In vivo imaging of glucose uptake and metabolism in tumors

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Tumors have a greater reliance on anaerobic glycolysis for energy production than normal tissues. We developed a noninvasive method for imaging glucose uptake in vivo that is based on magnetic resonance imaging and allows the uptake of unlabeled glucose to be measured through the chemical exchange of protons between hydroxyl groups and water. This method differs from existing molecular imaging methods because it permits detection of the delivery and uptake of a metabolically active compound in physiological quantities. We show that our technique, named glucose chemical exchange saturation transfer (glucoCEST), is sensitive to tumor glucose accumulation in colorectal tumor models and can distinguish tumor types with differing metabolic characteristics and pathophysiologies. The results of this study suggest that glucoCEST has potential as a useful and cost-effective method for characterizing disease and assessing response to therapy in the clinic.

Glucose is the primary source of energy in most organisms, where it is used in both aerobic and anaerobic respiration. Impaired or altered glucose consumption is associated with a range of pathological conditions, and the ability to noninvasively assess glucose uptake with [18F]-fluorodeoxyglucose ([18F]FDG) positron emission tomography (FDG-PET) has found wide clinical utility over the last 30 years. Here we propose a new way of detecting glucose uptake and metabolism that has no reliance on radiolabeled glucose analogs. Instead we use natural, nonradioactive glucose at physiologically reasonable quantities, which we image as it enters pathological tissues. Our technique is based on magnetic resonance imaging (MRI), which is a standard imaging modality that is available in most large hospitals, and it functions through a mechanism known as chemical exchange saturation transfer (CEST).

Tumors have a greater reliance on anaerobic glycolysis for energy production than normal tissues, a phenomenon that is known as the Warburg effect. This key discriminator of tumors from normal tissues, a phenomenon that is known as the Warburg effect. We propose a new way of detecting glucose uptake and metabolism in tumors by using the very large water signal rather than relying on the much smaller signal from glucose.

Comparison of glucoCEST with [18F]FDG autoradiography

GlucoCEST utilizes two properties of hydroxyl protons: first, when exposed to a magnetic field, the magnetic moments of hydroxyl protons precess at a different frequency as those of bulk tissue water and can therefore be selectively labeled using radiofrequency pulses; second, hydroxyl and water protons undergo exchange, thereby allowing magnetic labeling to be transferred from glucose to water and for glucose to be detected from the change in water signal in the MRI images (Fig. 1a). The CEST technique thus provides an amplification of detection by using the very large water signal rather than relying on the much smaller signal from glucose.

Having determined in vitro that glucose concentrations of only a few millimolar could be detected with glucoCEST (Supplementary Fig. 1), we aimed to determine the potential of glucoCEST in measuring regional glucose uptake in vivo. Thus we performed a gluco-CEST imaging experiment, the results of which we compared with those from [18F]FDG autoradiography 24 h later. We evaluated two subcutaneous human colorectal tumor mouse xenograft models, LS174T and SW1222, which have markedly different phenotypes.

In CEST experiments, the effect of exchangeable solute protons on the MRI water signal is visualized using the asymmetric magnetization transfer ratio (MTRasym) curve. We define a new parameter, the glucoCEST enhancement (GCE), as the change in area under the MTRasym curve from the area under a baseline curve (Fig. 1b). Baseline images of the area under the MTRasym curve reflect the influence of endogenous exchangeable protons, lipids and other exchange processes on the water signal. These contaminating effects are removed by subtracting the images before injection from those after injection using the GCE parameter.

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We used a gradient-echo CEST (GE-CEST) sequence to acquire images from a slice through the largest axial extent of each tumor at baseline and at 60 min after intraperitoneal (i.p.) bolus injection of glucose. A comparison of blood glucose measurements after i.p. and intravenous (i.v.) injections, and the subsequent glucoCEST response, suggested that the slow glucose kinetics after i.p. injection are preferable to the rapid changes after i.v. injection, with an increase in blood glucose concentration of approximately 5 mM (Supplementary Fig. 2). Images of glucose uptake from a cross-section through a mouse xenograft revealed a significantly greater GCE in SW1222 tumors compared to muscle (0.16 ± 0.04 (mean ± s.e.m.) and 0.04 ± 0.02%, respectively; \( P < 0.01 \)) (Fig. 2).

