APPLICATION NOTE

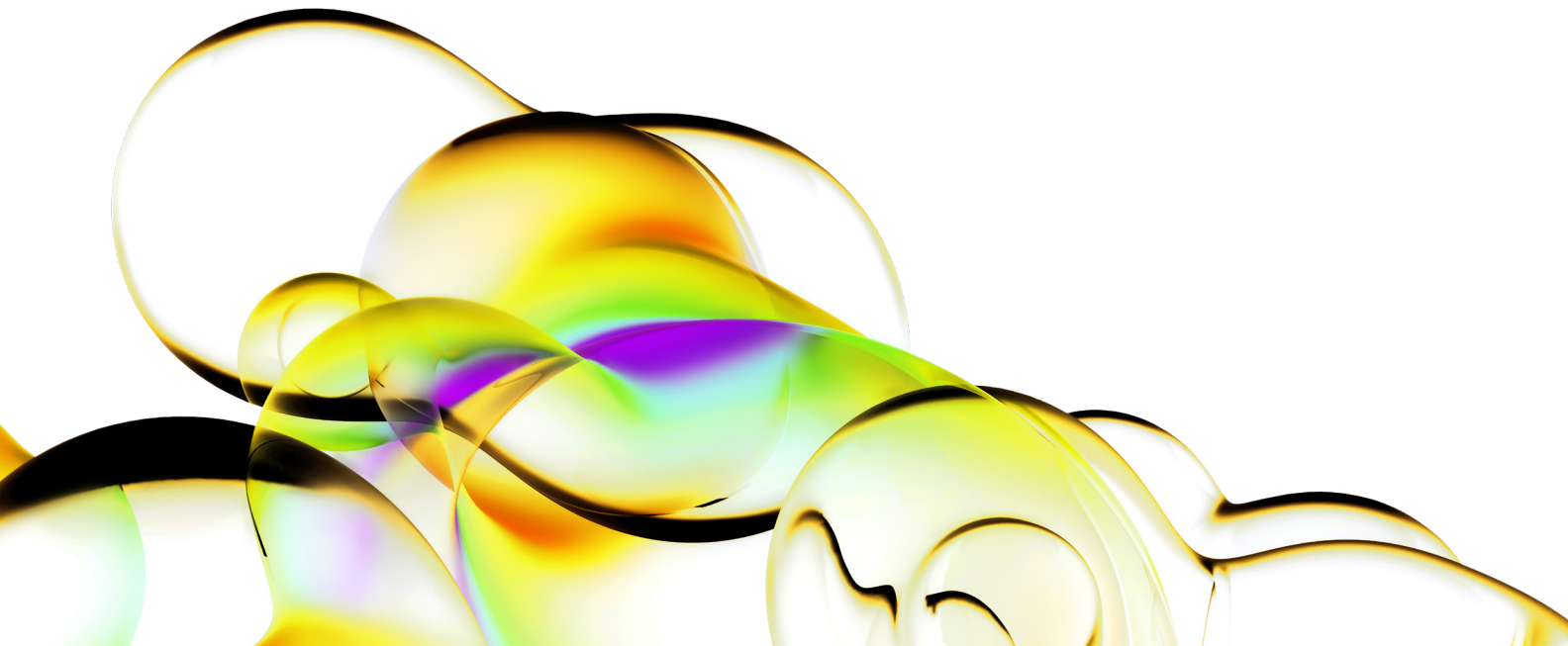
Advances in 3D optical imaging quantification and sensitivity.

Abstract

Advances in the detection and quantification for 3D optical tomography of bioluminescent and fluorescent reporters to quantify in terms of either cell number or absolute pmol concentration will be discussed. These methods include enhancing the detected signal levels using slight compression which reduces the amount of tissue light propagates through. Calibration techniques to improve signal location by reducing the excitation light artifacts, the amount of detected autofluorescence and techniques to quantify 3D reconstruction results in terms of biological activity will be demonstrated.

Introduction

Optical tomography of bioluminescent and fluorescent reporters in pre-clinical animal models is an important technology for the translational study of disease and drug development. However, the quality of 3D reconstructions can be limited by the sensitivity of detection. 3D results often lack quantification with biologically relevant units, therefore, new methods to improve detection and to quantify the results in terms of absolute cell number or dye molecules have been developed.



