NADH-Enzyme Fluorescent Lifetime Standards

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Abstract

Upregulation of mitochondrial gene expression + metabolic shift from glycolysis towards oxidative phosphorylation (OxPhos) → drug resistant cancer cells

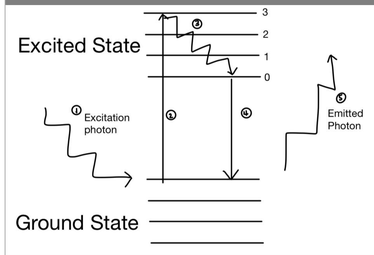
Hypothesis: We can determine which enzymes are behind this detectable shift
Alternate to the hypothesis: The FLIM signal is too complex to determine individual enzyme contributions

Test: Measure FLIM signals of NADH bound to pure enzymes in solution

Findings: Some enzymes have multiple component exponential decay when 100% of enzyme is NADH bound

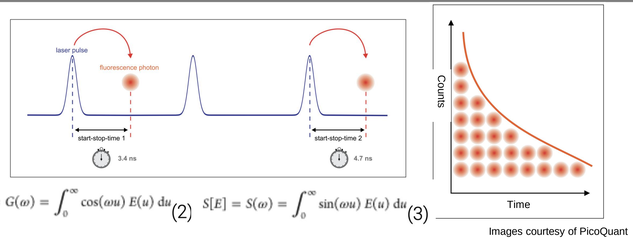
Fluorescence and FLIM

Principles of Fluorescence



Fluorescence Lifetime Imaging Microscopy(FLIM) measures how long a fluorescent molecule remains excited (time for step (2)) upon a photon promoting an electron (1) into an excited state, remaining in an excited vibrational state, relaxing down (2) to the lowest energy excited state, before returning to the ground state (3), and emitting a fluorescence photon (4) which is detectable.

FLIM Measurements, Decay Curves, and Fourier Transforms

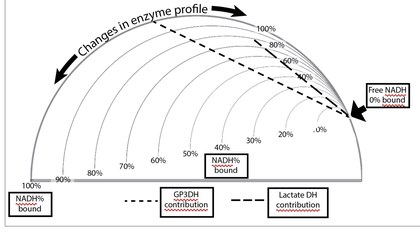


$$G[E] = G(\omega) = \int_0^\infty \cos(\omega u)\, E(u)\, du \quad (2) \qquad S[E] = S(\omega) = \int_0^\infty \sin(\omega u)\, E(u)\, du \quad (3)$$

Images courtesy of PicoQuant

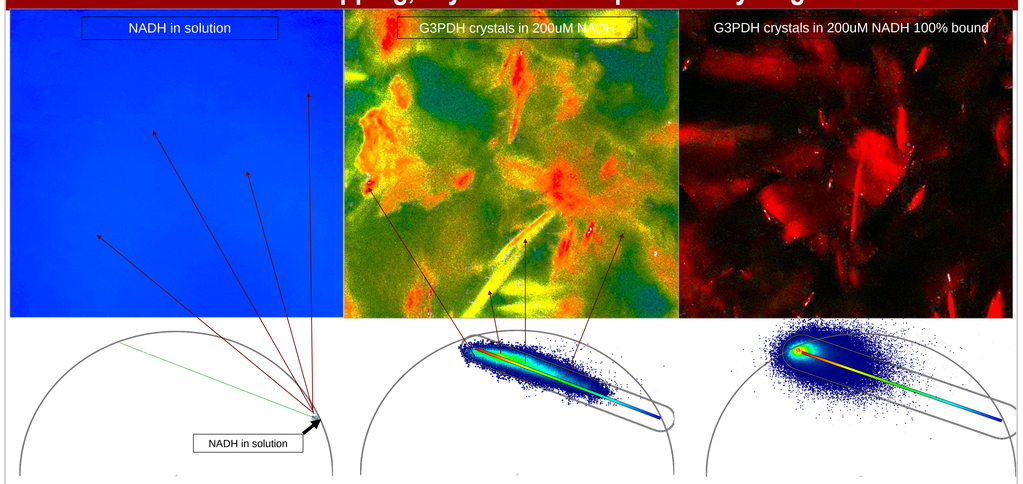
Our pulsed laser functions as a pacemaker and emitted photon arrival times are recorded to be placed into discrete time bins as FLIM measurement histogram. The fluorescence lifetime decay curve for each pixel in an image is calculated through Fourier transformation (equations 2 & 3), where cosine and sine coefficients are plotted as G and S coordinates respectively, in the phasor.

