Subcellular imaging in the live mouse

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Fluorescent proteins are available in multiple colors and have properties such as intrinsic brightness and high quantum yield that make them optimally suited for *in vivo* imaging with subcellular resolution in the live mouse. In this protocol, cancer cells in live mice are labeled with green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in the cytoplasm. GFP nuclear labeling is effected by linkage of GFP to histone H2B, and a retroviral vector is used for cytoplasmic labeling with RFP. Double-labeled cells are injected by various methods. High-resolution imaging systems with microscopic optics, in combination with reversible skin flaps over various organs, enable the imaging of dual-color labeled cells at the subcellular level in live animals. The double transfection and selection procedures described here take 6–8 weeks. Cancer cell trafficking, deformation, extravasation, mitosis and cell death can be imaged with clarity.

INTRODUCTION

Imaging of nuclear and cytoplasmic dynamics in the living animal has the potential to yield a wealth of information about the mechanisms of cell trafficking, invasion, cell division, cell death and numerous other *in vivo* cellular processes, some of which cause disease. Fluorescent proteins have properties of intrinsic brightness and high quantum yield, and they come in multiple colors making them optimally-suited to develop *in vivo* imaging technologies that have subcellular resolution in the live mouse[1]. In this protocol, cancer cells are labeled with GFP in the nucleus and RFP in the cytoplasm. GFP nuclear labeling is effected by linkage of GFP to histone H2B, and a retroviral vector is used for cytoplasmic labeling with RFP[2]. 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. Double-labeled cells are injected by various routes into the mice. Using high-resolution imaging systems with microscopic optics, dual-color labeled cells can be imaged at a subcellular level in live animals[2–4] (**Fig. 1**). It usually takes 6–8 weeks for gene transduction and selection of cells stably expressing both GFP and RFP. At such time, the cells are ready to be injected into the animals for imaging of both cytoplasm and the nucleus[2–4] (**Figs. 2** and **3**). Cancer cell death in the portal vein can be imaged where some cancer cell types rapidly die by first losing their cytoplasm, leaving behind naked nuclei[5] (**Figs. 4** and **5**). Apoptosis is readily visualized as nuclear fragmentation observed *in vivo*[5]. Mitosis could be imaged *in vivo* as well[2]. Subcellular imaging in live mice was effected by reversible skin flaps over various organs[2–4] (**Fig. 6**). Extravasation is imaged where it was observed that cancer cells exited from vessels by first protruding with cytoplasmic processes through which the cancer cell nucleus followed, enabling the whole cell to exit from the vessel[4] (**Fig. 7**). These methods lead to the possibility of the study of cell biology *in vivo* and for the development of visible drug targets for all disease types. Previous approaches to single-cell imaging *in vivo* have used single-color imaging that allows cellular behavior to be visualized *in vivo*[6,7]. In such experiments, however, the nuclei and cytoplasm are not distinguishable. The differential labeling of nucleus and cytoplasm in this protocol enables imaging of nuclear-cytoplasmic dynamics, which reveals cell-cycle events as well as apoptosis and the differential behavior of cytoplasm and nucleus during events such as extravasation.

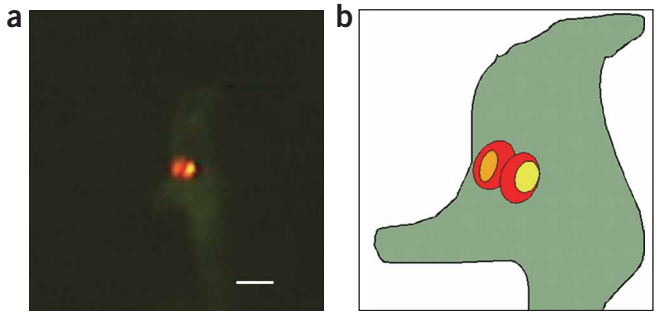

Figure 1 | Real-time whole-body image of mitotic cell in ear of a live mouse. (**a**) High-magnification image. Scale bar, 50 μm. (**b**) Schematic drawing of **a**. Image from ref. 2.

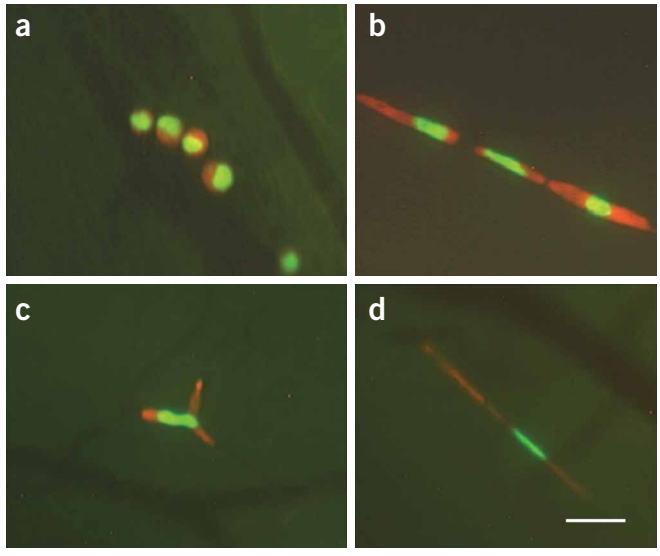

Figure 2 | Classification of the deformation of HT-1080-dual-color cells in the vessels in the skin. (**a**) Nondeformed cells are within a microvessel. (**b**) The cells and nuclei have elongated to fit a capillary. (**c**) A deformed cell is arrested at a capillary bifurcation. The nucleus is also deformed but remains intact. (**d**) Cytoplasmic fragmentation in a very thin capillary. Scale bar, 50 μm. Image from ref. 3.

