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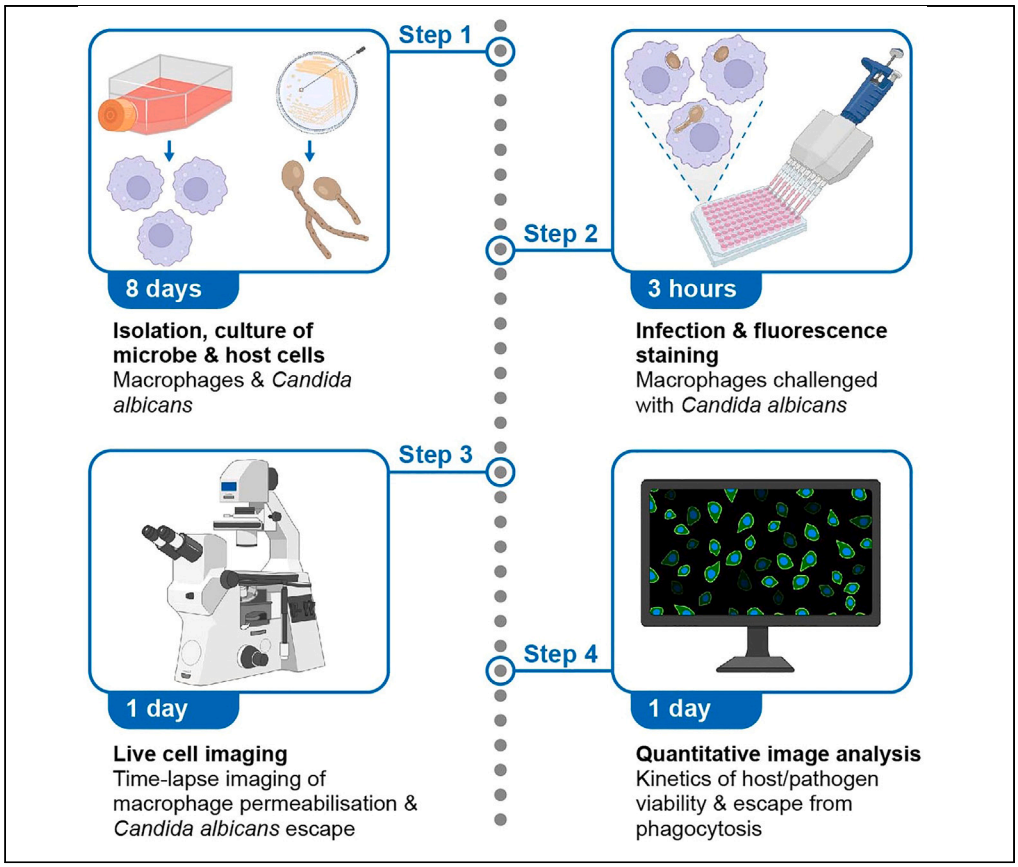
Quantitative live-cell imaging of *Candida albicans* escape from immune phagocytes



Population-level dynamics of host-pathogen interactions can be characterized using quantitative live-cell imaging. Here, we present a protocol for infecting macrophages with the fungal pathogen *Candida albicans in vitro* and quantitative live-cell imaging of immune and pathogen responses. We describe steps for detailed image analysis and provide resources for quantification of phagocytosis and pathogen escape, as well as macrophage membrane permeabilization and viability. This protocol is modifiable for applications with a range of pathogens, immune cell types, and host-pathogen mechanisms.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A quantitative live-cell imaging assay to measure microbial escape from phagocytes

Fluorescent labeling allows for parallel imaging of microbe and host mechanisms

Steps to quantify host cell permeabilization, microbial load, and escape kinetics

Detailed image analysis steps, along with example calculations and videos

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Quantitative live-cell imaging of *Candida albicans* escape from immune phagocytes

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SUMMARY

Population-level dynamics of host-pathogen interactions can be characterized using quantitative live-cell imaging. Here, we present a protocol for infecting macrophages with the fungal pathogen *Candida albicans in vitro* and quantitative live-cell imaging of immune and pathogen responses. We describe steps for detailed image analysis and provide resources for quantification of phagocytosis and pathogen escape, as well as macrophage membrane permeabilization and viability. This protocol is modifiable for applications with a range of pathogens, immune cell types, and host-pathogen mechanisms.

For complete details on the use and execution of this protocol, please refer to Olivier et al.[1]

BEFORE YOU BEGIN

Innate immune phagocytes are important effectors in immune clearance of clinically relevant bacterial and fungal pathogens.[2] Many bacteria and fungi have strategies to survive containment in the phagocytes, including within macrophage phagolysosomes. Furthermore, some pathogens are able to not only survive intracellularly in macrophages, but also escape into the extracellular environment.[3–5] These escape phenomena use pathogen factors, and also engage host signaling pathways that lead to immune cell damage and lysis and can also be associated with inflammation.[4,5] Therefore, a deeper understanding of these pathways could offer alternative therapeutic targets. This is important as many microbes are growing resistant to antimicrobial therapy.[6,7]

Live cell imaging has been used to understand innate immune interactions of pathogens.[8,9] Using image analysis, microscopy data can be harnessed to quantify several aspects of host-pathogen interactions,[10,11] including host cell membrane remodeling,[12] expression of virulence factors,[13] escape of the pathogen,[1] activation of inflammasome pathways,[14] and so on.

The development of a quantitative live cell imaging assay is best approached as an iterative process: this involves optimization of both the *in vitro* experiment and the image analysis pipeline in parallel.[15] In this protocol we describe a live cell imaging experiment that captures the outcome of the *in vitro* infection of macrophages with *Candida albicans*, a human fungal pathogen. The experimental conditions described here allow for accurate 2D image analysis of population-level interactions between macrophages and *C. albicans* in a time-resolved fashion, and can serve as a useful starting point for adapting the protocol to other host cells and microorganisms. Hundreds of

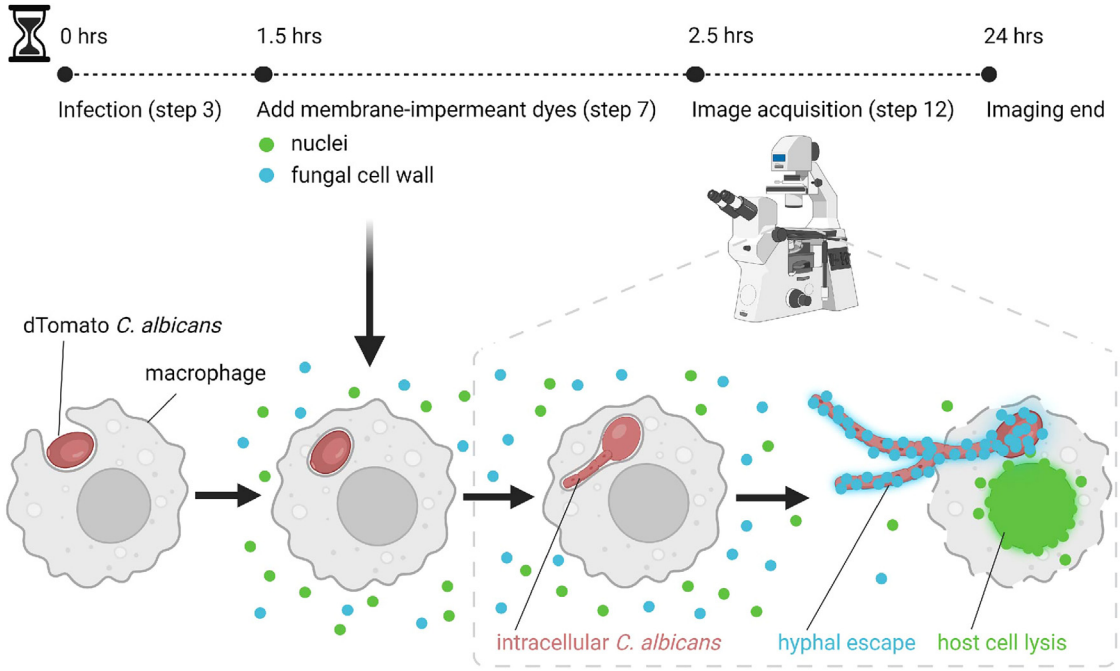

Figure 1. An overview of the hyphal escape assay infection and staining protocol

In this assay we use dTomato *C. albicans* and two fluorescent dyes CFW (blue dots) and DRAQ7 (green dots) to label respectively intracellular *C. albicans* (dTomato), escaped *C. albicans* hyphae (CFW) and the nuclei that have been permeabilized (DRAQ7). Created with BioRender.com. Adapted from previously published Figure with permission from Olivier et al., 2022.[1]

macrophages are imaged within a single field of view, and multiple imaging positions can be combined to collect data on thousands of cells per treatment condition. Cell processes can also be scrutinized at single-cell level: for example, the escape of individual hyphae, coinciding with lysis of the associated macrophage.[1] We also describe an image analysis pipeline that can be used to quantify host cell numbers, host cell permeabilization, amount of pathogen phagocytozed, amount of total viable pathogen and the escape kinetics of the pathogen. This image analysis pipeline employs the open-source image analysis software CellProfiler.[16]

We recently used this method to characterize the mechanisms that allow *C. albicans* to escape from macrophages.[1] Our method relied on the endogenous expression of a fluorophore to track fungal cells inside and outside of macrophages (dTomato-reporter *C. albicans*[17]), as well as a pathogen-specific fluorescent dye that is impermeant to the macrophage membrane (the cell wall stain calcofluor white (CFW) (key resources table, Figure 1). This double-labeling, along with strategically timed staining, was used to distinguish between intracellular and extracellular fungi (Figure 1). DRAQ7, a fluorescent DNA-binding dye, allowed us to capture macrophages permeabilization that occurs as fungal hyphae are escaping (Figure 1). Using this labeling method and live cell imaging, we recorded time-lapse image sequences that captured the progressive escape for several experimental conditions. As a working example in this protocol, we will compare *C. albicans* hyphal escape from macrophages when infected at 1:1 and 3:1 *Candida*:macrophage ratios.

Institutional permissions

The use of animals in this study was approved by the Monash University Animal Ethics Committee (approval numbers ERM14292 and ERM25488).

Isolation and culture of murine bone marrow-derived macrophages

Timing: 8 days

1. Extract marrow from mouse femurs and tibiae.
   a. Dissect tibia and femur bones from mice.
      i. C57BL/6J mice are euthanized aged 6–8 weeks by cervical dislocation ahead of extraction procedure.
      ii. Prepare for animal dissection, being careful to adhere to the safety standards set out by your institution: for example, in our institution the use of a biosafety cabinet is required for this step.
      iii. Sterilize all dissection tools using 70% ethanol solution.
      iv. Sterilize the lower limbs of the mouse with 70% ethanol solution.
      v. Remove skin round the lower limbs using tweezers and blunt-ended scissors; avoid rupture of organs in the pelvic region.
      vi. Cut through muscle and ligaments around the hip joint, exposing as much of the joint as possible.
      vii. Hyperextend the hip joint to remove the femur and cut away any ligaments or muscle that remained attached.

      ⚠ CRITICAL: there is a high risk of severing the femur: it is important to expose the head of the femur at the joint before hyperextending the limb.

      viii. Paper towels can now be used to remove flesh from tibia and femur bones.

      Note: Aim to remove as much flesh and tendons from the bones as possible.

      ix. Hyperextend the tibia and femur at the knee joint and separate these bones from each other.

      Note: Bones can be stored in sterile PBS on ice until marrow extraction: we recommend that this storage does not exceed two hours.

   b. Extract marrow from dissected bones.

      Note: Step 1b, and all subsequent steps that involve marrow, monocytes or macrophages, should be performed in a biosafety cabinet using aseptic technique. Dissection tools should again be sterilized using 70% ethanol solution.

      i. Warm BMDM differentiation medium (described in materials and equipment) to 37°C using a water bath.
      ii. Immediately prior to extraction, immerse each bone in 70% ethanol for 10 s, then in fresh sterile PBS for another 10 s at 20°C–25°C.
      iii. Use blunt-ended scissors to remove the proximal and distal epiphysis, leaving only the diaphysis of the bones.
      iv. Use a 10 mL syringe with a 21G needle to draw up 10 mL of BMDM differentiation medium.
      v. Hold a bone with tweezers over a 50 mL collection tube (conical, sterile polypropylene), then place the end of the needle into the shaft of the bone.
      vi. Carefully flush the contents of the bone into the 50 mL collection tube.

