METHODS IN MOLECULAR BIOLOGY™ 411

Reporter Genes

A Practical Guide

Edited by
Donald S. Anson

HUMANA PRESS

9

Subcellular Imaging of Cancer Cells in Live Mice

Robert M. Hoffman

Summary

Dual-color fluorescent cells, with one color in the nucleus and the other in the cytoplasm, enable real-time nuclear-cytoplasmic dynamics to be visualized in living cells in vivo as well as in vitro. To obtain the dual-color cells, red fluorescent protein (RFP) is expressed in the cytoplasm of cancer cells, and green fluorescent protein (GFP) linked to histone H2B is expressed in the nucleus. Nuclear GFP expression allows visualization of nuclear dynamics, whereas simultaneous cytoplasmic RFP expression allows visualization of nuclear-cytoplasmic ratios as well as simultaneous cell and nuclear shape changes. This methodology has allowed us to show that the cells and nuclei of cancer cells in the capillaries elongate to fit the width of these vessels. The average length of the major axis of the cancer cells in the capillaries increased to approximately four times their normal length. The nuclei increased their length 1.6 times. Cancer cells in capillaries over 8 μm in diameter were shown to migrate at up to 48.3 μm/h. With the use of dual-color fluorescent cells and the Olympus OV100, a highly sensitive whole-mouse imaging system with both macrooptics and microoptics, it is possible to achieve subcellular real-time imaging of cancer cell trafficking in live mice. Extravasation can also be imaged in real time. Dual-color imaging showed that cytoplasmic processes of cancer cells exited the vessels first, with nuclei following along the cytoplasmic projections. Dual-color in vivo cellular imaging was also used to visualize trafficking, nuclear-cytoplasmic dynamics, and the viability of cancer cells after their injection into the portal vein of mice.

Key Words: Green fluorescent protein; red fluorescent protein; in vivo imaging; nuclear-cytoplasmic dynamic imaging; cancer cell trafficking; metastasis.

1. Introduction

Visualization of microscopic cancer is essential for the understanding and control of cancer dormancy, growth, and colonization of distant sites *(1–3)*. Several approaches involving tumor-cell labeling have been developed for visualizing tumor cells in vivo. The *Escherichia coli* β-galactosidase (*lacZ*) gene has been used to detect micrometastases *(4)*. However, *lacZ* detection requires extensive histological preparation and sacrifice of the tissue or animal. There-

From: *Methods in Molecular Biology, vol. 411: Reporter Genes: A Practical Guide*
Edited by: D. Anson © Humana Press Inc., Totowa, NJ

fore, other techniques are required for real-time imaging and study of tumor cells in viable fresh tissue or living animals *(5)*.

The use of skin-fold chambers, exteriorization of organs, and subcutaneous windows inserted with semitransparent material *(6–8)* has yielded insights into microscopic tumor behavior. However, these techniques are only suitable for ectopic models *(6)* or for investigations with short periods of observation *(7,8)*. The difficulties of maintaining windows or other devices that are made with heavy, stiff, or other types of foreign materials limit the length of time that these tools can be used in vivo *(6,7)*. Cutaneous windows made with polyvinyl chloride film become opaque or detached after time, which precludes their use in long-term studies *(5,7)*.

To image noninvasively and follow the natural course or impediment of tumor progression and metastasis, high specificity and sensitivity, a strong signal, and high resolution are necessary. The green fluorescent protein (GFP) gene, cloned from the bioluminescent jellyfish *Aequorea victoria (9)*, was chosen to satisfy these conditions because it has great potential for use as a cellular marker *(10,11)*. GFP cDNA encodes a 283-amino acid monomeric polypeptide with M_r 27,000 *(12,13)* that requires no other *A. victoria* proteins, substrates, or cofactors to fluoresce *(14)*. Gain-of-function bright mutants of the GFP gene have been generated by various techniques *(15–17)* and have been humanized for high expression and signal *(18)*. Red fluorescent proteins (RFPs) from the *Discosoma* coral have similar features as well as the advantage of longer wavelength emission *(5,19–21)*.

Initial studies of tumor biology that used stable GFP expression focused on static images and examination of metastases *(22,23)*. The first use of GFP to image cancer cells in vivo was by Chishima et al. in our laboratory *(24)*. GFP was subsequently used to observe motility and shape changes of carcinoma cells in vivo *(25,26)*.

