Supplementary information

Improved in vivo two-photon imaging after blood replacement by perfluorocarbon
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Supplementary methods

Surgical preparation

The animals were kept in cages in a ventilated cabinet with standardized conditions of light (night/day-cycle 12h/12h), temperature and free access to food and water was always ensured. 12 hours and directly before the surgical preparation, animals were injected subcutaneously with 5 mg/kg dexamethasone to prevent brain edema. All surgical procedures and measurements were performed under isoflurane anesthesia (2.5% to 3.5% during surgery and 1.5% to 2% during imaging sessions). Isoflurane was delivered to the animal in a mixture of oxygen and air (30%/70%) before the transfusion with PFC and in pure oxygen during and after the transfusion to allow for sufficient arterial oxygen saturation. Surgery involved the cannulation of the right femoral artery and vein with PE-50 tubing containing saline and tracheotomy for artificial ventilation of the animal. The arterial catheter was used for the continuous monitoring of the arterial blood pressure and for withdrawal of blood and fluorocarbon during the blood exchange transfusion. The venous access was used for the systemic infusion of the fluorocarbon solution. After fixation of the animals head in a stereotactic frame (Kopf Instruments, Tujunga, CA, USA) the skull above the barrel cortex (1 mm caudal and 3 mm lateral from Bregma) was exposed after a midline incision and the disconnection of the temporal muscle from the skull. Using a dental drill (Bien Air Medical Technologies, Bienne, Switzerland) a craniotomy was performed and the dura was removed. Astrocytes were stained by

applying 100 μM sulforhodamine 101 (SR101; Molecular Probes, USA) in Ringer for 30 min topically (Nimmerjahn et al., 2004). Multicell bolus loading of layer II neocortical neurons with the calcium indicator Oregon Green 488 BAPTA-1 AM (Garaschuk et al., 2006) (OGB-1 AM; Molecular Probes, USA) was achieved by injecting 200 nl of OGB-1 AM dissolved in DMSO with 20% Pluronic 127 (Molecular Probes, USA), diluted to a final concentration of 1mM in calcium free Ringer solution, with a micropump system (UltraMicroPump III, World Precision Instruments, Sarasota, USA). Before dye injection, we identified the appropriate location by intrinsic optical imaging using a green super-luminescent LED (530 nm). The exposed brain was then covered with 2% Agarose (type III-A, Sigma, St. Louis, USA) in Ringer and with a round glass coverslip 5 mm in diameter. The coverslip was fixed to the skull with light curing dental cement (Tetric EvoFlow; Ivoclar Vivadent AG, Schaan, Principality of Liechtenstein) to reduce movements of the brain due to heartbeat and respiration. The animals' temperature was kept at 37 degrees with a heating blanket and blood gases were maintained in normal ranges by adjusting the ventilation parameters. At the end of the experiments, animals were euthanized with a bolus of intravenous pentobarbital (200 mg/kg).

Electrophysiology

Local field potentials (LFPs) were recorded using multielectrode silicon probes (Michigan Probes (NeuroNexus Technologies, Ann Arbor, MI, USA); 1 shank with 16 electrodes each, ~1 MΩ impedance, 100 μm spacing). The electrodes were inserted into barrel cortex to a depth of 1700 μm. All recordings were performed using a multichannel extracellular amplifier (MultiChannelSystems, Reutlingen, Germany; gain x5000, sampling frequency 20 kHz, band pass 1–5000 Hz). Voltage traces were band-pass filtered offline with digital

filters, at 1–200 Hz to yield LFPs. The latency was defined as the time point where the signal surpassed a predefined negative threshold (4x standard deviation of the baseline activity).

