Fluorescence Anisotropy of Cellular NADH as a Tool to Study Different Metabolic Properties of Human Melanocytes and Melanoma Cells

Yue Yuan, Yanfang Li, Brent D. Cameron, and Patricia Relue

Abstract—In this study, we wanted to see if fluorescence anisotropy could be used to detect changes in metabolism in cells with significant light scattering and absorption properties. Fluorescence anisotropy measurements of nicotinamide adenine dinucleotide (NADH) were performed with human melanocytes and melanoma cell lines. To demonstrate the feasibility of using fluorescence anisotropy for detecting metabolic changes, the electron transport chain was blocked using rotenone, inducing an accumulation of intracellular NADH. Total fluorescence increased in all cells as a result of rotenone treatment. Fluorescence anisotropy decreased in the rotenone-treated cells relative to the controls, suggesting an increased ratio of free to protein-bound NADH in the treated cells. In general, the fluorescence anisotropy of the melanocytes was significantly higher than that of the melanoma cell lines. Reflectance spectroscopy showed that the differences in fluorescence anisotropy between the cell types were not due to differences in scattering and absorption properties. Intrinsic cellular NADH fluorescence was experimentally extracted by ratioing polarized fluorescence to polarized reflectance. NADH binding, measured as the ratio of fluorescence intensity at 430 and 465 nm, showed more protein-bound NADH in the melanocytes than in the melanoma cells, consistent with the fluorescence anisotropy measurements.

Index Terms—Cancer, fluorescence spectroscopy, metabolism, polarized fluorescence, reflectance spectroscopy.

I. INTRODUCTION

NADPH (nicotinamide adenine dinucleotide phosphate) is an important coenzyme involved in the energy metabolism of living cells. NADPH binds to proteins associated with both aerobic and anaerobic metabolism, as shown in Fig. 1. In anaerobic metabolism (glucose to lactate), two protein-binding sites are present for NADH binding [1]. In contrast, in aerobic metabolism (glucose to H2O and CO2), eight protein-binding sites are present for NADH binding [1]. The ratio of free to protein-bound NADH reflects different metabolic states in cells associated with different levels of aerobic and anaerobic metabolism [2]–[4].

Glycolysis and respiration are tightly coupled in normal cells. However, cancer cells lack the ability to integrate energy metabolism between glycolysis and respiration. Metabolic changes of cancer cells include enhanced glycolysis, excessive lactate production, and reduced respiratory capacity [2], [5]–[8]. Rapidly growing cancer cells can derive about 60% of their energy from glycolysis and 40% of their energy from oxidative phosphorylation [9], whereas normal cells derive less than 10% of their energy from glycolysis and more than 90% of their energy from oxidative phosphorylation [10]. The shift in the energy metabolism of cancer cells from respiration to aerobic glycolysis may reduce the interaction of NADH with NADH-linked dehydrogenases, leading to a higher ratio of free to protein-bound NADH in the cancer cells. Further, the NADH-linked dehydrogenases involved in glycolysis are free in the cytosol [1], and most of the NADH-linked dehydrogenases involved in respiration are located in mitochondria, and two of them, namely, pyruvate dehydrogenase and the complex I, are attached to the inner mitochondrial membrane [1]. The high viscosity of the mitochondrial matrix space [11] and the association of citric...

Fig. 1. Summary of NADH binding with associated enzymes involved in aerobic and anaerobic metabolism. Glucose conversion to pyruvate is common to both metabolic pathways. From pyruvate, anaerobic metabolism yields lactate; two NADH binding sites exist within anaerobic metabolism. For aerobic metabolism, pyruvate enters the mitochondria where energy is produced via the TCA and the ETC. Eight NADH binding sites exist in the aerobic metabolic pathways. Enzymes for NADH binding are: (1) glyceraldehyde-3-phosphate dehydrogenase; (2) lactate dehydrogenase; (3) pyruvate dehydrogenase complex; (4) isocitrate dehydrogenase; (5) α-ketoglutarate dehydrogenase complex; (6) malate dehydrogenase; (7) complex I of the electron transport chain; (8) cytosolic malate dehydrogenase; and (9) cytosolic glycerol-3-phosphate dehydrogenase.

