

Detection of reductive stress in brain endothelial cells using FLIM imaging: new approaches to assessing endothelial dysfunction

© N.A. Kolotyeva¹, S.V. Novikova¹, N.A. Rozanova¹, V.I. Makarov², A.V. Ryabova², A.K. Berdnikov¹, A.N. Lukyanchuk^{1,3}, R.S. Mudarisova¹, L.V. Nikolaeva³, Yu.K. Komleva¹, A.B. Salmina¹

¹ Russian Center of Neurology and Neurosciences, Moscow, Russia

² Prokhorov Institute of General Physics, Russian Academy of Sciences, Moscow, Russia

³ Prof. V.F. Voino-Yasenetsky Krasnoyarsk State Medical University, Ministry of Health of the Russian Federation, Krasnoyarsk, Russia

e-mail: kolotyeva.n.a@neurology.ru

Received November 13, 2025

Revised December 02, 2025

Accepted March 24, 2026

Neurodegenerative processes are exacerbated by changes in the cell's redox potential. Reductive stress, the predominance of reduced forms of coenzymes such as NADH, NADPH, and GSH, disrupts the functions of cellular organelles, such as mitochondria. The aim of the study is to identify the pathways through which reductive stress affects cerebral endothelial cells. For the first time, this study investigated the effect of reductive stress on mitochondrial function and the metabolic profile of cerebral endothelial cells *in vitro* using fluorescence microscopy with fluorescence lifetime measurement (FLIM). A 24-hour incubation with 500 μ M dithiothreitol (DTT) did not alter mitochondrial membrane potential or the total pool of NAD⁺ and NADH, but increased the concentration of superoxide anion, indicating the onset of oxidative stress. Studies using phase-resolved FLIM revealed an increase in the short-lived NADH component as early as 1 hour after incubation, indicating metabolic shifts. The results suggest an adaptation of cellular metabolism toward enhanced glycolysis, which, with prolonged exposure, may disrupt the permeability of the blood-brain barrier and accelerate neurodegenerative processes. The data reveal early mechanisms of metabolic restructuring in cerebral endothelial cells under oxidative stress and demonstrate the potential of FLIM in identifying therapeutic targets aimed at preserving the integrity of the blood-brain barrier.

Keywords: reductive stress, cerebral endothelium, FLIM, microscopy, mitochondrial function, NADH.

DOI: 10.61011/EOS.2026.05.63849.60-25

Introduction

In recent years, increasing attention in biomedical optics and neurophysiology is paid to plasticity of neurovascular unit (NVU) cells, a functional system that includes microcerebrovascular endothelial cells, astrocytes, pericytes, neurons and glial cells. This plasticity ensures dynamic adaptation of energy metabolism to local neuron needs and plays a key role in the central nervous system (CNS) homeostasis control [1–3]. A blood-brain barrier (BBB) whose selective permeability is controlled by coordinated activity of all NVU components is of particular importance in the NVU structure. BBB impairment is an early and critical pathogenesis marker of neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease [4,5]. An important mechanism of abnormal BBB permeability is the development of mitochondrial dysfunction in cerebral endothelium cells leading to reduction of energy production and violation of barrier integrity [6].

Disturbance of mitochondria activity results in a change of reduction-oxidation (redox) homeostasis in cells. Along with a traditionally studied oxidative stress caused by reactive oxygen intermediate (ROI) excess, importance is being increasingly attached to a reductive stress — a condition featuring abnormal accumulation of reduced coenzymes: reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH)

and reduced glutathione (GSH) [7]. Such hyperreduced state leads to an „electronic jam“ in the mitochondrial respiratory chain, which can induce reverse electron current and, paradoxically, enhance ROI production [8]. Thus, the reductive and oxidative stresses are not mutually exclusive, but mutually enhance destructive processes underlying the neurodegeneration [9–11].

However, direct measurement of the reductive stress is associated with considerable methodological difficulties. Key redox pairs — NAD⁺/NADH, NADP⁺/NADPH and GSSG/GSH vary dynamically, which cannot be detected by standard biochemical methods without cell disruption. Moreover, there is no single specific biomarker allowing the reductive stress to be unambiguously identified [12]. In these conditions, optical methods based on detecting autofluorescence of endogenous metabolites are of particular value.

Fluorescence lifetime imaging microscopy (FLIM) demonstrates the greatest informativity among such methods. FLIM allows the redox state of individual cells to be evaluated quantitatively by the fluorescence properties of pyridine (NADPH) and flavin (FAD) nucleotides in real time without introducing external probes [13,14]. Unlike fluorescence intensity, fluorescence lifetime doesn't depend on the fluorophore concentration and absorbance of medium, making FLIM a selection tool for investigating metabolic heterogeneity in complex cell systems such as NVU.

Despite the growing interest in the reductive stress, its role in pathophysiology of cerebral endothelium that forms the structural base of BBB is still virtually unexplored. This is the first time we have used FLIM for evaluating the state of endothelial cells in experimentally induced reductive stress *in vitro*. The objective of the study is to identify metabolic and redox changes in cerebral endothelium cells that may precede the violation of BBB integrity and serve as the basis for searching new biomarkers of initial neurodegenerative disease stages.

