Fluorescence lifetime microscopy of NADH distinguishes alterations in cerebral metabolism in vivo

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Abstract: Evaluating cerebral energy metabolism at microscopic resolution is important for comprehensively understanding healthy brain function and its pathological alterations. Here, we resolve specific alterations in cerebral metabolism in vivo in Sprague Dawley rats utilizing minimally-invasive 2-photon fluorescence lifetime imaging (2P-FLIM) measurements of reduced nicotinamide adenine dinucleotide (NADH) fluorescence. Time-resolved fluorescence lifetime measurements enable distinction of different components contributing to NADH autofluorescence. Ostensibly, these components indicate different enzyme-bound formulations of NADH. We observed distinct variations in the relative proportions of these components before and after pharmacological-induced impairments to several reactions involved in glycolytic and oxidative metabolism. Classification models were developed with the experimental data and used to predict the metabolic impairments induced during separate experiments involving bicuculline-induced seizures. The models consistently predicted that prolonged focal seizure activity results in impaired activity in the electron transport chain, likely the consequence of inadequate oxygen supply. 2P-FLIM observations of cerebral NADH will help advance our understanding of cerebral energetics at a microscopic scale. Such knowledge will aid in our evaluation of healthy and diseased cerebral physiology and guide diagnostic and therapeutic strategies that target cerebral energetics.

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1. Introduction

Healthy brain function consists of intricate, relentless electrochemical signaling between neurons, astrocytes, and cerebral microvasculature, and these energetically-demanding processes rely critically upon oxidative metabolism and a tightly-regulated supply of metabolites such as oxygen and glucose [1]. For many pathological conditions, including stroke, epileptic seizures, and neurodegenerative diseases, the onset and progression of these debilitating disorders involve disruptions to energy metabolism, including impairments to metabolite supply and mitochondrial function [2–6]. Reliably characterizing alterations in cerebral metabolism is useful for developing potential biomarkers for assessment of disease progression and prospective treatment efficacies. Consequently, the critical need exists to develop robust techniques for measuring cerebral metabolism with high spatial resolution in vivo, where morphological and functional connections remain preserved.

Over the past several years, a wealth of reports have emerged to demonstrate fluorescence lifetime imaging (FLIM) of the reduced form of the intrinsic electron-shuttling molecule, nicotinamide adenine dinucleotide (NADH), which participates in multiple steps of both anaerobic glycolysis and aerobic oxidative metabolism. Fluorescence lifetime imaging (FLIM) provides information regarding the microenvironmental conditions around the fluorophore [7,8]. The technique relies on analyzing the time-resolved fluorescence decay profile of the fluorophore, and its interpretation is insensitive to changes in fluorophore concentration or excitation and emission intensity [9,10]. Coupling FLIM with 2-photon microscopy (2PM) provides the additional benefits of high axial and lateral resolution, increased penetration depth, and the ability to simultaneously measure multiple fluorophores [11–13]. Time-resolved decay profiles of NADH autofluorescence permit distinction of multiple fluorescence lifetime components associated with different ‘NADH species,’ which reportedly reflect differences in NADH enzymatic binding. Investigators have demonstrated variations in FLIM-based observations of NADH in response to pharmacological and physiological disruptions and disease progression in cell cultures, tissue slices, and in the liver in vivo [13–18]. We previously demonstrated in vivo 2PM-based FLIM (2P-FLIM) measurements of cerebral NADH in anesthetized rats, and observed how cerebral NADH fluorescence can be resolved into 4 distinct lifetime components whose amplitudes change
rapidly with anoxia and recovery [19]. These reports suggest that lifetime-based analysis of NADH fluorescence shows great promise for distinguishing variations in metabolism with much higher specificity than more conventional intensity-based measurements of NADH fluorescence. However, a detailed understanding of the underlying biochemical significance of FLIM-based observations of NADH is currently lacking and limits its rigorous interpretation.

The objective of this study was to assess the potential for 2P-FLIM measurements of NADH to distinguish distinct alterations in cerebral metabolic activity and demonstrate its utility as a biomarker for cerebral pathologies. Pharmacological reagents known to manipulate metabolism were locally administered to the cortical surfaces of anesthetized rats to interfere with specific reactions involved in anaerobic glycolysis and aerobic oxidative metabolism. Apart from the established association between the 2 shortest-lifetime components and unbound, ‘free NADH’ [9], our results revealed that direct relationships do not exist between specific intracellular enzymes and resolvable lifetime components of NADH. Nevertheless, we observed that inducing bottlenecks at distinct pathways of glycolysis and oxidative metabolism significantly and uniquely alter the relative proportion and total amounts of 4 resolvable NADH components when compared to baseline metabolic activity. Classification models were developed based on the NADH-FLIM observations to characterize separate experiments involving bicuculline-induced focal seizures. The observations demonstrate that minimally invasive 2P-FLIM measurements of cerebral NADH are useful for detecting changes in metabolic activity and mitochondrial function, and can be potentially used to study brain energetics in a wide range of neuropathologies.

