Color-coded Real-time Subcellular Fluorescence Imaging of the Interaction between Cancer and Host Cells in Live Mice

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Abstract. *Stromal cells are essential for tumor growth. Stromal cells interact with cancer cells during tumor growth and progression. We report here the development of a tri-color imageable mouse model to visualize the interaction between host cells and cancer cells. To observe subcellular cancer cell dynamics in vivo, HT-1080 human fibrosarcoma cells were labeled in the nucleus with histone H2B-green fluorescent protein (GFP) and with retroviral red fluorescent protein (RFP) in the cytoplasm. HT-1080-GFP-RFP cells were sprinkled over a skin-flap in transgenic GFP immunocompetent mice. After 24 h, the mice were imaged with an Olympus IV100 laser scanning microscope. HT-1080-GFP-RFP cells were visualized surrounded by host-derived lymphocytes and macrophages both expressing GFP. It was possible to observe host GFP macrophages contacting, engulfing, and digesting dual-color HT-1080-GFP-RFP cells in real time. The dual-color cancer cells were readily visible after being engulfed in the GFP macrophages. Other cancer cells were visualized being killed by lymphocytes. The results of this study show that differentially labeling cells with spectrally-distinct fluorescent protein can allow subcellular-resolution imaging of cell–cell interactions between host and cancer cells.*

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Our laboratory pioneered the use of green fluorescent protein (GFP) for *in vivo* imaging in 1997 (1). With the use of GFP, it was possible to observe individual cancer cells in fresh unstained tissue or even a live animal for the first time. Fluorescent proteins were used to visualize primary tumor growth, tumor cell motility and invasion, metastatic seeding and clononization, angiogenesis, and the interaction between the tumor and its microenvironment (2-7). The host and the tumor could be differentially labeled with fluorescent proteins. For example, a transgenic mouse expressing GFP in all of its cells, or in specific cells such as endothelial cells, transplanted with tumor cells expressing red fluorescent protein (RFP) enabled the interaction between the tumor and host cells to be visualized in real time (7, 8). For single-cell resolution, reversible acute skin flaps have been used over many parts of the body (skin, brain, lung, liver, *etc.*) (9, 10).

We report here the development of a tri-color imageable mouse model to visualize the interaction between host cells and cancer cells. To observe subcellular cancer cell dynamics, HT-1080 human fibrosarcoma cells were labeled in the nucleus with histone H2B-GFP and with retroviral RFP in the cytoplasm. Nuclear GFP expression enabled visualization of nuclear dynamics. Simultaneous cytoplasmic RFP expression enabled determination of nuclear-cytoplasmic ratios, as well as simultaneous cytoplasmic and nuclear shape changes. Thus, total cellular dynamics could be visualized in living dual-color cells in real time. Using the HT-1080-GFP-RFP cells and the GFP-expressing transgenic mice, GFP-lymphocyte and GFP-macrophage reactions with HT-1080-GFP-RFP cells were visualized at the subcellular level in live mice as described in the present report.

Materials and Methods

Production of RFP and histone H2B-GFP retrovirus. For RFP retrovirus production, the *Hind III*/*Not*I fragment from pDsRed2 containing the full-length RFP cDNA was inserted into the *Hind III*/*Not*I site of pLNCX2 that has the neomycin resistance gene to establish the pLNCX2-DsRed2 plasmid. For vector production, PT67 packaging cells (Irvine Scientific, Irvine, CA, USA), at 70% confluence, were incubated with a precipitated mixture of LipofectAMINE reagent (Life Technologies, Inc., Grand Island, NY, USA), and saturating amounts of pLNCX2-DsRed2 plasmid. The cells were examined by fluorescence microscopy 48 hours' post-transduction. For selection of a clone producing high amounts of an RFP retroviral vector (PT67-DsRed2), the cells were cultured in the presence of 200-1,000 μg/ml of G418 (Invitrogen, Carlsbad, CA, USA), increased stepwise. For histone H2B-GFP retrovirus production, the histone H2B gene was kindly provided by Professor Geoff Wahl (Salk Institute, San Diego, CA, USA). This gene has no stop codon and was linked to the 5´-end of the EGFP gene. The histone H2B-GFP fusion gene was inserted at the site of *Hind* III/*Cal*I of the pLHCX vector that has the hygromycin-resistant gene and the pLHCX histone H2B-GFP plasmid was thereby reconstructed. To establish a clone producing high amounts of a histone H2B-GFP retroviral vector, the pLHCX histone H2B-GFP plasmid was transfected to PT67 packaging cells by the same method for PT67-DsRed2. The transfected cells were cultured in the presence of 200-400 μg/ml of hygromycin, increased stepwise.