In order to enhance signal levels, and support multi-modality imaging, a mouse bed was developed which can slightly compress an animal. Direct comparisons show that compression can pick up deep-tissue signals that were previously undetectable. The compression bed also reduces the complexity of the boundary condition (rough fur, etc) facilitating 3D reconstructions in complicated models. Reconstructions for a compressed and uncompressed animal models will be shown co-registered to microCT data for cross validation.

Sensitivity of fluorescence detection can further be improved through an imaging method called normalized transmission fluorescence (NTF) efficiency. The sensitivity of fluorescent imaging is limited by autofluorescence. Transillumination can be used to trap the autofluroescent signal on the opposite side from the detector. However, transillumination imaging can create a pattern of the emission light that includes contributions from tissue heterogeneity and animal surface topography. To reduce this contribution from the detected signal, the fluorescent emission image is normalized by a transfer function comprised of a transmission image measured with the same emission filter and an open excitation filter. Eliminating the excitation light contribution from the measurement improves the robustness of the reconstruction algorithm by reducing artifacts and giving better signal localization and sensitivity.

Lastly, absolute quantitation of tomographic optical images utilizing a microwell plate calibration technique will be presented. In this technique measurements of known serial dilutions of luminescent or fluorescent cells, or fluorescent dye molecules are used in order to generate quantification databases of photons per second per cell or extinction coefficients per cell or per molecule. These databases are then used to determine the number of cells or dye molecules from the tomographic sources reconstructed inside an animal.

Methods and equipment

The optical imaging system shown in Figure 1 (IVIS® Spectrum, Revvity, Inc.) is designed with a highly sensitive CCD camera in order to detect both fluorescent and bioluminescent signals. For fluorescent imaging, the system has the capability to emit excitation light through trans-illumination (from the bottom) or epi-illumination (from the top). The instrument is equipped with several narrow band excitation filters and emission filters that allow for flexibility in probe selection and can also assist in significantly reducing autofluorescence using spectral unmixing algorithms (not shown here). For 3D diffuse bioluminescent and fluorescent reconstructions, the surface topography of the animal is determined using a laser galvanometer which delivers structured light across the imaging plane.

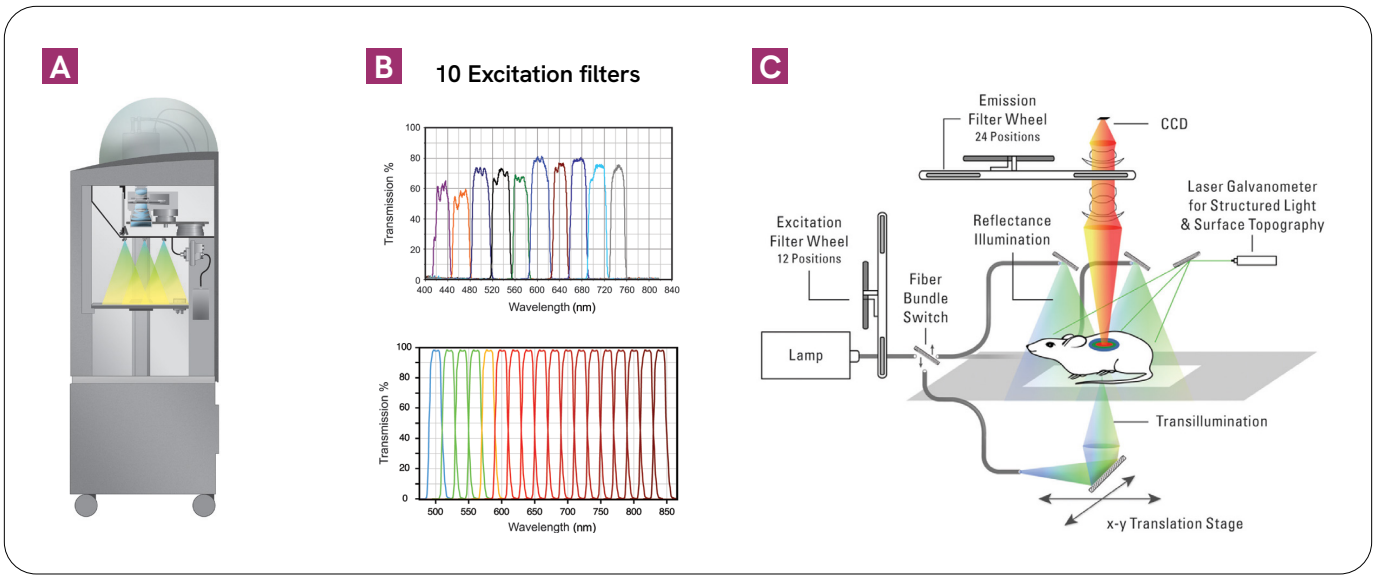


Figure 1: A) The IVIS Spectrum optical imaging system. B) Graph of the excitation and emission filter wavelengths. C) Schematic showing the optical components along with the excitation (epi-illumination or transillumination) (blue green) and emission (red-orange) light paths.