      Note: at this step you should see the color of the bone change from light pink to a clear white: this indicates that the marrow has been extracted. If this color change has not been observed, we recommend repeating the step by reusing medium expelled into the 50 mL collection tube.

      vii. Repeat Steps 1bi-vi for all other bones.

Note: The same 10 mL of medium with expelled marrow can be used to flush the remaining bones for a mouse (each animal will have a total of four bones to flush: two tibiae and two femurs). Ensure that expelled marrow is not pooled from different mice.

viii. Transfer the 10 mL of medium with expelled marrow to a T-25 tissue culture flask (Corning, catalog number 430639).
ix. Incubate the extracted marrow for 18–24 h at 37°C in a 5% CO_2 incubator.

Note: during this incubation step, differentiated cells, including macrophages and fibroblasts, will adhere to the surface of the culture flask.[18] The aim is to collect non-adherent monocytes the following day.

2. Differentiate monocytes to macrophages.
   a. Warm BMDM differentiation medium (described in materials and equipment) to 37°C using a water bath.
   b. Using a serological pipette, collect approximately 10 mL of the non-adherent layer of monocytes from the T-25 tissue culture flasks incubated for 18–24 h.
   c. Transfer this monocyte suspension to 90 mL of BMDM differentiation medium (described in materials and equipment).
   d. Using the serological pipette, homogenize the 100 mL solution of monocytes in BMDM differentiation medium.

Note: BMDM differentiation medium contains macrophage colony-stimulating factor (M-CSF), obtained through culture of L929 cells.[19]

   e. Aliquot the suspension of monocytes into Petri dish plates (polystyrene, sterile) 10 mL per plate.
   f. Incubate these Petri dishes (10 total) in a 37°C, 5% CO_2 incubator for seven days.

Note: We recommend using a dissection microscope (10× objective) to view the morphology and density of the cells. Note the monocytic appearance of the cells.

3. Seed macrophages into microplates.

⚠ CRITICAL: precision in macrophage seeding is vital for reproducibility in infection assay outcomes.

   a. After seven days of incubation, use a wide-field microscope (10× objective) to view the morphology of the bone marrow-derived macrophages (BMDMs).

Note: Compared to the monocytes observed on day one, amoeboid morphologies with adherence to Petri dish surfaces should be observed.

   b. Detach BMDMs from the surface of Petri dishes.
      i. Collect and discard spent BMDM differentiation media from Petri dishes.
      ii. Rinse the surfaces of Petri dishes using sterile PBS: gently add 10 mL of sterile PBS to the Petri dish, swirl the plate, and discard the sterile PBS.

Note: During this step, non-adherent cells, for example erythrocytes, are removed.[18]

      iii. Repeat this rinse three times.
      iv. Add 5 mL of BMDM differentiation media to the Petri dish surface and use a cell scraper to gently detach cells.

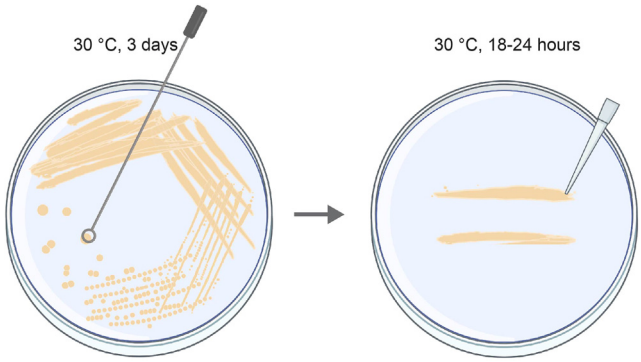

Figure 2. Preparing *C. albicans* culture for use in BMDM infection assays

C. albicans should be streaked (to obtain single colonies, which are used to streak out patches. These *C. albicans* patches are resuspended in sterile PBS for BMDM infection assays. Created with BioRender.com.

⚠ CRITICAL: the regions of detached BMDMs are visible by eye. Ensure that all BMDMs are detached.

v. Add this BMDM suspension to a 50 mL collection tube (conical, sterile polypropylene) using a serological pipette.

c. Homogenize the BMDM suspension: use the serological pipette to mix the suspension by repeatedly drawing up and expelling the entire suspension.

⚠ CRITICAL: we have found that BMDM viability is not affected by this mode of mixing. Repeat this mixing step at least five times for the best results. If the suspension is not homogenized the cell density calculations will not be accurate, seeded macrophages will be uneven and overconfluent. If clumps of cells are observed during cell counting, repeat this step until the BMDM suspension is homogenized.

d. Use a hemocytometer or automatic cell counter to calculate cell density and adjust the density of this cell suspension to 5×10^5 cells/mL.

e. Homogenize this solution immediately prior to seeding.

f. Aliquot 100 μL of this suspension into 96 well plates (clear, flat-bottom, tissue culture-treated).

Note: This results in a seeding density of 5×10^4 cells per well.

g. Incubate the microplate with seeded macrophages in at 37°C, 5% CO_2 for 18–24 h.

Culture of *C. albicans*

Timing: 5 days

A *C. albicans* strain expressing the dTomato fluorophore driven by the enolase (*ENO1*) promoter[17,20] was used to image *C. albicans* inside macrophages, see key resources table. We thank Mihalis Lionakis for sharing the strain with us.

4. Streak *C. albicans* onto agarose plates from 15% glycerol stocks frozen at −80°C.
   a. Use aseptic technique to streak *C. albicans* from frozen stock onto YPD + uridine agarose plates (described in materials and equipment).
   b. Perform the streak in a way that ensures that individual colonies (derived from a single parent cell) will be obtained.

Note: we recommend the four-quadrant streak technique (Figure 2).

c. Incubate these plates at 30°C for three days.

5. Prepare *C. albicans* for infection assay.
   a. Pick a colony from the *C. albicans* plate and patch onto a new YPD + uridine agarose plate by gently in a straight line approximately 5 cm long (Figure 2).

   Note: Several *C. albicans* colonies can be patched in this way.

   b. Incubate plates at 30°C 18–24 h, the day before performing the infection assay.
   c. Use a sterile P200 pipette tip to transfer a patch of *C. albicans* into 1 mL of sterile PBS in a 1.5 mL microcentrifuge tube.
   d. Using a hemocytometer or automatic cell counter, determine the concentration of this *C. albicans* cells suspension.

   Note: For cell counting, we usually prepare a 1000× dilution of the suspension: add 100 μL of the first suspension to 900 μL sterile PBS (10× dilution) then add 10 μL of the 10× dilution to 990 μL of sterile PBS (1000× dilution).

   e. Count and adjust the *C. albicans* cell density to 10^8 cells/mL in sterile PBS.

   ⚠ CRITICAL: We recommend that this suspension is prepared no longer than 30 min prior to use in the infection assay. The aim is for macrophages to be infected with *C. albicans* in yeast cell form, as phagocytosis of hyphal *C. albicans* may be incomplete. We recommend that the morphology of *C. albicans* is monitored at the stage of counting (Before you begin Step 5e) and infection (Step-by-step method details, after Step 5) using a light microscope.

Image analysis software

Timing: 1 day

Acquired live cell data may be saved in a proprietary file format (for example, .czi or .lif for Zeiss and Leica microscopes, respectively) which contain acquisition metadata as well as all time-lapse images for a range of positions. File format conversion software can be used to change these file formats into file formats that can be used by image analysis software. Here we describe an example, using a PC: converting a LIF (Leica Image File) to individual TIF files using the Bio-Formats "bftools" command line package, developed by Open Microscopy Environment.[21] These instructions may differ slightly depending on the operating system used.

6. Convert proprietary image file formats into individual TIF files that can be used by CellProfiler (image analysis software used in this protocol).

   Note: a list of other image file formats that are readable by Bio-Formats have been provided by developers.[22] More comprehensive instructions on how to use bftools is also available online.[23]

   a. Download the bftools command line package.[24]
   b. Extract the contents of the bftools zip folder to your PC's main drive (e.g., C:).
   c. Open the Command Prompt by looking up "cmd" in the Windows search bar.
   d. In Command Prompt, in the line that starts with "C:\Users\...", type:

```
> cd C:\bftools
```

Note: This will navigate you to the bftools folder you extracted in **Step 6b**.

e. Create a destination folder for your TIF image files.

Note: This folder can be given any name, for the purposes of this example will be called "lif_to_TIF".

f. To convert your LIF file to TIF files, type the following in the line that starts with "C:\bftools>":

Note: spaces in command lines are important. Note how there is a space before each of the lines specified above, and in between the two quotation marks separating the LIF file location and destination folder.

```
> bfconvert "C:\directory of your .lif file, i.e. Documents\LIF_file\livecelloutputfile.lif'' "C:\directory of your destination folder, i.e. Documents\lif_to_TIF\image_P%%s_T%%t_C%%c.tif''
```

Note: This command will create a TIF file for each channel and time point in your LIF file. If the error "java is not recognized as an internal or external command" is seen, consult Trouble-shooting 1. We collected two LIF files for each experiment: 1) CellTracker Green images for the first time point only and 2) DRAQ7, dTomato, CWF and bright-field images at every time point. When exporting these image subsets, we named the first image subset "CtG" and the second "hea", for "hyphal escape assay". This means that we performed the LIF to TIF conversion twice, first for CellTracker Green images:

```
> bfconvert "C:\Documents\LIF_file\livecelloutputfile.lif" "C:\Documents\lif_to_TIF\CtG_P%%s_T%%t_C%%c.tif''
```

And then again for hyphal escape assay images.

```
> bfconvert "C:\Documents\LIF_file\livecelloutputfile.lif" "C:\Documents\lif_to_TIF\hea_P%%s_T%%t_C%%c.tif''
```

This resulted in two different naming conventions. These naming conventions are important for the image analysis pipeline (step-by-step method details Steps 15–24) described later. To create time-lapse clips instead of individual .TIF files for each time point, modify Step 6f as follows.

```
> bfconvert "C:\directory of your .lif file, i.e. Documents\LIF_file\livecelloutputfile.lif" "C:\directory of your destination folder, i.e. Documents\lif_to_TIF\image_P%%s_C%%c.tif""
```

This command will create a single .TIF file for each channel in your .lif file.

7. Download CellProfiler image analysis software, see key resources table.

Note: we used CellProfiler 2.1.1. More updated versions of this software are now available.

8. Prepare the CellProfiler template project files that are needed for the imaging analysis pipeline.

Note: This pipeline is made up of three analyses: A, B and C (Figure 3), requiring three template project files. We have provided the settings required for these template project files in Tables S1, S2, S3, and S4, with added detail on important steps. For further guidance see Methods videos S1, S2 and S3, where we preview the preparation and testing of this analysis pipeline.

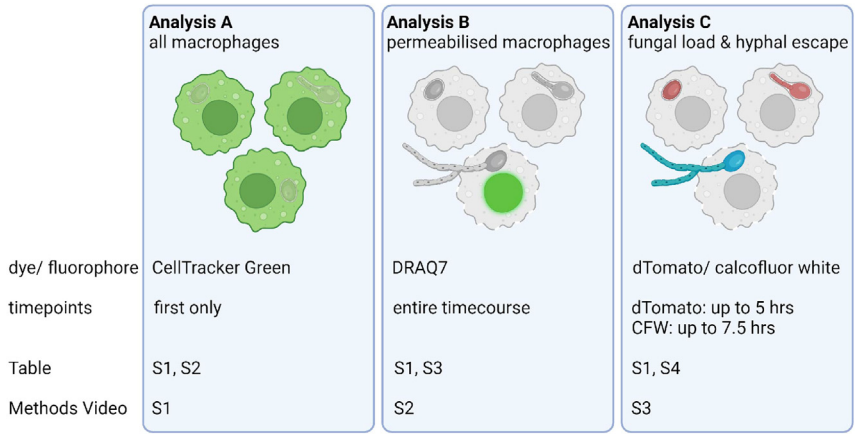

Figure 3. Image analysis pipeline resources for pathogen macrophage escape assay

A summation of resources accompanying the step-by-step image analysis instructions for Analysis A, B and C. In Data S1 we provide the data output from an example experiment (challenging macrophages with *C. albicans* at an 1:1 and 3:1 multiplicities of infection, or "MOI") where these analyses have been performed. Time points here refer to hours post-infection of macrophages with *C. albicans*. In "expected outcomes" we discuss the quantification of escaped and total *C. albicans* hyphal area over time (Analysis C). Due to photobleaching (dTomato) and overlapping hyphae (CFW) at later time points, we recommend quantification of hyphal areas for time points up to 5 (dTomato) and 7.5 (CFW) hours.

a. Prepare the template project file for Analysis A using the settings provided in Tables S1 and S2.

