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Two-photon autofluorescence dynamics imaging reveals sensitivity of intracellular NADH concentration and conformation to cell physiology at the single-cell level

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Abstract

Reduced nicotinamide adenine dinucleotide, NADH, is a major electron donor in the oxidative phosphorylation and glycolytic pathways in cells. As a result, there has been recent resurgence in employing intrinsic NADH fluorescence as a natural probe for a range of cellular processes that include apoptosis, cancer pathology, and enzyme kinetics. Here, we report on two-photon fluorescence lifetime and polarization imaging of intrinsic NADH in breast cancer (Hs578T) and normal (Hs578Bst) cells for quantitative analysis of the concentration and conformation (i.e., free-to-enzyme-bound ratios) of this coenzyme. Two-photon fluorescence lifetime imaging of intracellular NADH indicates sensitivity to both cell pathology and inhibition of the respiratory chain activities using potassium cyanide (KCN). Using a newly developed noninvasive assay, we estimate the average NADH concentration in cancer cells ($168 \pm 49 \mu\text{M}$) to be ~ 1.8 fold higher than in breast normal cells ($99 \pm 37 \mu\text{M}$). Such analyses indicate changes in energy metabolism and redox reactions in normal breast cells upon inhibition of the respiratory chain activity using KCN. In addition, time-resolved associated anisotropy of cellular autofluorescence indicates population fractions of free (0.18 ± 0.08) and enzyme-bound (0.82 ± 0.08) conformations of intracellular NADH in normal breast cells. These fractions are statistically different from those in breast cancer cells (free: 0.25 ± 0.08 ; bound: 0.75 ± 0.08). Comparative studies on the binding kinetics of NADH with mitochondrial malate dehydrogenase and lactate dehydrogenase in solution mimic our findings in living cells. These quantitative studies demonstrate the potential of intracellular NADH dynamics (rather than intensity) imaging for probing mitochondrial anomalies associated with neurodegenerative diseases, cancer, diabetes, and aging. Our approach is also applicable to other metabolic and signaling pathways in living cells, without the need for cell destruction as in conventional biochemical assays.

Keywords

NADH; Hs578T; Hs578Bst; two-photon FLIM; associated anisotropy; dehydrogenase

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1. Introduction

Reduced nicotinamide adenine dinucleotide, NADH, is an essential cofactor for oxidation-reduction (redox) reactions and energy metabolism in living cells [1–3]. In the cytoplasm of eukaryotic cells, glycolysis involves ten reactions that include the reduction of NAD⁺ during the oxidation of glyceraldehyde 3-phosphate, which is catalyzed by glyceraldehyde 3-phosphate dehydrogenase. The net transformation reaction of one glucose molecule into two molecules of pyruvate includes the generation of two NADH and two ATP [3]. Under aerobic conditions, the high-energy electrons from the cytosolic NADH are shuttled to the mitochondria using glycerol-3-phosphat and malate-aspartate shuttles [3,4]. In the electron transport chain (ETC) of the inner membrane of mitochondria, NADH (fluorescent) is oxidized to NAD⁺ (not fluorescent), which eventually leads to the majority of adenosine triphosphate (ATP) production via the oxidative phosphorylation pathway [1–3]. Under anaerobic conditions, however, NAD⁺ is regenerated by the reduction of pyruvate to lactate, which is catalyzed by lactate dehydrogenase (LDH) [5]. Anaerobic glycolysis is often predominant in tumors and causes elevated levels of lactic acid as well as increased LDH activity [6]. It has also been shown that cancer cells exhibit impaired mitochondrial metabolism, which skews the activities of key enzymes such as LDH and mitochondrial malate dehydrogenase (mMDH) [5,7,8]. The concentration and distribution of intrinsic NADH in living cells are sensitive to cell physiology [9,10] and pathology [11]. As a result, there is a great potential for cellular NADH as a natural biomarker for a range of cellular processes such as apoptosis [12], redox reactions [12,13], and mitochondrial anomalies associated with cancer [1,5,14,15] and neurodegenerative diseases [16]. Conventional biochemical methods have provided the bulk of information concerning NADH concentration in cell lysates as a snapshot of the metabolic and redox state of cells, but without morphological context [11,12,17,18].

Contrary to biochemical techniques, however, fluorescence-based approaches provide a non-invasive alternative for autofluorescence imaging in live cells or tissues. The pioneering work of Britton Chance has opened the door for using cellular autofluorescence as a biomarker for respiratory activities and mitochondrial functionality using 360 nm excitation [19–21]. Further studies have shown a blue shift in NADH emission as a result of binding with proteins in several cell lines, including keratinocytes [22], bronchial tissues [23], and brain slices [9].

Unfortunately, such a shift is difficult to use as an indicator for quantitative analysis of free and bound NADH due to the broad spectral profile of NADH [9]. It is worth mentioning that reduced nicotinamide adenine dinucleotide phosphate (NADPH) is used almost exclusively for reductive biosynthesis of fatty acids and steroids, whereas NADH is used primarily for ATP generation [3,12]. Unfortunately, it is difficult to differentiate between NADH and NADPH spectroscopically due to their similar photophysical properties. However, some evidence exists that intracellular NADH levels are higher than that of NADPH [17,24]. There are some inconsistencies, however, concerning the sensitivity of these pyridine nucleotides autofluorescence to cell pathology as a function of experimental techniques and cell lines [25,26]. While the use of one-photon excitation is a common practice for fluorescence microscopy [27], it suffers from extended photobleaching, scattering, and limited penetration depth in turbid biological samples [28]. In addition, ultraviolet (UV) excitation of NADH and tryptophan residues in proteins may cause DNA mutations [29] and cellular photodamage [30]. Multi-photon microscopy overcomes some of these challenges [31–37], with the two-photon (2P) excitation cross-section of NADH reported recently [38–40]. Currently, 2P-fluorescence microscopy of intrinsic NADH has been used to monitor energy metabolism in macrophages, pancreatic islet cells, skeletal muscle cells [41–43], brain slices [9,40], and cardiomyocytes [38].

Fluorescence lifetime imaging microscopy (FLIM) is sensitive to the conformational changes and surroundings of a fluorophore [44]. For example, frequency-domain FLIM has been shown

to be sensitive to free and enzyme-bound NADH conformations in solution [45]. Recently, 2P-FLIM studies of human breast cell (MCF10A) [10] and hair cells in isolated cochlear preparations [46] were reported for monitoring the metabolic activities using cellular autofluorescence. However, it is generally difficult to assign multiple exponential fluorescence decays to specific molecular origins [47]. To overcome such challenges, Vishwasrao et al. used a combination of time-resolved fluorescence and anisotropy measurements of intrinsic NADH in brain slices to monitor cellular response to hypoxia [9]. Using sample scanning mode, free and three enzyme-bound species of intrinsic NADH were identified in brain tissues to explain the observed multiexponential fluorescence decays and associated anisotropies [9]. Tissues, however, are more complex environment that may include other fluorescent species such as collagen and elastin [36,48]. In addition, imaging throughout thick tissues may suffer from scattering and other optical artifacts due to refractive index mismatch [49], which may influence polarization anisotropy imaging.

In this report, a noninvasive two-photon fluorescence dynamics assay is used to convert intracellular NADH fluorescence to actual concentration as well as molecular conformation (i.e., free and enzyme-bound molar fractions) in living cells. In this assay, 2P-FLIM is used to quantify the fluorescence quantum yield variation within individual cells. In addition, 2P-fluorescence anisotropy imaging is used to obtain the population fractions of free and enzyme-bound NADH using cellular autofluorescence. Human normal (Hs578Bst) and cancerous (Hs578T) breast cells are used as a model system with the epithelial Hs578T cell line is derived from a rare infiltrating ductal carcinoma of a 74-year-old female patient. Unlike most breast cancer cell lines, which express estrogen receptors, Hs578T represents the scarce transformed breast cell lines that are estrogen receptor negative. The corresponding myoepithelial normal cells were obtained from the same patient at a location peripheral to the tumor [34]. In addition to cell pathology, the functional response of cellular NADH to potassium cyanide (KCN) is also examined in the normal Hs578Bst cells. As a control, the binding kinetics of NADH with both mMDH and LDH, in solution, is also investigated using the same assay. These comparative studies in solution, under controlled NADH-enzyme mixing, enabled us to (i) understand the observed differences between the intracellular NADH fluorescence lifetime, as compared with the free cofactor in solution, (ii) mimic observed associated anisotropy of intracellular NADH, and (iii) directly quantify the population fraction of intracellular free and enzyme-bound NADH using the inherent differences in their molecular sizes.

