

Dynamic Subcellular Imaging of Cancer Cell Mitosis in the Brain of Live Mice

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Abstract. *The ability to visualize cancer cell mitosis and apoptosis in the brain in real time would be of great utility in testing novel therapies. In order to achieve this goal, the cancer cells were labeled with green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in the cytoplasm, such that mitosis and apoptosis could be clearly imaged. A craniotomy open window was made in athymic nude mice for real-time fluorescence imaging of implanted cancer cells growing in the brain. The craniotomy window was reversibly closed with a skin flap. Mitosis of the individual cancer cells were imaged dynamically in real time through the craniotomy-open window. This model can be used to evaluate brain metastasis and brain cancer at the subcellular level.*

Brain cancer and metastatic spread of cancer to the brain is associated with poor prognosis (1). Present treatment strategies to treat brain metastasis show only low response rates and small increases in median survival (2). For certain malignancies for which new therapies are effective (3), the central nervous system can become a sanctuary site, such as for melanoma and other types of cancer (4, 5).

Our laboratory pioneered *in vivo* imaging based on green fluorescent protein (GFP) expression in tumor cells (6). Using GFP imaging, we have observed spontaneous metastasis to the brain in three orthotopic nude mouse model systems of human cancer: the PC-3 human prostate cancer cell line (7); the LOX human melanoma cell line (8); and a spinal cord glioma model using the U87 human glioma cell line (9). Cruz-Munoz *et al.* (1) have developed sublines of melanoma with a predilection for brain metastasis.

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Kienast *et al.* (10) used multiphoton laser scanning microscopy to image cancer cells in the brain using a chronic cranial window. However, these authors used single-labeled red fluorescent protein (RFP)-expressing cancer cells which were not useful for determining cell cycle position or steps in apoptosis, which would be especially useful with respect to monitoring of treatment.

In the present study, we used cancer cells labeled with GFP in the nucleus and RFP in the cytoplasm to perform subcellular imaging of cancer cells in the brain. The two different-color genetic reporters can signal cell cycle position, as well as apoptotic responses to treatment. Using a craniotomy open window, we dynamically imaged mitosis of cancer cells in live mice in real time.

Materials and Methods

Cells. Lewis lung carcinoma (LLC) and U87 human glioma (U87) GFP-RFP cells were established by transfection with retroviral RFP and H2B-GFP vectors, as previously described (11) and maintained in (Dulbecco's Modified Eagle Medium (DMEM) (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Animals. Athymic nude mice (*nu/nu*), 4-6 weeks of age, were used in this study (AntiCancer Inc., San Diego, CA, USA). Mice were kept in a barrier facility under HEPA filtration. Mice were fed with an autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principals and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under Assurance number A3873-1.

Craniotomy open window. Mice were anesthetized with a ketamine mixture (10 µl ketamine HCL, 7.6 µl xylazine, 2.4 µl acepromazine maleate, and 10 µl H₂O) *via s.c.* injection. After fixing the mice in a prone position, a 1.5 cm incision was made directly down the midline of the scalp. The scalp was retracted and the skull was exposed. Using a skin biopsy punch (Acuderm Inc., Ft. Lauderdale, FL, USA), a 4 mm diameter craniotomy was made over the right parietal bone (Figure 1a and b). The bone fragment was removed

