

# Real-time subcellular imaging in live animals: New visible targets for cancer drug discovery

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*This feature describes new in vivo imaging technology to visualize and identify novel targets for cancer drug discovery. AntiCancer Inc has developed dual-color fluorescent cells, with one color in the nucleus and another color in the cytoplasm, that enable visualization of real-time nuclear-cytoplasmic dynamics in living cells in vivo and in vitro, as well as nuclear dynamics and simultaneous cell and nuclear shape change. To obtain the dual-color cells, red fluorescent protein (RFP) was expressed in the cytoplasm of human and rodent cancer cells, and green fluorescent protein (GFP) that was linked to histone H2B was expressed in the nucleus. The migration velocities of the dual-color cancer cells in the capillaries were measured by capturing images of the fluorescent cells over time. The cells and nuclei in the capillaries were shown to elongate to fit the width of these vessels. Cancer cells in capillaries that were more than 8  $\mu\text{m}$  in diameter were found to migrate. During extravasation, real-time imaging demonstrated that cytoplasmic processes of certain cancer cells exited the vessels first, with the nuclei then following along the cytoplasmic projections. Both the cytoplasm and nuclei underwent deformation during extravasation. Different cancer cell lines appear to vary strongly in their ability to extravasate. With the dual-color cancer cells and a highly sensitive whole-mouse imaging system (Olympus OV100), the subcellular dynamics of cancer metastasis can now be visualized in live mice. Further developments in subcellular imaging in live animals is expected to result in a new 'in vivo cell biology' that will provide visible targets for cancer and other diseases.*

**Keywords** Dual-color imaging, extravasation, GFP, green fluorescent protein, nuclear-cytoplasmic dynamics, red fluorescent protein, RFP

## Introduction and background in *in vivo* imaging technology

A technology for whole-body imaging of tumor growth and metastases in mice has been developed using tumor cells that express green fluorescent protein (GFP) and red fluorescent protein (RFP). Fluorescence imaging presents many new possibilities in research, including real-time studies of tumor progression, metastasis and drug-response evaluation. With these fluorescent tools, single cells from

tumors and metastases can be imaged. GFP technology has also been used for the real-time imaging and quantification of angiogenesis.

Initial studies of tumor biology using stable GFP expression focused on metastases by examining static images. The first use of GFP expression to characterize cancer cells *in vivo* was by Chishima *et al* (*Cancer Res* (1997) 57(10):2042-2047), and its first use to observe motility and shape changes of carcinoma cells in intact tumors *in vivo* was described by Farina *et al* (*Cancer Res* (1998) 58(12):2528-2532). Chishima *et al* (*Cancer Res* (1997) 57(10):2042-2047) and Huang *et al* (*Clin Exp Metastasis* (2002) 19(4):359-368) demonstrated that GFP-transduced cancer cells allowed for the imaging of tumor cells in blood vessels. To examine cell behavior during intravasation, Wyckoff *et al* (*Cancer Res* (2000) 60(9):2504-2511) used GFP imaging to view the transduced cells in time-lapse images within a single optical section, using a confocal microscope. *In vivo* imaging of the primary tumors indicated that both metastatic and non-metastatic cells are motile and exhibit protrusive activity. Metastatic cells demonstrated a greater orientation toward blood vessels, while non-metastatic cells fragmented when interacting with vessels.

GFP expression has also revealed other features of cells. Using intravital GFP imaging, Naumov *et al* (*J Cell Sci* (1999) 112(Pt 12):1835-1842) were able to visualize fine cellular details, such as pseudopodial projections, even after extended periods of *in vivo* growth. Mook *et al* (*Hepatology* (2003) 38(2):295-304) observed the initial arrest of GFP colon cancer cells in sinusoids of the liver after portal vein injection, caused by size restriction. Tumor cells were found to divide only intravascularly during the first 4 days of the study. Al-Mehdi *et al* (*Nat Med* (2000) 6(1):100-102) observed the steps in early hematogenous metastasis of tumor cells expressing GFP in subpleural microvessels in intact, perfused mouse and rat lungs. Metastatic tumor cells were found to attach to the endothelia of pulmonary precapillary arterioles and capillaries, while extravasation of tumor cells was rare. Early tumor colonies were observed to grow entirely within the blood vessels.

