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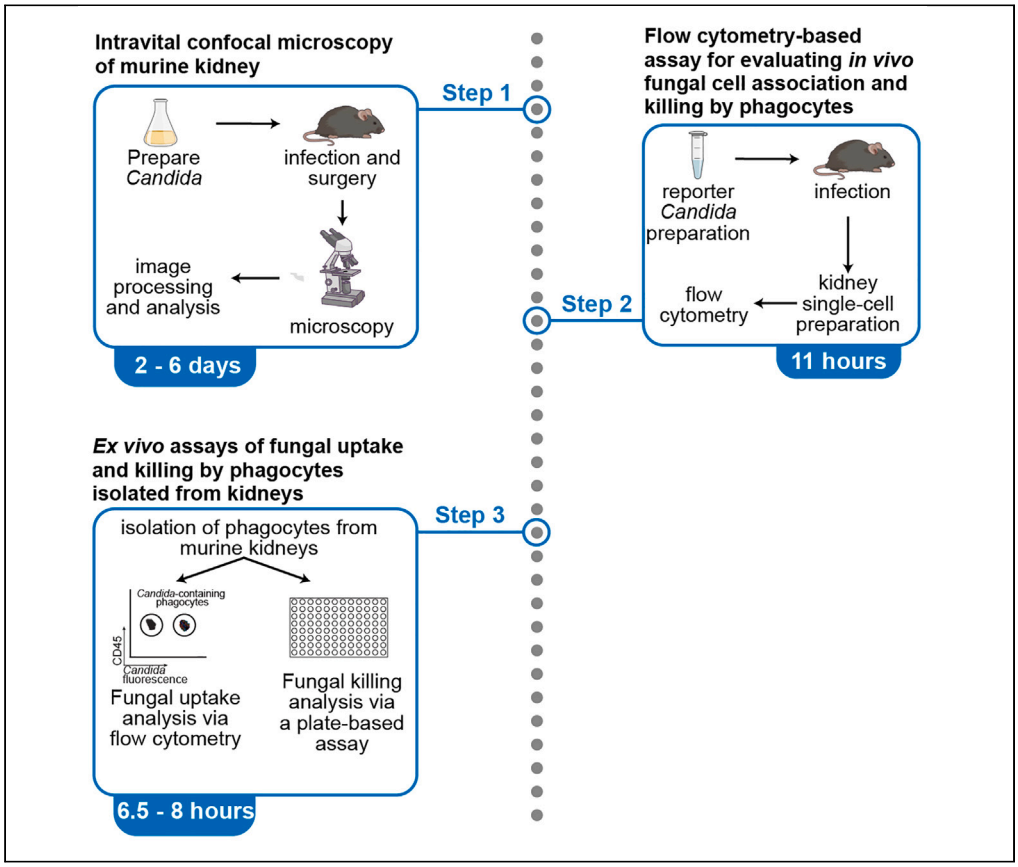
Evaluation of murine renal phagocyte-fungal interactions using intravital confocal microscopy and flow cytometry



Myeloid phagocytes are essential for antifungal host defense during systemic candidiasis. Here, we present a protocol for assessing phagocyte-fungal interactions *in vivo* in the kidney, the primary target organ of the murine systemic candidiasis model. We describe steps for intravital confocal microscopy and flow cytometry. We also detail a kidney tissue dissociation procedure to obtain highly pure functional phagocytes for utilization in downstream *ex vivo* fungal uptake and killing assays.

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

Jigar V. Desai,
Michail S. Lionakis

jigarkumar.desai@hmh-cdi.org (J.V.D.)
lionakism@niaid.nih.gov (M.S.L.)

Highlights

Quantitative intravital microscopy of *Candida albicans*-infected murine kidney

Flow cytometry-based analysis of renal phagocytes' antifungal effector functions

Renal phagocyte isolation and *ex vivo* effector function analysis

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Evaluation of murine renal phagocyte-fungal interactions using intravital confocal microscopy and flow cytometry

Jigar V. Desai[1,2,3,*] and Michail S. Lionakis[1,4,*]

[1]Fungal Pathogenesis Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

[2]Present address: Center for Discovery & Innovation, Hackensack Meridian Health, Nutley, NJ 07110, USA

[3]Technical contact

[4]Lead contact

*Correspondence: jigarkumar.desai@hmh-cdi.org (J.V.D.), lionakism@niaid.nih.gov (M.S.L.)
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SUMMARY

Myeloid phagocytes are essential for antifungal host defense during systemic candidiasis. Here, we present a protocol for assessing phagocyte-fungal interactions *in vivo* in the kidney, the primary target organ of the murine systemic candidiasis model. We describe steps for intravital confocal microscopy and flow cytometry. We also detail a kidney tissue dissociation procedure to obtain highly pure functional phagocytes for utilization in downstream *ex vivo* fungal uptake and killing assays.

BEFORE YOU BEGIN

The protocol below describes the specific steps for analyzing the interactions of renal myeloid phagocytes with *Candida albicans*, an opportunistic fungus that causes mucosal and life-threatening systemic infections in immunocompromised hosts.[1] We provide details for performing intravital confocal microscopy of the murine kidney after infection with fluorescent *C. albicans*. Subsequently, we outline the steps for image processing and analysis to obtain quantitative parameters depicting phagocyte-*Candida* interactions *in vivo*. In parallel, to comprehensively evaluate such interactions, we provide a flow cytometry-based approach to assess fungal uptake and intracellular killing by the renal phagocytes *in vivo* and outline the steps to isolate purified populations of renal neutrophils and macrophages for *ex vivo* analysis of fungal uptake and killing.

1. For intravital microscopy, our protocol utilizes a dTomato-expressing *Candida albicans* strain (see the key resources table). The steps used to grow *C. albicans* for its downstream use are outlined under "Preparation and growth of *C. albicans*".
2. To perform microscopy, we utilized Leica SP8 or STELLARIS 8 inverted confocal laser scanning microscopes, equipped with 4 laser lines and detectors. However, any confocal microscope with a similar configuration can be used.
3. The flow cytometry-based approach to assessing fungal uptake and killing *in vivo*, is adapted from an approach initially described for the opportunistic inhaled mold *Aspergillus fumigatus* by Hohl and colleagues.[2] Similar to their approach, this protocol requires preparation of the reporter *C. albicans* strain and its use in murine systemic infection experiments.
4. For flow cytometry, a 5-laser BD LSR Fortessa II was utilized; an equivalent analyzer that can detect the fluorophores outlined below can also be utilized to successfully execute the protocol. Alternatively, a customized panel of antibodies can be designed, based on the available instrument configuration and can be successfully used to execute the protocol.

5. All the reagents, buffers, and media should be handled aseptically, under a biosafety cabinet.

Institutional permissions

The mice used in developing this protocol were maintained in specific-pathogen-free conditions at American Association for Laboratory Animal Care-accredited facilities at the National Institute for Allergy and Infectious Diseases (NIAID), and Center for Discovery and Innovation, Hackensack Meridian Health (CDI-HMH), in accordance with the Institutional Animal Care and Use Committee (IACUC) approved protocols, LCIM14E (NIAID) and 293 (CDI-HMH). Individuals utilizing this protocol must obtain authorization from their respective institutional authorities.

