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Imaging Tumor Cell Movement In Vivo

David Entenberg^{1,3}, Dmitriy Kedrin^{1,3}, Jeffrey Wyckoff¹, Erik Sahai², John Condeelis¹, and Jeffrey E. Segall¹

¹Albert Einstein College of Medicine, Bronx, New York

²London Research Institute, London, United Kingdom

Abstract

This unit describes the methods that we have been developing for analyzing tumor cell motility in mouse and rat models of breast cancer metastasis. Rodents are commonly used both to provide a mammalian system for studying human tumor cells (as xenografts in immunocompromised mice) as well as for following the development of tumors from a specific tissue type in transgenic lines. The Basic Protocol in this unit describes the standard methods used for generation of mammary tumors and imaging them. Additional protocols for labeling macrophages, blood vessel imaging, and image analysis are also included.

Keywords

imaging; metastasis; mammary tumor; image analysis

INTRODUCTION

Understanding of the contribution of genetics to cancer has expanded greatly over the past several decades. However, studies of the functional significance of changes in oncogenic gene expression in vivo, as well as the effects of drugs on the oncogenic targets in vivo at the cellular level, have been limited by lack of technologies and associated techniques that would allow for dynamic high-resolution analysis. Moreover, the invasiveness of primary tumor cells makes a significant contribution to malignancy. Local invasion can enable tumor cells to spread from the primary tumor through neighboring connective tissues, and is responsible for poor patient prognosis in tumors that cannot easily be surgically removed (e.g., glioblastoma and head and neck cancer). Local invasion can also be important for the spread of tumor cells from the primary tumor into blood vessels or lymphatics in many other tumor types, including breast cancer. Although in vitro studies of cell motility and invasion are useful starting points for beginning to understand the mechanisms that regulate invasiveness, the complexity of the in vivo microenvironment, with its wide variety of cell types, vessels, and extracellular matrix, is not yet possible to reproduce in vitro. Thus, it is important to evaluate tumor cell invasiveness using in vivo assays. In particular, the ability to image tumor cells as they are invading and interacting with stromal tissue allows the direct evaluation of tumor cell motility and opens up experimental evaluation of specific mechanisms for tumor cell invasion.

This unit describes the methods that the authors of this unit have been developing for analyzing tumor cell motility in mouse and rat models of breast cancer metastasis. Rodents

are commonly used in order to provide a mammalian system both for studying human tumor cells as xenografts in immunocompromised mice and for following the development of tumors from a specific tissue type in transgenic lines. Basic Protocol 1 describes the standard methods used for the generation of mammary tumors and imaging them. Additional protocols for simultaneous multi-fluorophore imaging (Alternate Protocol 1), microscope setup (Support Protocol 1), labeling vasculature and macrophages (Support Protocols 2 and 3), second harmonic imaging (Support Protocol 4), and image analysis (Basic Protocol 2 and Support Protocol 5) are also included.

NOTE: All protocols using live animals must be reviewed and approved by the local Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL 1 GENERATION AND IN VIVO IMAGING OF MAMMARY TUMORS

MTLn3, a highly metastatic rat mammary adenocarcinoma cell line, is often used by the authors' laboratories (Neri et al., 1982). The cells must be transfected with a fluorescent protein so that they may be imaged *in vivo*. Custom-built, and increasingly, commercial systems may come with multiple simultaneously acquiring detectors. These detectors give the ability to image several colors at one time, enabling a fluorescent tumor cell line to be used in combination with other fluorescently labeled cells or volume filling dyes (Fig. 19.7.1). The choice of fluorophores must thus be made carefully. While there are many options, the optimal choice must be made based upon both the number and type of cells and dyes to be imaged, as well as the filter sets present in the microscope system. The authors of this unit have found one combination of filters that have proven to be extremely versatile and allow many combinations of fluorophores to be imaged with good spectral separation. These are discussed further in the microscope setup protocol. Even in cases where significant spectral overlap exists, there are techniques which may be employed to separate the signals when the fluorophores are well spatially separated. This technique is described in Alternate Protocol 1.

Finally, most two-photon excitation spectra are very broad, covering nearly 100 nm in width. This, in combination with some fluorophores having long Stokes-shift emission spectra, enables a select few fluorophores that cover a good portion of the visible spectrum (blue, green, yellow, orange) to be excited with a single wavelength (Kawano et al., 2008; Tillo et al., 2010). These combinations often utilize a large excitation peak below 760 nm found in many fluorescent proteins and, while this technique can prove to be useful, care must be taken to validate this use of the proteins, as excitation at these high-energy, low wavelengths can cause significant photobleaching and damage to cells.

A more robust and flexible approach utilizes multiple laser beams to excite fluorophores separately. This approach can even employ an optical parametric oscillator (OPO) to extend the wavelength range beyond the portion of the visible spectrum mentioned and into that of the red and far-red probes which have recently been developed (Drobizhev et al., 2009; Piatkevich et al., 2010). Use of lower energy far-red wavelengths has the added benefit of lower photobleaching, phototoxicity, reduced scattering, and deeper penetration.

For any fluorescent protein utilized, the cell lines used must stably express the protein, because the growth that occurs to form an imageable tumor involves many cell generations. The following protocol is specifically used for MTLn3 and its derivatives. A discussion of alternatives for other lines is provided in (Wyckoff et al., 2011).

Female 5- to 6-week-old BALB/c SCID mice or 7- to 8-week-old Fischer 344 rats are used. The animals are injected with 0.1 ml of cell suspension in the mammary fat pad, under the second nipple anterior from the tail, and tumors are allowed to grow for 3 to 4 weeks.

