donor and an immunosuppressed patient (after hematopoietic stem cell transplantation) phagocytosing resting conidia for 0.5 h, 2 h and 4 h. (**B**) Phagocytosis of resting fungal conidia was determined for clinically relevant *Aspergillus* and *Mucorales* species after 2 h co-incubation. Data were obtained from 5 (*Aspergillus*) or 3 (*Mucorales*) different donors in 5 or 3 independent experiments each. Error bars indicate SD. Abbreviations: A. *Aspergillus*, L. *Lichtheimia*, M. *Mucor*, R. *Rhizopus*

DISCUSSION:

This protocol presents a fast flow cytometric method to measure interaction of *A. fumigatus* conidia with a large number of primary human leukocytes that is not possible in common microscopic protocols. Imaging cells with a microscope and manually counting internalized conidia is cumbersome and can realistically be done for a few hundred cells only. Flow cytometry overcomes this problem by measuring thousands of cells within minutes. A hurdle common to both approaches is the distinction of phagocytosed and adherent conidia in or on cells, respectively. In microscopy, the dye calcofluor white is often used for staining adherent conidia but its usage is limited to microorganisms with a chitin-containing cell wall.

This protocol in contrast used distinct fluorophores for characterization of interaction events that allows the addition of further cell markers, such as the lineage markers CD45, CD14 and CD66b. Thus, it is also possible to discriminate phagocytosis of pathogens by monocytes and neutrophils in a single sample. While the choice of markers and fluorophores for cell identification can be adapted to the needs of the experiment and the capacities of the available cytometer, the usage of the specific APC anti-FITC antibody mentioned in this protocol is recommended as it was the most reliable antibody in our hands.

Since formaldehyde-fixed conidia are not internalized equally well, native spores are recommended for phagocytosis assays. However, native conidia will start or continue swelling during the co-incubation with human leukocytes and eventually germinate. Typically, *A. fumigatus* conidia germinate after about 8-9 h in glucose-containing media at 37 °C. The

combination of swelling time and co-incubation time with phagocytes should not exceed this time frame as germination causes the loss of FITC on the conidia surface. More important, germlings cannot be phagocytosed anymore by monocytes or neutrophils. Instead, these phagocytes accumulate around and stick to germlings that produce cell clusters that clog the cytometer, if not removed. Similarly, 4 h swollen conidia tend to generate clusters among themselves and with cells. Often these clusters cannot be separated mechanically anymore and the cells within are lost for flow cytometric analysis.

Although gating is straightforward and easy in the beginning, the more conidia are internalized by cells, the blurrier gating may become. Using MOIs > 2 increases phagocytosis at initial time points but gating issues might arise earlier as well. Therefore, MOIs should be carefully determined with the specific cells and pathogens of interest.

A limitation of this protocol is in the duality of the cell population with adherent conidia (FITC+ APC+) that might also be harboring cells with adherent as well as internalized conidia. A possibility for further discrimination is the application of imaging flow cytometry[15] that allows visual imaging of all cells measured in the flow cytometer.

Due to the unspecific FITC labeling of the conidial cell wall, this method is easily transferable to other fungi of interest such as clinically relevant molds of the genus *Mucorales* or the yeast *Candida albicans*. Moreover, also cell-wall bearing bacteria can be FITC-labeled. The universal counterstaining with the anti-FITC antibody allows fast and easy measurement of phagocytosis of all these pathogens alike by a large number of human leukocytes.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1 Brown, G. D. et al. Hidden killers: human fungal infections. *Science Translational Medicine.* **4** (165), 165rv113 (2012).
2 Brakhage, A. A., Bruns, S., Thywissen, A., Zipfel, P. F., Behnsen, J. Interaction of phagocytes with filamentous fungi. *Current Opinion in Microbiology.* **13** (4), 409-415 (2010).
3 Heinekamp, T. et al. Interference of Aspergillus fumigatus with the immune response. *Seminars in Immunopathology.* **37** (2), 141-152 (2015).
4 Slesiona, S. et al. Persistence versus escape: *Aspergillus terreus* and *Aspergillus fumigatus* employ different strategies during interactions with macrophages. *PLoS One.* **7** (2), e31223 (2012).

