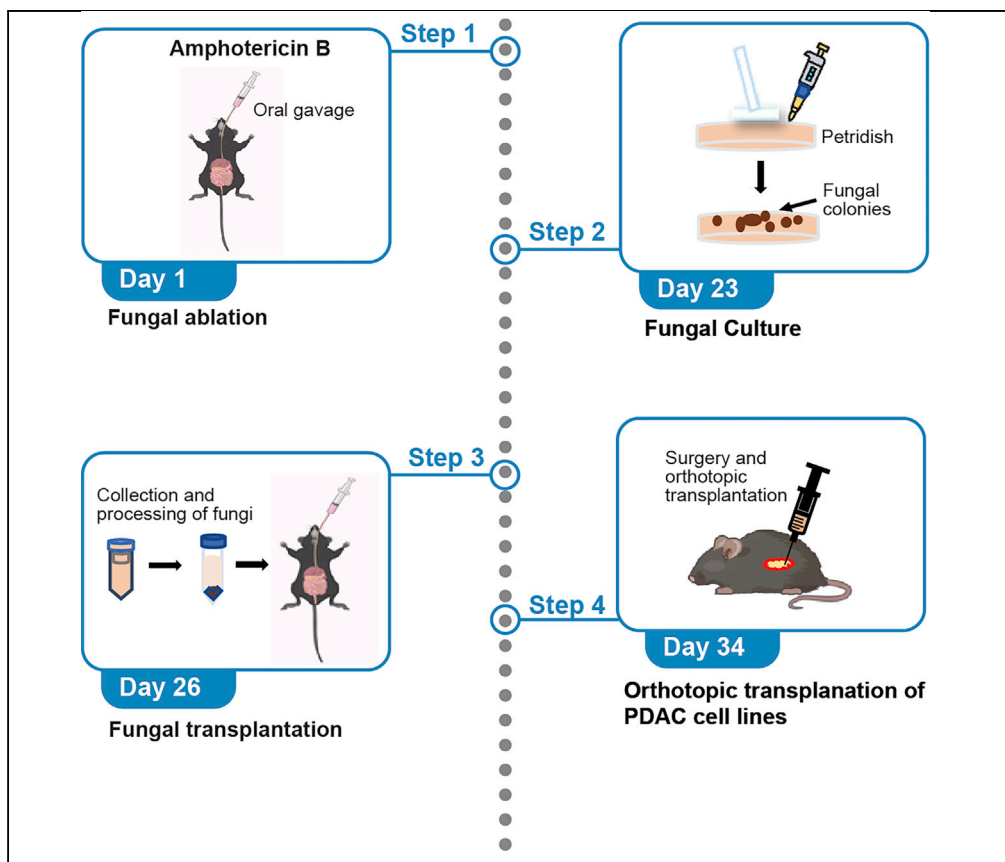


Protocol

Fungal ablation and transplantation of specific fungal species into PDAC tumor-bearing mice



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Highlights

Fungal ablation in mice with Amphotericin B treatment

Detailed steps for fungal culture of *Alternaria alternata* and *Malassezia globosa*

Transplantation of *Alternaria alternata* and *Malassezia globosa* via oral gavage

Intratumor and gut mycobiome is linked to pancreatic ductal adenocarcinoma (PDAC) tumorigenesis; however, an optimal approach to culture and transplant fungus into mouse for *in vivo* studies is missing. This protocol describes culture steps of *Alternaria alternata* and *Malassezia globosa* and their subsequent transplantation into a PDAC mouse model via oral gavage. The utilization of the fungal culture method will allow for consistent growth and expansion of specific fungal species for downstream processing.

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

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Protocol

Fungal ablation and transplantation of specific fungal species into PDAC tumor-bearing mice

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<https://doi.org/10.1016/j.xpro.2022.101644>

SUMMARY

Intratumor and gut mycobiome is linked to pancreatic ductal adenocarcinoma (PDAC) tumorigenesis; however, an optimal approach to culture and transplant fungus into mouse for *in vivo* studies is missing. This protocol describes culture steps of *Alternaria alternata* and *Malassezia globosa* and their subsequent transplantation into a PDAC mouse model via oral gavage. The utilization of the fungal culture method will allow for consistent growth and expansion of specific fungal species for downstream processing.

For complete details on the use and execution of this protocol, please refer to Alam et al. (2022).