Materials and methods

Primary cerebral endothelial cell cultures were obtained and incubated in accordance with a standard procedure [15] with modification (RU2774603C1) [16]. Material was taken from 14 day old Wistar rats under anesthesia. Brain extraction followed by removal of meninges and large cerebral vessels was performed by rolling on sterile filter paper. Then the cerebral cortex was isolated and large vessels were removed in cold Hanks' solution (PanEco, Russia). The ground cortex was centrifuged for 3 min at 1000 rpm. Two-fold volume of 25% bovine serum albumin solution (BSA, Sigma, USA) was added to the precipitate and mechanical dissociation was performed by pipetting. The obtained cell suspension was centrifuged for 10 min at 2000 rpm at room temperature. The lower precipitate layer containing microvessel fragments was placed into a 15 mL conical tube. Mechanical dissociation and centrifuging procedures were repeated thrice to increase the isolation purity. Then the precipitate underwent enzymatic treatment by 0.1% collagenase II solution (PanEco, Russia) during 35 min at 37 °C in a roller. After completion of incubation, several milliliters of warm Hanks' solution were added and then centrifuging was performed at 1000 rpm for 5 min. The precipitate was re-suspended in Modified Eagle Medium (DMEM-F12, GIBCO Invitrogen Corporation, USA) made up with 20% fetal calf serum (HyClone, Austria), 2 mM glutamine (GlutaMAX, Gibco, UK) and 16.6 mM glucose, and plated in plastic Petri dishes of 40 mm in diameter (Medpolimer, Russia) pre-coated with matrigel (Corning, USA). The culture medium was changed twice a week. Inoculation was performed in an CO₂ incubator (RWD Life Science, China) at 37 °C, 5% CO₂ and relative humidity of 98%. After formation of a confluent monolayer, cells were plated on 96-well culture plates (Servicebio, China) with 100 µL of suspension per well, another part was poured on cover slips in Petri dishes with 200 µL of suspension each with a density of 2×10^5 cells/mL. Cell counting and viability evaluation were performed using the Countess automatic counter (Invitrogen). Current culture state was controlled by the phase-contrast microscopy method using the Olympus CKX41 microscope (Japan).

Immunophenotyping of the cerebral endothelial cell culture was performed by immunofluorescent staining with achievement of 90% confluency of a monolayer grown on a 96-well plate or a slip. Cell fixation was carried out

using cooled 5% paraformaldehyde solution (PFA, Sigma, Germany) in 0.01 M sodium phosphate buffer (PBS; ECO servis, Russia) during 15 min at room temperature. Then, the samples were washed five times in phosphate-buffered saline. To enhance cell diffusion into cells, the samples were treated with 0.1% Triton X-100 (Calbiochem Biochemicals, USA) for 10 min at +4 °C. To prevent nonspecific antibody binding, cells were blocked in 5% goat serum solution (Sigma, Germany) for 60 min. Cells were incubated with primary mouse anti-CD31 antibodies (1:500, BF0611, Affinity Biosciences) during 6 h at +4 °C in a humidified chamber. Primary and secondary antibodies were diluted in IHCDiluent (Leica) according to the manufacturer's procedure. Samples were washed five times in PBS to remove unbound primary antibodies. Then the samples were incubated with secondary Goat anti-mouse IgG H&L antibodies (Alexa Fluor 594) (1:350, ab150116, Abcam) in 0.1% Triton X-100 solution with PBS during 2 h on a turntable at room temperature. Unbound secondary antibodies were removed by washing in PBS solution. Cells were placed under a cover slip with contrast nucleus 4',6-diamidin-2-phenylindole dihydrochloride (Fluoroshield with DAPI; Sigma) during 15 min. EVOS M7000 system (Termo Fisher Scientific, USA) was used for visualization. Relative number of target label expressing cells was counted in 5 fields of view. Images were analyzed in ImageJ, version 1.47 (Research Services Branch, USA).

Reductive stress induction was carried out upon achievement of 90% confluency of endothelial cell monolayer in serum-free medium (DMEM-F12 (GIBCO Invitrogen Corporation, USA) with addition of 2 mM glutamine (GlutaMAX, Gibco, UK) and 16.6 mM glucose). Dithiothreitol (DTT) with a final concentration of 500 µM was used as a reductive stress inductor, cells were incubated at 37 °C during 1 h and 24 h [8,17]. The chosen DTT concentration was measured during preliminary dose-dependent experiments with cell viability evaluation using the MTS assay. Three groups were formed: experimental — cerebral endothelial cells with medium substituted for serum-free one with DTT, 500 µM; control — cerebral endothelial cells with medium substituted for serum-free one without DTT; intact — cells cultivated in standard conditions without medium substitution for serum-free one and DTT.

