

The Cyan Fluorescent Protein Nude Mouse as a Host for Multicolor-coded Imaging Models of Primary and Metastatic Tumor Microenvironments

ATSUSHI SUETSUGU^{1,2,3}, MOHAMED K. HASSANEIN^{1,2}, JOSE REYNOSO¹,
YOSUKE OSAWA³, MASAHITO NAGAKI³, HISATAKA MORIWAKI³,
SHIGETOYO SAIJ³, MICHAEL BOUVET² and ROBERT M. HOFFMAN^{1,2}

¹AntiCancer, Inc. San Diego, CA, U.S.A.;

²Department of Surgery, University of California, San Diego, CA, U.S.A.;

³Gifu University Graduate School of Medicine, Gifu, Japan

Abstract. *The tumor microenvironment (TME) has an important influence on tumor progression. For example, we have discovered that passenger stromal cells are necessary for metastasis. In this report, we describe six different cyan fluorescent protein (CFP) multicolor TME nude mouse models. The six different implantation models were used to image the TME using multiple colors of fluorescent proteins: I) Red fluorescent protein (RFP)- or green fluorescent protein (GFP)-expressing HCT-116 human colon cancer cells were implanted subcutaneously in the CFP-expressing nude mice. CFP stromal elements from the subcutaneous TME were visualized interacting with the RFP- or GFP-expressing tumors. II) RFP-expressing HCT-116 cells were transplanted into the spleen of CFP nude mice, and experimental metastases were then formed in the liver. CFP stromal elements from the liver TME were visualized interacting with the RFP-expressing tumor. III) RFP-expressing HCT-116 cancer cells were transplanted in the tail vein of CFP-expressing nude mice, forming experimental metastases in the lung. CFP stromal elements from the lung were visualized interacting with the RFP-expressing tumor. IV) In order to visualize two different tumors in the TME, GFP-expressing and RFP-expressing HCT-116 cancer cells were co-implanted subcutaneously in CFP-expressing nude mice. A 3-color TME was formed subcutaneously in the CFP mouse, and CFP stromal elements were visualized interacting with the RFP-*

and GFP-expressing tumors. V) In order to have two different colors of stromal elements, GFP-expressing HCT-116 cells were initially injected subcutaneously in RFP-expressing nude mice. After 14 days, the tumor, which consisted of GFP cancer cells and RFP stromal cells derived from the RFP nude mouse, was harvested and transplanted into the CFP nude mouse. CFP stromal cells invaded the growing transplanted tumor containing GFP cancer cells and RFP stroma. VI) Mouse mammary tumor (MMT) cells expressing GFP in the nucleus and RFP in the cytoplasm were implanted in the spleen of a CFP nude mouse. Cancer cells were imaged in the liver 3 days after cell injection. The dual-color dividing MMT cells and CFP hepatocytes, as well as CFP non-parenchymal cells of the liver were imaged interacting with the 2-color cancer cells. CFP-expressing host cancer-associated fibroblasts (CAFs) were predominantly observed in the TME models developed in the CFP nude mouse. Thus, the CFP nude mouse adds another color to the pallet of the TME, allowing multiple types of color-coded cancer and stromal cells to be imaged simultaneously. The multi-colored models described in this report provide new opportunities to study the cellular interactions in the live primary and metastatic TME.

The tumor microenvironment (TME) is comprised of endothelial cells, fibroblasts, perivascular cells, inflammatory cells, and other cell types (1). Angiogenesis and lymphangiogenesis occur in the TME and are regulated by a variety of molecules released by cancer cells, as well as host stromal cells (2, 3). The cancer-associated fibroblast (CAF) is the most prominent cell type within the stroma of the TME (4-7). CAFs promote cancer cell growth and increase angiogenesis, invasion, and metastasis (8-10). Macrophages migrating to tumor stroma are called tumor-associated macrophages (TAMs). Although TAMs may have antitumor activity (11), they also promote tumor progression and invasion (12), including intravasation (13).

Correspondence to: Robert M. Hoffman, Ph.D., AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111 U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, email: all@anticancer.com

Key Words: GFP, RFP, CFP, transgenic nude mice, dual-color cancer cells, cancer-associated fibroblasts, color-coded imaging, HCT-116 human colon cancer cells, mouse mammary tumor cells, tumor microenvironment (TME).

The TME strongly regulates tumor behavior. For example, we discovered that passenger stromal cells are necessary for metastasis (14). After splenic injection of cancer cells, splenocytes co-trafficked with the cancer cells to the liver and enabled metastatic colony formation. Human colon cancer cells that express green fluorescent protein (GFP) linked to histone H2B in the nucleus and red fluorescent protein (RFP) in the cytoplasm (HCT-116-GFP-RFP) were injected into either the portal vein (PV) or spleen of transgenic nude mice expressing GFP and imaged at the subcellular level *in vivo*. Extensive clasmocytosis (destruction of the cytoplasm) of the cancer cells occurred within 6 hours after PV injection, and essentially all the cancer cells died. In contrast, splenic injection of the cancer cells resulted in the aggressive formation of liver and distant metastases. GFP spleen cells were found in the liver metastases that resulted from intrasplenic injection of the cancer cells in the transgenic GFP nude mice. When GFP spleen cells and the RFP cancer cells were co-injected into the PV, liver metastasis resulted that contained GFP spleen cells. Cancer cells alone injected into the PV were unable to form liver metastases. These results demonstrated that passenger stromal cells are necessary to form metastasis (14). This pioneering observation was confirmed by Duda *et al* four years later (15).

