



In vivo imaging with fluorescent proteins: the new cell biology[☆]

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Summary

We propose a new cell biology where the behavior of cells can be visualized in the living animal. An example of the new cell biology is dual-color fluorescence imaging using red fluorescent protein (RFP)-expressing tumors transplanted in green fluorescent protein (GFP)-expressing transgenic mice. These models show with great clarity the details of tumor–stroma interactions and especially tumor-induced angiogenesis, tumor-infiltrating lymphocytes, stromal fibroblasts and macrophages. Another example is the color coding of cells with RFP or GFP such that both cell types can be simultaneously visualized *in vivo*. Stem cells can also be visualized and tracked *in vivo*. Mice in which the regulatory elements of the stem-cell marker nestin drive GFP enable nascent vasculature to be visualized interacting with transplanted RFP-expressing cancer cells. Nestin-driven GFP expression can also be used to visualize hair follicle stem cells. Dual-color cells expressing GFP in the nucleus and RFP in the cytoplasm enable real-time visualization of nuclear-cytoplasm dynamics including cell cycle events and apoptosis. Multiple-color labeling of cells will enable multiple events to be simultaneously visualized *in vivo* including gene expression, ion fluxes, protein and organelle trafficking, chromosome dynamics and numerous other processes currently still only studied *in vitro*.

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Introduction

For the past half-century, cell biology and molecular biology have been carried out on cells growing in dishes and by the extracellular analysis of cellular components including genes and proteins. The advent of green fluorescent protein (GFP) as

reporter gene is enabling a paradigm change in cell biology and molecular biology. In the first phase of revolution, molecular processes such as gene expression and ion fluxes were visualized in living cells *in vitro* (Li et al., 1999). Spectrally distinct fluorescent proteins, especially red fluorescent proteins (RFPs), are being isolated by groups throughout the world (Wiedenmann et al., 2000,

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2002; Labas et al., 2002; Karasawa et al., 2003; Gilmore et al., 2003) which enable simultaneous multi-color "rainbow imaging" (Baubet et al., 2000) of numerous process in the living cell both *in vitro* and *in vivo*.

We have developed imaging of tumors and metastases in mice by use of cancer cells expressing GFP. These mice present many new possibilities for research including real-time studies of tumor progression, metastasis dynamics, and drug-response evaluations. We introduced the GFP gene into a large series of human and rodent cancer-cell lines *in vitro*, which stably express GFP after transplantation to rodents. With this fluorescent tool, single cells can be imaged and tracked *in vivo* (Yang et al., 2002).

Okabe et al. (1997) produced transgenic mice with GFP under the control of a chicken β -actin promoter and cytomegalovirus enhancer. With the GFP host mouse, it became possible to visualize all the host cells that can interact with the tumor. All of the tissues from these transgenic mice, with the exception of erythrocytes and hair, fluoresce green.

We have developed a simple yet powerful technique for delineating morphological events of tumor-host interactions with dual-color fluorescence. The method clearly images implanted tumors and adjacent stroma, distinguishing unambiguously the host and tumor-specific components of the malignancy. Dual-color fluorescence imaging is effected by using red fluorescent protein (RFP)-expressing tumors growing in GFP-expressing transgenic mice. This model shows with great clarity the details of tumor-stroma interactions, especially tumor-induced angiogenesis and tumor-infiltrating lymphocytes. The GFP-expressing tumor vasculature, both nascent and mature, is readily distinguished interacting with RFP-expressing cancer cells. GFP-expressing dendritic cells were observed contacting RFP-expressing cancer cells with their dendrites. GFP-expressing macrophages were observed engulfing RFP-expressing cancer cells. GFP lymphocytes were observed surrounding cells of the RFP tumor, which eventually regressed (Yang et al., 2003).

In order to develop a method to color-code cells *in vivo*, we have established stable, bright GFP- or RFP-expressing HT-1080 human fibrosarcoma clones. The implantation of mixtures of HT-1080-GFP and -RFP clones enable simultaneous real-time dual-color imaging in the live animal. The cells seeded the lung at high frequency with subsequent formation of pure green and pure red colonies as well as mixed yellow colonies. The lung metastases are visualized by external fluorescence imaging in live animals through skin-flap windows over the chest wall. Real-time metastatic growth of the two different colored clones in the same lung was

externally imaged with resolution and quantification of green, red, or yellow colonies in live animals. The color coding enabled determination of whether the colonies grew clonally or were seeded as a mixture with one cell type eventually dominating, or whether the colonies grew as a mixture. The simultaneous real-time dual-color imaging of metastatic colonies gives rise to the possibility of color-coded imaging of clones of cancer cells carrying various forms of genes of interest (Yamamoto et al., 2003a, b).

In transgenic mice with GFP under the control of nestin regulatory sequences, nestin-expressing cells, marked by GFP fluorescence, appear in the population of hair follicle stem cells. The relatively small, oval-shaped, nestin-expressing cells in the bulge area surround the hair shaft and are interconnected by short dendrites. Expression of the unique protein, nestin, in neural stem cells, hair follicle stem cells as well as nascent blood vessels suggests their possible relation (Li et al., 2003; Amoh et al., unpublished data).

Intravital imaging has shown that carcinoma cells in a primary breast tumor can move at up to 10 times the velocity of cells *in vitro*. Metastatic cancer cells moving along linear paths in association with extracellular-matrix fibers had the highest velocity. Breast carcinoma cells showed solitary amoeboid movement. Cells in metastatic breast tumors were observed to be attracted to blood vessels. Cell polarity towards blood vessels was correlated with increased intravasation and metastasis (Condeelis and Segall, 2003).

Material and methods

Transgenic animals

C57/B6-GFP-expressing mice. Transgenic C57/B6-GFP mice (Okabe et al., 1997) were obtained from the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. The C57/B6-GFP mice express GFP under the control of the chicken β -actin promoter and cytomegalovirus enhancer. All tissues from this transgenic line, with the exception of erythrocytes and hair, fluoresce green under excitation light. The GFP gene, regulated as described above, was crossed into nude mice on a C57/B6 background (Yang et al., 2003).

