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The Use of Fluorescent Proteins for Intravital Imaging of Cancer Cell Invasion

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Abstract

The analysis of cancer cell behavior in the primary tumor in living animals provides an opportunity to explore the process of invasion and intravasation in the complex microenvironment that is present in vivo. In this chapter, we describe the methods that we have developed for performing intravital imaging of mammary tumors. We provide procedures for generating tumors through injection of tumor cell lines, and multiphoton imaging using a skin-flap tumor dissection and a mammary imaging window.

Keywords

Intravital imaging; Multiphoton imaging; Live animal imaging

1. Introduction

The malignancy of cancer is due in large part to local and distant spread (1). In tumors whose locations make it difficult for surgery to completely remove them, such as glioblastoma (2) and head and neck cancer (3), local invasion can lead to local recurrence. For other cancers which quite often can be fully removed, such as breast cancer, cells which have spread to distant organs prior to surgery can form metastases in multiple sites, making further treatment very difficult. Currently most therapeutics are based on blocking tumor cell growth. Thus, an understanding of the processes of local tumor cell invasion and intravasation (which enables distant metastasis) may allow the development of new treatments which can reduce the malignancy of cancer.

Although 2D and 3D in vitro studies of cell motility and invasion are useful to understand basic mechanisms that regulate tumor cell movement, given the complexity of the tumor microenvironment, it is important to directly evaluate cancer cell motility in vivo. Specific features of the in vivo tumor microenvironment that are difficult to mimic in vitro include the presence of a variety of cell types, a range of extracellular molecules and fibers which are poorly defined, and blood vessels which have varying diameter and cell types associated with them.

Important stromal cell types that can affect tumor cell behavior include tumor-associated macrophages (4), neutrophils (5), fibroblasts (6, 7), and bone marrow-derived stem cells (8).

Extracellular fibers of varying diameter and composition run through the tumor and can potentially provide pathways for invasion (9, 10), hinder tumor cell movement, or provide a level of stiffness that can stimulate signaling for growth and invasion (11).

Blood vessels and lymphatics provide opportunities for transport to distant sites (12, 13). The arrangement of endothelial and smooth muscle cells in tumors is disorganized and more permeable compared to the vasculature of normal tissue (14, 15).

Mouse models of cancer provide an accessible mammalian system for studying cancer invasion in vivo. Using immunocompromised mice, human cancers can be transplanted either direct from human tumors as fragments or from cell lines (16). The advantage is that the focus is on the most clinically relevant (human) cancer, but the stromal effects cannot include potential contributions from T and B cells or other immune cells depending on the precise immunodeficiency model that is utilized (17). Conversely, transgenic models make use of specific oncogenes to induce tumors in the organ of interest in fully immunocompetent animals (18), with the limitations of using highly promoted oncogenes that may be unusual for human tumors as well as taking much longer and more sporadically to form a tumor than many xenograft models.

Both immunocompetent and immunodeficient mouse models have been utilized for intravital imaging of tumor cell invasion. In both cases, a key component is the expression of fluorescent proteins in the cells of interest (16). For xenograft transplants into immunodeficient mice, expression of fluorescent proteins is quite straightforward for cell lines, enabling relatively rapid testing of various proteins with specific functional or localization properties. However, imaging of stromal cells is challenging, with macrophages being the major cell type that has been imaged due to their uptake of i.v. injected fluorescent dextran (19). With transgenic animals, labeling of both tumor cells and stromal cells with fluorescent proteins is possible. As the appropriate transgenic strains become available, the label can be crossed into immunodeficient lines as well (20–23).