An animal restraining bed (Mouse Imaging Shuttle (MIS)) equipped with gas anesthesia connections has been developed to transport subjects between the optical and microCT imaging platforms via docking stations in order to acquire precise animal positioning (Figure 2). This device also has the option to slightly compress the subject in order to enhance the optical signal levels. By closing the lid of the MIS, the mouse is slightly compressed to a fixed height.

Several different sized containers were developed in order to achieve different compression heights. The CT docking station was developed with a fiducial design using a triangular encoded pattern allowing for accurate automatic registration of the CT volume to the optical coordinates even when only a portion of the subject is scanned.

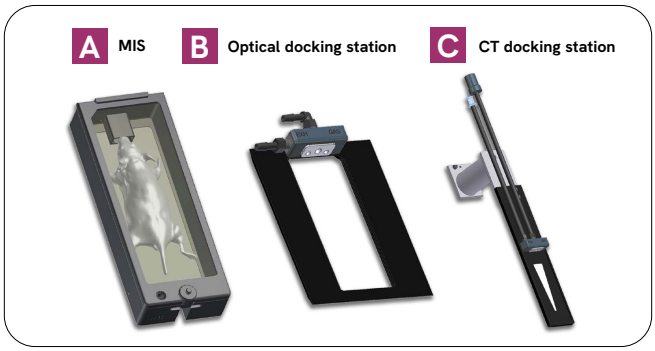


Figure 2: Mouse Imaging Shuttle (MIS) and docking stations. A) CAD drawing of the MIS showing subject placement. B) Optical docking station showing inlet and outlet anesthesia supply. C) CT docking station showing anesthesia connectors and triangular position encoded fiducial used for automatic co-registration.

Figure 3: The Quantum FX µCT was used for cross validation. The Mouse Imaging Shuttle was used to transport the animal between the IVIS Spectrum and the Quantum FX.

The standalone microCT system (Quantum, Revvity, Inc.) uses ultrafast and low dosage CT technology and is therefore suitable for longitudinal non-invasive studies (Figure 3). The microCT system was used to cross validate 3D optical reconstruction results by co-registering functional optical signals with its associated anatomical pathology.

Experimental results

Well plate quantification

To generate a database for absolute quantification, images of known serial dilutions of luminescent cells or fluorescent dye molecules needs to analyzed. Figure 4 is a 2:1 dilution of human colorectal carcinoma HTC116-luc2 bioluminescent cells. The well plate data is used to determine the Total Flux per cell and is used by the 3D tomographic recontruction to extrapolate the total number of cells from the 3D bioluminescent signal. In this example, the Total flux/cell was found to be 1.41e+02 p/s/cell.

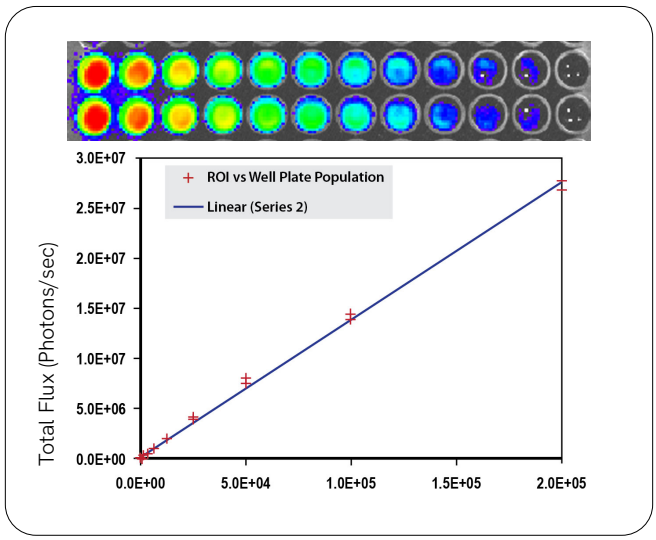


Figure 4: Serial dilution (2:1) of human colorectal carcinoma HTC116-luc2 bioluminescent cells with a starting concentration of $2x10^5$ cells. For this cell line the Total flux/cell was determined to be 1.41e+02 p/s/cell.