Employing the technique of GFP expression, metastatic and non-metastatic tumor cells have been determined to differ in movement. Using multiphoton microscopy and GFP labeling, Wang *et al* (*Cancer Res* (2002) 62(23):6278-6288) determined five major differences in carcinoma cell behavior between metastatic and non-metastatic primary breast tumors, involving the extracellular matrix, cell motility and chemotaxis. Goswami *et al* (*Cancer Res* (2005) 65(15):5278-5283) demonstrated that macrophages promote the invasion of GFP-labeled breast cancer cells. However, nuclear-cytoplasmic dynamics could not be visualized in these studies, as the entire cancer cells were labeled with GFP.

This feature discusses the use of dual-color fluorescence imaging in cancer cells, an approach that allows for a greater elucidation of the behavior of these cells.

### Recent promising developments in *in vivo* imaging technology

Yamamoto *et al* (*Cancer Res* (2004) 64(12):4251-4256) reported on the genetic engineering of dual-color fluorescent cells, with one color in the nucleus and another in the cytoplasm. The dual-color cells enable real-time nuclear-cytoplasmic dynamics to be visualized in living cells *in vivo*, as well as *in vitro*. To obtain the dual-color cells, RFP was expressed in the cytoplasm of HT1080 human fibrosarcoma cells, and GFP that was linked to histone H2B was expressed in the nucleus. Nuclear GFP expression enabled a visualization of nuclear dynamics, while the simultaneous cytoplasmic RFP expression enabled a visualization of nuclear-cytoplasmic ratios, as well as of simultaneous cell and nuclear shape changes. Thus, this approach enables the observation of total cellular dynamics in living dual-color cells in real time. When dual-colored cancer cells were used in conjunction with a highly sensitive new whole-mouse macro-micro imaging system (Olympus OV100), real-time dynamic subcellular imaging in live mice became possible (*Cancer Res* (2006) 66(8):4208-4214).

Using the recently developed dual-color imaging technology, new potential visible targets for drug discovery have been identified. Some of these targets are highlighted below.

### Real-time observation of nuclear cytoplasmic deformability in the brain

After common carotid-artery injection of HT1080-dual-color cells, the cells were visualized through the skull of mice via a scalp flap in a brain microvessel. The cell body and nucleus of the dual-color tumor cells were observed to be greatly elongated to fit in small diameter microvessels, revealing a tendency for an extreme deformability of both the nucleus and cytoplasm (*Cancer Res* (2004) 64(12):4251-4256).

### Whole-body real-time imaging of mitosis

Real-time images of mitotic cells were captured in the ear of a live mouse 12 h after the injection of HT1080-dual-color cells. The cells appeared to be extravasated and were rounded in shape, similar to the shape of 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 this animal (*Cancer Res* (2004) 64(12):4251-4256).

### Visualization of nuclear cytoplasmic dynamics in lung metastasis

Micrometastases of HT1080-dual-color cells on excised mouse lungs have also been visualized. The cells appeared to be distorted to enable a close contact among nuclei, suggesting that a close relationship exists among the cells (*Cancer Res* (2004) 64(12):4251-4256).

### Real-time deformability of cancer in vessels

Real-time observations of nuclear-cytoplasmic deformability in the capillaries of HT1080-dual-color cells have also been possible. The cells were injected into the hearts of mice for which the epigastric cranialis vein was wired shut. A skin flap was created without damaging the epigastric cranialis artery and vein, and was then spread and fixed on a stand. Because of this stable fixation, thousands of cells could be visualized easily in capillaries and in microvessels in the skin of the live mouse under fluorescence microscopy. The wired epigastric cranialis vein was observed to be clogged with round cells. Some cells were trapped because of the size restriction in capillaries, where the cells and nuclei were found to be highly deformed.

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. A disturbance of the blood supply to the skin does not occur during the skin flap procedure, because the epigastric cranialis artery is not injured in the process. In addition to these advantages, the skin flap can be completely reversed, and the mice need not be sacrificed. In this study, the skin flap was reversed after 24 h.