RFP and histone H2B-GFP gene transduction of fibrosarcoma cells. To establish dual-color cells, clones of HT-1080 human fibrosarcoma cells expressing RFP in the cytoplasm (HT-1080-RFP) were initially established. Briefly, HT-1080 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP packaging cells for 72 hours. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 hours' post-transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 μg/ml of G418. The level of G418 was increased stepwise up to 800 μg/ml. HT-1080-RFP cells were isolated with cloning cylinders using trypsin/EDTA and amplified by conventional culture methods. To establish dual-color cells, HT-1080-RFP cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-histone H2B-GFP packaging cells and culture medium. To select the double transformants (Figure 1), cells were incubated with hygromycin 72 hours after transfection and the level of hygromycin was increased stepwise up to 400 μg/ml.

Mice. Five female transgenic C57/B6-GFP mice were used in this study (11). Mice were kept in a barrier facility under HEPA filtration. Mice were fed with an autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, CA, USA). All animal studies were conducted in accordance with the principles and procedures outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals under PHS Assurance Number A3873-01.

Real-time visualization of HT-1080-GFP-RFP cells in live mice. GFP-transgenic immunocompetent mice (11) were anesthetized with a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H_2O) *via s.c.* injection. An arc-shaped incision was made in the abdominal skin, and then subcutaneous connective tissue was separated to free the skin flap without injuring the epigastric cranialis artery and vein (10). The skin-flap was spread and fixed on a flat stand. A total of 100 μl medium containing 5×10^5 HT-1080-GFP-RFP cells (Figure 1) were sprinkled over the surface of the skin-flap of the mice. Twenty-four hours later, the inside surface of the skin flap was directly imaged.

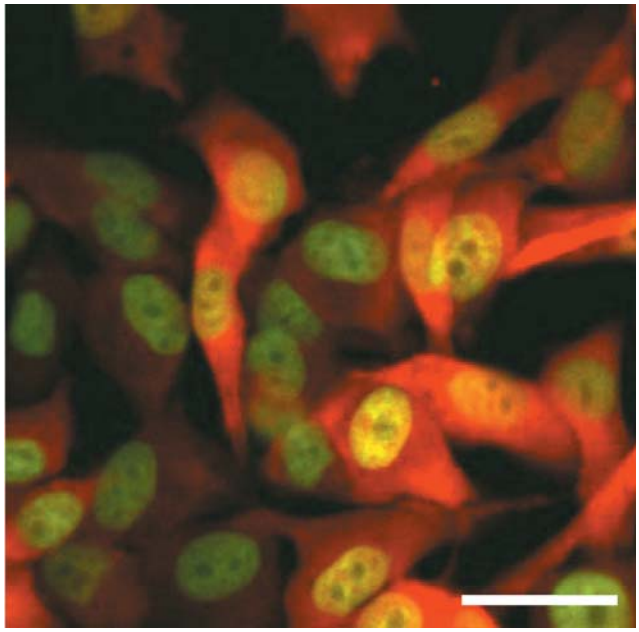

Figure 1. *Green fluorescent protein (GFP)- and red fluorescent protein (RFP)-expressing human fibrosarcoma cells (HT-1080-GFP-RFP cells) in vitro. GFP is localized in the nuclei due to linkage with histone H2B. Retroviral DsRed2 is expressed in the cytoplasm. See the Materials and Methods section for details. Bar=50 μm.*

Fluorescence optical imaging and data analysis. An IV100 laser scanning microscope (Olympus Corp., Tokyo, Japan) was used with a ϕ3.5 mm Micro Probe Objective lens (Olympus Corp) and IV-10 ASW software (Olympus Corp.) (12, 13). An argon laser (488 nm) and a DPSS laser (561 nm, Melles Griot, Carlsbad, CA, USA) were used for GFP and RFP excitation, respectively. Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 software.

