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CHARACTERIZING FLUORESCENCE LIFETIME OF NAD(P)H IN HUMAN LEUKEMIC MYELOID CELLS AND MONONUCLEAR CELLS

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The aim of this *ex vivo* study was to explore the potential of using the fluorescence lifetime of intracellular reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) as a label-free indicator to characterize the differences between human leukemic myeloid cells and normal mononuclear cells (MNC). The steady-state and time-resolved autofluorescence of two human leukemic myeloid cell lines (K562, HL60) and MNC were measured by a spectrofluorimeter. According to excitation–emission matrix (EEM) analysis, the optimal emission of NAD(P)H in these cells suspensions occurred at 445 nm. Furthermore, the fluorescence lifetimes of NAD(P)H in leukemic myeloid cells and MNC were determined by fitting the time-resolved autofluorescence data. The mean fluorescence lifetimes of NAD(P)H in K562, HL60, and MNC cells were 5.57 ± 1.19, 4.45 ± 0.71, and 7.31 ± 0.60 ns, respectively. There was a significant difference in the mean lifetime of NAD(P)H between leukemic myeloid cells and MNC ($p < 0.05$). The difference was essentially caused by the change in relative concentration of free and protein-bound NAD(P)H. This study suggests that the mean fluorescence lifetime of NAD(P)H might be a potential label-free indicator for differentiating leukemic myeloid cells from MNC.

Keywords: Leukemic myeloid cells; normal mononuclear cells; autofluorescence; nicotinamide adenine dinucleotide; lifetime; differentiation.

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1. Introduction

Leukemia, a type of cancer found in blood and bone marrow, is characterized by an abnormal increase of immature white blood cells. Myelogenous leukemia is a main type of leukemia and characterized by the proliferation of myeloid tissue and increase in the number of granulocytes, myelocytes, and myeloblasts in the circulating blood. Fluorescence *in situ* hybridization (FISH) flow cytometry and real-time quantitative reverse transcription polymerase chain reaction (RQ-PCR) are traditional methods for the clinical diagnosis of myeloid leukemia.[1–3] However, these methods are limited to a specific chromosome or fusion gene as the marker, such as BCL-ABL fusion gene for RQ-PCR.

Time-resolved autofluorescence has been used as a rapid and label-free optical method to characterize normal and malignant cells.[4,5] In recent years, the time-resolved autofluorescence spectroscopy of reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) has been successfully used to differentiate malignant cells from normal cells, which include lung cancer cells,[4] breast cancer cells,[6,7] and cervical carcinoma cells.[5] However, there is a few discussion about the diagnosis of leukemia using the fluorescence lifetime of NAD(P)H. NAD(P)H is a sensitive endogenous indicator of the cellular energy metabolism and its fluorescence lifetime is dependent on its microenvironment. In intracellular microenvironment, NAD(P)H molecules coexist in both free and protein-bound forms. Typically, the fluorescence lifetime of free NAD(P)H is less than 1.0 ns and the mean lifetime around 0.4 ns, whereas the fluorescence lifetime of protein-bound NAD(P)H is increased to 2.5 ns or longer.[8,9] Previous studies show that the fluorescence decay curve of NAD(P)H in cells or tissues can be fitted by a triple-exponential function, with the short lifetime ranging from 0.4 to 0.6 ns, the intermediate lifetime ranging from 2.0 to 3.6 ns, and the long lifetime ranging from 6.0 to 15.0 ns.[9–11] The short lifetime component is attributed to free NAD(P)H, and the intermediate and long lifetimes to protein-bound NAD(P)H.[8,11,12]

In this study, the feasibility of using fluorescence lifetime of NAD(P)H to diagnosis leukemia was investigated. We proposed that the fluorescence lifetime of NAD(P)H might be a potential label-free indicator for characterizing the differences between leukemic myeloid cells and MNC. The steady-state and time-resolved autofluorescence of leukemic model cells (e.g., K562 and HL60) and MNC were measured using spectrofluorimeter. In addition, the fluorescence lifetimes of NAD(P)H derived from the time-resolved autofluorescence of cells were used to establish a label-free indicator for characterizing leukemic myeloid cells and MNC.

