

# Improved *in vivo* two-photon imaging after blood replacement by perfluorocarbon

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Two-photon microscopy is a powerful method in biomedical research that allows functional and anatomical imaging at a subcellular resolution *in vivo*. The technique is seriously hampered by absorption and scattering of light by blood, which prevents imaging through large vessels. Here, we demonstrate in the rat cerebral cortex that blood replacement by perfluorocarbon emulsion, a compound also used in human critical care medicine, yields superior image quality, while preserving neuronal integrity. Shadows of large superficial vessels disappear completely and cells can be imaged underneath them. For the first time, it is possible to image complete populations of neurons and astrocytes in the upper layers of neocortex *in vivo*.

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**Abbreviations** PFC, perfluorocarbon; SR101, sulforhodamine 101; OGB-1 AM, Oregon Green BAPTA 1 AM;  $P_{tO_2}$ , tissue  $P_{O_2}$ ; ROI, region of interest.

*In vivo* two-photon-excited fluorescence laser-scanning microscopy (two-photon microscopy) is a remarkably successful method for physiological research. In combination with fluorescence staining, it allows the imaging of brain cortex (as well as other organs) with subcellular resolution (Denk *et al.* 1990; Helmchen *et al.* 1999). One of the main disadvantages of fluorescence microscopy is the limited penetration depth of light into tissue. In the brain, light scattering by tissue is accepted to be the main factor determining the maximal achievable imaging depth (Helmchen & Denk, 2005). However, the scattering by red blood cells needs to be considered too, as it can also limit the penetration depth of the excitation beam.

The combined effect of scattering and absorption by blood, and in particular by haemoglobin, can easily be seen in two-photon microscopy images where blood vessels cast a 'shadow' on the tissue below. A possible explanation for the relatively low interest in addressing this issue is the fact that there is seemingly not much room for improvement. This is true unless a surrogate oxygen-carrying molecule can replace haemoglobin.

For many years, the development of a potential blood substitute has been an important topic of research in human critical care medicine. A large number of possible

candidates have been suggested, some of them being based on haemoglobin or modified haemoglobin, others on artificial molecules with a high oxygen transport capacity. The latter class consists essentially of perfluorocarbons (PFCs). PFCs, liquid at room temperature, are chemically and biologically inert synthetic perfluorinated hydrocarbon compounds that carry oxygen in its physically dissolved form. There are numerous very encouraging results from preclinical and clinical trials, in which PFCs were successfully applied as blood substitutes (Riess, 2001; Keipert, 2006). Since PFCs are hydrophobic, they need to be formulated as a PFC-in-water emulsion. Such emulsions scatter light in the visible spectrum (and therefore appear opalescent). However, the absorption of light in the relevant fluorescence excitation and emission wavelength range is negligible. The aim of the present study was to combine PFC transfusion and *in vivo* two-photon microscopy to circumvent absorption and scattering by blood and thus improve image quality.

Part of this work has been published in abstract form (Weber *et al.* 2008).

## Methods

Experiments were performed in 12 adult male Sprague-Dawley rats ( $n=8$  for two-photon microscopy,  $n=2$  for electrophysiological recordings

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combined with blood gas measurements,  $n=2$  for blood gas measurements only) weighing 150–250 g. The local veterinary authorities approved all experimental procedures.

### Surgical preparation

All surgical procedures and measurements were performed under isoflurane anaesthesia (2.5–3.5% during surgery and 1.5–2% during imaging sessions) and involved a craniotomy, tracheotomy and catheterization of femoral vessels. All relevant physiological parameters were carefully monitored throughout the experiment. A detailed description of the procedures and parameters is given as Supplemental material (available online only).