Nestin-GFP-expressing transgenic mice

Nestin is an intermediate filament gene that is a marker for central nervous system progenitor cells and

neuroepithelial stem cells, hair follicle stem cells (Li et al., 2003) and blood vessels (Sugawara et al., 2002). Transgenic mice carrying enhanced GFP under the control of the nestin second-intron enhancer were also used as hosts to visualize nascent angiogenesis in transplanted RFP-expressing tumors (Li et al., 2003).

Expression vectors

The pLNCX₂ vectors were purchased from Clontech Laboratories (Palo Alto, CA, USA). The pLNCX₂ vector contains the neomycin resistance gene for antibiotic selection in eukaryotic cells. The RFP gene (DsRed2; Clontech Laboratories) was inserted in the pLNCX₂ vector at the Egl II and Not I sites (Yamamoto et al., 2003b).

RFP retroviral vector production

For retroviral transduction, PT67, an NIH3T3-derived packaging cell line, expressing the 10 Al viral envelope, was purchased from Clontech Laboratories. PT67 cells were cultured in DME (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-products, Calabasas, CA, USA). For vector production, PT67 cells at 70% confluence, were incubated with a precipitated mixture of DOTAPTM reagent (Boehringer, Mannheim, Germany), and saturating amounts of pLNCX₂-DsRed2 plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy at 48 h post-transfection. For selection of brightly fluorescing cells producing high titer retroviral supernatants, the RFP-expressing packaging cells were cultured in the presence of 500 µg/ml-2000 µg/ml G418 increased in a step-wise manner (Life Technologies, Grand Island, NY, USA) for seven 7 days to select a clone producing high amounts of RFP retroviral vector (Yamamoto et al., 2003b).

GFP retroviral vector production

The pLEIN retroviral vector (Clontech Laboratories expressing GFP and the neomycin resistance gene on the same bicistronic message was used as a GFP expression vector in PT67 cells, as described above for RFP retroviral vector production (Yang et al., 2003).

GFP and RFP gene transduction of cancer cell lines

For GFP or RFP gene transduction, 20% confluent cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and

RPMI 1640 or other culture media (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gemini Bio-products) for 72 h. Fresh medium was replenished at this time. Cancer cells were harvested with trypsin/EDTA and sub-cultured at a ratio of 1:15 in a selective medium, which contained 50 µg/ml G418. To select brightly fluorescent cells, the level of G418 was increased to 800 µg/ml in a step-wise manner. Clones expressing GFP or RFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) using trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent (Yamamoto et al., 2003a,b).

RFP-expressing cutaneous melanoma model

Six-week-old male C57/B6-GFP or nestin-GFP-expressing mice were injected subcutaneously with 10⁶ RFP-expressing mouse B16F0 melanoma cells. Cells were first harvested by trypsinization and washed 3 times with cold serum-containing medium, then kept on ice. Cells were inoculated by intradermal injection of the dorsal skin of the animal in a total volume of 50 µl within 40 min of harvesting (Yang et al., 2003).

RFP-expressing orthotopic prostate cancer models

Six-week-old male C57/B6-GFP immunocompetent mice were injected orthotopically with a single dose of 5 × 10⁵ RFP-expressing rat Dunning prostate cancer cells. Similarly, C57/B6-GFP nude mice were injected with 10⁶ PC-3-RFP human prostate cancer cells. Cells were first harvested by trypsinization and washed 3 times with cold serum-containing medium, then kept on ice. The cells were injected in a total volume of 30 µl within 40 min of harvesting. Bladder and prostate were exposed after a lower midline abdominal incision. The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery. All procedures of the operation described above were performed with a 7 × magnification stereomicroscope (Yang et al., 2003).

Tumor tissue sampling

Tumor tissue biopsies were processed from 3 days to 4 weeks after inoculation of cancer cells. Fresh tissue were cut into pieces of 1 mm³ in volume and pressed on slides for fluorescence microscopy. For analyzing tumor angiogenesis, tissues were

digested with trypsin/EDTA at 37°C for 5 min before examination. After trypsinization, tissues were put on pre-cleaned microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and covered with a second microscope slide (Yang et al., 2003).

Whole-body imaging

Whole-body imaging (Hoffman, 2002; Yang et al., 2000, 2001, 2002, 2003) was performed in a fluorescent light box illuminated by fiberoptic lighting at 470 nm (Lighttools Research, Encinitas, CA, USA). Emitted fluorescence was collected through a long-pass GG475 filter (Chroma Technology, Battleboro, VT, USA) on a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ, USA). High resolution images of 1024/724 pixels were captured directly on an IBM PC or continuously through video output on a high resolution Sony VCR model SLV-R1000 (Sony, Tokyo, Japan). Images were processed

for contrast and brightness and analyzed with the use of Image Pro Plus 4.0 software (Media Cybernetics, Silver Springs, MD, USA; Yang et al., 2002).

Fluorescence imaging microscopy

A BH 2-RFCA fluorescence microscope equipped with a mercury 100 W lamp power supply (Olympus, Tokyo, Japan) was used. To visualize both GFP and RFP fluorescence at the same time, excitation light was produced through a D425/60 band pass filter with a 470 DCXR dichroic mirror. Emitted fluorescence light was collected through the long pass GG475 filter. High-resolution images of 1024/724 pixels were captured by a C5810 3-chip cool color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ, USA) and directly stored on an IBM PC. Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus 4.0 software (Yang et al., 2003).

