

# Imaging Nuclear–Cytoplasmic Dynamics in Primary and Metastatic Colon Cancer in Nude Mice

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**Abstract.** *Background:* Colon cancer frequently results in metastasis to the liver, where it becomes the main cause of death. However, the cell cycle in primary tumors and metastases is poorly understood. *Materials and Methods:* We developed a mouse model of liver metastasis using the human colon cancer cell line HCT-116, which expresses green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in the cytoplasm (HCT-116-GFP-RFP). HCT-116 GFP-RFP cells were injected into the spleen of nu/nu nude mice. *Results:* HCT-116-GFP-RFP cells subsequently formed primary tumors in the spleen, as well as metastatic colonies in the liver and retroperitoneum by 28 days after cell transplantation. Using an Olympus FV1000 confocal microscope, it was possible to clearly image mitosis of the dual-colored colon cancer cells in the primary tumor as well as liver and other metastases. Multi-nucleate cancer cells, in addition to mono-nucleate cancer cells and their mitosis, were observed in the primary tumor and metastasis. Multi-nucleate HCT-116-GFP-RFP cells were also observed after culture of the primary and metastatic tumors. A similar ratio of mono-nucleate, multi-nucleate, and mitotic cells grew from the primary and metastatic tumors in culture, suggesting similarity of the nuclear–cytoplasmic dynamics of primary and metastatic cancer cells, further emphasizing the stochastic nature of metastasis. *Conclusion:* Our results demonstrate a similar heterogeneity of nuclear–cytoplasmic

dynamics within primary tumors and metastases, which may be an important factor in the stochastic nature of metastasis.

Colon cancer is the third most frequent cancer-related cause of death in the world (1). Colon cancer frequently results in metastasis to the liver, which becomes the primary cause of death in many cases (2, 3). The main pathway of colon cancer liver metastasis is *via* the portal vein, but the detailed nuclear–cytoplasmic dynamics of cancer cells in primary and metastatic colon cancer is incompletely understood (4).

To enable imaging of nuclear–cytoplasmic dynamics *in vivo*, a library of dual-color fluorescent cancer cells with green fluorescent protein (GFP), linked to histone H2B and expressed in the nucleus, and red fluorescent protein (RFP) expressed in the cytoplasm was previously genetically engineered (5-7). Nuclear GFP expression enables visualization of nuclear behavior, whereas simultaneous cytoplasmic RFP expression enables visualization of cytoplasmic behavior. Thus, total nuclear–cytoplasmic dynamics can be visualized at high resolution, including of individual chromosomes in some cases, in living dual-color cells in real time (8).

We previously reported that approximately 90% of cancer cells in center and 80% of total cells of an established tumor are in the G<sub>0</sub>/G<sub>1</sub> phase. Cytotoxic agents killed only proliferating cancer cells at the surface and, in contrast, had little effect on quiescent cancer cells, which are the vast majority of an established tumor. The results suggest why most drugs currently in clinical use, which target cancer cells in S, G<sub>2</sub> and M phases, are mostly ineffective on solid tumors (9).

We also previously observed that cancer cells in G<sub>0</sub>/G<sub>1</sub> phase migrated more rapidly and further than cancer cells in S, G<sub>2</sub> or M phases. Cancer cells ceased migrating when they entered S, G<sub>2</sub> or M phases and resumed migrating after cell division when the cells re-entered G<sub>0</sub>/G<sub>1</sub> phases. Migrating cancer cells were also resistant to cytotoxic chemotherapy, since they were preponderantly in G<sub>0</sub>/G<sub>1</sub> phase, where cytotoxic chemotherapy is not effective (10).

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*Key Words:* Dual color cancer cells, GFP, RFP, mitosis, colon cancer, nude mice, liver metastasis, imaging.

