Real-time Non-invasive Spectral Imaging of Orthotopic Red Fluorescent Protein-expressing Lung Tumor Growth in Nude Mice

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Abstract. *Background/Aim. Orthotopic implantation of cancer allows metastasis to occur. The most patient-like metastatic orthotopic models are developed with surgical orthotopic implantation using intact tissue in order to preserve the natural tissue structure of the tumor which contains both cancer cells and stroma. Materials and Methods. In the present study, we performed a simple thoracotomy by making an intercostal incision between the fourth and fifth ribs on the left side of the chest of nude mice. Lung tumor fragments expressing red fluorescent protein were then implanted on the left lung. Results. It was possible to monitor tumor formation in the lung non-invasively by spectral imaging using the Maestro system with a liquid tunable filter. The model described here has high tumorigenicity in the lung (100%) and a low mortality rate (5%). Conclusion. This imageable nude mouse model using surgical orthotopic implantation of lung cancer will be useful for all types of longitudinal studies.*

The usual xenograft models for human lung cancer use tumor implanted subcutaneously into nude mice. However, it is very rare for subcutaneously-growing tumors to metastasize (1). Lung cancer cells have also been implanted under the renal capsule (2). However, these types of models are not sufficiently representative of the clinical situation. McLemore *et al*. implanted lung cancer cells *via* intrabronchial injection (3, 4); disaggregated fresh tumor specimens were also implanted intrabronchially by McLemore *et al*. The tumors grew intrabronchially much more extensively than when transplanted subcutaneously. Intrabronchial tumors have only low rates of metastasis, with only 1% metastasizing to the left lung, 2% to the trachea, 6% to the peritracheal area and only 3% to lymph nodes, liver, or spleen (4). McLemore *et al*. (3) also injected lung cancer cells into the pleural space resulting in local growth with low rates of metastasis.

Our laboratory has used histologically-intact tumor tissue for surgical orthotopic implantation (SOI). SOI can use surgical patient specimens and the resulting tumor exhibits the natural history of cancer of the human patients. These models are termed patient-derived orthotopic xenografts (PDOX) (5-19). SOI is also useful for implantation of cancer cell lines (1).

We have previously described lung cancer models where tumor tissue is implanted into the left lung by a thoracotomy procedure (20). The present report demonstrates a lung cancer model using SOI with a 100% tumor take rate, 95% survival and non-invasive longitudinal fluorescence imaging of lung tumor growth made possible by fluorescent protein expression and a liquid tunable filter for spectral separation.

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Key Words: Lung cancer, surgical orthotopic implantation, nude mice, high tumor take rates, noninvasive imaging, spectral separation.

Materials and Methods

Mice. Nude mice (AntiCancer, Inc., San Diego, CA, USA), 5-6 weeks old, were used in the study. Test animals were bred and maintained at AntiCancer Inc. in a high-efficiency particulate arrestance (HEPA)-filtered environment for the experiment. Cages, food and bedding were autoclaved. Animals were housed in a barrier facility on a HEPA-filtered rack under standard conditions of a 12-hour light/dark cycle. Animals were housed at no more than five individuals per cage. The animals were fed an autoclaved laboratory rodent diet.

All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by isoflurane inhalation (Henry Schein, Melville, NY, USA). The response of animals during surgery was monitored to ensure adequate depth of anesthesia. Ibuprofen (7.5 mg/kg orally in drinking water every 24 hours for 7 days post-surgery) was used in order to provide analgesia postoperatively in the surgically-treated animals. The animals were observed on a daily basis and humanely sacrificed by CO_2 inhalation when they met the following humane endpoint criteria:

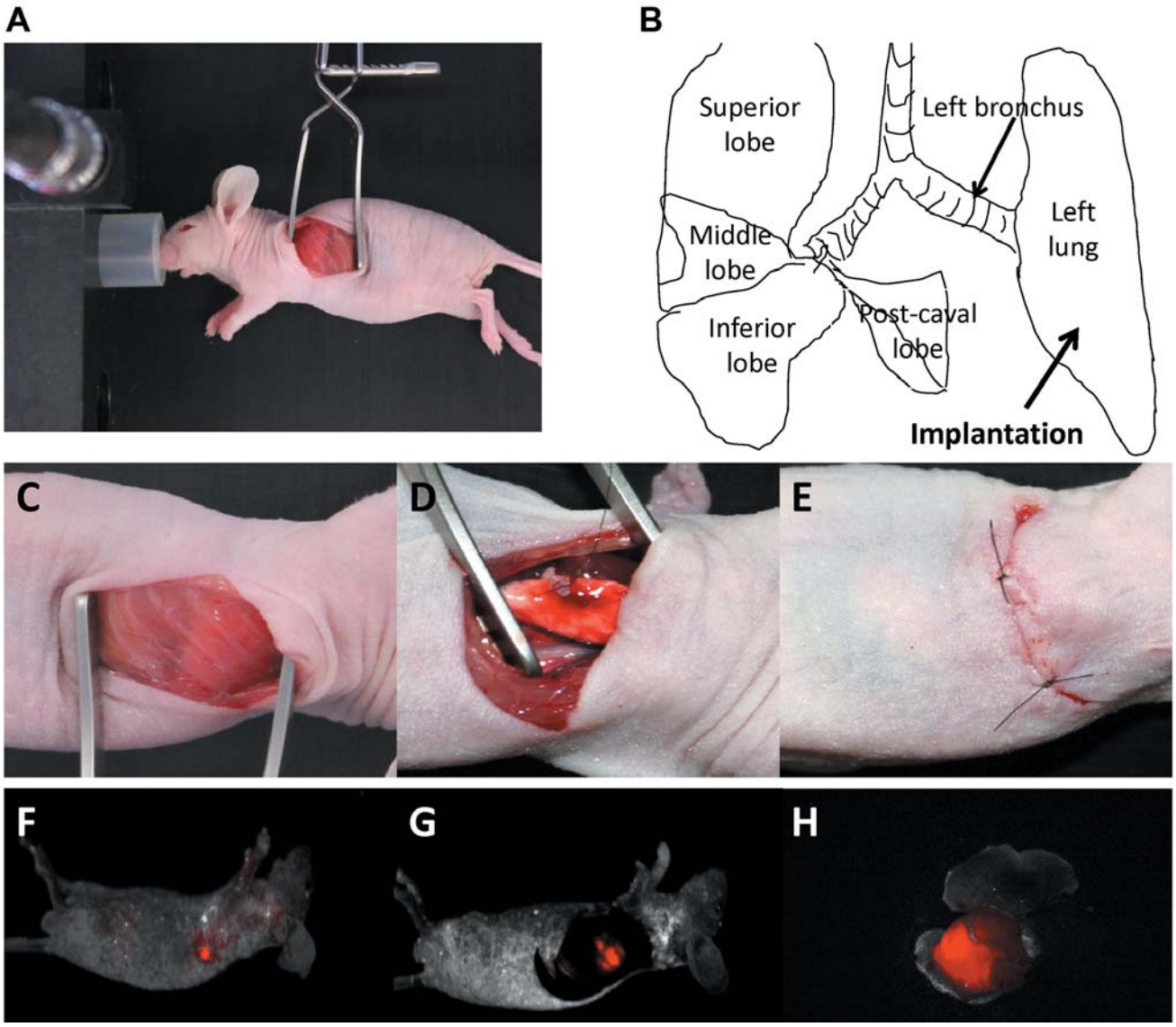

Figure 1. *Surgical orthotopic implantation of lung cancer. A: The animals were anesthetized with isoflurane inhalation. The surgical area was sterilized with iodine and alcohol. A transverse incision approximately 1.5 cm long was made along the left chest wall of the nude mouse using a pair of surgical scissors. B: Anatomical location of the implantation point on the left lung. C: An intercostal incision was made between the third and the fourth ribs and the left lung was exposed. D: The left lung was elevated with wet gauze. Two Lewis lung carcinoma (LLC)-RFP tumor fragments were transplanted to the surface of the left lung with 8-0 surgical sutures (nylon). E: The chest and skin were closed with 6-0 surgical silk sutures. The left lung was re-inflated by intrathoracic puncture using a 3 cc syringe with a G×$^{1}/_{2}$ needle to draw out the remaining air in the chest cavity. F: Non-invasive image of the tumor on the day 7 after implantation. G: Open image of (F) after removal of skin only. H: Resected lung with LLC tumor. Tumor was located exactly in the left lung.*

prostration, skin lesions, significant body weight loss, difficulty breathing, epistaxis, rotational motion and body-temperature drop.