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acid cycle (TCA) and electron transport chain (ETC) enzymes with one another [12], [13] and with the inner mitochondrial membranes [11] significantly reduce the mobility of NADH; thus, the metabolic shift in cancer cells may provide a much more relaxed environment for NADH motion.

NADH is a natural fluorophore that emits in the blue end of the visible light spectrum upon ultraviolet (UV) light excitation. The fluorescence properties of cellular NADH were first studied by Chance and coworkers as an optimal indicator of intracellular oxidation–reduction states and a useful tool to study mitochondrial function in energy-linked processes [14], [15]. Since Chance’s pioneering work, cellular NADH fluorescence has been widely studied for its ability to probe cell metabolic conditions [4], [16], proliferation [17], differentiation [18], transformation [2], [3], [19], [20], and drug response [21], [22].

The properties of the free and protein-bound NADH in living cells have been studied by the methods of fluorescence spectral analysis [19], [23], [24], fluorescence lifetime measurements [16], [20], [23], [25]–[28], and time-resolved fluorescence anisotropy decay [29]. When bound to protein, the wavelength of maximum NADH emission is shifted from 460 to 440 nm [30]. This emission property has been applied to study the ratio of free to protein-bound NADH in cells through spectral fitting analysis of the fluorescence emission of the cells [2]–[4]. When NADH binds to protein, the fluorescence lifetime is generally increased [25], [26], [29]. However, the fluorescence lifetime of protein-bound NADH varies depending on the protein to which the NADH binds [27], [29]. Some NADH/protein complexes, in fact, have fluorescence lifetimes comparable to or even shorter than the free NADH [28], [29], thus, increasing the challenge of using fluorescence lifetime measurements to discriminate between free and protein-bound NADH. Time-resolved fluorescence anisotropy decay has been used to address this problem since anisotropy-based methods target the rotational mobility of a fluorophore [29]. The large difference in rotational diffusion resulting from the difference in size between the free NADH and the NADH/protein complexes provides an excellent contrast for time-resolved fluorescence anisotropy measurements, allowing successful resolution of free and protein-bound NADH sharing comparable lifetimes [29].

In solution, rotational diffusion is a dominant cause of fluorescence depolarization [31]. Thus, fluorescence anisotropy, which is a measure of retained polarization, increases when a fluorophore associates with a larger molecule or is observed in a solution with higher viscosity [31]. However, in cells and tissue, both scattering and absorption can significantly contribute to the depolarization of both excitation and emission light, and hence, the measured fluorescence anisotropy.

To test the feasibility of using fluorescence anisotropy measurements to detect changes in NADH during metabolic perturbation, rotenone was employed to block mitochondria-associated energy metabolism in cells. Rotenone blocks electron transport and induces NADH accumulation in mitochondria, which in turn, increases NADH fluorescence intensity [2], [19], [26]. Fluorescence lifetime measurements have shown that rotenone-treated endothelial cells have a decreased fluorescence lifetime as compared to control cells, suggesting an accumulation of free NADH within the mitochondria [26]. Therefore, rotenone-treated cells are expected to have higher fluorescence emission and lower fluorescence anisotropy as compared to control cells.

An additional component of this study was to determine if differences in metabolic properties between normal human skin melanocytes and melanoma cells could be detected with fluorescence anisotropy measurements. The fluorescence signal from these cell types is very different due to differences in scattering, absorption, the total quantity, and the state of NADH (free or protein bound). Although the fluorescence anisotropy is independent of the total quantity of NADH, it is dependent on the state of NADH as well as by both scattering and absorption behaviors. Absorption and scattering properties of the cell suspensions were studied using reflectance spectroscopy. After correction for variation in scattering and absorption, fluorescence anisotropy between cell types was compared.