Results and Discussion

Interaction of host mouse cells and HT-1080-GFP-RFP cells. Twenty-four hours after sprinkling HT-1080-GFP-RFP cells over the surface of the skin flap of the transgenic GFP mice, numerous host-derived GFP-expressing lymphocytes and macrophages were seen interacting with the HT-1080-GFP-RFP cells (Figure 2).

Visualization of the interaction between host macrophages and HT-1080-GFP-RFP cells. Macrophages, which have

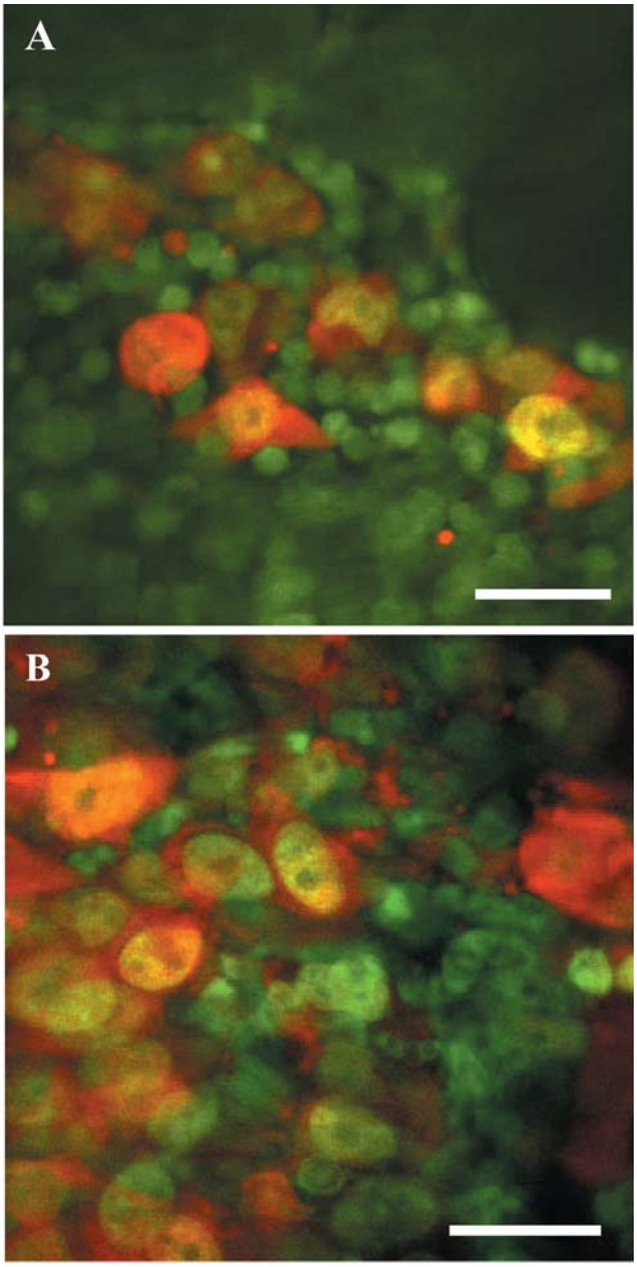

Figure 2. *Visualization of the interaction between GFP host macrophages and lymphocytes with human HT-1080-GFP-RFP cancer cells in live mice 24 hours after HT-1080-GFP-RFP cells were sprinkled over the surface of a skin flap of transgenic GFP mice. A: HT-1080-GFP-RFP cells can be seen surrounded by lymphocytes. B: Numerous host-derived GFP-expressing lymphocytes and macrophages can be seen interacting with HT-1080-GFP-RFP cell aggregates. Bar=50 μm.*