Note: in Analysis A, we count the number of CellTracker Green-positive macrophages (Figure 3). Note that CellTracker Green imaging is only performed for the first time point, as detailed in Step-by-step method details **Step 22**. The objective of this analysis is simply to count the total number of macrophages present in the field of view at the beginning of the live cell imaging period.

b. Prepare the template project file for Analysis B using the settings provided in Tables S1 and S3.

Note: in Analysis B we quantify the total number of permeabilized macrophages for each time point (Figure 3).

c. Prepare the template project file for Analysis C using the settings provided in Tables S1 and S4.

Note: in Analysis C we measure the area (μm^2) of dTomato-positive *C. albicans*. We also measure the area of escaped, CFW-positive *C. albicans* hyphae. The area values obtained through this analysis are used to calculate the escape-to-phagocytosis ratios of *C. albicans* following macrophage encounter. Use settings provided in Tables S1 and S4 to prepare this template project file.

Live cell microscopy training

This experiment requires access to, and proficiency in, live cell microscopy. Note that while we used a Leica live cell imaging system (see Table S5 for image acquisition details), there are many other live cell microscopes available that can produce image datasets that are suitable for this experiment. We recommend that the user become familiar with live cell image acquisition setup prior to attempting

this experiment. Initially, imaging of uninfected macrophages can serve as a useful steppingstone for training.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Calcofluor white stain	Sigma-Aldrich	18909
Fetal bovine serum	Serana	FBS-AU-015
Penicillin-Streptomycin	Sigma-Aldrich	P4333
Peptone	US Biological	73049-73-7
Yeast extract	US Biological	8013-01-2
Uridine	Sigma-Aldrich	58-96-8
CellTracker Green CMFDA dye	Thermo Fisher Scientific	C7025
DRAQ7 dye	Abcam	ab109202
Experimental models: Organisms/strains		
C. albicans: CAF2.1-dTom-NATr: URA3/ura3::λimm434, IRO1/iro1::λimm434, ENO1/eno1::dTom-SAT1	Lionakis et al.[17]	YCAT1127
Mouse: wild-type C57BL/6J, male or female, aged 6–8 weeks	Monash Animal Research Platform (Melbourne, Australia)	RRID: IMSR_JAX:000664
Software and algorithms		
Bio-Formats	[24]	The Open Microscopy Environment: https://www.openmicroscopy.org/bio-formats/
CellProfiler 2.1.1	McQuin et al.[16]	CellProfiler: https://cellprofiler.org/previous-releases
ImageJ 2.0.0-rc-69	Schindelin et al.[25]	ImageJ: https://imagej.net/ij/download.html
GraphPad Prism 9.0.0	GraphPad Software, San Diego, California, USA	GraphPad: https://www.graphpad.com/features

MATERIALS AND EQUIPMENT

BMDM differentiation medium

Reagent	Final concentration	Amount
RPMI 1640 pH 7.4 with glucose	10 mM (glucose)	31.375 mL
HEPES (1 M)	12.5 mM	625 μL
L-cells supernatant[19]	20%	10 mL
Fetal bovine serum	15%	7.5 mL
penicillin-streptomycin (10 000 Units)	100 U/L	0.5 mL
Total	**N/A**	50 mL

Fetal bovine serum should be heat inactivated prior to use, by heating at 56°C for 30 min. Medium can be stored at 4°C prior to use. Medium should be heated to 37°C prior to use and used within two weeks of preparation.

YPD + uridine agarose medium

Reagent	Final concentration	Amount
Peptone	20 g/L	10 g
Yeast extract	10 g/L	5 g
Agarose	20 g/L	10 g
Glucose	20 g/L	10 g
Uridine	80 g/L	40 g
ddH_2O	N/A	500 mL
Total	**N/A**	500 mL

Combine peptone, yeast extract and agarose powders with 450 mL of ddH_2O and autoclave, then cool to 50°C. Combine glucose and uridine powders with 50 mL of ddH_2O, dissolve, then combine with cooled peptone and yeast extract solution.

- Infection medium: add 1 mL of BMDM differentiation medium with 10 μL (1:1 *Candida*:macrophage ratio) or 30 μL (3:1 *Candida*:macrophage ratio) of 10^8 cells/mL suspensions of *C. albicans* in sterile PBS.

This medium should be used within 5 min of preparation.

STEP-BY-STEP METHOD DETAILS

Infection of macrophages with *C. albicans*

Timing: 3 h

Note: the preparation of the *C. albicans* suspension and seeded macrophages is described in the Before you begin section of this protocol.

In this step we aim to achieve the following: a) stain macrophage membranes with CellTracker Green, b) infect macrophages with *C. albicans* at the required MOIs (1:1 and 3:1 *C. albicans*: macrophage), c) allow phagocytosis to occur and d) add CFW and DRAQ7 to the medium, to detect escaped *C. albicans* hyphae and permeabilized macrophages respectively.

1. Observe seeded macrophages using a light microscope.

Note: a confluent monolayer of macrophages should be seen (Figure 4A). The infection assay should not proceed if macrophages are over- or under-confluent.

⚠ CRITICAL: A key goal of this assay is to measure the escape of *C. albicans* after phagocytosis by macrophages. To achieve this, virtually all of the *C. albicans* cells added need to be phagocytozed by macrophages. If the seeding density is too low (i.e., large spaces are seen in between cells) it is likely that incomplete phagocytosis of *C. albicans* cells will be seen. Non-phagocytozed, extracellular *C. albicans* cells will form hyphae which interfere with the intended hyphal escape analysis. Additionally, the cell density of macrophages and resulting ratio of infection with *C. albicans* cells need to be consistent among biological repeats.

2. Replace the culture medium of macrophages with 100 μL per well of FBS-free BMDM differentiation medium with 1 μM CellTracker Green CMFDA dye.
3. Incubate the macrophages at 37°C and 5% CO_2 for 30 min.

⚠ CRITICAL: care should be taken when replacing the culture medium of macrophages at each step, because the macrophage monolayer can be disrupted by scraping of pipette tips along the bottom of wells, or fast pipetting (Figure 4B). A multichannel pipette may be used to synchronize media exchange among wells.

Optional: This assay can be adapted to investigate the interactions between a number of host cells and pathogens, including bacteria and fungi. Mechanisms can be further investigated using chemical compounds or genetically modified host cells or pathogens. The timing of the addition of such a chemical compound depends on the aim of the experiment (Figure 4C).

4. Immediately prior to infection, resuspend *C. albicans* in BMDM differentiation medium at the required concentration to make infection medium.
   a. Add either 10 μL (1:1 ratio of infection) or 30 μL (3:1 ratio of infection) of the 10^8 cells/mL *C. albicans* suspension (described in the before you begin section) to 1 mL of BMDM differentiation medium.

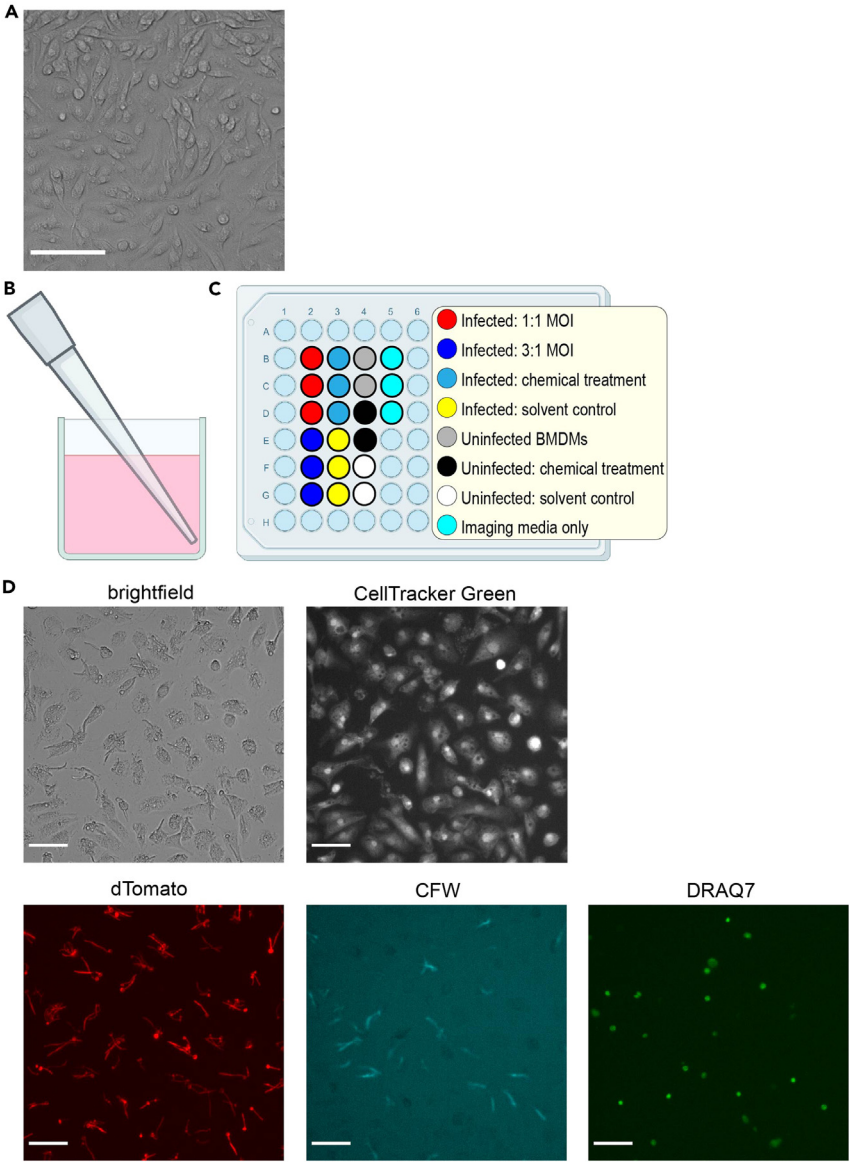

Figure 4. Preparing BMDMs for infection assay: confluency, media exchange and assay design

(A) Prior to infection, a confluent monolayer of BMDMs should be observed. Adjustments to image described in Table S6. Scale bar = 100 μm.

(B) We recommend pipetting along the corner of wells and avoiding direct contact with the bottom of wells to preserve the monolayer of cells.

(C) A typical BMDM infection assay layout, with three technical repeats per treatment condition. Chemical treatment is shown as an example. Created with BioRender.com.

(D) Preview of each channel at the first time point. Examples shown are bright field (macrophages and *C. albicans*), CellTracker Green (viable macrophages), dTomato (all *C. albicans*), CFW (escaped *C. albicans* hyphae only) and DRAQ7 (permeabilized macrophages). Scale bar = 50 μm.

b. Vortex mix this suspension for at least 10 s.

5. Replace the medium of seeded macrophages with the infection medium, 100 μL per well.

Note: this is considered to be 0 h post-infection. Take note at which time infection has occurred and use a timer to ensure that the steps that follow are done precisely when needed.

6. Centrifuge the 96-well microplates with infected BMDMs at 500 x *g* for 3 min at 20°C–25°C.

Note: this centrifugation step is important as it synchronizes the encounter of *C. albicans* cells across the population of seeded macrophages.

7. Incubate the infected BMDMs at 37°C and 5% CO_2 for 1.5 h to allow for phagocytosis to occur.
8. To remove *C. albicans* cells that were not phagocytozed, rinse each well with 150 µL of sterile PBS three times.
9. Add the imaging culture medium to each well: BMDM differentiation medium with 0.6 mM DRAQ7 and 10 µg/mL CFW.

Note: both DRAQ7 and CFW are fluorescent dyes that are impermeant to the BMDM cell membranes (Figure 1). This means that the nuclei of BMDMs are only stained when the membrane is permeabilized. Similarly, the hyphae of escaping *C. albicans* are only stained with CFW if BMDM membranes are permeabilized.