MATERIALS

REAGENTS

- Immunocompetent and immunodeficient mice (e.g., Charles River Labs, Taconic Labs or Harlan Teklad)
- GFP mice (Jackson Laboratories)
- Cell lines to be transfected with genes coding for fluorescent proteins (American Type Culture Collection (ATCC))
- EGFP gene (Clontech)
- RFP (DsRed-2) gene (Clontech)
- Retroviral vector pLEIN (Clontech)
- Retroviral vector pLNCX (Clontech)
- Retroviral vector pLHCX (Clontech)
- Supernatants of PT67-GFP cells, PT67-RFP cells and PT67 H2B-GFP cells (Clontech)
- Growth medium (normal and selective), appropriate for cell culture (e.g., Invitrogen or Irvine Scientific)
- HEPES buffer (20 mM, pH 7.2) (Sigma)
- Lipofectamine Plus transfection kit (Invitrogen)
- Anesthetic reagents (ketamine, xylazine, acepromazine maleate, isofluorane) (Butler Animal Health Supply)
- Nair (Carter-Wallance)
- G418 Neomycin (Invitrogen)
- PT67 packaging cells (Clontech)
- Hygromycin (Invitrogen)
- Lipofectamine Plus transfection kit (Invitrogen)

EQUIPMENT

- OV100 Whole Mouse Imaging System (Olympus)
- Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems)
- Lightools Fluorescent Imaging System (Lightools Research)
- Sony VCR model SLV-R1000 (Sony)
- Image Pro Plus 3.1 software (Media Cybernetics)
- 1 ml 27G2 latex-free syringe (Becton Dickinson)
- 25-µl Hamilton syringe (Fisher Scientific)

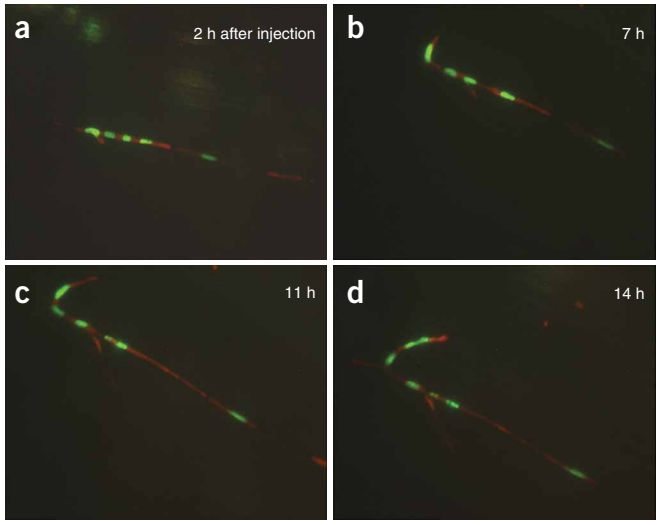

Figure 3 | Migration velocity of HT-1080-dual-color cell in capillaries. The corner of the capillary was regarded the starting point of migration. (**a**–**d**) Migration of cells in capillaries from 2 to 14 h. Image from ref. 3.

- D470/40 excitation filter (Chroma Technology)
- GG475 emission filter (Chroma Technology)
- Cloning cylinders (Bel-Art Products)
- Hemocytometer (Reichert Scientific Instruments)
- Blunt-end hook (Fine Science Tools)
- 33-gauge needle (Fine Science Tools)