5 Busetto, S., Trevisan, E., Patriarca, P., Menegazzi, R. A single-step, sensitive flow cytofluorometric assay for the simultaneous assessment of membrane-bound and ingested *Candida albicans* in phagocytosing neutrophils. *Cytometry A.* **58** (2), 201-206 (2004).
6 Lowe, D. M. et al. A novel assay of antimycobacterial activity and phagocytosis by human neutrophils. *Tuberculosis (Edinb).* **93** (2), 167-178 (2013).
7 Nuutila, J., Lilius, E. M. Flow cytometric quantitative determination of ingestion by phagocytes needs the distinguishing of overlapping populations of binding and ingesting cells. *Cytometry A.* **65** (2), 93-102 (2005).
8 Saresella, M. et al. A rapid evaluation of phagocytosis and killing of *Candida albicans* by CD13+ leukocytes. *Journal of Immunological Methods.* **210** (2), 227-234 (1997).
9 Fattorossi, A., Nisini, R., Pizzolo, J. G., D'Amelio, R. New, simple flow cytometry technique to discriminate between internalized and membrane-bound particles in phagocytosis. *Cytometry.* **10** (3), 320-325 (1989).
10 Heinzelmann, M., Gardner, S. A., Mercer-Jones, M., Roll, A. J., Polk, H. C., Jr. Quantification of phagocytosis in human neutrophils by flow cytometry. *Microbiology and Immunology.* **43** (6), 505-512 (1999).
11 Perticarari, S., Presani, G., Mangiarotti, M. A., Banfi, E. Simultaneous flow cytometric method to measure phagocytosis and oxidative products by neutrophils. *Cytometry.* **12** (7), 687-693 (1991).
12 Sveum, R. J., Chused, T. M., Frank, M. M., Brown, E. J. A quantitative fluorescent method for measurement of bacterial adherence and phagocytosis. *Journal of Immunolological Methods.* **90** (2), 257-264 (1986).
13 de Boer, E. C., Bevers, R. F., Kurth, K. H., Schamhart, D. H. Double fluorescent flow cytometric assessment of bacterial internalization and binding by epithelial cells. *Cytometry.* **25** (4), 381-387 (1996).
14 Hartung, S. et al. Fast and Quantitative Evaluation of Human Leukocyte Interaction with Aspergillus fumigatus Conidia by Flow Cytometry. *Cytometry A.* **95** (3), 332-338 (2019).
15 Fei, C., Lillico, D. M. E., Hall, B., Rieger, A. M., Stafford, J. L. Connected component masking accurately identifies the ratio of phagocytosed and cell-bound particles in individual cells by imaging flow cytometry. *Cytometry A.* **91** (4), 372-381 (2017).