### Two-photon microscopy

The barrel cortex (whisker somatosensory cortex) was stained by topical application of the astrocytic marker sulforhodamine 101 (SR101) (Nimmerjahn *et al.* 2004) and injected with the calcium indicator Oregon Green BAPTA 1 AM (OGB-1 AM) (Stosiek *et al.* 2003) ( $n=8$  animals). Two-photon microscopy was performed using a custom-built microscope with a  $\times 20$  or  $\times 40$  water immersion objective (0.95, 0.8 NA; Olympus, Japan). Fluorescent dyes were excited at 870 nm (150 fs pulse width) with a titanium sapphire laser (Mira 900-F laser; Verdi-5 pump; Coherent, USA). The microscope's motorized stages were controlled by custom-written LabVIEW motion control software. For scan mirror control and photomultiplier data acquisition, further LabVIEW software was used (courtesy of Fritjof Helmchen and colleagues). Calcium signals (128  $\times$  128 pixels) were acquired at a frequency of 8 Hz. Anatomical imaging was performed at a resolution of 256  $\times$  256 or 512  $\times$  512 pixels with averaging over four frames.

### Sensory stimulation

Single principal whiskers were stimulated using a piezoelectric ceramic wafer that produced brisk rostral to caudal deflections with an amplitude of approximately 1 mm. Multiple whiskers were stimulated with 30 ms long air puffs (40 p.s.i.) that were applied with a computer-controlled solenoid valve at a distance of 30 mm from the whiskers.

### Blood transfusion

While remaining under the microscope, animals were transfused with a mixture containing 50% perfluorocarbon (PFC) emulsion, 30% human albumin solution (Human Albumin, 50 g l<sup>-1</sup>; Baxter) and 20%

Ringer solution. It was complemented by glucose to reach a final concentration of 10 mM glucose. The PFC preparation as well as the optical properties of PFC are described in detail in the Supplemental material. The mixture was constantly agitated and maintained at body temperature ( $\sim 37^\circ\text{C}$ ). The mix was injected into the venous catheter while we drew blood from the arterial catheter with micropumps in a push–pull fashion achieving a transfusion rate of 1.9 ml min<sup>-1</sup>. This process was interrupted every 50 cycles to check the animal's condition and to measure haematocrit level. The transfusion was stopped after 30–50 ml of PFC/albumin/Ringer were injected. The first 6–8 ml of blood drawn from the animal were centrifuged to isolate plasma of which 2–3 ml were re-injected after the transfusion was finished. This procedure allowed us to decrease the haematocrit level to about 2–7%.

## Results

### Two-photon microscopy before and after PFC transfusion

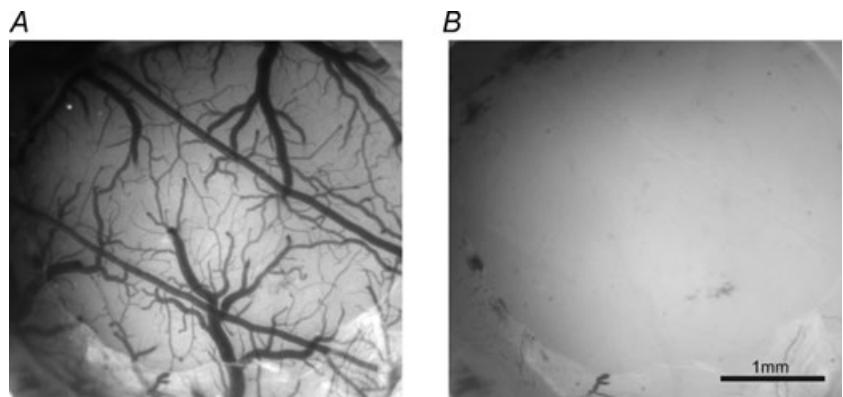
Wide-field imaging using a  $\times 2.5$  objective and a CCD camera under green light illumination (530 nm) shows the dense surface vascularization of the somatosensory cortex (Fig. 1A). PFC transfusion of the animal dramatically reduced the amount of red blood cells in the cortical vasculature of that same area as can be seen in Fig. 1B.

Figure 2 shows two-photon images of the astrocytes in the somatosensory cortex at a depth of 150  $\mu\text{m}$  before and after PFC transfusion. Dark areas in the pre-transfusion images are caused by blood vessels that absorb and scatter light (Fig. 2A). These dark areas clearly disappeared after replacing a substantial amount of red blood cells with PFC (Fig. 2D). The haematocrit level was reduced from 43% to 4% in this animal, permitting us to image cells that were located underneath large cortical vessels. After PFC transfusion, the quality of images both parallel and perpendicular to the cortical surface substantially improved in regions beneath large blood vessels (Fig. 2B–C before PFC transfusion, Fig. 2E–F after PFC transfusion).

