# Imaging Nuclear - Cytoplasm Dynamics of Cancer Cells in the Intravascular Niche of Live Mice

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**Abstract.** *We have previously shown that cancer cells can form an intravascular niche where they can proliferate and undergo apoptosis as well as traffic and extravasate. In the present study, green fluorescent protein (GFP) was expressed in the cytoplasm of HT-1080 human fibrosarcoma cells, and red fluorescent protein (mCherry), linked to histone H2B, was expressed in the nucleus to further investigate intravascular cancer cell nuclear-cytoplasmic dynamics. Nuclear mCherry expression enabled visualization of nuclear dynamics, whereas simultaneous cytoplasmic GFP expression enabled visualization of nuclear-cytoplasmic ratios as well as simultaneous cell and nuclear deformation. Cancer cells were injected in the epigastric cranialis vein in an abdominal flap of nude mice to enable subcellular in vivo imaging. The cell cycle position of individual living cells was readily-visualized by the nuclear-cytoplasmic ratio and nuclear morphology. Real-time induction of apoptosis was observed by nuclear size changes and progressive nuclear fragmentation. Intra- and extra-vascular mitotic cells were visualized by imaging. One hour after cell injection, round and elongated cancer cells were observed in the vessels. Three hours after injection, invadopodia-like structures of the cancer cells were observed. Five hours after injection, dual-color cancer cells began to divide within the vessel. By 10 h, some intravascular cancer cells underwent apoptosis. Deformed new blood vessels in the tumor were observed 10 days later. Extravascular cancer cells were imaged dividing in the tumor at day 14 after injection. The subcellular in vivo imaging approach described in the present report provides new visual targets for trafficking and proliferating intravascular cancer cells as well as extravasating and invading cancer cells.*

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Intravascular cancer cells are critical in the metastatic process (1-5). We have previously demonstrated many aspects of the dynamics of intravascular cancer cells. We showed that, in order to traffic in narrow blood vessels such as capillaries, cancer cells deform. To visualize the cytoplasmic and nuclear dynamics of cancer cells migrating in capillaries, red fluorescent protein (RFP) was expressed in the cytoplasm, and green fluorescent protein (GFP), linked to histone H2B, was expressed in the nucleus. Highly elongated cancer cells and nuclei in capillaries were imaged *via* a skin flap in living mice. The cells and their nuclei in the capillaries elongated in order to migrate in vessels 8 μm in diameter and could migrate up to 48.3 μm/h (4).

In larger blood vessels, the nuclear and cytoplasmic behavior of dual-color cancer cells was imaged in real time as the cancer cells migrated or adhered to the inner vessel surface. During extravasation, dual-color imaging demonstrated that cytoplasmic processes of the cancer cells exited the vessels first, with nuclei following along the cytoplasmic projections. Both cytoplasm and nuclei underwent deformation during extravasation. After extravasation, some of the cancer cells adhered and proliferated on the outer vessel surface (5).

When dual-color human colon cancer and mouse mammary tumor cells were injected in the portal vein of nude mice, extensive clasmocytosis (destruction of the cytoplasm) of the human colon cancer cells occurred within 6 hours. Apoptosis was readily-visualized in the dual-color cells by their altered nuclear morphology. In contrast, dual-color mouse mammary cancer cells injected into the portal vein mostly survived in the liver of nude mice 24 h after injection. Many surviving mouse mammary cancer cells showed invasive figures with cytoplasmic protrusions. The cells grew aggressively and formed colonies in the liver. However, when the host mice were pre-treated with cyclophosphamide, the human colon cancer 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 human colon cancer cells, but could not effectively kill the mouse mammary cancer cells (6). Dual-color human fibrosarcoma cells when injected into the epigastric cranialis vein of nude mice rapidly died. However, cyclophosphamide pre-treatment also resulted in intravascular proliferation, extravasation, and extravascular colony formation of the human fibrosarcoma cells (7).

In another previous study, we observed that prior to the detection of extra-vascular metastases, human prostate cancer cells resided initially inside the blood vessels of the liver and the lung, where they proliferated and expressed Ki-67 and exhibited matrix metalloprotenase (MMP) activity. Extravasation occurred earlier in the lung than in the liver (8).

In the present study, human fibrosarcoma cells labeled in the nucleus with histone H2B-mCherry and retroviral GFP in the cytoplasm were injected in the epigastric cranialis vein of nude mice. Real-time nuclear-cytosplasmic dynamics were imaged by confocal microscopy in live mice *via* a skin flap (9) demonstrating diverse behavior of intravascular-residing cancer cells.