Figure 1

Figure 1: Scheme of the method and gating strategy

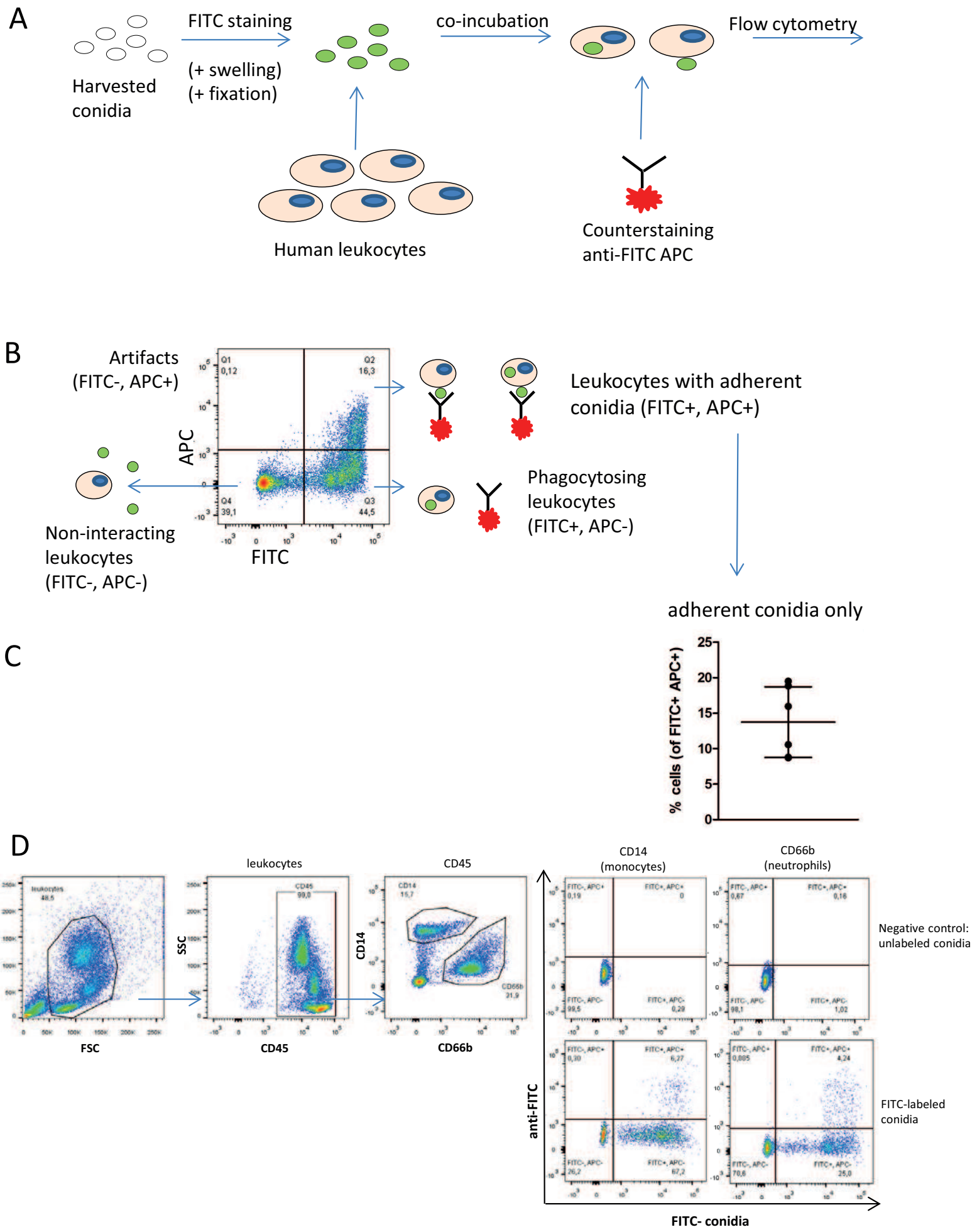
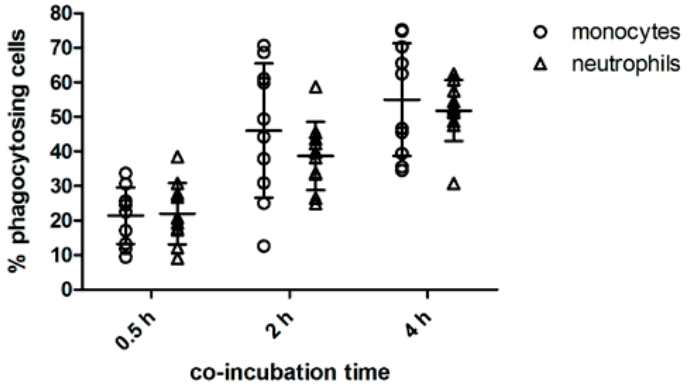


Figure 2: Phagocytosis depends on several conditions

A



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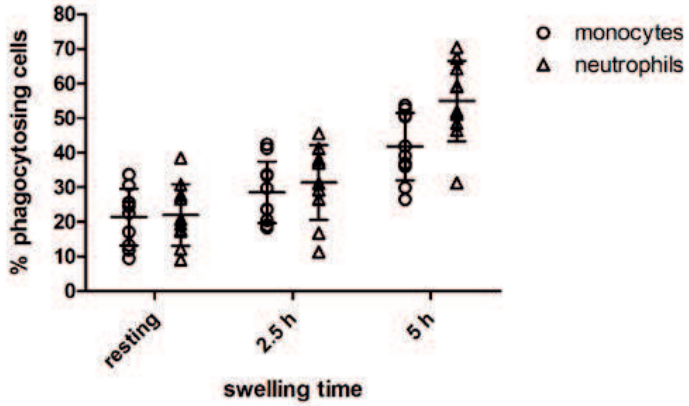

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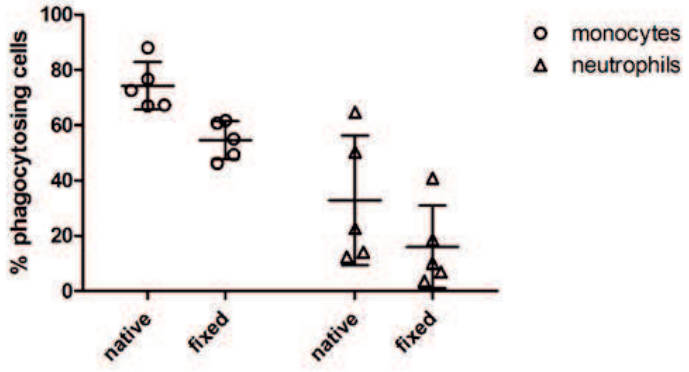