Mouse cancer cell line. The Lewis lung carcinoma (LLC) red fluorescent protein (RFP)-expressing cell line was used (21-24). For RFP gene transduction, 70% confluent murine LLC cells were incubated with a 1:1 mixture of RFP retroviral supernatants of PT67 packaging cells (Clontech, Mountain View, CA, USA) and RPMI-1640 medium for 72 h. Fresh medium was replenished at this time. LLC cells were harvested with trypsin-EDTA 72 h post transduction and subcultured at a ratio of 1:15 into selective medium that contained 200 μg/ml G418 (Life Technologies, Grand Island, NY, USA). The level of G418 was increased to 1000 μg/ml stepwise in order to select the brightest fluorescent cells (22-24).

Subcutaneous xenografts. Tumor stocks of the LLC-RFP cells were established by subcutaneously injecting the LLC-RFP tumor cells into the right flank of nude mice at a cell density of 1×10^{6}/100 μl/mouse. Strong RFP expression of the implanted tumor tissue was confirmed by fluorescence imaging at 470 nm excitation before harvest.

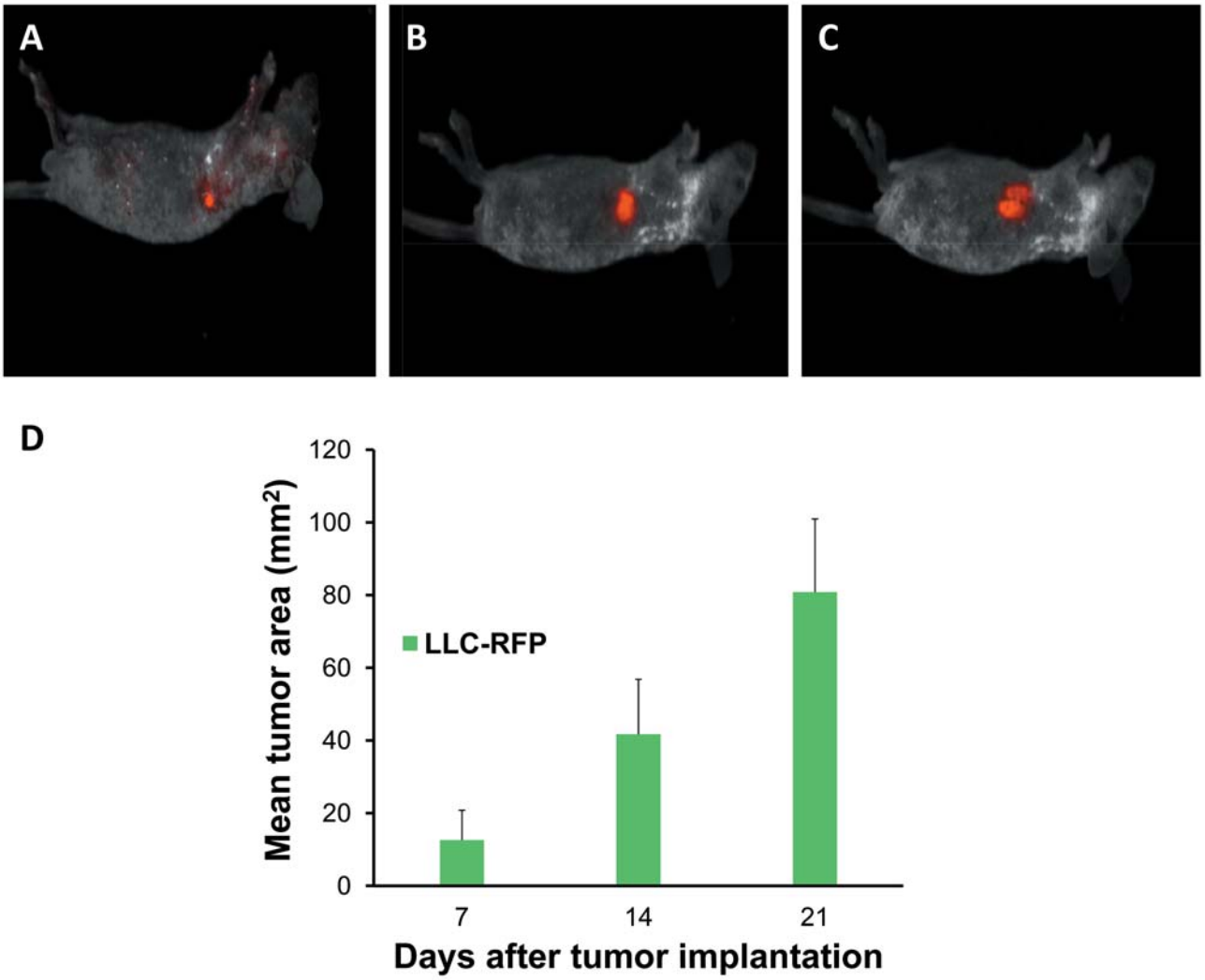

Figure 2. *Longitudinal non-invasive imaging of lung tumor growth. Lung tumor growth was monitored non-invasively on days 7 (A), 14 (B) and 21 (C). Total tumor area was calculated from the images (D). Statistically-significant tumor growth was observed at day 21 after implantation compared to day 7 ($p<0.05$).*

Surgical orthotopic implantation (SOI). The stock tumors were harvested from the subcutaneous site and placed in RPMI-1640 medium. Necrotic tissues were removed and viable tissues, with bright RFP fluorescence, were cut into 1-1.5 mm^3 pieces. The animals