2. Materials and methods

2.1 Animal preparation

2.1.1 Surgical preparation

Animals were prepared under a protocol approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital and conducted in accordance with Massachusetts General Hospital and ARRIVE guidelines, as described previously. Under isoflurane anesthesia (1-2% inhalation), male Sprague Dawley rats (250 – 350g, Charles River) underwent tracheotomy, cannulation of the femoral artery and vein, and craniotomy over the somatosensory cortex. Following surgical preparation, animals remained anesthetized and immobilized by continuous intravenous infusion of alpha-chloralose (25 mg/kg/hr, Sigma Aldrich) and Rocuronium Bromide (2 mg/kg/hr, Mylan), respectively. A ventilator and flow meter were used to adjust breathing parameters, while a heating blanket and rectal temperature probe maintained body temperature at 37°C. Arterial blood pressure and heart rate were monitored continuously, and blood gas levels were measured routinely from femoral artery samples with a blood gas analyzer (Rapidlab 248, Bayer Healthcare).

2.1.2 Delivery of reagents to the brain surface

As illustrated in Fig. 1(a), a specialized perfusion system was developed to locally administer reagents to the cortical surface through the sealed cranial window. In normal imaging experiments involving a cranial window, the exposed brain is covered with clear agarose gel and sealed with acrylic dental cement and a glass cover slip. For this study, metabolic inhibitors needed to be delivered directly to the cortex to avoid physiological complications or toxicity associated with more systemic administration routes. Therefore, a perfusion system was developed, similar to that reported in Cao, et al, in which PE50 tubing was positioned on either side of the exposed brain and secured with acrylic [20]. The acrylic functioned as a reservoir with ~1 mm height surrounding the exposed brain, and the tubing served as inlet (one tube) and outlet channels (two tubes) for fluid flow driven by a gravity-feed within the reservoir. The volume within the reservoir was filled with artificial
cerebrospinal fluid (ACSF). A syringe was used to guide ACSF through the tubing to eliminate the presence of air bubbles and confirm that fluid flowed smoothly through the perfusion channels. The reservoir was then sealed using a glass coverslip and more dental acrylic. This configuration allowed for reagents to be topically delivered to the brain surface by slow perfusion over the craniotomy. Sealing the area with a coverslip isolated the reagents to the brain surface, eliminating the risk of contaminating our microscope objective or surrounding environment with potentially hazardous metabolic reagents. To minimize physical perturbation of the brain tissue, extreme care was taken to avoid the presence of bubbles or large variations in pressure or temperature within the chamber. Flow rates were limited to below 0.5 ml/min by adjustment of the gravity feed heights and adjusting resistance on the inlet tube. Each animal was subjected to a single type of metabolic manipulation, with approximately 1-2 ml of inhibitor solution administered a single time for each manipulation. At least 3 animals were used to test each reagent. Physiological signals (blood pressure, heart rate, expired pCO2, body temperature) were monitored continuously before, during, and after the reagent administration and were found not to change appreciably (< 15%). For select experiments involving the induction of focal seizure activity, screw electrodes were inserted into the skull near the craniotomy to monitor EEG activity. The screws were positioned such that the tip surface slightly penetrated the skull, allowing gentle physical contact with the brain surface. EEG signals were amplified with a differential amplifier (DAM-80, World Precision Instruments) and recorded at a rate of 1000 Hz [21].

Fig. 1. (a) Perfusion system for local delivery of metabolic inhibitors to cortical surface under a sealed cranial window, with (for select experiments involving induced focal seizure activity) screw electrodes for EEG recordings (b) table of tested pharmacological reagents and their corresponding influence on metabolic activity or pathology.

For each experiment, topical perfusion was initially tested by applying Sulfurhodamine 101 (SR101, Sigma Aldrich) dye to the cortical surface for fluorescent labeling of cortical astrocytes [22]. Approximately 1 ml of SR101 was delivered to the surface. After 6 minutes, the excess SR101 was washed away by flushing the system with ACSF. Approximately 1 – 2 ml of solution was applied to the cortical surface a single time (with concentrations given in Fig. 1(b)). Delivery of reagents to the cortical surface was ensured by administering volumes in great excess of the perfusion system's total volume (~0.75 ml) and closely monitoring the volume of discarded fluid from the perfusion system.

