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Intravital Imaging of Human Melanoma Cells in the Mouse Ear Skin by Two-Photon Excitation Microscopy

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Summary

Noninvasive imaging of reporter gene expression by two-photon excitation (2PE) laser scanning microscopy is uniquely suited to perform dynamic and multi-dimensional imaging down to single-cell detection sensitivity *in vivo* in deep tissues. Here we used 2PE microscopy to visualize green fluorescent protein (GFP) as a reporter gene in human melanoma cells implanted into the dermis of the mouse ear skin. We first provide a step-by-step methodology to set up a 2PE imaging model of the mouse ear's skin and then apply it for the observation of the primary tumor and its associated vasculature *in vivo*. This approach is minimally invasive and allows repeated imaging over time and continuous visual monitoring of malignant growth within intact animals. Imaging fluorescence reporter gene expression in small living animals by 2PE provides a unique tool to investigate critical pathways and molecular events in cancer biology such as tumorigenesis and metastasis *in vivo* with high-spatial and temporal resolutions.

Keywords

Melanoma; Metastasis; Cancer cell migration; Angiotropism; Blood vessel; Dermis; Mouse ear; Two-photon microscopy; Multiphoton; Intravital imaging; Reporter gene expression

1. Introduction

Metastases, the development of secondary malignant growths at a distance from a primary site of cancer, are the main cause of cancer-associated death. How and why cancer cells leave their original location, travel throughout the body and home on a new site to form new tumors has been difficult to observe in real time. That is in part because the various steps

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involved in the metastatic process are ephemeral events that are happening deep inside the body and also because tumor cells are usually difficult to distinguish from normal tissue.

Endowing tumor cells with fluorescence reporter gene expression enables direct observation of tumor growth and metastasis formation by various optical methodologies. Of particular importance, two-photon excitation (2PE) laser scanning microscopy (also referred to as multiphoton microscopy) has expanded the application of fluorescence reporter gene expression deep into intact tissues (1) and has emerged as a powerful approach to image tumor cell behavior *in vivo* (2, 3). 2PE is a nonlinear process in which two low-energy photons combine energy to induce a higher-energy electronic transition in a fluorescent molecule (4). The near-simultaneous absorption of two photons of light of roughly half the energy is usually achieved with an ultrafast, mode-locked Ti:Sapphire pulsed laser emitting in the near IR region of the spectrum (690–1040 nm). This excitation process has a number of unique advantages. First, the localization of fluorescence excitation is confined within a femtoliter size 3D focal volume (5). The localized excitation volume also provides innate confocality without the need of a spatial confocal pinhole in front of the detector. This limits the out-of-focus background excitation which greatly improves image contrast and reduces photobleaching. Secondly, the use of deep red and near IR excitation wavelength afford deeper penetration into scattering samples allowing imaging of thicker biological specimen compared with conventional one-photon confocal microscopy. For all these reasons, 2PE microscopy is especially valuable for non-invasive deep imaging of live tissues.

The mouse ear is relatively thin and flat. It's skin offers an ideal location to perform 2PE intravital microscopy to follow stable fluorescent protein reporter expression in dynamic processes from immune responses to tumor development (6, 7). Here, we describe the use of the mouse ear skin in an experimental model of orthotopic injection of human melanoma cells with stable green fluorescent protein (GFP) expression. We present a general method to immobilize the mouse ear for imaging both the tumor and its associated vasculature *in vivo* in an undisturbed natural context with single cell resolution. This noninvasive and highly selective imaging of growing tumors, made possible by GFP fluorescence reporter expression, enables the tracking of tumor growth and metastasis formation in transplanted animals. 2PE microscopy imaging should accelerate the validation of cell-based reporter gene approaches for translation into preclinical small animal models.

2. Materials

All reagents are of the best analytical grade and all solutions should be prepared in Milli-Q water (resistivity of 18 M Ω -cm).

2.1 Reagents, General Supplies and Equipment

1. DyLight® 594 Lycopersicon esculentum (Tomato) Lectin, (Vector Laboratories, Burlingame, CA, cat. no. DL-1177).
2. 0.5 mL Lo-Dose™ Insulin Syringe U-100 28G^{1/2} Micro-Fine™ IV Needle (0.36 × 13 mm), (BD Becton Dickinson, Franklin Lakes, NJ, cat. no. 329465).