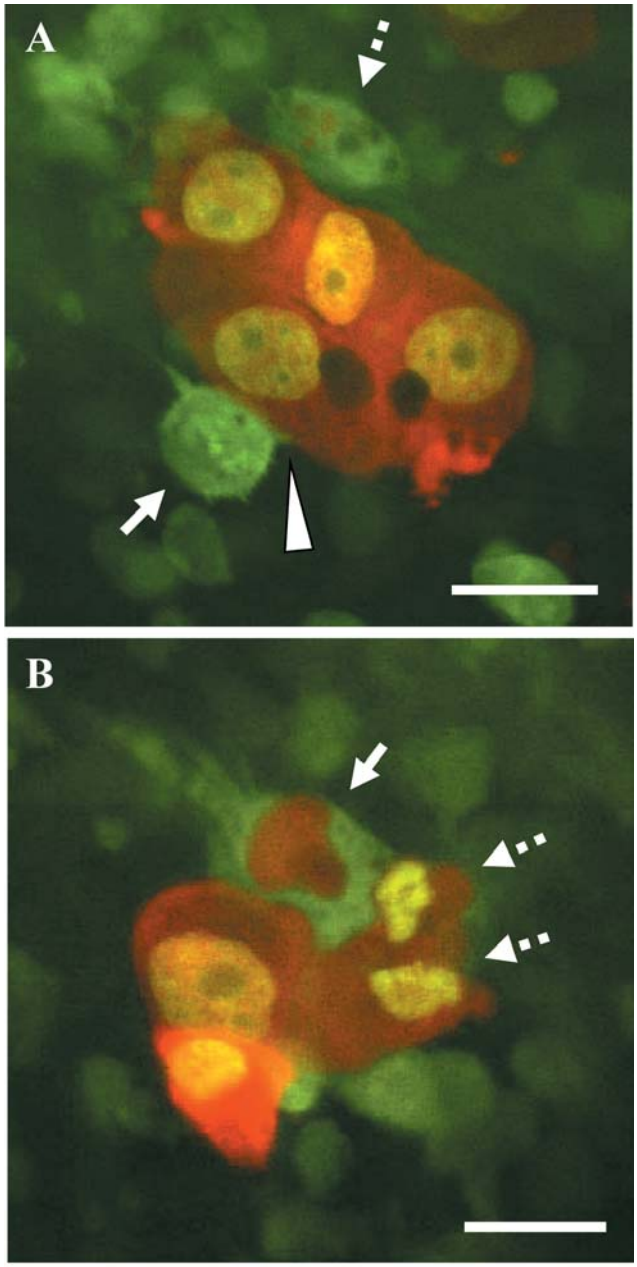

Figure 3. *Visualization of host macrophage-cancer cell interaction in live mice. A: Host GFP macrophages were visualized contacting HT-1080-GFP-RFP cells (arrow, broken arrow). Macrophage processes can be seen extending into the HT-1080-GFP-RFP cell (arrowhead). Another macrophage (broken arrow) contained cytoplasmic fragments after digesting an HT-1080-GFP-RFP cell. B: A macrophage (arrow), engulfing the RFP-expressing cytoplasm of an HT-1080 cell shown in contact with additional HT-1080-GFP-RFP cells (broken arrows). Bar=20 μm.*

both scavenger and antigen-presenting functions, play an important role in defending against tumors. Host-derived GFP macrophages were visualized contacting HT-1080-GFP-RFP cells (Figure 3A). Macrophage processes were seen extending into the HT-1080 cell. A macrophage that had digested an HT-1080 cell was visualized. In Figure 3-B, a macrophage which had engulfed an HT-1080 cell was visualized contacting other HT-1080-GFP-RFP cells.

