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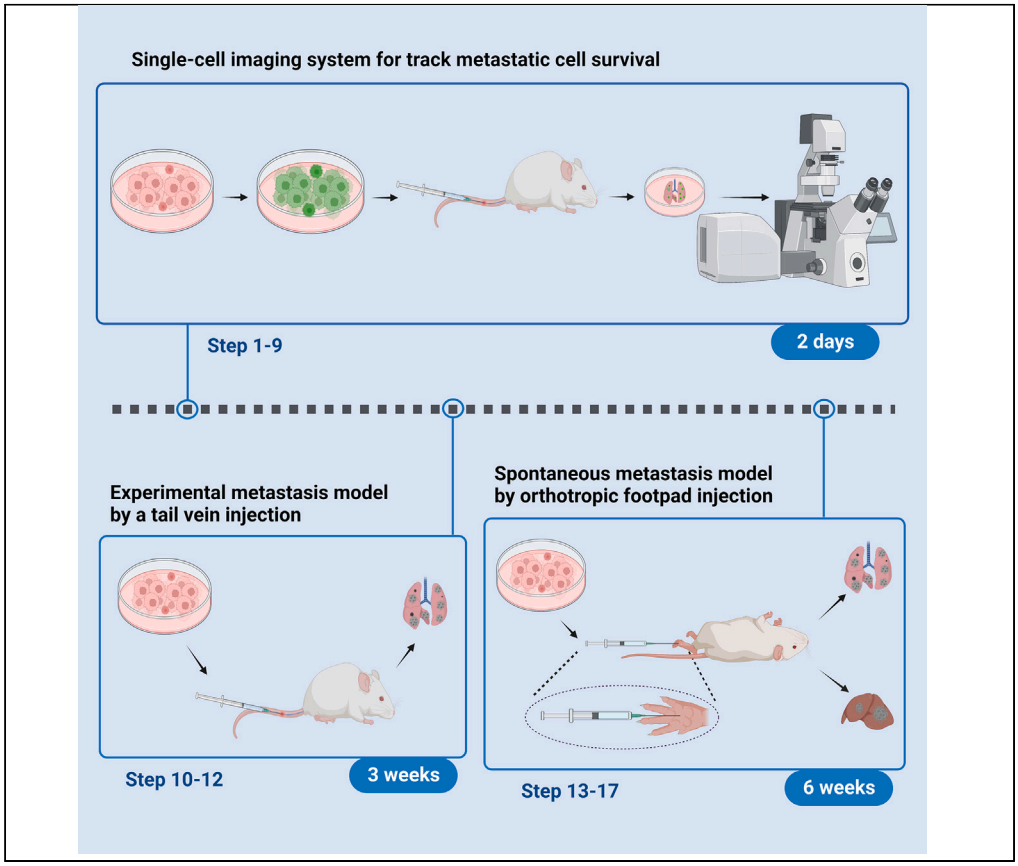
Using the single-cell imaging system and orthotropic footpad injection to establish mouse models for experimental and spontaneous melanoma metastasis



Metastasis, a complex process, is responsible for most deaths in patients with cancer. Clinically relevant research models are indispensable to advancing our understanding of metastatic mechanisms and developing new treatments. We here describe detailed protocols to establish mouse models for melanoma metastasis using the single-cell imaging system and orthotropic footpad injection. The single-cell imaging system permits the tracking and quantification of early metastatic cell survival, while the orthotropic footpad transplantation mimics aspects of the complex metastatic process.

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

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Highlights

The single-cell imaging system for tracking metastatic cell survival *in vivo*

Tail vein injection for establishing experimental melanoma metastasis model

The orthotropic footpad injection for building up spontaneous melanoma metastasis model

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Using the single-cell imaging system and orthotropic footpad injection to establish mouse models for experimental and spontaneous melanoma metastasis

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SUMMARY

Metastasis, a complex process, is responsible for most deaths in patients with cancer. Clinically relevant research models are indispensable to advancing our understanding of metastatic mechanisms and developing new treatments. We here describe detailed protocols to establish mouse models for melanoma metastasis using the single-cell imaging system and orthotropic footpad injection. The single-cell imaging system permits the tracking and quantification of early metastatic cell survival, while the orthotropic footpad transplantation mimics aspects of the complex metastatic process.
For complete details on the use and execution of this protocol, please refer to Yu et al.[1,2]

BEFORE YOU BEGIN

Before beginning, we need to prepare the following essential elements.

