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Sensitive detection of intracellular environment of normal and cancer cells by autofluorescence lifetime imaging

Kamlesh Awasthi [a], Daiki Moriya [b], Takakazu Nakabayashi [c], Liming Li [b], Nobuhiro Ohta [a,*]

[a] Department of Applied Chemistry and Institute of Molecular Science, National Chiao Tung University, 1001, Ta-Hsueh Road, Hsinchu 30010, Taiwan
[b] Department of Bio- and Material Photonics, Chitose Institute of Science and Technology, Chitose 066-8655, Japan
[c] Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-ku, Sendai 980-8578, Japan

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ABSTRACT

Intracellular fluorescence lifetime images of the endogenous fluorophores of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), which are well known as autofluorescence chromophores, were obtained from rat normal fibroblast cells (WFB) and H-*ras* oncogene-transfected cancer cells among WFB (W31). The average lifetime of the NADH and FAD autofluorescence was shorter in cancer cells than in normal cells, indicating that the difference in metabolism between healthy and cancer cells alters the conditions for coenzymes such as NADH and FAD and that the autofluorescence lifetime measurement of NADH and FAD is applicable for the noninvasive diagnosis of cancer cells. The pico- and nano-second time-resolved fluorescence spectra of NADH obtained with different time windows were similar in normal and cancer cells, indicating that every fluorescence decay component gives the same spectrum in both cell types. These results as well as the fluorescence lifetime images of exogenous fluorophores stained with sodium pheophorbide a in normal and cancer cells suggest that the difference in the fluorescence lifetime between normal and cancer cells cannot be attributed to a difference in the intracellular pH or refractive index but to the difference in the bound condition between proteins and NADH or FAD under the different intracellular environments of normal and cancer cells.

1. Introduction

Fluorescence microscopy is widely used in biochemical, biophysical and biomedical research, and various fluorescent molecules and proteins have been developed to enable selective intracellular imaging of a single cell. However, cells contain endogenous fluorophores that exhibit autofluorescence, and microscopy using autofluorescence enables the preservation of biological conditions without having to spend time on staining and problems associated with toxicity. Thus, interest in the imaging of label-free autofluorescence as a noninvasive diagnostic technique has increased [1–11].

Cancer research is a dynamic field that encompasses not only biological and medical sciences but also optics and spectroscopic sciences; rapid advances in the understanding of the biological features of various cancers are continuously being made, which translates into better detection and therapy. Recent research has shown that cancer cells undergo a metabolic switch [12]. Noncancerous (healthy) cells metabolize glucose to carbon dioxide for the production of ATP; this process begins in the mitochondrial tricarboxylic acid cycle with the oxidation of glycolytic pyruvates to produce nicotinamide adenine dinucleotide (NADH), and then oxidative phosphorylation is fueled to maximize ATP production with the minimal production of lactate. When oxygen is limited, the same cells produce ATP through glucose metabolism in the cytoplasm by reducing glycolytic pyruvates to lactate; this is called anaerobic glycolysis. In contrast to healthy cells, cancer cells develop a unique glucose metabolic system followed by the production of large amounts of lactate, regardless of the availability of oxygen; this is called aerobic glycolysis [13–16]. This selectivity for glucose metabolism produces not only large amounts of lactate but also excessive protons (H^+) in cells. Excessive H^+ deregulates the intra- and extracellular pH, which is a hallmark of cancerous tissues [17–22]. The difference in metabolic processes between healthy and cancer cells is thus expected to alter the conditions for coenzymes such as NADH.

NADH, whose structure is shown in Fig. 1a, is a well-known autofluorescent chromophore [23] that exhibits an intense absorption spectrum with a peak at around 340 nm and emits blue fluorescence, which arises from the reduced form of nicotinamide. This enables the fluorescence of NADH to be distinguished if light of suitable wavelengths is selected for both excitation and emission. In fact, the autofluorescence of NADH is widely used to examine the intracellular environment [1–3,6–8], and it is expected that the difference in intracellular conditions between cancer cells and normal cells may be distinguishable by measuring autofluorescence.