2.1.3 Metabolic inhibitors

Figure 1(b) lists the different reagents tested and their reported effects on metabolism. Sodium pyruvate, Iodoacetic acid, 2-Deoxy-D-glucose, Sodium fluoroacetate, Sodium arsenite, Rotenone, Potassium cyanide, Carbonyl cyanide-

| Reagent                  | Inhibited Process                      |
|--------------------------|----------------------------------------|
| Control (ACSF)           | None                                   |
| 2-Deoxy-D-glucose 15mM   | Glycolysis                             |
| Iodoacetic Acid (IAA) 1.5mM | Glycolysis                             |
| Sodium fluoroacetate 1.25 mM | TCA/ Kreb's Cycle                     |
| Sodium arsenite 1.25 mM  | TCA/ Kreb's Cycle                      |
| Rotenone 10 µM           | Electron Transport Chain               |
| Potassium cyanide 5 mM   | Electron Transport Chain               |
| FCCP 10 µM               | Oxidative Phosphorylation              |
| Brucine 1 mM             | Focal Seizure Model                    |
(trifluoromethoxy)phenylhydrazone (FCCP), and Bicuculline methiodide were all purchased from Sigma-Aldrich (St. Louis, MO). All reagents were dissolved in ACSF immediately before the experiment and warmed to 37°C before administration through the perfusion system. The concentrations of pharmacological agents are provided in Fig. 1(b) and were chosen to match, or slightly exceed, concentrations utilized in published reports to disrupt metabolism in cultured cells and isolated mitochondria [4,23–28].

2.2 Optical imaging

2.2.1 Custom multimodal microscope

2P FLIM measurements of cerebral NADH were performed using our multimodal imaging system custom designed for in vivo imaging, described previously [19]. Briefly, excitation was provided by scanning a pulsed, tunable Titanium-Sapphire laser (Mai Tai, Spectra Physics, 80 MHz repetition rate, ~360 fs pulse duration at the sample) tuned to 740 nm across the field of view with galvanometer-based scanner mirrors (Cambridge Technology, Inc). Laser intensity was adjusted using an electro-optic modulator (ConOptics). Incident power varied with each measurement, however it remained well below 50 mW at the sample for all measurements. A water-immersion high numerical aperture objective (Olympus XLumPlan Fluor, 20X, 1.00 NA, 2 mm working distance) was used to deliver and collect excitation and emission light, respectively. Emission light was separated using a series of dichroic mirrors, IR blockers, and bandpass filters and detected with a custom-designed detector array with high collection efficiency, as detailed in [29]. A hybrid PMT with high detection efficiency and minimal afterpulsing (HPM-100-40 Becker & Hickl, GmbH) was used to collect NADH emission, and SR101 fluorescence was detected using a photo-multiplier tube (PMT). SR101 fluorescence of astrocytic cell bodies provided morphological validation that the same fields of view were imaged before and after metabolic manipulations. Compared to NADH autofluorescence, the emission peak for SR101 is red-shifted by over 100 nm. We verified previously that SR101 fluorescence is not seen by our NADH detection channel [19].

2.2.2 Imaging protocol

Data acquisition was performed using commercial time-correlated single photon counting hardware (SPC-150, GVD-120, DCC-100, Becker & Hickl GmbH) and control software (SPCM, Becker & Hickl GmbH). Excitation intensity was adjusted to yield photon count rates of approximately 500,000 counts per second for these measurements, as indicated by the count rates continuously displayed in the SPCM software. Although incident power varied with cortical depth, it remained well below 50 mW for all measurements. FLIM measurements consisted of repetitive 256 x 256 pixel raster scans performed over a ~200 x 200 µm field of view at frame intervals of up to ~900 ms for 120 s. The measurements yielded time resolved fluorescence profiles with 256 temporal channels (Δt =50 ps binning intervals), yielding 256x256x256 data cubes.

For each animal experiment, images were initially collected in at least 3 locations under baseline metabolic conditions. A solution (~1-2 ml) containing either one of the metabolic inhibitors or a control solution of ACSF was then applied over the cortical surface to modulate metabolic function. After waiting ~4 minutes for application, followed by ~5 minutes to diffuse into the cortex, measurements were recorded at the same locations where baseline measurements were recorded.

2.2.3 Data analysis

After masking out blood vessels and 3x3 pixel binning, lifetime fits were calculated at each pixel within the field of view. As described previously [19], data were processed using custom designed software developed in Matlab employing metrics derived from FLIM data as detailed in Vishwasrao et al and Yu et al [13,14]. To characterize the system’s
instrument response function (IRF) and to aid with data processing, FLIM measurements were recorded of dissolved NADH (0.5 mM in saline) at the beginning or conclusion of each experiment. An iterative procedure was used to compute the IRF and to fit the fluorescence decay of NADH solution as the sum of two decaying exponentials [12].