1. Preparation of cancer cell lines.
   a. Culture mouse B16F1 cells stably expressing PTEN wildtype (WT), PTEN G129E (PTENΔL), or PTEN C124A (PTENΔLP) mutants, as well as empty vector control and 37-7cells,[3] in DMEM media with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin.
   b. Culture human melanoma A375 expressing shRNA for IGF1R or Entpd5 in MEM with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.1 mM MEM Non-Essential Amino Acids Solution, 1 mM Sodium Pyruvate, 1x MEM Vitamin Solution (100x).

   Note: All cell lines were grown under 37°C with 5% CO_2 in a cell culture incubator.

2. Injection of cells.

   Note: There are six different injection strategies based on the injected cell number, methods and hosts.

   a. Inject B16F1 cells at 5 × 10^5 into FVB/BL6 (F1 cross), C57/cBrd or 1 × 10^5 into athymic nude mice via tail vein for experimental metastasis.

b. Inject 37–7 cells at 2 × 10^5 into FVB or 1 × 10^5 into athymic nude mice via tail vein for experimental metastasis.
c. Inject human melanoma A375 panel cells via the tail vein or footpad into NOD SCID Gamma (NSG) mice at 1 × 10^6 for experimental and spontaneous metastasis.

3. Preparation of animals: obtain quarantined and pathogen free 4–6 weeks old female or male FVB/BL6 F1, C57/cBrd, athymic nude, or NSG mice.

Note: our mice were purchased from Charles Rivers (Germantown), or Jackson Laboratory-Bar Harbor, ME.

Institutional permissions

All mouse procedures were performed according to National Institutes of Health guidelines. The animal studies were conducted under animal study protocols approved by the National Cancer Institute-Bethesda Animal Care and Use Committee (ACUC) in the United States of America. Animals were randomly allocated into experimental groups. Animals had free access to water and food and were housed in a controlled environment with a 12 h–12 h light-dark cycle, constant temperature (21 °C), and relative humidity (20%–40%).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Green CMFDA	Invitrogen	Cat# C7025
70% alcohol	Fisher Chemical	N/A
10% Buffered formalin phosphate	Fisher Chemical	L23006
DMEM with glucose	Thermo Fisher/Life Technologies	Cat# 11965092
MEM with glucose	Thermo Fisher/Life Technologies	Cat# 11095080
Penicillin-streptomycin	Thermo Fisher/Life Technologies	Cat# 15070063
Glutamine	Thermo Fisher/Life Technologies	Cat# 25030081
MEM vitamin solution	Thermo Fisher/Life Technologies	Cat# 11120052
Sodium pyruvate (100 mM)	Thermo Fisher/Life Technologies	Cat# 11360070
MEM non-essential amino acids solution (100X)	Thermo Fisher/Life Technologies	Cat# 11140076
FBS	Thermo Fisher/Life Technologies	Cat# A5256701
Experimental models: Cell lines		
Mouse: B16F1 (passage 1 to 10)	ATCC	Cat# CRL-6323
Mouse: 37-7 (passage 2 to 10)	Yu Y, et al.[3]	Cancer Res 2002
Mouse: RMS772 (passage 2 to 10)	Yu Y, et al.[4]	Nat Med 2004
Human: A375p (passage 7)	MD Anderson Medical Center	Gift from Dr. Isaiah Fidler
Human: A375sm (passage 12)	MD Anderson Medical Center	Gift from Dr. Isaiah Fidler
Human: A375c5 (passage 7)	MD Anderson Medical Center	Gift from Dr. Isaiah Fidler
Human: A375c28 (passage 7)	MD Anderson Medical Center	Gift from Dr. Isaiah Fidler
Experimental models: Organisms/strains		
FVB/BL6	In house	N/A
C57BL/6-cBrd (C57/6 albino)	Charles Rivers Laboratories	Code:562
NSG	Charles Rivers Laboratories	N/A
Athymic nude	Charles Rivers Laboratories	Code:553
Recombinant DNA		
pGFP-PTEN WT	Yu Y et al.[1]	N/A
pGFP-PTEN ΔL	Yu Y et al.[1]	N/A
pGFP-PTEN ΔLP	Yu Y et al.[1]	N/A
pBabe-PTEN WT	Addgene	#10785

