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Real-time imaging of individual fluorescent-protein color-coded metastatic colonies *in vivo*

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Abstract

We have established stable, bright green fluorescent protein (GFP)- or red fluorescent protein (RFP)-expressing HT-1080 human fibrosarcoma clones. These cell lines showed similar cell proliferation rates and high-frequency experimental lung metastasis. The HT-1080-GFP and -RFP clones enable simultaneous real-time dual-color imaging in the live animal. HT-1080 cells were transduced with retroviral vectors containing GFP or RFP and the neomycin resistance gene. Stable transformants were selected stepwise with G418 up to 800 μl/ml. Subsequently, high GFP- or RFP-expressing clones, HT-1080-GFP or HT-1080-RFP, respectively, were selected. 3×10^6 cells from each clone were mixed and injected into the tail vein of SCID mice. The cells seeded the lung at high frequency with subsequent formation of pure green and pure red colonies as well as mixed yellow colonies with different patterns visualized directly on excised lungs. The lung metastases were also visualized by external fluorescence imaging in live animals through skin-flap windows over the chest wall. Lung metastases were observed on the lung surface of all mice. SCID mice well tolerated multiple surgical procedures for direct-view imaging via skin-flap windows. Real-time metastatic growth of the two different colored clones in the same lung was externally imaged with resolution and quantification of green, red, or yellow colonies in live animals. The color coding enabled determination of whether the colonies grew clonally or were seeded as a mixture with one cell type eventually dominating, or whether the colonies grew as a mixture. The simultaneous real-time dual-color imaging of metastatic colonies described in this report gives rise to the possibility of color-coded imaging of clones of cancer cells carrying various forms of gene of interest.

Abbreviations: GFP – green fluorescent protein; RFP – red fluorescent protein

Introduction

Whole-body imaging of mice with primary and metastatic tumors genetically-labeled with the green fluorescent protein (GFP) and red fluorescent protein (RFP), has been shown to be enabling, powerful, and simple [1]. The bright intrinsic fluorescence of GFP and RFP is due in part to the high quantum yield of these fluorophores [2, 3]. For *in vivo* imaging, it is necessary that GFP or RFP genes be stably-transduced in the tumor cells such that they become brightly fluorescent [4]. This can be accomplished by selection of such fluorescent tumor cells *in vitro* [4] and *in vivo* as well [5].

In mice with primary and metastatic tumors stably-expressing fluorescent proteins, the whole-body imaging of tumor growth and metastasis can utilize simple equipment [6]. A light box can be interfaced with a CCD camera with an appropriate filter that will display the images on a monitor that can be captured digitally by a PC [6].

In order to visualize smaller tumors and metastases, the animal can be put on a fluorescence dissecting microscope having a light source and appropriate filters [6]. The animals can be irradiated at 470 nm for long periods without harming them or bleaching the GFP or RFP fluorescence. Images can be processed with standard software. The imaging procedures can be repeated as often as necessary without harming the animal enabling real-time tumor growth and metastasis visualization.

Optical imaging based on fluorescence expression of GFP allows for a high ratio of the fluorescence signal from the tumor compared to the auto-fluorescence from any background tissue. The GFP signal is so strong and selective that external images of GFP-expressing tumors and their

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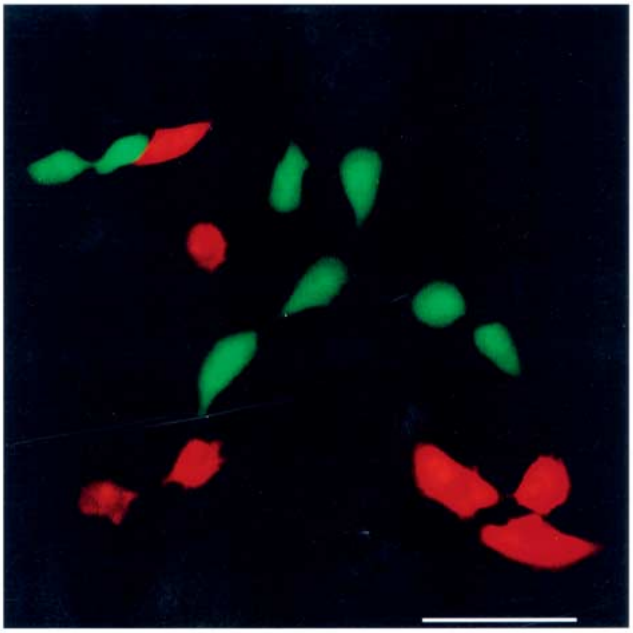

