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

AACR ANNUAL MEETING 2022 New Orleans — American Association for Cancer Research

Poster #2472

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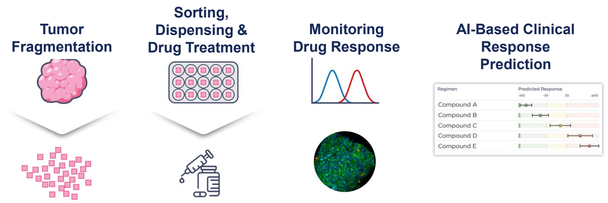
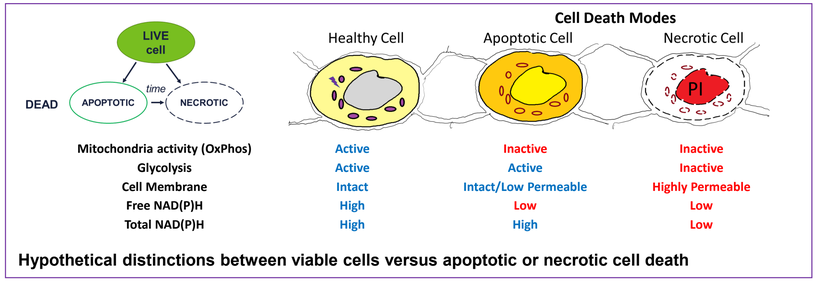
INTRODUCTION

- 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) and sensor-based ground truth training.



The Elephas Live Tumor Fragment (LTF) Platform

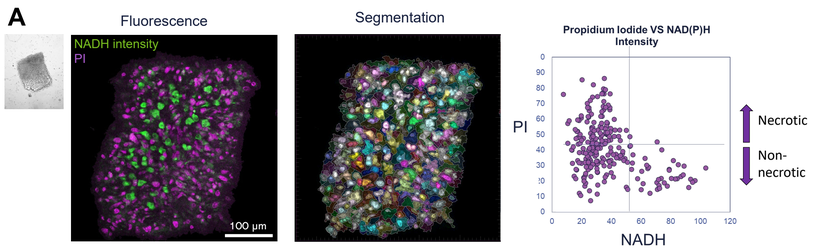


Hypothetical distinctions between viable cells versus apoptotic or necrotic cell death

METHODS

We cut live EMT6 or CT26 tumors into rectangular fragments that were then sorted and cultured in glass-bottomed multi-well plates in optimized conditions. We treated LTFs with the multi-kinase inhibitor, staurosporine (STS), the alkylating agent, cisplatin, or heat shock. Before and after treatment, we imaged LTF structure and metabolic status based on the intrinsically fluorescent metabolic co-factors nicotinamide dinucleotides (NAD(P)H) fluorescence intensity and lifetime using multiphoton fluorescence lifetime imaging microscopy (MP-FLIM). As a ground truth viability reference, fragments were stained using a nuclear probe propidium iodide (PI). To determine cell apoptosis, we used a red-fluorescent Caspase 3/7 sensor. We analyzed the data by fitting fluorescence decay curves with dual or triple exponents to generate images of lifetime parameters, including the mean (τ_m) and individual component lifetimes and amplitudes (α_1 is the shorter lifetime component). Finally, we co-registered the extrinsically labeled and autofluorescent 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.

