Label Free Imaging for Rapid Assessment of Tumor Viability in Live Tumor Fragments

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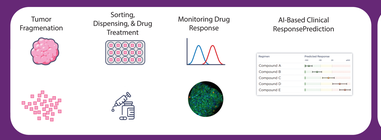
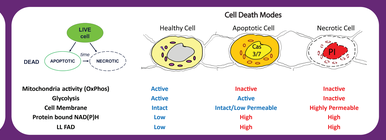
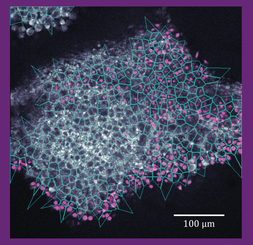
Introduction

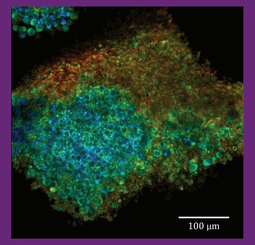
- Currently there are limited useful biomarkers to predict response to cancer therapies including checkpoint inhibitors.
- We have developed the novel human Live Tumor Fragment (hLTF) Cybrid™ platform that preserves the tumor micro-environment, including immune cells, to assess *ex vivo* response to cancer therapies.
- We have devised a multiphoton microscopy-based, label-free live/dead predictor (LDP) that circumvents the need for exogenous fluorescent probes, enabling rapid and repeated viability assessment.

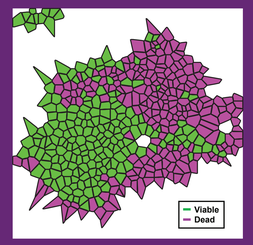




Cell Viability in Tissue Fragments Using a Label-Free Live/Dead Predictor (LDP)







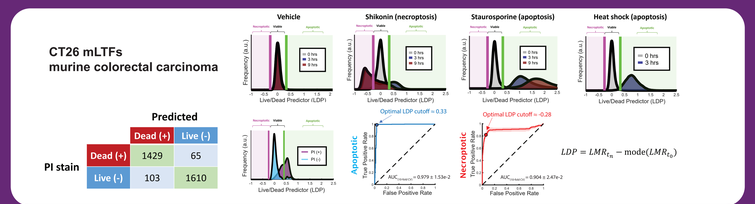
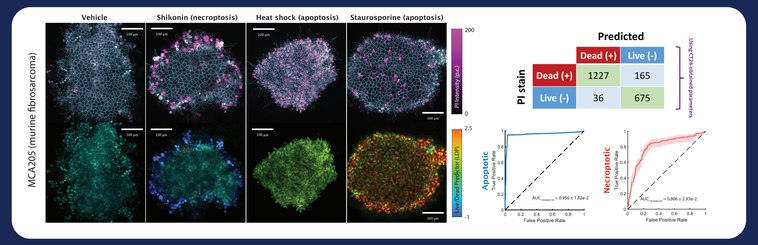
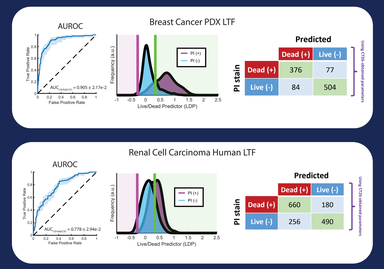
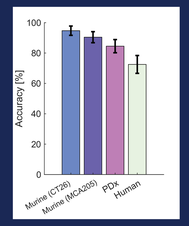


