Mapping metabolism of liver tissue using two-photon FLIM

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Abstract: Although fluorescence lifetime imaging microscopy (FLIM) has been extensively applied to study cellular metabolism in the liver, there is neither an established approach to analyze the data, nor have appropriate protocols been developed to maintain the optical metabolic characteristics in the ex vivo liver tissue sample. Here, we show that a tri-exponential decay fitting model for the fluorescence signal from nicotinamide adenine dinucleotide (NAD(P)H) and the use of ex vivo samples allows the most appropriate processing of the FLIM data. Moreover, we determine the medium that maintains the initial metabolic state of hepatocytes (liver cells), most effectively. Our results should be particularly relevant for the interrogation of liver samples, not only in laboratory research, but also in clinical settings in the future.

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1. Introduction

Modern methods of optical bioimaging offer promising tools for solving various biomedical problems. In particular, recent studies have demonstrated the possibility of investigating the structural and functional state of the liver using multiphoton microscopy methods. There have been a number of papers on studies related to metabolic changes in hepatocytes in the presence of various hepatic pathologies [1–6].

Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) are effective tools for studying cellular metabolic dynamics in both in vivo and ex vivo conditions. Due to its non-invasiveness, high sensitivity and the lack of need to use exogenous stains the FLIM method is a unique tool for visualization of the physico-chemical parameters, such as viscosity, temperature and pH of the environment at the cellular level [7]. Evaluation of metabolic activity using FLIM is performed on the basis of analysis of the fluorescence lifetimes of endogenous fluorophores, such as nicotinamide adenine dinucleotide (NAD(P)H). NADH and its oxidized form (NAD+) are a redox couple in the cycle of reactions of glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation. NAD is associated with ATP production in the cytosol by glycolysis, and in the mitochondria by oxidative phosphorylation, while its phosphorylated analogue NADPH is involved in the lipid, amino acid and nucleotide biosynthetic pathways, the glutathione cycle, and in defense against reactive oxygen species [8].

Being involved in cell respiration, the coenzyme NADH exists in cells in two forms, protein-bound and free, and their ratio depends on the metabolic state of the cells. The bound and free forms have different fluorescence lifetimes. While free NADH has fluorescence lifetime of around 0.3-0.4 ns, its bound counterpart produces fluorescence with the typical lifetimes from 1.2 to 2.5 ns, depending on the specific protein to which NADH is bound [9]. It is worth mentioning...
that the bound form of NADPH has longer fluorescence lifetime compared to the bound form of NADH and amounts to around 4.4 ns [10]. The fluorescence lifetime of the free form of NADPH is identical to the fluorescence lifetime of NADH.

To describe the fluorescence decay of NAD(P)H, one typically uses bi-exponential model, where the short and long lifetime components \( t_1 \) and \( t_2 \) correspond to the free and bound forms of NAD(P)H, respectively. The relative amplitudes \( a_1 \) and \( a_2 \) in this case are the contributions of the free and bound forms of the coenzyme, respectively [11].

It is known, that fluorescence decay curve of NAD(P)H may serve as an indicator of metabolic changes [12]. For example, the increase of the fraction of free NAD(P)H mainly correlates with a shift from oxidative phosphorylation (OXPHOS) process towards anaerobic glycolysis. In glycolysis, NAD(P)H is not bound to proteins (free), resulting in a high amount of fast decay component \( (a_1) \), whereas during OXPHOS there is a large amount of protein-bound NAD(P)H, resulting in a large amount of slow decay component \( (a_2) \) typically associated with more active OXPHOS.

The FLIM option is now available in a large number of clinical systems [13–15] but the range of tissues that can be investigated in this way is limited mainly to the skin and the oral mucosa [16,17]. Thus, investigation of the applicability of modern bioimaging methods for intraoperative cases becomes extremely important [18,19].

However, there is still no unequivocal opinion about which model of the exponential decay fitting and which conditions for metabolic imaging of liver tissue are preferable \( (\text{in vivo or ex vivo}) \), in respect of further translation into the clinic.