2. Materials and Methods

2.1. *Cells and cell culture*

Two human leukemic myeloid cell lines (K562 and HL60) were purchased from the Shanghai Institute of Cytobiology (Institute of Chinese Academic Medical Science, China). K562 cells (M6 AML) were grown in RPMI 1640 medium (GIBCO BRL) supplemented with 10% newborn calf serum (NBCS, GIBCO BRL), 75 μg/mL penicillin and 100 μg/mL streptomycin. HL60 cells (APML) were cultured in RPMI-1640 medium (GIBCO BRL) supplemented with 20% NBCS, 75 μg/mL penicillin, and 100 μg/mL streptomycin. All the cell lines were incubated at 37°C and 5% CO_2. Cells were maintained in log phase with viability >95%.

Normal bone marrow cells were obtained from healthy donors ($n = 5$) at the Fujian Institute of Hematology, Union Hospital of Fujian Medical University. The research protocol was approved by the Ethics Committee of Union Hospital of Fujian Medical University, and an informed consent form was obtained from each subject. Briefly, 5 mL of bone marrow was collected from donors and diluted with 5 mL PBS, mixed with 5 mL lymphocytes separation medium, and centrifuged at 700 g for 20 min. The MNC layer was collected and washed twice and resuspended in PBS for fluorescence measurement. All the experimental procedures were completed within 6 h.

2.2. *Steady-state fluorescence spectroscopy*

In order to ascertain the optimal excitation and emission wavelength of NAD(P)H in leukemic myeloid cells and MNC for the time-resolved fluorescence measurement, the excitation and emission matrix (EEM) of cell suspensions (1×10^6 cells/mL) were recorded on a spectrofluorimeter (FLS920, Edinburgh Instrument Ltd., UK). The fluorescence

emission spectra were obtained over a range of excitation wavelengths between 260 to 500 nm in 10 nm increments. For each excitation wavelength, the fluorescence spectra were recorded from 300 to 700 nm in 5 nm increments and with the first emission wavelength red-shifted by 20 nm from the excitation. The excitation and emission bandwidths were set at 2.0 and 1.0 nm, respectively. The integration time for each wavelength was 0.3 s.

2.3. *Time-resolved fluorescence spectroscopy*

Since the fluorescence lifetime of NAD(P)H is fairly short, i.e., <1.0 ns for free NAD(P)H, in order to obtain reliable data from cell suspension a picosecond pulsed diode laser (405 nm, 0.5 mW; Edinburgh Instruments Ltd.) was used as excitation light source. Combined with the time-correlated single photon counting technique, the accuracy of fluorescence lifetime measurement for the spectrofluorimeter can be obtained with a range of 100 ps. The time-resolved autofluorescence spectra of NAD(P)H for cell suspensions of leukemic myeloid cells and MNC (1×10^6 cells/mL) were recorded at the fluorescence emission wavelength of 445 nm. In order to effectively block the excitation light for fluorescence detection, a 420 nm long-pass filter (Huihong Photoelectric Instrument Ltd., China) was placed in front of the entrance of emission monochromator. In addition, the instrumental response function (IRF) of the spectrofluorimeter was determined by using the ludox solution as the scattering sample, and the obtained data was used to correct instrument response for each measurement.

2.4. *Data processing*

Time-resolved spectra data were analyzed using the F900 software (Version 6.83, Edinburgh Instruments Ltd.), which implements multi-exponential fluorescence decay fitting using the following mathematical model:

$$I(t) = A + \sum_{i}^{n} \alpha_i e^{-t/\tau_i}, \tag{1}$$

where $I(t)$ is the fluorescence intensity at time t after the excitation pulse, A is an additional background, n is the total number of decaying components in the exponential sum. The variables τ_i and α_i are the fluorescence lifetimes and corresponding relative amplitudes, respectively. The mean fluorescence lifetime was calculated from the following equation[13]:

$$\langle \tau \rangle = \sum_{i=1}^{n} \alpha_i \tau_i^2 \Big/ \sum_{i=1}^{n} \alpha_i \tau_i. \tag{2}$$

Data were presented as means $\pm$ S.D. of at least five independent measurements in each case. Statistical significance was determined by the unpaired t-test using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA), with $P < 0.05$ considered as statistically significant difference.

