

Chapter 4

Dual-Color Imaging of Tumor Angiogenesis

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Summary

Angiogenesis is a critical step in the process of tumor metastasis. Many models have been used to study this process, but they have been artificial and do not reflect the actual process that takes place in the human being. Our laboratory has developed realistic models of angiogenesis based on orthotopic transplantation of human tumors in mice. In order to make angiogenesis visible in real time, our laboratory has developed mouse models in which the blood vessels are labeled with green fluorescent protein (GFP) such that they can be visualized by vascularizing tumors expressing red fluorescent protein (RFP). A particularly valuable model is a nude mouse in which the promoter from the stem-cell-marker protein, nestin, drives the expression of GFP. In such transgenic mice, the nascent blood vessels, in contrast to the mature blood vessels, express GFP. This model, in which human tumors expressing RFP are implanted, has been used to test drugs for their antitumor and antiangiogenic activity. We have observed for the first time the high antiangiogenic efficacy of cancer drugs such as gemcitabine and doxorubicin. These models should prove very valuable in the discovery of new antiangiogenesis drugs.

Key words: GFP, RFP, Orthotopic transplantation, Nascent blood vessels, Angiogenesis, Anti-angiogenesis drugs.

1. Introduction

1.1. Treating Cancer with Antiangiogenesis Drugs

Over a century ago it was observed that the growth of human tumors is often accompanied by increased vascularity (1). The existence of tumor-derived factors responsible for promoting new vessel growth was postulated over 65 years ago (2), and a few years later it was proposed that tumor growth is crucially dependent on the development of a neovascular supply (3, 4).

The concept of treating tumors by inhibiting their ability to recruit new blood vessels (angiogenesis) was pioneered by Folkman (5). It is thought that solid tumors cannot grow beyond

microscopic sizes without forming new blood vessels, and will remain dormant, or even regress, if angiogenesis is prevented or is not induced (6). Tumors may stimulate angiogenesis (7). Both genetic and environmental events (such as lack of oxygen) appear to contribute to the switching on of new blood vessel growth. Genetic events that could induce angiogenesis include the ras oncogene activation and inactivation of the p53 tumor-suppressor gene (8, 9). The endothelial cells of new blood vessels have receptors that bind to vascular endothelial growth factors (VEGFs), a family of inducers of angiogenesis. These receptors make the endothelial cells of newly formed blood vessels selectively sensitive to agents such as antibodies directed against VEGF or the receptors themselves (see below).

Ferrara and Kerbel (4) noted that despite some initial setbacks and negative clinical trial results, major progress has been made over the past few years in targeting angiogenesis for human therapy. In 2004, the US Food and Drug Administration (FDA) approved bevacizumab (Avastin), a humanized anti-VEGF-A monoclonal antibody, for the treatment of metastatic colorectal cancer in combination with 5-fluorouracil (FU). This followed from a phase III study showing a survival benefit (10).

Boehm et al. (11) reported that chronic, intermittent therapy of three different mouse tumors with an inhibitor of angiogenesis, endostatin, did not lead to any traces of acquired resistance. For each treatment cycle, the tumor was allowed to grow (or regrow) until it reached ~3% of the body weight of the mouse. Endostatin was then infused daily until the tumors regressed. Tumors regressed even after the sixth successive round of endostatin therapy with no apparent resistance (6). In contrast, standard chemotherapy, using maximum doses of the drug cyclophosphamide, resulted in partial resistance by the third cycle of treatment, and complete resistance by the fourth cycle (6). Resistance to antiangiogenic therapy is emerging (12) and thus a better understanding of pathways that may mediate tumor angiogenesis in various circumstances is necessary (4).