BEFORE YOU BEGIN

Recent studies in pancreas, colon and head and neck cancer have demonstrated the role of the gut and intratumor fungi in tumor progression. However, the mechanism of fungal mediated tumor progression is not very clear because of the lack of a standard method for *in vitro* culture and transplantation of fungal spores to mice. To address this gap, we have developed a protocol that describes the fungal culture, downstream processing, and transplantation of fungi via oral gavage to understand the role of fungi in tumor progression. The protocol describes an easy culture technique for *Alternaria* and *Malassezia* spp. but can be adapted for other fungi as well.

Institutional permissions

The protocol requires the generation of a mouse orthotopic pancreatic cancer model and fungal transplantation in mice. Institutional ethical and biosafety approval is required before beginning this procedure. The procedure must comply with animal surgery guidelines, including the use of aseptic surgical techniques to prevent possible infection as well as the use of appropriate anesthesia and analgesia to minimize pain. The protocol also requires biosafety measures for the containment of fungal species limiting any human and mice infection. Animal experiments and fungal transplantation in this procedure were approved by Institutional Animal Care and Use Committee (IACUC) at Roswell Park Comprehensive Cancer Center, protocol number 1416M/1417M.

Requirements

Fungal species: *Alternaria alternata* (ATCC, 36376) and *Malassezia globosa* (ATCC, MYA-4889).

Fungal culture media: V8 media for *Alternaria alternata* and mDixon media for *Malassezia globosa*.

Equipment: BSL2 laminar airflow, bacterial shaker, incubator, spreader for plating, gavage syringe, Hamilton syringe, mouse surgical kit, heating pad, surgical blade, surgical wound clips, surgical suture, and glass bead sterilizer.



Preparation of reagents, media, and stock solution

⌚ Timing: 1 h

1. V8 media
 - a. Mix the V8 media ingredients and add up to 900 mL of water in a 2-L flask.
 - b. Adjust the pH to 7.2 and then adjust the volume to 1 L.
 - c. Cover the flask with foil and autoclave the mix for 15 min at 121°C.
 - d. Monitor the temperature as the solution cools and when the temperature reaches ~50°C, pour ~10 mL into each Petri dish.
 - e. Place the plates in a plastic bag and seal it.
 - f. Store at 4°C for a 1 month.
2. mDixon media
 - a. Mix the mDixon media ingredients and add up to 900 mL of water in a 2L flask.
 - b. Adjust the pH to 6. Make the volume up to 1L.
 - c. Cover the flask with foil and autoclave the mix for 15 min at 121°C.
 - d. Monitor the temperature as the solution cools. When the temperature reaches ~50°C, pour ~10 mL into each Petri dish.
 - e. Place the plates in a plastic bag and seal it.
 - f. Store at 4°C for a 1 month.
3. Media for mouse PDAC cell culture
 - a. To 500 mL of RPMI 1640 with L-glutamine add 50 mL of fetal bovine serum and 5 mL Pen/Strep and Store at 4°C for 1 month.

Note: For more detailed information refer to the [key resources table](#).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Matrigel-Phenol red-free, LDEV-free	Corning	Cat# 356255
IVIS brite D-Luciferin Potassium Salt Bioluminescent Substrate	PerkinElmer	Cat#122799
Doxycycline	Millipore Sigma	Cat# D9891
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Cat# 26140079
Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine	Corning	Cat# 10-040-CV
DPBS	Corning	Cat# 21031CV
Tween 40	Fisher Scientific	Cat#T25315G
Ox-bile	Millipore Sigma	Cat#70168
Peptone	Millipore Sigma	Cat#70173
Malt extract	Millipore Sigma	Cat#70167-500G
Glycerol	Millipore Sigma	Cat#G5516
Oleic acid	Millipore Sigma	Cat# O1008
Agar	Millipore Sigma	Cat# A1296
V8 juice	Wegmans	CB2981268
CaCO3	Millipore Sigma	Cat# 239216
Amphotericin B	Fisher Scientific	Cat#ICN19504394
Penicillin-streptomycin 100×	Corning	Cat# 30-002-CI
Experimental models: Cell lines		
AK-B6 mouse PDAC cell line	From Ron DePinho	PMID:32046984