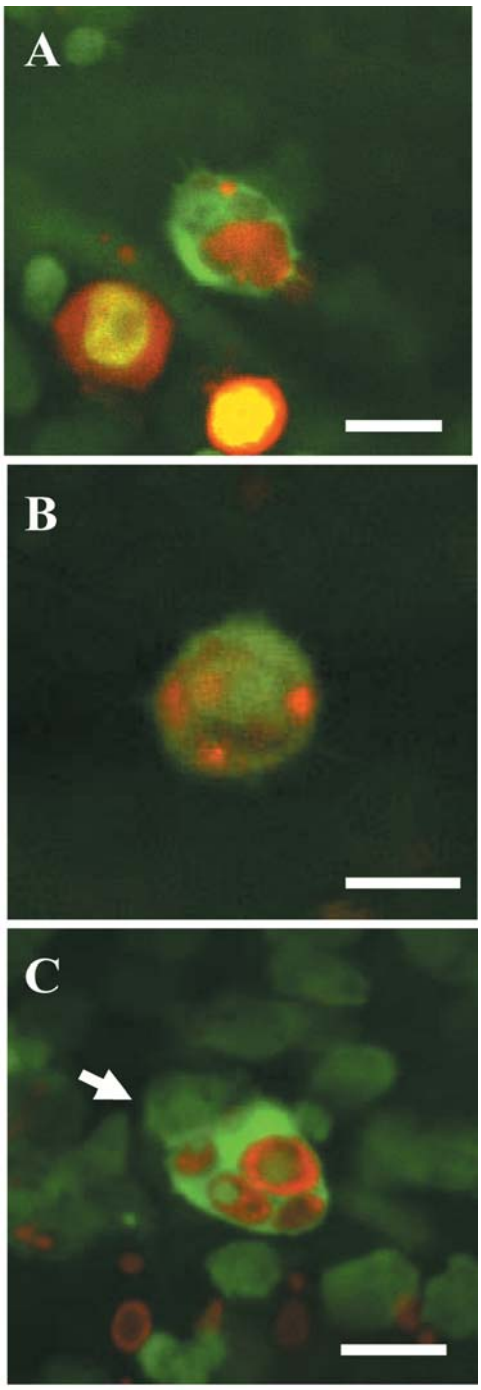

Figure 4. *Imaging a macrophage phagocytosing cancer cells in live mice. The macrophages had engulfed and digested HT-1080-GFP-RFP cells. A: A fragment of RFP-expressing cytoplasm from an HT-1080-GFP-RFP cell can be seen inside the macrophage. Many microspikes of the macrophage can be seen. B: The RFP-expressing cytoplasm of an HT-1080-GFP-RFP cell was digested by a macrophage and fragmented. C: The macrophage engulfed four HT-1080-GFP-RFP cells at a time. Each GFP nucleus of the HT-1080-GFP-RFP cells, which was surrounded by RFP cytoplasm, could be clearly visualized. A macrophage can be seen contacting a lymphocyte, possibly to present antigens (arrow). Bar=20 μm.*