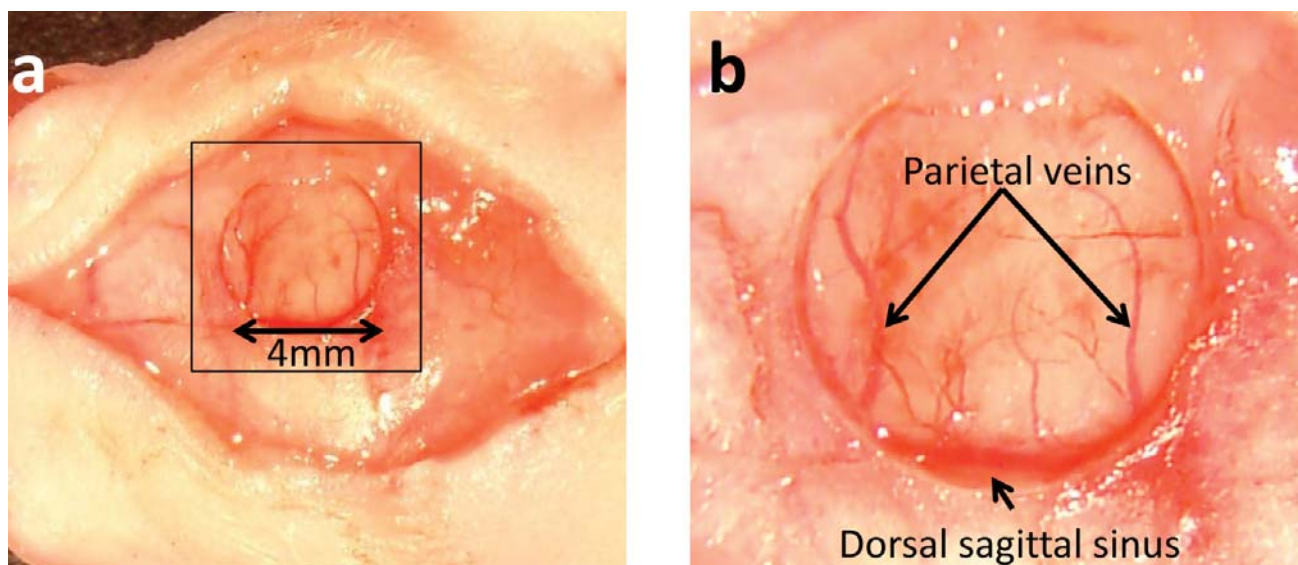


Figure 1. Craniotomy open window. a: Using a skin biopsy punch, a 4 mm diameter craniotomy was made over the right parietal bone. b: The brain tissue and veins were observed through the craniotomy open window.

carefully in order not to injure the meninges and brain tissue. The craniotomy open window was covered only by the scalp after each imaging session. The incision was closed with a 6-0 surgical suture (ETHICON, Inc., Somerville, NJ, USA). Thus, only scalp retraction was needed in order to image single cancer cells in the brain. All mice were kept in an oxygenated warmed chamber until they recovered from anesthesia (12, 13).

Internal carotid artery injection of cancer cells. Mice were anesthetized with a ketamine mixture *via s.c.* injection. The mice were then fixed on a board with rubber bands in a supine position. A thermoplate (Olympus Corp., Tokyo, Japan) was put on the board in order to maintain constant body temperature throughout the experiment. After the neck was sterilized using 70% ethanol, a 2.0 cm longitudinal incision was made in the skin at the center of the neck. The artery was exposed by blunt dissection from the common carotid artery at the point of division into the internal and external carotid arteries. After the external artery was clamped, dual-color Lewis lung carcinoma (LLC) cells (1×10^6 cells in 20 μ l) were slowly injected with a 31-gauge needle into the right internal carotid artery. Immediately after injection, the injected site was pressed with a swab to prevent bleeding and leakage of injected cancer cells. The skin was closed with a 5-0 surgical suture (ETHICON, Inc.) (12, 13).

Stereotactic injection of cancer cells in the brain. The mice were anesthetized with a ketamine mixture *via s.c.* injection. After the craniotomy open window was made, dual-color U87 cells (1×10^5) were injected in 1 μ l stereotactically into the mouse brain using a 10 μ l Hamilton syringe. Cells were injected at the middle of the craniotomy open window to a depth of 0.5 mm.

Fluorescence imaging. The Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD

camera (Olympus), were used for imaging live mice (12-14) and brain cross sections. An Olympus FluoView FV1000 confocal laser microscope (Olympus Corp., Tokyo, Japan) (15) was used for *ex vivo* imaging. High-resolution images were captured directly on a personal computer (Fujitsu Siemens Computers, Munich, Germany). Images were analyzed with the use of Cell[®] software (Olympus Biosystems).

Statistical analysis. The experimental data are expressed as the mean \pm SD. Statistical analysis was performed using the Student's *t*-test or the Kruskal-Wallis test. Kaplan-Meier analysis with a log-rank test was used to determine survival and differences between treatment groups. A *p*-value of less than 0.05 indicated a significant difference.

