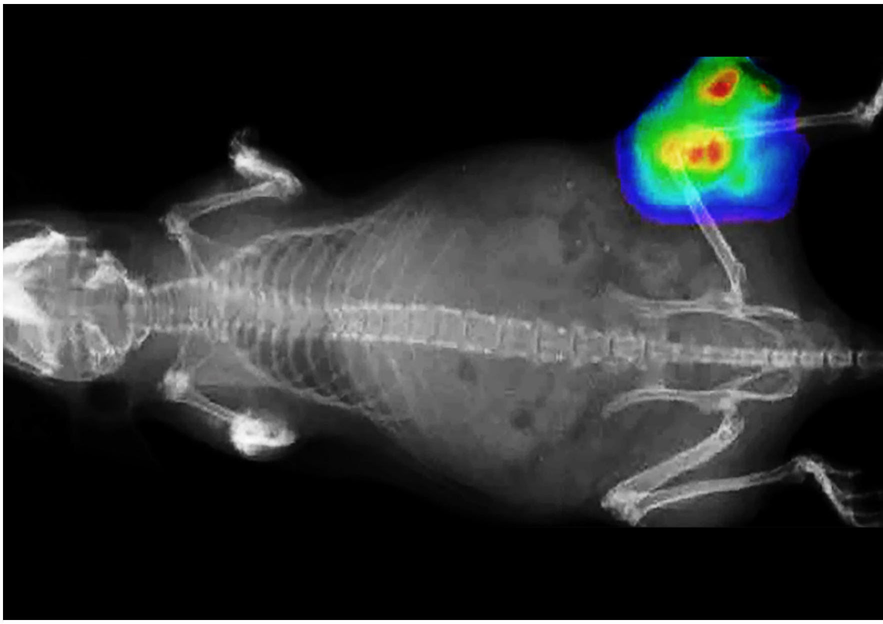

APPLICATION NOTE

Preclinical *In Vivo* Imaging

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Quantitative Analysis of Bone Erosion Using High-Resolution X-Ray Imaging

Introduction

Bone erosion is a pathological condition characterized by breaks in the cortical bone surface and loss of the adjacent trabecular bone. Several pathological processes can lead to bone erosion, including malignant tumors, abnormal metabolic processes such as hyperparathyroidism, and chronic inflammatory diseases such as rheumatoid arthritis. As the bone contains hydroxyapatite mineral with high electron density, bone erosion was first characterized using X-ray radiography. Thus, in clinical settings, bone erosion is routinely detected using X-ray based imaging technologies, such as conventional 2D planar X-ray and more advanced computed tomography (CT), which can provide highly-detailed 3D images of bones. Preclinical bone erosion animal models can also be imaged using X-ray based imaging technologies, however, since laboratory animals are much smaller than humans, it requires higher resolution to visualize bone erosion. Preclinical CT offers high-resolution 3D imaging for such purposes, but it is not widely available and often requires access to an animal imaging core facility. On the other hand, planar X-ray imaging has several advantages for preclinical bone assessment, including better accessibility to instrumentation, higher throughput (ability to image more than one animal at a time), and ease-of-use (less training required compared with CT). However, many currently available preclinical X-ray imagers lack sufficient resolution to visualize subtle bone density changes in small laboratory animals.

In light of this need, PerkinElmer developed the IVIS® Lumina X5 2D planar imaging platform that combines highly sensitive bioluminescence, wide-ranging fluorescence and high-resolution X-ray modalities into a small, compact system that fits on the benchtop. This application note highlights the optical/X-ray imaging capability of the IVIS Lumina X5 and Living Image® software for obtaining high quality images for quantitative analysis in a mouse bone erosion model, with the ease and speed of 2D imaging.

