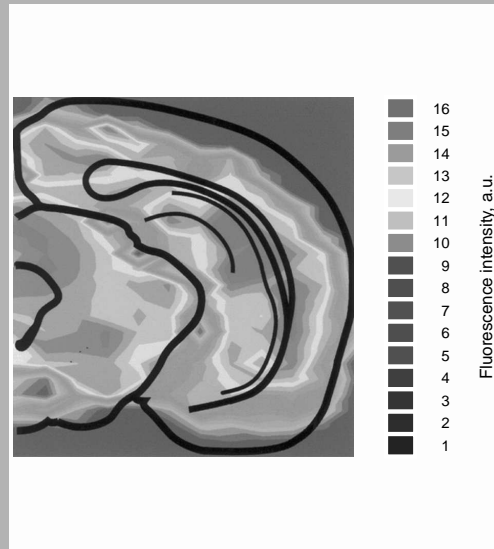


Abstract: There is an increasing need for continuously monitoring changes in brain metabolism and neuronal activity, respectively. The aim is to improve our understanding of mechanisms involved in physiological as well as pathophysiological and behavioural responses and to characterise drug actions. Changes of NADH concentration in the brain can be regarded as an index of changes in mitochondrial activity, which is closely related to neuronal activity. During the last decade the determination of NADH fluorescence by laser-induced fluorescence spectroscopy has become a method of choice in the study of mitochondrial metabolism in neuroscience. By now, small optical probes, providing excellent temporal and spatial resolution and the development of reliable and robust laser-based fluorescence detectors allow a widespread use in preclinical research. Besides *in vitro* studies, especially the assessment of changes in the NADH fluorescence *in vivo* has been shown to provide valuable information on brain function. Several applications are given, ranging from studying drug action or the extent of brain lesion to the measurement the time course of NADH concentration in a brain region of an awake and behaving laboratory rat. Theoretical aspects, opportunities, and limitations that have to be considered during the implementation of fluorescence spectroscopy are described. It is concluded, that measurement of NADH fluorescence by laser-induced fluorescence spectroscopy is a suitable tool for investigation of functional processes in the brain.



Distribution and intensity of the NADH fluorescence in coronal rat brain slices containing the hippocampus. The data represent the intensity of the NADH fluorescence in arbitrary units and are shown as averaged data (n=5) from each measuring point, respectively

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Applications of laser-induced fluorescence spectroscopy for the determination of NADH in experimental neuroscience

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1. Introduction

Experimental methods in neuroscience research range from characterization of the molecular and cellular basis

of neuronal activity to non-invasive or minimal-invasive studies of living and behaving animals.

Over the years the emphasis of methodology in neuroscience has shifted from structural to functional measure-

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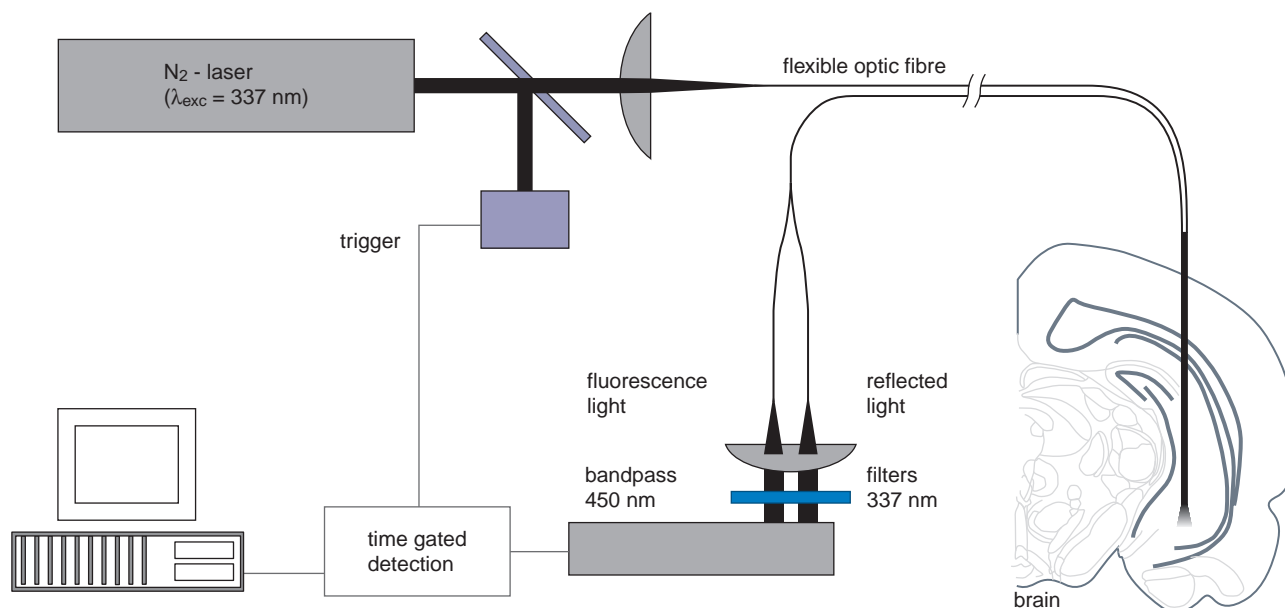


