In vivo functional NADH imaging with single-cell resolution

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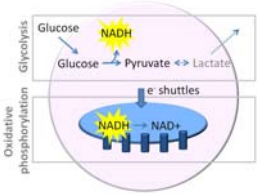
Background

β-nicotinamideadenine dinucleotide (NADH) is the principal electron carrier in glycolysis, the Krebs cycle and the mitochondrial respiratory chain. NADH is generated during glycolysis and is oxidized in the electron transport chain, establishing a potential across the inner mitochondrial membrane and enabling the production of ATP. Therefore, the ratio of NADH/NAD+ depends on the balance of oxidative phosphorylation and non-oxidative glycolysis.

Earlier 1-photon studies *in vivo* established that on a macroscopic level NADH auto-fluorescence of brain tissue *decreases* in response to cortical stimulation, cortical spreading depression and seizures throughout the duration of the stimulus as long as blood flow is not compromised, and *increases* in response to hypoxia or ischemia[1-5]. A simplified schematic diagram on the left shows that fluorescent NADH is produced during glycolysis in the cytosol and oxidized to non-fluorescent NAD+ during respiration in the mitochondria.



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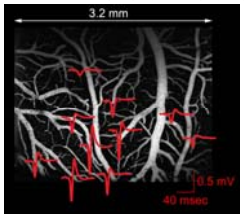
Methods

Animals: Rats were initially anesthetized with isofluorane and ventilated with a mixture of air and oxygen. Cannulas were inserted in the femoral artery and vein. Isofluorane was discontinued, and anesthesia was maintained with 50 mg*kg^{-1} intravenous bolus of a-chloralose followed by continuous intravenous infusion at 40 mg*kg^{-1}*h^{-1}. HR, BP and *t* were continuously monitored. Respiration was aimed to maintain pCO_2 between 35 and 45 mmHg.

Cortical exposure: An area of skull overlying SI was exposed, the skull and dura mater were removed, and the space between the exposed brain surface and the coverglass was filled with ~1 % (w/v) agarose (Sigma) in ACSF.



Mapping the cortical response: We used a silver ball electrode to map the response to forepaw stimulation. During 2-photon data collection the stimulus consisted of 5-s long train of electrical pulses (300 μs, 3 Hz, 1 mA) delivered to a contralateral to the exposed cortex forepaw.

Figure 1. Example of surface potential mapping. Recorded potentials (red traces) are overlaid on Maximum Intensity Projection (MIP) image of the cortical vasculature.

Dyes: After mapping the neuronal response using surface potentials, calcium indicator OGB1 was microinjected in the center of the responsive area. SR101 was applied to the surface. To visualize the vasculature, ~0.3 ml of 5 % (w/v) solution of 2 MDa fluorescein-conjugated dextran (FD-2000S, Sigma) in saline was injected i.v. in some experiments.

Imaging: Images were obtained using 4-channel Ultima 2-photon microscopy system from Prairie Technologies (www.prairie-technologies.com). We used a 4x objective (Olympus XLFluor4x/340, NA=0.28) to obtain images of the surface vasculature across the entire cranial window to aid in navigating and a 20x water-immersion objective (Olympus, XLUMPlanFI20x NA=0.95) for high-resolution and functional imaging. The laser was tuned to 740 nm. Filters used for detection of the emitted fluorescence are illustrated in Figure 2 below. Data analysis was performed in Matlab environment.

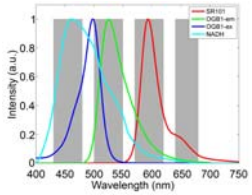


Figure 2. Emission spectra and filters. Emission spectra of NADH (cyan), OGB1 (green) and SR101 (red) are shown in relation to bandpass filters used for the measurements (gray rectangles). The excitation spectrum of OGB1 (1-photon excitation spectrum is shown in blue) significantly overlaps with NADH. As a result, OGB1 absorbs NADH fluorescence preventing simultaneous imaging.