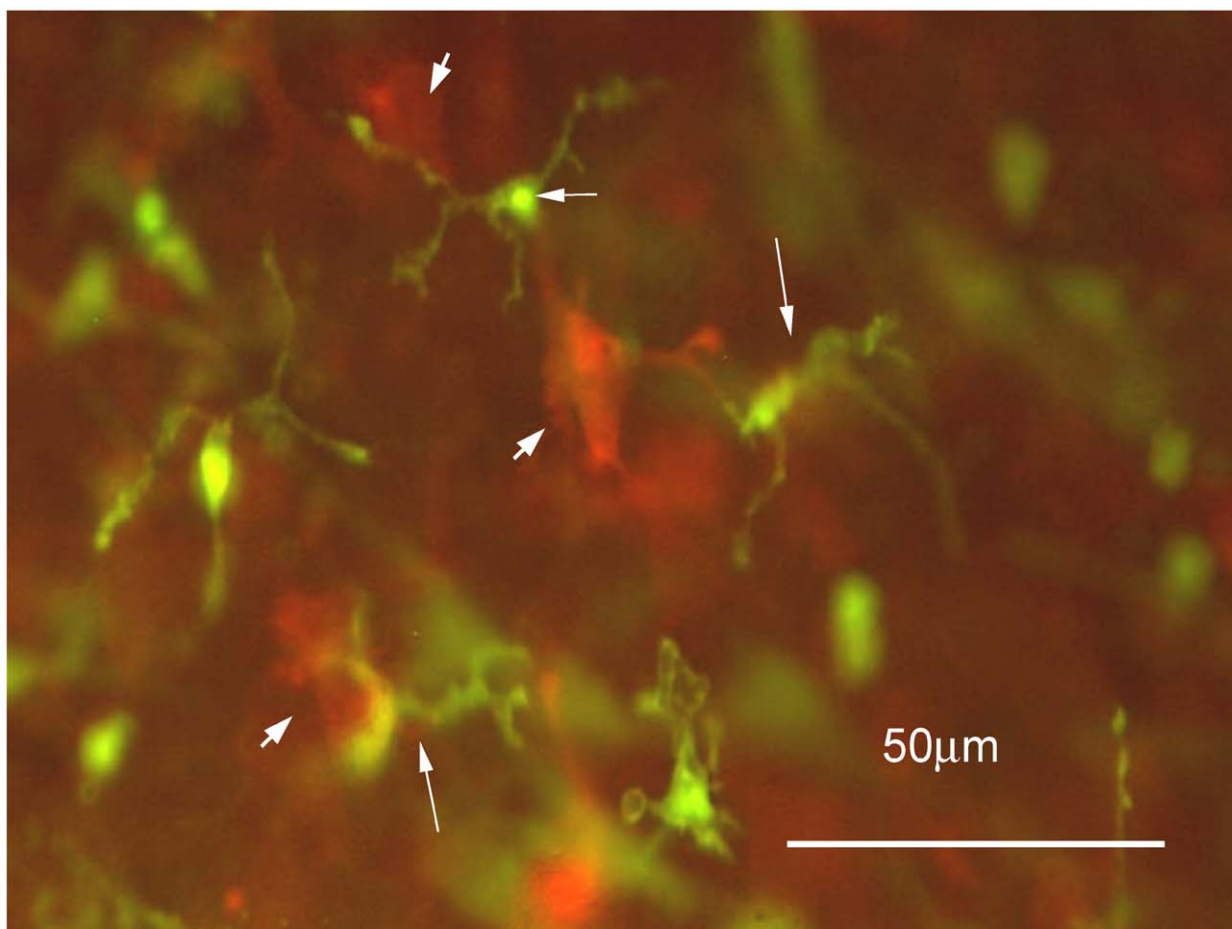


Figure 1. Visualization of interactions of host dendritic cells and cancer cells in fresh tumor tissue. Many host-derived GFP-expressing dendritic cells intimately contacting B16F10-RFP melanoma cells with their dendrites (arrows) are visualized under dual-color fluorescence microscopy. Dendritic cell-lymphocyte clusters are present in fresh tissue (arrowheads) obtained at 3 weeks after tumor implantation. Scale bar, 50 μm. After Yang et al. (2003).

Animal care

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

Results

Dual-color visualization of interactions of host dendritic cells and cancer cells

Figure 1 shows many host-derived GFP-expressing dendritic cells (DCs) intimately contacting B16F10-

RFP melanoma cells with their dendrites (Yang et al., 2003).

Visualization of angiogenesis in live tumor tissue

Figure 2 shows the visualization of the onset of angiogenesis and its development in live tumor tissue. Host-derived GFP-expressing fibroblast cells and endothelial cells are shown forming new blood vessels in the RFP-expressing B16F10 melanoma (Yang et al., 2003).

Visualization of host macrophage cancer cell interactions

Figure 3 shows a host macrophage expressing GFP at 35 days after orthotopic implantation of human PC-3 prostate carcinoma cells in a transgenic nude

