Fluorescence decay kinetics and imaging of NAD(P)H and flavins as metabolic indicators

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Abstract. The intrinsic fluorescence of various cell cultures in the blue and green spectral range has been attributed mainly to hydrated nicotinamide adenine dinucleotide (NADH) and flavin molecules. Their fluorescence decay curves were measured with subnanosecond resolution. The reduced coenzymes NADH and hydrated nicotinamide adenine dinucleotide phosphate NADPH, both showed a biexponential decay pattern in solution with similar time constants, but different relative intensities of the two components. They could thus be distinguished from one another as well as from their oxidized forms. The NAD(P)H fluorescence of *Saccharomyces cerevisiae* was located within the cytoplasm and its organelles and was by about a factor 4 higher for respiratory-deficient than for intact yeast strains. Intracellular flavin fluorescence showed a triexponential behavior—probably due to a superposition of protein-bound and free flavin molecules. The lifetime of the shortest component varied within the time range of 0.20 to 0.50 ns between respiratory-deficient and intact yeast strains, and the relative intensity of this component was most pronounced for the intact strain DL1. Time-resolved fluorescence seems therefore to be an appropriate method to probe the function of the respiratory chain and—in the further step—to differentiate between various types of cells and tissues in medical diagnosis or environmental research.

Subject terms: biomedical optics; intrinsic fluorescence; metabolic indicators; flavins; picosecond kinetics; microscopy.

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1 Introduction

Fluorescent coenzymes such as hydrated nicotinamide adenine dinucleotide (NADH) or flavin molecules are very sensitive bioindicators of metabolic functions such as the degradation of glucose or respiration. Although their fluorescence properties in cells and solutions are well documented,[1–5] their application in medical diagnosis so far has been limited,[6–9] and almost nothing has been reported about their use in the environmental field.

In previous articles, the fluorescence intensities of yeast strains[10,11] and human fibroblasts[12] were correlated with the function of the mitochondrial respiratory chain. For respiratory deficient cells, the intensity of NADH emission was a factor of 2 to 4 higher, and that of flavin emission up to a factor of 2 higher, than for respiratory intact cell lines. The purpose of the present paper is

- to visualize the intracellular intensity distributions and correlate them with the substructures of the cells
- to measure the fluorescence decay patterns after excitation by ultrashort laser pulses, because fluorescence lifetimes are very sensitive parameters for the states of oxidation or intracellular binding. In addition, time-resolved spectroscopy might be an appropriate method to differentiate between NADH and hydrated nicotinamide adenine dinucleotide phosphate NADPH that show the same fluorescence spectra, but are involved in different metabolisms. In those cases where NADH and NADPH could not be differentiated, the abbreviation "NAD(P)H" was used for both coenzymes.

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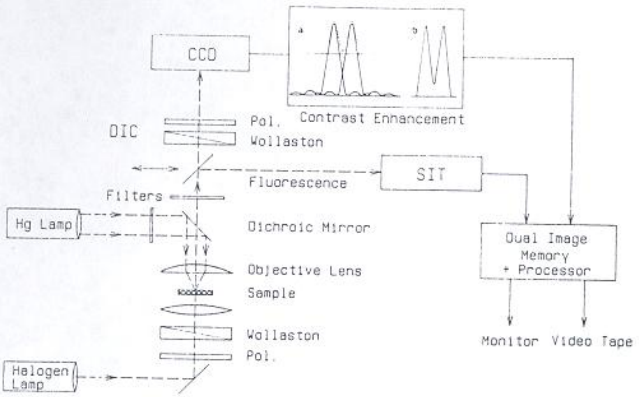

Fig. 1 Combined DIC and fluorescence microscopy: (a) Resolution and contrast enhancement by raising the threshold of the CCD camera and (b) detecting only the high-intensity part of the diffraction patterns. Beamsplitter and filters are removable.

2 Materials and Methods

2.1 *Materials*

Aqueous solutions of NADH and NADPH (10^{-3} M) as well as their oxidized forms NAD and NADP (10^{-1} M) were prepared from their sodium salts (Fluka, Neu-Ulm). The concentrations of the reduced forms were in the range, those of the oxidized forms were considerably above the physiological concentrations in tissue.[9] Aqueous solutions of riboflavin monophosphate (identical to oxidized flavin mononucleotide, in the following abbreviated as FMN, Fluka) were prepared at concentrations between $2 \cdot 10^{-4}$ M and 10^{-3} M.

