CT-aided Bioluminescence Tomography for Precise, High-Sensitivity Signal Quantification

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Introduction

Bioluminescent tomography (BLT) is a biomedical imaging modality for three-dimensional (3D), *in vivo*, non-invasive characterization of cellular and molecular phenomena. Similarly to its two-dimensional counterpart (2D bioluminescence imaging), it leverages the detection of light photons that are emitted after the oxidation of a substrate (luciferin) by an enzyme (luciferase). However, as opposed to the planar images of 2D bioluminescence imaging (BLI), BLT provides volumetric images and is therefore able to reconstruct the 3D spatial and intensity distribution of bioluminescent molecules. This can be achieved through the acquisition of a multispectral image (i.e., multiple scans acquired using as many different light filtrations), which is then reconstructed using dedicated algorithms. To work optimally, these algorithms should incorporate models of the light transport through tissue, accounting for the underlying optical parameters of the animal/tissue being imaged. This type of structural knowledge, essential for accurate reconstruction, can be obtained by using a concurrent modality able to capture anatomical details of the animal being imaged.

One of MILabs BLT technologies leverages the power of X-ray micro-computed tomography (micro-CT) to obtain structural information about the animal. Thanks to its high-resolution capabilities, MILabs micro-CT can depict anatomical information with high spatial detail and incorporate this information into an exclusive reconstruction algorithm that can correct tissue-specific light absorption and scattering. As a result, the bioluminescent signal is reconstructed accounting for the heterogeneous tissue distribution within the animal, resulting in high accuracy, high sensitivity, and precise 3D localization even in deep tissue regions. Furthermore, MILabs BLT uses a dedicated animal bed which is designed to hold up to three mice, allowing for the simultaneous acquisition and consecutive reconstruction of multiple animals in one single scan, reducing the overall imaging time and allowing higher throughput.

The potential of MILabs BLT has already been demonstrated in an independent study, which shows the ability to monitor cell pharmacokinetics and tumor volume non-invasively, longitudinally, and more accurately compared to 2D BLI. [1] In this white paper, we also demonstrate the ability of MILabs BLT to provide images with a higher sensitivity and a higher precision in signal quantification.

Materials and Methods

All animal experiments were approved by LAGeSo, and all procedures were conducted according to the local rules and regulations. For this study, a mouse with two subcutaneous colorectal cancer HT29 xenograft tumors was employed. A second mouse with no tumor growth was also included in all the experiments as a control.

The mice were anesthetized (4% isoflurane) and subsequently placed in a prone position on a heated three-mouse bed (MILabs) dedicated to optical imaging. The animals were kept under anesthesia for the duration of the experiment (1% isoflurane, 200 mL/min).

After induction, the bed was placed on the corresponding focus level in the docking station with a mounted optical imaging module for 2D imaging (Figure 1). The following parameters were used during BLI image acquisition: iris f/0.95, exposure time 2 minutes, preamplification 2x, binning 2x2. No emission filter was used. A photographic image of the mice was acquired using the standard camera settings.

Immediately after 2D BLI acquisition, the optical imaging module was moved to the main system (Figure 1) featuring a MILabs micro-CT (U-CT), and the animals underwent BLT acquisition. During bioluminescence acquisition, four images with as many different emission filters (586 nm, 615 nm, 631 nm, 661 nm) plus an unfiltered image were acquired, followed automatically by a CT scan (total body step-and-shoot protocol, full scan angle with 0.75 degrees angular step, no binning, exposure time 42 ms, voltage 55 kV, current 0.26 mA, total beam filtration of 0.5 mm of Aluminum). After the acquisition, the CT scan was reconstructed with a standard Feldkamp algorithm with Hann filtering at an isotropic voxel size of 280 µm, and the bioluminescence data was reconstructed automatically with the dedicated reconstruction software.

After image acquisition and reconstruction, three preclinical imaging scientists (referred