Subsequently, Chishima et al. *(24)* and Huang et al. *(27)* showed that GFP-transduced cancer cells allowed the imaging of tumor cells in blood vessels. To examine cell behavior during intravasation, Naumov et al. *(8)* used GFP imaging to visualize fine cellular details such as pseudopodial projections, even after extended periods of in vivo growth. Wyckoff et al. *(28)* have used GFP imaging to view these cells in time-lapse images within a single optical section using a confocal microscope.

Mook et al. *(29)* visualized GFP colon cancer cells in sinusoids of the liver. Al-Mehdi et al. *(30)* observed the steps in early hematogenous metastasis of tumor cells expressing GFP in subpleural microvessels in intact, perfused mouse and rat lungs.

Using multiphoton microscopy and GFP labeling, Wang et al. *(31)* examined differences in carcinoma cell behavior between the nonmetastatic and metastatic primary breast tumors. Goswami et al. *(32)* have shown that macrophages promote the invasion of GFP-labeled breast cancer cells. However, nuclear-cytoplasmic dynamics could not be visualized in the trafficking cells because the cancer cells were entirely labeled with GFP *(1)*. The visualization of nuclear cytoplasmic dynamics in living cells could enable the real-time study of the normal and malignant cell cycle and apoptotic behavior in vivo as well as in vitro. Chambers et al. *(33)* and Naumov et al. *(8)* used GFP-tagged tumor cells and intravital imaging to visualize individual cells. These studies visualized the shape of metastatic tumor cells in vivo but could not visualize nuclear-cytoplasmic dynamics, or nuclear shape changes, because the nucleus and cytoplasm could not be distinguished *(5)*.

A fusion protein of GFP and yeast histone H2B was shown to localize in yeast nuclei *(34)*. Subsequently, a fusion protein of GFP and human histone H2B (H2B-GFP) was shown to be incorporated into nucleosome core particles of HeLa cells without perturbing cell cycle progression *(35)*. H2B-GFP allowed high-resolution imaging of both mitotic chromosomes and interphase chromatin in live cells *(5,36)*.

With only H2B-GFP labeling of cells, an overlay with differential interference contrast images along with the GFP fluorescence images was necessary to visualize nuclear-cytoplasmic morphology. We have used H2B-GFP and RFP to differentially label the nucleus and cytoplasm of human HT-1080 human fibrosarcoma cells. This strategy allows the visualization of the cell cycle, apoptosis, and nuclear deformability in live cells in real time. This dual-color tagging strategy also allows real-time observation of nuclear-cytoplasmic dynamics in vivo as well as in vitro *(5)*.

Using the dual-colored cancer cells and a highly sensitive small-animal macro-imaging/microimaging system, the Olympus OV100, real-time dynamic subcellular imaging of cancer cell trafficking in live mice is possible. We described how this in vivo subcellular imaging technology can be used to visualize the cytoplasmic and nuclear dynamics of intravascular tumor cell migration and extravasation in live mice *(1)*.

This chapter assumes that the investigator has expertise in microsurgery in the mouse. A useful reference for those learning this field is *Experimental Microsurgery (36a)*.

2. Materials

2.1. Transduction of Cancer Cells

1. PT67 LHCX2-H2B-GFP producer cells *(5)* (*see* **Note 1**).
2. PT67 LNCX2-DsRed2 producer cells *(5)* (*see* **Note 1**).
3. Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific, Santa Ana, CA).

4. Heat-inactivated fetal bovine serum (Gemini Bio-products, Calabasas, CA).
5. G418 (Life Technologies).
6. Hygromycin (Life Technologies).
7. Cloning cylinders (Bel-Art Products, Pequannock, NJ).

2.2. Mice

1. Athymic nu/nu nude mice. Mice should be kept in a barrier facility under HEPA filtration and fed with autoclaved rodent food.
2. Tecklad LM-485 autoclaved laboratory rodent diet (Western Research Products, Orange, CA).

2.2. Delivery of Tumor Cells

1. Ketamine anesthetic: 10 µL ketamine HCl, 7.6 µL xylazine, 2.4 µL acepromazine maleate, and 10 µL H_2O.
2. Dissecting instruments:
   a. Blunt-end hook (Fine Science Tools, Foster City, CA).
   b. 33-gage Needle (Fine Science Tools).
   c. 30-gage Needle (Fine Science Tools).
   d. Dissecting microscope, (MZ6; Leica, Deerfield, IL).
   e. 6-0 Suture (Ethicon, Somerville, NJ).
   f. 1 mL Latex-free syringe (Becton Dickinson).

