

Oxidation–reduction states of NADH *in vivo*: From animals to clinical use

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Abstract

Mitochondrial dysfunction is part of many pathological states in patients, such as sepsis or stroke. Presently, the monitoring of mitochondrial function in patients is extremely rare, even though NADH redox state is routinely measured in experimental animals. In this article, we describe the scientific backgrounds and practical use of mitochondrial NADH fluorescence measurement that was applied to patients in the past few years. In addition to NADH, we optically measured the microcirculatory blood flow and volume, as well as HbO₂ oxygenation, from the same tissue area. The four detected parameters provide real time data on tissue viability, which is critical for patients monitoring.

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1. Introduction

It has been 50 years since the publication of the seminal work of Chance and Williams on the mitochondrial metabolic state *in vitro* (Chance and Williams, 1955b). The discovery of pyridine nucleotides by Harden and Young a century ago (Harden and Young, 1906) was 30 years later followed by the complete description of their structure by Warburg and collaborators (Warburg et al., 1935). In 1955, the seminal work of Chance and Williams (1955a) defined for the first time the metabolic states of isolated mitochondria *in vitro*, and correlated these states to the oxidation–reduction levels of respiratory enzymes. The physiological significance of these metabolic states was elaborated by Chance and collaborators between 1956 and 1959. This formed the basis for the present studies of the response of mitochondrial respiratory carriers to early

stages of apoptosis (Arnoult, 2006; Green, 2006; Kroemer et al., 1997).

Close to 1000 relevant publications have appeared since the initial studies of NADH fluorescence *in vitro* and *in vivo* (Chance et al., 1955, 1962). Several key experiments have shown that this technology could be used for metabolic mapping of animal brain. It could also be used to measure specific activation in localized human brain areas. More recently, this technique was adapted for clinical applications (in intra-operative and intensive care units). In this article we summarize the half century of research on NADH redox state monitoring *in vitro* and *in vivo*, including its clinical applications that have become practical in the last few years (Deutsch et al., 2004; Mayevsky et al., 1996, 2004b, 2005).

1.1. The history of NADH monitoring

According to Chance et al. (1973): “The accumulation of evidence since the pioneer work of David Keilin on cytochromes (Keilin, 1966) and Otto Warburg on “Atmungs-

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ferment” (Warburg, 1949) as the keystones of cellular oxygen utilization led us to the study of the redox states of electron carriers in isolated mitochondria as a function of oxygen concentration and to develop techniques to measure the states of anoxia and normoxia in living tissues at the level of isolated and *in situ* mitochondria.”

Although Warburg isolated DPNH and TPNH, he was apparently reluctant to imagine that those molecules were part of the respiratory chain. He also studied their optical properties and identified the 340 nm absorption band as being characteristic of the reduced form. The idea that these might also belong to Keilin’s respiratory chain was not considered and in fact, was to some extent abhorrent to Warburg to imagine that he should support Keilin’s views on the cytochrome chain.

Thus, the observation, made spectroscopically in Chance’s difference spectrum of the respiratory chain showing prominent peaks of what was soon to be called NADH and a trough due to flavoprotein, and the finding that they were, respectively, over 40 and over 10 times greater concentration than Keilin’s cytochrome *c*, was in a sense an unwelcome addition by Keilin. In my (Britton Chance) personal contact with Keilin, he never discussed these two components as new or overwhelmingly present as members of his respiratory chain (taking note that Slater’s preparation of Keilin and Hartree’s respiratory chain was carried out in such a way that no trace of NADH survived the rigorous sand grinding and acid extraction). In fact, personal contact with Keilin subsequent to the award of the Copley Medal, evoked no response whatsoever concerning these novel components, which certainly must have been prominent in his mind – nor in any conversation with him did he discuss Otto Warburg’s discovery of the chemistry of these two components.

The blue autofluorescence of cells and tissues was recognized from the first fluorescence microscopy studies and was termed “autofluorescence.” No attempt was made to characterize it or to do what must have been obvious to recent researchers, to follow Keilin’s most famous experiment, i.e. that on shaking a yeast cell suspension, the absorption bands of cytochrome disappeared as indeed did the absorption band of NADH, which would have brought an early realization of the relationship of NADH to the cytochrome electron carriers. Chance and Legallias (1951) described a rapid and sensitive spectrophotometer for reaction kinetics detection, and showed the applicability of this new technique to the measurements of pyridine nucleotide enzymes of muscle homogenates or intact cells (Chance, 1952).

The discovery of the optical properties of the mitochondrial reduced Nicotinamide Adenine Dinucleotide – NADH (old names – DPNH diphosphopyridine nucleotide or PN-pyridine nucleotide), led to very intensive research since the early 1950s. The reduced form of this molecule, NADH, absorbs light at 320–380 nm and emits fluorescent light in the 420–480 nm range. Since the oxidized form, NAD⁺, does not absorb light in this range, it became pos-

sible to evaluate the redox state of the mitochondrion by monitoring the UV absorbance or blue fluorescence of NADH.

The work of Chance and collaborators from the Johnson Research Foundation at the University of Pennsylvania in Philadelphia, led to the development of the technology and theory of mitochondrial function based on NADH redox state monitoring *in vitro* and *in vivo*. Since, multitudes of papers have been published on the state of NADH in the mitochondrial matrix. In 1951, Theorell and Bonnichsen discovered the shift in the absorption spectrum of DPN when bound to alcohol dehydrogenase (Theorell and Bonnichsen, 1951). This suggested the possible changes in NADH properties upon protein binding, which have become the subject of several recent reports.