10. Immediately proceed to the live cell imaging step.

⚠ CRITICAL: At this stage, *C. albicans* will already be making hyphal cells that can be in the process of escaping from macrophages. It is therefore important to proceed with live cell imaging set up as soon as possible to capture early permeabilization of BMDMs and *C. albicans* hyphal escape. Aim for the first live cell imaging time point to be no later than at 2.5 h post-infection (since first addition of infection media to macrophages, Step 5).

Live cell imaging of *C. albicans*-challenged macrophages

Timing: 1 day

In this step we acquire live cell images that capture the escape of *C. albicans* from macrophages. The 96-well microplate is mounted on a stage inside a microscopy incubator that maintains cells at 37°C and 5% CO_2 for the duration of the live cell imaging run.

⚠ CRITICAL: ensure that the microscope incubation chamber is pre-heated to 37°C, with CO_2 supplied at 5%, ahead of image acquisition.

Note: the 96-well microplate used was clear, flat-bottomed and tissue culture-treated. We used Falcon microplates (catalogue number 353072).

A detailed overview of microscopy specifications used in our experiments is provided in Table S5. Here we outline the main steps applicable to a range of live cell imaging systems.

11. Mount the 96-well plate to the microscopy stage and seal the microscope incubator.

Note: Ensure that the room is dark

12. Set up imaging positions within the 96-well microplate.
    a. Within each well, program two positions for image acquisition.

Note: We recommend randomizing the selection of these positions if possible. Ensure that the field of view for each position does not include the edge of the well.

    b. Save the position information file in a convenient file location.

Note: This ensures that this step does not need to be repeated if the software crashes. Also note that some microscopy software includes functions that automate the process of programming imaging positions within microplate wells. We encourage the use of this functionality as it decreases bias that can be introduced when selecting imaging positions. Also remember to include the imaging of uninfected macrophages and media-only controls (Figure 4C).

13. Acquire images of CellTracker Green fluorescence for all imaging positions.
    a. Set up a channel that captures CellTracker Green fluorescence (we used a FITC filter cube, Figure 4D; Table S5).
    b. Set up a second autofocus channel.

Note: we recommend using either bright-field or a far red (for example, a Y5 filter cube) channel for autofocus. In this way we limit exposing the sample to blue light, which can lead to phototoxicity.

    c. Preview image acquisition for each channel and optimize illumination settings accordingly.

Note: when optimizing illumination settings for each channel, we initially aimed for a good balance of laser intensity and gain to obtain a linear range of signal detection. However, this was found to lead to phototoxicity and/or photobleaching. We therefore adjusted illumination settings to acquire images of lower brightness, prioritizing cell viability.

    d. Once CellTracker Green fluorescence has been captured for all positions, save the image file.
14. Acquire *C. albicans* hyphal escape images at 30 min intervals, for 18–24 h.

Note: The interval at which images are acquired can be modified to suit the experimental question.

    a. Set up a bright-field channel, along with channels that capture DRAQ7, dTomato and CFW fluorescence (Figure 4D).
    b. Preview image acquisition settings for each channel, as described in Step 13c.
    c. Check over autofocus settings.

Note: here we recommend using the channel for DRAQ7 fluorescence (Y5 filter cube) for autofocus. The autofocus settings for our experiments are described in more detail in Table S5.

    d. Specify the time intervals for image acquisition.
    e. Start live cell image acquisition, observing image acquisition for the entirety of the first time point.

Note: it is important to check that each image is in focus, and that the time it takes to collect images from all positions occurs within the specified time frame (such as 30 min). If it takes longer to collect images within each time point, not all images will be collected, or time points may be further apart than specified in image acquisition settings. Decreasing the time taken to focus may reduce image acquisition time considerably. However, decreasing the autofocus time may also compromise focus accuracy, which may lead to out-of-focus images.

Image analysis: Generating illumination-correction images

Timing: 20 min

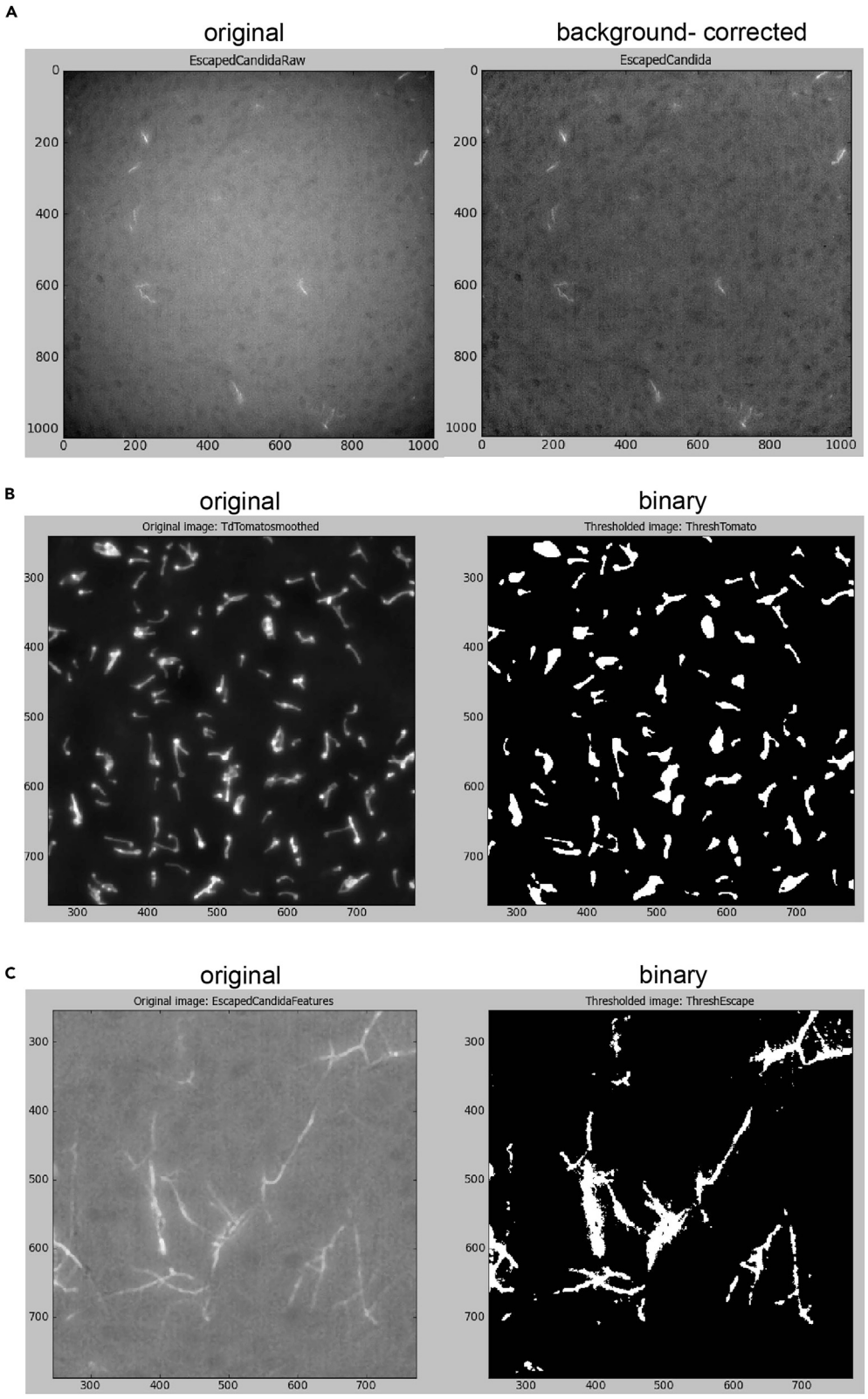
A
original
background- corrected
EscapedCandidaRaw
EscapedCandida
B
original
binary
Original image: TdTomatosmoothed
Thresholded image: ThreshTomato
C
original
binary
Original image: EscapedCandidaFeatures
Thresholded image: ThreshEscape

Figure 5. Examples of image binarization and vignetting correction

(A) Removing vignetting from an image of escaping (CFW- stained) hyphae using the "ImageMath" module in CellProfiler. The image on the right is the corrected version of the same image on the left, which has an uneven background.

(B and C) Converting dTomato (B) and CFW (C) images into binary images, using the CellProfiler "ApplyThreshold" module. Images on the right are binarized versions of the original dTomato- expressing hyphae (B) and CFW- stained (C) hyphae images on the left. Adapted from previously published Figure with permission from Olivier et al., 2022.[1]

Captured live cell images were used in the *C. albicans* hyphal escape image analysis pipeline (Figure 3). The accurate quantification of *C. albicans* escape phenomena depends on accurate cell segmentation performed in this pipeline. Illumination correction to remove non-uniform brightness (such as vignetting) is needed to achieve consistent thresholding results across the field of view. Using images that are representative of this uneven illumination ("illumination-correction" images, generated using media-only controls, Figure 4C), we corrected for image vignetting (Figure 5A).

15. Select images of empty wells (imaging culture media only, no *C. albicans* or macrophages) for each channel.

    Note: We recommend capturing at least six such images per experiment: with two imaging positions per experiment, this requires three wells in a 96-well microplate (Figure 4C). For each channel, open at least four of these images in ImageJ (see key resources table).

16. Use these images to produce a median projection image (an image that is the product of the median pixel intensity value from all images).
    a. In ImageJ, click on "Image," in the menu bar, then select "Stacks" and choose "Images to stack."
    b. Click "OK" when prompted (the name of the stack is not crucial in this context).

    Note: This action combines the selected images into a single stack.

    c. Now select "Stacks" and choose "Z project."
    d. You will be asked to specify the project type. Select "median" to perform a median projection of the stack. Click "OK" to proceed.
17. Apply a blurring effect.
    a. Using the "Process" menu, select "Filters" followed by "Gaussian Blur".
    b. Set the radius to 16 and confirm your choice by clicking "OK".
18. Locate the maximum intensity value in the image, the highest brightness level.
    a. Using the "Analyze" menu, choose "Set measurements," and ensure that "min & max gray value" is checked.
    b. Click "OK" to confirm the settings.
    c. Now, in the "Analyze" menu, select "Measure."
    d. Take note of the maximum intensity value obtained: this value is important for the subsequent calculations.
19. Normalize the intensity values of the image.
    a. Using the "Process" menu, select "Math," and choose "Divide."
    b. Enter the maximum value noted down in the previous step.

    Note: This division operation generates a new image where the intensity values are close to 1 at the center (maximum) and decrease towards the image edges, which will help us to account for vignetting effects in our image analysis pipeline.

20. Save this image.
    a. Go to the "File" menu, select "Save as," and choose the TIF format.

b. Provide an appropriate name, such as "IllumCor" followed by the month and year, to ensure easy identification of the file.

21. Repeat Steps 15–20 for each channel (CFW, DRAQ7, dTomato and CellTracker Green).

Note: for each analysis we recommend using illumination correction images generated within the same experiment. We strongly advise against using outdated illumination correction images, or images acquired from a different microscope.

Image analysis: Quantifying *C. albicans* fungal load, hyphal escape and macrophage membrane permeabilization

Timing: 1 day

Using the live cell imaging data generated from our example infection experiment (Figure 4C) we can quantify the total number of macrophages (permeabilized and viable) as well as the area of phagocytozed and escaped *C. albicans* (Figure 3). Here we describe how the image analysis software CellProfiler (see key resources table) can be used to perform these measurements.

Note: we provide settings and notes on image analysis modules used within CellProfiler in Tables S1, S2, S3, and S4, and refer where these apply in the instructions below. We encourage the reader to adapt these modules as needed for their imaging data. As a rule of thumb, image analysis needs at least as much time investment as the wet lab experiments performed to obtain the data. While the automation of cell counting and area estimates allows for thousands of images to be analyzed in a matter of hours, each image analysis step must be carefully tested. The output from image analysis is dependent on the quality of the data, and thorough overview of input images is required prior to analysis.

22. Perform analysis A (Figure 3).

Note: In this step we use CellTracker Green staining of macrophages, acquired only at the first time point, to quantify the total number of macrophages in the field of view. For this step we will use the CellProfiler project settings provided in Table S1 and S2. These image analysis steps are also demonstrated in Methods video S1.

a. Import all CellTracker Green ("CtG" images, as detailed in the LIF to TIF conversion in Step 6 of the before you begin section) TIF files into the "Images" module by dragging and dropping the files into the window.