Figure 1- Live murine CT26 tumors were cut into fragments (300 X 300 X 300 microns), sorted, and cultured in glass-bottomed multi-well plates in optimized conditions. We imaged LTF structure and metabolic status based on the intrinsically fluorescent metabolic co-factors nicotinamide dinucleotides (NAD(P)H), excited at 740nm, and flavin adenine dinucleotide (FAD) excited at 880nm. Multi-channel fluorescence intensity and lifetime were obtained using a custom four channel, multiphoton imaging microscope (MP-FLIM). As a ground truth viability reference, fragments were stained using two probes: a nuclear localizing probe, propidium iodide (PI), which indicates cell membrane degradation associated with necroptosis and necrosis, and a red-fluorescent Caspase 3/7 sensor (Thermo Fisher) to indicate apoptosis. Where necessary, overlapping dye spectra were deconvolved using a phasor analysis of the data with data segmenting based on cluster analysis. To determine lifetime parameters of the intrinsic signals, phasor analysis was done to obtain the relative percent contribution of the longer or shorter lifetime component for NAD(P)H or FAD (α_i is the shorter lifetime component). The lifetime metabolic ratio (LMR) was calculated from the α_i[%]. NAD(P)H/ α_i[%] FAD. Data derived from intrinsic NADH signals (obtained at 740nm), and FAD signals (obtained at 880nm) in CT26 LTFs were used to segment the image into individual Voronoi cells seeded from nuclear segmentation. LMR histogram data from the fragments was normalized by taking the mode of the resultant histogram data at baseline and setting this value to 0 across the time course. The normalized LMR value was determined for each Voronoi cell. Ground truth signals were determined for each Voronoi cell based on the presence or absence of PI or the caspase 3/7 sensor. CT26 fragments were subjected to varying treatments including: Addition of the multi-kinase inhibitor Staurosporine to induce apoptosis; Addition of Shikonin to induce necroptosis; and subjecting the fragment to heat shock. Area under the receiver operating characteristic (AUROC) logistic regression (10-fold cross-validation) analysis enabled us to set two threshold values on the normalized LMR (hereafter **Live/Dead Predictor [LDP]**) histogram which showed best correlation between LMR and PI staining on Voronoi cells. We have called these numbers the LDP thresholds. Values of the LDP thresholds were classified as follows: Voronoi cell values above 0.33 indicates apoptosis leading to cell death, and below -0.28 indicates necroptosis leading to cell death. Using these threshold values, confusion matrices were generated to determine the accuracy relative to PI staining.

Validation of LDP Across Species and Tumor Types







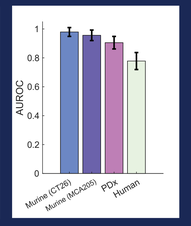
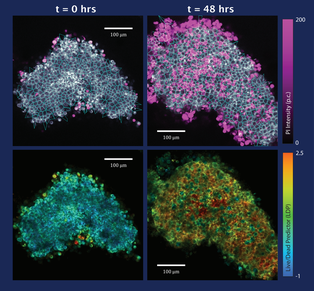


Figure 2 - Three channel lifetime data (blue, green, and red emission) was obtained at two separate wavelengths, 740nm and 880nm on CT26 murine LTFs, MCA205 LTFs, patient derived xenografts, and human renal cell carcinoma (RCC) LTFs. Data from intrinsic NADH signals (obtained at 740nm) and FAD signals (obtained at 880nm) and PI were used to segment the image and analyze the data as described in Figure 1. Representative hRCC samples showing high viability were selected to set baseline normalization for this tissue type. Utilizing the LDP values determined in Figure 1 generated from the murine CT26 model system, Voronoi cells were classified based on values obtained from the LDP histogram. PI staining was used to provide a ground truth for testing the LDP. Confusion matrices were generated with multiple tissue samples and the data plotted. AUROC data for the images was also obtained using ten-fold cross-validation.

Toxic Drug Activity is Quantified Over Time Using the LDP



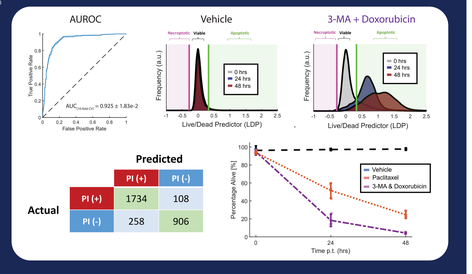


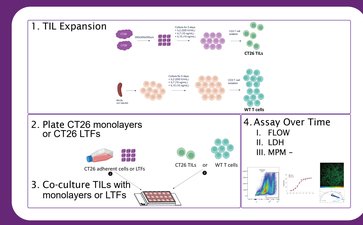
Figure 3 – To demonstrate the utility of the LDP, longitudinal experiments were undertaken in murine CT26 fragments treated with either paclitaxel or with a combination of 3-methyladenine (3-MA) and doxorubicin for 48 hours. Shifts in the LDP relative to time 0 baseline were observed as the cells were killed, and the percentage of dead tissue was determined using the LDP predictor values defined in Figure 1. A confusion matrix was constructed, using PI staining as cell death ground-truth, to determine the accuracy of the analysis across tumor and tissue types.

