# Noninvasive Imaging *In Vivo* With Fluorescent Proteins From Centimeters To Micrometers

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## ABSTRACT

Whole-body imaging with fluorescent proteins has been shown to be a powerful technology with many applications in small animals. Our laboratory pioneered *in vivo* imaging with fluorescent proteins (1) including noninvasive whole-body imaging (2). Whole-body imaging with fluorescent proteins depends in large part on the brightness of the protein. Brighter, red-shifted proteins can make whole-body imaging more sensitive due to reduced absorption by tissues and less scatter. Non-invasive imaging with fluorescent proteins has been shown to be able to quantitatively track tumor growth and metastasis, gene expression, angiogenesis, and bacterial infection (3) even at subcellular resolution depending on the position of the cells in the animal. Interference by skin autofluorescence is kept to a minimum with the use of proper filters. To noninvasively image cancer cell/stromal cell interaction in the tumor microenvironment and drug response at the cellular level in live animals in real time, we developed a new imageable three-color animal model. The model consists of green fluorescent protein (GFP)–expressing mice transplanted with dual-color cancer cells labeled with GFP in the nucleus and red fluorescent protein (RFP) in the cytoplasm. Various in vivo phenomena of tumor-host interaction and cellular dynamics were imaged, including mitotic and apoptotic tumor cells, stromal cells interacting with the tumor cells, tumor vasculature, and tumor blood flow as well as drug response. This imageable technology should lead to many new insights of in vivo cancer cell biology.

**Keywords**: green fluorescent protein, red fluorescent protein, color-coded imaging, non-invasive subcellular imaging

## 1. INTRODUCTION

**1.1 Features of GFPs.** The discovery, cloning and gene transfer of green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, has enabled a revolution in cell biology. GFP has been shown to be able to be genetically linked with almost any protein providing a permanent and heritable label in live cells to study protein function and location (3). Many different colors of fluorescent proteins have now been produced in the laboratory or found in nature. With multiple colors, many processes can be visualized simultaneously in cells. Thus, cells can be multiply labeled for imaging in live cells that heretofore could be performed only on fixed and stained cells. What previously could only be seen on gels and blots, can now be visualized in real-time in living cells expressing fluorescent proteins (4).

**1.2 Features of RFPs.** Whole-body imaging is more effective when the fluorescent protein emits at longer wavelengths which are absorbed less by tissues and by physiological molecules such as hemoglobin and are also less scattered (3). Red-emitting fluorescent proteins were first described in the late 1990s. The first such protein was isolated and cloned from the coral *Discosoma sp.* obtained from an aquarium shop in Moscow (5) and termed DsRed. After extensive modification by mutagenesis, a very bright red protein was eventually isolated, termed DsRed2 with an emission wavelength peak of 588. DsRed2 has shown to be very enabling for whole-body imaging and has been used to non-invasively follow cancer metastasis in real time (6) in nude mice as well as whole-body image dual-color models of tumors expressing DsRed2 growing in transgenic GFP nude mice as hosts (7).

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In 2004, a report appeared (8) describing a series of red-shifted proteins obtained by mutating DsRed. These proteins, termed mCherry, mRaspberry, mPlum, and mTomato, had emission maxima as long as 649 nm. However, these mutants have low quantum yields, thereby reducing their brightness.

A very bright, red-shifted fluorescent has recently been described by Shcherbo et al (9) originating from the sea anemone *Entacmaea quadricolor*. Katushka has many favorable properties in addition to its absorption and emission peaks including a rapid maturation time of 20 minutes. Importantly, an extinction coefficient of 65,000 $M^{-1}$ $cm^{-1}$ and quantum yield of 0.34, make Katushka the brightest fluorescent protein with an emission maximum beyond 620 nm. In cells, Katushka demonstrated no visible aggregates or other toxic effects **(Hoffman, R.M. A better fluorescent protein for whole-body imaging. Trends in Biotechnology 26, 1-4, 2008).**

**1.3 Method of Choice for Whole-Body Imaging.** The features of fluorescent-protein-based imaging, such as a very strong and stable signal enable noninvasive whole-body imaging down to the subcellular level (10), especially with red-shifted fluorescent protein, make it far superior to luciferase-based imaging. Luciferase-based imaging, with its very weak signal (11), precluding image acquisition and allowing only photon counting with pseudocolor-generated images, has very limited applications (12). For example, cellular imaging *in vivo* is not possible with luciferase. The dependence on circulating luciferin makes the signal from luciferase imaging unstable (12). The one possible advantage of luciferase-based imaging is that no excitation light is necessary. However, far-red absorbing proteins such as Katushka greatly reduce any problems with excitation, even in deep tissues, as shown by Shcherbo (9).