were anesthetized with isoflurane inhalation. The surgical area was sterilized with iodine and alcohol. A transverse incision approximately 1.5 cm long was made along the left chest wall of the nude mouse using a pair of surgical scissors. An intercostal incision was made between the third and the fourth ribs and the left lung was exposed (Figure 1A). Two LLC-RFP tumor fragments were transplanted to the surface of the lower level of the left lung with 8-0 surgical nylon sutures (Figure 1C-E). The chest wall and skin were closed with 6-0 surgical silk sutures (Figure 1). The left lung was re-inflated by intrathoracic puncture using a 3 cc syringe with a 25 G×1½ needle to draw out the remaining air in the chest cavity. All procedures of the operation described above were performed with a 7× magnification microscope (Olympus Corp., Tokyo, Japan under HEPA-filtered laminar flow hoods. The operation was completed within 1 minute in order to avoid mortality. A low mortality rate (1/20) was obtained.

Imaging. The Maestro™ fluorescence imaging system (Perkin-Elmer-CRi, Woburn, MA, USA) uses a liquid crystal tunable filter (LCTF) for spectral separation (25, 26). The LCTF is optically coupled to a CCD camera. Multispectral images are acquired, with images typically spaced every 10 nm throughout the desired spectral range (26, 27).

Statistical analysis. All statistical analyses were performed using SYSTAT 12.0 (SYSTAT, Inc., Chicago, IL, USA). The experimental data are expressed as the mean ± SD. The two-tailed Student's *t*-test was used for statistical analysis. The Kaplan–Meier test was used to analyze survival.

