**WORKFLOW FOR REAL-TIME IN-VIVO CHERENKOV-EXCITED LUMINESCENCE IMAGING DURING RADIOTHERAPY**

Ethan LaRochelle, Thayer School of Engineering at Dartmouth College
ethan.phillip.m.larochelle.th@dartmouth.edu
Jennifer Shell, Thayer School of Engineering at Dartmouth College
Jason Gunn, Thayer School of Engineering at Dartmouth College
Michael Jermyn, DoseOptics
Sergei Vinogradov, Department of Biochemistry and Biophysics at University of Pennsylvania
Brian Pogue, Thayer School of Engineering at Dartmouth College

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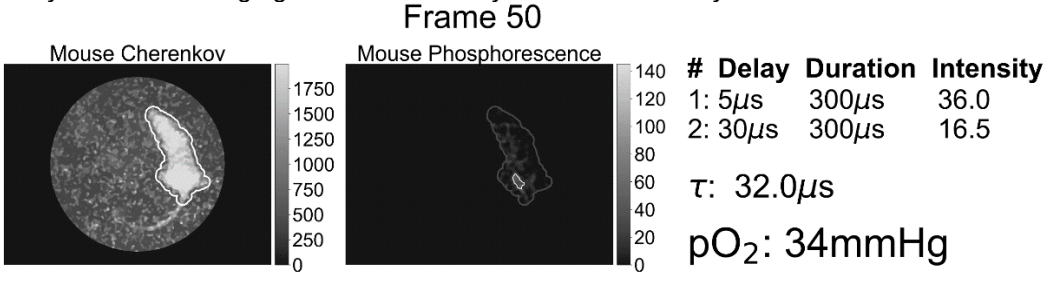
Radiotherapy is a common method for treating tumors, however, radiosensitivity can vary between tumor types or within the tumor microenvironment. The ability to deliver oxygen is crucial for the generation of reactive oxygen species resulting in increased localized cytotoxic effects. Alternatively, hypoxic tumors are thought to indicate a poor prognosis and may benefit from more aggressive treatments, yet identifying tumor hypoxia early in the course of a multi-week fractionated dose regimen is currently impractical. Using a time-gated imaging system and oxygen-sensitive phosphorescent compound (PtG4) we are able to estimate *in vivo* $pO_2$ distribution at a rate of 2.6 estimates per second, which corresponds to 50+ values during a common 2Gy dose fraction. While our previous work has reported using Cherenkov-excited luminescence to estimate *in vivo* $pO_2$ during external beam radiotherapy, the dose required was often greater than a standard fraction and camera acquisition parameters required modification during treatments, resulting in interrupted workflows. The current method utilizes custom control software which cycles through camera timing parameters during acquisition. Python code using the web-based user interface JupyterLab allows for interactive analysis of the resulting image stack without the need to pay expensive licensing fees for scientific computing packages. Using open source libraries, the analysis code is able to split the image stack into respective Cherenkov excitation and phosphorescence images, which can then be further automatically segmented to find regions of interest including the subject and phosphorescent region. The intensity of the regions in the phosphorescence images are used to estimate the compound lifetime, which can then be used in the Stern-Volmer relationship to estimate $pO_2$. This entire process does not compromise clinical workflow and is able to provide a $pO_2$ estimate within minutes after delivering the fractionated dose, providing clinicians early feedback about trends in tumor hypoxia. The current method has been validated with both direct injection of 50$\mu$M PtG4 in Matrigel in a mouse flank, and 24hrs post IV injection of mouse with MDA-MB-231 tumor implanted in the flank. The mouse with the direct injection was imaged under anesthesia and while awake and mobile to test the ability of the automated segmentation algorithm (Figure below). While the signal from the IV injection was less intense, simultaneous imaging using the previously reported method and current method resulted in similar lifetime estimates. While oxygen-sensitive PtG4 exhibits a lifetime between 16$\mu$s under atmospheric oxygen and 47$\mu$s when deprived of oxygen, other compounds have also been investigated. Europium chelate nanoparticle (~600$\mu$s), Iridium-based small molecules (~5$\mu$s), Si nanoparticles (~60$\mu$s), and UV-sensitive tattoo inks (~15$\mu$s) have all been imaged using Cherenkov-excitation. Camera time-gating can be utilized to discriminate these compound when mixed in the same field, allowing for additional tools in the realm of contrast enhancement during radiotherapy imaging. Ongoing studies with PtG4 and other compounds are being conducted to further improve system sensitivity and refine imaging workflows so they are more clinically translatable.




*Automatic segmentation to determine $pO_2$ of PtG4 implanted in Matrigel in the flank of an unanesthetized freely-moving nude mouse. (Left)Cherenkov image of the mouse where the white outline was determined programmatically. (Right) Phosphorescence image collected 5$\mu$s after the X-ray pulse, where the white outline shows the region more than one standard deviation above the mean mouse phosphorescence. (Right)Two phosphorescence intensities can be used to find a lifetime of 32$\mu$s which corresponds to a $pO_2$ of 34 mmHg.*