* Corresponding author.
E-mail address: nohta@nctu.edu.tw (N. Ohta).

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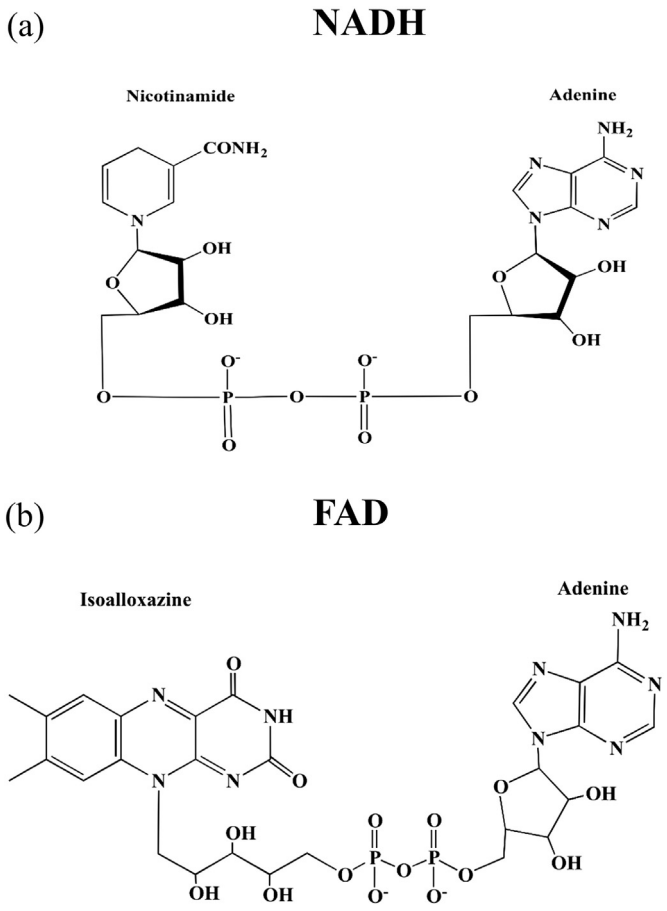

Fig. 1. Molecular structure of NADH (a) and FAD (b).

FAD, whose structure is shown in Fig. 1b, is another important cofactor that exists mostly as a component of flavoproteins, which catalyzes a variety of one- or two-electron redox reactions in living systems [24,25]. The isoalloxazine moiety of FAD has absorption and fluorescence spectra in the visible region. FAD is also a useful endogenous fluorophore that can be used for the noninvasive imaging of living cells in addition to NADH. Most flavoproteins exhibit very weak fluorescence because of the efficient fluorescence quenching of photoexcited flavin chromophores by its surroundings [26,27]. Only a few flavoproteins that include FAD, such as lipoamide dehydrogenase (LipDH), exhibit marked autofluorescence in cultured cells [28–32].

These fluorophores of NADH and FAD provide information about specific intracellular locations and functions. For example, mitochondrial NADH has been examined by absorption/fluorescence spectroscopic and imaging methods and it was shown that the redox ratio (NAD^+/NADH ratio) could serve as a sensitive indicator of cellular metabolism [33]. It was also reported that NADH fluorescence intensity differs between tumor cells and normal cells. A significant difference in the fluorescence spectrum between normal cells and malignant tissues was also reported [34]. However, quantitative analysis of the intracellular environment based on fluorescence intensity measurements is very difficult because fluorescence intensity depends on various experimental conditions, including excitation light intensity, excitation light wavelength, and the optical alignment. Fluorescence lifetime microscopy (FLIM) solves these problems [35–42] because its emission lifetime is an intrinsic parameter, which is independent of absorption intensity or irradiation light intensity and of other factors that limit the intensity measurements. Autofluorescence lifetime measurements are thus highly reliable and are also less invasive than other methods.