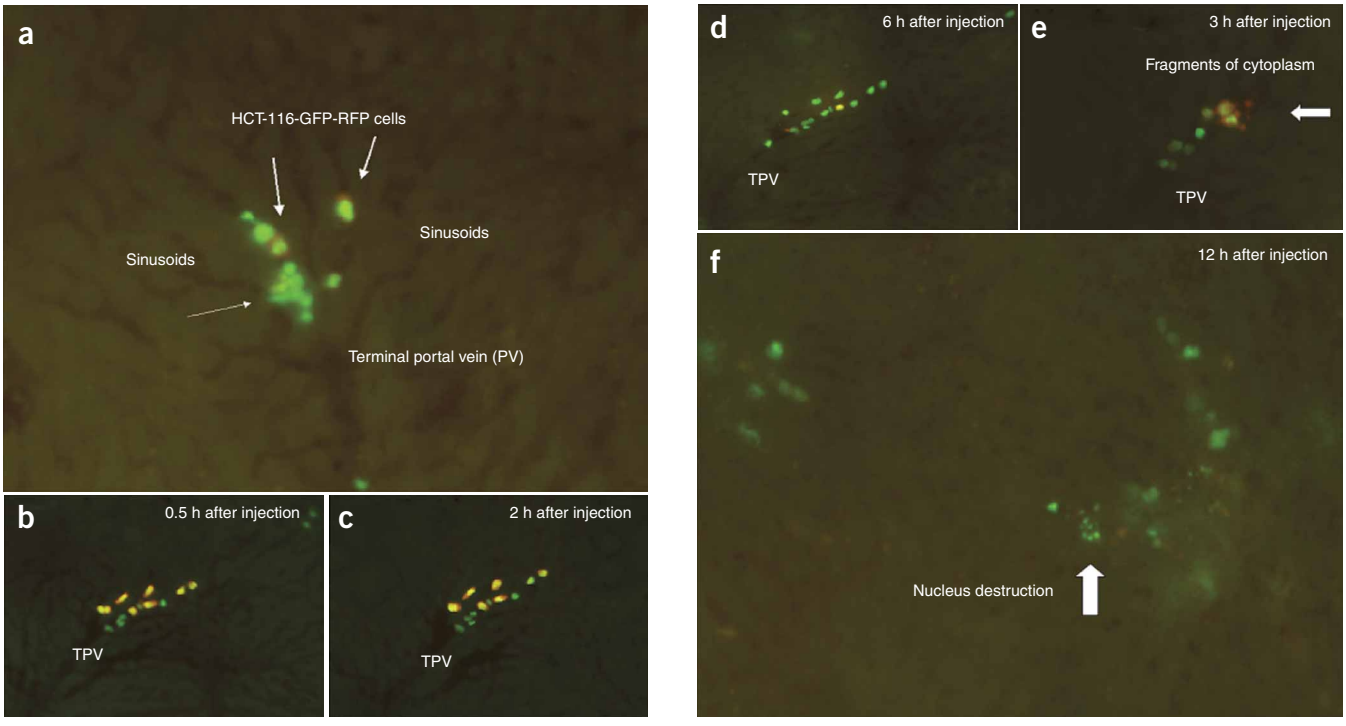

Figure 4 | Fate of HCT-116-GFP-RFP human colon cancer cells after portal vein injection. The liver surface was imaged for dual-color fluorescence in the Olympus OV100 whole-mouse imaging system. (**a**–**f**) Time-course imaging after injection of HCT-118-GFP-RFP cells. Destruction of RFP-expressing cytoplasm of the cancer cells began rapidly. The number of apoptotic cells with fragmented nuclei in the liver increased within 12 h. Fragmentation of GFP nuclei is clearly seen. In **a**, groups of cells are visible. Individual cells can be visualized in **b**–**f**. Image from ref. 5.

EQUIPMENT SETUP

Whole-body imaging equipment The Olympus OV100 Whole Mouse Imaging System (Olympus), containing an MT-20 light source and a DP70 CCD camera, can be used for whole-body and skin-flap imaging in live mice at variable magnification. The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging as well as microimaging with high light-gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Five individually optimized objective lenses, parcentered and parfocal, provide a 10^5-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has lenses mounted on an automated turret with a high magnification range of ×1.6 to ×16 and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images are captured directly on a PC (Fujitsu Siemens). Images are processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 (Corel) and Cell (Olympus Biosystems). Many other fluorescence imaging systems can also be used to acquire subcellular images with the double-labeled cells. For example, a Leica fluorescence stereo microscope (model MZ16) equipped with a mercury 50-W lamp power supply can be used. Selective excitation of GFP is produced through a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence is collected through a long-pass filter (GG475; Chroma Technology). Under anesthesia, the experimental animals can be examined with the microscope and the images can be acquired with a Hamamatsu C5810 three-chip cooled color charge-coupled-device camera (Hamamatsu Photonics Systems). Images can also be processed for contrast and brightness and analyzed with the use of Image Pro Plus software (Media Cybernetics). High-resolution images of 1,024 × 724 pixels can be captured directly on a PC or continuously through video output on a high-resolution VCR (e.g., Sony model SLV-R1000).

PROCEDURE

GFP retrovirus production

1| Use the pLEIN or equivalent retroviral vector expressing enhanced GFP or equivalent GFP and the neomycin resistance gene, on the same bicistronic message, as a GFP expression vector.

2| Use PT67, an NIH3T3-derived packaging cell line expressing the 10 Al viral envelope, to produce retrovirus. Culture PT67 cells in DMEM medium supplemented with 10% heat-inactivated FBS. It takes approximately 3 d for the cells to reach ~70% confluence after seeding 3 × 10^5 PT67 cells in a 25-mm^2 flask with DMEM medium containing 10% fetal calf serum.

3| For vector production, use packaging cells (PT67) (Clontech), at 70% confluence. Plate PT67 cells on a 60-mm dish at 60–80% confluence 12 h before transfection. Use 10 µg of pFB-GFP (Clontech) with the Lipofectamine Plus transfection kit. Add 7 µl of pre-complexed pFB-GFP DNA in 87 µl of serum-free medium and then add 6 µl Lipofectamine reagent in a tube; mix and incubate at room temperature (22–26 °C) for 15 min.

4| Dilute 4 µl of Lipofectamine in 96 µl serum-free medium in a second tube. Mix and incubate at RT for 15 min.