II. MATERIALS AND METHODS

A. Experimental Setup

The experimental setup for fluorescence and reflectance spectroscopy is shown in Fig. 2. A 100 W mercury arc lamp (Osram, Danvers, MA) was used as a light source. Excitation light was provided using a 360 nm excitation bandpass filter with a 20 nm bandwidth (Chroma Technology, Rockingham, VT). A 390-nm dichroic mirror was used to reflect the excitation light. Two silica-core optical fibers (Thorlabs, Newton, NJ) with diameter of 1 mm were used to deliver excitation light and collect fluorescence emissions. The numerical aperture of the fibers was 0.22 ± 0.02. The distance of the fiber tip to the sample surface was 1 cm for both the illumination and collection fibers. The angle between the illumination and collection fibers was 45°. The polarizer was a UV passing glass linear polarizer.
(Edmund Optics, Barrington, NJ). The analyzer consisted of a pair of film linear polarizers (Edmund Optics, Barrington, NJ) oriented orthogonally to one another. The film polarizers transmitted wavelengths greater than 380 nm. A custom-designed holder was used for the two fibers, the polarizer and the analyzer to ensure reproducible positioning. The collection fiber was connected to a USB2000 fiber optic spectrometer (Ocean Optics, Dunedin, FL) as the detector. Both the filter wheel and the spectrometer were controlled by a PC. Fluorescence and reflectance spectra collected by the spectrometer were displayed and stored on the PC. To collect non-polarized spectra, the linear polarizers were removed from the light path. For reflectance spectroscopy, a 6.5 W LS-1 tungsten halogen light source (Ocean Optics, Dunedin, FL) with a 1.0 neutral density filter was used. A silica-core optical fiber with diameter of 1 mm was used to deliver the halogen light.

B. Cell Culture

Human melanocyte cell strain HEMn_LP (Cascade Biologics, Portland, OR) was cultured with Medium 154 (Cascade Biologics, Portland, OR). Human melanoma cell lines WM793 and WM115 (The Wistar Institute, Philadelphia, PA) were cultured with Dulbecco’s modified Eagle’s medium (DMEM, ICN Biomedicinal Inc, Aurora, OH) supplemented with 10% fetal bovine serum (Gibco) and 5 µg/mL insulin (Sigma). Both WM793 and WM115 are vertical growth phase melanoma. All cells were routinely cultured in a 37 °C humidified 5% CO₂ incubator (NAPCO).

For spectra collection, cultured cells were trypsinized when they reached near or 100% confluence. The cells were washed three times with phosphate buffered saline (PBS). The third wash was measured to account for background fluorescence. Cell concentrations were measured with a hemocytometer and cell viabilities were determined by trypan blue exclusion assay.

C. Rotenone Treatment

The cells were resuspended in their culture media after trypsinization. The cell concentrations of the suspensions were 8 × 10⁶ to 1 × 10⁷ cells/mL for the melanocytes, 8 × 10⁶ cells/mL for WM115, and 1 × 10⁷ cells/mL for WM793. A 1 mL aliquot of cell suspension was split evenly into two sterile 1.5 mL centrifuge tubes. Cells were allowed to settle for 2–3 min, and 2.5 µL of supernatant was removed from each tube. A 2.5 µL aliquot of 1 mM rotenone (Sigma-Aldrich) stock solution was added and mixed with the cell suspension in one tube. As the rotenone stock solution was made by dissolving rotenone in ethanol, 2.5 µL of ethanol was added and mixed with the cell suspension in the other tube as a control. The cell suspensions were incubated for 10 min at room temperature and gently swirled to prevent cell precipitation. Following the incubation, the cells were spun down, washed three times with PBS, and finally resuspended in 0.5 mL PBS in each tube for fluorescence measurements. Cell viabilities were checked before and after the rotenone treatment and after the fluorescence measurements.

D. Fluorescence and Reflectance Spectroscopy of Cell Suspension

Due to the different scattering and absorption properties of the cell suspensions, the melanocytes and melanoma cells were resuspended in the PBS at different concentrations ranging from 5 × 10⁶ to 6 × 10⁷ cells/mL. For the spectral measurement of each cell suspension, 0.2 mL of cell suspension was transferred into a 0.2 mL custom-made mini petri dish. After waiting for 5 min to allow the cells to settle, sequential polarized fluorescence spectra were collected with the polarization orientation between the polarizer and analyzer set as parallel, perpendicular, perpendicular, and parallel. The two parallel polarized fluorescence spectra were compared to determine if photobleaching occurred. Polarized reflectance spectra were collected in the same manner immediately following the collection of the polarized fluorescence spectra. Nonpolarized reflectance spectra were collected by removing the polarizers from the light path. The nonpolarized reflectance spectra were used to study the changes of absorption of the cell suspensions of different cell types at different concentrations. Cell viabilities were checked before and after all of the fluorescence and reflectance spectra were collected.

