Color-coded fluorescence imaging of tumor-host interactions

Robert M Hoffman[1,2] and Meng Yang[1]

[1]AntiCancer, 7917 Ostrow Street, San Diego, California 92111, USA, and [2]Department of Surgery, University of California, San Diego, 200 West Arbor Drive, San Diego, California 92103-8220 USA. Correspondence should be addressed to R.M.H. (all@anticancer.com).

Published online 3 August 2006; doi:10.1038/nprot.2006.119

Fluorescent proteins have the properties of being very bright with high quantum yield and are available in many colors. Tumor-host models consist of transgenic mice expressing green fluorescent protein (GFP) in essentially all cells and tissues or expressing GFP selectively in specific tissues such as blood vessels. Particularly useful are the corresponding nude mice transgenic for GFP expression, as they can accept human tumors. When tumor cells expressing red fluorescent protein are implanted in mice expressing GFP, various types of tumor-host interactions can be observed, including those involving host blood vessels, lymphocytes, tumor-associated fibroblasts, macrophages, dendritic cells and others. The 'color-coded' tumor-host models enable imaging and therefore a deeper understanding of the host cells involved and their function in tumor progression. Approximately 4–8 weeks are needed for these procedures.

INTRODUCTION

Tumor–host cell interactions in the tumor microenvironment have profound effects on tumor progression. This protocol describes methods for imaging tumor-host interactions with the use of fluorescent proteins. Fluorescent proteins have the properties of being very bright with high quantum yield and are available in many colors[1]. The origins of these proteins are sea animals such as jellyfish and corals, in which they accomplish essential protective and other functions. These fluorescent proteins have distinct emission peaks and can be readily distinguished in the live animal. Conveniently, they are excited by blue light, so only one light source is needed. We have exploited those properties to produce 'color-coded' tumor-host model systems that offer the possibility of imaging tumor-host interactions at the cellular level[2–5]. Such models consist of transgenic mice expressing green fluorescent protein (GFP) in essentially all cells and tissues, with the GFP under the control of the β-actin promoter[2,3,6], or expressing GFP selectively in specific tissues such as blood vessels[4,5]. In the latter case, expression of GFP is driven by the enhancer of the gene encoding nestin, which is active in nascent blood vessels[7]. Particularly useful are the corresponding nude mice transgenic for GFP, as they can accept human tumors[3,5]. Tumor cells are labeled with red fluorescent protein (RFP). When tumor cells expressing RFP are implanted into mice expressing GFP (as in **Fig. 1**), various types of tumor interactions can be observed[2–5]. In fresh tissue specimens, tumor vessels expressing GFP can be visualized vascularizing tumors expressing RFP in primary and metastatic sites (**Fig. 2**). Dendritic cells expressing GFP can be seen in close contact with tumor cells with their dendritic processes (**Fig. 3**). Stromal fibroblasts expressing GFP can be seen in contact with multiple cancer cells through their pseudopodia. Lymphocytes expressing GFP can be observed in the process of rejecting tumor cells from growing in immunocompetent mice. Macrophages expressing GFP can be observed engulfing tumor cells expressing RFP (**Fig. 4**). The color-coded tumor-host models will enable imaging and therefore help elucidate to a much greater extent the function of the stromal host cells involved in tumor progression. The stromal cells can now also provide visible targets for new classes of drugs.

MATERIALS

REAGENTS

- Restriction enzymes *Hin*dIII and *Not*I
- RFP cDNA (pDsRed2; Clontech)
- Plasmid pLNCX2
- 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)
- Growth medium (normal and selective) appropriate for cell culture, such as DMEM (Invitrogen; Irvine Scientific)
- Fetal bovine serum (FBS; Gemini Biological Products)
- Lipofectamine PLUS (Invitrogen)
- G418 neomycin (Invitrogen)
- Polysulfonic filter, 4.5 μm
- Polybrene
- Trypsin-EDTA (Fisher Scientific) and trypsin
- Mice expressing GFP ('GFP mice'; Jackson Laboratories; Japan SLC, Hamamatsu, Japan)
- Immunocompetent and immunodeficient mice (Charles River; Taconic; Harlan Teklad) ▲ CRITICAL 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. Animals are kept in a barrier facility under high-efficiency particulate air filtration. Mice are fed an autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products).