The studies done by Chance and collaborators between 1952 and 1954 resulted in a major milestone in NADH monitoring methodology. The technique, presented by Chance (Chance, 1954) and by Chance and Connelly (1957), using a double beam spectrophotometer, enabled the determination of the appropriate wavelengths for respiratory enzymes measurements. The detailed descriptions of the respiratory chain and oxidative phosphorylation in the mitochondria were published in 1955 (Chance et al., 1955; Chance and Williams, 1955a,b,c,d,e). These findings have established our basic knowledge of mitochondrial function *in vitro* (Chance et al., 1955; Chance and Williams, 1955c,e).

The fact that NADH was monitored by the difference in the absorption spectrum of its reduced form, limited the technique to the study of mitochondria *in vitro* and very thin tissue samples (e.g. muscle) or cell suspensions. To create a method more specific than absorption spectroscopy, NADH measurements applied fluorescence spectrophotometry in the reflectance mode in the near-ultraviolet (UV-A) region. The first detailed study using fluorescence spectrophotometry of NADH in intact Baker yeast cells and Algal cells was published in 1957 by Duysens and Ames (Duysens and Ames, 1957). However, it failed to establish the crucial coupling of these signals to the intracellular mitochondria.

In the next 5 years (1958–1962), monitoring of NADH fluorescence expanded significantly. In a preliminary abstract, Chance et al. (1958) reported simultaneous fluorimetric and spectrophotometric measurements of the reaction kinetics of bound pyridine nucleotides (PN) in the mitochondria. In the same year, Chance and Baltscheffsky (1958) presented the preliminary results of measuring the fluorescence of intramitochondrial PN. This study proved the connection between the mitochondrial metabolic state and the redox state of NADH, measured by spectral fluorimetry in isolated rat liver mitochondria (Chance and Williams, 1955e).

The correlation between the enzymatic PN assay and sensitive spectrophotometry was investigated by Klingenberg et al. (1959) using rat liver, heart, kidney and brain.

In 1959, Chance and collaborators were able to expand NADH fluorometry application to various experimental models, ranging from isolated mitochondria to intact tissues. The nature of intramitochondrial pyridine nucleotides was analyzed in connection to the ADP–ATP cycle (Chance, 1959). In order to monitor NADH localization in intact cells, Chance and Legallias (1951) developed a unique differential microfluorimeter with a very high spatial resolution. This approach was used to identify the intracellular locale of NADH fluorescence signals in a variety of cells (Chance and Thorell, 1959; Perry et al., 1959). The next step was to apply the fluorimetric technique to animal tissues of higher organization. Together with the late Jobsis, Chance (1959) measured *in vitro* changes in muscle NADH fluorescence after stimulation. Chance and Thorell (1959) came to a very significant conclusion that “The oxidation and reduction state of mitochondrial pyridine nucleotide without a measurable change of cytoplasmic fluorescence suggest that compartmentalization of mitochondrial and cytoplasmic pyridine nucleotide occurs *in vivo*, at least in the grasshopper spermatid.” Chance and Hollunger (1960) found that NADH could be generated through reverse electron transfer, contributing from the substrate to the citric acid cycle redox state as high a redox potential as succinate fumarate. This novel pathway is still under investigation.

The *in vivo* NADH monitoring approach was extensively researched during 1962. The “classical” paper on *in vivo* NADH monitoring was published in that year (Chance et al., 1962). The authors were able to simultaneously monitor the brain and kidney of an anesthetized rat using two microfluorimeters. Table 1 presents the major milestones in the initial development of NADH monitoring.

1.2. Current state of NADH monitoring

Today we are facing a new era of possibilities to commonly monitor mitochondrial function *in vivo* in hospitalized patients. The best approach to evaluate intracellular or intramitochondrial O₂ levels is to measure the spectroscopic signatures of the various components of the respiratory chain (Chance et al., 1973). The latter publication

concluded that, “For a system in a steady state, NADH is at the extreme low potential end of the chain, and this may be the oxygen indicator of choice in isolated mitochondria and tissues as well.” The same conclusion was reached by the late Lübbers: “the most important intrinsic luminescence indicator is NADH, an enzyme of which the reaction is connected with tissue respiration and energy metabolism” (Lubbers, 1995).

Many approaches have been developed to monitor tissue oxygen concentration. Some were based upon colloid-coated oxygen micro-electrodes of Davies and Brink, that were successfully commercialized by Leland Clark. Some used oxygen quenched luminescence of porphyrins (Vanderkooi et al., 1973). However, their intracellular location has been difficult to determine and their sensitivity to oxygen does not approach that of the mitochondrial NADH.

The involvement of the mitochondria in many pathophysiological processes and clinical conditions has become apparent during the past ten years. Nevertheless, very little was done to monitor mitochondrial function *in vivo* in clinical environments. For example, in recently published reviews, the relationship between mitochondrial and neuronal survival was described, but *in vivo* monitoring of NADH was not mentioned (Nichols and Budd, 2000; Sims and Anderson, 2002). In reviewing relevant achievements and perspectives, Scheffler omitted the contribution of *in vivo* monitoring of mitochondrial function, i.e. NADH fluorescence (Scheffler, 2000). Only by simultaneous measurements of the redox state of NADH and the desaturation of hemoglobin, did it become clear, in the quantitative studies of Schindler of the remarkable sensitivity of yeast NADH to oxygen tension, that the affinity of the cytochrome chain for oxygen depended upon its turnover number and varied from 10⁻⁸ to 10⁻¹⁰ moles/liter for physiological turnover numbers. This linked the metabolic state of isolated mitochondria and mitochondria in yeast cells to the possibilities for oxygen quantification in cells and tissues.