2.3. Imaging of Tumor Cells

1. Phosphate-buffered saline (PBS; Irvine Scientific).
2. Olympus OV100 small animal imaging system (*see* **Note 2**).

3. Methods

3.1. RFP and Histone H2B-GFP Gene Transduction of Cancer Cells

For RFP and H2B-GFP gene transduction, 70% confluent human cancer cells were used. To establish dual-color cells, clones of cancer cells expressing RFP in the cytoplasm were initially established.

1. Incubate cancer cells with a 1:1 mixture of retroviral supernatant from PT67-RFP cells and normal growth medium for 72 h (*see* **Note 1**).
2. Aspirate media and replace with normal growth medium.
3. At 72 h posttransduction, harvest cells and subculture cells at a ratio of 1:15 in growth medium containing 200 to 800 µg/mL G418.
4. Isolate clones of transduced cancer cells, identified by fluorescence microscopy, with cloning cylinders and amplify by conventional culture methods.

For establishing dual-color cells, the process is then repeated on a clone of cancer cells expressing high levels of RFP using retroviral supernatants from PT67 H2B-GFP cells and selecting with 200 to 400 µg/mL of hygromycin. The resulting clones should be checked for stably expressed GFP in the nucleus and RFP in the cytoplasm using fluorescence microscopy.

3.2. Real-Time Visualization of Dual-Labeled Cells In Vivo

Examples are given for the analysis of cells in the brain, ear, and capillaries. Cells should be harvested by trypsinization and washed twice with serum-free medium before injection. Cells should be used within 40 min of harvesting.

3.2.1. Imaging of Cells in the Brain of Live Mice

To visualize cell dynamics in the brain of living mice, cells were injected in the common carotid artery. All procedures of the operation described are performed with a x7 dissection microscope

1. Anesthetize nude mice with ketamine mixture via s.c. injection.
2. Make a longitudinal skin incision on the neck.
   a. After exposing the submandibular gland, cut it in the middle and retract to each side.
   b. Separate the right sternohyoid muscle, right sternomastoideus muscle, and connective tissue with a blunt instrument.
   c. After isolation of the right common carotid artery, gently release the artery from surrounding connective tissue.
   d. Place light tension on the proximal site of the artery with a blunt-end hook.
3. Inject a total of 200 µL of medium containing 2×10^5 dual-color cells into the artery using a 33-gage needle. Immediately after injection, press the injected site with a swab to prevent bleeding or leakage of injected tumor cells. Close the skin with a 6-0 suture.

Visualize (*see* **Note 2**) tumor cells in the brain through the skull via a skin-flap window.

1. Anesthetize the animals with the ketamine mixture via s.c. injection.
2. Make an arc-shaped incision in the scalp, and separate the s.c. connective tissue to free the skin flap.
3. The skin flap can be opened repeatedly to image (*see* **Note 2**) tumor cells in the brain through the nearly transparent mouse skull and can be simply closed with a 6-0 suture. This procedure greatly reduced the scatter of fluorescent photons (*5*).

For examples of the sort of results that can be obtained with this technique, *see* **ref. 5**.

3.2.2. Noninvasive Imaging of Cells in the Ear

1. Anesthetize nude mice with ketamine mixture via s.c. injection.
2. Inject dual-color cells intradermally into the ear of the mouse using a 27-gage needle.

3. Observe the surface of the ear of the intact animal under fluorescence microscopy *(5)* (*see* **Note 2**).

For examples of the sort of results that can be obtained with this technique, *see* **ref. 5**.

3.2.3. Imaging of Cells in Blood Vessels

To visualize cell dynamics in vessels in live mice, cells are injected into the heart. During the period of measurement, the animal is kept under anesthesia and kept warm. To determine migration velocities, measurements are taken at the initial time and 2 h later. Blocking off the epigastric cranialis vein traps the cells in the vein and forces them into the surrounding capillaries at the observation site (abdominal skin flap).