Note: This includes CellTracker Green images for all positions as well as the illumination correction image for the CellTracker Green channel.

⚠ CRITICAL: it is important to check that each .TIF file name contains information about the position, time point and channel. For example, an image with the name "P22_T0_C0" means that this is image is of position 22 at the first time point, of channel = 0 (only one channel is acquired on this occasion). The generation of TIF images with this file naming convention is discussed further in the before you begin section, Step 6.

b. In the metadata module, check that channel, position, time series and image type information are captured for all images.

Note: Images should appear as "ctg" and "None" in the "Type" column of CellProfiler for CellTracker Green and illumination correction images, respectively.

c. In the NamesAndTypes module, check that each CellTracker Green image is listed next to the same illumination correction image.

Note: the order of the images does not matter at this stage of the analysis.

d. Preview vignetting correction and cell segmentation.
   i. Select the eye icon next to the ImageMath and IdentifyPrimaryObject modules to be "open".
   ii. Select "Start Test Mode" in the bottom left corner of the program interface.
   iii. Select "Step" to progress through the image analysis modules.
   iv. To progress through all image analysis modules, select "Run".

Note: at this point, windows appear with image analysis previews. If input images appear dark, use the "Subplots" menu to adjust the contrast of a previewed image: for example, by selecting "Image contrast" then "Log normalized". The third image, showing outlines of where cells will be segmented, is a useful visualization of segmentation. Green outlines represent objects that fall within the acceptable cell size range, purple outlines represent objects that are too small or large to be classified as a cell.

e. Preview the segmentation of macrophages from each treatment condition within an experiment.

Note: for example, for the experimental setup in Figure 4C, we would optimize the segmentation using 8 images: one from each treatment condition.

f. When segmentation of all images has been validated, select "Exit Test Mode".

Note: cell segmentation can be impacted by uneven fluorescence staining, irregular cell morphologies or overlap of cells (as this is two-dimensional image data). For this reason, a small number of cells cannot be segmented successfully (less than 5%), even with optimal segmentation settings. If cells are seeded too densely (increased overlap) or there is low signal-to-noise ratio in the captured images, this can lead to decreased segmentation accuracy, and more optimization of sample preparation and image acquisition steps may be required. Cell thresholding and segmentation parameters provided in this protocol (Tables S2, S3, and S4) are exemplary only, as these are affected by image acquisition settings and the morphology of cells used in the infection experiment. Image analysis settings should be validated for every experiment: this is done by adjusting cell segmentation parameters (in particular, object diameter and thresholding value) to produce the best segmentation results. For example, if a single macrophage appears to be segmented multiple times, the object diameter values may be too small. On the other hand, if several macrophages appear to be segmented as a single cell, the object diameter values may be too large.

g. In the ExportToSpreadsheet module, nominate a destination folder for export of the spreadsheet file under "Output file location".
h. Select "Press to select measurements" to confirm that the correct measurements are set to be exported.

Note: See Table S2 for recommendations on which measurements to export.

i. With your image analysis settings now finalized, save this project file in a location that is separate to the template file.
j. Press "Analyze Images" to begin analysis.

Note: for faster processing of your images, deselect the "eye" icons next to the analysis modules.

23. Perform analysis B (Figure 3).

Note: in this step we use DRAQ7 staining to quantify the number of macrophages that are permeabilized over time. For this step we will use the CellProfiler project settings provided in Table S1 and S3. These image analysis steps are also demonstrated in Methods video S2. This analysis involves a similar process to the CellTracker Green analysis (**Step 22, a-j**) described above, with the following exceptions:

a. In the "Images" module, drag-and-drop all hyphal escape analysis TIF image files, along with the DRAQ7 illumination correction image.

Note: these are all hyphal escape assay "hea" images, as detailed in the LIF to TIF conversion in Step 6 of the before you begin section. We can use the necessary subset of these images by classifying which of these files are of DRAQ7-positive macrophages in the "NamesAndTypes" module.

b. In the Metadata module, check that hyphal escape assay images are of "hea" type, while the illumination correction image appears as "None".
c. As with Step 22c, each DRAQ7 image ("hea", channel 0) should be listed alongside the same illumination correction image in the "NamesAndTypes" module.
d. Test all cell segmentation parameters, as described in Step 22d-e.

Note: not all macrophages will be DRAQ7-positive. Images across several time points need to be tested in addition to a range of positions. Also consider that DRAQ7-positive nuclei will appear smaller than CellTracker-positive cell membranes, and that the expected object diameter needs to be adjusted accordingly in the "IdentifyPrimaryObjects" module. As done for Analysis A, this requires optimization by the user for a given experiment, and the values provided in Table S3 are exemplary only.

e. Prepare the export of data, save the project file and run the analysis as described in Step 22g-j.

24. Perform analysis C (Figure 3).

Note: In this step we quantify the total area of dTomato and CFW-positive *C. albicans*. Similar to analysis A and B, vignetting is corrected for in images before thresholding and area measurements occur. More information on each analysis step, along with template file specifications, is provided in Table S1 and S4. These image analysis steps are also demonstrated in Methods video S3.

a. Add TIF "hea" images to the "Images" module, as described in Step 22a.

Note: two illumination correction images should be added (to correct vignetting for both dTomato and CFW images).

b. As described in Step 22b, check that the regular expression extracts relevant metadata from image file names in the "Metadata" module.
c. Using the "NamesAndTypes" module, assign channel identifies to the dTomato and CFW images.

Note: in our experiments, these images corresponded to channels 1 and 2, respectively (see Table S4). Also note that it is important to distinguish the dTomato and CFW illumination correction images in this module. For example, we assigned the names "IlluminationCorrectionC1" and "IlluminationCorrectionC2" to these respective channels (Table S4).

d. As described for Steps 22d-e, test vignetting correction and cell segmentation settings.

Note: compared to analysis A and B, analysis C contains several additional modules. Each of these modules should be validated before commencing analysis. The end goal is to obtain binarized images that best represent dTomato (Figure 5B) and CFW-positive (Figure 5C) *C. albicans* cells, as seen in the fluorescence images (these binarized images are previewed using the "ApplyThreshold" modules). Accurate binarized representations of *C. albicans* cells are needed to estimate total area of dTomato and CFW-positive *C. albicans* cells. The process of validating these imaging settings is previewed in Methods video S3, and more detail on analysis modules provided in Table S4.

e. Prepare the export of data, save the project file and run the analysis as described in Step 22g-j.

Note: More details on these module settings are provided in Table S4.

EXPECTED OUTCOMES

Here we will discuss the outcomes of our shared hyphal escape experiment example: comparing two multiplicities of *C. albicans* infection (3:1 and 1:1 MOI, Figure 4C). Using this data, we can investigate how these fungal loads affect macrophage membrane permeabilization and the efficiency of hyphal escape. We will refer to the outputs of Analysis A, B and C as described in the step-by-step details for image analysis. The calculations for these quantitative outputs are described in quantification and statistical analysis.

Images of *C. albicans* hyphal escape from macrophages

The captured live cell images demonstrate that *C. albicans* cells are first contained inside macrophages, before extending hyphae escape and extend at later time points (Figure 6). Each imaging channel provides a different view of this phenomenon. We preview all hyphae, phagocytozed inside macrophages and escaped, in images of dTomato-positive *C. albicans* (Figure 6, top panel). These hyphae pick up the CFW stain when they escape, providing a view of escaped hyphae (Figure 6, middle panel). At the first imaging time point (2.5 h) it is evident that the majority of *C. albicans* cells remain contained inside macrophages (dTomato-positive, CFW-negative, Figure 6). We therefore used the area of dTomato-positive *C. albicans* at the first time point as a measure of total phagocytozed *C. albicans* (see quantification and statistical analysis). Finally, lysis of macrophages is indicated by DRAQ7-positivity, which increases over time (Figure 6, bottom panel). When viewing these images as time-lapse videos it is clear that DRAQ7-positive nuclei appear in close temporal and spatial proximity to escaping hyphae (Methods video S4), as we have shown previously.[1]

Evaluation of acquired live cell images is an important first step in the investigation of experimental outcomes. Before proceeding to imaging analysis, it should be verified that the captured images are in focus, with consistent illumination and field-of-view positioning. At the same time these images can be used to confirm that the assay conditions were optimal. For example, it can be established whether seeded macrophages had good confluency and phagocytozed *C. albicans* cells well (Figure 6, top panel, 2.5 h). A high number of DRAQ7-positive macrophages at the first imaging time point (2.5 h) is indicative of low macrophage viability that could have been caused by sub-optimal

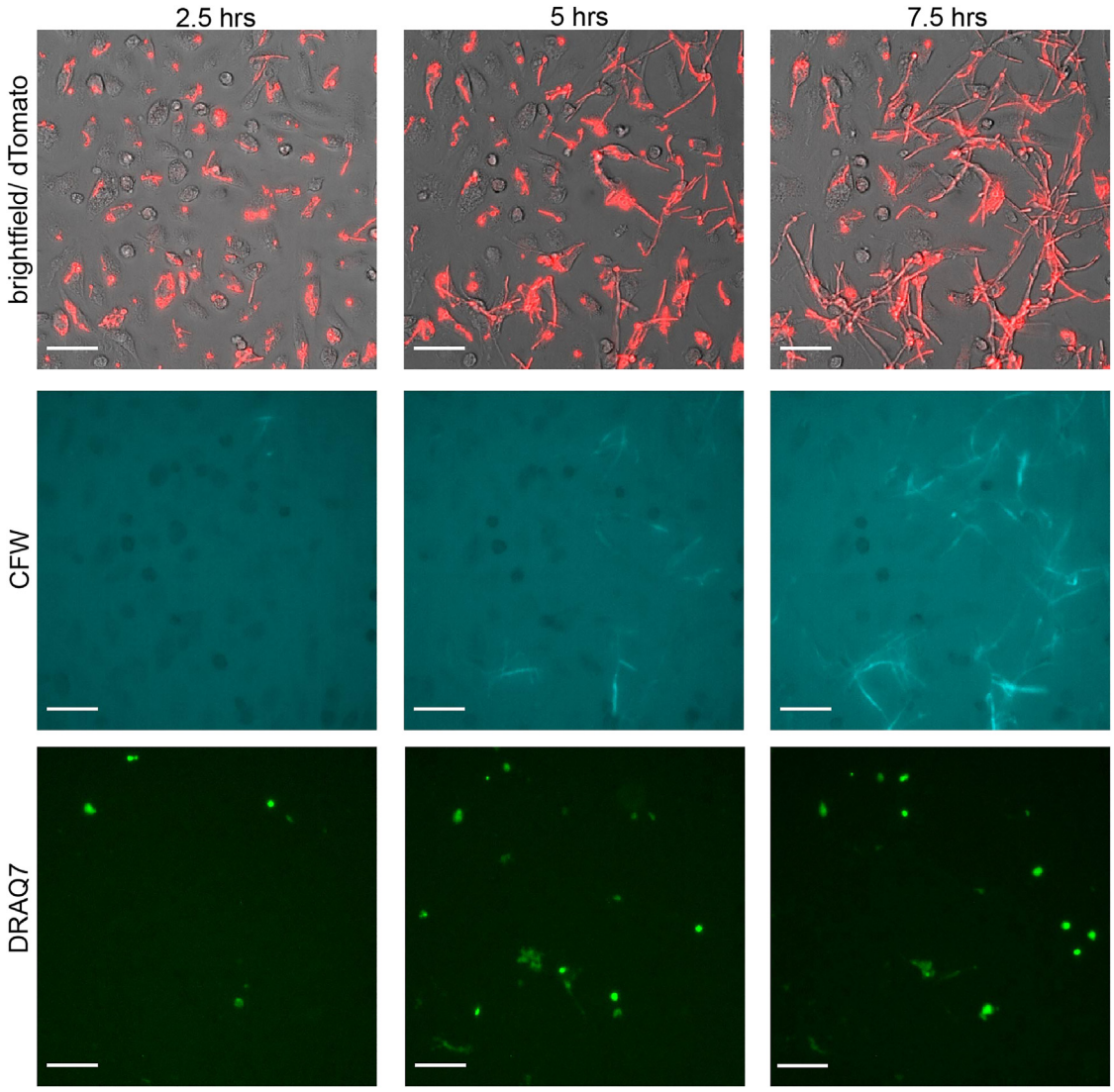

Figure 6. The escape of *C. albicans* hyphae and macrophage membrane permeabilization captured by live cell microscopy

Macrophages challenged with *C. albicans* (MOI 1). Top panel: images of dTomato-positive *C. albicans* captures both phagocytozed and escaped hyphae. Middle panel: CFW-positive *C. albicans* shows hyphae that are no longer contained by macrophages. Bottom panel: DRAQ7-staining of lysed macrophage nuclei. Adjustments to images described in Table S6. Scale bar = 50 μm.

culture conditions (pH, temperature, CO_2, nutrient availability). Acquired live cell images also provide useful qualitative insight into escape phenomena. For example, we can use images to understand if *C albicans* is successful in forming hyphae inside the macrophage phagolysosome, and whether these hyphae escape or remain contained.