Prepare YPD (yeast extract, peptone, dextrose) broth media

Timing: 3–4 h

6. In a glass bottle, add 50 *g* of Difco YPD (Yeast extract, Peptone, Dextrose) broth powder to deionized water.
7. Sterilize by autoclaving using the liquid cycle with a temperature of 121°C for 40 min.
8. Subsequent to sterilization, allow the broth to cool down to room temperature and then aseptically supplement it with penicillin and streptomycin, at final concentrations of 100 units/mL and 100 μg/mL, respectively.
9. The prepared YPD broth will be used to grow *Candida albicans*.

Note: Sterilized broth can be stored at room temperature for up to 6 months.

Prepare YPD agar plates

Timing: 3–4 h

10. Add 65 *g* of Difco YPD agar powder to deionized water.
11. Sterilize by autoclaving, as above.
12. Subsequent to sterilization, allow the liquid to cool down to 45°C–50°C and then aseptically supplement it with penicillin and streptomycin at final concentrations of 100 units/mL and 100 μg/mL, respectively.
13. Pour 15–20 mL of media into bacteriological petri dishes, under a biosafety cabinet, and allow them to cool down and dry for up to 30 min to let the moisture escape.
14. Place the petri dish lids and store the plates, refrigerated, upside down, in provided plastic sleeves.
15. The prepared YPD-agar plates will be used to grow and isolate *Candida albicans* colonies for subsequent passage into liquid YPD broth, as outlined below.

Note: YPD agar plates can be stored refrigerated for up to 1–2 months.

Preparation and growth of *Candida albicans*

Timing: 5 days

16. From a vial of a frozen *C. albicans* stock, streak the fungus onto a YPD (Yeast extract, Peptone, Dextrose) agar plate, supplemented with penicillin/streptomycin, and allow the fungus to grow for 48 h at 30°C to obtain single colonies.
17. Pick a single colony to inoculate 5 mL of YPD broth supplemented with penicillin/streptomycin in a 50 mL conical tube and allow it to grow at 30°C for 16–20 h with continuous shaking at 220 rpm.

Note: To allow air exchange during growth, do not tighten the cap completely; the cap can be loosely threaded on the tube and secured with an adhesive tape.

18. Perform a serial passage by inoculating 5 μL of the grown fungal culture as above to a fresh 50 mL conical tube containing 5 mL YPD broth supplemented with penicillin/streptomycin.
19. Repeat a serial passage as above.
20. After these two serial passages, centrifuge the *Candida*-containing suspension at 350 × *g* at 4°C for 5 min, and wash twice with phosphate-buffered saline, pH 7.4.
21. Determine the yeast cell concentration using a hemocytometer and *Candida* is now ready to use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor 647 anti-mouse Ly-6G antibody (1:100)	BioLegend	Cat# 127610, RRID: AB_1134159
Alexa Fluor 647 rat IgG2a, κ isotype ctrl antibody (1:100)	BioLegend	Cat# 400526, RRID: AB_2864284
Alexa Fluor 488 anti-mouse F4/80 antibody (1:100)	BioLegend	Cat# 123120, RRID: AB_893479
Alexa Fluor 488 rat IgG2a, κ isotype ctrl antibody (1:100)	BioLegend	Cat# 400525, RRID: AB_2864283
BV421 rat anti-mouse CD31 antibody (1:100)	BD Biosciences	Cat# 562939, RRID: AB_2665476
BD Horizon BV421 rat IgG2a, κ isotype control (1:100)	BD Biosciences	Cat# 562602, RRID: AB_11153860
Rat anti-mouse CD16/CD32 (1:100)	BD Biosciences	Cat#553142, RRID: AB_394657
Brilliant Violet 605 anti-mouse CD45 antibody (1:100)	BioLegend	Cat# 103155, RRID: AB_2650656
Brilliant Violet 605 rat IgG2a, κ isotype ctrl antibody (1:100)	BioLegend	Cat# 400539, RRID: AB_11126979
PerCP/Cyanine5.5 anti-mouse Ly-6G antibody (1:100)	BioLegend	Cat# 127616, RRID: AB_1877271
PerCP/Cyanine5.5 anti-mouse CD3ε antibody (1:100)	BioLegend	Cat# 100328, RRID: AB_893318
PerCP/Cyanine5.5 anti-mouse CD19 antibody (1:100)	BioLegend	Cat# 152406, RRID: AB_2629815
PerCP/Cyanine5.5 anti-mouse CD335 (NKp46) antibody (1:100)	BioLegend	Cat# 137610, RRID: AB_10641137
PerCP/Cyanine5.5 rat IgG2a, κ isotype ctrl antibody (1:100)	BioLegend	Cat# 400531, RRID: AB_2864286
APC/Cyanine7 anti-mouse/human CD11b antibody (1:100)	BioLegend	Cat# 101226, RRID: AB_830642
APC/Cyanine7 rat IgG2a, κ isotype ctrl antibody (1:100)	BioLegend	Cat# 400523, RRID: AB_2923252
MHC class II (I-A/I-E) monoclonal antibody (M5/114.15.2), eFluor 450, eBioscience (1:100)	Thermo Fisher Scientific	Cat# 48-5321-82, RRID: AB_1272204
Rat IgG2b kappa isotype control (eB149/10H5), eFluor 450, eBioscience (1:100)	Thermo Fisher Scientific	Cat# 48-4031-82, RRID: AB_1272017
PE/Cyanine7 anti-mouse F4/80 antibody (1:100)	BioLegend	Cat# 123114, RRID: AB_893478
PE/Cyanine7 rat IgG2a, κ isotype ctrl antibody (1:100)	BioLegend	Cat# 400522, RRID: AB_326542
Alexa Fluor 700 rat anti-mouse Ly-6C (1:100)	BD Biosciences	Cat# 561237, RRID: AB_10612017
BD Pharmingen Alexa Fluor 700 rat IgM, κ isotype control (1:100)	BD Biosciences	Cat# 561207, RRID: AB_10611563
Chemicals, peptides, and recombinant proteins		
Corning 100 mL Penicillin-Streptomycin solution, 100x	Corning	Cat# 30-002CI
Liberase TL research grade	MilliporeSigma	Cat# 5401020001
DNase I	MilliporeSigma	Cat# 10104159001
EDTA (0.5 M), pH 8.0	Quality Biological	Cat# 351-027-101
HEPES buffer (1 M), pH 7.3	Quality Biological	Cat# 118-089-721
ACK lysing buffer	Quality Biological	Cat# 118-156-721
Fetal bovine serum - premium, heat inactivated	R&D Systems	Cat# S11150H
10% Sodium azide	Teknova	Cat# S0209
Bovine serum albumin	Fisher Scientific	Cat# BP9706100
Corning RPMI 1640	Corning	Cat# 10-041-CV
alamarBlue cell viability reagent	Thermo Fisher Scientific	Cat# DAL1100
Triton X-100	Thermo Fisher Scientific	Cat# A16046.AE
Percoll	Cytiva	Cat# 17089101
Paraformaldehyde, 4% in PBS	Thermo Fisher Scientific	Cat# J61899.AK
Biotin-XX, SE (6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, succinimidyl ester)	Thermo Fisher Scientific	Cat# B-1606

