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Abstract. Early detection of cancer is crucial for the successful diagnostics of its presence and its subsequent treatment. To improve cancer detection, we tested the progressive multimodal optical imaging of U87MG cells in culture. A combination of steady-state spectroscopic methods with the time-resolved approach provides a new insight into the native metabolism when focused on endogenous tissue fluorescence. In this contribution, we evaluated the metabolic state of living U87MG cancer cells in culture by means of endogenous flavin fluorescence. Confocal microscopy and time-resolved fluorescence imaging were employed to gather spectrally and time-resolved images of the flavin fluorescence. We observed that flavin fluorescence in U87MG cells was predominantly localized outside the cell nucleus in mitochondria, while exhibiting a spectral maximum under 500 nm and fluorescence lifetimes under 1.4 ns, suggesting the presence of bound flavins. In some cells, flavin fluorescence was also detected inside the cell nuclei in the nucleoli, exhibiting longer fluorescence lifetimes and a red-shifted spectral maximum, pointing to the presence of free flavin. Extra-nuclear flavin fluorescence was diminished by 2-deoxyglucose, but failed to increase with 2,4-dinitrophenol, the uncoupler of oxidative phosphorylation, indicating that the cells use glycolysis, rather than oxidative phosphorylation for functioning. These gathered data are the first step toward monitoring the metabolic state of U87MG cancer cells. [DOI: 10.1117/1.JBO.20.5.051017]

Keywords: endogenous flavin fluorescence; fluorescence lifetime imaging; spectrally resolved confocal microscopy; U87MG cells; cancer; glycolysis.

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1 Introduction

Early cancer detection is crucial for the successful diagnostics of its presence and thus its subsequent treatment. Cancer is well known to be linked with alterations in cell metabolism, characterized by higher demand for nutrients and, consequently increased metabolic activity and accumulation of metabolites in cancer cells.[1,2] In recent years, a number of new approaches for the imaging of cells and tissues has been developed, particularly the use of unstained, endogenously fluorescing samples.[3] An increase in flavin adenine dinucleotide (FAD) fluorescence intensity and lifetime was observed in invading metastatic cells.[4] Autofluorescence from bronchial tissue has been investigated as a mean of lung cancer detection. Loss of autofluorescence was observed in carcinogen-transformed bronchial epithelial cells, and was proposed to be the result of changes in the fluorophore oxidation state.[5]

Multimodal optical imaging of flavin fluorescence is a promising method to record metabolic changes in the suspected tissues. A combination of steady-state spectroscopic methods with a time-resolved approach provides more precise insight into native metabolism when focused on tissue autofluorescence.[6] Fluorescence lifetime imaging microscopy (FLIM) allows direct visualization of fluorescing molecules and their environment in living cells.[7–9] Fluorescence lifetime is sensitive to the fluorophore environment, such as viscosity, energy transfer, etc. Directly visualizing the fluorescence lifetime of flavins in living cells allows evaluation of changes in their metabolic oxidative state.

Flavins, by ensuring the transfer of electrons in the mitochondrial electron transport chain (ETC), play a crucial role in the cellular energetics.[10] Despite the identification of several enzymes with flavin cofactors, flavins coupled to the mitochondrial ETC are the most important contributors to the flavin fluorescence in cells. Flavins are natural fluorophores excitable with blue/green light and they emit in a wide spectral band from approximately 490 to 530 nm.[11] Their endogenous fluorescence predestines them for noninvasive monitoring in living cells and tissues. Spectral analysis of the flavin fluorescence showed the emission maxima between 490 and 500 nm for flavins coupled to ETC and a maximum near 530 nm for free FAD molecules.[12] Time-resolved characterization of flavins demonstrated the presence of multiple fluorescence decay lifetimes: shorter fluorescence lifetimes (0.5 to 2 ns) for the bound flavins and longer (2 to 3 ns) for the free FAD.[7,13,14] In this work, we evaluate the time- and spatially resolved characteristics of flavin fluorescence in U87MG, the cellular model system of human glioblastoma.[15] In recent years, time-resolved imaging of flavoproteins was employed in numerous applications, ranging from cardiovascular, via cancer, up to neuronal cells and tissues, as well as to cell cultures[16,17] (see Ref. 18 for reviews).