Transmembrane mitochondrial potential in the cerebral endothelial cell culture was evaluated in reductive stress conditions and in the control using a fluorescent potential-sensitive probe — tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma, USA). Fluorescence intensity of the TMRE probe characterizes a change in the mitochondrial membrane potential and energy supply of cells [18]. Cells were incubated with TMRE with a service concentration of 500 nM during 15 min at 37 °C and then washed thrice in modified Ringer-Locke's solution. Mitochondrial fluorescence intensity was measured on the SpectraMax Reader (Molecular Devices, USA) using SoftMax Pro 7 with 560/590 nm emission excitation in fluorescence mode. For normalization of fluorescence values, each value was

divided by the mean fluorescence of the control in the corresponding plate and multiplied by 100%.

Mitochondrial production of superoxide anion in living cells was measured using the MitoSOX™ Green high-selective fluorescent probe (MSG, Invitrogen, Thermo Fisher Scientific). This probe is specifically accumulated in mitochondria and selectively oxidized by superoxide, which is followed by the appearance of bright-green fluorescence. MSG is not reactive with other reactive oxygen or nitrogen intermediates, and its oxidation is inhibited by superoxide dismutase. Cells were incubated with 1 μ M MSG during 30 min at 37 °C in the CO₂ incubator, then washed thrice in warm HBSS buffer containing Ca²⁺ and Mg²⁺. Fluorescence was detected on the SpectraMax Reader (Molecular Devices, USA) using SoftMax Pro 7 with 488/510 nm emission excitation. For normalization of fluorescence values, each value was divided by the mean fluorescence of the control in the corresponding plate and multiplied by 100%.

Content of total NAD⁺ and NADH in the endothelium cell culture was measured using a commercial NAD⁺/NADH Colorimetric Assay Kit (Elabscience, China). The method is based on a chromogenic enzymatic reaction where NAD⁺ is reduced to NADH, NADH in turn reduces WST-8 to orange formazan with a maximum absorption peak at 450 nm that was measured on the Inno-S spectrophotometer (LTeK, China). Formation of formazan is proportional to the total amount of NAD⁺ and NADH in cell lysate. Measurement range is 0.02–5.0 μ M. NAD⁺ and NADH content was normalized to protein concentration by dividing the mean fluorescence of each sample by the mean protein concentration in the sample.

FLIM and data analysis methods

Time-resolved fluorescent images of autofluorescence were recorded with two-photon excitation by Chameleon Ultra II femtosecond titanium-sapphire laser (Coherent, USA) at 740 nm. Measurements were performed on the LSM-710 NLO confocal system (Zeiss) with the FLIM module (Becker & Hickl, Germany) connected to it. The system included the SPC-150 time-correlated single-photon counting (TCSPC) module and the GaAsP HPM-100-07 hybrid photodetector; primary assembly and raw data quality control were carried out in SPCImage (Becker & Hickl, version 8.x). FB450-40 and FB550-40 bandpass filters (Thorlabs, USA) were used for wavelength division multiplexing for NADH and FAD channels, respectively; actual analysis wavelength ranges were limited approximately to 410–490 nm for NADH and to 510–590 nm for FAD. For each test sample, 2–3 FLIM images were recorded; each image included at least 15 mature cerebral endothelial cells and was used for independent analysis.

All measurements were organized within three test sample groups: „DTT“ — cells were subjected to dithiothreitol (DTT) treatment in conditions described in Section „Reductive Stress Induction“; „Control“ — cells were measured

in the serum-free medium and used as a reference control; „Intact“ — cells were measured in DMEM complete culture medium.

Preliminary processing of FLIM images was performed in SPCImage: quality evaluation by the number of detected photons per pixel and noise pixel threshold rejection, background correction adjustment and basic filtration were performed. Time characteristics of fluorescence in NADH and FAD spectral ranges were interpreted by the phasor analysis without parametric fitting according to Ranjit et al protocol. (Nat. Protoc. 2018). In this representation, each pixel is described by the G and S coordinates — real and imaginary parts of the first harmonic component of the Fourier transform of fluorescence decay function. In addition to the phasor analysis without fitting, parametric quantities — relation of component amplitudes and mean lifetimes, were also extracted from the time curves for qualitative evaluation of the metabolic status and simplified biological interpretation. In particular, biexponential lifetime deconvolution was performed for NADH in SPCImage (Becker & Hickl) with a priori set components $\tau_1 \approx 0.4$ ns (short component, „free“ NADH) and $\tau_2 \approx 2.5$ ns (long component, „bound“ NADH); preexponential factors a_1 and a_2 were obtained from fitting results and a_1/a_2 was calculated. Increase in this parameter was interpreted as the growth of relative contribution of the short component (increase in the proportion of „free“ NADH relative to bound one), which in terms of biology is widely viewed as an increase in the NAD⁺ and NADH cytosolic pool and as a sign of metabolism shift towards glycolytic paths or reductive state. For clarity and comparability with phasor coordinates, a_1/a_2 was averaged over ROI (intensity-weighted average) and used as a „metabolic index“ for descriptions of results and curves.