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Streptavidin conjugated to Alexa Fluor 633	Thermo Fisher Scientific	Cat# S21375
Hoechst 33258 pentahydrate	Thermo Fisher Scientific	Cat# H3569
Propidium iodide	Sigma-Aldrich	Cat# P4170
Difco YPD broth	BD Biosciences	Cat# 2421810
Difco YPD agar	BD Biosciences	Cat# 242710
Experimental models: Organisms/strains		
Mouse: 8–12 weeks old BALB/cJ mice (males or females)	Jackson Laboratory	Stock# 000651
Candida albicans: CAF2-1-dTomato	Laboratory of Michail S. Lionakis	N/A
Candida albicans: SC5314	Laboratory of Michail S. Lionakis; also, commercially available from ATCC (MYA-2876)	Cat# MYA-2876
Software and algorithms		
Fiji	NIH	https://imagej.net/software/fiji/
Imaris v9.2	Oxford Instruments	https://imaris.oxinst.com/
FlowJo v10	BD	https://www.flowjo.com/
Other		
Universal mounting frame K100-Set	Leica Microsystems	Cat# 11532998
Corning borosilicate cover glass, No. 1.5, (50 mm × 22 mm)	Fisher Scientific	Cat# 12-553-468
Cole-Parmer Essentials cell spreaders, sterile, polypropylene, individual wrap	Cole-Palmer	UX-04396-52
3M Transpore surgical tape	Fisher Scientific	Cat# 18-999-381
Fisherbrand absorbent underpads	Fisher Scientific	Cat# 14-206-65
SomnoSuite low-flow anesthesia system with RightTemp module and RoVent ventilator	Kent Scientific	Cat# SS-01, SS04-Module
Puralube ophthalmic ointment	Fisher Scientific	Cat# NC2004680
Aquasonic 100 ultrasound transmission gel	VWR Scientific	Cat# 68200-710
Falcon bacteriological Petri dishes with lid	Fisher Scientific	Cat# 08-757-100D
BD needle 5/8 in. single use, sterile, 25 G	BD	Cat# 305122
Cell strainer 70 μm	Alkali Scientific	Cat# MT5070
Cell strainer 40 μm	Alkali Scientific	Cat# MT5040
Tubes with 35 μm cell strainer	VWR Scientific	Cat# 21008-948
CellPro vacuum filtration flasks PES membrane	Alkali Scientific	Cat# V50022

MATERIALS AND EQUIPMENT

- Phosphate buffered saline (PBS), 1X and 10X.
- Sodium bicarbonate buffer (0.1 M, pH 9.5): Dissolve 3.56 *g* Na_2CO_3 and 8.4 *g* $NaHCO_3$ in 1 L de-ionized water, and filter sterilize.

  Note: Sodium bicarbonate buffer can be stored at room temperature for up to 1 year.

- Tris-HCl (1 M, pH 8): Dissolve 121.14 *g* Tris base in 900 mL deionized water. Adjust pH with 10 N HCl, make up the volume to 1 L, and filter sterilize.

  Note: Tris-HCl can be stored at room temperature for up to 1 year.

- Kidney digestion buffer[3]:

Reagent	Final concentration	Amount
RPMI tissue culture medium with 25 mM HEPES	N/A	9.1 mL
Liberase TL	0.2 mg/mL	0.4 mL
DNase	0.1 mg/mL	0.5 mL

Note: The digestion buffer should be prepared fresh and kept on ice.

Note: Liberase TL was prepared at a stock concentration of 5 mg/mL by adding 1 mL sterile water to a vial containing 5 mg of the enzyme. The reconstituted enzyme can be aliquoted in sterile microtubes and stored at −20°C for up to 1 year.

Note: DNase was prepared at a stock concentration of 2 mg/mL by adding 50 mL sterile water to a vial containing 100 mg of the enzyme. The reconstituted enzyme can be aliquoted in sterile microtubes and stored at −20°C for up to 1 year.

- RPMI 1640 culture medium containing 25 mM HEPES, and supplemented with penicillin and streptomycin (RPMI + P/S).

Note: The above media should be kept refrigerated and can be stored for up to 6 months.

- RPMI 1640 culture medium containing 25 mM HEPES and 10% fetal bovine serum (RPMI + P/S + FBS).

Note: The above media should be kept refrigerated and can be stored for up to 6 months.

- Hank's buffered salt solution (HBSS) supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA).

Note: HBSS with EDTA can be stored refrigerated for up to 1 year.

- FACS buffer:

Reagent	Final concentration	Amount
1X PBS	N/A	500 mL
Bovine serum albumin	0.5% w/v	2.5 *g*
10% sodium azide solution	0.05% w/v	0.25 mL

Note: Filter sterilize using a 0.22 μm polyethersulfone (PES) membrane vacuum filtration flask.

⚠ CRITICAL: Sodium azide is a harmful chemical and should be handled with care. Wear appropriate personal protective equipment to prevent skin contact and operate under a chemical fume hood to prevent inhalation.

Note: Sterilized FACS buffer can be stored refrigerated for up to 6 months.

- Percoll: First mix 9 parts Percoll with 1 part 10X PBS. Use this to prepare 70% and 40% Percoll solutions by diluting in FACS buffer.

Note: Percoll to be used for experiments can be reconstituted a day before and can be stored refrigerated.

- Sorting buffer: 1X phosphate-buffered saline (PBS) supplemented with 5% heat-inactivated fetal bovine serum. Filter sterilize, and to reduce the chances of contamination, the sorting buffer can be aliquoted in sterile 50 mL conical tubes.

Note: Sorting buffer can be stored refrigerated for up to 6 months.

- MACS buffer:

Reagent	Final concentration	Amount
1X PBS	N/A	500 mL
Bovine serum albumin	0.5% w/v	2.5 *g*
0.5 M EDTA	2 mM	2 mL

Note: MACS buffer can be stored refrigerated for up to 6 months.

- 0.02% Triton-X-100: Prepare 0.2% Triton-X-100 by diluting in deionized water, followed by 10-fold dilution to the working concentration.

Note: 0.02% Triton-X-100 can be stored at room temperature for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Intravital confocal microscopy of murine kidneys

Timing: 2–6 days

Timing: 1 h (for step 1)

Timing: 30 min (for step 2)

Timing: 2–4 h (for step 3)

Timing: 1–5 days (for step 4)

This step outlines the procedures for microscope stage insert and mouse surgical preparation, microscopy, image processing, and analysis.

1. Preparation of the microscope stage.

Note: This sub-step outlines how to prepare a custom piece and place it on the microscope stage insert. This design ensures that the exposed kidney is positioned efficiently and reduces movement artifacts caused by the mouse heartbeat and breathing during imaging.

a. Take a disposable L-shaped polypropylene cell spreader.
b. Cut out the horizontal short end of the "L" using a scalpel or sharp scissors (Figure 1A).
c. Start carving out the middle portion (approximately 0.5 cm) using a scalpel, with horizontal motion, parallel to the long axis of the spreader (Figure 1A).
d. Stop the carving when the thickness at the deepest part is about half of the original.
e. Once the carved spreader is ready, prepare the stage insert by affixing a cover glass to the center of the stage insert's opening using surgical tape (Figure 1B).
f. Affix the carved cell spreader toward the top of the cover glass using surgical tape.

⚠ CRITICAL: Please use caution while carving using the scalpel to avoid injury. The top and side views of an assembled stage insert are depicted in Figure 1C, while Figure 1D depicts the mouse placement on the stage insert for downstream imaging.