In order to image the TME, the cancer cells and the host elements must be distinguished. Transgenic nude mice expressing various fluorescent proteins, including GFP (16), RFP (17), and cyan fluorescent protein (CFP) (18, 19), are very useful as hosts for human tumors since stromal and cancer cells can be color-coded.

We previously imaged GFP-expressing CAFs recruited by metastatic tumors in the liver of transgenic GFP nude mice. A desmin-positive area increased around and within the liver metastasis over time, suggesting CAFs were recruited by the liver metastasis and have a role in tumor progression (20). Egeblad *et al.* (21) observed that CFP was useful for imaging the TME. We report here the power of the CFP nude mouse as a host for multicolor imaging of the primary and metastatic TME.

Materials and Methods

Cell culture. HCT-116 human colon cancer cells and mouse mammary tumor 060562 (MMT) (22) cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine from Gibco-BRL, Life Technologies Inc. (Grand Island, NY, USA). All media were supplemented with penicillin and streptomycin (Gibco-BRL). All cell lines were cultured at 37°C with 5% CO₂ (23).

Fluorescent protein gene transduction of cancer cells. Cancer cells were labeled with RFP or GFP. Clones expressing RFP or GFP were initially established. In brief, cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP or PT67-GFP packaging cells and RPMI 1640 (Irvine Scientific, Irvine, CA, USA) containing 10% FBS for 72 h. Fresh medium was replenished at

this time. Cells were harvested with trypsin/EDTA 72 h post-transduction and sub-cultured at a ratio of 1:15 into selective medium, which contained 200 µg/ml G418 (Invitrogen, Carlsbad, CA, USA). The level of G418 was increased stepwise up to 800 µg/ml (24).

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 *EGFP* gene (Clontech Laboratories, Mountain View, CA, USA) (25). 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 the histone H2B GFP retroviral vector, the pLHCX histone H2B-GFP plasmid was transfected in PT67 packaging cells. The transfected cells were cultured in the presence of 200 to 400 µg/ml hygromycin (Life Technologies) for 15 days to establish stable PT67 H2B-GFP packaging cells (26, 27, 28).

Establishment of dual-color cancer cells. For establishing dual-color cells, the RFP-expressing cancer cells, produced as described above were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 H2B 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 (26, 27, 28).

CFP mice. Transgenic nude mice, expressing CFP under the control of chicken β-actin promoter coupled with the cytomegalovirus (CMV) immediate early enhancer, were used (29).

RFP mice. Transgenic nude mice, expressing RFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer, were used (30).

Animal care. Transgenic nude mice expressing CFP or RFP were bred and maintained in a HEPA-filtered environment at AntiCancer, Inc. (San Diego, CA, USA) with cages, food and bedding sterilized by autoclaving. The animal diets were obtained from Harlan Teklad (Madison, WI, USA). Ampicillin (5.0%, w/v; Sigma, St. Louis, MO, USA) was added to the autoclaved drinking water. All surgical procedures and imaging were performed with the animal anesthetized by intramuscular injection of 0.02 ml of a solution of 50% ketamine, 38% xylazine and 12% acepromazine maleate. 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 PHS Assurance number A3873-01.

Subcutaneous cancer cell implantation. Mice were anesthetized as described above. HCT-116-GFP or HCT-116-RFP cells (1×10⁶) were injected subcutaneously with a 1 ml 27G/2 latex-free syringe (BD Biosciences, Bedford, MA, USA).

Two-color stromal model. Mice were anesthetized as described above. HCT-116-GFP colon cancer cells (1×10⁶) were injected subcutaneously in RFP nude mice and allowed to grow for 14 days. The tumors were excised from the RFP mice, cut into 3 mm cubes, and transplanted subcutaneously into CFP nude mice.