Some of our studies of in vivo tumor properties using intravital imaging have used confocal microscopy. Single-photon confocal microscopy can provide greater flexibility in terms of excitation of multiple fluorophores (due to the relatively lower cost of using multiple lasers and a wider range of wavelengths available) but provides more limited depth penetration (24). Multiphoton microscopy allows deeper penetration, as well as the detection of some (but not all) matrix fibers using second harmonic generation (SHG) (9, 25, 26). We currently use multiphoton microscopy to allow relatively deep (>100 μm) imaging into tumors in animals with relatively little bleaching or damage. We originally used a skin-flap method to expose the tumor surface for imaging, which is difficult to use for multiple imaging sessions. We then developed a mammary imaging window to evaluate tumor cell motility over multiple sessions, in combination with the photoconvertible protein Dendra2 (27). In this chapter, we provide technical details for generating and observing mammary tumors

using intravital imaging, including injection of cancer cells, skin flap dissection, and utilization of a mammary imaging window.

2. Materials

We describe below materials required for orthotopic (i.e., mammary gland) growth (see Subheading 2.1), cancer cell imaging by the skin-flap method (see Subheading 2.2), generating mammary imaging windows (see Subheading 2.3), window implantation (see Subheading 2.4), and microscope setup (see Subheading 2.5).

2.1. Generating the Tumors

In our work, MTLn3 rat mammary adenocarcinoma and MDA-MB-231 human mammary adenocarcinoma cell lines are frequently used. These well-characterized cell lines have been transfected with vectors driving constitutive expression of GFP for visualization of organelles of individual cells. It is, however, possible to restrict expression of fluorescent markers to a specific organelle (nucleus, Golgi, etc.) depending on the study design.

Materials required for orthotopic (i.e., mammary gland) tumor growth and imaging (for both skin-flap methods as well as mammary imaging window methods) are listed below:

1. MTLn3 (rat) or MDA-MB-231 (human) adenocarcinoma cell lines (available from authors) or an alternative.
2. Solution 1: For cell detachment. Phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} containing 2 mM ethylenediamine tetraacetic acid (EDTA) pH 8. Autoclaved or filter sterilized using 0.22 μm pore size.
3. Solution 2: For resuspension and injection of MTLn3 cells. PBS with Ca^{2+} and Mg^{2+} , containing 0.2% (w/v) bovine serum albumin (BSA). Filter sterilized using 0.22 μm pore size.
4. Solution 3: For resuspension and injection of MDA-MB-231 (4173) cells. PBS with Ca^{2+} and Mg^{2+} , containing 0.2% (w/v) BSA and 20% (v/v) collagen (type 1). Filter sterilized using 0.22 μm pore size.
5. Immunodeficient mice, i.e., female 5–6-week-old BALB/c SCID/Ncr mice (National Cancer Institute). MTLn3 cells can also be injected into immunocompetent Fisher 344 rats since they are derived from this strain.
6. 70% (v/v) ethanol.
7. 15-cm tissue-culture dishes.
8. 15-mL conical polypropylene centrifuge tubes, sterile.
9. Cell scrapers.
10. Centrifuge.
11. 1-mL syringes with 25-G needles for cell injection.

2.2. Animal Preparation for Imaging via Skin Flap

An oxygen/anesthesia system and equipment are used when imaging with the mammary imaging window in conjunction with the custom-imaging box described below.

1. Oxygen/anesthesia vaporizer apparatus (we use Forane Vaporizer, model 100, from SurgiVet).
2. Rodent-sized anesthesia box.
3. Compressed oxygen (100% pure, for anesthesia delivery/mixture).
4. Isoflurane anesthesia, USP.
5. Anesthesia delivery/breathing circuit (tubing, nose cone, scavenging system).
6. Heated stage or heater/blower system for microscope box.
7. 70% ethanol solution in dH₂O.
8. Surgical gloves.
9. Rodent-sized surgical scissors (sterilized).
10. Rodent-sized surgical forceps (sterilized).
11. Sterile cotton swabs (or forceps to reposition fat-pad covering tumor imaging surface).
12. Optional: Magnification glass with light.
13. Heating pad for surgical procedure.
14. Sterile tape for securing animal and anesthesia equipment to microscope stage.