Many patients treated with VEGF inhibitors, especially in combination with chemotherapy, may survive longer, but they eventually succumb to their disease. VEGF may be replaced by other angiogenic pathways as the disease progresses. For example, VEGF-unrelated pathways may become activated. Other members of the VEGF family such as the lymphangiogenic factors VEGF-C and VEGF-D, may bind to and activate VEGFR-2 after proteolytic cleavage (13). Other possible mechanisms for acquired resistance to antiangiogenic drugs (14, 15) include selection and overgrowth of tumor-cell variants that are hypoxia resistant and thus less dependent on angiogenesis (16). The recruitment of bone-marrow-derived angiogenic cells may also provide a potential mechanism of resistance to antiangiogenic strategies (4).

Furthermore, Ferrara and Kerbel (4) noted that recent studies suggest that in some cases endothelial cells associated with tumors are not a genetically stable, nontransformed, compartment (as long assumed) and instead may provide a further mechanism of resistance to antiangiogenic therapies. Streubel et al. (13) reported that a significant percentage of the endothelial cells in human B-cell lymphomas harbor lymphoma-specific chromosomal abnormalities, suggesting that endothelial cells in B-cell lymphomas may be in part tumor related. Genetic instability in these endothelial cells could lead to resistance to anti-angiogenesis agents.

1.2. Previous Models Used to Visualize Angiogenesis

The discovery and evaluation of anti-angiogenic substances initially relied on methods such as the chorioallantoic membrane assay (17, 18), the monkey iris neovascularization model (19), the disk angiogenesis assay (20), and various models that use the cornea to assess blood vessel growth (21–26). Although they are important for understanding the mechanisms of blood vessel induction, these models do not represent tumor angiogenesis and are poorly suited to drug discovery.

Subcutaneous tumor xenograft mouse models have been developed to study tumor angiogenesis, but these require cumbersome pathological examination procedures such as histology and immunohistochemistry. Measurements require animal sacrifice and therefore preclude ongoing angiogenesis studies in individual, live, tumor-bearing animals. Moreover, subcutaneous tumor xenografts are not representative models of human disease.

Tumors transplanted in the cornea of the rodents (27–29) and rodent skin-fold window chambers have also been used for angiogenesis studies (30–36). The cornea and skin-fold chamber models provide a means for studying tumor angiogenesis in living animals. However, quantification requires specialized procedures, and the sites do not represent natural environments for tumor growth. The cornea and skin-fold window chamber tumor models do not allow metastatic angiogenesis to occur, which may involve mechanisms of angiogenesis (37) that are qualitatively different from those occurring in ectopic models.

1.3. Dual-Color Fluorescent Proteins in Tumor–Host Models to Differentially Visualize the Tumor and Blood Vessels

Okabe et al. (38) produced transgenic mice with GFP under the control of a chicken β -actin promoter and cytomegalovirus enhancer. All of the tissues from these transgenic mice, with the exception of erythrocytes and hair, fluoresce green. Fluorescent proteins have been shown to be very useful for imaging in tumors including the formation of nascent vessels. We have developed unique mouse models to image tumor angiogenesis with fluorescent proteins, which are described in this chapter.

Tumor cells to be transplanted in the GFP mouse were made visible by transforming them with the red fluorescent protein

(RFP) (39). In order to gain further insight into tumor–host interaction in the living state, including tumor angiogenesis, we have visualized RFP-expressing tumors transplanted in the GFP-expressing transgenic mice under dual-color fluorescence microscopy. The dual-color fluorescence made it possible to visualize the tumor growth in the host by whole-body imaging as well as to visibly distinguish interacting tumor and host cells in fresh tissue. The dual-color approach affords a powerful means of both visualizing and distinguishing the components of the host–tumor interaction (40).

Dual-color images of early events in tumor angiogenesis induced by a B16F10 mouse melanoma in the transgenic GFP-expressing mouse were acquired in fresh tissue preparations. Host-derived GFP-expressing fibroblast cells and endothelial cells form nascent blood vessels were visualized clearly against the red fluorescent background of the RFP-expressing mouse melanoma. Host-derived GFP-expressing mature blood vessels within the RFP-expressing mouse melanoma also became visible. The images were acquired 3 weeks after subcutaneous injection of B16F10-RFP melanoma cells in the GFP mouse (40).