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
C57BL/6J	The Jackson Laboratory	Cat# JAX: 000664 RRID: IMSR_JAX:006362
<i>Alternaria alternata</i>	ATCC	Cat# 36376
<i>Malassezia globosa</i>	ATCC	Cat# MYA-4889
Software and algorithms		
IVIS Spectrum Imaging System	PerkinElmer	N/A
Leica	Leica Microsystems	N/A
Other		
Mouse surgical kit	Roboz Surgical	N/A
Centrifuge	Eppendorf	Cat# 5810R
Falcon® 100 mm TC-treated Cell Culture Dish	Corning	Cat#353003
Heating pad	Braintree Scientific	Cat#50-195-4002
Surgical lamp	AmScope	Cat# HL250-AY
Laminar air flow cabinet	Thermo Fisher Scientific	Cat# 13-261-222
Single edge surgical blade	Razor Blade Co.	Cat# 94-0120
Surgical wound clips	Reflex 7	Cat# 203-1000
Stoelting™ Dry Glass Bead Sterilizer	Fisher Scientific	Cat# 10-000-632
COATED VICRYL® (polyglactin 910) Suture	Vicryl	Cat# J492G
Countess II	Thermo Fisher Scientific	N/A
Cell scraper	Corning	Cat# CLS3008
Fine-mesh sieve strainer	Fisher Scientific	Cat# NC1033784
IVIS Spectrum In Vivo Imaging System	PerkinElmer	Cat# 124262

MATERIALS AND EQUIPMENT

V8 media		
Reagents	Final concentration (in percent)	Amount
V8 juice	20	200 mL
CaCO ₃	0.3	3 g
Agar	1.5	15 g
DI water	N/A	Up to 1 L

*Store at 4°C and use within 1 month.

Modified Dixon (mDixon) media		
Reagents	Final concentration (in percent)	Amount
Malt extract	3.6	36 g
Desiccated ox bile	2	20 g
Tween 40	0.1	10 mL
Peptone	0.6	6 g
Glycerol	0.2	2 mL
Oleic acid	0.2	2 mL
Agar	1.5	15 g
DI water	N/A	Up to 1 L

*Store at 4°C and use within 1 month.

Media for mouse PDAC cell culture	
RPMI 1640 with L-glutamine	500 mL
Fetal bovine serum (FBS)	50 mL
Pen/Strep	5 mL

*Store at 4°C and use within 1 month.

STEP-BY-STEP METHOD DETAILS

⌚ Timing: 2 months

This Protocol consists of 4 steps:

Fungal culture: Culture media for *Alternaria Alternata* and *Malassezia globosa*. Requirement: Incubator, and BSL2 laminar air flow cabinet.

Fungal ablation in mice with Amphotericin B treatment.

Fungal re-population: Transplantation of *Alternaria alternata* and *Malassezia globosa* via oral gavage.

Orthotopic PDAC tumor model in C57BL/6J background.

Requirement: It requires a surgical suite and supportive surgical instruments.

Section 1: Fungal culture

⌚ Timing: 3 days

1. Obtain the fungal species *Alternaria alternata* and *Malassezia globosa* from ATCC.

⚠ **CRITICAL:** Institutional biosafety clearance is required for acquiring the fungal species. All the precautions must be taken while handling and storing the fungal samples received from ATCC.

2. Prepare V8 broth media and V8 agar plate for culturing *Alternaria alternata*, one day before starting the experiment.

3. Prepare mDixon broth media and mDixon agar plates for culturing *Malassezia globosa*, one day before starting the experiment.

4. Revive and culture *Alternaria alternata* (Figure 1):

a. Open an ampoule of *Alternaria alternata* by snipping the top half and cracking the glass.

⚠ **CRITICAL:** This procedure needs to be done in a laminar airflow cabinet.

b. Add 1 mL of sterile water to the ampoule and pipette up and down until the fungal pellet is dissolved into a suspension.

c. Transfer the fungal suspension into a 15 mL falcon tube filled with 5 mL of autoclaved DI water.

d. Keep the fungal suspension for 12–16 h at 22°C to restore the fungal growth.

e. After 12–16 h of incubation at 22°C spread the fungal spore to V8 agar plates and incubate for 48–72 h at 26°C in a humidified aerobic condition.

5. Revive and culture *Malassezia globosa* (Figure 2):

a. Thaw the frozen *Malassezia globosa* ampoule at 25°C in a dry bath for 5 min.

b. Break open the ampoule and spread plate 100 µL of the fungal suspension into mDixon agar plates.

c. Incubate the fungal spread plate at 30°C for 48–72 h.

Note: Keep observing the plates every 24 h for fungal growth. Also, make sure there is no fungal overgrowth as shown in (Figure 5).