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
pBabe-PTEN ΔL	Addgene	#10771
pBabe-PTEN ΔLP	Addgene	#10931
pBabe-IGF1R	Addgene	#11212
Software and algorithms		
GraphPad Prism 6 software	GraphPad	N/A
ImageScope V 10.0 software	Aperio Technologies	N/A
Openlab V3.1	Improvision/PerkinElmer	N/A
Other		
LEICA-DM IRB fluorescent inverted microscope	LEICA	N/A
1 mL syringe with 27-GA/1/2-inch needle (tail vein injection)	Fisher Scientific	BD309623
Sterile Luer Lock Tip 10 cc/mL syringe (insufflation of the lung)	Terumo Medical Corporation	BD302995
20-GA x 1.16-inch Angio-catheter	Becton Dickinson, Infusion Therapy Systems Inc.	BD 381703
Sterile latex free intravenous extension set 30-inch long	Hospira, Inc.	1265628
Tissue culture hood	Thermo Fisher Scientific	N/A
Tissue culture dishes and flasks	Fisher Scientific	N/A
Sterile surgical drapes, 18-inch x 26-inch long	Fisher Scientific	NC2032042
Micro dissecting forceps straight, serrated	Fisher Scientific	10-001-161
Super cut iris scissors, straight, 12.5-cm long	Fisher Scientific	N/A
Sutures, vicryl absorbable, 4-0, 18-inch long	Ethicon	Cat# SKU: J494G
Insulin syringes 3/10 mL/cc with 30-G needle (for footpad injection)	BD (Becton, Dickinson and Company)	Cat# SKU: 328291
CO_2 chamber for euthanizing mouse	Fisher Scientific	N/A
Heat light	Fisher Scientific	N/A
Mouse restraint device	Fisher Scientific	N/A

STEP-BY-STEP METHOD DETAILS

Protocol for a single-cell imaging system to track metastatic cell survival *in vivo*

Timing: 2 days

This section describes the protocol of the single-cell image system to track the fate of fluorescently labeled single tumor cells reaching the lung. Upon completing the section, the early survival of metastatic tumor cells arriving in the lung has been determined and quantified. The protocol can be applied to any tumor cells.

1. Label B16F1 tumor cells: 2 h.

Note: Before labeling tumor cells, dissolve the lyophilized CMFDA in DMSO to a stock concentration of 10 mM. Dilute the stock solution to a working concentration of 5 μM in a serum-free medium.

a. Tumor cell line preparation.
   i. Culture tumor cells to a confluency of 60%–70% (3 dish-175 cm).

Note: Make sure to have enough cells for *in vivo* injections the next day.

   ii. Remove the cultural media.
   iii. Wash cells with Phosphate-Buffered Saline (PBS) 2 times.
b. Gently add 5 mL of 5 μM CMFDA working solution in a 175 cm dish.
c. Incubate for 30 min under growth conditions of 37°C and 5% CO_2 in a cell culture incubator.

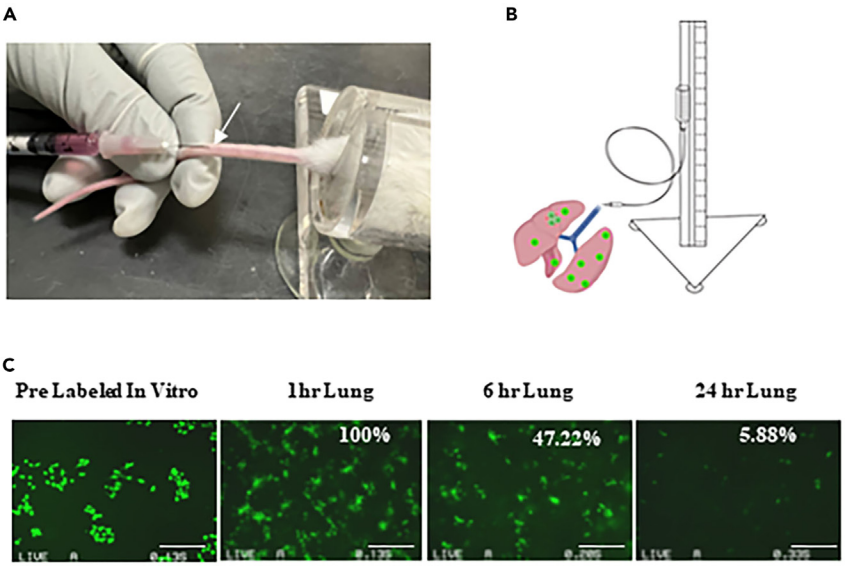

Figure 1. Diagram of the single-cell imaging system to track the survival of metastatic cells reaching the lung

(A) Tail vein cell delivery.

(B) Lung insufflation.

(C) Representation of images of labeled tumor cells reached the lung at 1-, 6- and 24-h time points after injection. The total number of labeled tumor cells at the 1-h time point was presented 100%. The percent of metastatic survival was determined by normalizing the mean number of cells at 6 or 24 h with the mean number of cells at 1 h for each mouse lung. Scale bars: 200 μm.

d. Gently remove the CMFDA working solution, and wash with PBS 2 times.
e. Add cell culture media and incubate for another 30 min in the same cell culture condition in a cell culture incubator.
f. Image labeled cells as a pre-labeled *in vitro* group using green (emission 517 nm and excitation 492 nm) filters under fluorescence microscopy.
g. Collect the labeled cells in 5×10^6 cells/mL in serum-free media and keep them on ice for tail vein injection into mice.