Note: The carved piece can be prepared ahead of time and can be reused. For reuse, please ensure effective cleaning with soap water, followed by decontamination via spraying with

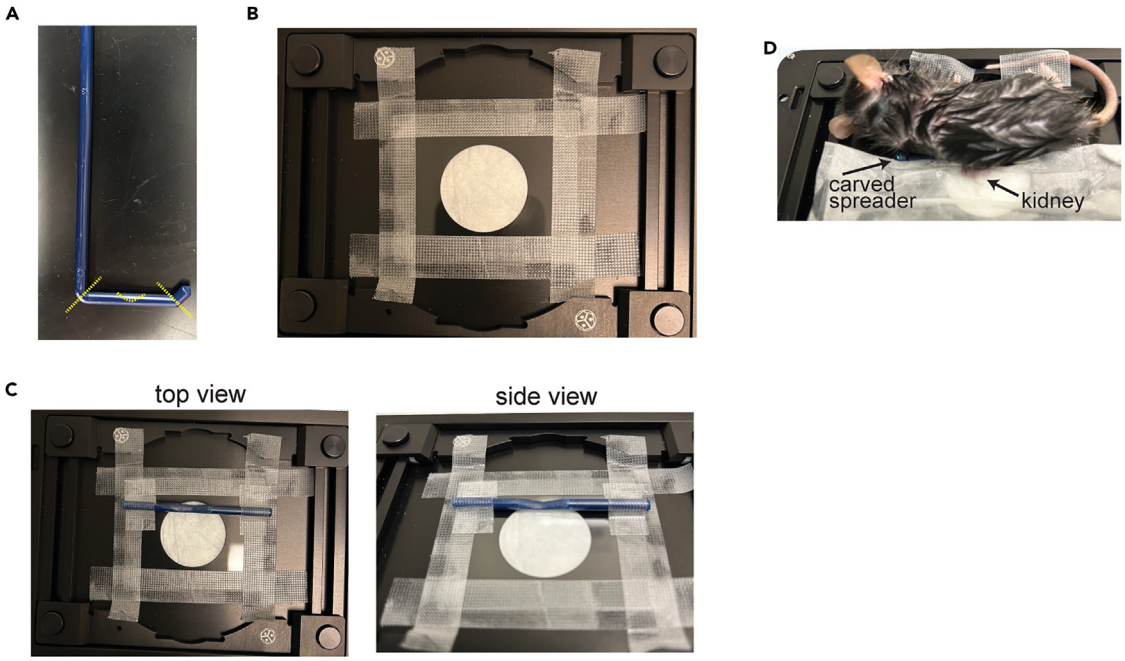

Figure 1. Microscope stage insert and mouse placement for renal intravital microscopy

(A) Polypropylene cell-spreader, where the yellow dotted lines mark the areas for cutting and carving.

(B) Top view of the microscope stage insert.

(C) Images of the microscope stage insert after affixing the carved cell spreader.

(D) Image of the mouse placed on the microscope stage insert, with arrow-heads depicting the exteriorized kidney over the custom-carved spreader.

70% ethanol after each use. For subsequent reuse, decontaminate again by spraying with 70% ethanol and affix on the cover glass and stage insert as depicted in Figure 1C.

2. Preparation of the antibody cocktail and *Candida albicans* for injection.

Note: We use anti-mouse antibodies against F4/80, Ly6G, and CD31 to stain renal macrophages, neutrophils, and endothelium, respectively. This sub-step details the preparation of the antibody cocktail and the fungus for retro-orbital injection into mice. Alternatively, the antibodies/fungus can also be injected intravenously through the lateral tail-vein, if the users have access to a warming device (for safely warming the mouse for vasodilation) and appropriate restrainer in their microscope room.

a. In our experience, injecting 5 μg of anti-CD31, 2.5 μg of anti-F4/80, and 2 μg of anti-Ly6G, 30 min before the surgery is sufficient to ensure efficient staining.
b. Calculate the volume of antibodies, based on their stock concentrations.
c. Mix the antibodies in a single sterile microtube, and make up the volume to 50 μL with sterile 1X PBS.
d. Prepare *C. albicans*, as described under "preparation and growth of *Candida albicans*."
   i. Dilute *Candida* to 5×10^7 colony forming units (CFU) per mL in 1X PBS, so that 100 μL will deliver 5×10^6 CFU.

3. Microscopy of the infected kidney.

Note: This sub-step details how to prepare a mouse for surgery, surgically expose the kidney, and then position it on the microscope stage insert for imaging.

a. Switch on the microscope and associated equipment, including the temperature control modules, at least 30 min before initiating the surgery.

b. Prepare the work surface by placing absorbent under pads over the work area and the mouse warming pad.
c. Sanitize the work surface by spraying with 70% ethanol.
d. Anesthetize the mouse by placing it in an induction chamber of an isoflurane vaporizer-based system.
e. Adjust isoflurane to 3% and wait for the induction of anesthesia.

Note: For the anesthesia systems that use compressed oxygen, adjust the oxygen flow rate to 1.5–2 L/min along with the isoflurane concentration as specified above.

f. Once the mouse is anesthetized, remove it from the induction chamber and place it over the work area, above the warming pad.
g. Continue delivering the anesthesia by placing a nose cone, secured in place using surgical tape.
h. After ensuring appropriate anesthesia depth by toe-pinching, inject the antibody cocktail, prepared as specified under "Preparation of the antibody cocktail and *Candida albicans* for injection", via retro-orbital injection.
i. Remove the nose cone, place the mouse back in the cage, and allow it to recover from anesthesia.
j. Wait for 30 min before proceeding further with surgery for microscopy.

⚠ CRITICAL: It is necessary to allow 30 minutes for efficient labeling of the cells within the renal tissue, before proceeding further with surgery for microscopy.

k. During the 30 min wait period, set up the acquisition parameters on the microscope.

Note: We have used Leica STELLARIS 8 confocal microscope equipped with 4 HyD S detectors, 4 laser lines (405/488/561/638 nm), and a 25X water-immersion objective. However, the steps below can also be used with any other confocal laser scanning microscope of equivalent configuration.

   i. From the software, switch on the lasers, adjust the detector gains to an initial value of 100, and specify the acquisition of different fluorophores on specific detectors.
   ii. Specify acquisition parameters to acquire an image with appropriate voxel size.

l. After 30 min of intravenous antibody injection, anesthetize the mouse again by placing in the induction chamber and setting isoflurane to 3%.
   i. Once the mouse is anesthetized, remove it from the induction chamber.
   ii. Continue delivering the isoflurane by placing a nose cone, secured in place using surgical tape.
m. Inject 5×10^6 CFU of *C. albicans* in 100 μL volume, via retro-orbital injection, at the injection site not previously used for the antibody cocktail injection.
   i. Note down the time of infection.
n. Place the mouse over the work surface, supine on its right side, above the warming pad.
o. Ensure that the mouse is under deep anesthesia via toe pinch and insert a rectal probe for continuous temperature monitoring; ideally, the temperature is maintained between 35°C–36.5°C.
p. Adjust isoflurane to 1%, and apply the ophthalmic ointment to prevent ocular drying. A stable anesthesia can be visually inspected by monitoring the breathing at 55–60 breaths per minute.
q. Sanitize the left flank of the mouse and make a small (about 1 cm) skin incision to expose the underlying abdominal wall.

Note: Underneath the exposed abdominal wall, the kidney could be visualized inferior to the spleen.

r. With the help of forceps, lift the membranous abdominal wall overlying the kidney and utilize the hemostatic forceps to pinch about 0.5–1 cm of the lifted membrane.

Note: This helps in stemming bleeding from the excision of any vessels running along the abdominal wall in the next step.

s. Excise the abdominal wall using fine scissors and forceps, which would expose part of the retroperitoneum.
t. Gently push and exteriorize the kidney through the small opening.
u. Apply pre-warmed PBS, and work carefully using a PBS-saturated cotton-tipped applicator to handle the kidney and use fine scissors to remove the surrounding fat.

