

Multi-color fluorescence imaging of sub-cellular dynamics of cancer cells in live mice

Robert M. Hoffman*

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

Department of Surgery, University of California, 200 West Arbor Drive, San Diego, CA 92103

ABSTRACT

We have genetically engineered dual-color fluorescent cells with one color in the nucleus and the other in the cytoplasm that enables real-time nuclear-cytoplasmic dynamics to be visualized in living cells in the cytoplasm *in vivo* as well as *in vitro*. To obtain the dual-color cells, red fluorescent protein (RFP) was expressed of the cancer cells, and green fluorescent protein (GFP) linked to histone H2B was expressed in the nucleus. Mitotic cells were visualized by whole-body imaging after injection in the mouse ear. Common carotid artery or heart injection of dual-color cells and a reversible skin flap enabled the external visualization of the dual-color cells in microvessels in the mouse where extreme elongation of the cell body as well as the nucleus occurred. The migration velocities of the dual-color cancer cells in the capillaries were measured by capturing individual images of the dual-color fluorescent cells over time. Human HCT-116-GFP-RFP colon cancer and mouse mammary tumor (MMT)-GFP-RFP cells were injected in the portal vein of nude mice. Extensive clasmocytosis (destruction of the cytoplasm) of the HCT-116-GFP-RFP cells occurred within 6 hours. The data suggest rapid death of HCT-116-GFP-RFP cells in the portal vein. In contrast, MMT-GFP-RFP cells injected into the portal vein mostly survived and formed colonies in the liver. However, when the host mice were pretreated with cyclophosphamide, the HCT-116-GFP-RFP cells also survived and formed colonies in the liver after portal vein injection. These results suggest that a cyclophosphamide-sensitive host cellular system attacked the HCT-116-GFP-RFP cells but could not effectively kill the MMT-GFP-RFP cells. With the ability to continuously image cancer cells at the subcellular level in the live animal, our understanding of the complex steps of metastasis will significantly increase. In addition, new drugs can be developed to target these newly visible steps of metastasis.

Keywords: Green fluorescent protein; red fluorescent protein; cancer cells; *in vivo*; subcellular imaging

1. INTRODUCTION

Our laboratory introduced the use of GFP for *in vivo* imaging in 1997. With the use of GFP, individual cancer cells could be observed in fresh unstained tissue for the first time (1). In 2000, we demonstrated the first whole-body imaging using GFP (2).

In 2004, we developed cancer cells that were double labeled with GFP in the nucleus and RFP in the cytoplasm (3). With the double-labeled cells, mitotic and apoptotic cells were imaged in live animals. We also imaged cancer cells that underwent cytoplasmic and nuclear deformation in small capillaries in the brain in the live animal (3).

In 2005, using the dual-color cancer cells, we imaged and quantified the maximum nuclear and cytoplasmic deformation of cancer cells in small capillaries after injection in the heart. We observed that the whole cancer cell could stretch to approximately 4 times its original length to fit in a small capillary, while the nucleus could stretch only to approximately 1.5 times its original length. After injection of dual-color cancer cells, the velocity of cancer cell movement was roughly estimated by acquiring an image of the cancer cells in small capillaries at one time-point and then imaging them in the same capillary two hours later (4). We described real-time imaging of the deformation of cancer cells and their nuclei *in*

* Email: all@anticancer.com; phone 858-654-2555; fax 858-268-4175; www.anticancer.com

vivo. In addition to the deformability, we imaged migration of HT-1080-dual-color cells in microvessels and capillaries in real time (4).

We also visualized the early trafficking of dual-colored cancer cells injected into the portal vein of nude mice. We imaged the fate of different cancer cell types in the portal circulation (5).

With the ability to image cancer cells at the subcellular level in the live animal, our understanding of the complex steps of metastasis will significantly increase. In addition, new drugs can be developed to target these newly visible steps of metastasis.

2. MATERIALS AND METHODS

2.1 Production of RFP retroviral vector

For RFP retrovirus production, the *Hind* III/*Not*I fragment from pDsRed2 (Clontech Laboratories, Inc., Palo Alto, CA), containing the full-length RFP cDNA, was inserted into the *Hind* III/*Not*I site of pLNCX2 (Clontech Laboratories, Inc.) that has the neomycin resistance gene to establish the pLNCX2-DsRed2 plasmid. PT67, an NIH3T3-derived packaging cell line (Clontech Laboratories, Inc.) expressing the 10 A1 viral envelope, was cultured in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-products, Calabasas, CA). For vector production, PT67 cells, 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 post-transduction. For selection of a clone producing high amounts of a RFP retroviral vector (PT67-DsRed2), the cells were cultured in the presence of 200–1000 µg/ml G418 (Life Technologies, Inc.) for 7 days (3).