Results

Real-time imaging of dual-color labeled cancer cells in the brain. Single cancer cells expressing GFP in the nucleus and RFP in the cytoplasm in the brain were observed through a craniotomy open window (Figure 1) in live mice using the OV100 Small Animal Imaging System. The GFP nuclei and RFP cytoplasm of single dual-color LLC cells, which were injected in the brain *via* the internal carotid artery, and single dual-color human U87 glioma cells, which were injected stereotactically in the brain, were imaged (Figure 2a). Colonies of both cell types were also similarly observed in the brain (Figure 2a).

The OV100 imaging system enables imaging of cancer cells up to a depth of approximately 100 μ m in the brain of live mice through the craniotomy open window. Depth of imaging with the OV100 was calibrated by *ex vivo* imaging methods, such as FV1000 confocal laser microscopy of

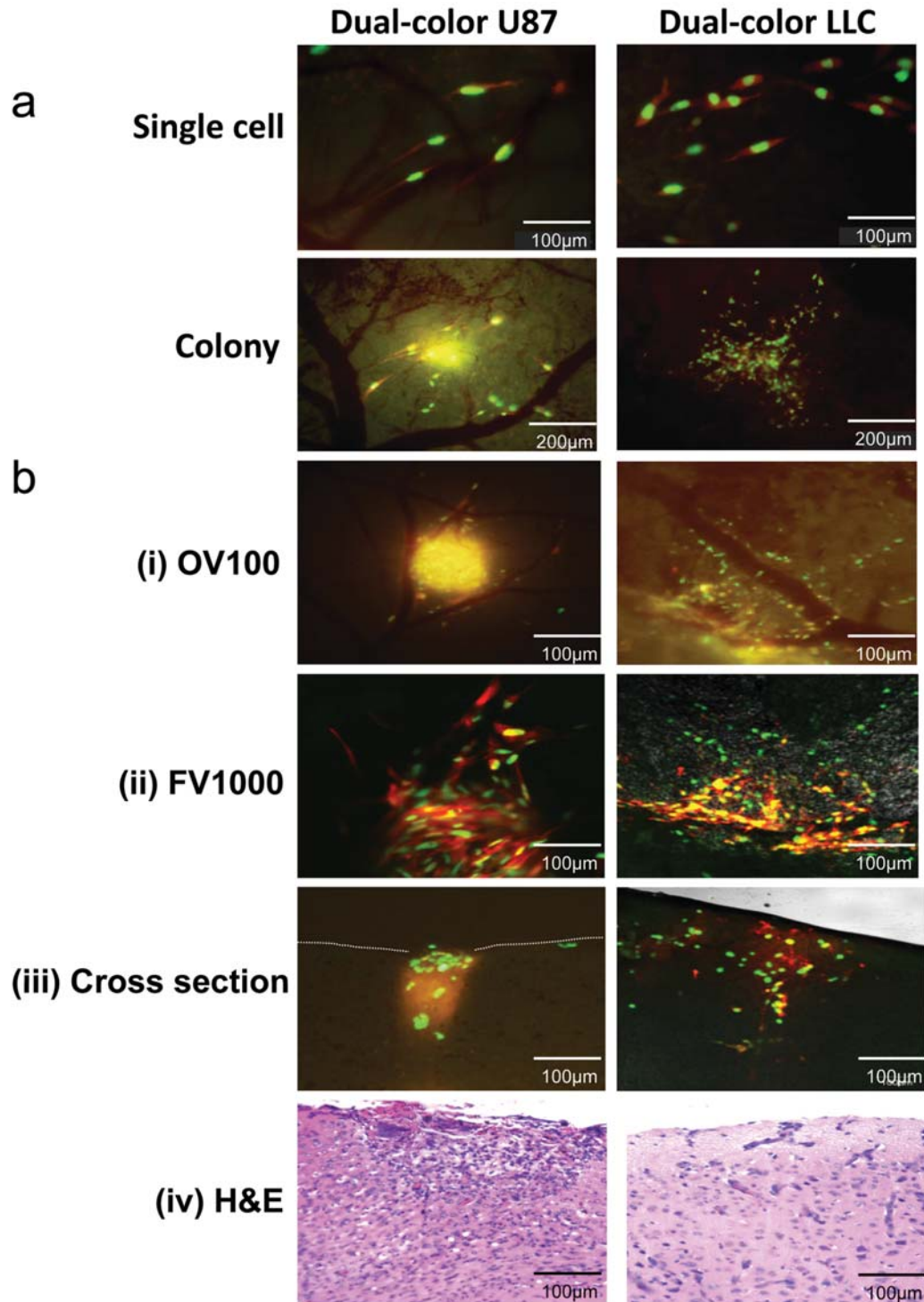


Figure 2. Dual-color U87 human glioma (U87) cells, which were injected stereotactically, and Lewis lung carcinoma (LLC) cells, which were injected into internal carotid artery, in the brain were observed through the craniotomy open window in live mice. **a**: Dual-color U87 and LLC cells with green fluorescent protein (GFP)-expressing