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High-Resolution In Vivo Imaging of Fluorescent Proteins Using Window Chamber Models

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Abstract

Fluorescent proteins enable in vivo characterization of a wide and growing array of morphological and functional biomarkers. To fully capitalize on the spatial and temporal information afforded by these reporter proteins, a method for imaging these proteins at high resolution longitudinally is required. This chapter describes the use of window chamber models as a means of imaging fluorescent proteins and other optical parameters. Such models essentially involve surgically implanting a window through which tumor or normal tissue can be imaged using existing microscopy techniques. This enables acquisition of high-quality images down to the cellular or subcellular scale, exploiting the diverse array of optical contrast mechanisms, while also maintaining the native microenvironment of the tissue of interest. This makes these techniques applicable to a wide array of problems in the biomedical sciences.

Keywords

Window chamber; Intravital imaging; Fluorescence; Fluorescent protein; GFP; Microscopy; In vivo imaging

1. Introduction

1.1. History and Background of Fluorescent Proteins and Application of the Window Chamber Model

Green fluorescent protein (GFP) was discovered by Shimomura et al. while attempting to discern the mechanism of autofluorescence in the *Aequorea* jellyfish (1). The mechanism was unlike any others known at that time in that a chemiluminescent calcium-binding protein, aequorin, emits a blue photon upon binding to calcium. This blue photon is then transferred to GFP via fluorescence resonance energy transfer (FRET) to provide the green bioluminescence characteristic of the animal. After much painstaking effort, the GFP protein was sequenced and cloned, and fortuitously found to autocatalytically form its active chromophore, making it suitable for use in virtually any species as a molecular marker. Subsequently, mutants and alternative fluorescent proteins have provided a whole palette of colors spanning the visible and, recently, near infrared wavelengths (2, 3). It has by now become an indispensable tool of biology, useful for investigating protein localization, protein interactions (via FRET), cell localization, interrogation of signaling pathways, and more (4). It has also been adapted for use as a genetically-encoded biosensor for such diverse measurands as calcium concentration, pH, proximity, and protease activity (5)

Given the diverse and wide ranging tool set that fluorescent proteins can potentially provide, it is desirable to have an effective means of imaging them in vivo. A variety of techniques exist, including invasive imaging, diffuse optical (deep tissue) imaging, or simply using

confocal or multiphoton imaging transcutaneously or directly. In this chapter, we focus on window chamber techniques, which have the advantages of providing direct access for high-resolution microscopy, while also enabling longitudinal monitoring of the same site.

The development of the window chamber model for the investigation of cancer in vivo has proven an invaluable resource in the elucidation of real-time tumor inception, growth, adaptation, and treatment response. A critical advancement in the optical interrogation of living tissue came in 1928 with J.C. Sandison's observations of blood vessel growth through a transparent chamber implanted in a rabbit ear (6). In 1939, this technique was later adapted to carcinoma studies by Ide and Warren (7) in one of the first direct observations of angiogenesis and its influence on tumor growth. The window chamber model was further refined in 1943 by Glenn Algire, with his publication of a number of novel window chamber designs (8)—among them, the dorsal window chamber model, which is widely used in tumor xenograft and allograft animal studies to this day (9). As an example, Fig. 1 illustrates the use of fluorescent protein reporters of hypoxia-inducible factor-1 (HIF-1) upregulation in response to radiotherapy (RT) (10). The use of the window chamber in conjunction with fluorescent protein reporters enabled a detailed understanding of the mechanism and temporal dynamics with which HIF-1 is upregulated post-therapy and has spurred several studies investigating the use of HIF-1 inhibitors as a means of improving the therapeutic efficacy of RT (11, 12).

1.1.1. Animal and Tumor Models—A variety of genetically-engineered animals and tumor models exist that incorporate fluorescent proteins for imaging. This includes transgenic animals that express GFP in their germ line, which enables in vivo imaging of host tissue. Another common approach is to use standard animal strains, with a genetically-engineered cell line introduced into the mature animal in the form of tumor-cell xenografts, or other transplants.