Preparation of perfluorocarbon emulsion

The formulation of the PFC emulsion consisted of perfluorooctyl bromide ($C_8F_{17}Br$, PFOB; 70% w/v, 47% v/v; AtoFina, Paris, France) emulsified with egg yolk phospholipids (Egg PC; 4% w/v; Asahi, Japan) and stabilized with a semifluorinated alkane, ($C_6F_{13}C_{10}H_{21}$, F6H10; 2.45% w/v; equimolar with Egg PC) in a phosphate buffer (0.052% w/v NaH_2PO_4, H_2O; 0.355% w/v Na_2HPO_4, $7H_2O$; 0.25% w/v NaCl). The mixed fluorocarbon-hydrocarbon diblock compound synthesized in the lab was thoroughly purified by column chromatography.Emulsification was achieved under high pressure (10000 kPa, 30 min) with a laboratory processor (Microfluidizer M-110, Microfluidics Corp., Newton, MA, USA). In order to avoid fluorohydrocarbon impurities, PFOB has been distilled twice and detoxified by chromatography on an alumina column followed by filtration on a 0.22 µm Millipore filter prior to use. The emulsion was then heat-sterilized (121°C, 103 kPa, 15 min). The PFC emulsion is stable for more than two years at room temperature.

Supplementary results

Optical properties of the PFC emulsion

The maximal achievable depth for two-photon imaging is determined by instrumental parameters such as available laser power and the detector collection efficiency, the two-

photon cross section of the fluorophore as well as the scattering and absorption mean free path for excitation and emission. The latter is determined by scattering and absorption by tissue and blood. Attenuation of the incoming and emitted light is described by the Lambert-Beer law: $T= I/I_0= \exp(-\mu z)$ where the attenuation coefficient $\mu= \mu_s+ \mu_a$ is the sum of contributions from scattering μ_s and absorption μ_a. The mean free path l for ballistic photon transport is $1/\mu$. One of the important advantages of multi-photon imaging is that the excitation laser can be operated in the near-infrared (in our case λ=870 nm) where absorption from hemoglobin in red blood cells is very low μ_a <0.3 mm^{-1}. Thus the penetration depth of the required ballistic photons is mainly limited by scattering in tissue and blood. In brain gray matter, the scattering mean free path in the near-infrared is typically in the order of 200 micrometers (Helmchen and Denk, 2005), $\mu_s\approx5$ mm^{-1}, which allows one to reach depths of several hundred micrometers. Unfortunately, scattering by red blood cells is much stronger. For whole blood scattering coefficients in the range of μ_s=80-300 mm^{-1} have been reported limiting the penetration of near-infrared light to only tens of micrometers (Faber et al., 2004; Roggan et al., 1999). Light absorption of hemoglobin on the other hand is high in the range of wavelengths between 500 and 600 nm where light is emitted with values $\mu_a\approx10$ mm^{-1} for whole blood and $\mu_a\approx1$ mm^{-1} for tissue, respectively. The fluorophores used in this study show peak emission at 520 nm and 590 nm. As a direct consequence, in the vicinity of and especially below blood vessels, a large fraction of the emitted light is absorbed as soon as the path length to the detector exceeds 100 microns.

To rationalize the advantages of using the PFC blood substitute we have performed a detailed optical characterization of the PFC emulsion. We first studied the properties of individual emulsion droplets on a 1:100 diluted PFC-emulsion using photon correlation

spectroscopy (3DDLS spectrometer from LSInstruments, Fribourg, Switzerland, λ=633 nm) following the approach of Brown and Pusey (Brown and Pusey, 1974). We obtained a number weighted mean particle size of R=108nm and a polydispersity of 20% without any detectable fraction of particles larger than this. Using the first Born (or Rayleigh-Gans-Debeye) approximation we calculated the wavelength dependence of the scattering coefficient μ_s up to an arbitrary prefactor and the scattering anisotropy parameter g, defined as the mean cosine of the scattering angle (van de Hulst, 1981). In the limit of sufficiently large optical density (in our case $m_t L \gg 5$ (Kaplan et al., 1993)) the total transmission coefficient is given by $T \propto [1+4/(3m_t L)]^{-1} \sinh^2(g/m l_a)/\sinh(L/l_a)$ with a constant $\gamma \approx 2$ and $l_a^2 = 3m_a/m_s$, $\mu_t = (1-g)\ \mu_s$ (Ishimaru, 1978). We measured the optical transmission as a function of the path length L at three different wavelengths (λ=532 nm, 633 nm, 785 nm) by gradually filling a metal cylinder, diameter 44 mm, with a 100% PFC emulsion up to a filling height of L≈25 mm. As a reference standard we used a suspension of polystyrene latex nanoparticles (particle radius 110 nm, volume fraction 1.5%, μ_s calculated numerically from Mie theory). From the measurements on the non-absorbing polystyrene suspension, we estimated the detection limit of our experiment to $\mu_a \approx (4\pm1)\cdot10^{-3}$ mm^{-1}.