2. Materials and methods

2.1 Cell culture and treatment

Breast cancer cell line (Hs578T), its non-transformed counterpart (Hs578Bst), and the recommended culture media were obtained from American Type Culture Collection (ATCC). Cancer cells were grown in Dulbecco's modified eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (PS). Normal cells were grown in modified DMEM, supplemented with 10% (v/v) FBS, 1% (v/v) PS and 30 ng/mL epidermal growth factor (Sigma). Cells were cultured in T-75 flasks (BD Biosciences) in a 37°C incubator with 5% CO₂ and allowed to reach 70–90% confluence before passage. Cells were plated into glass bottom Petri dishes (MatTek Corporation) and incubated overnight before imaging. Tyrodes buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 20 mM HEPES and 5 mM glucose) was used to replace the media and to wash the cells three times prior to imaging of intracellular NADH autofluorescence. Cell morphology was monitored before and after each measurement using differential interference contrast (DIC) microscopy to ensure cell integrity. Both normal and cancer cells underwent a maximum of ten passages. To monitor the drug-induced fluorescence change of NADH in normal cells, the electron transport chain inhibitor, potassium cyanide (KCN, Fluka), was dissolved in phosphate buffered solution, PBS (pH 7.4),

at a final concentration of 5 mM in the Petri dish where the cells were incubated and imaged. 2P-FLIM images were recorded subsequently after ~ 8 min following KCN application.

Rhodamine 123 (Rh123; Invitrogen) was used as a mitochondrial marker [50] to examine cell morphology and mitochondrial distribution of breast cancer and normal cells. For our solution studies, free NADH was purchased from Sigma without further purification. NADH was dissolved in Tris buffer (100 mM, pH 8.5), and stored as a stock solution of 500 μ M. Both mMDH and L-LDH, purchased as crystalline suspensions in ammonium sulfate solution from Sigma, were dialyzed three times against Tris buffer (100 mM, pH 8.5) at 4 °C, and then concentrated by centrifugation. Concentrations of LDH and mMDH were determined by measuring absorbance in a UV-Vis spectrophotometer (DU800, Beckman Coulter). The extinction coefficients (at 280 nm) are $7.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ (mMDH) and $16.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ (LDH), respectively [51]. NADH concentrations of 226 μ M (for mMDH) and 183 μ M (for LDH) were titrated with variable enzyme concentration to ensure detectable NADH fluorescence signal.

2.2 Confocal and two-photon fluorescence micro-spectroscopy

The experimental setup and data analysis were described in detail elsewhere [52,53]. Briefly, time-resolved fluorescence lifetime (at magic angle of 54.7°) and anisotropy experiments were performed using a femtosecond (~120 fs at 76 or 4.22 MHz, 740 nm) laser system (Coherent) consisting of a solid-state laser pumped with a 10-W diode laser. Laser pulses were steered toward a modified laser scanning confocal system (Olympus) before being focused on the sample using a dichroic mirror (670DLPC) and a microscope objective (60 $\times$, 1.2NA, water immersion). The epi-fluorescence was detected simultaneously using two microchannel plate photomultiplier tubes (R3809U-50; Hamamatsu) following a beam splitter, Glan-Thompson polarizers, and filters (690SP and BGG22: 350–550 nm filter, Chroma). The detected signals were amplified and then fed into a single photon counting module (SPC830; Becker & Hickl). Data acquisition and non-linear least-square fitting analysis were carried out using SPCImage (Becker & Hickl).

2.3 Quantitative calculation of intracellular NADH concentration

The protocol for monitoring extrinsic fluorophore concentration *in vivo* with 2P-FLIM and intensity images was reported earlier [53]. In brief, the time-averaged 2P-fluorescence from a given pixel (x, y), $\langle F_{2P}(x, y) \rangle$, is defined as [37,39]:

$$\langle F_{2P}(x, y) \rangle = \frac{8n\lambda^3 \Phi(x, y) \xi(\lambda) C(x, y) \sigma_{2P} g_P \langle I(t) \rangle^2}{2R_P \tau_P (\pi NA)^3}, \quad (1)$$

where Φ and C are the fluorescence quantum yield and concentration of the fluorophore in a given pixel, respectively. The observed signal is also dependent on detection efficiency ($\xi(\lambda)$) and the 2P-excitation cross section (σ_{2P}) of a fluorophore, as well as the excitation pulses (full width half maximum, τ_P , and repetition rate R_P , and the time-averaged intensity $\langle I(t) \rangle$). The dimensionless g_P -factor depends on the temporal profile of the laser pulse (e.g., $g_P = 0.66$ for a Gaussian pulse shape) [37]. The spatial profile of two-photon pulsed excitation can be written as $8n(\lambda/\pi NA)^3$ under thick-sample approximation [39]. The detection efficiency and other system parameters can be difficult to quantify. Thus, we used free NADH at a known concentration as a reference for quantitatively imaging the spatial distribution of NADH concentration in living cells. Because the fluorescence quantum yield is linearly proportional to the fluorescence lifetime (τ_f) in a given pixel ($\Phi(x, y) = k_r \tau_f(x, y)$, where k_r is the radiative rate constant), the dependence of the 2P-fluorescence signal on concentration can be simplified as:

$$\langle F_{2P}(x, y) \rangle = \tau_{fl}(x, y) \psi(x, y) C(x, y), \quad (2)$$

where the system parameter, $\psi(x, y) = 4n\lambda^3 k_r \zeta(\lambda) \sigma_{2P} g_p I^2 / \pi^3 NA^3 R_P \tau_P$, can be cancelled out using the free NADH in solution with a known concentration as a reference, such that [54]:

$$C_{NADH}^{cell}(x, y) = \frac{F_{2P}^{cell}(x, y)}{F_{2P}^{sol}(x, y)} \frac{\tau_{NADH}^{sol}}{\tau_{NADH}^{cell}(x, y)} C_{NADH}^{sol}, \quad (3)$$

For simplicity, we assume that the sensitivity of the radiative rate constant (k_r) to the cellular environment is negligible, even though a slight blue spectral shift of NADH has been observed upon enzyme-binding [9]. The accuracy of this approach can be further improved by considering the refractive index variation throughout live cells [55,56], as well as accurate knowledge of the two-photon excitation cross-section of both free and enzyme-bound NADH [38,40].

2.4 Data analysis

Fluorescence lifetime analysis is performed using the SPCImage software package (Becker & Hickl), where the deconvoluted fluorescence intensity decay per pixel, $F_{2P}(x, y; t)$, is generally fit as [52,53]:

$$F_{2P}(x, y, t) = \sum_i a_i(x, y) \cdot \exp[-t/\tau_i(x, y)], \quad (4)$$

where a_i and τ_i are the amplitude and lifetime of the i^{th} fluorescence component, respectively,

and $\sum_i a_i$ is normalized to unity. For a multi-exponential fluorescence decay, the average

fluorescence lifetime is give by $\bar{\tau}_{fl} = \sum_i a_i \tau_i$, while the relative contribution of the i^{th} decay component to the total fluorescence signal can be calculated as follows [47,54]:

$$F_i(x, y, t) = a_i(x, y) \tau_i(x, y) / \sum_i a_i(x, y) \tau_i(x, y) \quad (5)$$

All 2P-FLIM images reported here were detected at magic-angle (54.7°) polarization to eliminate rotational effects [47]. Typical 2P-FLIM images were recorded using 740 nm, ~ 120 fs pulses, 76 MHz (with an average power ≤ 3 mW), 256×256 pixels, 64 time channels per pixel, and 259 ps/channel. For presentation purpose, the resolution of the color-coded lifetime bar is chosen to highlight the autofluorescence lifetime (i.e., quantum yield) heterogeneity throughout the cell. The corresponding pixel-lifetime histogram for each FLIM image is also provided to reflect the overall distribution of average lifetime per pixel throughout the cell. DIC images were recorded before and after the FLIM measurements to assess the cellular viability and photodamage, which is negligible [33,36,37] but should not be ruled completely [46]. In pseudo-single point lifetime measurements, the laser pulses (4.22 MHz, average power ≤ 300 μ W) were scanned over the entire cell(s) and the autofluorescence signal was recorded in 1024 channels, with ~ 12.2 ps/channel, and thus higher signal-to-noise ratio as well temporal resolution.

