

In Vivo Fluorescence Imaging of Tumor Proliferation Using Pre-labeled Cancer Cells and a Targeted Probe

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Application Overview

Optical molecular imaging techniques have emerged as valuable tools for pre-clinical oncology research in mouse models of cancer.¹⁻⁵ Diseased tissues and cells are typically detected during in vivo fluorescence imaging by one of three means: (1) near-infrared (NIR) injectable probes that are either targeted^{1,3,4} or activated by^{2,6} tumor cells, (2) tumor cells that are exogenously labeled in culture with NIR dyes⁷ and subsequently injected in vivo, or (3) cell lines that have been genetically modified, typically through viral vectors, to express fluorescent protein reporters that allow in vivo detection.^{5,8} We demonstrate here an imaging experiment that merges two of these strategies. Exogenously labeled tumor cells were implanted and subsequently detected by an injectable, targeted probe with a different emission wavelength.

Exogenous cell labeling provides a convenient means to transfer fluorescent molecules to cells, thus enabling their detection. This can be accomplished through a receptor-ligand/antibody-epitope interaction. Alternatively, lipophilic agents, like DiR (Invitrogen Inc., Eugene, OR USA), which non-specifically enter cell membranes and typically give better labeling. Non-receptor-mediated endocytosis may also be utilized as cells that will absorb dye into internal endosomal compartments. The X-SIGHT 761 Nanoparticle (Carestream Health, Inc., Woodbridge, CT USA) utilizes a polycationic surface to facilitate endocytotic uptake and cell labeling.⁹ Our own data indicate that DiR and X-SIGHT 761 provide comparable cell labeling.

After animal injection, labeled cells can be monitored until their proliferation reaches a point where subsequent daughter cells do not contain enough fluorophore to overcome threshold-detection levels in a given region. It is for this reason that, depending upon the rate of division of the cells used, cell labeling is rarely effective for long time-course longitudinal imaging past two or three weeks.

Injectable probes are a family that encompasses targeted, activatable, or free fluorophores. Targeted probes typically consist of a fluorophore conjugated to a targeting ligand, e.g., an antibody that interacts and/or binds to a biomacromolecule of interest, thus increasing the fluorophore concentration at the target site. An activatable probe generally consists of a fluorophore conjugated to a quenching molecule that prevents its visualization. The fluorescent quencher is typically attached to the fluorophore via a peptide linker with a consensus sequent that can be recognized by specific enzymes. Once the peptide is cleaved, the quencher dye disassociates, leaving only the active fluorophore to serve as an indicator of the presence of the enzyme. Free fluorescent dyes and nanoparticles can sometimes be used to non-specifically localize to compartments in the body such as the leaky and retentive vascular networks within tumors. The facile detection, longitudinal use, ease of synthesis, and commercial probe availability are all benefits of injectable fluorescent agents.

Genetically manipulated cell lines expressing fluorescent protein probes can be introduced into test subjects or various animal models. These studies are useful as subsequent daughter cells also express the fluorophore and can be longitudinally monitored

without any loss in signal intensity per cell. Despite this method's benefits, it also has its limitations. First, establishing a stable cell line or animal model often requires extensive resources and time. Second, fluorescent protein expression may not be either constitutive or consistent, and third, occurrence of reporter silencing is always a possible outcome.

In this study, we utilized two fluorescent labeling methods to perform tumor imaging: (1) exogenous cell labeling, for early localization and detection, and (2) targeted probe injection, for localization of late-stage tumor development at the conclusion of the study. The approach looked to combine the two methods to compensate for the time point that each method may fail to give reliable data. By combining labeling methods, it was hypothesized that the cell labeling method would maximize visualization and quantitation of early-stage tumor tissue, while the injectable probe would do the same for later-stage tumor tissue that had undergone too many mitotic divisions to possess sufficient quantities of NIR fluorescent tags. Also, a multimodal animal rotation system (MARS) (Carestream Molecular Imaging, Carestream Health, Inc., Woodbridge, CT USA) was used to optimize quantitation by driving the mouse to specific angular positions to optimize optical signal capture.