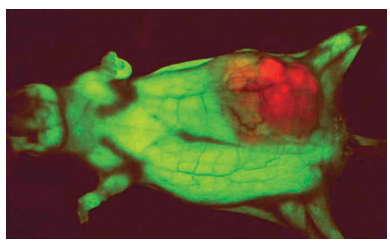

Figure 1 | Whole-body image of orthotopically growing HCT 116 RFP$^+$ human colon cancer in a nude GFP mouse. Image was acquired in a fluorescence light box with a CCD camera 10 weeks after orthotopic implantation of HCT 116 RFP$^+$ cells. Original magnification, ×1. Image from ref. 2.

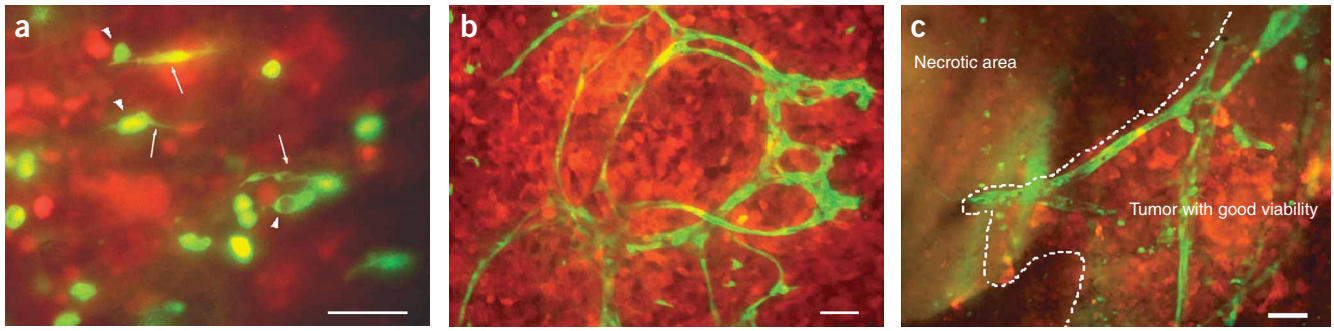

Figure 2 | Visualization of angiogenesis in live tumor tissue 3 weeks after s.c. injection of B16F10 RFP$^+$ melanoma cells in a transgenic GFP mouse. (**a**) Host-derived GFP-expressing fibroblast cells (arrows) and endothelial cells (arrowheads) form new blood vessels in the RFP-expressing B16F10 melanoma. (**b**) Well developed, host-derived GFP-expressing blood vessels in an RFP-expressing mouse melanoma. (**c**) GFP-expressing tumor vasculature can be readily identified in the area where the tumor tissue maintains good viability; however, only remnants of GFP-expressing vasculature can be visualized in the necrotic area. Scale bars, 50 μm. Images from ref. 2.

- Anesthetic reagents (ketamine HCl, xylazine, acepromazine maleate; 'ketamine mixture'; Butler Animal Health Supply)
- Nair (Carter-Wallance)
- Doxorubicin
- NaCl, 0.9%
- Optimum cutting temperature blocks
- Antibody to rat immunoglobulin (anti–rat immunoglobulin) and anti–mouse immunoglobulin horseradish peroxidase detection kits (BD PharMingen)
- Monoclonal anti-CD31 (CBL1337; Chemicon)
- Monoclonal anti-nestin (rat 401; BD PharMingen)
- Substrate-chromogen 3,3′-diaminobenzidine

