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Metal plasmon-coupled fluorescence imaging and label free coenzyme detection in cells

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ABSTRACT

Flavin adenine dinucleotide (FAD) is a key metabolite in cellular energy conversion. Flavin can also bind with some enzymes in the metabolic pathway and the binding sites may be changed due to the disease progression. Thus, there is interest on studying its expression level, distribution, and redox state within the cells. FAD is naturally fluorescent, but it has a modest extinction coefficient and quantum yield. Hence the intrinsic emission from FAD is generally too weak to be isolated distinctly from the cellular backgrounds in fluorescence cell imaging. In this article, the metal nanostructures on the glass coverslips were used as substrates to measure FAD in cells. Particulate silver films were fabricated with an optical resonance near the absorption and the emission wavelengths of FAD which can lead to efficient coupling interactions. As a result, the emission intensity and quantum yield by FAD were greatly increased and the lifetime was dramatically shortened resulting in less interference from the longer lived cellular background. This feature may overcome the technical limits that hinder the direct observation of intrinsically fluorescent coenzymes in the cells by fluorescence microscopy. Fluorescence cell imaging on the metallic particle substrates may provide a non-invasive strategy for collecting the information of coenzymes in cells.

1. Introduction

The coenzymes of reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) are key metabolites in cellular energy conversion [1,2]. Typically, the process occurs through an electron transfer within the mitochondrial pool, in which NADH and FAD may work as electron acceptor and donor, respectively, and some energy is saved in the form of two adenosine triphosphate (ATP) molecules during electron transfer to molecular oxygen. The coenzymes in cells are relevant to the occurrence of many diseases. For instance, compared with normal cells, cancer cells are known to have an increased metabolic demand because of faster cell division, so the tumor cells sometimes possess larger amounts of coenzymes [3]. In some cases, the coenzyme levels can be considered as biological markers for some diseases including tumors [4–6]. It is also known that coenzymes can bind with some enzymes in the metabolic pathway. The binding sites are altered by the disease progression [7,8]. Thus, the concentration and distributions of coenzymes within the cells are important for studying the mechanisms of diseases.

It is possible to estimate the amounts and map the distributions of coenzymes in cells by cellular autofluorescence because both NADH and FAD are naturally fluorescent [9,10]. This method may also offer an opportunity for a non-invasive measurement under label-free conditions. However, as intrinsic fluorophores, the coenzymes have relatively low extinct coefficients and quantum yields [11,12]. Hence, the emissions from the coenzymes are too weak to be discriminated from the cellular backgrounds that are arisen from cellular species and water scattering as well as the emissions from other intrinsic fluorophores in cells [13]. As a result, there is an essential need to develop a novel strategy for significantly increasing the emission signals by the coenzymes in the fluorescence cell imaging.

The use of near-field interactions of fluorophores with metal nanoparticles has progressed dramatically in recent years [14–16]. For the metal nanoparticles with subwavelength sizes, there are collective oscillations of free electrons on the surfaces induced by incident light [17,18]. These electron oscillation produced local electromagnetic fields around the metal nanoparticles are called plasmon resonances. If a fluorophore is localized nearby a

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metal nanoparticle within a near-field distance of about 50 nm, the emission by the fluorophore can be enhanced by 1–3 orders of magnitude due to the interaction of the fluorophore with the metal nanoparticle [19,20]. Enhanced optical properties by the metal nanoparticle have been widely used to develop bioassays with improved detection sensitivity of a wide range of biomedical and clinical analyses [21–24]. In the present paper, we used the near-field interactions with metal substrates to enhance the emissions from coenzymes in cells. Increased emission signals from the coenzymes due to the near-field interactions may provide an opportunity to isolate the emission of FAD from the cellular background in order to estimate their amounts and mapping their distributions in cells [25,26].

Our previous results reveal that because of low quantum yields, single FAD molecules could not be determined by fluorescence imaging on glass coverslips but the single molecule detection (SMD) becomes practical on the silver islands film (SIF) [27]. On the basis of the results, we incubated the cell lines on the silvered slides to exam the FAD fluorescence relative to the cellular backgrounds. Importantly, through the near-field interactions of FAD with plasmon resonances from the metal nanostructures can dramatically increase the radiative rate of fluorescence by the coenzymes leading to shortened fluorescence lifetime of FAD in cells [28,29]. Enhanced intensity and reduced lifetime of FAD in cells may greatly reduce the interference from the other-component cellular autofluorescence and improve the isolation of emissions by FAD in fluorescence microassays.