## Materials and Methods

*GFP and histone H2B-mCherry transduction of fibrosarcoma cells.* For GFP and H2B-mCherry transduction, 70% confluent human HT-1080 fibrosarcoma cells were used. To establish dual-color cells, clones of HT-1080 expressing GFP in the cytoplasm (HT-1080-GFP) were initially established. In brief, HT-1080 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-GFP cells and RPMI 1640 (Irvine Scientific, Santa Ana, CA, USA) containing 10% fetal bovine serum for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h post-transduction and sub-cultured at a ratio of 1:15 into selective medium, which contained 200 μg/ml G418. The level of G418 was increased stepwise up to 800 μg/ml. HT-1080-GFP cells were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) using trypsin/EDTA and amplified by conventional culture methods. For establishing dual-color cells, HT-1080-GFP cells were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 H2B-mCherry 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 (10-12).

*Cell culture.* Dual-color HT-1080 cells, expressing GFP in the cytosplasm and mCherry in the nucleus, were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine from Gibco-BRL, Life Technologies Inc. (Grand Island, NY, USA). All media were supplemented with penicillin and streptomycin (Gibco-BRL). The cell line was cultured at 37˚C with 5% $CO_2$.

*Mice.* Nude (*nu/nu*) mice (AntiCancer Inc., San Diego, CA, USA) were bred and maintained in a HEPA-filtered environment with cages, food, and bedding sterilized by autoclaving. The animal diets were obtained from Harlan Teklad (Madison, WI, USA). Ampicillin (5.0%, w/v; Sigma, St. Louis, MO, USA) was added to the autoclaved drinking water. All surgical procedures and imaging were performed with the animal anesthetized by intramuscular injection of 0.02 ml of a solution of 50% ketamine, 38% xylazine and 12% acepromazine maleate (Butler-Schein, Dublin, OH, USA). All animal studies were conducted in accordance with the principles of and procedures outlined in the NIH guide for the care and use of laboratory animals under assurance number A3873-1.

*Real-time visualization of HT-1080 dual-color cells in vessels in live mice.* To visualize cell dynamics in vessels in live mice, cells were injected into the epigastric cranialis vein in an abdominal flap (5). Nude mice were anesthetized with the ketamine mixture *via s.c.* injection. HT-1080-dual-color cells ($2\times10^5$) were injected into the epigastric cranialis vein. A skin flap was spread and fixed on a flat stand. The inside surface of the skin flap was directly observed with the Olympus Fluovivo FV1000 confocal microscope (13). HT-1080-dual-color cells were imaged at 1, 3, and 10 h to determine the migration of the cancer cells. During the interval between imaging sessions, 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. The skin was then closed with a 6-0 suture. The skin flap could be opened repeatedly to image HT-1080 cancer cells.

## Results and Discussion

*Real-time visualization of mitosis and apoptosis of HT-1080-GFP-mCherry cells in vitro.* The selected HT-1080 dual-color cells have bright GFP and RFP fluorescence *in vitro* (Figure 1A). Red fluorescence was localized in the nuclei; green fluorescence was localized in the cytoplasm. All cells in the population expressed both GFP and mCherry, indicating stability of both transgenes (Figure 1A). Figure 1B shows a series of images of a single HT-1080 GFP-mCherry cell during mitosis at 5-min intervals. A metaphase cell progressing through anaphase and cytokinesis was visualized by the mCherry-expressing nucleus and GFP-expressing cytoplasm in real time. Apoptotic HT-1080 dual-color cells with fragmented nuclei were visualized (Figure 1C). Progressive fragmentation of a nucleus of a single cell was observed in real time in images taken every 5 minutes (Figure 1C).

*Imaging of proliferating intravascular cancer cells.* One hour after injection in the epigastric cranialis vein of an abdominal flap, the majority of the HT-1080 GFP-mCherry cells remained in blood vessels without extravasation. The vessels expanded, apparently in order to maintain blood flow. The dual-color cancer cell observed in a large vessel were rounded and the nucleus was oval (Figure 2A, left panel). HT-1080 cells in a small-diameter capillary deformed to fit in the capillary (Figure 2A, right panel). HT-1080 dual-color cells were observed in microvessels in live mice 3 h after cell injection. The vessel was filled with HT-1080 cells. The HT-1080 cells migrated along the inner vessel wall (Figure 2B, left panel). At high magnification, cellular protusions could be seen that appeared to be invadopodia (right panel). The