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Overview of the IVIS Lumina X5 System

Versatile Planar Imager Capable of High-Throughput Optical and High-Resolution X-Ray Imaging

The IVIS Lumina X5 combines 2D bioluminescence and fluorescence imaging with integrated high-resolution x-ray for visualizing anatomical details. To improve imaging throughout, the IVIS Lumina X5 has a highly sensitive 1-inch CCD camera providing a larger 20 x 20 cm field of view (FOV) sufficient for imaging 5 mice or two rats at a time. In addition to bioluminescence imaging, the IVIS Lumina X5 is equipped with 26 filters for imaging fluorescent signal ranging from green to near-infrared. The X-ray module consists of a micro-focused X-ray source and a large, independently-deployed scintillator, making it possible to acquire either 3- or 5-mouse X-ray images or zoomed-in high-resolution images of a single mouse or smaller selected regions. With optical image overlay capability at every X-ray resolution, it is easy to view underlying biological and anatomical changes at the same time. This sets a new standard in multimodal 2D imaging resolution and will help researchers explore new applications and extract more biologically relevant information from the animal models. In addition to the improved optics for better image sensitivity and quality, the system offers a set of 'Smart' animal handling accessories that were designed with improved performance and throughput in mind. Detachable Smart loading trays allow the researcher to prepare and position animals on the benchtop docking station before placing the tray into the IVIS system. The tray magnetically attaches to the gas-delivery manifold in the imaging chamber. Once connected, the tray provides gas anesthesia and vacuum during imaging. In addition, fiducials built into the tray allow Living Image software to automatically recognize and draw Regions of Interest (ROIs) providing automated animal identification. When used with the next generation anesthesia unit (RAS-4, PerkinElmer), all tray parts along with strong vacuum capabilities prevent excess gas from escaping, minimizing user exposure to anesthetic gas. Most importantly, the Smart Tray is X-ray compatible and can be used for high-resolution imaging without signal interference. As an integrated solution, Living Image software installed on the system offers an intuitive user interface that guides users from image acquisition, organization and data analysis. An imaging application for tumor-induced bone erosion analysis is demonstrated in the following sections.

Figure 1. IVIS Lumina X5 system, Smart Tray accessories and docking station for high-throughput imaging.

The Knee Bone Erosion Model and Imaging Study Overview

In order to demonstrate IVIS Lumina X5 optical/X-ray imaging capacities, a tumor-induced bone erosion model was established using the mouse breast cancer 4T1 cell line (Bioware® Brite 4T1-Red-Fluc cell line, PerkinElmer) which is known for bone metastasis and destructive growth. The 4T1-Red-FLuc cells used in the study express a red-shifted firefly luciferase for bioluminescence imaging. To induce osteolytic tumor growth, 0.5 million 4T1-Red-FLuc cells were injected into the left knees of nu/nu mice on day zero. Tumor growth and bone erosion in the knee bones were assessed by longitudinal bioluminescence imaging (BLI)/X-ray imaging on day 1, 7, 10 and 14. Of note, at each imaging timepoint, we first used the high-throughput mode to image all 5 mice and then used the high-resolution mode to examine knees for signs of bone erosion. The overlaid BLI images provided a direct assessment of tumor growth, while the X-ray images revealed bone erosion and destruction. Figure 2 illustrates the study design and representative images from each timepoint.