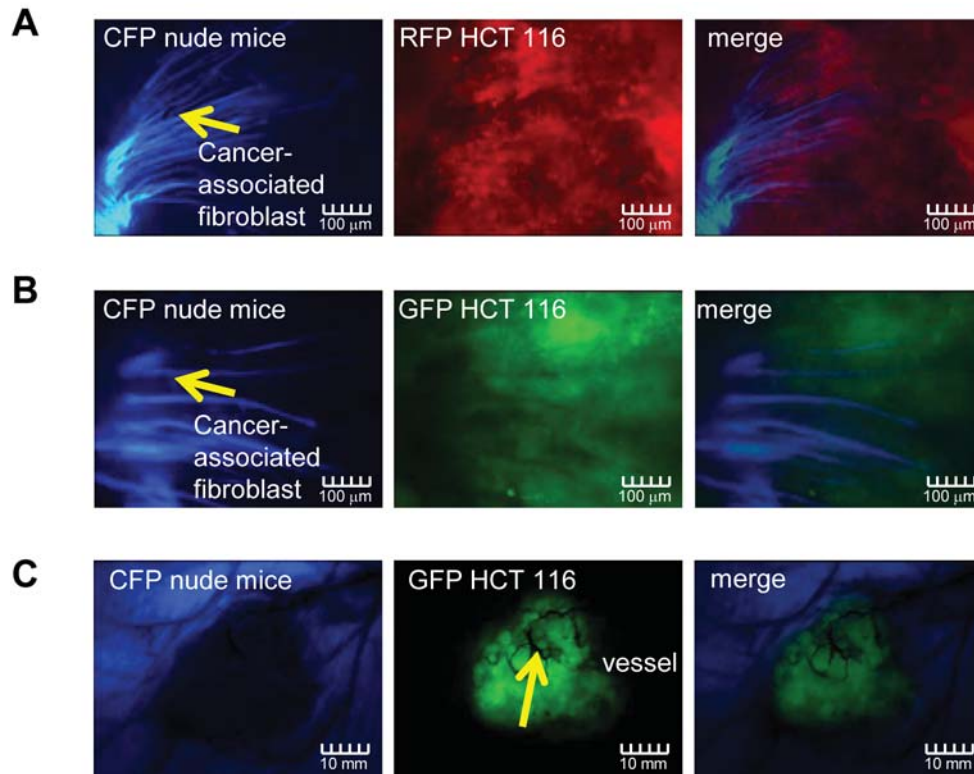


Figure 1. A: HCT-116-RFP human colon cancer growing subcutaneously in the cyan fluorescent protein (CFP) nude mouse. The panel is a high-magnification image obtained with the MVX10 fluorescence microscope. The panel represents a cross-section of the subcutaneous tumor mass. Cancer-associated fibroblasts (CAFs) from the host CFP mouse are seen invading the tumor mass (yellow arrow). Many CAFs were observed in the tumor. (Bar=100 μ m). B: HCT-116-GFP human colon cancer expressing GFP in a CFP nude mouse. The panel represents a cross-section of subcutaneous tumor mass, taken with the MVX10 fluorescence microscope at high magnification. CAFs from the host CFP mouse are seen invading the tumor mass (yellow arrow). Many CAFs were observed in the tumor. (Bar=100 μ m). C: HCT-116-GFP human colon cancer growing subcutaneously in a CFP nude mouse. Yellow arrow shows neovascularization. (Bar=10 mm).

Transplantation of cancer cells in the spleen. Six-week-old CFP nude mice were used as the host for HCT-116 RFP cells or dual-color mouse mammary tumor (MMT) cells expressing GFP in the nucleus and RFP in the cytoplasm. HCT-116 RFP cells or dual-color MMT cells were first harvested by trypsinization and washed three times with cold serum-free medium, then resuspended with serum-free RPMI medium 1640. CFP nude mice were anesthetized as described above. The cancer cells (2×10^6) were then injected into the spleen of CFP nude mice with a 1 ml 27G1/2 latex free syringe (BD Biosciences) during open laparotomy. The cancer cells subsequently formed liver metastases.

Tail-vein injection of cancer cells. Six-week-old CFP nude mice were used. HCT-116 RFP cells (1×10^6) were injected into the tail vein of CFP nude mice, where they subsequently formed lung metastases.

Animal imaging. Imaging was performed using the long-working-distance MVX10 *in vivo* fluorescence microscope with high numerical aperture objectives for variable magnification imaging in live mice from macro- to subcellular (Olympus Corp., Tokyo, Japan) (31) or a FV1000 confocal fluorescence microscope (Olympus Corp.) (32).

Results and Discussion

Visualizing the subcutaneous TME with RFP- or GFP-expressing cancer cells in the CFP nude mouse. HCT-116 human colon cancer cells expressing RFP (HCT-116-RFP) were implanted subcutaneously in CFP-expressing nude mice. Tumor growth was followed over 14 days. The long working distance MVX10 fluorescence microscope acquired images of aggressive fibroblast migration to the tumor site as a host reaction. Two-color fluorescence microscopy visualized the tumor host reaction clearly. Cross-sectional imaging of the deeper tumor tissue showed that the tumor mass (red fluorescence) was surrounded by host elements (blue fluorescence) (Figure 1A).