Of great interest in tissue function is the response of mitochondria to ADP, for example in muscle contraction and in neuronal brain function. Under *in vitro* conditions, Chance and Williams defined the metabolic state of the mitochondria by changing ADP, substrate and oxygen,

Table 1
Milestones in the early period of mitochondrial NADH monitoring

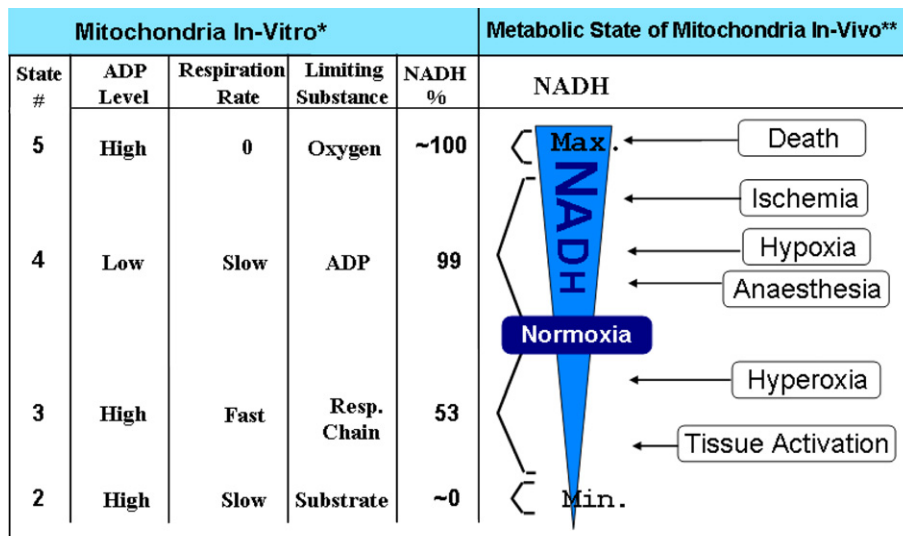
Year	Discovery	Author(s)
1905	Involvement of adenine containing nucleotides in yeast fermentation	Harden and Young (1906)
1935	Description of the complete structure of “Hydrogen transferring Coenzyme” in erythrocytes	Warburg et al. (1935)
1936	Definition of the two cofactors DPN and TPN	Warburg (1949)
1951	A shift in the absorption spectrum of DPNH with Alcohol dehydrogenase	Theorell and Bonnicksen (1951)
1951	Development of a rapid and sensitive Spectrophotometer	Chance and Legallias (1951)
1952	Monitoring of pyridinenucleotide enzymes	Chance (1952)
1954	Development of the double beam spectrophotometer	Chance (1954)
1957	The first detailed study of NADH using Fluorescence spectrophotometry	Duysens and Ames (1957)
1958	Measurement of NADH fluorescence in isolated mitochondria	Chance and Baltscheffsky (1958)
1959	Measurement of muscle NADH fluorescence <i>in vitro</i>	Chance and Jobsis (1959)
1962	<i>In vivo</i> monitoring of NADH fluorescence from the brain and kidney	Chance et al. (1962)

and measuring the redox state of NADH, flavoproteins and cytochromes. As shown in Fig. 1, NAD is converted to the reduced form NADH to 99% in the resting state 4. When ADP is added to the mitochondrial suspension, NADH is oxidized to a level of 53% which was defined as the active state 3. The addition of ADP led to an increase in the activity of the respiratory chain in order to synthesize more ATP and resulted in increased oxygen consumption. When O₂ is depleted from the mitochondrial suspension (state 5), NADH increased to the maximal level of 100%. Since it is difficult to conduct the same experiments under *in vivo* conditions, we performed many tests *in vivo* where the level of NADH was recorded in various metabolic and pathophysiological situations. As it is seen in Fig. 1 (right), the resting level of NADH *in vivo* is between states 3 and 4. A normoxic organ or tissue in this state could be induced to state 5 by anoxia (0% oxygen) or cardiac arrest. Tissue activation of various organs may lead to oxidation of NADH toward state 3, though it is very difficult or even impossible to determine the exact minimal level of NADH *in vivo*. Preliminary results (Mayevsky, 1976) suggested that the resting metabolic state of the brain *in vivo* is between states 3 and 4 (3.5-3.6), based on the changes in NADH.

1.3. From NADH fluorescence measurement as a single parameter to multiparametric monitoring

We will now describe two ways in which the studies of isolated mitochondria were expanded to monitoring of important tissues, particularly, the heart and the brain.