Using a nonlinear least squares algorithm, the IRF and in vivo lifetime measurements of NADH were fitted using the following expressions:

\[ I_{\text{theoretical}}(t) = I_{\text{offset}} + \sum_{i=1}^{N} \alpha_i \exp \left( -\frac{t}{\tau_i} \right) \]  

\[ I_{\text{measured}}(t) = IRF(t) \otimes \left( I_{\rho}(t) + I_{\text{theoretical}}(t) \right) \]  

where \( I_{\text{offset}} \) represents a weak, constant baseline intensity component that results from detector noise or ambient light. \( I_{\rho}(t) \) denotes an ultrafast component in the detector response, believed to arise from faintly-detected backscattered excitation light. We model the theoretical NADH fluorescence decay observed in the living brain as the sum of 4 fluorescence lifetime components (\( N = 4 \)), where \( \alpha_i \) and \( \tau_i \) represent the amplitude and lifetime of each component \( i \). As we contend that the first two components correspond to unbound NADH in different folding conformations [12,19], the values of \( \tau_1 \) and \( \tau_2 \) are set to those computed for the fluorescence decay of NADH dissolved in saline, and a nonlinear least squares fitting algorithm is used to calculate the remaining values. We consider 5000 total photons (after 3 x 3 binning) as the approximate minimum number of photons to perform these “quasi-global,” multi-exponential lifetime fits.

The contribution from \( I_{\text{offset}} \) is generally negligible compared to those of the 4 NADH components. Consequently, by integrating \( I_{\text{theoretical}}(t) \) over time, the overall fluorescence signal can be expressed as

\[ F = \text{Fluorescence} = \sum_{i=1}^{4} \alpha_i \tau_i \]  

where the contribution of each NADH component to the overall fluorescence signal is its amplitude-weighted lifetime \( \alpha_i \tau_i \). Computed results therefore focus on variations of amplitude-weighted lifetime, the average lifetime \( \langle \tau \rangle \):

\[ \langle \tau \rangle = \frac{\sum_{i=1}^{4} \alpha_i \tau_i}{\sum_{i=1}^{4} \alpha_i} \]  

as well as the fractional fluorescence, reflecting the normalized contribution of each component to the overall fluorescence:

\[ f_i = \text{Fractional Fluorescence}_i = \frac{\alpha_i \tau_i}{\sum_{i=1}^{4} \alpha_i \tau_i} \]  

NADH lifetimes, amplitudes, weighted lifetimes, and fractional fluorescence values were computed in the astrocytic and neuronal cell bodies and neuropil. The computed values were then averaged over all pixels. For each applied inhibitor, the two-sided Wilcoxon rank sum test was used to determine whether mean values measured after inhibition differed significantly from baseline conditions.

2.2.4 Classification models

To evaluate the utility of NADH lifetime measurement for precisely characterizing metabolic disruptions, the observed changes of NADH lifetime metrics were utilized to develop classification models for characterizing metabolic perturbations. Using the Statistics Toolbox in Matlab, experimental data were grouped into 4 different classes: inhibitors of either...
glycolysis, the TCA cycle, the ETC, or uncoupling of oxidative phosphorylation (OXPHOS). For each measurement, pixel-averaged values for $<\tau>$, $\alpha_i\tau_i$, fractional fluorescence, and changes in fluorescence intensity were used as training data for classification models based on Linear Discriminant Analysis (LDA), K-nearest neighbors (KNN), or naïve Baye's (NB) classification algorithms [30]. The quality of the models was evaluated by determining the resubstitution accuracy, defined as the fraction of correctly-classified data sets from the training data. The models were applied to classify the type of metabolic inhibition induced in the focal seizure experiments.

3. Results

Fluorescence decay profiles of intrinsic NADH were measured with high spatial resolution in vivo in anesthetized rats before and after application of several well-characterized modulators of metabolism (Fig. 2(a)). Fluorescence signal from astrocytes labelled with Sulphorhodamine 101 (SR101) dye was detected in a second channel, providing morphological information and ensuring that identical fields of view were imaged before and after metabolic manipulation (Figs. 2(b)-2(e)). While previous investigations have resolved organelles such as mitochondria and nuclei in cultured cell monolayers using higher magnification optics [14,31], distinguishing mitochondria in intricate 3-D tissue environment would be a considerably greater challenge, particularly in densely-packed cells with complex morphologies such as neurons and astrocytes. Consequently, this study focused on characterizing larger fields of view (~200 x 200 µm) without resolving subcellular compartments, in accordance with the long-term goal of characterizing the complex interactions between multiple cells and metabolite-supplying microvasculature within intact brains.

In principle, a conflict exists between NADH autofluorescence and spectrally-identical nicotinamide adenine dinucleotide phosphate (NADPH). Endogenous fluorescence could arise from a mix of both NADH and NADPH. In metabolically-active brain tissue, however, NADPH is widely believed to contribute minimally to the fluorescence signal, due to its much lower concentration in brain tissue [32,33], low quantum yield [34], and insensitivity to metabolic perturbation [13,35,36].

