Multi-Channel MRI Labeling of Mammalian Cells by Switchable Nanocarriers for Hyperpolarized Xenon

Stefan Klippel[1,2], Christian Freund[2] and Leif Schröder[1]

1) ERC Project BiosensorImaging
Leibniz-Institut für Molekulare Pharmakologie (FMP)
13125 Berlin (Germany)
E-mail: lschroeder@fmp-berlin.de
Homepage: https://www.fmp-berlin.de/schroeder

2) Protein Biochemistry Group, Freie Universität Berlin
14195 Berlin (Germany)

Supporting information

1. Preparation of PFOB nanoemulsion

The PFOB nanoemulsion has been prepared similar to the procedure described by Pines and coworkers[1]. In brief, 420 µL PFOB (Sigma-Aldrich) and 315 µL Pluronic F-68 (100 mg/ml, Sigma-Aldrich) have been mixed within 10 mL of purified, deionised water. The mixture was emulsified by high-pressure homogenisation (EF-C3 Avestin Emulsiflex) at a working pressure of 1500 bar (sample was kept an ice bath while being processed). The nanoemulsion was finally sterile filtrated (0.2 µm syringe filter) and stored undiluted at 4°C prior to use. After preparation the mean nanodroplets diameter was around 200 nm as analyzed by dynamic light scattering (droplet concentration: 16 nM).

2. Cell labeling and NMR/MRI sample preparation

Mouse fibroblasts (L929) were cultivated as monolayer's in DMEM supplemented with 10 % FBS (Sigma Aldrich, Taufkirchen, Germany) under standard conditions (in T-flasks, 37 °C, 5 % CO_2). The cells have been unspecifically labeled with the tested contrast agents (CrA, PFOB nanoemulsion) by coincubation for 18 h. The contrast agents were diluted in DMEM (10 % FCS, 1 % DMSO) at concentrations given within the experimental sections.

For cell labeling with cryptophane-A (CrA) the incubation procedure was optimized in a previous study[2] to generate a maximal intracellular cryptophane concentration without impairing the cellular viability. Within this study a reduced viability of fibroblasts was observed for CrA concentration exceeding 60 µM while incubation for 18 h with 50 µM CrA (as used within our manuscript) is unproblematic.

For cell labeling with PFOB the incubation procedure was optimized with keeping the CrA parameters in mind: the aim was a) to generate a high intracellular PFOB concentration without impairing the cellular viability and b) to achieve a comparable MRI contrast intensity as for CrA labeled cells. This was motivated since comparable contrast intensities for both contrast agents allows for comparing their individual sensitivity with respect to the achieved intracellular concentrations. We started with preparing a PFOB stock solution (16 nM) according to the protocol used by Pines and coworkers[1]. The cell incubation time was set to 18 h for PFOB labeling in order to use the identical incubation time as used for CrA labeling. This time frame is in accordance with other ex vivo labeling of cells with nanoemulsions

including PFOB [3–5]. The stock solution was diluted within cell culture medium. We found that a 1:10 dilution (resulting in an incubation concentration of 1.6 nM PFOB) had no toxic effect on cells (checked by trypan blue staining) while the cellular uptake of PFOB achieved for this incubation condition gave a contrast intensity that was comparable to cell labeling with CrA. For NMR/MRI experiments with cells in suspension, fibroblasts were labeled as monolayer's, washed in PBS, harvested and resuspendet in fresh DMEM (10 million cells/mL).

For NMR/MRI experiments with alginate-encapsulated cells, the labeling scheme differs for each of the tested contrast agents. In case of CrA, cells were immobilized within alginate beads prior to CrA-labeling. After encapsulation the cells were transferred into spinner vessels (stirring speed: 30 rpm) and incubated with 50 µM CrA for 18 hours.

In case of PFOB, fibroblasts were first labeled as monolayers with 1.6 nM of a PFOB nanoemulsion (diameter: 200 nm). After PFOB-labeling, cells were washed in PBS and encapsulated within alginate beads.

Cell encapsulation in alginate beads was done as described earlier[2]. In brief: cells have been harvested followed by washing and re-suspending in Hank's Balanced Salt Solution (HBSS, Sigma Aldrich). The suspension of cells was further mixed with an equal volume of HBBS containing 2% alginate (alginic acid sodium salt from brown algae, Sigma Aldrich) and 2% mannitol. The cell-alginate solution (20 million cells/mL, 1% alginate, 1% mannitol in HBBS) was trickled into a solution containing 1.5% $CaCl_2$ and 1% mannitol by the use of a custom made droplet generator[6]. The processed alginate beads are characterized by a uniform diameter of around 1 mm and an average cell density of ca. 10000 cells per bead. The alginate beads were washed three times in HBBS, 1% mannitol before they were transferred into the perfusion system.