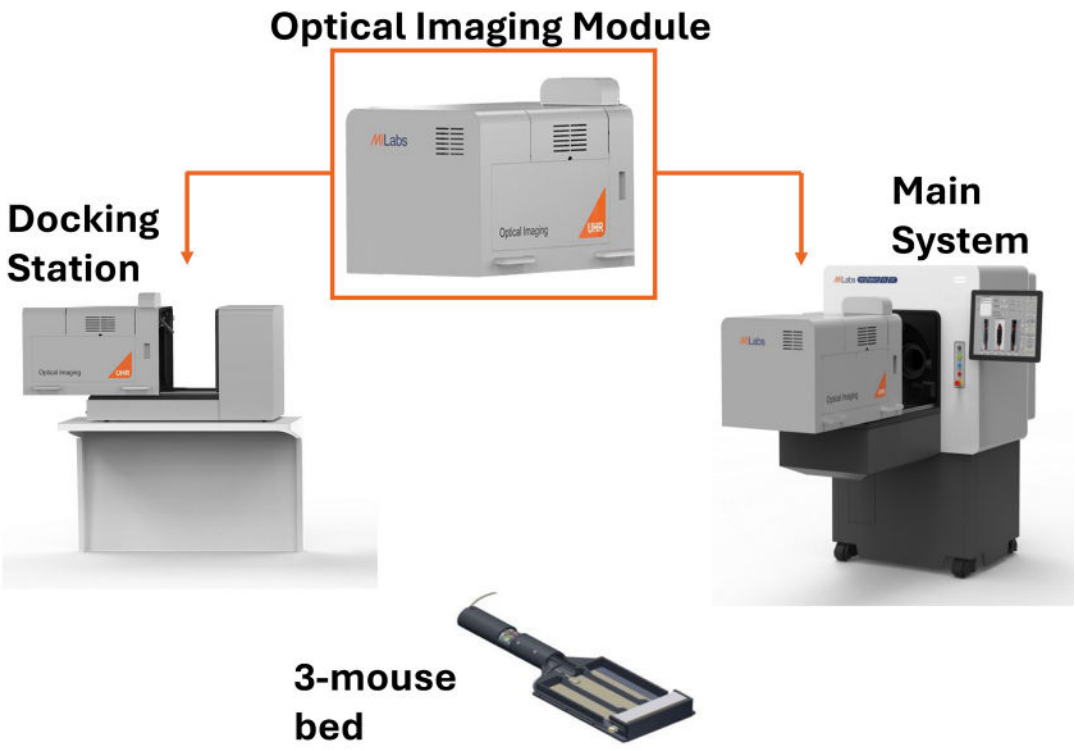

Figure 1. MILabs three-mouse bed, optical imaging module, docking station (used for 2D BLI acquisition) and main system featuring the micro-CT (used for BLT acquisition).

from now on to as *readers*) with experience in bioluminescence imaging were asked to independently segment the two tumors in both BLI and BLT images and extract with a dedicated software the total luminescent signal (i.e., the sum of all the voxels) from each tumor. In the case of BLI, the segmentation was performed in two dimensions, with the luminescent image overlayed on the photographic reference image. In the case of BLT, the segmentation was performed in three dimensions, with the luminescent 3D volume overlayed on the reconstructed CT image. Each reader was asked to repeat the segmentation three times, with at least one day in between each round. The readers had no time limit and were instructed to use, at their discretion, the whole imaging information available (2D luminescence and photographic reference in BLI, 3D luminescence and CT in BLT) to delineate the tumors and obtain the segmentation results.

After all results were obtained, the coefficient of variation was used to calculate the intra- and inter-reader variability in total signal quantification based on differences in tumor segmentation. The mean across the tumor total signal measurements was also calculated (per reader, and across readers), to investigate the signal strength achievable in BLI and BLT images (which is correlated to the sensitivity of the modality). For this latter measurement, all signal measurements were normalized to the maximum value (separately for BLI and BLT), to encompass the difference in pixel and voxel value scales of the two modalities, and thus ensure a quantitative comparison.

To avoid any bias, all analysis experiments were performed with the same software (Imalytics Preclinical 3.0, Gremse-IT, Aachen, Germany)

Results and Conclusion

The (normalized) total tumor signal was higher, per tumor, in BLT compared to 2D BLI (Table 1), highlighting the higher sensitivity of the former. This suggests that BLT should be preferred in all cases where accurate signal quantification is needed, but also in studies where the structure of interest is located deep inside the animal body (and thus signal collection becomes more challenging).

In terms of uncertainty in signal quantification, BLT showed a lower coefficient of variation compared to 2D BLI, both intra- and inter-reader (Table 2). This suggests that signal quantification measurements are more reproducible in BLT, despite it being a three-dimensional modality where measurements are performed over a volume, and not over an area (and, therefore, inherently subjected to a higher chance of an increased absolute variability).