⚠ CRITICAL: Handle the exteriorized kidney gently and avoid touching it directly with the forceps. Utilize the PBS-soaked cotton applicator to handle the kidney.

v. With the kidney appropriately exteriorized, place the mouse on the microscope stage insert, with the kidney placed on the cover glass.
w. Once the kidney is laid over the cover glass and sufficiently separated using the affixed cell spreader, place a PBS-soaked Kimwipe over the kidney.
    i. Every 10–15 min, apply pre-warmed PBS to the kidney to avoid dehydration.

Note: Alternatively, aqueous hydrogel (e.g., Aquasonic 100 ultrasound transmission gel) can also be used, via direct application over the PBS-soaked Kimwipe.

x. Carefully transfer the stage insert carrying the mouse to the microscope and proceed ahead with microscopy.
y. Locate the kidney using the bright-field and epifluorescence settings of the microscope using an eyepiece.
z. Once located, switch to the confocal scanning mode and adjust the laser power and detector gains for each channel for an optimal signal-to-noise ratio while keeping the saturated pixel area to less than 5% of the entire image field.

⚠ CRITICAL: At this point, it is advisable to assess if excessive motion is absent. If the entire image exhibits significant movements away from the imaging plane, retrieve the anesthetized mouse from the microscope stage and gently reorient the mouse and the exteriorized kidney. Such maneuvering can aid in reducing motion. If motion still persists, then additional surgical manipulation will be necessary. Specifically, surgically remove any additional connecting tissue between the kidney and the retroperitoneum; exercise extreme care to avoid accidentally damaging the renal hilum.

    i. Specify the acquisition of image stacks with 50–90 μm thickness.
    ii. Readjust the laser power and detector gains, if necessary, to obtain optimal signal-to-noise ratio at deeper image planes.
    iii. Specify the parameters including image size, time interval, and step size in the z-direction.

Note: In our experience, image stacks with an XYZ voxel size of 0.3 × 0.3 × 2 μm, with 2–2.5 minutes of temporal resolution, have been sufficient for extracting quantitative parameters of phagocyte-fungal cell interactions. We have successfully imaged the kidneys for up to 4 hours. Upon completion of the imaging, euthanize the mouse without recovery from the anesthesia, as per the institutionally approved guidelines.

4. Image processing and analysis.

Note: The duration of quantitative analysis can vary based on the complexity of the temporal image stack. Given that the imaging is anticipated to produce datasets of several gigabytes in size, a dedicated workstation equipped with at least 128 GB of RAM and a multi-core processor are essential. From a software standpoint, using both FIJI (available for free) and Imaris (commercially available) facilitates the required processing and analysis, as detailed below.

a. Export the "XYZT" time series (hyperstack) dataset to FIJI, correct minor drift across time points using the "Correct 3D Drift" plug-in,[4] and save the dataset in TIFF format.
b. For subsequent analysis in Imaris, use the "Imaris File Converter" to convert the saved TIFF file to IMS format.
c. Open the IMS file in Imaris for three-dimensional rendering, processing, and analysis.
d. To perform segmentation, utilize the "Surface Creation" module to create surfaces for the stained cells.
    i. Upon initialization of the "Surface Creation" module, specify a region of interest (ROI) in the beginning, and check the box to process the entire image afterward.

Note: In our experience, this works well with memory-efficient processing, especially when toggling back and forth is needed for surface creation.

    ii. Once the ROI is selected, specify the channel for which the surfaces are to be created and proceed forward by specifying "Thresholding" via "Absolute intensity" or "Background Subtraction". In the subsequent step, manually select the threshold to a level that delineates the signal on the cells, while avoiding the non-specific background.
    iii. In the subsequent step, set the "Quality" threshold appropriately to discard low-quality structures, and the "Number of Voxels" above 100–200 to exclude artifacts arising due to smaller autofluorescent structures.
    iv. The surfaces will now represent the segmented cells, for which different aspects, including intensity profile, sphericity, velocity, and other quantitative parameters can be exported in a comma-separated values file.
e. To quantify the association of *Candida* with renal phagocytes, the time series is scanned to identify the *Candida* structures which are observed to be stably associated with the phagocytes in at least 3 consecutive frames.
    i. For quantifying *Candida* association across different mice uniformly, a uniform temporal window (e.g., from 1-h post-infection to 2-h post-infection) is chosen, and the stably associated *Candida* structures are then enumerated.

Note: For such stably associated *Candida*, their analysis across different timeframes also provides information on their fate and growth rates.

Note: If there are multiple hyperstacks (time series) for a single field of view, they should be first concatenated using FIJI. In such a scenario, first, register the hyperstacks, and then proceed ahead with concatenation.

Note: Imaris renders the imaging data in 3D upon opening the image stack, while for FIJI, "3D Viewer"[5] or "BigDataViewer"[6] plug-ins can be utilized to render and explore the imaging data.

Flow cytometry-based assay for evaluating *in vivo* fungal cell association and killing by phagocytes

Timing: 11 h

Timing: 3 h (for step 5)

Timing: 8 h (for step 6)

This step describes the preparation of the reporter *Candida albicans* strain and its use in a murine model of systemic candidiasis to assess renal phagocytes for their capacity to associate with and kill the fungus. We have used this approach for *Candida albicans* and renal phagocytes,[7] but it can be easily adapted to other *Candida* species and organs in addition to the kidneys.

5. Preparation of reporter *Candida albicans*.
   a. Prepare dTomato-expressing and the non-fluorescent *Candida albicans* strains as outlined above,
   b. Resuspend the *Candida* at 1 × 10^9 CFU/mL in 1 mL 50 mM sodium bicarbonate buffer, pH 9.5.

⚠ CRITICAL: The labeling of a non-dTomato-expressing *C. albicans* with Alexa Fluor 633 is necessary, as it will be used as a compensation control during flow cytometry.

   c. Spin down at 375 × *g* at room temperature for 5 min and resuspend in 1 mL 50 mM sodium bicarbonate buffer, pH 9.5.
   d. Add 50 μL of biotin-XX-SE (10 mg/mL). Thus, the final concentration will be 0.5 mg/mL.
   e. Cover the tube with aluminum foil to protect the fluorescent fungus from light, and put the tube in a microtube shaker. Incubate with shaking at 200 rpm for 2 h at room temperature.
   f. After 2 h of incubation, spin down at 375 × *g* at room temperature for 5 min, remove the supernatant, and wash twice with 0.1 M Tris-HCl (pH 8). After the final wash resuspend in 1 mL 1X PBS.
   g. Add 40 μL of streptavidin-AF633 (0.5 mg/mL). Thus, the final concentration will be 0.02 mg/mL.
   h. Cover the tube with aluminum foil and incubate for 30 min at room temperature, with shaking at 200 rpm.
   i. Wash twice with 1X PBS, count using a hemocytometer again, resuspend at 5 × 10^7 CFU/mL, and infect the mice with 5 × 10^6 CFU, via lateral tail vein injection.

