Effect of Fixation and Mounting on Fluorescence Lifetime of Cellular Autofluorescence

Xinyi Wang, Yonghui Xie, Maojia Huang, Longfang Yao, Yulan Wang, Yiyan Fei, Jiong Ma, and Lan Mi

***Abstract*—Fluorescence lifetime measurements are often performed on live as well as fixed cells and tissues. Fixation and mounting processes are routinely used in cellular research or clinical diagnosis. In this paper, the effects of fixation and mounting on the fluorescence lifetime of cellular autofluorescence were studied by fluorescence lifetime imaging microscopy over time. Two endogenous fluorescent fluorophores, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), showed different results between live cells and fixed cells. The average lifetime of NADH in live HeLa cells was about 1.02 ns, while maintained about 1.57 ns during the fixation periods of 14 days. The average lifetimes of FAD in live and fixed HeLa cells within 11 days were similar around 1.75 ns but increased to 2.10 ns after 12 days. The free and bound states of the two kinds of fluorophores were further analyzed. It was found that the bound-FAD had two different groups, which was related to the cell division cycle. The effect of mounting medium on fluorescence lifetimes was also studied, indicating glycerol has a negative impact on the fluorescence lifetime compared with neutral balsam.**

***Index Terms*—Autofluorescence, fixed cells, fluorescence lifetime, mounting medium.**

I. Introduction

FLUORESCENCE lifetime imaging microscopy (FLIM) is a noninvasive tool to analyze the metabolic properties of cells and tissues [1], [2]. The pathways of cellular energy metabolism are mainly glycolysis and oxidative phosphorylation, which can be monitored basing on the primary endogenous fluorophores, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) [3]. Cellular metabolic imaging is based on the fact that the fluorescence lifetimes of NADH and FAD in free and protein-bound states are quite different. Bound-NADH and bound-FAD lifetimes depend on the binding-protein and vary in a range of several hundred picoseconds [4]. There are a number of works devoted to the analysis of their lifetime changes and the relative percentage contribution of free and bound states.

Based on the metabolic status of cells and tissues, FLIM were used to evaluate oncological processes in tumor cells [5], [6], stem cell differentiation [7]–[9], oxidative stress [10], differential diagnosis of tissues on breast cancer [11], lung cancer [12], and cervical cancer [13]. These fluorescence measurements were often performed on fixed cells [5], [6], [10] as well as fixed tissues [11]–[13] since the easier type of samples we can access in most medical research were fixed relative to fresh excision tissues or biopsies.

Fixation and mounting is a routine procedure in cell biology and pathological research [14], which provides good tissue and protein preservation for a relatively long time as long as 2 weeks. But fixation may cause change of fluorescent signals and the microenvironments of the endogenous fluorophores [15]. Unfortunately, the effect of the procedure on fluorescence lifetime of the metabolic cofactors NADH and FAD has been poorly studied to date. Ganguly *et al.* reported the fluorescence lifetime of EYFP tagged with serotonin$_{1A}$ in CHO cells reduced in fixed cells than live cells and fixation also has a drastic effect on the fluorescence anisotropy [16]. Joosen *et al.* showed that the fixation by formaldehyde or methanol did not affect the fluorescence lifetime of GFP variants but several mounting media decreased the lifetime by up to 20% [17]. In both studies [16], [17] the fixation and mounting procedure reduced the fluorescence lifetime. But Poulon *et al.* revealed that the fluorescence lifetimes of NADH and FAD in fixed samples were longer than those of fresh samples [18]. Therefore, details on the changes of endogenous fluorophores and their free or protein-bound states need to be further studied.

In this work, the effects of the fixation and mounting processes on the fluorescence lifetime of NADH and FAD were examined. The free and protein-bound states of the two kinds of fluorophores were analyzed for both live and fixed cells over time.

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II. Materials and Methods

A. Cell Culture

The human cervical cell line (HeLa) cells or human pulmonary adenocarcinoma (A549) cells were grown on 20 mm round coverslips in 12-well plates or in Petri dishes. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA) and A549 cells in RPMI-1640 medium (Roswell Park Memorial Institute 1640, Gibco; New York, NY, USA) with 10% (v/v) fetal bovine serum (Sijiqing Inc., Hangzhou, China) in a fully humidified incubator at 37 °C with 5% CO_2 until reaching 80% confluence. Live cells in Petri dishes were imaged directly, and cells on coverslips were observed after fixation.