Materials

MTLn3 cells (available from authors; jeffrey.segall@einstein.yu.edu)

MTLn3 growth medium (see recipe)

2 mM EDTA (Invitrogen, cat. no. 15575-038) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS; Invitrogen; also see *APPENDIX 2A*)

Dulbecco's phosphate-buffered saline (with calcium and magnesium; DPBS; Invitrogen; also see *APPENDIX 2A*)

Female 5- to 6-week-old BALB/c SCID/Ncr mice (National Cancer Institute), Fisher 344 rats (The Jackson Laboratory), or transgenic mouse tumor models (e.g., MMTV-pyMT; The Jackson Laboratory)

70% (v/v) ethanol

Isoflurane, USP (AERRANE, Baxter)

15-cm tissue culture dishes (Falcon)

15-ml conical polypropylene centrifuge tubes, sterile

Cell scrapers

Centrifuge (e.g., Sorvall GLC-1)

1-ml syringes with 25-G needles for cell injections

Oxygen/anesthesia apparatus (Forane Vaporizer, model 100, from SurgiVet)

100% compressed oxygen tanks to be used in combination with vaporizer

Surgical instruments

In vivo imaging microscope setup (Support Protocol 1)

Multiphoton apparatus setup (Support Protocol 4)

Surgical tape (optional)

Additional reagents and equipment for tissue culture techniques including counting cells (*UNIT 1.1*), housing of immunocompromised mice (Donovan and Brown, 2007), imaging blood vessels (Support Protocol 2 or 3; optional), inhalant anesthesia of rodents (Donovan and Brown, 2001), euthanasia of rodents (Donovan and Brown, 2006a), and image analysis and quantitation (Basic Protocol 2)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be carried out in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Grow and prepare cells for injection

1. Grow MTLn3 cells in MTLn3 growth medium to 50% to 70% confluence in a 15-cm tissue culture dish.
 UNIT 1.1 describes basic cell culture techniques.
2. Under sterile conditions, rinse the dishes three times, each time with 10 ml of 37°C sterile CMF-DPBS containing 2 mM EDTA.
 Since the cells are tightly attached to the dish, it is possible to aspirate all of the medium and gently add fresh medium to the side of the dish without losing many cells.
3. Add 12 ml of CMF-DPBS/2 mM EDTA and place at room temperature to detach cells.
 Depending on the thoroughness of the previous rinse steps, this can take from 10 to 30 min. Once 75% of the cells are detached after tapping the dish on a surface, the cells are ready to be collected.
 It may also be possible to use trypsin/EDTA without adverse effects on the cells. In this case, care must be taken not to over-trypsinize. Neutralize the trypsin with some serum, and then wash the cells. Also see UNIT 1.1.
4. Add 6 ml DPBS containing Ca and Mg and detach the remaining cells by gently rinsing.
5. Collect the cells into two 15-ml sterile tubes, scraping the dish to collect the matrix and adding it to the tube as well. Take an aliquot and count cells using a hemacytometer (UNIT 1.1).
 Cell counting may be performed during the subsequent centrifugation step.
6. Centrifuge cells 5 min at 180 to 200 × *g*, room temperature.
7. On the basis of the cell count performed on the aliquot taken in step 5, determine the appropriate volume for resuspending the pellet to obtain a cell density of ~1 × 10⁷ cells/ml. Resuspend cells in sterile DPBS at 1 × 10⁷ cells/ml, and recount (UNIT 1.1) to confirm actual density.
 Dilutions as low as 5 × 10⁶ cells/ml can also be used, which produce tumors slightly more slowly.
8. Transfer cell suspension to a sterile 1.5 ml microcentrifuge tube and keep on ice until injection.

Inject cells for xenograft

9. With two people working together, one to hold the animal and another to inject the cells using a 1-ml syringe fitted with a 25-G needle, prepare for injection of cells.

As one person prepares the mouse by picking it up by the back of the neck and holding the back leg with another hand, the second person takes the cell suspension off the ice and fills the syringe.

10. Spray the area at the second lowest nipple with 70% ethanol and insert the needle subcutaneously at a shallow angle in the mammary fat pad. Inject 100 μ l of cell suspension and notice a slight bulge in the skin above the injected area.

At this step it is important that the needle only penetrate the skin and stay within the mammary fat pad, so no cells spread into the peritoneum. To ensure this, keep the angle of injection very shallow.

Injection of Fischer 344 rats is done in the same manner, but injection volume can be increased to 150 to 200 μ l.

11. Keep the animals in the barrier-room facility (Donovan and Brown, 2007) until the tumors are large enough to image (0.5 to 1 cm diameter for mice or up to 1.5 cm for rats, typically 3 to 4 weeks). Check tumor size on a regular basis to make sure it is growing.

The tumor should be palpable after 1.5 weeks. After 3 to 4 weeks, the tumor should be about 5 to 7 mm in diameter (mice) or 1 cm in diameter (rats), at which time the animals are ready to be imaged.

Smaller tumors are harder to image due to lack of good imaging surfaces. This is because the tumor has not yet grown through to the edge of the fat pad. Adipocytes significantly interfere with multiphoton imaging by acting as a lens and distorting the point spread function. Larger tumors tend to become necrotic, also destroying adequate imaging surfaces on the tumor. Additionally, large tumors can invade the peritoneum, preventing skin-flap surgery from being adequate for imaging purposes.

Many groups have used implantable windows (Jain et al., 2011) to avoid these issues and to enable serial imaging over significantly longer time periods while minimizing artifacts introduced by surgery. For mammary tumors, the authors of this unit have developed an implantable window which has proven to be highly useful in combination with photo-convertible proteins (Gligorijevic et al., 2009). Alternatively, transgenic animals, such as those described by (Wyckoff et al., 2004), can be used to image tumors. For this purpose, animals are grown until tumors reach a size adequate for imaging. Palpable tumors are large enough to image in transgenic animals, but due to staging of tumor progression, intravasation, and metastasis do not occur generally until late-stage carcinoma. Therefore, each tumor model will be different in determining the best time for imaging.

Prepare animal for imaging

12. Place the animal under anesthesia with 5% isoflurane in O₂ using an oxygen/anesthesia apparatus.

Isoflurane is to be administered to the animal via an oxygen mask prior to and during surgery, and while collecting imaging data.
13. *Optional:* Inject a fluorescent-dextran to image blood vessels and perivascular macrophages (see Support Protocols 2 and 3).

14. Place the animal on the surgery stage and swab the tumor area with 70% ethanol. Perform a medial skin incision to expose the tumor.
15. Clear the area around the tumor from fascia and fat while maintaining blood vessel supply as much as possible and keeping bleeding to a minimum.