Proteins such as Katushka as well as photo-activatible (13) and photoconvertible fluorescent proteins (14), provide powerful tools for future whole-body imaging experiments. Imaging instrumentation such as these with variable magnification (15) or scanning lasers (10) and multiphoton microscopy (16) make fluorescent proteins tools of choice for whole-body imaging. Whole-body imaging with fluorescent proteins can now reach the subcellular level using cells labeled in the nucleus with GFP and RFP in the cytoplasm (please see below) (10). However, there are misconceptions in the literature suggesting fluorescent protein-based imaging is inferior to luciferase (17-19). The results described here should greatly clarify this subject.

**1.4 Tumor microenvironment.** Cancer cells coexist in a complex association with host-stromal tissue cells. The stroma provides the vascular supply to the tumor in the angiogenesis process as well as many other cell types and functions. The factors that regulate the development of the stromal elements, as well as the influences these constituents have on the tumor, are poorly understood. The lack of information about the interaction between tumor and stroma can be attributed in part to lack of suitable models (20). Tumor progression is a multistep process accompanied by the accumulation of mutations in cancer cells. However, it is now becoming clear that the tumor microenvironment is also critical for malignancy, which is in part the product of interaction between different cancer and host cell types (21).

The heterogeneous and structurally complex nature of the interactive tumor microenvironment is little understood. The relative amount of stroma and its composition vary considerably from tumor to tumor and vary within a tumor over the course of tumor progression. The interaction between cancer cells and stromal cells largely determines the phenotype of the tumor. For example, recent studies have shown that the growth, invasiveness, and angiogenesis of human breast tumor xenografts in mice depend on the presence of stromal fibroblasts (22).

The tumor microenvironment is a potential therapeutic target. Advantages to targeting the stroma cells are that the cells are genetically stable unlike cancer cells and are therefore less likely to develop drug resistance (23, 24). For example, anti–vascular endothelial growth factor antibodies, which inhibit formation of new blood vessels in the tumor, are used to treat colorectal cancer (25).

**1.5 Color-coded imaging.** We have developed a simple yet powerful technique for delineating the morphologic events of tumor-induced angiogenesis and other tumor-induced host processes with dual-color fluorescence. The method clearly imaged implanted tumors and adjacent stroma, distinguishing unambiguously the host and tumor-specific components of the malignancy in fresh tissue samples. The dual-color fluorescence imaging was effected by using RFP-expressing tumors growing in transgenic mice that express GFP in essentially all nucleated cells. This model showed with great clarity the details of the tumor-stroma interaction, especially tumor-induced angiogenesis and tumor-infiltrating lymphocytes (7).

**1.6 Non-invasive subcellular imaging in mice.** To noninvasively visualize cellular and subcellular events in the tumor microenvironment in real time in the live mouse, we used a laser scanning microscope with a 0.3-mm-diameter stick objective that is up to 2 cm in length. The system allows an imaging depth that extends to at least 200 μm. This novel imaging system, coupled with the use of the dual-color cancer cells and transgenic GFP mouse, has enabled noninvasive in vivo imaging of the cancer and stromal cells in the tumor microenvironment at the subcellular level, the subject of a current report (21).

## 2. MATERIALS AND METHODS

**2.1 Production of RFP retroviral vector.** For RFP retrovirus production, the HindIII/NotI fragment from pDsRed2 (Clontech Laboratories, Inc.), containing the full-length RFP cDNA, was inserted into the HindIII/NotI site of pLNCX2 (Clontech Laboratories) that has the neomycin resistance gene to establish the pLNCX2-DsRed2 plasmid. PT67, an NIH3T3-derived packaging cell line (Clontech Laboratories) expressing the 10 Al viral envelope, was cultured in DMEM (Irvine Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-products). For vector production, PT67ce lls, at 70% confluence, were incubated with a precipitated mixture of LipofectAMINE reagent (Life Technologies, Inc., Grand Island, NY) and saturating amounts of pLNCX2-DsRed2 plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h after transduction. For selection of a clone producing high amounts of a RFP retroviral vector (PT67-DsRed2), the cells were cultured in the presence of 200 to 1,000 μg/mL G418 (Life Technologies) for 7days (21).