Figure 1 (online color at www.lphys.org) Schematic drawing of the experimental setup for the measurement of laser induced NADH fluorescence in rat brain

ments. There is an increasing need to continuously monitor changes in regional brain function in laboratory animals to improve our understanding of the mechanisms involved in physiological and behavioural responses.

It is generally accepted that the major part of the energy produced in the neurons is spent for normal physiological function (activity) of neuronal cells and it could be shown that energy demand and aerobic energy metabolism are strongly related to neuronal activity [1]. Therefore, determining changes in the use of energy shown by shifts in metabolic activity allows an indirect assessment of neuronal activity.

During the last years an impairment of cellular metabolism caused by mutations of the mitochondrial DNA is thought to play an important role in the development of neurodegenerative disorders with differential clinical features like Morbus Parkinson or Alzheimer disease [2]. The fact that neurons are highly dependent on oxidative energy metabolism has suggested a unified pathogenetic mechanism, based on an underlying dysfunction in mitochondrial energy metabolism.

For that reason the assessment of the cellular metabolism under basal conditions and following physiological or pharmacological stimuli could offer an insight in the functional regulation of regional metabolism in the central nervous system.

Since the pioneering work of Chance and co workers (e.g. [3,4]) the measurement of naturally occurring fluorophores in the central nervous system has emerged as a method to determine the metabolic status in the tissue.

While there are several endogenous fluorophores in neuronal tissue like aromatic amino acids as tryptophan, neurotransmitters such as serotonin and cholecystokinin [5–7], as well as substances involved in mitochondrial metabolism as nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NAD(P)H) [4,8] or flavoproteins [9], only NADH, NAD(P)H and flavoproteins vary in their fluorescence related to changes in mitochondrial metabolism.

The reduced nicotinamide adenine dinucleotide (NADH) is a main substrate for the energy transfer in the first complex of the mitochondrial respiratory chain, located in the inner membranes of the mitochondria.

The ratio of NADH to the oxidised NAD^+ depends on the metabolic status in the cells [10]. Changes in NADH concentrations in the tissue reflect the intracellular redox status and the utilisation of energy.

A rise in the concentration of NADH indicates a reduced activity with lesser consumption of the energy-rich electron donor NADH, whereas an increased activity causes a higher consumption of NADH, leading to a diminished NADH level. Determination of changes in the concentration of NADH allows an assessment of the energy state of cells, and can be used as a marker of the cellular metabolism.

The selective determination of the reduced NADH is possible by using its spectroscopic properties. Only reduced NADH but not the oxidised form NAD^+ absorbs in the UV spectrum at 340 nm. The induced blue fluorescence signal has a maximum at 465 nm.

The potential advantages of using the NADH fluorescence to monitor neuronal activity and/or mitochondrial metabolism are obvious. Since NADH is endogenous in the tissue, there is no need to stimulate electrically or to use additional markers like pH-sensitive dyes, which may cause their own effects [11–14].