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2 Materials and Methods

2.1 *Cell Cultures*

Hypodiploid adherent human glioblastoma-astrocytoma cell line U87MG (ATCC, HTB-14™)[19,20] was cultured up to the 30th passage at 37°C in a humidified incubator with 5% carbon dioxide. Dulbecco's modified Eagle medium (DMEM, PAA Laboratories) medium with 10% foetal bovine serum and 1% penicillin/streptomycin (both Gibco Invitrogen), called in this paper the complete DMEM, was employed. For the experiments, cells were harvested by trypsinization for 5 min, centrifuged at 100 g for 5 min and resuspended in fresh complete DMEM. Cells (30,000) in 1 ml of complete DMEM were seeded onto a round coverslip (30 mm of diameter) in a Petri dish and incubated for 24 to 48 h to allow the cell attachment. The coverslip was then transferred to a custom-made recording chamber filled with complete DMEM for microscopy recordings.

2.2 *Spectrally Resolved Confocal Imaging*

Confocal fluorescence images were taken with the laser scanning confocal microscope Axiovert 200 laser scanning microscope (LSM) 510 Meta (Zeiss, Germany) equipped with C-Apochromat 40×, 1.2 NA, 488-nm laser, using a 16 channel META detector.[12] A long pass (LP) 505-nm emission filter was used for recording the flavin fluorescence, while BP 535 to 590 nm, BP 500 to 550 nm, and BP 500 to 530 nm were employed for measurement of the di-8-ANEPPS (or Mitotracker orange), rhodamine 123, and acridine orange fluorescence, respectively. Spectrally resolved images were gathered with a 10- to 11-nm step size. Three-dimensional (3-D) images were recorded with a 1-μm step size.

2.3 *Fluorescence Lifetime Imaging Microscopy*

Fluorescence lifetime images were recorded using time-correlated single photon counting (TCSPC).[21,22] In these experiments, a 475 nm, picosecond laser diode (BDL-475, Becker&Hickl, Germany) was used as an excitation source with ~1 mW output power. The laser beam was reflected to the sample through the epifluorescence path of the Axiovert 200 LSM 510 Meta (Zeiss, Germany) inverted microscope with C-Apochromat 40×, 1.2 NA. The emitted fluorescence was separated from laser excitation using LP 500 nm and detected by a HPM 100-40 photomultiplier array (Becker&Hickl, Germany) employing an SPC-830 TCSPC board.

2.4 *Solutions and Drugs*

Fluorescence probes for individual organelles were selected based on their distinct spectral and lifetime characteristics; di-8-ANEPPS, rhodamine 123, or Mitotracker orange (all Life Technologies, Germany), and acridine orange (Sigma-Aldrich, Germany) were applied at concentrations of 5, 1, 1, and 0.5 μM to cells seeded onto a glass coverslip for 30 min in the incubator to determine the presence of plasma membrane, mitochondria, and nucleoli, respectively. The excess of the unbound probes was then cleared by washing with fresh complete DMEM twice. For metabolic modulation, cells were treated for 30 min in the incubator with 2,4-dinitrophenol (DNP, 50 μM), or 2-deoxyglucose (10 μM; both Sigma-Aldrich). All measurements of cells were performed in the complete DMEM medium.

2.5 *Data Analysis*

Data were analyzed using SPCImage software (Becker&Hickl, Germany), Origin 7.0 (OriginLab), or ZEN software (Zeiss, Germany). Linear unmixing was achieved using ZEN software, employing reference spectra recorded by the separate staining of cells with individual probes. The fluorescence intensity was evaluated as summed photon counts/pixel belonging to the cell area by a custom-made software.

3 Results

3.1 *Time- and Spectrally Resolved Properties of Endogenous Flavin Fluorescence of U87MG Cells*

Fluorescence of U87MG cells was studied in their native state. In most cells, an unstained round nucleus region was noted. Endogenous flavin fluorescence was present in the whole body of the cells, but was predominantly located in the cytosol around the cell nucleus [Fig. 1(a)]. Only in some cells was the flavin fluorescence also observed inside the cell nucleus in the round subnuclear organelles [Fig. 1(c)].

Time-resolved fluorescence was evaluated by FLIM. Fluorescence lifetimes of flavins in U87MG cells, estimated by the triple exponential decay model, demonstrated two fluorescence lifetimes in the extra-nuclear localization [Fig. 1(b)], first between 0.6 and 0.8 ns and the second around 1.3 ns. In the nucleus, the endogenous fluorescence showed longer lifetimes between 1.9 and 2.2 ns [Fig. 1(d)].