Similarly for FAD, the mean lifetime τ_{mean} was calculated as intensity-weighted average over components obtained through three-exponential fitting (typical τ_i for FAD: $\tau_1 \approx 0.25$ ns, $\tau_2 \approx 1.4$ ns, $\tau_3 \approx 5.0$ ns). For NADH, the mean lifetime was calculated as $\tau_{\text{mean}} = (a_1\tau_1 + a_2\tau_2)/(a_1 + a_2)$, for FAD — the equivalent weighted sum over three components was used. Changes in τ_{mean} are used as an additional shift marker of the redox state: direction and amount of change in τ_{mean} were visualized together with phasor centroids to demonstrate shift paths in the phasor space (for example, transition towards shorter/longer mean lifetimes corresponds to centroid movement along the arc of the phasor semicircle).

All amplitude coefficients and τ_{mean} were obtained at the pixel level and then aggregated over ROI/image; aggregation used the intensity weight to consider the contribution of bright pixels. As the phasor analysis is initially used as a method without fitting, the use of a_1/a_2 and τ_{mean} was treated as a complementary analysis that increases the biological interpretability of observed shifts. Comparison of phasor analysis data and parametric metrics served as an additional inference robustness measure: main trends (of G and S centroid variation) were confirmed by the direction and amount of change of a_1/a_2 and τ_{mean} .

Phasor diagram plotting, density and centroid calculation, and visualization algorithms are implemented in the Julia programming language using KernelDensity and Makie libraries. Intensity filtering was additionally used before statistical processing: 1% to 25% of upper points were selected by intensity (magnitude of filtration varied depending on the quality of particular set of images) to minimize the effect of background noise and artifacts.

Statistical data analysis and plotting of curves were performed using GraphPad Prism 10 (GraphPad Software, USA) and the R programming language. Normality of distribution and data dispersion equality were validated using the Shapiro-Wilk and Levene tests, respectively. For group comparison in the case of normal data distribution and adherence to homoscedasticity condition, one-way ANOVA test was used and followed by Tukey's post hoc test. If the data didn't meet the normality or homoscedasticity assumptions, the nonparametric Kruskal-Wallis test followed by the Dunn post hoc test were used for group comparison.

Pairwise comparison of phasor distributions between groups (DTT vs Control, Intact vs Control, DTT vs Intact) used the nonparametric Mann-Whitney U test; multivariate comparisons, if required, used MANOVA followed by multiple comparison correction. Besides the p values, the distribution overlap metric ranging from 0 to 1, where 1 corresponds to full overlap, was calculated to evaluate the practical relevance.

Data is represented as $M \pm SD$ or as the $\pm IQR$ median, where M is the mean, SD is the standard deviation, IQR is the interquartile range depending on the shape of distribution; differences were considered to be statistically significant at $p < 0.05$, unless otherwise specified.

Results

Reductive stress in the experimental animal cerebral endothelial cell culture was simulated using dithiothreitol (DTT), a low-molecular reducing agent, the main function of which is in prevention of disulfide bonding in protein molecules, especially intramolecular and intermolecular bonds between protein cysteine residues. Immunostaining method showed the prevalence of CD31 immunopositive cells (88%) expressing cell adhesion membrane proteins that are typical of cerebral microvascular endothelium, which confirms the quality of obtained cell cultures, and they can be used for evaluation of reductive stress effects.

It has been found that the content of total NAD⁺ and NADH in the endothelium culture with addition of DTT was $0.075 \pm 0.01 \mu\text{mol/g}$ of protein, which is higher by 55% than in the control ($0.034 \pm 0.01 \mu\text{mol/g}$ of protein), however, these data are statistically insignificant ($p = 0.174$) (Figure 1).

Incubation with DTT during 24 h doesn't significantly change the transmembrane mitochondrial potential in the cerebral endothelial cell culture (Figure 2, *a*) in all groups of interest ($p = 0.2843$). There is reliable increase in the mitochondrial production of superoxide in cells incubated

with addition of DTT (123.7 ± 38.24) compared both with the control (99.83 ± 20.95 , $p = 0.0004$) and intact (119.3 ± 59.25 , $p = 0.0408$) groups (Figure 2, *b*). Thus, the reductive stress simulation initiates the development of oxidative stress, however, this is not followed by a functional change in the cerebral endothelium mitochondria *in vitro*.

Redox disbalance induced by a disbalance between pro-oxidant and antioxidant cell systems can lead to a shift towards reduced forms of pyridine nucleotides — NAD⁺ level reduction and NADH accumulation. To evaluate the consequences of such disbalance in cerebral endothelial cells, a high-sensitive method was used — fluorescence lifetime imaging microscopy (FLIM) allowing the redox state to be quantified on the basis of autofluorescence of

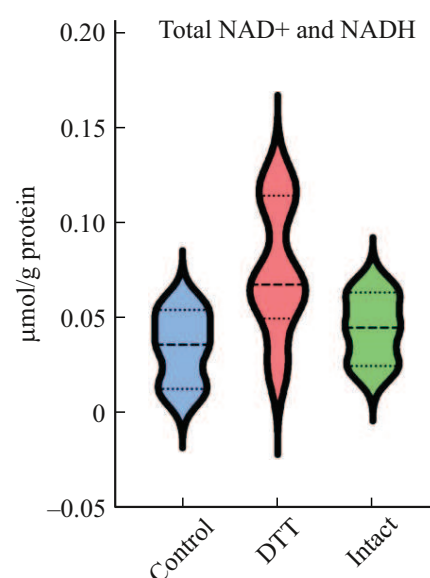


Figure 1. Content of the total NAD⁺ and NADH pool in the cerebral endothelial cell cultures with reductive stress induction *in vitro*. The data are shown as the $\pm IQR$ median.

