A Genetically Encoded Reporter for Diffusion Weighted Magnetic Resonance Imaging

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SUPPLEMENTARY METHODS

Lentiviral packaging and transfection

Lentiviral packaging was performed in HEK 293T cells by transfecting 22 μg of packaging plasmid to expresses the capsid genes from a CMV promoter along with 22 μg of insert plasmid harboring the gene of interest (AQP1-IRES-EGFP, AQP4-IRES-EGFP, or GFP) flanked by LTR sequences, and 4.5 μg of VSV-G plasmid that expresses the vesicular stomatitis virus G protein to enable broad tropism of the lentiviral particles. Transfection was achieved using 25 kDa linear polyethyleneimine (Polysciences, Warrington, PA) at a concentration of 2.58 mg PEI/mg DNA. Approximately 24 hours post transfection, the culture medium was supplemented with sodium butyrate at 10 mM concentration to induce expression of the packaging genes. Virus production was allowed to proceed for 48–60 hours following which the virus-laden supernatant was collected, centrifuged at 500 x g to remove residual HEK 293T cells, mixed with 1/10th the volume of Lenti-X concentrator (Clontech, Mountain View, CA), and incubated at 4°C for at least 24 hours. Lentiviral particles were subsequently sedimented by centrifugation at 1500 x g for 45 minutes at 4°C and resuspended in 1–2 mL of Dulbecco's Modified Eagle Medium. Resuspended viral particles were immediately used to transfect CHO, CHO-TetON or U87 cells to generate stable cell lines. For this, the cells were first grown to 70-80% confluency in 6-well plates. Spent medium was aspirated from the wells and replaced with 1 mL lentivirus suspension together with 8 μg/mL polybrene. The cells were spinfected at 2000 x g for 90 minutes at 30°C, following which the plates were returned to the 37°C incubator for 48 hours to allow gene expression.

Determination of cell viability

Cell viability was determined by staining with ethidium homodimer-1 (Thermo Fisher) that specifically stains nucleic acids in cells in which the membrane integrity has been compromised. For quantifying cell viability, AQP1 and GFP expressing cells were grown in 6-well plates for 48 hours, trypsinized, and resuspended in 100 μL PBS supplemented with ethidium homodimer-1 at 4 μM final concentration. The cell-dye mixture was allowed to incubate at 4°C for 1 hour in a rotary shaker. Subsequently, 10 μL of the cell suspension was loaded in a disposable hemocytometer (C-chip DHC S02, Incyto) and total number of cells was estimated by imaging the hemocytometer chamber using bright field microscopy. Dead cells stained red and were estimated using fluorescence imaging with a Cy3 filter set. Viability was calculated as the fraction of cells that did not stain using ethidium homodimer-1.

Western blotting

For quantitative western blotting to estimate AQP1 expression levels, CHO TetON cells were first induced for 48 hours using, 0, 0.01, 0.1, and 1 μg/mL doxycycline. Membrane fractions were then isolated using ProteoExtract native membrane protein extraction kit (EMD Millipore, Billerica, MA) and concentrated ~30-fold using a 10 kDa centrifugal filter. Proteins were resolved on a denaturing SDS PAGE gel, transferred to a PVDF membrane, and aquaporin expression was probed using mouse anti-FLAG primary antibodies and horse radish peroxidase conjugated goat anti-mouse secondary antibodies. Signal detection was achieved using the Clarity chemiluminescent substrate (Biorad, Hercules, CA) using an exposure time of 1–10 s. AQP1 expression was quantified from a calibration curve of known quantities (100-400 ng) of FLAG-tagged bacterial alkaline phosphatase (Sigma Aldrich) that was simultaneously loaded, stained, and imaged on the same blot. In Figure 2c, the quantities of this standard are labelled as equivalent cellular molar concentrations.