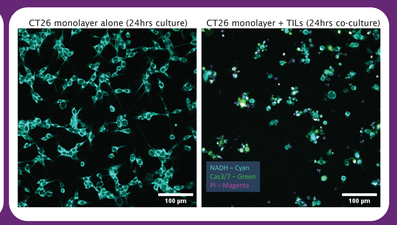
Acknowledgements

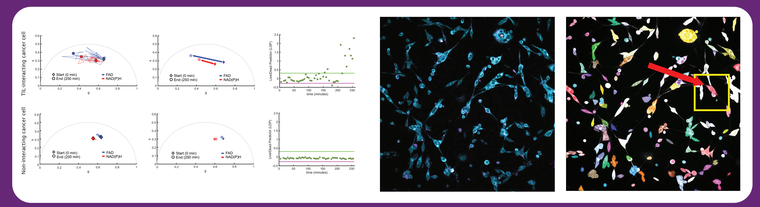
We would like to thank the University of Wisconsin Carbone Cancer Center BioBank, supported by P30 CA014520, for use of its facilities and services. Tissue samples were provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute.

For a timelapse video of mobile CD8 TILs within our human live tumor fragments (hLTFs™), scan this QR code!

Localized T cell Cytotoxicity Can be Determined Using the LDP







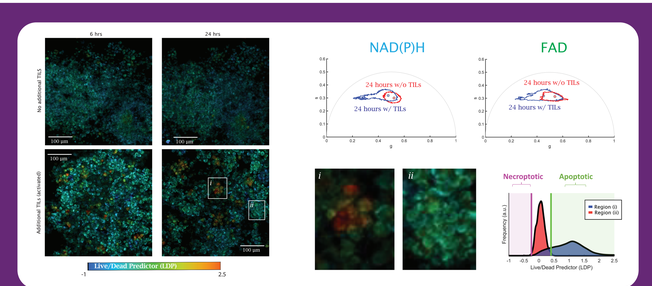


Figure 4 – T cell-induced cytotoxicity via granzyme B-induced apoptosis was explored using tumor infiltrating lymphocytes (TILs) isolated from CT26 syngeneic tumors in both CT26 monolayers and CT26 LTFs. TILs were isolated from CT26 fragments using negative selection for T cells. Briefly, CT26 fragments were incubated for 24 h to allow the efflux of TILs into the surrounding media. After the incubation period, the fragments were filtered from the media and the remaining cells in the media were subjected to negative selection for T cells following the standard protocol from Stem Cell Technologies. Isolated CD3+ TILs were either used immediately for multiphoton microscopy (MPM) experiments or activated for 48-72 h with PMA/Ionomycin prior to MPM experiments. To characterize the metabolic signature of a target tumor cell that encounters an activated TIL, we first co-cultured isolated TILs with a monolayer of CT26 cells. We tracked TILs and determined specific points in time when TILs come into contact with target cells. For those cells with a contact event, we performed phasor analysis for NAD(P)H and FAD and calculated the LDP to quantify changes in time. These signals often preceded observed cellular blebbing and had exceeded an LDP value of 0.33, indicating that the cell had died. Death signatures were further validated using the presence or absence of PI and/or a caspase 3/7 sensor. Additional experiments were also conducted in CT26 LTFs. Single CT26 LTFs were placed in conical well plates (Insphero) and co-cultured with PMA/Ionomycin-activated TILs. After the addition of TILs, fragments were imaged periodically over a 24-hour period at imaging depths of 50 or 75 microns into the sample. TILs were observed to associate with the periphery of the LTF and then gradually infiltrate the fragment over several hours. After 24 hours, we were able to observe whole tissue changes via phasor plots relative to the control tissue. We observed punctate regions of significantly changed LMR over time. Zooming in on these areas, cell death was indicated by the LDP in 50 X 50 micron areas. To provide an orthogonal verification for the observed TIL cytotoxicity, we performed flow cytometry and LDH release assays on the TIL-CT26 co-cultures. Using flow cytometry, we quantified the percent of dead CD45- cells to measure CT26 cell death. Additional experiments were performed using verified labeled CD8 positive TILs, scan the QR code for additional data.

Conclusion

- Here we present a first-ever methodology to assess T cell- and chemotherapy-mediated cell death in live tumor fragments without the use of probes.
- Using multiphoton microscopy, we can rapidly and accurately predict live/dead status of cells in LTFs (AUROC 0.91 in murine models and 0.78 in human tumors).
- We show that therapy-induced cell death can be repeatedly assessed in LTFs over a 48-hr time course.
- This novel approach bolsters the Cybrid™ platform with critical spatial context and provides an additional tool towards the goal of personalized cancer treatment.