

Color Coding Cancer Cells with Fluorescent Proteins to Visualize *In Vivo* Cellular Interaction in Metastatic Colonies

NORIO YAMAMOTO^{1,2,3}, MENG YANG¹, PING JIANG¹, MINGXU XU¹, KENSUKE YAMAUCHI^{1,2,3}, HIROYUKI TSUCHIYA³, KATSURO TOMITA³, A.R. MOOSSA² and ROBERT M. HOFFMAN^{1,2}

¹AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111;

²Department of Surgery, University of California, 200 W. Arbor Dr., San Diego, CA 92103-8220, U.S.A.;

³Department of Orthopedic Surgery, School of Medicine, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa, 920-8641 Japan

Abstract. We report here *in vivo* visualization of cancer cell interaction using stable sublines of green fluorescent protein (GFP)- and red fluorescent protein (RFP)-expressing HT-1080 human fibrosarcoma cells. These color-coded fibrosarcoma cell lines show similar cell proliferation and lung metastasis potential. The color-coded cells were mixed at a ratio of 1:1 and were injected into the tail vein of severe combined immunodeficient (SCID) mice. The resulting experimental lung metastases were simultaneously imaged and their ratio was determined by color-pixel analysis. Fluorescence color coding of cancer cells enables visualization of the interaction of cancer cells and can be used to distinguish cancer cells of any genotype or phenotype.

Fluorescent protein imaging enables the visualization of metastasis both *ex vivo* and by whole-body imaging (1-12). We have previously developed a simple, rapid, and high-resolution fluorescence method to determine clonality of metastasis (1). Green fluorescent protein (GFP) and red fluorescent protein (RFP) were used to tag clones of the HT-1080 human fibrosarcoma cell line. Upon *i.v.* injection of red and green fluorescent HT-1080 cells, fluorescent metastatic colonies result on the lung that are either pure red or pure green, indicating clonality, or yellow indicating mixed origin. GFP and RFP fluorescence enabled simultaneous dual-color imaging of the metastatic lung colonies (1).

We describe here quantitative results obtained by image analysis that determined the ratio of red to green metastatic colonies after *i.v.* injection of equivalent mixtures of color-coded HT-1080 fibrosarcoma cells.

Correspondence to: Robert M. Hoffman, AntiCancer, Inc., San Diego, CA 92111, U.S.A. Tel: 858-654-2555, Fax: 858-268-4175, e-mail: all@anticancer.com

Key Words: GFP, RFP, *in vivo* imaging, metastasis.

Materials and Methods

Production of GFP and RFP Retrovirus (1). The pLEIN retroviral vector (Clontech Laboratories, Inc., Palo Alto, CA, USA) expressing GFP and the neomycin resistance gene on the same bicistronic message was used as a GFP expression vector. PT67, an NIH3T3-derived packaging cell line, expressing the 10 AI viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in DMEM (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, Calabasas, CA, USA). For vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim, Indianapolis, IN, USA) and saturating amounts of pLEIN plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h after transduction. For selection, the cells were cultured in the presence of 500-2000 µg/ml G418 (Life Technologies, Inc., Grand Island, NY, USA) for 7 days to select for a clone producing high amounts of a GFP retroviral vector (PT67-GFP).

For RFP retrovirus production, the *HindIII/NotI* fragment from pDsRed2 (Clontech), containing the full-length RFP cDNA, was inserted into the *HindIII/NotI* site of pLNCX2 (Clontech) that has the neomycin resistance gene to establish the pLNCX2-DsRed2 plasmid. The PT67 cells, at 70% confluence, were incubated with a precipitated mixture of Lipofectamine reagent (Life Technologies, Inc.) and saturating amounts of pLNCX2-DsRed2 plasmid as described above for GFP vector production. For selection of a clone producing high amounts of a RFP retroviral vector (PT67-DsRed2), the cells were cultured in the presence of 200-1000 µg/ml G418 for 7 days (10, 11).