1. Anesthetize nude mice with ketamine mixture via s.c. injection.
2. Expose the ribs using a 1-cm midline incision.
   a. Using a 30-gage 1/2 needle and a 1-mL latex-free syringe, inject a total of 100 to 200 μL of medium containing 1 to 5×10^6 dual-color cells into the heart ventricle.
   b. This is done by inserting the needle into the second intercostal space 2 mm left of the sternum and aimed centrally. The spontaneous, pulsatile entrance of bright red oxygenated blood into the needle hub indicates proper positioning.
   c. The cells should be injected over 1 to 2 min.
3. Immediately after injection, make an arc-shaped incision in the abdominal skin, and then separate the s.c. connective tissue to free the skin flap without injuring the epigastric cranialis artery and vein.
4. Expose the epigastric cranialis vein of the mouse and block with a 6-0 suture.
5. Spread the skin flap and fix on a flat stand.
6. Visualize cells (*see* **Notes 2** and **3**).
7. During the intervals between imaging, occasionally spray PBS on the inside of the skin flap to keep the surface wet (*see* **Notes 4–6**).

For examples of the sort of results that can be obtained with this technique, *see* **refs. *1*** and ***26***.

4. Notes

1. Any vector that expresses high levels of the relevant fluorescent proteins can be used. A selective marker on each vector simplifies isolation of transduced cells, although this could also be achieved by fluorescence-activated cell sorting (FACS) or cloning/FACS analysis.
2. 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), was used for subcellular imaging in live mice. 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 the 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 were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of PaintShopPro 8 and Cell® (Olympus Biosystems) *(1)*.
3. The images should include the cell in its blood vessel, as well as the surrounding vessels, which can be used as a map to relocate the cell in its vessel at later time points.
4. The skin flap can be completely reversed *(26)*.
5. For motility analysis, the epigastric cranialis vein was not wired.
6. For motility analysis, cells were reimaged after 2 h.
7. For imaging cancer cell trafficking in blood vessels, images were acquired in real time.
8. For analysis of extravasation, images were acquired every hour after injection with the skin flap open, or every 12 h by opening and closing the skin flap.
9. Using the skin flap for observation of cells in capillaries has important advantages. The skin can be spread stably on a stand, such that motion from the mouse's heartbeat or breathing has no influence on imaging. Disturbance of the blood supply for the skin does not occur during the skin flap procedure, because the epigastric cranialis artery is not injured during the procedure. In addition to these advantages, the skin flap could be completely reversed such that the mice need not be sacrificed. In our study, the skin flap was reversed after 24 h *(26)*.

Acknowledgments

These studies were supported in part by National Cancer Institute grants CA103563 and CA099258.

References

1. Yamauchi, K., Yang, M., Jiang, P., et al. (2006) 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.
2. Tsuji, K., Yamauchi, K., Yang, M., et al. (2006) Dual-color imaging of nuclear-cytoplasmic dynamics, viability, and proliferation of cancer cells in the portal vein area. *Cancer Res.* **66,** 303–306.
3. Chambers, A. F., Groom, A. C., and MacDonald, I. C. (2002) Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer* **2,** 563–572.
4. Lin, W. C., Pretlow, T. P., Pretlow, T. G. II, and Culp, L. A. (1990) Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression. *Cancer Res.* **50,** 2808–2817.