**2.2 Production of histone H2B-GFP vector.** The histone H2B gene has no stop codon, thereby enabling the ligation of the H2B gene to the 5′-coding region of the Aequoria victoria EGFP gene (Clontech Laboratories). The histone H2B-GFP fusion gene was then inserted at the HindIII/ClaI site of the pLHCX (Clontech Laboratories) that contains the hygromycin resistance gene. To establish a packaging cell clone producing high amounts of a histone H2BGFP retroviral vector, the pLHCX histone H2B-GFP plasmid was transfected in PT67cells using the same methods described above for PT67-DsRed2. The transfected cells were cultured in the presence of 200 to 400 μg/mL hygromycin (Life Technologies) for 15 days to establish stable PT67H2B-GFP packaging cells (21).

**2.3 RFP gene transduction of cancer cells.** For RFP gene transduction, 70% confluent mouse mammary tumor (MMT) cells or Lewis lung carcinoma (LLC) cells were used. To establish dual-color cells, clones of cancer cells expressing RFP in the cytoplasm were initially established. In brief, cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI 1640 (Mediatech, Inc.) containing 10% FBS for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h after transduction and subcultured at a ratio of 1:15 in selective medium, which contained 200 μg/mL G418. The level of G418 was increased stepwise up to 800 μg/mL. RFP-expressing cancer cells were isolated with cloning cylinders (Bel-Art Products) using trypsin/EDTA and amplified by conventional culture methods (21).

**2.4 Establishment of dual-color cancer cells.** For establishing dual-color cells, RFP-expressing cancer cells were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67H2B-GFP cells and culture medium. To select the double transformants, cells were incubated with hygromycin 72 h after transfection. The level of hygromycin was increased stepwise up to 400 μg/mL. Clones of dual-color cancer cells were isolated with cloning cylinders under fluorescence microscopy. These clones were amplified by conventional culture methods. These sublines stably expressed GFP in the nucleus and RFP in cytoplasm (21).

**2.5 Transgenic GFP nude mice.** Transgenic C57/B6-GFP mice were originally obtained from Prof. Masaru Okabe (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). C57/B6-GFP mice express GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer. All of the tissues from this transgenic line, with the exception of erythrocytes and hair, express GFP. Ten-week-old transgenic GFP female C57/B6 mice were crossed with 6- to 8-week-old BALB/c nu/nu or NCR nu/nu male mice (Harlan). Male F1 mice were crossed with female F1 C57/B6 GFP mice to obtain GFP nude mice. When female F2 immunocompetent GFP mice were crossed with male GFP nude mice, or when F2 GFP nude male mice were back-crossed with female F1 immunocompetent GFP mice,

~50% of their offspring were GFP nude mice. GFP nude mice can be consistently produced by the methods described above (26).

**2.6 Footpad injection model.** Dual-color cancer cells were washed thrice with serum-free cold medium. GFP nude mice were injected with $1 \times 10^6$ cells in a total volume of 20 AL serum-free medium into the right hind footpad. Cells were injected within 30 min of harvest (21).

**2.7 Laser scanning microscope.** The Olympus IV100 microscope is a scanning laser microscope. A 488-nm argon laser was used. The novel stick objectives (as small as 1.3 mm) were designed specifically for this laser scanning microscope. The very narrow objectives deliver very high resolution images (27). A PC computer running FluoView software (Olympus Corp.) was used to control the microscope. All images were recorded and stored as proprietary multilayer 16-bit Tagged Image File Format files (27).

All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals under assurance A3873-1. Animals were kept in a barrier facility under HEPA filtration. Mice were fed an autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products).

## 3. RESULTS AND DISCUSSION

**3.1 Noninvasive color-coded imaging of tumors at the subcellular level in the live unperturbed mouse.** Tumors that developed from dual-color cancer cells in the footpad of GFP nude mice are visualized at the cellular and subcellular level in their microenvironment. The cancer cells are the MMT and LLC. The cancer cells are double-labeled expressing GFP in the nucleus and RFP in the cytoplasm. The nuclei of the cancer cells appear yellow because they are surrounded by RFP cytoplasm. The green cells are the host cells because they express GFP only. Although both tumor types contain numerous green stromal cells, the MMT tumor seems more enriched in stromal cells. In both tumors, cancer cells are interdispersed between the stromal cells. The stromal cells form a very significant portion of the tumor. The stromal cells seem more heterogeneous in the Lewis lung tumor. Dividing cancer cells can be visualized by their two juxtaposed nuclei. Blood vessels are prominently seen in the MMT tumor. They appear dark due to absorption of light by hemoglobin in the red cells that do not express GFP. These are the first images of tumors in the unperturbed live animal at subcellular resolution (21).