This must be done carefully and meticulously to avoid destroying the vasculature or cutting into the tumor itself.

16. Transfer the animal to a prewarmed imaging stage (30°C) with the exposed tumor placed on the coverslip (objective) for imaging.

As mentioned above, fat cells act as lenses within the tumor and distort the tight focus required for efficient multiphoton excitation. Thus, fat should be removed as much as possible, without disrupting the blood flow to the tumor, to allow for better image quality.

Proper fixturing of the tumor to be imaged is crucial to ensure stable, drift-free imaging over several hours. A fixturing technique developed by the authors is illustrated in Figure 19.7.2. After surgical exposure of the tumor, the surrounding skin is stretched across a $0.75 \times 0.5 \times 0.125$ -in. rigid plastic-backed piece of foam (a plastic-backed computer mouse pad works well for this application) and the edges attached using surgical staples. After the mouse is placed on the microscope stage, a $0.25 \times 0.25 \times 1.5$ -in. piece of rubber is placed between the tumor and the mouse's body along the body line. This isolates the skin flap from the body and reduces movement of the sample due to breathing. To avoid compression of the tumor on the microscope stage, two small pieces of rubber ($0.5 \times 0.75 \times 0.25$ in.) are placed at either end of the foam to create a bridge suspending the tumor. The ends of this bridge are then secured with two longer pieces of rubber to the microscope stage and fixed in place using adhesive tape over the foam and rubber. An additional benefit of this technique is that the rubber forms a chamber which can be filled with PBS to keep the tissue hydrated and maintain optical contact with the coverslip. The open side can then be blocked with one additional piece of rubber to reduce evaporation of the PBS.

17. During imaging, sequentially lower the isoflurane level in steps of 0.25% every 20 to 30 min to maintain a constant low breathing rate.

If breathing is too rapid, distortions might make acquired data difficult to interpret. To avoid this, the anesthesia levels should be adjusted and/or the position of the animal on the imaging platform should be adjusted to minimize drift and breathing artifacts. With time, less anesthesia will be required to maintain a low breathing rate. Typical final isoflurane levels are 0.75% to 1.25%.

Even with excellent fixturing, maintenance of breathing and minimization of drift and movement of the animal is crucial for successful time-lapse data collection. A balance must be struck between the desire for deep anesthesia to minimize motion due to breathing and shallow anesthesia to minimize impact on the vitality of the mouse and the cells of interest. Too much anesthesia will kill the animal. The best way to strike this balance is by using inhalable anesthesia and precisely controlling its level. This ability to manipulate

the breathing is why using inhalable anesthesia is a better choice than injectable anesthesia.

The impact of anesthesia on the physiology of the animal goes beyond just breathing, however. For example, mice and rats lose their ability to regulate body temperature under anesthesia, and the use of purified compressed oxygen means that the inhaled gases contain no moisture. Both of these can have the effect of throwing the animal far out of homeostasis. For this reason, it is important, particularly for longer time-lapse imaging sessions, to provide humidified heat (Ewald et al., 2011).

Perform intravital microscopy

Multiphoton microscopy produces images that are optical sections of the tissue. Thus, each image is from a single x - y imaging plane. Full, three-dimensional information about the tumor is gathered using a z -series of individual x - y images collected by moving the height of the stage or the objective lens.

Many systems come equipped with a variety of objectives. While this often makes sense for wide-field illumination systems where the magnification and resolution are completely determined by the objective lens, in point scanning systems, these parameters can also be controlled by the deflection range of the scanning system and the number of pixels acquired. Thus, a 20 $\times$ objective lens with the same numerical aperture as a 40 $\times$ lens can offer the same image magnification and resolution if the scan parameters are chosen correctly. The use of a single low-magnification, high-numerical-aperture lens significantly simplifies the optical setup of the laser beam path and can eliminate beam-expansion lenses, which must resize the beam to properly fill the back aperture of each lens. Further, use of this lens allows rapid switching between histologic, large-field-of-view-type imaging and high-magnification-high resolution single cell imaging. Only the ability to visualize samples at the higher magnification through the ocular is sacrificed. For intravital imaging setups, this is often not a problem, as the amount of scatter from tissue limits the usability of the ocular anyway.

As with all types of microscopy, the full resolution capabilities of a microscope can only be attained by acquiring data under Nyquist conditions. For a 25 $\times$ 1.05-NA objective lens acquiring a 500 $\times$ 500- μ m field of view, this translates into acquiring images with $\sim$ 1500 $\times$ 1500 pixels and z -step sizes of 0.5 μ m. Since most multiphoton setups acquire images at about 1 frame per second, using these conditions to acquire a z stack of 150 μ m would require $\sim$ 5 min, 300 images, and 150 MB of storage space per channel acquired. Thus, trying to acquire full-field 4-D image sets (z stack–time lapses) at this resolution proves to be quite impractical and will not match the time-lapse rate required to capture cellular movement within the tissue ($\sim$ 4 μ m/min). We typically acquire full-field images using 512 $\times$ 512 pixels and 5- μ m z steps to capture overall cellular dynamics and interactions, and then switch to the Nyquist conditions mentioned above on any identified sub-regions of interest.

18. Focus in on fluorescence of the tumor, looking for areas with flowing blood vessels by viewing through the eyepiece in epifluorescence mode.

Since tumors often consist of densely packed, bright cells when viewed through the ocular, it can be difficult to distinguish individual cells and details through the eyepiece, and hence difficult to identify regions of interest. A field diaphragm present in the epi-illumination path can be used reduce the overall brightness of the sample as well as the amount of scattered, out-of-focus light. Stopping down this aperture can thus reveal imageable regions close to the surface of the tumor.

19. Upon finding an area that has bright fluorescent cells close to the surface and flowing vessels, switch to imaging via the multiphoton mode.

For one-color GFP imaging, the animal is placed under anesthesia as described above and imaged. On average, each imaging sequence lasts 20 to 30 min, and the same animal can be maintained on the microscope for 4 hr or more.