Basically, this technique has been established by Chance and co-workers [3,4]. Various fluorometric methods for the determination of NADH have been developed using high-powered light sources as xenon lamps, tungsten-halogen lamps or mercury arc lamps (for a comprehensive review see [15]). Usually the stationary fluorescence signal is measured at 450 nm by means of microscopes or CCD cameras. A drawback of these techniques was the limitation of the fluorescence measurement to the surface of the brain [16] or to *in vitro* preparations of brain structures (e.g. [17,18]). Later flexible fibre-optic light guides have been applied eliminating the limitations of a rigid optical set-up [19,20].

The rapid development of the laser technique permitted the usage of lasers (nitrogen laser) instead of xenon- or mercury lamps as the light source for the excitation of NADH [21–23] in combination with small diameter glassy fibres allowed the determination of changes in NADH fluorescence in deeper brain areas *in vivo* as well as *ex vivo* [24–27].

During the last 15 years the development of small and efficient lasers enabled the construction of compact, robust and transportable fluorescence detection systems with time-gated signal recognition for a better discrimination of fluorescence processes that have fluorescence lifetimes different from that of the fluorophore of interest, useful for medical research and diagnostic purposes [22,23] (Fig. 1).

For example, advantage is taken of the temporal specificity of the NADH fluorescence in order to achieve a higher selectivity against other fluorophores with longer fluorescence lifetimes like flavins by placing the electronic gate exactly on the raising edge and the peak of the fluorescence trace.

Thus, these fluorescence detectors in combination with short pulsed lasers permit a continuous measurement of NADH fluorescence. The usage of small-diameter optical fibres in connection with the known penetration depth of the laser (0.5 mm) [28] allows the determination of intracellular NADH with high spatial resolution.

In general, the fluorescence measured by this technique seems to be mostly derived from the NADH molecules bound to the mitochondrial membrane, since the inclusion of a time gate and the filters used for the measurement excludes most other fluorescent substances with different fluorescence spectra. However, NAD(P)H has similar optical properties as NADH and the fluorescence produced by both pyridine nucleotides can not discriminated easily. In mitochondria the concentration of NADH and the yield of the NADH derived fluorescence is higher than that of NAD(P)H [15,26,29,30]. While NADH is also distributed in the cytoplasmic fraction, it is well known that the lifetime and the yield of fluorescence of protein-

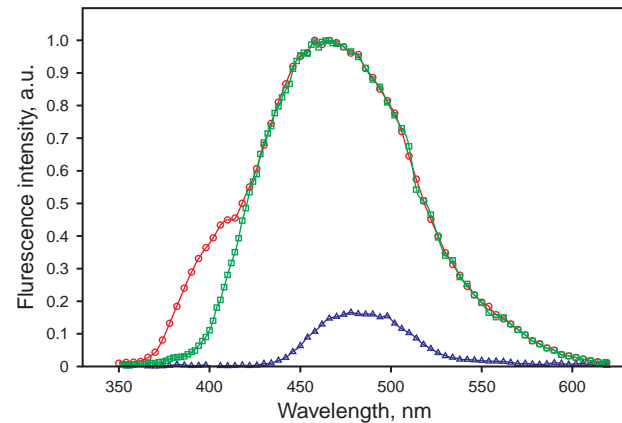


Figure 2 (online color at www.lphys.org) Example of a fluorescence spectrum of the hippocampus (\square) and a slice of the same hippocampal region with some blood seeped into the tissue (\triangle) in comparison to the spectrum of an aqueous NADH solution (5×10^{-5} M, \circ) measured using an acousto-optic tuneable filter

bound NADH are increased significantly compared to the fluorescence of non-bound NADH [31,32].

Further evidence that the fluorescence measured stems from NADH but not from other fluorophores is given by the fact that fluorescence spectra obtained from different brain regions like the cortex [17] or the hippocampus [33] and the emission spectrum of NADH in solution show no apparent differences with their emission maximum at $\lambda = 465$ nm (Fig 2).