Fluorescence and reflectance spectra were imported into MATLAB. Polarized spectra for reflectance and fluorescence were calculated by averaging the two parallel or two perpendicular polarized spectra collected in each experiment. The fluorescence anisotropy (FA) and degree of linear polarization (DLP) were calculated as

\[
FA = \frac{I_{f//} - I_{f\perp}}{I_{f//} + 2I_{f\perp}}
\]

(1)

\[
DLP = \frac{I_{r//} - I_{r\perp}}{I_{r//} + I_{r\perp}}
\]

(2)

where \( I \) is the intensity of a spectrum, and \( f \) or \( r \) refer to fluorescence or reflectance spectra, respectively, and \( // \) or \( \perp \) represent either parallel or perpendicular alignment of the polarizer and analyzer [31].

For experiments with rotenone treatment, the total fluorescence spectrum, \( I_f(\lambda) \), was calculated from the parallel and perpendicular spectra as

\[
I_f(\lambda) = I_{f//}(\lambda) + 2I_{f\perp}(\lambda).
\]

(3)

The total fluorescence was calculated by integrating the total fluorescence spectrum \( I_f(\lambda) \) over the wavelength range from 430 to 550 nm. The change in the fluorescence due to rotenone treatment was calculated as the difference between the total fluorescence of the rotenone-treated cells and the control cells divided by the total fluorescence of the control cells.

III. RESULTS

A. Effects of Rotenone Treatment on Cell Fluorescence

Fluorescence spectra with polarized excitation at 360 nm were collected from melanocyte, WM115, and WM793 cell suspensions on different days. No background fluorescence was detected and no photobleaching was observed during the
B. Reflectance Spectra of the Cell Suspensions

Differences in pigment levels between the melanocytes and melanoma cells used in this study resulted in a large difference in absorption between the cells. To examine these differences, nonpolarized reflectance spectra were collected from the melanocyte and melanoma cell suspensions at concentrations ranging from $5 \times 10^6$ to $6 \times 10^7$ cells/mL. The reflectance spectra collected for each cell type and concentration were averaged over the measurements of different batches of cells collected on different days. Each average spectrum was then normalized against its peak intensity. The normalized reflectance spectra are shown in Fig. 4. All three cell types show stronger absorption at higher cell concentrations, which results in a red shift of the reflectance spectra. The red shift was much more pronounced for the melanocytes than for the two melanoma cell lines.

The peak wavelength was also determined for each spectrum and averaged over all spectra collected for each cell type and concentration. The values of peak wavelength are summarized in Table III. The peak wavelength was red shifted with increasing concentration of the cell suspensions for each cell type. All three cell types showed a significant red shift of the peak wavelength as the cell concentration increased from $2 \times 10^5$ to $6 \times 10^7$ cells/mL ($P < 0.05$). The melanocytes also showed a significant red shift between $1 \times 10^5$ and $2 \times 10^7$ cells/mL ($P < 0.05$). However, no statistically significant shift in peak wavelength was observed for the cell concentrations from $5 \times 10^6$ to $1 \times 10^7$ cells/mL. The lack of red shift at the low cell concentrations suggests that absorption is considerably reduced.

No statistical difference was seen between the peak wavelengths of WM115 and WM793 at any concentration, suggesting similar absorption by these two melanoma cell lines. However, the peak wavelength of the melanocytes was significantly higher than either of the two melanoma cell lines at the cell concentrations of $6 \times 10^7$ ($P < 0.0001$) and $2 \times 10^7$ cells/mL ($P < 0.05$), suggesting a much stronger absorption by the melanocytes than the melanoma cells at these high cell concentrations. There was no statistically significant difference in the peak wavelength between the melanocytes and melanoma cells at the concentrations of $1 \times 10^7$ and $5 \times 10^6$ cells/mL, suggesting that the melanocytes and melanoma cells shared a similar absorption at these low cell concentrations.

