Longitudinal far red gene-reporter imaging of cancer metastasis in preclinical models: a tool for accelerating drug discovery

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Abstract: In this short communication, we demonstrate for the first time, the use of far red fluorescent gene reporter, iRFP to longitudinally and non-invasively track the in vivo process of lymphatic metastases from an orthotopic site of mammary implantation through lymphatic vessels and to draining lymph nodes. Potentially useful to accelerate cancer drug discovery as an in vivo screening tool to monitor the pharmacological arrest of metastasis, we show that the custom as well as commercial small animal imaging devices have adequate performance to detect the gene reporter in stably expressing metastatic cancer cells.

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OCIS codes: (000.1430) Biology and medicine; (040.1520) CCD, charge-coupled device; (110.3080) Infrared imaging.

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1. Introduction

While most cancer deaths occur to metastasis to distant organs, cancer therapeutics are typically tested using preclinical cancer models that are largely irrelevant [1, 2]. Transplantation models, whether xenografts or allografts, depend upon subcutaneous or
orthotopic implantation of cancer cells to form a primary lesion, which may or may not metastasize typically through the regional lymph nodes before reaching the circulatory system and seeding distant organs. Another transplant model of metastatic dissemination involves direct injection into the systemic circulation, largely bypassing lymph nodes for metastases in several organs, including liver and lung. Genetically engineered mice models involve spontaneous or inducible cancers that can recapitulate the metastasis cascade seen in humans, but like the transplantation models, it is difficult to longitudinally track the processes of metastases much less the spontaneous primary lesion formation within an intact animal.

The detection of lymph node metastases in humans remains elusive requiring lymph node biopsy or dissection and subsequent pathological examination as part of tumor node metastasis staging. In animals, bioluminescence imaging of implanted cells expressing luciferase allows detection of macroscopic lesions and imaging of fluorescent protein reporter has enabled real time tracking of individual cells with intravital microscopy techniques. But there has been little work to use these technique to track lymph node metastases within intact animals. Previously, we have used DsRed, a red excited fluorescent protein reporter, and iRFP [3], a far red fluorescent gene reporter as a pathologically confirmed “ground truth” for ex vivo determination of cancer-positive lymph nodes in an orthotopic xenograft model of metastatic prostate cancer [4, 5]. However, autofluorescence that arises from excitation of red excited fluorescent proteins can reduce contrast preventing longitudinal imaging of the early stages of metastases through the lymphatic channels. In this short communication, we demonstrate for the first time, the use of wide-field, fluorescence planar imaging of intact orthotopic xenografts of MDA-MB-231 expressing iRFP to longitudinally show the processes of metastases through the lymphatic channels draining into axillary lymph nodes. Due to the far red excitation spectra and the high fluorescent yields, the use of iRFP enables high contrast for in vivo imaging. The model described herein could be used to quantitatively test the efficacies of anti-cancer and anti-metastasis drug candidates. Furthermore, we use solid working standards previously developed by us [6] to compare the performances of the camera systems that could be similarly utilized to image longitudinally the processes of lymphatic metastasis. Specifically, we sought to determine whether the signal strength of iRFP is sufficiently bright for in vivo imaging using a common electron multiplying charge coupled device (EMCCD), intensified CCD (ICCD), as well as a commercial IVIS small animal imaging system. Our work demonstrates that this model could be widely adopted in several laboratories for longitudinal imaging of the processes of metastases through lymphatic vessels.

2. Materials and methods

2.1 MDA-MB-231-iRFP infection and cell culture

To transfect MDA-MB-231 cells expressing iRFP gene reporter, MDA-MB-231 cells were cultured as monolayer in DMEM-F12/10% fetal bovine serum (FBS, BioExpress, Kaysville, UT, USA). At near confluency, the culture was transected with piRFP plasmid (Addgene, Cambridge, MA, USA) by Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) as suggested by the manufacturer. Transfected cells were grown under 0.8 mg/ml G418 selection in DMEM-F12/10% FBS growing medium. Transfectoma cells that survived the antibiotic selection were then sorted through flow cytometry outfitted with 690 nm/730 nm (excitation/emission) wavelengths to obtain the population of high iRFP expressers.