Figure 3

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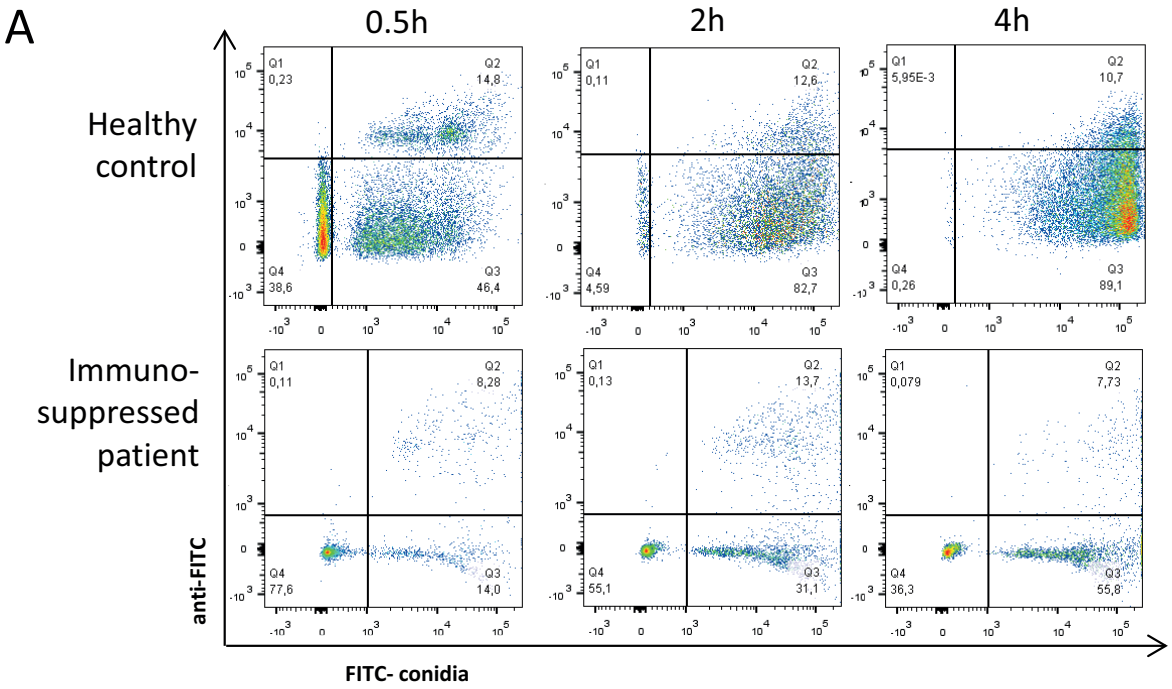
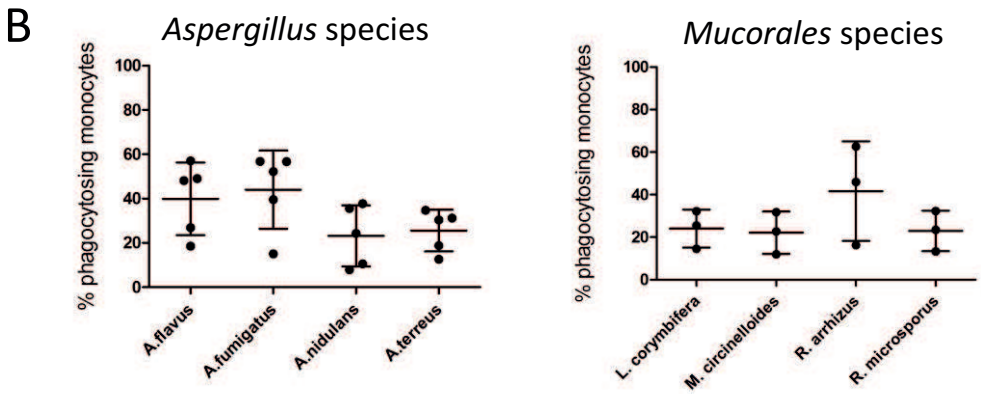
Figure 3: Application of phagocytosis