C. Polarized Reflectance Spectra of the Cell Suspensions

To determine scattering properties of the three cell types as a function of concentration, polarized reflectance spectra were collected immediately after collection of the polarized fluorescence spectra. The DLP was calculated using (2) as described in the Section II. The DLP provides information about multiple scattering within a turbid media, where increased scattering tends to depolarize incident polarized light to a greater extent, leading to a lower DLP. The DLP was averaged over different batches of cells of the same type and concentration collected on different days. As shown in Fig. 5, the DLP decreased as the cell concentration increased for the three cell types, indicating that as the cell layer increased in thickness, depolarization of the backscattered light increased.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Cell Viabilities Before Rotenone Treatment and After Fluorescence Measurement</th>
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<tbody>
<tr>
<td>Cell type</td>
<td>Before rotenone treatment (%)</td>
</tr>
<tr>
<td>Melanocyte</td>
<td>96.8±4.5 (n=2)</td>
</tr>
<tr>
<td>WM115</td>
<td>96.9±0.3 (n=4)</td>
</tr>
<tr>
<td>WM793</td>
<td>97.5±1.8 (n=4)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Table II</th>
<th>Change in Total Fluorescence for the Different Cell Types With and Without Rotenone Treatment</th>
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<tbody>
<tr>
<td>Cell type</td>
<td>$F_C$</td>
</tr>
<tr>
<td>Melanocyte</td>
<td>45,450±13,053</td>
</tr>
<tr>
<td>WM115</td>
<td>74,057±9,930</td>
</tr>
<tr>
<td>WM793</td>
<td>46,460±11,138</td>
</tr>
</tbody>
</table>

Integration times were the same for control and treatment groups and were 1.5 s for the melanocytes, 0.8 s for WM115, and 1 s for WM793 cells.

$F_C$ - Total fluorescence of the control cells;
$F_{RT}$ - Total fluorescence of the rotenone-treated cells.
TABLE III
EFFECT OF CELL CONCENTRATION ON THE PEAK WAVELENGTH OF THE REFLECTANCE SPECTRA OF THE CELL SUSPENSIONS

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Concentration of cell suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6×10^5 cells/mL</td>
</tr>
<tr>
<td>Melanocyte</td>
<td>680.8±9.1 (n=4)</td>
</tr>
<tr>
<td>WM115</td>
<td>622.9±3.8 (n=4)</td>
</tr>
<tr>
<td>WM793</td>
<td>621.9±4.3 (n=4)</td>
</tr>
</tbody>
</table>

Values represent average peak wavelength (in nm) and statistical results are based on a student’s t test.

As shown in Fig. 6(a) and (b), the DLP of the melanocytes was higher than that of the two melanoma cell lines at the two highest concentrations. These are the same concentrations where the melanocytes showed significantly stronger absorption than the melanoma cells. Similar results were reported in the literature [32], [33], where the authors suggest that stronger absorption results in a higher DLP due to the absorption of highly scattered photons with long optical pathlengths. At low cell concentrations (< 1×10^7 cells/mL), absorption by the cells was relatively weak, and the absorption of the melanocytes and the two melanoma cells was not statistically different (see Table III). When comparing the cells with similar absorption, such as the WM115 and WM793 cells at equal concentration, the DLP is not the same. This suggests that the difference in DLP is not associated with absorption, but with differences in scattering. Therefore, the difference in DLP seen between the melanocytes and melanoma cells when their absorptions are equivalent cannot be attributed to absorption.

The DLP of WM115 cells was always lower than that of WM793 cells at the same concentrations, suggesting a stronger scattering of WM115 cells than WM793 cells. At the highest cell concentrations where absorption was significant, the DLP of the melanocytes was higher than that of the two melanoma cell lines [Fig. 6(a) and (b)]. However, as the concentration was reduced to 1×10^7 cells/mL, the DLP of the melanocytes [Fig. 6(c)] was between that of the two melanoma cell lines, suggesting that absorption no longer played a significant role in the DLP. The DLP of the melanocytes at the concentration of 1×10^7 cells/mL was practically the same as that of WM115 cells at 7×10^6 cells/mL [see Fig. 6(d)], suggesting that the same scattering property could be obtained by lowering the cell concentration for WM115 cells.