Proofs for the mitochondrial origin of the fluorescence measured are changes of the fluorescence intensity following manipulation of the electron transport chain. Blocking the cytochrome C oxidase of the complex IV by cyanide (NaCN) causes a maximal reduction in the electron transport chain, including the NADH molecules [24,34]. Transient inhibition of the respiratory chain complex I by rotenone also caused an increase in NADH fluorescence [18]. The NADH fluorescence recovered with washout.

On the opposite, uncoupling of the oxidative phosphorylation using 2,4-dinitrophenol leads to a decreased NADH fluorescence, which recovered slowly, indicating a longer lasting increase in NADH utilisation *in vitro* [25] and *in vivo* [27].

The effects of NaCN, rotenone, and 2,4-dinitrophenol on the NADH fluorescence are completely consistent with a mitochondrial origin of the fluorescence measured.

Taken all together the laser-induced fluorescence spectroscopy should be a promising method to monitor changes in the concentration of mitochondrial NADH and therefore in metabolic activity *in vitro* and *in vivo*.

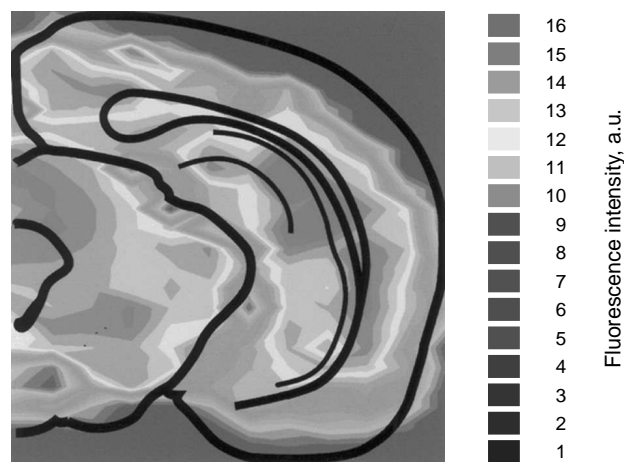


Figure 3 Distribution and intensity of the NADH fluorescence in coronal rat brain slices containing the hippocampus. The data represent the intensity of the NADH fluorescence in arbitrary units and are shown as averaged data ($n=5$) from each measuring point, respectively

2. NADH fluorescence *in vitro*

Most of the experiments determining changes in NADH autofluorescence intensity in cell cultures or brain slices are primarily accomplished using microscopy systems. Different microscopy systems seem to offer diverse capabilities. Determination of NADH fluorescence in slice preparations using confocal microscopy is a well established method for the assessment of the coupling of neuronal activity, energy metabolism and mitochondrial function in brain areas *in vitro* [9,35,36] and a standard protocol for the measurement of NAD(P)H fluorescence in hippocampal slices has been published previously [18]. A recent study demonstrated that NADH autofluorescence imaging can be used to monitor the spatial and temporal properties of neuronal activity in hippocampal slices [37].

Another microscopy technique employing multiphoton excitation examining deeper into the tissue is possibly more sensitive than confocal microscopy [38–40]. Multiphoton microscopy of NADH fluorescence enables identification of the fluorescence signals originating either in the cytoplasm or the mitochondria of astrocytes and neurons in hippocampal brain slices [41]. The authors could show that activity-dependent glycolytic and oxidative metabolic responses in the central nervous system are highly compartmentalised.

For a more mesoscopic view experiments were also performed *in vitro* exploiting a setup with larger optical fibres (100 μm diameter) optimised to detect or locate presumed metabolic changes not at the cellular but at the larger tissue level with high temporal resolution [25].

In preparation for *in vivo* experiments we examined the regional distribution of NADH fluorescence in the rat brain

and it could be shown that the NADH fluorescence and therefore the corresponding NADH content in the CNS is not homogeneous but differs strongly between brain-structures [33]. The observed differences in the NADH concentrations enable the discrimination of structures as e.g. the hippocampus, the cortex, the periaqueductal gray, for example, or the differentiation of lesioned and healthy tissue in the brain (Fig. 3).