We returned the mice to their cages and allowed them to recover for 24 h, after which we administered between 10 and 15 MBq of \(^{18}\text{F}\)FDG (Fig. 3). The median activity at 60 min after administration was significantly higher in SW1222 than LS174T tumors (\( P < 0.01 \)). Similarly, we measured a significant difference in the median GCE between the two tumor types and a statistically significant correlation between the median \(^{18}\text{F}\)FDG and glucoCEST measurements (\( r^2 = 0.70, P < 0.01 \)). \(^{18}\text{F}\)FDG and GCE images, both acquired at 60 min after injection, also showed similar regional uptake (Fig. 4).
Relationship between glucose uptake, perfusion and hypoxia

Composite fluorescence microscopy images of perfusion and hypoxia (using Hoechst 33342 and pimonidazole staining, respectively) revealed SW1222 tumors to be more uniformly perfused and less hypoxic than LS174T tumors (with 5% ± 3% (mean ± s.d) and 35% ± 12% hypoxia, respectively) (Fig. 4). We found no correlation between the spatial distributions of pimonidazole and Hoechst 33342 staining (P > 0.05). A pixel-by-pixel comparison of [18F]FDG and pimonidazole uptake after image registration and regridding revealed a significant correlation in LS174T tumors (P < 0.01) but not in SW1222 tumors. We found no correlation on a pixel-by-pixel basis between Hoechst 33342 and FDG concentration for either of the two tumor types.

Generally, pixel-by-pixel comparisons of GCE and histological images were challenging because of nonlinear deformations occurring during tissue processing, so we instead compared the averages from whole-tumor sections. From this analysis we found that GCE and pimonidazole uptake were correlated in LS174T tumors, although at a lower level of statistical significance (P < 0.05), and we found no correlation in the averaged signal from SW1222 tumors. We found no correlation between GCE and Hoechst uptake in either tumor type.

Compartmentalization of tumor glucoCEST measurements

To investigate the relationship between the delivery of glucose to tumor tissue and its metabolism by tumor cells, we performed a glucoCEST experiment, which we followed immediately with dynamic contrast-enhanced (DCE) MRI acquisition in which we monitored the uptake of a bolus of gadolinium-diethylenetriamine pentacetate (Gd-DTPA, a commonly used MRI contrast agent) with time as it was delivered to the tumor (n = 4 SW1222 tumors and n = 4 LS174T tumors). Gd-DTPA is a useful marker of delivery, as after systemic injection it extravasates into tissue through gaps in blood vessel walls but cannot traverse cell membranes. Thus, a measurement of the uptake of Gd-DTPA in a tumor reflects the local blood flow, vascular permeability and volume of the extracellular space. Equally, the measured enhancement in a glucoCEST experiment could reflect each of these tissue properties, in addition to an intracellular glucose fraction, potentially in a metabolized form (for example, as hexoses and pentoses of the glycolytic pathway).

Comparison of DCE MRI area under the curve (AUC) images with glucoCEST images showed some similarities between the regional uptake of Gd-DTPA and glucose (Supplementary Fig. 3), although the differences between the two measurements were arguably more apparent than the similarities, and we found no significant correlation between them (Fig. 3d, P > 0.05).

In a further group of tumors (n = 5 SW1222 tumors and n = 5 LS174T tumors), we administered [U-13C]glucose i.p. at the same dose as that used in the glucoCEST experiments. At 60 min after injection, we freeze clamped and resected the tumors. We acquired 1H-decoupled 13C nuclear magnetic resonance (NMR) spectra of the aqueous phase separated from each tumor fragment (Fig. 5a). We detected glucose and glucose-6-phosphate from doublets in the C16 region of the 13C spectra, with a relative chemical shift of 0.13 p.p.m.9–11. The relative area of these peaks gave a ratio of labeled glucose-6-phosphate to glucose of 42% ± 18% (mean ± s.e.m.) and 38% ± 21% in SW1222 and LS174T tumors, respectively. In phantoms, glucose and glucose-6-phosphate produced similar z spectra, whereas fructose-6-phosphate and fructose-6-biphosphate produced smaller signals (Fig. 5b and Supplementary Fig. 4).