1.2. Orthotopic Mammary Window Chamber Model

Orthotopic and ectopic organ environments differentially influence cancer cell gene expression, tumor growth, invasiveness, angiogenesis, metastasis, drug delivery, and sensitivity to therapeutic agents in many tumor types (13–15). Orthotopic breast cancer models with rodent syngeneic tumors or human xenografts have been widely used in the studies of hormone dependency, novel metastasis models, molecular targets, growth factors, angiogenesis, tumor growth, and gene therapy (16–21)

For intravital imaging of orthotopic breast cancer, we have established a rodent mammary window model, which combines the unique features of a tissue window with growth in an orthotopic organ environment, which allows, continuous, and noninvasive monitoring of tumor growth and angiogenesis (22). There is significant overlap with the dorsal skin-fold model, so we focus on an overview of the procedures, while highlighting the differences in technique.

1.3. Imaging

Once the window chamber has been implanted, imaging can be carried out using any of the established techniques for fluorescence microscopy, including wide field, confocal, or multiphoton fluorescence. The primary advantages of confocal and multiphoton being better resolution and depth-resolved imaging. The following protocol describes wide-field fluorescence imaging, but any of these techniques can be used with similar approaches.

2. Materials

2.1. Materials for Dorsal Flank Model and Common Materials for Both Window Types

2.1.1. Cell Culture—The cancer cells to be used for inoculation should be screened for pathogens before they are introduced into animals. If using cells from cryo-storage, the cells should be plated out and passed at least once prior to inoculation. The number of cells needed for injection may vary significantly depending upon the particular cell line and animal strain. Nude mice, for example, will readily take a wide variety of cancer-cell lines. 10,000 cells per animal often achieves a near-100% tumor rate with highly tumorigenic cell lines such as the 4T1 mammary carcinoma line. For less tumorigenic cell lines, or when using animals strains with a competent immune system, however, larger quantities of cells may be needed (up to $>10^7$). Some cell lines will not grow well in certain strains, or may require special conditions (e.g., matrigel cell suspensions, implantable estrogen pellets). It is important to research the cells and animals intended for use in order to avoid wasting time and animals on failed surgeries.

2.1.2. Anesthetics and Analgesics—The concentrations and doses listed here are for mice. These doses provide a recommendation for animal protocol development, but it is important to ensure that all drug administration adheres to your IACUC guidelines (see Note 1).

1. Ketamine/xylazine is recommended for window chamber surgeries. Pentobarbital can also be used for surgeries (for hairy mice that need shaving and depilatory creams), although it is prone to a higher incidence of accidental overdose. Ketamine/xylazine should be diluted in sterile saline solution to a concentration of 10 mg/mL ketamine and 1 mg/mL xylazine. The administered dose should be 80–120 mg/kg ketamine, and 8–12 mg/kg xylazine i.p. for mice. At this concentration and dose, this results in approximately 0.01 mL/kg body weight, or 0.2 mL for a 20-g mouse. If using pentobarbital, it should be diluted in sterile saline to a concentration of 10 mg/mL, and the administered dose should be 75–90 mg/kg i.p. for mice.
2. Buprenorphine is used for postsurgical pain management. It is diluted to a concentration of 15 µg/mL and administered at a dose of 100 µg/kg subcutaneously (s.c.) for mice, or 50 µg/kg for rats. A second dose can be given after 8–12 h, if necessary.

2.1.3. Surgical Equipment and Accessories—Figure 2 shows the surgical equipment, numbered as described below. These items should be placed in a surgical tray, wrapped in 40 in. ×40 in. sterilization wrap, and steam or dry-heat sterilized prior to surgery:

- 1 Glass microsyringe (25–100 µL volume).
- 2 Mayo scissors.
- 3 Iris scissors.
- 4 Conjunctival scissors.
- 5 Jeweler type forceps.

¹Inadequate depth of anesthesia is one of the most common problems associated with this procedure. It is important to remember that sensitivity to anesthesia can vary across mouse strains, and even individual mice of the same strain can have significantly different sensitivities. The doses listed here are adequate in most cases, but they may require adjustment based on experience or prior knowledge of sensitivities. Be sure that your doses are in compliance with all approved animal protocols.

- 6 Mosquito forceps.
- 7 Needle holder.
- 8 C-clamp.
- 9 Cotton gauze.
- 10 Metal paper clip.
- 11 6 in. ×6 in. squares cut from a piece of spare sterilization wrap (one square per animal).