2D Bioluminescent signal enhancement with compression and 3D quantification

Slight compression of an animal can increase the detected surface signals. Figure 5 shows the bioluminescent results from a Balb/c male mouse that had a direct injection of 5.0E+05 HCT 116-luc2 cells into the right lung. The animal was injected IP with 150 µL of D-luciferin 10 minutes prior

to imaging. The 2D bioluminescent images were taken before and after compression using the 13.4 mm MIS. 3D reconstructions co-registered to the CT data are also shown. When the animal was compressed 8.1 mm an increase of 1.7 was obtained in the measured surface radiance.

The 3D bioluminescent reconstruction co-registered to CT data nicely illustrates the accuracy of the reconstructed signal location. In both the 3D views, coronal and transaxial cross sections, the reconstructed signal is shown originated from the right lung. Quantification of the reconstructed signals gave a slight overpredition of 5.8E+05 cells for the uncompressed animal and 5.1E+05 cells for the compressed animal (actual concentration is 5.0E+05 cells).

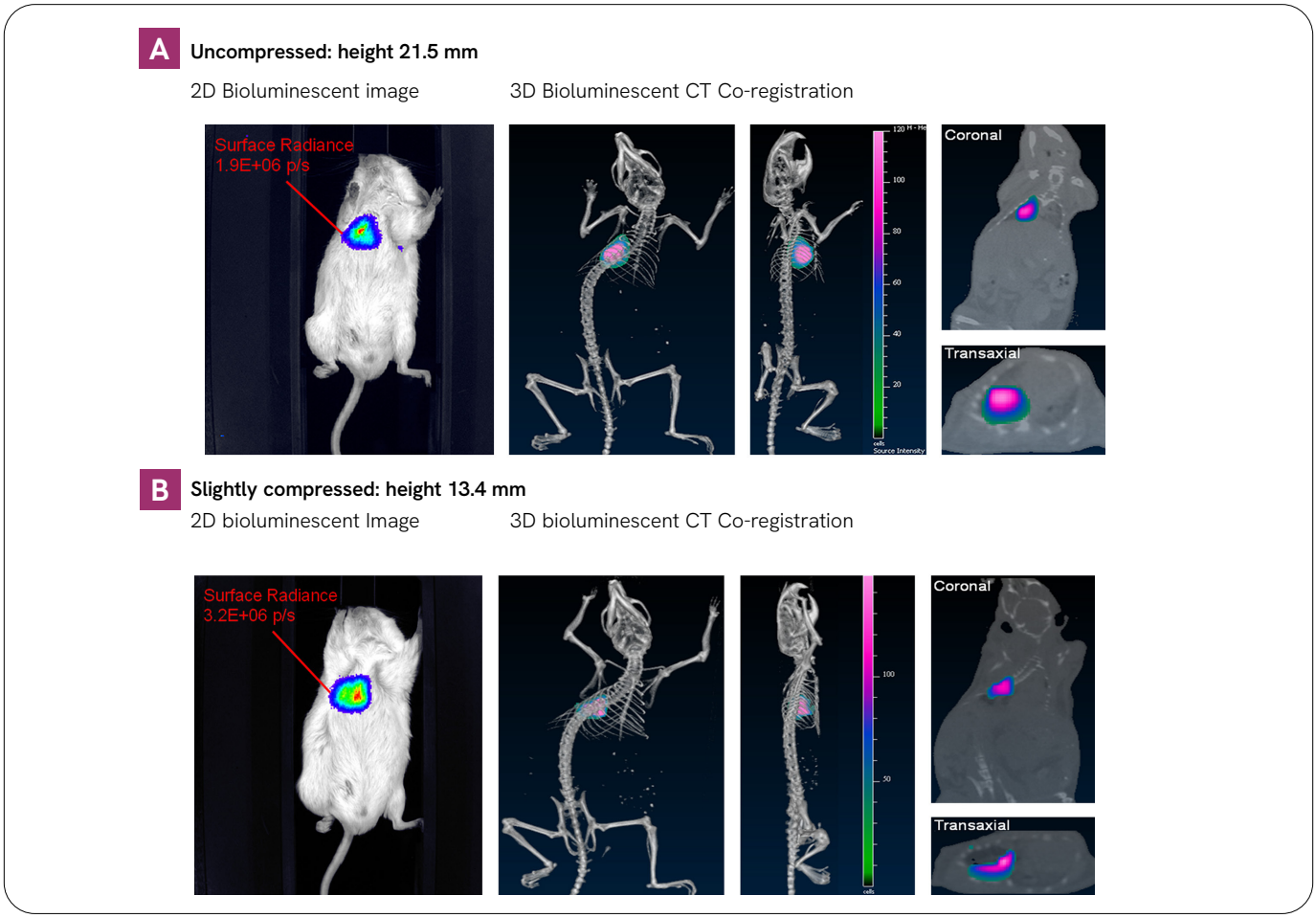


Figure 5: Bioluminescent surface signals and 3D reconstructions from an A) uncompressed and B) slightly compressed male mouse with a direct injection of 5.0E+05 HCT 116-luc2 cells in the right lung. The 3D reconstructed source signal is co-registered to CT data.

Normalized transmission fluorescent, NTF efficiency