A GFP nude mouse was obtained by crossing nontransgenic nude mice with the transgenic GFP C57/B6 mouse (41). In the nude mice, the organs all brightly expressed GFP, including the heart, lungs, spleen, pancreas, esophagus, stomach, and duodenum. RFP-expressing human cancer cell lines, including PC-3-RFP prostate cancer, HCT-116-RFP colon cancer, MDA-MB-435-RFP breast cancer, and HT1080-RFP fibrosarcoma were transplanted to the transgenic GFP nude mice. All of these human tumors grew extensively in the transgenic GFP nude mouse. Dual-color fluorescence imaging enabled visualization of human tumor–host interaction by whole-body imaging and at the cellular level in fresh and frozen tissues. The GFP mouse model should greatly expand our knowledge of human tumor–host interaction (41).

1.4. Imaging of Nascent Angiogenesis Using Nestin-Driven GFP Transgenic Mice

The intermediate filament protein, nestin, marks progenitor cells of the CNS. Such CNS stem cells are selectively labeled by placing GFP under the control of nestin regulatory sequences in transgenic mice (nestin-driven GFP [ND-GFP]). In the ND-GFP transgenic mice, the hair follicle stem cells express ND-GFP. The hair follicles are linked by a network of ND-GFP-expressing blood vessels which appear to originate from the hair follicle stem cells (42).

We visualized tumor angiogenesis by dual-color fluorescence imaging in ND-GFP transgenic mice after transplantation of the murine melanoma cell line B16F10-expressing RFP. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumor. Immunohistochemical

staining showed that the blood vessel-specific antigen CD31 was expressed in ND-GFP-expressing nascent blood vessels. ND-GFP expression was diminished in the vessels with increased blood flow. Progressive angiogenesis during tumor growth was readily visualized by GFP expression. Doxorubicin inhibited the nascent tumor angiogenesis as well as tumor growth in the ND-GFP mice transplanted with B16F10-RFP (43).

The nestin ND-GFP gene was crossed into nude mice on the C57/B6 background to obtain ND-GFP nude mice. ND-GFP was expressed in the brain, spinal cord, pancreas, stomach, esophagus, heart, lung, blood vessels of glomeruli, blood vessels of skeletal muscle, testes, hair follicles, and blood vessel network in the skin of ND-GFP nude mice. Human lung cancer, pancreatic cancer, and colon cancer cell lines as well as a murine melanoma cell line and breast cancer tumor cell line expressing RFP were implanted orthotopically, and a RFP-expressing human fibrosarcoma was implanted s.c. in the ND-GFP nude mice. These tumors grew extensively in the ND-GFP mice. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumors, visualized by dual-color fluorescence imaging. Immunohistochemical staining showed that CD31 was expressed in the ND-GFP-expressing nascent blood vessels (44).

Dual-color fluorescence imaging tumor visualized angiogenesis in the ND-GFP transgenic nude mice after orthotopic transplantation of the MIA PaCa-2 human pancreatic cancer line expressing RFP. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumor. The density of nascent blood vessels in the tumor was readily quantitated. Gemcitabine significantly decreased the mean nascent blood vessel density in the tumor as well as decreased tumor volume. These results demonstrated for the first time that gemcitabine is an inhibitor of angiogenesis as well as tumor growth in pancreatic cancer (45).

In another study, nascent angiogenesis of pancreatic cancer liver metastasis in the ND-GFP transgenic nude mice was imaged. Human pancreatic cancer cells were visualized by RFP. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing liver metastasis. The density of nascent blood vessels in the tumor was readily quantitated. Gemcitabine significantly decreased the mean nascent blood vessel density in the pancreatic liver metastases (46).

Angiogenesis of sarcoma formed by the HT-1080 human fibrosarcoma cell line expressing RFP was also imaged in the ND-GFP mice. Tumor cells were injected into either the muscle or the bone. Nestin was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumors, including the surrounding tissues. Doxorubicin significantly decreased the mean nascent blood vessel density in the tumors as well as

decreased tumor volume. Thus, the dual-color model of the ND-GFP nude mouse and RFP sarcoma cells is also useful for the visualization and quantitation of bone and soft tissue tumor angiogenesis and evaluation of angiogenetic inhibitors for such tumors (47).