T_1 and T_2 weighted MRI of aquaporin expression in cell pellets

T_1 weighted images were acquired using a Rapid Acquisition with Relaxation Enhancement (RARE) sequence with the following parameters: T_E = 9.6 ms, RARE factor = 4, N_{EX} = 2, matrix size = 128 x 256, FOV = 8 x 5 cm^2, slice thickness = 1.5 mm, and receiver bandwidth = 50,505.1 Hz. Variable T_R times were used including 146.19, 321.47,

519.98, 748.83, 1018.9, 1348.72, 1771.99, 2363.81, 3355.44, and 7500 ms. T_1 values were estimated from $S/S_0 = 1 - e^{\frac{-T_R}{T_1}}$, where S_0 is the equlibrium magnetization. T_2 weighted images were acquired using a Car-Purcell-Meiboom-Gill pulse sequence with the following parameters: T_E = 11 ms, T_R = 1.5 s, number of echoes = 63, number of excitations = 4, matrix size = 256 x 256, FOV = 8 x 5 cm^2, slice thickness = 1.5 mm, and receiver bandwidth = 50,505.1 Hz. T_2 relaxation rates were estimated by fitting the first 19 echoes to $S/S_0 = e^{\frac{-T_E}{T_2}}$. All images were analyzed using custom macros in ImageJ (NIH) and least squares regression fitting was performed using OriginLab.

Histological analyses of brain tissue

For histological analyses of brain tissue, mice were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight), and transcardially perfused first with PBS containing heparin (10 units/mL, Sigma Aldrich, St. Louis, MO) and subsequently with 4% w/v paraformaldehyde (Sigma Aldrich, St. Louis, MO). Following perfusion, the brain was harvested and fixed in 4% w/v paraformaldehyde for 2 hours at room temperature and washed 3 times with PBS. Axial brain sections of 100 µm-thickness were obtained using a vibratome (Leica Biosystems, Buffalo Grove, IL). Free-floating sections were incubated for 30 minutes at room temperature with a 1 µM solution of TO-PRO-3 Iodide nuclear stain in PBS (Thermo Fisher Scientific, Waltham, MA). Stained sections were washed three times with PBS and mounted on glass slides with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA) and imaged using a confocal microscope with GFP and Cy5 specific filter sets.

Monte carlo simulations of water diffusion in cells

We developed a model for restricted water diffusion and exchange in cells, building on the previously described Karger and Szafer[1-3] models of tissue water diffusion. We modeled our experimental cell pellets as a face-centered cubic lattice packed with 108 spherical cells with water molecules distributed randomly throughout the lattice at $t = 0$. Cell radii were sampled from a normal distribution with a mean of 6.8 µm and a standard deviation of 1.2 µm. We set the simulation time step $\tau = 50\ \mu s$ and at each time step, water molecules were propagated in a 3D random walk with step size given by $N\sqrt{\pi/2}\ \sqrt{2D\tau}$ in each direction. Here, N is sampled from a random normal distribution and D is the free diffusion coefficient of water at 12.9°C (the bore temperature of our MRI scanner) in the intracellular compartment (554.7 $\mu m^2/s$) or in the extracellular space (1664.2 $\mu m^2/s$)[4]. If a water molecule encounters a membrane, the propagation step is recalculated and the molecule either transmitted or reflected off the membrane with a probability given by $1 - 4P\sqrt{\tau/6D}$, wherein P is the membrane permeability and D is the free diffusion coefficient of water in the intracellular compartment. Diffusion paths were simulated in Python and the apparent diffusion coefficient (ADC) was calculated using Matlab as described in the Szafer model[1]: $ADC(\Delta) = -\lim_{q\to 0^+} \frac{ln\langle e^{-q\frac{\sum x^2}{2}}\rangle}{q\Delta}$ where $\sum x^2$ represents the sum square displacement of a water molecule from its starting position and q is given by $(\gamma\delta g)^2$ where γ is the gyromagnetic ratio, g is the gradient strength and δ is the duration of the pulsed diffusion gradient. We note that b-value is calculated as: $b = q.(\Delta - \delta/3)$. In the first set of simulations (ADC *vs.* permeability), we varied the cell permeability from .034 to 0.39 µm/s and calculated ADC(Δ) for each value of cell permeability. In the second set of simulations (ADC *vs.* fraction of AQP1-expressing cells) the permeability of AQP1-expressing cells and control cells were fixed at 0.14 µm/s and 0.039 µm/s respectively, in accordance with previously published values[5]. We incrementally varied the fraction of cells expressing AQP1 and for each composition, simulated 3 x 10^4 (nonunique) random arrangements of AQP1 expressing and control cells to exclude geometry or arrangement dependent bias in the results. ADC(Δ) was estimated corresponding to varying fractions of AQP1 expressing cells in the population.