Quantification of macrophage membrane permeabilization (DRAQ7 positivity) over time

The percentage of total macrophages positive for DRAQ7 (which correlates with cell lysis) can be plotted over time (Figure 7A) using the quantitative output of Analysis A and B, as described in step-by-step method details steps 22 and 23. In our shared example it can be seen that the kinetics of macrophage permeabilization is affected by fungal load (Figure 7A). These kinetics can reveal information on the mechanisms of macrophage cell death following *C. albicans* challenge, as previously described.[1,14,26–28]

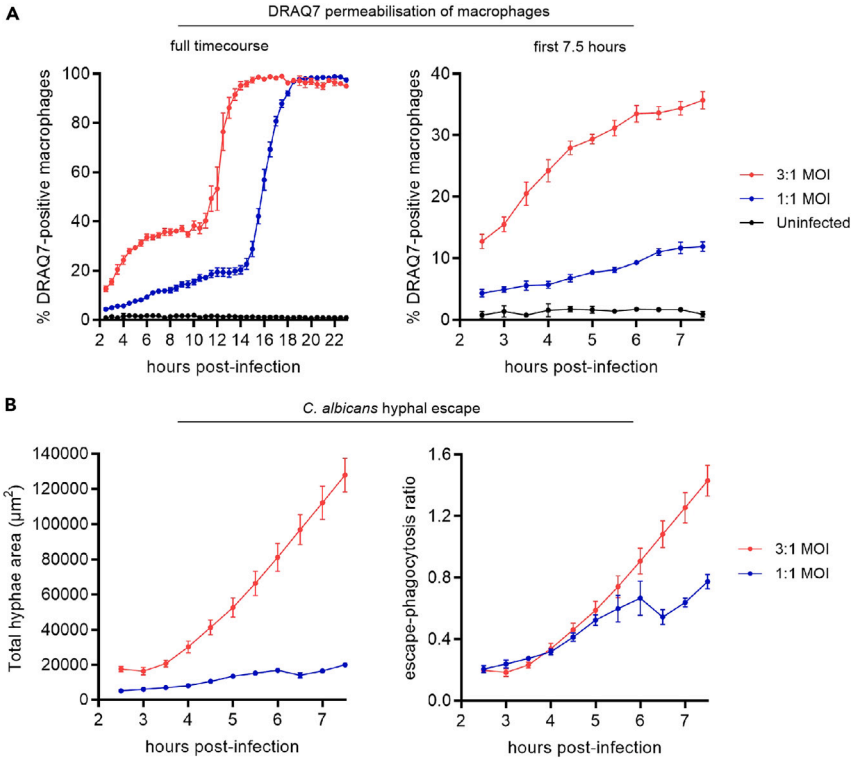

Figure 7. Quantification of macrophage DRAQ7 permeabilization and *C. albicans* hyphal escape over time from live cell imaging experiments

For each time point, error bars represent the standard error of the mean of two (uninfected macrophage control) or three (3:1 and 1:1 MOI infections) technical repeats. A: Permeabilization of macrophages to the nuclear dye DRAQ7 over a 23-h time course (left panel) or limiting to the first 7.5 h only (right panel). The data in the two panels is from the same experiment. Note that the y-axis differs in the two panels, going up to 100% in the left panel and up to 40% in the right panel. B: Hyphal escape quantified using the total area of CFW-positive hyphae (left panel) or the ratio of these areas to dTomato areas captured during the first time point (a measure of total phagocytozed *C. albicans* cells, right panel).

Quantification of *C. albicans* hyphal escape over time

C. albicans hyphal escape from macrophages can be quantified using the output of Analysis C (see step-by-step method details step 24). In our example experiment (Figure 4C), there appears to be more CFW-positive *C. albicans* hyphae up to 7.5 h post-infection as a result of 3:1 MOI infections, compared to 1:1 MOI infections (Figure 7B). The increase in CFW-positive *C. albicans* area over time is a product of an increase in the number of escaping hyphae as well as the growth of those hyphae. At earlier time points, the increase in CFW-positive *C. albicans* area is predominantly a result of new escaping hyphae appearing (Figure 6, 5 h). Escaping hyphae continue to grow, adding to the total area of CFW-positive *C. albicans* (Figure 6, 7.5 h). This is also apparent when comparing the area of dTomato-positive *C. albicans* (all hyphae) with CFW-positive *C. albicans* (escaped hyphae only) over time (Figure 8A, right panel). A more rapid increase in CFW-positivity, compared to dTomato-positivity, illustrates that dTomato *C. albicans* becomes "unshielded" from the CFW stain over time (Figure 8A, right panel). As escaped (CFW-positive) hyphae continue to grow, they overlap and dominate the field of view (Figures 6 and 8B). As our analysis quantified hyphal area in two dimensions, an overlap of hyphae leads to an underestimation of escaped hyphal areas at later time points. For this reason, we restricted hyphal escape measurements to the first 7.5 h post-infection (Figure 7B).

The ratio of the CFW-positive hyphae to the total area of phagocytozed *C. albicans* (dTomato-positive *C. albicans* at the first time point) can also be quantified: see quantification and statistical

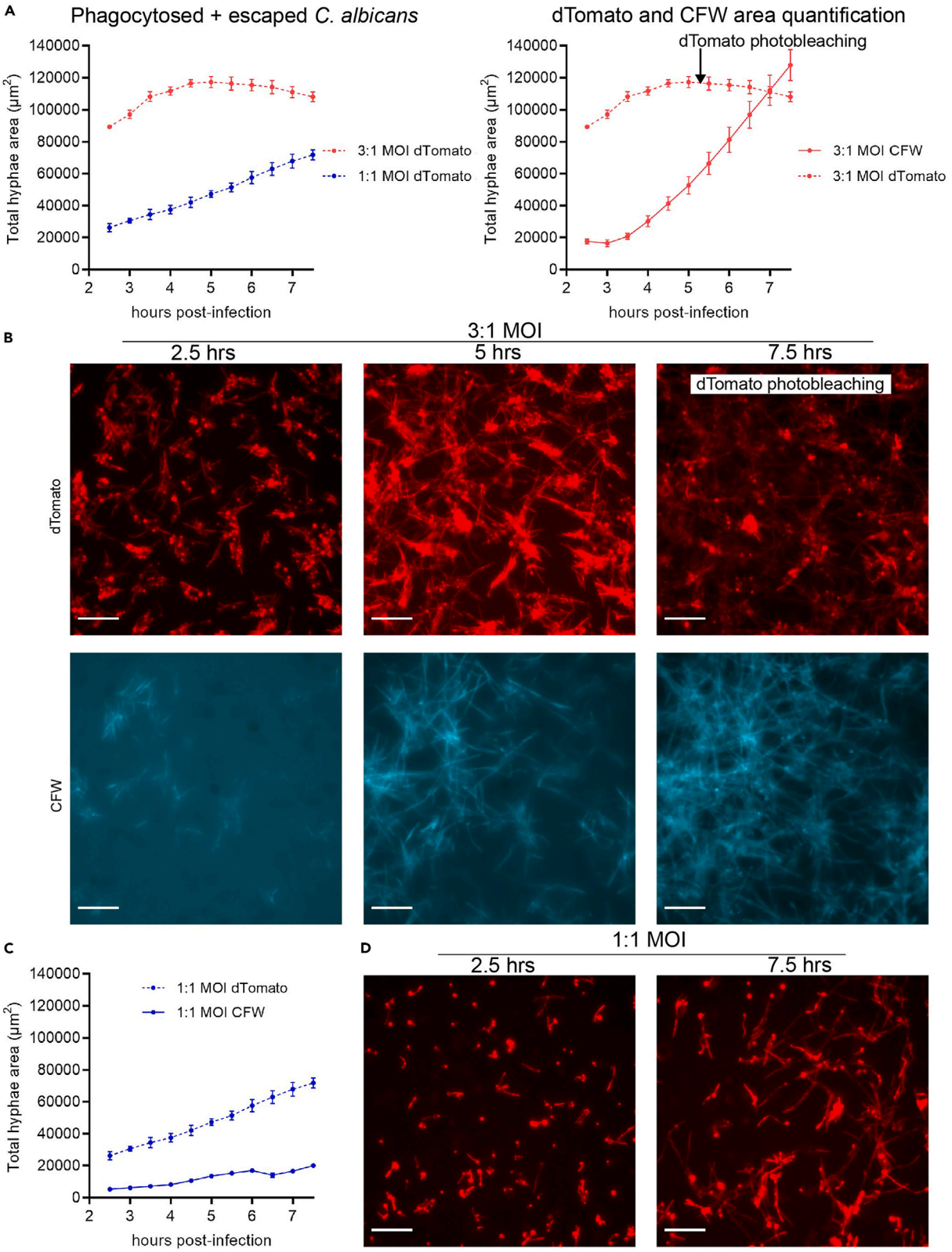
A
Phagocytosed + escaped C. albicans
Total hyphae area (µm²)
hours post-infection
3:1 MOI dTomato
1:1 MOI dTomato
dTomato and CFW area quantification
dTomato photobleaching
3:1 MOI CFW
3:1 MOI dTomato
B
3:1 MOI
2.5 hrs
5 hrs
7.5 hrs
dTomato photobleaching
dTomato
CFW
C
1:1 MOI dTomato
1:1 MOI CFW
D
1:1 MOI
2.5 hrs
7.5 hrs

Figure 8. Quantification of total (dTomato-positive) *C. albicans* hyphae over time

For each time point, error bars represent the standard error of the mean of three technical repeats. A, left panel: comparing total hyphal areas for 1:1 and 3:1 MOI infections. A, right panel: comparing the area of dTomato-positive and CFW-positive hyphae for 3:1 MOI infections. An arrow illustrates the point at which dTomato photobleaching is evident. B: an example of dTomato-positivity compared to CFW-positivity for a 3:1 MOI infection over time. At 7.5 h post-infection, photobleaching of the dTomato signal is evident. Scale bar = 50 μm. C: comparing the area of dTomato-positive and CFW-positive hyphae for 1:1 MOI infections. D: dTomato-positivity for 1:1 MOI infections at 2.5 and 7.5 h post-infection. Adjustments to images described in Table S6. Scale bar = 50 μm.

analysis. The escape-to-phagocytosis ratios of 1:1 and 3:1 MOI infections are comparable up to 5 h post-infection (Figure 7B, right panel). More biological repeats would be essential to add strength to this observation, as discussed in quantification and statistical analysis.

Quantification of total *C. albicans* hyphae area over time

The output of Analysis C (step-by-step method details Step 24) can also be used to plot the area of all hyphae (contained in macrophages and escaped) over time (Figure 8A, left panel). Using this data we estimated that dTomato positivity of 3:1 MOI infections at the first time point is approximately 3.33 times higher than that of 1:1 MOI infections (see quantification and statistical analysis) at the first imaging time point. The difference in total hyphae quantified at the first time point is therefore explained largely by the MOI. From 2.5–4 h post-infection, these areas increase as a result of hyphal growth both inside and outside macrophages (Figure 8A, left panel).