Thus, fluorescent proteins are very useful for imaging angiogenesis. Their intrinsic brightness, nontoxicity, and multiple colors make them the genetic reporters of choice to image angiogenesis. Particularly powerful are mouse models in which the vessels express one-color protein and the tumor expresses another. It is expected that these models will lead to a deeper understanding of the real-time angiogenesis process and to the discovery of safe and more effective antiangiogenesis agents for metastatic cancer.

2. Materials

2.1. Reagents

1. Restriction enzymes HindIII and NotI
2. RFP cDNA (pDsRed2; Clontech)
3. Plasmid pLNCX2 (Clontech). This plasmid is derived from Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV) elements for retroviral gene delivery and expression. Upon transfection into a packaging cell line, pLNCX2 can transiently express, or integrate and stably express, a transcript containing Ψ + (the extended viral packaging signal), and a gene of interest under direction of the human CMV promoter.
4. PT67 packaging cells (Clontech); 3T3 cells for viral titering; cell lines to be transfected with genes encoding fluorescent proteins, such as B16F0 melanoma cells (American Type Culture Collection)
5. Growth medium (normal and selective) appropriate for cell culture, such as DMEM (Invitrogen; Irvine Scientific)
6. Fetal bovine serum (FBS; Gemini Biological Products)
7. Lipofectamine PLUS transfection kit (Invitrogen)
8. G418 neomycin
9. Polysulfonic filter, 4.5 μ m
10. Polybrene
11. Trypsin-EDTA and trypsin
12. Mice expressing GFP ("GFP mice"; Jackson Laboratories; Japan SLC, Hamamatsu, Japan)
13. Immunocompetent and immunodeficient mice (Charles River; Taconic; Harlan Teklad)

14. Anesthetic reagents (ketamine HCl, xylazine, acepromazine maleate; “ketamine mixture”; Butler Animal Health Supply)
15. Nair (Carter-Wallace)
16. Doxorubicin
17. NaCl, 0.9%
18. Optimum cutting temperature blocks
19. Antibody to rat immunoglobulin (antirat immunoglobulin) and antimouse immunoglobulin horseradish peroxidase detection kits (BD PharMingen)
20. Monoclonal anti-CD31 (CBL1337; Chemicon)
21. Monoclonal anti-nestin (rat 401; BD PharMingen)
22. Substrate-chromogen 3,3'-diaminobenzidine

2.2. Equipment

1. 60-mm culture dishes; 25-mm culture flasks; 96-well plates
2. Humidified incubator at 37 °C and 5% CO₂
3. Cloning cylinders (Bel-Art Products)
4. 27G2 latex-free syringe, 1 mL (Becton Dickinson)
5. 8-0 surgical sutures
6. Leica fluorescence stereo microscope, model LZ12, with a mercury 50-W power supply, and MZ6 stereo microscope (Leica)
7. D425/60 band-pass filter and 470 DCXR dichroic mirror
8. D470/40 emission filter and GG475 emission filter (Chroma Technology)
9. C5810 three-chip cooled color charge-coupled device (CCD) camera (Olympus) (Hamamatsu Photonics Systems) or DP70 CCD camera (Olympus)
10. Image-Pro Plus 4.0 software (Media Cybernetics)
11. Personal computer (PC; IBM or Fujitsu-Siemens)
12. VCR (Sony, model SLV-R1000)
13. Blue LED flashlight (LDP LLC)
14. Coolpix camera (Nikon)
15. Fluorescent lightbox with fiberoptic lighting at 470nm (FluorVivo Imaging System; INDEC Systems, Inc.)
16. OVI100 Small Animal Imaging System (Olympus) with an M20 light source (Olympus Biosystems) and 470-nm excitation light
17. Paint Shop Pro 8 (Corel) and Cell^R (Olympus Biosystems)
18. Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-W lamp power supply
19. Leica CM1850 cryostat