3. Results

3.1. *Autofluorescence spectra of leukemic myeloid cells and MNC*

The typical EEM profiles of K562, HL60 cells, and MNC measured at the concentrations of 1×10^6 cells/mL are shown in Fig. 1. The plots were shown on a log contour scale, where each contour corresponded to levels of equal fluorescence intensity. The results showed that the primary autofluorescence peaks were observed at excitation–emission wavelength pairs of 290–335, 340–445, and 460–545 nm, respectively. According to previous studies,[14,15] these fluorescence peaks were attributed to fluorescence emission from tryptophan, NAD(P)H and flavin adenine dinucleotide (FAD), respectively. The same three peaks were observed in EEMs of all types of cell lines. Although the maximal emission peak was generated under the excitation of 340 nm, the emission profiles obtained under 340 nm and 405 nm were similar ($\lambda_{em} = 445$ nm), the line of 405 nm pulsed laser was used for obtaining the time-resolved autofluorescence because of its availability.

3.2. *Time-resolved autofluorescence of NAD(P)H in leukemic myeloid cells and MNC*

The representative fluorescence decay curves of leukemic myeloid cells and MNC are shown in Fig. 2. According to previous studies,[8,11,12] the lifetime of intracellular NAD(P)H could be resolved by three lifetime components model, in which the short lifetime is attributed to free NAD(P)H, and