References

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SUPPLEMENTARY FIGURES

Figure S1

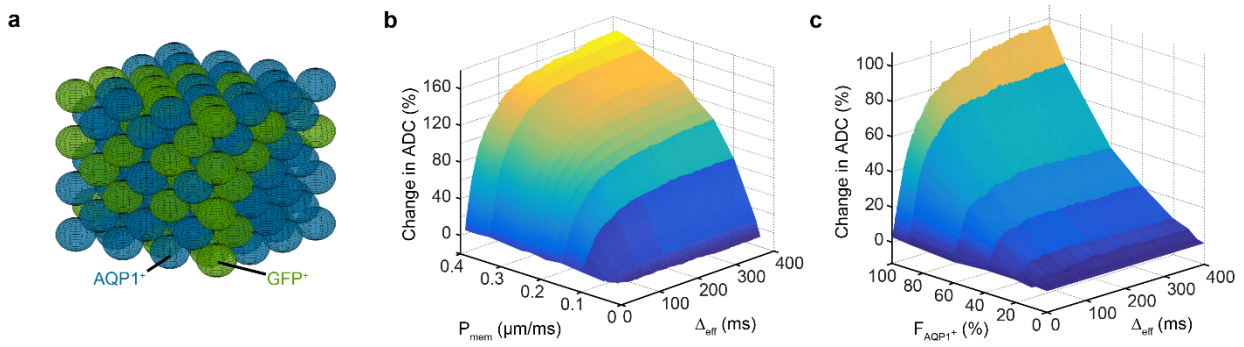

Figure S1. Monte Carlo simulations of water diffusion in AQP1+ and GFP+ (control) cells as a function of cell membrane permeability (P_{mem}), effective diffusion time (Δ_{eff}), and percentage of AQP1-labeled cells (F_{AQP1+}). We modeled cellular tissue as a face-centered cubic lattice packed with 108 spherical cells and with water molecules distributed randomly throughout the lattice at $t = 0$. (a) Mixed populations of AQP1+ and GFP+ cells were modeled by randomly distributing AQP1+ and GFP+ cells in the lattice to simulate 3 x 10^4 (nonunique) random arrangements of heterogeneous cell populations corresponding to varying fractions of AQP1+ cells. (b) ADC increases with increasing cell permeability, with the percent change in the ADC (measured relative to control cells with a basal permeability of 0.035 μm/s[5]) being most pronounced at longer diffusion times, consistent with the role of AQP1 in enhancing water diffusion across the cell membrane. (c) ADC increases in a nonlinear fashion with increase in the fraction of AQP1-labeled cells in a mixed population comprising AQP1+ and GFP+ cells. AQP1+ cells are assigned a permeability coefficient of 0.14 μm/s while GFP-expressing control cells are assigned a basal permeability 0.035 μm/s in accordance with literature values[5].

Figure S2

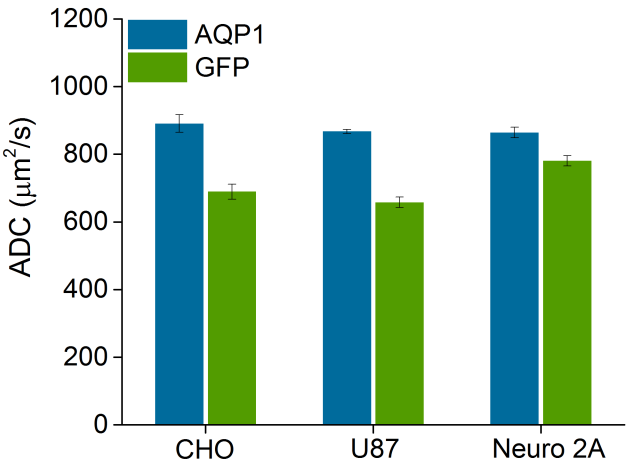

Figure S2. Apparent diffusion coefficient of water in AQP1 and GFP-expressing cells measured at short diffusion times (Δ_{eff} = 18 ms) typically used in DWI. Percent change in ADC on account of AQP1 expression is smaller at short diffusion times. Error bars represent standard error of mean (SEM) for 4 biological replicates.