An image processing algorithm was also developed to calculate steady-state 2P-fluorescence anisotropy images [52,53] for mapping the dipole-moment orientation of fluorescent molecules under local restrictions of the cellular environment. 2P-fluorescence anisotropy, $r(x, y; t)$, is obtained using two fluorescence intensity images recorded simultaneously at parallel, $I_{//}(x, y, t)$, and perpendicular, $I_{\perp}(x, y, t)$, polarizations with respect to the electric vector of the excitation laser, and is defined as [47,54,57]:

$$r(x, y; t) = \frac{I_{//}(x, y; t) - GI_{\perp}(x, y; t)}{I_{//}(x, y; t) + 2GI_{\perp}(x, y; t)} \quad (6)$$

The G -factor, which indicates the sensitivities of the detection channels to polarization, is estimated using the tail-matching approach [57] with either free NADH or coumarin in solution. In these anisotropy images, the steady-state anisotropy per pixel, which yields the orientation angle (δ) between the absorbing and emitting dipoles [47,57], is given by:

$$r(x, y) = 2\gamma \frac{3\cos^2\delta - 1}{2(2\gamma + 3)}, \quad (7)$$

where γ is the number of excitation photons ($\gamma = 1$ for 1P and $\gamma = 2$ for 2P) [57]. Time-resolved fluorescence anisotropy, $r(t)$, of an ensemble of non-interactive fluorophores with different excited-state lifetimes and hydrodynamic volumes, can be described as an associated anisotropy [9,47,57,58]:

$$r(t) = \frac{\sum_i^m a_i e^{-t/\tau_i} \cdot \beta_i e^{-t/\phi_i}}{\sum_i^m a_i e^{-t/\tau_i}}, \quad (8)$$

where the pre-exponential factor (β_i) represents the initial anisotropy of the i^{th} species with its size-dependent rotational time constant (ϕ_i). As mentioned above (Eq. 5), the contribution of the i^{th} species to the total fluorescence signal is dependent on both the amplitude fraction (a_i) and fluorescence lifetime component (τ_i). The time-resolved fluorescence in both pseudo-single point (no pixel-to-pixel resolution) and FLIM are measured independently using magic-angle polarization (54.7°) detection. The associated anisotropy curve of a fluorophore, in two different environments, can alternatively be written as a function of its ground-state molar fraction [59]. In this case, one would expect that small, fast rotating species (e.g., free NADH) are dominant at early times (≤ 300 ps) of the associated anisotropy decay. In contrast, the large species (e.g., enzyme-bound NADH) would rotate much slower and therefore dominate at later times during rotational diffusion. The rotational time of a spherically-shaped molecule is related to the hydrodynamic volume by the Stokes-Einstein equation [47,54,57]:

$$\phi(x, y) = \frac{\eta(x, y)V(x, y)}{k_B T}, \quad (9)$$

where η , V , k_B and T are the viscosity of the local environment, the hydrodynamic volume, the Boltzmann constant and the absolute temperature, respectively.

3. Results

3.1 Confocal microscopy reveals distinct morphology of breast cancer cells

Confocal and DIC imaging of Rh123-stained mitochondria in both normal and cancer breast cells (Fig. 1) were used for qualitative assessment of the mitochondrial distribution and cell morphology in both cell lines. Sub-confluent normal Hs578Bst cells exhibit an apparently smaller nuclear-to-cytoplasmic size ratio with a spindle-like cell shape (Fig. 1A and 1B), as compared with the polygonal shaped Hs578T cancer cells (Fig. 1C and 1D). Cancer cells also exhibit a peri-nuclear mitochondrial distribution as compared with normal cells that have a fibrous-shaped, inter-connected, and extended mitochondrial distribution.

3.2 Cellular autofluorescence exhibits a heterogeneous fluorescence lifetime (i.e., quantum yield) as revealed by 2P-FLIM

Fluorescence lifetime is a sensitive probe to changes in the molecular conformation and surrounding environment. 2P-FLIM also allows for the conversion of a fluorescence intensity image to a molecular concentration image by quantifying the variation of fluorescence lifetime (i.e., quantum yield) within living cells in a calibrated microscope. Care must be taken in FLIM analysis, which depends on the signal-to-noise per pixel, pixel binning, and fitting parameters such as offset and scattering contribution. To overcome these challenges, we used the same fitting procedure for comparative FLIM analysis when possible. In addition, pseudo-single point measurements serve as a point of reference.

Typical laser scanning 2P intensity and FLIM images of normal breast cells are shown in Fig. 2A and Fig. 2B, along with their pixel-lifetime histogram (Fig. 2E). The corresponding 2P-FLIM image histogram (Fig. 2E, open circles) reveals a heterogeneous average lifetime distribution of intracellular NADH and can be fit using double Gaussian with lifetime bands at 1.0 ns (Gaussian distribution width, $\omega_1 = 0.26$ ns, amplitude = 4778, 53 %) and 0.67 ns ($\omega_2 = 0.27$ ns, amplitude = 4285, 47%). Using this approach, we estimate the average lifetime per image of normal cells to be 0.9 ± 0.2 ns ($n = 5$). Using pixel-to-pixel (3 images, 20 pixels per image) analysis in 2P-FLIM images, the 2P-autofluorescence of intracellular NADH autofluorescence decays biexponentially (apparent mitochondrial NADH: $\tau_1 = 0.6 \pm 0.1$ ns, $\alpha_1 = 0.71 \pm 0.04$, $\tau_2 = 3.4 \pm 0.5$ ns, $\alpha_2 = 0.29 \pm 0.04$, $n = 33$ pixels, and $\bar{\tau}_{fl} = 1.3 \pm 0.2$) in Hs578Bst cells (Fig. 3A and 3B). Using high resolution 2P-FLIM imaging, the cytosolic autofluorescence ($\tau_1 = 0.6 \pm 0.1$ ns, $\alpha_1 = 0.75 \pm 0.05$, $\tau_2 = 3.3 \pm 0.6$ ns, $\alpha_2 = 0.25 \pm 0.05$, and $\bar{\tau}_{fl} = 1.2 \pm 0.1$) also decays biexponentially. In both the cytoplasm and apparent mitochondria, the relative contribution of the species with a longer lifetime, as calculated using Equation 5, contribute ~ 69% of the detected autofluorescence signal, as compared with ~ 31% contribution from the other species with a short lifetime (Fig. 3B). Using pixel-to-pixel intensity analyses (4 images, 20 pixels per image), the apparent mitochondrial NADH autofluorescence in normal cells is dominant (86 ± 10 %), with a minor contribution (14 ± 6 %) from the cytosol (Fig. 3C).