Methods

In vivo Imaging Model

Mice were challenged with 200 μ L CT26.CL25 cells (CRL-2638™) colon carcinoma cells that express beta-galactosidase (ATCC, VA USA). Cells were injected subcutaneously into the right flank of a male, athymic nude mouse (Nu/Nu, 6 weeks old, Taconic Farms, Germantown, NY USA), resulting in approximately 2.5×10^5 viable cells injected in the right flank. Cells were exogenously labeled with X-SIGHT 761 prior to injection. Cell cultures in RPMI-1640 media were incubated in a 1 μ M solution of X-SIGHT 761 (Carestream Health, Woodbridge, CT USA) for 4 h at 37°C, were washed 3 times with 1X PBS, and resuspended in RPMI-1640 media. Following this cell labeling procedure, an 86% viability of the cells was observed by a Trypan Blue exclusion test of cell viability. The tumor was allowed to grow to a palpable size. At 19 days post injection of tumor

cells, a single bolus of a novel tumor probe, which we will call "Tetra," was injected intravenously by way of the lateral tail vein. The mouse was then imaged 22 days after grafting of tumor cells. At the time of imaging, the mouse was approximately 8 weeks of age with an overall tumor measurement of 7 x 19 mm.

In vivo Fluorescence Imaging

The mouse was anesthetized in an induction chamber with 2% aerosolized isoflurane and placed into a Carestream In-Vivo Multispectral FX PRO (MS FX PRO) imaging system configured with a MARS. A protocol was administered that consisted of 6 images at each angle, ranging from 0° to 270° in increments of 5°. All images consisted of a 16-bit image collected by a cooled CCD camera. Near-infrared fluorescence (NIRF) and deep-red fluorescence images were taken to separately image X-SIGHT 761 (ex: 750 nm \pm 10 nm, em: 830 nm \pm 17.5 nm, f-stop 2.5, 2 x 2 binning, 10 s exposure) and Tetra (ex: 650 nm \pm 10 nm, em: 700 nm \pm 17.5 nm, f-stop 2.5, 2 x 2 binning, 10 s exposure) signals, respectively.

For anatomical co-registration of fluorescence signal, an X-ray image was taken (f-stop 2.5, 10 s exposure, 2 x 2 binning, 0.4 mm aluminum filter), as well 3 separate reflectance images, to render a Red/Green/Blue (RGB) composite image (ex: 440 nm \pm 10 nm, 540 nm \pm 10 nm, 600 nm \pm 10 nm, no-emission filter, 0.175 s exposure, f-stop 4, no binning). For precise co-registration, all images were taken with a 100 mm field of view (FOV) and a focal plane of 10.1 mm.

Image Analysis and Presentation

Semi-quantitative analysis was performed using Carestream MI Software version 5.0.5.29 (Carestream Health, Inc., Woodbridge, CT USA). Region of interest (ROI) analysis was completed on Tumor 1 in the X-SIGHT 761 channel by thresholding an ROI to 50% of its maximum intensity using the auto-ROI tool in MI software. The location of this ROI was transposed onto the Tetra filter pair images using the copy and paste function. The Tumor 2 signal was measured by drawing a free-form manual ROI. The liver signal was obtained by thresholding the liver region to 70% of its maximum pixel intensity. Non-target values for both image sets were obtained by drawing a large free-form ROI away from the target signals. Data was exported and figures were compiled in Microsoft Excel®.

Image presentation was completed in ImageJ software (v.1.44i, NIH, <http://imagej.nih.gov/ij/download.html>). All 16-bit tiff images were batch exported from MI software. Fluorescence images were then false colored (X-SIGHT 761 in red, Tetra in blue), and X-SIGHT 761/Tetra and X-SIGHT 761/Tetra/X-ray overlays were compiled (Fig. 1).

Results/Discussion

Imaging of the mouse revealed two separate subdermal tumor masses that were connected by a bridge of tumor tissue and designated “Tumor 1” and “Tumor 2.” These distinct masses could be localized in the RGB composite image (not shown), and their respective fluorescence was measured (Figs. 1 and 2). Tumor 1 was deduced to be the established primary-tumor region because it possessed high signal intensity from X-SIGHT 761 in the NIRF images. The visualization of Tumor 1 using X-SIGHT 761 was distinct with a target/non-target (T/NT) ratio peak of 12.6 (see Fig. 2). As the

tumor matured and developed a secondary mass, Tumor 2, endocytic concentration of X-SIGHT 761 dropped below threshold-detection levels, and no signal was seen in the NIRF images. This inherent limitation of pre-cell labeling, which is an inability to visualize and quantify late-stage tumor tissue, was overcome by the addition of a Tetra compound. The Tetra compound labeled both tumors almost equally (Fig. 2) and yielded peak T/NT values of 2.56 and 2.66, respectively.