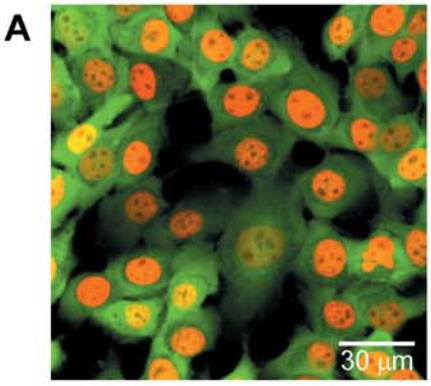
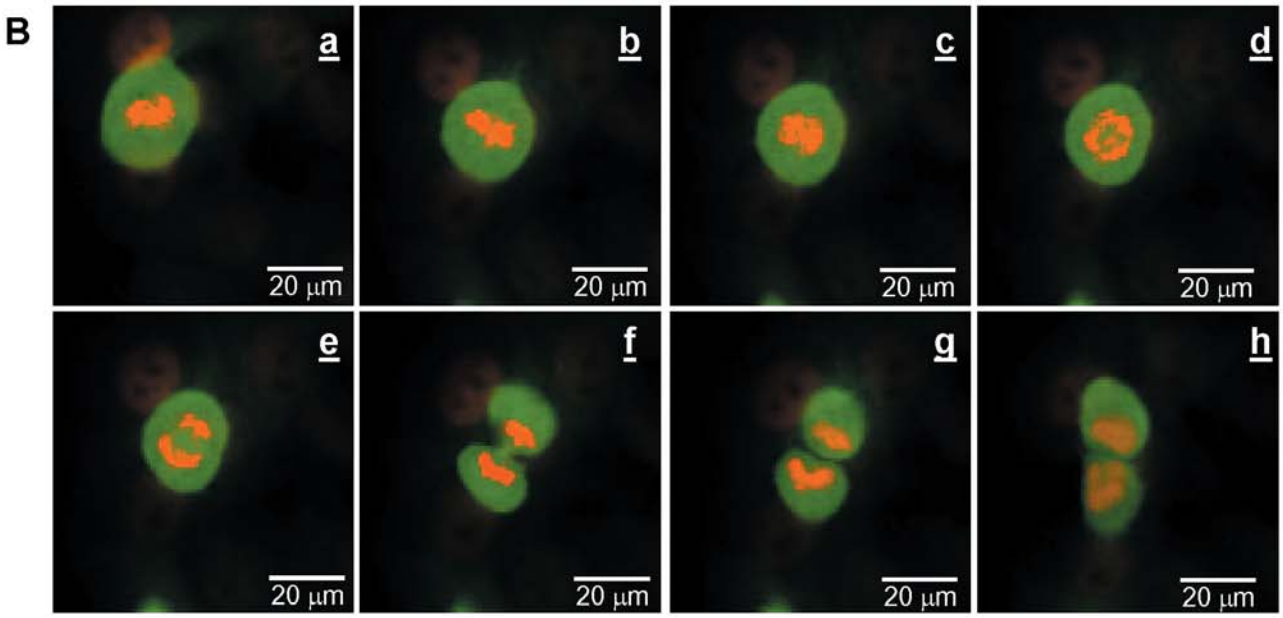