HCT-116-GFP cancer cells were also implanted subcutaneously in CFP-expressing nude mice, and tumor growth was followed for 14 days. The advantage of the GFP-CFP two-color model is that the neovascularization of the tumor mass was clearly visualized with the MVX10

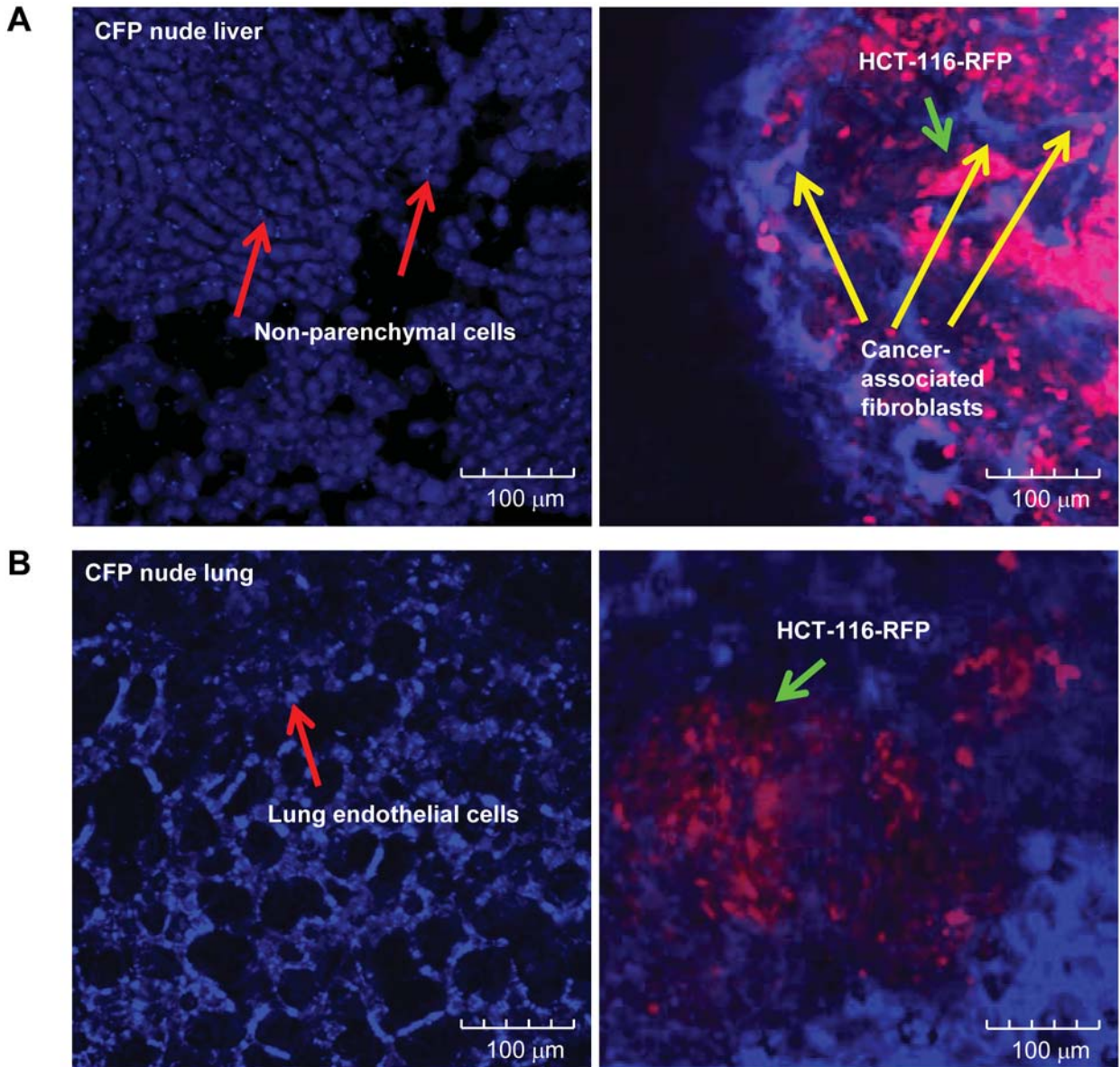


Figure 2. A: Liver metastasis of HCT-116-RFP cancer cells after splenic injection. Non-parenchymal liver cells have strong cyan fluorescent protein (CFP) fluorescence (red arrows). CFP hepatocytes had weak CFP fluorescence. However, the hepatocyte nucleus has bright CFP fluorescence. The left panel is an image of the normal liver of the CFP mouse. Red arrows indicate the bright CFP non-parenchymal liver cells. The right panel is an image of the liver metastasis of HCT-116-RFP cancer cells formed after splenic injection. Cancer-associated fibroblasts (CAFs) (yellow arrows) are seen invading the tumor mass formed by the HCT-116-RFP cancer cells (green arrow). (Bar=100 μ m). B: Image of the lung metastasis of HCT-116-RFP cancer cells formed after tail vein injection. The left panel is an image of a normal CFP mouse lung. The red arrow indicates the bright CFP lung endothelial cells. The right panel is an image of the lung metastasis of HCT-116-RFP cancer cells formed after tail vein injection (green arrows). All images were acquired with an FV1000 confocal microscope (Olympus Corp.). (Bar=100 μ m).

microscope (Figure 1C). When the tumor tissue was cross-sectioned to investigated deeply into the tumor tissue, CFP-expressing CAFs that invaded the tumor mass were clearly visualized with the MVX10 microscope (Figure 1B). The relationship between the tumor tissue and the invading fibroblasts was imaged by color-coded fluorescence imaging.