2. Experimental

All reagents and spectroscopic grade solvents were used as received from Fisher or Sigima/Aldrich. Nanopure water (>18.0 MΩ cm^{-1}) purified using Millipore Milli-Q gradient system, was used in all experiments.

2.1. Preparation of silver island films

The silver island films were fabricated as described previously on the glass coverslips by reduction of metal precursors with a mild reduction agent [27,30]. The absorbance of silver islands was in a range of near 0.2. To improve the chemical stability of metal nanoparticles and reduce their toxicity to the cultured cells, the metal island films on the glass coverslips were coated with the monolayers of the amino acid-like ligand 2-mercapto-propionylamino acetic acid 2,5-dioxo-pyrrolidin-1-ylester (tiopronin) [28,29].

2.2. Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and immobilized on the glass coverslips or SIF slides as described [31]. The cell lines were immobilized on glass coverslips or silver island films with the tiopronin monolayer coating. The immobilized cells were washed with 10 mM PBS buffer at pH 7.4, and dried in air for fluorescence cell imaging.

2.3. Spectra measurements

Ensemble absorption spectra of SIF substrates were recorded using a Hewlett Packard 8453 spectrophotometer. Fluorescence cell imaging measurements were recorded on a time-resolved confocal microscope (MicroTime 200, PicoQuant), which consists of an inverted confocal microscope coupled to a high-sensitivity optics and electronics. A single mode pulsed laser diode (470 nm, 100 ps, 10 MHz) was used as the excitation source. An oil

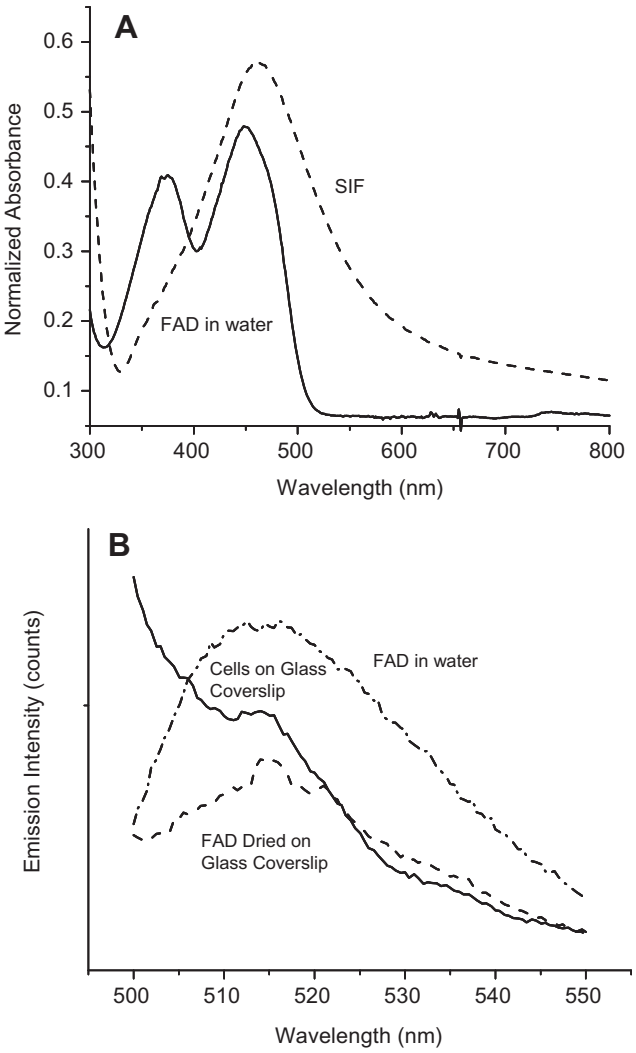

Fig. 1. (A) Absorption spectrum of extrinsic FAD in aqueous solution and plasmon resonance of silver island film on the glass coverslip. (B) Ensemble emission spectra of extrinsic FAD in aqueous solution, extrinsic FAD in PVA membrane on the glass coverslip, and dried HeLa cell sample on the glass coverslip. Ensemble spectra were determined upon excitation at 470 nm.

immersion objective (Olympus, 100×, 1.3 NA) was used to focus the laser beam on the sample and collect the emission from the sample. The emission signals passed a dichroic mirror and focused on a 75-μm pinhole for a spatial filtering and were recorded on a single photon avalanche diode (SPAD) (SPCM-AQR-14, Perkin-Elmer Inc.). A bandpass filter with 540/50 nm was used to eliminate the residual emission signals. The data were collected with a TimeHarp 200 board and stored in time-tagged time-resolved mode (TTTR).