5| Combine pre-complexed DNA and diluted Lipofectamine reagent; then mix and incubate at RT for 15 min.

6| While the complexes are forming, replace medium on the cells with 800 µl serum-free DMEM. Add the DNA-Lipofectamine reagent complex to the dish with cells containing fresh DMEM. Mix the complexes into the medium gently; incubate in a humidified incubator at 37 °C and 5% CO_2 for 4 h.

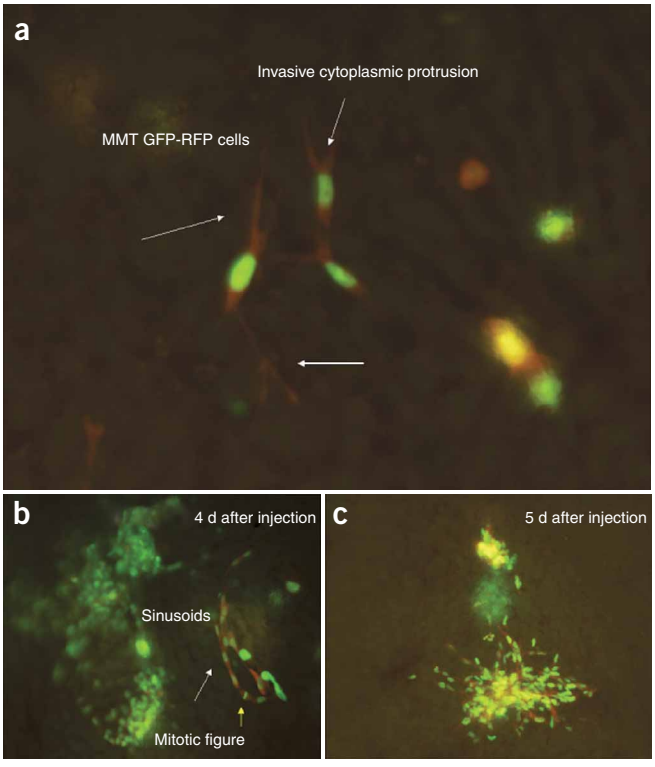

Figure 5 | Fate of MMT GFP-RFP mouse mammary tumor cells after portal vein injection (see **Fig. 4** for imaging details). (**a**) Most MMT GFP-RFP cells maintained normal cell structure and survived >24 h after injection. (**b**, **c**) Many MMT GFP-RFP cells, which impacted into sinusoids near the terminal portal vein after injection, survived and grew into colonies. Image from ref. 5.

7| After 4 h incubation, increase volume of medium to 5 ml. Incubate in the same conditions for 24 h.

8| After 24 h incubation, clone the packaging cells by limit dilution in 96-well plates.

9| Examine the cells by fluorescence microscopy 48 h post-transduction.

10| For selection, culture the cells in the presence of 500–2,000 µg ml^{-1} of G418 to select for a clone producing high amounts of a GFP retroviral vector (PT67-GFP). Culture the cells for 1–2 d in each concentration of G418. High-viral-titer production clones of GFP PT67 cells are identified with 3T3 cells used for virus titering. Clones with titer higher than 10^6 plaque-forming units per ml are used for GFP vector production.