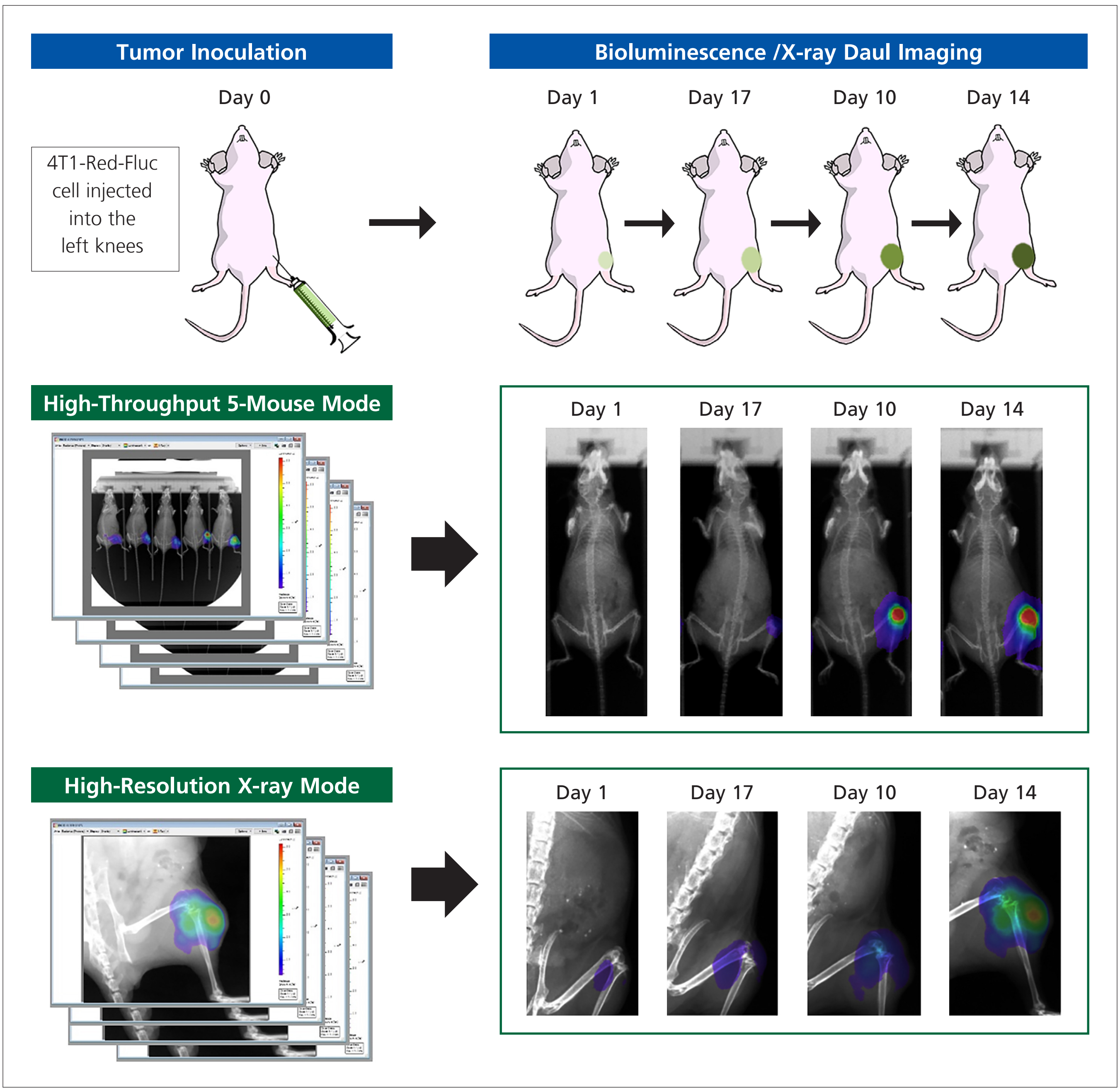

Figure 2. Knee erosion mouse model preparation and imaging timepoints.

Longitudinal Imaging of Knee Bone Erosion

The imaging results summarized in Figure 3 clearly indicate significant tumor growth of 4T1-Red-Fluc cells in the knee region over 14 days (Figure 3A) and by quantified bioluminescence (Figure 3B). This tumor growth induced extensive bone mass loss and joint destruction, generally correlating to tumor size/ bioluminescence. The 5-mouse X-ray images provided sufficient resolution and were used to generally identify potential subjects with knee bone degradation by zooming in to the knee regions (Figure 3C, lower panels). Nevertheless, it is recommended to use the high-resolution mode for more accurate visualization of bone erosion as it offers much higher resolution (Figure 3C, upper panels).

