# Quantitative Analysis of Bone Erosion in Small Laboratory Animals Using High-resolution X-ray images

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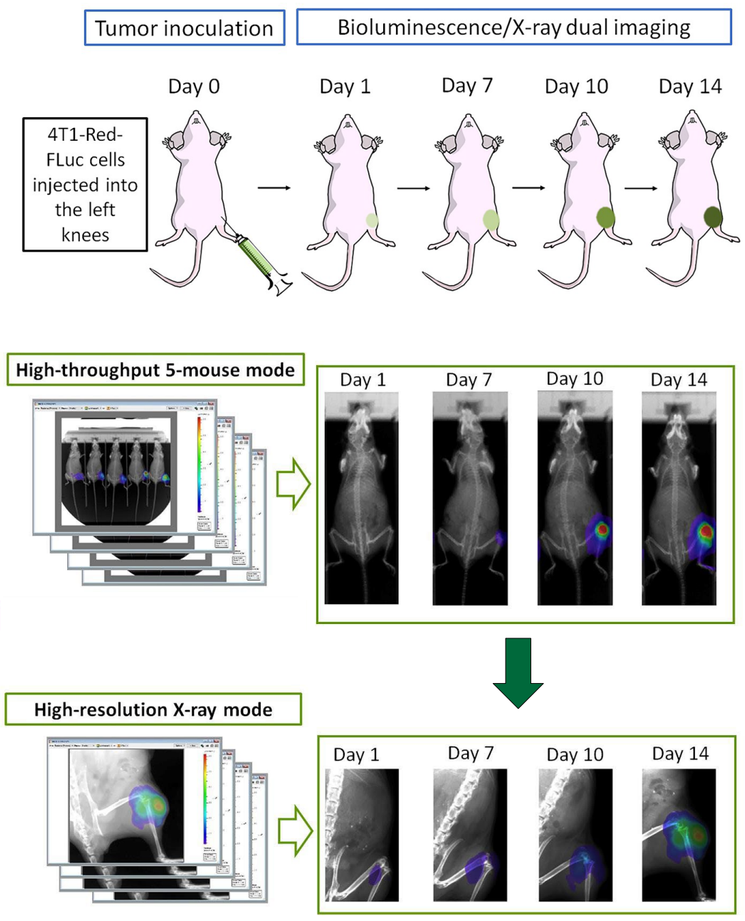
## 1 Abstract

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 tumor, abnormal metabolic processes such as hyperparathyroidism, and chronic inflammatory disease such as rheumatoid arthritis. As the bone contains hydroxyapatite mineral with high electron density, bone erosion was first characterized using X-ray radiography and has been routinely detected in clinical setting using X-ray based imaging technologies, such as conventional 2D planar X-ray and more advanced 3D computed tomography (CT). Pre-clinical imaging of bone erosion in animal models can also be achieved using planar X-ray or CT imaging. However, since laboratory animals are much smaller than human, it requires higher resolution to visualize bone erosion and subtle density changes. Pre-clinical CT indeed offers high- resolution 3D for such purposes, but it is not widely available and usually requires access to an animal imaging core facility. Planar X-ray imaging has several advantages for pre-clinical bone imaging, including better accessibility, higher throughput and ease-of-use. However, most currently available pre-clinical X-ray imagers lack sufficient resolution to visualize subtle bone density changes in small laboratory animals.

In this study, we take advantage of the IVIS™ Lumina X5 imaging system which has a highly sensitive CCD camera for bioluminescence imaging and a large scintillator panel for high-quality X-ray images. The imager can generate X-ray images at more than 21 lp/mm, allowing accurate visualization of subtle bone structural changes. We performed longitudinal, high-resolution planar X-ray imaging of bone erosion in nu/nu mice inoculated with mouse IVISbrite™ 4T1 Red F-luc breast tumor cells into the knees. As the cells also express firefly luciferase, the tumor growth could be monitored by bioluminescence imaging. In addition, the level of bone erosion induced by tumor growth was quantified using the line profile tool currently available in the Living Image® controller software that comes with every IVIS Lumina X5 installation. Unlike other third-party software, Living Image™ directly accesses the raw intensity data at each pixel, and any adjustments in contrast, brightness or opacity only affect image visualization 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 imaging analysis.