Control : NADH fluorescence increases during breath hold

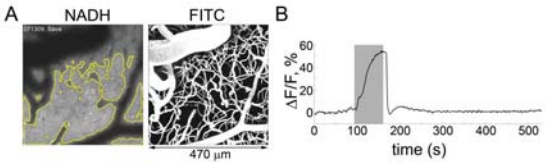


Figure 3. ***A,*** Corresponding NADH and FITC images. FITC was injected i.v. following the NADH measurements. ***B,*** Time-course of NADH fluorescence change during a 60 sec breath hold extracted from the mask within the yellow contour in the NADH image.

Two-photon NADH imaging with cellular resolution

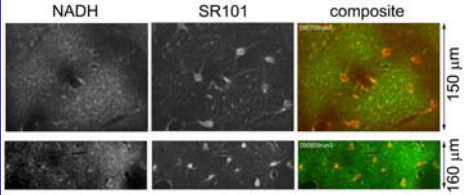


Figure 4. Astrocytes have higher NADH fluorescence than the surrounding neuropil. Two examples of corresponding 2-photon NADH and SR101 images from cortical layer I are shown. A combined image of NADH and SR101 in pseudo color (green and red, respectively) is shown on the right.

NADH fluorescence in cortical tissue transiently decreases in response to somatosensory stimulation

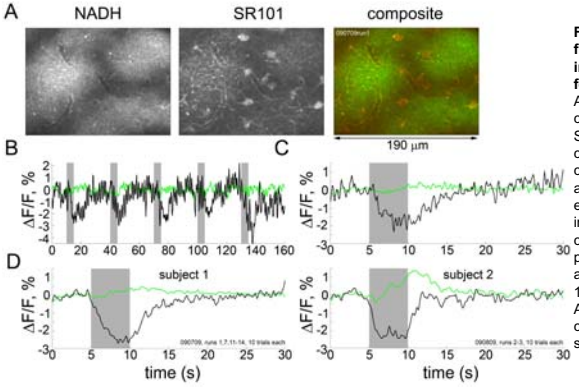


Figure 5. NADH fluorescence decreases in response to the forepaw stimulus. ***A-C,*** A single example of corresponding NADH and SR101 images from cortical layer I (A), time-courses of NADH (black) and SR101 (green) extracted from the entire image during 5 consecutive stimulus presentations (B), and the averaged response over 10 stimulus trials (C). ***D,*** Averaged response time-courses from 2 individual subjects.

The stimulus causes strong arteriolar vasodilation response and calcium increase in neurons but not in astrocytes

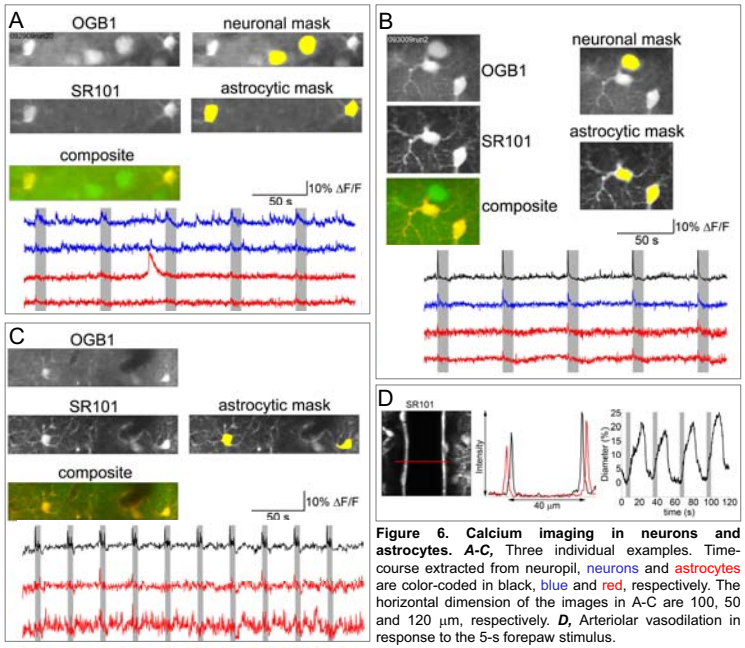


Figure 6. Calcium imaging in neurons and astrocytes. ***A-C,*** Three individual examples. Time-course extracted from neuropil, neurons and astrocytes are color-coded in black, blue and red, respectively. The horizontal dimension of the images in A-C are 100, 50 and 120 μm, respectively. ***D,*** Arteriolar vasodilation in response to the 5-s forepaw stimulus.