2. Injection of labeled tumor cells: 30–45 min

Note: After labeled cells are collected, 1×10^6 cells (200 μL of 5×10^6 cells/mL) are immediately delivered to mice (5 mice per condition group) by tail vein injection using a 27 1/2-GA needle.

a. Prepare the mice: place mice under a heating light to maintain body temperature.
b. Load the syringe with a 27/ 1/2-GA needle and draw the tumor cells into a sterile syringe.
c. Locate the vein.
   i. Gently restrain the mouse by holding its tail using a mouse restraining device.

Note: The tail vein can usually be seen through the skin as a thin blue line.

   ii. Grasp the tail firmly just below the area where you will be injecting and disinfect the area with alcohol.
d. Insert the needle into the vein at a shallow angle (less than 30 degrees) and advance it about 1–2 mm (Figure 1A).
e. Aspirate to confirm that the needle is in the vein, then slowly inject 200 μL of cells into the vein of the mouse. Be sure to inject slowly and avoid causing damage to the vein.

Note: Aspiration is tricky. A gently negative pressure applies to the plunger to avoid introducing possible bubbles.

f. Remove the needle gently and apply pressure to the injection site with a sterile gauze pad to stop any bleeding. Continue until all groups of mice are completed (10–20 mice per group).
g. Monitor the mice closely for any signs of distress or adverse effects following the injection.

Note: Tail vein injections should only be performed by trained personnel with appropriate ethical approval and in compliance with institutional and regulatory guidelines.

3. Euthanize the mice at 1, 6, or 24 h after injection. Five mice were injected per cell line per time point.

Note: Euthanize the mice by CO_2 inhalation with an initial 30%–60% flow rate for 2–3 min to minimize stress to the mouse breathing and lungs, then increase the CO_2 flow rate by 70%–90% for an extra 2 min. The euthanasia steps are critical to prevent any lung hemorrhage and alveoli breakage caused by a high flow rate of CO_2.

4. Lung insufflation (Figure 1B). 10–30 min.
   a. Set up the insufflation apparatus using a 20 mL syringe connected to a 35 mL Extension Set Priming Volume, DEHP-Free and Latex-Free Male and Female luer locks with Slide Clamp to monitor PBS volume and flow rate.
   b. Consistency of insufflation of the lungs to the right size without under or over insufflation with PBS (approximately volume per lung 0.75–1.0 mL).
   c. Spray the mice with 70% alcohol before placing them in the tissue culture hood for lung extraction.
   d. A mid-transverse skin incision is made at the mid-abdominal area and the skin should be pulled over in opposite directions (cranial-caudal) to expose the thoracic and abdominal cavities.
   e. Make an incision on the ventral midabdominal line alba close to the xiphoid process, then follow with an incision into the abdominal cavity to expose the mouse diaphragm.
   f. Cut into the mouse diaphragm without lacerating the lungs.
      i. Make a bilateral incision along the sternum to the first rib cage.
      ii. Pull the sternum cranially to expose the trachea.
   g. Canulate the trachea at the intercartilaginous ligament with a 20G × 1″ IV Angio-catheter approximately 1/2 inch.
      i. Remove the stylet needle and advance the catheter.
      ii. Place a single loose suture with 2-0 Vicryl underneath the proximal trachea and leave the suture loose.
   h. Connect the extension IV set primed with PBS into the IV catheter and hold it in place.

Note: the lungs will be expanded approximately 30–45 seconds after insufflation is completed.

      i. Lock the IV set with the slide clamp.
      ii. Slightly pull IV catheter.
      iii. Gently tie up the distal trachea with 2-0 Vicryl suture without rupturing it to avoid any leakage of insufflated PBS.

Note: This will prevent the mouse lung from collapsing.

5. Dissect the mouse lungs surgically.
   a. Cut the trachea distally without compromising the suture on the trachea.

b. Use the trachea or hold the mouse heart with a thumb dressing forceps to dissect the lungs out using straight iris scissors.
c. Cut the lung mediastinal ligamentum.
d. Cut the attachment of the diaphragm that connects to the mouse liver and gently remove the lungs.
e. Place and rinse the mouse lung in cold PBS for 30–45 s to get rid of any blood debris.