LABEL-FREE IMAGING OF TUMOR FRAGMENT VIABILITY



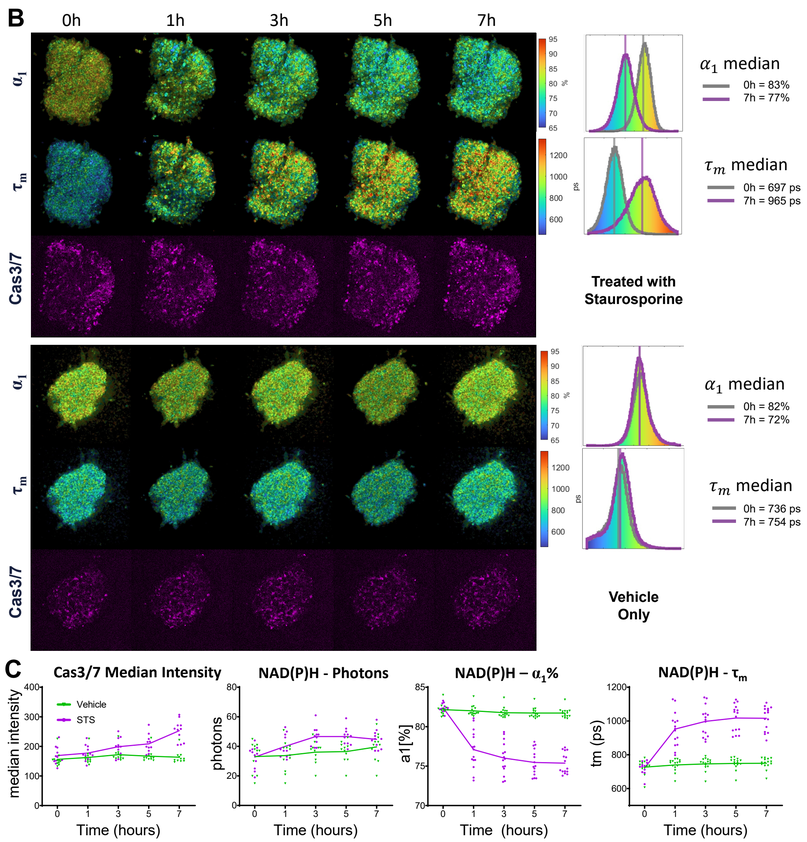


Figure 1. NAD(P)H fluorescence lifetime microscopy imaging of necrotic (A) versus apoptotic (B, C) cell death. A: EMT6 LTFs were UV-illuminated and treated with 0.3125 µM cisplatin, causing cell death. After 48 h, NAD(P)H intensity and lifetime were imaged using MP-FLIM. Necrotic cells were identified by nuclear staining with propidium iodide (PI). Cell bodies were segmented based on combined NAD(P)H and PI channels using Imaris software. In the graph, each dot represents one cell. Consistent with our hypothesis, cells with high NAD(P)H fluorescence intensity were PI-negative, and vice versa, cells with high PI fluorescence were NAD(P)H-low. **B:** One CT26 LTF group was treated with 1 µM staurosporine (STS), and the other was untreated or vehicle-treated as control. The same fragments were repeatedly imaged over time by MP-FLIM, and apoptosis was detected using a red-fluorescent Caspase 3/7 sensor. **C:** Quantification of STS treatment data. Each data point represents a single fragment.

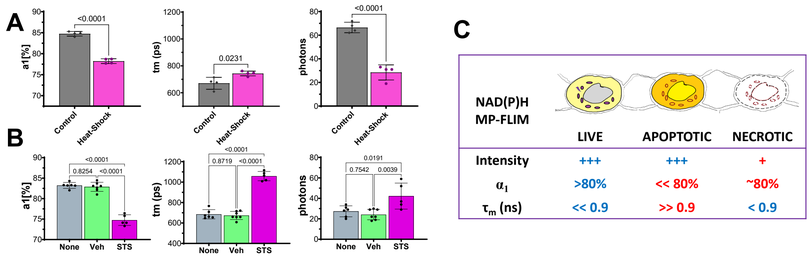


Figure 2. Quantitative method for Live vs Apoptotic Dead vs Necrotic Dead tissue classification using FLIM. A: Statistical analysis of MP-FLIM of necrotic fragment death by heat treatment. **B:** Statistical analysis of MP-FLIM of LTFs treated for 24 h with STS. **C:** MP-FLIM-based Cell Viability Classification Matrix for mouse EMT6 tumor model. In a general method, specific threshold values (e.g, α_1 = 80% and τ_m = 0.9 ns) may slightly differ from those for EMT6 and are calibrated by using controls such as by heat shock and STS treatment.

LABEL-FREE LIVE/DEAD MODEL TRAINING

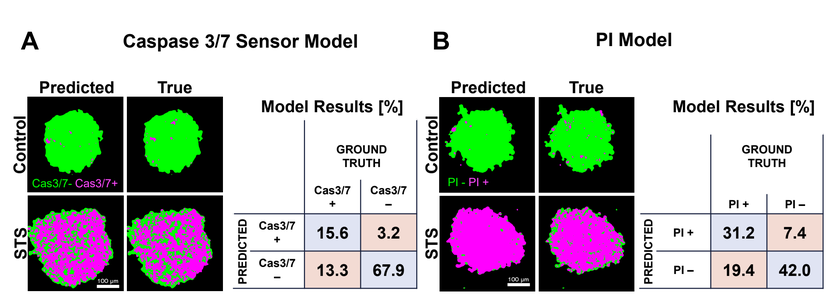


Figure 3. Classifier model trained using MP-FLIM parameters to predict apoptotic (Caspase3/7 sensor elevated) (A) or necrotic (PI elevated) (B) cell regions for cell segmentation-independent viability recognition. A: Predicted apoptotic state (**+**: green, **–** : magenta) for Control (top) and STS-treated (bottom) groups; True indicates the actual apoptotic state; model confusion matrix indicating 83.5% accuracy. **B**: PI-based model results; left panel shows Predicted necrotic state (**+**: green, **–** : magenta) for Control (top) and STS-treated (bottom) groups; True panel indicates the actual necrotic state; right panel highlights model confusion matrix indicating 73.2% accuracy. Preliminary model results highlight the potential for an MP-FLIM-based model, coupled with robust cell segmentation, to accurately identify treatment-responsive cells and regions.

LABEL-FREE IMAGING OF TISSUE COMPOSITION

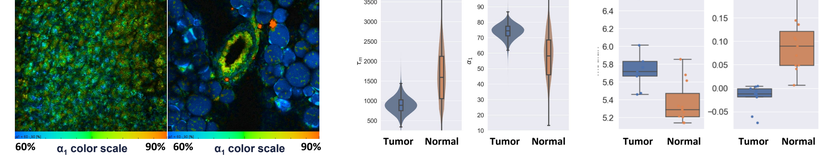


Figure 4. Label-free tumor vs. normal analysis of a mouse breast tumor using MP-FLIM entropy metrics. A: Tissue fragments were cut from EMT6 breast tumor or normal mouse breast tissues and imaged by MP-FLIM of NAD(P)H. Intrinsic contrast revealed cellular and tissue structures. Compared to normal breast tissue, tumor mean lifetime was lower, the short lifetime component was higher, median entropy of NAD(P)H images was higher, and entropy distribution negatively skewed for CT ($p<0.01$), enabling tumor to be distinguished from its corresponding normal tissue of origin.

SUMMARY

- Using a combination of MP-FLIM imaging of NAD(P)H fluorescence brightness and lifetime parameters, we can distinguish live cells from apoptotic and necrotic cells.
- Using MP-FLIM, we can distinguish tumor from normal tissue in living tumor fragments without use of chemical 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.
- The label-free MP-FLIM approach will also enable evaluation of the biological effect of treatments on the viability of cells in tumor fragments.