Figure 6 is a pictorial diagram showing the process involved in obtaining a normalized transmission fluorescent (NTF) image. A set of two images are acquired: a fluorescent image taken with an excitation/emission filter pair and a transmission image taken with an emission filter only (no excitation filter). The fluorescent image is then divided by the transmission image). The NTF efficiency image results in improved sensitivity by reducing the geometric contribution and effects of tissue heterogeneity.

2D fluorescent signal enhancement with compression and 3D quantification

For the fluorescent example shown in Figure 7, a CF750 pillow was implanted in the left peritoneum of a male Nu/nu mouse. NTF efficiency measurements were taken before and after compression. When the animal was compressed 11.9 mm the surface efficiency signal showed a 2.3 increase.

Also in Figure 7 are the 3D NTFLIT fluorescent reconstructions co-registered to CT data. The micro CT system was able to detect the fluorescent pillow and is outlined by the red box. For the uncompressed animal, the CT data showed the reconstructed souce location is approximately 1.3 mm off (center-to-center) in the transaxial cross section and slightly less for the sagittal cross section. For the compressed example, however, the reconstruction yields good localization.

The reconstructed fluorescent signals were quantifed in terms of the number of dye molecules using a similar well plate technique as described above, however the well plate data is not shown here for brevity. In this example, the CF750 pillow contained a concentration of 0.01 pmoles of dye. The reconstructed yeiled a concentration of 0.009 and 0.008 pmoles for the uncompressed and compressed reconstructions, respectively.