Figure S3

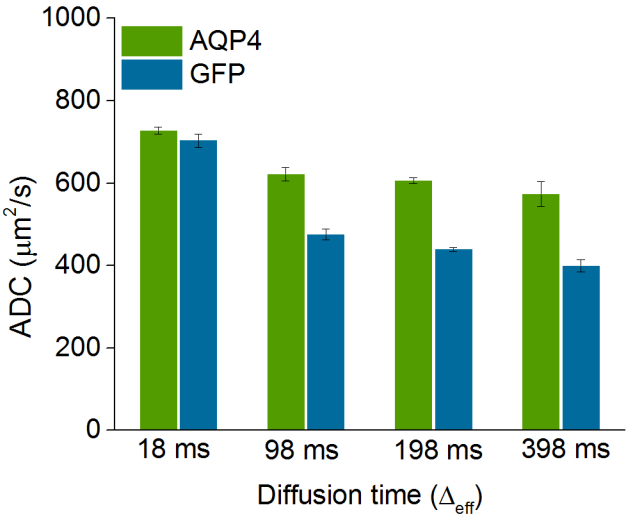

Figure S3. AQP4 is a genetically encoded reporter for diffusion weighted MRI. AQP4 expression enhances water diffusion in CHO cells relative to GFP controls, albeit not to the same extent as AQP1. Error bars represent standard error of mean (SEM) for 4 biological replicates.

Figure S4

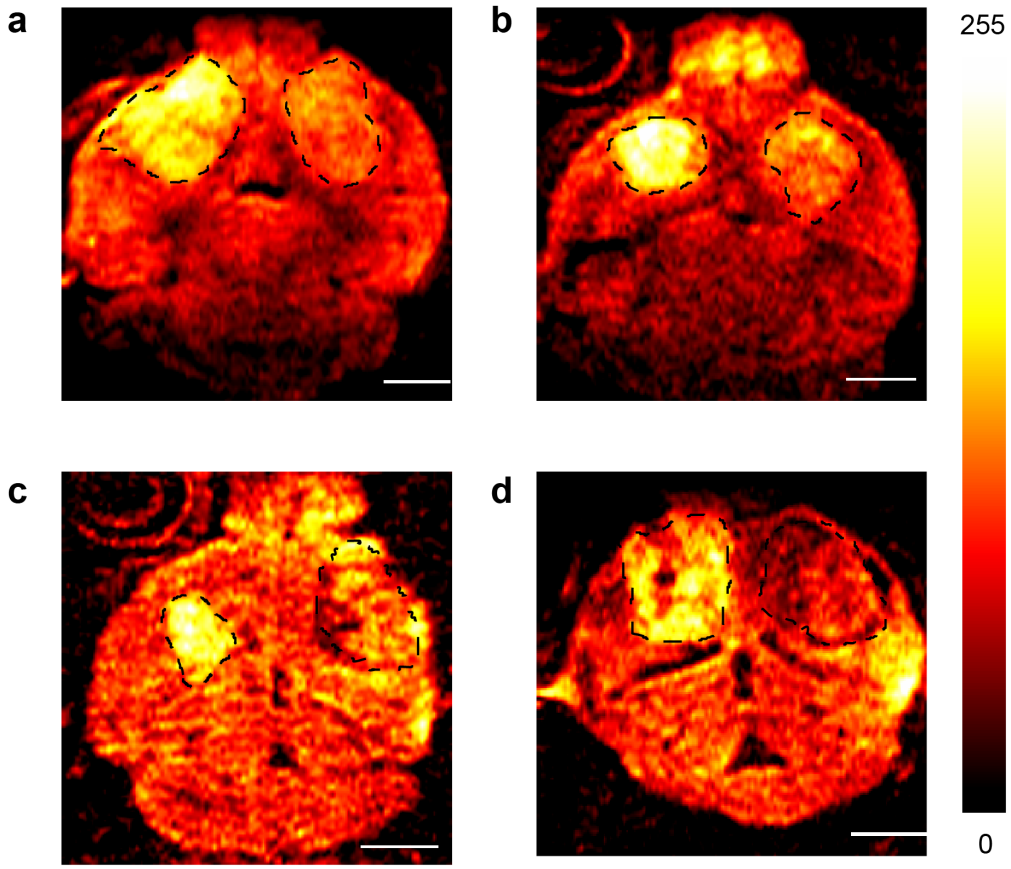

Figure S4. Diffusion weighted images of horizontal sections of the mouse brain with bilateral tumor xenografts, acquired 48 hours following intraperitoneal injection of doxycycline. AQP1 expressing tumors (right striatum) are visibly dimmer than the contralateral tumors (left striatum), which express GFP. Diffusion weighted images were acquired using an EPI DWI sequence with Δ_{eff} = 98 ms and b = 1000 s/m^2. Dashed lines indicate the tumor ROI(s). Scale bar is 3 mm.