GFP and RFP gene transduction of HT-1080 fibrosarcoma cells (1). For GFP or RFP gene transduction, 70% confluent human HT-1080 human fibrosarcoma cells (American Type Culture Collection, Manassas, VA, USA), were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-GFP or PT67-DsRed2 cells and RPMI 1640 (Mediatech, Inc., Herndon, VA, USA) containing 10% fetal bovine serum for 72 h. Fresh medium was replenished at this time. Cells were harvested by trypsin/EDTA 72 h after transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 µg/ml G418. The

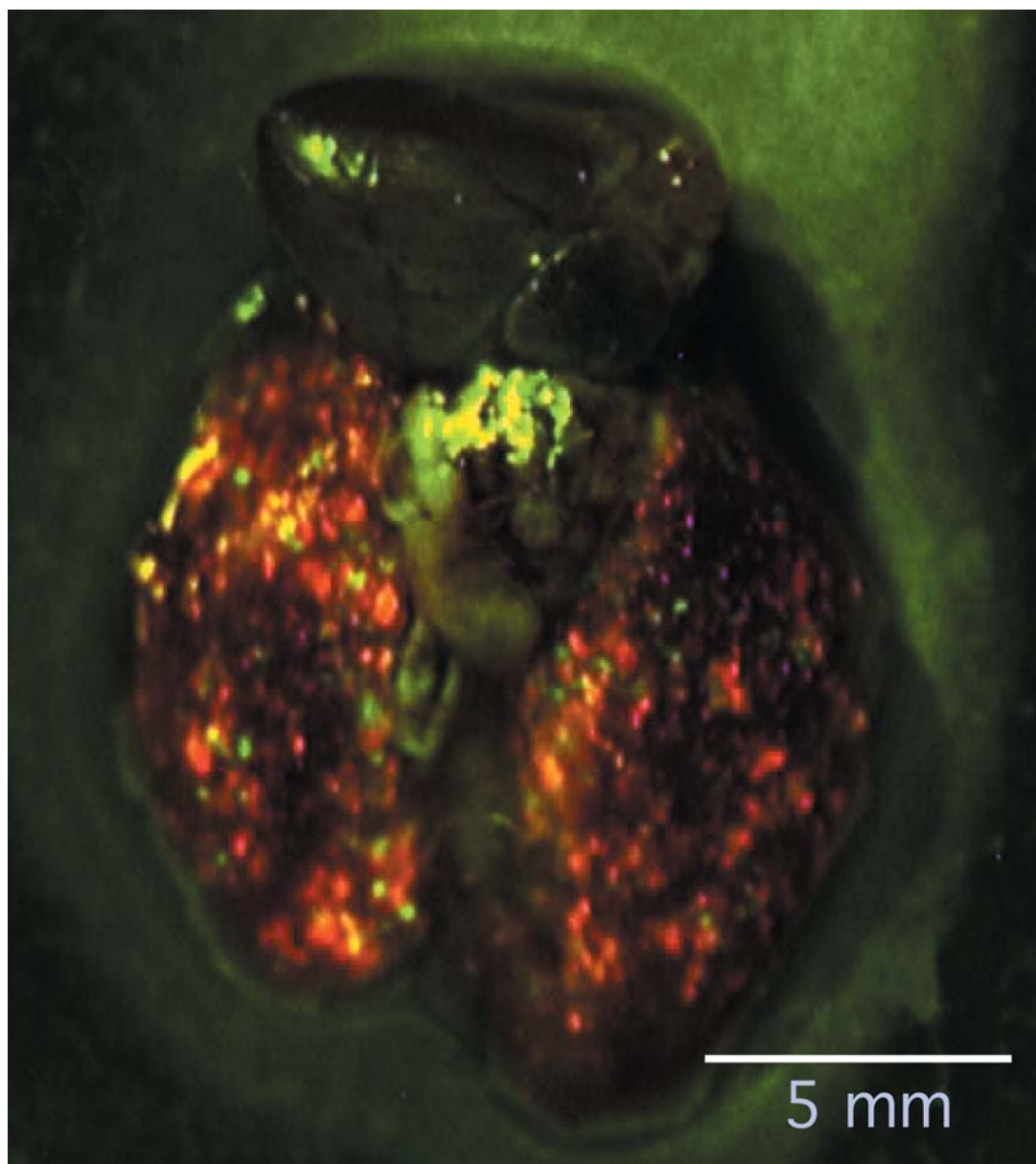


Figure 1. Experimental lung metastasis with color-coded cancer cells. HT-1080-GFP cells and HT-1080-RFP cells were harvested by trypsinization. SCID mice were injected with 6×10^6 cells (3×10^6 cells from each clone) in a total volume of 200 μ l into the tail vein. Two weeks after cell injection, the lung was removed and tumor colonies on the surface were observed under fluorescence microscopy.

level of G418 was increased stepwise up to 800 μ g/ml. Clones of HT-1080 expressing high levels of GFP (HT-1080-GFP) or RFP (HT-1080-RFP) were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) using trypsin/EDTA and amplified by conventional culture methods.