3. Confocal laser scanning microscopy

Cellular uptake and localisation of CrA and PFOB was validated by laser scanning confocal microscopy. PFOB nanodroplets were fluorescence labelled by incubation with lipophilic dialkylcarbocyanine (Vybrant® DiI, life technology's) for 1 h at 37 °C under agitation (10 µL DiI / 1 ml PFOB nanoemulsion).[7] Unbound dialkylcarboxycyanine was removed from the nanoemulsion by centrifugation (1500 g, 20 min). CrA was fluorescence labelled by covalently attaching fluorescein (CrA-FAM) as described earlier.[2]

Microscopy of surface-attached fibroblasts was done by growing 400000 cells for 18 h on glass coverslips (30 mm, pretreated with 100 µg/mL poly-L-lysine). The cells were incubated with 75 µM of CrA-FAM (1% DMSO) or 1.60 nM PFOB-DiI nanodroplets (diameter: 200 nm) dissolved in DMEM (10 % FCS) for 18 h at 37 °C.

For microscopy of alginate encapsulated cells, labeling with the respective fluorescent contrast agents (75 µM CrA-FAM or 1.6 nM PFOB-DiI, diameter: 200 nm) was done similar to as described in section 2 (Cell labeling and NMR/MRI sample preparation).

Imaging was done on a LSM 510 (Carl Zeiss Microimaging GmbH, Jena, Germany) microscope using an x 100/1.3 numerical aperture oil objective (static cells) or an x 10/0.3 numerical aperture water objective (cells in alginate beads). Fluorescence signals were recorded using a 488 nm argon laser/ BP 505-550 nm for CrA-FAM and a 543 nm HeNe laser/LP 560 nm for PFOB-DiI. Images were processed using ZEN 2009 light Edition (Carl Zeiss Microimaging GmbH).

4. Intracellular concentration

The intracellular concentration of both contrast agents was determined by measuring their fluorescence intensity in cell lysates.[8] To do so, cells have been incubated with fluorescence-labeled versions of both tracers under incubation conditions (18 h, 37 °C) identical to the respective MRI experiment (CrA-FAM: 50 µM, PFOB-DiI: 1.6 nM / diameter: 200 nM). PFOB nanodroplets were fluorescence labelled by incubation with lipophilic dialkylcarbocyanine (Vybrant® DiI, life technology's) for 1 h at 37 °C under agitation (10 µl DiI / 1 ml PFOB nanoemulsion).[7] Unbound dialkylcarboxycyanine was removed from the nanoemulsion by centrifugation (1500 g, 20 min). CrA was fluorescence labelled by covalently attaching fluorescein (CrA-FAM) as described earlier.[2]

Cells were incubated as monolayers with either PFOB-DiI or CrA-FAM dissolved in DMEM, 10 % FCS, 1% DMSO. Cell lysates were made by suspending cells in Lysation-buffer (1 % Triton, 2 % SDS in PBS) for 30 min at 4 °C. Fluorescence measurements were performed on a Safire Micro plate reader (TECAN Deutschland GmbH, Crailskirchen, Germany).

For calculations several simplifications have been made: We calculated the cell-averaged concentration of both contrast agents for a cell volume that was measured to be 3300 fL for spherical L929 fibroblasts in alginate. As shown earlier, the uptake of unmodified CrA is 2.5 fold higher compared to the fluorescence labeled construct CrA-FAM due to a higher hydrophobicity.[2] Based on a measured intracellular CrA-FAM concentration of 15 µM, the concentration of CrA is ca. 40 µM. For PFOB we assumed a stable droplet size of 200 nm following cellular internalization. Further on we imply that labeling of PFOB with fluorescence dye DiI has no effect on the uptake of the nanodroplets. The intracellular concentration of PFOB is 80 nM in this case. The given concentrations are the mean value of three independent experiments.