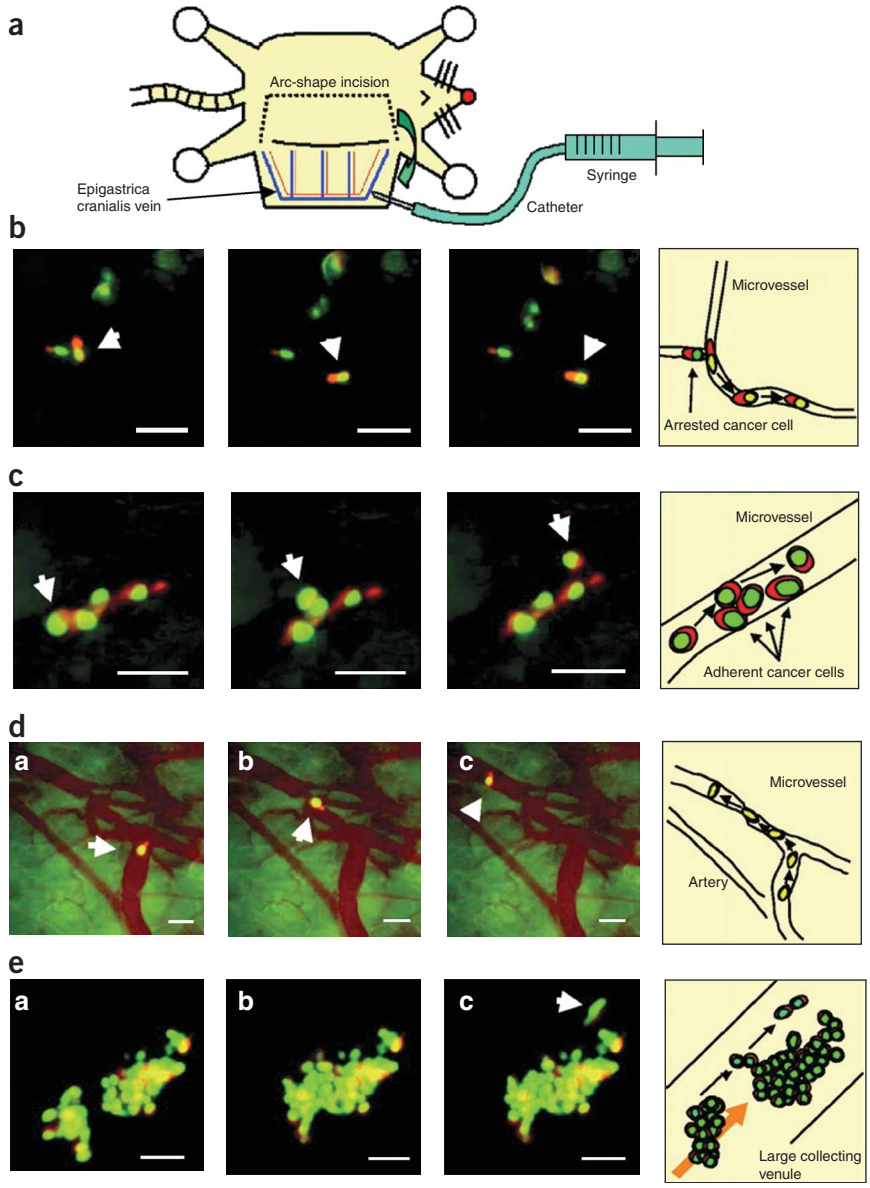

Figure 6 | Intravascular trafficking of HT-1080-GFP-RFP human fibrosarcoma cells. (**a**) Schematic diagram of the skin flap model in live mice for imaging intravascular trafficking and extravasation. An arc-shaped incision was made in the abdominal skin, and then the skin flap was spread and fixed on a flat stand. Dual-color HT-1080 cells were injected into the epigastric cranialis vein through a catheter. Immediately after injection, the inside surface of the skin flap was directly observed. (**b**) A dual-color HT-1080 cell crawls smoothly along the vessel wall without rolling in a capillary (arrow). The nucleus and cytoplasm are slightly stretched. The nucleus is in the front of the cell while the cell is crawling. When the cell advances into a part of the capillary where the diameter is smaller than of deformation limit of the cell, the cell does not advance any farther. Scale bar, 100 μm. (**c**) A dual-color HT-1080 cell, trafficking at low velocity, advances between other cells and the vessel wall. The moving cancer cell contacts the other cells (arrow). The cell deforms slightly and continues to move without adhesion. Scale bar, 100 μm. (**d**) One cancer cell migrating in the post-capillary with slow velocity. The cytoplasm is at the head of the cell while the cell is moving in a large vein, but the nucleus is at the head in a small vein. Images were taken every 3.3 s. Scale bar, 50 μm. (**e**) Multicellular aggregate collides with another aggregate that is already attached to the vessel wall. The two aggregates attach and form a larger aggregation. Some cells (arrow) escape from the aggregate because of weak adhesion and recommence movement. Images were taken every 1.04 s. Scale bar, 100 μm. Images were acquired in real time with the Olympus OV100. Right, schematics of **b**–**e**. Image from ref. 4.

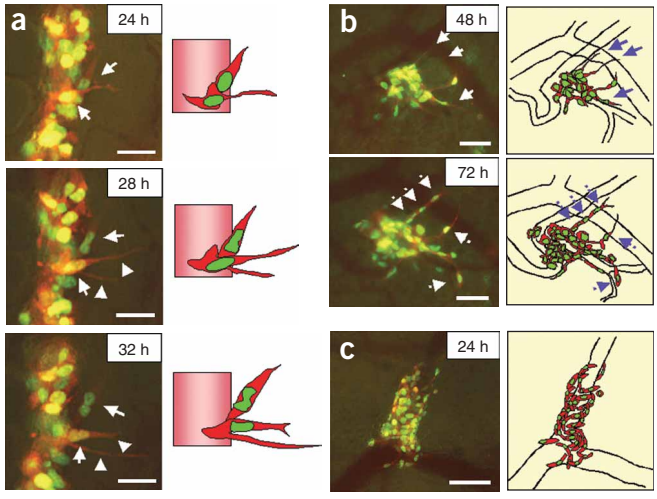

Figure 7 | Time-lapse imaging of extravasation of MMT mouse mammary GFP-RFP tumor cells. (**a**) 12 h after injection, the skin flap is opened and fixed on a flat stand. Images are acquired every hour for 24 h with the skin flap open. Two MMT cells are visualized in the process of extravasation 24 h after injection (arrows). The cancer cells extend fine cytoplasmic projections into the host tissue at the onset of extravasation. One of the cells extends two fine cytoplasmic projections into the host tissue (arrowhead). The nuclei then migrate along the cytoplasmic projection until the whole cell is out of the vessel. Subsequently, the whole cell extravasates. Scale bar, 20 μm. (**b**) 48 and 72 h after injection. Cytoplasmic processes are extended along the vessel wall 48 h after injection (arrows). Cells extravasate in the same direction of the cytoplasmic projections (broken arrows). Images were acquired every 24 h by opening and closing the skin flap. Scale bar, 50 μm. (**c**) Invasion and proliferation of MMT cells around a vessel after extravasation. Scale bar, 50 μm. Images were acquired with the Olympus OV100. Right, schematics of **a**–**c**. Image from ref. 4.