6. The lungs (L1, L2. L3, L4, L5) are then immediately transferred into a 50 mL conical tube containing 35 mL PBS at 4°C supplemented with penicillin 100 U/mL/streptomycin 10 μg/mL for surface fluoresce microscopy imaging on a Leica DM IRB Widefield Fluorescence Microscope equipped with a filter set appropriate for imaging green fluorescent protein (GFP).

Note: Each mouse lung was imaged one time only per each time point at 1 h, 6 h, and 24 h by taking 10 random surface fluorescent pictures of each single mouse lung (L1, L2, L3, L4, L5) using Improvision's Openlab 3.1 scientific image acquisition software, then all lung pictures were saved until ready to be analyzed and quantified. In addition, Improvision's Openlab 3.1 scientific image acquisition software was programmed to read and analyze each event at a size of 10 photons or greater, any event smaller than 10 photons was not read or analyzed; this was considered an artifact or background noise (Figure 1C).

7. After imaging each timepoint (1 h, 6 h, 24 h), place all five lungs in a 50 mL conical tube filled with 35 mL of 10% formaldehyde buffered for H&E analysis.
8. Images analysis.
   a. Use Improvision's Openlab 3.1 scientific image acquisition software to analyze and quantify the total sum of the surface fluorescent area in the 10 images of each single mouse lung (L1, L2, L3, L4, L5) per timepoint.
   b. Use Microsoft Excel to graph the average number of all five-lung surface fluorescent events (L1, L2, L3, L4, L5) at 1 h, 6 h, 24 h.
   c. Subtract the average number of each timepoint at 6 h, 24 hr from the average baseline number at 1 h timepoint.

Note: Quantification of lung fluorescent events was validated using both enumeration of surface fluorescent event counts and mean fluorescent area.

9. Statistical analysis: Nonparametric t-test and ANOVA were used for image analysis. Statistical analyses were performed using GraphPad Prism version 3.0 for Macintosh (GraphPad Software). The percent metastatic survival is determined by normalizing the mean number of cells at 6 h or 24 h with the mean number of cells at 1 h for each mouse lung (Figure 1C). Statistical significance was defined as $P \leq 0.05$.[1,5–7]

Establishment of experimental melanoma metastasis model by tail vein injection

Timing: 3 weeks

This section describes the procedure to develop the experimental metastasis model by tail vein injection. Upon completion of the section, the metastatic potential of tumor cells has been measured quickly and easily. The protocol can be applied to any tumor cells.

10. Tumor cells preparation: 1 h.

Note: Tumor cell lines should be cultured to a 70%–85% confluency.

   a. Trypsinize tumor cells, and centrifuge down with 1200 rpm for 5 min.
   b. Wash the tumor cells with 10 mL serum-free media.

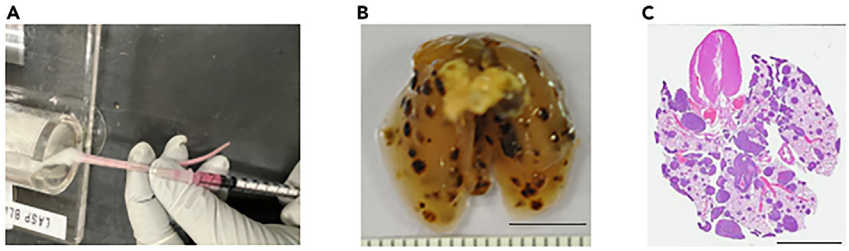

Figure 2. Diagram of experimental metastasis model by tail vein injection
(A) Tail vein injection of 5 × 10^5 B16F1-PTENΔLP tumor cells.
(B) Lung metastases 3 weeks after injection.
(C) Images of a lung section with micrometastases. Scale bars: 1 cm.

c. Resuspend the tumor cells in serum-free media at a concentration of 2.5 × 10^6/mL for B16F1, and 5 × 10^6 for A375 cells and keep the cells on ice for injection as soon as possible.

11. Tumor cells injection: 1–2 h.
    a. Prepare the mice: place mice under a heating light to maintain body temperature.
    b. Load the syringe with a 27/ 1/2-GA needle and draw the tumor cells into a sterile syringe.
    c. Locate the vein.
        i. Gently restrain the mouse by holding its tail using a mouse restraining device.

    Note: The tail vein can usually be seen through the skin as a thin blue line.

        ii. Grasp the tail firmly just below the area where you will be injecting and disinfect the area with alcohol.
    d. Insert the needle into the vein at a shallow angle (less than 30 degrees) and advance it about 1–2 mm (Figure 2A).
    e. Aspirate to confirm that the needle is in the vein, then slowly inject 200 μL of cells into the vein of the mouse.