Visualizing the TME of liver and lung metastasis with RFP cancer cells in the CFP nude mouse. HCT-116-RFP cancer cells were implanted in the spleen of CFP-expressing nude mice. Metastatic colonies were subsequently found in the liver (Figure 2A). When HCT-116-RFP cancer cells were transplanted in the tail vein of CFP nude mice, they formed

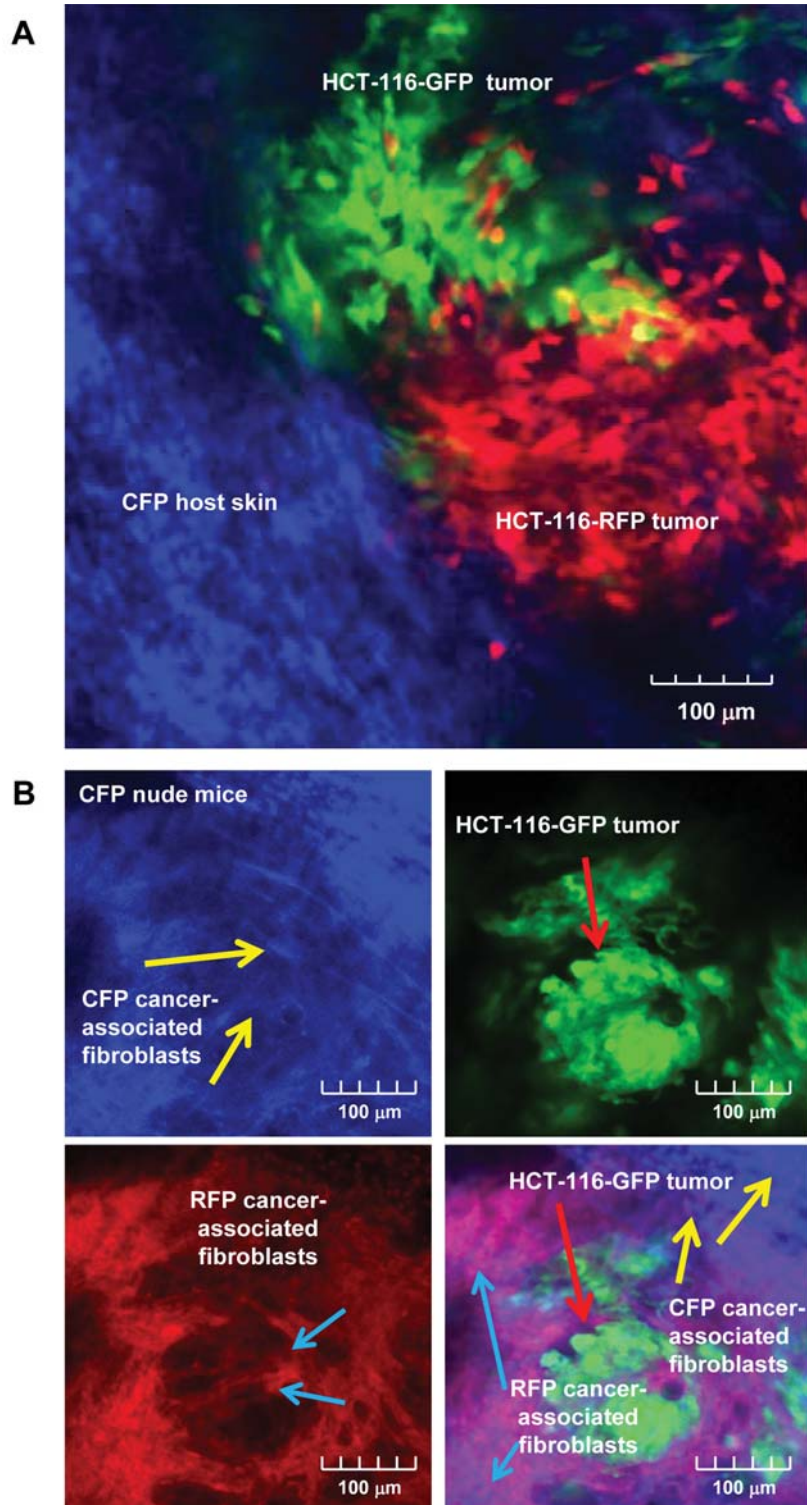


Figure 3. A: HCT-116 human colon cancer cells expressing either RFP or GFP were co-injected subcutaneously in a CFP-expressing nude mouse. The red and green fluorescence of the tumor mass and the blue fluorescence of the host tissue were observed. The image was obtained with an FV1000 confocal microscope. (Bar=100 μ m). B: HCT-116-GFP human colon cancer cells were implanted in an RFP mouse. The tumor was harvested after 21 days and implanted in a CFP nude mouse. RFP-expressing stroma cells (blue arrows) derived from the RFP nude mouse, and CFP stromal cells (yellow arrows) were observed in the GFP-expressing tumor (red arrows). The images were obtained with an FV1000 confocal microscope. (Bar=100 μ m).