Name of Material/ Equipment	Company	Catalog Number
Adhesive foil	Brand	701367
anti-CD14 V500	BD Biosciences	561391
anti-CD45 BUV395	BD Biosciences	563792
anti-CD66b PerCP-Cy5.5	BD Biosciences	562254
anti-FITC APC	ThermoFisher Scientific	17-7691-82
Cell culture plate, 12-well	Greiner Bio-one	665180
Cell scraper	Bioswisstech	800020
Cell strainer, 30 µm	Miltenyi Biotech	130-098-458
Cytometer	BD Biosciences	
Detergent	Sigma Aldrich	P1379
Drigalski spatula	Carl Roth	PC59.1
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	ED3SS-500g
Erythrocyte lysis buffer		
Fetal Calf Serum (FCS)	Biochrom AG	S 0115
Fluorescein isothiocyanate (FITC)	Sigma Aldrich	F3651-100MG
Formaldehyd	Carl Roth	PO87.3
Malt agar (1.5%)		
Na_2CO_3	Carl Roth	8563.1
Petri dish	Greiner Bio-one	633180
Phosphat Buffered Saline (PBS)	ThermoFisher Scientific	189012-014
RPMI 1640	ThermoFisher Scientific	61870010
Rotator	Miltenyi Biotech	130-090-753
Round-bottom tube, 7.5 mL	Corning	REF 352008
Software for data acquisition and analysis	BD Biosciences	
V-bottom plate, 96 well	Brand	781601

Comments/Description

clone M5E2
clone HI30
clone G10F5

clone NAWESLEE

SmartStrainer
LSR Fortessa II, lasers: 488 nm (blue), 405 nm (violet), 355 nm (UV) and 640 nm (red)
Tween 20, 0.01% in PBS

2 mM in PBS

0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA

10% in RPMI 1640

0.1 mM in Na_2CO_3 /PBS solution

Histofix
malt extract (40 g), yeast extract (4 g), agar (15 g), Aqua dest. (1 L), adjust pH to 5.7-6.0, sterilise at 121
0.1 M in PBS

without Calcium, without Magnesium

RPMI 1640 Medium, GlutaMAX Supplement

MACSmix Tube Rotator

FACSDiva 8.0

untreated surface

°C for 35 minutes

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Title of Article: Measuring phagocytosis of Aspergillus fumigatus conidia by human Leukocytes using flow cytometry

Author(s): Susann Hartung, Christopher Rauh, Mai Thi Ngoc Hoang, Susanne Jahreis, Silke Rummler, Andreas Hochhaus, Marie von Lilienfeld-Toal

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CORRESPONDING AUTHOR

Name:	Susann Hartung
Department:	Infections in Hematology and Oncology
Institution:	Leibniz Institute for Infection Biology and Natural Product Research
Title:	Dr.
Signature:	S. Hartung
Date:	06/06/2019

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Dear Dr. Wu,

Thank you very much for considering our manuscript "Measuring phagocytosis of Aspergillus fumigatus conidia by human leukocytes using flow cytometry" for publication. We are also thankful for the reviewers' as well as the editorial comments as we feel they improve the manuscript substantially.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
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6. Please do not abbreviate journal titles for references.
7. Please specify the antibody in the protocol.
8. Please define all abbreviations before use.
9. Please specify the composition of all buffer or media in the protocol.

The manuscript was carefully checked for formatting, requested units, abbreviations and commercial language. It was proofread by three people. All antibodies used are specified in the Table of Materials and Reagents and were not further detailed in the protocol to avoid commercial language.

The references were updated and buffer and media compositions were added to the protocol.

Copyright permission was uploaded as .doc file as requested and citations added to the respective figures although the figures intended for publication with JOVE are not the same as in the previous publication.

Reviewers' comments:

Reviewer #1:

Major Concerns:

The double labeling strategy with FACS has been repeatedly published to identify conidia of A.fumigatus binding to phagocytes vs conidia phagocytosed. Here an antibody against FITC is used instead of the classical anti-conidium antibody. This ms does not bring anything new special (except

the use of an antiFITC antibody but at the end it is always the same strategy)
Discriminate phagocytosis of pathogens by monocytes and neutrophils in a single sample is not an issue for aspergillosis since these phagocytes are not present together and targets different morphotypes.
The authors mention that the limitation of this protocol is in the duality of the cell population that harbor cells with adherent as well as internalized conidia. But they did not explore the putative solutions (even though they mention one ref)

Upon intake of *A. fumigatus* conidia to the human lung, alveolar macrophages constitute the first line of cellular defense. However, if cytokines are released to acquire further innate leukocytes to combat conidia, monocytes as well as neutrophils are recruited from the blood stream. Thus, the analysis of phagocytotic capabilities of monocytes and neutrophils towards conidia might still hold relevance.