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 *A. victoria EGFP* gene (Clontech Laboratories, Inc.). The histone H2B-GFP fusion gene was then inserted at the *Hind*III/*Cla*I site of the pLHCX (Clontech Laboratories, Inc.) that has 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 PT67 cells using the same methods described above for PT67-DsRed2. The transfected cells were cultured in the presence of 200–400 µg/ml hygromycin (Life Technologies, Inc.) for 15 days to establish stable PT67 H2B-GFP packaging cells (3).

2.3 RFP and histone H2B-GFP gene transduction of cancer cells

For RFP and H2B-GFP gene transduction, 70% confluent human cancer cells were used. To establish dual-color cells, clones of cancer cells expressing RFP in the cytoplasm (HT-1080-RFP) were initially established. In brief, The cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI 1640 (Mediatech, Inc., Herndon, VA) containing 10% FBS for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h post-transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 µg/ml G418. The level of G418 was increased stepwise up to 800 µg/ml. HT-1080-RFP cells were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) using trypsin/EDTA and amplified by conventional culture methods (3).

For establishing dual-color cancer cells, they 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. Dual-color cancer cells were isolated with cloning cylinders under fluorescence microscopy. These two clones were amplified by conventional culture methods. These sublines stably expressed GFP in the nucleus and RFP in cytoplasm (3).

2.4 Real-time visualization of deformability of dual-color cancer cells in brain microvessels of live mice

To visualize cell dynamics in the brain of living mice, cells were injected in the common carotid artery. Nude mice were anesthetized with a ketamine mixture (10 µl ketamine HCl, 7.6 µl xylazine, 2.4 µl acepromazine maleate, and 10 µl H₂O)

via s.c. injection. A longitudinal skin incision was made on the neck. After exposing the submandibular gland, it was cut in the middle and retracted to each side. The right sternohyoid muscle and right sternomastoideus muscle and connective tissue were separated with a blunt instrument. After isolation of the right common carotid artery, the artery was gently released from surrounding connective tissue. Light tension was put on the proximal site of the artery with a blunt-end hook (Fine Science Tools, Inc., Foster City, CA). A total of 200 μ l of medium containing 2×10^5 HT-1080-dual-color-1 cells were injected in the artery using a 33-gauge needle (Fine Science Tools). Immediately after injection, the injected site was pressed with a swab to prevent bleeding or leakage of injected tumor cells. The skin was then closed with a 6-0 suture. All procedures of the operation described above were performed with a $\times 7$ dissection microscope (MZ6; Leica, Deerfield, IL) (3).

Tumor cells in the brain were visualized through the skull via a skin-flap window. The animals were anesthetized with the ketamine mixture. An arc-shaped incision was made in the scalp, and s.c. connective tissue was separated to free the skin flap. The skin flap could be opened repeatedly to image tumor cells in the brain through the nearly transparent mouse skull and simply closed with a 6-0 suture. This procedure greatly reduced the scatter of fluorescent photons (3).

2.5 Real-time visualization of deformation of dual-color cancer cells in abdominal vessels in live mice

To visualize cell dynamics in vessels in live mice, cells were injected into the heart. Five nude mice were anesthetized with a ketamine mixture (10 μ L ketamine HCl, 7.6 μ L xylazine, 2.4 μ L acepromazine maleate, and 10 μ L H₂O) via s.c. injection. A total of 200 μ L of medium containing 5×10^6 HT-1080-dual-color cells were injected into the heart. To observe the shapes of the dual-color cancer cells within the microvessels before arrest, the epigastric cranialis vein of the mouse was wired with a 6-0 suture (Ethicon Inc., Somerville, NJ) before cell injection. Immediately after injection, an arc-shaped incision was made in the abdominal skin, and then s.c. connective tissue was separated to free the skin flap without injuring the epigastric cranialis artery and vein. The skin flap was spread and fixed on the flat stand. The inside surface of the skin flap was directly observed under fluorescence microscopy. After making the skin flap, HT-1080-dual-color cells were imaged immediately and 2 hours later to determine the migration velocities of the cells. During the interval between imaging, PBS (Irvine Scientific) was occasionally sprayed on the inside of the skin flap to keep the surface wet. The skin flap could be completely reversed (3).