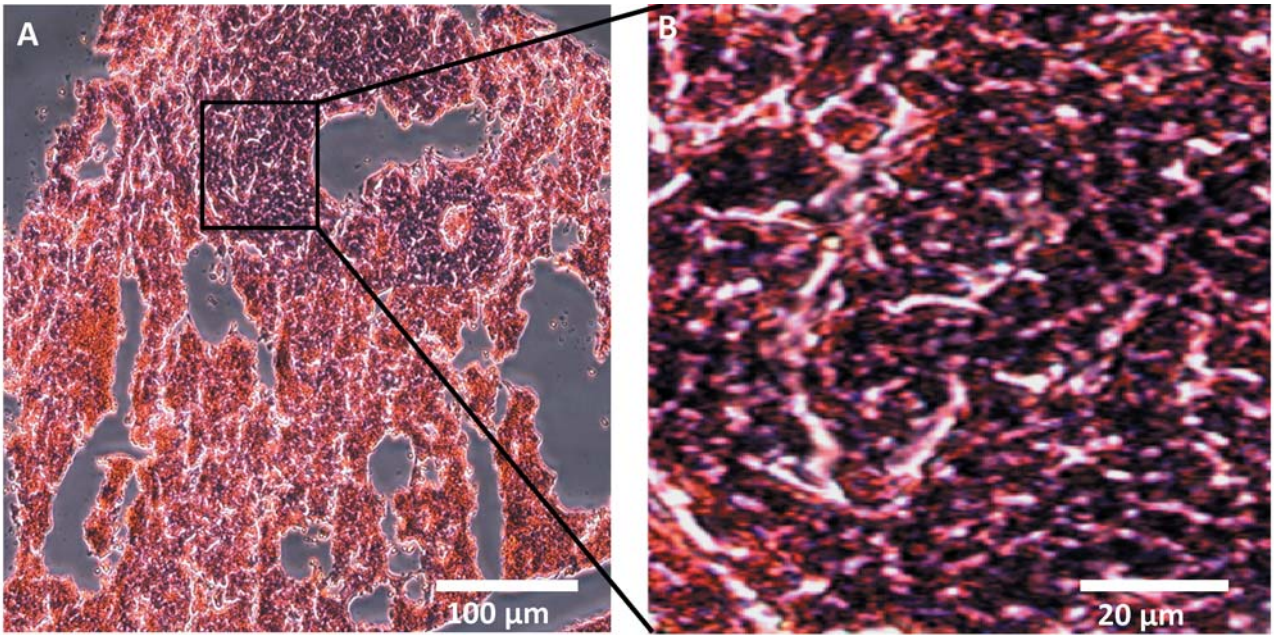

Figure 3. *H&E staining of tumor. Tumors were removed and processed for H&E staining. Slides were observed microscopically. Magnification A: ×200 (Bar=100 µm). B: ×400 (Bar=20 µm).*

Results and Discussion

High tumor take rate with low mortality. A tumor-take rate of 100% (19/19) was obtained for SOI of LLC in nude mice. Only one mouse died from the procedure.

Imaging tumor growth on the lung. Statistically-significant growth was observed at day 21 after tumor implantation compared to day7 ($p<0.05$) (Figure 2). Non-invasive spectral-separation imaging clearly visualized tumor growth on the lung. The tumor grew exponentially over time (Figure 2). The tumor was removed on day 21 and processed for H&E staining (Figure 3), which shows the high malignancy of the LLC-RFP tumor.

Noninvasive imaging of lung cancer growth confers a great advantage. For example, it enables drug efficacy to be monitored multiple times without surgical procedures. It has been previously stated that fluorescent proteins are is not suitable for deep-tissue imaging (28, 29). However, the present report demonstrates that spectral separation imaging, especially with a tunable filter, enables very bright images of the LLC-RFP tumor on the lung to be non-invasively obtained, which is located deep in the body.

Imaging of tumor growth, progression and metastasis with fluorescent proteins in mouse models is a powerful technology. A limit to fluorescent protein imaging has previously been non-invasive deep-seated tumors, such as on the lung. In the present study, the Maestro spectral-separation fluorescence imaging system with a liquid crystal tunable filter enabled noninvasive detection of lung cancer growth (30).

Conclusion

The orthotopic implantation of lung cancer exhibited high tumorigenicity (100%) and a low mortality rate (5%). Bright RFP expression of the tumor and spectral-separation enables non-invasive imaging for long-term follow-up.

The LLC-RFP model has important advantages over luciferase models in that the strong RFP signal, which is approximately 1000 times stronger than that of luciferase (31), allows real-time imaging of metastasis in any organ, as can be seen in the present report where a deep-seated tumor in the lung was imaged non-invasively. Fluorescence imaging with fluorescent proteins is far superior to photon-counting with luciferase, whose signal is so weak only photon counts, without real images, is possible.

Dedication

This article is dedicated to the memory of A. R. Moossa, MD.

References

1 Hoffman RM: Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. Investigational New Drugs *17*: 343-359, 1999.

2 Bogden AE, Cobb WR, Lepage DJ, Haskell PM, Gulkin TA, Ward A, Kelton DE and Esber HJ: Chemotherapy responsiveness of human tumors as first transplant generation xenografts in normal mouse: Six-day subrenal capsule assay. Cancer *48*: 10-20, 1981.