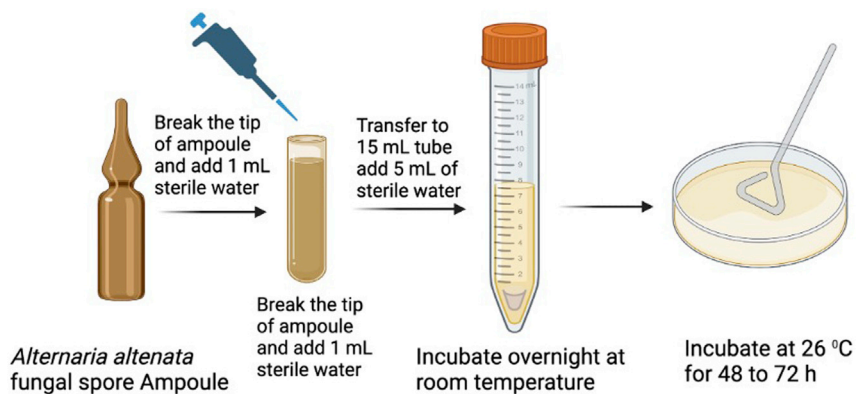


Figure 1. Schematic showing the revival of *Alternaria alternata* fungal spores and plating on V8 agar media plate

△ CRITICAL: Fungal species can cause allergy and dermatitis; direct skin contact must be avoided. Also, avoid using a bacterial incubator to prevent any cross-contamination.

Section 2: Fungal ablation with amphotericin B treatment

⌚ Timing: 26 days

6. Prepare Amphotericin B stock and working solution:
 - a. Prepare Amphotericin B stock solution: Dissolve Amphotericin B powder in DMSO at a concentration of 30 mg/mL and store at -20°C .
 - b. Prepare Amphotericin B working solution: Make 1 mg/mL working solution by diluting 1 mL Amphotericin B stock solution in 29 mL of PBS and store at -20°C .
7. Purchase mice and keep under normal conditions.

Note: For this protocol, mice were purchased from The Jackson Laboratory (C57BL/6J, Stock #000664) and kept under normal conditions (22°C , fed with a 5% chow diet, and allowed to acclimate to day/night cycle for 7 days).

8. Autoclave oral gavage syringe for 15 min at 121°C a day before Amphotericin B treatment.
9. Obtain the special cages and water bottles before starting the experiment.

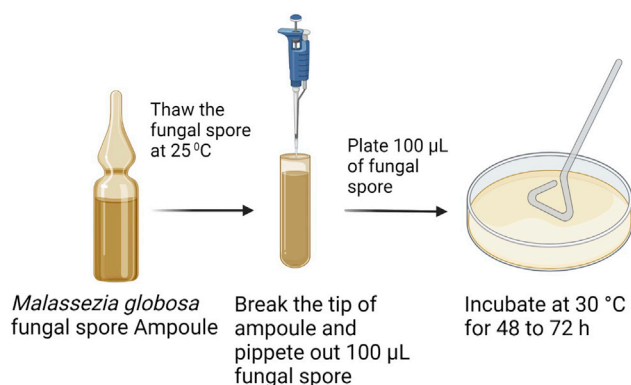


Figure 2. Schematic showing the revival of *Malassezia globosa* fungal spores and plating on mDixon agar media plate

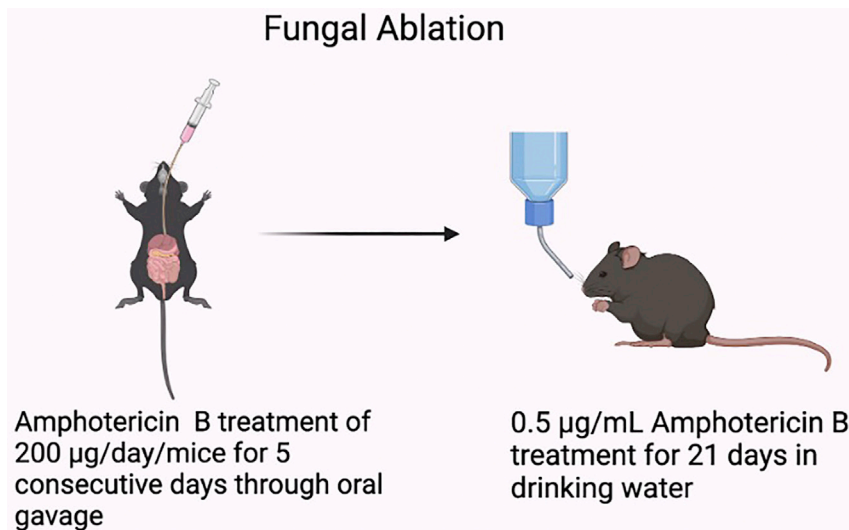


Figure 3. Schematic showing fungal ablation strategy with the treatment of Amphotericin B

Note: For this protocol specialized water restriction cages are used to restrict the automated water supply to the cages; water bottles are used to supply Amphotericin B treated water.