2.6 Morphologic analysis of cancer cell deformation

For morphologic analysis, we examined cells in microvessels and capillaries in mice. During the period of the measurement, the animals were kept under anesthesia. The animals were kept warm and the skin flap was kept hydrated with saline solution. Images were taken at the initial time. The image included the cell in its vessel as well as the surrounding vessels which were used as a map to relocate the cell in its vessel 2 hours later when the next images were captured. Usually, 10 to 20 cells were followed in a given experiment. The lengths of the major and minor axes of the whole cells and the nuclei were measured using Image ProPlus 3.1 software, where (A) was the length of the major axis and (B) was the length of the minor axis of the whole cell. For the nuclei, (a) is the length of the major axis and (b) is the length of the minor axis (4).

2.7 Motility analysis *in vivo*

For motility analysis, the epigastric cranialis vein was not wired. All other procedures were the same as for morphologic analyses. Motility analysis was done using Image ProPlus 3.1 software. Images that were taken immediately after injection and 2 hours later were used to determine migration velocities of the dual-color cancer cells. Fluorescent optical imaging and data analysis. Images were captured directly with a Hamamatsu C5810 3CCD camera (Hamamatsu Photonics, Bridgewater, NJ). For microimaging, a Leica fluorescent stereo microscope (model MZ16) was coupled with the Hamamatsu camera. High-resolution images (1024 \times 724 pixels) were captured directly on an IBM PC. Images were processed for contrast and brightness and analyzed with the use of Image ProPlus 3.1 software (4).

2.8 Whole-body real-time visualization of mitosis

To visualize mitosis by whole-body imaging, mice were anesthetized with the ketamine mixture, and HT-1080 dual-color cells were injected in the ear of the mouse. The surface of the ear of the intact animal was directly observed under fluorescence microscopy (3).

2.9 Injection of cells in the portal vein

The nude mice were anesthetized with the ketamine mixture injected into the peritoneal cavity. Human HCT-116-GFP-RFP colon cancer cells and mouse mammary tumor (MMT)-GFP-RFP cells ($0.125-1.0 \times 10^6$ cells/50 μ L) were injected in the portal vein of nude mice during open laparotomy (5).

2.10 Pretreatment of animals with cyclophosphamide

In some experiments, the host mice were pretreated by portal vein injection with cyclophosphamide (100 mg/kg) 6 days and 1 day before injection of HCT-116-GFP-RFP cells into the portal vein (5).

2.11 Visualization of cell trafficking in liver of living mice

At laparotomy, a glass slide was put on the exteriorized liver of the mice to regulate motion. The mice were observed with an Olympus OV100 whole-mouse imaging system. The images were taken immediately after cell injection, and 0.5, 1, 6, 12, and 24 hours, and daily thereafter (5).

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 of 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).

3. RESULTS

3.1 Cell proliferation rates of parental, HT-1080-RFP, and HT-1080-RFP-GFP dual-color human fibrosarcoma cells

The selected HT-1080-dualcolor cells have bright GFP and RFP fluorescence *in vitro*. Green fluorescence is localized in the nuclei; red fluorescence is localized in the cytoplasm. All cells in the population expressed both GFP and RFP, indicating stability of both transgenes. There was no difference in the proliferation rates of parental HT-1080, HT-1080-RFP, HT-1080-dual-color-1, or HT-1080-dual-color-6 clones determined in monolayer culture, indicating that expression of GFP and/or RFP did not affect cell cycle progression (3).

3.2 Whole-body real-time imaging of cancer cell mitosis

Real-time images of mitotic cells could be captured in the ear of a live mouse 12 h after the injection of HT-1080-dual-color-1 cells. The cells appeared to be extravasated and were rounded, similar to dividing cells in culture. The shape of each nucleus, the high nuclear-cytoplasmic ratio, and the boundary of the cells were clearly visualized by dual-color whole-body imaging in the living animal (3).

3.3 Real-time observation of cancer cell nuclear cytoplasmic deformability in the brain

After common carotid-artery injection of HT-1080-dual-color cells, they were visualized through the skull via a scalp flap in a brain microvessel. The cell body and nucleus of the dual-color tumor cells were greatly elongated to fit in small diameter microvessels, showing the extreme deformability of both the nucleus and cytoplasm (3).