While a clear discrimination between neuronal and glia cells is not possible, most of the NADH fluorescence measured should stem from cells with a high oxidative metabolism, excluding mostly the glia cells whose metabolism is mainly a glycolytic one.

3. NADH fluorescence *in vivo*

While measurement of the mitochondrial metabolism *in vitro* permits the determination of some aspects of the spatial and temporal properties of neuronal activity in hippocampal slices (e.g. [37]), assessment of changes in the NADH fluorescence *in vivo* in an intact brain offers the possibility to measure changes in cellular metabolism under more normal conditions during physiological and possibly behavioural responses with pathways and feedback mechanisms still intact. It has to be considered that the optic properties and therefore, the intensity of the NADH fluorescence measured, *in vivo* might be affected by small movements of the brain surface caused by hemodynamic effects and possibly even by ventilation [15] i.e. physiological factors excluded in the *in vitro* assays. In our experiments we found no measurable effects on the NADH fluorescence caused by ventilation. Faster changes in the optical properties caused by the pulse beat (280–450 beats/minute) could be excluded by sampling the NADH fluorescence for 5 seconds, therefore averaging eventually occurring heartfrequency-related changes.

Changes in the central blood flow, as vasodilation or vasoconstriction, may cause a slight movement of the brain tissue around the blood vessels and the cortical surface below the trepanations. It is widely known that vasodilators as nitroglycerine [42,43] or nimodipine [44] and vasoconstrictors as endothelin-1 [45] change the cerebral blood flow, too. These changes of the vessel volume alter the geometric properties of the cerebral tissue below the optical probe resulting in variations in the intensity of the NADH fluorescence and the scattered light measured [27].

Additionally, artefacts included by the blood itself have to be considered. It is known that haemoglobin absorbs both excitation light as well as the emitted NADH fluorescence and various blood corpuscles may also change the optical characteristics of the tissue and therefore affect the scattered and reflected light. We found repeatedly a total loss of fluorescence and backscattered light measured when the tip of the optical probe was in contact with blood, caused either by a remaining blood film on the brain surface or by the accidental contact with

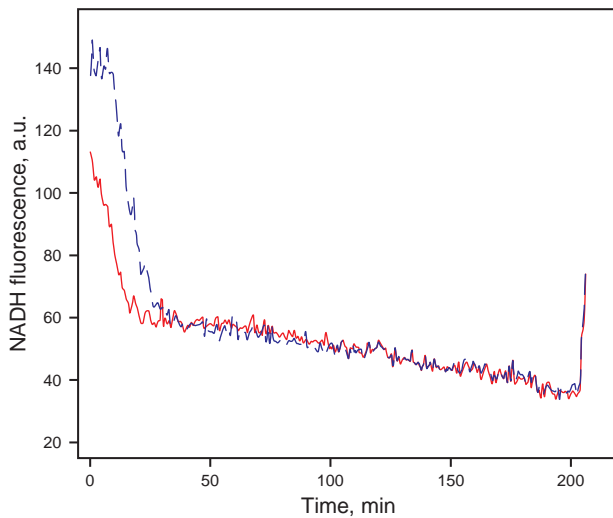


Figure 4 (online color at www.lphys.org) Comparison of two compensation methods to exclude changes in the optic properties of the tissue *in vivo* based on the measurement of the reflected light of 337 nm in the same animal. Both methods, the subtraction of the reflected light from the NADH fluorescence (broken line) as well as the ratio of the measured NADH fluorescence and the reflected light (solid line) lead to similar results (Data shown in arbitrary units)

a capillary during the insertion of the probe towards the target brain region [33] (Fig. 2).

To exclude or at least minimize the effects of changes in the tissue optical properties various compensation methods have been developed during the last 40 years (e.g. [10,15,17,20]). The compensation methods rely either on variations in a tissue derived fluorescence like riboflavin or on the parallel measurement of changes in the reflected excitation light. To the present knowledge there is not the one-and-only compensation method. Own experiments showed that different compensation methods based on the measurement of the reflected light of 337 nm lead to similar results (Fig. 4).