Studies of several Chance’s collaborators (Asakura et al., 1974; Steenbergen et al., 1977) made striking advances in time resolved flash photography of metabolic/hypoxic events in the perfused heart. At the same time, Barlow et al. (1979) developed a flying spot system that operated through a cranial window in human subjects, indicating the beneficial effects of the ECIC operation in several patients. Another example is the study of the effect of adrenaline in the kidney and the brain (Chance et al., 1962). The authors described the effect of nor adrenaline on NADH fluorescence measured in the Brain and the Kidney simultaneously. They showed “large and rapid increases in fluorescence in the kidney and a decrease in the brain that is smaller and longer lasting.” The investigators speculated that “presumably the nor adrenaline affects the circulation of the Kidney much more markedly than that of the Brain.” It took 40 years to experimentally prove the cause for the discrepancy between brain and kidney responses to nor adrenaline, measured by NADH fluorescence, and its connection to the effect on microcirculation. Recently we simultaneously measured NADH fluorescence and microcirculatory blood flow in 4 organs (Brain, Kidney, Liver and Testis) of rats exposed to adrenaline (Kraut et al., 2004). We found that the responses of the brain and kidney were opposite due to the well known response differentiation of the more vital (Brain) and less vital organ (Kidney) to emergency situations (adrenaline is a sympathetic stimulant). This mechanism is also known as the “Brain sparing effect” in newborns (Clerici et al., 2001) and adults (Barber et al., 1994; Ganong, 1991). Fig. 2 demonstrates this effect.



*According to Chance & Williams 1955 (Nature, Vol.176:250-254)

**According to Mayevsky and Chance 1982 (Science, Vol.217:537-540)
 Mayevsky 1984 (Brain Res. Rev., Vol. 7:49-68)

Fig. 1. The relationship between mitochondrial function *in vitro* and NADH levels *in vivo* under various conditions.

As shown in Fig. 2, the relationship between microcirculatory blood flow – TBF and NADH fluorescence, measured in the brain and the kidney, was completely opposite after adrenaline injection. The sympathetic stimulation by nor epinephrine (marked as NE, Fig. 2a) led to a significant decrease in TBF (hypo perfusion) and a large increase of NADH in the kidney, despite the increase in systemic blood pressure (signal not shown). In the brain, which is a more vital organ, the blood flow increased and NADH became slightly oxidized (the fluorescence signal decreased). The difference between the brain and the kidney becomes clear when the responses of the two organs are plotted in the same figure (Fig. 2b). As it is seen, after NE injection, the brain became better perfused and oxidized, moving right and down on the x - and y -axis, respectively. The kidney became hypoperfused and the NADH was less oxidized (see the signal decrease on the x -axis and the increase in NADH).

The monitoring of microcirculatory tissue blood flow (TBF) together with mitochondrial NADH, reveals the flow redistribution between the organs activated by nor adrenaline. It became clear that the more parameters are measured, the better interpretation can be given to the very complex pathophysiological situations. In view

of this, the multiparametric monitoring system based of diverse optical principles was developed. Each parameter could be measured by the available technologies that have emerged during the past three decades (Mayevsky and Rogatsky, 2007). Nevertheless, for clinical applications, all parameters were combined into a compact unit that would suite the clinical environment. Fig. 3 illustrates the principles of optical spectroscopy that enable measurements of four different physiological parameters using the same fiber optic probe located on the surface of the monitored tissue.

Clearly, the normal mitochondrial function is the most significant factor in O_2 balance in the tissue. Nevertheless, in order to properly assess energy metabolism at the cellular and microcirculation levels, it is necessary to measure additional parameters representing the O_2 supply to the tissue (tissue pO_2), and other vascular parameters. The monitored intravascular parameters include microcirculatory blood flow and the level of hemoglobin oxygenation. The fourth parameter, i.e. the tissue reflectance, representing the optical properties of the monitored tissue, is also needed for the correct evaluation of NADH fluorescence (Mayevsky and Chance, 1982).

2. Methods

2.1. The multiparametric monitoring system (MMS)

Much information can be gathered from simultaneous measurements of hemoglobin dynamics in the visible area along with mitochondrial dynamics measured by NADH fluorescence. Two lines of development have been pursued: (1) The use of spectrophotometer–fluorometer with a rotating disk, developed by Norman Graham and the Johnson Foundation, have allowed simultaneous measurements of NADH, flavoprotein, oxy- and deoxyhemoglobin. (2) Further development has been performed of multiple optical fibers put in contact with the tissue, for example the brain, particularly for long term observations of the brain through a cranial window or through fiber optic contact to the dura.

The new MMS device is based on the monitoring principles of the 4 parameters shown in Fig. 3, and it will be described in the next few paragraphs.

2.1.1. Mitochondrial function

Mitochondrial function is evaluated by monitoring NADH fluorescence served as a good indicator of a decrease in oxygen availability in cellular compartments (Chance et al., 1973).

The tissue is excited by UV light (375 nm) and the emitted fluorescence is measured (420–480 nm). The principles of this method were established 50 years ago and the major advances introduced since then, were the new type of the light source and the use of optical fiber bundles to connect the tissue to the fluorometer. Also, a correction for hemodynamic artifacts has been adopted (Chance et al., 1973). The main light source used in the past was Hg arc lamp