Furthermore there is the significant issue of the need to develop an effective methodology for the preservation of liver tissue samples. Despite the success of attempts to introduce multiphoton microscopy methods into the clinic, searching for a method of preserving liver tissue, which would most effectively maintain the specific metabolic activity of cells, remains a pressing issue. Until the time when this becomes available, most studies of NAD(P)H using FLIM will be performed on formalin-fixed [20], frozen tissue sections [21,22] or biopsy samples transported without using any specifically tested conditions [23], that may not accurately represent the metabolic state of the live, intact tissue. Recently, Lukina et al. have developed preservation protocols that allow maintenance of consistent fluorescence lifetimes and free/bound ratios of NAD(P)H as measured \( \text{in vivo} \) for at least 3 hours after excision of samples from colorectal cancer patients. However, the metabolic and synthetic activity of tumor cells and of hepatocytes differs significantly. Therefore, additional investigation of the protocols proposed in the work of Lukina et al. is necessary in order to determine the optimal conditions for the preservation of liver tissue, specifically [24].

Using a rat model we find the most appropriate fitting model for the analysis of liver tissue. Furthermore, we determine optimum conditions for the preservation of liver tissue in terms of its metabolic status.

### 2. Materials and methods

#### 2.1. Animal model

All experiments were approved by the Ethics Committee of the Privolzhsky Research Medical University (Nizhny Novgorod, Russia). The experiments were performed on male Wistar rats at 3 months of age with a body weight of 300–500 g. Before starting the study each animal was weighed. The rats were then anesthetized with Zoletil at a concentration of 80 mg/kg. For \( \text{in vivo} \) analysis, laparotomy was performed and the edges of the wound were fixed with a surgical dilator for better visualization of the organ. The surgical procedures were performed under aseptic conditions. Body temperature was controlled by placing the animals on a heating pad set to 37°C. For \( \text{ex vivo} \) analysis, following laparotomy, the rats were sacrificed and a tissue sample (size 0.5 × 0.5 cm) was taken for further research. The tissue samples were washed with physiological saline. The selection of an adequate model for studying the liver using multiphoton microscopy
was performed on 10 rats (for each of the *in vivo* and *ex vivo* studies). A study of preservation methods was conducted on 3 rats for control purposes with each type of medium.

### 2.2. Two-photon fluorescence microscopy and FLIM

*Ex vivo:* The two-photon fluorescence intensity and lifetime images of NAD(P)H were obtained using an LSM 880 laser scanning confocal microscope (Carl Zeiss, Germany) equipped with a time correlated single photon counting system (TCSPC, Simple-Tau 152, Becker & Hickl GmbH, Germany). A Ti:sapphire femtosecond laser operating at 80 MHz and having a pulse duration of 140 fs was used for excitation of NAD(P)H fluorescence at 750 nm. The fluorescence emission of NAD(P)H between 450 and 490 nm was selected by a combination of a 490LP dichroic mirror and a ET475/50 bandpass filter (Chroma, US) and detected by a hybrid detector (HPM-100-40, Becker&Hickl GmbH, Germany). The average power at the samples was about 6 mW. A C-Apochromat 40x/1.2 water immersion objective was used for image acquisition. The field of view was 213 × 213 µm (512 × 512 pixels). Acquisition time of the images was 60s.

We collected 10 intensity and fluorescence lifetime images of NAD(P)H as well as second harmonic images for each animal. SPCImage software (Becker&Hickl GmbH, Germany) was used for data analysis. On average 10,000 photons were collected per decay curve and the fit was performed using a bi- or a tri-exponential decay model, depending on the experimental data.

The *in vivo* investigation of liver tissue was carried out using multiphoton tomography at a temperature of +18°C. Specifically, we used an MPTflex multiphoton tomography (JenLab, Germany) equipped with a tunable wavelength Ti:Sa MaiTai laser (MaiTai, SpectraPhysiscs, USA) and an SPC-150 time-correlated single photon counting (TCSPC)-based FLIM module (Becker & Hickl GmbH, Germany). Images were obtained using a 40x oil immersion lens with a numerical aperture of 1.3, resulting in a field size of 213 × 213 µm. Fluorescence emission was recorded by two PMT detectors in the range of 373–387 nm and 409–660 nm. Excitation at 750 nm was used for NAD(P)H imaging and SHG imaging of collagen. The average power at the samples was approximately 12 mW.

For both the *in vivo* and *ex vivo* models the fluorescence intensity images were processed with background correction using ImageJ software (National Institutes of Health, USA). Fluorescence lifetime analysis was performed using SPCImage software (Becker&Hickl GmbH, Germany) for the cytoplasm of the cells. The fluorescence decay curves were fitted using both bi- and tri-exponential decay models.