Figure 1. Stable high GFP expressing human fibrosarcoma cells (HT-1080-GFP) and RFP expressing human fibrosarcoma cells (HT-1080-RFP) *in vitro*. Human fibrosarcoma cells (HT-1080) were transduced with GFP and the neomycin resistance gene or RFP and the neomycin resistance gene in retrovirus vectors. High GFP-expressing cells (HT-1080-GFP) or RFP-expressing cells (HT-1080-RFP) were selected with G418 up to 800 μg/ml. Please see 'Materials and methods' for details. Bar, 100 μm.

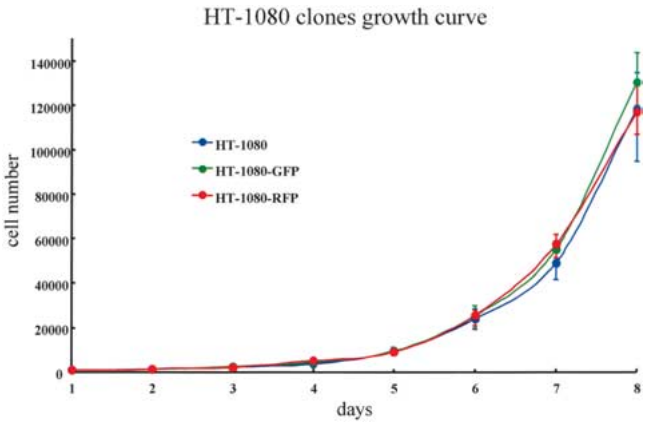

Figure 2. Cell proliferation rate of parental and fluorescent protein expressing clones. Three dishes for each clone (parental HT-1080, HT-1080-GFP, HT-1080-RFP) were used at each time-point to count the cell number over one week. Cells were trypsinized, stained with trypan blue, and counted in a hemocytometer. Filled circle shows averaged cell number for each group.