In the present study, the difference in the intracellular environment between normal cells and cancer cells was examined using autofluorescence lifetime microscopy and autofluorescence measurements of NADH and FAD. As reported in our previous paper [43], FLIM images of exogenous fluorophores of talaporphfin sodium (TPS) and sodium-pheophorbie a (NaPh) stained in Wistar-King A rat fetus fibroblast (WFB) cells and W31 cells, which are malignant cells transformed from WFB, were successfully measured, and it was shown that FLIM images can be used to distinguish cancer cells and normal cells with TPS or Na-Ph-a. However, photoirradiation of these dyes is considered to give some damage to cells even for normal cells, which is known from the fluorescence quenching by photogradiation. On the other hand, intracellular coenzymes such as NADH and FAD are intrinsically fluorescent, which allows for the accurate and noninvasive imaging of the metabolic activities in cells and tissues. Thus, autofluorescence measurement of endogenous fluorophores of NADH and FAD is a label-free technique, and so we don't have to worry about deterioration as well as toxicity of fluorophores. Further, visualization of NADH and FAD by FLIM may make it possible to distinguish the intracellular environments and activities between healthy and cancer cells, allowing for the structural analysis of organelles in a noninvasive manner [6,44,45]. It is also expected that the detection of NADH and FAD autofluorescence in healthy and cancerous tumor cells will enhance our understanding of the environments within these cells. To the best of our knowledge, no information on autofluorescence lifetime image of cancer cells and normal cells which have the same origin as well as on comparison between these images has been reported. To discuss the cause of the differences in the NADH and FAD autofluorescence lifetime between healthy and cancer cells, FLIM images of exogenous fluorophores in healthy and cancer cells are also shown, as a reference.

2. Experimental Methods

2.1. Cell Culture

Normal cells (WFB) and cancer cells (W31) were grown in a 5% CO_2 humidified atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM, D5796, Sigma) supplemented with 2×10^5 U dm^{-3} penicillin G, 200 mg of streptomycin sulfate, and 10% fetal bovine serum (FBS), with the same manner as the one reported elsewhere [43,46]. The cell culture medium was replaced by calcium- and magnesium-free phosphate-buffered saline [PBS(−)] medium just before the measurements of the NADH and FAD autofluorescence lifetime. The optical measurements for the population of WFB and W31 cells were performed by collecting the cells into a cuvette having a 1 mm optical path. The number of a population of WFB and W31 cells was qualitatively estimated to be 10^4–10^8 on the assumption that the volume of a cell was 3×10^3 μm^3 and cell were tightly packed after the complete precipitation on the cuvette bottom [47]. For the measurements of the exogenous fluorophores, commercially available sodium pheophorbide a (NaPh; Chlorophyll Research Institute) was used. NaPh was dissolved in FBS-free DMEM, and the solution was added to the cultured cells. Next, the cells were incubated for 30 min and washed twice with calcium- and magnesium-free PBS(−). We started the emission measurements just after the exchange of PBS(−) medium.

2.2. Fluorescence Lifetime Imaging

FLIM images of endogenous fluorophores of NADH and FAD as well as exogenous fluorophores of NaPh in normal cells (WFB) and cancer cells (W31) were measured using an inverted microscope (TE2000E, Nikon) through an objective lens (40×) with a time-correlated single photon counting (TCSPC) system (Becker & Hickel GmbH, SPC-830) [47]. The second harmonic output of 380, 450, and 405 nm from a mode-locked Ti:sapphire laser (Tsunami, Spectra Physics; pulse duration of approximately 100 fs, repetition rate of 81 MHz) was used as excitation light for NADH, FAD, and NaPh, respectively. Autofluorescence signals of NADH and FAD as well as fluorescence signals of NaPh in