EQUIPMENT

- Culture dishes, 60 mm (Fisher Scientific); flask, 25 mm; plates, 96-well
- Humidified incubator at 37 °C and 5% CO_2
- Cloning cylinders (Bel-Art Products)
- 27G2 latex-free syringe, 1 ml (Becton Dickinson)
- 8-0 surgical suture
- Leica fluorescence stereo microscope, model LZ12, with a mercury 50-Watt power supply, and MZ6 stereo microscope (Leica)
- D425/60 band-pass filter and 470 DCXR dichroic mirror
- D470/40 emission filter and GG475 emission filter (Chroma Technology)
- C5810 three-chip cooled color charge-coupled device (CCD) camera (Olympus) (Hamamatsu Photonics Systems) or DP70 CCD camera (Olympus)
- Image-Pro Plus 4.0 software (Media Cybernetics)
- Personal computer (PC; IBM or Fujitsu-Siemens)
- VCR (Sony, model SLV-R1000)
- Blue LED flashlight (LDP LLC)
- Coolpix camera (Nikon)
- Fluorescent lightbox with fiberoptic lighting at 470 nm (Lightools Fluorescent Imaging System; Lightools Research)
- OV100 Small Animal Imaging System (Olympus) with an M20 light source (Olympus Biosystems) and 470-nm excitation light
- Paint Shop Pro 8 (Corel) and cell^R (Olympus Biosystems)
- Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-Watt lamp power supply
- Leica CM1850 cryostat

REAGENT SETUP

Nude GFP mice Use a gene encoding GFP under the control of either the β-actin promoter, resulting in ubiquitous GFP expression, or the enhancer 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 F_1 mice with female F_1 C57BL/6 GFP mice.

EQUIPMENT SETUP

Whole-body imaging equipment 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. Five individually optimized objective lenses, parcentered and parfocal, provide a 10^5-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 high magnification range of ×1.6 to ×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-Watt 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

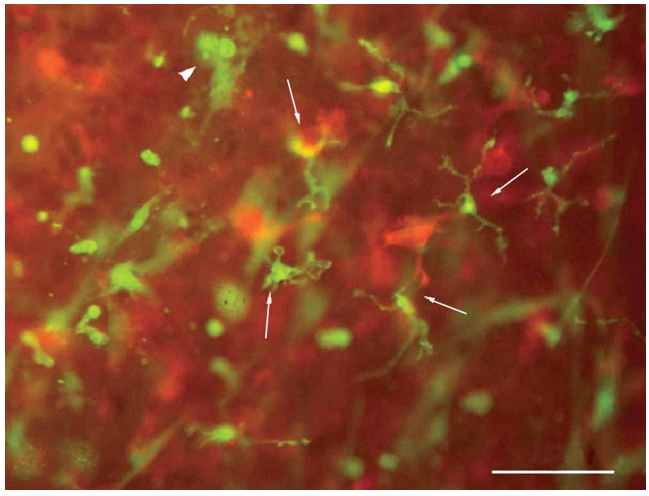

Figure 3 | Visualization of the interaction of host dendritic cells and tumor cells in fresh tumor tissue. Many host-derived GFP-expressing dendritic cells directly contacting B16F10 RFP$^+$ melanoma cells with their dendrites (arrows) are visualized. Dendritic cell–lymphocyte clusters can be seen in certain regions of the image (arrowhead) 3 weeks after tumor implantation. Scale bar, 50 μm. Image from ref. 2.