Physiologically occurring changes in NADH fluorescence, e.g. in the cerebral cortex, are of relatively small magnitude (sometimes less than one percent of the baseline level) [16,46,47], which complicates the usage of NADH autofluorescence as a routine method. Not to neglect is the fact that most of the *in vivo* fluorescence measurements were carried out in anaesthetised animals with reduced metabolic activity. Therefore, these animals would use NADH at a lower rate, which could lead to a higher NADH/NAD ratio.

These obstacles could be a reason for the limited use of the determination of the NADH autofluorescence *in vivo*, such as monitoring pathophysiological processes like hypoxia, spreading depression or epileptiform activity [24,48–51].

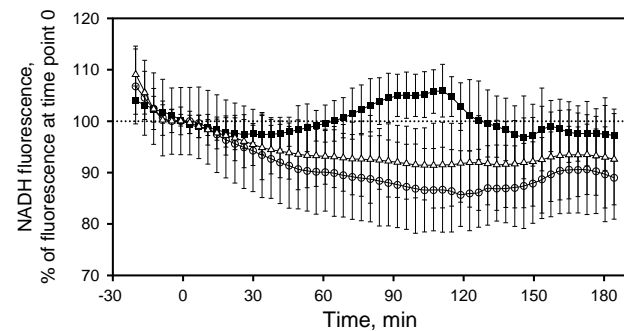


Figure 5 Change in the intensity of NADH fluorescence in the ventral hippocampus following administration of a serotonin_{1A} receptor agonist (■) alone and following pre-treatment with a selective and silent antagonist (△) compared to vehicle treated animals (○). Data are corrected for scattered light and expressed as mean \pm SEM. (n=7-10)

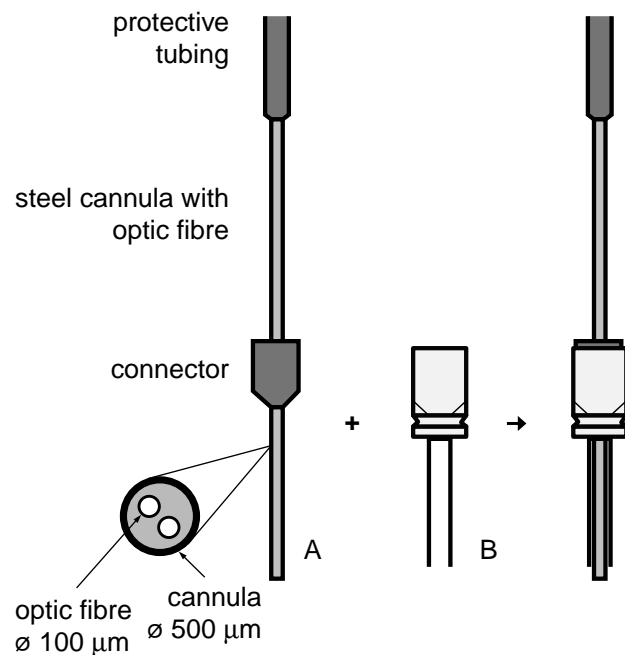


Figure 6 Schematic drawing of the optical probe (A) and the guide cannula (B) for the measurement of laser induced NADH fluorescence in freely moving animals

The introduction of fairly robust and easy to operate fluorescence detectors with negligible need for daily maintenance [22,23] allows a more extensive determination of the metabolic state *in vivo* in standard laboratory practice.

We were able to determine changes in mitochondrial activity following pharmacological and pathophysiological manipulations in the cortex and in the ventral hippocampus of anaesthetised rats [27].