3.4 Real-time observations of cancer cell nuclear-cytoplasmic deformability in capillaries

The cells were injected into the heart of mice whose epigastric cranialis vein was wired to close it off. The skin flap was made without injuring the epigastric cranialis artery and vein, and then spread and fixed on a stand. Because of this stable fixation, thousands of cells could be easily visualized in capillaries and in microvessels in the skin in the live mouse under fluorescence microscopy. The wired epigastric cranialis vein was clogged with round cells. Some cells were trapped due to size restriction in capillaries, where the cells and nuclei were highly deformed (4).

We classified the shapes of the cells into four categories. One included the cells in microvessels, where the cells were spherical and the nuclei were oval. Another category included the cells that were elongated in capillaries, where the nuclei were also elongated. Another category was that of cells that were elongated and bifurcated at the corner of the capillaries. The other category was that of cells that were so elongated that the cytoplasm disconnected. The nuclei, however, remained intact although they were very elongated (4).

3.5 Quantitation of cancer cell and nuclear deformation

The lengths of the major and minor axes of the whole cells and the nuclei were measured. The average lengths of the axes of the whole cells in capillaries were 3.97 times as long as in microvessels. As for the nuclei, the average lengths were 1.64 times as long. The lengths of the minor axes of the whole cells in capillaries were equal to those of the nuclei (4).

3.6 Real-time observation of cancer cell nuclear-cytoplasmic clasmatosis

Clasmatosis or cytoplasmic fragmentation was observed in the cells immediately after injection (13). The cytoplasm was removed from many cells leaving naked nuclei. Nuclei were also fragmented or stretched dramatically (5).

3.7 Motility analysis in capillaries

Two hours after taking the first images, the same cells were imaged again to determine the migration velocities of the cells. The bifurcated corners of the capillaries were used as markers to calculate the distance migrated. The distances from the marker to the centers of the nuclei were measured and subtracted. Approximately 25% of the cells observed bifurcated at the corner of the capillaries and could not migrate and were therefore excluded from our calculations. Of the 45 cells that were not at the corner of the capillaries, 20 cells migrated and 25 cells did not migrate. The average migration velocity of the 20 cells that migrated was 13.2 $\mu\text{m}/\text{hour}$ (1.1-48.3). A 1 μm difference in distance could be distinguished from the captured images of the dual-color fluorescent cells (4).

The diameters of the capillaries were noted when calculating migration velocity. The minimum diameter of the capillary that allowed cell migration was approximately 8 μm . Sixteen out of 20 cells in capillaries over 8 μm in diameter migrated up to 48.3 $\mu\text{m}/\text{hour}$. In contrast, 21 out of 25 cells in capillaries less than 8 μm in diameter could not migrate at all (4).

3.8 Fate of human HCT-116-GFP-RFP colon cancer cells in the portal vein

After the HCT-116-GFP-RFP human colon cancer cells were injected into the main portal vein, cells accumulated in the terminal portal veins. Initially, many HCT-116-GFP-RFP cells impacted into sinusoids just after injection. Most HCT-116-GFP-RFP cells remained in the sinusoids near the terminal portal vein. Thirty minutes after injection of HCT-116-GFP-RFP in the portal vein, 70% of the cells were viable. However, by 2 hours, only 50% of the cells were viable, and by 6 hours, only 10% of the cells were viable. Viability was readily observed as the dead cells were stripped of their RFP-expressing cytoplasm leaving only the GFP nucleus. The number of apoptotic cells rapidly increased within the portal vein within 12 hours of injection. Fragmentation of GFP nuclei could be clearly visualized (5).

3.9 Fate of mouse MMT-GFP-RFP mammary cells in the portal vein

MMT-GFP-RFP mouse mammary tumor cells injected into the portal vein had a very different fate in the liver compared with HCT-116-GFP-RFP cells. Although there was rapid cell destruction of some MMT-GFP-RFP cells similar to HCT-116-GFP-RFP cells, many MMT-GFP-RFP cells survived to 24 hours and beyond after injection. Many MMT-GFP-RFP cells became invasive with cytoplasmic protrusions within 48 hours after injection. MMT-GFP-RFP cells subsequently formed micrometastasis in the liver from days 2 to 5 (5).