Regarding the issue of a double-positive population, combining microscopy and flow cytometry, as already reported by Fei et al. (Cytometry A. 91 (4), 372-381, 2017) provides a sophisticated way to automatically determine percentages of cells with adherent conidia and cells with ingested and adherent conidia. If such a device is unavailable, manual microscopy is an alternative. Going for the latter, we further checked the double-positive population. Please see the reply to reviewer 2 on this aspect.

Reviewer #2:

Manuscript Summary:

A straightforward description of a standard protocol for measuring the phagocytosis of fungal spores by human blood monocytes.

Major Concerns:

No major concerns with the protocol or the written article.

Minor Concerns:

Microscopic analysis of the double positive population should be performed to provide a general quantitative assessment of how many macrophages or neutrophils have spores exclusively on their surface (e.g. non-phagocytic cells). This would provide an understanding (e.g. 10% of the population) of how many leukocytes failed to internalize their targets at the times examined. In addition, how sensitive is this assay for measuring meaningful changes in the phagocytic potential of human leukocytes? Do PBLs from immunocompromised donors demonstrate reduced abilities to engulf these pathogens? If so, can the current protocol be used to accurately detect a reduced phagocytic potential? Overall, I think this would be a very important aspect of the study.

As to distinguish the double positive population (FITC+ APC+) of cells with attached and potentially phagocytosed conidia, we took the chance to analyze this population microscopically and found

between 8 and 20% of these cells to harbor only attached conidia (see new Figure 1 C). To point out the fact in the manuscript, the respective part from the Representative Results sections now reads:

"FITC-labeled conidia that are attached to the cells but not internalized, render cells also positive for FITC but also provide a target for the anti-FITC antibody which makes these cells double positive for FITC and APC (FITC+, APC+). When analyzed microscopically, this population contained up to 20% of cells with attached conidia only in our experiments."

The reviewer was also enquiring after usage of the assay to detect changes in the phagocytic potential of human leukocytes. We have tested leukocytes from immunocompromised donors (having undergone allogenic stem cell transplantation) with our assay and found their ability to engulf *A. fumigatus* conidia diminished. Because these data in their entirety will be presented in a separate manuscript being in preparation, we present an example here in the new Figure 3. At all tested time points phagocytosis of patient monocytes lacked behind healthy cells (Figure 3 A).

Reviewer #3:

Manuscript Summary:

The authors describe a protocol for assessment of phagocytosis of Aspergillus conidia by primary human phagocytes with flow cytometry

Major Concerns:

The protocol is well described and seems straightforward

Minor Concerns:

The use of FITC-labeling for conidia of other fungi (e.g. Mucorales) is not so efficient as compared to Aspergillus and this protocol might not be applicable in general to other molds. If the authors have data on other molds it would be nice to provide them

In addition, calculation of phagocytosis rate and index should be provided and explained for the general reader and the performance of the assay with different MOI should be given

Reviewer 3 asked for the application of the technique to pathogens other than *A. fumigatus*. To address this issue we tested other clinically relevant molds of the genus *Aspergillus* and several Mucorales species. As shown in the new Figure 3 B, all these species can be investigated with our phagocytosis assay.

The phagocytosis ratio and index are quantities that relate the number of phagocytosed pathogens to either the total number of pathogens present or number of (host) cells, respectively. However, determination of exact numbers of phagocytosed and adherent conidia by flow cytometry is difficult. The green fluorescence is conveyed to the cells by ingestion of a single FITC-labelled conidium as well as by ingestion of several conidia. FITC fluorescence intensities vary between cells in a continuous gradient that does not allow a reliable correlation between number of phagocytosed conidia and fluorescence intensity.

As with all experimental parameters, such as the applied cells, the specific pathogen or the incubation time, MOIs have to be titrated to suit the particulars of the experiment. In our case, MOI 2

was a reliable ratio of phagocytes and conidia within the range of investigated swelling and incubation times. A note regarding using different MOIs was added in the discussion.

"Although gating is straight forward and easy in the beginning, the more conidia are internalized by cells, the blurrier gating may become. Using MOIs > 2 increases phagocytosis at initial time points but gating issues might arise earlier as well. Therefore, MOIs should be carefully determined with the specific cells and pathogen of interest."

We hope that the improvements made to manuscript due to the reviewer's helpful advice now make it acceptable for publication in the Journal of Visualized Experiments.

Yours sincerely,

Susann Hartung

On behalf of the authors

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