Spectrally resolved confocal images were taken from 500 to 670 nm with a step size of 10 to 11 nm. These images allowed estimation of the flavin fluorescence spectra in U87MG cells. In the cytosol, flavin fluorescence showed spectra that did not reach a maximum when measured from 500 nm, with the fluorescence values decreasing at rising wavelengths [Fig. 1(e), black]. In the nucleus, the spectra of endogenous fluorescence were red-shifted [Fig. 1(e), gray]; the differential spectrum [Fig. 1(f)] showed a maximum at 530 nm.

An estimated fluorescence spectral maximum under 500 nm,[12] as well as a short fluorescence lifetime[3] of the flavin fluorescence in the cytosol of U87MG cells in culture is in agreement with the presence of the bound flavins. On the other hand, longer fluorescence lifetimes and red-shifted fluorescence spectra of the subnuclear autofluorescence point to the presence of free flavins.

3.2 *Spatial Localization of Flavin Fluorescence in U87MG Cells*

To monitor the morphology of U87MG cells, cell membranes were stained with di-8-ANEPPS probe. 3-D images of the cells, recorded in a z-stack with a step size of 1 μm [Fig. 2(a)], demonstrated that these cells, when attached on glass coverslips, have thicknesses in the order of micrometers.

To compare the spatial distribution of flavin fluorescence with that of individual organelles in U87MG cells, organelle-specific probes were employed. On one hand, rhodamine 123 served to localize mitochondria in U87MG cells [Fig. 2(b)]. The probe showed mitochondria in the whole body of the cells, primarily localized around the nucleus, but also in lamellopodia ensuring the cell-to-cell communication. This result points to the cytosolic localization of the endogenous flavin fluorescence. On the other hand, acridine orange, known for