These items should also be packaged and sterilized prior to surgery. Items with plastic and nonstainless components should be gas sterilized separately:

- 12 Petri dish.
- 13 Plexiglass viewing stage.
- 14 Nut driver.
- 15 Retaining-ring pliers.
- 16 Two large metal binder clips.
- 17 Leather hole puncher with 1/8 in. hollow punch (or a 16-G needle can also be used).
- 18 Window chambers with nuts, glass cover slips, and retaining rings, seen in Fig. 3

The following items can be purchased as prepackaged sterile units:

- 19 18 in. ×26 in. sterile field.
- 20 Skin marker.
- 21 4-0 Monosof or silk suture.
- 22 Disposable surgical blade.
- 23 1-mL syringe.
- 24 Two 30-G needles.
- 25 Surgical gloves.

The following items do not need to be presterilized:

- 26 Water-circulating heating blanket.
- 27 Fiber-optic lamp.
- 28 Hot plate.
- 29 Glass-bead sterilizer.
- 30 Plexiglass surgical platform.
- 31 Paraffin heating pad.
- 32 Absorbent paper.
- 33 Exidine solution.
- 34 70% Ethanol solution.
- 35 Vortexer.

- 36 Antibiotic ointment.
- 37 Ophthalmic ointment.
- 38 Surgical mask.
- 39 Hair removal lotion (Nair) and electric shaver (for non-nude mice only).

2.2. Mammary Window Chamber Materials

2.2.1. Mammary Window Materials—Most surgical equipment and supplies are shared in common with the dorsal skin-fold window chamber model, with the notable exception of the window itself. This is shown in Fig. 4

Glass windows have better optic quality and scratch resistance but are a bit thicker and heavier. Acrylic or polycarbonate windows are lighter, but optical quality and scratch resistance are not as good as glass. The window sizes for rats and mice are 12 and 8 mm in diameter, respectively.

2.2.2. Animals and Tumors—Depending on tumor types and study designs, different female rats or mouse strains can be used. For the most commonly used syngenic rat mammary-adenocarcinoma line, R3230Ac, the Fischer 344 rat is desired. Since this tumor grows best with tumor-fragment implants from donor rats rather than cancer-cell inoculation, retired female breeders or lactation-weaned rats are preferable because of a well-developed nipple sinus where tumor fragments are implanted. However, any female rat or mouse can be used depending on tumor types.

2.2.3. Anatomical Consideration—Rats and mice have six pairs of mammary glands, and they are referred to by location as cervical, cranial-thoracic, caudal-thoracic, abdominal, cranial-inguinal, and caudal-inguinal, or by numbers, anterior to posterior as L1, R1, L2, R2, etc. (23). The mammary glands are compound tubuloalveolar glands comprised of a highly branched system of ducts and terminal secretory alveoli arranged in lobules. Each gland in the rat and mouse has a single lactiferous duct entering the nipple. The duct widens to form the nipple sinus, which then opens onto the surface by way of the nipple canal. The nipple, nipple canal, and nipple sinus are lined by squamous epithelium continuous with the epidermis. The second to fifth pairs of mamma on either side can be used for window surgery, but for convenience of intravital microscopy, mammary glands R4 or R5 are most commonly used.

2.3. Imaging Materials

2.3.1. Equipment

- *Fluorescence microscope and camera.* This can be any type of fluorescence microscope, inverted or upright. For the mammary window, breathing artifacts are more of a concern. A stage with an adjustable restraining device is preferable (22), although an inverted microscope is better for the mammary window to minimize breathing artifacts and enable the mouse to remain upright. Ideal objectives depend on the application, but can range from 2.5× for a broad field of view suitable for imaging large segments of the window, up to 60× or higher, suitable for imaging at a subcellular resolution. Filter cubes with the appropriate filter sets for each fluorophore of interest should be in place, as well as a sensitive CCD camera. Standard filter sets are available from several manufacturers that are capable of imaging overall intensity spanning the typical excitation/emission wavelength range of common fluorescent protein variants. A more ideal solution is to acquire hyperspectral data sets using a liquid-crystal tunable filter (LCTF), acousto-optic

tunable filter (AOTF), or similar system. This enables pixel-by-pixel measurement of the emission spectrum as a function of wavelength, which facilitates quantitative discrimination of multiple fluorophores using spectral discrimination techniques. For bright light epi-illumination, a cold flexible fiber optic lamp can be used. A ring lamp attached to the lens will provide more even illumination (see Note 7).