Figure 2G and H shows that depth penetration below big vessels is dramatically improved after PFC transfusion for both the red (Fig. 2G) and the green channel (Fig. 2H). Depth penetration in areas not obscured by big surface vessels is not changed significantly. PFC transfusion increases the average fluorescence in the range 0–400  $\mu\text{m}$  below big surface vessels by a factor of  $8.8 \pm 0.4$  (mean  $\pm$  S.E.M.) for the red channel and by a factor of  $11.2 \pm 0.7$  (mean  $\pm$  S.E.M.) for the green channel. The decay curves closely resemble those obtained for cortical areas without big surface vessels.

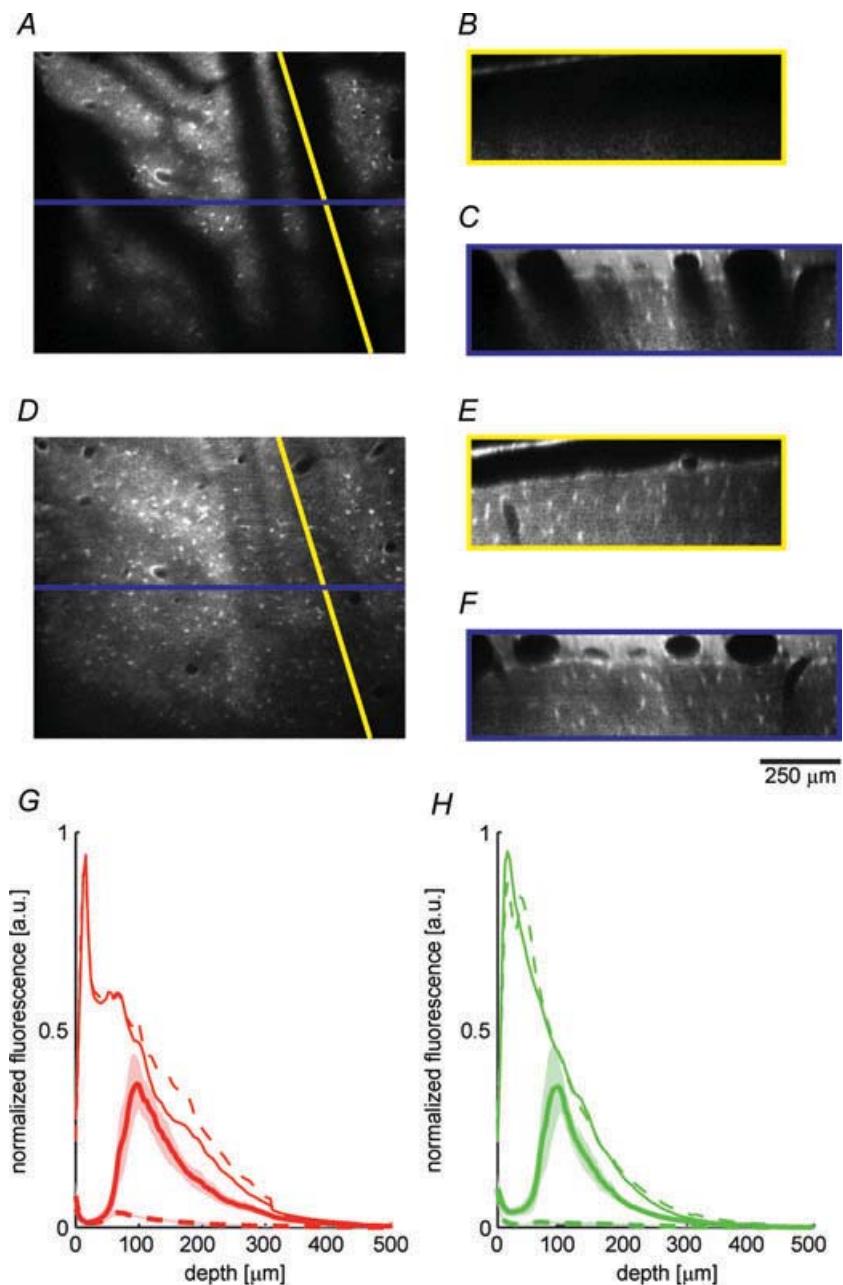
**Figure 1. Vessel pattern of the somatosensory cortex before and after PFC transfusion**

Wide-field images taken with a CCD camera and a  $\times 2.5$  objective at 530 nm illumination. A, before PFC transfusion, showing the typical effect of absorption and scattering of light by the superficial vessels. B, after PFC transfusion, the vascular absorption is very low.