3. WillCo-dish® Glass Bottom dishes (WillCo Wells B.V., Amsterdam, The Netherlands, cat. no. GWSB-5040).
4. MakerBot Replicator Desktop 3D Printer (MakerBot® Industries, LLC Brooklyn, NY USA).
5. UV hand-held lamp.
6. Curved splinter forceps.
7. Cotton-tipped applicators (Covidien, cat. no. 8884540400).
8. Kimwipes delicate task wipes (Kimtech Science, cat. no. 34155).
9. Water bath and heating mat set for 37°C.

2.2 Cells and Cell Culture Reagents

1. C8161 GFP human melanoma cell line (8).
2. Dulbecco's modified Eagle's medium (DMEM-500 mL, GIBCO® by Life Technologies, cat. no. 11995-065) supplemented with 10% (50 mL) inactivated fetal calf serum (Gemini Bio-products, Sacramento, CA, cat. no. 100-106), 5 mL of L-Glutamine 200 mM (100X, GIBCO® by Life Technologies, cat. no. 25030-081), 5 mL of Penicillin-Streptomycin (10,000 units, GIBCO® by Life Technologies, cat. no. 15140-122) and 4 µL of 2-βMercaptoethanol at 14.3 M (Sigma-Aldrich, cat. no. M7522).
3. Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺ (1X, DPBS-500 mL, GIBCO® by Life Technologies, cat. no. 14190-144).
4. 1X Solution of 0.25% Trypsin-EDTA (GIBCO® by Life Technologies, cat. no. 25200-056).
5. Vented 75 cm² - 250 mL Falcon tissue culture flasks (Fisher Scientific, cat. no. 13-680-65).
6. 15 mL Falcon tubes with conical bottoms (Fisher Scientific, cat. no. 14-959-49B).
7. 50 mL Falcon tubes with conical bottoms (Fisher Scientific, cat. no. 14-432-22).
8. Plastic Pasteur pipettes (Celltreat Scientific Products, Shirley, MA, cat no. 229285).
9. Cell counter and phase hemacytometer (VWR international, cat no. 15170-263).
10. CO2 cell culture incubator (SANYO, cat. no. MCO-36AIC) set up at 37°C.
11. Tissue culture hood biosafety cabinet (The Baker Company, SterilGARD III, cat. no. SG-403a).
12. Fluorescence microscope (Leica Microsystems Inc., Exton, PA, model no. DMIL) equipped with a GFP filter cube (Excitation BP 450-490 nm, Dichroic RSP 510 nm, Emission LP 515 nm).

2.3 Experimental Animals and Reagents

1. Athymic nude, nu/nu mouse strain JAX 002019 NU/J (The Jackson Laboratory, Bar Harbor, ME) (*see* Note 1).
2. Artificial tears (Lubricant Ophthalmic Ointment), (Akorn Animal Health, Lake Forest, IL, cat. no. 17478-162-35).
3. Nair (Hair remover lotion), available in any drugstore and pharmacy.
4. Isoflurane, USP (Isothesia-250 mL, cat. no. 029405, Henry Schein® Animal Health (*see* Note 2).
5. Oxygen supply (provided by a central distribution system or by pressurized tanks).
6. Anesthetic vaporizer (Summit Medical Equipment Company, Bend, OR).

2.3 Two-Photon Excitation (2PE) Laser Scanning Microscopy

1. Confocal upright microscope with fixed stage (Leica Microsystems Inc., Exton, PA, model no. DM6000 CFS).
2. Tunable (690–1040 nm) Ti:Sapphire pulsed laser providing > 2.4 W average power and < 70 fs pulse width (Spectra-Physics, Santa Clara, CA, model no. Mai Tai® DeepSee™).
3. High-sensitivity non-descanned (NDD) detectors (Leica Microsystems Inc., Exton, PA, model no. HyD SMD).
4. Water immersion objective (Leica HC PL IRAPO 20X/0.75).
5. Dual-band filter set for NDD detection of GFP and DyLight® 594: BP 525/50 nm (GFP), Dichroic RSP 560 nm, BP 624/40 nm (DyLight® 594) (Leica Microsystems Inc., Exton, PA).
6. 4' × 6' optical table (TMC, Peabody, MA).

2.4 Intravital Ear Stage Platform

1. Custom-built 3D-printed ear-imaging stage platform (9). (*see* Note 3).
2. Heating pad and temperature controller set for 37°C (Watlow, Riverside, CA).

3. Methods

Please note that all experiments using laboratory animals should be conducted in accordance with the relevant ethics guidelines and regulations in place at your institution.