2.3. Production of Imaging Window

The mammary imaging window consists of a glass coverslip mounted atop a tissue-culture grade plastic platform designed for implantation over a murine mammary gland or mammary tumor, located proximal to the hind leg. Cancer cells can be injected under the window to monitor the initial steps of tumor growth. Alternatively, the window can be inserted over spontaneously grown mammary tumors either in transgenic animals, such as the MMTV-PyMT strain, or xenograft tumors formed by orthotopic implantation. See refs. 26–28 for details.

1. Tissue-culture grade plastic (tissue-culture dishes can be utilized).
2. Cyanoacrylate glue.
3. Bunsen burner.
4. 23-G needle.
5. Dremel tool for sanding and shaping of the plastic parts.
6. 8-mm diameter, circular glass cover-slips.
7. 70% Ethanol.

2.4. Mammary Imaging Window Implantation

1. Anesthesia (Avertin).
2. Betadine.
3. Ethanol solution (70%) in dH₂O.
4. Hair removal cream and small animal shaver.
5. Sterile dissecting microscissors.
6. Sterile dissection microforceps.
7. Suturing thread (nonwicking) and needle.
8. Suturing forceps.
9. Sterile Q-tips.
10. Sterile cloth.
11. Heating pad (90°F).
12. Cyanoacrylate glue.
13. Sterile gauze.
14. Ophthalmic ointment.
15. TMP-SMX antibiotic mix: sulfamethoxazole 0.6 mg/mL, trimethoprim 0.12 mg/mL.
16. Analgesia, Flunixin (Banamine): Used at 2.0–2.5 mg/kg body-weight.
17. Surgical gown, hairnet, gloves, and mask.

2.5. Imaging Microscope Setup

We use several different multiphoton microscopy systems for intravital imaging in mice and rats. These include one of the first available multiphoton turnkey systems, the BioRad Radiance 2000 (manual laser tuning, two PMT detectors), the Olympus FV1000-MPE (computer-tuned laser, four PMT detectors), equipped both with confocal and multiphoton capabilities, which has a 15-W Tsunami (Spectra-Physics) Ti Sapphire laser. With these two microscopy systems we are able to image second harmonic generated excitation, CFP and Texas Red simultaneously. We have found that imaging at 880 nm allows for the best simultaneous imaging of these fluorophores. We also use a custom-built dual-laser multiphoton system with an optical parametric oscillator (OPO). With this microscope, we are able to image the above-mentioned chromophores along with Red Dendra and all other red fluorescent proteins, including those fluorescing into the far red (26).

3. Methods

The ultimate goal of intravital imaging is to capture the behavior of cells (in our case cancer cells) in their native microenvironment. In our group, two methods are used to access the tumor for imaging. Both methods make use of either tumors of transgenic origin or tumors

generated from orthotopic implantation. The first method makes use of a surgically-created skin flap to expose the tumor surface. This method allows for imaging sessions lasting up to several hours (see Note 1). The second method described utilizes a custom-built mammary imaging window that is subcutaneously implanted over a growing tumor, but can also be inserted first over the mammary gland, with cancer cells injected afterward. The window allows multiple imaging sessions using the same animal and tumor over a period of several days (see Note 2). For detailed video instructions on window implantation and its use for mammary imaging *in vivo*, see (28). Both the skin flap and window techniques have their pros and cons in terms of preparation time, types of data acquired, number of animals per experiment, and flexibility in experimental design. All are important considerations when choosing the appropriate approach for testing new hypotheses (see Note 3).

3.1. Growth and Generation of Tumors for In Vivo Imaging

In our work, we have utilized fluorescent derivatives of both MTLn3 cells—a highly metastatic rat mammary adenocarcinoma cell line and the human MDA-MB-231 breast cancer cell line. Here, we describe the method for preparing and injecting MTLn3 and MDA-MB-231 cells. Our group utilizes 5–6-week-old BALB/c SCID mice or 7–8-week-old Fischer 344 rats (used for MTLn3 cells). The animals are injected with a cell suspension into the mammary fat pad under the fourth (abdominal) nipple. MTLn3 tumors typically reach suitable imaging size within 3–4 weeks, while MDA-MB-231 require approximately 8 weeks of growth.