To control the cell cycle, cells were incubated with 0.8 μg/ml colchicine (≥95%, Genview) for 7 h or by serum starvation for 20 h before observation to block the cell cycle in M-phase [19] or G1-phase [20], respectively. The untreated cells were set as the control group.

B. Fixation and Mounting

Fixation was performed by incubating the cells with 4% (v/v) paraformaldehyde for 10 min. Cells were then washed by phosphate buffered saline (PBS) for three times and mounted with neutral balsam (Aladdin), or 56% (v/v) glycerol. Fluorescence measurement was applied after fixation and mounting 1 to 14 days. The only exception was the fixed cells without mounting, which were stored in PBS, measured immediately after fixation and denoted as 0 day in the following.

C. Fluorescence Lifetime Imaging Microscopy (FLIM)

Fluorescence lifetime images of live or fixed cells were acquired by a single photon counting module on a laser scanning confocal microscope (Olympus, FV300/IX 71, Japan) equipped with two picosecond lasers (BDL-405-SMC and BDL-488-SMN, Becker & Hickl, Berlin, Germany) and a water immersion objective (60×, NA = 1.2). The fluorescence signal of NADH was excited by the 405 nm laser and obtained with a band-pass filter of 447 ± 30 nm. The fluorescence of FAD was excited by 488 nm and detected with a 496 nm long-pass filter. The lifetime signal was detected by a photomultiplier tube (PMC-100-1, Becker & Hickl, Berlin, Germany) and processed by a time-correlated single photon counting (TCSPC) system (SPC-150, Becker & Hickl, Berlin, Germany). As reported previously [13], synchronized fluorescence lifetime data collection was achieved on a pixel-by-pixel basis by using the *x* and *y* laser scanning signals generated by the confocal scan unit. Each FLIM image of 256 × 256 pixels was acquired in 90–120 sec and 8–12 different areas were imaged for each sample. Altogether 100–120 cells were analysed for each statistical scatter point. Every experiment was conducted and measured independently at least three times. Every area was imaged only once to avoid photobleaching.

D. Data Analysis

Time-decay data of each pixel was fitted with multi-exponential decay models using the commercial SPCImage software package (Becker & Hickl GmbH, Berlin, Germany). Based on the fitting, the weighted mean lifetime of each pixel is calculated as $t_m = \sum_{i=1}^{n} a_i t_i$, where t_i is the lifetime of the fluorescent component, a_i is the corresponding amplitude of the exponential component. The goodness-of-fit χ^2 values were used to evaluate the fitting. To clarify how many exponential components should be used, both the double-exponential and three-exponential models were tried. It was found that double-exponential function was a better fit for both NADH and FAD with χ^2 values closer to one. As shown in Supplemental Figure S1, fitting the data with two components delivered a perfect χ^2 value of 1.00, but fitting with three components delivered the χ^2 value of 0.99. Moreover the third lifetime component is extremely long and with a low contribution. So in this study, the decay curves were fitted by a double-exponential model as a number of works reported in [8], [13], [20], where the short and long lifetime components (t_1 and t_2) and their relative contributions (a_1 and a_2, $a_1 + a_2 = 1$) were estimated.

Among the components, the lifetimes of free NADH (fast component t_1 for NADH) and free FAD (slow component t_2 for FAD) were fixed as 0.58 ns and 2.90 ns. The two lifetime values were obtained by measuring the aqueous solution of NADH (7.5 mM, Sigma) and FAD (0.75 mM, Aladdin) respectively. Since the fluorescence of NADH or FAD is a combination of free and bound states, one component of free state can be fixed as the measured values for analyzing and the other component demonstrated the bound states. In this work, the χ^2 values were 1.3 ± 0.3 and the residuals showed no noticeable systematic variations.

III. Results

A. Effect of Fixation Period on Fluorescence Lifetime

Fig. 1(a) and (b) showed the FLIM images of NADH and FAD in live or fixed HeLa cells mounted with neutral balsam. As the statistical curves shown in Fig. 1(c), the average lifetime of NADH in live cells was about 1.02 ns and rose up to 1.46 ns after fixation by paraformaldehyde, and then maintained about 1.57 ns during the fixation periods of 1–14 days. While the average lifetime of FAD in live cells was 1.53 ns and increased to 1.65 ns when fixed by paraformaldehyde, then stayed around 1.76 ns for 11 days. The average lifetime of FAD of fixed cells had a sharp rise of 0.34 ns since the 12th day. The results indicated that fixation with paraformaldehyde and mounting with neutral balsam had a weak effect on the fluorescence lifetime of NADH in two weeks, whereas the processes had a significant influence on FAD after 12 days fixation. Similar results were observed for A549 cells (see Supplemental Figure S2).