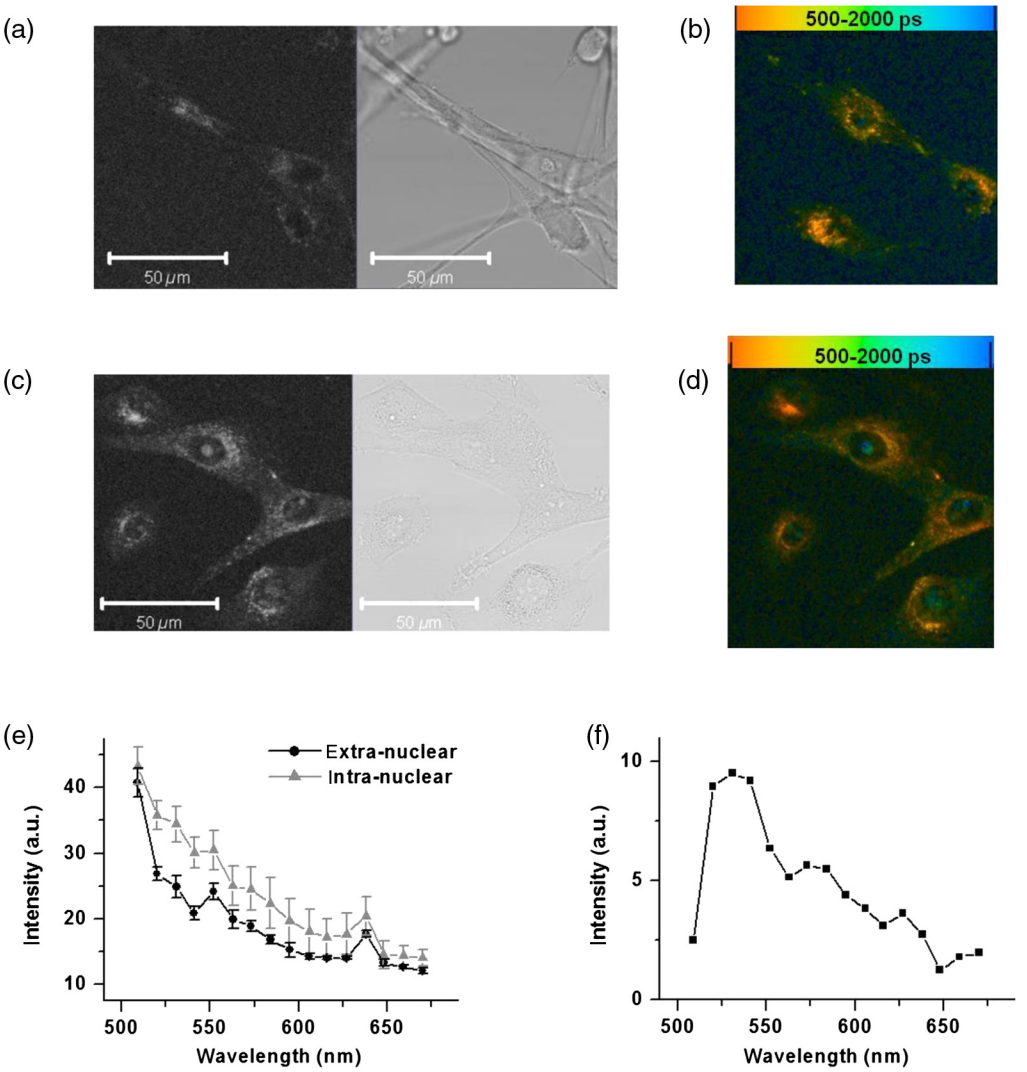

Fig. 1 Characterization of fluorescence lifetime and spectral properties of flavin fluorescence of U87MG cells in culture. (a) Fluorescence (left) and transmission (right) image of unstained U87MG cells recorded with confocal microscopy and (c) an example of the cells featuring the flavin fluorescence inside an unstained nucleus. (b) Fluorescence lifetime image of the flavin fluorescence outside the cell nucleus in the cytosol and (d) an example of the flavin fluorescence lifetime image outside versus inside the cell nucleus. (e) Spectra recorded from spectrally resolved confocal images of U87MG cells outside versus inside the cell nucleus ($n = 4$ for both) and (f) differential spectra from (e).

marking the cell nucleoli in U87MG cells,[23] clearly stained these subnuclear organelles inside the unstained nucleus [Fig. 2(c)]. Spatial localization of acridine orange fluorescence corresponded to intranuclear autofluorescence localization. In addition, we noted presence of lysosomes in the U87MG cells using the Lysotracker green fluorescence probe, observed as fluorescent points inside the cell cytosol (data not shown) that did not correspond to the recorded cell autofluorescence. Altogether, the gathered data indicate that the localization of the extra-nuclear flavin fluorescence in U87MG cells is most likely derived from mitochondria, while the intranuclear flavin fluorescence is located inside the nucleoli.

In order to better demonstrate mitochondrial localization of the extra-nuclear flavin fluorescence, double staining with membrane probe di-8-ANEPPS and the mitochondrial one, Mitotracker orange, was performed. Spectrally resolved confocal microscopy was used to record cell images together with their spectra. Spectra recorded in cells treated with individual probes [Fig. 3(a, left)] were used as reference spectra for linear unmixing. Mitotracker orange, localized in mitochondria, had a spectral maximum at 580 nm, while di-8-ANEPPS, marking the plasma membrane, featured two emission peaks at 580 and 630 nm. The spectral unmixing of flavins versus di-8-ANEPPS was then employed to show the intracellular under-membrane presence of flavins, located predominantly around the unstained nucleus [Fig. 3(a, right)]. Spectral unmixing of the flavin fluorescence versus that of Mitotracker orange [Fig. 3(b)] revealed colocalization of the two markers, demonstrating mitochondrial localization of the extra-nuclear flavins [Fig. 3(c)]. Gathered data support the extra-nuclear flavin fluorescence being derived from mitochondria.

3.3 *Metabolic Regulation of Flavin Fluorescence in U87MG Cells*

With the aim of studying the flavin fluorescence sensitivity to metabolic changes and to identify the preferred metabolic