3. Results and discussion

The silver island films that were fabricated on the glass coverslips were demonstrated to have the silver nanoparticles of 100–500 nm across and 70 nm high covering ca. 20% of the surface of glass coverslips [27,30]. The metal islands were coated with the monolayers of the amino acid-like ligand 2-mercapto-propionylamino acetic acid 2,5-dioxo-pyrrolidin-1-ylester to improve their chemical stability and reduce their toxicity to the cultured cells [29,30]. Like on the glass coverslips, HeLa cells were observed to

incubate and immobilize well on the silver substrates suggesting that the silver nanoparticles express relatively low toxicity to the cells.

Absorption spectrum of the SIF substrate showed a maximum of plasmon resonance band from the silver nanoparticles at 460 nm that can consist with the absorbance bands of FAD molecules (Fig. 1A). Upon excitation at 470 nm, the ensemble fluorescence spectrum of extrinsic FAD in aqueous solution expressed a maximum of the emission band at 520 (Fig. 1B). The absorption band of the SIF substrate is relatively broad and overlaps strongly with the both the absorption and emission bands from FAD, suggesting that the plasmon resonances by the SIF substrate can interact efficiently with FAD at the excitation and emission wavelengths.

The ensemble emission spectrum of FAD in solid state was also examined. In the experiment, the extrinsic FAD was co-dissolved with 1 wt.% polyvinyl alcohol (PVA, MW 31,000) in water following by casting the solution on the glass coverslips. Upon excitation at 470 nm, the ensemble emission spectrum of FAD in the PVA film exhibits a broader emission band relative to the spectrum in solution and has a maximum of the emission band at 516 nm (Fig. 1B) close to the maximum of emission band in solution. On the basis of this observation, we expect that the emission spectrum of intrinsic FAD molecules in cells can be observed by the fluorescence measurements.

HeLa cells were immobilized on the glass coverslips or SIF substrates [31], and the immobilized cells were dried in air. Under the same conditions as the extrinsic FAD, the ensemble fluorescence spectrum was measured, which shows a significant rise of emission from FAD in cells at 514 nm in spite of a presence of strong scattering decay due to cellular background (Fig. 1B). This observation also demonstrates that the emission from the intrinsic FAD molecules within cells can be detectable, and furthermore, can interact with the metal substrates more strongly relative to the cellular background.

Fluorescence cell imaging measurements were recorded as both emission intensity and lifetime images on a time-resolved confocal microscope. Representative images on the glass coverslips and SIF substrates were presented in Fig. 2. According to the ensemble