2.2 Orthotopic MDA-MB-231 animal model

To provide the most relevant model of tumor growth and lymph node metastasis, we orthotopically implanted MDA-MB-231-iRFP cells (1.0 × 10⁶) into the 4th right lower mammary pad of Nu/Nu female mice at 8 weeks of age (CharlesRiver, Wilmington, MA, USA). Mice were maintained in a pathogen-free environment and fed AIN-93G (OpenSource Diets, New Brunswick, NJ, USA) for reducing food background fluorescence. All animal studies were conducted following approval from the University of Texas Health Science
Center Animal Welfare Committee as part of a larger ongoing study to assess agent targeting of cancer cells within the lymphatic vascular space. Herein, we report the unexpected results that show iRFP imaging of the process of metastasis through the lymphatic space which is visualized in approximately 50% of animals.

2.3 iRFP imaging of in transit and lymph node metastases

Between 4 and 7 weeks after implantation, animals were anesthetized with isoflurane and imaged weekly to provide longitudinal assessment of in transit lymphatic metastases and lymph node metastasis using a 16-bit EMCCD (Photon Max 512, Princeton Instruments, Trenton, NJ). Briefly excitation of iRFP is provided by a 690 nm light from a laser diode filtered through a “clean up” filter (690FS10-25, optical density > 4, FIR-Xray, Andover, Salem, NH) for illumination of the animal with a 10 cm in diameter field of view with incident \( \sim 1\text{mW/cm}^2 \). The collection of iRFP fluorescence at 720 nm occurs through two 720 nm band pass filters (720FS10, optical density > 4, FIRXray, Andover, Salem, NH) for image registration on an EMCCD camera. Typical imaging views consisted of ventral, right, and left lateral sides with and without the primary implantation site covered to enable the detection of iRFP signals within the limited dynamic range of the 16-bit detectors. While we could not access conventional, commercial small animal imaging systems and investigational clinical systems for animal imaging due to common animal barrier and IACUC housing constraints, we sought to assess whether the IVIS imaging system (IVIS Lumina II, Caplier Life Sciences, Waltham, MA) as well as our clinical ICCD system [7] could potentially likewise detect iRFP metastases in the lymphatic channels using the solid working standards.

2.4 Camera performance assessment using solid working standards

Unfortunately, there is no stable working standard to consistently compare camera performance that would enable investigators to assess whether imaging performed on one fluorescence imaging system could be equally performed on another. Liquid phantoms containing proteins and organic dyes are unsatisfactory working standards, since they typically are single use, often unstable, and are not robustly reproducible. Previously, we developed stable, far red and near-infrared fluorescent solid working standards for characterizing the sensitive collection and detection of fluorescence emanating from a multiply scattering medium in order to compare fluorescence imaging devices [6]. Specifically we embedded QDots 800 (Q21771MP, Invitrogen, Carlsbad, CA) in increasing pM – nM concentrations with TiO2, and polyethylene in the wells of a 96 well assay plate to create stable, fluorescent working standards that emitted dim fluorescence >720 nm when excited with 690 nm excitation light for assessment of fluorescent contrast and SNR as previously described [6]. Contrast was computed from the ratio of the 720 nm fluorescent signal averaged across wells with and without QDots 800 while SNR was computed from their difference in fluorescent intensity divided by the standard deviation of the intensities from the well without QDots 800. Measurements were performed on the ICCD, EMCCD, as well as for comparison to the IVIS commercial system installed with CCD camera in order to compare image performance at 720 nm and speculate on the performance of a common small animal imaging system routinely used in preclinical drug discovery studies. Specifically we sought to assess suitability of the ICCD and CCD systems for collecting fluorescence from iRFP relative for the performance of the EMCCD system used in the in vivo studies. All measurements of working standards were conducted at 200 ms integration times.

3. Results and discussion

Figure 1 shows a typical series of longitudinal scans 4, 5, 6, and 7 weeks after orthotopic implantation using the EMCCD fluorescence imaging system. Background is minimized with feeding of chlorophyll-free chow, enabling visualization of the right and left lymphatic channels (arrows) draining cells from the right fourth mammary gland by weeks 6 and 7. Only when the primary tumor is covered, do the lymphatic channels become visible, owing to the limited dynamic range associated with charge coupled devices. Pathology confirmed that
the iRFP cells were located within the lymph nodes as well as within the lymphatic vessel channels, as depicted in Fig. 2.