### 2.3. Determination of optimal conditions for the preservation of liver tissue samples to maintain the metabolic status of cells

To develop an optimal method for preserving liver tissue samples, we analyzed 6 preservation techniques that were selected on the basis of literature data analysis: DMEM / DMEM medium on ice (on a refrigerant at -20°C); Custodiol / Custodiol on ice; 10% BSA / 10% BSA on ice. The choice of the methods was based on the previous protocols for tissue sample preservation used for three types of tumors (mouse colon cancer, Lewis lung carcinoma and murine melanoma) [24]. For the selection of optimal conditions for effective preservation of liver tissue we investigated *ex vivo* liver tissue samples of 0.5 × 0.5 cm using multiphoton microscopy and FLIM. The images were obtained at one hour intervals over a period of three hours. For control measurements we used liver tissue samples that were not immersed in the media.

### 2.4. Statistical analysis

To conduct a comparative analysis of the two fitting models, as well as to test the various preservation methods, we analyzed 200 regions of interest (ROI) in the cell cytoplasm for each rat (20 ROI for each FLIM image). The mean values and the standard deviation (SD) were
calculated for all the FLIM parameters under investigation. The charts were created in Excel (Microsoft, USA) or Prism 7 (GraphPad, USA).

3. Results

3.1. Selection of the optimal model for FLIM data processing

Initially, we performed a selection of the most appropriate fitting model for the fluorescence decay of NAD(P)H. The analysis was carried out under ex vivo conditions using the bi-exponential and tri-exponential fitting models. The ex vivo conditions were selected in order to provide the possibility of conducting the most accurate analysis.

The bi-exponential fitting allowed investigation of the fluorescence lifetime parameters of the free and bound forms of NAD(P)H and their contributions:
- $t_1$ (ps) – fluorescence lifetime of the short component – free form of NAD(P)H;
- $t_2$ (ps) – fluorescence lifetime of the long component – bound form of NAD(P)H;
- $a_1$ (%) is the contribution of the free form of NADH;
- $a_2$ (%) is the contribution of the bound form of NADH.

To assess the contribution of NADPH, the FLIM data were processed using a tri-exponential decay model. With tri-exponential fitting, the fluorescence lifetime parameters of the free and bound forms of NADH and NADPH were studied:
- $t_2$ (ps) – lifetime of the long component – bound form of NADH;
- $t_3$ (ps) – lifetime of the phosphorylated form – bound form of NADPH;
- $a_1$ (%) is the contribution of the free form of NADH;
- $a_2$ (%) is the contribution of the bound form of NADH;
- $a_3$ (%) is the contribution of the bound form of NADPH.

The fluorescence lifetime of the free form of NADH was fixed at 400ps due to the high conservatism of the fluorescence lifetime values of this molecule in a free state [21].

The analysis of the data using the bi-exponential model delivered fluorescence lifetimes values for the bound form of NAD(P)H of 3600 ± 160 ps, which are rather high compared to the known values from the literature (1500–2200 ps) [9,25]. Analysis of the validity of the selected fitting model was also performed on the basis of the average value of the parameter $\chi^2$, which reflects the goodness of the fit (Fig. 1). Depending on the fluorophore’s conformation and its environment, several mathematical models have been developed to describe the experimentally obtained decay curves. The fit of a particular model to the experimental decay was achieved via a program algorithm that minimized the difference between the experimental and simulated data and found the global minimum in the fitting function. The acceptance criteria for the goodness of the fit included a satisfactory reduced chi-squared value ($\chi^2$) [26]. $\chi^2$ values were shown to amount to 1.3 ± 0.24 and 1.1 ± 0.02 for bi-exponential and tri-exponential decay models, respectively. Thus, we found that it was the phosphorylated form that was contributing to the fluorescence lifetime signal. In connection with the obtained results, we further analyzed the NAD(P)H fluorescence lifetimes using a tri-exponential fitting model.

3.2. Selection of optimal model for the analysis of liver tissue

The liver is covered with a capsule consisting of collagen. For in vivo studies without capsule removal this will presumably contribute to the obtained data. In order to select the optimal model, we tested four models. These were: in vivo analysis with intact liver capsule, in vivo analysis with the liver capsule removed, ex vivo analysis with intact liver capsule and ex vivo analysis with the liver capsule removed.