**3.2 Noninvasive color-coded scanning imaging of unperturbed tumors in the live animal.** The LLC-GFP-RFP tumor being scanned from the outer surface to the inner. The outer surface is mostly GFP host cells. Numerous host GFP dendritic as well as other cell types can be seen at the surface. Other stromal cells seem prominent as scans are made at 10-μm intervals. At 80-Am depth, cancer cells become more predominant. Mitotic cancer cells can be discerned by juxtaposed nuclei. Stromal cells are observed in close proximity to cancer cells throughout the tumor (21).

**3.3 Noninvasive color-coded imaging of the effects of chemotherapy in real time on cancer and stromal cells.** In the MMT tumor model, the cancer cells are sensitive to doxorubicin (10 μg/kg, i.v.). The cancer cells lose their spindle shape, and the nuclei tend to contract soon after treatment. Tumor blood vessels could be visualized in the MMT tumor in the live mouse. Twelve hours after treatment with doxorubicin, the cytoplasm seemed to fragment away from the tumor cells, and the number of tumor cells was significantly reduced. The GFP host stromal cells become highly elongated in some cases. In the Lewis lung tumor, cancer cells are sensitive to taxol (5 μg/kg, i.v.) and cisplatin (10 μg/kg, i.v.). Color-coded interacting cancer and stromal cells and tumor blood vessels were imaged before chemotherapy. On day 5 after treatment with taxol, the cytoplasm seemed to fragment from the cancer cells, and the number of cancer cells was significantly reduced by day 7 after treatment. In the case of cisplatin, the nuclei of the cancer cells condensed. The shape of the cancer cells changed by day 2 after treatment, and they become surrounded by lymphatic-like GFP host stromal cells. On day 3 after treatment, the number of cancer cells was dramatically reduced. The three-color imaging model described here allows for the first time the ability to noninvasively observe drug response at the subcellular level in tumors in the living animal (21).

**3.4 Noninvasive color-coded imaging of tumor blood flow before and after chemotherapy.** Before treatment, numerous GFP-expressing host cells can be seen in the tumor blood flow. After treatment, the number of GFP-

expressing host cells in the blood flow was greatly reduced, and RFP cytoplasmic fragments were observed, which were stripped from the cancer cells due to chemotherapy (21).

The results described here are a significant improvement from the inserted-window models of Jain et al. (28) or of the skin flap models we have previously developed to image tumors (7, 15). A striking observation is the interdispersal of cancer cells among the host stromal cells in the tumor microenvironment. Such observations were made possible by using the Olympus IV100 Intravital Laser Scanning Microscope, a ubiquitously-expressing GFP mouse as a host, and cancer cells expressing RFP in the cytoplasm and GFP in the nucleus that appears yellow due to the surrounding RFP cytoplasm. We also showed the usefulness of this model to noninvasively image chemotherapy of tumors in real time at the subcellular level, which distinguishes efficacy on cancer and stromal cells. Tumor blood flow was readily imaged, and striking changes were observed after chemotherapy. The ultra-narrow objectives of this scanning imager enable the very high resolution whole-body images obtained in a current study (21).

The new technology described in this report enables clear color-coded distinction of cancer and stromal cells without perturbing the tumor or the animal. Future experiments will develop techniques to differentially label cancer and stromal cells in cancer patients' surgical specimens in order to transplant and image cancer cell/stromal cell interactions in patient tumors as we did for the tumor cell lines in the present study. The patient specimens will give further opportunity to screen for new drugs that specifically target stromal cells that play a critical role in tumor behavior, in particular, malignancy (21).

The results described here are also an improvement on the study previously done with the IV100 where the ''stick'' objective was inserted into the animal (27). Although this was a useful application of this instrument, the present report shows that the IV100 can be used for whole-body, completely noninvasive color-coded imaging at the subcellular level with very high resolution (21).

This new technology opens many new possibilities for studying cancer cell/stromal cell interactions in the microenvironment noninvasively at the cellular level and as a new approach for in vivo cell biology and drug discovery.

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