Focal Points
Application Note FP-178

Biodistribution Monitoring Using the iBox Explorer^2 Imaging Microscope

Quantitative and Qualitative Measurement of Near Infrared Dye Distribution in a Nude Mouse

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Introduction

Biodistribution studies are critical in the investigation of novel pharmacological agents, yielding vital information about tissue distribution of drug formulations. For example, one formulation may cause increased uptake of a therapeutic agent in a non-specific manner, potentially resulting in adverse events when dosed in patients. Thus, such studies can guide development of improved formulations.

Biodistribution is also used to characterize non-pharmaceutical agents. For example, information about localization can assist in characterizing the effects of intrinsic physical and chemical properties upon movement and sequestration within a living organism (hydrophobicity, isomerism, molecular weight, presence of functional groups, etc.). Understanding how modification of intrinsic properties affects distribution can further guide development of the molecule as well as provide insight into which specific applications to funnel the novel agent.

Fluorescence imaging plays a pivotal role in tracking the biodistribution of novel agents, particularly in an *in vivo* model. Fluorescent markers, either in the form of genetic reporters like green fluorescent protein (GFP), organic/ inorganic dyes or nanoparticles, can be attached to the molecule of interest. In the case of GFP, the genetic marker is transfected into a host (cell line, mouse, virus, etc.) and co-expressed with the protein of interest. For targeting to specific extracellular, intracellular or cell-surface antigens, fluorescent markers can also be conjugated to antibodies and administered via several methods (intravenous, intraperitoneal, etc).

The iBox® Explorer™^{2} Imaging Microscope is a versatile tool capable of monitoring the distribution of fluorescent material throughout organs and tissues of small animals *in vivo*. The wide angle optics and selectable magnifications allow for a range of studies to interrogate biological phenomena from the macroscopic (cm) to microscopic (µm) scale. In addition, the iBox Explorer^2 is well suited for imaging the distribution of dye in a whole animal, allowing:

- Non-invasive monitoring of fluorescence *in vivo*
- Rapid detection of low fluorescent signals with a high sensitivity CCD camera
- Anesthesia and warming of experimental animals for safe and rapid imaging
- Macro to micro detection for detailed visualization at the cellular level

To highlight the capabilities of whole animal imaging, a biodistribution study was conducted using a near infrared (NIR) dye conjugated to an antibody. A dose of the conjugate was administered in an anesthetized animal and the distribution of the dye monitored via fluorescence imaging over a 4-hour period. Qualitative and quantitative data analysis revealed a predilection of conjugate accumulation within the liver, sites of trauma and neck lymph nodes of a nude, non-tumor bearing mouse.

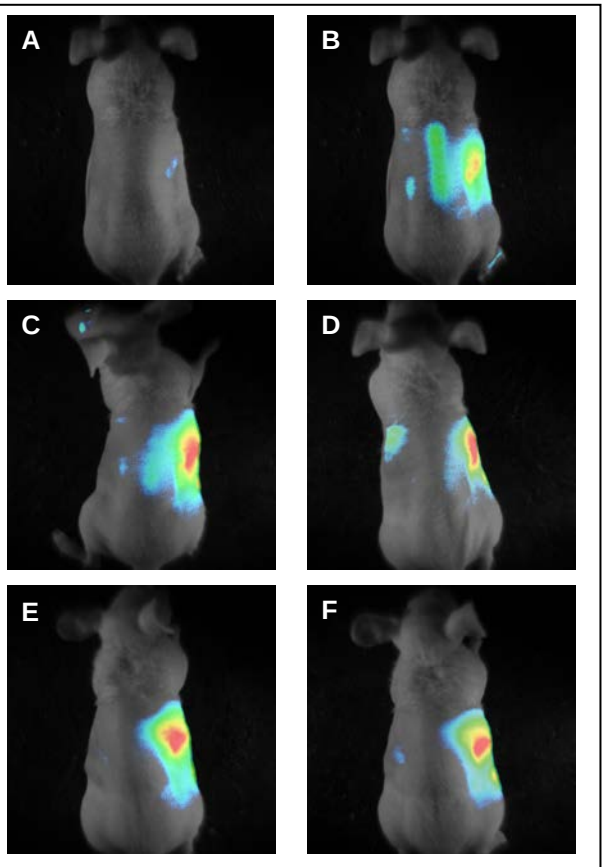


Figure 1. Distribution of DyLight 755 conjugated to anti-CEA MAb in a nude mouse over 4 hours. The images above correspond to the following time points, post injection:
A: 0 hrs; B: 10 mins; C: 1 hr; D: 2 hrs; E: 3 hrs; F: 4 hrs.