⚠ CRITICAL: As a control, infect one mouse with a non-fluorescent *C. albicans*. This will serve as a "negative control" and will aid in drawing gates to select for *Candida*-positive phagocyte populations.

   j. After 2 h post-infection, euthanize the mice, and harvest kidneys for single-cell preparation.
6. Single-cell preparation from kidneys, and flow cytometry.
   a. While working under a biosafety cabinet, aseptically transfer the kidneys (both kidneys from a single mouse) in a 35 mm petri dish.
   b. Inject kidney digestion medium with a 25G needle (about 1 mL per kidney) directly into kidneys, at the poles along the long axis, until they inflate slightly.
   c. With a sterile scalpel, mince the kidneys into 1–2 mm small pieces, and transfer to a 50 mL sterile conical tube.
   d. Add 9 mL kidney digestion medium to the tube, transfer to a 37°C water bath, and incubate for 20 min, with gentle shaking of 100–120 rpm.
   e. After 20 min, remove the tubes from the water bath, place them on ice, and add ice-cold 20 mL RPMI containing 10% FBS and 25 mM HEPES to halt digestion.
   f. Pass the digested kidney pieces, along with the medium, through a 70 μm cell strainer.
      i. For efficient dissociation, using a 25 mL serological pipette, initially take the kidney pieces along with 5 mL of the medium, and pass them through the strainer.
      ii. Smash the kidney pieces atop the strainer using a sterile syringe plunger (a plunger from a 3 mL syringe works well for this purpose).

iii. Pass the remaining medium through the strainer.

g. Add 15 mL ice-cold RPMI containing 10% FBS and 25 mM HEPES to 45 mL and centrifuge at 350 x g for 5 min.

h. Remove the supernatant and lyse the red blood cells (RBC) by resuspending the pellet in 5 mL of ACK lysing buffer and incubating for 30 s at room temperature.

i. After 30 s, halt the RBC lysis by adding 25 mL of ice-cold HBSS containing 2 mM EDTA.

j. Pass the digested kidney suspension through a 40 μm cell strainer, centrifuge at 375 × *g* for 5 min, and remove the supernatant.

k. Resuspend the digested kidney pellet in 8 mL 40% Percoll solution and overlay the suspension on 3 mL 70% Percoll, placed in a 15 mL conical tube.

l. Centrifuge the gradients at 939 × *g* at room temperature for 30 min while keeping the brake off.

m. After 30 min, carefully remove the tubes and harvest the cell suspension from the 40% and 70% Percoll interphase in a fresh 15 mL conical tube.

n. Wash once with ice-cold FACS buffer, followed by 1X PBS.

o. Count the cells, resuspend at 10^7 cells per mL in 1X PBS, and stain the dead cells using the LIVE/DEAD Fixable Blue Dead Cell Stain, as per manufacturer's instructions.

p. Incubate for 10 min on ice, protected from light.

q. To block non-specific binding, add Rat anti-mouse CD16/CD32 at 1:100 dilution, bovine serum albumin at 0.5% final concentration (blocking reagent).

r. Incubate for 10 min on ice, protected from light.

s. Stain the cells by adding the following antibody cocktail at the dilution specified, in the presence of the blocking reagent from the step above, and incubate for 30 min on ice, protected from light.

Antibody	Dilution
MHC Class II (I-A/I-E) Monoclonal Antibody eFluor 450	1:100
Brilliant Violet 605 anti-mouse CD45 Antibody	1:100
PerCP/Cyanine5.5 anti-mouse Ly-6G Antibody	1:100
PerCP/Cyanine5.5 anti-mouse CD3ε Antibody	1:100
PerCP/Cyanine5.5 anti-mouse CD19 Antibody	1:100
PerCP/Cyanine5.5 anti-mouse CD335 (NKp46) Antibody	1:100
APC/Cyanine7 anti-mouse/human CD11b Antibody	1:100
PE/Cyanine7 anti-mouse F4/80 Antibody	1:100
Alexa Fluor 700 Rat Anti-Mouse Ly-6C	1:100

⚠ CRITICAL: Do not use any antibodies in PE and APC channels to accommodate dTomato and Alexa Fluor 633 fluorescence from the fungal reporter strain, respectively.

t. After 30 min, add ice-cold FACS buffer to 4 mL in each tube and centrifuge at 350 × *g* for 5 min.

u. Remove the supernatant, dissociate the pellet, and wash twice using 4 mL FACS buffer.

v. After the final wash, resuspend the cell pellet in 300–500 μL of FACS buffer and pass it through a 35 μm cell strainer fixed atop the flow cytometry tube. The cells are now ready for analysis using a flow cytometer.

⚠ CRITICAL: Perform flow cytometry on the same day, without adding any fixatives as those decrease the fluorescent intensity of dTomato. We also advise acquiring >500,000 events to accurately quantify the percent of fungal cell association and killing by myeloid phagocytes.

⚠ CRITICAL: For compensation, utilize dTomato-expressing *Candida* and Alexa Fluor 633 coupled "non-dTomato-expressing" *Candida*, along with the single-stained and unstained

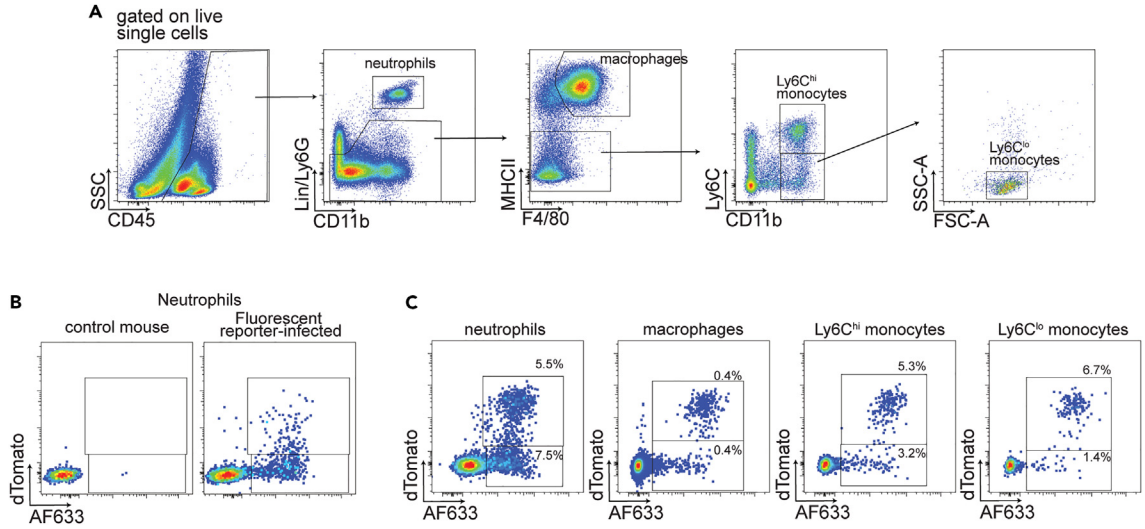

Figure 2. Flow cytometry-based analysis of myeloid phagocytes harvested from infected kidneys

(A) Gating strategy.

(B) Representative plots depicting the kidney neutrophils from mice infected with "non-labeled, non-fluorescent" and "fluorescent reporter" *Candida albicans*.

(C) Representative plots depicting the indicated myeloid phagocytes in association with viable (dTomato$^+$ AF633$^+$), and dead (dTomato$^-$ AF633$^+$) *C. albicans*.

compensation beads. It is also critical to infect a mouse with a non-fluorescent *C. albicans* strain; as shown in Figure 2B, this mouse serves as a "negative control" for identifying and drawing the gates around the leukocyte populations which harbor "live" or "dead" fungi.

Ex vivo assays of fungal uptake and killing by phagocytes isolated from kidneys

Timing: 6.5–8 h

Timing: 5 h (for step 7)

Timing: 1.5 h (for step 8)

Timing: 3 h (for step 9)

This step details isolating macrophages and neutrophils from kidneys using fluorescence-activated cell sorting (FACS) and their use in assays of fungal uptake and killing.