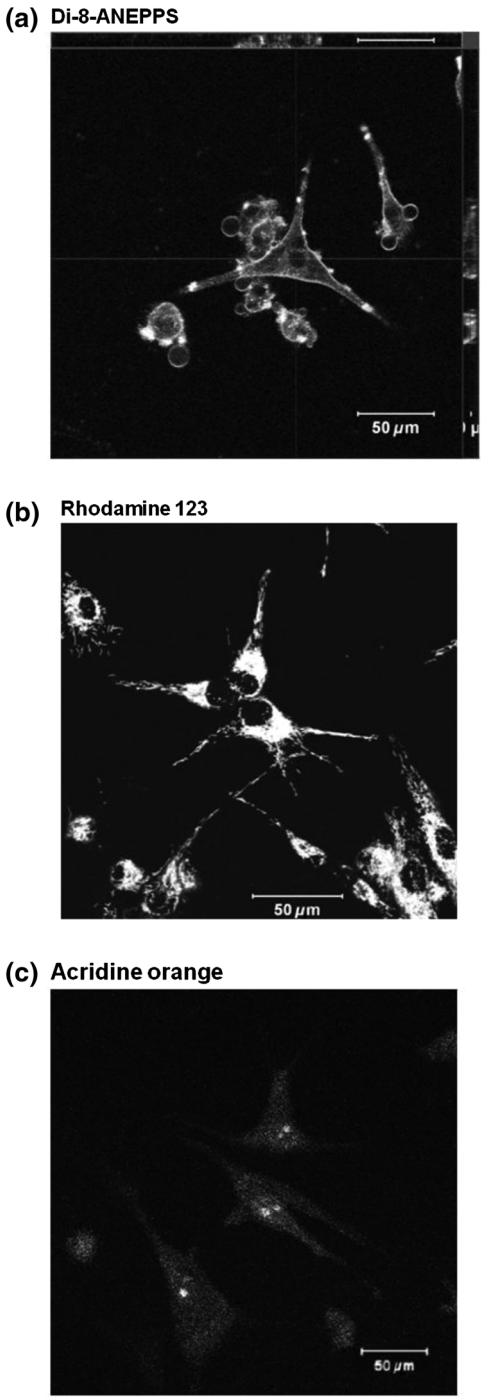

Fig. 2 Presence of organelles in U87MG cells. (a) Three-dimensional image (1 μM in z-stack) of the cell membrane staining with di-8-ANEPPS. (b) Mitochondria stained with rhodamine 123. (c) Nucleoli inside cell nucleus stained with acridine orange.

pathway employed by U87MG cells, metabolic modulators were employed. The fluorescence responses were studied by confocal microscopy and FLIM (Fig. 4). When compared to control conditions [Fig. 1(a)], the application of DNP (50 μM), an uncoupler of a respiratory chain,[24] failed to increase the flavin fluorescence in U87MG cells [Fig. 4(a, left)], as expected for this metabolic modulator. It instead decreased the fluorescence intensity. On the other hand, the application of 2-deoxyglucose (10 μM), capable of blocking glycolytic pathways,[25] abolished flavin fluorescence in these cells [Fig. 4(b, left)]. The findings were confirmed by comparison of the summed photon counts per pixel belonging to the cell area [Fig. 4(c)]. We recorded no change in the normalized fluorescence spectra with the two modulators (data not shown). The fluorescence lifetime distribution did not show an apparent modification after application of DNP [Fig. 4(a, right)], or 2-deoxyglucose [Fig. 4(b, right)]. Gathered observations point to the fact that U87MG cells are fueled by glycolysis rather than oxidative phosphorylation, in agreement with pathways preferentially employed by cancer cells.[26]

4 Discussion

In the present study, we investigated the properties of endogenous flavin fluorescence in the U87MG cell line in order to evaluate their metabolism. For the first time, we have performed FLIM of flavins directly in U87MG cells. We demonstrated that: (1) Flavin fluorescence in U87MG cells is predominantly localized outside the nucleus in the cell mitochondria and is characterized by a shorter fluorescence lifetime and a spectral maximum under 500 nm, suggesting the presence of bound flavins. (2) Some flavin fluorescence is also localized in nucleoli inside nucleus, characterized by a longer mean fluorescence lifetime and red-shifted spectra, indicating the presence of free flavins. (3) The cytosolic flavin fluorescence is sensitive to glycolytic inhibition, pointing to glycolysis rather than oxidative phosphorylation as the dominant process for energy production in U87MG cells.

U87MG cell line is the hypodiploid human glioblastoma–astrocytoma cell line employed as a model for testing the presence and responsiveness of cancer cells.[19,20] These cells serve as a useful model for testing anticancer drugs, namely the hypericin/mitochondrial complexes and their role in photodynamic therapy.[27] Importantly, these cells can also be employed as xenografts transplanted in model animals[28–31] for the study of the tumor growth. The development of sensitive methods for non-invasive monitoring of their endogenous metabolic state is, therefore, crucial for the improvement of such studies.