From UV_VIS spectroscopy, we find that the scattering coefficient μ_s of the PFC emulsion drops from $\mu_s \approx 1$ mm^{-1} at emission wavelengths around 550 nm to $\mu_s \approx 0.3$ mm^{-1} at the excitation wavelength of 870 nm (Fig. 1a). At the same time anisotropy factor g decreases from 0.52 to 0.16 (whole blood g≈0.97) (Hammer et al., 2001). Diffuse transmission experiments confirm these findings as shown by the open symbols in Supplementary Figure 1a and furthermore allow us to estimate an upper bound for the absorption coefficient $\mu_a < 0.01$ mm^{-1} (Supplementary Figure 1b). We can thus conclude that both the

scattering and absorption coefficient of the PFC emulsion are orders of magnitude smaller than the intrinsic scattering and absorption coefficients of whole blood (Faber et al., 2004). Therefore, even at the very low hematocrit levels of 2-7% present in our study, scattering and absorption from vessels are largely dominated by the residual red blood cells and the influence of the PFC emulsion on the scattering and propagation of visible (VIS) and near infrared (NIR) light is negligible.

Physiological parameters before and after PFC transfusion

Animals were transfused with a mixture containing 50% perfluorocarbon emulsion, 30% human albumin solution and 20% Ringer solution complemented by glucose to reach a final concentration of 10 mM. Before the transfusion, blood was drawn from the animal, centrifuged to isolate 2-3 ml of plasma for intravenous reinjection after the transfusion. Blood gases, pH and arterial blood pressure were monitored before, during and after the PFC transfusion. Table 1 shows blood parameters such as CO_2 pressure, pH and arterial blood pressure, which did not change significantly after the transfusion as already reported by others (Nomura et al., 2000). In particular, there was no need to constantly infuse phenylephrine after transfusion in order to maintain arterial blood pressure in a range similar to that before the transfusion (Yang et al., 2008). On the contrary, arterial oxygen pressure significantly increased. The transfused animals were monitored for as long as data acquisition required and no degradation of their physiological parameters was observed in this period of time (average 140 min; ranging from 60 to 360 min).

Table 1

Physiological parameters.

	before transfusion (mean ± SD)	after transfusion (mean ± SD)	p-value (paired t-test)
pO_2 (kPa)	26.1 ± 0.9	50 ± 20	0.04
pCO_2 (kPa)	5 ± 1	5 ± 1	0.59 *
pH	7.34 ± 0.06	7.36 ±0.04	0.67 *
arterial blood pressure (mm Hg)	89 ± 7	79 ± 13	0.23 *
hematocrit	0.44 ± 0.03	0.07 ± 0.01	< 0.0001

* Not significant. Weight = 247 ± 21 g (n = 4; mean ± SD) Physiological parameters were monitored during 2 hrs after the end of the transfusion. Average transfusion length is 55 min (n = 12; range: 20 - 126 min).

Local field potential recordings before and after PFC transfusion

We recorded extracellular electrical signals in the somatosensory cortex to confirm the responsiveness of neuronal structures after PFC transfusion (n=2 animals). Local field potentials recorded from layer IV showed that neurons responded normally to the applied tactile stimulus. Basic waveform shapes before and after PFC transfusion were similar as can be seen in Supplementary Figure 2 (blue dashed line before, red line after transfusion and blue solid line normalized to peak negative amplitude of the red trace). The latency of the first negative peak onset was not significantly altered by the PFC transfusion procedure (Student's t-test for independent samples, df=180, p=0.65), confirming the results of a previous transfusion study (Nomura et al., 2000). We could observe an increase in amplitude of the peak negative response after the transfusion (before: -0.14 mV ± 0.04 SD, after: -0.2 mV ± 0.05 SD). This finding may be associated with a lighter state of anesthesia resulting from a reduced number of red blood cells and a difference in isoflurane binding

properties between blood and blood substitute (Cuignet et al., 2002; Lockwood et al., 1997).