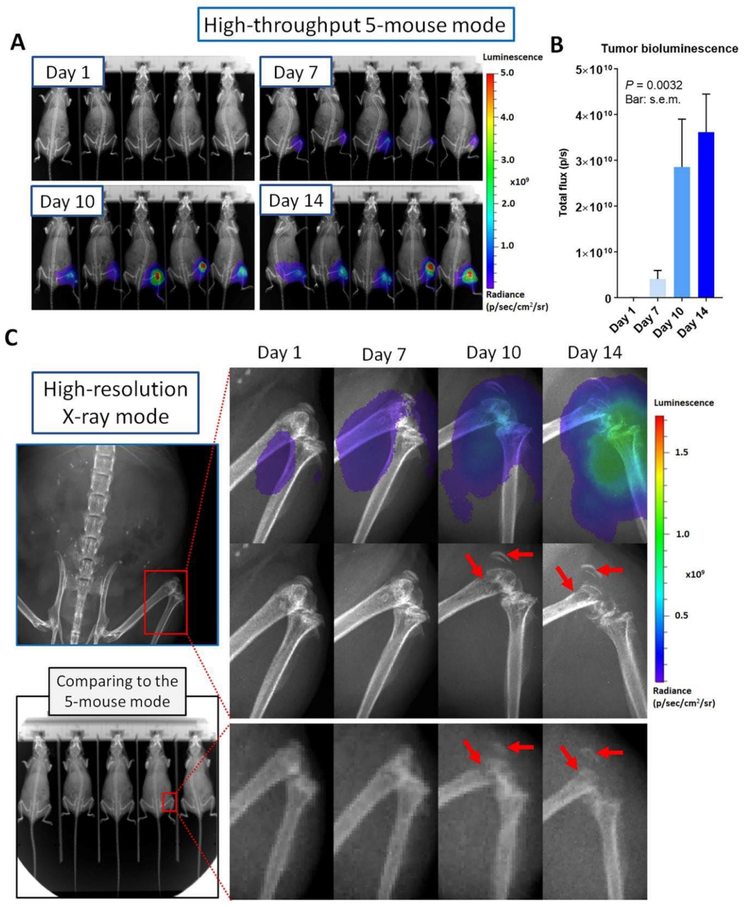
This proof-of-concept study shows the utility of acquiring quantitative tumor bioluminescence data combined with the quantitative analysis of tumor-induced bone changes using high-resolution X-ray images. Although a robust tumor-induce knee erosion model was used to demonstrate this point, combined optical imaging and quantitative X-ray can be useful for other types of imaging studies, and preliminary research suggests utility in other bone-turnover applications, including arthritis and ovariectomy-induced osteoporosis.

## 2 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 (IVISbrite 4T1 Red F-lluc cell line, Revvity) which is known for bone metastasis and destructive growth. The 4T1 Red F-luc cells used in the study expresses a red-shifted firefly luciferase for bioluminescence imaging. To induce osteolytic tumor growth, 0.5 million 4T1 Red F-luc cells were injected into the left knees of nu/nu mice on day 0. 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.

## 3 Longitudinal Imaging of Knee Bone Erosion




The imaging results summarized in Figure 3 clearly indicate that 4T1 Red F-lluc tumors grow significantly in the knee region over 14 days as seen in images (Figure 3) and by quantifying 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).