- *Wavelengths.* Filters used for wavelength selection will depend on the source of contrast used. GFP is compatible with fluorescein or FITC filters, which are common on fluorescence microscopes. RFP is compatible with rhodamine or TRITC filter sets. Most manufacturers sell filter sets optimized for a variety of fluorescent protein variants.
- *Imaging mount.* This is used to secure the window chamber in place during imaging. For the dorsal window, this can be as simple as a metal sheet with three holes drilled in to allow placement of the three window frame bolts to be fed through and secured. A mammary window requires use of a strap to secure the animal in place over the objective.
- *Heating pad.* A thermally-regulated heating pad designed for veterinary use is ideal. Alternatives include heated paraffin pads designed to maintain a constant suitable temperature as they undergo a phase transition from liquid to solid.
- *Anesthesia equipment.* This depends on the method of anesthesia, but for isoflurane anesthesia a vaporizer, scavenger, gas tank, and regulator are needed, as well as tubing and a nose cone to deliver the gas to the animal.

3. Methods

3.1. Dorsal Skin-Fold Window Chamber and Common Procedures

Note. Surgeries are most efficiently performed as a two-person team. While one person prepares the cells, the other can prepare the surgical area. During surgeries, one person can anesthetize and prepare the animal and handle the cells while the other person remains sterile and performs the surgeries.

3.1.1. Preparation

Cells

1. Immediately prior to surgery, remove the media from plates containing cancer cells and incubate them in trypsin until the majority of cells detach.
2. Dilute the trypsinized cells in standard media, pipetting repeatedly to break apart large clumps.
3. Pipet the cell suspension into a sterile conical tube and centrifuge at $680 \times g$ for 5 min.
4. Aspirate the supernatant and resuspend the cells in a minimal quantity of phenol red-free media.
5. Count the cells and dilute the cell suspension to a concentration such that a 10–20- μ L volume contains the desired number of cells for inoculation.
6. Pipet approximately 500 μ L of the cell suspension into a sterile microcentrifuge tube, and place the tube on ice.

⁷Inverted or upright microscopes are suitable for imaging the dorsal skin-fold model, but an inverted microscope is preferred for the mammary fat pad to minimize motion artifact.

Surgical Area: Surgery should be performed in a laminar-flow HEPA-filtered hood or other isolated environment with an accessible electrical outlet. Once a sterile environment has been set up, care should be taken not to unintentionally introduce any nonsterile objects into the area. Any sterile item or surface that accidentally comes into contact with a nonsterile object should be considered contaminated and must be resterilized or replaced. A complete setup is shown in Fig. 5

1. Place the fiber-optic lamp to the far end of the surgical area and turn the lamp on to its highest setting.
2. Place the hot plate in an accessible corner of the surgical area.
3. Place the glass-bead sterilizer in an accessible location immediately adjacent to the surgical area and turn it on.
4. Thoroughly clean and disinfect all exposed surfaces within the surgical area using Sporicidin or similar hospital grade antiseptic. Clean and disinfect the Plexiglass surgical platform and place it to the side.
5. Place the wrapped and sterilized surgical tray in the middle of the surgical area. Fully unwrap the tray and use the inner surface of the sterilization wrap to set up a broad, sterile working surface. Do not touch the inner surface of the wrap or the tray as you are preparing your field. The wrap should not cover the hot plate or obstruct the fiber lamp.
6. Placing your hands underneath the wrap (in contact with only the nonsterile outer surface), position the surgical tray to the side of the surgical area corresponding to the surgeon's dominant hand.
7. While handling only the outer packaging, hold items 13–24 over the surgical tray and open them (without touching the sterile inner contents), allowing the items to fall into the tray.
8. Open the Petri dish packaging, and without touching the inner portion of the dish, place it on the hot plate. Using a syringe, fill the Petri dish with a few milliliters of sterile saline, and set the temperature of the hot plate to heat the saline to approximately 37°C.
9. Microwave the paraffin pad for a few minutes until the wax is partially liquefied. Knead the pad to distribute the heat evenly across the surface.
10. Place the heated pad directly on the sterilization wrap within your surgical area. It should be placed towards the center of the working surface and about half a forearm's length from the front edge.
11. Place the Plexiglass surgical platform on top of the paraffin wax pad. You may need to place an item beneath the wax pad to elevate it to a level such that it is in contact with the inner surface of the platform. This will ensure that the platform remains heated during surgery. Remember that although the surgical platform is disinfected, it is not aseptic. Do not allow any sterile items to come into contact with it.
12. Using binder clips, attach the Plexiglass viewing stage to the far end of the platform such that it slopes downward towards the user. The clips and the outer portion of the viewing stage will become nonsterile as you set this up. Ensure that the forward-facing, sloped portion of the viewing stage remains sterile.
13. Position the fiber lamp's illumination source directly behind the Plexiglass surgical platform, facing forward and slightly upwards.