20. While imaging in multiphoton mode, move around the tumor until a good imaging area is obtained.

Good imaging fields for cell invasion and intravasation are areas around flowing vessels with well-defined cell boundaries and extracellular matrix. Flowing vessels can be visualized by labeling with a fluorescent dextran or by viewing vessels through the oculars of the microscope.

The authors find that focusing at the outer tumor surface (where cells are just invading the matrix) and generating a z-series stepping into the tumor until the image degrades to a point where cell morphology is not well defined, gives the best images for analysis. Many systems now can vary the laser power or detector gain as a function of z-position (known as “progressive” gain or laser power), allowing the use of more power or higher gain to image greater depths without damaging or overexposing the tissue closer to the surface.

21. For the outermost plane of the z-series, focus on the outside of the tumor by visualizing the collagen fiber network in the second harmonic channel (Fig. 19.7.3A).

Depending on the goal of the imaging session, the size of the z-series that is selected is constrained by: (1) the desired quality of each image, (2) the time-lapse interval desired between images at a particular plane, and (3) the z-axis resolution desired. The quality of each image is determined by the brightness of the fluorescence and the gain setting of the detectors; higher gain creates higher noise.

This noise may be countered by frame or line averaging. For brightly labeled tumors, no averaging may be needed, but for dimly labeled tumors, 2, 4, or even 8 averages may be required and hence become time limiting. The time-lapse interval required is determined by the dynamics of the structures that are being imaged. Subcellular shape changes at high-magnification may require 10- to 30-sec time intervals. General tumor cell tracking using lower magnification may be adequate at 1- to 3-min intervals.

22. Set up the time-lapse z-series (generally 5 μm steps for 150 μm and 1 min intervals for 30 min) and start collecting images.

For time-lapse multiphoton imaging, as many z planes should be taken as is possible given the above constraints on image quality and time-lapse interval. Especially during the first 30 min of imaging, there can be significant (typically $>10 \mu\text{m}$) drift. This z-series collection can compensate to some degree for this drift, but in the authors' experience, visualizing a cell traveling from one plane in a z-series to the neighboring plane usually generates an artifact for the time interval during which the shift occurs. For detailed tracking of individual cells,

2.5- to 5- μ m step sizes are best. For experiments in which one wishes to sample through as great a depth as possible to compare behavior at the periphery versus the interior of the tumor, 10- μ m or larger steps may be required.

23. Euthanize the animal (Donovan and Brown, 2006a) at the end of data collection.
24. Perform image analysis (Basic Protocol 2).

SUPPORT PROTOCOL 1 IN VIVO IMAGING MICROSCOPE SETUP

The authors use one of two microscope setups, depending upon the application. The first is a commercially available multi-photon microscope (FluoView MP, Olympus) equipped with four simultaneously acquiring detectors on an inverted IX81 stand. This system utilizes a 15-W, computer-controlled femtosecond-pulsed laser (Mai Tai, Spectra Physics) equipped with a group velocity dispersion (GVD) compensator. This laser gives an output of greater than 500 mW over the range from 690 to 1040 nm. As mentioned above, a 25 $\times$ 1.05-NA objective lens allows a wide range of magnifications to be attained at high resolution.

The second microscope utilized by the authors is a custom-built two-laser multiphoton microscope (Entenberg et al., 2011) built around an inverted microscope stand (IX-71, Olympus) and similarly equipped with four simultaneously acquiring detectors and a GVD compensator. The two lasers on this system include both a manually tuned, 15-W, femtosecond-pulsed laser (Millennia-Tsunami, Spectra Physics) whose output can be used between 780 and 1040 nm, and a computer-controlled femtosecond pulsed laser (Mai Tai, Spectra Physics) whose output is either used directly (780 to 1040 nm) or as a pump beam for an OPO with an output in the range of 1100 to 1600 nm. As discussed above, the presence of two lasers in this system gives a greater degree of flexibility in wavelength and intensity tuning for imaging multiple fluorophores simultaneously, access to more simultaneous combinations of probes, and deeper penetration depth.

On both systems, an environmental enclosure with a heated air blower surrounds an automated *x-y* stage. This is important not only for maintaining the vitality of the animal, as mentioned above, but also because, as the body temperature falls from its physiologic norm, a reduction of cell movement is observed. This is true for tumor cells and is particularly so for other cell types such as immune cells. Care must be taken, however, not to maintain too high a temperature, as this increases the animal's metabolism, reducing the efficacy of anesthesia and causing the animal to hyperventilate and awaken. For these reasons, we recommend maintaining the chamber temperature at 30°C.

For microscope systems without environmental enclosures, an alternative heating method that can be employed is to cover the animal during imaging with a heating pad maintained at temperature by a circulating water flow. Use of electric heating pads is discouraged unless tight control of the temperature is maintained using PID temperature controllers to eliminate the large temperature swings common to these devices.

The filter-set combination that the authors have found most versatile consists of (all from Semrock): 447/60 (SHG and blue), 520/35 (green), 580/60 (red), and 697/75 (far red). The multiphoton instrumentation can be calibrated so that the images taken on separate days are of the same intensity, both by measuring and adjusting the laser intensity at the microscope input before each imaging session and by acquiring a *z* stack of calibration fluorescent beads and comparing their signal to that of a standard *z* series performed when the microscope has been well aligned. Laser power and gain settings to be used during imaging sessions are determined by the brightness of the fluorophores utilized. An image should have a good dynamic range to be considered usable for data interpretation. Depending on the precise

measurements and depth of image stack desired, it may be useful in some cases to saturate the signal from cells in the uppermost slice to allow adequate signal from cells in deeper slices or to employ progressive gain or laser power.

SUPPORT PROTOCOL 2 LABELING VASCULATURE AND MACROPHAGES

To provide information about blood flow in and around the tumor, the blood vasculature can be labeled by tail-vein injection of a fluorophore such as a fluorescently labeled dextran or quantum dots. When quantum dots or a large molecular weight dextran is used, the fluorophores remain in the blood stream and are only slowly cleared. This enables clear labeling of the vasculature for the duration of the experiment. Use of lower-molecular-weight dextrans (~70 KDa range) allows for escape of some of the dye from the blood stream over time. This dye is then picked up and aggregated by perivascular macrophages within the first 2 hr after injection. At longer times after injection, other macrophages pick up the dye as well.