Comparative 2P-FLIM measurements on cancer cells were also conducted (Fig. 2C and 2D) to examine the sensitivity of intrinsic NADH dynamics to cell pathology, using the same experimental and analytical approaches. Double Gaussian fit of the corresponding average lifetime histogram (Fig. 2E, solid circles) of the 2P-FLIM image yields mean lifetimes of 0.93 ns (amplitude = 3724, $\omega_1 = 0.23$ ns) and 0.55 ns (amplitude = 3902, $\omega_2 = 0.32$ ns). As a result the estimate average lifetime per image is 0.7 ± 0.2 ns ($n = 5$). In addition, pixel-to-pixel fluorescence decay analysis of 2P-FLIM images Supplementary Fig. S1) reveals that 2P-fluorescence decays of intrinsic NADH per pixel decays bi-exponentially (apparent mitochondrial NADH: $\tau_1 = 0.52 \pm 0.05$ ns, $\alpha_1 = 0.72 \pm 0.05$, $\tau_2 = 2.4 \pm 0.3$ ns, $\alpha_2 = 0.28 \pm 0.05$, $n = 23$ pixels, and $\bar{\tau}_{fl} = 1.0 \pm 0.1$). The observed trend of longer NADH lifetime distribution in breast normal ($n = 8$) versus cancer ($n = 12$) cells is consistent among these representative data

and is statistically significant (Student's *t*-test, $p < 0.05$). In addition, the species with a shorter lifetime contributes $38 \pm 2\%$ of the total autofluorescence signal as compared with $62 \pm 7\%$ of the long-lifetime species.

To overcome the low temporal resolution and signal-to-noise ratio of 2P-FLIM, pseudo-single point lifetime measurements were also performed. In this modality, the laser pulses (740 nm, 4.2 MHz, average power of $\leq 300 \mu\text{W}$) were scanned over the whole cell (i.e., without the pixel-to-pixel resolution) and the autofluorescence signal was continuously collected and stored in 1024 channels (instead of 64 channels in FLIM images). The autofluorescence of normal cells ($n = 6$) decays as triexponential, with an average lifetime of 0.83 ± 0.05 ns (Table 1, Supplementary Fig. S2). The observed triexponential decay pattern of intrinsic NADH agrees with recent studies on 3T3-L1 adipocytes and 3T3 fibroblast cells [44] using ultraviolet excitation. The autofluorescence of cancer cells also decays as a triexponential ($n = 6$), with an estimated average lifetime of 0.75 ± 0.05 ns (Table 1, Supplementary Fig. S2). Student's *t*-tests show that the average autofluorescence lifetime in normal and cancer cells, using pseudo-single point measurements is slightly different ($p \sim 0.05$). Under the same experimental conditions, the single point 2P-fluorescence of free NADH (pH 7.4) decays as a biexponential, with $\tau_1 = 0.36$ ns ($a_1 = 0.82$), $\tau_2 = 0.75$ ns ($a_2 = 0.18$), and $\tau_{\text{fit}} = 0.43$ ns, which is consistent with literature values [9] as well as 2P-FLIM measurements ($\tau_1 = 0.34$ ns, $a_1 = 0.79$, $\tau_2 = 0.89$ ns, $a_2 = 0.21$, and an average lifetime of ~ 0.46 ns). The multiexponential fluorescence decays of free NADH are likely due to different molecular conformations (e.g., extended versus folded) [9,60], which makes the assignment of cellular NADH as free and enzyme-bound more difficult using only fluorescence lifetime measurements [9]. In addition, the heterogeneity of local pH, microviscosity, and refractive index [55,56] in cell environment may contribute to the observed changes in intracellular NADH autofluorescence lifetime.

3.3 Electron transport chain inhibitor (KCN) induces a functional intracellular NADH response

We further conducted 2P-FLIM measurements on normal breast cells ($n = 4$) under resting conditions and pharmacological manipulation of the ETC using the complex IV inhibitor KCN (Fig. 4). Figure 4 shows representative 2P intensity (Fig. 4A) and FLIM (Fig. 4B) images before and after KCN treatment (8 min, Fig. 4B). These FLIM images were analyzed using the same fitting constraints (e.g., pixel binning 5, threshold 15 counts, weighted pixel intensity, and improved matrix calculations). In addition to the heterogeneity of intracellular NADH lifetime (Fig. 4A and 4B), we have also carried out pixel-to-pixel analyses of the time-averaged autofluorescence intensity (3 images, 15 pixels per image) in both apparent mitochondria and the cytoplasm (Fig. 4E). The relative changes of cytosolic and mitochondrial autofluorescence intensity are $(43 \pm 8)\%$ and $(39 \pm 12)\%$, respectively, upon the respiratory chain inhibition using KCN (Fig. 4F). Such functional response of breast normal cells (HTB125) to ETC inhibition also indicate that the 2P-autofluorescence can be assigned to intrinsic NADH, under our excitation/detection conditions [9,10,13,38,61].

3.4 Intracellular NADH concentration imaging in intact, live cells using 2P-autofluorescence dynamics assay

Intracellular NADH concentration is an important biochemical criterion for many physiological and pathological events in cellular metabolism that are indispensable to life. 2P-FLIM of cellular autofluorescence indicates the variation in fluorescence lifetime (i.e., quantum yield) in the heterogeneous cell environment. As a result, it is essential to account for the differences in fluorescence quantum yield prior to converting fluorescence intensity images to concentration images in living cells. Using two-photon intensity and lifetime imaging in a calibrated microscope, concentration images of intrinsic NADH in normal (Fig. 5A) and cancer breast cells were obtained. As shown in this representative image of a photoselected cross-

section of a normal breast cell, the relative concentration of intracellular NADH varies significantly from pixel to pixel throughout the cell. Free NADH (PBS, pH 7.4) of known concentration was used, under the same experimental conditions, as a reference for calibrating our microscope. The averaged NADH concentration in breast cancer cells is $168 \pm 49 \mu\text{M}$ ($n = 7$), compared to $99 \pm 37 \mu\text{M}$ ($n = 7$) in normal counterpart (Fig. 5B). The concentration between normal and cancer cells are statistically different (Fig. 5B; Student's *t*-test, $p < 0.05$). Recently, Kasischke et al. [40] reported an enhancement (by a factor of ten) of the two-photon excitation cross-section of mMDH-bound NADH. Accordingly, these concentration estimates, at the single-cell level, should be weighted by the molar fraction of enzyme-bound NADH in living cells (see below).

3.5 Autofluorescence anisotropy provides direct evidence for multiple molecular conformations of intracellular NADH at the single-cell level

Since NADH is a cofactor for enzymes that catalyze redox reactions in cells, it is important to quantify the population fractions of free and enzyme-bound conformations under different physiological conditions. There have been recent attempts to use FLIM alone as a contrasting factor between NADH conformations [10,13]. However, direct correlation between different fluorescence decay components and structural conformations is not straightforward [9], especially with multiexponential decays of both free and enzyme-bound NADH in solution (see below). Here, we use complementary steady-state autofluorescence anisotropy imaging to assess the restrictive nature of the cellular microenvironment to intrinsic NADH. Importantly, we also employ pseudo-single point, time-resolved 2P-autofluorescence anisotropy on living cells for direct assessment of free and enzyme-bound NADH populations at the single-cell level for the first time, to the best of our knowledge.

From the steady-state perspective, typical 2P-autofluorescence polarization images (i.e., parallel and perpendicular with respect to the laser polarization) are shown in Fig. 6. These parallel (Fig. 6A) and perpendicular (Fig. 6B) polarization images of cellular NADH were recorded simultaneously to minimize possible fluctuations in laser intensity and cell movement. The steady-state 2P-anisotropy images (Fig. 6C), calculated using a MATLAB-based algorithm, reveal heterogeneous (Fig. 6C, color code) orientation order and environmental restrictions of intracellular NADH in living cells. The estimated average (from cell to cell) initial anisotropies per cell are 0.32 ± 0.05 ($n = 6$) and 0.30 ± 0.03 ($n = 6$) for normal and cancer cells, respectively. These average values of the steady-state 2P-autofluorescence anisotropy are significantly lower than the theoretical maximum, 0.57 [57], which may be attributed to the rotational flexibility of intrinsic NADH [62], intramolecular energy transfer in cellular microenvironments, and optical depolarization due to the high NA objective [63, 64]. On average, the corresponding angle between the absorption and emission dipoles of NADH are $33 \pm 3^\circ$ (normal, $n = 6$) and $34 \pm 2^\circ$ (cancer, $n = 6$).