Cell proliferation rate determination (1). Each fluorescent-tagged HT-1080 clone (HT-1080-GFP or HT-1080-RFP) and parental clone (HT-1080) was seeded at a density of 1×10^3 cells/dish in 100-mm dishes in RPMI 1640 (day 1). The dishes were kept in an incubator at 37°C and 5% CO₂. Every other day, from days 2 to 8, three dishes

for each clone were used to count cells. Resuspended cells collected by conventional methods were stained with trypan blue (Sigma) for viability. Viable cells were counted with a hemocytometer (Reichert Scientific Instruments, Buffalo, NY, USA).

Tail-vein-injection experimental-metastasis model (1). SCID mice were injected with 2×10^6 cells (1×10^6 RFP and 1×10^6 GFP cells) in a total volume of 200 μ l into the tail vein. Cells were injected within 30 min of harvest. Two weeks after cell injection, the mice were sacrificed and the lungs removed to evaluate the lung colonies for pure or mixed color under fluorescence microscopy.

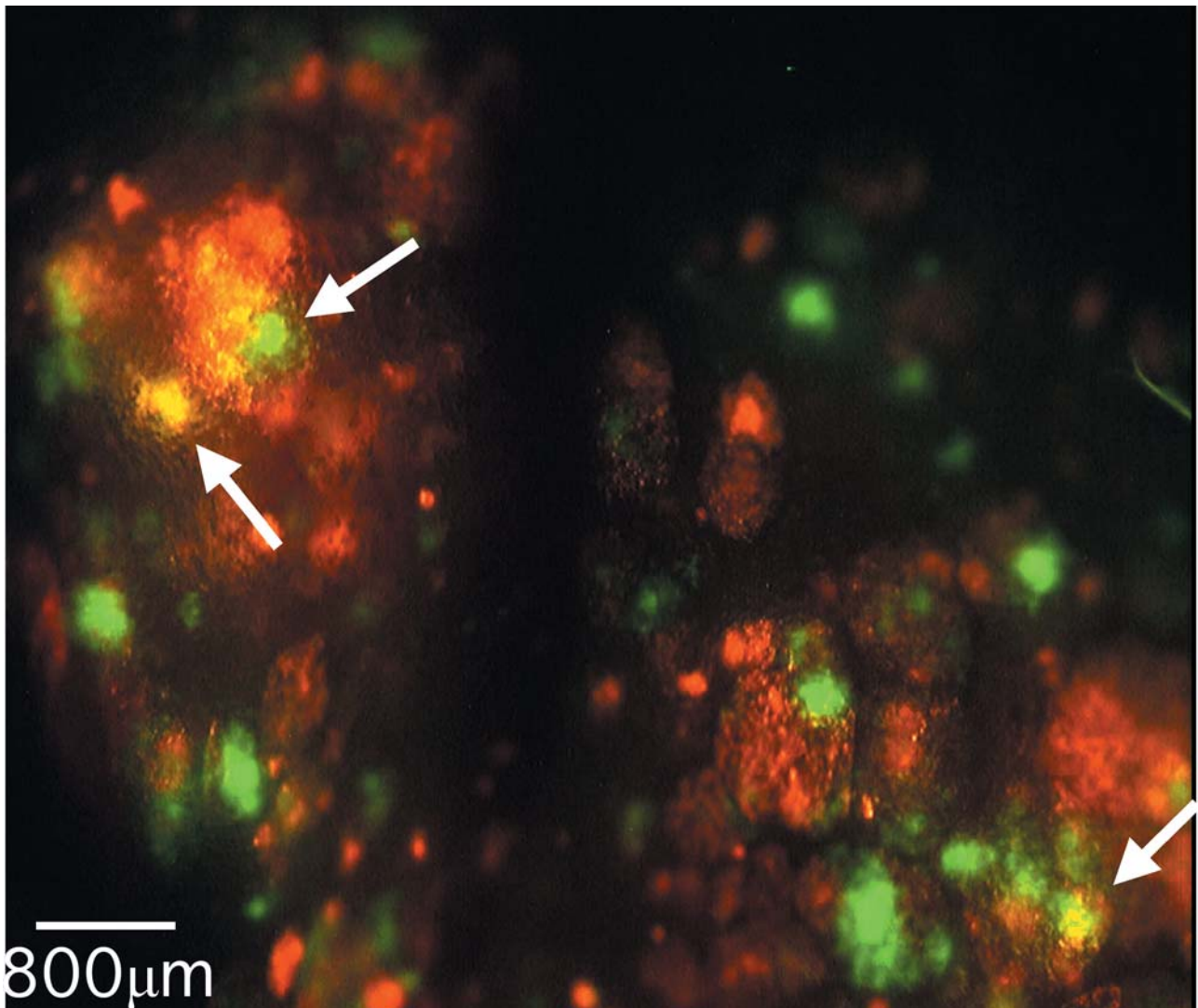


Figure 2. High-magnification fluorescent images of color-coded metastatic colonies on the lung. Numerous pure clonal colonies which were red or green as well as mixed colonies which were yellow were observed. See Figure 1 for details.

Fluorescence optical imaging. Images were captured directly with a Hamamatsu C5810 3CCD camera (Hamamatsu Photonics, Bridgewater, NJ, USA). For macroimaging, a fluorescence light box (Lighttools Research, Encinitas, CA, USA) was used. For microimaging, a Leica fluorescence stereo microscope model LZ12 was coupled with the charge-coupled device (CCD) camera. This microscope was equipped with a GFP filter set and a mercury lamp with a 50-W power supply. Images were processed for contrast and brightness and analyzed with the use of Image ProPlus 3.1 software. High-resolution images of 1024 x 724 pixels were captured directly on an IBM PC (1).

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

Results and Discussion

Cell proliferation potentials in parental, GFP- and RFP-expressing clones. As we reported previously (1), stable and bright GFP or RFP fluorescent HT-1080 cells were isolated *in vitro* after transfection with GFP and RFP genes, respectively. There was no difference in the proliferation rate of parental HT-1080 and the HT-1080-GFP or HT-1080-RFP cells in monolayer culture (data not shown).

Color-coded experimental metastases. Two weeks after cell injection, the lung was removed and tumor colonies on the surface were visualized under fluorescence microscopy. At low magnification, numerous fluorescent metastatic colonies were