Supplementary Figure legends

Supplementary Figure 1. Optical properties of blood and perfluorocarbon emulsion.

(a) Scattering coefficients μ_s of oxygenated (red) and deoxygenated (blue) whole blood are in the range of 200-300 mm^{-1} [both adapted from (Faber et al., 2004)]. The values for the undiluted PFC emulsion are considerably lower. The symbols represent the measured scattering coefficient of a fluorocarbon-in-water emulsion. The green line is a Mie-calculation (in arbitrary units) assuming a Schultz-distributed mono-modal emulsion of unknown refractive index with a number weighted mean particle size of R=108 nm and polydispersity of 20% (standard deviation). The open symbols are results for μ_s obtained independently from diffuse transmission measurements at selected wavelengths using large sample volumes up to 40 ml.

(b) Absorption coefficient μ_a of oxyhemoglobin (red), deoxyhemoglobin (blue) [both adapted from (Faber et al., 2004)]. Vertical lines depict the excitation wavelength as well as the peak emission for the green and red fluorophores used in this study. Open symbols are estimated absorption coefficients of a fluorocarbon-in-water emulsion (symbols) based on diffuse transmission experiments. The error bar covers the range from the absorption coefficient of water up to measured values (close or within the detection limits of our experiment).

Supplementary Figure 2. Amplitude and shape of local field potential responses after whisker stimulation.

Blue dashed line is the average (n=180) of field potential responses in one animal resulting from the air puff stimulation of several whiskers recorded at a depth of 500 μm in barrel cortex before blood substitution. Solid red line shows the evoked field potential after PFC transfusion (n=180). The blue solid line is the pre-transfusion response normalized to the peak negative amplitude of the red trace. The basic waveform shape of the two solid traces is similar.

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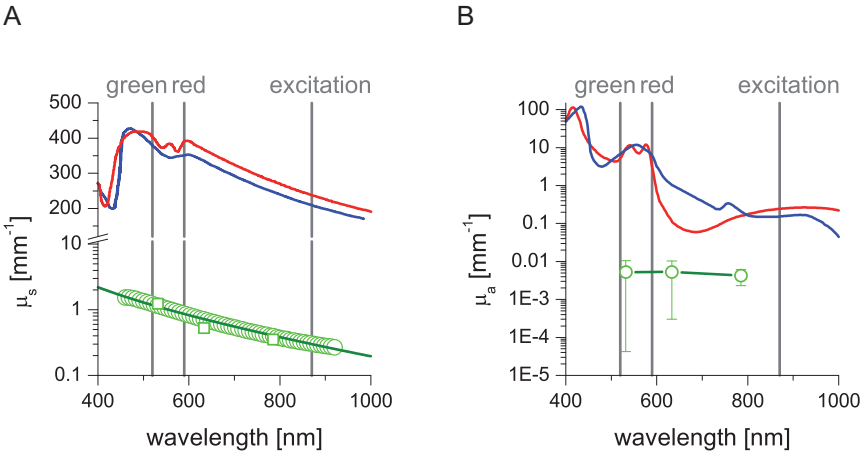
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Figure S1

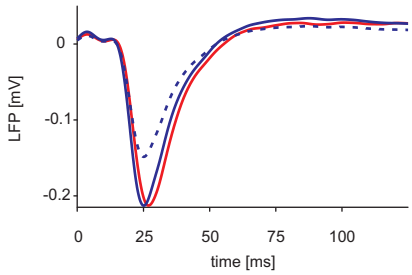

Figure S2