Animals: It is important to remember that the animal's core body temperature will drop while it is under anesthesia. Steps must be taken to ensure that the animal does not become dangerously hypothermic. Throughout the entire course of anesthesia, the animal should remain in contact with a heated surface, and body temperature should be regularly monitored (see Note 2).

1. Power-on the water-circulating heating blanket and cover it with the absorbent paper. Allow it to warm to approximately 37°C, which should be calibrated by measuring the actual temperature on the metal surface. Placing a thin metal surface on the heating blanket will improve thermal conductance and help maintain the anesthetized animal's body temperature more efficiently (see Note 5a).
2. Prepare a working solution of either ketamine/xylazine or pentobarbital anesthesia at the concentrations listed in Subheading 2. You will need approximately 1 mL of either anesthetic for every three animals (allowing for redosing).
3. Prepare a working solution of buprenorphine for postsurgical pain management. At the concentration listed in Subheading 2, you will need approximately 0.15 mL per animal.
4. Weigh the animal and administer the proper dose of anesthesia with an i.p. injection (see Note 1).
5. Isolate the animal until the anesthesia takes effect. Once the animal is down, place it on the heating pad in a sternally recumbent position with limbs spread.
6. Apply ophthalmic ointment to the eyes to prevent corneal desiccation during surgery.
7. If using a non-nude mouse, use the electric shaver to shave the hair from the base of the tail to the nape and from limb to limb on both sides. Apply hair-removal lotion and follow the product instructions to remove remnant hair. Skip this step if using nude mice.
8. Using cotton gauze, apply 2% chlorhexidine disinfectant solution to the animal's visible torso and tail. Wipe away with cotton gauze soaked with 70% ethanol. Repeat this process a total of three times. Avoid recontaminating the skin afterwards.

3.1.2. Surgery

1. Don sterile surgical gloves and surgical mask. Throughout the surgery, your gloved hands should not come into contact with any nonsterile object. In the case of contamination, replace the gloves.
2. Using the Mayo scissors, cut out a rectangular portion in one of the folds of the sterile field, approximately the size of the forward-sloping portion of the Plexiglass viewing stage. Drape the sterile field over the surgical platform so that only the sterile portion of the viewing stage is visible and protruding through the hole in the sterile field.
3. Take one of the 6 in. × 6 in. squares cut from the sterilization wrap and fold it diagonally. Cut a slit, approximately 1.5 in. long, along the diagonal axis.

²It is important to ensure that all animals are large enough to accommodate the window chamber prior to beginning surgeries. Mice generally need to be greater than 20 g.

^{5a}Mice that are inadequately heated throughout the surgery may become dangerously hypothermic. Make sure to periodically check the animal's body temperature.

4. With the skin marker, lightly trace a line along the length of the animal's spine. On either side of this line, make a small mark to note the highest-rising point on the animal's back. This will serve as a guide for the correct placement of the window chamber.
5. Take the 6 in. ×6 in. square and drape it over the animal such that the slit you cut runs parallel to the spine and the highest point on the back is at the center of the slit. Touching only the wrapping, maintain the animal in this position while rolling up the loose corners of the square until the animal is held snugly in place. Secure the rolled up corners with a sterilized paper clip.
6. Place the animal on the sterile field within the surgical area, and gently pull the loose skin on the back through the slit in the wrapping, producing a skin fold. Ensure the skin is evenly stretched up from either side and folded along the marked line running parallel to the spine. The marked area corresponding to the peak of the back should be centered.
7. Using the needle driver, suture the skin fold to the c-clamp in four places, stretching the skin evenly and creating a taut area for window placement. Hang the c-clamp from the top of the viewing stage so that the surgical area is well illuminated.
8. Hold the screw-less half of a window chamber flush against the skin fold in a position such that the window fully fits within the area of the skin fold. Use the marker to mark the positions of the screw holes on the skin flap, and mark the area for the window by tracing a circle along the circumference of the window hole.
9. Remove the c-clamp from the viewing stage. Using the marks for the screw holes as a guide, punch a hole through both sides of the skin flap at each one of the marked locations using a leather hole puncher or a 16-G needle.
10. Return the c-clamp to the viewing stage. With the iris scissors in your dominant hand, cut away the forward-facing half of the skin flap along the circumference of the circle marked on the skin. Use a mosquito forceps in your other hand to hold the skin in place as you cut. You will need to cut away the connective tissue beneath the skin to remove the skin disk.
11. Holding a conjunctival scissors in your dominant hand and a jeweler forceps in the other, pull up and cut away any residual connective tissue in the area, leaving a thin layer of translucent fascia covering the visible dermis of the back-skin fold. Take care not to puncture this dermal layer, as this will lead to visible lesions within the window (see Notes 3 and ⁴).
12. Throughout the rest of the surgery until the cover slip is secured, control bleeding by flushing the exposed wound with saline (using the warmed saline on the hot plate and the 1-mL syringe) and absorbing fluids with a sterile cotton gauze.
13. Remove the c-clamp from the viewing stage. Take the half of the window chamber with the attached screws and insert the screws through the holes you punched in the skin fold. You may need to clear away connective tissue with the iris scissors to fully insert the screws through the skin fold.