The shapes of the cells in this study were classified into four categories. The first category included the cells in microvessels, where the cells were round and the nuclei were oval. The second category included the cells that were elongated in capillaries, where the nuclei were also elongated. The third category comprised cells that were elongated and bifurcated at the corner of the capillaries. The final category included cells that were elongated to the extent that the cytoplasm disconnected; however, the nuclei of these cells remained intact despite being elongated (*Cancer Res* (2005) 65(10):4246-4252).

### Quantitation of cancer cell and nuclear deformation

The lengths of the major and minor axes of the intact HT1080-dual-color cells and the nuclei were measured. The average lengths of the major axes of the whole cells in capillaries were 3.97-fold greater than the length of the axes in microvessels. The average lengths of the major axes of the nuclei in the capillaries had a 1.64-fold length increase compared to the nuclei in the microvessels. The lengths of the minor axes of the whole cells in capillaries were equal to those of the nuclei. The lengths of the major and minor axes of the nuclei and the whole cells therefore deformed to fit the width of the capillaries. The nucleus appears to be less deformable than the cytoplasm, possibly resulting from the difference in the cytoskeleton of the cytoplasm and the nucleus (*Cancer Res* (2005) 65(10):4246-4252).

### Real-time observation of nuclear-cytoplasmic clasmatosis

Clasmatosis, or cytoplasmic fragmentation, was observed in the HT1080-dual-color cells immediately following their injection into the mouse hearts. The cytoplasm was removed from many cells, resulting in naked nuclei. The nuclei were

also observed to be either fragmented or stretched dramatically (*Cancer Res* (2005) **65**(10):4246-4252).

### **Motility analysis in capillaries**

At 2 h after obtaining the first images of the cells, the same cells were imaged again to determine their migration velocities. The bifurcated corners of the capillaries were used as markers to calculate the distance that had been migrated: the distances from the marker to the centers of the nuclei were measured and subtracted. Approximately 25% of the observed cells were found to bifurcate at the corners of the capillaries and could not migrate, and were therefore excluded from the calculations. Of the 45 cells that were not present at the corners of the capillaries, 20 cells were observed to migrate and 25 cells did not migrate. The average migration velocity of the 20 migrating cells was 13.2  $\mu\text{m}/\text{h}$  (range of 1.1 to 48.3  $\mu\text{m}/\text{h}$ ). The captured images of the dual-color fluorescent cells allowed for the resolution of a 1- $\mu\text{m}$  difference in distance.

In calculating migration velocity, the diameters of the capillaries were measured. The minimum capillary diameter that allowed for cell migration was approximately 8  $\mu\text{m}$ , with 16 out of 20 cells in the capillaries > 8  $\mu\text{m}$  in diameter migrating at a velocity  $\leq$  48.3  $\mu\text{m}/\text{h}$ . In contrast, 21 out of 25 cells in capillaries < 8  $\mu\text{m}$  in diameter could not migrate at all (*Cancer Res* (2005) **65**(10):4246-4252).

### **Dynamic subcellular imaging of intravascular trafficking of dual-color cancer cells in vessels in live mice**

Real-time intravascular trafficking of HT1080-GFP-RFP cells was observed in the epigastric vein in a skin flap of live mice using the Olympus OV100 imaging system. In larger vessels, some cells were observed to attach to the vessel walls. Some of the cells subsequently detached and began trafficking. When the cancer cells entered narrower capillaries, both the whole-cell and nucleus were visualized to stretch and deform greatly.

Intravascular aggregation of cancer cells is believed to be an important step in metastasis. Aggregate formation and disaggregation was observed in real time in this study. One aggregate was visualized to collide with another aggregate, which was already attached to the vessel wall. Aggregates were also observed to sometimes attach to each other, with some aggregates becoming increasingly larger with repeated collisions. The cellular adhesion in the aggregates was not strong, however, and some cells were able to escape in the bloodstream. The dual color labeling of the nucleus and cytoplasm allowed for the distinction of the individual cells and nuclei in the emboli (*Cancer Res* (2006) **66**(8):4208-4214).