Imaging with the MARS system revealed optimal angles for detection of tumor signals. A rotation angle of 105° was shown to be the optimal angle for detection of Tumor 1 fluorescence, both utilizing X-SIGHT 761 and Tetra, and 130° was the optimal angle for detection of Tumor 2 (see Fig. 2). A single capture compromise between 115-125° could be taken to image both tumors simultaneously. Tumor 1 had a 50% peak signal detection range of 100°, while Tumor 2 had a peak signal detection range of 80°, suggesting that the width of Tumor 1 was greater.

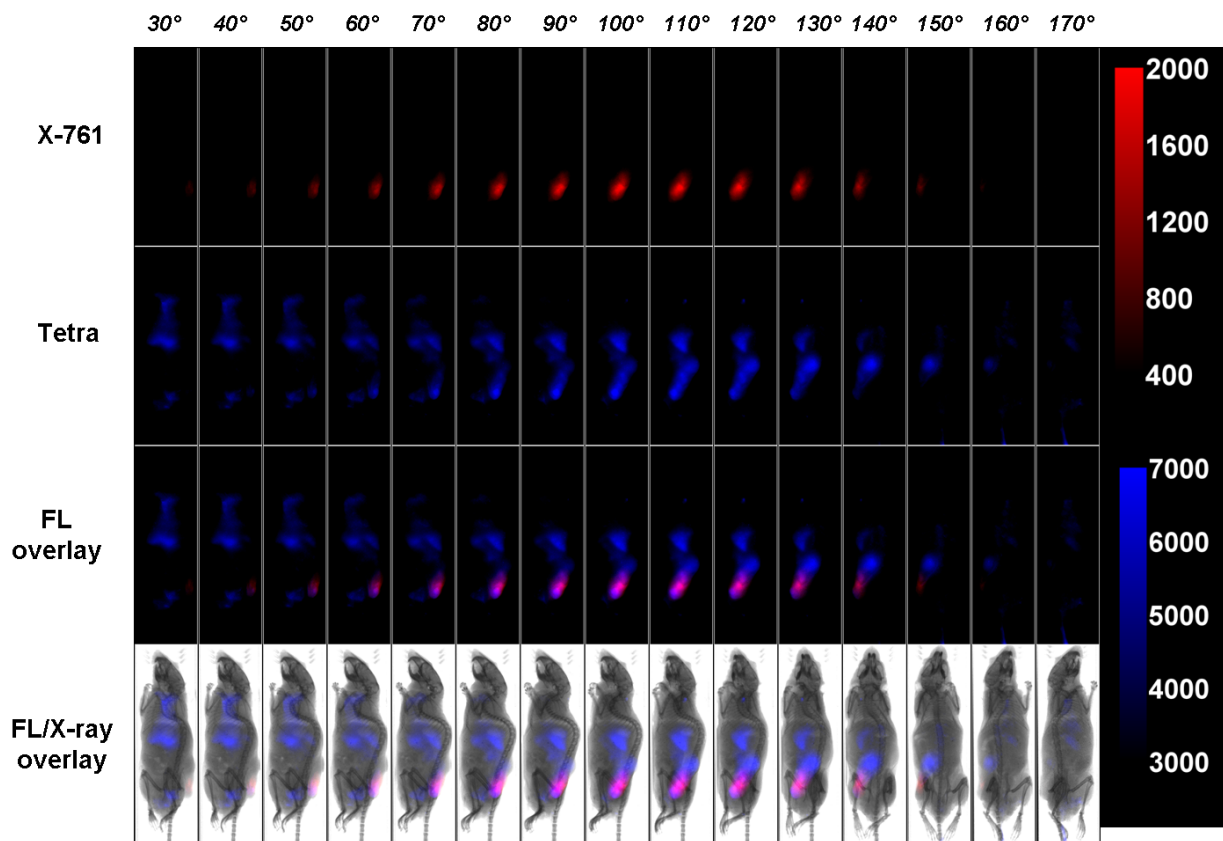


Figure 1. Image montage of single-channel images of X-SIGHT 761 (red) and Tetra (blue), fluorescence overlay of X-SIGHT 761 + Tetra, and fluorescence/X-ray overlay of X-SIGHT 761 + Tetra + X-ray at 10° incremental rotations across the detectable range of each fluorophore reporter