A variety of reagents were locally administered to the cortical surface to impede glycolytic and oxidative ATP synthesis at different reaction steps, as illustrated in Fig. 2(a). Using the SR101 image for morphological guidance, extreme care was taken to ensure that identical fields of view were recorded before and after metabolic inhibition. Inducing these bottlenecks at distinct stages yielded variations from baseline in the metrics computed from endogenous NADH FLIM measurements. Applying artificial cerebrospinal fluid (ACSF) solution alone served as a control experiment and induced no appreciable changes. Baseline physiological signals across all animals appeared normal (arterial pO$_2$: 113.5 ± 18.9 mmHg, arterial pCO$_2$ 36.9 ± 3.9 mmHg, blood pH 7.38 ± 0.02, arterial blood pressure: 100.8 ± 15.5 mmHg, heart rate 290 ± 76.2 bpm, core body temperature: 38.0 ± 3.8 °C), and deviated by less than 15% during and after metabolic manipulation.
The "constant-fraction discriminator" (CFD) rate parameter in the SPCM acquisition software (Becker & Hickl, GmbH) operated as a real-time observable to verify that topical administration modulated metabolism at the measured cortical depths. During FLIM acquisition, the CFD rate parameter updated continuously at a rate of ~1 Hz and reflected the number of NADH fluorescence photons detected during each raster scan. Variations in the CFD rate parameter indicated changes in NADH intensity and served as a real-time, quantitative indicator of metabolic alterations before and after reagent administration. With the exception of the control group, each inhibitor induced a distinct change on the CFD metric.
relative to baseline value starting approximately 2-3 minutes after administration. Although
the CFD rate parameter was not recorded during the acquisition, the CFD rate trends were
monitored regularly during the experiment. Trends in the CFD rate parameter were similar to
the NADH intensity changes displayed in Fig. 3(c).

Using nonlinear least squares fitting techniques described in section 2.2.3, lifetime values
of cerebral NADH in vivo under baseline conditions were calculated to be 0.406 ns
(component c1; attributed to free NADH), 1.102 ns (c2; attributed to free NADH), 2.228 ±
0.010 ns (c3), and 4.487 ± 0.020 ns (c4). For all metabolic manipulations tested herein,
lifetime values were found not to deviate by more than 10% of their baseline values. The
amplitudes of each component varied considerably, and the extents of these amplitude
variations depended upon the specific metabolic perturbation.

Figure 3(a) displays representative fluorescence decay profiles of NADH in the cerebral
cortex after inhibiting glycolysis, the TCA cycle, the electron transport chain, and oxidative
phosphorylation. Each profile depicts the global fluorescence decay after normalizing to their
respective peak values. Compared to the baseline temporal profile of NADH fluorescence,
each method of metabolic inhibition induced distinct variations to the slope and/or peak
value.

peak value to 49% of its corresponding baseline while slightly increasing the slope. As
seen in Fig. 3(b), each form of metabolic inhibition resulted in resolvable decreases in the
computed average lifetime \(<\tau>\) from the baseline value of 1.193 ± 0.005 ns. With the
exception of the control experiment, all changes in \(<\tau>\) were significantly different from the
baseline case, as determined by the Wilcoxon rank sum test. Inhibiting glycolysis or
uncoupling oxidative phosphorylation yielded modest reductions to ~1.15 ns, while disrupting
the TCA cycle and ETC resulted in substantially more reduced \(<\tau>\) to ~1.09 ns and ~1.04 ns,
respectively. Compared to in vivo measurements of NADH in the cerebral cortex, the
fluorescence observed from a cuvette of NADH dissolved in saline demonstrates a much
sharper, precipitous decay. While each inhibitor significantly lowered the average lifetime
compared to the observed baseline in vivo, the average lifetime value of NADH in solution
was still drastically lower at approximately 0.47 ns.

Inhibiting different steps of glycolytic and oxidative ATP metabolism induces varying
effects on NADH intensity as well as the relative amounts of the 4 observable species
resolved with NADH FLIM, as shown in Fig. 3(c) and Fig. 4. Intensity differences in Fig.
3(c) were computed by integrating the time-resolved fluorescence profiles at each pixel
corresponding to neuropil and neuronal and astrocytic cell bodies. The glycolysis inhibitors,
2-Deoxy-d-glucose (2DG; hexokinase blocker) [24] and glyceraldehyde 3-phosphate
dehydrogenase inhibitor (IAA) [25], were administered along with 1.5 mM pyruvate to
sustain downstream oxidative metabolism. Administration of the 2DG (15mM) and IAA
(1.5mM) had varying effects on the NADH intensity. Glycolysis inhibition did not
significantly alter NADH intensity from baseline levels. As Fig. (4) illustrates, IAA induced
significant reductions in amplitudes of fluorescence lifetime components 2 (c2) and 4 (c4)
and reductions of amplitude-weighted lifetimes for c2, c3, and c4. Both glycolysis inhibitors
increased the fractional fluorescence of c1 by a significant, yet relatively modest, amount (13-
15%).
Fig. 3. (a) Representative time-resolved profiles of endogenous NADH fluorescence in the rat cortex after pharmacological manipulation, summed over the entire field of view and normalized (b) and computed average lifetime of NADH fluorescence in solution and in vivo under varied metabolic conditions. (c) Pharmaceutically-induced changes to NADH intensity from baseline levels, computed by integrating the time-resolved fluorescence profiles in pixels corresponding to astrocytic cell bodies and neuropil * indicates significantly different from baseline in vivo measurement. Error bars indicate standard error across all pixels over all measurements.
Impeding the TCA/Kreb’s cycle, where the majority of NADH is created by reducing NAD⁺, induced changes distinct from those incurred by blocking other steps of metabolism. TCA cycle inhibition was performed by administering either the aconitase-blocker Sodium fluoroacetate [37] or Sodium meta-arsenite, which reportedly inhibits both pyruvate and α-ketoglutarate dehydrogenases [38,39]. Figure 3(c) shows that fluoroacetate and arsenite reagents induced significant reductions in the NADH intensity by 15% and 30%, respectively, relative to baseline. Figure 4 shows that both fluoroacetate and arsenite also caused significant decreases in the amplitudes and weighted-lifetimes of c2, c3, and c4. The influence of each TCA inhibitor on the fractional fluorescence was found to be different, with fluoroacetate yielding increases in the fractional fluorescence of c1 and c2 and decreases in c3.
and c4, while arsenite caused a significant increase in the fractional fluorescence of c1 and decreased c2.