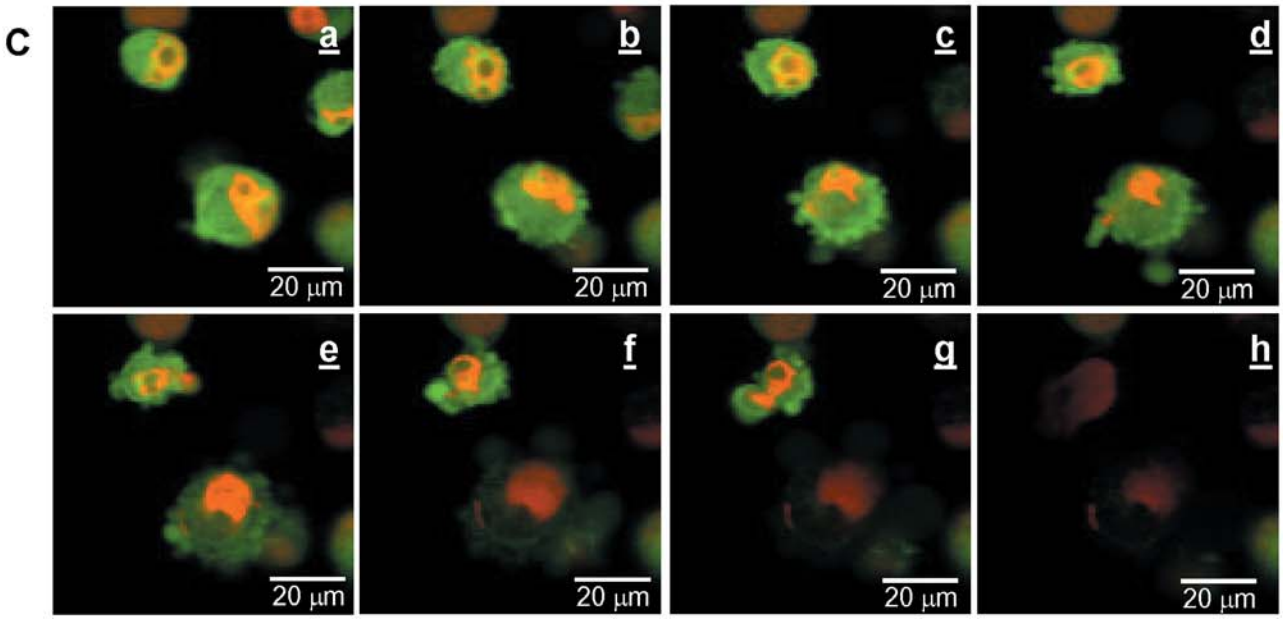
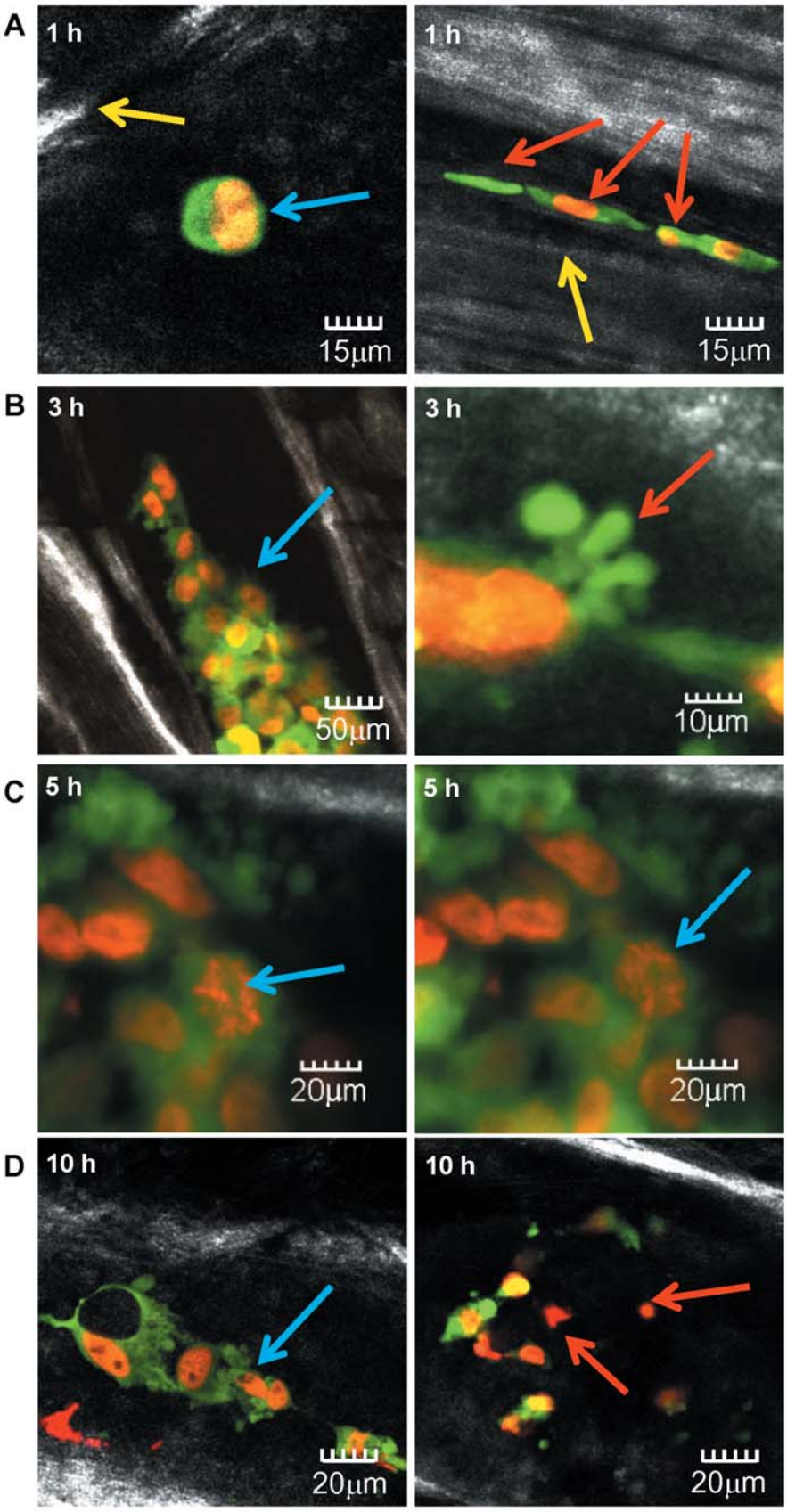