The NIR DyLight (Thermo Fisher Scientific) was selected because its physico-chemical properties facilitated immediate labeling of amine-containing molecules (e.g., antibodies) due to the presence of a NHS ester reactive group. Anti-Carcino Embronic Antigen (CEA) antibody was selected as the conjugated protein due to the high expression of CEA in certain cancer cell lines. Thus, this study would serve as a proof-of-principle experiment for future studies in tumor-bearing mice.

Material & Methods

Antibody Preparation and Conjugation

A monoclonal antibody to Carcino Embryonic Antigen (CEA) was placed in PBS buffer at a pH of 7.5 with 0.09% sodium azide at a concentration of 5.7 mg/ml and spiked with 10%v/v 0.67 borate buffer concentration. A determined amount of dye (Thermo Fisher Scientific), reconstituted at 10 mg/ml in dimethyl formamide, was added to a borate spiked antibody solution and vortexed for 30 seconds to one minute. The dye-protein mixture was then incubated for one hour in the dark at room temperature. After incubation, the conjugates were purified using the Pierce Dye removal Resin (100ul of 50% slurry) in a 5 micron Harvard column.

Experimental Protocol

An athymic nude mouse (AntiCancer, Inc, San Diego, CA, USA) was anesthetized subcutaneously with 40 microliters of a ketamine mixture (ketamine/xylazine). The conjugate, with a peak excitation of 754nm and a peak emission of 776nm, was diluted in 100 microliters of phosphate buffered saline and injected via the left retro-orbital sinus. Human anti-CEA antibody was chosen for this study to serve as a baseline for future experiments involving injecting the same conjugate in high CEA-expressing tumors. The mouse was then placed in the prone position on the iBox Explorer^{2}s imaging stage and images were captured with the highly sensitive CCD camera after 10 minutes. To ensure euthermia, the imaging stage has a built-in warming plate that maintained the temperature of the mouse at 37°C.

The mouse was briefly anesthetized with 2-3% isoflurane before each subsequent imaging time point to minimize motion artifact, then imaged in both a prone and supine position every hour for a total of 4 hours. Using the iBox Anesthesia System (UVP, LLC), vaporized inhaled anesthesia and 500 mL/min of oxygen were delivered using a non-rebreathing apparatus. After the 4 hour period, a series of images was captured with the peritoneum retracted. For an *ex vivo* analysis, several vital organs (heart, lungs, kidneys, spleen, liver/gall bladder and stomach/intestines) were resected and imaged.

Lighting and Filters

Excitation light was filtered with a 720nm, bandpass 40nm (720bp40) filter and emission light was filtered with a 780lp nm filter. Excitation light was supplied by the BioLite™ Xe 150W MultiSpectral Light Source and delivered to the darkroom through fiber optic cables. The sample was illuminated via side lighting above the imaging stage, providing high contrast and a wide field of illumination. At higher magnifications, in which deeper interrogation of tissue is required, coaxial lighting (excitation light channeled through the system's optics) provides intense light focused on a narrow region of the stage. This form of lighting is ideal for probing cellular phenomena. For this experiment, side lighting was utilized as a wide field of illumination was required.