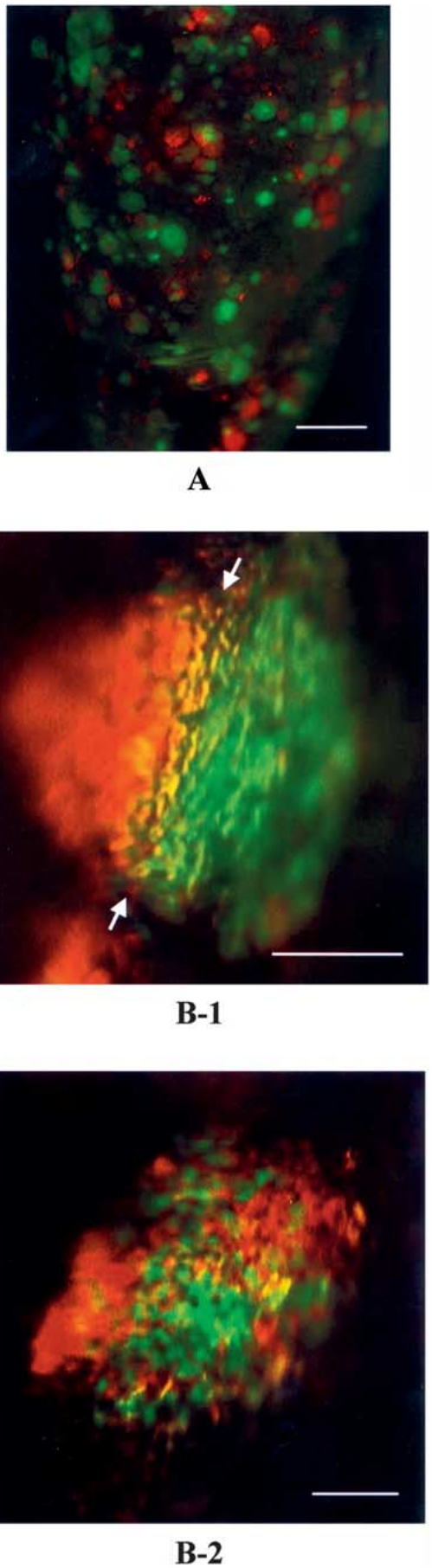

Figure 3. Dual-color fluorescent images of metastatic colonies on the extracted lung. Numerous small metastases could be detected on the lung under fluorescence microscopy. (A) Low magnification view. Bar, 2 mm. (B-1, 2) High magnification views. White arrows indicate the border of green and red colonies. Bars, 200 μm. See 'Materials and methods' for details. Five of five mice implanted with tumor had lung metastases and were used for this experiment.

metastases were acquired in freely-moving animals [6]. RFP may even have greater imaging capability due to its longer emission wavelength [2].

GFP demonstrates highly important advantages as a reporter over other optical approaches to imaging. The fluorescence intensity of GFP is very strong since the quantum yield is approximately 0.9 [3]. The protein sequence of GFP has also been 'humanized' which allows for high-level expression in mammalian cells. In addition, the fluorescence is relatively unaffected by the external environment since the chromophore is protected by the three-dimensional structure of the protein [7]. The excitation wavelength is at a relatively long wavelength (470 nm) [3, 4] which does not quench the fluorescence thereby allowing for long-term measurements.

In vivo GFP fluorescence is mainly limited by light scattering which, as noted above, has been overcome by skin flaps [8] such that single cells can be imaged external to the animal. Longer wavelength fluorescent proteins, such as RFP used in the present study, can be used to reduce scatter *in vivo* as well [3].

In contrast to GFP and RFP, the luciferase reporter requires anesthetized, restrained animals in order to collect sufficient photons to construct a pseudo-image and imaging must be carried out in a virtually light-free environment [9]. The animals must also be injected with the luciferin substrate that must reach every tumor cell uniformly so reliable imaging can be obtained. In addition, luciferase activity is not stable *in vivo* after luciferase injection [10]. Luciferase-based imaging precludes studies that would be perturbed by anesthesia, restraint, or substrate injection also makes high-throughput screening not feasible. Expression of firefly luciferase (Luc) can be used to visualize tumor cell growth and regression in response to various therapies in mice. However, detection of Luc-labeled cells *in vivo* was limited to the order of 10^3–10^4 human tumor cells [9] in contrast to single cell sensitivity and resolution for GFP and RFP. The much higher signal afforded by GFP and RFP allows unrestrained animals to be imaged without any perturbation or substrate; all that is needed is irradiation with blue light, which is not damaging to tissue. The great advantage of the GFP as a reporter is that the introduction of a substrate is not required [1], unlike firefly luciferase [9].

This study takes advantage of GFP and RFP tumor imaging to develop a new dual-color imaging model to distinguish metastatic colonies in the lung including live animals.

Materials and methods

Production of GFP and RFP retrovirus

The pLEIN retroviral vector (Clontech Laboratories, Inc., Palo Alto, California), expressing enhanced green fluorescent protein (GFP) and the neomycin resistance gene on the same bicistronic message, was used as a GFP expression vector [7]. PT67, an NIH3T3-derived packaging cell line, expressing the 10 Al viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in DME medium (Irvine Scientific, Santa Ana, California) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-products, Calabasas, California). For GFP vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of DOTAP^{TM} reagent (Boehringer Mannheim, Indianapolis, Indiana), 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 post-transduction. For selection, the cells were cultured in the presence of 500–2,000 μg/ml of G418 (Life Technologies, Grand Island, New York) increased over 7 days to select for a clone producing high amounts of a GFP retroviral vector (PT67-GFP) [4].