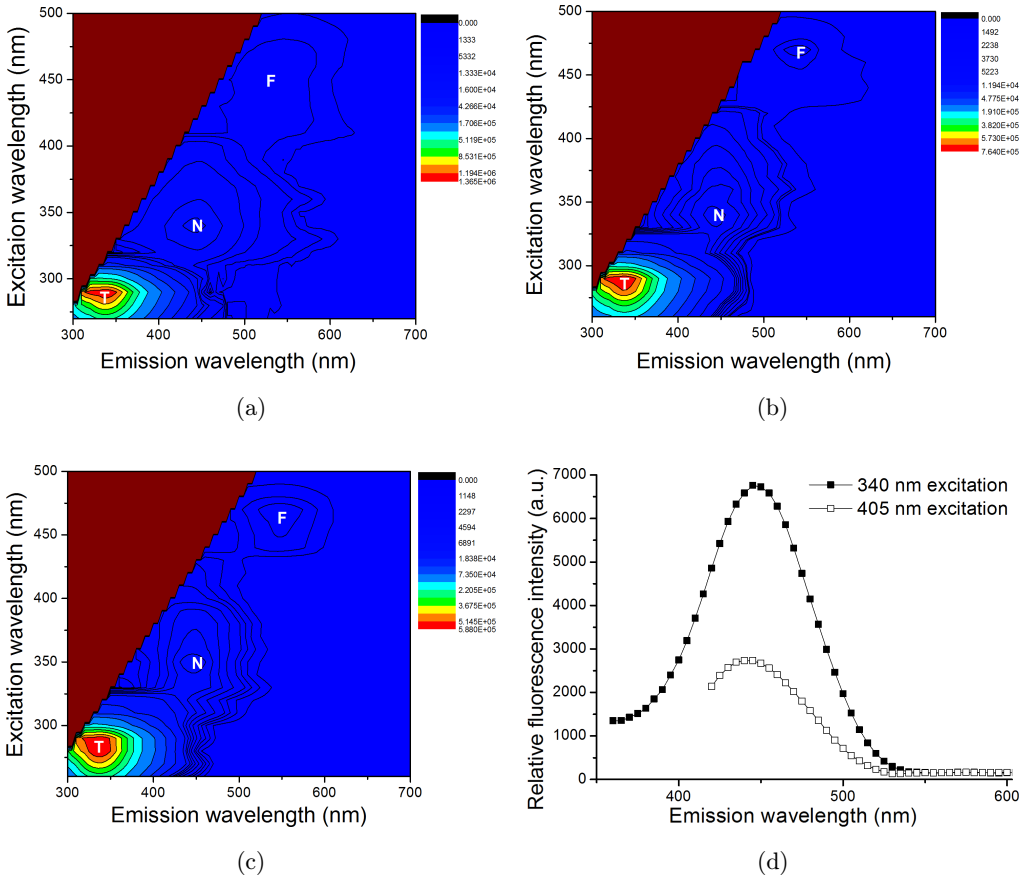

Fig. 1. Autofluorescence EEMs of (a) K562, (b) HL60 cells, (c) MNC and (d) two examples of the special cases ($\lambda_{ex} = 340$ and 405 nm) from MNC. Autofluorescence peaks "T", "N", and "F" are attributed to tryptophan, NAD(P)H, and FAD, respectively.

the intermediate and long lifetimes to protein-bound NAD(P)H. The time-resolved data were fitted well by a triple-exponential decay model, with the chi-square (χ^2) closed to 1.0. In this case, three

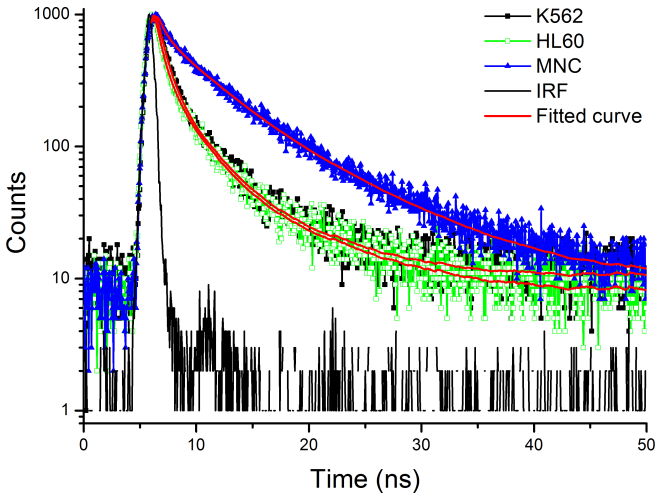

Fig. 2. Time-resolved autofluorescence spectra of K562, HL60 cells, and MNC ($\lambda_{ex} = 405$ nm and $\lambda_{em} = 455$ nm).

different pools of NAD(P)H lifetimes were determined for leukemic myeloid cells and MNC, respectively.

The lifetimes of each pool (τ_i), the corresponding relative amplitudes (α_i) and mean lifetimes for leukemic myeloid cells and MNC are listed in Table 1. Lifetimes were presented as means ± S.D. of five independent fitting results for each type of cells. First, the mean lifetimes of K562, HL60 cells, and MNC were 4.45 ± 0.71, 5.57 ± 1.19, and 7.31 ± 0.60 ns, respectively. The unpaired t-test result showed that there was a statistical difference in the mean lifetimes between leukemic myeloid cells and MNC ($P < 0.05$). However, there was no significant difference between the mean lifetimes of K562 and HL60 cells ($P > 0.1$). Secondly, three different pools of NAD(P)H lifetimes (τ_1, τ_2, and τ_3) were resolved for leukemic myeloid cells and MNC. The short, intermediate, and long lifetimes (τ_1, τ_2, and τ_3) ranged from 0.56 to 0.66, 2.85 to 3.91, and 10.51 to 12.62 ns, respectively. No significant difference was found in each lifetime pool

Table 1. Lifetime data determined by fitting the time-resolved autofluorescence spectra ($n = 5$).

	Lifetimes (ns)			Corresponding relative amplitudes				
Cell type	τ_1	τ_2	τ_3	α_1	α_2	α_3	Mean lifetime (ns)	χ^2
K562	0.66 ± 0.06	2.85 ± 0.51	12.62 ± 2.54	0.69 ± 0.05	0.25 ± 0.02	0.06 ± 0.03	4.45 ± 0.71	<1.27
HL60	0.56 ± 0.06	3.10 ± 0.72	10.51 ± 2.41	0.70 ± 0.03	0.24 ± 0.01	0.06 ± 0.02	5.57 ± 1.19	<1.21
MNC	0.56 ± 0.15	3.91 ± 0.42	11.99 ± 2.23	0.49 ± 0.07	0.37 ± 0.09	0.14 ± 0.05	7.31 ± 0.60	<1.10

between leukemic myeloid cells and MNC ($P > 0.05$). Finally, there was a significant difference in the relative amplitudes (α_i) between leukemic myeloid cells and MNC ($P < 0.05$), which indicated that there was an increase in α_1 and a decrease in both α_2 and α_3 in leukemic myeloid cells, as compared with that in MNC (see Table 1).