The transient decrease in NADH fluorescence is observed both in neurons and astrocytes

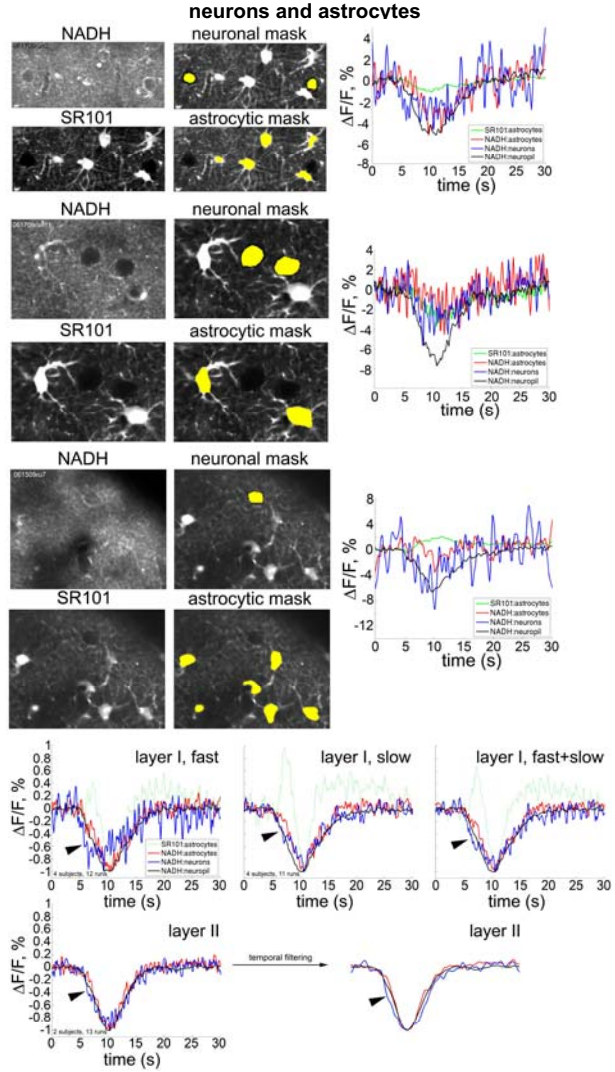


Figure 7. Evoked NADH fluorescence changes in nearby neurons and astrocytes. ***A-C,*** Three individual examples. Each time-course is an average of 9 stimulus trials. The horizontal dimension of the images in A-C are 75, 50 and 65 μm, respectively. ***D,*** Time-courses averaged across subjects.

Conclusions

1. **Functional NADH signal changes can be imaged with 2-photon microscopy *in vivo* on the level of single neurons and astrocytes.**
2. **Under conditions of uncompromised perfusion and adequate oxygen supply NADH fluorescence *decreases* in response to somatosensory stimulation. This is consistent with earlier reports using 1-photon illumination and CCD detectors[2].**
3. **A decrease in NADH fluorescence in response to stimulation is observed both in neurons and astrocytes. This finding suggests that in both compartments a transient metabolic response is dominated by oxidative phosphorylation rather than glycolysis.**
4. **The same stimulus leads to robust vasodilation and calcium increase in neurons and neuropil but not in astrocytes.**
5. **The lack of astrocytic calcium increase despite the large evoked arteriolar vasodilation does not support the current hypothesis of astrocytic regulation of blood flow through calcium-dependent pathways in healthy cerebral cortex.**

Acknowledgements

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