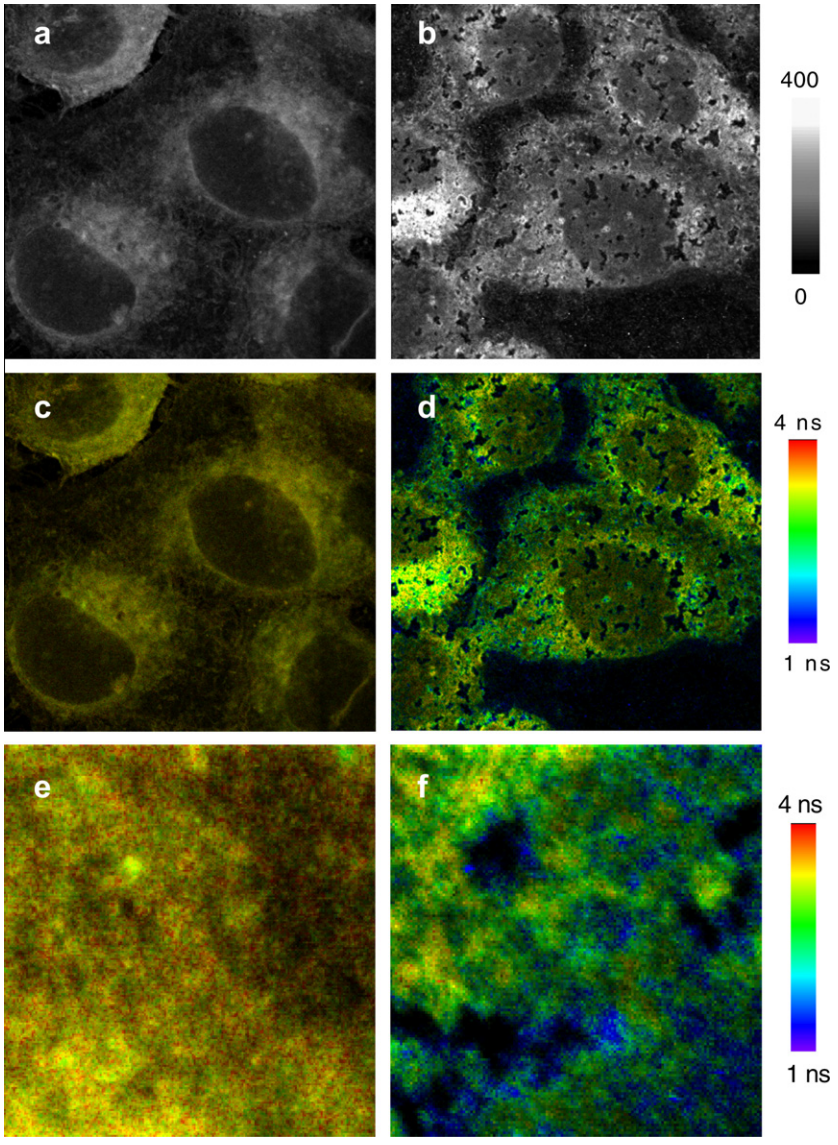

Fig. 2. The images of (a) and (b) represent fluorescence intensity cell images of HeLa cell lines on the (a) glass coverslip and (b) silver island substrate and the images of (c) and (d) represent the corresponding fluorescence lifetime cell images. The cell samples were collected upon excitation with a laser of 470 nm. The scales are 80 × 80 μm and the resolutions of diagrams are 500 × 500 pixel with an integration of 0.6 ms/pixel. The images of (e) and (f) represent the focused areas of lifetime cell images on the glass and metal substrates. The scales of diagrams are 5 × 5 μm and the resolutions are 100 × 100 pixel with an integration of 0.6 ms/pixel.

emission spectra in Fig. 1b, we can anticipate that the emissions through the current bandpass filter would be mostly due to the intrinsic FAD within the cells. It is noticed that the overall brightness of the cell image on the glass (Fig. 2a) is significantly lower than that on the silvered slides (Fig. 2b) demonstrating the presence of near-field interactions. Using the PicoQuant analysis software, the distributions of the emission intensity over these cell images were obtained, showing a maximum at 240 on the glass and a maximum at *ca.* 450 on the silvered slides (Fig. 3A). This result indicates that the emission intensity over the cell images on the silvered slides is 2.8-fold higher than that on the glass coverslips.

The ratio of emission intensity by the coenzyme over the emission of cellular backgrounds is an important parameter to evaluate the isolation of coenzyme emission from other cellular autofluorescence. In this case, the areas close to the cell nucleus were briefly selected representing the concentrated presence of FAD in cells. Moreover, the areas of cell nucleus were used to represent the cellular backgrounds. The ratio of emission intensity by FAD over the cellular backgrounds hence can be estimated. It is shown that on the glass slides, the ratio value is 3.0, and on the silvered slide, the ratio value is 4.5, significantly higher than the value on the glass slide, indicating that the metal slides can provide a higher enhancement of the emissions from the coenzymes above the cellular background due to the near-field interactions.

It is also known that the overall emission intensity throughout the cell images may provide a great opportunity to insight the changes of energy study in cells [1,2]. Although we did not perform measurements on additional cell lines, we believe that the increased coenzyme emission intensity on the metal substrates can offer a new strategy to determine the amounts of FAD in cells more sensitive and accurate.