5. Yamamoto, N., Jiang, P., Yang, M., et al. (2004) 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.
6. Brown, E. B., Campbell, R. B., Tsuzuki, Y., et al. (2001) In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nat. Med.* **7,** 864–868.
7. Ciancio, S. J., Coburn, M., and Hornsby, P. J. (2000) Cutaneous window for in vivo observations of organs and angiogenesis. *J. Surg. Res.* **92,** 228–232.
8. Naumov, G. N., Wilson, S. M., MacDonald, I. C., et al. (1999) Cellular expression of green fluorescent protein, coupled with high-resolution in vivo video-microscopy, to monitor steps in tumor metastasis. *J. Cell Sci.* **112,** 1835–1842.
9. Prasher, D. C., Eckenrode, V. K., Ward, W. W., et al. (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111,** 229–233.
10. Chalfie, M., Tu, Y., Euskirchen, G., et al. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.
11. Cheng, L., Fu, J., Tsukamoto, A., and Hawley, R. G. (1996) Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. *Nat. Biotechnol.* **14,** 606–609.
12. Cody, C. W., Prasher, D. C., Westler, W. M., et al. (1993) Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. *Biochemistry* **32,** 1212–1218.
13. Yang, F., Moss, L. G., and Phillips, G. N. Jr. (1996) The molecular structure of green fluorescent protein. *Nat. Biotechnol.* **14,** 1246–1251.
14. Morin, J. and Hastings, J. (1971) Energy transfer in a bioluminescent system. *J. Cell Physiol.* **77,** 313–318.
15. Cormack, B., Valdivia, R., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33–38.
16. Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* **14,** 315–319.
17. Delagrave, S., Hawtin, R. E., Silva, C. M., et al. (1995) Red-shifted excitation mutants of the green fluorescent protein. *Biotechnology* **13,** 151–154.
18. Heim, R., Cubitt, A. B., and Tsien, R. Y. (1995) Improved green fluorescence. *Nature* **373,** 663–664.
19. Zolotukhin, S., Potter, M., Hauswirth, W. W., et al. (1996) A 'humanized' green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J. Virol.* **70,** 4646–4654.
20. Gross, L. A., Baird, G. S., Hoffman, R. C., et al. (2000) The structure of the chromophore within DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci. USA* **97,** 11,990–11,995.
21. Fradkov, A. F., Chen, Y., Ding, L., et al. (2000) Novel fluorescent protein from *Discosoma* coral and its mutants possesses a unique far-red fluorescence. *FEBS Lett.* **479,** 127–130.

22. Hoffman, R. M. (2002) Green fluorescent protein imaging of tumour growth, metastasis, and angiogenesis in mouse models. *Lancet Oncol.* **3,** 546–556.
23. Condeelis, J. and Segall, J.E. (2003) Intravital imaging of cell movement in tumours. *Nat. Rev. Cancer* **3,** 921–930.
24. Chishima, T., Miyagi, Y., Wang, X., et al. (1997) Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression. *Cancer Res.* **57,** 2042–2047.
25. Farina, K. L., Wyckoff, J. B., Rivera, J., et al. (1998) Cell motility of tumor cells visualized in living intact primary tumors using green fluorescent protein. *Cancer Res.* **58,** 2528–2532.
26. Yamauchi, K., Yang, M., Jiang, P., et al. (2005) Real-time in vivo dual-color imaging of intracapillary cancer cell and nucleus deformation and migration. *Cancer Res.* **65,** 4246–4252.
27. Huang, M. S., Wang, T. J., Liang, C. L., et al. (2002) Establishment of fluorescent lung carcinoma metastasis model and its real-time microscopic detection in SCID mice. *Clin. Exp. Metastasis* **19,** 359–368.
28. Wyckoff, J. B., Jones, J. G., Condeelis, J. S., and Segall, J. E. (2000) A critical step in metastasis: in vivo analysis of intravasation at the primary tumor. *Cancer Res.* **60,** 2504–2511.
29. Mook, O. R. F., Marle, J. V., and Vreeling-Sindelarova, H. (2003) Visualization of early events in tumor formation of eGFP-transfected rat colon cancer cells in liver. *Hepatology* **38,** 295–304.
30. Al-Mehdi, A. B., Tozawa, K., Fisher, A. B., Shientag, L., Lee, A., and Muschel, R. J. (2000) Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. *Nat. Med.* **6,** 100–102.
31. Wang, W., Wyckoff, J. B., Frohlich, V. C., et al. (2002) Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res.* **62,** 6278–6288.
32. Goswami, S., Sahai, E., Wyckoff, J. B., et al. (2005) Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res.* **65,** 5278–5283.
33. Chambers, A. F., Schmidt, E. E., MacDonald, I. C., Morris, V. L., and Groom, A. C. (1992) Early steps in hematogenous metastasis of B16F1 melanoma cells in chick embryos studied by high-resolution intravital videomicroscopy. *J. Natl. Cancer Inst.* **84,** 797–803.
34. Flach, J., Bossie, M., Vogel, J., et al. (1994) A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. *Mol. Cell. Biol.* **14,** 8399–8407.
35. Kanda, T., Sullivan, K. F., and Wahl, G. M. (1998) Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol.* **8,** 377–385.
36. Manders, E. M., Visser, A. E., Koppen, A., et al. (2003) Four-dimensional imaging of chromatin dynamics during the assembly of the interphase nucleus. *Chromosome Res.* **11,** 537–547.
36a. Lee. S. (1987) *Experimental microsurgery.* Igaka-Shoin, New York.