Respiratory deficient (MK2, MK20, GDVO) and intact strains (DL1, D273/10B) of *Saccharomyces cerevisiae*, which were described in a previous article,[10] were measured as suspensions (time-resolved spectroscopy) or monolayers (microscopy) in the spectral ranges of NAD(P)H and flavin emission.

2.2 *Experimental Methods*

Combined differential interference contrast (DIC) and fluorescence microscopy were used to visualize the substructures and fluorescence intensity distributions of individual yeast cells (Fig. 1). Because the diameter of single cells was only about 5 μm, the resolution and the contrast of the CCD camera (Sony) were enhanced by raising the offset of the camera and spreading the intensity range (DIC images, Fig. 1). In comparison with the Rayleigh criterion, the resolution was thus improved by about a factor of 2, and objects of less than 200 nm in diameter could be visualized.[13] The fluorescence was excited by a high-pressure mercury lamp and detected using a dichroic mirror, a long-pass filter, and a highly sensitive SIT camera (Hamamatsu Photonics, C 2400-08). Excitation wavelengths of 365 and 436 nm were used for the NAD(P)H and flavin molecules, respectively. The cutoff wavelength of the long-pass filter was 410 nm for NAD(P)H and 470 nm for flavin emission. Fluorescence and DIC images were integrated and stored in a dual image processor (Hamamatsu, C1901 Mark II).

Fluorescence decay kinetics were measured using a novel frequency doubled laser diode (Hamamatsu, LDH 038, wavelength 390 nm, pulse duration 40 ps, repetition rate adjusted to 1 MHz) and a single photon-counting device, as demonstrated in a previous paper.[11] Both NAD(P)H and flavin molecules were excited simultaneously within the cells. From the broad and overlapping emission bands,[11] the spectral range of 450 ± 20 nm was selected for the detection of NAD(P)H, and 500 ± 20 nm was selected for the detection of flavins by appropriate interference filters (Schott Glass Technologies Inc.).

3 Results and Discussion

Figure 2 shows the DIC and fluorescence images of the respiratory-deficient yeast strain GDVO and the intact strain D273/10B. The nucleus can be identified as a trough in the DIC images. Both the nucleus and cytoplasm show some substructures that may be attributed to nucleoli, and to mitochondria or lysosomes, respectively. Fluorescence mainly arises from the cytoplasm, and it remains the subject of further investigations as to whether there is some preferential emission from the mitochondria. The figure demonstrates that the fluorescence of the defective strain GDVO is considerably higher than that of the intact strain D273/10B. The NAD(P)H fluorescence of the respiratory-deficient strains MK2 and MK20 was similar to GDVO and about a factor of 4 higher than for the intact strains DL1 and D273/10B (as verified by pixel integration over individual cells). Flavin emission (excited at 436 nm) was lower than NAD(P)H fluorescence, and the differences between respiratory-deficient and intact strains were less pronounced.

Figure 3 shows the fluorescence decay curves of reduced NAD(P)H and oxidized NAD(P) in a semilogarithmic scale. The reduced forms can be identified from their biexponential behavior with the time constants $T_1 = 0.5$ to 0.6 and $T_2 = 1.5$ to 2.0 ns. (In the literature,[4] only one component with about 0.4 ns has been reported so far.) The relative intensity of the long-lived component is about 10% for NADH and 25% for NADPH. In principle, it is therefore possible to differentiate between these reduced coenzymes according to their decay patterns. The decay times obtained for the oxidized forms are different (see Table 1), but the fluorescence of these forms only plays a role, if their concentrations are by a factor of about 100 higher than those of the reduced coenzymes. The fluorescence decay curves of all strains of *Saccharomyces cerevisiae* measured at 450 ± 20 nm showed a triexponential decay pattern with lifetimes of $T_1 = 0.20$ to 0.30, $T_2 = 1.4$ to 2.4, and $T_3 = 6$ to 8 ns. Only T_2 is in agreement with NAD(P)H solutions, whereas T_1 is by a factor of 2 shorter. This difference, as well as the occurrence of the long-lived component, may result from the binding of NAD(P)H molecules to proteins with a concomitant change of the fluorescence quantum yield.[4] In addition, a contribution of flavin fluorescence in this spectral range cannot be excluded.