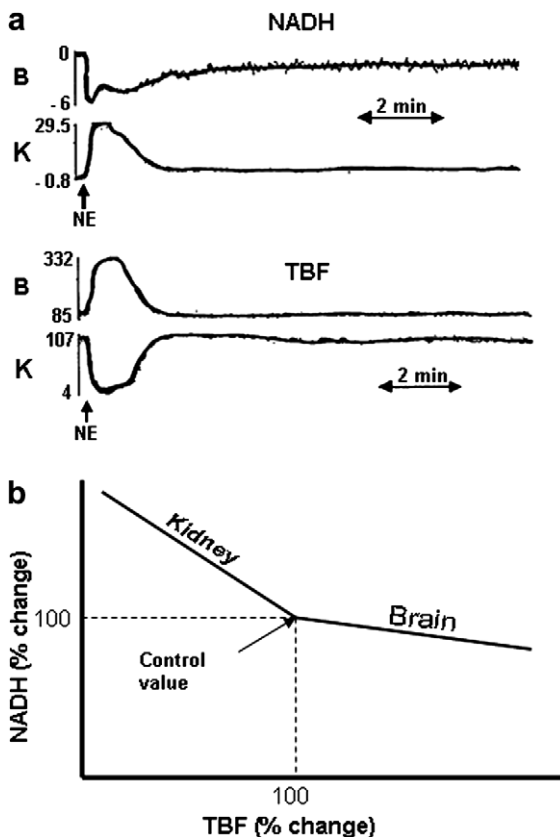


Fig. 2. The effect of intravenous injection of nor epinephrine on NADH fluorescence and microcirculatory blood flow (TBF) in the brain and kidney of an anesthetized rat. (a) Real-time analog signals. (b) Correlation between the parameters.

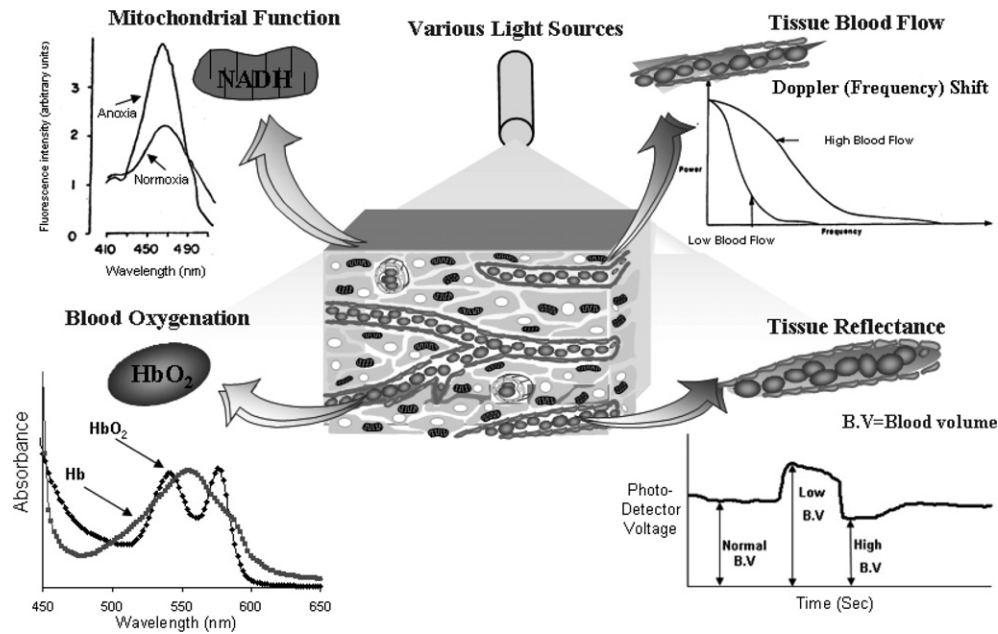


Fig. 3. The principles of tissue vitality monitoring by the multiparametric monitoring system – MMS. Three of these parameters were monitored from the intravascular compartment, while the NADH parameter indicates the function of an intracellular organelle – the mitochondrion.

having a strong emission line at 366 nm absorbed by the reduced NADH but not by its oxidized form (NAD^+). The introduction of the 375 nm Light Emitting Diode (LED) of Nichia as a new light source in the present instrument (MMS), significantly reduced the size and the price of this component, the factors crucial for a possible medical device.

The use of optical fibers led to a more flexible connection between the monitored tissue and the fluorometer (Chance et al., 1973; Mayevsky, 1984). This enabled the monitoring of the non-anesthetized brain (Mayevsky, 1984), and monitoring in different locations in the same organ or the same animal (Mayevsky and Chance, 1982).

2.1.2. Tissue reflectance

The continuous changes in tissue absorption properties affect the intensity of tissue reflectance. This parameter was used as a correction measure for artifacts in the fluorescence signal (Chance et al., 1973; Jobsis et al., 1971; Mayevsky et al., 2004b). As seen in Fig. 3, the reflectance signal (R) is mainly affected by changes in blood volume (Mayevsky, 1984; Mayevsky and Chance, 1982). When ischemia is induced, the decrease in blood volume will lead to an increase in the R signal. During the recovery from ischemia, the hyperemic response (an increase in blood volume) will be recorded as a decrease in the R signal.

2.1.3. Tissue blood flow

The monitoring of microcirculatory tissue blood flow using the laser Doppler flowmetry approach (LDF) started 30 years ago. Since, it has been widely used in various animal models and clinical applications (Shepherd, 1990). The

main advantage of the LDF approach is that it provides real time data, due to the optical nature of the signal. A great number of studies (over 5000 publications) in experimental animals and patients indicate its applicability – for more details see (Mayevsky et al., 2004b).