Materials

Mouse with xenograft-induced tumor (Basic Protocol 1)

70% (v/v) ethanol

20 mg/ml dye labeled-dextran (70 kDa, Invitrogen) in DPBS (Invitrogen; also see [APPENDIX 2A](#))

Mouse restrainer (Donovan and Brown, 2006b)

1-ml syringe with 26-G needle

Superglue

Additional reagents and equipment for mouse restraint (Donovan and Brown, 2006b)

1. Warm up the tail vein of the tumor-bearing animal by immersing the tail for 1 to 2 min in warm (50° to 60°C) water.

This causes the tail vein to dilate so that a 26-G needle can be inserted. The animals can also be placed 4 to 8 in. (~10 to 20 cm) away from a lamp with a 150-W light bulb for 5 min.

2. Place mouse into restrainer and close the restrainer so the tail is easily accessible.
3. Wipe the tail with 70% ethanol and rotate it to position the lateral vein for injection. Stabilize the vein by holding the tail between thumb and middle finger and securing the vein with the index finger.
4. Prepare a 1-ml syringe with a 26-G needle containing 200 μ l of 20 mg/ml fluorescent dextran. Inject the mouse by orienting the bevel of the needle facing upward and piercing gently through the skin parallel to the vein, 1 to 1.5 in. (~2.5 to 4.25 cm) from the base of the tail.

Successful insertions into the vein are indicated by easy sliding of the needle into the vein and injection of the solution without resistance or accumulation at the needle tip.

5. Inject slowly so that the 200 μ l of the dextran solution enters the vein.

If a large force is required for injection of the solution, the needle is not correctly inserted into the vein. In this case, attempt additional

injections, moving slightly closer to the base of the tail with each attempt.

6. Place the mouse back into the cage for later imaging.

The animals are left for 1 to 2 hr. Because the ability to image macrophages depends on their ability to take up dextran molecules by phagocytosis, the authors have found that this time interval is adequate for labeling.

7. Image the tumor.

See Basic Protocol 1 for imaging steps.

SUPPORT PROTOCOL 3 BLOOD VESSEL IMAGING USING AN INDWELLING CATHETER

In the preceding protocol, a fluorescent marker is injected prior to the start of imaging. However, in some cases it can be advantageous to begin perfusion after imaging has begun, or to perfuse an additional dose of fluorescent marker some time after the initial dose. For these cases, an alternate procedure can be performed employing an indwelling intravenous catheter in either the jugular vein of the rat or mouse (described in the protocol below) or in the tail-vein. After introduction of the marker into the circulation, it can be visualized using the techniques described in Basic Protocol 1.

This procedure does require some practice because catheter insertion is not trivial. It is also important to note that since multiphoton imaging requires an additional nonsurvival surgery, the incision for the catheter insertion should be kept to a minimum. Alternatively, direct catheterization of the tail vein can be performed.

Additional Materials (also see Basic Protocol 1)

Mouse with xenograft-induced tumor (Basic Protocol 1)

Heparinized PBS solution (Baxter, cat. no. FKB0953G)

Indwelling tail vein catheter (no. MTV-01, SAI Inc.; <http://www.sai-infusion.com>)

1-ml syringes

Waterproof marker (e.g., Sharpie)

Prepare catheter

1. Prepare indwelling catheter by pulling out the guide wire and attaching a 1-ml syringe filled with heparinized PBS.

A catheter designed for the tail vein is used, but it will be inserted into the jugular vein for this protocol.

Insert the catheter

2. Insert the catheter into the guide needle so that 4 mm of catheter extends out the tip of the needle. Mark the catheter using a Sharpie where it inserts into the rear of the needle, as a guidepoint for the depth of insertion in to the vein.
3. With the animal anesthetized using 5% isoflurane (see Basic Protocol 1), spray the right thorax area with 70% ethanol.

4. Make a small incision to expose the left external jugular vein and the overlying muscle layer.
5. Insert the guide needle through the muscle into the overlying jugular vein.
6. Push the catheter 4 mm into the vein and, while holding the catheter in the vein with a small forceps, pull out the guide needle over the catheter.
7. Place a drop of glue (e.g., Superglue) over the site of insertion of the catheter into the vein to hold it in place.

Test the catheter

8. Allow the glue to dry for several seconds and push a few microliters of PBS through the syringe to see if there is any leakage (an indicator of a bad insertion).
9. Try drawing blood to make sure that the catheter is patent.
If correctly inserted, blood should be drawn into the catheter. However, in some cases, the catheter tip will be against the wall of the vessel and it may be difficult to draw blood.

Inject the dextran

10. Prepare another 1-ml syringe filled with 20 mg/ml fluorescent dextran. Switch the catheter from the PBS syringe to the 20 mg/ml fluorescent dextran syringe and prepare the animal for imaging (Basic Protocol 1).
11. After an area containing a flowing blood vessel has been discovered, inject the fluorescent dextran into the vessel and observe the fluorescence by collecting a time-lapse image of the area (Basic Protocol 1).

SUPPORT PROTOCOL 4 SECOND HARMONIC FIBER IMAGING

A valuable benefit of multiphoton microscopy is the ability to use the second harmonic scattering effect (Zipfel et al., 2003) to generate images of extracellular matrix fibers (ECM) around the carcinoma cells of the tumors. The size, amount, and condition of the ECM fibers that are imaged can be determined by measuring the signal intensity and morphology of the images generated by the second harmonic (Ahmed et al., 2002). However, it is important to appreciate that such images label only a very restricted subset of matrix molecules that may be present. Typically, only large, type I collagen fibers are likely to be imaged. With alternative wavelength comparisons, elastic fibers may also be identified (Zipfel et al., 2003), but other structures including small fibers, basement membrane, fibronectin fibers, and other cell types are not imaged. The authors are able to excite the second harmonic signal with wavelengths of 760 to 960 nm and 1100 to 1250 nm, and because our work is typically more concerned with imaging of cells, the excitation wavelength is selected based on the cell to be imaged. The second harmonic image can typically be separated from the rest of the fluorophores using the channel subtraction technique described in Alternate Protocol 1.