**D. Polarized Fluorescence Spectra of the Cell Suspensions**

Polarized fluorescence spectra were collected from the three cell types at different cell concentrations. Cell viabilities were checked before and after each measurement and were always greater than 90%. Fluorescence anisotropy was calculated using (1) as described in the Section II, and the FA was averaged over different batches of cells for each cell type and concentration. As shown in Fig. 7(a)–(c), fluorescence anisotropy decreased as the cell concentration increased for the three cell types, suggesting a dependence of fluorescence anisotropy on the scattering of the cell suspensions. The fluorescence anisotropy,
like the DLP, was remarkably higher for the melanocytes than for the two melanoma cell lines at high cell concentrations (>2 × 10⁷ cell/mL). This difference could partly be due to the much stronger absorption of the melanocytes relative to the melanoma cells at the high cell concentrations. However, the fluorescence anisotropy of the melanocytes was still appreciably higher than that of the two melanoma cell lines at the lowest cell concentrations [Fig. 7(d)], where the two melanoma cell lines showed similar fluorescence anisotropy. The DLP measurements [Fig. 6(d)] showed that all three cell types had similar scattering properties at these cell concentrations. Therefore, the higher fluorescence anisotropy for the melanocytes is not due to differences in scattering and/or absorption of the cell suspensions. As a result, the higher fluorescence anisotropy for the melanocytes could indicate a higher proportion of protein-bound NADH and/or a more stringent microenvironment for NADH in the melanocytes than in the melanoma cells.

E. Fluorescence Intensity Ratio of Melanocytes and Melanoma Cells

Fluorescence emission of the protein-bound NADH is blue shifted as compared to that of the free NADH. It was suggested that the ratio of fluorescence intensity at 410 and 465 nm could provide a sensitive test of NADH binding [19]. Due to an artifact in our data at 410 nm that was caused by the mini petri dish, we instead compare the ratio of fluorescence intensity at 430 and 465 nm. Cell concentrations were 1 × 10⁷ cells/mL for the melanocytes and WM793 cells and 7 × 10⁶ cells/mL for WM115 cells. Polarized fluorescence spectra were defined as the difference between the parallel and perpendicular fluorescence spectra. Polarized reflectance spectra were defined as the difference between the parallel and perpendicular reflectance spectra. Fluorescence ratio spectra were obtained by normalizing the polarized fluorescence spectra with its corresponding polarized reflectance spectra. This normalization was experimentally shown to recover intrinsic fluorescence of fluorophores from turbid media [34]. As shown in Fig. 8, fluorescence ratio spectra were averaged over six batches of cells for each of the three cell types and normalized against the maximum intensities. The emission peaks were around 420 nm. However, the fluorescence ratio spectrum of the melanocytes showed an appreciable blue shift as compared to those of the two melanoma cell lines.

The ratios of fluorescence intensity at 430 and 465 nm for the melanocytes and melanoma cells are shown in Table IV. The fluorescence intensity at each wavelength (i.e., 430 or 465 nm) was obtained by integrating the fluorescence ratio spectra over a wavelength range of ±1 nm about the wavelength. The fluorescence intensity ratio was averaged over six batches of cells for each cell type. The melanocytes demonstrated a significantly higher fluorescence intensity ratio than the two melanoma cell lines (P < 0.05), while no statistical difference was seen between the two melanoma cell lines. The higher fluorescence intensity ratio of the melanocytes suggests that
the NADH binding was higher in the melanocytes than in the melanoma cells, which is consistent with the higher fluorescence anisotropy of the melanocytes as compared to the melanoma cells.

IV. DISCUSSION

To evaluate the feasibility of using fluorescence anisotropy of cellular NADH for tracking metabolic changes in living cells, rotenone treatment was applied to induce metabolic changes in the cells. Rotenone inhibits the complex I of the electron transport chain [35], and thus, blocks the transfer of electrons through the electron transport chain, which in turn, arrests the oxidation of NADH at the site of the complex I. Therefore, NADH accumulates in the mitochondria with rotenone treatment. Studies have demonstrated an increase of NADH fluorescence emission after rotenone inhibition in various types of cells [2], [19], [26]. In addition, spectral fitting analysis of the fluorescence of rotenone-treated fibroblasts has revealed an increased free/bound NADH ratio as compared to the untreated cells [2]. Fluorescence lifetime imaging has also demonstrated a decreased fluorescence lifetime for rotenone-treated endothelial cells compared to control cells, which was attributed to the accumulation of free NADH within the mitochondria, and the rest of NADH is protein-bound and contributes to about 80% of the fluorescence emission [27]. As the total matrix NADH level increases, the pool of the protein-bound NADH saturates, whereas the pool of free NADH increases linearly as the total NADH level increases [27]. The mitochondrial NADH level increases with rotenone inhibition. Thus, the protein-bound NADH pool in the mitochondria could saturate, while the free NADH pool in the mitochondria could still linearly increase. Therefore, in our experiments with rotenone treatment, the increase in overall fluorescence could be attributed to an increase in both free and protein-bound NADH, whereas a relative increase in the free NADH pool could account for the decreased fluorescence anisotropy seen in all cell types.