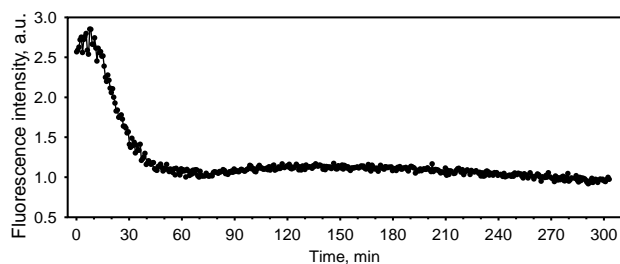


Figure 7 Example for the change in the intensity of NADH fluorescence in the ventral hippocampus of a freely moving rat over the time elapsed following insertion of the optical probe. Data are corrected for scattered light

At first the application of the NADH fluorescence *in vivo* was confined to the surface of the brain but with the development of protected and durable optical probes the assessment of metabolic changes in deeper brain areas was possible. A brain region of interest is the hippocampus, a serotonergic projection area, involved in the modulation of various behaviours, like anxiety-related behaviour. Serotonergic drugs are used in neuropharmacological research and in the clinic. In a first study we could show that administration of an agonist at a subtype of the serotonin receptor family, the serotonin_{1A} receptor, with anxiolytic activity and known inhibitory effects on the electrical activity of neurons in the hippocampus causes a reversible increase in the intensity of hippocampal NADH fluorescence in pharmacologically relevant doses [52]. The increase in NADH, to our knowledge the consequence of decreased metabolic, and therefore neuronal, activity, could be prevented by pre-treatment with a selective antagonist at this serotonin receptor, indicating a specific action of the agonist at a neuronal receptor (Fig. 5). To our knowledge this is the first demonstration of effects of a psychoactive drug on metabolic activity in deep brain structures by the laser-induced fluorescence spectroscopy *in vivo*.

4. NADH fluorescence in freely moving animals

Determination of NADH fluorescence in conscious animals aims at a combined analysis of neuronal (mitochondrial) activity and behavioural (functional) analysis.

To ensure reliable measurements the animal should be able to move freely in the experimental arena with the optical probe securely attached. First attempts to determine changes in the cerebral NADH autofluorescence in non-anaesthetised animals were made throughout the sleep-wake cycle, showing that the oxidative energy balance seems to be related to sleep states [26].

Our practical approach for the measurement of NADH fluorescence by laser-induced fluorescence spectroscopy is

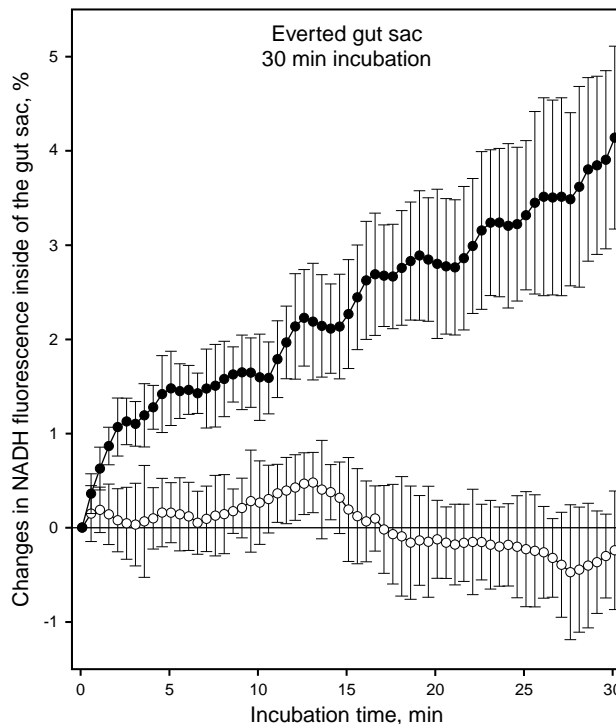


Figure 8 Change in the intensity of NADH fluorescence in the interior of the everted gut sac during 30 minutes incubation in a NADH solution (10 mg/l, ●) compared to an incubation in a NADH-free vehicle solution (○). Data are expressed as mean \pm SEM. (n=10)

developed from the microdialysis technique in freely moving animals. The optical probe fits into a commercially available guide cannula for microdialysis probes (Fig. 6), which was implanted into the brain stereotactically and fixed to the skull with stainless steel screws and cold curing resin one week before the experiments.

