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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

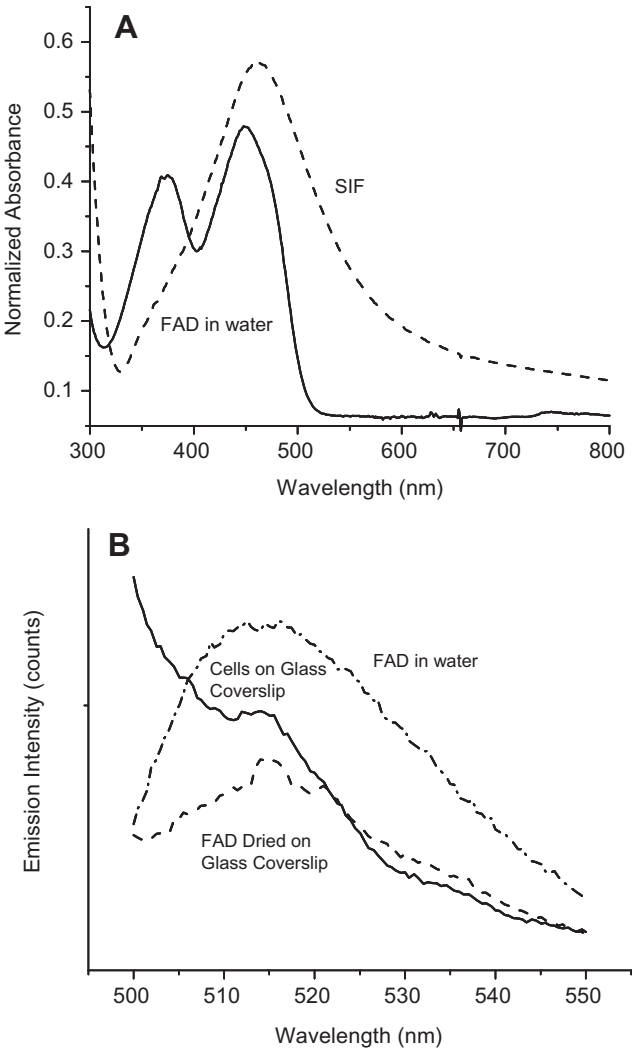

Fig. 1. (A) Absorption spectrum of extrinsic FAD in aqueous solution and plasmon resonance of silver island film on the glass coverslip. (B) Ensemble emission spectra of extrinsic FAD in aqueous solution, extrinsic FAD in PVA membrane on the glass coverslip, and dried HeLa cell sample on the glass coverslip.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