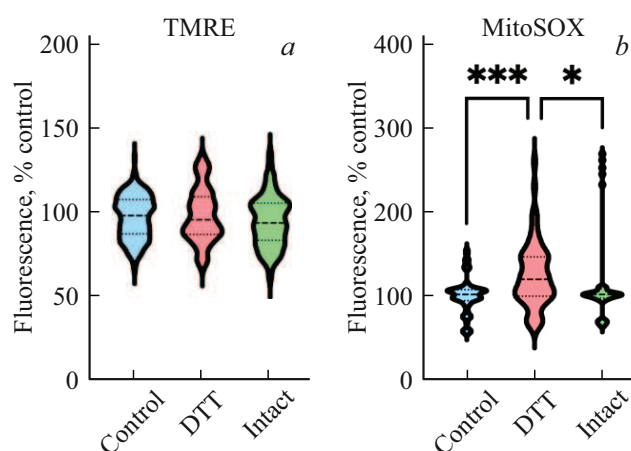


Figure 2. (a) Transmembrane mitochondrial potential level; (b) evaluation of mitochondrial production of reactive oxygen intermediates in the cerebral endothelial cell culture in reductive stress simulation conditions (DTT, 24 h). Data are represented as the $\pm IQR$ median; *** — $p < 0.001$, * — $p < 0.05$.

metabolic coenzymes, NADH and FAD. Figure 3 shows phasor diagrams reflecting changes in the NADH and FAD wavelength ranges before and after incubation with DTT.

FLIM maps and phasor diagrams (Figure 3) show that after incubation with the DTT reducing agent, in particular, of mitochondria located near the nucleus, a short-lived component signal, NADH lifetime (marked in red in the images), is enhanced. Local increase in the short component contribution is interpreted as an increase in the proportion of „free“ NADH and, accordingly, as an increase in NAD^+/NADH — a typical marker of the reductive stress. Parallely observed „blur“ and loss of sharpness of mitochondrial boundaries can reflect not only the NADH accumulation in the organelle matrix, but also partial buffering or movement of NADH into the cytosol, which agrees with violation of nucleotide pool compartmentalization in mitochondrial homeostasis disorder.

Key observation is that similar changes — enhancement of the short NADH component and reduction of morphological mitochondria differentiation, are also detected in the control group incubated in the serum-free medium. This is indicative of a considerable contribution of incubation conditions (in particular, the absence of serum factors) to formation of redox disbalance: „free“ culture itself can induce a phenotype partially mimicking the effect of reducing agents, which calls for strict control of the medium composition during interpretation of FLIM parameters as abnormality markers.

For FAD, reduction of τ_{mean} in both groups (DTT and Control) is observed on the presented data. Reduction of τ_{mean} of FAD is correlated to the metabolic shift towards enhancement of NAD^+ regeneration mechanisms, primarily glycolytic regeneration (for example, through lactic dehydrogenase activity), and thus reflects compensatory metabolic cell switching in response to excess of reduced equivalents. Total changes in NADH and FAD form a „metabolic shift“ picture combining the local reductive stress and system activation of glycolytic paths.

Practical importance of the findings is in the fact that the FLIM phasor maps functional heterogeneity of the redox state in the endothelial cell population in an adequate and spatially resolved manner and sensitively identifies both direct reductive effects (DTT) and background effects induced by the incubation conditions. For clear differentiation of adaptive metabolic switching and true mitochondrial dysfunction, additional control measurements are required, in particular, direct evaluation of the NAD^+ and NADH pool, lactate measurements, functional mitochondria tests (OCR, membrane potential) and morphological organelle validation.

Discussion

The study is the first to evaluate the functional state of mitochondria and metabolic profile of cerebral endothelial cells during reductive stress simulation *in vitro* using DTT as a reductive agent. It is shown that the 24 h incubation

with DTT doesn't lead to certain changes of transmembrane mitochondrial potential and total content of NAD^+ and NADH in cerebral endothelium cells, however, there is an increase in mitochondrial production of superoxide anion, suggesting that signs of the oxidative stress occur in connection with maintaining the structural and functional integrity of mitochondria in redox disbalance conditions. Altogether, this corresponds to the concept of the interconnected development of the reductive and oxidative stresses in cells [8], including in terms of age-dependent endothelial cell damage [19].

The obtained data prove high informativity of phasor FLIM for detecting early metabolic rearrangements in cerebral endothelial cells: as early as 1 h after DTT exposure a significant increase is noted in the contribution of the short-lived NADH component — a parameter which is traditionally interpreted as a reflection of a „free“ (including cytosolic) coenzyme pool and, accordingly, as an indicator of increasing NAD^+/NADH . Such spatially-resolved interpretation without lifetime fitting is adequately described in the phasor analysis methodology and serves as a reliable tool for analysis without τ distribution fitting in tissues and cultures [20].