nuclei and red fluorescent protein (RFP)-expressing cytoplasm and colonies of dual-color cancer cells in the brain visualized through a craniotomy window in live mice (representative images were chosen from five mice for each cell line). **b**: In order to determine the depth of (i) OV100 imaging of cancer cells in the brain in live mice, various ex vivo imaging methods were used, including (ii) the FV1000 confocal laser microscope; (iii) fluorescence imaging of brain cross-sections (broken line indicates surface of the brain); and (iv) histological analysis (hematoxylin and eosin staining). From these data, it was determined that the OV100 imaged up to a depth of 100 µm in the brain through the craniotomy open window. This depth is sufficient to observe cancer-cell behavior inside the brain parenchyma. (Scale bars: **a**, upper=100 µm; lower=200 µm; **b**, 100 µm).

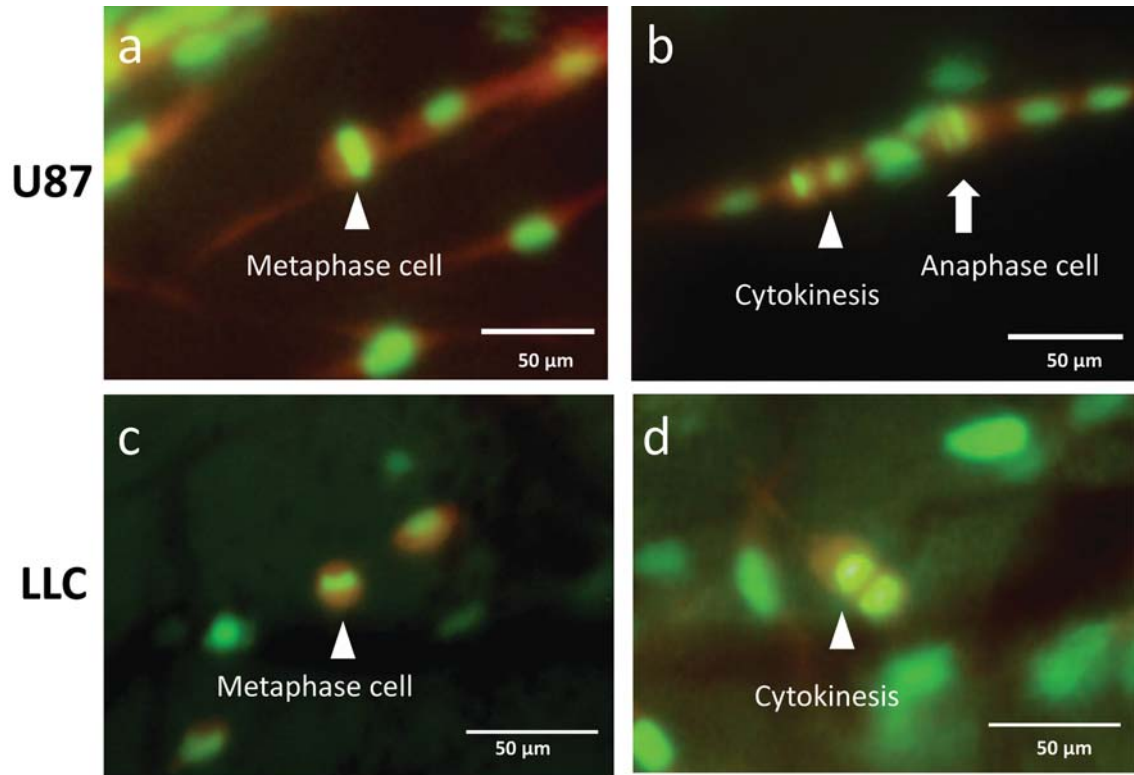


Figure 3. Imaging of mitosis of dual-color cancer cells in the brain of live mice through a craniotomy window. a: Metaphase dual-color U87 cell. b: Cytokinesis (arrowhead) and anaphase cell (arrow) of dual-color U87 cells. c: Metaphase dual-color LLC cell. d: Cytokinesis of dual-color LLC cell (representative images of five mice for each cell line are shown).