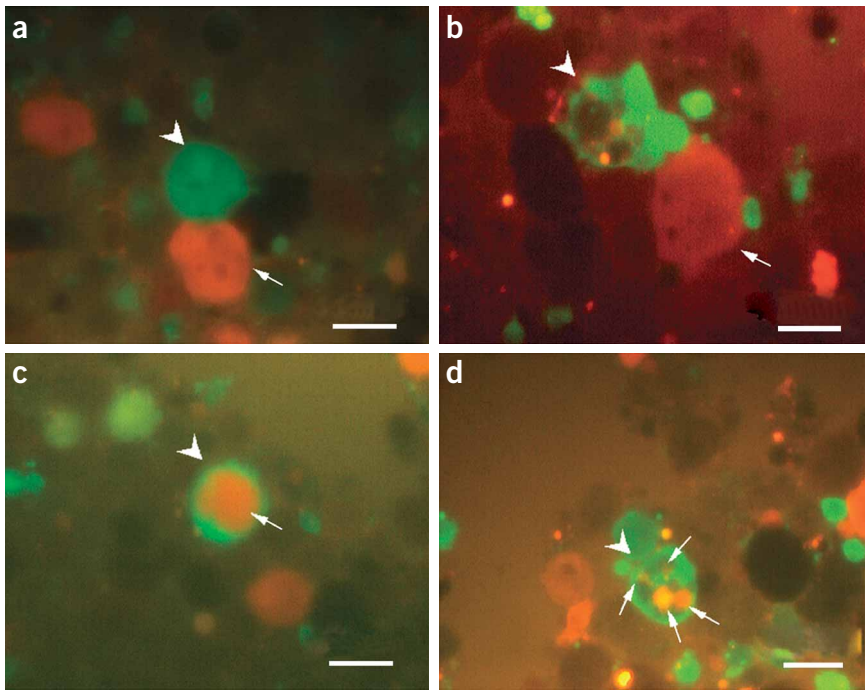

Figure 4 | Visualization of macrophage-tumor interactions. (**a**) Host GFP$^+$ macrophage (arrowhead) contacting a PC-3 RFP$^+$ human prostate cancer cell (arrow). (**b**) GFP$^+$ macrophage (arrowhead) engulfing an RFP$^+$ cancer cell (arrow). (**c**) RFP$^+$ cancer cell (arrow) engulfed by a GFP$^+$ macrophage (arrowhead). (**d**) RFP$^+$ cancer cell (arrows) digested by a GFP$^+$ macrophage (arrowhead). Scale bars, 20 μm. Images from ref. 2.

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 × 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).

PROCEDURE

RFP retrovirus production

1| Insert the *Hin*dIII–*Not*I fragment from pDsRed2, containing full-length RFP cDNA, into the *Hin*dIII–*Not*I 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 10 Al viral envelope, to produce retrovirus. Culture PT67 cells in DMEM medium supplemented with 10% heat-inactivated FBS. It takes approximately 3 d for the cells to reach about 70% confluence after 3 × 10^5 PT67 cells are seeded in a 25-mm^2 flask with DMEM medium containing 10% FBS.

! CAUTION The National Institutes of Health and Center for Disease Control have designated retroviruses as 'Level 2' organisms. This requires the maintenance of a Biosafety Level 2 facility, including working in a limited-access area, posting biohazard warning signs, minimizing the production of aerosols, decontaminating potentially infectious waste before disposal and taking precautions with sharps. For more information on Biosafety Level 2, see the following reference: *Biosafety in Microbiological and Biomedical Laboratories*, Third Edition (May 1993) HHS Pub. #(CDC) 93-8395 (US Department of Health and Human Services, PHS, CDC, NIH).

3| For vector production, use PT67 packaging cells at 70% confluence. Plate PT67 cells on a 60-mm culture dish at 60–80% confluence 12 h before transfection. Use 10 μg of 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 °C to 26 °C (room temperature).

4| Dilute 4 μl Lipofectamine reagent in 96 μl serum-free medium in a second tube. Mix and incubate for 15 min at room temperature.

5| Combine the DNA prepared in Step 3 and diluted Lipofectamine reagent, then mix and incubate for 15 min at room temperature.

6| While the complexes are forming, replace medium on the cells with 800 μl serum-free DMEM. Add the DNA-Lipofectamine complex to the dish with cells containing fresh DMEM. Mix the complexes into the medium gently; incubate for 4 h at 37 °C in 5% CO_2.

7| After 4 h of incubation, increase volume of medium to 5 ml and incubate for 24 h at 37 °C in 5% CO_2.

8| After 24 h of incubation, clone the packaging cells by limiting dilution in 96-well plates.

9| For selection of a PT67 packaging cell clone producing large amounts of RFP retroviral vector (PT67-DsRed2), culture the cells in the presence of 200–1,000 μg ml^{-1} of G418. Culture cells for 1–2 d in each concentration of G418. 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.

▲ CRITICAL STEP Increasing the amount of G418 in a stepwise way is very important for inducing transgene expression. This procedure ensures high production of RFP^+ retrovirus.

RFP gene transduction of tumor cell lines

10| 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.

11| For retroviral infection, collect conditioned medium from packaging cells (PT67–DsRed2) and filter medium through a 0.45-μm polysulfonic filter. Add virus-containing filtered medium to target cells. Add polybrene to a final concentration of 8 μg ml^{-1}. Incubate cells for 24 h at 37 °C.