B. Bound-NADH and Bound-FAD

The lifetime of bound-NADH (slow component t_2 for NADH) in live cells was 3.48 ns, and the contribution of bound-NADH (a_2 for NADH) was as low as 13%. The lifetimes of bound-NADH in fixed cells were much shorter than those of live cells, between 2.89 ns and 3.13 ns (Fig. 2(a)), and bound state contributed about 33–40% of all NADH in fixed cells within 14 days (Fig. 2(b)).

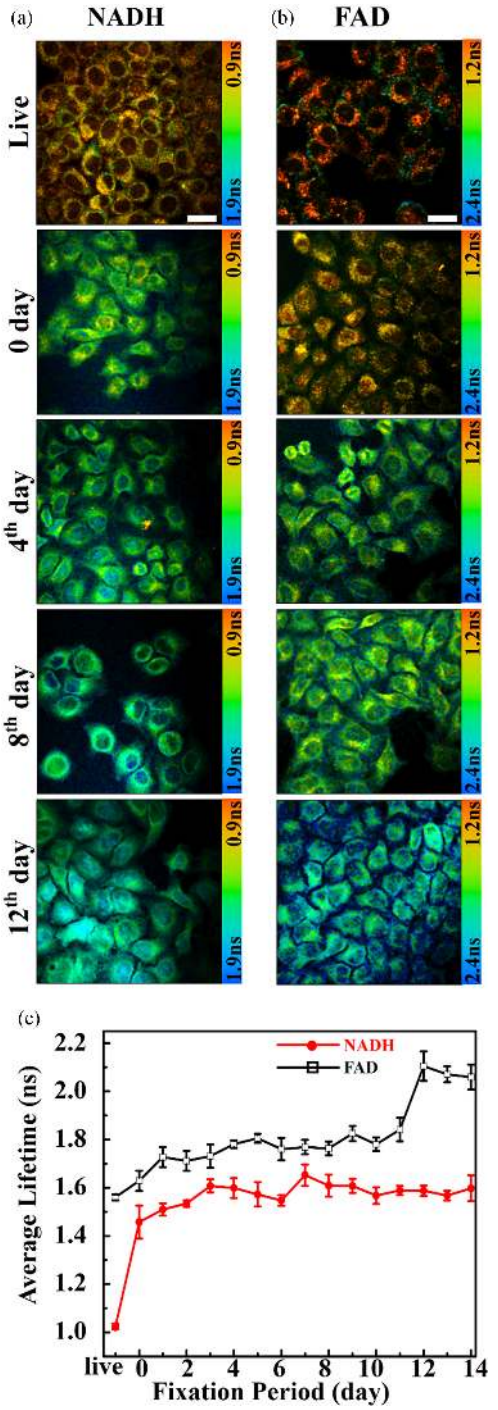

Fig. 1. FLIM images of (a) NADH and (b) FAD in HeLa cells, scale bar: 20 μm; (c) Average fluorescence lifetime of NADH (solid circle) and FAD (hollow square) in live cells or fixed cells mounted by neutral balsam with different fixation periods.

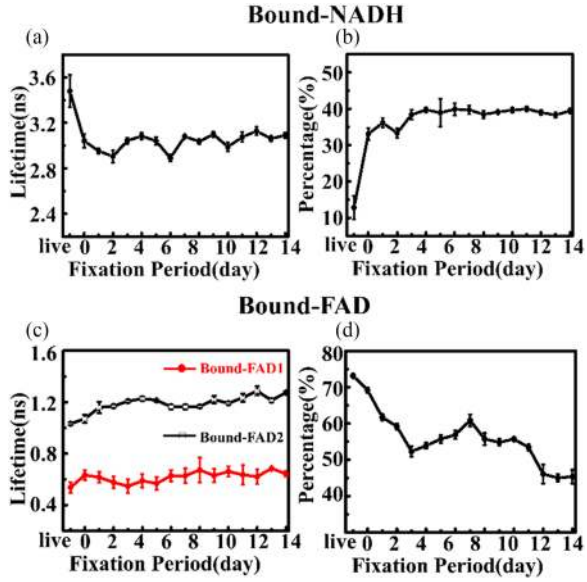

Fig. 2. Fluorescence lifetime of (a) bound-NADH and (c) bound-FAD in live or fixed HeLa cells with different fixation periods. Percentage of (b) bound-NADH and (d) bound-FAD in live or fixed cells.