From the rotational dynamic perspective, however, time-resolved autofluorescence anisotropy of native NADH reveals an associated anisotropy, which directly indicates the presence of two emitting species with different hydrodynamic volumes and fluorescence properties, at the single-cell level (Fig. 7A). The associated anisotropy curves in breast cancer normal (Fig. 7A) and normal cells ($n = 10$) are described using Eq. 8 and the fitting parameters are summarized in Table 2. At early times of rotational diffusion, the contribution of free NADH is prevalent compared with the diffusion of enzyme-bound molecules that dominates at a later time due to the molecular size differences (Fig. 7A). In these analyses, the slow (>30 ns) rotational time is much longer than the corresponding average autofluorescence lifetime (0.75 – 1.5 ns) of cellular NADH and, therefore, less accurate. The rotational time constants of free and enzyme-bound NADH were almost fixed with a variable amplitude fraction. In addition, the magic-angle fluorescence decay parameters of cellular NADH autofluorescence were used as

measured to minimize the number of floating fitting parameters. The results are compared with control experiments on NADH as a function of the mMDH (Fig. 7B) and LDH (Fig. 7C) and concentrations (see below).

3.6 Enzyme-NADH binding in solution mimics the rotational diffusion of cellular autofluorescence

To elucidate the underlying mechanism of autofluorescence lifetime enhancement in living cells, single point (i.e., no laser scanning) time-resolved fluorescence measurement were carried out on NADH (PBS, pH 7.4, at room temperature) as a function of LDH and mMDH concentrations. In addition, complementary time-resolved fluorescence polarization anisotropy was conducted under controlled molar fractions of NADH and enzymes. These control measurements provide a point of reference concerning the associated anisotropy of cellular NADH and the analyses of free and enzyme-bound populations. In these experiments, we used fixed NADH concentrations (226 μM and 182 μM for mMDH and LDH titrations, respectively) that would yield detectable two-photon fluorescence with good signal-to-noise ratio, while the concentration ratio of the enzymes (mMDH or LDH) was varied accordingly. NADH was titrated with mMDH and LDH at [NADH]:[enzyme] concentration ratios ranging from 16:1 to 1:1 for both time-resolved 2P-fluorescence and anisotropy measurements. Under the excitation and detection conditions used here, only the 2P-fluorescence of NADH (both free and enzyme-bound) was measured (i.e., no fluorescence from free mMDH or LDH was detected)

The 2P-fluorescence of free and enzyme-bound NADH decays as multiexponential (Table 1 and Supplementary Fig. S2) with an average lifetime that increases as the binding sites of mMDH and LDH become occupied (e.g., at [NADH]:[enzyme] $\sim$ 30:1 to 2:1 ratio). The equilibrium constant ($\sim$ 3.8 μM) of NADH with mMDH has been reported [65]. When the two binding sites of mMDH [9,66] are fully occupied, the changes in 2P-fluorescence and lifetime of NADH-mMDH complex are 2 fold larger (Supplementary Fig. S2) compared with the free cofactor in solution [9]. At a [NADH]:[mMDH] ratio of 16:1, which closely mimics the associated anisotropy of cellular autofluorescence (Fig. 7A), the 2P-fluorescence decays as a triexponential with $\tau_1 = 276$ ps ($a_1 = 0.42$), $\tau_2 = 650$ ps ($a_2 = 0.51$), $\tau_3 = 1.57$ ns ($a_3 = 0.06$), and $\bar{\tau}_f \sim 550$ ps. Similar studies were carried on NADH-LDH binding kinetics (Table 1 and Supplementary Fig. S2C), revealing a sigmoidal enhancement of average fluorescence lifetime as a function of LDH concentration. The 2P-fluorescence and lifetime enhancement of NADH increases by a factor of three when fully bound with LDH. The lifetime enhancement due to enzyme-binding of NADH is more pronounced than the viscosity effects, as measured in PBS with $\sim$ 22% glycerol (data not shown).

Time-resolved fluorescence anisotropy of NADH-mMDH, with all binding sites occupied, decays as a single exponential with a rotational time of $\sim$ 30 ns and an estimated hydrodynamic radius of $\sim$ 137 nm³ (Eq. 9). The rotational time constant of mMDH (molecular weight $\sim$ 70 kDa [67]) is much slower than that of free NADH ($\sim$ 665 Da), in agreement with Vishwasrao et al [9]. Below binding-site saturation, however, the mixture of free and enzyme-bound NADH, with both mMDH (Fig. 7B) and LDH (Fig. 7C), exhibits associated anisotropy features that resemble those of cellular autofluorescence (Fig. 7A). For example, the associated anisotropy decays of a mixture of NADH and mMDH ([NADH]:[mMDH] $\sim$ 16:1) is best described by $\tau_1 = 0.43$ ns, $a_1 = 0.43$, $\tau_2 = 0.80$ ns, $a_2 = 0.48$, $\phi_1 = 0.14$ ns, $\beta_1 = 0.28$, $\phi_2 = 29.5$ ns, and $\beta_2 = 0.41$ (Fig. 7B, Table 2). Using extended observation time and more data points on the titration curve, our associated 2P-anisotropy results of NADH, as a function of mMDH concentration, agree with recent studies by Vishwasrao et al. [9]. Similar measurements were carried out on NADH as a function of LDH concentrations ([NADH]:[LDH] = 1:1 to 16:1). At a concentration ratio of [NADH]:[LDH] = 8:1, for example, the associated anisotropy

resembles the cancer cell autofluorescence, with $\tau_1 = 0.37$ ns, $a_1 = 0.62$, $\tau_2 = 1.02$ ns, $a_2 = 0.37$, $\phi_1 = 0.16$ ns, $\beta_1 = 0.26$, $\phi_2 = 37$ ns, and $\beta_2 = 0.41$ (Fig. 7C, Table 2). Importantly, Deng et al. have identified four NADH binding sites in pig heart LDH with a dissociation constant of ~ 6.8 μ M [68], which we used to calculate the corresponding molar fractions of free and LDH-bound NADH (see below).

4. Discussion

Intracellular NADH is a ubiquitous cofactor that participates in many metabolic reactions, especially energy metabolism, in eukaryotic cells [3,18,24]. Changes in intracellular NADH concentration and the ratio of oxidized (NAD^+) to reduced (NADH) cofactor are usually associated with a cell transformation [10,13–15,24,69]. In most cancer cells, there is a reduced amount of NADH undergoing oxidation in the mitochondria due to the mutation of enzyme complexes and subsequent uncoupling of the ETC [14]. As a compensatory mechanism for ATP production, cancer cells exhibit an elevated glycolytic rate, i.e., the “Warburg effect” [5], leading to larger pools of cytosolic NADH compared to normal cells. As a result, preserving morphological context during energy metabolism studies in living cells is particularly important since oxidation-reduction reactions in living cells depend on the spatial distribution of NADH [9].

DIC and confocal microscopy images of Rh123-stained Hs578T and Hs578Bst cells exhibit apparent differences in cell morphology and mitochondrial distribution (Fig. 1). The cancer cell line Hs578T shows an increased nuclear-to-cytoplasmic ratio, which might be attributed to an increased proliferation rate in cancer cells [4,70,71]. The increased nuclear size and nucleocytoplasmic ratio have also been reported in other cancer cell lines such as human gastric carcinoma [70], human liver cancer and the dysplastic liver [72] cell lines. The observed perinuclear mitochondrial distributions in the transformed breast cells (Hs578T) agree well with previous reports on breast cancer (MCF-7) and human lung carcinoma (A549) cells [73]. These perinuclear mitochondrial features have been attributed to ATP demands for detoxification and high motility in carcinoma cells [73].