³When performing incisions, avoid severing major vessels. The placement of the window can be altered somewhat to avoid large vessels. If severing a major vessel is unavoidable, allow a few minutes for clotting to occur before continuing with the surgery. Flush the window area with sterile saline to remove any blood which may inhibit visibility through the window.

⁴When cutting away the excess fascia, it is important to leave a layer in place so that the cell solution can be injected between the fascia and the dermis. If too much fascia has been removed, no bubble will form, and the cell solution will not be held in place. If this is the case, try injecting in an area where the fascia remains intact.

14. Manipulate the forward facing skin such that the dermal layer of the far side of the skin fold is fully visible within the window area.
15. Insert the other half of the window chamber through the protruding screws to clamp the skin fold in place. Securely fasten the two halves together with the nuts and nut driver, but ensure that the window is not fastened so tightly as to cut off circulation to any region of tissue.
16. Holding the skin taut, suture the window chamber in place using the suture holes in the window chamber frame. Use multiple knots to ensure that the suture remains in place for the duration of the window chamber study. (If the suture comes off, it can be replaced at a later point in the study using aseptic technique).
17. Once the window is secured, use the disposable surgical blade to cut away the sutures holding the mouse to the c-clamp.
18. Position the window chamber such that the exposed surface is level. Use the syringe and gauze to flush out and remove any remaining fluid within the window.
19. Attach one of the 30-G needles to the glass microsyringe.
20. Retrieve the microcentrifuge vial containing the cancer cells, and use the vortexer to break apart any large cell aggregates (in order to maintain sterility, this step is best performed by an assistant).
21. Draw the cell suspension into the glass microsyringe, noting the volume (ideally 10–20 μ L) needed for your calculated cell-inoculation number.
22. Inserting the needle at the shallowest possible angle with the bevel up, inject the appropriate volume of the cell suspension between the dermal layer and the overlying fascia while avoiding major blood vessels. Bending the needle tip at a 45° angle with the needle driver helps achieve a shallower angle of entry. With a successful injection, a small bubble should appear. If no bubble is visible, attempt the injection again.
23. Attach the other 30-G needle to a 1-mL syringe, and draw it full with warm saline.
24. With the very end of the needle placed level with the window surface and slightly within the window area, place one of the glass cover slips on top such that it is positioned directly over the window, but with one edge supported by the needle tip.
25. Inject saline into the window until it begins to spill over. Remove the needle tip, allowing the cover slip to fall in place over the window. The cover slip should now seal off the saline-covered dermis, and no air bubble should be visible within the window. If an air bubble remains, push up on the window opening on the far skin fold to remove the cover slip, and repeat the process.
26. Once the window is in place with no air bubbles, use the retaining-ring pliers to flex one of the retaining rings, place it flush above the cover slip, and release the pliers leaving the retaining-ring in place to secure the cover slip.
27. Remove the animal from its wrapping and allow it to recover on the heating blanket.
28. As the anesthesia wears off, inject the appropriate dose of buprenorphine subcutaneously for post-surgical pain management. The mouse can be returned to its cage once it regains mobility.

29. If performing additional surgeries, replace the sterile field. Metal surgical tools can be sterilized with the glass-bead sterilizer, although it is important to remember to allow the tools time to cool in order to avoid burning the animal.
30. After all surgeries have been performed return the animals to the appropriate animal facility.
31. Observe the animals daily for the next few days for signs of distress.