For 3:1 MOI infections however, a plateau in dTomato-positivity is seen at 5 h-post infection (Figure 8A, left panel). This is explained by photobleaching: a loss of fluorescence caused by damage to the dTomato fluorophore expressed by hyphae. This is demonstrated by comparing the dTomato signal of hyphae at 5 and 7.5 h, where fading is evident (Figure 8B). The corresponding images of CFW-positivity shows that *C. albicans* hyphae have increased in number and length (Figure 8B). As a result, the area of escaped (CFW-positive) hyphae exceed that of dTomato-positive hyphae at 7 h post-infection (Figure 8A, right panel). In this shared example, photobleaching is not seen for infections with the lower fungal load of 1:1 MOI (Figures 8C and 8D). As a result, the areas of dTomato-positive and CFW-positive *C. albicans* hyphae continue to increase over time (Figure 8C). This quantification of total hyphal (dTomato) area is therefore only useful for the first 5 h post-infection. The possibility of photobleaching should be considered for all channels.

QUANTIFICATION AND STATISTICAL ANALYSIS

Throughout this paper we have discussed an example infection assay, where macrophages are challenged with *C. albicans* at 1:1 and 3:1 MOI (Figures 6, 7, and 8). The quantitative outputs of Analysis A-C for this experiment have been provided in Data S1 and a smaller subset of these values (1:1 MOI and uninfected conditions only, values from two positions within the same well imaged for each) provided in Tables 1, 2, and 3. These datasets contain calculations for the macrophage DRAQ7 permeabilization and *C. albicans* hyphal area values plotted in Figures 7 and 8. If quantitative values from some positions are zero, or several orders of magnitude larger compared to values from other time points, errors in image analysis may have occurred. An example of this is provided in our experimental output: see CFW values for position 6 (Table 2, Data S1). Problem solving for this is discussed in troubleshooting 2.

The data from this experiment is considered as a single biological repeat. At least three of these biological repeats should be performed before a result can be interpreted with certainty. Within our example assay, the macrophage permeabilization and hyphal area values from each well is considered a single technical repeat. As discussed in step-by-step method details Step 12, images should be acquired from i) at least two positions in each well and ii) at least two (ideally, three) wells per treatment condition.

Table 1. Example of macrophage DRAQ7 permeabilization data

	1:1 MOI				Uninfected			
Time (h)	position 5	position 6	Avg.	%	position 13	position 14	Avg.	%
2:30	10	22	16	4.644412	1	3	2	0.358744
3:00	9	26	17.5	5.079826	5	3	4	0.717489
3:30	12	30	21	6.095791	3	3	3	0.538117
4:00	13	32	22.5	6.531205	3	6	4.5	0.807175
4:30	18	34	26	7.54717	10	6	8	1.434978
5:00	18	39	28.5	8.272859	7	7	7	1.255605
5:30	16	45	30.5	8.853411	7	7	7	1.255605
6:00	15	48	31.5	9.143687	11	9	10	1.793722
6:30	16	54	35	10.15965	10	9	9.5	1.704036
7:00	19	50	34.5	10.01451	10	10	10	1.793722
7:30	20	55	37.5	10.88534	7	7	7	1.255605
Total seeded	319	370	344.5		564	551	557.5	

- For each time point, the number of DRAQ7-positive macrophage nuclei from each position within a well is averaged, then divided by the total number of macrophages to obtain a percentage of DRAQ7-permeabilization (Table 1, Data S1). The total number of macrophages is determined by the number of CellTracker Green-positive macrophages (Analysis A, Figure 3) or the maximum number of DRAQ7-positive macrophages across the imaging time course (Analysis B, Figure 3; Table 1, Data S1). For uninfected controls, the total number of macrophages can only be estimated using positivity for CellTracker Green.

$$Percentage\ of\ DRAQ7\ -\ positive\ macrophages\ = 100 \times \left(\frac{DRAQ7\ -\ positive\ nuclei}{total\ number\ of\ macrophages}\right)$$

- dTomato-positive and CFW-positive pixel area values were obtained for each imaging position, at each time point. To determine what area values were in μm^2, pixel values were multiplied with the inverse of the resolution (pixels per micron) squared. An example of this value conversion is demonstrated in Table 3 and Data S1.

$$\mu m^2 area\ values\ =\ number\ of\ pixels\left(\frac{1}{resolution\ in\ pixels\ per\ micron^2}\right)$$

$$In\ our\ shared\ example, 1 pixel = 1.5444 \mu m^2, \text{see Table S5}$$

$$So \mu m^2 area\ values\ =\ number\ of\ pixels\left(\frac{1}{1.5444^2}\right)$$

- The dTomato and CFW hyphal area values from two positions were then averaged for each well (one technical repeat). To obtain the escape-to-phagocytosis ratio, the CFW area value from each time point was divided by the dTomato area value at the first time point (an approximation of the total area of *C. albicans* that was phagocytozed, see Data S1 and Table 3).

$$escape\ -\ to\ -\ phagocytosis\ ratio\ =\ \left(\frac{area\ of\ CFW\ -\ positive\ hyphae}{area\ of\ dTomato\ -\ positive\ hyphae\ at\ the\ first\ timepoint}\right)$$

Table 2. Example of dTomato and CFW area data

	1:1 MOI							
	Position 5				Position 6 (error in CFW area values)			
Time (h)	CFW (pixels)	CFW (μm^2)	dTomato (pixels)	dTomato (μm^2)	CFW (pixels)	CFW (μm^2)	dTomato (pixels)	dTomato (μm^2)
2:30	11303	4741.319	50766	21295.03	**1047719**	439491.2	58176	24403.34
3:00	12477	5233.781	65785	27595.12	9292	3897.756	73501	30831.78
3:30	14964	6277.014	68042	28541.87	**1047754**	439505.9	78802	33055.42
4:00	18062	7576.546	76752	32195.5	**1047803**	439526.5	88438	37097.47
4:30	23591	9895.819	84956	35636.86	**1047549**	439419.9	99730	41834.18
5:00	29975	12573.74	102375	42943.69	27661	11603.08	118779	49824.74
5:30	37247	15624.16	109825	46068.77	37612	15777.27	125978	52844.54
6:00	44209	18544.54	118658	49773.99	**1047594**	439438.8	129108	54157.49
6:30	28984	12158.04	131201	55035.45	27636	11592.59	135758	56947
7:00	34849	14618.26	141545	59374.5	33798	14177.39	150757	63238.69
7:30	44094	18496.3	156972	65845.73	43069	18066.34	157796	66191.37

- We can also use dTomato area values to validate the accuracy of the MOIs performed in the experiment. In the shared example, the 3:1 MOI *C. albicans* infection was determined to have 3.3 times the amount of *C. albicans* hyphal area at the first imaging time point as follows (values provided in Data S1):

$$Average1:1MOI\ dTomato\ area(pixels) = 69143 + 73591 + 73440 + 58025 + 50766 + 58176 = 63856.83 pixels$$

$$Average3:1MOI\ dTomato\ area(pixels) = 198937 + 220436 + 210600 + 227781 + 207717 + 212229 = 212950 pixels$$

$$Fold\ difference, 3:1 and 1:1 MOI\ fungal\ load = \left(\frac{Avg3:1MOI\ dTomato\ area(pixels)}{Avg1:1MOI\ dTomato\ area(pixels)}\right)\left(\frac{212950}{63856.83}\right) = 3.33$$

To plot DRAQ7 permeabilization and hyphal area data from multiple biological repeats in one graph, technical repeats should be averaged to provide a single representative value for that biological repeat per time point. For example, a plot from four biological repeats should have four values per time point. To determine if differences in DRAQ7, dTomato, CFW or escape-to-phagocytosis ratio values are significant, paired t-test comparisons on time points of interest, assuming Gaussian distribution, should be performed.

Table 3. Quantification of *C. albicans* escape

	1:1 MOI		
Time (h)	Total hyphal area (dTomato μm^2)	Escaped hyphae area (CFW μm^2)	escape-phagocytosis ratio
2:30	21295.03	4741.319	0.222649
3:00	27595.12	5233.781	0.245775
3:30	28541.87	6277.014	0.294764
4:00	32195.5	7576.546	0.355789
4:30	35636.86	9895.819	0.464701
5:00	42943.69	12573.74	0.590454
5:30	46068.77	15624.16	0.7337
6:00	49773.99	18544.54	0.870839
6:30	55035.45	12158.04	0.570933
7:00	59374.5	14618.26	0.686463
7:30	65845.73	18496.3	0.868573

LIMITATIONS

Imaging data acquired using this protocol are two-dimensional: while the infected macrophages are a monolayer, we noticed that a small number of *C. albicans* hyphae extend vertically from this monolayer. These hyphae are not within the imaging plane and, therefore, out-of-focus. Acquiring z-stack images could overcome this limitation. When optimizing image analysis steps for Analysis C, we found that *C. albicans* cells could not be segmented consistently due to irregular cell morphologies: for example, yeast versus hyphae and hyphae of different orientation and shape. Instead, we found that quantifying the total area of *C. albicans* cells (dTomato or CFW) was a more precise measure of fungal load. Machine learning image analysis techniques of z-stack images could overcome this limitation.

The example discussed in this protocol used murine bone marrow-derived macrophages. We have performed similar live cell imaging experiments using other macrophage cell types, including human monocyte-derived macrophages and differentiated THP-1 cells.[14] While suitable for other host cells, this protocol would be best applied to adherent cells. While qualitative observations can be made using this protocol, it is best applied to observe population-level effects over several hours, post-infection. To image a 24-h time course of escape events from hundreds of macrophages per field of view, we employed wide-field microscopy at 30-min intervals. This relatively low spatial and temporal resolution means that limited conclusions can be made about cause and consequence relationships between host cell lysis and escape events. However, the *in vitro* infection protocol presented here can be used in tandem with other microscopic techniques of higher spatial and temporal resolution: for example, confocal or lattice light sheet microscopy.

TROUBLESHOOTING

Problem 1

Step 6f describes the use of Bio-Formats bftools to convert proprietary image file formats to TIF files. Bio-Formats bftools makes use of the Java programming language. Access to Java versions is provided as an "environment variable" on your PC. If Java cannot be found by bftools an error message will appear as below.

```
C:\Users\STAR protocols reader> cd C:\bftools

C:\bftools> bfconvert "C:\Live cell analysis\Example experiment\experiment.lif" "C:\Live
cell analysis\Example experiment\lif to TIF\hea_P%%s_T%%t_C%%c.tif"

'java' is not recognized as an internal or external command, operable program or batch file.
```

Potential solution

- This error message means that the system does not know where to find Java. The location can be defined in your system's user variables: search "edit the system environment variables".
- Under the "Advanced" tab, select "Environment Variables".
- Under "User variables for…", check that there is a variable called "JAVA_HOME". The variable value should be the location of the latest version of java installed on your system, for example: "C:\Program Files\java".
- Under "User variables for…", double click on the Path variable. If not present, add the following variable: "%JAVA_HOME%\bin".

Note: the solution is described as applicable to Windows 11.