In most cells, the recorded flavin fluorescence had extranuclear localization characterized by short fluorescence lifetimes under 1.5 ns and spectra with maximum under 500 nm. We previously identified bound flavins in living cardiac cells as having a spectral maximum at 490 nm[12] and short fluorescence lifetimes.[21] These observations point to the presence of bound flavins in U87MG cell mitochondria. Mitochondrial localization is expected, as it is well known that endogenous flavin fluorescence is mostly correlated with mitochondria.[32]

In some cells, endogenous fluorescence was also noted in the cell nucleolus. This fluorescence had longer fluorescence lifetimes and red-shifted spectra. Our previous studies demonstrated that free flavins have a red-shifted spectral maximum at 530 nm[12] and fluorescence lifetimes around 2 ns.[21] Spectral- and time-resolved characteristics of the endogenous fluorescence in nucleoli thus corresponded to the free flavins. Nuclear flavins were previously described as relevant in nuclear redox activities, namely as a depository for nuclear flavin synthesis.[33] Our observation of nucleolar flavin fluorescence may indicate that the cells are synthesizing free flavins as a consequence of an unknown metabolic trigger. As this fluorescence was only observed in about 20% of all control cells, further experiments are needed to understand what can trigger this phenomenon.

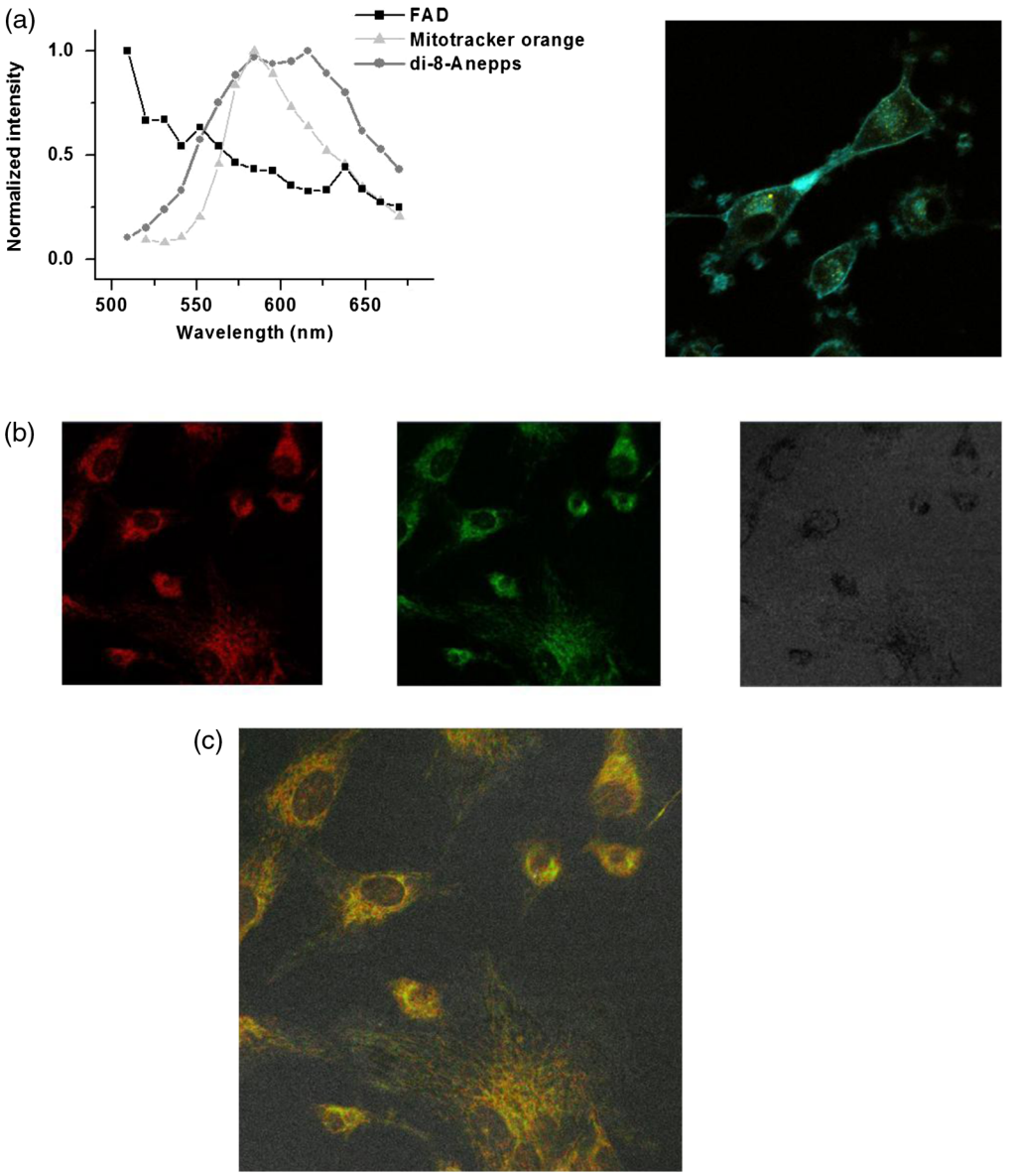

Fig. 3 Comparison of flavin and mitochondrial localization in U87MG cells. (a) Reference spectra used for spectral linear unmixing of the cytosolic flavin fluorescence, the membrane probe di-8-ANEPPS and the mitochondrial probe Mitotracker orange, recorded in single-stained cells (left). Unmixed image of the membrane probe di-8-ANEPPS and cytosolic flavin fluorescence, based on the reference spectra featured in (a, left) (right). (b) Unmixed image of the mitochondrial probe Mitotracker orange (left) and cytosolic flavin fluorescence (middle), versus background (right), based on the reference spectra featured in (a). (c) Collocalized unmixed image of images featured at (b).