3.2. Mammary Window Procedures

3.2.1. Surgical Procedure—The basic procedures in rats and mice are similar, except for the window size. The surgical tools specific to this protocol are shown in Fig. 6.

- 1 Animals are anesthetized with sodium pentobarbital given i.p. at 45 mg/kg body weight for rats and 80 mg/kg for mice. Animals are kept warm using a circulating water blanket. Set temperature at 37°C.
- 2 For hairy rodents, the anterior aspect of lower thorax and abdomen is shaved and depilated with Nair (Carter-Wallace, Inc., New York, NY). Skin is wiped with Chlorhexidine (Baxter-Healthcare, Co., Deerfield, IL) followed by alcohol, alternately for three times.
- 3 Surgery is performed with aseptic technique with the aid of a dissecting microscope. A circular incision with a diameter of 8 mm for rats or 5 mm for mice, is made on the skin around the nipple. For better marking, a clinically-used skin-biopsy puncher, with an 8 or 5 mm diameter, can be used for rats or mice, respectively.
- 4 The thin layer of skin around the base of the nipple is removed within the circular incision.
- 5 The nipple is cut at its base and the nipple sinus is exposed. The lining epithelium is carefully removed.
- 6 Cancer cells or tumor fragments are implanted into the nipple sinus (see below). Cancer-cell suspensions from tissue culture or tumor fragments from a donor rat should be prepared before the surgery starts.
- 7 Pre-gas sterilized window disk (see Subheading 2) is placed into the wound, with the tumor implant located at the center, as shown in Fig. 7.
- 8 The skin edge should cover the periphery of the disk. The disk is sutured to the skin edge using modified subepidermal sutures (5-0 Monosoft® nylon suture). Neosporin ointment is applied around the wound.
- 9 The analgesic buprenorphine HCl can be injected after surgery, at 0.05 or 0.01 mg/kg s.c., for mice and rats, respectively.
- 10 Animals should be monitored and kept on the warm blanket until fully recovered from anesthesia.

3.2.2. Tumor Transplantation into the Window—Different techniques can be used for tumor implantation. For better monitoring of cancer-cell morphological change and tumor–host interactions, single-cell suspensions of fluorescent protein-expressing cancer cells is preferable. The cancer cells can be injected into the mammary tissues before the window disk is mounted. Tumor fragments of R3230 Ac, derived from subcutaneously-implanted tumors in a donor animal can be transplanted into the nipple sinus of Fischer 344 rats, which yields faster tumor growth. For cancer-cell inoculation, half-confluent cells are trypsonized

and washed with PBS twice, immediately prior to surgery. Viable cell numbers are counted, using trypan blue exclusion, with a hemocytometer. Defined concentrations of viable cell suspensions are made by resuspending cell pellets with PBS. A cell-suspension tube can be stored on ice prior to transplantation (see Note 5b).

- 11 Cancer-cell injection: 10–20 μL of cancer cells are injected into the mouse mammary gland at an appropriate cell density depending on cancer-cell type and mouse strain. For the murine mammary carcinoma cell line 4T1 used in Balb/C mice, the usual cell density is $1 \times 10^6/\text{mL}$.
- 12 Cancer cells in Gelfoam transplants: GFP-R32330Ac cells can be used for cancer-cell transplantation. Three microliters of GFP-R32330Ac cancer cells, at $1 \times 10^7/\text{mL}$, are soaked into a 1-mm³ piece of absorbable gelatin sponge (Gelfoam, Pharmacia & Upjohn, Kalamazoo, MI), which is placed into the nipple sinus.
- 13 Tumor fragments of R3230Ac: A 0.5-mm³ piece of tumor tissue from a donor animal, with tumor growing subcutaneously on the thigh, is placed into the nipple sinus for the first generation of the mammary window. Fragments from orthotopically-transplanted R3230Ac tumors in donor rats are used for later surgeries. The methods for tumors fragment implantation, from donor rats bearing subcutaneous tumors, have been reported previously (24). Briefly, donor animals are anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL) at 50 mg/kg i.p. and tumor tissue is removed aseptically. After removal, tumor tissue is rinsed with sterile saline, cut to 0.5 mm³ fragments in filtered DMEM, and maintained in DMEM on ice, not longer than 2 h before being implanted into the nipple sinus.