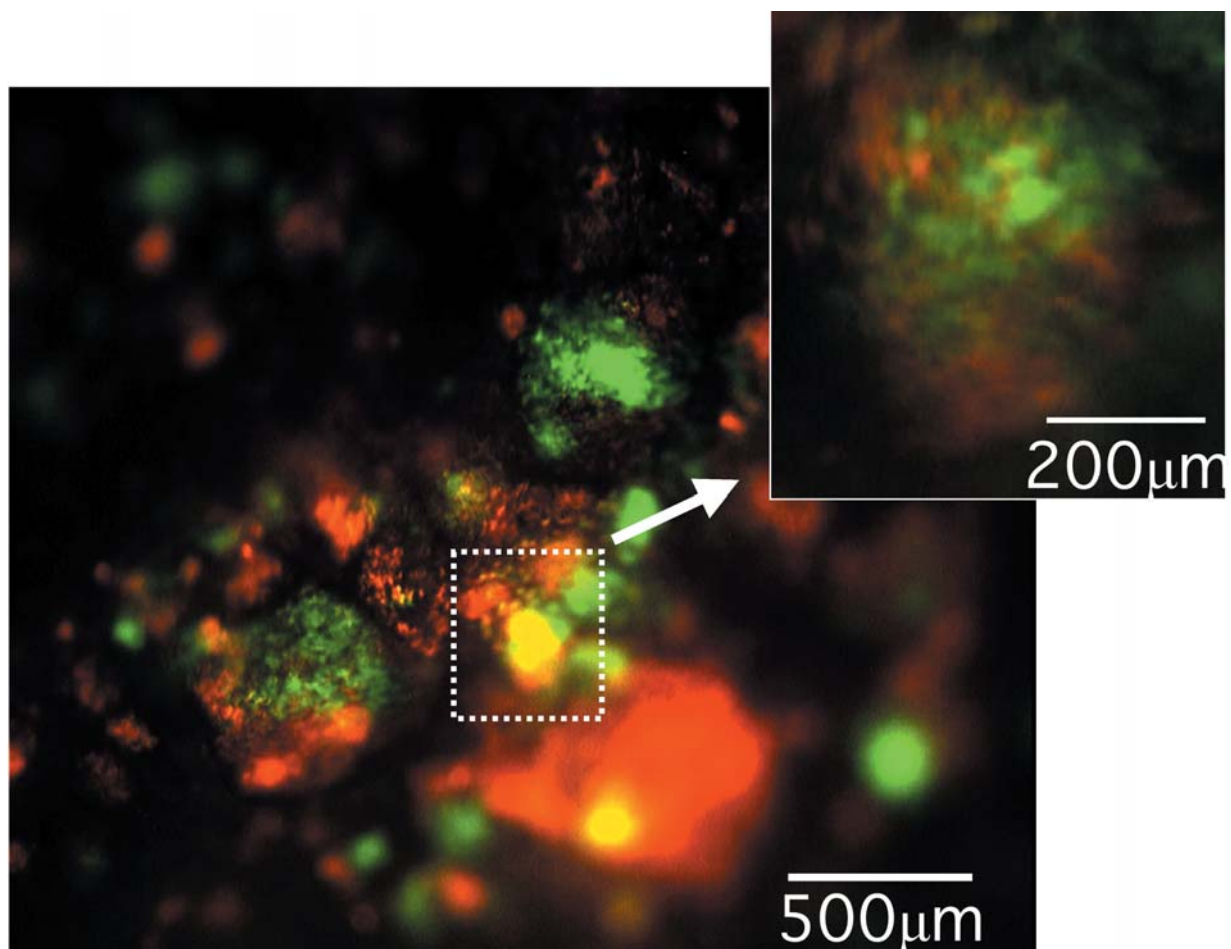


Figure 3. Metastatic-colony analysis at the cellular level using color-coded cells. High-magnification view of yellow fluorescent colony showing the individual