**Figure 2. Structural imaging of cortical astrocytes *in vivo* before and after PFC transfusion**

A, two-photon image of astrocytes (SR101 staining) taken at a depth of 150  $\mu\text{m}$  before PFC transfusion. Shadows resulting from absorption and scattering by red blood cells disappear after PFC transfusion (D). B, C, E and F, Z-sections reconstructed from image stacks were taken at the location marked with the blue and yellow line in A and D. Astrocytes located below large vessels were not visible before PFC transfusion. Contrast has been optimized in each individual frame in the stack. G, depth penetration in the red channel was measured at fixed laser intensity before (dashed line) and after the transfusion (continuous line) below a big surface vessel about 50  $\mu\text{m}$  thick (thick lines) and in an open area without vessel at the surface (thin lines). All traces are averages over 4 stacks collected in 3 animals ( $\pm$  S.E.M. shaded area; average region of interest (ROI) is 800 pixels). All curves were normalized to the peak surface intensity measured in the open area before and after transfusion. The transfusion does not significantly change depth penetration in the open area (compare thin lines) but dramatically increases depth penetration below vessels (thick lines). H, same as in G for the green channel.



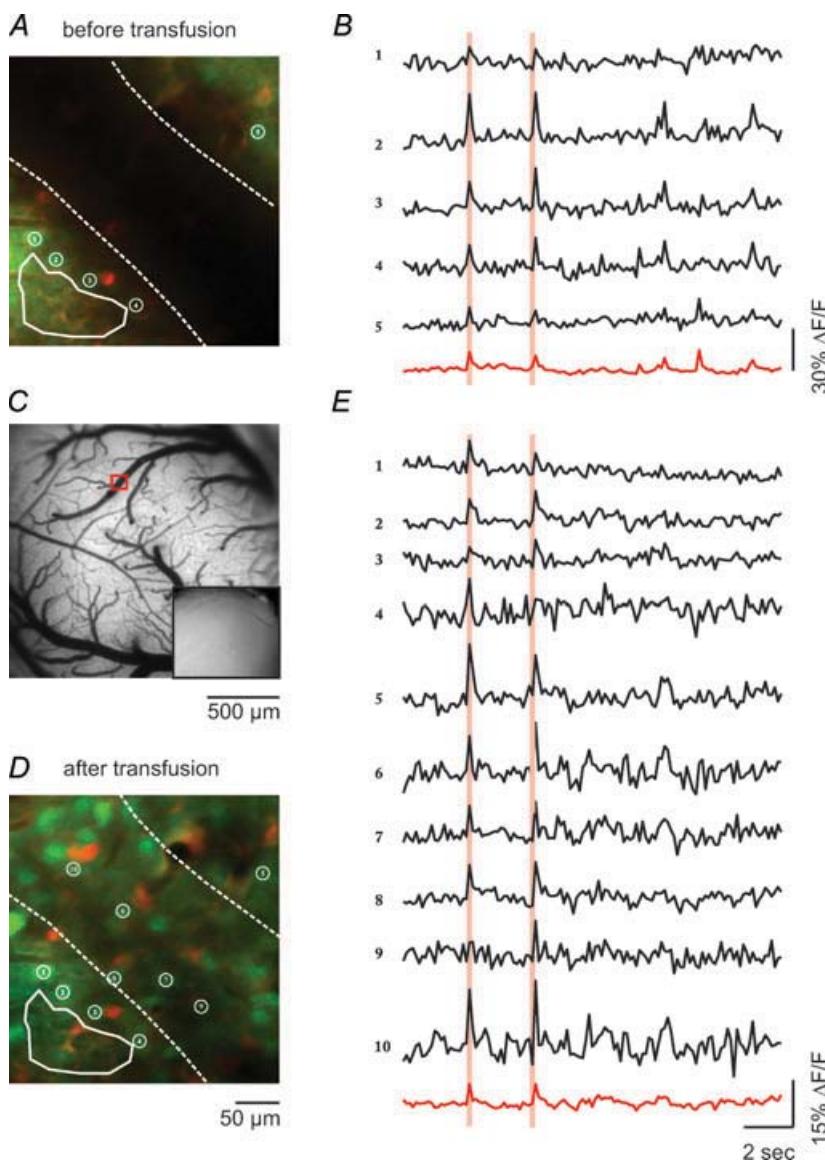
## Functional calcium imaging of neurons before and after PFC transfusion