Phasor Roadmap



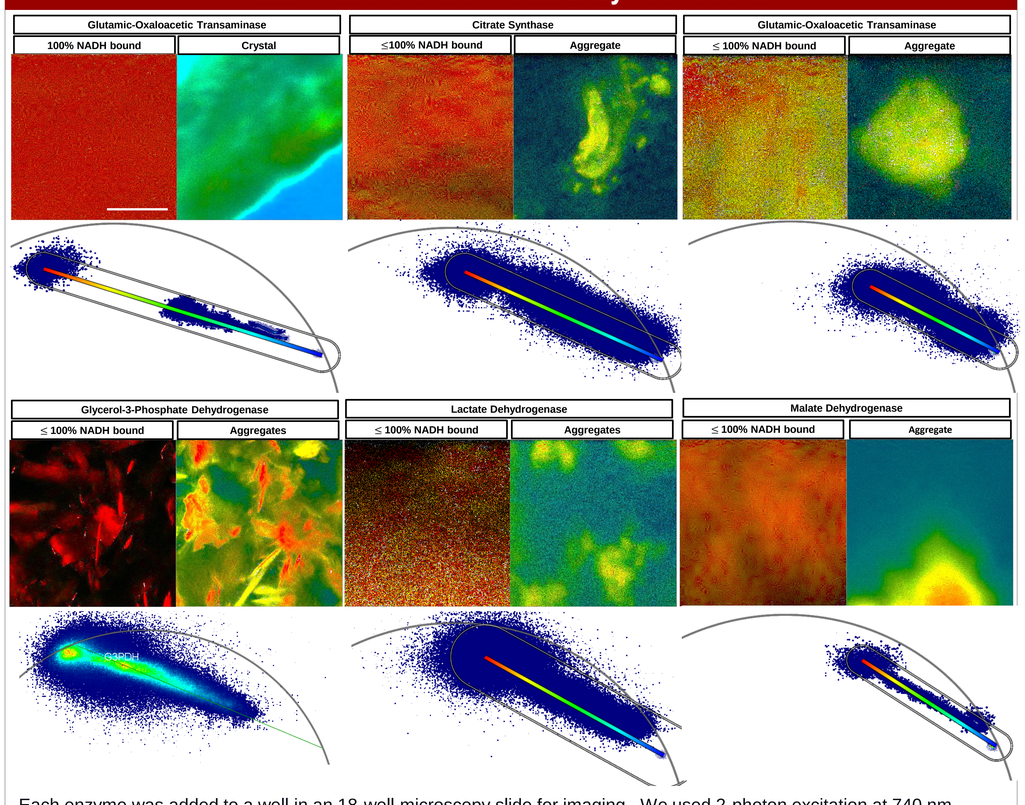
The FLIM phasor has two major landmarks for metabolic imaging. The arrow pointing to Free NADH is in a constant location at 0.4ns on the unit circle. The edge of the unit circle is the putative position of NADH when 100% of it is bound to enzyme. The isolines represent all positions where %s bound lies.