Images of the autofluorescence intensity of NADH and SHG are presented in Fig. 2. It can be seen that in vivo analysis of the structure of the liver tissue and assessment of the level of fibrogenesis are difficult as a result of the presence of artifacts due to the animal’s movement.
Fig. 1. Comparison of bi-exponential and tri-exponential decay fitting models. (A) FLIM images of NAD(P)H in normal liver tissue. Fluorescence lifetime of the bound form of NADH (t2) and goodness of the fit parameter ($\chi^2$). Images obtained using LSM. Collection time of the image was 60s. (B) Comparison of the distribution of values for the $\chi^2$ parameters for the two fitting models.
To compare the four models, we analyzed the fluorescence lifetimes of the bound forms of NADH and NADPH, as well as the contributions of the free and bound form of NADH both with the native capsule and without it (Fig. 3, 4).

The images obtained during in vivo investigation are fuzzy and acquire distortions associated with the animal movement. In addition, the number of accumulated photons is significantly lower (300–1000 photons per decay curve) compared to the analysis from the ex vivo model. This is critical as, at least 10000 photons per decay curve are required for an accurate analysis of the FLIM data using tri-exponential fitting model. Attempting to accumulate such a number of photons over a period of approximately one minute means that the position of the tissue in focus will shift with this time. Thus, such a data analysis is not sufficiently accurate and is incomplete.

By comparing these two models, in vivo and ex vivo, we can conclude that the preferable method for further investigation of the state of the liver is by obtaining a signal from the ex vivo tissue samples without capsules.

In particular, the values of the fluorescence lifetimes and their contributions for the in vivo model with a native capsule differ most significantly. Such inadequate values are most likely due to the high contribution of the fluorescence lifetime of the collagen that makes up the capsule. In addition, there is also a contribution from the flavin signal (FMN, riboflavin, and the long component of FAD) in the case of the in vivo investigations performed on the MPTflex. Unfortunately, the MPTflex does not allow selection of a spectral band specific only for NAD(P)H. Due to the ability to select more appropriate filters when using the LSM, there is no such issue with signals from flavins and, therefore, the ex vivo data, both with capsule and even more so without it, corresponds most closely to the data published previously [9, 25]. The fluorescence data for each of the investigated models are presented in Fig. 4.
3.3. Selection of the optimal method of preservation and delivery of ex vivo samples while maintaining metabolic activity

We conducted an analysis of six media to maintain the metabolic status of the hepatocytes of ex vivo samples: Custodial/Custodial on ice, BSA/BSA on ice and DMEM/DMEM on ice. It was found that the technique based on preservation with BSA on ice most effectively maintained the metabolic status of a tissue sample. In the case of preservation with BSA the fluorescence lifetimes and their contributions are comparable to the control values for 0 hour; in addition, the parameters did not significantly change during the entire study time. The results of the analysis of the metabolic status of hepatocytes during preservation in each of the media are presented in Fig. 5.
Fig. 4. Comparison of the NAD(P)H fluorescence lifetimes of the bound forms of NADH and NADPH, as well as the contributions of the free and bound forms of NADH – with and without capsule, under *in vivo* and *ex vivo* conditions. The short component (t1) was fixed at 400ps. Mean ± SD.

Fig. 5. Evaluation of the FLIM parameters under the application of various conditions for the preservation of *ex vivo* samples. Mean ± SD.
4. Discussion

Currently, no definitive technique has been developed for analyzing the metabolic status of liver tissue both in its normal state and in pathological conditions. In this work, using a rat model we have selected appropriate decay fitting, an optimal model, as well as the method of tissue preservation for the most effective maintenance of the metabolic status of liver tissue.

To begin, a suitable fitting model was determined. It was found that the most appropriate model was one based on tri-exponential fitting. Despite most studies on metabolic imaging of liver tissue using bi-exponential decomposition analysis, our results have shown that there was a high contribution from NADPH, which is associated with biosynthetic processes in the cells, including through lipogenesis and the pentose phosphate pathway.