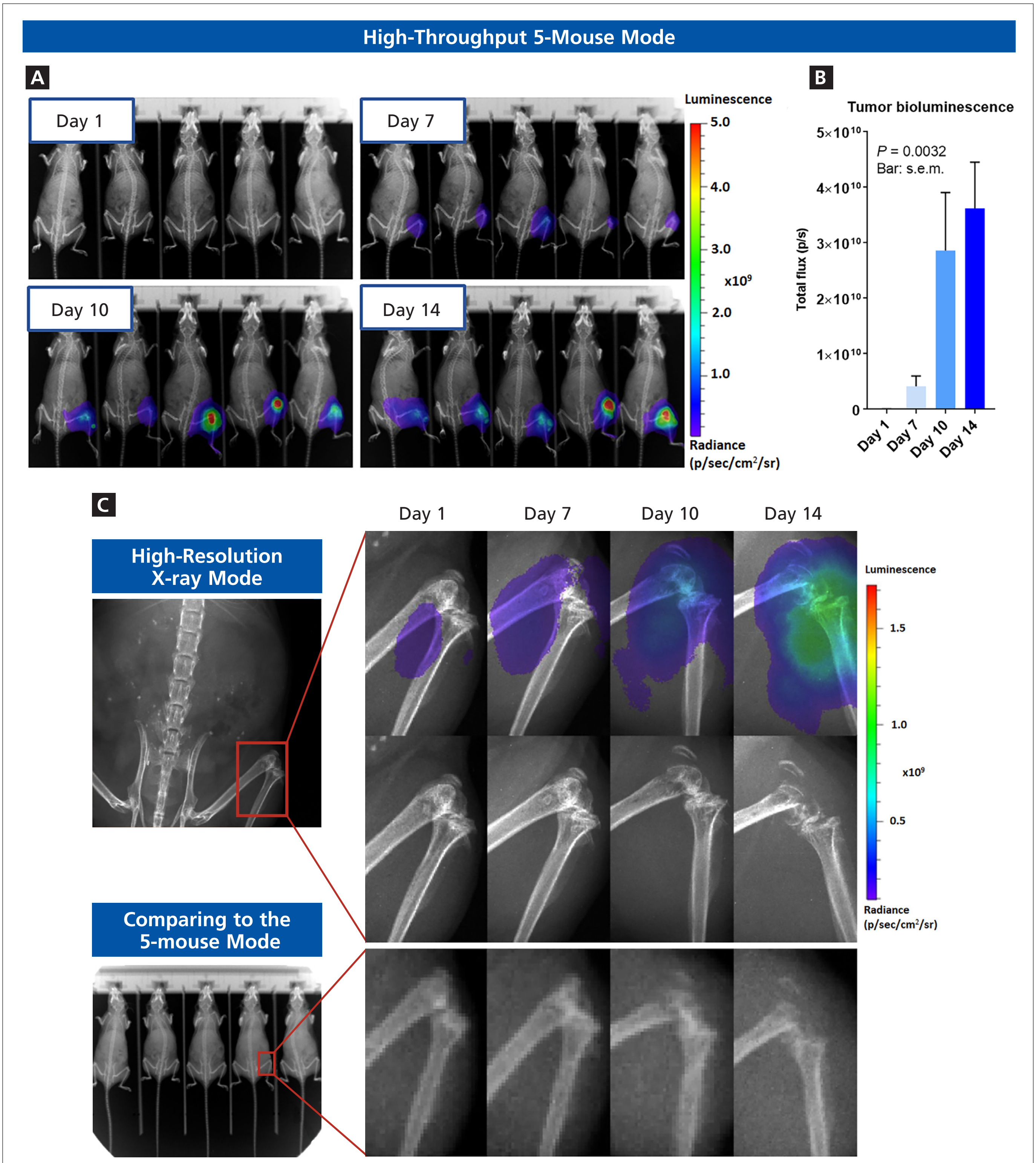

Figure 3. Dual BLI/X-ray imaging of tumor-mediated bone erosion in mouse knee.