The 2P-autofluorescence under our experimental conditions (740 nm excitation and 450 ± 50 nm detection) is attributed to intrinsic NADH following previous reports on a number of biological models [9,38,40,46,74]. The experimental evidence supporting such autofluorescence assignment to intracellular NADH includes the cell response to hypoxia [9], sodium cyanide (NaCN) [38,46], and carbonyl cyanide 4-(trifluoromethoxy)phenyl hydrazone (FCCP) treatments [38,74]. In addition, intracellular NADH is mostly co-localized with the mitochondria. While it is possible that intracellular flavin adenine dinucleotide (FAD) could be excited at 740 nm [38], its contribution to the cellular NADH autofluorescence was negligible under our detection conditions [9]. The functional response of normal breast cells to an ETC inhibitor (Fig. 4) supports such an argument [38]. As mentioned above, however, differentiating between intracellular NADH and NADPH is not possible using our microspectroscopy techniques due to their similar photophysical properties. In addition, the intracellular NADH level is likely to be higher than that of NADPH [17,24].

Intracellular NADH autofluorescence lifetime imaging (Fig. 2 and Fig. 3) reveals a heterogeneous environment in both breast normal and cancer cells. The multiexponential decays of cellular autofluorescence lifetime also indicate the presence of different excited states, likely due to the presence of multiple molecular conformations (e.g., free and enzyme-bound NADH) [9,10,13] and a heterogeneous cell environment. The intermediate (0.65 ± 0.08 ns, amplitude $\sim 48\%$) and slow (3.45 ± 0.07 ns, amplitude $\sim 14\%$) decay components of cellular NADH autofluorescence using pseudo-single point measurements agree well with the spatially resolved FLIM studies (0.7 ± 0.2 ns, $\sim 63\%$ and 2.7 ± 0.3 ns, 38%) in normal cells. Using 2P-

FLIM, these two autofluorescence lifetimes in breast cancer (MCF7) cells have been assigned to free and enzyme-bound NADH [9,10,13]. However, different fluorescence lifetimes from multiexponential decays may not necessarily correlate to specific molecular conformations. For example, the fastest decay component (e.g., 97 ± 38 ps in normal cells) in pseudo-single point, magic-angle measurements is absent in FLIM analysis due to the low temporal resolution. In addition, the 2P-fluorescence of NADH (PBS, pH 7.4) decays as a multiexponential as a function of mMDH and LDH concentrations (Table 1, Supplementary Figure S1). The folded conformation of free NADH, mitochondrial swelling, and excited-state processes such as charge transfer may contribute to such a fast lifetime component [9,57]. The 2P-autofluorescence lifetime distribution and pixel-to-pixel analysis in FLIM images indicate a significantly shorter lifetime in cancer cells as compared with normal cells ($p < 0.05$). These FLIM parameters generally agree with other biochemical and spectroscopic studies of NADH in transformed (MCF-7) and non-transformed (MCF-10A) cells [10,61,75]. The cellular environment, however, is rather complex with variable local pH, microviscosity, and refractive indices [55,56], which may influence the autofluorescence lifetime of intracellular NADH. As a result, care must be taken in strictly assigning the observed heterogeneity in intracellular NADH lifetime to molecular conformations (free versus enzyme-bound). It is also not clear how minor photobleaching during intrinsic NADH in living cells or tissues [46] may contribute to the observed fluorescence lifetime changes.

Using 2P-FLIM imaging of intracellular NADH in living cells in a calibrated microscope, we were able to convert fluorescence intensity into concentration images (Fig. 5A) after accounting for the lifetime (i.e., quantum yield) heterogeneity in living cells. It is worth mentioning that, in a given cell, the relative concentration of intracellular NADH can be as high as a few 100s μM in some pixels (Fig. 5A). Despite the large cell-to-cell variation ($n = 7$), the average concentration of cellular NADH in breast cancer ($168 \pm 49 \mu\text{M}$) cells is about 1.8 times higher than in normal cells ($99 \pm 37 \mu\text{M}$) (Fig. 5B; Student's t -test of $p < 0.05$). These observed trends are consistent with previous studies on other normal and malignant cell lines and tissues using biochemical and spectroscopic methods [11,61,69]. For example, Uppal and Gupta have reported approximately 2 fold higher NADH fluorescence in malignant human breast cancer tissues to be higher than in their normal counterpart [11]. Using UV micro-spectrofluorimetry and biochemical cycling assays, Villette et al. have also reported enhanced NADH fluorescence intensity in normal and cancerous esophageal epithelium cells [69].

The observed response of intracellular NADH autofluorescence to KCN treatment (Fig. 4) is consistent with the ETC inhibition, which interrupts the oxidation of NADH to NAD^+ . Since the intracellular level of NADH (fluorescent) and NAD^+ (not fluorescent) are equilibrated under resting conditions, one may assume that the observed increase in NADH concentration (i.e., $\delta[\text{NADH}]$), under physiological manipulation of the same cell, would correspond to a decrease in $[\text{NAD}^+]$ by the same amount. Under respiration chain inhibition using KCN, the relative changes of cytosolic and mitochondrial autofluorescence intensity are $43 \pm 8\%$ and $39 \pm 12\%$, respectively (Fig. 4F). We may also assume that the intracellular NADH concentration in normal breast cells ($\sim 99 \mu\text{M}$) is increased by the same amount ($\sim 140 \mu\text{M}$). Accordingly, the intracellular NAD^+ is likely decreased by $\sim 41 \mu\text{M}$, which indicates about $\sim 41\%$ changes in the redox state of normal breast cells upon ETC inhibition. One may reach a similar conclusion using KCN effects on the pixel-to-pixel lifetime analysis. In this case, we would assign the fast and slow decay components correspond to free and enzyme-bound intracellular NADH, respectively. These rough approximations, however, should be considered as such due to other physiological effects of KCN treatment. This argument is supported by the observed changes on mitochondrial and cytosolic NADH, which suggest that KCN effect on cellular function may extend beyond the respiration chain inhibition.

For a mixture of fluorophores, time-resolved fluorescence anisotropy is sensitive to both the hydrodynamic volume, conformations, and surrounding environment [57]. As a result, these anisotropy measurements are one of the most direct approaches for quantifying the population fractions of free and enzyme-bound NADH [9]. Intracellular NADH autofluorescence, in both breast normal and cancer cells, reveals an associated anisotropy at the single-cell level (Fig. 7A). These results provide direct evidence that the cellular autofluorescence is collectively emitted by a mixed population of NADH with different hydrodynamic volumes (e.g., free and enzyme-bound) and fluorescence properties. The observed associated anisotropy of cellular autofluorescence can be described satisfactorily using only two species of distinct fluorescence lifetime and rotational time constants (Table 2), which we assign as free and enzyme-bound NADH. Conceptually, one would think of these associated anisotropy features as free NADH dominating at early times due to its rapid rotation and the enzyme-bound cofactor appearing at longer rotational times due to its relatively larger size. Based on these associated anisotropy analyses [9,57], the population fraction of intracellular free NADH is $f_{free} = 0.18 \pm 0.08$ ($n = 9$) in normal breast cells, which is significantly larger than the enzyme-bound fraction ($f_{bound} = 0.82 \pm 0.08$). These fractions are statistically different (Student's t -test, $p < 0.05$) from those in breast cancer cells where $f_{free} = 0.25 \pm 0.08$ ($n = 7$) and $f_{bound} = 0.75 \pm 0.07$. These results provide direct quantitative evidence of free and enzyme-bound NADH at the single-cell level, where cancer Hs578T cells reveal a statistically significantly larger fraction of free NADH than normal cells. Such an enhancement in intracellular NADH levels in transformed cells is attributed to compromised enzymatic activities in the ETC of mitochondria and, perhaps, an increase in the glycolytic rate [1,5,7,8,13–15,24]. These single-cell studies also support the findings by Vishwasrao et al. [9] in brain tissues, regardless of the inherent biocomplexity of the latter model. Since intracellular NADH is a necessary coenzyme for a range of reduction-oxidation reactions, a number of NADH-enzyme complexes are likely to exist [9,76] under a given physiological condition. Using our autofluorescence dynamics assay alone, however, we are unable to speculate about the nature of an enzyme-bound species of intrinsic NADH.