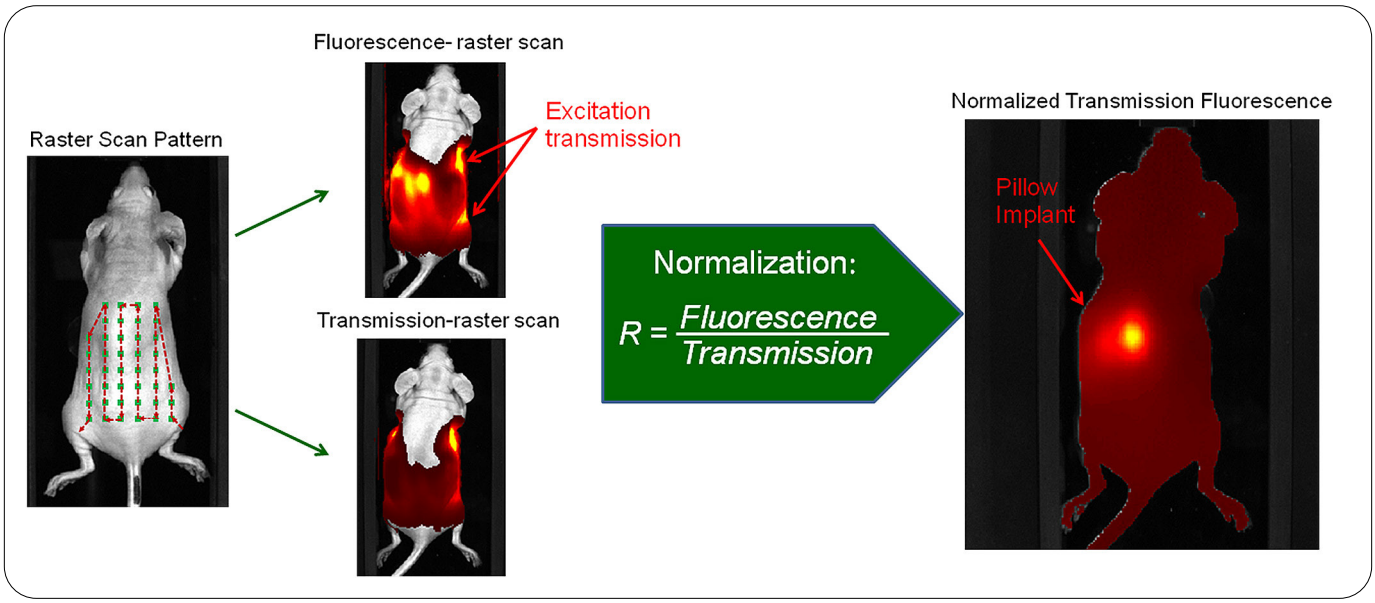


Figure 6: Diagram showing the process involved in obtaining a NTF image where the fluorescent image is divided by a transmission image taken at the emission wavelength (no excitation filter).

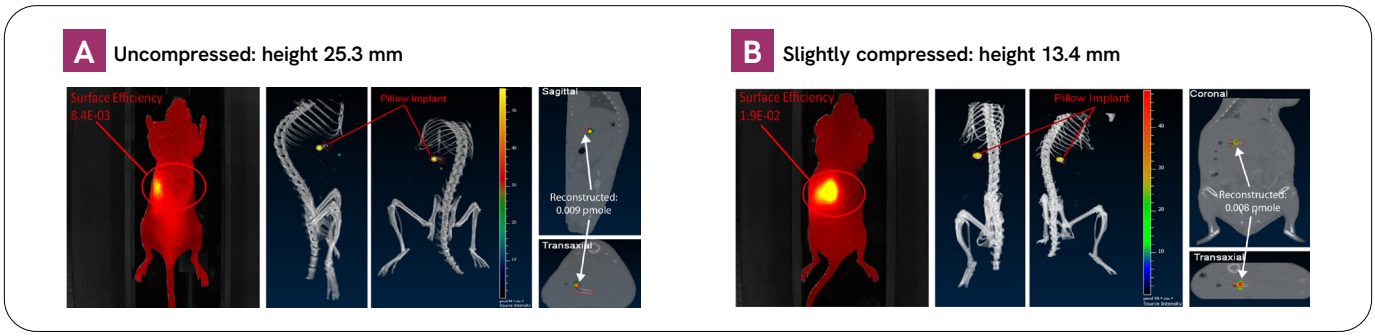


Figure 7: Fluorescent surface signals and 3D reconstructions from a A) uncompressed and B) compressed Nu/nu male mouse with a left peritoneum pillow implant containing 0.01 pmole of CF750. The 3D reconstructed source signal is co-registered to CT data.

Conclusions

Slight compression of an animal showed an increase in the surface signal levels by minimizing the amount of tissue light propagates through. Normalized transmission fluorescent efficiency measurements improved signal localization by reducing excitation light artifacts from the tissue heterogeneity. By analyzing the bioluminescent or fluorescent markers in a well plate prior to the *in vivo* application, the 3D reconstructed results can be quantified in biologically relevant units of cell number for bioluminescent measurements or dye molecules for fluorescent measurements. Using the micro-CT system, localization of the 3D reconstructions was validated.

References

1. C. Kuo, O. Coquoz, T.L. Troy, H. Xu and B.W. Rice, "Three-Dimensional reconstruction of *in vivo* Bioluminescent Sources Based on multi-spectral Imaging," J. Biomed. Opt. 12, 024007 (2007).
2. C. Kuo and B.W. Rice, "Bayesian Techniques in Fluorescent Tomography," Biomedical Optics annual meeting, OSA Technical Digest, BSuE46 (2008).
3. H. Xu, A. Christensen, C. Kuo, D. Nilson, E. Lim, J. Whalen, J. Kim, N. Zhang, R. Singh, S.J. Oldfield, T.L. Troy and B. Rice, "Co-registration of *in vivo* Optical Tomography and Micro-CT for Longitudinal Studies," Word Molecular Imaging Congress, 0323B (2010).

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com