12| Replace medium with DMEM and 10% FBS after 24 h of incubation and check for RFP-expressing cells by fluorescence microscopy.

13| Collect tumor cells with trypsin-EDTA and subculture them at a ratio of 1:15 in selective medium, which contains 50 μg ml^{-1} G418.

14| To select brightly fluorescent cells, increase G418 to a concentration of 800 μg ml^{-1} in a stepwise way. Culture cells for 1–2 d in each concentration of G418.
▲ CRITICAL STEP Increasing G418 in a stepwise way is very important for inducing expression of the transgene. This procedure ensures high expression of RFP.

15| Isolate clones expressing RFP with cloning cylinders using trypsin-EDTA and amplify them in DMEM in the absence of the selective agent. Further select cells for brightness and stability.
▲ CRITICAL STEP This step ensures that the cells will stably express RFP in the absence of antibiotic selection, which is the case *in vivo*. It takes 7–10 d to obtain a sufficient number of cells for imaging studies.

16| 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).

(A) Cell injection to establish an experimental metastasis model

(i) Collect fluorescent protein–expressing tumor cells by trypsinization for 3 min at 37 °C with 0.25% trypsin.
(ii) Wash cells three times with cold serum-free medium using a tabletop centrifuge at 500*g*.
(iii) Resuspend cells in approximately 0.2 ml serum-free medium.
(iv) 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 in the lateral tail vein or subcutaneously using a 1-ml 27G2 latex-free syringe.
▲ CRITICAL STEP Cells in suspension may lose viability over time and therefore should be injected as soon as possible.
(v) For liver colonization, inject fluorescent protein–expressing cells directly into the portal vein in anesthetized mice (details on inducing anesthesia are presented below).

(B) Surgical orthotopic implantation to establish a spontaneous metastasis model

(i) Induce anesthesia with a 'ketamine mixture' (10 μl ketamine HCl, 7.6 μl xylazine and 2.4 μl acepromazine maleate, injected s.c.).
(ii) Use a microscope (Leica MZ6) with magnification of about ×6 to about ×40 for all procedures of the operation.
(iii) Isolate fluorescent protein–expressing tumor fragments (1 mm^3) from subcutaneously growing tumors, formed by injection of RFP-expressing tumor cells (Step 16A), by mincing tumor tissue into 1-mm^3 fragments. After proper exposure of the target organ, implant three tumor fragments per transgenic GFP mouse.
(iv) With 8-0 surgical suture, penetrate the tumor fragments and suture the fragments onto the target organ.
▲ CRITICAL STEP Orthotopic implantation of tumor fragments results in higher spontaneous metastatic rates than injection of a cell suspension[8].
(v) Keep mice in a barrier facility under high-efficiency particulate air filtration.

(C) RFP-expressing cutaneous melanoma model

(i) Collect RFP-expressing mouse B16F0 melanoma cells by trypsinization for 3 min at 37 °C with 0.25% trypsin.
(ii) Wash cells three times with cold serum-containing medium using a table-top centrifuge at 500*g* for 5 min at room temperature, and then keep on ice.
(iii) 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 μl of cell culture medium within 40 min of collection.

17| 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).

(A) Microscopy

(i) Use a Leica fluorescence stereo microscope (model LZ12) equipped with a mercury 50-Watt lamp power supply or its equivalent.

(ii) Produce selective excitation of GFP via a D425/60 band-pass filter and 470 DCXR dichroic mirror.

(iii) Collect emitted fluorescence through a long-pass filter (GG475) on a Hamamatsu C5810 three-chip cooled color CCD camera or its equivalent.

(iv) Process images for contrast and brightness with the use of Image-Pro Plus 4.0 software or its equivalent.

(v) Capture high-resolution images of 1,024 × 724 pixels directly on an IBM PC or continuously through video output on a high-resolution Sony VCR, model SLV-R1000 or its equivalent.

(vi) For C57BL/6 mice, remove hair with Nair or by shaving before images are obtained.