BASIC PROTOCOL 2 MULTIPHOTON TIME-LAPSE IMAGE ANALYSIS USING ImageJ AND CUSTOM PLUGINS

Commercially available software applications such as Imaris and Volocity can be quite helpful in providing visualizations of 3-D time lapse movies and offer the capability of real-time rotation of time lapse 3-D stacks and changing perspectives. Thus, if the user's budget

allows, such programs can be quite convenient, but the specific details of data processing are program specific.

We have found ImageJ (Schneider et al., 2012), a free image-processing program developed by Wayne Rasband at the NIH, to be a tremendously valuable image analysis and manipulation program. ImageJ is written in Java, is open source, and has an ever increasing user base. In addition to the standard, built-in functionality of ImageJ, it is possible to write macros and plugins to customize and extend the capabilities of the application. The remainder of this protocol describes our use of ImageJ, which we typically use for analysis of 3-D time lapse data sets.

It is useful to have a computer with as much RAM as possible for generating movies of optical sections from 4-dimensional acquisitions (*z* series time lapses). A good guide is to have twice the total amount of RAM as the size of the data to be loaded. In addition to having RAM physically installed on the computer, ImageJ must be configured (under Edit → Options → Memory & threads) to be able to access this memory. For extremely large data sets, ImageJ has the capability of loading “virtual” stacks wherein the images are read off of the hard disk as they are needed for display. Caution should be exercised using this feature, however, because to utilize this feature the files must be accessed by ImageJ in a particular order (i.e., the naming of the files must match an expected convention) and some processing operations require that all of the images be loaded into memory at once anyway.

The discussion below involves three typical procedures that are performed:

1. Generating time-lapse movies from time-lapse *z*-series.
2. Eliminating *x-y* drift in time-lapse movies.
3. Marking areas of interest in time-lapse movies.

Generating Time-Lapse Movies from Time-Lapse *z*-Series

When image data are first loaded from disk into ImageJ, the data are stored in memory and presented to the user as a single stack of images. While this presentation is sufficient for a single *z* stack or a single time series within a single channel, it does not aid in the visualization of multi-dimensional image data (e.g., *z* stack, time-lapse, multi-channel data). For this, ImageJ has the capability of converting the stack of images into a “Hyperstack,” a logical ordering of the underlying stack into channels, *z* slices and timepoints. When properly defined as a hyperstack, the data may be easily navigated using sliders for time, depth, and channel located at the bottom of the image data.

The authors of this unit typically generate sequences of *z*-series data in which one *z* series is acquired every minute or so: a level of time resolution adequate for imaging even highly motile tumor and immune cells (up to 10 $\mu\text{m}/\text{min}$).

Procedure

1. Start ImageJ.
2. Import the data set into ImageJ.

The exact procedure for this step will depend on individual microscope image acquisition software. As an example, when data from the Olympus Fluoview are saved as separate tiff files, the data may be imported by dragging the folder onto the ImageJ program window and choosing to load the images as a stack from the pop-up window that results. A hyperstack is then created by selecting Image → Hyperstack

→ Stack to Hyperstack from the drop-down menu, specifying the number of channels, z-slices, and time frames, and selecting the sort order “xytzc.” For other systems, macros can be written to shuffle the z-series slices into time-lapse movies in each image plane.

3. Transform the data set into a hyperstack of the correct dimensionality.
4. Visually inspect each slice for moving cells and record their behavior patterns (e.g., movement on or off matrix fibers, interaction with host cells, movement towards blood vessels, etc.).

For sparse image data (images with just a few, or with well separated cells in each z-slice), this visual inspection process can be greatly sped up by using a maximum intensity projection of 2 or more z slices and inspecting the projected data. Regions of interest can be readily identified at a particular x, y, and t location in the projection and then relocated in the corresponding z stack within the full 4-D data set.

5. Use the Reduce Dimensionality function found under the Hyperstack pull down menu to extract a single z-slice as a separate hyperstack.

This function can also be used to reduce the number of time points or the number of channels as well.

6. Save the extracted hyperstack as a TIFF stack, or as an .avi movie.

Eliminating x-y Drift in Time-Lapse Movies

Philippe Thévenaz has written a valuable series of image registration plugins that can reduce/eliminate x-y drift from color time-lapse sequences (Thevenaz et al., 1998). This involves first downloading a plugin called TurboReg, and then downloading StackReg. In each case, the downloads are .zip files, which should be extracted to the plugins folder of ImageJ. This will generate two directories within the plugins folder named StackReg and TurboReg. It is necessary to restart ImageJ after these folders are created. To use the plugin, open the movie (only have one movie open at a time) from which drift is to be eliminated and then go to the plugins drop-down menu in ImageJ. Move to the StackReg line and click on the StackReg box that appears. Various transformations can be chosen, but we have found the default choice (rigid body) is most often best. More information is available at the download site (<http://bigwww.epfl.ch/thevenaz/stackreg>). Note that this plugin runs quite slowly: it can easily take a minute to step through a 30-frame movie. It registers each frame relative to the previous frame, and thus with time, black areas appear on the sides opposite the direction of drift of the movie. The program may not be able to accommodate rapid, large changes in position.

In cases with large position changes that StackReg cannot follow, one can first use TransformJ (Meijering et al., 2001; again available from the plugins section of the ImageJ Web site) to shift sets of slices of the movie in the x-y plane in order to reduce the jumps enough for StackReg to track them. Changes in z-axis are extremely difficult to compensate for, requiring more extensive z-series collection (perhaps every 1 to 3 μm , depending on the scale of magnification) and then manually assembling images from the appropriate planes of each z-series.

Items to put in Plugins folder—StackReg folder: <http://bigwww.epfl.ch/thevenaz/pointpicker/>.

TurboReg folder: <http://bigwww.epfl.ch/thevenaz/pointpicker/>.

Procedure

7. Start ImageJ.
8. Open movie to be registered.
9. Select StackReg from the plugins drop-down menu.
10. Select Rigid body transformation.
11. Wait until the progress bars in the ImageJ command bar stop appearing and the movie has stepped through all of its frames.