1. MTLn3 or MDA-MB-231 cells are grown to 80% confluence in a 15-cm tissue-culture dish.
2. The dish is rinsed three times with 5–7 mL of prewarmed (37°C) solution 1. After rinsing, add another 7 mL of solution 1 and incubate the cells at 37°C until detachment, which takes 10–30 min depending on cell density. Cells can be dislodged by gently tapping the side of the plate. Alternatively, trypsin/EDTA can be used to detach cells.
3. Once the cells have detached, harvest and transfer them to a sterile 15-mL conical tube. Scrape the plate to collect the cell matrix and transfer the mixture to the same 15-mL conical tube.
4. Add 7 mL of solution 2 to the plate to wash and collect the remaining matrix. At this point an aliquot of cell suspension can be taken for counting.

¹The skin flap technique is very flexible in terms of tumor size (29). Furthermore, it does not require a lengthy surgery or postoperative recovery time in preparation for imaging. However, data are acquired over an imaging session during which the animal is under anesthesia and its physiological processes may be somewhat impaired. Conclusions are made by combining data not only from several animals, but also from different tumor microenvironments from within the same tumor.

²The flexible nature of the imaging-window design provides the researcher with several options for introducing fluorescent protein-expressing cells for imaging. One important advantage of the window system is that cancer cells (or other cell types), with stable or transient expression of fluorescent proteins, can be delivered to the imaging environment during any stage (i.e., into either a mammary fat pad or growing tumor) and imaged immediately. Furthermore, through the use of photoconvertible proteins, the imaging window allows the researcher to follow cell fate over days from within the same tumor microenvironment.

³It is important to note that the skin flap and mammary imaging window methods do not image the same side of the tumor—the skin flap exposes the side of the tumor adjacent to the peritoneum, while the window exposes the side oriented towards the skin. Therefore, differences exist in the amount of collagen and fat in the outer layer of the tumor, hence the microenvironment will also differ.

5. Pellet the cells by centrifugation for 5 min at 180–200×g at room temperature. Upon completion, aspirate the supernatant.
6. The cells are resuspended in solution 2 to a concentration of not more than 10^7 cells/mL (10^6 cells/100 μ L). If injecting MDA-MB-231 cells, resuspend in solution 3, which contains 20% collagen (the collagen is an important component in the initial stages of tumor formation for this cell line). This suspension is kept on ice until injection into the mammary fat pad, which should not be more than 30 min after resuspending them. Alternatively, a 50% (v/v) Matrigel-cell suspension mixture can be injected.
7. Keep the cells suspended and load the mixture into a 1-mL syringe fitted with a 25-G needle.
8. The area around the fourth abdominal nipple is sprayed with 70% ethanol. Inject 100 μ L of the cell suspension under the skin into the mammary fat pad (for rats the volume can be increased to 200 μ L).
9. To inject conscious animals, it is best to have two researchers working as a team. One person restrains the animal by simultaneously grasping the base of the tail and the scruff of the neck with one hand, while holding and extending the hind leg with their other hand to expose the nipple area. The second researcher performs the injection by slowly inserting the needle into the skin under the nipple. A proper injection into the mammary fat pad should induce the formation of a small raised area under the nipple. This raised area will not appear if the injection misses the fat pad. Alternatively, the animal can be anesthetized and a single researcher can perform the injection.
10. The animals are kept in a barrier-room facility until the tumors are large enough to image. Typically, this is 5–7 mm in diameter when using the mammary imaging window and 5–20 mm for the skin-flap technique.

3.2. Preparation of the Animal for Imaging Using the Skin Flap Method

Animals with tumors of diameter 5–20 mm can be used for imaging using the skin-flap method.