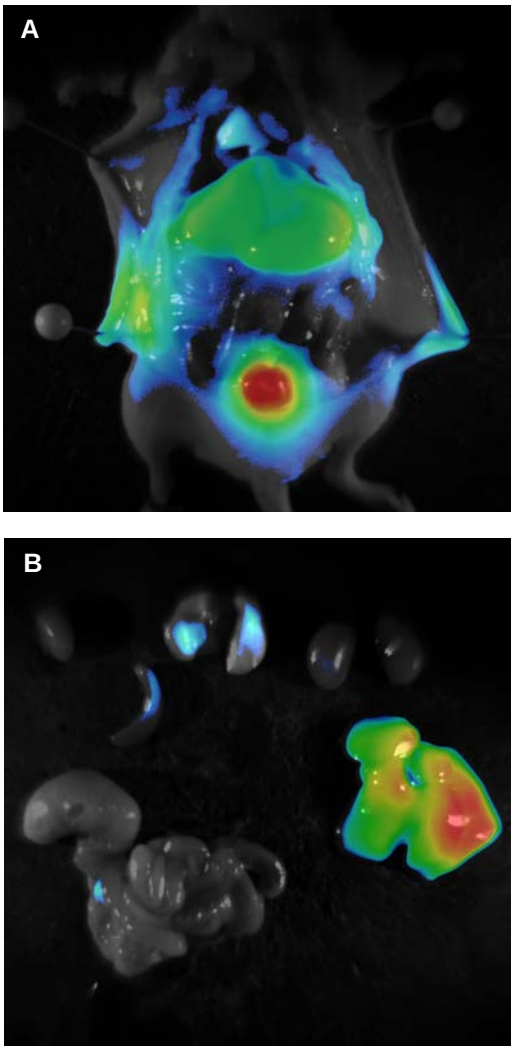


Figure 2. Image A corresponds to a digital zoom of the abdomen, highlighting the liver (center, top), bladder (center, bottom) and a region of local inflammation (center, left). *Ex vivo* analysis (Image B) shows that the liver has the highest emission in the NIR range, suggesting the greatest accumulation of dye. Dye was excited using a 720bp40nm filter and emitted light was captured with a 780lp filter.

Image Editing

Monochrome images were captured to obtain maximum sensitivity for later analysis. Two images were acquired at each time point using a bright field (white light) and an NIR channel. These images were then selected for overlay using the VisionWorks®LS software composite feature. The NIR filtered image was pseudocolored with an intensity map. Colored areas with the greatest intensity were labeled red. Decreasing intensity ranged from yellow to blue with the lowest intensity represented by the color blue. The pseudocolored image was then overlayed on the bright field image.

Software Analysis

Quantitation was performed using the VisionWorksLS area density function to monitor the distribution of dye. To determine the pixel intensity of the fluorescent signal, an unaltered monochrome image containing the fluorescent marker was selected. The area of fluorescence was highlighted using the selection tool. Background signal was also selected with a similar tool and subtracted from the original signal. The mean pixel density was calculated by averaging the total fluorescent signal intensity and dividing the value by the total pixel area. This process was completed for selected organs (liver, neck lymph node) at each time point, and the collected data was exported to Microsoft Excel for further analysis.

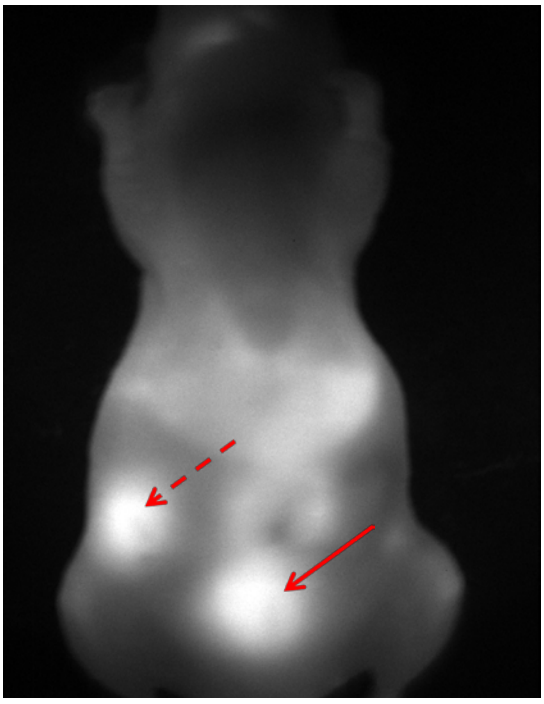


Figure 3. NIR-filtered monochrome image of conjugate accumulation in several vital organs. NIR conjugate is seen accumulating in the neck lymph nodes (top of image). The liver (crescent-shaped organ inferior to the rib cage) shows a high fluorophore density. In addition, two fluorescent masses are present within the bladder (solid arrow) and at a site of inflammation (dashed arrow).

Results

The NIR dye conjugated to anti-CEA monoclonal antibody (MAb) is seen concentrating in the liver of the mouse (Figure 1) over a 4 hour period. At time 0 there was no detectable NIR signal. At 10 minutes post-injection, dye rapidly accumulated in the liver as well as in the vasculature running along the vertebral column. Post-experiment surgical exposure (Figure 2) revealed presence of dye in the liver, bladder and a region of inflammation in the subcutaneous tissue. In addition, *ex vivo* analysis of the vital organs confirmed that much of the dye had been sequestered by the liver.