3.2.3. Postoperative Care—To protect the window disk from being damaged by animals after surgery, a lightweight rat Elizabethan collar (Harvard Apparatus, Holliston, MA) is applied before animals emerged from anesthesia. For mice, an 18-mm wide adhesive bandage (Band-Aid by Johnson & Johnson Medical Inc., Arlington, TX) can be applied for the first 2 days. Animals should be kept in a cage with a wire floor to minimize wound contamination for the first 5 days. The collars and bandages can be removed 2 days after surgery (see Note 6).

3.3. Imaging Methods

1. *Prepare the imaging equipment.* A fluorescent lamp should be turned on and warmed up for the recommended time, typically 5–20 min. A warming pad is required to maintain the animal's body temperature during imaging under anesthesia. The camera should also be turned on and allowed to stabilize at its operating temperature over a similar time frame. A thermally-regulated heating pad, designed for veterinary use, should be allowed to warm up prior to anesthetizing the animal.

^{5b}Different cell lines have different capacities for tumor formation, and this also varies greatly among mouse strains. Failure of a tumor to grow may be due to an inherent incompatibility between a cell line and mouse strain, although other cell lines may simply require higher cell concentrations. It is important to research the cell line of interest to ensure it is compatible with the proposed strain. If a tumor still fails to grow despite known compatibility, try increasing the number of injected cells.

⁶Continue to monitor the animals at frequent intervals post-surgery. If the window chamber becomes grayish or translucent, it is likely that the tissue being held by the frame is suffering from lack of circulation. If it becomes cloudy or whitish, it may be infected. During future surgeries, ensure that the window chamber is not being tightened to the point of inhibiting circulation. Maintain proper aseptic technique.

2. *Anesthetize the animal.* Perhaps the best option is to use 1–2.5% isoflurane + oxygen, which enables a safe and easily controllable depth of anesthesia. The isoflurane dose can be adjusted based on the animals breathing rate and motion. Alternatively, medical air can be used, if tissue oxygenation/hypoxia is being studied, to avoid perturbing the oxygen tension of the tissue. A well-ventilated room and a scavenging system are required to prevent gas build up. Alternatively, injectable anesthesia may be used following the anesthesia protocols described above (see Subheading 2.1.2) (see Note 1).
3. *Prepare the animal for imaging.* The animal should be placed on an imaging mount designed to hold the window securely in place. The window cover glass should be placed directly facing the objective. The warming pad should be placed such that the animal is in direct contact during imaging. Rectal temperature should be monitored to ensure the animal is not hypothermic. Eye drops should be placed on the animal's eyes to prevent drying. A cotton swab wetted with ethanol should be used to clean the window prior to imaging.
4. *Acquire images.* Images are acquired, as with any fluorescence sample, adjusting exposure time, light intensity, gain, aperture size, and other settings specific to each microscope, to ensure adequate signal levels. Important points to observe include: (1) quantitative analysis requires that the signal is not saturated, (2) reducing the illumination power levels minimizes photobleaching, (3) appropriate controls are needed to distinguish autofluorescence or signal bleed-through from true signal, and (4) the animal's temperature and breathing rate should be carefully monitored during imaging.
5. *Post-imaging.* The animal is removed from anesthesia and monitored while body temperature is maintained until it is alert and able to stand.

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Fig. 1.

Fluorescent proteins are used to study the induction of HIF-1 after radiotherapy. A GFP construct with an hypoxia responsive element is used to report HIF-1 activity, while a constitutively-active RFP construct with a cytomegalovirus (CMV) promoter reports overall tumor extent. White light imaging shows the vascular morphology and reformation over time. It can be seen that HIF-1 activity increases significantly post-RT, while the vasculature becomes dilated. Reprinted from (10) with permission from Elsevier.

Fig. 2.
Surgical equipment used in implanting dorsal skin-fold window.

Fig. 3.
Dorsal window chamber frames.

Fig. 4. Window choices: Metal/glass or polycarbonate disk can be used. **(a)** Metal ring with glass cover slide (1) stainless steel ring with holes, (2) internal retaining ring to fix cover glass into the ring, and (3) assembled window. **(b)** Acrylic or polycarbonate disk with holes for suturing.

Fig. 5.
The prepared surgical workstation.

Fig. 6.
Sterile surgical instrument, suture, skin puncher, and acrylic disks in sterile saline.

Fig. 7.
The window disk has been inserted into the circular wound and is being sutured with the skin edge.