Most often, including hepatocytes, a bi-exponential decay fitting model is used to analyze the metabolic cellular status. In particular, using a bi-exponential decay fitting, Wang et al. performed in vivo metabolic imaging of the liver for hepatocellular carcinoma and ischemia-reperfusion injury [2]. They demonstrated that, the parameters $t_m$, $t_1$ and $t_2$ had changed with the development of pathologies. Specifically, they found that the lifetime $t_2$ amounted to $3120 \pm 291.8\,\text{ps}$ for normal tissue, which is a rather high value that does not correlate with generally accepted values from the literature. These results may indicate the presence of a third component contributing to the long component ($t_2$). The values of $t_2$ in the liver with cholangiocarcinoma and with ischemia-reperfusion syndrome, decrease to $2541.7 \pm 341.8\,\text{ps}$ and $2641.3 \pm 355.2\,\text{ps}$, respectively. Most likely this is due to a decrease in synthetic activity in the presence of such pathologies. Analysis of the contribution of the third component in the fluorescence decay allows indirect evaluation of the level of cellular synthetic activity [27] and assessment of the intensity of antioxidant defense [28]. Therefore, in order to take into account the changes in the synthetic activity of the liver cells, we believe that the use of tri-exponential decay fittings is more appropriate.

Moreover, only the amplitude weighted mean fluorescence lifetimes ($t_m$) have been discussed in most of the investigations in liver, brain, skin and other biological samples [2, 21, 29–30]. However, the $t_m$ depends on a variety of parameters, and this complicates the interpretation of the results. An important aspect is that, in most works, the bi-exponential decay fitting was based only on the lifetimes data, and not on the contributions that reflect the specific shift of metabolic pathways, which actually correlate with the functional activity of the cells [31]. We propose an approach that takes into account not only the lifetimes of the various forms of NADH, but also their contributions to the cellular metabolic state.

The tri-exponential fit with the evaluation of the contribution of each specific form of NAD(P)H rather than fluorescence lifetime maybe relevant in cases when the contribution of the phosphorylated form of NADH is non-negligible. For example, there is increased contribution of protein-bound NADPH in adipocytes that is associated with lipogenesis [32]. Therefore, evaluation of the contribution to the total fluorescence signal from NADPH could deliver additional biochemical information in this case.

Another area where the estimation of the NADPH contribution can be important is the study of neurodegenerative diseases. It is known that increased activity of NADPH oxidase result in the increase in the production of the reactive oxygen species (ROS) due to the reduction of the NADPH content. The increased production of ROS consequently will cause a loss of dopaminergic neurons in the substantia nigra of the brain and this may lead to Parkinson’s disease [33]. Therefore, an analysis of FLIM data that we have implemented for liver, may prove to be useful in the interpretation of the FLIM data obtained during the investigation of changes in NADPH content in neurodegenerative diseases.

We should note, that one of the alternative methods widely used for FLIM data analysis is based on the phasor plot [34]. While nonlinear curve fitting that we use require some level of expertise in the choice of the fitting model, phasor analysis requires less initial assumptions of
the components contributing to fluorescence decay profiles and requires no iterative calculations. Consequently it is not computationally demanding, and thus, less time consuming compared to the least square fitting analysis. However, one have to be careful comparing the results obtained with phasor approach and nonlinear curve fitting. While the contribution of the fast (a1) and slow decay components (a2 and a3) in nonlinear curve fitting is linear to the relative photon contribution of the fluorophore with the fast/slow fluorescence lifetimes, interpretation of a1, a2 and a3 obtained in the phasor analysis is more complicated due to mathematical distortions caused by the Fourier transformation. Phasor plot applied for the analysis of the NAD(P)H FLIM resulted in the explosion of papers on metabolic FLIM [35–37].

Overall, conventional analysis of NADPH content in cells is currently done using an expensive and invasive method of mass-spectrometry. Therefore, alternative approaches that allow to evaluate the contribution of this co-enzyme could be of interest for biochemists.

Our next step was therefore to conduct a study of the metabolic state of liver cells based on two models, those of in vivo and ex vivo samples, in order to determine the methodology that is the most convenient to implement while correctly reflecting the cellular metabolic state in both animal models, as well as in clinical settings in the future. Since he purpose is to study the liver parenchyma intraoperatively, both in the organ with preserved blood flow and on the remote part of the liver, an analysis of these approaches is extremely important.