Preliminary experiments confirm the suitability of the experimental setup for the use in awake and behaving animals. Probably due to cell leakage caused by the insertion of the optical probe through the cerebral tissue, there is at the beginning of the experiment a massive NADH fluorescence, which declines rapidly during the first thirty minutes following insertion of the probe. From this point the fluorescence measured remained fairly steady throughout the experiment (Fig. 7). It has to be taken into account, that the animal was habituated to the experimental arena.

A torque proof optical fibre allows us in the near future the determination of cerebral NADH fluorescence in animals during a behavioural test and therefore the assessment of physiological occurring changes in central metabolism.

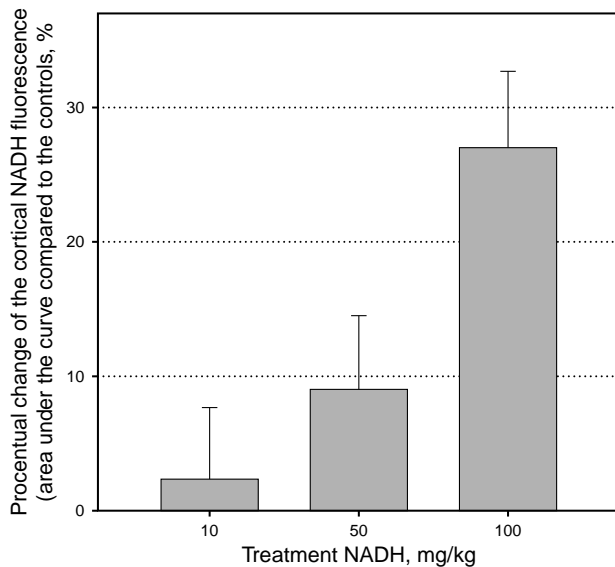


Figure 9 Change in the intensity of cortical NADH fluorescence following sublingual/buccal administration of a NADH solution compared to the cortical NADH fluorescence in vehicle treated animals (100%). Data are corrected for scattered light, represent the area under the curve and are expressed as mean \pm SEM. (n=9-10)

5. A special application for the determination of NADH fluorescence

For several years NADH has been used as an advertised “nutraceutical” or “life style” drug. NADH as a drug is supposed to improve several disorders of the central nervous system, like Parkinson disease, cognitive decline, or even major depression. However, publications about central effects of NADH are equivocal and the therapeutic evidence is inconsistent or of limited-quality and patient-oriented [53,54]. Surprisingly, NADH given intraperitoneally showed also a mnemotrophic effect in old rats [55] and an antidepressant-like effect in the rat forced swim test [56]. So the question arose whether or not NADH, given peripherally, would have an effect on the NADH fluorescence in the CNS. In a first *in vitro* experiment with an isolated organ it was determined that NADH could be absorbed in the small intestine. By inserting the optical probe in to the centre of the well established simple model of the “everted gut sac” developed by Wilson and co workers (1954) we could measure a time dependent absorption of NADH into the small intestine, using the laser-induced fluorescence spectroscopy (Fig. 8).

In a subsequent study we found a significant dose-related rise of the measured NADH fluorescence intensity in the cortex of anaesthetised rats following intravenous and intraperitoneal injection [58] as well as following sublingual/buccal administration of a NADH solution (Fig. 9). Naturally, it is not verified that the NADH molecules that

cause the increased fluorescence in the cortex are identical with the injected NADH molecules. However, the assessment of the bioavailability of NADH shows a useful application of the laser fluorescence spectroscopy in neuropharmacological studies.

6. Conclusion

Laser-induced fluorescence spectroscopy of the intrinsic and mainly mitochondrial bound NADH is a reliable method which allows the spatial and temporal characterization of functional changes in the brain associated with metabolic changes *in vitro* and *in vivo*.

To our knowledge, presently no other imaging technique for the visualization of functional regulation in the living brain provides a comparable spatial and temporal resolution.

Measurements of NADH in awake and freely moving animals are in principle possible and should be carried out in further investigations.

In the future the combination of the laser-induced fluorescence spectroscopy with other techniques like *in vivo* microdialysis or functional magnetic resonance imaging will provide novel important data on brain regional changes in neuronal function in response to physiological and pharmacological interventions.

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