Tumor/normal and live/dead classification in live tumor fragments using label-free multiphoton microscopy

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Introduction: We have developed a live tumor fragment (LTF) platform for predicting clinical response to cancer drugs. The success of this approach depends on screening tissue fragments derived from biopsies and excisions before drug treatment to select those that have acceptable levels of viability and tumor content. To enable this screening, we are developing label-free methods for integrated assessment of LTF histology, viability, and metabolic status using intrinsic multiphoton-excited fluorescence lifetime microscopy (MP-FLIM).

Methods: We cut live EMT6 tumors and mammary fat pad into rectangular fragments that were then sorted and cultured in glass-bottomed multi-well plates. To induce cancer cell death, we treated one group with a therapeutic agent, e.g., the multi-kinase inhibitor, staurosporine, or the alkylating agent, cisplatin. Before and after treatment, we imaged LTF structure and metabolic status based on the intrinsically fluorescent metabolic co-factors nicotinamide dinucleotides (NAD(P)H) and flavin adenine dinucleotides (FAD) fluorescence intensity and lifetime using dual-excitation multiphoton microscopy. As a ground truth viability reference, we then stained and re-imaged the fragments using nuclear and cell viability probes such as Hoechst, SYTOX, TMRE and/or propidium iodide (PI). We analyzed the data by fitting fluorescence decay curves with dual or triple exponents to generate images of lifetime parameters, including the mean and individual component lifetimes and amplitudes. Finally, we co-registered the extrinsically labeled and auto fluorescent lifetime images for 3D spatial analysis using commercial and custom software. We verified the methods using monolayer cell cultures and ATP luminescence and flow cytometry viability assays. Image spatial heterogeneity was quantified utilizing entropy metrics. Intrinsic contrast from multiphoton imaging revealed cellular and tissue structures.

Results: The median entropy was higher and its distribution negatively skewed for EMT6 ($p<0.01$), allowing us to distinguish tumor from its corresponding normal tissue of origin. In viable cells, nuclei were distinguishable from the cytoplasm via higher cytoplasmic NADH signal. Upon loss of cell viability, nuclear NADH signals increased, with characteristically short lifetimes, and the overall NADH intensity decreased, reducing the contrast between nuclei and their surrounding cytoplasm. We detected an increase in the whole fragment mean lifetime (τm $p<0.002$) and concomitant decrease in the short lifetime component ($\alpha 1$ $p< 0.002$) within 24 hours of staurosporine exposure.

Conclusion: We have demonstrated that MP-FLIM can distinguish tumor from normal and live from dead in LTFs without labels. This approach will facilitate selection of fragments with acceptable viability and tumor content for subsequent drug treatment as part of a platform built for personalizing cancer therapy.

Control/Tracking Number: 5084-AACR

Session Category: Tumor Biology