Also evident in the 13C spectra were lactate and amino acids, such as glutamine, glutamate, taurine and alanine, resulting from the glutamine synthesis pathway. Notably, each of these amino acids contains amide groups that have a strong CEST effect in vitro from the exchange between amide protons and water (Fig. 5c). Conversely, lactate, which was evident in the 13C spectra, has a negligible CEST effect compared with that of glucose, with in vitro measurements revealing a lactate area under the MTRsym curve of 4% that of glucose. Similarly, in phantom experiments, the average CEST effect from glutamine, glutamate, taurine and alanine was 83% ± 5% that found in glucose but was centered on a single resonance at 3.5 p.p.m., as compared with the three glucose resonances, which were situated between 1 p.p.m. and 4 p.p.m.
DISCUSSION

We present a new technique named glucoCEST, which we used to detect the uptake of exogenously administered, unlabeled glucose in tumors. Raised glucose uptake is a hallmark of solid tumors, and its noninvasive assessment would be of key importance in the clinic, with potential applications that include tumor detection, monitoring tumor progression and evaluating response to therapy. Although we focused on tumors in this study, it is anticipated that glucoCEST could find utility in other conditions, such as Alzheimer’s disease or stroke.

Our experiments revealed that glucoCEST was sufficiently sensitive to allow the detection of millimolar changes in tumor hydroxyl group concentrations after injection. As glucoCEST measures the change in signal from baseline after glucose injection, endogenous amounts of molecules with exchangeable protons (of either amide or hydroxyl groups) do not contribute to the glucoCEST effect and are assumed to not catalyze glucose-based hydroxyl proton exchange. However, byproducts of glucose metabolism after intracellular uptake of the administered glucose could contribute to the glucoCEST signal.

We compared glucoCEST with [18F]FDG autoradiography, which provides a highly sensitive and specific measure of FDG uptake. Although FDG is an analog of glucose with slightly differing pharmacokinetics, it provides the closest gold-standard technique for comparison. In accordance with the result from the glucoCEST experiments, [18F]FDG autoradiography revealed a significantly lower uptake of glucose in LS174T than SW1222 tumors, and we observed a marked spatial accordence between the FDG and glucose concentration maps. Moreover, the median tumor glucose and FDG uptake values were significantly correlated, thereby providing a validation of the glucoCEST technique. These results also help inform on the compartmentalization of the signal measured using glucoCEST, as FDG can only be metabolized into its primary phosphorylated state, at which point it becomes ‘trapped’ in the cell. Because of the avid accumulation of glucose by tumor cells, a measurement at 60 min after administration probably contains a substantial portion of FDG that has accumulated in the intracellular compartment.

13C NMR spectroscopy of tumor fragments administered [U-13C]glucose revealed the presence of labeled glucose in both tumor types, which could be either intracellular or extracellular in origin, alongside measurable quantities of glucose-6-phosphate. Our in vitro measurements of the CEST effect caused by the first four molecular species in the glycolytic pathway show that intracellular stages of glycolysis can be detected with glucoCEST, with glucose-6-phosphate giving an approximately equal enhancement as glucose. The concentration of intracellular glucose has been found previously to depend strongly on the concentration of extracellular glucose, with one study estimating the intracellular glucose concentration to be half of that outside the cell. Similarly, researchers from another study showed that the concentration of intracellular glucose depends on the rate of glucose uptake and metabolism, which in turn depends on cell type, with intracellular glucose concentrations ranging from very low (<0.07 mM) to as high as those of extracellular glucose (>5 mM).

Thus, although neither 13C NMR nor glucoCEST can distinguish between intracellular and extracellular free glucose, a contribution by intracellular glucose to the glucoCEST signal is possible, and an attenuation of the signal from extracellular glucose could occur as a result of the lower pH of the extracellular fluid. Indeed, from phantom measurements (Supplementary Fig. 5), intracellular and extracellular pH values of 7.2 and 6.9, respectively, could result in a 16% lower signal from the extracellular compartment per mole of glucose. These data, together with the lack of spatial concordance with Gd-DTPA–enhanced maps, suggest that glucoCEST does not simply report on vascular delivery of glucose but also on cellular uptake and metabolic activity.