Inhibition of the ETC, where NADH becomes oxidized back to NAD\(^+\), yielded the most pronounced changes to observable metrics of NADH FLIM. ETC activity was inhibited by applying either rotenone, a blocker of NADH dehydrogenase (aka Complex I) [28], or potassium cyanide (KCN), a blocker of cytochrome c oxidase (aka Complex IV) [40], where molecular O\(_2\) acts as the final recipient of electrons to form water. Rotenone and KCN yielded pronounced increases of 58\% and 67\% in NADH intensity, respectively, as well as the amplitudes and weighted-lifetimes of all 4 components. While both reagents significantly increased the fractional fluorescence of c1 and decreased c2, KCN also caused the fractional fluorescence of c4 to decrease.

FCCP was used to uncouple ATP phosphorylation from the ETC, resulting in unregulated, increased oxidation of NADH back to NAD\(^+\). Uncoupling phosphorylation caused NADH intensity to decrease by 30\%, and the amplitudes and weighted lifetimes of all 4 components also decreased significantly relative to baseline conditions. Uncoupling aerobic respiration induced similar effects on NADH intensity and weighted lifetimes as impairment of the TCA cycle. However, unlike impaired TCA activity, uncoupling did not significantly alter the fractional fluorescence of any component.

As an example of a pathological condition that modulates metabolism and could be implemented with our experimental protocol, we induced focal seizure activity by topical administration of gamma-Aminobutyric acid-A receptor (GABA\(_A\)) antagonist Bicuculline methiodide (BMI). Seizure activity was verified by simultaneously recording electroencephalography (EEG) activity using screw electrodes embedded in the rat skull [21]. For each trial, BMI (~1 ml, 1 mM) was perfused over the brain surface, and the solution stayed there for the remainder of the experiment. Focal seizure activity ensued within minutes after perfusion began and continued for the duration of the recordings, lasting at least 1 hour. Seizure activity provoked a substantial decrease in \(\tau\) to \(\sim 1.05\) ns and a large increase in NADH intensity relative to baseline values. Significant increases were also observed in amplitudes and weighted lifetimes of all 4 components. An increase in fractional fluorescence of c1 was observed, while decreases in c2 and c3 also occurred. These metabolic changes appear similar, yet not identical, to those encountered with disruption of the electron transport chain.

Classification models were developed to test the ability of computed NADH FLIM metrics to precisely characterize disruptions at distinct stages of energy metabolism. Furthermore, the models were used to identify the metabolic behavior that accompanies focal seizure activity. Experimental data were categorized as disruptions of either glycolysis (2DG, IAA), the TCA cycle (fluoroacetate and arsenite), the ETC (KCN and rotenone), or uncoupling of oxidative phosphorylation (FCCP). Models based on Linear Discriminant Analysis (LDA), K-nearest neighbors (KNN), or naïve Baye's (NB) classification were developed using the Statistics Toolbox in Matlab using combinations of mean lifetime (\(\langle \tau \rangle\)), amplitude-weighted lifetimes (\(a_\tau_i\), i: 1-4), fractional fluorescence, and changes in fluorescence intensity (\(\Delta I\)) as input data [30]. Results from the BMI-induced seizure experiments were used to test the performance of each classification model. Different combinations of parameters were tested as model predictors. The combination of \(\Delta I\), \(\langle \tau \rangle\), and amplitude weighted lifetimes showed suitable separation of different classes, as seen in Fig. 5, and yielded resubstitution accuracies of 70\%, 85\%, and 70\% for LDA, KNN, and NB models, respectively. With these parameters, the models classified 92\%, 75\%, and 92\% of the induced seizure trials as disruption of the ETC.
4. Discussion