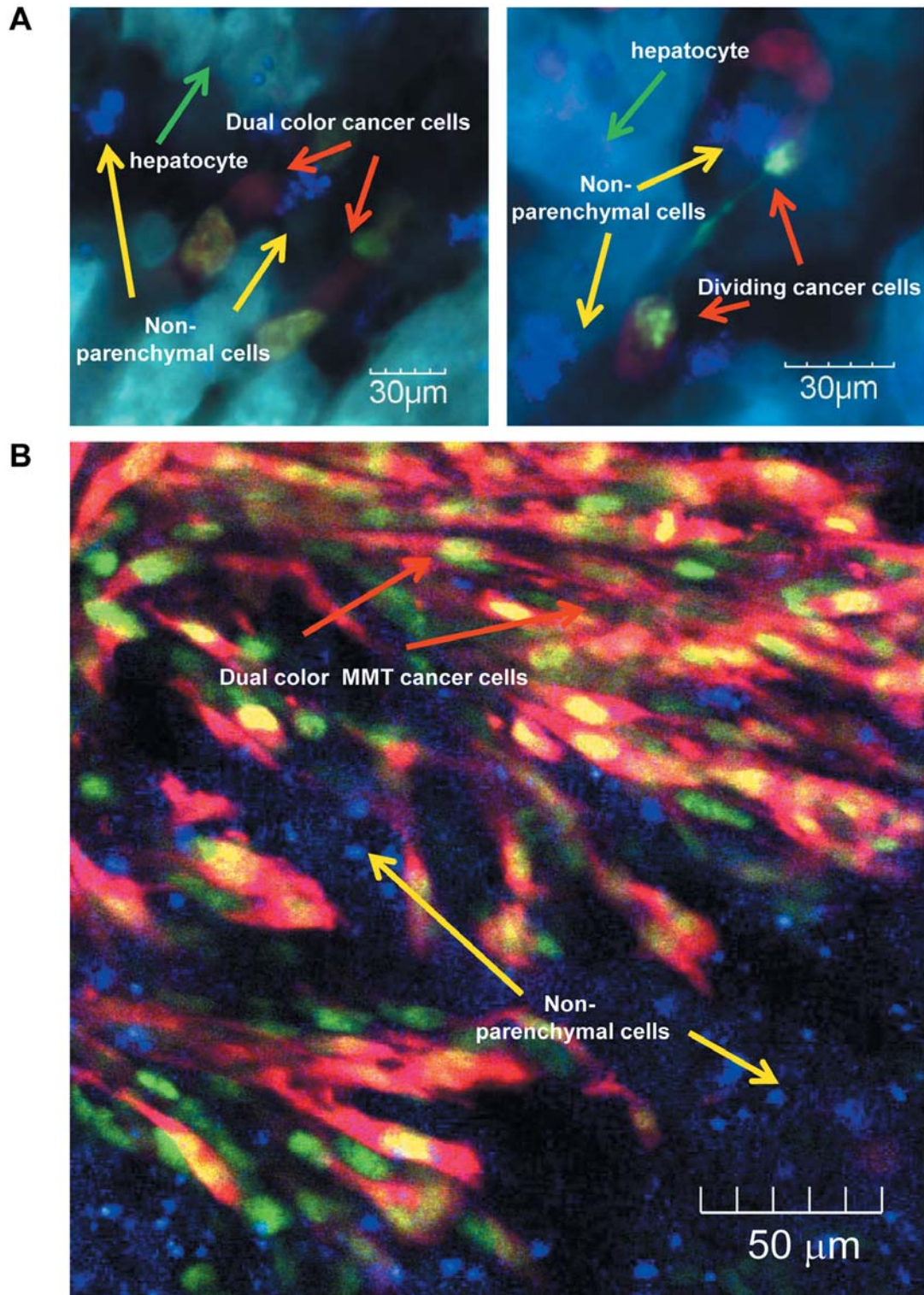


Figure 4. Dual-color MMT cells with GFP in the cytoplasm and RFP in the nucleus, growing in the liver of a CFP nude mouse after splenic injection. A: Dual-color MMT cells (red arrows) were observed in a sinusoid of the liver 3 days after splenic injection. Non-parenchymal liver cells had strong CFP fluorescence (yellow arrows). Hepatocytes had weaker CFP fluorescence (green arrows). The image was obtained with an FV1000 confocal microscope. (Bar=30 μ m). B: Dual-color MMT cells formed tumors in the liver of a CFP mouse 28 days after splenic injection. Hepatocytes, non-parenchymal liver cells (yellow arrows) and dual-color MMT cancer cells (red arrows) were visualized simultaneously. The image was taken with an FV100 confocal microscope. (Bar=50 μ m).

tumors in the lung (Figure 2B). In both liver and lung, CFP-expressing CAFs were visualized by confocal fluorescence imaging, invading the RFP tumor mass as a host reaction. Two-color fluorescence imaging with the Olympus FV1000 confocal microscope enabled clear imaging of cancer-cell growth at the metastatic site (red fluorescence) and the host reaction (blue fluorescence) (Figure 2A, B).