7. Isolation of macrophages and neutrophils from kidneys.

Note: Given that kidneys typically have few infiltrating neutrophils at a steady state, we advise collecting kidneys 2–4 days post-infection for neutrophil isolation intended for functional analyses. Conversely, macrophages can be extracted from both infected and uninfected kidneys.

a. Prepare single-cell suspension from kidneys as described in the section above.
b. As outlined above, incubate the cells with Rat anti-mouse CD16/CD32 and bovine serum albumin (blocking reagent) to block the non-specific binding.

c. Perform cell staining using the antibody cocktail for phagocyte sorting, at the dilution specified, and incubate for 30 min on ice, protected from light.

Antibody	Dilution
MHC Class II (I-A/I-E) Monoclonal Antibody eFluor 450	1:100
Brilliant Violet 605 anti-mouse CD45 Antibody	1:100
PE anti-mouse Ly-6G Antibody	1:100
PerCP/Cyanine5.5 anti-mouse CD3ε Antibody	1:100
PerCP/Cyanine5.5 anti-mouse CD19 Antibody	1:100
PerCP/Cyanine5.5 anti-mouse CD335 (NKp46) Antibody	1:100
APC/Cyanine7 anti-mouse/human CD11b Antibody	1:100
PE/Cyanine7 anti-mouse F4/80 Antibody	1:100

d. After 30 min, add ice-cold sorting buffer to 4 mL in each tube and centrifuge at 350 × *g* for 5 min.
e. Remove the supernatant, dissociate the pellet, and wash twice using 4 mL sorting buffer.
f. After the final wash, resuspend the cell pellet in 300–500 μL of sorting buffer and pass it through a 35 μm cell strainer, fixed atop the flow cytometry tube. The cells are now ready for FACS.

Alternatives: For neutrophil isolation, magnetic-activated cell sorting can be used alternatively. To this end, we have used the Miltenyi "anti-Ly6G microbeads, UltraPure" and "QuadroMACS Separators". For this purpose, instead of resuspending the cells in "sorting buffer" at STEP 3, resuspend them in MACS buffer, and perform the cell separation as specified on the product insert of "Miltenyi anti-Ly6G microbeads, UltraPure" and detailed elsewhere.[8,9]

8. *Ex vivo* analysis of neutrophil-fungal association.

Note: The capacity of neutrophils to associate with *C. albicans* can be analyzed using opsonized or unopsonized *Candida*. While we employ dTomato-expressing *C. albicans* for this protocol, the subsequent steps can be adapted for any fluorescent fungal strain.

a. Resuspend *C. albicans* in RPMI + P/S at 2.5 × 10^9 CFU/mL.
b. Prepare tubes to perform opsonization.
   i. Aliquot 50 μL of RPMI + P/S containing 10% normal mouse serum in capped round-bottom polystyrene tubes. These tubes will contain "opsonized *C. albicans*".
   ii. For "unopsonized *C. albicans*", aliquot 50 μL of RPMI + P/S containing 10% heat-inactivated fetal bovine serum in capped round-bottom polystyrene tubes, as opposed to normal mouse serum used above for preparing "opsonized *C. albicans*" .
c. Add 2 μL of *C. albicans* from the 2.5 × 10^9 CFU/mL suspension (i.e., 5 × 10^6 CFU) to each of the "opsonized *C. albicans*" tubes containing 10% normal mouse serum, and "unopsonized *C. albicans*" tubes, containing 10% heat-inactivated fetal bovine serum.
d. Incubate on ice for 30 min. While the above incubation is ongoing, resuspend neutrophils at 10^8 cells/mL in RPMI + P/S, and keep them on ice.
e. After 30 min, add 50 μL of neutrophils from the 10^8 cells/mL suspension (i.e., 5 × 10^6 neutrophils) to each of the *C. albicans* containing tubes.
f. Transfer the capped tubes to a 37°C water bath, and incubate for 30 min, with gentle shaking of 100–120 rpm.
g. After 30 min, transfer tubes on ice, add 1 μL of the APC/Cy7-coupled anti-mouse CD11b antibody to each tube and analyze the cells via flow cytometry.
h. The neutrophils associated with *C. albicans* can be identified as "$CD11b^+$ $dTomato^+$", while the bystander neutrophils will be "$CD11b^+$ $dTomato^-$".

9. *Ex vivo* analysis of macrophage-mediated fungal killing.

Note: This sub-step outlines the analysis of macrophages for their ability to kill *C. albicans* yeast-form cells, adapted from the protocol described initially in Lionakis et al.[10] We have also successfully used the protocol with neutrophils, the details for which can be found in Swamydas et al.[11]

a. Seed 10^5 macrophages in 50 μL of 1X PBS to 96-well flat-bottomed tissue culture plates, and incubate at 37°C in a humidified incubator with 5% CO_2 for 30 min.
   i. Seed the macrophages in a manner that allows technical triplicates per mouse.
b. Resuspend *C. albicans* in 1X PBS containing 10% normal mouse serum at 10^6 CFU/mL.
c. Incubate for 30 min on ice to allow opsonization.
d. Transfer 50 μL (i.e., 5×10^4 CFU) to each well containing macrophages.
   i. Additionally, in separate non-macrophage-containing wells, transfer 2-fold serial dilutions of 100 μL of *C. albicans*, in triplicate, in 1X PBS with 5% normal mouse serum, starting at 5×10^4 *C albicans* per well.

Note: These known standards are useful in assessing the assay performance and calculating the degree of fungal killing.

e. Incubate at 37°C in a humidified incubator with 5% CO_2 for 3 h.
f. After 3 h, remove the plate, centrifuge at 350 × *g* for 5 min, discard the supernatant carefully, and lyse the macrophages using 0.02% Triton-X-100.
g. Wash the remaining *C. albicans* twice with 1X PBS, and after the final wash add alamarBlue reagent.
h. Incubate at 37°C in a humidified incubator with 5% CO_2 for 18 h, and measure the fluorescence, indicative of viable *C. albicans*.
i. For calculating the macrophage fungal killing capacity, compare the *C. albicans* viability in the presence of macrophages, relative to the non-macrophage-containing wells.

EXPECTED OUTCOMES

Appropriate execution of the intravital microscopy approach is expected to allow real-time visualization of fungal-phagocyte interactions *in vivo* in the kidney. The downstream analyses will provide quantitative insights into such interactions. Methods video S1 depicts a *Candida albicans* yeast cell in the kidney where it filaments. $Ly6G^+$ neutrophils (in gray color) rapidly accumulate at the site and destroy the fungus.

Successful execution of "Flow cytometry-based assay for evaluating fungal association and killing *in vivo*" will enable the identification of phagocytes associated with viable or dead *C. albicans*. The digestion of two kidneys per mouse is expected to yield >1 million cells, which upon staining with the above-specified antibodies allows for the identification of different phagocyte subsets. Specifically, neutrophils were identified as $CD45^+$ $CD3^-$ $CD19^-$ $NKp46^-$ $Ly6G^+$ $CD11b^+$ subsets. Macrophages were identified as $CD45^+$ $CD3^-$ $CD19^-$ $NKp46^-$ $Ly6G^-$ $CD11b^+$ $MHCII^{hi}$ $F4/80^+$ subsets. $Ly6C^{hi}$ monocytes were identified as $CD45^+$ $CD3^-$ $CD19^-$ $NKp46^-$ $Ly6G^-$ $CD11b^+$ $MHCII^{lo}$ $Ly6C^{hi}$, while $Ly6C^{lo}$ monocytes were $CD45^+$ $CD3^-$ $CD19^-$ $NKp46^-$ $Ly6G^-$ $CD11b^+$ $MHCII^{lo}$ $Ly6C^{lo}$ SSC^{lo}.