Parallel reduction of τ_{mean} for FAD and coordinated shifts of phasor positions are indicative of coordinated shift of the metabolic profile towards an increase in NAD^+ regeneration paths, primarily glycolytic regeneration, which shall be treated as adaptive metabolic cell switching with reductive equivalent excess, rather than unambiguously as a primary acute dysfunction of the respiratory chain. Similar transitions in the metabolic status in various metabolic stresses have been also described earlier when using the phasor FLIM for distinguishing metabolic states [21].

It is critical that a similar picture (increase in the contribution of the short NADH component and decrease in τ_{mean} of FAD) was also observed in the control group incubated in the serum-free medium. This emphasizes a considerable contribution of incubation conditions (in particular, the absence of serum factors) to formation of the redox disbalance and indicates that the medium composition shall be strictly controlled when interpreting the FLIM parameters in redox stress models.

Finally, it should be noted that reduction of τ_{mean} not always unambiguously reflects only redistribution of „free“/„bound“ NADH: recent studies demonstrate that changes of τ_{mean} can also partially depend on a change of the total NADH pool, which introduces additional aspects in FLIM data interpretation. In particular, visualization of subcellular changes of the NADH pool using FLIM has been recently demonstrated in experimental studies, which shall be considered when analyzing the reduction of τ_{mean} in reductive stress models [14].

Thus, cerebral endothelial cells retain viability and functional integrity of mitochondria in experimental reductive stress simulation *in vitro*, however, their energy metabolism quickly changes towards enhanced glycolysis. This switching is apparently a compensatory response to reduced