▲ CRITICAL STEP Increasing the level of G418 in a stepwise manner is very important to induce the expression of the transgene. This procedure assures high-level production of GFP retrovirus.

RFP retrovirus production

11| To establish the pLNCX2-DsRed2 plasmid, insert the *Hin*dIII/*Not*I fragment from pDsRed2, containing the full-length red fluorescent protein cDNA, into the *Hin*dIII/*Not*I site of pLNCX2 that has the neomycin resistance gene.

12| Incubate PT67 cells at 70% confluence. Lipofectamine reagent is used as described in Steps 3–8 to transfect the pLNCX2-DsRed2 vector into the PT67 cells.

13| For selection of a clone producing high amounts of a RFP retroviral vector (PT67-DsRed2), culture the cells in the presence of 200–1,000 μg ml^{-1} of G418. Culture the cells for 1–2 d in each concentration of G418.
▲ CRITICAL STEP This procedure assures high-level production of RFP retrovirus as described in Step 10.

Production of histone H2B-GFP vector

14| Insert the histone H2B-GFP fusion gene at the *Hin*dIII/*Cla*I site of the pLHCX retrovirus that has the hygromycin resistance gene.

15| To establish a packaging cell clone producing high amounts of a histone H2B-GFP retroviral vector, transfect the pLHCX histone H2B-GFP plasmid in PT67 cells using the same methods described above for PT67-DsRed2 (Step 12).

16| Culture the transfected cells in the presence of 200–400 μg ml^{-1} hygromycin to establish stable PT67 H2B-GFP packaging cells. The amount of hygromycin is increased stepwise as described above for G418.
▲ CRITICAL STEP This procedure assures high-level production of histone H2B-GFP retrovirus.

RFP or GFP gene transduction of tumor cell lines

17| For RFP or GFP gene transduction, use 20% confluent cancer cells. Plate the target cells 12–18 h before infection with GFP or RFP retrovirus, at a cell density of 1–2 × 10^5 per 60-mm plate.

18| For retroviral infection, collect conditioned medium from packaging cells (PT67/pFB GFP or PT67/pLNCX2-DsRed-2) and filter medium through a 0.45-μm polysulfonic filter. Add virus-containing filtered medium to target cells. Add polybrene to a final concentration of 8 μg ml^{-1}. Incubate cells for 24 h at 37 °C.

19| Replace medium with DMEM and 10% FCS after 24 h incubation.

20| Check for GFP- or RFP-expressing cells under fluorescence microscopy.

21| Harvest tumor cells with trypsin/EDTA and subculture them at a dilution ratio of 1:15 in selective medium, which contains 50 μg ml^{-1} of G418.

22| To select brightly fluorescent cells, increase the level of G418 to 800 μg ml^{-1} in a step-wise manner. Culture the cells for 1–2 d in each concentration of G418.
▲ CRITICAL STEP Increasing the level of G418 in a stepwise manner is very important to induce the expression of the transgene. This procedure assures high-level production of GFP or RFP in the cells.

23| Isolate clones expressing GFP or RFP with cloning cylinders using trypsin/EDTA and amplify them in the same medium as above in the absence of selective agent. Further select cells for brightness and stability.
▲ CRITICAL STEP This step assures that the cells will stably express GFP or RFP in the absence of antibiotic selection, which is the case *in vivo*.

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

24| To establish dual-color cancer cells, use clones of cancer cells expressing RFP in the cytoplasm at 70% confluence. Incubate RFP cancer cells, produced as described above (Steps 17–23), with the retroviral-containing medium supernatants of PT67 H2B-GFP cells and the above culture medium. To obtain the double transformants, incubate cells with hygromycin 48 h after transfection and select as described above (Step 16).

Visualization of cancer cells in live mice

25| A number of options are available for subcellular imaging of tumor cells in live mice following transduction of dual-color tumor cell lines. These options include real-time visualization of deformation of cancer cells in small vessels, real-time visualization of deformability of cancer cells in the brain and real-time imaging of nuclear-cytoplasmic dynamics of trafficking cancer cells. Cancer cell dynamics can also be visualized in the portal vein area (**Box 1**).

(A) Real-time visualization of deformation of cancer cells in small vessels in live mice.

(i) To visualize cell deformation in vessels in live mice, inject cells into the heart. To do this, anesthetize nude mice with a ketamine mixture (10 µl ketamine HCl, 7.6 µl xylazine, 2.4 µl acepromazine maleate and 10 µl H_2O) via s.c. injection. Inject a total of 200 µl of medium containing 5×10^6 dual-color cells into the heart.

(ii) To observe the shapes of the dual-color cancer cells within the microvessels, wire the epigastric cranialis vein of the mouse with a 6-0 suture before cell injection. Immediately after cell injection, make an arc-shaped incision in the abdominal skin, and then separate s.c. connective tissue to free the skin flap without injuring the epigastric cranialis artery and vein.