2.3. Nude GFP Mice

Use a gene encoding GFP under the control of either the β -actin promoter, resulting in ubiquitous GFP expression, or the promoter of the gene encoding nestin, resulting in selective GFP expression, including expression in nascent blood vessels in tumors. To obtain nude GFP mice, first cross 6-week-old female C57BL/6 GFP mice with 6- to 8-week-old BALB/c homozygous nude (*nu/nu*) or NCR *nu/nu* male mice, then cross male F₁ mice with female F₁ C57BL/6 GFP mice (48). Before working with animals proper ethical documentation must be obtained, and nude mice require special housing facilities and diet (*see Note 1*).

2.4. Whole-Body Imaging Equipment

We use an Olympus OV100 Small Animal Imaging System with an MT-20 light source and DP70 CCD camera (Olympus) for whole-body imaging in live mice at variable magnification. The optics of the OV100 fluorescence imaging system have been especially developed for “macroimaging” as well as “microimaging” with high light-gathering capacity. The instrument incorporates a unique combination of high-numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 10⁵-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has lenses mounted on an automated turret with a magnification range of $\times 1.6$ to $\times 16$ and a field of view ranging from 6.9 nm to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiple fluorescent reporters in small animals. High-resolution images are captured directly on a PC (Fujitsu-Siemens). Images are processed for contrast and brightness and are analyzed with the use of Paint Shop Pro 8 and cell^R.

Many other fluorescence imaging systems can also be used for dual-color tumor–host imaging. For example, a Leica fluorescence stereo microscope (model LZ12) equipped with a mercury 50-W lamp power supply can be used. Selective excitation of GFP is produced via a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence is collected through a long-pass filter (GG475). Anesthetized animals can be examined with a microscope and images can be acquired with a Hamamatsu C5810 three-chip cooled color CCD camera. Images can also be processed for contrast and brightness and can be analyzed with the use of Image-Pro Plus software. High-resolution images of 1,024 \times 724 pixels can be captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR. Simpler systems such as a light box with appropriate filters and camera or even a blue light LED flashlight with appropriate filters can be used for macroimaging (discussed below) (48–51).

3. Methods

3.1. RFP Retrovirus Production

1. Insert the *HindIII*–*NotI* fragment from pDsRed2, containing full-length RFP cDNA, into the *HindIII*–*NotI* site of pLNCX2, which contains a neomycin-resistance gene, to establish the pLNCX2-DsRed2 plasmid.
2. Use PT67, an NIH3T3-derived packaging cell line expressing the 10A1 viral envelope, to produce retrovirus.
3. Culture PT67 cells in DMEM medium supplemented with 10% heat-inactivated FBS. It takes ~3 days for the cells to reach ~70% confluence after 3×10^5 PT67 cells are seeded in a 25-mm² flask with DMEM medium containing 10% FBS.
4. For vector production, when PT67 packaging cells reach 70% confluence in the 25-mm² flask, replat cells in a 60-mm culture dish at 60–80% confluence 12 h before transfection.
5. Use 10 µg pLNCX2-DsRed2 DNA and the Lipofectamine PLUS transfection kit. Add 7 µL pLNCX2-DsRed2 DNA to 87 µL serum-free medium in a tube and then add 6 µL Lipofectamine reagent. Mix and incubate for 15 min at 22–26 °C (room temperature).
6. Dilute 4 µL Lipofectamine reagent in 96 µL serum-free medium in a second tube. Mix and incubate for 15 min at room temperature.
7. Combine the DNA prepared in step 3 and diluted Lipofectamine reagent, then mix and incubate for 15 min at room temperature.
8. While the complexes are forming, replace medium on the recently plated cells with 800 µL of serum-free DMEM. Add the DNA–Lipofectamine complex to the dish with cells containing fresh DMEM. Mix the complexes in the medium gently; incubate for 4 h at 37 °C in 5% CO₂.
9. After 4 h incubation, increase volume of medium to 5 mL and incubate for 24 h at 37 °C in 5% CO₂.
10. After 24 h incubation, clone the packaging cells by limit dilution in 96-well plates.
11. For selection of a PT67 packaging cell clone producing large amounts of RFP retroviral vector (PT67-DsRed2), culture the cells first in the presence of 200 µg/mL G418. Every 1–2 days, increase the concentration of G418 to 400, 600, 800, then 1,000 µg/mL (*see Note 2*).
12. Clones of PT67-DsRed2 cells with high viral titer production are identified with 3T3 cells used for virus titering. Clones with a titer higher than 1×10^6 plaque-forming units per mL are used for RFP vector production.