5. NMR/MRI experiments

All NMR/MRI experiments has been performed as described earlier[2]. In brief:

Xenon hyperpolarization:

Xenon-129 was hyperpolarized within a custom-designed continuous-flow setup by spin-exchange optical pumping.[9] By using a gas mixture consisting of 5 % Xe (26.4 % natural abundance of ^{129}Xe), 10 % N_2 and 85 % He a ^{129}Xe spin polarization of approximately 25 % was achieved. The polarizer pressure (3.5 bar overpressure) was used to bubble the gas mixture either directly into the sample solution by a spectrometer-triggered bubble dispenser or it was delivered indirectly via a custom-designed bioreactor setup working under continuous perfusion with hp-xenon saturated medium.

Xenon delivery by direct bubbling:

For each scan the gas was bubbled for 10 sec (at 0.1 standard liters per minute (SLM)) followed by a delay of 2 sec (collapse of remaining gas bubbles) before signal acquisition. The formation of foam was reduced by adding 0.02-0.1 % anti-foam agent (pluronic® L81, BASF corporation, Ludwigshafen, Germany).

Xenon delivery by perfusion:

Immobilized cells has been perfused with xenon saturated medium HBB (HBSS, Sigma Aldrich) for 60 s at a flow rate of 7.5 ml/min. After xenon delivery the medium flow through the sample was stopped for NMR saturation (in case of Hyper-CEST) and signal acquisition.

Hyper-CEST-NMR/MRI:

Measurements were performed on a 9.4 T NMR spectrometer (Bruker Biospin, Ettlingen, Germany) equipped with gradient coils for imaging applications. A double resonant probe (^{129}Xe and ^{1}H) with an inner diameter of 10 mm was used for excitation and detection. After the delivery of hp-xenon, CEST weighting was achieved by applying a continuous wave saturation pulse at a frequency off-resonant from the subsequently detected Xe in solution pool. For each experiment, frequency, length and power of the saturation pulse were adjusted individually (parameters given in the main text).

MR images were acquired using a fast Hyper-CEST-adapted Rapid Acquisition with Relaxation Enhancement (RARE[10]) readout (in plane geometry: field of view = 20 x 20 mm^2,

matrix size = 32 x 32, in-plane resolution = 625 µm, slice thickness = 20 mm, centric *k*-space encoding, bandwidth = 5 kHz, echo time = 10 ms, acquisition time = 320 ms, rare factor = 32). Image processing was done using MATLAB® (R2012a, MathWorks, Natick, MA). CEST-spectra were fitted by using an exponential Lorentzian function[11] in OriginPro® 8.6.OG (OriginLab, Northampton, MA).

6. MR-image post-processing

The average of either 20 (direct bubbling, supplementary Figure S1) or 2 (indirect perfusion, supplementary Figure S2) magnitude-images detecting xenon in solution is shown for off-resonant saturation (irradiating at 23 ppm) and on-resonant saturation (xenon@PFOB in cells: irradiating at 107 ppm or xenon@CrA in cells: irradiating at 70 ppm). The CEST effect was evaluated by calculating the percentage reduction of single-pixel intensities due to on-resonant saturation for both applied on-resonant frequencies individually. This allows an analysis independent from slightly different xenon delivery throughout the sample phantom. Areas within the calculated CEST-effect images that do not belong to the sample phantom were masked as shown within the figures. The masked CEST-effect images (32 x 32 pixels) were resized by cubic spline interpolation to the resolution of the underlying proton MR-images (256 x 256 pixels). The contrast within the resulting difference images is false-color coded with respect to the frequency of the applied on-resonance scans which is related to the individual cell tracers (red: on-resonant saturation at 107 ppm, green: on-resonant saturation at 70 ppm).