(iii) Spread and fix the skin flap on a flat stand. Image the inside surface of the skin flap directly.

(iv) During the interval between imaging, occasionally spray PBS on the inside of the skin flap to keep the surface wet. The skin flap can also be completely reversed.

(v) During the period of the measurement, keep the animal under anesthesia. Keep the animal warm and keep the abdominal skin flap hydrated with saline solution.

(vi) Take images at the initial time point. The image includes cells in their blood vessels as well as the surrounding vessels which are used as a map to relocate the cell in its vessel 2 h later when the next images are captured. Follow 10 to 20 cells in a given experiment.

(vii) Measure the lengths of the major and minor axes of the whole cells and the nuclei using Image ProPlus 3.1 software.

(B) Real-time visualization of deformability of dual-color cancer cells in the brain of live mice.

(i) To visualize cell dynamics in the brains of living nude mice, inject cells in the common carotid artery. To do this, first anesthetize the nude mice with the ketamine mixture via s.c. injection.

(ii) For carotid injection, make a longitudinal skin incision on the neck. After exposing the submandibular gland, cut in the middle and retract to each side.
▲ **CRITICAL STEP** Perform this and all remaining steps of the operation with a ×6.3–40 dissection microscope (MZ6; Leica) to ensure proper exposure of and injection into the carotid artery.

(iii) Separate the right sternohyoid muscle and right sternomastoideus muscle and connective tissue with a blunt instrument.

(iv) After isolation of the right common carotid artery, release the artery gently from surrounding connective tissue.

(v) Put light tension on the proximal site of the artery with a blunt-end hook.

(vi) Inject a total of 200 µl of medium containing 2×10^5 dual-color cells in the artery using a 33-gauge needle.

(vii) Immediately after injection, press the injection site with a swab to prevent bleeding or leakage of injected tumor cells.
▲ **CRITICAL STEP** Bleeding will interfere with imaging and therefore must be prevented. Leaking of cells must be prevented so that cells can be imaged in microvessels in brain.

(viii) Close the skin with a 6-0 suture.

(ix) To image the cells, make a skull skin-flap in the mouse. To do this, make an arc-shaped incision in the scalp, and separate s.c. connective tissue to free the skin flap.

(x) Re-open the skin flap repeatedly to image tumor cells in the brain through the nearly transparent mouse skull and simply close with a 6-0 suture. This procedure greatly reduces the scatter of fluorescent photons.

(C) Mouse model for imaging real-time nuclear-cytoplasmic dynamics of trafficking cancer cells.

(i) To image nuclear-cytoplasmic dynamics of trafficking cancer cells in live mice, inject dual-color cancer cells directly into the epigastric cranialis vein. To do this, first anesthetize nude mice with a ketamine mixture via s.c. injection.

(ii) To form a skin flap, make an arc-shaped incision in the abdominal skin.

(iii) Separate the subcutaneous connective tissue to free the skin flap without injuring the epigastric cranialis artery and vein.

(iv) Spread and fix the skin-flap on a flat stand.

BOX 1 | PORTAL VEIN INJECTION MODEL TO STUDY CANCER CELL VIABILITY

This procedure takes approximately 30 min to complete and can visualize cancer cell viability as well as nuclear and cytoplasmic dynamics in the portal vein (PV) area. Dynamics and viability can be compared with different cell lines. Drugs can be tested for their effects on the cancer cells and on the host's cancer-cell killing mechanism in the PV area[5].

1. Anesthetize the nude mice first with the ketamine mixture (described above) injected into the peritoneal cavity.
2. Inject human HCT-116-GFP-RFP colon cancer cells and mouse MMT-GFP-RFP mouse mammary tumor cells ($0.125 \sim 1.0 \times 10^6$ cells per 50 µl) in the PV of nude mice during open laparotomy. Viability is monitored by intactness of the cells such that the cytoplasm has not separated from the nucleus and lack of apoptosis. Cells appear to die in the PV area by loss of cytoplasm (clasmocytosis).
3. Cut and open the middle abdomen (minilaparotomy, 10–15 mm).
4. Locate the appendix and iliocolic vein in the mesentery, which joins to the supramesenteric vein (SMV) and PV.
5. Insert a 28G needle (0.5 cc insulin syringe) into the iliocolic vein under a fluorescence dissecting microscope and inject cancer cells carefully. Confirm the flow of injected cancer cells into the PV under fluorescence microscopy.
6. Withdraw the needle and press the insertion point for 20–30 s to stop bleeding.
7. Suture the abdomen using 6-0 sutures.

(v) Inject a total of 30 µl medium containing 5×10^5 dual color cells into the epigastric cranialis vein.
(vi) For imaging cancer cells in blood vessels, acquire images in real time.
(vii) For extravasation, acquire images every hour after injection with the skin flap open or every 12 h by opening and closing the skin flap. Image the inside surface of the skin-flap directly.