Figure 3 shows a representative picture of dye accumulation in the abdomen of an anesthetized mouse at 3 hours post-injection. In the supine position, the outline of the liver is seen in the upper abdomen, represented as a crescent-shaped mass inferior to the rib cage. Two additional fluorescent hot spots are present in the lower abdomen. The first identifiable fluorescent mass is the bladder. The second fluorescent mass is seen near the right hind leg at the site of the anesthesia injection. This mass likely represents the accumulation of dye into the site of trauma due to vasodilation induced by pro-inflammatory factors released after anesthetic injection.

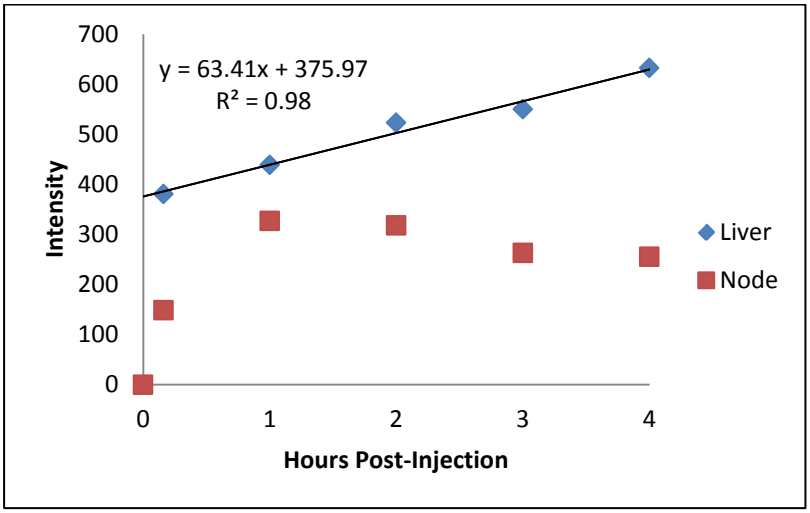
Quantitative analysis confirms a steady increase in the liver over the course of the 4-hour period. In addition, a roughly stable concentration of dye in a neck lymph node was observed (Figure 4). After a rapid accumulation increase in the liver within the initial 10 minutes post-injection, the liver intensity curve shows a linear increase in mean density (intensity units/pixel^2) over the course of the 4-hour protocol. Regression analysis shows a linear increase in concentration over time (r^2=0.98). This data suggests that the liver is an important organ for clearance of the dye conjugate from the bloodstream. Presence of the dye within the neck nodes also suggests involvement of the reticuloendothelial system in uptake of the dye-antibody conjugate. Alternatively, there may be high CEA expression in both of these organs, resulting in the binding of the anti-CEA antibody.

Conclusion

The anti-CEA antibody-conjugated NIR dye readily accumulates in both the liver and the lymph node of a nude mouse. Dye is rapidly taken up by the liver and shows a linear increase in intensity over time, suggesting sequestration of dye in this organ in order to clear the conjugate from the systemic circulation. It is also possible that accumulation of the dye is a result of its unique chemical structure. Furthermore, presence of dye in the neck lymph nodes suggests expression of the surface antigen CEA or non-specific binding of this antibody to the reticular cells. Taken together, these data show possible mechanisms for uptake of dye in this nude mouse model and can form the foundation for future biodistribution studies.

The iBox Explorer[2] is an ideal system for imaging distribution of fluorescent markers within small animals for pre-clinical experimental protocols. The wide-angle lens allows for the imaging of a whole animal and can accommodate up to two mice. In addition, the cooled, scientific grade CCD rapidly captures crisp, publication quality images. A bright xenon arc lamp provides intense excitation light from the visible to NIR range, accommodating a host of *in vivo* studies and fluorescent markers. Finally, an application-specific filter set can be tailored for imaging virtually any pre-clinical fluorescence study.

iBox Explorer[2] Imaging Microscope



HOUR	0.00	0.16	1.00	2.00	3.00	4.00
LIVER	0.00	380.45	438.43	523.35	549.64	632.20
NODE	0.00	148.94	327.01	318.09	262.94	255.42

Figure 4. Graphical representation of fluorescence intensity of two organs over 4 hours. Liver intensity, as represented by intensity units per pixels squared, increases linearly. Regression analysis confirms the linear increase in signal intensity within the liver. Accumulation of dye into the representative right neck lymph node is observed peaking after one hour.

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