3.1. Cell Culture

1. Subculture cells in vented 75 cm² tissue culture flasks in order to achieve 70–80% confluence of actively growing cells (Fig. 1A).
2. Remove medium with a Pasteur pipette and wash the cells with 10 mL of DPBS.

3. Add 0.5 mL of trypsin to detach cells and incubate 4 min at 37°C. Monitor the enzymatic digestion with a light microscope.
4. Stop digestion by adding 10 mL of fresh complete medium. Transfer the cells into a 15 mL tube.
5. Count cells with hemacytometer.
6. Centrifuge the solution at room temperature for 10 min at 1100 rpm.
7. Discard the supernatant by aspirating it off carefully with a Pasteur pipette (Fig. 1B).
8. Resuspend the cell pellet in 20–100 µl of DPBS. Adjust final volume to achieve a cell concentration of about 5000 cells per µl (*see* Note 4).
9. Fill an insulin syringe with up to 10–20 µl of the cell suspension to be injected in the ear.

3.2. Intradermal Injection in the Mouse Ear

1. Anesthetize the mouse using a mixture of 4–5% isoflurane and oxygen under a steady flow (*see* Note 2).
2. Place the mouse on a heating pad set to 37°C to maintain its body temperature at 37°C throughout the procedure (*see* Note 5).
3. With a cotton-tip applicator, gently apply artificial tears to both eyes to prevent dryness.
4. While the mouse is lying flat on its belly, insert the conical end of a 1.5 mL microcentrifuge tube inside the ear such that the mouse ear is wrapped flat against the tube wall (Fig. 1C).
5. Once the mouse ear is properly and firmly positioned, gently rest the needle of the insulin syringe on the mouse ear with the bevel up (Fig. 1C).
6. Carefully pierce the mouse skin while maintaining the needle bevel up as flat as possible, almost parallel to the ear (*see* Note 6).
7. Slowly inject all or part of the syringe content.
8. A transient bulge should be visible immediately after the injection.
9. Slowly remove the needle to minimize the loss of some of the injected cell suspension.
10. Allow the mouse to recover on the heating pad.
11. Imaging can start on day 1 after injection and/or whenever desired thereafter.
12. Tumor growth under the ear skin can be monitored with a UV hand-held lamp (Fig. 2).

3.3. Pre-Imaging Preparation of the Mouse Ear

1. If labeling of the blood vessels is desired (optional), red lectin can be injected in the vein at the base of the dorsal side of the mouse ear (Fig. 3A) using an insulin syringe and the microcentrifuge tube holding method described above (Fig. 1C) (*see Note 7*).
2. Removal of the hair on the mouse ear can also be performed at this point to facilitate later imaging by 2PE (optional for nude mice).
3. Using a PBS pre-moistened cotton-tip applicator, carefully apply the hair removal cream to the dorsal side of the mouse ear (Fig. 3B).
4. After 1–2 min, gently remove the cream with a wet cotton-tip applicator (*see Note 8*).

3.4. Mouse Ear Mounting onto the Imaging Platform

1. The custom 3D-printed stage holder (Fig. 4A) is placed directly on top of the 37°C heating pad on the microscope stage (*see Note 3*).
2. Place 2 strips of double-sided tape on each side of the slit of the ear stage (Fig. 4B).
3. Carefully position the anesthetized mouse in the 3D-printed holder.
4. Maintain a continuous flow of low dose anesthesia (2% isoflurane and oxygen) (*see Note 5*).
5. Using flat curved forceps, gently pull the mouse ear through the slit and secure it flat on the tape (*see Note 9*).
6. Place a small drop of water on top of the ear to keep it moist.
7. Place a 50 mm WillCo-dish glass bottom dish on top of the ear and fill it with water (Fig. 4B).

3.5. Intravital 2PE Imaging

1. Carefully lower the 20X water objective into the water-filled viewing chamber (*see Note 10*).
2. Keep monitoring anesthesia and mouse body temperature closely (*see Note 11*).
3. Identify the location of the tumor and start imaging by turning on live preview.
4. Imaging settings will vary depending of the 2PE microscope used. We typically use the following settings on a 2PE Leica DM6000 CFS microscope equipped with 2 NDD HyD detectors: 832 pixels × 832 pixels resolution; 24 kHz scan frequency (6 lines and 20 frames averaging); digital zoom 1–4X; 169 μm × 169 μm scan field area.
5. Sequentially scan the 2 fluorophores with the appropriate 2PE wavelength: 910 nm for GFP and 800 nm for DyLight® 594 Lectin (Fig. 5).