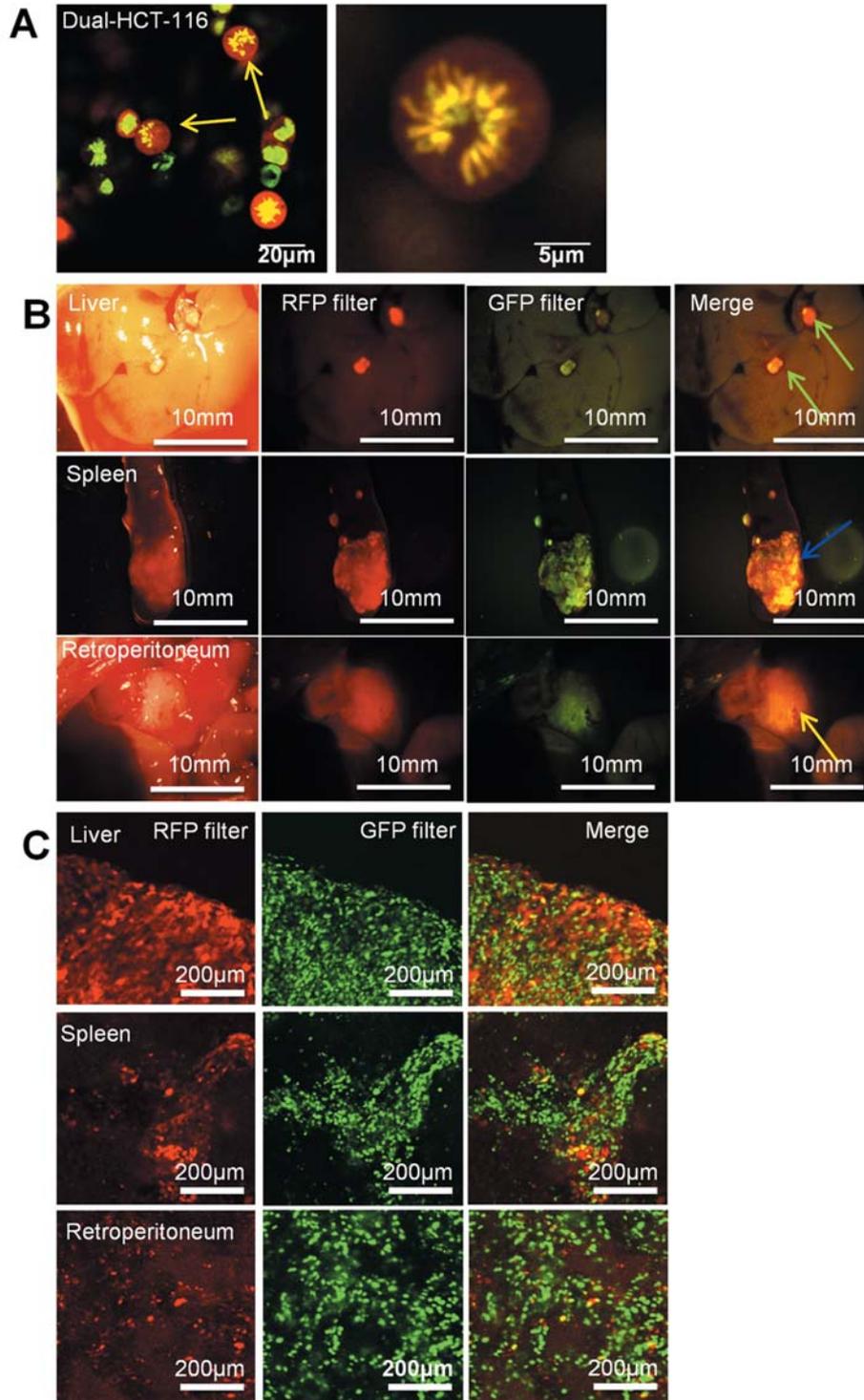


Figure 1. A: Left panel: Low-magnification images of dual-color HCT-116 colon cancer cells expressing green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in the cytoplasm in vitro (Bar=20  $\mu$ m). HCT-116 dual-color cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin and streptomycin. Yellow arrows indicate mitotic cells. Right panel: High-magnification images of a mitotic HCT-116 dual-color cell. Metaphase chromosomes are clearly visualized (Bar=5  $\mu$ m). Images of live cells were captured with an Olympus FV1000 confocal microscope. B: Bright-field and fluorescence low-magnification images of primary and metastatic tumors in nude mice. Green arrows indicate liver metastasis. Blue arrows indicate splenic tumor (injection site). Yellow arrow indicates retroperitoneal metastasis (Bar=10  $\mu$ m). All images were captured with an Olympus SZX7 microscope. C: High-magnification images of dual-color HCT-116 cells in each organ. Upper panel: Liver metastasis. Middle panel: Splenic tumor. Lower panel: Retroperitoneal metastasis.