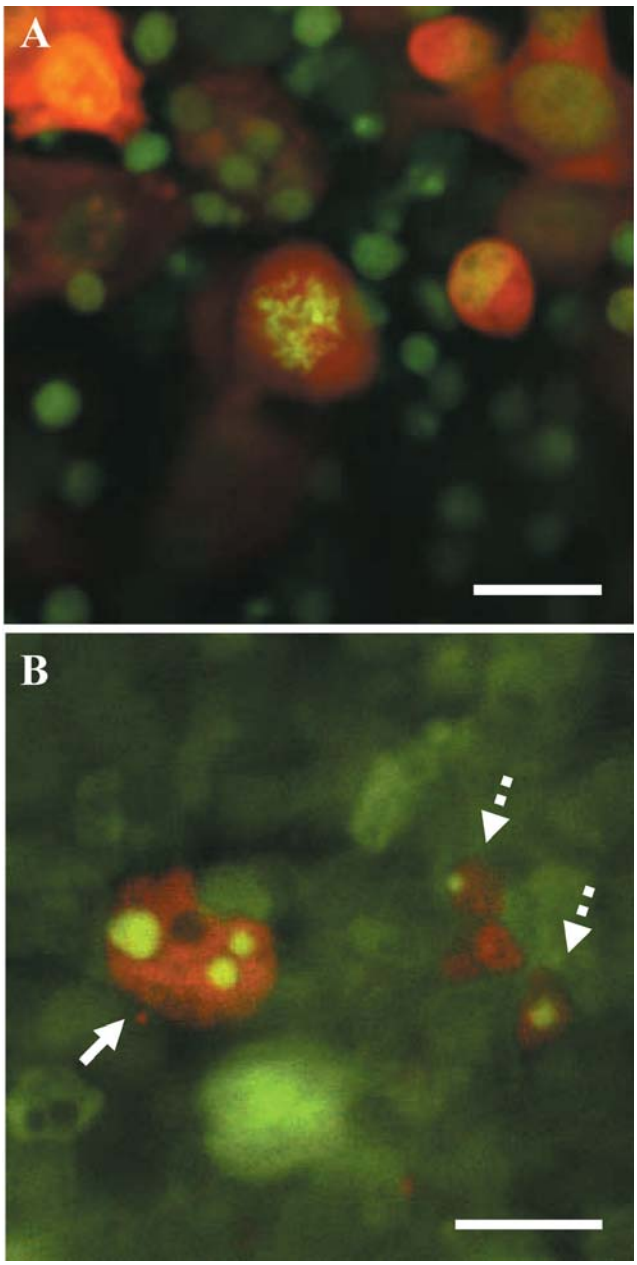

Figure 5. *Visualization of lymphocyte-mediated cancer-cell killing in live mice. A: Twenty-four hours after HT-1080-GFP-RFP cell transplantation, a GFP nucleus was seen to be fragmented within a whole cell. B: GFP nucleus fragmented into three parts in the HT-1080 cell (arrow). The whole cell had not yet broken. Two apoptotic bodies, consisting of condensed fragmented nuclei and segregated cytoplasm, can be seen (broken arrows). Bar=20 μm.*

Visualization of phagocytosis of cancer cells by macrophages. Phagocytosis is a special form of endocytosis in which large particles are ingested *via* phogosomes. Figure 4 shows a macrophage phagocytosing RFP cytoplasm derived from an HT-1080-GFP-RFP cell. The nucleus could not be seen in the macrophage (Figure 4A). After the macrophage had

phagocytosed the HT-1080 cell, the RFP cytoplasm was digested into fragments (Figure 4B). Another macrophage was imaged engulfing four HT-1080-GFP-RFP cells at once (Figure 4C). Each HT-1080 nucleus was clearly identified. The macrophage was also contacting a lymphocyte, possibly in order to present antigens (Figure 4C).

Visualization of host-induced cancer cell death. The GFP nucleus of an HT-1080-GFP-RFP cell was fragmented within the cell 24 hours after *in vivo* seeding (Figure 5A, B). Nuclear fragmentation indicates the early stage of apoptosis. The whole cell had not yet fragmented. Two apoptotic bodies, consisting of condensed fragmented nuclei and segregated cytoplasm, were observed. The two apoptotic bodies were much smaller than the non-apoptotic HT1080-GFP-RFP cells.

With the GFP transgenic mice and the dual-color cancer cells labeled with GFP in the nucleus and RFP in the cytoplasm, we were able to visualize the interaction between host macrophage and lymphocytes and HT-1080-GFP-RFP cells at the subcellular level in live mice.

Macrophages were visualized contacting, engulfing, and digesting the HT-1080 cells within 24 h after *in vivo* seeding. In addition, a lymphocyte was visualized contacting a macrophage that was engulfing four HT-1080 cells. This suggests that the macrophage was presenting antigens to the lymphocyte. Lymphocytes were also visualized killing cancer cells.

This model can provide a powerful tool for further understanding the interaction between host and cancer cells in live mice.

Conflict of Interest

None of the Authors have a conflict of interest with this study.

Acknowledgements

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