Functional two-photon imaging of neuronal calcium transients in the somatosensory representation of the rat's whiskers before and after blood cell replacement was performed in the area that had been microinjected before with OGB-1 AM. The exact location of this representation was determined before the injection using intrinsic optical imaging (data not shown). Neuronal signals resulting from tactile stimulation led to calcium transients in stained cells, reflected in fluorescence increases of the calcium indicator dye OGB-1 AM (green). Fluorescence traces of individual cells can be seen in Fig. 3B and E. The dark areas resulting from absorption and scattering were still visible in the green as well as the red channel before transfusion. PFC transfusion, however, allowed functional imaging of cells that were previously undetectable, while

preserving the responsiveness of the cortical neurons (see Fig. 3A and B compared to Fig. 3D and E). In addition to the stimulus-evoked activity, we were still able to observe spontaneous calcium transients after the PFC transfusion (not shown).

## Discussion

In the present study we introduce a combination of blood replacement by perfluorocarbon (PFC) transfusion with *in vivo* two-photon imaging of neocortical neurons and astrocytes. The optical properties of PFC (see online Supplemental material) can offer substantially improved imaging performance due to reduced scattering and absorption. The scattering coefficient of the undiluted emulsion is in the order of  $\mu_s \approx 1 \text{ mm}^{-1}$  or lower over the whole visible spectrum range and therefore scattering by PFC is negligible with respect to scattering in tissue and



**Figure 3. Optical recording of calcium transients *in vivo* before and after transfusion with perfluorocarbon**

A, layer 2/3 neurons (green, stained with OGB-1 AM) and astrocytes (red, stained with SR101) in the rat somatosensory cortex imaged before transfusion ( $185 \mu\text{m}$  deep; haematocrit 44%). Dotted lines denote the border of a big cortical vessel. B, single trial calcium responses recorded from individual cells (black traces; ROIs consist of 12 pixels;  $8 \text{ Hz frame}^{-1}$ ) and from the neuropil (red trace) upon repeated stimulation of several whiskers (vertical red bars denote the time point of stimulation). The numbers refer to the cells labelled in A. The neuropil signal is measured within the white polygon drawn in A. C, cortical surface before transfusion. The same area is shown after transfusion in the inset. The square indicates the position of the area imaged along a big vessel (A, B, D and E). D, same area as in A but imaged after PFC transfusion (haematocrit 3%). Note that the shadow of the cortical vessel has disappeared and that additional cells can now be imaged. E, single trial calcium responses recorded from individual cells (black traces) and from the neuropil (red trace) after transfusion. Cells 1–5 correspond to cells 1–5 in A. Cells 6–10 are additional cells that can now be imaged below the vessel. The numbers refer to the cells labelled in D. The neuropil signal below the vessel is comparable to the signal observed in the chosen ROI (not shown).

by residual blood cells. Moreover, the colourless emulsion does not absorb light in the visible range, which increases the amount of emitted fluorescence light reaching the detector.

The comparison of the optical properties of whole blood and the blood replacement rationalizes the advantages of our approach for *in vivo* two-photon imaging. We could, for the first time, record functional and structural fluorescence images of the brain *in vivo* that are normally obscured by the vascular network of the neocortex. We show that this method allows functional calcium imaging of neuronal populations that are located below large veins and arteries oriented parallel to the cortical surface. The integrity of neuronal function was assessed on a single neuron level and on a larger scale encompassing several hundreds of neurons with two-photon calcium imaging and local field potential recordings (see online Supplemental material) of the same cells or cortical area before and after PFC transfusion. Neurons in the primary somatosensory cortex remain responsive to a tactile stimulus after the exchange of red blood cells by PFC.