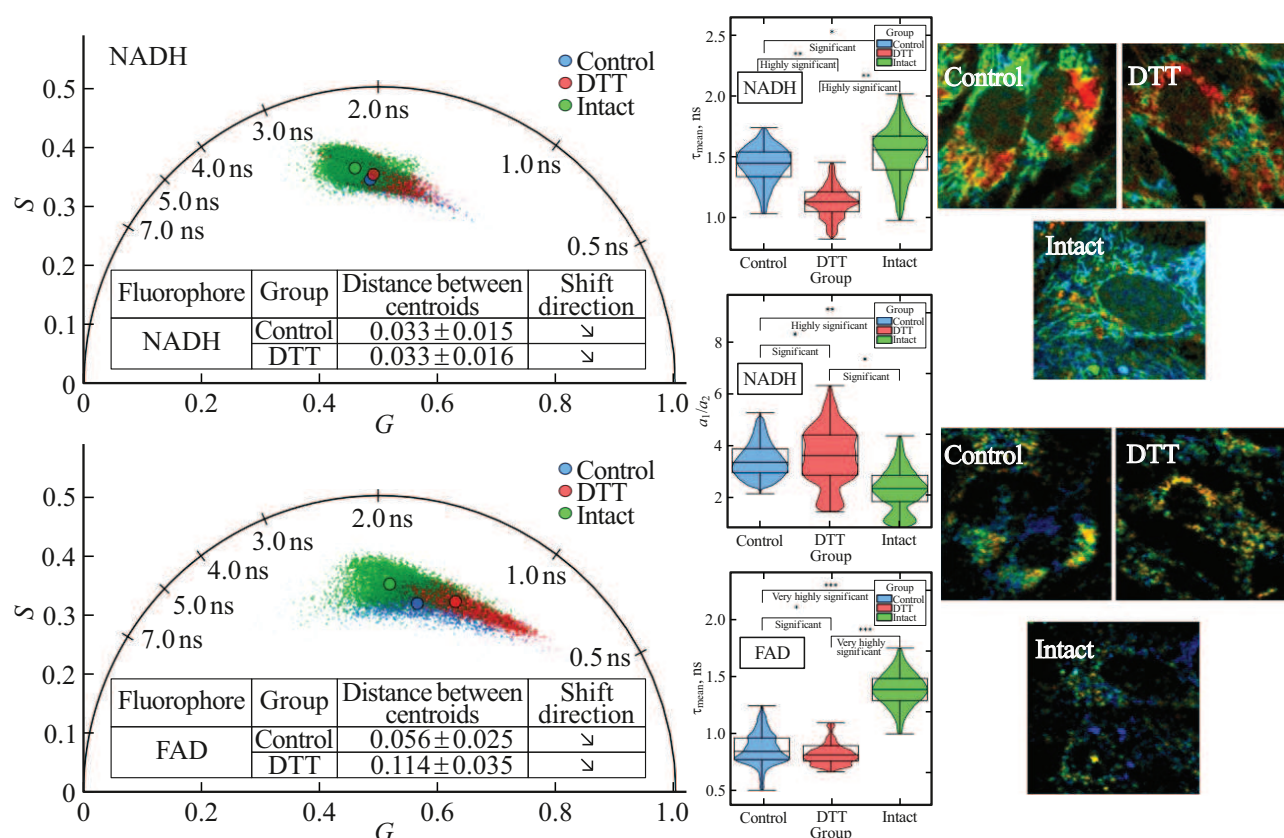


Figure 3. Phasor and parameter analysis of FLIM data for NADH (NADH, upper panes) and FAD (FAD, lower panes) in cerebral endothelial cells in three experimental conditions („Intact“ — medium with serum, „Control“ — serum-free medium, „DTT“ — dithiothreitol treatment). Left — G – S phasor diagrams with pixel distribution by groups (blue — Control, red — DTT, green — Intact) and marked intensity-weighted centroids; a semicircle corresponds to monoexponential decay, divisions on the arc serve as a reference for equivalent lifetimes. Nested tables in left panes show Euclidean distances between group centroids and total shift direction (arrows). On the right, there are representative spatial lifetime maps (phasor-mapping) for the „Intact“, „Control“ and „DTT“ conditions (lifetime color coding) and corresponding key metric distribution diagrams: intensity-weighted τ_{mean} for NADH and FAD and a_1/a_2 for NADH (a_1/a_2 reflects the amplitude ratio of short and long NADH components). Data are shown as $M \pm SD$ or as $\pm IQR$ depending on the shape of distribution; *** — $p < 0.001$ and overlap < 0.2 ; ** — $p < 0.001$ and overlap 0.2 – 0.4 or $p < 0.01$ and overlap < 0.3 ; * — other combinations with $p < 0.05$.

equivalent excess, however, in case of prolonged action, can become a risk factor facilitating BBB permeability impairment. It should be emphasized that energy crisis in endothelial cells of cerebral microvessels is treated as one of the key pathogenetic mechanisms of BBB damage and progression of neurodegenerative diseases [22–25].

Conclusion

This study used the fluorescence-lifetime imaging microscopy for the first time to show that cerebral endothelial cells exhibit plasticity to exogenously induced reductive stress: while retaining the mitochondrial membrane potential and in connection with oxidative damage, they demonstrate quick metabolic rearrangement — energy metabolism shift towards glycolysis. This indicates that compensatory metabolism rearrangement, rather than mitochondrial dys-

function, is a key early marker of redox disbalance in the BBB endothelium. Obtained data emphasize high FLIM sensitivity to subclinical changes of redox homeostasis. It is important that FLIM can detect changes in the redox state long before morphological or functional signs of cell damage appear.

Despite the general progress in understanding the redox regulation, reductive stress in NVU and BBB cells is still understudied. Key constraints include the absence of standardized *in vitro* and *in vivo* reductive stress models specific for CNS cells; difficulties in interpretation of exogenous reducing agent effects due to the influence of incubation conditions (in particular, serum-free medium); limited availability of methods for accurate real-time evaluation of the redox status and mitochondrial function. Promising applications include development of physiologically relevant NVU/BBB models, employment of genetically encoded redox sensors for dynamic monitor-

ing of NAD⁺/NADH and GSSG/GSH *in vitro* and *in vivo*.

Thus, the FLIM spectroscopy is a tool for noninvasive, quantitative and spatially-resolved monitoring of redox-metabolic status in NVU and BBB cells. The results of the study expand the understanding of BBB redox plasticity and offer a potential for development of new optical biomarkers of early neurodegenerative disease stages. In future, FLIM integration with genetically encoded sensors and physiologically relevant NVU models will allow the development of therapeutic agents and personalized neuroprotection.

Compliance with ethical standards

All laboratory animal experiments were carried out with strict adherence to ethical standards established by the laws of the Russian Federation, Basel Declaration principles, and are approved by the local ethics committee of Russian Center of Neurology and Neurosciences (minutes № 10-9/23 as of 20.12.2023).

Funding

The study was funded from the budget within state order „Aberrant metabolic plasticity of neurovascular unit cells in cerebrovascular pathology“, № 1023101100004-9-3.1.8;3.1.4.

Conflict of interest

The authors declare no conflict of interest.