In both BLI and BLT, the quantification of the signal

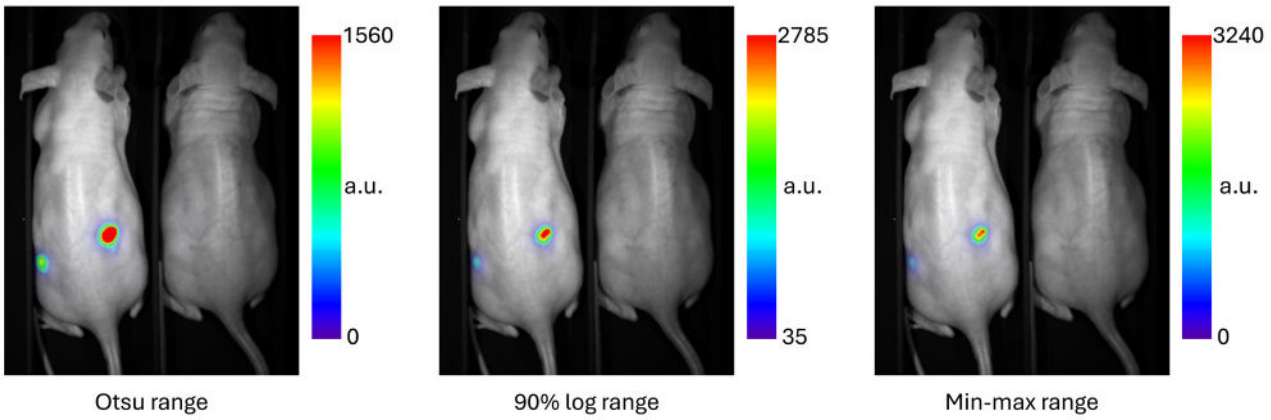

Figure 2. Examples of three different color scales used to visualize the 2D BLI image of the experimental and control mice. As apparent, different settings of the color scale yield different apparent signal intensities, which ultimately hamper the precision in tumor segmentation.

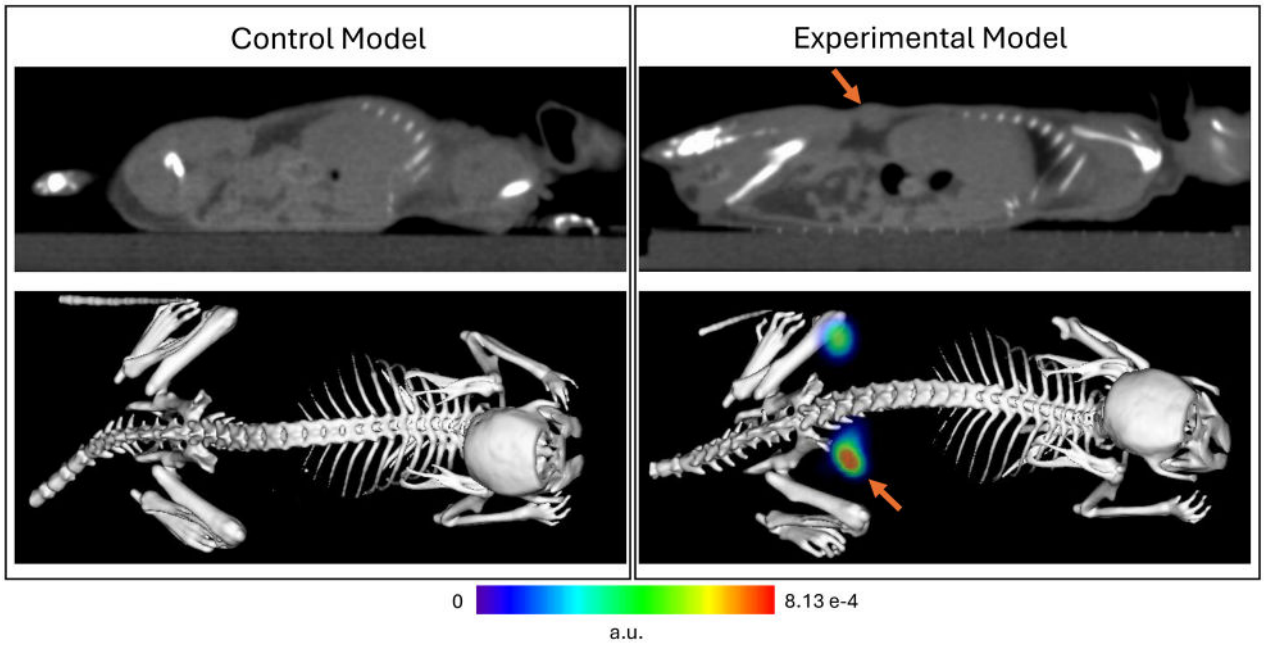

Figure 3. Sagittal view of the CT scan (top row) and 3D rendering of the BLT image (bottom row) for the control and experimental animal models. The bioluminescence signal was automatically overlaid to the mice skeleton as seen in CT to provide anatomical reference. The arrow points to one of the subcutaneous tumors, clearly visible in the CT image of the affected animal.