Phasor Pixel Mapping, Glycerol-3-Phosphate Dehydrogenase



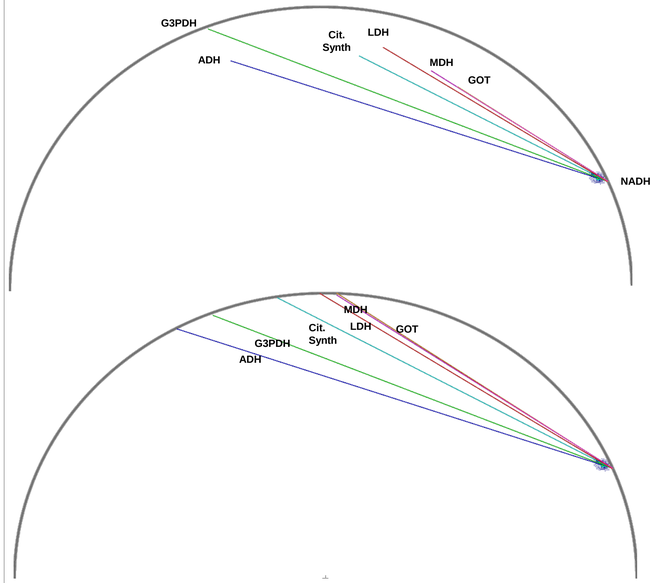
Each pixel in the FLIM image corresponds to a pixel in the phasor plot, and the phasor position in the circle gives insight into the metabolic state of living tissues and cells when FLIM-phasor analysis is applied to live-imaging experiments.

FLIM Phasors of Individual Enzymes in buffer



Each enzyme was added to a well in an 18-well microscopy slide for imaging. We used 2-photon excitation at 740 nm wavelength and detected photons from 400-475 nm, both corresponding to the excitation and emission, respectively, of NADH. The color scale bars in all but the G3PDH phasor (which is shown in the top panel) displays the lifetimes along the axis and the extent of the linear distributions from 0% bound (blue end) to 100% boundf (red end). We used a 63x, 1.2NA water immersion lens and a 6x zoom. The scale bar is 50 um and applied to all images

Results



Future Directions/Conclusion

If we followed a similar protocol as performed in another lab (Leben et. al 2019) by extending the linear distributions of enzymes until we reached the universal circle, and we display the results in the lower phasor diagram. However, we found that, even at 100% NADH bound to enzyme, the end of the linear distribution does not end at the edge of the universal circle, but rather, inside the unit circle. This means that only a minority of metabolic enzymes have a single exponential decay when NADH is bound to them. More importantly, we show that the previous work by ourselves and others maybe falsely reading too much into their metabolic FLIM data. Up until now, where we thought NADH was only 50-70% bound to enzyme, we maybe looking at 100% bound NADH to particular up- or down-regulated enzymes.

The next steps of this research project: 1) image more enzyme lifetimes, 2) image multiple enzymes together, and 3) image enzymes in vivo with fluorescent-tagged enzymes. This will enable us to better understand phasor analysis of metabolic FLIM.

Relevant Articles

Wang P, Hecht F, Ossato G, Tille S, Fraser SE, **Junge JA****. Complex wavelet filter improves FLIM phasors for photon starved imaging experiments**.** Biomed Opt Express. 2021. 12(6):3463-3473. doi: 10.1364/BOE.420953.

Leben, R.; Köhler, M.; Radbruch, H.; Hauser, A.E.; Niesner, R.A. Systematic Enzyme Mapping of Cellular Metabolism by Phasor-Analyzed Label-Free NAD(P)H Fluorescence Lifetime Imaging. *Int. J. Mol. Sci.* **2019**, *20*, 5565. https://doi.org/10.3390/ijms20225565

Stringari C, Edwards RA, Pate KT, Waterman ML, Donovan PJ, Gratton E. Metabolic trajectory of cellular differentiation in small intestine by Phasor Fluorescence Lifetime Microscopy of NADH. Sci Rep. 2012;2:568. doi: 10.1038/srep00568. Epub 2012 Aug 10. PMID: 22891156; PMCID: PMC3416911.

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