Figure 1. *Stable high GFP-mCherry-expressing dual-color HT-1080 human fibrosarcoma cells in vitro. Cancer cells were initially transduced with GFP and the neomycin-resistance gene. The cells were subsequently transduced with histone H2B-mCherry and the hygromycin-resistance gene. Double transformants were selected with G418 and hygromycin, and stable clones were established. See Materials and Methords for details. (A) HT-1080 dual-color human sarcoma cancer cells (Bar=30 μm). (B) Visualization of time course of mitosis in vitro. HT-1080-dual-color cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Images were captured from the same cell under FV1000 confocal microscopy at various time points: A, 0 time; B, 5 min; C, 10 min; D, 15 min; E, 20 min; F, 25 min; G, 30 min; H, 35 min (Bar=20 μm). (C) Visualization of time course of apoptosis in vitro. Real-time high magnification images of apoptotic HT-1080 dual-color cells. A, 0 time; B, 5 min; C, 10 min; D, 15 min; E, 20 min; F, 25 min; G, 30 min; H, 35 min (Bar=20 μm).*

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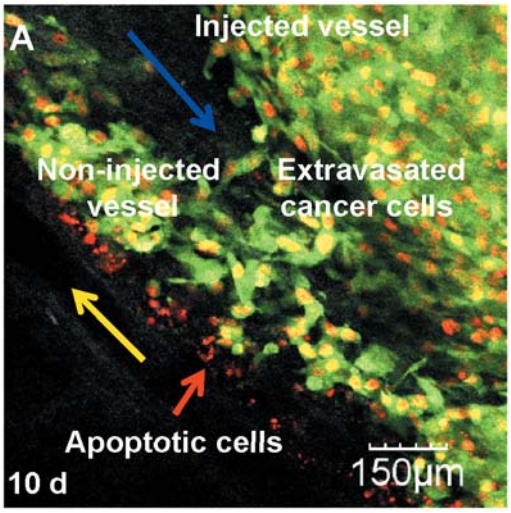


dual-color cancer cells began to divide within the vessel 5 h after cell injection (Figure 2C). Some cancer cells attached to the inner vessel wall. By 10 h, some intravascular cancer cells underwent apoptosis (Figure 2D). Nuclear fragments expressing mCherry were observed in the vessel (Figure 2D).

*Imaging of intravascular and extravascular cancer cells*. Within 10 days after cell injection in the epigastric cranialis vein, HT-1080 cells extravasated and subsequently grew around the outer surface of the blood vessels (Figure 3A). The colonies outside of the vessels continued to proliferate and increase in size. Blood flow in the injected vessel was observed (Figure 3A).

← Figure 2. *Imaging of dual-color HT-1080 cells in a live mouse. To visualize cancer cell dynamics in vessels in live mice, dual-color HT-1080 cells were injected in the epigastric cranialis vein in an abdominal skin flap. (A) Imaging of dual-color HT-1080 cells in small and large blood vessels 1 h after cell injection. A dual-color HT-1080 cell in a large vessel is round and the nucleus is oval (left panel). The cells and nuclei deformed by elongation to fit in a small-diameter capillary (right panel) (Bar=15 μm). Yellow arrow indicates the blood vessel wall. Blue arrow indicates a single rounded HT-1080 dual-color cell. Red arrows indicate elongated HT-1080 dual-color cells. (B) Imaging of HT-1080 dual-color cells in a microvessel in live mice 3 h after cell injection. The vessel is filled with HT-1080 dual-color cells. Blue arrow indicates embolus of HT-1080 cells (Bar=50 μm) (left panel). Red arrow indicates cells that have protrusions that appear to be invadopodia (Bar=10 μm) (right panel). (C) Images of a mitotic HT-1080 cell in a vessel 5 h after cell injection. Blue arrows indicate dividing HT-1080 cells (Bar=20 μm) (left and right panels). (D) Image of attached HT-1080 dual-color cells on the inner vessel wall (blue arrow) (Bar=20 μm) (left panel). By 10 hours, some intravascular cancer cells underwent apoptosis (Bar=20 μm). Red arrow indicates apoptotic HT-1080 nuclear fragments (right panel).*