References

- [1] O.L. Lopatina, Y.K. Komleva, N.A. Malinovskaya, Y.A. Panina, A.V. Morgun, A.B. Salmina. *Front. Immunol.*, **11** (2020). DOI: 10.3389/fimmu.2020.585294
- [2] A.B. Salmina, E.V. Kharitonova, Y.V. Gorina, E.A. Teplyashina, N.A. Malinovskaya, E.D. Khilazheva, A.I. Mosyagina, A.V. Morgun, A.N. Shuvaev, V.V. Salmin, O.L. Lopatina, Y.K. Komleva. *Int. J. Mol. Sci.*, **22** (9), 4661 (2021). DOI: 10.3390/ijms22094661
- [3] A.B. Salmina. *Neurochem. J.*, **17** (3), 325–337 (2023). DOI: 10.1134/S1819712423030157
- [4] E.A. Pozhilenkova, O.L. Lopatina, Y.K. Komleva, V.V. Salmin, A.B. Salmina. *Rev. Neurosci.*, **28** (4), 397–415 (2017). DOI: 10.1515/revneuro-2016-0071
- [5] U.H. Langen, S. Ayloo, C. Gu. *Annu. Rev. Cell Dev. Biol.*, **35** (1), 591–613 (2019). DOI: 10.1146/annurev-cellbio-100617-062608
- [6] D.N. Doll, H. Hu, J. Sun, S.E. Lewis, J.W. Simpkins, X. Ren. *Stroke*, **46** (6), 1681–1689 (2015). DOI: 10.1161/STROKEAHA.115.009099
- [7] Y. Wang, J. Wu, J. Wang, L. He, H. Lai, T. Zhang, X. Wang, W. Li. *Mitochondrion*, **69**, 71–82 (2023). DOI: 10.1016/j.mito.2023.01.007
- [8] W.-X. Ma, C.-Y. Li, R. Tao, X.-P. Wang, L.-J. Yan. *OMCL*, 1–11 (2020). DOI: 10.1155/2020/5136957
- [9] J.J. Heindel, B. Blumberg, M. Cave, R. Machtinger, A. Mantovani, M.A. Mendez, A. Nadal, P. Palanza, G. Panzica, R. Sargis, L.N. Vandenberg, F. vom Saal. *Reprod. Toxicol.*, **68**, 3–33 (2017). DOI: 10.1016/j.reprotox.2016.10.001
- [10] E.J. Anderson, M.E. Lustig, K.E. Boyle, T.L. Woodlief, D.A. Kane, C.-T. Lin, J.W. Price, L. Kang, P.S. Rabinovitch, H.H. Szeto, J.A. Houmard, R.N. Cortright, D.H. Wasserman, P.D. Neuffer. *J. Clin. Invest.*, **119** (3), 573–581 (2009). DOI: 10.1172/JCI37048
- [11] Y.-H. Wu, H.-L. Hsieh. *Antioxidants*, **12** (10), 1816 (2023). DOI: 10.3390/antiox12101816
- [12] A. Krakowiak, S. Pietrasik. *Biology*, **12** (6), 875 (2023). DOI: 10.3390/biology12060875
- [13] T.S. Blacker, Z.F. Mann, J.E. Gale, M. Ziegler, A.J. Bain, G. Szabadkai, M.R. Duchen. *Nat. Commun.*, **5** (1), 3936 (2014). DOI: 10.1038/ncomms4936
- [14] A. Song, N. Zhao, D.C. Hilpert, C. Perry, J.A. Baur, D.C. Wallace, P.M. Schaefer. *Commun. Biol.*, **7** (1), 428 (2024). DOI: 10.1038/s42003-024-06123-7
- [15] Y. Liu, Q. Xue, Q. Tang, M. Hou, H. Qi, G. Chen, W. Chen, J. Zhang, Y. Chen, X. Xu. *Microvasc. Res.*, **90**, 199–205 (2013). DOI: 10.1016/j.mvr.2013.08.004
- [16] M.R. Kapkaeva, A.B. Salmina, E.D. Khilazheva, D.N. Voronkov. *Sposob vydeleniya endoteliya mikrososudov mozga krysy [Elektronnyi resurs]*. URL: https://www.fips.ru/registers-doc-view/fips_servlet
- [17] L. Yan, W. Liu, H. Zhang, C. Liu, Y. Shang, Y. Ye, X. Zhang, W. Li. *J. Cell Sci.*, (2014). DOI: 10.1242/jcs.135293
- [18] R.C. Scaduto, L.W. Grotyohann. *Biophys. J.*, **76** (1), 469–477 (1999). DOI: 10.1016/S0006-3495(99)77214-0
- [19] M. Mladenov, L. Lubomirov, O. Grisk, D. Avtanski, V. Mitrokhin, I. Sazdova, M. Keremidarska-Markova, Y. Danailova, G. Nikolaev, R. Konakchieva, H. Gagov. *Antioxidants*, **12** (5), 1126 (2023). DOI: 10.3390/antiox12051126
- [20] S. Ranjit, L. Malacrida, D.M. Jameson, E. Gratton. *Nat. Protoc.*, **13** (9), 1979–2004 (2018). DOI: 10.1038/s41596-018-0026-5
- [21] C. Stringari, A. Cinquin, O. Cinquin, M.A. Digman, P.J. Donovan, E. Gratton. *PNAS*, **108** (33), 13582–13587 (2011). DOI: 10.1073/pnas.1108161108
- [22] L.S.S. Ferreira, C.S. Fernandes, M.N.N. Vieira, F.G. De Felice. *Front. Neurosci.*, **12** (2018). DOI: 10.3389/fnins.2018.00830
- [23] A. Sędzikowska, L. Szablewski. *Int. J. Mol. Sci.*, **22** (18), 9987 (2021). DOI: 10.3390/ijms22189987
- [24] O.L. Lopatina, Y.A. Panina, N.A. Malinovskaya, A.B. Salmina. *Rev. Neurosci.*, **32** (2), 131–142 (2021). DOI: 10.1515/revneuro-2020-0077
- [25] A.S. Averchuk, M.V. Ryazanova, T.I. Baranich, A.V. Stavrovskaya, N.A. Rozanova, S.V. Novikova, A.B. Salmina. *Bull. Exp. Biol. Med.*, **175** (3), 315–320 (2023). DOI: 10.1007/s10517-023-05859-2

Translated by E.Ilyinskaya