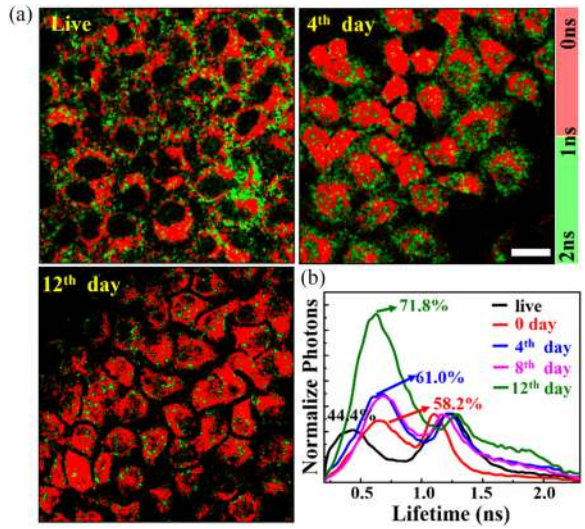

Fig. 3. (a) FLIM images of bound-FAD with two kinds of bound states in HeLa cells, scale bar: 20 μm. (b) The distribution curves of bound-FAD lifetimes in live or fixed HeLa cells after 0–14 days.

As for FAD, an example image showing the decay time of bound-FAD (fast component t_1 for FAD) was in Fig. 3(a). The lifetime histogram was presented accordingly in Fig. 3(b). The two peaks in Fig. 3(b) depicted two different groups of bound-FAD. Therefore t_1 for FAD could be separated into two groups, one below 1.0 ns and the other between 1.0 and 2.0 ns based on Fig. 3, denoted as bound-FAD1 and bound-FAD2. The two lifetime peaks were used to obtain the statistical scatter points in Fig. 2(c). One was around 0.62 ns and the other was around 1.20 ns (Fig. 2(c)). The lifetimes of both bound-FAD1 and bound-FAD2 increased marginally after fixation. The contribution of bound-FAD showed a rapid decrease from 73% in live cells to 45% after fixation for 14 days (Fig. 2(d)). It indicated that free FAD continued releasing from bound states after fixation. However, the average lifetime of FAD was persistent around 1.76 ns within 11 days (Fig. 1(c)), which could be resulted from the switch between the two bound-FAD types.

As exhibited in Fig. 3(a), the FLIM images of bound-FAD changed with the fixation period. The two types of bound-FAD were denoted bound-FAD1 and bound-FAD2, as red and green

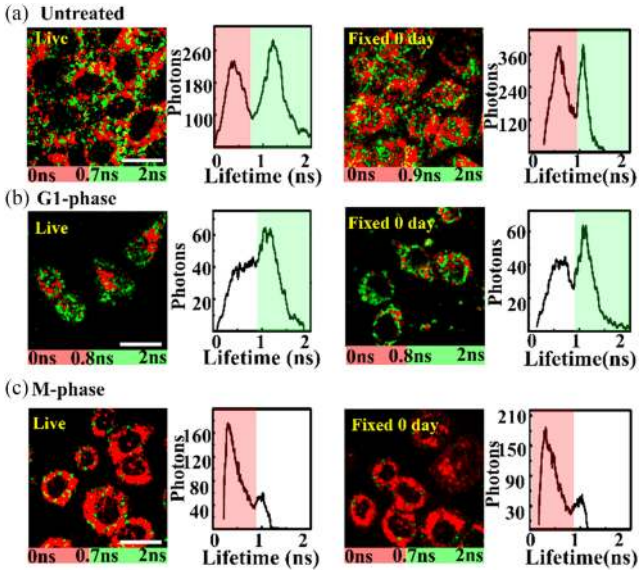

Fig. 4. FLIM images of bound-FAD in HeLa cells (a) control group, (b) in G1-phase, and (c) in M-phase. Scale bar: 20 μm.

colors in Fig. 3(a), whose distribution curves were shown in Fig. 3(b). For live cells, the contribution of bound-FAD1 was only 44.4%. The peaks of bound-FAD1 increased with time and its contribution was raised to 71.8% when stored for 12 days.