Aqueous solutions of flavin mononucleotide showed a monoexponential decay (Fig. 4) with a time constant of 5.2 ns. Similar decay times have been reported for other flavin species[14] that occur in cellular and particularly in mitochondrial metabolism. In contrast to this, the fluorescence decay curves measured for all strains of *Saccharomyces* at 500 ± 20 nm were triexponential (Fig. 4) with lifetimes of

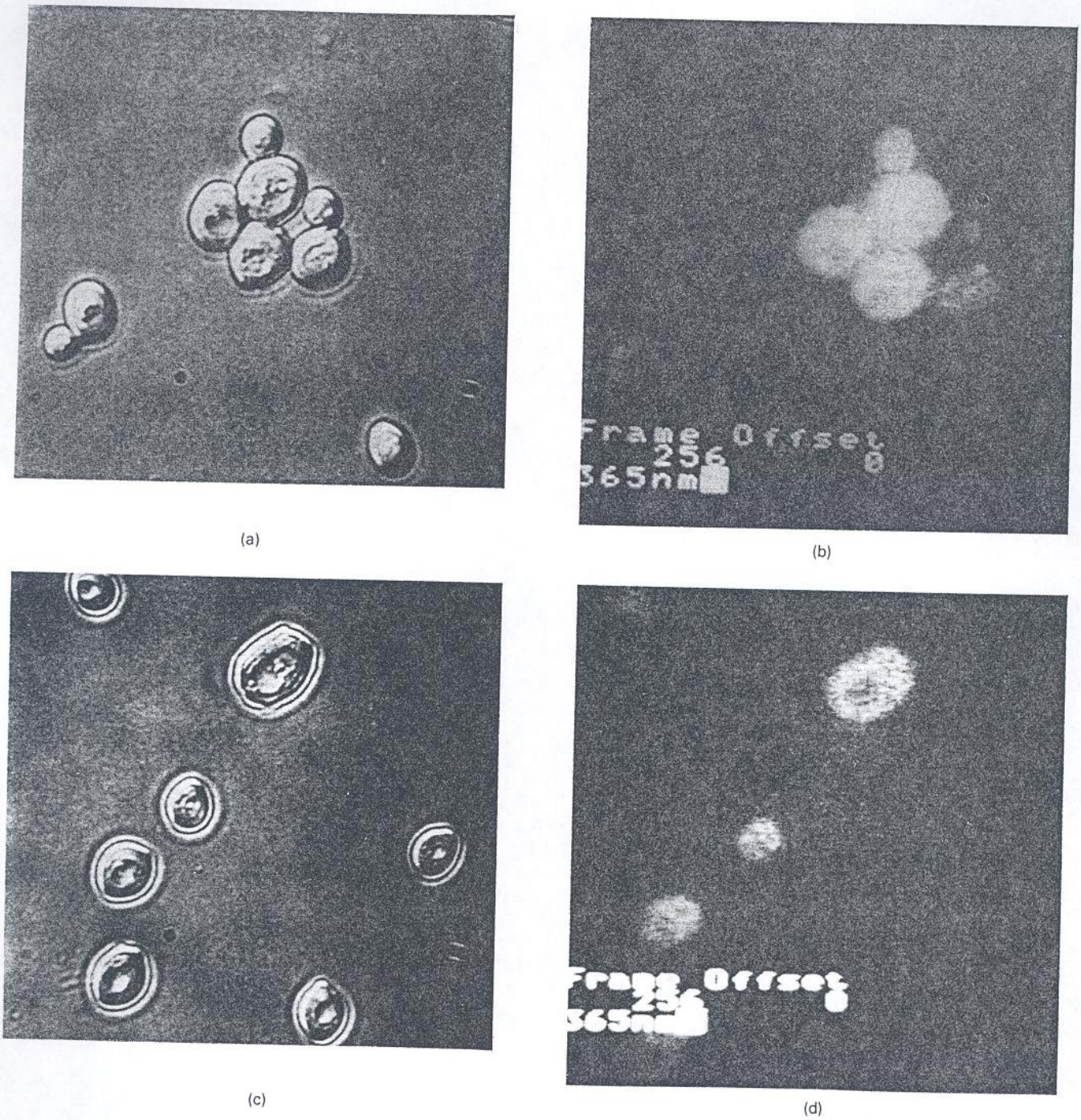

Fig. 2 (a) DIC and (b) fluorescence images of respiratory-deficient cells of the strain GDVO of *Saccharomyces cerevisiae*, (c) DIC and (d) fluorescence images of intact cells of the strain D273/10B. Fluorescence excitation at 365 nm, detection range 410 to 800 nm, and image size 60×60 μm (DIC) and 45×45 μm (fluorescence).

$T_1 = 0.20$ to 0.50, $T_2 = 2.0$ to 3.0, and $T_3 = 6$ to 8 ns (Table 1). Only the long-lived component may be attributed to free flavin molecules, whereas the shorter components seem to arise from flavoproteins[15,16] [and possibly from a small amount of superimposed NAD(P)H fluorescence]. The fluorescence lifetime of the short-lived component was lower for the respiratory intact strains DL1 and D273/10B ($T_1 = 0.20$ ns) than for the defective strains MK2, MK20, and GDVO ($T_1 = 0.36$ to 0.50 ns); the relative intensity of this component was highest for DL1 (47%) compared with all other strains (31 to 39%). This indicates that the ratio of free/protein-bound flavin molecules depends on the function of the respiratory chain. In medical diagnosis, the time-resolved detection of flavin fluorescence therefore may be used to differentiate between various types of tissue (e.g., tumor/surrounding host tissue). In the environmental field, intact