△ **CRITICAL:** Sterile water is required for the preparation of Amphotericin B in drinking water.

10. After the initial 7 days of acclimatization, treat the mice with Amphotericin B for 26 days (Figure 3).

Alternatives: Alternatively, other antifungal drug such as fluconazole can also be used for fungal ablation.

11. Divide the Amphotericin B treatment regimen into two courses.
 - a. For The first course treat with 200 µg amphotericin/mice for 5 consecutive days through oral gavage
 - b. Follow with a second course of 0.5 µg/mL of Amphotericin B, *ad libitum* for 21 days.

△ **CRITICAL:** The Amphotericin B needs to be freshly prepared and replaced every 3 days.

△ **CRITICAL:** Amphotericin B is weakly soluble in water; stock should be prepared in DMSO. The working stock should be prepared fresh in PBS before administering to mice.

Section 3: Fungal re-population

⌚ **Timing:** 7 days

12. Autoclave the oral gavage syringe a day before Amphotericin B treatment.
13. Grow *Alternaria alternata* and *Malassezia globosa* as described in [section 1: Fungal culture](#).

△ **CRITICAL:** Do not allow the fungal culture plates to become over confluent.

14. Autoclave a fine mesh sieve strainer (Fisher Scientific, NC1033784) for filtration of fungal spores.

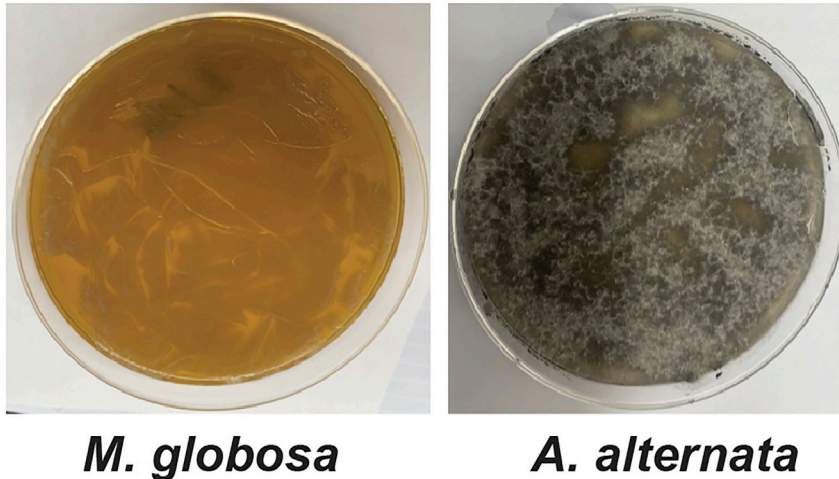


Figure 4. *Malassezia globosa* and *Alternaria alternata* culture plates showing fungal overgrowth

15. Take out the *Alternaria alternata* and *Malassezia globosa* culture plates after 72 h of incubation from the incubator and determine the growth.

Note: Avoid *Alternaria alternata* culture plates having a fungal overgrowth (Figure 4).

16. Add 5 mL of PBS to fungal culture plates containing *Alternaria alternata* and *Malassezia globosa* respectively.
17. Incubate at 22°C for 15 min to make sure the fungus loosens from the plates.
18. Using a cell scraper (Corning, CLS3008) gently scrap the fungal culture from the plates.
19. Transfer the fungal suspension to a 50 mL falcon tube and add 10 mL of PBS to the tube.
20. Pipet the fungus solution up and down 15–20 times with 10 mL serological pipet to break the clumps.

△ CRITICAL: Note that *Alternaria alternata* is a filamentous fungus and forms a clump while grown in culture dishes. To make single-cell suspension, a two-step procedure is required:

- a. The fungal suspension is passed through a sterile fine-mesh sieve strainer with gentle pressure using a 20 mL syringe plunger.
- b. Next, pass the fungal filtrate through a 70 μm cell strainer with gentle pressure using a 10 mL syringe plunger to get a single cell suspension.

△ CRITICAL: *Malassezia globosa* is a unicellular organism belonging to the yeast family and usually does not form clumps. Passing through a 70 μm cell strainer is enough to get a single cell suspension.

21. Determine colony formation unit/mL using a Thermo Fisher Countess II Cell Counter using the [manufacturer's protocol](#).
 - a. Centrifuge the fungal spore at 400 g at 4°C for 5 min, resuspended in 1 mL PBS.
 - b. Briefly, mix the fungi with trypan blue in 1:1 ratio and load 10 μL of the mix into the counting slide.
 - c. Measure 3 replicates and then average the values to get a fungal count.

