**Title**: Affordable Bioluminescence Pipeline for Sustained and Localized Imaging of Metastatic Tumor Nodules

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ABSTRACT

**Introduction**. Genetic labeling of cancer cells with bioluminescent reporters allows the detection and quantification of tumor burden in preclinical metastasis models. The gold standard for bioluminescence imaging is the IVIS, which overlays photographs with bioluminescence signals that can be efficiently quantified in the corresponding Living Image software. Although this system allows for the straightforward quantification of tumor burden in general regions of interest (ROI), the limited spatial resolution makes it difficult to localize specific tumor nodules anatomically. Visualizing the precise location of nodules can shed light on potential cell types that support tumor growth and metastatic routes. Herein, we aimed to improve the spatial assessment of bioluminescence imaging by extending the ex vivo bioluminescent signal duration with a new protocol and improving the imaging resolution with our own low-cost bioluminescence chamber and software.

**Method**. The standard ex vivo bioluminescence imaging protocol requires injecting mice with D-luciferin before euthanasia and resecting relevant organs quickly due to rapid signal dissipation. We overcame this restrictive time limitation by incorporating physiological principles in our modified ex vivo imaging protocol. High glucose cell culture media was incubated at 37°C and bubbled with a carbogen mixture containing 95% oxygen since oxygen reacts with the D-luciferin substrate to produce light. The oxygenated media was then aliquoted in plates and stored at 37°C until needed for the organs. Ten minutes before euthanasia, mice were injected at 150mg/kg with the standard D-luciferin dose (15 mg/ml). The organs were then collected, placed in the wells containing pre-warmed bubbled media, and covered with sufficient luciferin (~10% volume/well).

**Results**. The ex vivo bioluminescent signal of the same organ was measured over time using the traditional method without media, cold-bubbled media, or warm-bubbled media and normalized to the starting signal. The signal without media dissipated within 30 minutes, while the bubbled media containing additional D-luciferin substrate extended the signal for hours. This extra time was used to carefully remove the organs and pin them so specific tumor nodules, rather than whole organ ROIs, could be visualized and quantified. The resolution of the IVIS (2048 x 2048 pixels) makes it difficult to pinpoint individual nodules. Instead, we created our own hardware system, consisting of the pco-edge camera (resolution of 2496 x 2496 pixels), a ring light LED that is controlled by the USB-6002 and LabVIEW to automatically turn on for brightfield images, and off, within a dark chamber to remove background light. This process is automized through a LabVIEW program to take 4 darkfield images and 1 brightfield image in each iteration Together, the cost was ~1/10 that of the IVIS. Using LabView software, we coded an interactive platform, similar to Living Image, that allows users to set exposure time, binning, and the ratio of dark to light images taken. Overlays of the bioluminescence and brightfield images were generated in the free software FIJI ImageJ. After time was allotted to pin the mesentery using our procedure, it became clear that tumor nodules accumulated along the adipose branches. This finding was not apparent from the IVIS image of a quickly removed murine mesentery.

**Conclusion**. Our system has broad applications in oncological imaging and beyond. By integrating our ex vivo imaging method, bioluminescence chamber, and software analysis, we could distinguish individual tumor nodules in discrete ROIs, providing spatial insight into potential mechanisms of metastasis. Radiochemists who cannot use bioluminescent imagers in core facilities for safety concerns can follow our methodology to build similar chambers that can image animals with radioactivity. In future studies, we will integrate live imaging by connecting the chamber to isoflurane and explore real-time imaging of luciferase-labeled tumor cells.

Figure Legends

(A) Schematic of our imaging protocol, including prewarming the high-glucose media, bubbling the media with carbogen, aliquoting the media, injecting the mice with D-luciferin, and adding additional D-luciferin the pre-warmed, oxygenated wells.

(B) Quantification of the bioluminescence signal duration among organs resected and imaged without media, with cold bubbled media, or warm bubbled media imaged on the IVIS. The signal is normalized to the starting signal and analyzed in LivingImage.

(C) Image of our bioluminescence dark chamber hardware consisting of the pco-edge camera, ring light LED, and USB6002.

(D) The user view of the LabView software interface, consisting of buttons to control the binning, exposure time, and the number of images taken.

(E) Comparison between the IVIS50 image of a mouse mesentery taken within 10 minutes post resection and our image of the mouse mesentery taken 40 minutes post-resection after pinning using our protocol, hardware, and software.