While these results are encouraging, we also notice that the amplification of FAD emission in cells on the metal substrate is only 2.8-fold significantly lower than the 100-fold intensity increase we can obtain using single molecule detection to the extrinsic FAD [27]. In addition, the distribution curve of emission intensity histogram on the metal slides is noticed to be broader in comparison to that obtained on the glass slides (Fig. 3A). We believe this is due to the thickness of the cell and the valuable distance between FAD and metal nanoparticles. It is known that the cells have thickness over 1 or 2 μm on the coverslips. The near-field interactions typically occur within 100 nm from the metal surfaces. Hence, even though there is wide distribution of intrinsic FAD through the cells, only those of FAD molecules localized in the near-field range from the metal surfaces can interact with the plasmon resonances resulting in enhanced fluorescence. In the other words, there is only a portion of FAD in cell that is localized in the near-field range that can interact with the metal substrates.

The near-field interactions can typically increase radiative rate of a fluorophore and thus, result in a decreased lifetime [19,20]. When the lifetime of a fluorophore is shorter than the lifetime of cellular background (3–5 ns) in lifetime cell imaging, the emission

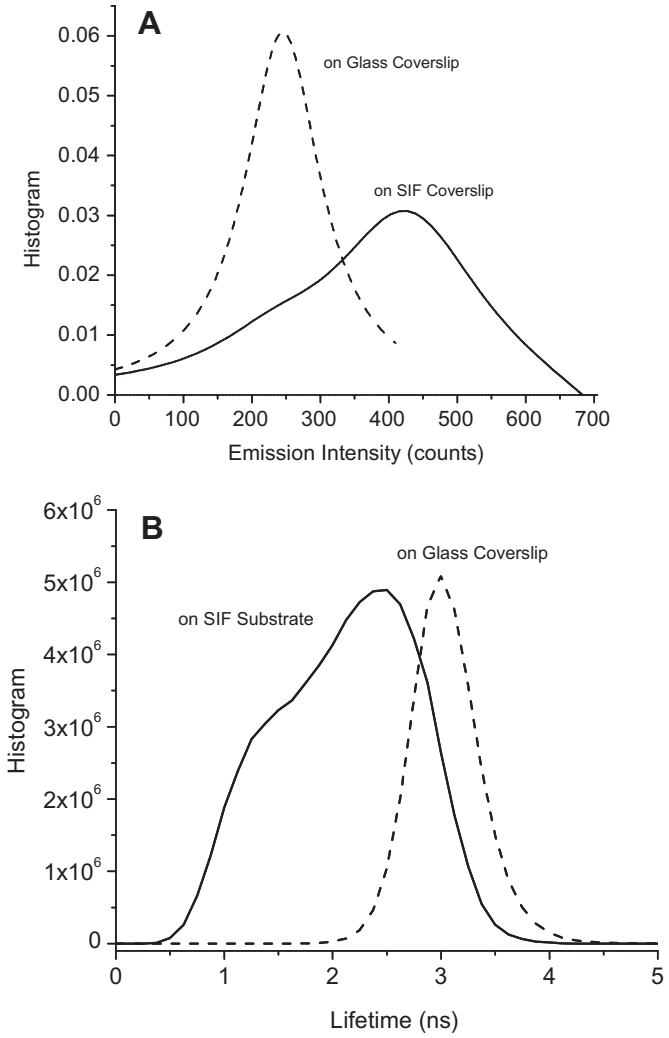

Fig. 3. Histogram distributions of (A) emission intensity and (B) lifetime throughout the entire cell images on the glass and metal substrate.