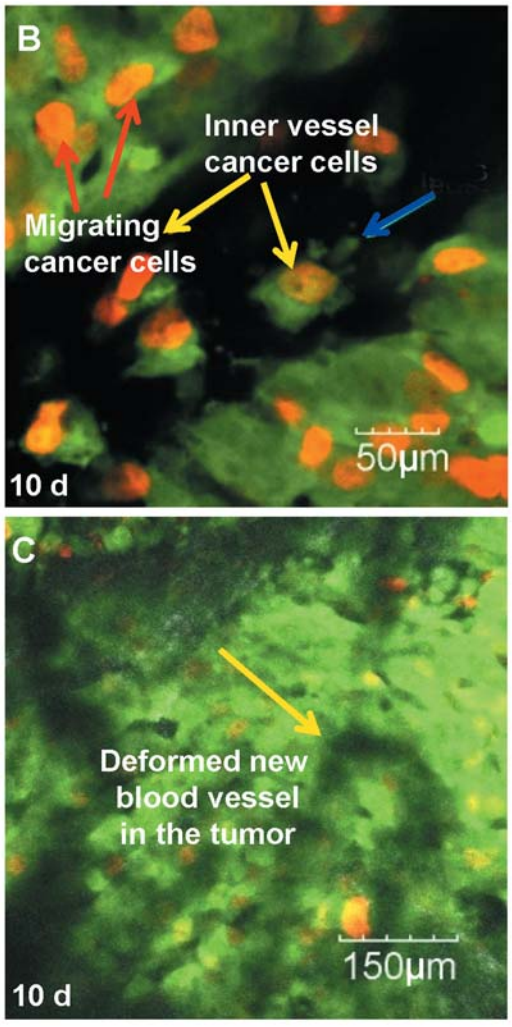


Figure 3. *Image of intra- and extra-vascular HT-1080 dual-color cells 10 days after injection. (A) Extravasated cancer cells were observed around the injected vessel 10 days after injection. mCherry-expressing fragmented nuclei of apoptotic HT-1080 cancer cells were observed at the edge of the tumor (Bar=150 μm). Blue arrow indicates blood flow of the injected vessel. Red arrow indicates apoptotic cells. Yellow arrow indicates blood flow of another vessel. (B) HT-1080 dual-color cells migrated along the inner vessel wall (Bar=50 μm). Yellow arrows indicate intravascular HT-1080 dual-color cells. Blue arrow indicates cancer cell protrusions. Red arrows indicate migrating HT-1080 cancer cells. (C) Deformed new vessels in the tumor were observed 10 days later (yellow arrow) (Bar=150 μm).*

Figure 3B shows intravascular HT-1080 cells. A deformed new blood vessel in the tumor was observed (Figure 3C).

Extra-vascular cancer cells were observed dividing in the tumor at day 14 (Figure 4A). HT-1080 cells were observed in the tumor at the single-cell level using confocal microscopy (Figure 4A). Figure 4B shows a series of images of a single extravasated HT-1080 GFP-RFP cell during mitosis at 2-min intervals in the tumor.