2.1.4. Microcirculatory blood oxygenation

For the monitoring of blood oxygenation, we utilized the 2-wavelengths reflectance technique to analyze light absorption in the blood, employing the difference in absorption spectra of oxyhemoglobin (hemoglobin saturated with oxygen) and deoxyhemoglobin. The principles of this technique were demonstrated years ago using a time-sharing system (Rampil et al., 1992). Generally, the absorption coefficient of oxyhemoglobin differs from that of deoxyhemoglobin in most of the spectrum (Prahl, 1999). However, at certain isosbestic wavelengths, the absorption curves cross and show the same value. The change in reflectance at an isosbestic wavelength is affected only by the blood volume and light scattering, while the change in reflectance at a non-isosbestic wavelength is also affected by the oxy–deoxy ratio. When subtracting the back-reflectance at these two wavelengths, one can consider the difference as a qualitative representation of hemoglobin oxygenation levels in small blood vessels. As Fig. 3 demonstrates, the former time-sharing system (Mayevsky et al., 2004a; Rampil et al., 1992) used two Hg-lamp emission bands to determine the oxy–deoxy ratio *in vivo* by measuring the reflectance at 585 nm (R_{585}) and 577 nm (R_{577}). Calculating $(R_{577} - R_{585})$ yields the relative change in oxyhemoglobin. Presently, we used the same principle with modern 530 nm (nearly isosbestic) and 470 nm

(non-isosbestic) super-bright LEDs. The relative change in oxyhemoglobin is therefore derived from (R470–R530).

Importantly, the two wavelengths must be closed and roughly within the same range of absorption coefficient. Thus, the absorption of the tissue may be considered constant and neutralized. The complete technical specifications of the light source unit (LSU) and the detection unit (DTU) were published (Mayevsky et al., 2005, 2006). The following gives their brief overview, and Fig. 4 shows a block diagram of the MMS device.

2.1.5. Light source unit (LSU)

The light source unit of the MMS comprises a 785 nm CW laser diode which serves for laser Doppler measurements, a 375 nm LED for NADH fluorescence excitation and for total back scattering (or reflection) measurements, a 470 nm LED and 530 nm LED for oxygenation measurements. Each discrete light source is fed by appropriate electronic drivers and controlled by dedicated software via a D/A converter.

2.1.6. Detection unit (DTU)

The detection system is based on two channels. In one channel, 785 nm light is collimated after emitting from the optic fibers, then filtered by a high-pass filter and focused onto the active area of a fast photodiode with a joined pre-amplifying circuit. The detected signal is processed by an analog Doppler processor and the results are digitized in the PC by an A/D converter. In the other channel, the light is collimated and filtered by a low pass filter to eliminate 785 nm reflection. Then the light is split by wavelengths via a dichroic mirror. The back-reflected 375 nm light is reflected by the dichroic mirror towards focusing lenses and onto the active area of a second pre-amplified photodiode. The NADH fluorescence at about 450 nm and the reflections at 470 and 530 nm pass through

the dichroic filter. These wavelengths pass through an interference filter specified for NADH emission and detected by a photomultiplier (PMT). Since the reflection intensities of 470 and 530 nm signals are much stronger than NADH fluorescence, the actual irradiation intensity of these light sources is reduced significantly to adjust the reflection intensity to be similar to the fluorescence level. This enables a single PMT detector to detect all the three wavelengths. This arrangement also eliminates the need for two separate excitation fibers. The detected signals are processed by the detection electronics that consist of sampling and holding circuits, synchronized with the respective excitation pulses. The output of the detection electronics is fed to the PC by an A/D converter.

In order to connect the experimental animal to the monitoring system, a needle type probe was used.

2.2. Animal preparation and experimental protocols

All experimental protocols were approved by the institutional animal care committee under the instruction of the National Institutes of Health. The experimental procedures were detailed previously (Mayevsky et al., 2005; Mayevsky and Chance, 1982). In order to demonstrate the performance of the MMS we used male Mongolian gerbils. The animals were anesthetized by Equithesin (E-th = Chloral hydrate 42.51 mg; Magnesium sulfate 21.25 mg; Alcohol 11.5%; Propylene glycol 44.34%; Pentobarbital 9.72 mg) IP injection of 0.3 ml/100 gram body weight. The animals were kept anesthetized during the operation and during the entire monitoring period, by IP injections of E-th 0.03 ml every 30 min. The addition of small volumes of E-th every 30 min kept the animals in a stable state. Body heat was measured by a rectal probe (YSI) and was regulated to be in the range of 35–37 °C using a heating blanket.

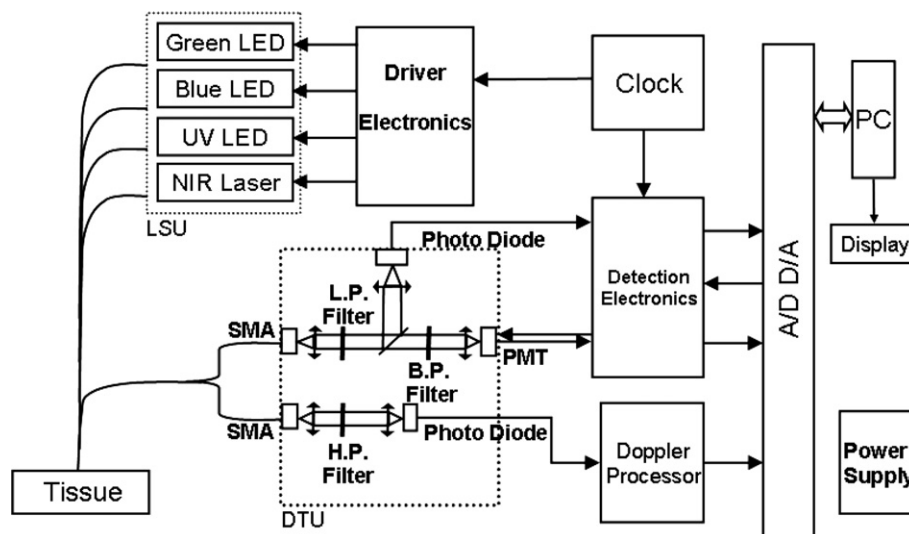


Fig. 4. Block diagram of the multiparametric monitoring system (for details see text).