Evaluation of the cytosolic flavin fluorescence regulation revealed that the U87MG cells are employing the glycolytic pathway rather than the oxidative phosphorylation one. On one hand, cytosolic flavin fluorescence inhibition by 2-deoxyglucose and, on the other hand, the lack of the capacity of DNP to raise the flavin fluorescence, indicate the predominance of the glycolytic pathway. This observation is in agreement with the expected behavior of cancer cells,[26] where the metabolic switch from oxidative phosphorylation to glycolysis has been well documented.[1,2]

The present study has its limitations that need to be addresses in future studies. Precise quantification of the fluorescence lifetimes from the time-resolved images is a complex task, as we currently do not have an approach to precisely establish mono- and/or multiexponential decay in the each pixel of the image, based on the localization of the fluorescent molecules in the specific regions of the cell. Advanced analytical methods need to be tested in the future to improve quantification of the lifetimes and their distribution, and thus their comparison in distinct regions and/or in different cells. Also, the cell cultures do not perfectly match the native environment of cancer cells:[34] *in vivo*, these are often localized inside collagen networks that play an important role in the metastatic activity of tumors.[4,35] Such networks were not taken into consideration and, in the future, we propose to test the use of 3-D cultures[34] based on the presence of collagen networks, mimicking the natural density of tissues in living