3 McLemore TL, Eggleston JC, Shoemaker RH, Abbott BJ, Bohlman ME, Liu MC, Fine DL, Mayo JG and Boyd MR: Comparison of intrapulmonary, percutaneous intrathoracic, and subcutaneous models for the propagation of human pulmonary and nonpulmonary cancer cell lines in athymic nude mice. Cancer Res *48*: 2880-2886, 1988.

4 McLemore TL, Liu MC, Blacker PC, Gregg M, Alley MC, Abbott BJ, Shoemaker RH, Bohlman ME, Litterst CC, Hubbard WC, Brennan RH, McMahon JB, Fine DL, Eggleston JC, Mayo JG and Boyd MR: Novel intrapulmonary model for orthotopic propagation of human lung cancers in athymic nude mice. Cancer Res *47*: 5132-5140, 1987.

5 Fu X, Besterman JM, Monosov A and Hoffman RM: Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens. Proc Natl Acad Sci USA *88*: 9345-9349, 1991.

6 Fu X, Guadagni F and Hoffman RM: A metastatic nude-mouse model of human pancreatic cancer constructed orthotopically from histologically intact patient specimens. Proc Natl Acad Sci USA *89*: 5645-5649, 1992.

7 Fu X and Hoffman RM: Human ovarian carcinoma metastatic models constructed in nude mice by orthotopic transplantation of histologically-intact patient specimens. Anticancer Res *13*: 283-286, 1993.

8 Fu X, Le P and Hoffman RM: A metastatic-orthotopic transplant nude mouse model of human patient breast cancer. Anticancer Res *13*: 901 904, 1993.

9 Furukawa T, Kubota T, Watanabe M, Kitajima M, Fu X and Hoffman RM: Orthotopic transplantation of histologically intact clinical specimens of stomach cancer to nude mice: correlation of metastatic sites in mouse and individual patient donors. Int J Cancer *53*: 608-612, 1993.

10 Kaushal S, McElroy MK, Luiken GA, Talamini MA, Moossa AR, Hoffman RM and Bouvet M: Fluorophore-conjugated anti-CEA antibody for the intraoperative imaging of pancreatic and colorectal cancer. J Gastrointest Surg *12*: 1938-1950, 2008.

11 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Moriwaki H, Bouvet M, Saji S and Hoffman RM: Multi-color palette of fluorescent proteins for imaging the tumor microenvironment of orthotopic tumorgraft mouse models of clinical pancreatic cancer specimens. J Cell Biochem *113*: 2290-2295, 2012.

12 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Saji S, Moriwaki H, Bouvet M and Hoffman RM: Imageable fluorescent metastasis resulting in transgenic GFP mice orthotopically implanted with human-patient primary pancreatic cancer specimens. Anticancer Res *32*: 1175-1180, 2012.

13 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Saji S, Moriwaki H, Bouvet M and Hoffman RM: Non-invasive fluorescent-protein imaging of orthotopic pancreatic-cancer-patient tumorgraft progression in nude mice. Anticancer Res *32*: 3063-3068, 2012.

14 Hiroshima Y, Maawy A, Sato S, Murakami T, Uehara F, Miwa S, Yano S, Momiyama M, Chishima T, Tanaka K, Bouvet M, Endo I and Hoffman RM: Hand-held high-resolution fluorescence imaging system for fluorescence-guided surgery of patient and cell-line pancreatic tumors growing orthotopically in nude mice. J Surg Res *187*: 510-517, 2014.

15 Metildi CA, Kaushal S, Luiken GA, Talamini MA, Hoffman R and Bouvet M: Fluorescently-labeled chimeric anti-CEA antibody improves detection and resection of human colon cancer in a patient-derived orthotopic xenograft (PDOX) nude mouse model. J Surg Oncol *109*: 451-458, 2014.

16 Hiroshima Y, Maawy A, Metildi CA, Zhang Y, Uehara F, Miwa S, Yano S, Sato S, Murakami T, Momiyama M, Chishima T, Tanaka K, Bouvet M, Endo I and Hoffman RM: Successful fluorescence-guided surgery on human colon cancer patient-derived orthotopic xenograft mouse models using a fluorophore-conjugated anti-CEA antibody and a portable imaging system. J Laparoendosc Adv Surg Tech A *24*: 241-247, 2014.