▲ **CRITICAL STEP** Hair is highly autofluorescent, so improper removal of hair will result in low-quality images.

(B) Flashlight imaging

(i) Use a blue LED flashlight (LDP LLC) with an excitation filter (midpoint wavelength peak of 470 nm; Chroma Technology) and a D470/40 emission filter for whole-body imaging of GFP mice with RFP-expressing tumors growing in or on internal organs.

▲ **CRITICAL STEP** Correct filters are necessary to eliminate tissue autofluorescence.

(ii) Acquire images with a digital camera such as a Nikon Coolpix or a simple CCD camera and store on a PC as described above.

(iii) For C57BL/6 mice, remove hair with Nair or by shaving before images are obtained.

▲ **CRITICAL STEP** Hair is highly autofluorescent, so improper removal of hair will result in low-quality images

(C) Light-box imaging

(i) Do whole-body imaging in a fluorescent light box illuminated by fiberoptic lighting at 470 nm.

(ii) Collect emitted fluorescence through a GG475 long-pass filter on a Hamamatsu C5810 three-chip cooled color CCD camera or its equivalent. (Use of separate band-pass filters for RFP or GFP emission allows a monochrome camera to be used.)

(iii) Capture high-resolution images of 1,024 × 724 pixels directly on an IBM PC or its equivalent.

(iv) Process images for contrast and brightness with the use of Image-Pro Plus 4.0 software or its equivalent.

(v) For C57BL/6 mice, remove hair with Nair or by shaving before images are obtained.

▲ **CRITICAL STEP** Hair is highly autofluorescent, so improper removal of hair will result in low-quality images

(D) Chamber imaging

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

(ii) 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 five objective lenses.

(iii) Capture images on a PC (Fujitsu-Siemens), and process images for contrast and brightness with Paint Shop Pro 8 and cell^R.

(iv) For C57BL/6 mice, remove hair with Nair or by shaving before images are obtained.

▲ **CRITICAL STEP** Hair is highly autofluorescent, so improper removal of hair will result in low-quality images.

Tumor tissue sampling

18| Obtain tumor tissue biopsies from 3 d 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 mm^3 or less) with a scalpel. 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.

19| Cut fresh tissue into pieces of about 1 mm^3 and gently press onto slides for fluorescence microscopy. This procedure is done manually on normal slides.

20| To analyze tumor angiogenesis, digest the tissues with trypsin-EDTA for 5 min at 37 °C before examination.

21| After trypsinization, put tissues on precleaned microscope slides and cover with another microscope slide.

Fluorescence microscopy

22| Use an Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-Watt lamp power supply or its equivalent.

23| 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.

24| Collect emitted fluorescence light through a GG475 long-pass filter.

25| 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.

26| Process images for contrast and brightness using Image-Pro Plus 4.0 software or its equivalent.

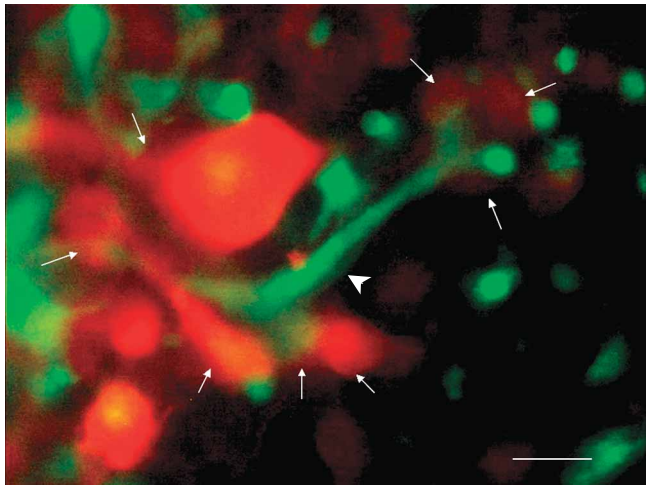

Figure 5 | Interactions (arrows) of host stromal GFP+ fibroblast cell (arrowhead) and Dunning RFP+ rodent prostate cancer cells in live tumor tissue. Scale bar, 20 μm. Image from ref. 9.