To further understand the switch between the two bound-FAD types, the cells were blocked in G1-phase or M-phase and compared with the untreated group (Fig. 4). The lifetime distribution of bound-FAD showed significant difference in G1-phase and M-phase. The bound-FAD2 with longer lifetime around 1.2 ns was dominating when the cells were in G1-phase, while in M-phase cells most bound-FAD was contributed by the bound-FAD1 with shorter lifetime around 0.6 ns.

C. *Effect of Mounting Media on Fluorescence Lifetime*

The results of cells mounted by glycerol were different compared with those of mounted by neutral balsam. As shown in Fig. 5, the average lifetimes of NADH and FAD in fixed HeLa cells when mounted by glycerol within 2 days was similar as those of cells mounted by neutral balsam. However, the lifetime fluctuation kept increasing since the 2nd day. This could be caused by the fluorescence of glycerol whose intensity was around one tenth of NADH signal when excited by the 405 nm laser. In contrast, the signal of neutral balsam was two orders lower than the NADH signal. Thus, neutral balsam is a better mounting medium than glycerol if the fixed cells need to be measured in a relatively long time.

IV. Discussion

A few studies had shown that fixation or mounting procedure could lead to the change of fluorescence lifetime [16], [17], [21], [22] as well as fluorescence intensity [18], [23], [24]. They observed enhanced fluorescence intensity in fixed samples than live or fresh samples [10], [23], [25]. Similar results were observed in this work. The fluorescence intensity of NADH in

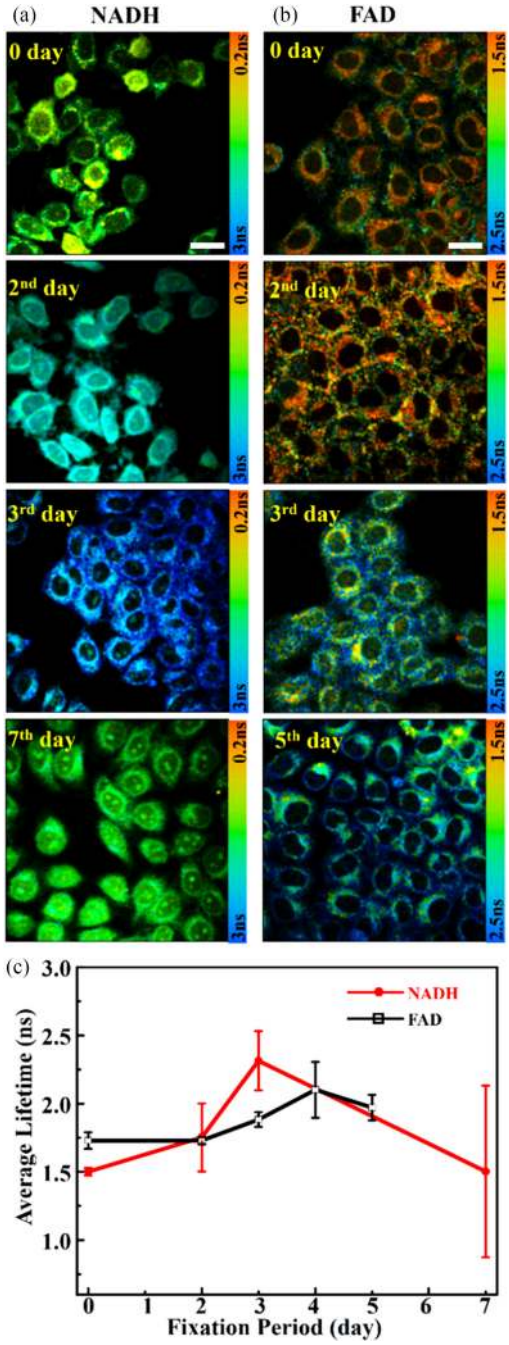

Fig. 5. FLIM images of (a) NADH and (b) FAD in fixed HeLa cells mounted by glycerol. Scale bar: 20 μm; (c) Average fluorescence lifetime of fixed cells mounted with glycerol within 7 days.

fixed cells was much higher than that of in live cells, while the intensity of FAD in fixed cells was a little higher than that of live cells.