17 Hiroshima Y, Zhao M, Maawy A, Zhang Y, Katz MH, Fleming JB, Uehara F, Miwa S, Yano S, Momiyama M, Suetsugu A, Chishima T,Tanaka K, Bouvet M, Endo I and Hoffman RM: Efficacy of Salmonella typhimurium A1-R versus chemotherapy on a pancreatic cancer patient-derived orthotopic xenograft (PDOX). J Cell Biochem *115*: 1254-1261, 2014.

18 Hiroshima Y, Maawy A, Zhang Y, Murakami T, Momiyama M, Mori R, Matsuyama R, Katz MH, Fleming JB, Chishima T, Tanaka K, Ichikawa Y, Endo I, Hoffman RM and Bouvet M: Metastatic recurrence in a pancreatic cancer patient derived orthotopic xenograft (PDOX) nude mouse model is inhibited by neoadjuvant chemotherapy in combination with fluorescence-guided surgery with an anti-CA 19-9-conjugated fluorophore. PLoS One *9*: e114310, 2014.

19 Hiroshima Y, Zhang Y, Zhang M, Maawy A, Mii S, Yamamoto M, Uehara F, Miwa S, Yano S, Murakami T, Momiyama M, Chishima T, Tanaka K, Ichikawa Y, Bouvet M, Murata T, Endo I and Hoffman RM: Establishment of a patient-derived orthotopic xenograph (PDOX) model of HER-2-positive cervical cancer expressing the clinical metastatic pattern. PLOS ONE *10*:, e0117417, 2015.

20 Wang X, Fu X and Hoffman RM: A new patient like metastatic model of human lung cancer constructed orthotopically with intact tissue *via* thoracotomy in immunodeficient mice. Int J Cancer *51*: 992 995, 1992.

21 Zhang Y, Zhang N, Su S, Hoffman R and Zhao M: Salmonella typhimurium A1-R tumor targeting in immunocompetent mice is enhanced by a Traditional Chinese Medicine herbal mixture. Anticancer Res *33*: 1837-1843, 2013.

22 Hoffman RM and Yang M: Subcellular imaging in the live mouse. Nature Protocols *1*: 775-782, 2006.

23 Hoffman RM and Yang M: Color-coded fluorescence imaging of tumor-host interactions. Nature Protocols *1*: 928-935, 2006.

24 Hoffman RM and Yang M: Whole-body imaging with fluorescent proteins. Nature Protocols *1*: 1429-1438, 2006.

25 Levenson R, Yang M and Hoffman RM: Whole-body dual-color differential fluorescence imaging of tumor angiogenesis enhanced by spectral unmixing. Proc Am Assoc Cancer Res *45*: 46, 2004.

26 Mansfield JR, Gossage KW, Hoyt CC and Levenson RM: Autofluorescence removal, multiplexing, and automated analysis methods for *in vivo* fluorescence imaging. J Biomed Opt *10*: 41207, 2005.

27 Levenson RM and Mansfield JR: Multispectral in biology and medicine: Slices of life. Cytometry A *69*: 748-758, 2006.

28 Ntziachristos V, Ripoll J, Wang LV and Weissleder R: Looking and listening to light: the evolution of whole-body photonic imaging. Nat Biotechnol *23*: 313-320, 2005.

29 Kocher B and Piwnica-Worms D: Illuminating cancer systems with genetically engineered mouse models and coupled luciferase reporters *in vivo*. Cancer Discovery *3*: 616-629, 2013.

30 Zhang Y, Hiroshima Y, Ma H, Zhang N, Hoffman RM and Zhao M: Complementarity of variable-magnification and spectral-separation fluorescence imaging systems for noninvasive detection of metastasis and intravital detection of single cancer cells in mouse models. Anticancer Res *35*: 661-668, 2015.

31 Ray P, De A, Min JJ, Tsien RY and Gambhir SS: Imaging tri-fusion multimodality reporter gene expression in living subjects. Cancer Res *64*: 1323–1330, 2004.

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