[U-13C]glucose NMR also revealed the incorporation of glucose carbon molecules into amino acids such as glycine, glutamine, taurine and alanine. Under the assumption that the presence of additional glucose causes an increase in the concentration of these molecules, the large CEST effect associated with these molecules from amide proton exchange (rather than hydroxyl proton exchange) could provide...
an alternative intracellular contribution to the glucoCEST signal. It is conceivable that the differing shapes of the spectra associated with hydroxyl and amide proton exchange may allow these two phenomena to be modeled and separated in vivo, which would potentially allow glycolysis and gluconeogenesis to be separately probed. Thus, the combined effects of (i) extended vascular delivery of glucose, (ii) the presence of extracellular glucose, intracellular glucose and phosphorylated sugars in the glycolytic pathway, (iii) increases in amino acid concentration from gluconeogenesis and (iv) a lower extracellular pH lead to the conclusion that the source of the glucoCEST signal could be attributed to both intracellular and extracellular compartments, which explains its accordance with \( ^{18} \)RFDG autoradiography measurements.

We also found a correspondence with pimonidazole staining for hypoxia in LS174T tumors, which agrees with previous measurements in the same model by authors of a previous study, who showed that this correspondence was due to the expression of the glucose transporters GLUT-1 and GLUT-4 (ref. 20). We did not find this relationship in SW1222 tumors, which were much less hypoxic. It is also important to note the lack of correlation between either FDG or glucoCEST signal and Hoechst 33342 staining (a measure of perfusion), which would indicate that the measured glucoCEST signal is not limited by vascular delivery, as also argued above in relation to Gd-DTPA uptake. This result is particularly relevant, as the glucoCEST signal is potentially sensitive to physiological changes induced by hyperglycemia such as increased blood flow and decreased pH. Additional measurements of both properties after glucose injection showed that neither blood flow nor pH changed for the dose of glucose administered (1.1 mmol per kg body weight), thereby confirming that the observed glucoCEST signal was not caused by concomitant effects due to hyperglycemia (defined as a blood glucose concentration >20 mM (ref. 21)) (Supplementary Fig. 6).

The major findings of this study are therefore that glucoCEST has the sensitivity to discriminate between differing tumor phenotypes and that glucoCEST and \( ^{18} \)RFDG may be able to provide similar yet complimentary information. These conclusions have strong implications for the future utility of glucoCEST, as the ability to discriminate between tumors of differing pathophysiology would be of particular importance for diagnosis, prognosis and potentially assessing response to therapy.

Some challenges associated with the clinical translation of glucoCEST remain to be overcome, such as the lower field strengths used in the clinic, which would reduce the chemical shift between glucose and water and thereby increase the coalescence time, during which the chemical exchange rate would be faster than the chemical shift. However, the effect of this difference on the glucoCEST signal is not straightforward to predict and must be experimentally evaluated on clinical systems. The potential loss of contrast-to-noise ratio caused by lower field strengths may be compensated in part by the advanced, rapid imaging technologies available on clinical scanners. Although no clinical implementations of accelerated CEST acquisition schemes have yet been reported, techniques such as echo-planar imaging, steady-state free precession and fast-scan echo have been used extensively in magnetization transfer imaging, which is a closely related technique, and could be readily applied to a CEST measurement22–24. Similarly, as glucoCEST relies on the measurement of a sufficient contrast between signals from different selective saturations, signal averaging, in conjunction with rapid imaging techniques, would enable such differences to be more easily discriminated within a clinically acceptable examination time.