High-resolution X-ray Images Show Knee Bone Erosion as a Result of Tumor Growth

Figure 4A summarizes the osteolytic effects of a 4T1-Red-FLuc tumor in a representative mouse as visualized by the high-resolution X-ray images. At early stages (day 1 and 7), there was no obvious visible bone loss. In contrast, starting on day 10, the tumor gradually displaced the patella (knee cap, yellow arrows). Considerable bone loss occurred in the metaphysis and/or epiphysis regions (red arrows). Interestingly, some degree of marrow loss was observed in the diaphysis region near the tumor boundary (blue arrow) on day 14, suggesting abnormal mineral turnover activity unique to this microenvironment. The reduction in pixel intensity along the femoral axis was analyzed using the Line Profile tool (Living Image software), which allows the analysis of pixel intensity along a free-hand line drawn to capture regions of bone density change. To ensure analysis consistency, fixed-length 400-pixel lines were drawn from the diaphysis and "anchored" to the tip of the epiphysis (Figure 4A, point A to point B) for each imaging timepoint. For corroboration of results, high-resolution microCT imaging was performed using the Quantum GX2 system, confirming the significant bone loss and destruction in this tumor-bearing animal on day 14 (Figure 6B).

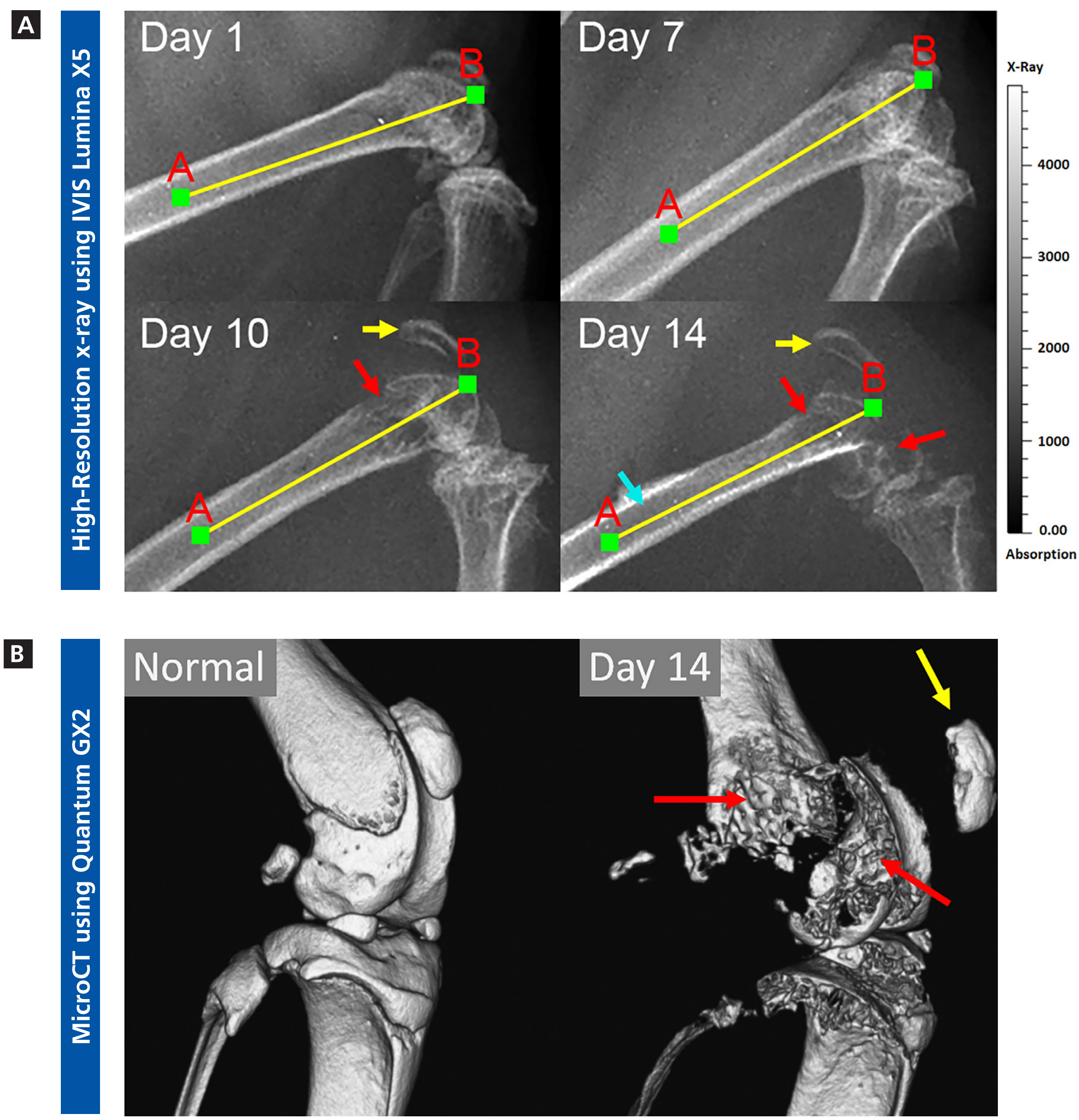

Figure 4. Use of Line Tool in Living Image software to draw lines along he femur axis for quantitative analysis (Figure 4A). Corraboration of results performed using the Quantum GX2 high-resolution microCT imaging system (Figure 4B).

Quantitative Analysis of Longitudinal Bone Density Changes

The pixel/intensity data from X-ray images was used for longitudinal analysis of bone density loss after tumor inoculation. To improve accuracy, the background intensity was determined and subtracted using the average pixel intensity of a short 50 pixel line drawn next to the knee (in the muscle/soft tissue) as a reference at each timepoint. Figure 5A shows the normalized line profiles in a 400-pixel femur range that encompasses the epiphysis to tumor border. Interestingly, the profiles showed effects of tumor growth in three distinctive femur regions. Figure 5B shows summary bar charts for average changes in different bone regions. The tumor border region (0-100 pixels) showed reduced density in the marrow at late stage, whereas the diaphysis region (100-300 pixels) was less affected by tumor growth. In addition, the meta-epiphysis region (300-400 pixel) showed dramatic bone loss (>75%) and knee destruction. Statistics were assessed with one-way analysis of variance (ANOVA) and Sidak's post-tests.