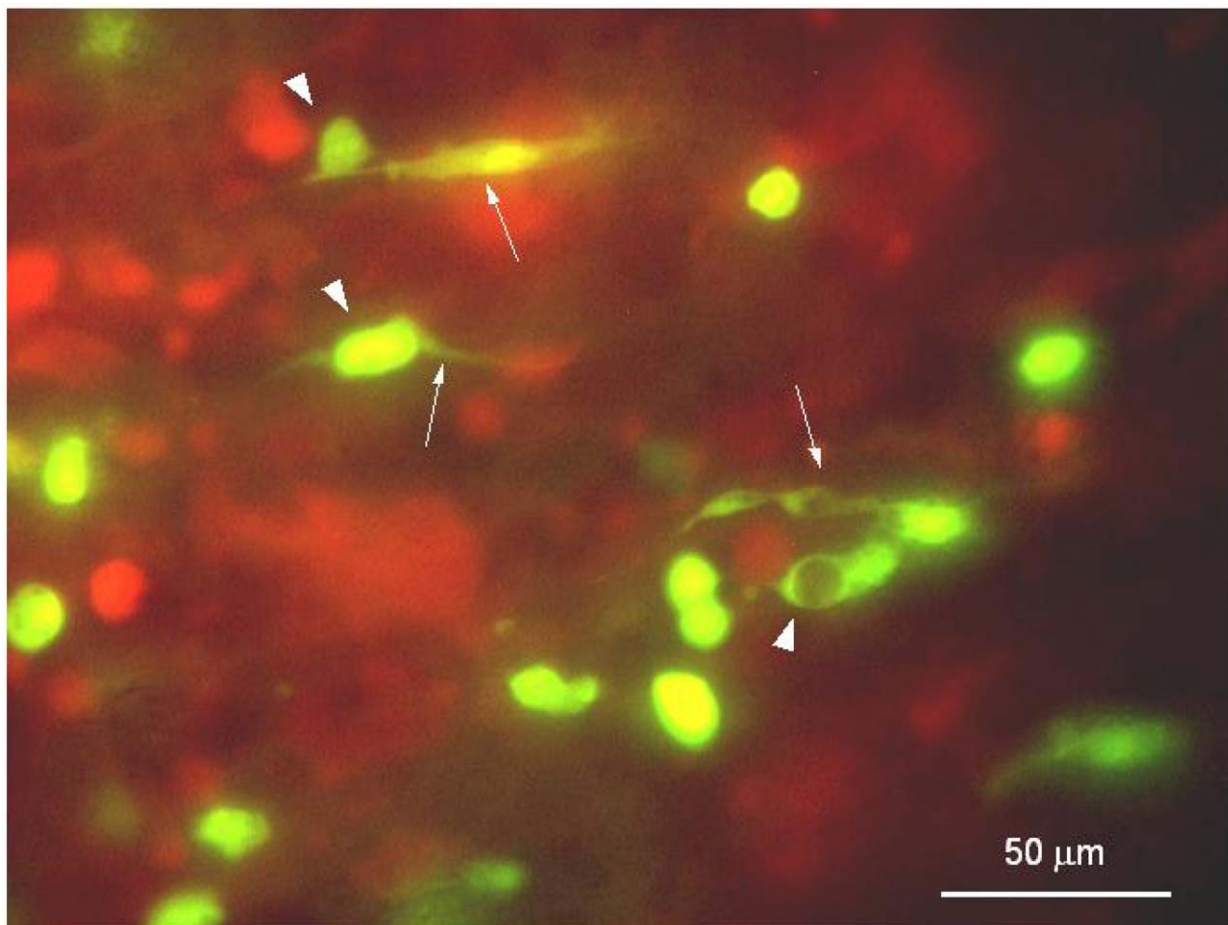


Figure 2. Visualization of angiogenesis in live tumor tissue at 3 weeks after s.c. injection of B16F10-RFP melanoma cells in the transgenic GFP mouse. Visualization of angiogenesis onset and development imaged in live tumor tissue. Host-derived GFP-expressing fibroblast cells (arrows) and endothelial cells (arrowheads) are forming new blood vessels in the RFP-expressing B16F10 melanoma. Scale bar, 50 μm . After Yang et al. (2003).

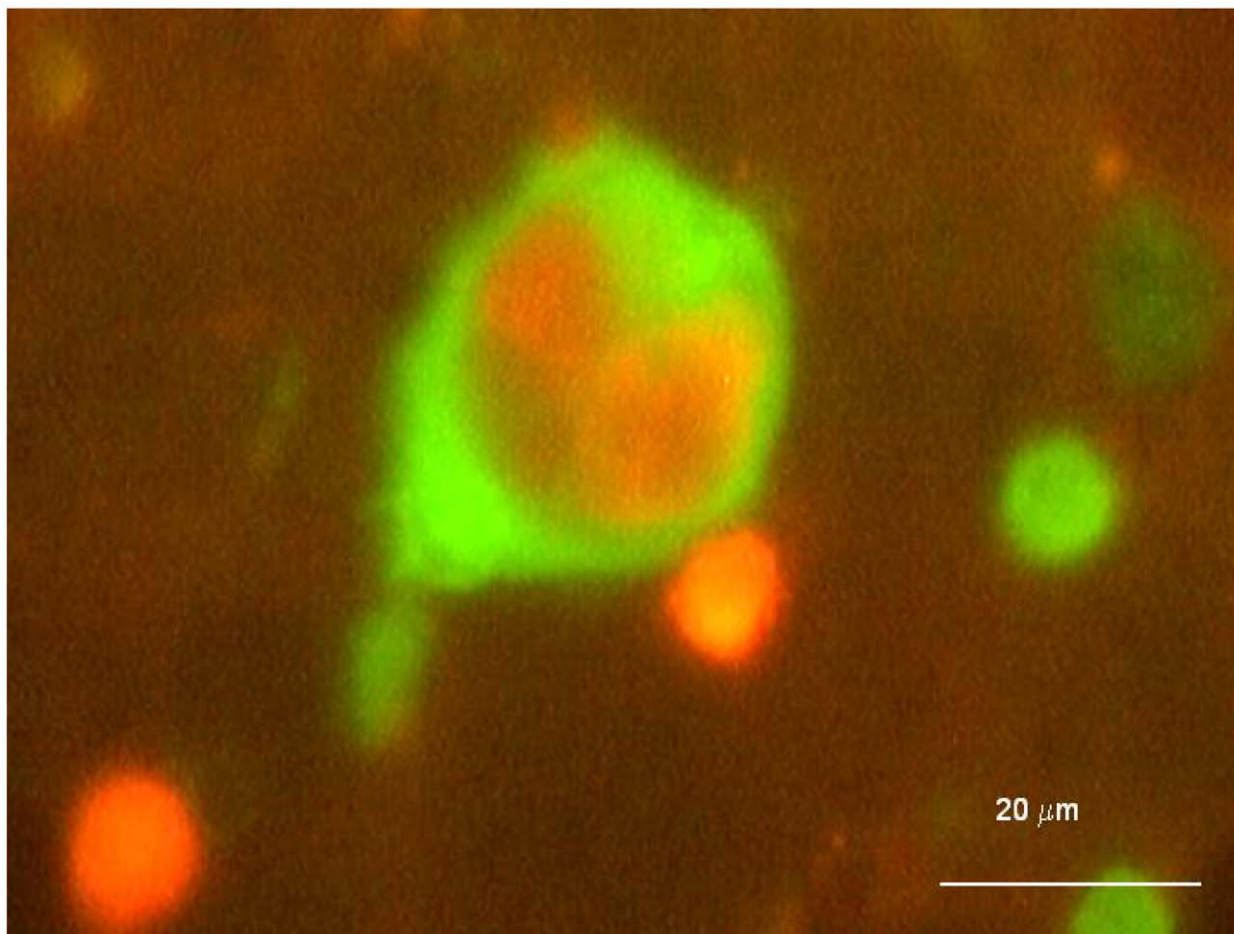


Figure 3. Macrophage cancer cell interaction in live tumor tissue. Image of fresh tissue shows host macrophage expressing GFP at 35 days after orthotopic implantation of human PC-3 prostate carcinoma cells in the transgenic GFP nude mouse. Dual-color fluorescence microscopy shows that the GFP-expressing host macrophage has engulfed the RFP PC-3 human prostate cancer cells. Scale bar, 20 μm . After Yang et al. (2003).

mouse expressing GFP in all its tissues. The host macrophages, identified by morphology and expressing GFP are observed under dual-color fluorescence microscopy after engulfing human PC-3 prostate cancer cells expressing RFP.