4. Discussion

Time-resolved autofluorescence has been used as a rapid and label-free optical method to characterize normal and malignant cells.[4,5] In this study, the triple-exponential decay model was used to fit the measured time-resolved autofluorescence spectra of leukemic myeloid cells and MNC. The mean fluorescence lifetimes of K562, HL60 cells, and MNC were 4.45 ± 0.71, 5.57 ± 1.19, and 7.31 ± 0.60 ns, respectively (see Table 1). A statistical difference in the mean lifetime was observed between MNC and K562 cells and between MNC and HL60 cells ($P < 0.05$). However, there was no significant difference in τ_1, τ_2, and τ_3 values between leukemic myeloid cells and MNC. The decrease in the mean lifetime in leukemic myeloid cells was mainly determined by the changes in α_i of leukemic myeloid cells. In this case, α_1 was increased but α_2 and α_3 decreased. Overall, α_1 and α_3 were more dominant in the changes between leukemic myeloid cells and MNC (see Table 1).

In general, the fluorescence lifetime of NAD(P)H in living cells could be resolved in three different pools: short lifetime (e.g., 0.4 to 0.6 ns) is attributed to free NAD(P)H, and intermediate (e.g., 2.0 to 3.65 ns) and long lifetime (6.0 to 15.0 ns) to protein-bound NAD(P)H.[8–11] Previous studies show that the relative amplitude (α_i) is a good proxy for the relative concentration of the corresponding lifetime pool (τ_i).[16–18] Specifically, α_1 represents the relative concentration of free NAD(P)H, and α_2 and α_3 represent the relative concentration of the protein-bound NAD(P)H.[16] Therefore, the decrease of the mean lifetime of NAD(P)H in leukemic myeloid cells observed in this study was attributed to the change of relative concentration of free and protein-bound NAD(P)H.

The increase of free NAD(P)H component and decrease of protein-bound NAD(P)H component in human cervical carcinoma cells has been reported.[5] Based on the analysis of steady-state autofluorescence spectra that implemented by using Gaussian deconvolution, Monici *et al.* have previously showed that in the HL60 cells the equilibrium between free and protein-bound NAD(P)H could shift towards the free form in comparison to the normal blood cells.[19] In this study, similar results were independently demonstrated by using the fluorescence lifetime measurement. The change of relative concentration of free and protein-bound NAD(P)H between normal and malignant cells was mainly related to the loss of binding sites for NAD(P)H in malignant cells.[20] The loss of binding sites for NAD(P)H in malignant cells could be related to their anaerobic metabolism nature.[8,21] The preliminary result indicates that the difference in the mean lifetime of NAD(P)H is attributed to the change of the intracellular conformation of NAD(P)H between leukemic myeloid cells and MNC, which could be used as a potential label-free indicator to characterize the differences between the leukemic myeloid cells and MNC. In the future work, the more subtypes of leukemic cells obtained from patients should be measured by time-resolved autofluorescence spectra. Furthermore, the relationship between these subtypes of leukemic cells and MNC should be further studied.

5. Conclusions

A significant difference in the mean autofluorescence lifetime of NAD(P)H was found between leukemic myeloid cells and MNC, which was mainly

attributed to the change in relative concentration of free and protein-bound NAD(P)H. The increase in relative concentration of free NAD(P)H could be related to the loss of binding sites for NAD(P)H in K562 and HL60 cells. The preliminary results suggest that the mean fluorescence lifetime of NAD (P)H might be used as a potential label-free indicator to characterize the differences between the human leukemic myeloid cells and MNC.

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