green and red cells in the mixed colony. See Figure 1 for details.

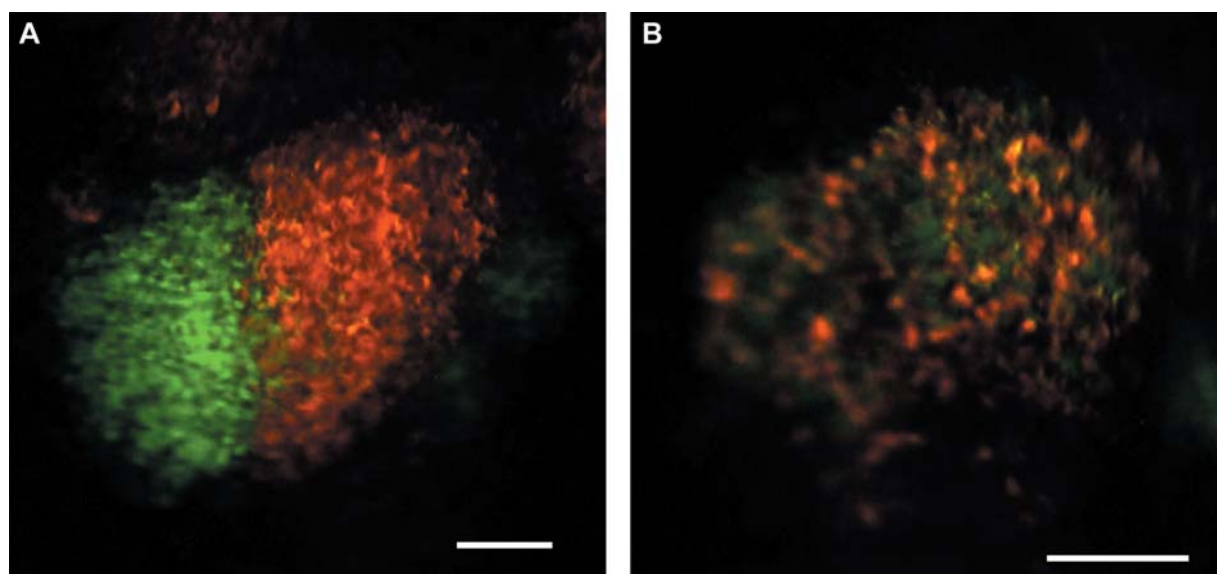
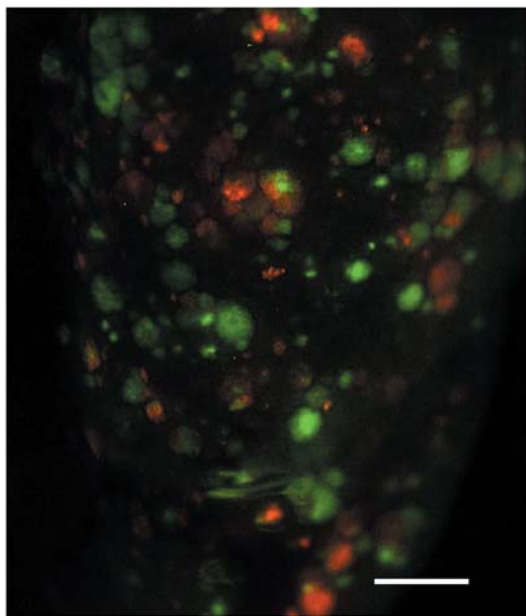
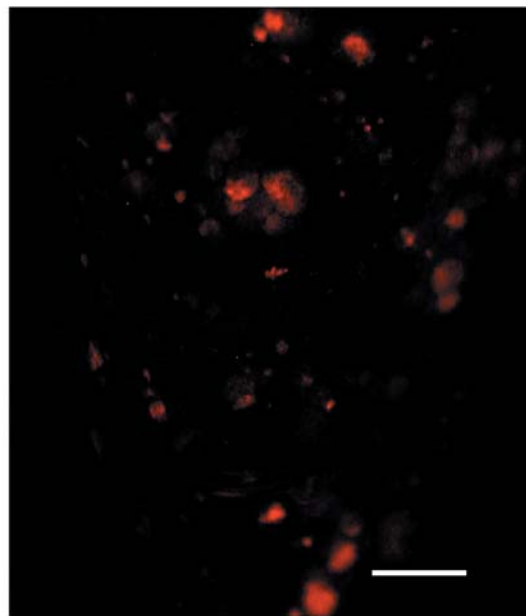