    Note: Be sure to inject slowly and avoid causing damage to the vein.

    f. Remove the needle gently and apply pressure to the injection site with a sterile gauze pad to stop any bleeding.

    Note: Continue until all groups of mice are completed (10–20 mice per group).

    g. Monitor the mice closely for any signs of distress or adverse effects following the injection.
12. Check tumor metastasis.
    a. Three weeks after the transplantation of tumor cells, euthanize the mice with CO_2.
    b. Dissect the mouse from the ventral side.
    c. Inject the fixing solution of 10% Buffered Formalin Phosphate into the lung through the trachea.
    d. Visually count the number of macro-metastases in the lung.
    e. Transfer the lung with tumor metastasis in the 10% Buffered Formalin Phosphate fixing solution.

Note: The fixed lungs should also be paraffin-embedded and sectioned for H&E staining for counting micro-metastasis under the microscopy (Figure 2).[1,8]

Note: Tail vein injections should only be performed by trained personnel with appropriate ethical approval and in compliance with institutional and regulatory guidelines.

Establishment of the spontaneous metastasis model by an orthotropic footpad injection

Timing: 6 weeks

This section describes that the spontaneous metastasis model by the orthotropic footpad injection can more completely mimic the actual complexity of the metastatic process and is more relevant to assess distant metastases.

13. Tumor cells preparation: 1–2 h.

Note: Tumor cell lines should be cultured to a 70%–85% confluency.

a. Trypsinize tumor cells, collect the tumor cells in 10 mL culture media, and centrifuge down with 1200 rpm for 5 min.
b. Wash tumor cells with 10 mL serum-free media, resuspend in serum-free media at a concentration of 5×10^7 cells/mL, and keep on ice for the next step as soon as possible.

14. Footpad injection: 1–2 h.
a. Fill a syringe with a 30-GA needle with the appropriate amount of cells to be administered.

Note: The amount that can be injected into a footpad is 20 μL per mouse.

b. Place the mouse in a restraining device with one hind foot isolated and extended by gently grasping the skin above the stifle.
c. Wipe the foot with alcohol to sterilize and remove debris before injecting.
d. Hold the toes of the extended foot before releasing the skin at the stifle.
e. Inject 20 μL tumor cells subcutaneously into the center of the hind footpad at a shallow angle (less than 30 degrees) and slowly advance it about 2–3 mm (Figure 3A).

Note: This is the critical step; any leakage will affect the result (Figure 3A).

f. Remove the needle and observe for bleeding.
g. Apply light pressure to the puncture site if bleeding occurs.

15. Return the animals to the cage.
16. Monitor the animal: Watch the animal closely for any signs of distress or adverse effects following the injection.
17. Check the metastasis (Table 1).
a. Six weeks after injection, euthanize the mice with CO_2.
b. Lay the ventral side up of the mouse and dissect it.
c. Inject the fixing solution of 10% Buffered Formalin Phosphate into the lung through the trachea and visually count the number of macro-metastases in the lung.
d. Count the number of metastases in other organs.
e. Fix the organs with tumor metastasis in the 10% Buffered Formalin Phosphate fixing solution.

Note: The organs with metastatic tumors can also be sectioned for H&E staining for counting micro-metastasis under a microscope (Figure 3).[1,2,9]

Note: Footpad injections should only be performed by trained personnel with appropriate ethical approval and in compliance with institutional and regulatory guidelines. Also, note