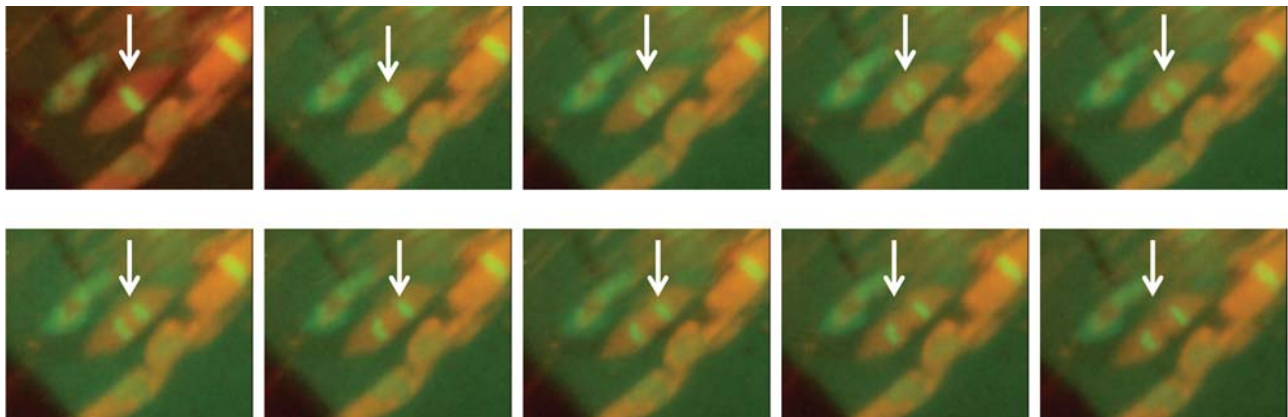


Figure 4. Time-course dynamic imaging of a dual-color LLC cell undergoing mitosis in the brain visualized through the craniotomy window with the OV100 imaging system. Arrows point to the same cell undergoing mitosis.

brain cross-sections, and histological analysis (Figure 2b). This depth of OV100 imaging is sufficient to observe cancer cells within the brain parenchyma. It was possible to carry out imaging for at least two weeks through the craniotomy open window. Mice survived for approximately one month, at which time they died from cancer progression. No side-

effects due to the craniotomy open window (for example infection, or behavioral changes) were observed.

Dynamic imaging of mitotic cancer cells in the brain of live mice. Mitotic U87 and LLC dual-color cancer cells was clearly imaged in the brain of live mice (Figure 3). Figure 3a

shows a metaphase U87 cell and Figure 3b shows anaphase and cytokinesis of U87 cells in the brain. Figure 4 shows time-course dynamic imaging of a dual-color LLC cell undergoing mitosis in the brain.

Discussion

Winkler *et al.* (16) and Kienast *et al.* (10) imaged RFP-labeled cancer cells in the brain using an implanted cranial window and multiphoton laser-scanning microscope in live mice. However, the RFP-labeled cancer cells that they used could not be imaged at the subcellular level, because with the single cellular label, the nuclei could not be distinguished from the cytoplasm, thus precluding imaging of apoptosis and mitosis. In contrast, in our study with double-labeled cancer cells and the craniotomy open window, nuclear–cytoplasmic dynamics of cancer cells were dynamically imaged in the brain in live mice. With these techniques, we were able to dynamically observe cancer cell mitosis in the brain of live mice (Figure 3).

In conclusion, we have developed a novel mouse brain imaging model in which cancer cells in the brain can be observed at the subcellular level in live mice. The dual-color cancer cells, craniotomy open window and Olympus OV100 imaging system, all used in the present study, enabled longitudinal real-time subcellular nuclear-cytoplasmic dynamic imaging of cancer cells in the brain over a two-week period (12, 13). With this imaging technology, we were able to observe, with very high resolution, nuclear and cytoplasmic dynamics during mitosis. This model can be used to evaluate new treatment modalities of brain metastasis and brain cancer at the subcellular level (12, 13). The imaging model opens up new possibilities for brain cancer or brain metastasis treatment.