### **Imaging the dynamics of cancer cell extravasation**

Extravasation has been imaged in real time using dual-color imaging technology. In contrast to HT1080 cells, MMT-GFP-RFP mouse mammary tumor cells have been found to extravasate frequently. MMT-GFP-RFP cells extravasated by first extending their cytoplasmic processes. The nuclei then followed along the extension, undergoing varying degrees of deformation to fit within the extended

cytoplasmic protrusion as they exited the blood vessel. The whole cell eventually extravasated, and was sometimes followed by other whole cells. MMT-GFP-RFP cells that initially extravasated remained in the proximity of the vessels, and appeared to surround the vessels at 24 h after injection (*Cancer Res* (2006) **66**(8):4208-4214).

### **Imaging the fate of post-extravasation cancer cells**

Previous studies with Lewis lung carcinoma (LLC) demonstrated that these cancer cells adhere to the basement membrane of lung tissue 24 h after injection in the tail vein of mice (*Cancer Res* (1995) **55**(12):2520-2523). Another study suggested that the time of extravasation of the LLC cells varies in different organs (*Am J Pathol* (1999) **155**(2):461-472). For example, the time of extravasation was 6 h in the liver and adrenal gland, 16 h in the lung, and 48 h in the brain (*Cancer Res* (2005) **65**(10):4246-4252). The time of extravasation varied depending on the structural complexity of the microcirculation in each organ.

In the skin vessels of the experiments discussed here, the extravasated LLC-GFP-RFP cells appeared to remain closely associated with the blood vessel. Extravasated LLC-GFP-RFP cells initially migrated along the vessels, a mechanism termed extravascular migratory metastasis. Lugassy *et al* (*Am J Pathol* (2004) **164**(4):1191-1198) suggested that some melanoma cells are able to migrate along the outside of vessels in a pericyte-like location, forming angio-tumoral complexes. In the present skin vessel experiments, cancer cell division was also observed to occur outside of the vessels. By 120 h after injection, LLC-GFP-RFP cells surrounded a large vessel. Most of the cells were highly elongated, and the major axes of the cells stretched to approximately 100  $\mu\text{m}$ . The cells and nuclei elongated in order to occupy as much area as possible on the vessel surface. The cancer cells, including their nuclei, elongated and extended their cytoplasmic processes in order to surround the vessel. The elongated cells appeared to become rounded before cell division, similar to the attached cells in culture (*Cancer Res* (2006) **66**(8):4208-4214).

In contrast to the LLC-GFP-RFP and MMT-GFP-RFP cells, HT1080 cells were observed to extravasate at a low frequency in the skin vessel experiments. HT1080-GFP-RFP cells did not extend cytoplasmic processes into the host tissue. Occasionally, a cell was observed to divide in a vessel, an observation that was also noted by Al-Mehdi *et al* (*Nat Med* (2000) **6**(1):100-102) in static imaging studies. Unlike the LLC and MMT cells, cell death was observed for HT1080-GFP-RFP cells in vessels, as observed at 8 h after injection. The cells remained rounded in shape, and cytoplasmic fragmentation occurred in the vessels. Even at 24 h after injection, the majority of the cells remained in vessels without extravasation. However, the vessels containing cancerous cells appeared to expand, perhaps as a result of an increase in intravascular pressure (*Cancer Res* (2006) **66**(8):4208-4214).

### **Conclusion**

With the development of the Olympus OV100 whole-mouse imaging system and dual-color fluorescent cells, the

behavior of cancer cells can now be imaged in live animals at the subcellular level in real time. Using this technology, each step of metastasis is expected to be observable in live animals. For the first time, the many steps of cancer metastasis can be visualized in such animals. Because metastasis is the lethal aspect of cancer, and the aspect about which the least is known and which most current drugs generally fail to address adequately, the development of effective anti-metastatic drugs is an urgent prerogative. The advances in subcellular imaging of cancer cell metastasis provide the possibility to identify new visible targets for anti-metastatic drug development. Future developments in subcellular imaging in live animals should result in a new 'in vivo cell biology' that will provide visible targets for many diseases.

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