Interactions of a host stromal fibroblast cell and cancer cells

Figure 4 shows the intimate contact of host-stromal fibroblast expressing GFP with at least 4 RFP-expressing Dunning prostate carcinoma cells expressing RFP.

Dual-color imaging of GFP- and RFP-expressing metastatic tumors

GFP-labeled and RFP-labeled HT-1080 human fibrosarcoma cells were used to determine clonality by simple fluorescence visualization of metastatic

colonies after mixed implantation of the red and green fluorescent cells. Resulting pure red or pure green colonies were scored as clonal, whereas mixed yellow colonies were scored as nonclonal. In a spontaneous metastasis model originating from footpad injection in SCID mice, 95% of the resulting lung colonies were either pure green or pure red, indicating monoclonal origin, whereas 5% were of mixed color, indicating polyclonal origin. In an experimental lung metastasis model established by tail vein injection in SCID mice, clonality of lung metastasis was dependent on cell number. Figure 5 visualizes the development of many mixed colonies of experimental lung metastasis with a large input cell number (Yamamoto et al., 2003a).

Fluorescence imaging of stem cells

We have recently shown that the stem cell marker nestin is expressed in hair follicle stem cells in

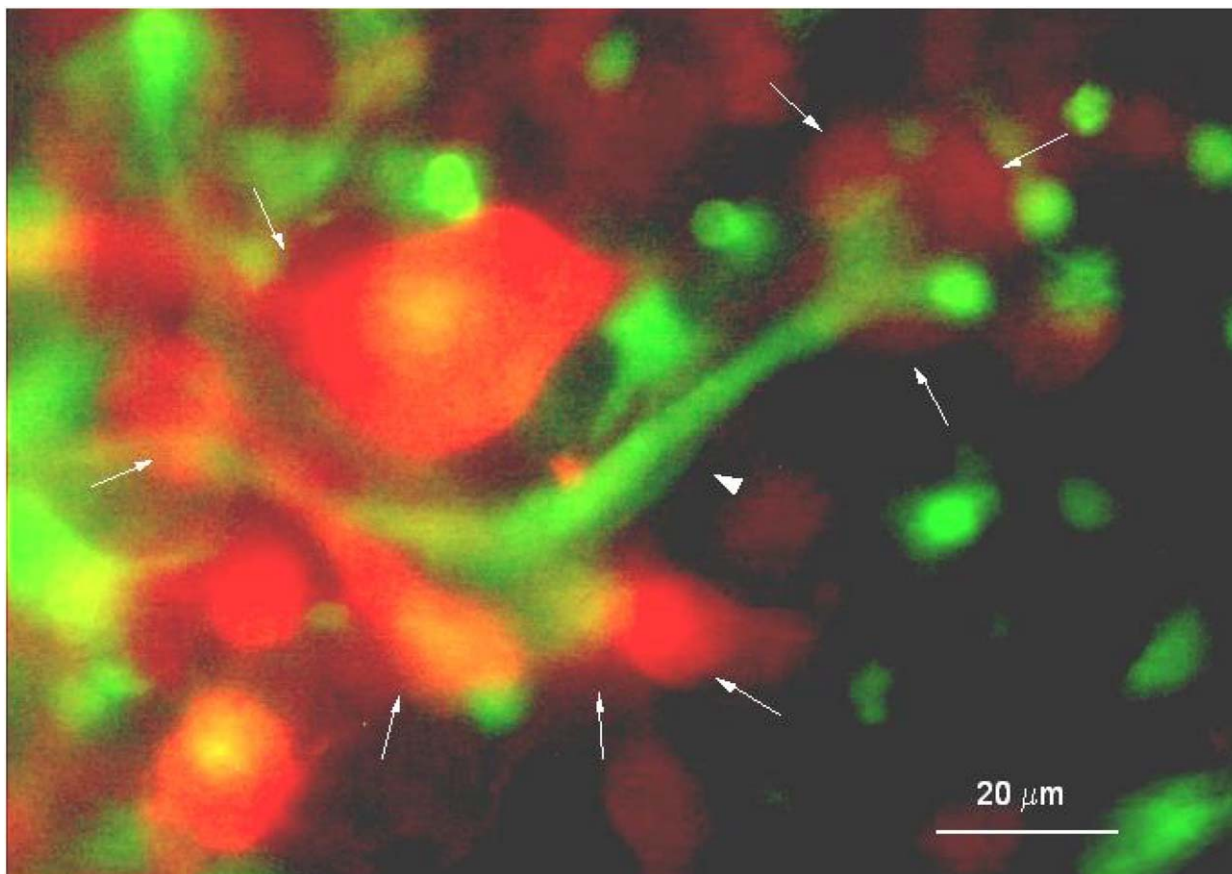


Figure 4. Interactions (arrows) of host stromal fibroblast cell (arrowhead) and cancer cells in live tumor tissue. Dual-color fluorescence microscopy of fresh tissue obtained at 3 days after orthotopic inoculation of 1×10^5 Dunning RFP-expressing rodent prostate cancer cells. Scale bar, 20 μm . After Yang et al. (2003).

nestin-driven GFP transgenic mice (Li et al., 2003). During early anagen or growth phase of the hair follicle, nestin-expressing cells, marked by GFP fluorescence in nestin-GFP transgenic mice, appear in the permanent upper hair follicle immediately below the sebaceous glands in the follicle bulge. This is where stem cells for the hair follicle outer root sheath are thought to be located. The relatively small, oval shaped, nestin-expressing cells in the bulge area surround the hair shaft and are interconnected by short dendrites. Figure 6 shows the hair follicle nestin-GFP expressing cells in the telogen phase of nestin-GFP transgenic mouse skin. GFP-nestin is also expressed in blood vessels of the skin of these mice. GFP expressing blood vessels in the nestin-GFP transgenic mouse were visualized by dual-color fluorescence microscopy in a transplanted RFP-expressing B16 melanoma. RFP tumor cells can be seen in the GFP blood vessels under both blue and green excitation fluorescence microscopy (Fig. 7).