Based on the assumption that enhanced glycolysis and reduced aerobic respiration of cancer cells may change the physiological properties of NADH, fluorescence anisotropy of the cellular NADH of the melanocytes and melanoma cells were collected to probe the different metabolic properties of the cells. In order to compare fluorescence anisotropy between the different types of cells, differences in optical properties of the cell suspensions, namely, absorption and scattering, need to be addressed. Reflectance spectroscopy was used to compare the optical properties of the melanocyte and melanoma cell suspensions. The melanocytes showed much stronger absorption than the melanoma cells at high cell concentrations (> 2 × 10⁷ cell/mL), resulting in a higher degree of linear polarization and higher fluorescence anisotropy for the melanocytes. The effect of absorption on the DLP and fluorescence anisotropy may be caused by the loss of highly scattered long pathlength photons in the backscattered light [32], [33]. The difference in absorption between the melanocytes and the melanoma cells was minimized with dilution of the cell suspensions, and the relatively weak absorption at the lowest cell concentrations might be eliminated through normalization. WM115 cells showed the strongest scattering and WM793 cells showed the weakest scattering among the three cell types. The different scattering properties may be caused by the different level and shape of the index mismatched structures in these cells.

When the cell concentrations were adjusted to minimize differences in scattering and absorption effects, the melanocytes showed an appreciably higher fluorescence anisotropy than either of the melanoma cell lines. Thus, this higher fluorescence anisotropy suggests a higher proportion of protein-bound NADH and/or a more stringent microenvironment for NADH in the melanocytes. The ratio of fluorescence intensity at 430 and 465 nm also supports higher levels of NADH binding in the melanocytes. As described in the Section I, two NADH binding sites exist for anaerobic metabolism, while eight NADH binding sites exist for aerobic metabolism. The mitochondrial matrix also provides a more mobility-restricted environment than the cytosol for proteins or even small molecules like NADH [11]. Thus, the metabolic shift of normal aerobic metabolism to enhanced glycolysis and reduced respiration may decrease the binding of NADH to metabolic proteins, and provide a more mobile microenvironment for the NADH.

Aerobic glycolysis, which is a characteristic of many tumors, is accompanied by significantly enhanced activities of glycolytic enzymes and reduced activities of the enzymes of the TCA cycle and the electron transport chain [8], [36]–[38]. Melanoma cells have shown the elevated expression of c-myc oncogene in comparison to melanocytes [39]. The activation of the c-myc oncogene results in an oncogenic transcription factor (c-Myc), which is capable of activating gene expressions for the glucose transporter, glycolytic enzymes, and the lactate dehydrogenase [40]–[42]. Decreased enzymes of the electron transport chain have also been observed in melanoma [43]. Therefore, the enhanced glycolysis and reduced respiration of melanoma cells may result in a lower proportion of NADH binding to proteins and a more mobile microenvironment for NADH, resulting in a decrease in fluorescence anisotropy as observed in this study. Several other studies on cellular NADH fluorescence have revealed reduced binding of NADH in cancer cells relative to their normal counterparts [2], [19], [28].

Since aerobic glycolysis is constitutively upregulated in tumors, cells derived from tumors typically maintain their metabolic phenotypes in culture under normoxic conditions [7]. Thus, the in vitro fluorescence anisotropy measurements of cellular NADH in cultured cells may mirror behavior of in vivo tissues, rendering the possibility of extending the fluorescence anisotropy method to in vivo imaging. Hypoxia in tumor tissues induces anaerobic glycolysis, which will further increase the contrast for the fluorescence anisotropy measurements. Progressively increased glycolytic rate and reduced respiration capacity are associated with cancer progression [44]–[46]. Therefore, fluorescence anisotropy measurement of cellular NADH may potentially allow discrimination of cancer cells at different stages of progression.
REFERENCES

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