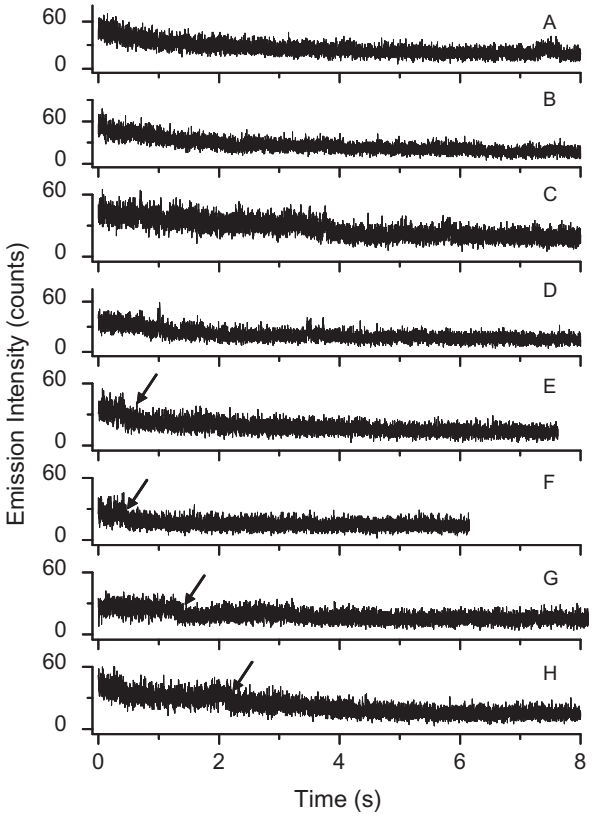

Fig. 4. Panels represent typical time traces collected from the small emission spots on the lifetime cell images on the metal substrate. Traces (A–D) can represent the decays from the emission spots containing multiple coenzymes whereas traces (E–H) can represent the single-step photobleaching from the single coenzyme molecules within the emission spots.

by the fluorophore can be isolated by fluorescence lifetime imaging microscope (FLIM) [12]. Herein, FLIM was also used to monitor the emissions of intrinsic FAD in cells. Lifetime cell images on the glass coverslip and silvered slide are presented in Fig. 2c and D, respectively. The cell image on the glass (Fig. 2c) showed a significant longer-component lifetime than the cell image on the metal (Fig. 2d). This decreased lifetime on the metal slide is consistent to increased emission intensity due to the near-field interactions. PicoQuant analysis software was used to calculate the distribution of lifetime over the cell image on the glass or silvered slide, showing a maximum of 3.0 ns on the glass and a maximum of 2.4 ns on the metal (Fig. 3B). This observation indicates that the near-field interaction on the metal substrate can shift the lifetime histogram over the cell image to shorter. It is also noticed that there is a clear rise on the lifetime distribution curve at 1.1 ns on the metal slide (Fig. 3B), which we believe is due to the intrinsic FAD in cells which are in close proximate to the metal substrate.

The reduced lifetime of FAD in cells on the metal slide allows the isolation of their emissions from the cellular backgrounds on the lifetime cellular images. This point is further proven when the observations are focused on smaller areas of the lifetime cell images (Fig. 2e and F). It is shown that the emissions by FAD on the glass coverslips remain vague and discrete (Fig. 2e), whereas the emissions by FAD on the silvered slides become almost completely isolable from the cellular backgrounds (Fig. 2f). Some small emission spots on these lifetime cell images were selected and irradiated with 470 nm laser for collecting their time-profiles. The results show that most emission spots display graduate changes from high to low on either glass or silvered slides, representing the presence of clusters of FAD within the spots (Fig. 4A–D). On the other hand, some small emissions spots on the metal slides are identified to display single-step or multiple-step photobleaching representing the presence of single FAD molecules or small number of FAD molecules within the spots (Fig. 4E–H). In contrast, the emissions from the single or small clustered FAD cannot be artificially identified on the glass slides. Thus, we predict that the metal substrates may offer an opportunity to insight the distributions of intrinsic coenzymes throughout the cells.

In this article, we investigate the fluorescence cell imaging on the metal substrates that may provide a non-invasive strategy for studying the coenzymes in cells. Because of their weak intrinsic emissions in cells, it is difficult to isolate the coenzyme emissions from the cellular background. But the near-field interactions on the metal substrates may help imaging of coenzymes in the cells by enhanced emission intensity. The near-field interactions on the metal substrate may also reduce the lifetime of FAD. Thus, the interference from the cellular backgrounds can be reduced on the emission intensity and lifetime images that can improve the isolation of FAD emissions from cellular background. In future work, we may consider resolving the cellular free and protein-bound state coenzymes through the lifetime analysis. There is a long history of concern about cancer cells being more active and needing more oxygen than normal cells. The observation in this study can be used to interpret the changes of the energy metabolism and redox reactions in the cancer cells, and furthermore will be used to study the mitochondrial anomalies.

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