3.10 Efficacy of cyclophosphamide pretreatment on portal vein viability of HCT-116-GFP-RFP cells

When the host mice were pretreated with cyclophosphamide, the HCT-116-GFP-RFP cells also survived and formed colonies in the liver after portal vein injection, in striking contrast to their rapid cell death in the untreated animals. These results suggest that a host cellular system attacked the HCT-116-GFP-RFP cells. Most HCT-116-GFP-RFP cells survived 24 hours after injection in the cyclophosphamide-treated mice and subsequently formed metastasis in the liver in three of five mice compared with none of the non-cyclophosphamide-treated mice. Some of the HCT-116-GFP-RFP micrometastases became vascularized by day 14 (5).

4. DISCUSSION

Using the skin flap for observation of cells in capillaries has important advantages. The skin can be spread stably on a stand, such that motion from the mouse's heartbeat or breathing has no influence on imaging. Disturbance of the blood supply for the skin does not occur during the skin flap procedure, because the epigastric cranialis artery is not injured

during the procedure. In addition to these advantages, the skin flap could be completely reversed such that the mice need not be sacrificed. In our study, the skin flap was reversed after 24 hours (4).

To observe the shape of the HT-1080-dual-color cells within blood vessels, we wired the epigastric cranialis vein closed before injection of the cells into the heart. We observed many spherical cancer cells in the microvessels in the skin. As for the cells in capillaries, almost all the cells and their nuclei deformed in order to conform to the diameter of the capillaries. The cancer cells and their nuclei deformed into three-pronged forks when the cells were arrested at capillary bifurcations (4).

The nucleus seems to be less deformable than the cytoplasm. This may be due to the difference between the cytoskeleton in the cytoplasm and the nucleus. In our study, the lengths of the major and minor axes of the nuclei and the whole cells deformed to fit the width of the capillaries (4).

There is an apparent limitation to cell and nuclear deformation when the cells are arrested in capillaries. We found many cancer cells whose cytoplasm seemed to fragment and separate from nuclei, a process called clasmotosis. We also found that nuclei could fragment. When the cells arrested in very narrow capillaries, the cytoplasm of many cells as well as their nuclei were destroyed (4).

Morris et al. (6) previously described clasmotosis of cells in the liver. We could visualize this phenomenon more clearly by using bright dual-color fluorescent cancer cells with the cytoplasm labeled with RFP and the nucleus labeled with GFP. HCT-116-GFP-RFP cells injected into the portal vein were all dead within 12 hours. Rapid cell death was also reported by Morris et al. (6). The liver may contain an innate rapid local defense for cells entering the liver portal veins. Our results suggest that such a host defense is sensitive to cyclophosphamide. However, MMT-GFP-RFP cells had a very different fate compared with HCT-116-GFP-RFP cells. The MMT-GFP-RFP cells survived and formed metastases in the liver of untreated nude mice.

The fate of injected malignant cells in the portal vein seems dependent on the species of origin. Mouse cells go on to form metastatic colonies whereas human colon cancer cells die. The difference is clearly due to the host response because prior treatment of the mice with cyclophosphamide obliterates the inhibitory response to human cells. This finding should be studied further.

Cyclophosphamide is a widely used cancer chemotherapy drug, including low-dose therapy. The results of the present study show that cyclophosphamide might, in certain instances, promote cancer cell growth as shown for the HCT-116 human colorectal cancer cells in the portal vein. Further studies into the sensitivity of host anticancer mechanisms to cyclophosphamide are indicated (5).

Bioluminescence imaging, magnetic resonance imaging, positron emission tomography, and single photon emission computed tomography have allowed detailed and dynamic views of tissues. However, these methods lack the spatial and/or temporal resolution to visualize single-cell dynamics *in situ*. Such information can be generated with fluorescence imaging. Newer techniques such as multiphoton microscopy can be combined with second harmonic generation imaging to visualize cellular behavior in the interstitium of solid organs (7).

For example, acquisition of protease-independent amoeboid dissemination was visualized for HT-1080 cells injected into the mouse dermis monitored by intravital multiphoton time-lapse microscopy (8). The transition from proteolytic mesenchymal toward nonproteolytic amoeboid movement shows a plasticity mechanism in cell migration (8). Such transitions in movement can now be imaged with the dual-colored cells described in the present report in order to visualize the role of the nucleus as well as the cytoplasm.

The dual-color cells, with GFP in the nucleus and RFP in the cytoplasm, along with new approaches to *in vivo* imaging in appropriate hosts provide a powerful tool to understand the mechanism of cancer cell migration and deformation as well as viability in vessels *in vivo*. With this new information, we can better understand the mechanism of metastasis and develop new drugs for these now visible targets.

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