1. The animal is placed under anesthesia with 5% isoflurane via the oxygen/anesthesia apparatus. For mice, isoflurane levels are immediately reduced to 2.5% upon achieving unconsciousness. Importantly, animals with heavier lung metastasis burdens will require lower isoflurane levels than animals with lungs free of metastasis. Labored or erratic breathing is a danger sign.
2. Anesthesia is maintained throughout surgery and the imaging session. If vessel labeling is used, 70 kDa Texas Red- or FITC-dextran can be introduced via tail vein injection prior to or immediately after the skin-flap surgery.
3. Prior to making the surgical incision, thoroughly swab and clean the skin at the incision site with 70% ethanol. Allow the solution to dry before making the incision in order to prevent seepage into the wound.

4. Skin is then incised medially to the tumor using an incision long enough to permit exposure of the tumor surface facing the medial line of the animal.
5. Since fat cells can alter the path of photons coming into and exiting the imaging plane, the area over the exposed imaging surface of the tumor should be cleared of fascia and fat. However, care should be taken to maintain the vasculature and minimize bleeding, which can potentially alter cellular behavior within the tumor microenvironment. An attempt should first be made to carefully push aside the fascia to expose a region on the tumor surface. If fat and fascia obscure the image quality, delicately microdissect away the tissue.
6. The animal is subsequently transferred to a prewarmed and preferably enclosed microscope stage. As air-conditioning and contact with metal stages can cause extensive loss of body heat, external heaters should be used to maintain an environment between 25°C and 30°C. The exposed tumor surface is placed against the upper surface of the coverslip directly over the objective lens. It is necessary at this point to secure the tumor to the stage in order to minimize breathing artifacts and drift caused by animal movement. We have found that the most efficient method is to isolate the tumor with surgical or laboratory tape by simultaneously securing both the skin flap/tumor and lower tail region of the animal to the stage. By providing the lungs room to expand and the upper torso freedom to absorb the movement, we can minimize the breathing artifacts. Care should be taken to minimize pressure on the tumor so as not to block the blood flow or compress the tumor as these will cause image drift during time-lapse acquisition. However, as pressure from the tape will introduce a small amount of drift along the Z-axis during imaging, the setup should be allowed to stabilize before the start of image collection.
7. Lower breathing rates minimize image drift and distortions, which can interfere with data analysis. As the imaging session progresses, the level of anesthesia deepens in the animal. As a result, during the imaging session it is advisable to progressively lower the isoflurane levels. By periodically lowering the isoflurane in steps of 0.25%, a constant breathing rate can be maintained while providing adequate tissue oxygenation and preventing suffocation. A proper breathing rate is defined by one or fewer distortions (breathing artifacts) per image scan when scanning at 166 lines/s.
8. After each z-stack time series is captured on a specific field, the tumor (microscope stage) can be repositioned to image additional fields. Multiple series can be captured as long as the animal's breathing and pulse are stable.

3.3. Mammary Imaging Window Construction (See Fig. 1)

1. The bottom of a tissue-culture dish is softened by heating with a flame (we typically use an alcohol lamp). Subsequently, a curved surface (2 cm diameter, 8 mm center height) is created by using a mold (e.g., an appropriately sized rounded Dremel bit) to press in to the softened plastic. The centerpiece of the curved surface, 1 cm in diameter, is then cut out using a hot scalpel.

2. Using a small, cone-shaped Dremel bit, a 6-mm hole is formed in the center of the curved plastic surface. Edges of the plastic base are smoothed by sanding with the Dremel and further filing.
3. The top of the plastic base is filed to make a flat surface (7 mm in diameter) for the glass coverslip.
4. A circular glass coverslip (8 mm) is glued using cyanoacrylate adhesive and the glue is allowed to dry for 15 min.
5. Eight suturing holes are made in the plastic by using a flame-heated 23-G needle.
6. The imaging window is cleaned with ethanol. A Q-tip can be used to clean the glass. If there are foggy spots present from the glue vapor, carefully use acetone with a Q-tip to remove them.
7. The window can be sterilized by overnight UV exposure in a tissue-culture hood. Ensure that both sides of the window are exposed to UV light.