Dual-color cells for imaging cell cycle position and apoptosis

We have established a dual-colored PC-3 human prostate cancer cell line, which stably expresses histone H2B-GFP in the nucleus and DsRed-2-RFP in the cytoplasm. Cells in different phases of the cell cycle were identified by nuclear-cytoplasmic ratios as determined by simultaneous dual-color real-time fluorescence imaging (Fig. 8). Live apoptotic cells were identified by their nuclear morphology. The dual-color cells are being used as a model for imaging apoptosis and cell cycle position *in vivo* in real time.

Imaging cell trafficking in graft-versus-host disease

Panoskaltis-Mortari et al. (2004) has developed a mouse system to track migration and homing of cells in a setting of bone-marrow-transplant-induced

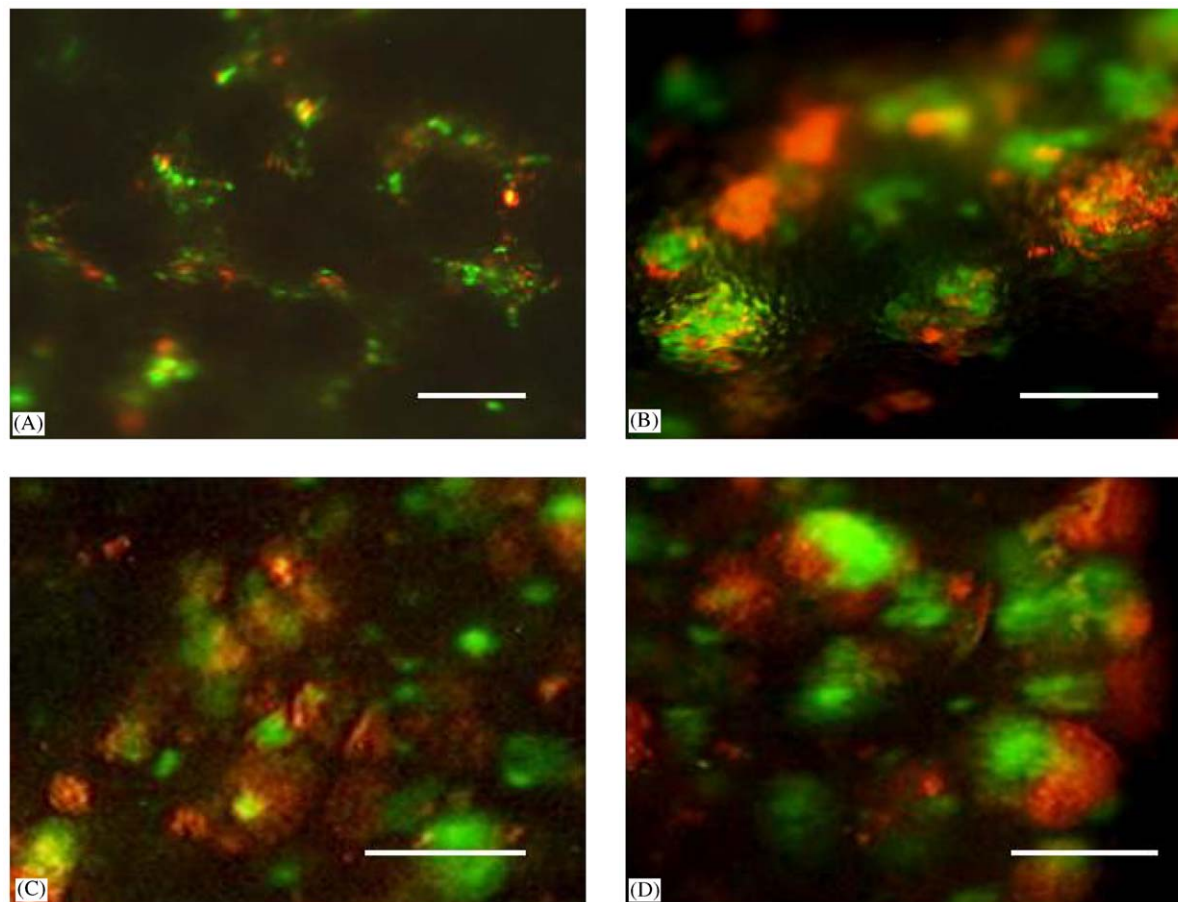
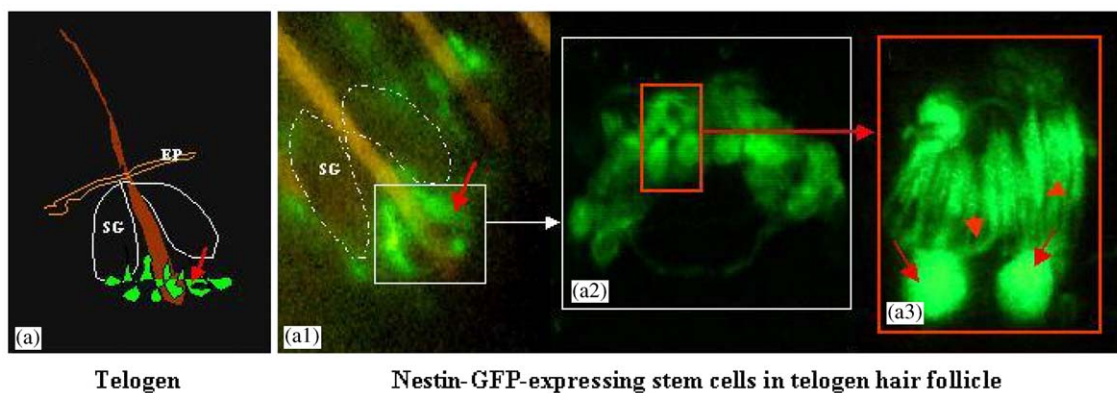


Figure 5. Development of colonies of experimental lung metastasis with large-input cell numbers. Metastases on the excised lung were imaged using fluorescence microscopy at each time point. (A) Immediately after tail vein injection (day 1). Bar, 200 μm . (B) Day 6. Bar, 250 μm . (C) Day 11. Bar, 300 μm . (D) Day 16. Bar, 400 μm . After Yamamoto et al. (2003b).