In concentration image analyses (Fig. 5), we used NADH (PBS, pH 7.4) of known concentration as a reference to calibrate our microscope under the same experimental conditions. We have also assumed similar two-photon excitation cross-sections of free and cellular NADH, which may not be accurate (Eqs. 2 and 3). Recently, Kasischke et al. [40] reported an enhanced (by a factor of ten) two-photon excitation cross-section of mMDH-bound NADH. Assuming 82% of the intracellular NADH population is enzyme-bound, the weighted average concentration in breast cancer (Hs578T) cells will be equivalent to $\sim 22 \mu\text{M}$. The corresponding intracellular NADH, weighted by 75% enzyme-bound cofactor, is $\sim 12 \mu\text{M}$ in breast normal (Hs578Bst) cells.

Our control measurements on NADH (pH 7.4), as a function of mMDH and LDH concentrations, complement our cellular autofluorescence studies and enable us to examine the structural conformations and 2P-excited-state lifetime enhancement upon enzyme binding. The multiexponential 2P-fluorescence decay of NADH (PBS, pH 7.4), as a function of mMDH and LDH concentration (Supplementary Figure S2A and S2B), suggest the presence of multiple structural conformations, as was proposed previously [68,77]. In addition, the fluorescence intensity (data not shown) and average lifetime of NADH-mMDH is twice (Supplementary Figure S2C) that of the unbound cofactor [78,79]. At fully occupied LDH sites [68,80], the average fluorescence lifetime is increased by 3 fold as compared with free NADH (Supplementary Figure S1C). The observed enhancement of NADH fluorescence in the protein environment is due to the unfolded conformation of adenine and nicotinamide ring [9,60,77]. It is worth mentioning that the slow component (1.57 ns, 6%) is significantly faster than that of cellular autofluorescence decays (~ 3.3 ns, $\sim 15\%$). Further, the average lifetime of cellular autofluorescence (0.75 – 0.83 ns), using pseudo-single point measurements, is slower than the

average lifetime (0.55 ns) of NADH in solution ($[\text{NADH}]:[\text{mMDH}] = 16:1$). Collectively, these comparative studies suggest a complex environment and conformation of intracellular NADH as compared with solution studies presented here.

In controlled NADH titrations with mMDH and LDH, time-resolved fluorescence anisotropy on a mixture of free and fully bound NADH-mMDH (i.e., below saturation) reveals an associated anisotropy behavior that mimics cellular autofluorescence. Two fluorescence anisotropy decay components are also resolved in solution studies. Based on the pre-exponential parameters, the free and mMDH-bound NADH fractions are $f_{\text{free}} \sim 0.4$ and $f_{\text{bound}} \sim 0.6$, respectively (at $[\text{NADH}]:[\text{mMDH}] = 16:1$). Using the equilibrium constant for NADH dissociation ($K_e \sim 3.8 \mu\text{M}$ [81]), this molar fraction of $[\text{NADH}]:[\text{mMDH}]$ in solution corresponds to a free-to-bound fraction of $\sim 13:1$, which could be considered as an upper limit for our cellular studies. The corresponding free and LDH-bound NADH populations are $f_{\text{free}} \sim 0.7$ and $f_{\text{bound}} \sim 0.3$, respectively, at $[\text{NADH}]:[\text{LDH}] = 8:1$. The accuracy of these numbers is limited by the number of fitting parameters, as well as the fast excited-state lifetime compared with the rotational diffusion of the slow, enzyme-bound species. However, the observed associated anisotropy of cellular autofluorescence and solution studies of NADH-enzyme mixing provides support to our conclusions.

In summary, our autofluorescence dynamics imaging assay indicates that the level of intracellular NADH and its conformation are sensitive to cell physiology to pathology. Using breast normal (Hs578Bst) and cancer (Hs578T) cells as a model system, our results indicate that the cellular autofluorescence lifetime is larger than that of free NADH in solution, which is attributed mainly to enzyme binding. The observed heterogeneity of the two-photon autofluorescence lifetime (i.e., quantum yield) throughout living cells was used in intensity-to-concentration image conversion. The estimated intracellular NADH level in the breast cancer cell model is almost twice that in the non-transformed counterpart. For the first time, the two-photon cellular autofluorescence exhibits associated anisotropy features, at the single-cell level, that directly indicate the presence of two NADH species of differing conformations (i.e., free and enzyme-bound). These findings and their interpretation in living cells are examined using free NADH (PBS, pH 7.4) under controlled mixing with mitochondrial malate dehydrogenase (mMDH) and lactate dehydrogenase (LDH) in solution. These studies demonstrate the sensitivity of intrinsic NADH dynamics imaging to cell physiology, as well as its potential application for sensing cellular respiration, apoptosis, and health problems associated with mitochondrial anomalies such as cancer, aging, and neurodegenerative diseases. Our analytical assay, which can be applied to other labeled biomolecules, provides a complementary, non-invasive approach to conventional biochemical techniques that require cell lysates and, therefore, the loss of morphological context.

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Fig. 1.

Normal and cancerous breast cells exhibit different morphology and mitochondrial distribution. Confocal and DIC images of Rh123-stained normal (Hs578Bst) cells exhibits spindle-like in shape, with the mitochondria distributed throughout the cell (A, B). Cancer cells (Hs578T), however, are polygonal in shape with a perinuclear mitochondrial distribution (C, D). The confocal images were recorded using 488 nm excitation and a 525/30 emission filter. The cells were incubated with 0.5 μ M mitochondrial marker Rh123 for ~ 15 minutes at 37°C and rinsed with PBS three times before imaging in Tyrodes buffer. Scale bar = 20 μ m.

Fig. 2.

Two-photon autofluorescence lifetime imaging reveals heterogeneous environment of the intracellular NADH, which is distinct in breast cancer and normal cells. The 2P-intensity (A, C) and FLIM (B, D) images of breast normal (A, B) and cancerous (C, D) cells reveals different autofluorescence lifetime distribution as shown in the corresponding pixel-lifetime histograms (E). The lifetime distribution histogram of normal (E, open circle) and cancer (E, solid circle) cells can be described as a double-Gaussian (see text). These images were recorded using 740 nm excitation (~ 3 mW at the sample), 256×256 pixels, 120 s collection time per image, and 690sp and BGG22 (475/150) emission filters. In these FLIM image analysis, similar pixel binning (3), threshold (15), scatter ($<3\%$, floating), biexponential fit, and pixel-intensity

weighting were used. [See Fig. 3 and Supplementary Fig. S1 for pixel-to-pixel analyses of autofluorescence intensity and lifetime in Hs578Bst and Hs578T, respectively.] Scale bar = 10 μm .

Fig. 3.

Pixel-to-pixel autofluorescence lifetime and intensity analyses of FLIM reveal two emitting species of intracellular NADH in normal Hs578Bst cells, which is predominantly in the mitochondria. (A) The two emitting species of intracellular autofluorescence have distinct excited-state lifetimes ($\tau_1 = 0.6 \pm 1$ ns, $\tau_2 = 3.4 \pm 0.5$, $n = 33$ pixels in 3 FLIM images). (B) While the amplitude fraction of the fast decay components is large ($\alpha_1 \sim 0.71 \pm 0.04$), its contribution (gray) to the overall fluorescence intensity is small ($F_1 = 31 \pm 4\%$) compared with $69 \pm 9\%$ from the species with a longer lifetime. (C) Using high zoom imaging, pixel-to-pixel time-averaged intensity analyses indicate that the intracellular NADH autofluorescence

in Hs578Bst is predominantly ($86 \pm 5\%$) from apparent mitochondria, as compared with $14 \pm 4\%$ from the cytosol. [See Supplementary Figure S1 for similar analyses on breast cancer cells.]