Figure 4. Dual-color fluorescence distinguishes fusion of pure clonal colonies and mixed non-clonal colony. A: Fusion of two pure lung colonies. B: Mosaic lung colony. Bars, 200 μm . See Figure 1 for details.



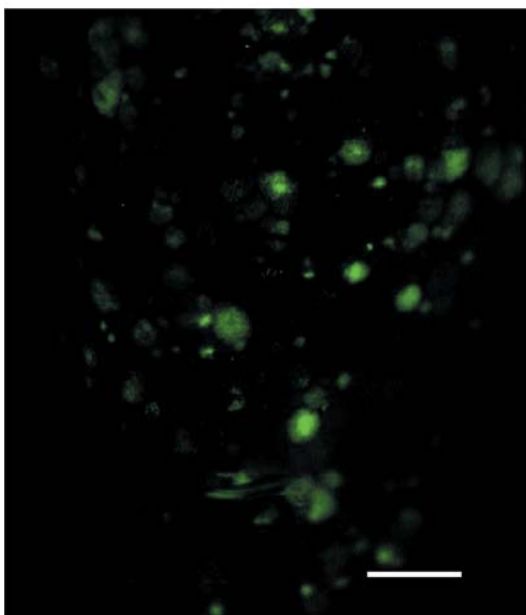
A

Green / Red = 1.11



C

110783 pixels



B

123232 pixels

Figure 5. Pixel analysis of color-coded metastatic lung colonies. An area of the lung where the majority of metastatic colonies were either red or green fluorescent was chosen for analysis. See Figure 1 for details. A: Dual-color image on indicated area of lung with green and red colonies. B: Image from green channel in area imaged in A. C: Image from red channel in area imaged in A. Pixel analysis demonstrates approximately equal numbers of red- and green-fluorescent metastatic colonies. Bars, 2 mm.

observed (Figure 1). At higher magnification, colonies could be readily visualized to be green, red or yellow fluorescent (Figure 2). The red and green colonies are pure and therefore clonal, and the yellow colonies are mixed and therefore non-clonal (1).

High-magnification fluorescence microscopy analysis of color-coded metastatic tumor cells. Under higher magnification fluorescence microscopy, the lung metastases could be visualized at the single cell level (Figure 3). Higher

magnification showed that a yellow metastatic colony was comprised of a mixture of red and green fluorescent cancer cells (Figure 3). Two clonal metastases, one containing only green cells and one containing only red cells, could be visualized growing together (Figure 4A). In contrast, a mixed-color non-clonal metastasis containing red and green fluorescent cells was also visualized (Figure 4B).