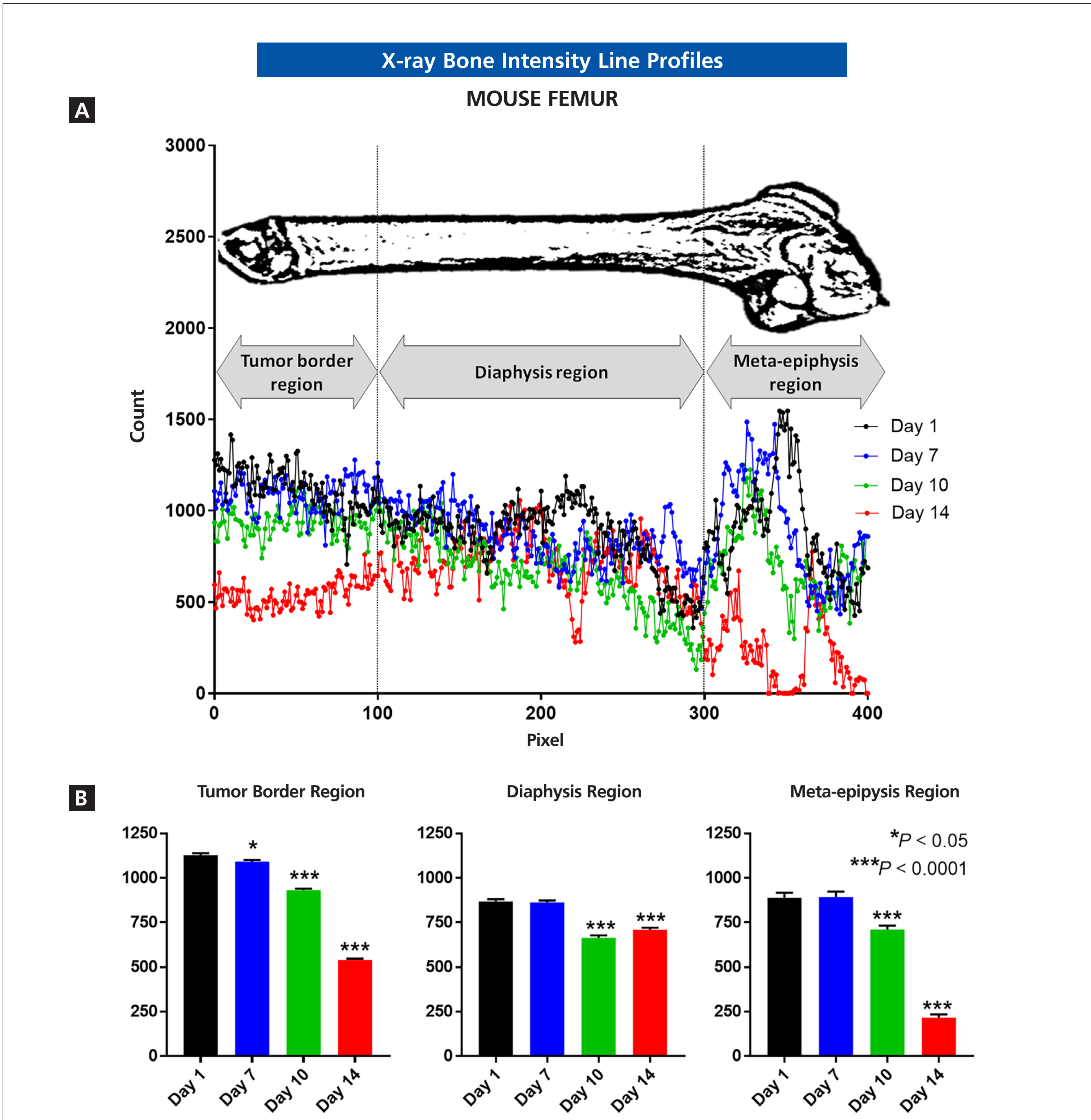

Figure 5. Normalized line profiles in a 400-pixel femur encompassing the epiphysis through the tumor border (Figure 5A). Line pixel intensity profiles of femur regions at varying time points (Figure 5B).

Conclusions

This application note provides an overview on evaluating bone disease using the IVIS Lumina X5 planar optical/X-ray imaging system. Together with the X-ray compatible Smart Tray accessories, the IVIS Lumina X5 provides a convenient imaging workflow for animal preparation and loading into the imager. As an example, a tumor-induced bone erosion model was used to illustrate the utility of this versatile system to acquire high-throughput or high-resolution BLI/X-ray hybrid images for quantitative tumor/bone analysis. The full-field imaging mode allowed simultaneous optical and X-ray imaging of five mice, which significantly increased the imaging throughput for identifying subjects with bone degradation.

The IVIS Lumina X5 also offers a high-resolution X-ray imaging mode for more accurate visualization of subtle bone structural changes. Taking advantage of the larger scintillator panel and the highly sensitive camera, the system can produce high-resolution X-ray images at more than 21 lp/mm (typically 25 lp/mm). For quantitative data output, the Line Profile tool in the Living Image software was used to generate pixel intensity line profiles across the eroded bone regions at various timepoints. It is of great importance to draw the lines in a consistent manner throughout the study in order to ensure assay accuracy. This method enables the quantitative assessment of longitudinal bone loss. Although a robust tumor-induce knee erosion model was used to assess X-ray imaging and analysis on the IVIS Lumina X5, this approach can be used with other types of bone research, and preliminary studies suggest utility in other bone-turnover applications, including arthritis and ovariectomy-induced osteoporosis. Of course, the high-quality planar X-ray images can be exported and analyzed using third party software, such as the open-source Image J, which is popular and widely available. However, Living Image has a distinctive advantage over the use of third party image analysis software. When an X-ray image is opened in Living Image, the software has direct access to the raw intensity data at each pixel, so you can adjust the contrast, brightness or opacity of the images without changing the pixel intensity numeric output. This offers full flexibility in visualization without adding any subjectivity in the quantification. Thus, Living Image is inherently more accurate and less biased in X-ray analysis.

This application note serves as an example of how the IVIS Lumina X5 imaging system can be used to perform bone research. The combination of bio-optical and X-ray imaging technologies in a relatively small footprint make the IVIS Lumina X5 a appealing solution for preclinical research.

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