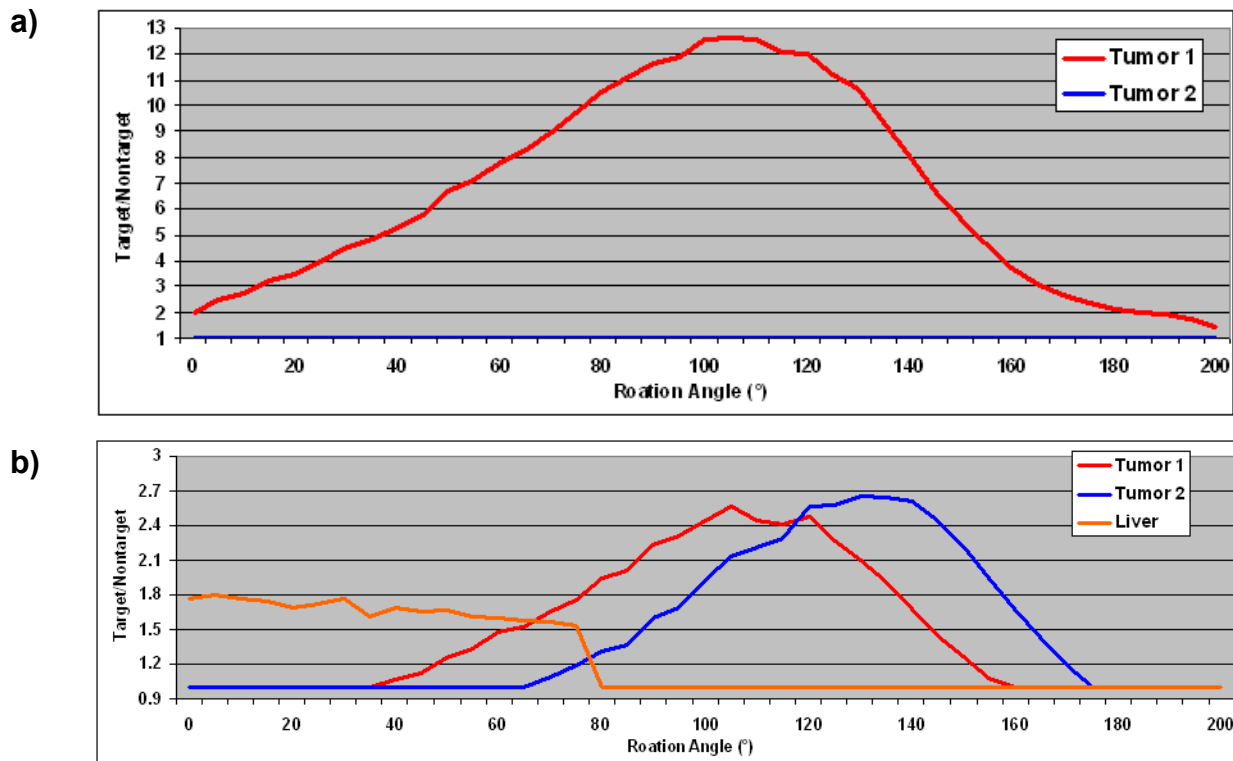


Figure 2. Normalized intensities for (a) X-SIGHT 761 and (b) Tetra-SQR at each rotational angle.

Conclusion

This application note describes an experimental protocol to image both early- and late-stage tumors without the use of a genetic reporter. An exemplary mouse was injected exogenously with tumor cells labeled with X-SIGHT 761 nanoparticles. An early tumor mass (Tumor 1) was effectively visualized using the X-SIGHT 761 pre-label, as was evidenced by a high T/NT ratio. Next, we injected a novel probe to detect a later-stage tumor (Tumor 2), while also imaging the first tumor with near equal fluorescence intensity (Tumor 1). Early-stage tumor imaging with the X-SIGHT pre-label and long time-point visualization with the injectable demonstrates a powerful one-two punch for in vivo fluorescent tracking of tumors. Additionally, use of the MARS module showed how fluorescent signal sensitivities could be optimized by imaging at precise animal orientations in which there were minimal amounts of diffusive tissue between tumor-associated signals and the MS FX PRO camera.

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