Three-color-coded TME imaging model with tumors of different color fluorescence in the CFP nude mouse. HCT-116 human colon cancer cells, expressing RFP or GFP, were simultaneously implanted subcutaneously in CFP-nude mice, and tumor growth was followed for 21 days (Figure 3A). This multicolor model enabled visualization by confocal microscopy of two tumors with different color fluorescence, as well as a detailed visualization of the host reaction (Figure 3A).

Three-color-coded TME imaging model with stroma of different color fluorescence in the CFP nude mouse. HCT-116-GFP human colon cancer cells were implanted subcutaneously in RFP nude mice. Tumor growth was followed for 14 days, at which point the tumor was excised from the mouse. The tumor contained HCT-116-GFP cancer cells and RFP-expressing stroma derived from the RFP nude mouse (Figure 3B). The dual-color tumor was then implanted in a CFP-nude mouse. With this model, it was possible to visualize two types of stromal reaction, expressing RFP or CFP in the TME using confocal microscopy (Figure 3B).

Four-color-coded TME imaging model in the liver of the CFP mouse. A four-color TME model was established. Dual-color MMT cells with GFP in the nucleus and RFP in the cytoplasm were imaged by confocal fluorescence microscopy in the sinusoids of the CFP liver 3 days after splenic injection (Figure 4A). Non-parenchymal liver cells had very bright CFP fluorescence. Hepatocytes had distinctly lighter CFP fluorescence and were readily distinguishable from non-parenchymal liver cells. Mitotic and dividing MMT-GFP-RFP cells were observed surrounded by non-parenchymal liver cells and hepatocytes (Figure 4B).

In this report, we describe six multicolor models to visualize the TME. Cancer cells expressing RFP and/or GFP were implanted in nude mice expressing CFP. The CFP nude mouse adds another color to the pallet to study the TME, allowing multiple types of color-coded cells to be imaged simultaneously. These multicolor models provide new opportunities to study the cellular interactions in the living TME using the powerful imaging tools of fluorescent proteins and confocal microscopy (22-26, 28, 33-37).

Conflict of Interest

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

Acknowledgements

This study was supported in part by National Cancer Institute grant CA132971.

References

- 1 Finger EC and Giaccia AJ: Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer Metastasis Rev* 29: 285-293, 2010.
- 2 Folkman J: How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial award lecture. *Cancer Res* 46: 467-473, 1986.
- 3 Folkman J: What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 82: 4-6, 1990.
- 4 Kalluri R and Zeisberg M: Fibroblasts in cancer. *Nat Rev Cancer* 6: 392-401, 2006.
- 5 Ostman A and Augsten M: Cancer-associated fibroblasts and tumor growth—bystanders turning into key players. *Curr Opin Genet Dev* 19: 67-73, 2009.
- 6 Anderberg C and Pietras K: On the origin of cancer-associated fibroblasts. *Cell Cycle* 8: 1461-1462, 2009.
- 7 Pietras K and Östman A: Hallmarks of cancer: Interactions with the tumor stroma. *Exp Cell Res* 316: 1324-1331, 2010.
- 8 Nyberg P, Salo T and Kalluri R: Tumor microenvironment and angiogenesis. *Front Biosci* 13: 6537-6553, 2008.
- 9 De Wever O, Demetter P, Mareel M, Bracke M: Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* 123: 2229-2238, 2008.
- 10 Räsänen K and Vaheri A: Activation of fibroblasts in cancer stroma. *Exp. Cell Res* 316: 2713-2722, 2010.
- 11 Lewis CE and Pollard JW: Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 66: 605-612, 2006.
- 12 Kitadai Y: Angiogenesis and lymphangiogenesis of gastric cancer. *J Oncol* 2010: 468725, 2010.
- 13 Condeelis J, Segall JE: Intravital imaging of cell movement in tumours. *Nat Rev Cancer* 3: 921-930, 2003.
- 14 Bouvet M, Tsuji K, Yang M, Jiang P, Moossa AR and Hoffman RM: *In vivo* color-coded imaging of the interaction of colon cancer cells and splenocytes in the formation of liver metastases. *Cancer Res* 66: 11293-11297, 2006.
- 15 Duda DG, Duyverman AM, Kohno M, Snuderl M, Steller EJ, Fukumura D and Jain RK: Malignant cells facilitate lung metastasis by bringing their own soil. *Proc Natl Acad Sci USA* 107: 21677-21682, 2010.
- 16 Okabe M, Ikawa M, Kominami K, Nakanishi T and Nishimune Y: 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 407: 313-319, 1997.
- 17 Vintersten K, Monetti C, Gertsenstein M, Zhang P, Laszlo L, Biechele S and Nagy A: Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 40: 241-246, 2004.
- 18 Hadjantonakis AK, Macmaster S and Nagy A: Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal. *BMC Biotechnol* 2: 11, 2002.
- 19 Tran Cao HS, Kimura H, Kaushal S, Snyder CS, Reynoso J, Hoffman RM and Bouvet M: The cyan fluorescent protein (CFP) transgenic mouse as a model for imaging pancreatic exocrine cells. *J Pancreas* 10: 152-156, 2009.

