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A Novel Anisotropy Imaging Technique for NAD(P)H Autofluorescence

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Autofluorescence based metabolic mapping has proven to be a useful tool in diagnostics for cancer and neurodegenerative diseases in the past two decades. These metabolic interpretations use either fluorescence intensity or fluorescence lifetime of a critical cofactor reduced Nicotinamide Adenine Dinucleotide [NAD(P)H], to estimate the redox state of the cell. The difference between these two types of measurements owing to their underlying physics gave rise to two independent metabolic imaging schemes known as optical redox imaging and autofluorescence lifetime imaging. Intensity-based NAD(P)H measurements directly measure the quantity of NAD(P)H in the system [1]. To decode the total NAD(P)H measured in the biological sample to its redox state, the NAD(P)H intensity values are compared relative to the quantity of Flavin Adenine Dinucleotide or FAD fluorescence intensity from the specimen. This ratio NADH/FAD will correlate with the redox state of the biological sample. In contrast, Fluorescence lifetime is an intensity-independent parameter, and the total amount of NAD(P)H in the cell or mitochondria doesn't change the NAD(P)H lifetime. However, NAD(P)H undergoes conformational changes when it is bound to an enzyme [2]. This conformational mobility increases the lifetime, and studies show that a higher fluorescence lifetime correlates with a higher Oxidative Phosphorylation and Electron Transport chain (ETC). These two methods have been widely used in biomedical imaging and have addressed diagnostic capability for cancer and neurodegenerative disorders [3]. When performing single cell measurements, both these methods use many subtle assumptions. For example, these techniques work on the premise that NADH is present in the region of interest at a higher concentration when compared to its triphosphate counterpart NADPH. Similarly, studies have postulated that the NAD(P)H based measurements are sensitive towards biological phenotype and physical factors such as viscosity, pH, temperature, and other microenvironmental factors [4]. To address these concerns, techniques need to adapt to accommodate fluorescence spectrum and polarization in addition to the lifetime and intensity [5]. Here we pursue the polarization imaging scheme for NAD(P)H fluorescence.

In this work, we illustrate a fast-imaging system capable of imaging up to 4 frames per sec(fps) steady state anisotropy data (intensity-based anisotropy calculation) and 1fps time-gated anisotropy decay (the lifetime histograms are used to separate intensity in pre-selected lifetime ranges). These fast anisotropy measurements allow one to study NADH based on the hindered anisotropy (r_∞) value and the depolarization decay in live cells. This system uses a 740nm multiphoton excitation from an ultrafast laser at 76MHz (~150fs, MIRA900, Coherent) and a XY galvanometric scanner (Cambridge) to scan the sample. Fluorescence is collected using a 60xWI Objective lens (Nikon) and two Hybrid PMTs (Picoquant PMA-40) separated using a polarizing beam splitter. The lifetime measurements are made using a rapid FLIM timing electronics (Timeharp260, PicoQuant). HeLa cells plated on a glass bottomed dish were imaged for 1 hour with temperature control (at 37degC) and without CO2 regulation to introduce pH stress to the cells [4]. The data presented in Figure 1 shows that the lifetime and anisotropy correlated in the

cells under pH stress. The steady state gated measurements and relative change in mean anisotropy calculated per cell is shown below. This time-gated method to characterize NADH binding using anisotropy in a real time fashion allow us to map the cell with relative changes in anisotropy in real time fashion.

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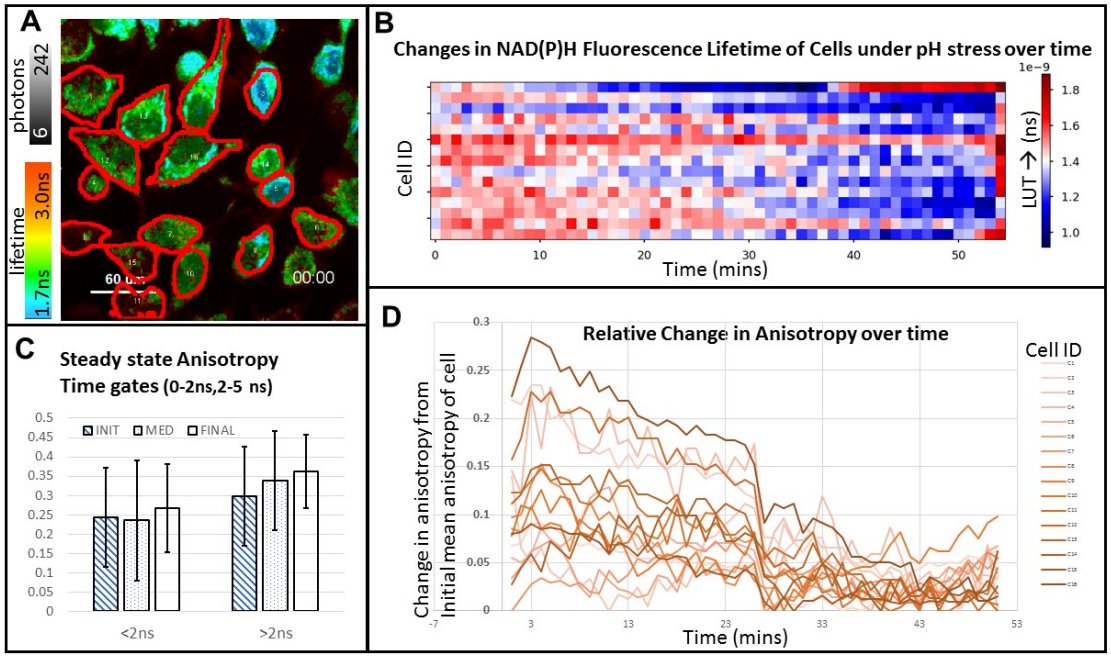

Figure 1. The change of Fluorescence lifetime correlate with the change in the relative anisotropy calculated per cell. Panel A shows the NAD(P)H image of a group of cells. The image is 90 um x90um and the color scale shows a lifetime range between 1.7ns and 3.0 ns. The intensity is overlaid on the lifetime image with a grey color scale from 6 to 242 photons per pixel per second. Each cell is segmented and followed over a time of 55 mins without CO2. The fluorescence lifetime of the cells under stress of pH decreases as seen panel B. The LUT color scales is from 1ns to 2ns. Almost all the cells reach a lower lifetime by 50 mins. C): The steady state lifetime (intensity based) was calculated for two time-gates (0-2ns) and (2-4) ns and the anisotropy is different for short and long lifetime species. D) The macro-time resolved anisotropy (without gates) calculation for these cells over this time period shows that the anisotropy drops similar to the lifetime values. Note that this graph is plotting the relative change in the anisotropy to minimalize the variations due to the initial mean anisotropy measured per cell.

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