3.2. RFP Gene Transduction of Tumor Cell Lines

1. For RFP gene transduction, use cancer cells that are 20% confluent. Plate cancer cells at a density of 1×10^5 to 2×10^5 cells per 60-mm plate 12–18 h before infection with RFP retrovirus.
2. For retroviral infection, collect conditioned medium from packaging cells (PT67–DsRed2) and filter it through a 0.45- μm polysulfonic filter. Add the virus-containing filtered medium to the target cancer cells.
3. Add polybrene to a final concentration of 8 $\mu\text{g}/\text{mL}$ and incubate the cells for 24 h at 37 °C.
4. Replace the medium with DMEM and 10% FBS after 24 h incubation and check for RFP-expressing cells by fluorescence microscopy.
5. Collect tumor cells with trypsin–EDTA and subculture them at a ratio of 1:15 in selective medium, which contains 50 $\mu\text{g}/\text{mL}$ G418.
6. To select for the most brightly fluorescent cells, increase G418 to a concentration of 800 $\mu\text{g}/\text{mL}$ (in 200 $\mu\text{g}/\text{mL}$ increments), by culturing cells for 1–2 days in each concentration of G418 (*see Note 3*).
7. Isolate clones expressing RFP with cloning cylinders using trypsin–EDTA and amplify them in DMEM in the absence of the selective agent (*see Note 4*). Further select cells for brightness and stability.

3.3. Tumor Models of Fluorescent Protein-Expressing Tumor Cells

3.3.1. Cell Injection to Establish an Experimental Metastasis Model

Use one of the following options to establish a tumor model of fluorescent protein-expressing tumor cells: i.v. cell injection (a), surgical orthotopic implantation (b), or inoculation of cells by intradermal injection (c).

1. Collect fluorescent protein-expressing tumor cells by trypsinization for 3 min at 37 °C with 0.25% trypsin.
2. Wash the cells three times with cold serum-free medium using a tabletop centrifuge at $500 \times g$.
3. Resuspend the cells in ~0.2 mL of serum-free medium.
4. Within 30 min of collecting cells, inject 1×10^6 tumor cells in a total volume of 0.2 mL into 6-week-old C57BL/6 GFP mice or nude (*nu/nu*) GFP mice via the lateral tail vein or subcutaneously using a 1-mL 27G2 latex-free syringe (*see Note 5*).
5. For liver colonization, inject fluorescent protein-expressing cells directly into the portal vein in anesthetized mice (details on inducing anesthesia are presented below).

3.3.2. Surgical Orthotopic Implantation to Establish a Spontaneous Metastasis Model (*See Note 6*)

1. Induce anesthesia with a “ketamine mixture” (10 μL ketamine HCl, 7.6 μL xylazine, and 2.4 μL acepromazine maleate) by s.c. injection.
2. Use a microscope (Leica MZ6) with magnification of about $\times 6$ to about $\times 40$ for all procedures of the operation.

3. Isolate fluorescent protein-expressing tumor fragments ($n \text{ mm}^3$) from subcutaneously growing tumors, formed by injection of RFP-expressing tumor cells by mincing tumor tissue into 1-mm^3 fragments. After proper exposure of the target organ, implant three tumor fragments per transgenic GFP mouse (*see Note 7*).
4. With 8-0 surgical sutures, penetrate the tumor fragments and suture the fragments onto the target organ.
5. Keep mice in a barrier facility under high-efficiency particulate air filtration.