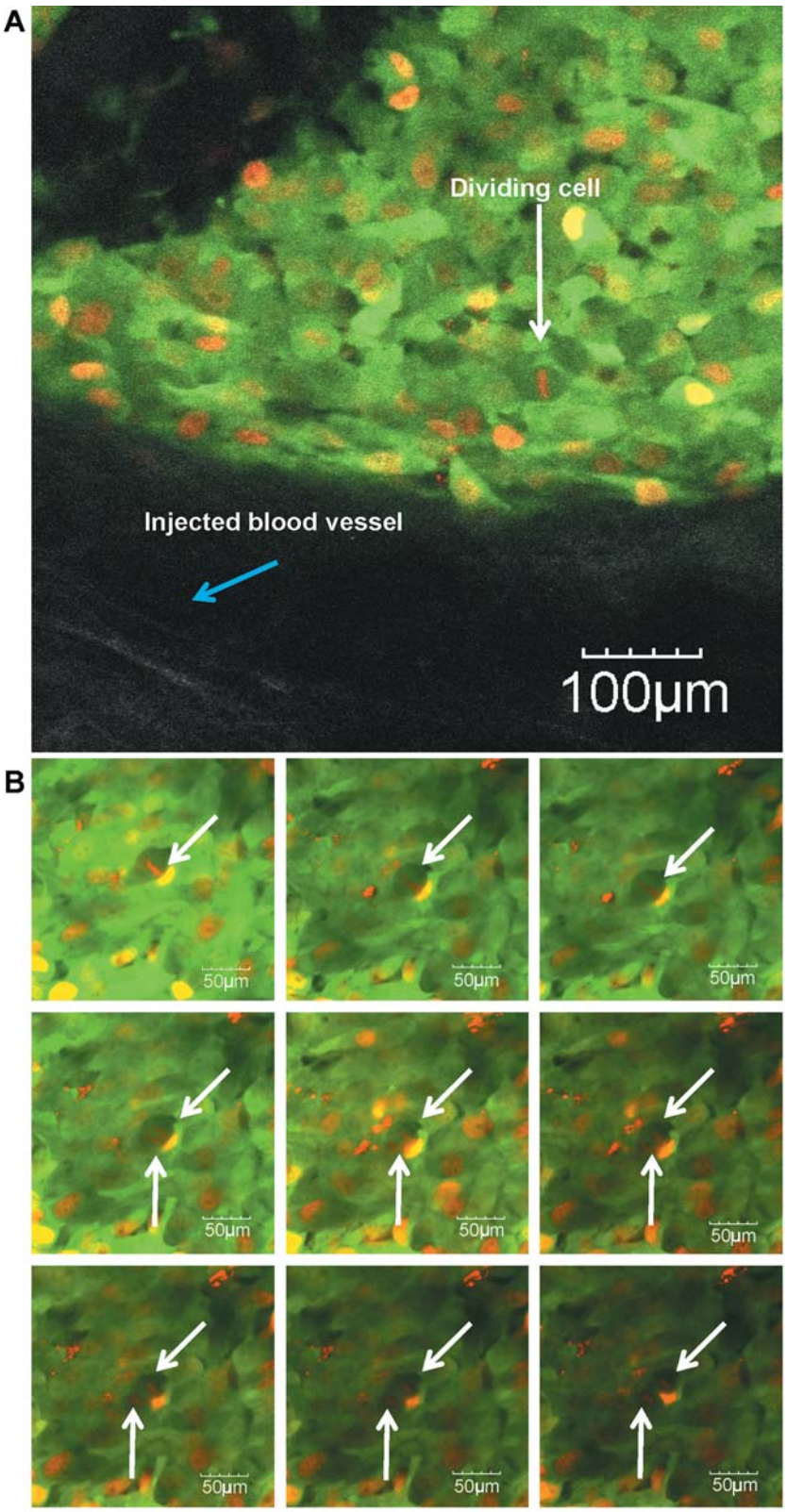


Figure 4. *Extra-vascular HT-1080 dual-color cells were dividing in the tumor on day 14 (Bar=100 μm). (A) Single HT-1080 dual-color cells were observed in the tumor. White arrow indicates dividing HT-1080 dual-color cell. (B) HT-1080 dual-color cell was dividing in the tumor (white arrows) (Bar=50 μm). Blue arrow is injected blood vessel.*