The gating strategy depicting the above phagocytes is shown in Figure 2A. Based on the dTomato and Alexa Fluor 633 fluorescence (Figures 2B and 2C), the phagocytes can then be categorized as bystander phagocytes not associated with *C. albicans* ($dTomato^-$ Alexa Fluor 633^-), phagocytes associated with dead *C. albicans* ($dTomato^-$ Alexa Fluor 633^+), and phagocytes associated with live *C. albicans* ($dTomato^+$ Alexa Fluor 633^+).

The percentage of cells associating with *C. albicans* is calculated as "percentage of phagocytes associated with dead or live *C. albicans* of the total phagocytes", while the percentage of cells

that have killed *C. albicans* is calculated as "percentage of phagocytes associated with dead *C. albicans* / percentage of phagocytes associated with dead or live *C. albicans*". For example, as shown in Figure 2B, the percentage of neutrophils associating with *C. albicans* is 13% (i.e., 7.5% + 5.5%), while the percentage of cells that have killed *C. albicans* is 57.7% (i.e., 7.5% of cells with dead *C. albicans* / 13% of cells associated with dead or live *C. albicans*).

The FACS-based phagocyte isolation typically yields 2–3 × 10^5 macrophages and 5–9 × 10^5 neutrophils, from the uninfected, and infected kidneys (two kidneys per mouse), respectively. The number of neutrophils will vary depending on the infecting inoculum.[3,10–12] Isolated neutrophils and macrophages can then be used in the fungal association and killing assays as described above, as well as, in additional functional analyses, as described previously.[7,10,11]

In summary, a combinatorial use of the above approaches can provide a comprehensive analysis of how myeloid phagocytes interact with *C. albicans* to promote its clearance *in vivo*.

LIMITATIONS

Since our intravital approach utilizes single photon confocal laser scanning microscopy, the imaging depth is limited to 90 μm. However, if a multiphoton microscope is available, with optimum imaging parameters and fluorophores, our approach can be utilized efficiently for imaging at an extended depth and a larger imaging volume of up to 200 μm.

Our "Flow cytometry-based assay for evaluating fungal association and killing *in vivo*" is tailored to evaluate phagocyte-fungal interactions 2 h after infection, offering insights into the initial stages of infection. As *C. albicans* forms filamentous pseudohyphae and hyphae, analysis of the later time points after infection will not be accurate, as Alexa Fluor 633 will be diluted with the growing cell wall of the filamentous growth.

For *ex vivo* analysis of phagocyte-fungal interactions, the number of phagocytes may vary based on the genetic background and infection status of the mice. Immunophenotyping via flow cytometry can provide an estimate of cellular proportions and can thus aid in the planning of experiments, as outlined above. In such instances, pooling of multiple mice may be necessary, resulting in enhanced cost and time.

TROUBLESHOOTING

Problem 1

Microscopy depicts excessive accumulation of neutrophils in areas that lack fungal presence (related to the STEP 3: Microscopy of infected kidneys, sub-steps "r" - "x".).

Potential solution

- This is a potential indicator of damage and micro-injury. Perform microscopy using uninfected animals to assess the impact of surgical technique and kidney handling. Uninfected kidneys should not show significant neutrophil accumulation in the imaging area. To avoid such micro-injuries, handle the kidney gently while performing the experiment. Additionally, check the laser power to assess if excessive power is inducing damage over time, leading to neutrophil accumulation. For directly visualizing whether necrotic areas are arising due to surgery or kidney preparation for imaging, real-time imaging can be performed using Hoechst 33258 and propidium iodide. To this end, inject 0.5 mg of Hoechst 33258 and 6.7 μg of propidium iodide into an anesthetized mouse via retroorbital injection. After 10 min, proceed with the kidney preparation for microscopy and perform microscopy. An ideal preparation should not have many propidium iodide-positive necrotic cells, while Hoechst 33258 will stain all the nuclei.

Problem 2

Motion artifacts interfering with segmentation (related to STEP 4: "Image processing and analysis", sub-step "c").

Potential solution

- The approach outlined above incorporates a procedure to correct translational motion artifacts by using the "Correct 3D drift" plug-in. Similarly, Imaris provides approaches to correct the motion artifacts, where in principle the image volumes are compared across time points to the initial reference frame, which acts as fiducial. In our experience, the initial time point for the image channel corresponding to the vasculature acts as an excellent fiducial reference frame. To this end, the users should perform segmentation via creating surfaces around the fluorescent vasculature and should track these surfaces over time. Once the surfaces and tracks are created, the drift correction can be performed using the "Correct Drift…" option, actionable under the created surface object.

Problem 3

Excessive motion artifacts during microscopy (related to STEP 3: "Microscopy of infected kidneys", sub-steps "r" - "x").

Potential solution

- While our approach incorporates post-acquisition image processing steps that can correct the motion artifacts, excessive motion resulting from an entire image volume shifting away from the imaging plane during acquisition poses a very difficult registration problem. Such a scenario should be assessed while acquiring the imaging data. If encountered while imaging, it is advisable to retrieve the anesthetized mouse from the microscope stage for reorienting the mouse and the exteriorized kidney. Such maneuvering can aid in reducing motion. If excessive motion still persists, then additional microsurgical manipulation directed towards removing the connecting tissue around the kidney, will be necessary, as mentioned above. In such a situation, extreme care should be exercised to avoid accidentally damaging the renal hilum.

Problem 4

During surface creation, surfaces are created around non-specific structures. (related to Step 4: "Image processing and analysis", sub-step "d").

Potential solution

- While creating surfaces in Imaris, carefully select the "Threshold", "Quality", and "Number of Voxels" parameters, and iteratively re-assess the surfaces for accuracy.
- In some instances, spurious surfaces may result from fluorescence bleed-through, potentially due to simultaneous acquisition. This can be avoided by performing "sequential" acquisition. However, as "sequential" scanning is not ideal, post-acquisition bleed-through correction can be performed using FIJI, and subsequently, the surface creation can be performed in Imaris.

Problem 5

An insufficient number of phagocytes are obtained after the digestion of kidneys (related to STEP 6, under the "Single-cell preparation from kidneys and flow cytometry", sub-steps "e" - "m").

Potential solution

- After the completion of the digestion step, use a syringe plunger to completely smash the kidney pieces through the cell strainer. Subsequently, pass the remaining RPMI + P/S + FBS, while continuing the plunger-based smashing over the cell strainer to completely transfer the digested tissue through the strainer.
- Work quickly during the RBC lysis step, as the lysis buffer can deleteriously impact the viability of other cell types.
- While creating the Percoll gradient, carefully overlay the 40% Percoll to avoid mixing of layers. Similarly, after centrifugation, care should be exercised while withdrawing the tubes from the centrifuge to not disturb the gradient and the interphase that harbors the immune cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michail S. Lionakis (lionakism@niaid.nih.gov).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Jigar V. Desai (jigarkumarkumar.desai@hmh-cdi.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze new code or datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102781.

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AUTHOR CONTRIBUTIONS

J.V.D. and M.S.L. designed the studies, analyzed the data, and wrote the manuscript. J.V.D. conducted the experiments. M.S.L. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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