Problem 2

In quantification and statistical analysis, we discuss how CellProfiler output values can be used to estimate the proportion of permeabilized macrophages and the escape-to-phagocytosis ratios of

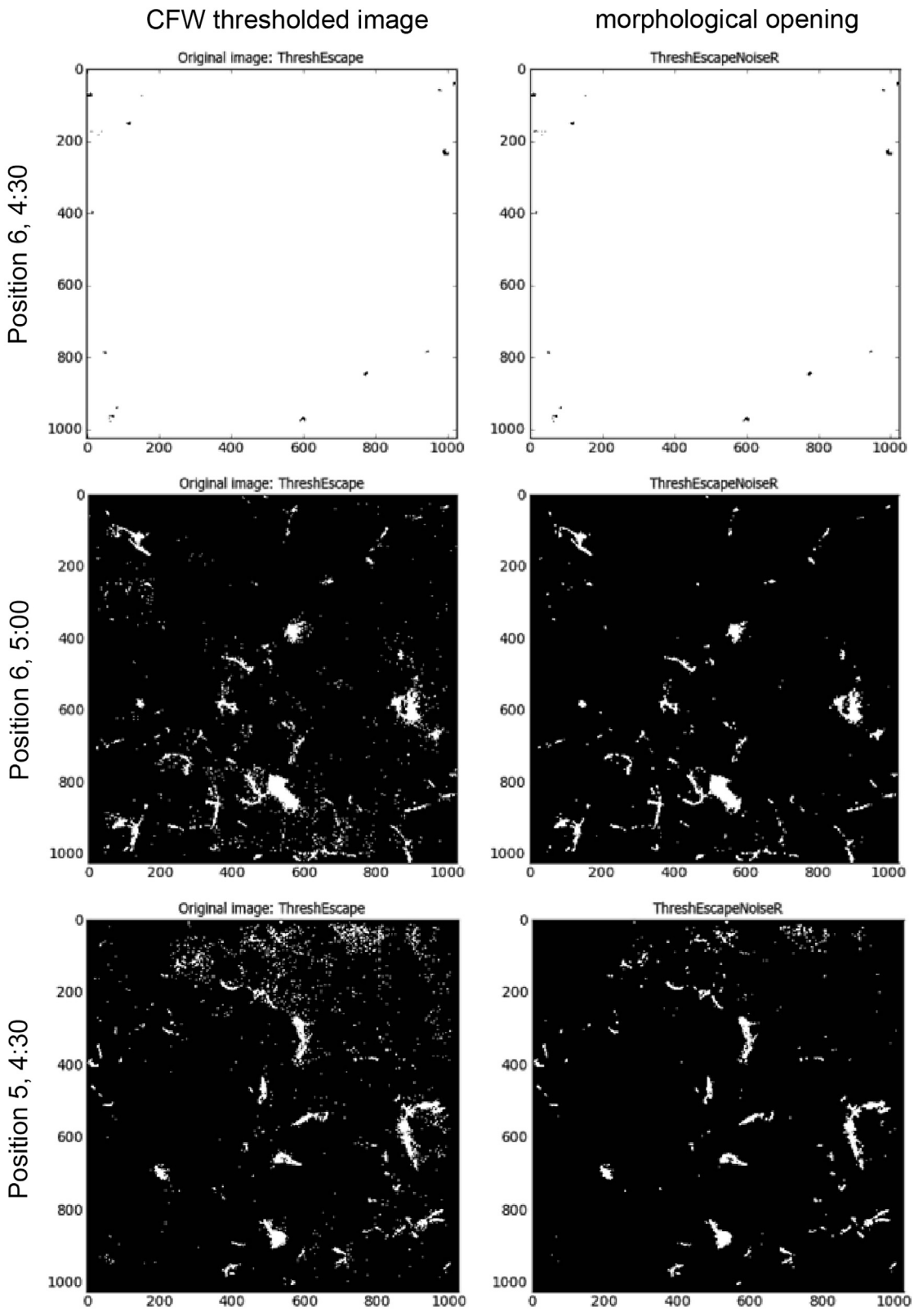
CFW thresholded image
morphological opening
Position 6, 4:30
Position 6, 5:00
Position 5, 4:30
Original image: ThreshEscape
ThreshEscapeNoiseR
0
200
400
600
800
1000

Figure 9. Error in thresholding of CFW-positive *C. albicans* hyphae

Screenshots of the "ApplyThreshold"(left column) and "Smooth" (right column) modules in CellProfiler from Analysis C (see Table S4, Methods video S3). The "ApplyThreshold" module is used to convert fluorescence images of CFW-positive C. albicans hyphae into binary images. Using the "Smooth" module, a morphological opening is performed which removes objects of low pixel values, corresponding to background, from the binary image. Top row: the entire field of view appears to pass the thresholding settings set for this CFW fluorescence image (position 6, 4:30). Middle row: at the same position, one time point later, thresholding appears normal (position 6, 5:00). Bottom row: thresholding appears normal for the other position within the same well, at the same time point (position 5, 4:30).

C. albicans cells. The quantified DRAQ7, dTomato or CFW area values for a particular position are 0 or approaching the maximum area value possible. For example, some (but not all) of the CFW pixel area values for position six (for 1:1 MOI infected macrophages) are near the theoretical maximum (Table 2). As we acquired images that are 1024 × 1024 pixels in size (Table S5), the maximum possible area value would be 1048576 pixels. These large pixel area values indicate that almost all of the field of view has passed the threshold value for CFW-positive hyphae, leading to the entire image being quantified as CFW-positive (Figure 9, top row).

Potential solution

- Test the image analysis settings for the affected images within CellProfiler, as described in step-by-step method details Step 22d-e, Step 23d and Step 24d.
- For example, we investigated the error in our dataset by testing the CellProfiler thresholding for position 6 at 4:30 h post-infection (Figure 9, top row). From the CFW area values in Table 2 it is clear that an unusually high CFW area is detected. Concurrent with this, most of the field of view appears as white, meaning that almost the entire image passes the thresholding parameters set for CFW-positive hyphae (Figure 9, top row). However, when we study the thresholding of the next time point for the same position (Figure 9, middle row), it appears that a binary image of CFW-positive hyphae is produced as normal. Similarly, thresholding appears successful for the other position within this well, at the same time point (Figure 9, bottom row). This corresponds to the CFW-positive hyphae area values obtained (Table 2, Data S1).
- These thresholding inconsistencies can be caused by even small fluctuations in image brightness or focus. If only one position is affected by a DRAQ7 count or hyphal area estimate, the values for that position can be excluded from analysis, and the remaining position used to represent that well (Tables 2 and 3, Data S1). Importantly, cell segmentation or thresholding settings should be consistent for all images within a single experiment. The redundancy in imaging positions within a well allows for erroneous image analysis outputs to be discarded.
- If these inconsistent outputs affect multiple positions, the cell counting or thresholding settings used may not be suitable for the dataset. To solve this, adjust these settings while previewing outputs, as described in step-by-step method details **Step 22d-e**, **Step 23d** and **Step 24d.**
- Technical problems with the microscope used may contribute to this problem: for example, an aging lamp, or autofocus errors.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ana Traven (ana.traven@monash.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Numerical data used to construct figures are provided as Data S1. Unedited microscopy images for all figures can be accessed, by request, from the lead contact.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102737.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.A.B.O. and A.T.; methodology, F.A.B.O.; formal analysis, F.A.B.O.; visualization, F.A.B.O.; resources, A.T.; writing – original draft, F.A.B.O.; writing – review and editing, F.A.B.O. and A.T.; supervision, A.T.; project administration, A.T.; funding acquisition, A.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Olivier, F.A.B., Hilsenstein, V., Weerasinghe, H., Weir, A., Hughes, S., Crawford, S., Vince, J.E., Hickey, M.J., and Traven, A. (2022). The escape of Candida albicans from macrophages is enabled by the fungal toxin candidalysin and two host cell death pathways. Cell Rep. *40*, 111374.

2. Silva, M.T. (2010). Neutrophils and macrophages work in concert as inducers and effectors of adaptive immunity against extracellular and intracellular microbial pathogens. J. Leukoc. Biol. *87*, 805–813.

3. Roy, C.R., and Mocarski, E.S. (2007). Pathogen subversion of cell-intrinsic innate immunity. Nat. Immunol. *8*, 1179–1187.

4. Erwig, L.P., and Gow, N.A.R. (2016). Interactions of fungal pathogens with phagocytes. Nat. Rev. Microbiol. *14*, 163–176.

5. Flannagan, R.S., Cosío, G., and Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat. Rev. Microbiol. *7*, 355–366.

6. Wiederhold, N.P. (2017). Antifungal resistance: current trends and future strategies to combat. Infect. Drug Resist. *10*, 249–259.

7. Huemer, M., Mairpady Shambat, S., Brugger, S.D., and Zinkernagel, A.S. (2020). Antibiotic resistance and persistence—Implications for human health and treatment perspectives. EMBO Rep. *21*, e51034.

8. Hoppe, A.D., Seveau, S., and Swanson, J.A. (2009). Live cell fluorescence microscopy to study microbial pathogenesis. Cell Microbiol. *11*, 540–550.

9. Bain, J., Gow, N.A., and Erwig, L.-P. (2015). Novel Insights into Host-Fungal Pathogen Interactions Derived from Live-Cell Imaging (Springer), pp. 131–139.

10. Peng, H. (2008). Bioimage informatics: a new area of engineering biology. Bioinformatics *24*, 1827–1836.

11. Nketia, T.A., Sailem, H., Rohde, G., Machiraju, R., and Rittscher, J. (2017). Analysis of live cell images: Methods, tools and opportunities. Methods *115*, 65–79.

12. Geoghegan, N.D., Evelyn, C., Whitehead, L.W., Pasternak, M., McDonald, P., Triglia, T., Marapana, D.S., Kempe, D., Thompson, J.K., Mlodzianoski, M.J., et al. (2021). 4D analysis of malaria parasite invasion offers insights into erythrocyte membrane remodeling and parasitophorous vacuole formation. Nat. Commun. *12*, 3620.

13. Dumont, A., Boucrot, E., Drevensek, S., Daire, V., Gorvel, J.P., Poüs, C., Holden, D.W., and Méresse, S. (2010). SKIP, the host target of the Salmonella virulence factor SifA, promotes kinesin-1-dependent vacuolar membrane exchanges. Traffic *11*, 899–911.

14. Tucey, T.M., Verma, J., Olivier, F.A.B., Lo, T.L., Robertson, A.A.B., Naderer, T., and Traven, A. (2020). Metabolic competition between host and pathogen dictates inflammasome responses to fungal infection. PLoS Pathog. *16*, e1008695.

15. Senft, R.A., Diaz-Rohrer, B., Colarusso, P., Swift, L., Jamali, N., Jambor, H., Pengo, T., Brideau, C., Llopis, P.M., Uhlmann, V., et al. (2023). A biologist's guide to planning and performing quantitative bioimaging experiments. PLoS Biol. *21*, e3002167.

16. McQuin, C., Goodman, A., Chernyshev, V., Kamentsky, L., Cimini, B.A., Karhohs, K.W., Doan, M., Ding, L., Rafelski, S.M., Thirstrup, D., et al. (2018). CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol. *16*, e2005970.

17. Lionakis, M.S., Swamydas, M., Fischer, B.G., Plantinga, T.S., Johnson, M.D., Jaeger, M., Green, N.M., Masedunskas, A., Weigert, R., Mikelis, C., et al. (2013). CX 3 CR1-dependent renal macrophage survival promotes Candida control and host survival. J. Clin. Invest. *123*, 5035–5051.

18. Assouvie, A., Daley-Bauer, L.P., and Rousselet, G. (2018). Growing murine bone marrow-derived macrophages. Methods Mol. Biol. *1784*, 29–33.

19. Rios, F.J., Touyz, R.M., and Montezano, A.C. (2017). Isolation and differentiation of murine macrophages. Methods Mol. Biol. *1527*, 297–309.

20. Gratacap, R.L., Rawls, J.F., and Wheeler, R.T. (2013). Mucosal candidiasis elicits NF-κB activation, proinflammatory gene expression and localized neutrophilia in zebrafish. Dis. Model. Mech. *6*, 1260–1270.

21. Linkert, M., Rueden, C.T., Allan, C., Burel, J.-M., Moore, W., Patterson, A., Loranger, B., Moore, J., Neves, C., MacDonald, D., et al. (2010). Metadata matters: access to image data in the real world. J. Cell Biol. *189*, 777–782.

22. Bio-Formats 5.7.1 documentation: supported formats. (2017). https://docs.openmicroscopy.org/bio-formats/5.7.1/supported-formats.html.

23. Hilsenstein, V.. https://github.com/VolkerH/Tutorial-on-bftools-and-bfconvert.

24. Bio-Formats 5.7.1 documentation: user information. (2017). https://docs.openmicroscopy.org/bio-formats/5.7.1/users/comlinetools/index.html.

25. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

26. Tucey, T.M., Verma, J., Harrison, P.F., Snelgrove, S.L., Lo, T.L., Scherer, A.K., Barugahare, A.A., Powell, D.R., Wheeler, R.T., Hickey, M.J., et al. (2018). Glucose Homeostasis Is Important for Immune Cell Viability during Candida Challenge and Host Survival of Systemic Fungal Infection. Cell Metab. *27*, 988–1006.e7.

27. Uwamahoro, N., Verma-Gaur, J., Shen, H.H., Qu, Y., Lewis, R., Lu, J., Bambery, K., Masters, S.L., Vince, J.E., Naderer, T., and Traven, A. (2014). The pathogen Candida albicans hijacks pyroptosis for escape from macrophages. mBio *5*, e00003–e00014.

28. Kasper, L., König, A., Koenig, P.A., Gresnigt, M.S., Westman, J., Drummond, R.A., Lionakis, M.S., Groß, O., Ruland, J., Naglik, J.R., and Hube, B. (2018). The fungal peptide toxin Candidalysin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes. Nat. Commun. *9*, 4260.