Telogen

Nestin-GFP-expressing stem cells in telogen hair follicle

Figure 6. Hair follicle nestin-GFP expressing cells in the telogen phase of the skin of a nestin-GFP transgenic mouse. The skin sample was prepared freshly immediately after excision from the back skin of a nestin-GFP transgenic mouse. The skin sample was then directly analyzed with fluorescence microscopy and confocal microscopy with the dermis side up after subcutaneous tissue was dissected out. (a) Cartoon of telogen hair follicle showing the position of nestin-GFP-expressing hair follicle stem cells. (a1) Low magnification fluorescence microscopy image showing the ring of bulge nestin-GFP-expressing stem cells (small white box; compare with cartoon in a). (a2) High magnification confocal microscopy image reflecting the small white box in (a1). Note the small, round or oval shaped nestin-GFP expressing cells in the bulge area of the hair follicle (small red box). (a3) High magnification fluorescence microscopy image of individual nestin-GFP-expressing stem cells shown in the red box in (a2). Note the unique morphology of the hair follicle stem cells and the multiple dendrite-like structures of each cell. Original magnifications: a1, 100 \times ; a2, 400 \times ; and a3, 1600 \times . After Li et al. (2003).

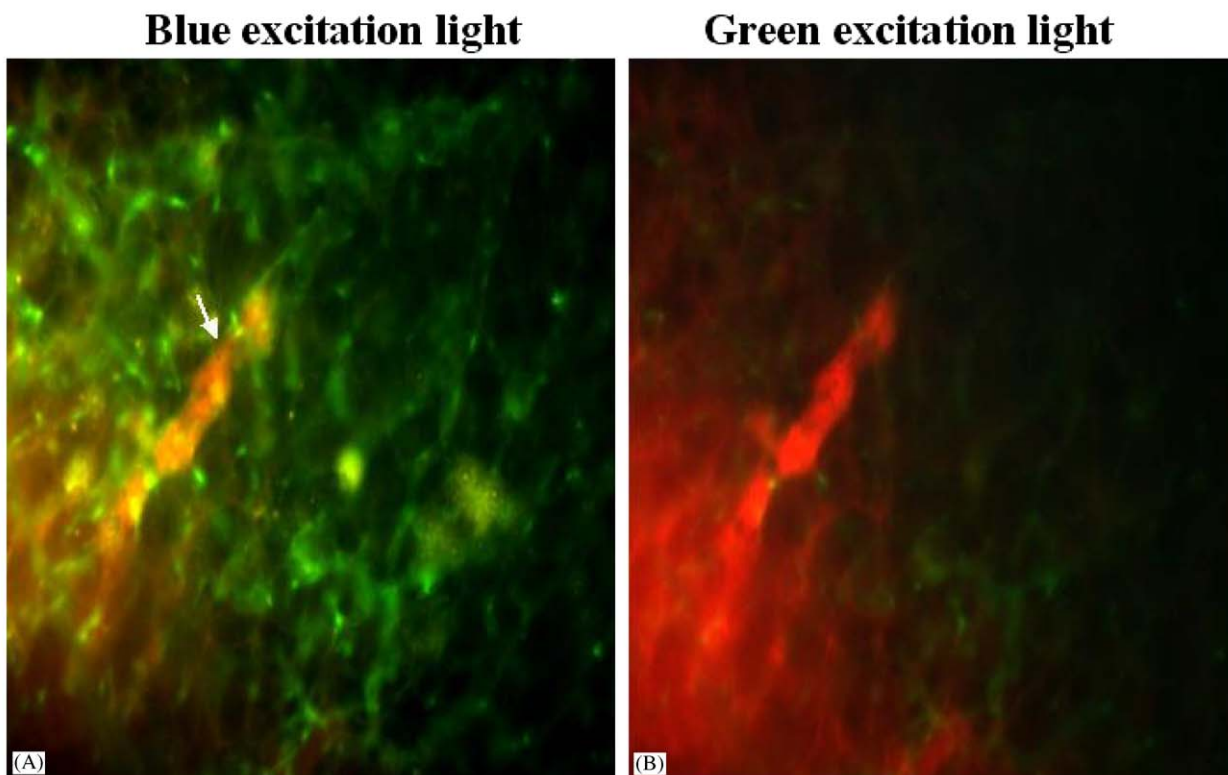


Figure 7. GFP-nestin-expressing vessels in RFP B16 melanoma. GFP-expressing blood vessels in the nestin-GFP transgenic mouse are visualized by dual-color fluorescence microscopy in a transplanted RFP-expressing B16 melanoma. RFP-expressing cancer cells are present in GFP-expressing blood vessels visualized under both blue (A) and green (B) excitation fluorescence microscopy in fresh tissue acquired at 7 days after transplantation (Amoh et al., unpublished data).

graft-versus-host disease (GVHD). GFP transgenic cells were used for transplantation. Whole body images of mice taken at various time points after GFP cell infusion revealed early migration of allogeneic cells to peripheral lymphoid organs with subsequent infiltration to GVHD target organs. Localization of the GFP cells was visualized on internal organs through the skin of shaved mice and were easily discernable.

Discussion

The new cell biology we envision is based on imaging functions of cells *in vivo*, including their interactions with similar and dissimilar cells. For example, the dual-color tumor–host model system allows visualization of tumor–host interactions by whole-body imaging as well as in fresh tissue. Both the tumor and the host cells are uniquely identified by their fluorescence color—RFP for the tumor, GFP for the host. The model has shown the specificity of various types of host cells for

the tumor. The dual-color tumor–host interaction model system allows observations of tumor–host interactions at the single-cell level in fresh tissue affording further insights in the role of host cells in tumor growth and progression (Yang et al., 2003). To specifically visualize angiogenesis, a nestin-GFP transgenic mouse was used since nascent blood vessels express nestin-GFP. Remarkable images were acquired in fresh tissue of an RFP-expressing melanoma residing in a GFP-expressing tumor blood vessel (Fig. 7; Amoh et al., unpublished data).