NADH FLIM has been applied to characterize metabolism in biological specimens with varying levels of complexity, including isolated mitochondria [41,42], cell cultures [14,43], tissue slices [13,16], and in vivo measurements [15,18]. It shows considerable promise for precisely evaluating stages of disease progression and treatment efficacy based on aberrant metabolic activity, particularly when coupled together with observations of flavin adenine dinucleotide (FAD) autofluorescence [15,44] or oxygen partial pressure (pO2). From our experience investigating cerebral metabolism in vivo, FAD fluorescence is weak and provides an inadequate signal to noise ratio, likely the result of low FAD concentration in cerebral cortex [10]. Additionally, a spectral conflict exists between NADH and the coumarin molecules of presently-available O2-sensitive contrast agent for 2P microscopy [45,46], which currently prevents simultaneous 2PM measurements of pO2 and NADH in tissue. Deciphering as much metabolic information from intrinsic NADH is therefore a highly-coveted goal for characterizing cerebral metabolism in vivo.

Investigators have emphasized for many years that lifetime-based measurements of NADH autofluorescence offer multiple advantages over more common intensity-based measurements, including resolution of bound and unbound molecular conformations and insensitivity to both NADH concentration [9] and changes in tissue optical properties [47]. Coupling FLIM with 2P microscopy makes the technique especially useful for high-resolution characterization of highly vascularized, scattering tissues such as the brain in vivo; however, attempts to rigorously interpret and validate the significance of FLIM-based NADH observations have not been reported until recently [48]. The common practice of multi-exponential fitting of FLIM data has enabled resolution of multiple enzyme-bound “species” of NADH. Relating these species with biochemical and metabolic processes is challenging, but essential to guide accurate interpretation of NADH FLIM measurements.
This study provides experimental results confirming the much-repeated hypothesis that NADH FLIM can identify specific alterations in cerebral metabolism in vivo, building upon our previous characterizations of NADH FLIM in the cerebral cortices of anesthetized rats [19]. Although the observed changes to NADH fluorescence intensity induced by our pharmacological manipulations are intuitively satisfying and generally in agreement with previous metabolic characterizations in isolated mitochondria and cell cultures [49,50], the intensity-based measurements alone are insufficient for identifying specific impairments to glycolysis, the TCA cycle, the ETC, or oxidative phosphorylation in the living brain. Intensity-based measurements of NADH lack the specificity to resolve impairments of glycolytic activity, or to resolve between inhibited TCA cycle activity and uncoupling of oxidative phosphorylation.

Our experimental fluorescence decay profiles in the rodent cerebral cortex are best modeled by multi-exponential decays consisting of 4 exponential terms (i.e. species, or components c1 – c4) and a small offset, consistent with other NADH FLIM reports investigating isolated liver mitochondria and brain slices [13,41]. Our results demonstrate that inhibiting glycolytic and oxidative metabolism at different reaction steps significantly and distinctly alters the relative amounts of each of these 4 components, reflecting shifts in the relative amounts of different enzyme-bound components of NADH in cerebral tissue.

Previous investigations have reported a range between 2 and 4 resolvable components contributing to NADH fluorescence [51]. The total number and relative amounts of components could vary with cell and tissue type. Given the vast number of intracellular enzymes to which NADH can bind, the resolvable components are now regarded as a weighted average of several enzymatic species [14,48,52]. In all observed biological specimens, it is generally accepted that the shortest lifetime component represents unbound, ‘free NADH,’ with a lifetime around 0.3-0.4 ns. In our analyses, we attribute the first 2 components of in vivo NADH (c1 and c2 in our data) with the two components detected in NADH solution, with lifetimes around 0.4 ns and 1.0 ns that reportedly represent different folding conformations of free NADH [13,52]. In agreement with previous reports, our data illustrate that free NADH constitutes a considerable portion of NADH autofluorescence as species c1, and its relative contribution increases as oxidative metabolism becomes inhibited [13,15,18]. The increase in c1 is particularly pronounced if inhibition occurs within the ETC, as seen in our results and reported by several others. The results of this study demonstrate that fractional fluorescence of c1 increases not only with inhibited ETC activity, but also with inhibition of glycolysis and the TCA/Kreb’s s cycle, indicating that free NADH does not directly correlate with either anaerobic or aerobic metabolism. While previous reports emphasize the utility for NADH FLIM to characterize differences between normal tissue and tissue with reduced ETC activity such as cancer cells demonstrating the Warburg effect [53], this study shows that NADH FLIM permits additional more specific resolution of metabolic alterations. By characterizing changes of the relative amounts of all 4 detected components and its average lifetime, our data demonstrate that specific metabolic bottlenecks in anaerobic and aerobic metabolism uniquely influence the metabolic signature of the 4 observable NADH components in brain tissue. Table 1 summarizes the notable, distinctive changes associated with impairments to glycolytic activity (with sustained downstream oxidative metabolism), the TCA cycle, the ETC, and uncoupling of ETC and ATP phosphorylation.
Table 1. Alterations in NADH FLIM metrics associated with impairments to different metabolic processes