Alternatives: Measurement can be done using a manual counting method using a hemocytometer.

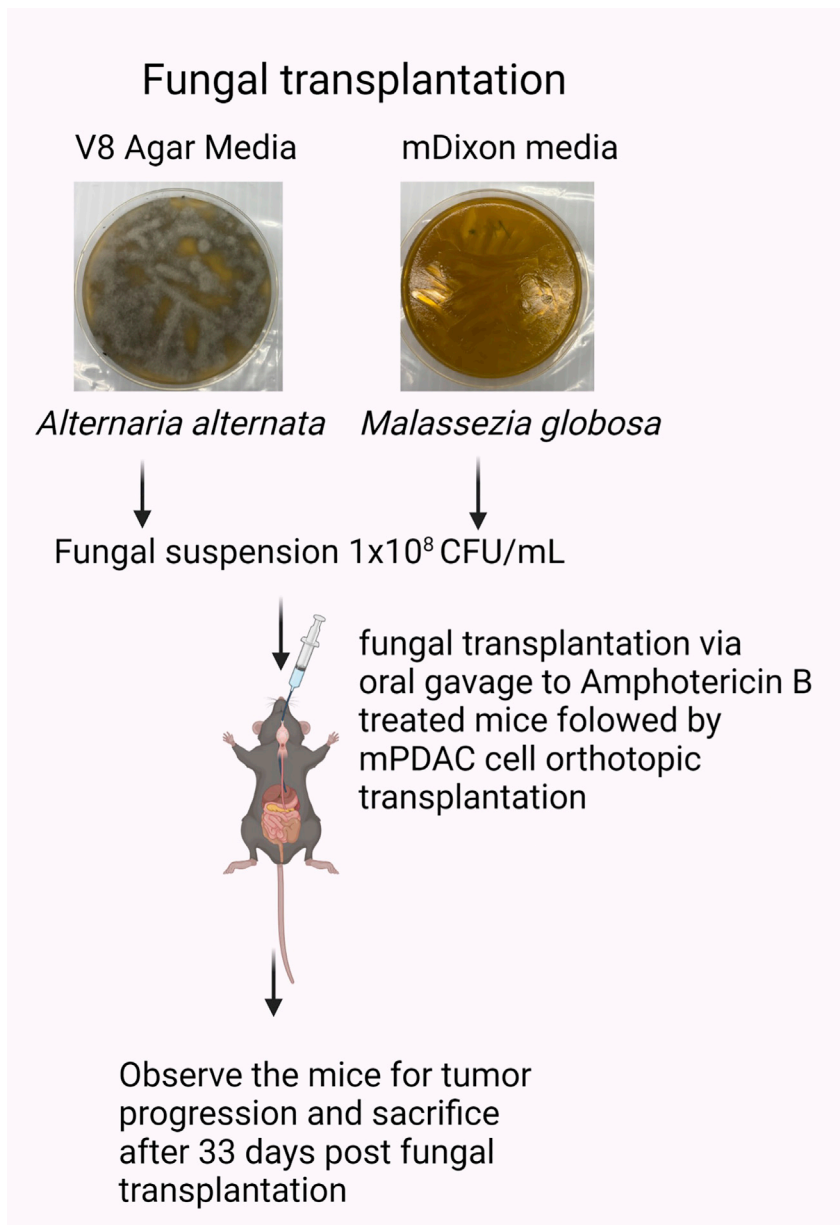


Figure 5. Schematic of major steps of fungal repopulation followed by orthotopic transplantation of PDAC cell lines into mouse pancreas

22. After Counting the fungal spore, adjust the volume of fungal suspension to produce a 1×10^8 spore/mL suspension for oral gavage.
23. Transfer 200 μ L of either *Alternaria alternata* or *Malassezia globosa* of fungal suspension at a concentration of 1×10^8 colony formation units (CFU)/mL, via oral gavage to mice (Figure 5).
24. Post fungal transplantation, switch the mice cages back to normal drinking water.

△ CRITICAL: Monitor mice for 7 days for any allergic symptoms or illness such as runny nose, cough, and red watery eye.

Section 4: Orthotopic PDAC tumor model

⌚ Timing: 26 days

25. Culture the mouse PDAC cell lines in PDAC cell culture media at 37°C and 5% CO₂ in humidified incubator.
26. On the day of surgery harvest the cells; count and make a single-cell suspension for orthotopic transplantation.
 - a. A week before the surgery thaw a vial of mPDAC cell lines and seed in a T-25 flask.
 - b. Split and feed the cells using standard techniques to expand the cells.
 - c. Determine the cell number based on the number of mice to be transplanted with mPDAC cells.