● TIMING

Steps 1–10: 2 weeks
Steps 11–13: 2 weeks
Steps 14–16: 2 weeks
Steps 17–23: ~2 weeks
Step 24: 6–8 weeks
Step 25: It takes approximately 30 min to prepare the animal for imaging including anesthesia, surgical procedures and cell injection. It takes only seconds to acquire images, which are visualized in real time on the computer screen.

? TROUBLESHOOTING

See **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Autofluorescence	Use of wrong filters	It is important to minimize autofluorescence of the tissue and body fluids by use of proper filters. Excitation filters should have a narrow band as close to 490 nm as possible to specifically excite GFP, whose peak is distinct from that of the tissue and fluid of the animal. In addition, proper band-pass emission filters should be used with a cut-off of approximately 515 nm (Chroma Technology).
Bleeding	Improper surgical procedures	Bleeding should be avoided at the surgical site since hemoglobin will absorb the incident excitation light.
Dehydration	Long-term procedures on an open animal	When using skin flap and other open procedures, it is essential to hydrate the animal by spraying normal saline on the open tissue.
Infection	Unclean instruments and environment	When using repeat procedures such as a skin-flap or other invasive procedures, it is critical to maintain a proper sterile operation field.

ANTICIPATED RESULTS

The procedures described in these protocols enable subcellular imaging of fluorescent protein–labeled cells in live mice. **Figure 1** demonstrates the whole-body imaging of mitotic dual color HT-1080 human fibrosarcoma cells, labeled with GFP in the nucleus and RFP in the cytoplasm. **Figure 2** demonstrates the various shapes that the dual-color HT-1080 cells can take in variously sized vessels. Note the extreme deformation of the cancer cells, especially the cytoplasm. **Figure 3** demonstrates migration of deformed dual-color HT-1080 cancer cells in very small diameter vessels. Note the very tight turns the migrating cancer cells

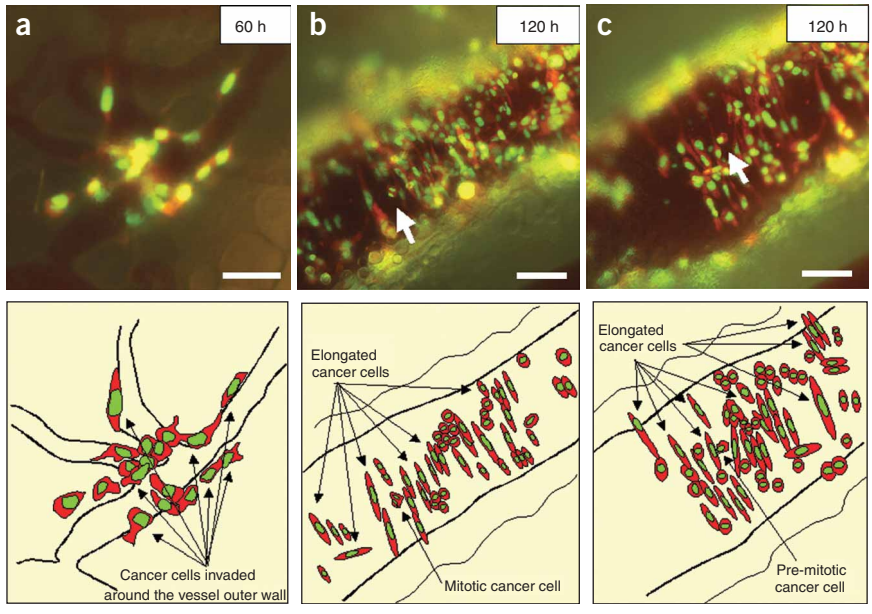

Figure 8 | Imaging of extravasation, invasion and proliferation of Lewis lung carcinoma (LLC) GFP-RFP cells. (**a**) LLC cells are observed to proliferate on the vessel outer wall 60 h after injection. (**b**,**c**) Extravasated LLC cells wind around a large vessel 120 h after injection. Most of the cells and nuclei are elongated with the major axes of the cells reaching 100 µm. Mitotic and premitotic cancer cells round up and nuclei are condensed (arrows). Scale bar, 100 µm. Images were acquired with the Olympus OV100. Bottom, schematics of **a**–**c**. Image from ref. 4.

can make. **Figure 4** shows the rapid loss of viability of dual-color HCT-116-GFP-RFP cancer cells after injection in the portal vein of nude mice. Note the loss of RFP-expressing cytoplasm in the dying HT-1080-GFP-RFP cells. **Figure 5** shows opposite behavior of dual-color mouse mammary tumor cells double labeled with GFP and RFP. These cells survive after injection in the portal vein and can form colonies in the liver. **Figure 6** shows trafficking of HT-1080-GFP-RFP cells in the vessel in a skin flap of a nude mouse. Note the variety of trafficking and arrest patterns of the cancer cells. **Figure 7** shows extravasating MMT-GFP-RFP cells. Note that the cells first protrude their cytoplasm as they exit the vessel and that the nucleus follows in the cytoplasmic protrusion. **Figure 8** shows that recently extravasated cells wrap around vessels and proliferate, a new concept for the role of blood vessels in metastasis. These results demonstrate the possibility of studying cell biology in the live animal and for developing new visible drug targets for the various processes of metastasis.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests (see the html version of this article for details).

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