- 20 Suetsugu A, Osawa Y, Nagaki M, Saji S, Moriwaki H, Bouvet M and Hoffman RM: Imaging the recruitment of cancer-associated fibroblasts by liver-metastatic colon cancer. *J Cell Biochem* 112: 949-953, 2011.
- 21 Egeblad M, Nakasone ES and Werb Z: Tumors as organs: complex tissues that interface with the entire organism. *Dev Cell* 18: 884-901, 2010.
- 22 Tsuji K, Yamauchi K, Yang M, Jiang P, Bouvet M, Endo H, Kanai Y, Yamashita K, Moossa AR and Hoffman RM: Dual-color imaging of nuclear-cytoplasmic dynamics, viability, and proliferation of cancer cells in the portal vein area. *Cancer Res* 66: 303-306, 2006.
- 23 McElroy M, Kaushal S, Bouvet M and Hoffman RM: Color-coded imaging of splenocyte-pancreatic cancer cell interactions in the tumor microenvironment. *Cell Cycle* 7: 2916-2921, 2008.
- 24 Kishimoto H, Aki R, Urata Y, Bouvet M, Momiyama M, Tanaka N., Fujiwara T and Hoffman RM: Tumor-selective adenoviral-mediated GFP genetic labeling of human cancer in the live mouse reports future recurrence after resection. *Cell Cycle* 10: 2737-2741, 2011.
- 25 Yamauchi K, Yang M, Hayashi K, Jiang P, Yamamoto N, Tsuchiya H, Tomita K, Moossa AR, Bouvet M and Hoffman RM: Imaging of nucleolar dynamics during the cell cycle of cancer cells in live mice. *Cell Cycle* 6: 2706-2708, 2007.
- 26 Hoffman RM and Yang M: Subcellular imaging in the live mouse. *Nat Protocols* 1: 775-782, 2006.
- 27 Yamamoto N, Jiang P, Yang M, Xu M, Yamauchi K, Tsuchiya H, Tomita K, Wahl GM, Moossa AR and Hoffman RM: Cellular dynamics visualized in live cells *in vitro* and *in vivo* by differential dual-color nuclear-cytoplasmic fluorescent-protein expression. *Cancer Res* 64: 4251-4256, 2004.
- 28 Glinsky GV, Glinskii AB, Berezovskaya O, Smith BA, Jiang P, Li X-M, Yang M and Hoffman RM: Dual-color-coded imaging of viable circulating prostate carcinoma cells reveals genetic exchange between tumor cells *in vivo*, contributing to highly metastatic phenotypes. *Cell Cycle* 5: 191-197, 2006.
- 29 Tran Cao HS, Reynoso J, Yang M, Kimura H, Kaushal S, Snyder CS, Hoffman RM and Bouvet M: Development of the transgenic cyan fluorescent protein (CFP)-expressing nude mouse for 'Technicolor' cancer imaging. *J Cell Biochem* 107: 328-334, 2009.
- 30 Yang M, Reynoso J, Bouvet M and Hoffman RM: A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. *J Cell Biochem* 106: 279-284, 2009.
- 31 Kimura H, Momiyama M, Tomita K, Tsuchiya H and Hoffman RM: Long-working-distance fluorescence microscope with high-numerical-aperture objectives for variable-magnification imaging in live mice from macro- to subcellular. *J Biomed Optics* 15: 066029, 2010.
- 32 Uchugonova A, Duong J, Zhang N, Koenig K and Hoffman RM: The bulge area is the origin of nestin-expressing pluripotent stem cells of the hair follicle. *J Cell Biochem* 112: 2046-2050, 2011.
- 33 Jiang P, Yamauchi K, Yang M, Tsuji K, Xu M, Maitra A, Bouvet M, Hoffman RM: Tumor cells genetically labeled with GFP in the nucleus and RFP in the cytoplasm for imaging cellular dynamics. *Cell Cycle* 5: 1198-1201, 2006.
- 34 Amoh Y, Li L, Katsuoka K, Hoffman RM: Multipotent hair follicle stem cells promote repair of spinal cord injury and recovery of walking function. *Cell Cycle* 7: 1865-1869, 2008.
- 35 Hoffman RM: The multiple uses of fluorescent proteins to visualize cancer *in vivo*. *Nat Rev Cancer* 5: 796-806, 2005.
- 36 Hoffman RM, Yang M: Color-coded fluorescence imaging of tumor-host interactions. *Nat Protocols* 1: 928-935, 2006.
- 37 Hoffman RM, Yang M: Whole-body imaging with fluorescent proteins. *Nat Protocols* 1: 1429-1438, 2006.

Received October 15, 2011

Revised November 22, 2011

Accepted November 23, 2011