The gerbils were anesthetized and secured in a head holder. After a midline incision of the skin, an appropriate hole was drilled in the parietal bone of the right hemisphere. The dura mater remained intact and an appropriate light guide holder was placed in the hole. Two stainless steel screws in the right parietal bone were used, with dental acrylic cement, to fixate the probes, which were positioned by a micromanipulator on the cortex. The two common carotid arteries were isolated just before brain surgery, and ligatures of 4–0 silk threads were placed around them.

The experimental animals were exposed to the following perturbations:

Anoxia: The animals were exposed to oxygen deficient atmosphere by spontaneous breathing of 100% N₂, for 25–30 s.

Ischemia: Reversible occlusion (~1 min) of one or 2 common carotid arteries (by constricting them with threads) led to brain ischemia in the gerbils.

3. Results

The MMS device was tested in a group of Gerbils subjected to changes in O₂ supply (anoxia, ischemia) as well as in neurosurgical patients. Fig. 5a presents typical results

measured in a gerbil exposed to 2 step ischemia followed by anoxia. The upper trace, TBF, is measured by the laser Doppler flowmetry (LDF). The 2nd and 3rd parameters are measured by fluorometry/reflectometry (Mayevsky and Chance, 1982). The NADH signal is calculated by subtracting the reflectance signal from the fluorescence signal, as done by many investigators – for details see (Mayevsky, 1984; Mayevsky and Chance, 1982). The lower trace represents the measurement of the hemoglobin oxygenation level calculated by subtracting the R530 signal from the R470 signal.

Ischemia was induced in two steps, namely, partial cerebral ischemia was achieved by unilateral carotid occlusion (Roccl) followed by occlusion of the second artery (Loocl) resulting in complete ischemia. Due to ischemia, the TBF and HbO₂ decreased while NADH increased, indicating lack of O₂ in the brain. The two stages of the ischemic episode were pronounced in all the signals and well correlated, because the Willis circle in the gerbil was not complete (Mayevsky et al., 2005).

Fig. 5b shows two responses measured from the brain of a comatose head injury victim, hospitalized in a neurosurgical intensive care unit. This patient was monitored by the multiparametric monitoring system (Mayevsky et al., 1996). The response shown in part b1 of Fig. 5 developed