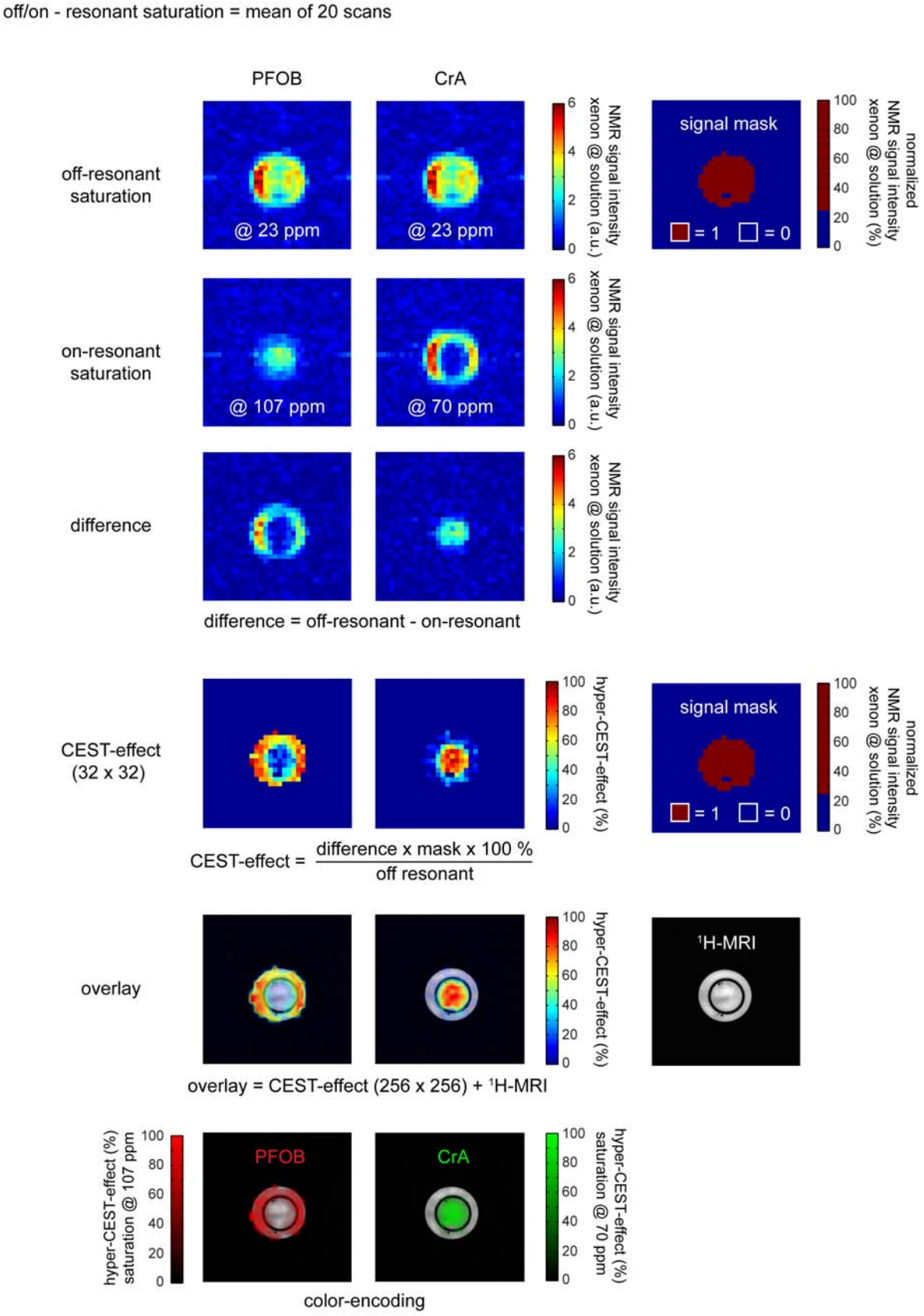

Figure S1: Post-processing of MR-images shown in Figure 3 (cw saturation for 8 sec with 10 µT).