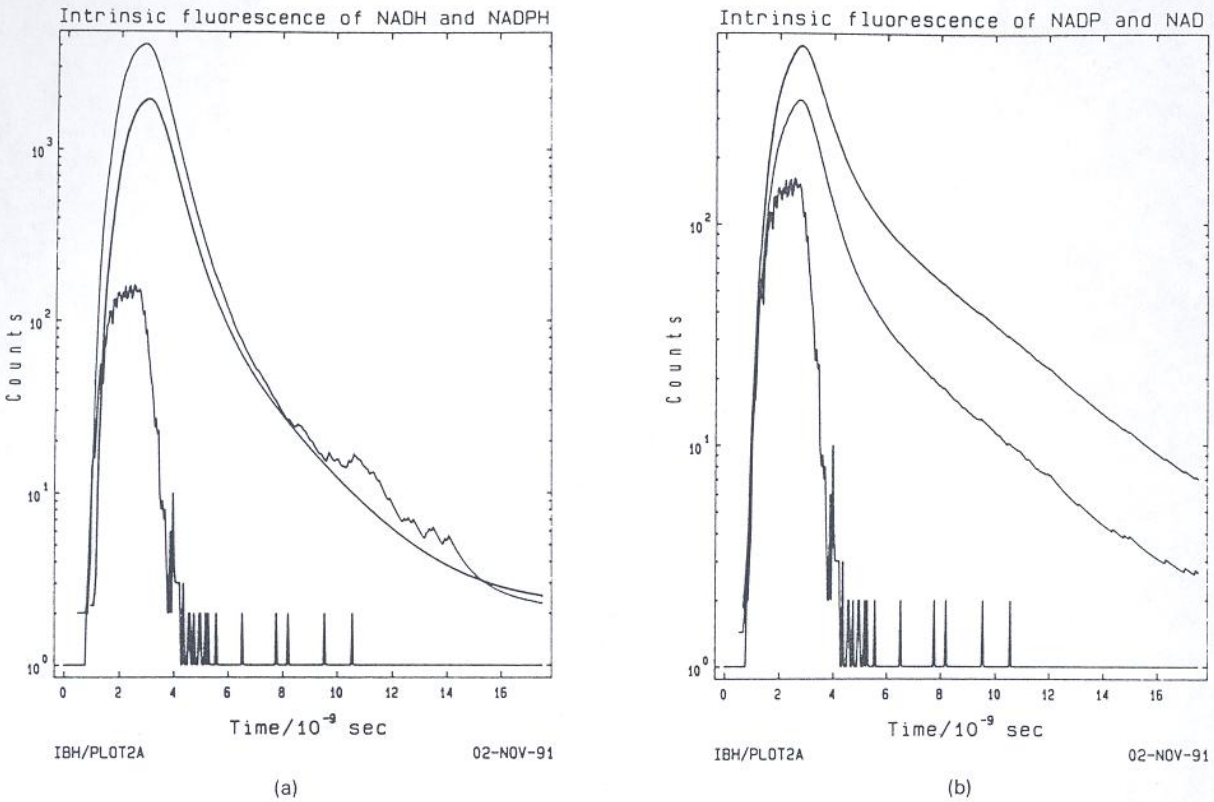

Fig. 3 (a) Fluorescence decay curves of the reduced coenzymes NADH (upper curve) and NADPH (lower curve) at a concentration of 10^{-3} M in aqueous solution; emission measured at 450 ± 20 nm. (b) Fluorescence decay curves of the oxidized coenzymes NADP (upper curve) and NAD (lower curve) at a concentration of 10^{-1} M in aqueous solution; detection range as in 3(a). The response curve of the apparatus is included in the figure (lowest curve).

Table 1 Fluorescence lifetimes (T_i) and relative intensities (I_i); emission measured at 450 ± 20 nm or 500 ± 20 nm, respectively.

		T_1 (ns)	I_1 (%)	T_2 (ns)	I_2 (%)	T_3 (ns)	I_3 (%)
NAD	(450 nm)	0.55-0.65	65	3.5-4.5	35	--	--
NADP	(450 nm)	0.60-0.70	50	4.0-4.8	50	--	--
NADH	(450 nm)	0.50-0.60	90	1.4-2.0	10	--	--
NADPH	(450 nm)	0.45-0.60	75	1.4-2.2	25	--	--