Measurement of GFP-expressing tumor blood vessel length and evaluation of antiangiogenetic agents

27| Give mice daily i.p. injections of doxorubicin (5 μg per gram body weight in a 2 mg ml^{-1} 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.

28| Anesthetize mice with the ketamine mixture and obtain biopsies on days 10, 14, 21 and 28 after implantation (Step 18 provides biopsy sample details).

29| Gently flatten the tumor tissue between the slide and coverslip.

30| Quantify angiogenesis in the tumor tissue by measuring the length of GFP-expressing blood vessels in all fields using fluorescence microscopy.

31| Obtain measurements in all fields at ×40 or ×100 magnification to calculate the total length of GFP-expressing blood vessels.

32| Calculate the vessel density by dividing the total length of GFP-expressing blood vessels (in mm) by the tumor volume (in mm^3).

Immunohistochemical staining

33| '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 μm with a Leica CM1850 cryostat.

34| Detect colocalization of GFP fluorescence, CD31 and nestin in the frozen skin sections of mice transgenic for nestin enhancer–driven GFP expression using the anti-rat immunoglobulin and anti-mouse immunoglobulin horseradish peroxidase detection kits following the manufacturer's instructions.

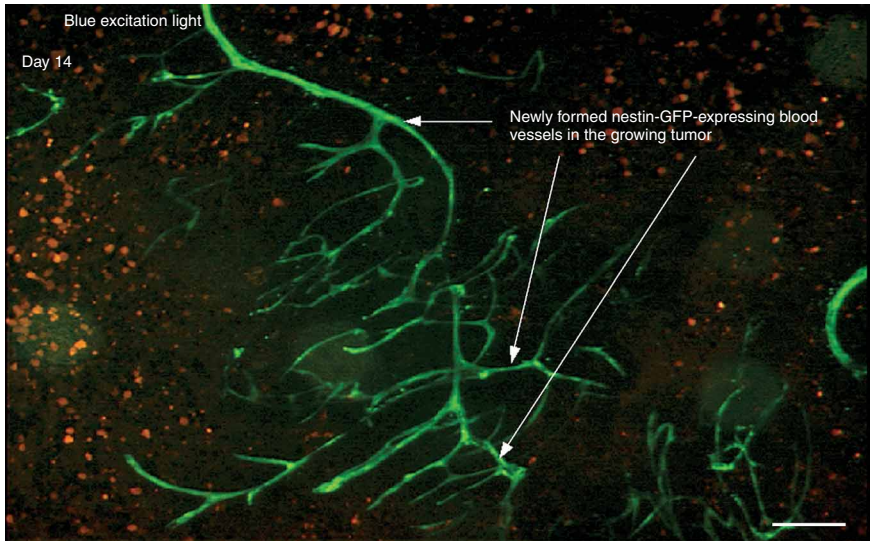

Figure 6 | Intermediate-stage angiogenesis. At day 14 after implantation of tumor cells, blood vessels expressing nestin-driven GFP are forming networks. Scale bar, 100 μm. Image from ref. 4.

35| 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.

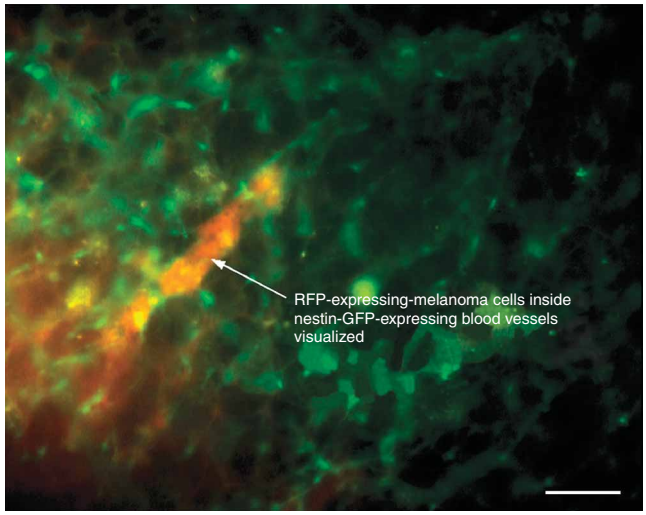

Figure 7 | Visualization of intravasated tumor cells. B16F10 RFP$^+$ tumor cells are inside blood vessels expressing nestin-driven GFP. Under a blue excitation light, the RFP$^+$ tumor cells in the GFP$^+$ vessels appear orange; vessels without tumor cells have green fluorescence. Scale bar, 100 μm. Image from ref. 4.