Despite the undeniable advantages of in vivo research, the main drawback of this technique is the presence of motion artifacts. The appearance of artifacts does not allow time for a sufficient accumulation of photons, thereby impairing the accuracy of the data obtained. In ex vivo samples, due to the absence of motion artifacts, there is a greater opportunity to accumulate the necessary number of photons, quite apart from the important benefit that carrying out metabolic imaging with ex vivo samples is easier to implement.

Furthermore, we have observed incorrect values of fluorescence lifetimes in some case of in vivo investigation. This result is due to flavin adenine dinucleotide (FAD) also contributing to the detected fluorescence signal in this excitation / emission spectral range. Despite FAD’s extremely low fluorescence signal, the presence of a small FAD fluorescence background might be possible in the MPTflex-detected wavelength range [38]. Thus, both the long components of NADH and FAD contribute to the t2 value. Using LSM, we could control the emission spectral range and, therefore, the received FLIM data is more specific for the molecules being studied.

The main drawback of models with a native capsule (both in vivo and ex vivo) is the contribution of the capsule’s collagen fluorescence lifetime. The problem arises because the fluorescence signal of liver tissue excited at 750 nm has contributions from collagen, elastin and NADH [39,40]. The lengthening of the fluorescence lifetime of the bound form of NADH in the case of liver still contained within its capsule is due to the contribution of the collagen fluorescence lifetime. Collagen produce fluorescence under blue excitation; it shows a broad emission up to 500 nm, depending on the excitation wavelength (270–370 nm) [41]. The blue emission of collagen has been attributed to tyrosine and dityrosine [42,43]. The collagen lifetime is longer than that of the bound form of NADH (at ~5300 ps) [42,44], and thereby distorting the measured values.

Thus, comparing the four models, it was found that performing ex vivo investigation without the capsule solves the problems of motion artifacts, insufficiency of number of photons per pixel and the contribution of the collagen fluorescence lifetime. We therefore conclude that the most effective approach for metabolic imaging in this field is through the surface of ex vivo samples without their capsule.

Finally, for adequate metabolic imaging of ex vivo samples of liver, it is necessary to select the optimal conditions for preservation of the liver tissue for the most efficient maintenance of the initial cellular metabolic status. First, it is necessary to select an appropriate period of time during which it should be possible to conduct an analysis without significant changes having
occurred to the metabolism of the liver tissue. From analyzing the changes in the metabolic state of hepatocytes in the process of “tissue dying” we estimated that up to 3 hours is the window available for analyzing a patient’s liver tissue sample whilst the metabolism still remains representative of the in vivo state.

Second, it is important to select a suitable medium for the most effective preservation of the initial metabolic state. Analysis of the state of liver tissue in various media and conditions showed that, of the media tested, only with preservation of the liver tissue in 10% BSA on ice, did the metabolic state of hepatocytes not change within 3 hours. A similar result was shown by Lukina et al., 2019 for tumor tissue [24]. A possible cause of metabolic changes in the other methods of tissue sample preservation are changes in the permeability of the cell membrane that lead to the transfer of ions and other components of the test medium into the cells, with the parallel transfer of cellular components to the environment. Such a process can lead to violations of the cellular redox state, the osmotic balance, and of general cell homeostasis. It is possible that 10% BSA, dissolved in phosphate-buffered saline (PBS), acts as a membrane stabilizer, helping to maintain membrane integrity, while storing the sample on ice reduces the diffusion rate. The protective effects of BSA on cellular membranes are well described in the literature [45,46].

5. Conclusion

In this work, we tested two decay fitting models, in order to determine the approach that would provide the most reliable FLIM data. It was found that, by using a tri-exponential decay fitting model, the obtained fluorescence lifetimes of NADH, as well as the goodness of the fit parameter values correspond most closely to the known published data. This result is due to the high synthetic activity of hepatocytes, which reflects the high contribution of the third component – the protein-bound form of NADPH. In addition, we determined that the most relevant research model for metabolic imaging is the use of ex vivo liver tissue samples without the liver capsule. Furthermore, we also selected the optimal medium for the preservation of liver tissue samples that provided the best maintenance of the initial metabolic state of the tissue. It was shown that the preservation of liver samples in 10% BSA on ice, does not change significantly the values of the fluorescence lifetimes and their contributions for 3 hours. The results we have obtained are relevant for conducting meaningful analyses to reflect the metabolic state of hepatocytes in fresh liver tissue, and are applicable both in experimental studies and for the further translation of the FLIM method into clinics.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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