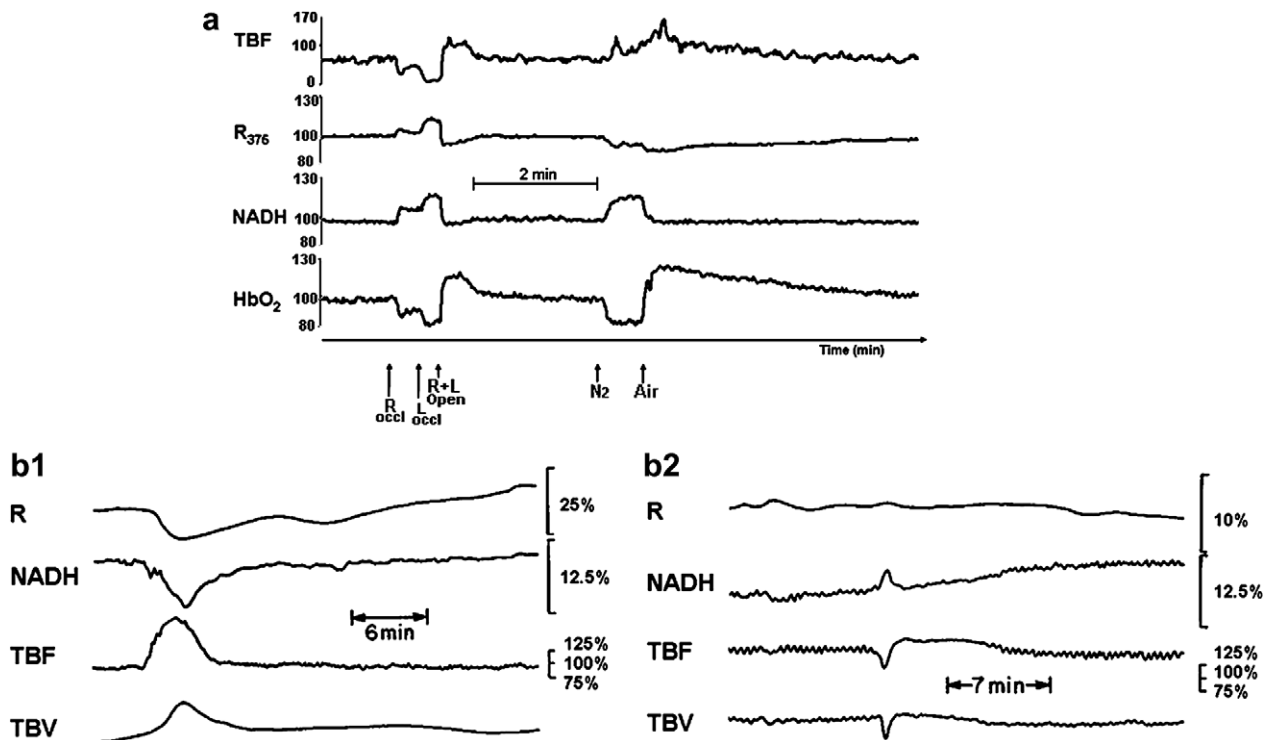


Fig. 5. (a) Effects of partial and complete ischemia (left) and anoxia (right) on the various parameters monitored from the cerebral cortex of an anesthetized gerbil. (b) Two spreading depression-like responses monitored in a neurosurgical patient. TBF – tissue blood flow; R, R375 – reflectance or total back-scattered light at a 375 nm excitation wavelength; NADH – net change in NADH calculated by subtracting R375 or R from Fluorescence at 450 nm (not shown); HbO₂ – microcirculatory blood oxygenation calculated by subtracting R530 from R470; TBV – Tissue blood volume also measured by a laser Doppler flowmeter. The Y-scale of all parameters, except TBF, is given as percent change of the signal calibrated to 100% at the initial stage of monitoring.

spontaneously, and it is very similar to classical cortical spreading depression (CSD) measured in experimental animals (Mayevsky and Rogatsky, 2007). Here, the cerebral blood flow (TBF) shows an increase while NADH becomes more oxidized, indicating an elevation in oxygen consumption due to changes in ionic homeostasis under CSD. The two parameters correlated to blood volume, show an increase (decrease in reflectance). Part b2 of Fig. 5 demonstrates another response typical for CSD, recorded in the same patient. In this response, the blood flow decreased while NADH increased, indicating that the brain had a problem compensating for the extra oxygen needed to overcome the disturbance in the ionic homeostasis during the CSD (Mayevsky, 1984; Mayevsky and Chance, 1982).

4. Discussion

This paper summarizes 100 years of research of the mitochondrial function *in vitro* and *in vivo*. It is clear (cf. Introduction) that NADH redox state measured by fluorometry is the best and a practical parameter to evaluate mitochondrial function and tissue oxygen balance. Nevertheless, due to the complexity of biological systems, monitoring a single parameter such as NADH redox state is insufficient. The limitation is that this parameter, when monitored alone, represents both the intracellular oxygen level and the balance between oxygen demand and supply. Therefore the addition of the parameters of tissue blood flow and hemoglobin oxygenation is highly desirable. The O_2 supply is affected by the dynamic changes in the microcirculatory blood flow and by the level of HbO_2 . The higher the flow and hemoglobin oxygenation, the better is the oxygen supply.

In addition to the in depth review of NADH measurements, the present article describes a new potential medical device, MMS, which enables the monitoring of microcirculatory hemoglobin oxygenation together with the parameters of tissue microcirculatory blood flow, reflectance and mitochondrial NADH redox state (Mayevsky et al., 1996). Tissue vitality is correlated to oxygen or energy balance defined as the ratio between O_2 supply and demand. Energy or oxygen supply mechanisms are identical in all tissues and therefore could be monitored by the same MMS technique in various body organs. In investigating the functional state of a tissue subjected to various pathophysiological conditions, measurements of the additional parameters can substantially improve tissue diagnosis.

As the present results show (Fig. 5), mitochondrial NADH is the most stable and representative parameter of tissue energy metabolism. It is affected by substrate and O_2 availability and ATP turnover, determined by the metabolic activity of the tissue. The interrelated CBF and HbO_2 are more sensitive to instability in the regulation of blood flow, which is influenced by many factors (NO , CO_2 , pH) and not only by oxygen consumption.

The significance of multiparametric tissue monitoring is demonstrated by the present typical results of concomitant TBF and NADH redox measurements (Fig. 6). Here, the

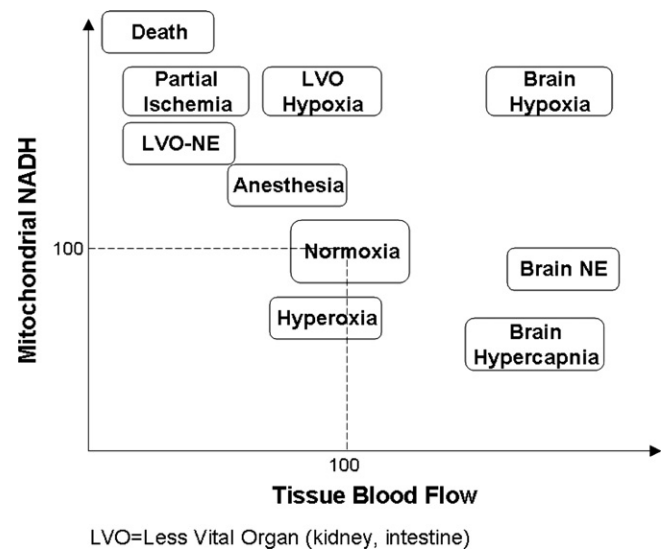


Fig. 6. The relationship between tissue blood flow and mitochondrial NADH redox state under various pathophysiological perturbations measured in the brain and other organs. NE – Nor epinephrine.

control levels of the normoxic tissue are defined as 100% and changes are expressed by an increase or decrease relative to the control values. Notably, the monitoring of one parameter (TBF or NADH) is insufficient, since various conditions can increase or decrease these values, making the diagnosis of an unknown clinical condition impossible.

The results accumulated in preliminary clinical studies and typical records (Fig. 5) suggest that the monitoring of the patients brain is practical and can provide critical information for patient care. Recently the MMS was applied in ICU patients where the vitality parameters were monitored in the urethral wall via a three way Foley catheter (Mayevsky et al., 2006).

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