For RFP retrovirus production [2], the *Hind* III / *Not* I fragment from pDsRed2 (Clontech), containing the full-length red fluorescent protein cDNA, was inserted into the *Hind* III / *Not* I site of pLNCX2 (Clontech) that has the neomycin resistance gene to establish the pLNCX2-DsRed2 plasmid. PT67 cells, at 70% confluence, were incubated with a precipitated mixture of L$_{IPOFECT}$AMINE^{TM} reagent (Life Technologies), and saturating amounts of pLNCX2-DsRed2 plasmid using similar methods 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 200-1,000 μg/ml of G418 increased over 7 days same as PT67-GFP.

GFP and RFP gene transduction of fibrosarcoma cells

For GFP and RFP gene transduction, 70% confluent HT-1080 cells, derived from human fibrosarcoma, were purchased from American Type Culture Collection (Rockville, Maryland). The HT-1080 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-GFP or PT67-RFP cells and RPMI 1640 (Mediatech, Inc., Herndon, Virginia) containing 10% fetal bovine serum for 72 h. Fresh medium was replenished at this time. Cells were harvested by trypsin/EDTA 72 h 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. 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, New Jersey) using trypsin/EDTA and amplified by conventional culture methods.

Cell proliferation rates of parental, GFP and RFP expressing clones

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 with growth medium (day 1). The dishes were kept in an incubator at 37 °C and 5% CO_2. Every other day (days 2–8), three dishes for each clone were used for cell counting. Briefly, resuspended cells collected after trypsinization were stained with trypan blue (Sigma, St. Louis, Missouri). Only

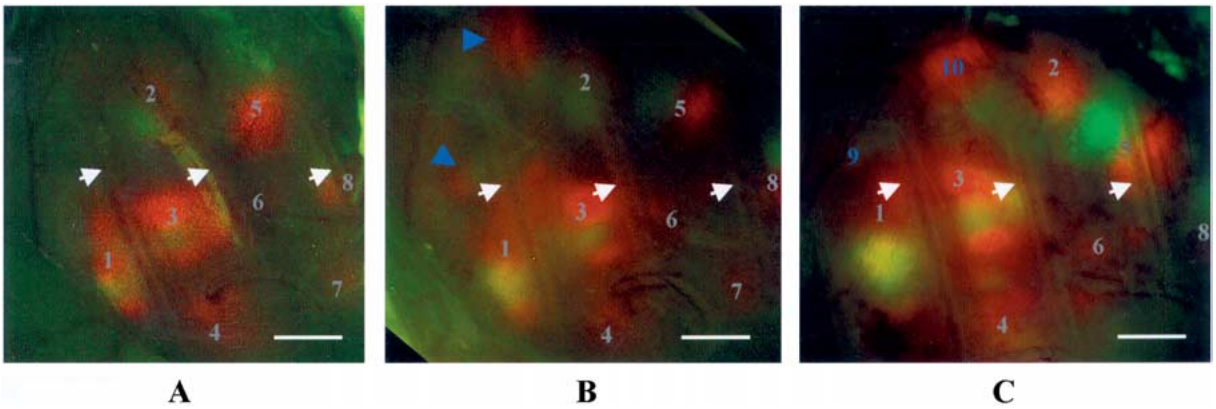

Figure 4. Time-course images of dual-color lung micrometastases in a living mouse. Dual-color micrometastases were observed under fluorescence microscopy via skin-flap windows. White arrows indicate ribs. Blue arrowheads indicate new colonies. Please note the enlargement of each colony over time. (A) Two weeks after footpad injection (day 15). (B) Day 18. (C) Day 21. Bars, 2 mm. See 'Materials and methods' for details.