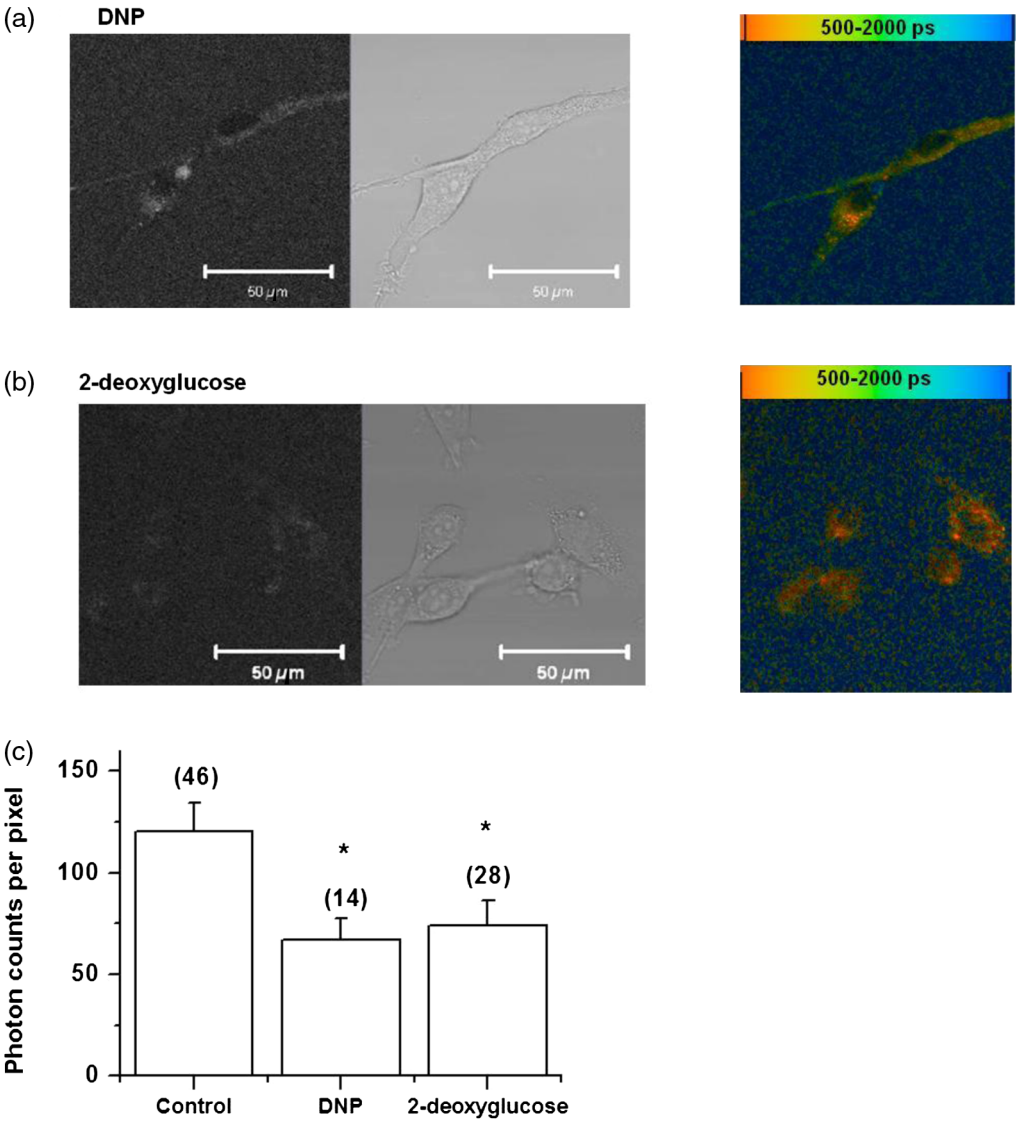

Fig. 4 Metabolic regulation in U87MG cells. Cells in the presence of (a) DNP (50 μM), the uncoupler of the respiratory chain and (b) 2-deoxyglucose (10 μM), the glycolytic pathway inhibitor, for 30 min. (a, b) Fluorescence (left), transmission (middle), recorded with confocal microscopy and time-resolved images recorded by FLIM (right). (c) Histogram of the recorded fluorescence intensity, control ($n = 46$ cells), DNP ($n = 14$ cells), 2-deoxyglucose ($n = 28$ cells). The data are reported as means $\pm$ SEM, $^*p < 0.05$.

organisms and providing the cells with a natural environment for nutrients uptake, cell-to-cell communication, multiplication and growth, etc. In addition, identification of all possible sources of the flavin fluorescence needs to be further addressed. We previously demonstrated the presence of the endogenous fluorescence derived from acylcoenzyme A dehydrogenase with a spectral maximum at 560 nm.[12] The spectral blip at 550 nm observed in the recorded spectra [Fig. 1(e), extra-nuclear], can also suggest implications of such flavin in U87MG. Finally, metabolic regulation also needs to be better understood. Flavin endogenous fluorescence is expected to be derived from the mitochondria;[32] however, little is known about contribution of extra-mitochondrial cytosolic flavin fluorescence in conditions with increased glycolysis. It is also noteworthy that mitochondria are an important target for oxidative stress in a broad range of pathologies, including cancer. We previously reported the capacity of oxidative stress to affect endogenous flavin fluorescence[26,36] and such effects should be further tested in U87MG cells.

Gathered data are the first step toward characterization of endogenous fluorescence in U87MG cells and its possible use as a sensor of the metabolic oxidative state of these cells in specific experimental conditions. We demonstrate the advantage of employing FLIM in addition to spectral recordings for better understanding of the molecular species underlying endogenous fluorescence in these cells.

5 Conclusions

The presented study demonstrates for the first time that the flavin fluorescence present endogenously in living U87MG cells in culture has spatial, spectral, and lifetime characteristics

corresponding predominantly to mitochondrial bound flavins, implicated in the glycolytic cycle. Some flavin fluorescence is also located in the cell nucleoli, with characteristics pointing to the presence of free flavins. The gathered data demonstrate the advantage of using time-resolved imaging for the characterization of the endogenous fluorescence in cancer cells. Understanding the precise role of the oxidative processes and their products in cancer cells is crucial for finding new noninvasive tools for biomedical diagnostics of this pathology.

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