Visualizing color-coded cancer cells interacting with each other is also part of the new cell biology (Fig. 5). Visualization of stem cells *in vivo* is now possible with fluorescent proteins including those that form hair follicles (Fig. 6; Li et al., 2003) and possible related cells that form blood vessels (Fig. 7).

Individual cells themselves can be multi-colored (Fig. 8). The example presented has a GFP-colored nucleus and RFP-colored cytoplasm (Jiang et al., unpublished data). Dual-color cells expressing GFP in the nucleus and RFP in the cytoplasm enable real-time visualization of nuclear–cytoplasm

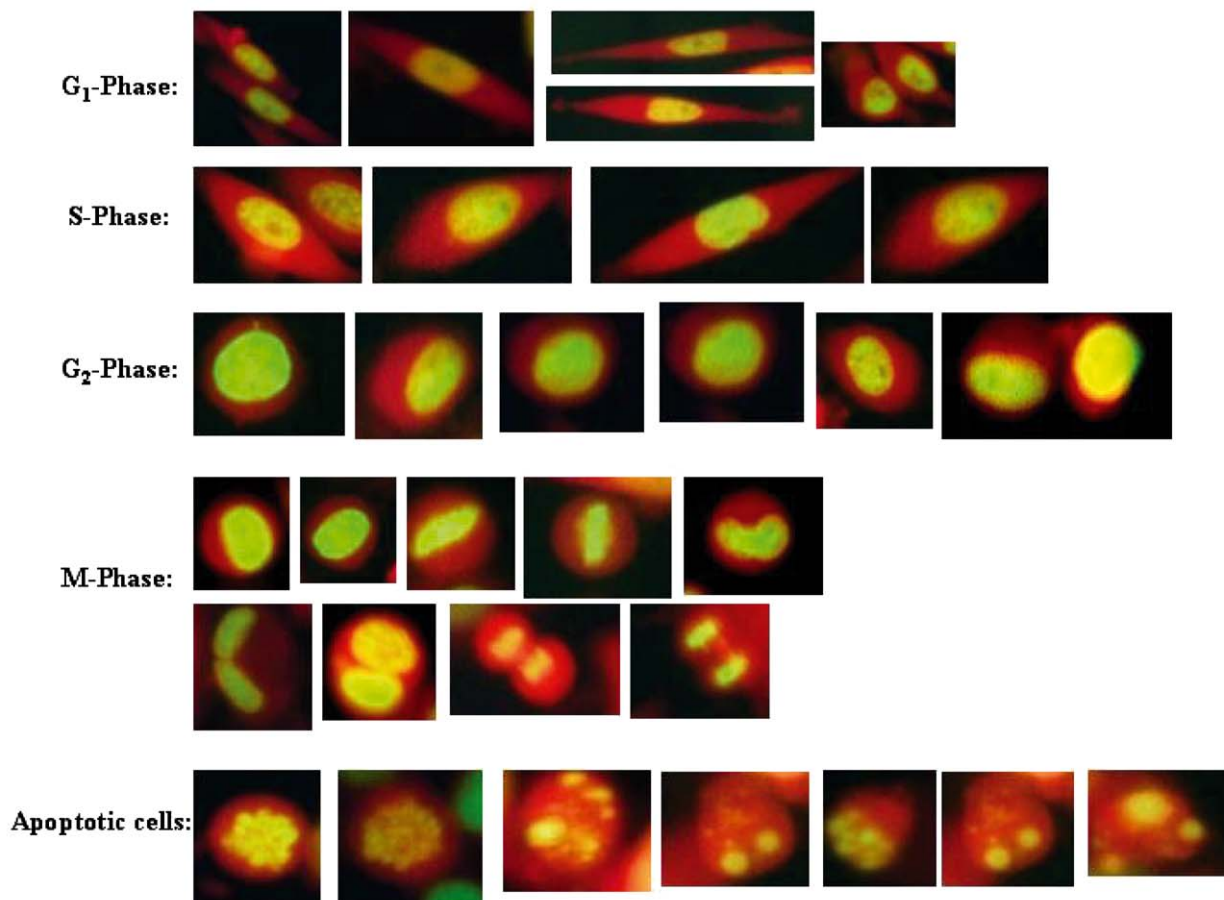


Figure 8. Live cell cycle analysis of dual-colored (RFP in cytoplasm/histone H2-B-GFP in nucleus) PC-3 human prostate cancer cells. Dual-colored PC-3 human prostate cancer cells, which stably express histone H2-B-GFP in the nucleus and DsRed-2-RFP in the cytoplasm are shown in various stages of the cell cycle as well as during apoptosis (Jiang et al., unpublished data).

interactions including cell cycle events and apoptosis. This dual-colored cell strategy can also be used to make the cells one color and the gene of interest linked to another color. With new spectrally distinct fluorescent proteins being cloned, “rainbow” cells allowing the real-time imaging of multiple events will be possible soon.

Other optical methods for *in vivo* imaging also exist including the use of luciferase. However, as stated by Panoskaltzis-Mortari et al. (2004) there are many features of luciferase imaging that make it limiting. The cost of the hardware prevents most laboratories from purchasing the equipment. The predominant drawbacks to luciferase is low spatial resolution and low sensitivity. The resolution issue, when using luciferase, is the inability to accurately identify the tissue that is emitting the signal. Furthermore, the sensitivity is so low that at least 1000 cells need to be present in one location in order for a signal to be detected by luciferase. In

addition, the substrate, luciferin, needed for luciferase, needs to be injected prior to luciferase imaging, may not reach all tissues consistently. Finally, the stability of the luciferase signal has been questioned (Burgos et al., 2003).

Multiple fluorescence labeling of cells will enable multiple events to be visualized *in vivo* including gene expression, ion fluxes, protein and organelle trafficking, chromosome dynamics and numerous other processes currently still only analyzed *in vitro*.

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