In order to position and secure the animal on the microscope stage, a custom-designed 1/8-in. thick plexiglass-imaging box was constructed and used in combination with the mammary imaging window (see Fig. 2). The mouse is placed inside of the box with the imaging windows glass coverslip projecting out from the underside. Precise placement of the window and coverslip against the microscope objective is achieved by adjusting two sliding doors. Vents have been constructed in the sides of the box to allow anesthesia to enter and exit while maintaining a constant flow. Finally, depending on the specific microscope and stage to be used, the design of the bottom of the imaging box can be altered to accommodate the different set-ups.

3.4. Mammary Imaging Window Implantation Method

1. As mentioned, the mammary imaging window can either be implanted over a normal mammary gland or a growing tumor. The optimal tumor size for window implantation is 5–7 mm, which assures that the tumor will be in contact with the coverslip during imaging and which permits space under the window for further tumor growth. It is also important to note the appearance of the skin over the tumor surface. In order to maximize the number of imaging sessions and data acquisition, tumors selected for the procedure should not be protruding or invading through the skin.
2. Sterile technique should be used throughout the surgery—optimally inside a positive airflow sterile hood. All instruments used should be sterilized prior to surgery. Full gown, mask, and gloves should be worn throughout the procedure.
3. The mouse is anesthetized by intraperitoneal injection of 2.5% Avertin (20 μ L/g body weight) in HBSS.
4. Hair should be removed by shaving the area above the tumor using a small animal shaver. The rest of the hair can be removed using hair removal cream.
5. The skin is disinfected using betadine and ethanol-dipped Q-tips. Ophthalmic ointment is applied to the eyes to protect from infection and drying.

6. First a 2-mm incision is made medial to the fourth (abdominal) nipple; this is where the tumor tends to form closest to the surface of the skin. The initial incision can be expanded, while simultaneously the underlying mammary fat pad is separated from the skin using blunt dissection with scissors and forceps until it is large enough to accommodate the imaging window. Care should be taken to avoid severing of major blood vessels supplying the tumor or surrounding area. This step is necessary to allow adequate space for the insertion of the imaging window. The imaging window is inserted such that there is skin on top of the imaging window base and sutured in place using nonabsorbable thread and a reverse cutting needle.
7. Tissue adhesive is used to fill in the suturing holes and secure the imaging window to the skin.
8. An analgesic should be administered in the immediate postoperative period for pain control.
9. TMP-SMX antibiotic mix is added into the cage water bottle for 3 days before and after the surgery.
10. The animal is allowed to recover over the next 3–4 days before the first imaging session is attempted. This allows for healing of the surgery area and thus minimizes inflammatory/tissue-remodeling artifacts. Imaging can subsequently be carried out up to 7 days (see Fig. 3).

3.5. Image Acquisition

While performing intravital microscopy, the data are collected as a 4D-data set (x , y , z series at each time point) of images at various scan speeds, adjusted depending on information to be obtained from analysis. The exact microscope parameters are highly dependent on the specific fluorophores used to obtain the data. Most commonly, we use a 20 $\times$ water or glycerol immersion objective, which presents a field of view 1 mm in diameter in epifluorescence mode. Depending on the microscope, this becomes an imaging field of 500–775 μ m in confocal or multiphoton mode. In confocal (single photon) microscopes, we use a laser power of 30–60 μ W at the sample, 4.9 μ s pixel dwell time, 1 Airy disk pinhole, and 50–100% PMT setting. For multiphoton microscopy, we use a laser with a 300-fs pulse width, 80-MHz repetition rate and <30 mW average laser power at the sample, 7.6 μ s pixel dwell time, and 50–100% PMT setting. The imaging procedure is as follows:

1. Initial field selection and focusing are performed in epifluorescence mode through the microscope eyepiece. Select the channel from which the brightest signal is emitted and focus on the tumor surface. We typically use GFP-expressing cancer cells and introduce labeled dextrans into the circulation. Either the GFP fluorescence of the cancer cells or an intravenously-injected fluorescent label can be used for focusing and field selection. The eyepiece allows for a larger field of view than the PMT detectors, and epifluorescence mode allows quick illumination of the whole field and several depths at once. The brightest cancer cells are typically those closer to the objective lens and will yield the best quality images.