The exact cortical area chosen for two-photon imaging is often biased, because large vessels need to be avoided. However, even after a careful choice of the field of view, many neurons remain undetectable because they are located beneath deeper cortical blood vessels. As a consequence, imaging of complete neuronal networks is not possible. This problem is more prominent in older *versus* younger animals and larger *versus* smaller species (e.g. rats *versus* mice). By applying PFC transfusion it is, for the first time, possible to image *in vivo* the complete population of neurons and astrocytes in the upper layers of neocortex. Imaging studies targeting a small brain region, e.g. neuronal representations on a columnar or subcolumnar scale (Kerr *et al.* 2007), will benefit from the possibility of eliminating the ‘shadow’ cast by the blood vessels on the area of interest. Furthermore, investigations of large areas with two-photon imaging will benefit from the possibility of acquiring data from complete populations of cells without interruptions (Lillis *et al.* 2008). Unlike haemoglobin, perfluorocarbon emulsions carry oxygen as a dissolved gas and thus the partial pressure of O<sub>2</sub> ( $P_{O_2}$ ) and O<sub>2</sub> content follow a linear relationship. Relatively high arterial  $P_{O_2}$  is necessary to maximize the O<sub>2</sub> transport capacity of PFC and it is therefore generally suggested that PFC emulsions are used in combination with breathing pure O<sub>2</sub> in clinical applications. The impact of PFC transfusion and the potentially high tissue  $P_{O_2}$  ( $P_{tO_2}$ ) on the cell metabolism has not been addressed in this work and needs further investigation. However, changing the O<sub>2</sub> content in breathing air would enable  $P_{tO_2}$  to be brought back to normal levels. Such conditions should ensure the proper function of neurons and result in normal receptive field properties. It should be noted

that the applied PFC transfusion protocol also leads to a removal of non-red blood cells and, as a consequence, to altered levels of factors released by these cells (e.g. cytokines). Although the effects of these factors on brain activity are not well understood, future PFC transfusion protocols could include the re-injection of the non-red blood cells (present in the buffy coat).

Interestingly, imaging depth was not significantly increased by PFC transfusion in this study. Therefore, our results also demonstrate that the maximal imaging depth in two-photon imaging is not critically determined by light absorption by haemoglobin and light scattering by red blood cells. Although absorption and scattering is considerably higher for blood than tissue (in the case of absorption the difference is orders of magnitude) the relatively low volume fraction of the cortical vascular system (around 3%) seems to limit its effect. In other words, the light scattering by cortical tissue is clearly the major limiting factor of imaging depth. However, it has to be stated that the instruments used in the present study were not tailored for deep tissue imaging. It may well be that the combination of PFC transfusion with advanced imaging techniques (Theer *et al.* 2003) could further increase imaging depth in two-photon imaging.

Finally, for the expanding field of *in vivo* two-photon imaging it will be mandatory to reduce the impact of absorption and scattering by red blood cells on the image quality of highly vascularized organs such as the liver or thymus. Given the success of PFCs as a blood substitute, their commercial availability can be expected in the near future.

In summary, *in vivo* two-photon microscopy can be substantially improved by replacing blood with PFC, due to the superior optical properties of the latter. This substitution allows the imaging of complete cellular networks, parts of these being normally obscured by blood vessels. In combination with the appropriate imaging hardware, this approach could also be beneficial for deep tissue two-photon imaging (Helmchen & Denk, 2005).

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### Author contributions

(1) F.H., F.S., M.P.K. and B.W. contributed to the conception and design; F.H., R.J., M.T.W., J.R., N.B.B., F.S., M.P.K. and B.W. contributed to the analysis and interpretation of data; (2) F.H., R.J., M.T.W., J.R., N.B.B., F.S., M.P.K. and B.W. contributed to drafting the article and revising it critically for important intellectual content; (3) F.H., R.J., M.T.W., J.R., N.B.B., F.S., M.P.K. and B.W. made the final approval of the version to be published.

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