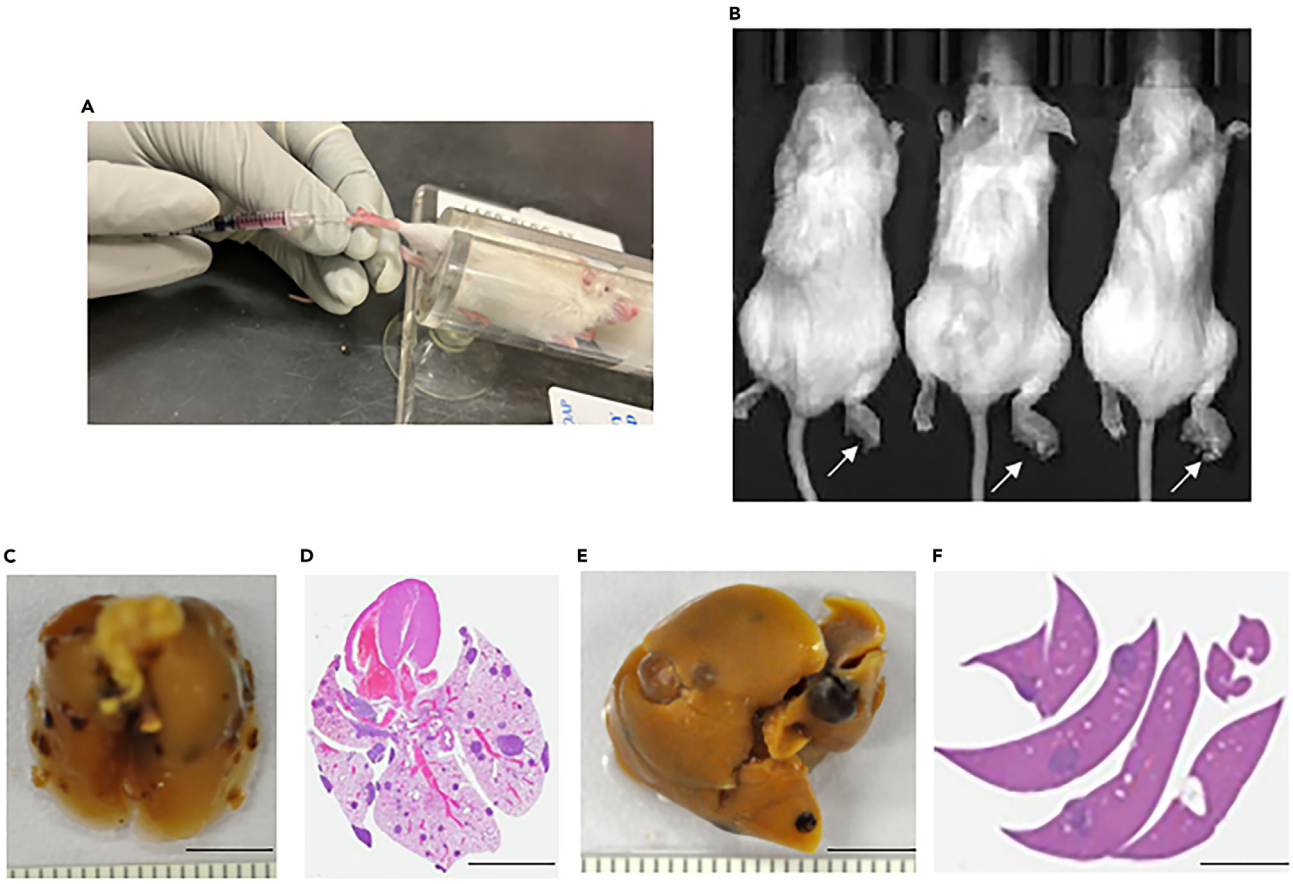

Figure 3. Diagram of the orthotropic spontaneous metastasis model by footpad transplantation

(A) 1 × 10^6 A375p ENTPD5 tumor cells were delivered by footpad injection.

(B) Representative image of mice with the local foot tumor (white arrow) at 6 weeks after footpad injection.

(C) Representative lung metastases of tumor cells at 6 weeks after footpad injection.

(D) Image of lung section with micrometastases.

(E) Image of liver metastases.

(F) Image of the liver section with micrometastases. Scale bars: 1 cm.

that the volume of injection should be limited to no more than 50 μL to avoid overloading the footpad, which can cause tissue damage and pain to the animal.

Analysis of the metastasis

Tumor numbers are obtained by visual inspection of tissues in mice euthanized 3 weeks (tail vein injection) or 6 weeks (footpad injection) post-transplantation. Micrometastases should be counted by a pathologist's evaluation after the dissection of the lung. Statistical analyses can be performed as follows: unpaired t-test (two-tailed) for all column datasets. The p-values of less than 0.05 are considered statistically significant.

EXPECTED OUTCOMES

The protocol of the single-cell image system can track the fate of fluorescently labeled single tumor cells arriving in the lung and quantify the early survival of tumor cells *in vivo* in 24 h. The experimental metastasis model by tail vein injection is an easy and quick assay for measuring the metastatic potential of tumor cells. However, our spontaneous metastasis model by the orthotropic footpad injection can more completely mimic the entire complexity of the metastatic process and is more relevant

Table 1. The outcomes of spontaneous metastasis models by the orthotropic footpad transplantation