Pixel analysis to determine ratio of color-coded colonies. Using image analysis, an area of the lung that had predominantly green and red colonies was quantified by pixel analysis to determine the ratio of green to red colonies. A ratio of 1.1/1 of green to red pixels was obtained (Figure 5), suggesting that both red and green fluorescing cells formed metastases with equal propensity.

These results demonstrated the utility of color coding cancer cells, in this case to observe their interaction to form pure or mixed metastatic colonies. Resolution was at the cellular level. Future experiments will use color-code cancer cells of different phenotypes and genotypes to visualize how the cells interact *in vivo*, in the live animal.

Acknowledgements

This study was supported in part by grant funding from the U.S. National Cancer Institute CA099258, CA103563 and CA101600.

References

- 1 Yamamoto N, Yang M, Jiang P, Xu M, Tsuchiya H, Tomita K, Moossa AR and Hoffman RM: Determination of clonality of metastasis by cell-specific color-coded fluorescent-protein imaging. *Cancer Res* 63: 7785-90, 2003.
- 2 Chishima T, Miyagi Y, Wang X, Yamaoka H, Shimada H, Moossa AR and Hoffman RM: Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression. *Cancer Res* 57: 2042-7, 1997.
- 3 Chishima T, Miyagi Y, Wang X, Tan Y, Shimada H, Moossa AR and Hoffman RM: Visualization of the metastatic process by green fluorescent protein expression. *Anticancer Res* 17: 2377-84, 1997.
- 4 Chishima T, Miyagi Y, Wang X, Baranov E, Tan Y, Shimada H, Moossa AR and Hoffman RM: Metastatic patterns of lung cancer visualized live and in process by green fluorescent protein expression. *Clin Exp Meta* 15: 547-52, 1997.
- 5 Chishima T, Yang M, Miyagi Y, Li L, Tan Y, Baranov E, Shimada H, Moossa AR, Penman S and Hoffman RM: Governing step of metastasis visualized *in vitro*. *Proc Natl Acad Sci USA* 94: 11573-11576, 1997.
- 6 Yang M, Hasegawa S, Jiang P, Wang X, Tan Y, Chishima T, Shimada H, Moossa AR and Hoffman RM: Widespread skeletal metastatic potential of human lung cancer revealed by green fluorescent protein expression. *Cancer Res* 58: 4217-21, 1998.
- 7 Yang M, Jiang P, Sun FX, Hasegawa S, Baranov E, Chishima T, Shimada H, Moossa AR and Hoffman RM: A fluorescent orthotopic bone metastasis model of human prostate cancer. *Cancer Res* 59: 781-6, 1999.
- 8 Yang M, Chishima T, Wang X, Baranov E, Shimada H, Moossa AR and Hoffman RM: Multi-organ metastatic capability of Chinese hamster ovary cells revealed by green fluorescent protein (GFP) expression. *Clin Exp Meta* 17: 417-22, 1999.
- 9 Yang M, Baranov E, Jiang P, Sun FX, Li XM, Li L, Hasegawa S, Bouvet M, Al-Tuwaijri M, Chishima T, Shimada H, Moossa AR, Penman S and Hoffman RM: Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. *Proc Natl Acad Sci USA* 97: 1206-11, 2000.
- 10 Yang M, Baranov E, Wang JW, Jiang P, Wang X, Sun FX, Moossa AR, Penman S and Hoffman RM: Direct external imaging of nascent cancer, tumor progression, angiogenesis, and metastasis on internal organs in the fluorescent orthotopic model. *Proc Natl Acad Sci USA* 99: 3824-9, 2002.
- 11 Yamamoto N, Yang M, Jiang P, Tsuchiya H, Tomita K, Moossa AR and Hoffman RM: Real-time GFP imaging of spontaneous HT-1080 fibrosarcoma lung metastases. *Clin Exp Meta* 20: 181-5, 2003.
- 12 Hoffman RM: Green fluorescent protein imaging of tumour growth, metastasis, and angiogenesis in mouse models. *Lancet Oncology* 3: 546-556, 2002.

Received September 21, 2004

Accepted October 25, 2004