Conflicts of Interest

None of the Authors have a conflict of interest regarding this study.

Acknowledgements

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References

- 1 Cruz-Munoz W, Man S, Xu P and Kerbel RS: Development of a preclinical model of spontaneous human melanoma central nervous system metastasis. *Cancer Res* 68: 4500-4505, 2008.
- 2 Tarhini AA and Agarwala SS: Cutaneous melanoma: Available therapy for metastatic disease. *Dermatol Ther* 19: 19-25 (2006).
- 3 Lin NU and Winer EP: Brain metastases: The HER2 paradigm. *Clin Cancer Res* 13: 1648-1655, 2007.
- 4 Mayer M: A patient perspective on brain metastases in breast cancer. *Clin Cancer Res* 13: 1623-1624, 2007.
- 5 Palmieri D, Chambers AF, Felding-Habermann B, Huang S and Steeg PS: The biology of metastasis to a sanctuary site. *Clin Cancer Res* 13: 1656-1662, 2007.
- 6 Hoffman RM: The multiple uses of fluorescent proteins to visualize cancer *in vivo*. *Nat Rev Cancer* 5: 796-806, 2005.
- 7 Yang M, Jiang P, Sun FX, Hasegawa S, Baranov E, Chishima T, Shimada H, Moossa AR and Hoffman RM: A fluorescent orthotopic bone metastasis model of human prostate cancer. *Cancer Res* 59: 781-786, 1999.
- 8 Yang M, Jiang P, An Z, Baranov E, Li L, Hasegawa S, Al-Tuwaijri M, Chishima T, Shimada H, Moossa AR and Hoffman RM: Genetically fluorescent melanoma bone and organ metastasis models. *Clin Cancer Res* 5: 3549-3559, 1999.
- 9 Hayashi K, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Bouvet M, Wessels J and Hoffman RM: A color-coded orthotopic nude-mouse treatment model of brain-metastatic paralyzing spinal cord cancer that induces angiogenesis and neurogenesis. *Cell Proliferation* 42: 75-82, 2009.
- 10 Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J and Winkler F: Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med* 16: 116-122, 2010.
- 11 Yamamoto N, Jiang P, Yang M, Xu M, Yamauchi K, Tsuchiya H, Tomita K, Wahl GM, Moossa AR and Hoffman RM: Cellular dynamics visualized in live cells *in vitro* and *in vivo* by differential dual-color nuclear-cytoplasmic fluorescent-protein expression. *Cancer Res* 64: 4251-4256, 2004.
- 12 Momiya M, Suetsugu A, Kimura H, Kishimoto H, Aki R, Yamada A, Sakurada H, Chishima T, Bouvet M, Endo I and Hoffman RM: Imaging the efficacy of UVC irradiation on superficial brain tumors and metastasis in live mice at the subcellular level. *J Cell Biochem* 114: 428-434, 2013.
- 13 Momiya M, Suetsugu A, Chishima T, Bouvet M, Endo I and Hoffman RM: Subcellular real-time imaging of the efficacy of temozolomide on cancer cells in the brain of live mice. *Anticancer Res* 33: 103-106, 2013.
- 14 Yamauchi K, Yang M, Jiang P, Xu M, Yamamoto N, Tsuchiya H, Tomita K, Moossa AR, Bouvet M and Hoffman RM: Development of real-time subcellular dynamic multicolor imaging of cancer-cell trafficking in live mice with a variable-magnification whole-mouse imaging system. *Cancer Res* 66: 4208-4214, 2006.
- 15 Uchugonova A, Duong J, Zhang N, König K and Hoffman RM: The bulge area is the origin of nestin-expressing pluripotent stem cells of the hair follicle. *J Cell Biochem* 111: 2046-2050, 2011.
- 16 Winkler F, Kienast Y, Fuhrmann M, Von Baumgarten L, Burgold S, Mitteregger G, Kretzschmar H and Herms J: Imaging glioma cell invasion *in vivo* reveals mechanisms of dissemination and peritumoral angiogenesis. *Glia* 57: 1306-1315, 2009.

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