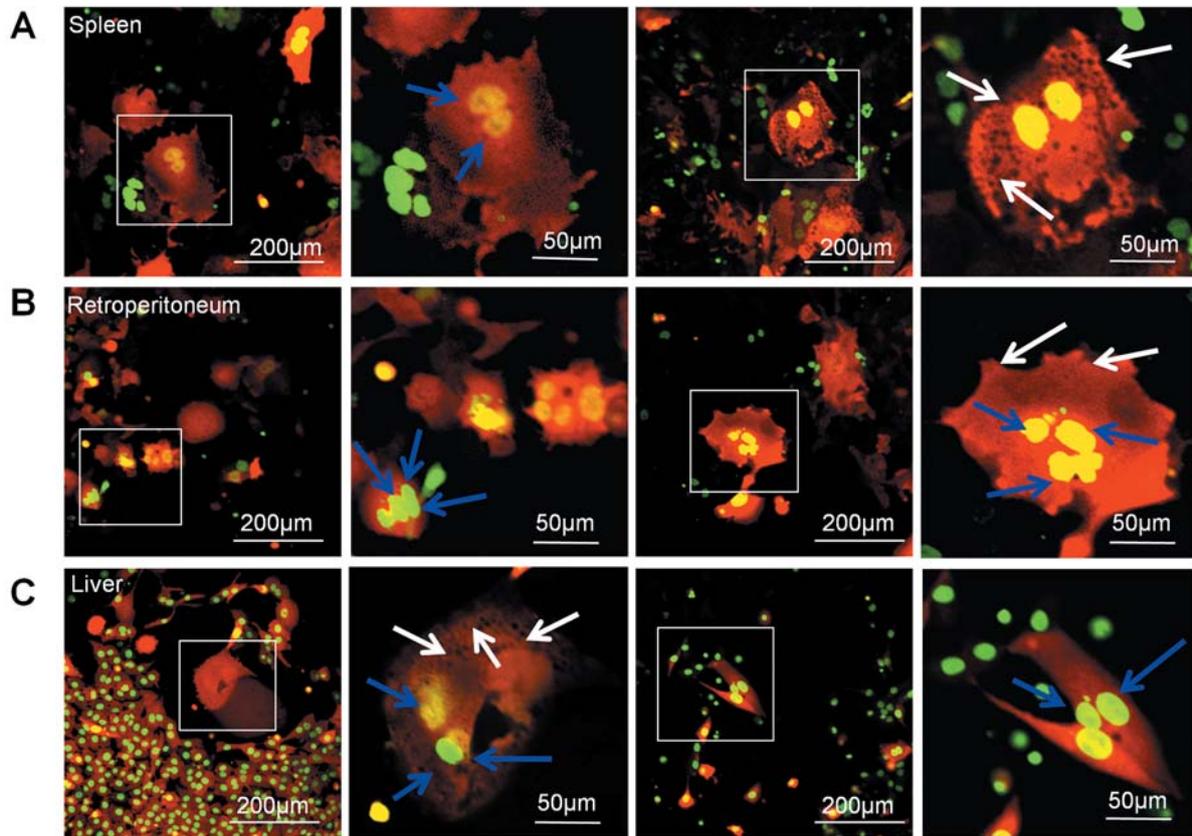


Figure 2. High-magnification images of cells cultured from tumors in each organ. A: Liver metastasis. B: Splenic tumor. C: Retroperitoneal metastasis. In each tissue, mitotic cells and multi-nucleated cells were observed. The inset contains multi-nucleated cells with high-magnification images of these cells shown in the adjacent panel. Blue arrows indicate multi-nucleated cells. White arrows indicate vacuolar degeneration. Multi-nucleated cells suggest the possibility of cancer-cell fusion. Images were captured with an Olympus FV1000 confocal microscope.

In the present study, we developed a nude-mouse model of liver metastasis using a human colon cancer cell line (HCT-116) which expresses GFP in the nucleus and RFP in the cytoplasm (HCT-116-GFP-RFP). Using dual color-coded confocal imaging, we analyzed nuclear–cytoplasmic dynamics in the HCT-116 primary tumor and metastases.

## Materials and Methods

**Cell line and culture conditions.** The metastatic human colon cancer cell line HCT-116 (11-14) was engineered to express GFP linked to histone H2B in the nucleus and RFP in the cytoplasm (HCT-116-GFP-RFP) (5-7, 10-15). The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin (Gibco-BRL). The cell line was cultured in an incubator at 37°C with 5% CO<sub>2</sub>, 95% air.

**Colon cancer metastasis model.** Six-week-old nude mice were used as the host. HCT116-GFP-RFP cells were harvested by trypsinization and washed three times with cold serum-free RPMI-1640 medium, then re-suspended in the serum-free medium. HCT-116-RFP-GFP

cells ( $2.0 \times 10^6$ ) were then injected in the spleen of nude mice. The cells subsequently formed tumors in the liver and retroperitoneum by 28 days after cell transplantation.

**Tumor imaging.** An SZX7 microscope, and FV1000 confocal microscope, both from Olympus Corp. (Tokyo, Japan), were used for intravital and *ex vivo* imaging.

**Study approval.** All experiments were conducted in accordance with the institutional guidelines of Gifu University and were approved by the Animal Research Committee and the Committee on Living Modified Organisms of Gifu University (approval number 26-37).