In some reports [16], [17], the lifetimes of GFP variants decreased after fixation and mounting. Different conclusions was reported by Poulon *et al.* [18] in the study of endogenous fluorescence of human tissues. They found the fluorescence lifetime increased about 0.48 ns for NADH and 0.68 ns for FAD in fixed samples when excited by 405 nm. In this study, we observed increased fluorescence lifetimes of NADH and FAD in fixed and

mounted cells, similar with the recent report of [18]. Probably, the effects of fixation and mounting media vary on different fluorescent molecules. It is well known that fixation by cross-linking/aldehyde reagents, in this study like paraformaldehyde, creates a network of biomolecules by linking amino groups via intermolecular bridging [17]. Therefore the fixation by aldehyde reagents can have direct effect on fluorescent proteins, such as CFP and GFP [26]. NADH and FAD are both redox coenzymes, which involved in enzymatic reactions in metabolism as a prosthetic group of proteins. It was hypothesised that the cross-linking reagents had effect on proteins, as a consequence the lifetimes of NADH and FAD changed with the fixation effect on different proteins.

NADH in free state has shorter lifetime nearly 0.3 and 0.5 ns [27] and longer lifetime around 1.7–2.9 ns [28] in protein-bound state. The lifetime of protein-bound NADH varies with different binding proteins. For FAD, the terms of "free" and "bound" is rather traditional. The different lifetime of FAD is caused not by protein-binding, but two conformation of the cofactor molecule [28]. One is closed, when aromatic rings of isoalloxazine and adenine are in extremely close proximity, with shorter lifetime. The other conformation is open, when the two aromatic rings are extremely separated from each other, with longer lifetime about 2–3 ns [27], [28].

In this study, the mean lifetime of NADH was immediately enhanced 0.44 ns by paraformaldehyde and then could be maintained for at least 14 days in neutral balsam. As for FAD, the paraformaldehyde and neutral balsam did not have significant effect on the lifetime for about 11 days. The difference between NADH and FAD could be caused by the emitting sources. Both free NADH and protein-bound NADH have relatively high quantum yield and similar emission spectrum. The majority of FAD fluorescence is emitted by lipoamide dehydrogenase (LipDH)-containing enzyme complexes [28], [8]. Previous studies have noticed the fluctuation of the dehydrogenase enzymes expression which correlates with DNA synthesis during cell cycle of cultured HeLa cells [29]. When the amount of isoenzymes changes with the cell cycle, the fluorescence lifetimes of bound-FAD could also change. Besides LipDH-containing enzymes, FAD fluorescence is partially emitted by flavoprotein and sodium dithionite. Various emitting source may result in two types of bound-FAD.

It was found that the autofluorescence measurements of fluorescence lifetime could be acquired on FAD in both live cells and fixed cells. Among them, the fixed samples are easier to access and can be stored for no more than 11 days. On the contrary, concerning NADH studies, the fluorescence in fixed cells should be measured with caution. Our results can be used to aid the calibration lifetime of NADH if fixation and mounting procedures are inevitable.

We observed the switch of two bound types of FAD was related with the cell division cycle. FAD is mainly formed during oxidative phosphorylation, which can vary significantly in different metabolic status. FAD in different conformational states is very sensitive and is a good indicator for invasive measurements of tumor detection, and stem cell differentiation.

V. Conclusion

In conclusion, fixation and mounting showed different effect on the fluorescence lifetime of NADH and FAD. For NADH, the lifetime was immediately enhanced by paraformaldehyde and then could be steady for at least 14 days in neutral balsam. For FAD, the fixation and mounting by neutral balsam did not have significant effect on the lifetime in 11 days. Similar results were also observed for the lifetime of bound-NADH and bound-FAD. In addition, two bound-FAD types were observed, which were revealed a relationship with the cell replication and division.

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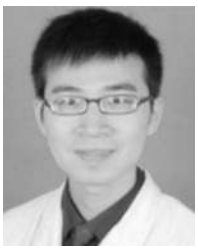

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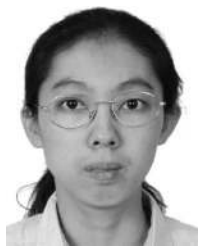

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