Fig. 1. Rows 1-4, iRFP fluorescence imaging (units in AU camera output) of animals 4 – 7 weeks after orthotopic implantation in right lower mammary gland showing primary tumor in ventral view (column 1, week 4 only); ventral view with primary tumor covered (column 2) showing lymphatic channels (arrows); lateral right view (Column 3, week 7 only) showing axillary lymph node (arrow); and left lateral view (column 4, week 7 only) showing faint lymphatic vessel (arrow). Images were collected using EMCCD camera system.

Fig. 2. H&E staining showing the tumor cells (left, arrows) in the lymphovascular spaces and the residual lymphocytes and clusters of metastatic carcinoma (right, arrows) in the metastatic lymph node seven weeks after orthotopic implantation. All images were taken at 200X magnification and scale bars represent 50 μm.
Figure 3 plots the SNR versus contrast associated with the QDots 800 fluorescence in the solid working standards for the ICCD and EMCCD that employed the same filters and excitation sources as well as IVIS imaging system. Consistent with our prior work [6], the ICCD demonstrates much higher measurement sensitivity in both SNR and contrast compared to the EMCCD, attributed to the intensifier amplification of both signal and noise, with subsequent chip integration to reduce the noise levels. It should be noted that the resolution of the intensified system is less than that of the EMCCD as can be expected from the intensified multiple channel plate as well as the fiber coupler between the intensifier and chip [7]. In order to provide adequate resolution in the small fields of view in preclinical imaging, we used the EMCCD over the ICCD that we routinely use in clinical studies involving wider fields of view. The results associated with the IVIS system operating in the Cy5.5 channel show comparable contrast, but improved SNR as compared to the results with the EMCCD system. Because QDots 800 is excited over a broader spectral range than iRFP, the broader band of excitation and detection of the IVIS system could be responsible for a higher signal that obtained from the EMCCD system that employed laser diode excitation. But nonetheless, the working standard comparison shows that the IVIS as well as the EMCCD camera systems have similar performance and can be expected to longitudinally detect iRFP in metastasizing cancer cells, and that the ICCD systems optimized for clinical imaging may not be necessary for detection of cancer metastases through lymphatic channels.

![Graph showing SNR versus contrast for QDots 800 fluorescence in ICCD, EMCCD, and IVIS.](image)

Fig. 3. Measured signal-to-noise ratio versus contrast measured on QDots 800 based fluorescent solid working standard using ICCD (open circles), EMCCD (filled triangles), and IVIS (open diamonds). Symbols denote a single measurement on the working standard.

While it may be optimal to develop working standards based upon the protein iRFP or an organic dye with similar spectra to iRFP, unfortunately proteins and dyes are notoriously unstable in solution or when embedded in a cured polyurethane solid (data not presented for brevity). Instead, we employ the broad spectra of QDots with albeit small absorption coefficient and fluorescence yield in the far-red as described in our prior work [6]. The stability of QDots in the solid working standards makes them suitable as a working standards that are stable for up to two years.

While our work is the first to use iRFP to monitor the process of lymphatic metastasis longitudinally and shows the utility for screening the anti-metastatic capabilities of cancer.
drugs, others have used iRFP preclinically to monitor infection, primary tumors, and metastatic lymph nodes [8]. Using an iRFP expressing strain of parasitic protozoan *Leishmania infantum*, Calvo-Alvarez et al [9] showed the ability to monitor *in vivo* infection longitudinally using the IVIS system in order to provide future *in vivo* screening of drug candidates. Deliolanis, et al [10] used fluorescence and optoacoustic tomography to detect *in vivo* the iRFP gene expression of implanted glioma brain tumors in animals and Agollah et al. [11], showed the ability to detect metastases of inflammatory breast cancer cells within lymph nodes, but did not show the locoregional and distant processes of metastases through the lymphatic vessels as sown in this study.

In summary, pathology confirms that the far red fluorescence of iRFP can be successfully imaged to track the transit of cancer cells through the lymphatic channels, enabling new preclinical opportunities to assess cancer metastasis longitudinally in response to drug candidates. The higher measurement sensitivity of the ICCD clinical systems employed in our clinical studies may not be needed to detect iRFP fluorescence. Instead, the performance of commercial IVIS imaging systems as well as the custom EMCCD system may be sufficient for detecting the transit of iRFP expressing cancer cells in peripheral lymphatic vessels that drain to axillary lymph nodes.

Acknowledgments

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