Marking Sites of Interest in Time-Lapse Movies

In order to extract statistical information from the image data, it is necessary to track objects as they move through the four-dimensional data set.

To this end, the authors have written a plugin, called ROI_Tracker (available upon request from the authors), which facilitates this extraction. The purpose of this plugin is to aid in the identification and marking of cells and other structures (regions of interest) within the four-dimensional data set, grouping these markings into “tracks” and then accumulating measurements associated with them. It provides the ROI average channel intensity and centroid location, track segment and cumulative lengths, and instantaneous velocity. These data can be exported for further statistical analyses. By choosing as one of the objects of interest a landmark object which is stationary within the sample volume, the statistics of the overall sample drift can be obtained and further analyses of motile cells can be referenced to this “drift track”. In this way, it may be possible to perform analyses without going through the x-y drift elimination procedure.

Items to put in Plugins folder—ROI_Tracker.jar file

Procedure

1. Start ImageJ.
2. Import the data set into ImageJ.
3. Transform the data set into a hyperstack of the correct dimensionality.
4. Select ROI_Tracker from the plugin drop-down menu.
5. Use a region of interest marker (point, ellipse, rectangle, or freehand) to outline or mark the object of interest
6. Click the Add ROI button.
7. Repeat for all time points and z slices containing the object of interest.
8. When ready to mark another object of interest, click the Add Track button and repeat steps 5 to 7.
9. When all tracks and points have been created, save the data by clicking the Save button and specifying a file name, and then export the data as a comma separated value (.csv) file by clicking the Export button and specifying a file name.

SUPPORT PROTOCOL 5 SEPARATION OF SPECTRAL OVERLAP

For fluorophores that have a narrow emission spectrum (e.g., quantum dots, Evans Blue), separation of signals simply consists of matching an emission filter to the fluorophore being

used. However, when multiple fluorophores with wide emission spectra are used together, the signal from a single fluorophore will tend to be present in an adjacent channel as well (e.g., CFP bleed-through into the green channel, YFP bleed through into the red channel). The authors' group has previously shown (Sahai et al., 2005) that when multiple fluorophores are imaged simultaneously (using either one or two lasers), it is possible, so long as the two signals are not spatially overlapping, to separate the signal from each source, even when there is significant overlap in their emission spectra. The process for separating these signals involves tuning the wavelength, laser power, and detector gains so that the signal levels for each fluorophore are equal. Under these conditions, the two channels may be subtracted from each other, and any signals present in both images (e.g., CFP) can be removed from the overlapped channel. The optimal imaging wavelength(s) for performing this separation is chosen by a desire for equalizing the fluorescence levels of the two fluorophores. For example, while CFP has its excitation maximum at 840 nm and GFP at 960 nm, the authors have found that imaging at 880 nm balances the fluorescence generated for these two fluorophores. Finally, laser power and detector gains must be set to fine tune the equalization. This is most easily done utilizing a channel overlay image on the microscope and adjusting the settings to attain a balanced color combination. For Blue-Green balancing, spectrally overlapping structures should look cyan; for Green-Red balancing they should appear yellow. The following example illustrates how to separate CFP, GFP, and SHG signals from the blue and green channels into three separate images.

Procedure

1. Start ImageJ.
2. Import the data set into ImageJ.

The exact procedure for this step will depend on individual microscope image acquisition software. Macros can be written to shuffle the *z*-series slices into time-lapse movies in each image plane.
3. Transform the data set into a hyperstack of the correct dimensionality.
4. Use the Split Channels function under the Image → Color drop-down menu to separate the channels into three hyperstacks.
5. Select the Image Calculator function found under the Process drop-down menu.
6. Subtract the blue channel image from the green channel image.

This will remove CFP objects from the green image and result in a GFP-only image. If desired, this resulting image may be renamed as the GFP image by selecting the Rename function under the Image drop-down.
7. Again, select the Image Calculator function found under the Process drop-down menu.
8. Subtract the green channel from the blue channel.

This operation removes all of the CFP objects from the blue channel. The presence of GFP objects in the green channel does not affect the resulting image because pixels with negative values after subtraction are set to zero in the resulting signed integer image. This image may be renamed as the SHG image.
9. Subtract the SHG image from the blue channel.

This operation removes all of the SHG objects from the blue channel.
This image may be renamed as the CFP image.

10. Merge the CFP, SHG and GFP images using the Merge Channels function under the Image → Color drop-down menu.

Each channel can now be assigned to a pseudo-color.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common stock solutions, see [APPENDIX 2A](#); for suppliers, see [SUPPLIERS APPENDIX](#).

MTLn3 growth medium

Minimal Essential Medium, α modification (α -MEM; Invitrogen) containing:

5% (v/v) fetal bovine serum (FBS; Fisher, cat. no. SH3008803IR)

1× penicillin/streptomycin (Invitrogen)

COMMENTARY

Background Information

In recent years, the use of multiphoton laser scanning microscopy, with its benefits of tissue penetration depth and high resolution, has shed light on many aspects of tumor microenvironment. Multiphoton fluorescence excitation uses a laser beam to focus photons of a wavelength two times longer than the excitation wavelength of the fluorophore at a single point (Wang et al., 2003; *UNIT 4.15*). This allows for greater penetration with less phototoxicity and bleaching during *z*-series collection.

Troubleshooting

Imaging tumors that are closer to the chest cavity is more difficult due to breathing possibly interfering with image collection. Animals with such tumors must be kept more sedated, and this may affect the length of the imaging session.

Data collection requires a large amount of hard drive space (500 MB to 1 GB/4D series), so make sure that the computer is up to date and able to handle large amounts of data for storage and transfer. A 64-bit processor computer is recommended.

Anticipated Results

Using the metastatic MTLn3 rat mammary adenocarcinoma cell line transfected with GFP (Wyckoff et al., 2000), and comparing to the nonmetastatic MTC cell line, it is anticipated that ~57% of blood vessels in tumors generated from the metastatic cell line have at least four cells oriented towards them, compared to ~24% for the nonmetastatic cell line. Other parameters, determined as the number of fields with associated events that were found to be distinct, were protrusion (62% versus 47%), translocation (17% versus 10%), and cell fragmentation (6% versus 32%).