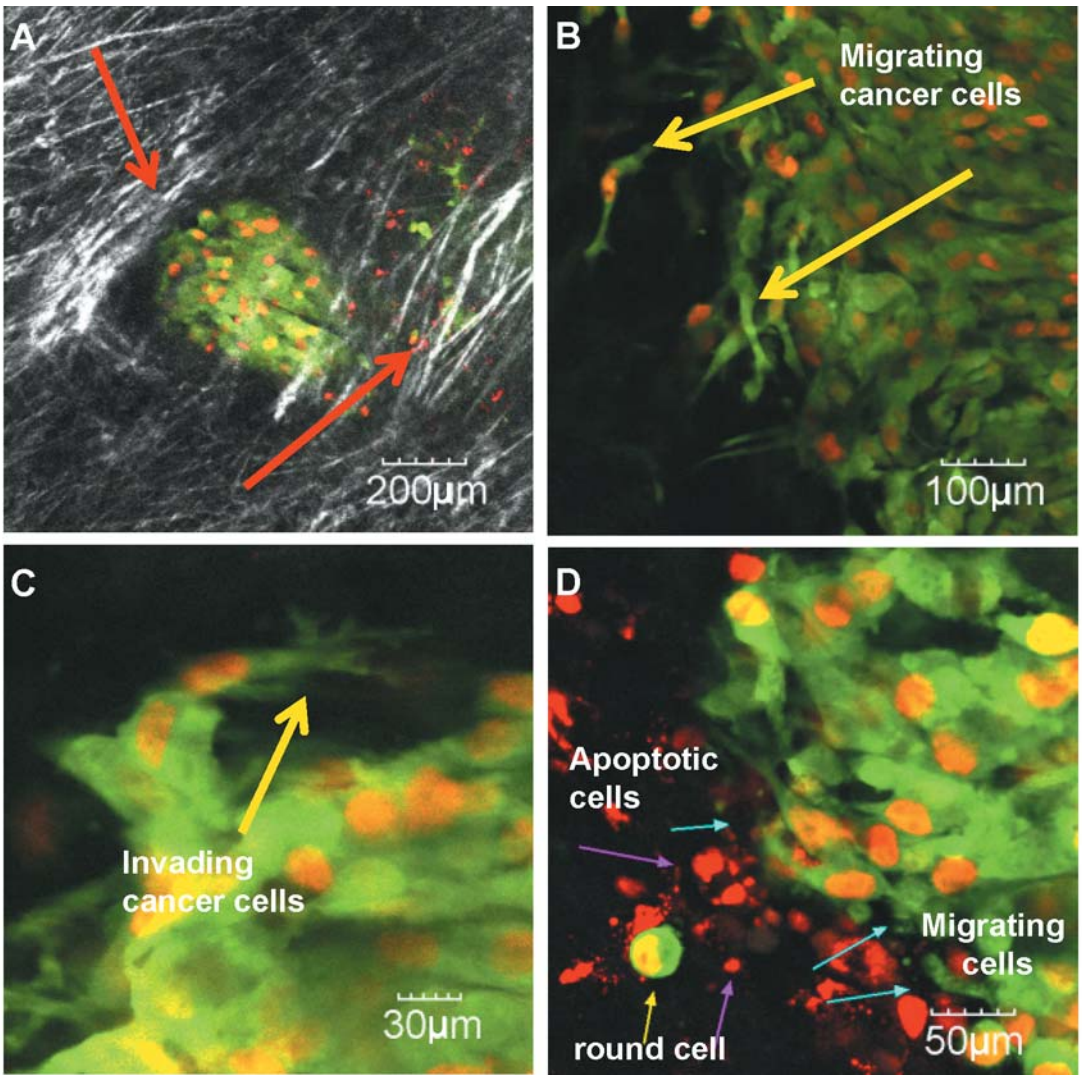


Figure 5. *An HT-1080 tumor formed by day 21. (A) Red arrows indicate collagen fibers in the tumor (Bar=200 μm). (B) HT-1080 dual-color cells invaded the skin tissue (yellow arrow) (Bar=100 μm). (C) Yellow arrow indicates invading HT-1080 dual-color cells (Bar=30 μm). (D) mCherry-expressing fragmented nuclei (purple arrows) of apoptotic HT-1080 dual-color cells were observed at the edge of the tumor where these were also migrating cancer cells (blue arrows) (Bar=50 μm). A rounded HT-1080 dual-color cell was observed (yellow arrow).*

An HT-1080 tumor was formed in the mice by day 21. HT-1080 cancer cells invaded the skin tissue. Collagen fibers were observed in the tissue (Figure 5A). Invading cancer cells were observed at the edge of tumor (Figure 5B). Invading HT-1080 cells were observed in the tumor at the single cell level using confocal microscopy (Figure 5C). Fragmented nuclei of apoptotic HT-1080 cells were observed around invading cancer cells at the edge of the tumor (Figure 5D). A single rounded cell was observed near apoptotic cells (Figure 5D).

The dual-color cancer cells and the Olympus FV1000 confocal microscope provide very high resolution of nuclear and cytoplasmic dynamics during intravascular cancer cell trafficking, proliferation, apoptosis, extravasation and post-extravasation proliferation. The cell imaging techniques described here will enable future studies to further understand the mechanisms of cancer invasion and metastasis at the cellular and subcellular levels *in vivo*.

Angiotropic extravascular migratory metastasis (EVMM) (14) is where cancer cells migrate along vessels such as observed in this report and as previously (4, 5). Angiotropism has been shown to be a prognostic factor for melanoma metastasis (14).

Cell migration through extracellular matrices *in vitro* has been shown to depend on cell deformability, in particular deformation of the nucleus (15). Our current and previous results suggest nuclear deformation is important in cell migration *in vivo* (4, 5).

## Conflicts of Interest

None of the Authors have any conflict of interest in regard to this study.

## Acknowledgements

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