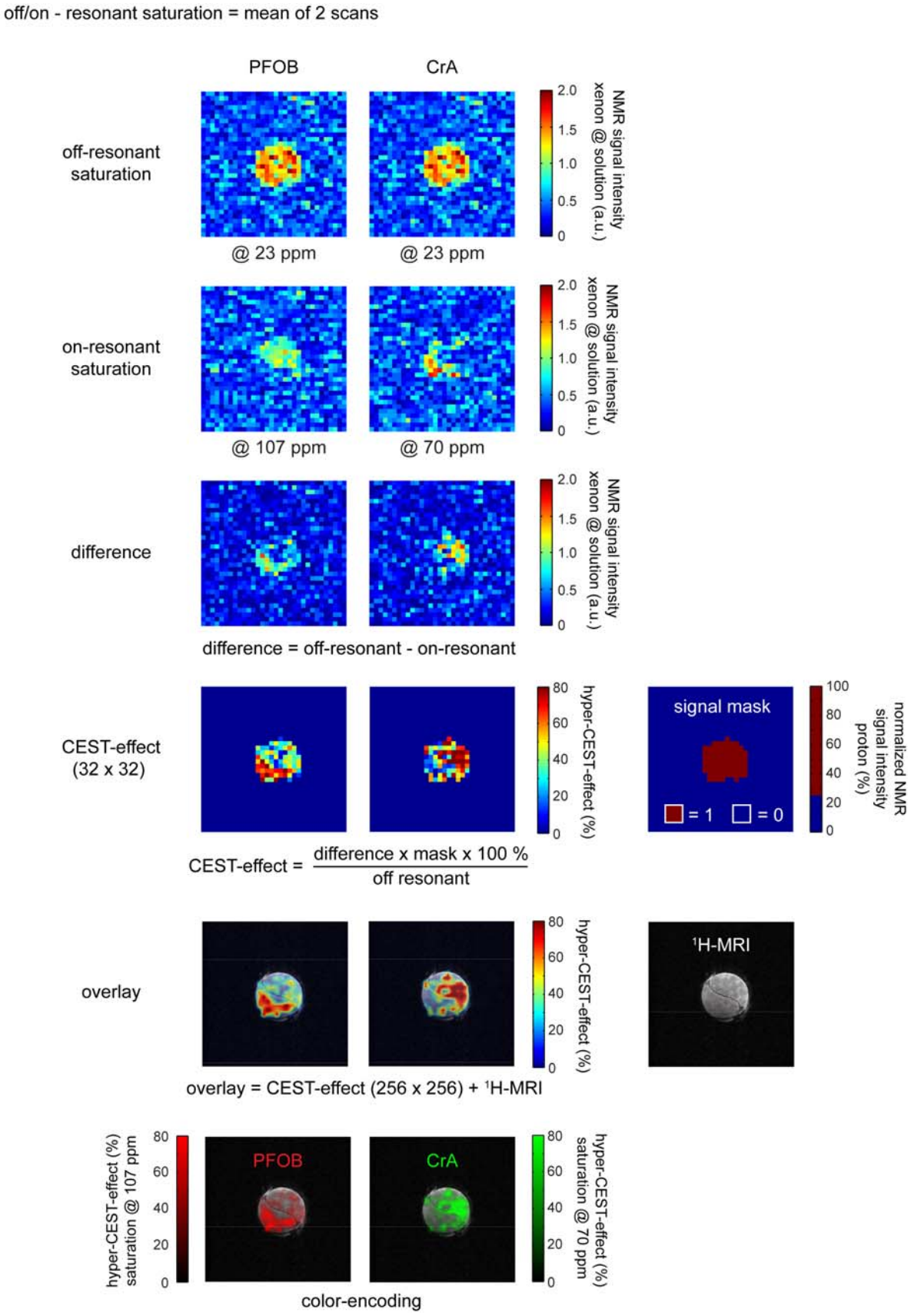

Figure S2: Post-processing of MR-images shown in Figure 4 (cw saturation for 10 sec with 10 µT).

6. Supporting references

(1) Stevens, T. K.; Ramirez, R. M.; Pines, A. *J. Am. Chem. Soc.* 2013**,** *135*, 9576–9579.

(2) Klippel, S.; Döpfert, J.; Jayapaul, J.; Kunth, M.; Rossella, F.; Schnurr, M.; Witte, C.; Freund, C.; Schröder, L. *Angew. Chem. Int. Ed.* 2014, *53*, 493–496.

(3) Partlow, K. C.; Chen, J.; Brant, J. A.; Neubauer, A. M.; Meyerrose, T. E.; Creer, M. H.; Nolta, J. A.; Caruthers, S. D.; Lanza, G. M.; Wickline, S. A. *FASEB J.* 2007, *21*, 1647–1654.

(4) Ahrens, E. T.; Flores, R.; Xu, H.; Morel, P. A. *Nat. Biotechnol.* 2005, *23*, 983–987.

(5) Ahrens, E. T.; Zhong, J. *NMR Biomed.* 2013, *26*, 860–871.

(6) León Fiszman, G.; Karara, A. L.; Finocchiaro, L. M. E.; Glikin, G. C. *Electron. J. Biotechnol.* 2002, *5*, 23–24.

(7) Ahrens, E. T.; Young, W.-B.; Xu, H.; Pusateri, L. K. *BioTechniques* 2011, *50*, 229–234.

(8) Seward, G. K.; Wei, Q.; Dmochowski, I. J. *Bioconjug. Chem.* 2008, *19*, 2129–2135.

(9) Walker, T. G.; Happer, W. *Rev. Mod. Phys.* 1997, *69*, 629–642.

(10) Hennig, J.; Nauerth, A.; Friedburg, H. *Magn. Reson. Med. Off. J. Soc. Magn. Reson. Med. Soc. Magn. Reson. Med.* 1986, *3*, 823–833.

(11) Zaiss, M.; Schnurr, M.; Bachert, P. *J. Chem. Phys.* 2012, *136*, 144106–144106–10.