Fig. 4. Intracellular NADH level is sensitive to the inhibition of complex IV in the ETC using KCN. These two-photon autofluorescence intensity (A, C) and FLIM (B, D) images were recorded before (A, B) and after (C, D: 8 min) the addition of KCN to normal breast cells (Hs578Bst) in a Petri dish. These images were recorded using 740 nm excitation and analyzed using similar fitting constraints (Binning 5, Threshold 15 counts, and Improved Matrix calculation). (E) Pixel-to-pixel analyses of the time-averaged autofluorescence intensity (23 pixels per image) in both apparent mitochondria (gray column) and the cytoplasm (white column). (F) The relative changes of cytosolic and mitochondrial autofluorescence intensity are $43 \pm 8\%$ and $39 \pm 12\%$, respectively, upon the respiratory chain inhibition using KCN.

Fig. 5. Intrinsic NADH concentration in living cells as revealed by 2P-autofluorescence intensity and lifetime images. A typical concentration image (A) of intracellular NADH in a breast normal cell under physiological conditions. This concentration imaging of cellular NADH was carried out after taking into consideration the fluorescence lifetime (i.e., quantum yield) variation in cellular compartments and using a calibrated microscope (see text). Using this assay, our statistical analysis (B) indicates that the average NADH concentration in breast normal cells is $99 \pm 37 \mu\text{M}$ ($n = 7$), compared with $168 \pm 49 \mu\text{M}$ ($n = 7$) in the transformed counterpart.

Fig. 6.

Steady-state 2P-fluorescence anisotropy imaging of cellular autofluorescence indicates a heterogeneous and restrictive cell environment surrounding intracellular NADH. Parallel (A) and perpendicular (B) polarized-autofluorescence images of a single cancer cell, using two-channels with a Glan-Thompson polarizer at each channel, were recorded simultaneously. These polarization-analyzed images were recorded in 256×256 pixels with 120 s acquisition time. An image processing routine was developed, using MATLAB software, for calculating the anisotropy images (C). The estimated average steady-state anisotropy of this image is ~ 0.32 . Similar steady-state anisotropy images of cellular autofluorescence in normal breast cells were also obtained (data not shown).

Fig. 7.

Time-resolved associated anisotropy of intrinsic NADH autofluorescence, at the single-cell level, indicates multiple emitting species with different hydrodynamic volumes and fluorescence properties. Typical time-resolved fluorescence anisotropy in a cancer cell is shown (A). These pseudo-single point measurements were performed by scanning the laser (740 nm) over the whole cell, while both parallel and perpendicular polarizations were recorded simultaneously using 1024 channels (12.4 ps/channel). The G-factor (1.14) was estimated by the tail-matching approach using a coumarin sample. Comparative measurements were also conducted on NADH titrated with mMDH (B; [NADH]:[mMDH] = 1: 0, 16:1, 8:1, 4:1, 2.7:1, and 2:1; from bottom up) and LDH (C; [NADH]:[LDH] = 1: 0, 32:1, 16:1, 8:1, 5.3:1, 4:1, and

2:1; from bottom up). The concentration ratios (B; [NADH]:[mMDH] = 16:1 and C; [NADH]:[LDH] = 8:1 to 5.3:1) gave the closest resemblance to the cellular autofluorescence curves (gray curve), which is also shown in both Fig 7B and 7C for visual comparison.

Summary of the fitting parameters for time-resolved autofluorescence of intrinsic NADH in both normal (Hs578Bst) and cancer (Hs578T) breast cells. These pseudo-single point autofluorescence measurements on living cells were carried out using magic angle (54.7°) detection. Comparative results for free NADH in PBS solution (pH 7.4, at room temperature) are also shown as a function of the relative concentration ratio of mMDH ([NADH] ~ 226 μM) and LDH ([NADH] ~ 183 μM).

Table 1

	τ_1/ps	a_1	τ_2/ps	a_1	τ_3/ns	a_1	τ_f/ns
<u>(A) Intracellular:</u>							
Hs578Bst ($n = 6$)	97(38) ^a	0.38(6)	648(75)	0.48(6)	3.45(7)	0.14(1)	0.83(5) ^b
Hs578T ($n = 6$)	75(11)	0.35(6)	564(14)	0.53(6)	3.4(2)	0.123(5)	0.75(5)
<u>Free NADH:</u>							
In PBS (pH 7.4)	356(9)	0.82(3)	754(9)	0.18(3)	-	-	0.43(3)
<u>[NADH]:[mMDH]</u>							
16:1	356	0.42	650	0.51	1.57	0.06	0.55
8:1	293	0.32	677	0.75	1.49	0.11	0.64
4:1	299	0.19	692	0.66	1.45	0.15	0.74
2.6:1	333	0.17	752	0.69	1.50	0.14	0.79
2:1	303	0.16	793	0.74	1.69	0.10	0.80
1.3:1	365	0.17	791	0.73	1.65	0.11	0.81
1:1	304	0.14	767	0.74	1.57	0.12	0.81
<u>[NADH]:[LDH]</u>							
32:1	233	0.44	508	0.52	1.59	0.04	0.43
16:1	213	0.43	533	0.1	1.62	0.06	0.46
8:1	227	0.42	603	0.47	1.66	0.11	0.57
5:1	243	0.39	671	0.45	1.67	0.16	0.67
4:1	263	0.35	728	0.45	1.7	0.20	0.75
2:1	324	0.23	999	0.58	1.97	0.19	1.02
1:1	338	0.22	1081	0.67	2.53	0.11	1.08

^aThe numbers in parentheses represent the standard deviation of the last digit(s).

^bThe fitting parameters shown here correspond to $\chi^2 \leq 1.2$ and homogeneous residual around its zero-value.

Table 2

Fitting parameters for time-resolved fluorescence associated anisotropy of intracellular NADH in breast normal (Hs578Bst) and cancer (Hs578T) cells using pseudo-single point measurements. The results on free and enzyme-bound NADH in solution (PBS, pH 7.4) are also shown for comparative purpose as a function of the relative concentration ratio of mMDH ([NADH] ~ 226 μ M) and LDH ([NADH] ~ 183 μ M).

	a_1	τ_2/ns	a_2	τ_2/ns	β_1	ϕ_1/ns	β_2	ϕ_2/ns
<u>Cellular NADH:</u>								
Hs578Bst ($n = 9$)	0.3(1) ^d	0.4(1)	0.7(1)	0.7(4)	0.42(5)	0.3(2)	0.36(1)	∞
Hs578T ($n = 7$)	0.4(1)	0.5(2)	0.6(1)	0.8(4)	0.36(6)	0.18(4)	0.40(3)	∞
<u>Free NADH:</u>								
In PBS (pH7.4)	--	--	--	--	0.29	0.27	--	--
<u>[NADH]:[mMDH]</u>								
16:1	0.52	0.43	0.48	0.80	0.28	0.14	0.41	29.5
8:1	0.33	0.43	0.67	0.80	0.26	0.12	0.43	30.0
4:1	0.15	0.43	0.85	0.80	0.10	0.03	0.45	27.9
2.6:1	0.08	0.43	0.93	0.80	0.05	0.04	0.46	26.8
2:1	--	--	--	--	--	--	0.45	30.2
1:1	--	--	--	--	--	--	0.44	30.0
<u>[NADH]:[LDH]</u>								
32:1	0.88	0.42	0.12	1.02	0.21	0.11	0.09	39.6
16:1	0.80	0.42	0.20	1.02	0.26	0.19	0.39	43.5
8:1	0.63	0.42	0.37	1.02	0.26	0.16	0.41	36.8
5:1	0.48	0.42	0.52	1.02	0.24	0.15	0.42	36.5
4:1	0.38	0.42	0.62	1.02	0.24	0.12	0.42	43.5
2:1 and 2:2	--	--	--	--	--	--	0.414	39.6

^dThe numbers in parentheses represent the standard deviation of the last digit(s). The solution measurements were repeated two times and the standard deviation is within 10 % of the stated fitting parameters.