Note: Each mouse receives 0.5×10^6 cells per injection.

- d. Continue to grow the cells in a T-175. A day before the surgery split the cells to ensure the cells are in log phase of growth on the day of surgery.
 - e. On the day of surgery trypsinize the T-175 flask with 5 mL of 0.25% Trypsin. Add 10 mL RPMI media and then centrifuge the mix at 300 g for 5 min.
 - f. Resuspend the cells in 10 mL of PBS and centrifuge again the mix at 300 g for 5 min.
 - g. Repeat step f.
 - h. Count and resuspend the cells at a concentration of 0.5×10^6 per 10 μ L of PBS. The cells are ready for transfer to mouse.
27. Thaw the Matrigel for 12–16 h at 4°C.

Note: Matrigel takes at least 4–5 h to thaw, keeping it at 4°C for 12–16 h is a preferred method to save some critical time on the day of surgery. Matrigel should always be kept on ice until used for injection.

28. Mix the cells and Matrigel at 1:1 ratio (50 μ L cells and 50 μ L Matrigel).

Note: Keep the Hamilton syringe on ice before drawing the Matrigel and cell mix. Mix the cells and Matrigel just before the surgery to avoid solidification of Matrigel in the syringe.

29. Set up the surgical table inside a biobubble or in a sterile location.

⚠ CRITICAL: Make sure the surgical instruments are sterilized, and the heating pad is turned on.

Note: After 26 days of Amphotericin B treatment and 7 days of fungal repopulation period, the mPDAC cell line is orthotopically transplanted to pancreas (Figure 6).

30. For orthotopic transplantation, anesthetize mice by placing them in the induction chamber of isoflurane system and turning the gas on at 2–3% with a flow rate of 0.8–1.0 liter/min.
31. Shave a 2 × 2 mm portion of the left abdomen at the splenic silhouette, located just below the rib cage, to facilitate transplantation.
32. Make an incision of 1 cm in the left abdomen such that the pancreas is gently exposed along with the spleen.
33. Inject mouse PDAC cells mixed with Matrigel (20 μ L of cells (5×10^5) mixed with Matrigel), slowly into the tail of the pancreas using a Hamilton syringe.
34. Put the pancreas back into the abdomen using a surgical probe.
35. Suture the peritoneal membrane using sterile 6-0 (polyglactin 910) absorbable suture (Vicryl).

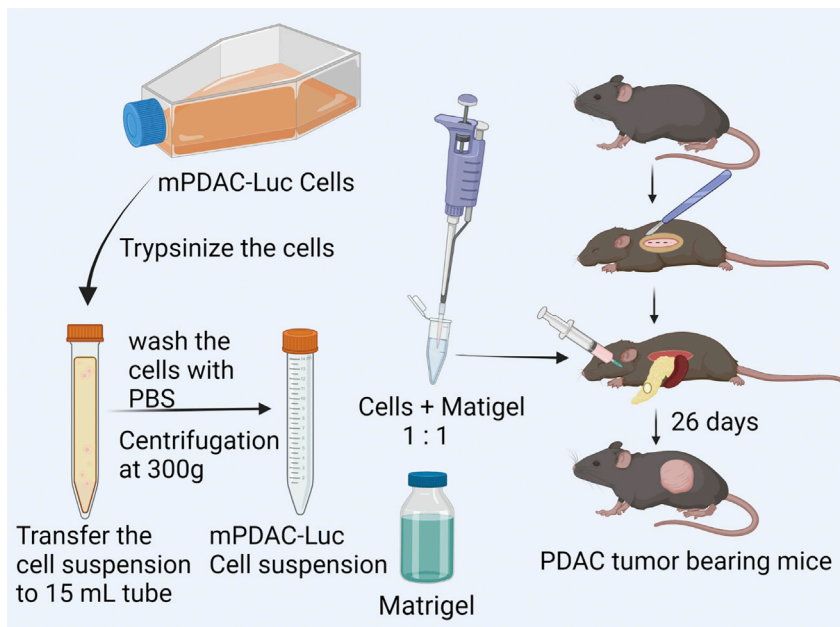


Figure 6. Schematic of the major steps of orthotopic transplantation of PDAC cell lines into mouse pancreas

36. Clip the outer skin with 2–3 surgical clips.
37. For the orthotopic model, image animals (IVIS Spectrum, PerkinElmer) 2 days after surgery to assess the successful implantation of the tumors.