Carcinoma cells in the metastatic MTLn3 cell line–derived tumors (Fig 19.7.3A) were found to be associated with and to locomote on collagen-containing fibers when compared to the nonmetastatic MTC cell line (Ahmed et al., 2002). Correlation of tumor cell behaviors with gene-expression analysis allows for a novel way of exploring cancer-related genes (Wang et al., 2005). From their experiments, the authors have found that the minimum motility machine genes are coordinately up-regulated in both metastatic tumors as compared to

nonmetastatic tumors and in the invasive population when compared to the metastatic tumor (Wang et al., 2004).

Using multicolor imaging (CFP and GFP cell populations with different metastatic potential in the same tumor), the authors were able to observe (Fig 19.7.3B) morphological differences, such as the movement speed between the two cell lines in the same microenvironment, and showed that motile cells often follow the same path (Sahai et al., 2005). Coinjection of parental MTLn3 cells with ErbB1-overexpressing MTLn3 cells showed that the ErbB1 overexpressor cells moved at a greater frequency than the parental cells and that more metastatic differences could be observed (Xue et al., 2006).

Host cells could be seen as shadows crawling over the background of the GFP fluorescence (Wyckoff et al., 2000). Some of these cells were determined to be macrophages by injecting Texas Red–dextran intravenously and allowing the macrophages to phagocytose the dextran (Fig. 19.7.3D). Imaging of tumor cell and macrophage cell interactions confirmed a paracrine loop involving EGF and CSF-1 and their corresponding receptors, which was discovered by using an in vivo invasion assay developed in the authors' laboratory (Wang et al., 2004). Tumor cells and macrophages could be imaged moving toward the bevel of a needle containing Matrigel and EGF and CSF-1, respectively (Wang et al., 2004).

Transgenic mice containing the PyMT antigen have been crossed with a *Cre-lox-β*–actin promoter-GFP to create mice with fluorescent mammary tumors (Ahmed et al., 2002). The PyMT-generated tumors have been shown to mimic human disease (Lin et al., 2003). Many of the morphological characteristics seen in the orthotopically injected models have been seen, including cell motility, invasion, and intravasation. The authors have also crossed the PyMT mice with other GFP-containing transgenics to observe the role of host cells in tumors. These include Tie2-GFP mice with fluorescent endothelial cells (Motoike et al., 2000), lys-GFP mice (Faust et al., 2000), and C-fms-GFP mice (Sasmono et al., 2003) that label macrophages and granulocytes.

Time Considerations

Depending on the cell line used, it can take several months for animals to develop tumors (this is mainly true for human cell lines). In the authors' experience, MTLn3, a fast-growing cell line, is ideal in this respect, with tumors developing in ~3 weeks.

Each animal can stay on the microscope for up to 4 hr, under ideal conditions; thus, it is important to manage the breathing rate (by gradually reducing the isoflurane levels) to make sure that each animal is able to breathe steadily and evenly. Generally, time-lapse *z*-series images are collected for 30 min; macrophages are labeled 2 hr prior to imaging, and vessels are perfused with dextran immediately prior to imaging.

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Figure 19.7.1.

Effective excitation spectra of various combinations of fluorophores common in multiphoton microscopy. Excitation by Tsunami, Mai Tai, or Opal are indicated by blue, red, or pink vertical lines, respectively. Excitation spectra of fluorophores are indicated as horizontal bars. (A) Relative output power spectra of Ti:Sapph lasers (Tsunami or Mai Tai Spectra Physics) and optical parametric oscillators (Opal, Spectra Physics). (B) CFP, GFP, and Quantum dots can be combined. The broad excitation spectra of quantum dots make them suitable for on systems employing a single laser. Their wide range of emission spectra makes them particularly useful in combination with each other, as well as with many fluorophores. (C) CFP, GFP, rhodamine and Evans Blue all have overlapping excitation spectra allowing their use with a single laser system. The long Stokes shifts of rhodamine and Evans Blue make them cleanly separable with the filter sets described in this chapter. (D) Use of a second Ti:Sapph femtosecond laser source enables the simultaneous imaging of CFP, Dendra2 green, and Dendra2 red. (E,F) Use of a Ti:Sapph femtosecond laser source in combination with an optical parametric oscillator allows the use of a variety of fluorophores whose emission spectrum lies in the red and far red (>650 nm). For the color version of this figure, go to <http://www.currentprotocols.com/protocol/cb1907>.

Figure 19.7.2.

Diagram of tumor fixturing setup. **(A)** Supine view of mouse with skin flap surgery indicating placement locations of rubber support pieces used on the microscope stage. Two small ($0.5 \times 0.75 \times 0.25$ -in.) pieces of rubber placed at the sides of the tumor act as a support for the tissue and prevent over compression of the tumor vasculature. A third thin strip of rubber ($0.25 \times 0.25 \times 1.5$ -in.) is placed between the body and the tumor to isolate the tumor from breathing motion. **(B)** Lateral view of mouse mounted on microscope stage. The two small pieces of rubber from (A) can be seen supporting and suspending the skin, with its foam backing, above the coverslip. Two anchoring strips of rubber then are affixed to the tumor with adhesive tape.

Figure 19.7.3.

Multiphoton imaging of MTLn3 xenotransplant tumor in mouse. (A) GFP-expressing tumor cells are shown in the green channel, and matrix second harmonic image is shown in both red and blue channels (for contrast). (B) Mix of GFP-expressing cells (green channel) with CFP-expressing cells (red and blue channels) in a single tumor. (C) Simultaneous imaging of GFP-expressing tumor cells (green) and blood vessels (red) following intravenous injection of Texas Red-dextran. (D) Imaging of macrophages labeled with Texas Red-dextran (red) in GFP-expressing MTLn3 tumor. Scale bars for A and C represent 50 μm , those for B and D represent 25 μm . For the color version of this figure, go to <http://www.currentprotocols.com/protocol/cb1907>.