Cell lines	No. injected cells	Time of metastasis	No. mice with metastasis / total mice	No. gross lung metastasis	No. gross liver metastasis
A375p C	1×10^6	6 weeks	4/10	0,0,0,0,0,0,1,1,1,1	0,0,0,0,0,0,0,0,0,0
A375p Entpd5	1×10^6	6 weeks	7/7	7,11,3,11,15,13,6	3,6,4,5,7,6,4
A375sm C	1×10^6	6 weeks	7/7	3,7,6,10,13,15,13	No mets
A375sm IGF1R-shRNA1	1×10^6	6 weeks	2/6	0,0,9,0,0,2	No mets
A375sm IGF1R-shRNA2	1×10^6	6 weeks	2/6	0,1,0,1,0,0	No mets
A375sm C	1×10^6	6 weeks	6/6	11,17,25,6,12,8	No mets
A375sm PHLPP1	1×10^6	6 weeks	10/10	9,3,1,9,3,1,2,8,1,2	No mets
A375sm PHLPP2	1×10^6	6 weeks	6/6	13,6,3,9,8,6	No mets

to assess distant metastases. Table 1 shows detailed outcomes of spontaneous metastasis models by the orthotropic footpad transplantation (Table 1).

LIMITATIONS

Although the experimental metastasis model by tail vein injection is an easy and quick assay for metastasis, the cancer cells are introduced directly into the circulation; this assay depicts only the late phases of the invasion-metastasis cascade. Therefore, a major drawback of this model system is that it fails to represent the earlier stages of the metastatic process, such as local invasion and intravasation. In contrast, the spontaneous metastasis model by an orthotropic footpad injection more faithfully mimics the whole metastatic process and is more relevant to assess distant metastases (e.g., lung, liver, and brain). However, it should be noted that not all tumor cell lines can form distant metastasis effectively in the orthotropic footpad injection model.

TROUBLESHOOTING

Problem 1

Weak signal of labeled cells (step 1).

Potential solution

Different cell lines have their own growth conditions and proliferation rates. The penetration of the dye into the cells may require modified conditions. Suggest running a high dye concentration of up to 25 μM or increasing the labeling time to 45 min.

Problem 2

Hard to push cells by the tail vein by tail vein injection (steps 2 and 11).

Potential solution

Needlepoint or needle hit or leave tail vein. Pull out the needle. Heat the mouse under heat light and make sure to see the blue tail vein and try again.

> *Note:* Restrain the mouse tail while occluding the vein with your nondominant hand and with the bevel of the needle facing upward and the needle almost parallel to the vein, slide the needle into the tail vein, then confirm the location by gently applying negative pressure to the plunger; if the needle is in the vein, you should see a flash of blood in the hub of the needle, if you do not see a flash of blood in the hub of the needle pull your needle back slightly without removing it from the tail, while keeping negative pressure in the syringe and redirect the needle until you see a flash of blood, then deliver the tumor cells slowly by pressing the plunger into the vein, if the needle is in the vein, there will be no resistance while injecting the tumor cells and the vein itself will blanch, if the needle is not in the vein, the fluid will cause blanching around the vein or a subcutaneous bleb. If unsuccessful, rest the mice for 5–10 minutes and

reheat the mice again for 3–5 minutes and try the opposite site of the lateral tail vein again. Also, tail vein injections should only be performed by trained personnel.

Problem 3

The mouse is found to die after tail vein injection immediately to two days (steps 2 and 11).

Potential solution

This may cause by tiny air bubbles or cell aggregation. Make sure to remove all air bubbles in the syringe and needle before injecting or filtering the cells.

Problem 4

Footpad tumors develop in sites where space for tumor expansion is restricted and may cause distress, inflammation and pain (step 17).

Potential solution

If the mouse has no other clinical signs, it may be treated with analgesics (bupSR S.Q. every 1–2 days at 1.2 mg/kg). This will provide temporary analgesia/pain relief while allowing the animals to remain in the study and develop metastases. Veterinarians should be consulted in this regard. However, if any footpad tumor size is reached 1 cm in any dimension and the animal progress or has difficulty attaining food/water, the mouse will be euthanized in our method.

Problem 5

No metastasis has been found visibly in mice after transplantation of 6 weeks (step 17).

Potential solution

Although orthotropic footpad injection could mimic the whole metastatic process, the limitation of this model is that not all tumor cell lines can form metastasis effectively. A pilot study should test and find a suitable injected number of cells and time to keep. Increasing the number of injected cells or keeping the mice longer will allow the metastatic cells to grow. Or, the section of fixed organs is checked for micrometastasis. As noticed, the tumor cells could easily form metastasis in the experimental metastasis model by tail veil injection, but they may not develop distant metastasis effectively in the orthotropic footpad injection model.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yanlin Yu (yuy@mail.nih.gov).

Materials availability

The plasmids and cell lines generated in this study are available from the authors at reasonable request.

Data and code availability

This study did not generate new unique data and code.

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AUTHOR CONTRIBUTIONS

Y.Y. designed experiments; A.M. and Y.Y. performed experiments, analyzed data, and wrote the manuscript; and G.M. critically reviewed and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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