Note: For IVIS imaging we inject the D-luciferin 150 mg Luciferin/kg body weight.

38. For a 10 g mouse, inject 100 μ L to deliver 1.5 mg of Luciferin via IP, wait for 5 min and then image the mice with 1 min exposure and 5 min lag time.

△ CRITICAL: Only orthotopic tumors of similar luciferase intensity are used for further study.

39. Monitor mice for tumor burden through MRI and/or bioluminescence imaging every week until euthanized.
40. On day 26 or earlier, if the mice are moribund, euthanize the mice, and harvest the tumor.
41. Weigh, measure and image the tumor for analysis.

△ CRITICAL: Prevent leakage during surgery by injecting a smaller volume of mPDAC cells and Matrigel mixture. Injection into the pancreas should form a small bubble; wait for 30–60 sec to let the Matrigel solidify and gently push the pancreas with the help of a blunt probe.

△ CRITICAL: During the oral gavage of fungi, injury to the trachea or GI tract must be avoided. If the fluid gets into the lung and there is a sign of asphyxiation the mouse needs to be euthanized immediately. Post fungal administration, Amphotericin B must not be used until the completion of the experiment.

EXPECTED OUTCOMES

This protocol is expected to produce two major outcomes- 1) culture of a specific fungal species and 2) introduction of fungal species into a mouse model. This protocol also provides a relatively easy

method to introduce fungus, using an already standardized technique of oral gavage, as well as supplementing the protocol with other methods, such as orthotopic syngeneic transplantation.

LIMITATIONS

Although it is expected that this protocol can be adapted for all fungal cultures and transplantation, however, it needs to be individually tested and a proper working protocol needs to be established. Also, fungal culture needs to be optimized by using a full suite of culture media and growth conditions. The usability of fungal culture within a BSL-2 facility is also subject to guidelines, as most of the pathogenic fungi cannot be cultured within the basic laboratory setting. For pathogenic fungus, appropriate approval and biosafety level should be determined by the institutional biosafety committee.

TROUBLESHOOTING

Problem 1

Contamination of the fungal culture.

Potential solution

This can be reduced by working in a BSL-2 hood, as well as taking proper PPE precautions. Contamination may be transferred from the exterior of the ampoule containing the fungal stock. This can be limited by cleaning the ampoule thoroughly before cracking it open the ampoule. Also, contamination can happen inside the incubator if the incubator is shared for bacterial culture. Clean the incubator thoroughly before the fungal procedure is initiated.

Problem 2

Slow growth of the fungi.

Potential solution

This can be limited by splitting fungal colonies into multiple plates. If the condition persists, try different growth conditions, especially optimization in temperature and nutrient conditions will largely sort out the growth condition.

Problem 3

Fungal agar media may contaminate the fungal spores during harvesting which will reduce the viability of the fungus.

Potential solution

While scraping the fungus, hydrate the fungus with PBS which will allow easy scraping of the fungus.

Note: To avoid contamination of fungal culture, it should not be placed in a bacterial incubator/shaker. The addition of antibiotics such as ampicillin and kanamycin can reduce bacterial contamination. Usage of antibiotics will require optimization of dose to prevent any alteration of fungal growth.

Problem 4

Fungal administration might induce severe allergy or anaphylactic shock in mice.

Potential solution

Limit the fungal load by decreasing the amount of CFU's administered or distributing the dosage given over multiple days to drastically reduce such adverse events.

Problem 5

Mice might develop dermatitis due to administration of *Malassezia globosa*.

Potential solution

Malassezia globosa is known to cause dermatitis in humans and animals. If mice develop dermatitis immediately inform the institutional veterinarian for treatment. Monitor the mice closely for a few days. If the problem persists and spreads to other animals in the same cage, all the mice need to be euthanized.

Problem 6

Spillage of fungus during oral gavage.

Potential solution

Proper mouse restraining method needs to be adopted to avoid movement of animals during oral gavage. Also, adequate PPE protection and gloves need to be used to avoid any contact with the fungus.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prasenjit Dey (prasenjit.dey@roswellpark.org).

Materials availability

This study did not generate new reagents.

Data and code availability

This study did not generate/analyze datasets and code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, P.D.; methodology, A.A., S.C., and E.L.; investigation, A.A., S.C., and E.L.; writing – review & editing, P.D., A.A., S.C., and E.L.; funding acquisition, P.D.; supervision, P.D.

DECLARATION OF INTERESTS

P.D. and A.A. have a patent pending on targeting IL33 and mycobiome in cancer. U.S. Provisional Patent Application Serial No. 63/238,531.

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