Saccharomyces cerevisiae	(450 nm)	0.20-0.30	30	1.4-2.4	40	6.0-8.0	30
Saccharomyces cerevisiae	(500 nm)	0.20-0.50 (*)	32-47 (*)	2.0-3.0	26-34	6.0-8.0	32-40
FMN	(500 nm)	--	--	--	--	5.2	100

(*) Lower time constants and higher relative intensities for intact than for respiratory deficient strains

and defective organisms may be distinguished, as shown in preliminary measurements of needles from intact and damaged spruces.[17]

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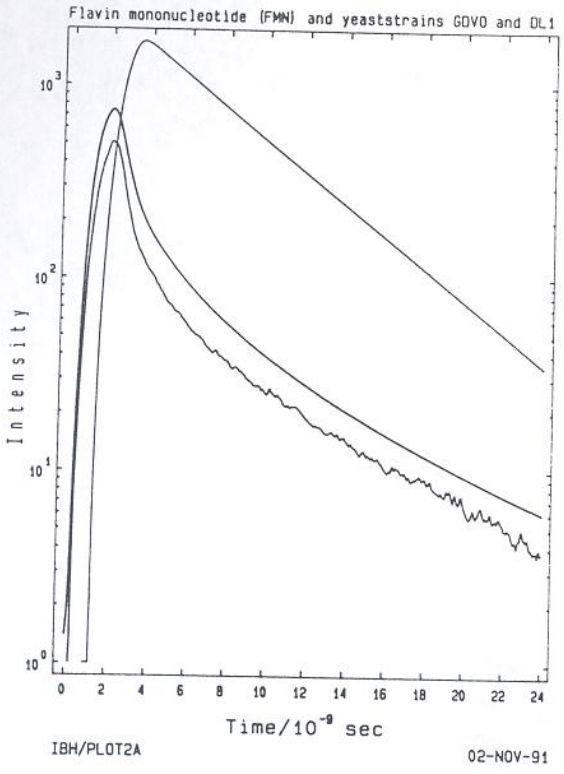

Fig. 4 Fluorescence decay curves obtained from FMN ($2 \cdot 10^{-4}$ M) in aqueous solution (upper curve) and *Saccharomyces cerevisiae* (middle curve—respiratory-deficient strain GDVO; lower curve—intact strain DL1); emission measured at 500 ± 20 nm.

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