| Impaired Process                  | Altered FLIM Metrics                                                                 |
|----------------------------------|---------------------------------------------------------------------------------------|
| Glycolysis                       | Intensity: No (significant) change Mean Lifetime $<\tau>$: ↓ 3\% Weighted lifetime, $\alpha\tau$<sub>i</sub>: $-$c<sub>1</sub>, ↓ c<sub>2</sub>, ↓ c<sub>3</sub>, ↓ c<sub>4</sub> * |
| TCA/Kreb’s Cycle                 | Intensity: ↓ 15 – 30\% Mean Lifetime $<\tau>$: ↓ 8\% Weighted lifetime, $\alpha\tau$<sub>i</sub>: $-$c<sub>1</sub>, ↓ c<sub>2</sub>, ↓ c<sub>3</sub>, ↓ c<sub>4</sub> |
| Electron Transport Chain         | Intensity: ↑ 58 - 65\% Mean Lifetime $<\tau>$: ↓ 12\% Weighted lifetime, $\alpha$↑ c<sub>1</sub>, ↑ c<sub>2</sub>, ↑ c<sub>3</sub>, ↑ c<sub>4</sub> |
| Coupling between ETC and ADP phosphorylation | Intensity: ↓ 28\% Mean Lifetime $<\tau>$: ↓ 3\% Weighted lifetime, $\alpha$↓ c<sub>1</sub>, ↓ c<sub>2</sub>, ↓ c<sub>3</sub>, ↓ c<sub>4</sub> |

Focal seizure activity involves enhanced neural activity and provokes drastic increases in both metabolic flux and cerebral blood flow (CBF). Reports have demonstrated that the localized application of GABA<sub>A</sub> antagonist BMI yields pronounced disinhibition, yielding increased excitatory signaling with consequent changes in metabolism and blood flow that vary with time and distance from the focal seizure location [54–56]. Studies continue to elucidate the specific details and mechanisms of focal seizure-induced neurovascular alterations in the center and surrounding areas [56,57]. Investigators have reported conflicting observations concerning the metabolic response to seizure activity in vivo. Some have observed that the increased CBF accompanying seizure activity provides sufficient oxygen to sustain the increased metabolic activity, yielding increased ETC activity and oxidation of NADH [58], while others have reported relative energy failures induced by inadequate oxygen supply [59–61]. Numerous discrepancies exist in the experimental methodologies, including animal models, methods of seizure induction, and duration and magnitudes of seizures. Our in vivo observations of NADH lifetime metrics in BMI-induced focal seizure studies support the hypothesis that, during prolonged seizure activity lasting several minutes, metabolic demand of oxygen exceeds the increased supply provided by the increased cerebral blood flow, ultimately resulting in impairments in ETC activity [62,63]. In addition to causing prolonged interictal spike activity, topical application of BMI yielded pronounced changes to NADH lifetime metrics, including a significant increase and decrease in the relative amounts of c1 and c2, respectively, and a notable decrease in the average lifetime. The changes are consistent with those associated with compromised ETC activity, specifically indicating that inadequate oxygen supply yields altered metabolic flux through bottlenecks at the ETC.

Classification models based on Linear Discriminant Analysis, K-nearest neighbors, and naïve Baye's classification were developed based on our experimental observations and predicted impaired ETC activity induced by focal seizure activity in 92\%, 75\%, and 92\% of the experimental data sets. Though the models appropriately demonstrate the utility of NADH FLIM for resolving different impaired metabolic processes, the classification methods can likely be improved through the use of more advanced algorithms, larger data sets, and by utilizing a subset of classifier variables to minimize confounds associated with high-dimensional data sets including poor sampling of the variable space. Future investigations will explore the utility of phasor analysis of FLIM data to potentially avoid confounds with lifetime-fitting and classification with high-dimensional data [17,64,65].

Noninvasive optical measurement of intrinsic NADH fluorescence intensity has been widely utilized for evaluating metabolism since groundbreaking mitochondrial characterization studies were performed over 50 years ago [50,66–70]. For in vivo investigations, however, the intensity-based measurement technique is confounded by optical
scattering and hemoglobin absorption, and its utility has been largely limited to identifying critical deficits in oxygen supply. To better understand the complex relationship between metabolic state and cellular and neurovascular signaling in the brain [71], characterizing cerebral metabolism with greater specificity is essential. While investigators have recently made remarkable advancements for characterizing metabolic parameters such as NADH-NAD + redox state using novel genetically-encoded sensors [72–74], the utility of these sensors for quantitative, 2PM-based in vivo studies remains limited at the present time [75].

Our data rigorously demonstrate that 2P-FLIM measurements of intrinsic NADH is a promising tool for reliably identifying and characterizing specific impairments in cerebral energy metabolism in vivo. Although it is currently constrained to the superficial cortical layers, the technique offers potential for exploring the etiologies and guiding treatments for a wide range of cerebral pathologies in pre-clinical models, including epilepsy, ischemia and reperfusion injury, neurodegenerative disorders, and mitochondrial disease.

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