3.3.3. RFP-Expressing Cutaneous Melanoma Model

1. Collect the RFP-expressing mouse B16F0 melanoma cells by trypsinization for 3 min at 37°C with 0.25% trypsin.
2. Wash cells three times with cold serum-containing medium using a tabletop centrifuge at $500 \times g$ for 5 min at room temperature, and then keep on ice.
3. Inject 6-week-old male C57BL/6 GFP mice or nude GFP mice with 1×10^6 RFP-expressing mouse B16F0 melanoma cells that were collected and washed. This is done by intradermal injection of cells into the dorsal skin of the mouse in a total volume of $50 \mu\text{L}$ cell culture medium within 40 min of collection.

3.4. Imaging

Use one of the following methods for whole-body imaging of mice: microscopy (a), flashlight imaging (b), light-box imaging (c), or chamber imaging (d).

3.4.1. Microscopy

1. Use a Leica fluorescence stereo microscope (model LZ12) equipped with a mercury 50-W lamp power supply or its equivalent.
2. Produce selective excitation of GFP via a D425/60 band-pass filter and 470 DCXR dichroic mirror.
3. Collect emitted fluorescence through a long-pass filter (GG475) on a Hamamatsu C5810 three-chip cooled color CCD camera or its equivalent.
4. Process images for contrast and brightness with the use of Image-Pro Plus 4.0 software or its equivalent.
5. Capture high-resolution images of $1,024 \times 724$ pixels directly on an IBM PC or continuously through video output on a high-resolution Sony VCR, model SLV-R1000 or its equivalent.
6. For C57BL/6 mice, remove hair with Nair or by shaving before images are obtained (*see Note 8*).

3.4.2. Chamber Imaging

1. Do whole-body imaging with an Olympus OV100 imaging system using 470-nm excitation light originating from an MT-20 light source.

2. Collect emitted fluorescence through appropriate filters configured on a filter wheel with a DP70 CCD camera. Variable magnification imaging can be done with a series of four objective lenses.
3. Capture images on a PC (Fujitsu-Siemens), and process images for contrast and brightness with Paint Shop Pro 8 and cell^R.
4. For C57BL/6 mice, remove hair with Nair or by shaving before images are obtained (*see Note 8*).

3.4.3. Tumor Tissue Sampling

1. Obtain tumor tissue biopsies from 3 days to 4 weeks after inoculation of tumor cells. Biopsies of tumor tissue can be obtained from anesthetized mice by removal of a small piece of tumor tissue (1 mm³ or less) with a scalpel.
2. Staunch bleeding by pressing the wound with sterile gauze. Alternatively, the mouse can be killed and the tissue can be collected and processed for analysis.
3. Cut fresh tissue into pieces of ~1 mm³ and gently press onto slides for fluorescence microscopy. This procedure is done manually on normal slides.
4. To analyze tumor angiogenesis, digest the tissues with trypsin-EDTA for 5 min at 37 °C before examination.
5. After trypsinization, put tissues on precleaned microscope slides and cover with another microscope slide.

3.5. Fluorescence Microscopy

1. Use an Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-W lamp power supply or its equivalent.
2. To visualize both GFP and RFP fluorescence at the same time, produce excitation light via a D425/60 band-pass filter and a 470 DCXR dichroic mirror (*see Note 9*).
3. Collect emitted fluorescence light through a GG475 long-pass filter.
4. Capture high-resolution images of 1,024 × 724 pixels with a Hamamatsu C5810 three-chip-cooled color CCD camera or its equivalent and store directly on an IBM PC or its equivalent.
5. Process images for contrast and brightness using Image-Pro Plus 4.0 software or its equivalent.