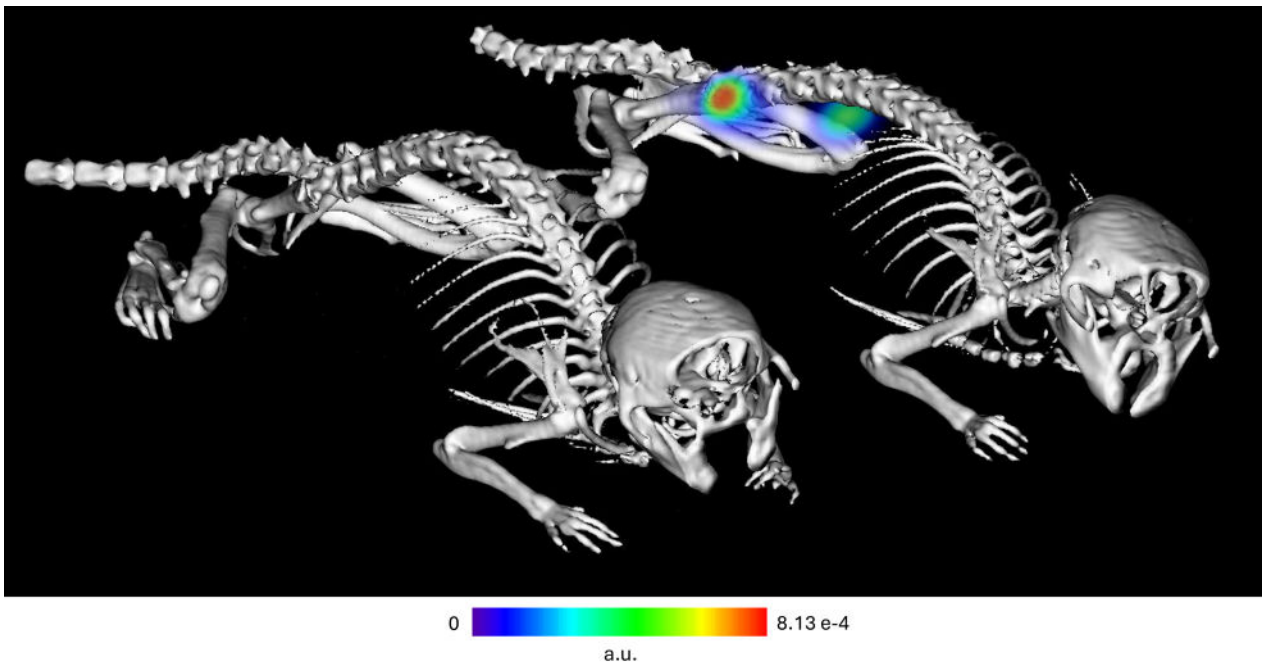

Figure 4. 3D rendering (oblique view) of the two animals (control and experimental model) acquired with BLT.

is derived from the result of the segmentation of the structure of interest. In 2D BLI, the segmentation results are highly dependent on the settings of the color scale (which are arbitrary), since no anatomical information about the inner body structure is present (Figure 2). While this does not hamper the ability to perform relative signal measurements (e.g., quantify the variation in an area or the total signal of a tumor longitudinally, or of two tumor models), absolute quantification becomes challenging.

In BLT, instead, not only true 3D measurements can be performed, but these also appear to be more consistent and reproducible. The higher precision of BLT is likely due to the presence of the CT reconstructed image, which provides anatomical information at high spatial detail and allows for a more objective segmentation of the structure of interest (in this case, the tumors, Figure 3-4).

These results suggest that BLT not only allows for stronger luminescence signal collection, but also for a higher quantification reproducibility, which is an essential prerogative for comparative studies, longitudinal studies, and any types of experiments that require accurate image quantification (and absolute measurements).

Finally, it is worth mentioning that, despite the superiority of BLT, 2D BLI still finds many applications in the preclinical imaging realm [2-5], and should still be considered as a baseline modality in all types of studies that require high-throughput, speed and simplicity in acquisition, ease in visual assessment and analysis, and relative signal quantification. In these scenarios, BLT should be included as a supplemental modality to bring signal visualization, analysis and quantification to the next level of precision, and thus to validate (and strengthen) the results obtained with 2D BLI.

Table 1. Normalized total tumor signal (left and right tumor). Results are reported in terms of average measurements within (and across) readers.

	2D BLI		BLT	
	Left Tumor	Right Tumor	Left Tumor	Right Tumor
Reader 1	0.05	0.65	0.34	0.84
Reader 2	0.20	0.69	0.54	0.94
Reader 3	0.12	0.82	0.38	0.87
All Readers Combined	0.13	0.72	0.42	0.88

Table 2. Intra- and inter-reader coefficient of variation in the measurements of the total tumor signal (left and right tumor). The inter-reader coefficients of variation (i.e., the last row in the table) were calculated as the standard deviation across all measurements (nine in total, three per reader), normalized by their mean.

	2D BLI		BLT	
	Left Tumor	Right Tumor	Left Tumor	Right Tumor
Reader 1	13%	10%	8%	4%
Reader 2	14%	17%	12%	7%
Reader 3	32%	16%	3%	7%
All Readers Combined	51%	17%	24%	8%

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