6. Imaging can be performed for several hours or days later.
7. At the end of the imaging session, gently remove the WillCo-dish glass bottom dish and the mouse ear with a moist cotton-tip applicator.
8. If the intent is to keep the mouse for further imaging, let it recover on the heating-pad.
9. If it is to be euthanized, follow official regulations and guidelines approved by your institution.

4. Notes

1. Maintenance and surgical procedures were consistent with UCLA Animal Research Council Guidelines.
2. Federal law restricts this drug to use by or on the order of a licensed veterinarian.
3. The custom-built 3D-printed ear-imaging stage platform serves to shield the imaging from artefacts induced by the breathing motion and heartbeat of the mouse (9).
4. It is especially important to obtain a single-cell suspension as any remaining cell aggregates will clog the injection needle.
5. We recommend monitoring the mouse body temperature during and after anesthesia using an animal rectal probe (World Precision Instruments, cat. no. RET-3) as it can drop very quickly.
6. If the injection angle is too steep, you may risk piercing through both sides of the ear.
7. Intravenous injections can also be administered *via* the tail vein but higher doses of lectin are needed.
8. Always apply a minimal amount of the depilatory cream for the shortest period of time that's necessary to satisfactorily remove the excess hair. The ear skin is extremely thin and leaving the hair cream for too long could cause inflammation and damage to the skin. The cream must be removed thoroughly but gently with a wet cotton-tip applicator.
9. It is essential to move the body of the mouse at the same time the ear is pulled through the slit to avoid tearing apart the thin skin. Also, great care should be taken to make sure the ear lays flat on the tape (no folds) to make a flat surface for the coverslip.
10. Periodically check the water level as it will eventually evaporate over time.
11. When imaging for several hours, it is imperative to monitor the levels of both oxygen and isoflurane in the vaporizer as not to run out of anesthesia and have to deal with an awakening mouse in the middle of an experiment.

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Fig. 1.

Procedure for intradermal injection of human melanoma cells in the mouse ear. **(A)** Human C8161 melanoma cells expressing GFP are grown into a semi-confluent mono-layer. Scale bar, 50 μm . **(B)** After trypsinization, C8161 cells are centrifugated and collected in a 1.5 mL microcentrifuge tube. Scale bar, 100 μm . **(C)** While the mouse is anesthetized and laying onto a heating pad, the conical end of 1.5 mL microcentrifuge tube is inserted inside the ear. 10–20 μL of the cell suspension is loaded into an insulin syringe equipped with a 28G^{1/2} needle. The needle is held as flat as possible bevel up and the cells are injected into the dermis of the anesthetized mouse. A transient bulge should be visible immediately after the injection. Scale bar, 1 cm.

Fig. 2. Tumors arising from the injection of C8161 human melanoma cells after 2 weeks. **(A)** Macroscopic bright-field image of site of injection (black arrow). **(B)** Fluorescence image showing the formation of tumors under the ear skin (green arrows). Scale bars, 1 cm.

Fig. 3. Pre-imaging preparation of the mouse ear. **(A)** Macroscopic bright-field image of the vasculature of the mouse ear showing the site of Dylight594-Tomato Lectin intravenous injection to label blood vessels (red arrow). **(B)** Close-up view of the mouse ear showing the green melanoma tumors under the skin upon UV exposure (green arrows). The dotted rectangle denotes the area to apply cream for hair removal before 2PE imaging. Scale bars, 5 mm.

Fig. 4. Procedure for immobilizing the mouse ear for intravital imaging. **(A)** 3D-printed plastic mouse ear stage. **(B)** Photograph of the 2PE imaging setup with the mouse ear fastened onto the stage. The ear is gently pulled through one of the slits and secured in place with double-sided tape. A drop of water is placed on top of the ear to prevent dehydration before affixing a 50 mm WillCo-dish glass bottom dish that provides a flat surface for imaging and a reservoir for the 20X water objective. The objective is then lowered in the water-filled viewing chamber. The heating pad sits directly under the plastic stage insert.

Fig. 5:
Two-color intravital imaging of melanoma tumors in the mouse ear by 2PE microscopy. **(A)** Optical section through about 70 μm of dermis showing melanoma tumor cells expressing GFP. **(B)** Blood vessels labeled with Dylight594 Tomato Lectin. **(C)** Overlay image of GFP and Tomato Lectin. In these figures, it is possible to observe a clear angiotropism of individual melanoma cells spreading along the vessels (10). Scale bars, 50 μm .