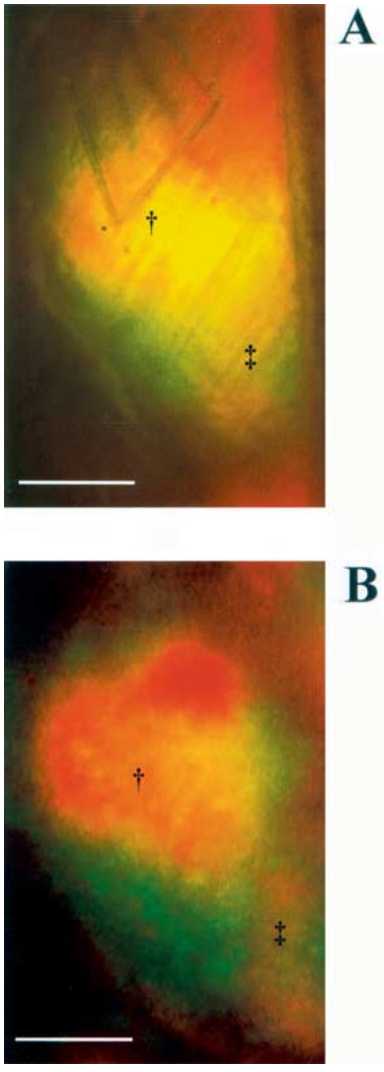

Figure 5. Comparison of imaging of lung metastases from living mice with skin-flap window to direct imaging. Dual-color colony was observed through the chest wall of live mice or directly in sacrificed mice with the chest wall removed. Five of five mice implanted with tumor had lung metastases and were used for this experiment. (A) Image captured in living mouse. (B) Image captured in same mouse after sacrifice with chest wall removed. Bars, 400 μm. † and ‡ indicate overlapping parts of green and red color colonies. See 'Materials and methods' for details.

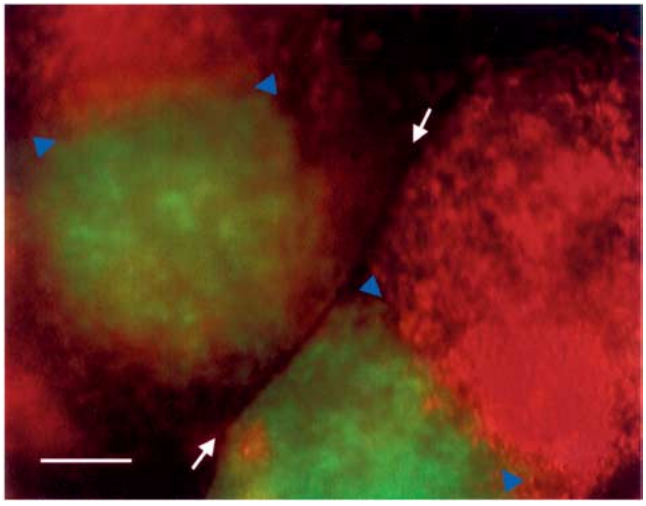

Figure 6. Boundary of dual-color colonies. The border of each color colony was clear on the extracted lungs. The borders had two patterns. White arrows indicate the border with clear band, which allows colonies to be distinguished from one another even of the same color. Blue arrowheads indicate the borders of green and red colonies without clear bands. Bar, 250 μm. See 'Materials and methods' for details.

viable cells were counted with a hemocytometer (Reichert Scientific Instruments, Buffalo, New York) subsequently.

Imaging of dual-color micrometastases in extracted lung

Six-week-old, female, severe combined immunodeficient (SCID) mice were used. HT-1080-GFP cells and HT-1080-RFP cells were first harvested by trypsinization and 3×10^6 cells from each clone were mixed well with each other. The cells were washed three times with serum free cold medium. SCID mice were injected with 6×10^6 cells (3×10^6 cells from each clone) in a total volume of 200 μl into the tail vein. Harvested cells were injected within 30 min. Ten days after cell injection, the lung was removed and tumor colonies on the surface were evaluated with a fluorescence microscope.

Dual-color tumor cells on the lungs were visualized through the chest wall. The animals were anesthetized with

a ketamine mixture. An arc-shaped incision was made in the skin, and s.c. connective tissue was separated to free the skin flap. The skin flap could be opened repeatedly to image tumor cells on the internal organs through the nearly transparent mouse body walls or skull and simply closed with an 6–0 suture. This procedure greatly reduced the scatter of fluorescent photons [8].