3.6. Measurement of GFP-Expressing Tumor Blood Vessel Length and Evaluation of Antiangiogenic Agents

1. Give mice daily i.p. injections of doxorubicin (5 µg/g body weight in a 2-mg/mL solution of 0.9% NaCl) or other drugs or 0.9% NaCl solution (vehicle controls) on days 0, 1, and 2 after implantation of tumor cells.
2. Anesthetize mice with the ketamine mixture and obtain biopsies on days 10, 14, 21, and 28 after implantation using sterile technique (*see Note 10*).

3. Biopsies are removed from the tumor with a scalpel.
4. Gently flatten the tumor tissue between the slide and coverslip.
5. Quantify angiogenesis in the tumor tissue by measuring the length of GFP-expressing blood vessels in all fields using fluorescence microscopy.
6. Obtain measurements in all fields at $\times 40$ or $\times 100$ magnification to calculate the total length of GFP-expressing blood vessels.
7. Calculate the vessel density by dividing the total length of GFP-expressing blood vessels (in mm) by the tumor volume (in mm^3).

3.7. Immunohistochemical Staining

1. "Snap-freeze" fresh tissue with liquid nitrogen, then orient and embed the frozen tissue in optimum cutting temperature blocks and store at -80°C . Cut the frozen sections to a thickness of $5\ \mu\text{m}$ with a Leica CM1850 cryostat.
2. Detect colocalization of GFP fluorescence, CD31, and nestin in the frozen skin sections of mice transgenic for nestin-driven GFP expression using the anti-rat immunoglobulin and anti-mouse immunoglobulin horseradish peroxidase detection kits following the manufacturer's instructions.
3. Use monoclonal anti-CD31 (1:50 dilution) and monoclonal anti-nestin (1:80 dilution) as primary antibodies. To identify the GFP-expressing tumor-infiltrating natural killer cells, macrophages, and dendritic cells, detect localization of GFP together with cell surface markers using immunohistochemical staining with monoclonal antibodies to NK1.1, CD111b, and CD11c, respectively.
4. Use staining with substrate-chromogen 3,3'-diaminobenzidine for antigen detection.

Notes

1. All animal studies are done in accordance with the principles and procedures outlined in the National Institutes of Health National Research Council's *Guide for the Care and Use of Laboratory Animals* (available at <http://oacu.od.nih.gov/regs/guide/guidex.htm>) under assurance number A3873-1. Nude mice must be kept in a barrier facility under high-efficiency particulate air filtration. Also, these mice must be fed an autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products).

2. Increasing the amount of G418 in a stepwise way is very important for inducing transgene expression. This procedure ensures high production of RFP⁺ retrovirus.
3. Increasing G418 in a stepwise way is very important for inducing expression of the transgene. This procedure ensures high expression of RFP.
4. Selecting for cancer cells stably and brightly expressing RFP in the absence of antibiotic, after antibiotic selection, ensures that the cells will stably express RFP in the absence of antibiotic selection. This is necessary since there will be no selection when the cells are transplanted into the mice. It normally takes 7–10 days in culture without selection to obtain a sufficient number of cells for imaging studies.
5. Some cells in suspension may lose viability rapidly over time and therefore should be injected as soon as possible.
6. Orthotopic implantation of tumor fragments results in higher spontaneous metastatic rates than injection of a cell suspension.
7. Bleeding should not occur if the surgery is properly performed. It must be avoided at the surgical site, as hemoglobin will absorb the incident excitation light and interfere with the fluorescent imaging.
8. Hair is highly autofluorescent, so improper removal of hair will result in low-quality images.
9. It is important to minimize autofluorescence of tissue and body fluids by using proper filters. Excitation filters should have a narrow band as close to 490 nm as possible to specifically excite GFP whose peak is distinct from that of the skin, tissues, and fluid of the animal. In addition, proper band-pass emission filters should be used with a cutoff of ~515 nm (Chroma Technology).
10. When using open-biopsy procedures or other surgical procedures that open the animal, it is essential to hydrate the animal by spraying sterile normal saline on the open tissue. Also, when repeating procedures such as an open biopsy or other invasive procedures, it is critical to maintain a properly sterile operation field and sterile instruments to avoid infection.

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