36| Use staining with substrate-chromogen 3,3′-diaminobenzidine for antigen detection.

● TIMING

Steps 1–9: 2 weeks
Steps 10–15: 7–10 d
Step 16: 2 weeks to 2 months
Step 17: Up to 30 min if anesthesia is induced; seconds to capture images
Steps 18–21: 2 h
Steps 22–26: 1 h
Steps 27–32: 2 h per time point
Steps 33–36: 6 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Autofluorescence	Use of wrong filters	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 approximately 515 nm (Chroma Technology).
Bleeding	Improper surgical procedures	Bleeding should be avoided at the surgical site, as hemoglobin will absorb the incident excitation light.
Dehydration	Long-term procedures on an open animal	When doing open-biopsy procedures, it is essential to hydrate the animal by spraying normal saline on the open tissue.
Infection	Unclean instruments and environment	When doing repeat procedures such as an open biopsy or other invasive procedures, it is critical to maintain a properly sterile operation field.

ANTICIPATED RESULTS

Figure 1 shows a whole-body image of a nude GFP mouse orthotopically implanted with an RFP-expressing colon tumor. **Figure 2** shows various stages of GFP$^+$ blood vessel formation in an RFP-expression melanoma. **Figure 3** shows GFP$^+$ dendritic cells interacting with an RFP-expressing melanoma cells. **Figure 4** shows GFP$^+$ macrophages engulfing human prostate cancer cells expressing RFP. **Figure 5** shows a GFP$^+$ stromal fibroblast interacting with many RFP$^+$ prostate cancer cells. **Figure 6** shows blood vessels expressing nestin-driven GFP in an RFP$^+$ melanoma. **Figure 7** shows RFP$^+$ melanoma cells inside blood vessels expressing nestin-driven GFP. **Supplementary Figures 1–5** online show GFP expression in the nude mouse system (**Supplementary Figs. 1–3**) and also demonstrate the effects of doxorubicin and gemcitabine on RFP-expressing carcinomas (**Supplementary Figs. 4,5**). The protocols described here should allow the color-coded imaging of all types of tumor-host interactions at the cellular level. These results will be very useful for understanding the function of stromal cells in tumor progression and for exploiting tumor cell–interacting stromal cells as visible drug targets.

Note: Supplementary information is available via the HTML version of this article.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

Published online at http://www.natureprotocols.com/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Hoffman, R.M. The multiple uses of fluorescent proteins to visualize cancer *in vivo*. *Nat. Rev. Cancer* **5**, 796–806 (2005).
2. Yang, M. *et al.* Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. *Proc. Natl. Acad. Sci. USA* **100**, 14259–14262 (2003).
3. Yang, M. *et al.* Transgenic nude mouse with ubiquitous green fluorescent protein expression as a host for human tumors. *Cancer Res.* **64**, 8651–8656 (2004).

4. Amoh, Y. *et al.* Hair follicle-derived blood vessels vascularize tumors in skin and are inhibited by doxorubicin. *Cancer Res.* **65**, 2337–2343 (2005).
5. Amoh, Y. *et al.* Nestin-linked green fluorescent protein transgenic nude mouse for imaging human tumor angiogenesis. *Cancer Res.* **65**, 5352–5357 (2005).
6. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. & Nishimune, Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313–319 (1997).
7. Amoh, Y. *et al.* Nascent blood vessels in the skin arise from nestin-expressing hair follicle cells. *Proc. Natl. Acad. Sci. USA* **101**, 13291–13295 (2004).
8. Hoffman, R.M. Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. *Invest. New Drugs* **17**, 343–359 (1999).
9. Hoffman, R.M. *In vivo* imaging with fluorescent proteins: The new cell biology. *Acta Histochem.* **106**, 77–87 (2004).