## 4 High-resolution X-ray images show knee bone

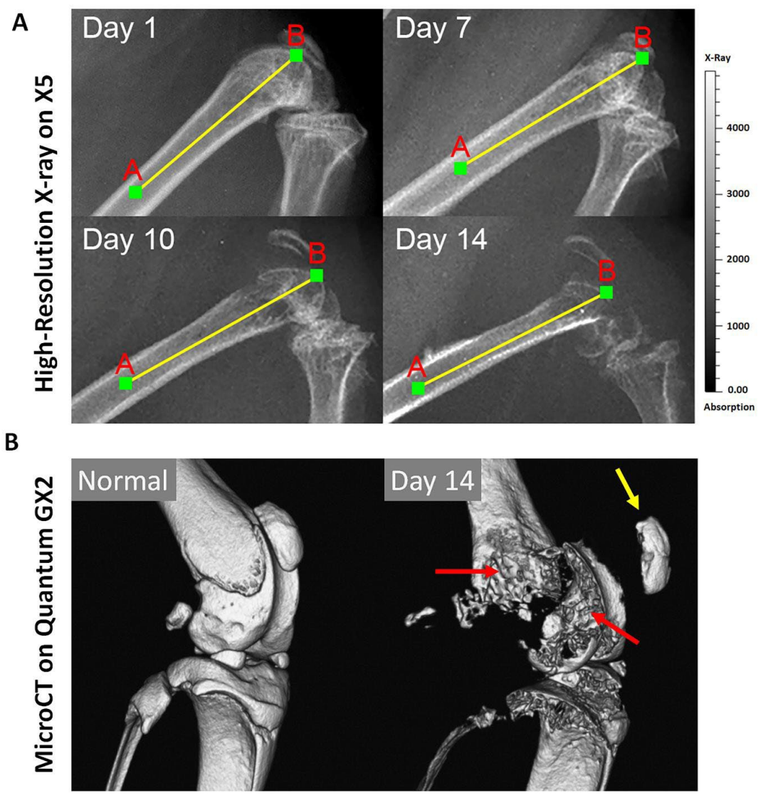
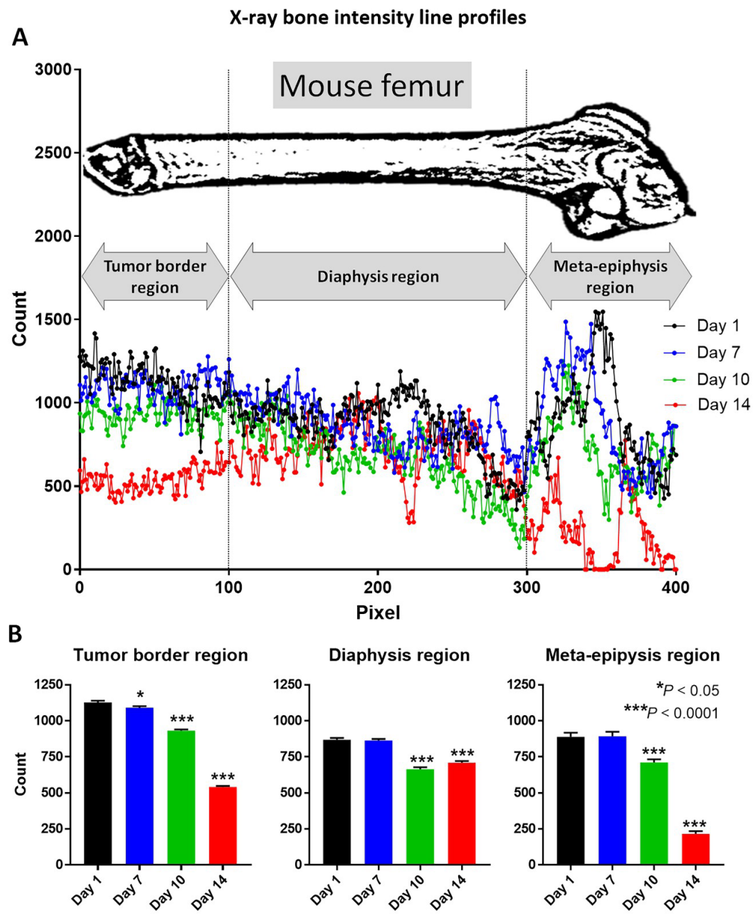



Figure 4A summarizes the osteolytic effects of a 4T1 Red F-luc tumor in a representative mouse as visualized by the high-resolution X-ray images. At early stages (day 1 and 7), there was no obvious visible bone loss. In contrast, starting on day 10, the tumor gradually displaced the patella (kneecap, 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 4B).

## 5 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. On the other hand, 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.

## 6 Summary

This study provides an overview of the utility for combined planar optical/X-ray imaging (IVIS Lumina X5, Revvity, Inc.) in detecting and quantifying both tumor growth and its effect on nearby bone. In particular, a high-resolution X-ray imaging mode was required to provide accurate visualization of subtle bone structural changes without the need for microCT imaging. This higher resolution was due to a larger scintillator panel and a highly sensitive camera, which produced high-resolution X-ray images at more than 21 lp/mm (typically 25 lp/mm). For quantitative data output, the Line Profile tool in Living Image software was used to generate pixel intensity line profiles across the eroded bone regions at various timepoints. This method enabled a flexible quantitative assessment of longitudinal bone loss across different bone regions. Although various third- party image analysis software can also be used for analysis, when an image dataset containing X-ray images is opened in Living Image, the software has direct access to the raw intensity data at each pixel, allowing adjustment of contrast, brightness and opacity of the images without changing the pixel intensity numeric output. This offered full flexibility in visualization/detection without any added subjectivity in the quantification.