2. After selecting and focusing on the initial field, the microscope is switched from visualization to data collection in multiphoton mode. Ideal areas for our experiments are bright fluorescent fields of view populated with vessels containing active blood flow. Red blood cells absorb excitation light due to their high hemoglobin content. Therefore, flowing red blood cells can be observed indirectly in epifluorescence as moving shadows, or directly if labeled dextran has been injected into the vasculature. Areas that should be avoided are those densely covered by adipocytes (the mammary fat pad), which heavily refract and scatter light, and those regions where red blood cells have leaked from damaged vessels onto the tumor surface. To capture cell motility, we typically average 30 min to capture a 4D data set. However, depending on the specific cellular manipulation, animal model, or experimental aims, this time can be extended further.
3. While in multiphoton mode, we select new fields by scanning at higher speeds while adjusting the stage to find the best area to image. After selecting a new field, the scan speed is decreased to allow a longer dwell time per pixel (which results in higher signal-to-noise images). In our experience, the areas with motile cells are found at the tumor–matrix borders and which possess blood vessels with active flow. We try to select the edge of a tumor outgrowth with well-defined cell boundaries, and which includes extracellular matrix fibers as cancer cells typically migrate along their length.
4. Optimally, the beginning of a z -stack should be set on the outer tumor surface, one or two slices above the tumor–matrix border, so that the local tumor microenvironment is intact. If the system used for imaging allows for varying the laser power in z , more power can be used to image at greater depths (though it is important to confirm that this does not affect the tissue), thus allowing for a greater number of slices recorded in a given z -stack.
5. Set up the time-lapse z -series (generally we use 10- μ m steps for a 100- μ m scan and 1-min intervals for 30 min of imaging time) and start collecting images.
6. At the end of the imaging session (or sessions for experiments using the mammary imaging window), the animal should be euthanized using protocols approved by the user's institute animal care committee.

3.6. Processing Intravital 4D Data

Until recently, most available image-processing tools offered were not optimized for intravital or 3D imaging. They were commonly designed for 2D images or time-lapse sequences without measurement of axial movements or ability to follow movement of the same cell throughout a stack. This task is somewhat complicated by difficulty in the display of 4D (3D information over time), as well as difficulty in analysis of 3D data due to decreased resolution in z as compared to x – y dimensions. Also, results of intravital imaging often include slight drift in one of the x , y , z dimensions as well as irregular breathing artifacts. However, as the number of groups imaging in vivo or in 3D cultures is growing, solving this issue becomes increasingly important. All techniques reported so far are based on the ability of the software to automatically segment and threshold images based on

fluorescence intensity. Unfortunately, this is a task that cannot be fully automated for cells in solid tissues, such as tumors, where the cells are packed closely to each other in three dimensions. Our typical quantitation procedure involves visually counting the number of moving cancer cells in all the slices in a 4D stack. Additional parameters that can be evaluated include movement of cancer cells on visible matrix fibers, movement of host cells (nonfluorescent cells seen as shadows against the fluorescent cancer-cell background), orientation around blood vessels, and matrix density.

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Fig. 1.

Mammary imaging window design. (a) Overview of the construction with two spacer rings attached, with a coverslip mounted on the top. (b) Front view of the coverslip. (c) Alternative design of the window, where the bottom ring is curved to better accommodate a growing tumor. (d) Bottom view of a completed window ready for implantation. Adapted from (27).

Fig. 2.

Imaging box used in combination with the mammary imaging window. (a) This general imaging box schematic can be optimized to fit any microscope stage. (b) A photograph of a currently-used version of the imaging box.

Fig. 3.

Example of use of the mammary imaging window. When using the mammary imaging window and imaging box, multiple imaging sessions can be done using the same animal. Using expression of a photoconvertible protein, Dendra2, one can monitor the same cell population over 7 days (168 h).