Comparison of images captured in living mice through the chest wall to direct imaging

To compare images in living mice through the chest wall and direct images from sacrificed mice with the chest wall removed, the mice were sacrificed after the final observation (day 21) and stored at −80 °C to prevent the shrinkage of lung. The frozen chest walls were gently removed and lung metastases were imaged.

Fluorescent optical imaging

Images were captured directly with a Hamamatsu C5810 3CCD camera (Hamamatsu Photonics, Bridgewater, New Jersey). For macro-imaging, a fluorescence light box (Lightools Research, Encinitas, California) was used. For micro-imaging, a Leica fluorescence stereo microscope model LZ12 was coupled with the 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. 1024 × 724 pixel high-resolution images were captured directly on an IBM PC [6].

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health 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 (Teckland LM-485, Western Research Products, Orange, California).

Results and discussion

Cell proliferation of parental, GFP- and RFP-expressing clones

The selected HT-1080 cells have a strikingly bright GFP or RFP fluorescence *in vitro* (Figure 1). All cells in the population expressed GFP or RFP, indicating stability of the transgene (Figure 1). There was no difference in the proliferation rate of parental HT-1080 and selected HT-1080-GFP or HT-1080-RFP clones determined in monolayer culture (Figure 2).

Patterns of dual-color micrometastases in lung

Ten days after tail-vein cell injection, the lungs were removed and tumor colonies on the surface were visualized under fluorescence microscopy. Even at low magnification, the brightly fluorescent colonies were clearly resolved (Figure 3A). The number of pure green fluorescent colonies were approximately equal to the red ones. At high magnification, there were two main patterns of mixed-color colonies that were comprised of HT-1080-GFP and HT-1080-RFP. In one pattern, green and red cells are readily distinguished (Figure 3B-1). The border of each color is seen as a yellow band (Figure 3B-1 arrow). The other pattern is mosaic growth (Figure 3B-2). The mosaic colonies suggest that green and red cells started to grow together in the same colony. The cells were readily observed at the single cell level.

Real-time imaging of dual-color colonies in live mice

Lung metastases were visualized growing via skin-flap windows starting two weeks after injection at days 15, 18, and 21 (Figure 4). New colonies were detected on day 18 (indicated by arrowhead). No mice had complications after skin-flap surgery (e.g., infection or massive bleeding). Pure green or red fluorescent colonies were well distinguished from each other with the same filter (GFP filter set). The overlapping parts of green and red fluorescent colonies were yellow. Newly established colonies were observed over time (Figure 4). Colony enlargement was also visualized over time (Figure 4).

Comparison of images of lung metastases in live mice through the chest wall to direct images

The images captured in live mice had high resolution (Figure 5A). Overlapping green and red fluorescent cells were imaged as yellow in the skin-flap window model. Similar images were seen after sacrifice and removal of the chest wall (Figure 5B). The high resolution images captured through the intact chest wall in the living animal were due to the following: The mice were well anesthetized such that breathing motion was minimal. In addition, the muscles that cover the lateral chest wall in the mouse (e.g., costal muscles) are not thick. In addition, the pleura itself is very clear and transparent.

There were two types of borders of dual-color colonies as seen in extracted lungs (Figure 6). One type had a clear band (indicated by white arrow in Figure 6) between adjacent colonies. Each colony could be distinguished even of the same color. This clear band seems to be a capsule formed around the colonies. The other pattern had no clear band at the border of dual-color colonies (indicated by blue arrowheads in Figure 6). This pattern may reflect the original cells of the colonies growing together during early colony formation.

In the present study, we have shown that we can image the time-course of lung metastasis formation and growth from two differently colored clones of a tumor cell line in the same live mouse. We could also determine whether the colonies were clonal or heterogeneous and distinguish them from other colonies. This work opens up the possibility of color-coded *in vivo* imaging where cells with different properties, including specific genes of interest, can be simultaneously imaged *in vivo* in real time.

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