## Results and Discussion

**Real-time visualization of nuclear–cytoplasmic dynamics *in vitro*.** Figure 1A shows a low-magnification image of cultured dual-color HCT-116-RFP-GFP cells captured by FV1000 confocal microscopy. Figure 1B shows a high-magnification image of HCT-116 dual-color cells during mitosis, with individual metaphase chromosomes clearly visualized.

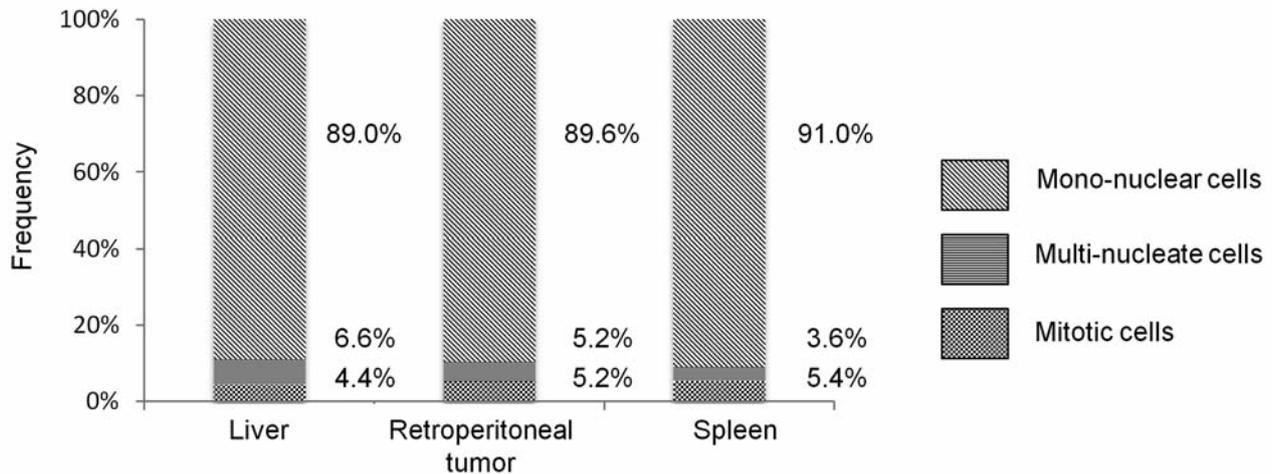


Figure 3. Five randomly-selected, low-magnification visual fields were quantified for the number of mono-nucleate, mitotic, and multi-nucleated HCT-116 cancer cells. The frequency of each cell type in the liver metastasis, spleen tumor, and retroperitoneal metastasis was plotted in the bar graphs. There were no significant differences in the ratios of the cell types between the different tumor sites.

**Nuclear–cytoplasmic primary and metastatic tumor growth.** Dual-color colon cancer cells were injected into the spleen of *nu/nu* mice. Dual-color-expressing tumors were observed in the spleen, retroperitoneum, and the liver by day 28 (Figure 1B). Tumor size in the liver was larger than in the spleen and retroperitoneum, Dual-color-expressing cancer-cell density in the liver metastasis was also higher than in the spleen and retroperitoneum, as the spleen and retroperitoneum contained fewer HCT-116-RFP-GFP cells (Figure 1C).

**Imaging of cancer cell dynamics ex vivo.** To compare cell-cycle dynamics and morphology of HCT-116-GFP-RFP tumors in the various organ sites, tumors were resected and cultured. Figure 2 shows images of cultured HCT-116-GFP-RFP cells from each organ. Very large ruffled cytoplasm with multiple cavities and multi-nucleated cells were observed in tumor cultures from each tumor-bearing organ (Figure 2A-C). Spindle-type cancer cells were observed only in the liver metastasis (Figure 2C, right panel). These 'abnormal' cancer cell types were not observed in the original HCT-116-GFP-RFP cell cultures before implantation in mice. To compare the primary and metastatic tumors, we counted the number of mono-nucleate cancer cells, multi-nucleated cancer cells, and mitotic cells in the tumors from each organ. There were no significant differences in the ratio of mono-nucleate cancer cells, multi-nucleated cancer cells, and mitotic cancer cells between the primary and metastatic tumors. The presence of spindle-shaped cancer cells in the liver metastasis may suggest stem-like cells as previously seen in the XPA1 pancreatic cancer cell line (16). The presence of multinucleated cancer cells suggests possible cell fusion (17).

The similarity of nuclear–cytoplasmic dynamics of the primary and metastatic tumors of HCT-116-GFP-RFP further emphasizes the stochastic nature of metastasis.

**Conflicts of Interest**

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

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