If it is sufficiently sensitive, glucoCEST offers a promising new tool for probing tumor glucose accumulation and metabolism, which naturally aligns with FDG-PET as an obvious candidate for comparison. Although FDG-PET uses trace amounts of FDG, we have shown that the physiological concentrations of glucose required for detection by glucoCEST are insufficient to produce any measurable changes in pH or blood flow. Scaling the dose used in this study to a 70-kg human would correspond to 14 g of glucose, which is approximately the same as found in half of a standard-sized chocolate bar. Oral administration could induce a greater insulin response than i.p. injection25 but would be easier to implement in the clinic than i.v. injection and would more closely mimic the slow enhancement protocol investigated in this study. A drinkable sugar solution could conceivably be ingested while a person is in the scanner, which is similar to the technique used in standard glucose tolerance testing in patients with diabetes26,27. This would enhance patient acceptance, although a period of fasting before the scan may also be required to stabilize baseline blood glucose concentrations, as is commonly performed with a range of medical examinations27,28.

GlucoCEST could thus offer a viable alternative to FDG-PET, particularly as \( ^{18} \)RFDG is expensive to manufacture and necessarily has an associated radiation dose, which limits the ability to perform repeated, longitudinal measurements and can prohibit examination of certain patient populations (such as young children or pregnant women27,28). Equally, as glucoCEST uses unlabeled glucose to provide image contrast, the logistics and infrastructure necessary for the production and delivery of \( ^{18} \)RFDG are removed, thereby simplifying the imaging procedure. FDG-PET is generally considered to be more expensive than MRI29,30, so glucoCEST could also offer considerable financial savings (although the estimated costs for the two modalities can vary substantially from country to country31). Furthermore, as the resolution of modern PET systems (typically of the order of 5 × 5 × 5 mm\(^3\) (ref. 32)) is much lower than the resolution afforded by clinical MRI scanners (typically around 1 × 1 × 3 mm\(^3\), which is a factor of 40 decrease in voxel size), glucoCEST could offer considerable improvements in spatial resolution over FDG-PET. Such improvements would allow for intratumor heterogeneity to be more effectively probed and smaller tumor masses to be evaluated, thereby allowing earlier characterization of disease and assessment of response to therapy.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.W.-S. designed and performed experiments, analyzed data, developed the methodology and wrote the paper. R.R. performed glucose tail-vein measurements, assisted with \textit{in vivo} experiments and developed the arterial spin labeling (ASL) post-processing software. F.T. and M.R. performed most phantom experiments and analyzed data. S.P.I. and R.B.P. developed and set up tumor xenograft models. V.R. performed histology and autoradiography measurements and analyzed data. H.G.P. performed \( ^{13} \)C NMR experiments. S.R. designed the bespoke apparatus for \textit{in vivo} imaging. M.G. assisted with \textit{in vivo} experiments. D.L.T., E.A., R.B.P.
X.G. and M.F.L. gave conceptual advice and assisted in the design of experiments. X.G. devised the initial glucoCEST concept and experiment. X.G. and M.F.L. jointly directed this research, helped perform experiments and contributed to the writing and editing of this manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Tumor models. All in vivo experiments were performed in accordance with the UK Home Office Animals Scientific Procedures Act, 1986 and United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines. Five-million LS174T or SW1222 human colorectal carcinoma cells were injected subcutaneously into the right flank of female MF1 nu/nu mice using a 27G needle and allowed to grow for 10–16 d.

GlucocEST MRI experiments. We acquired all MRI data using a horizontal-bore 9.4 T VNMRS system (Agilent, Oxford, UK). Before imaging, mice were fasted for 24 h and anesthetized in an induction box using 3% isoflurane in oxygen followed by 1.5% oxygen through a nose cone during MRI. Core body temperature was monitored and maintained using a physiological monitoring apparatus and a warm air blower. A solution of glucose was prepared in saline at a concentration of 140 mM, and 0.2 ml was administered either as a bolus, i.v. through a tail vein or i.p. using a nonmetallic pediatric cannula and a line running through the MRI scanner.

Tumors were localized using a T2-weighted fast-spin echo sequence (Supplementary Methods), from which we defined a slice through the largest extent of the tumor to use in subsequent imaging measurements. GlucocEST data were acquired using either a GE-CEST imaging sequence or an exchange-modulated point resolved spectroscopy (EXPRESS) sequence for single-voxel (whole-tumor) measurements. In the GE-CEST acquisition, lines from each image were acquired in a linear order such that for 128 phase-encoding steps, 100 saturation pulses were applied within the outer third of the k space, enabling a steady state to be reached (Supplementary Fig. 7). Data post-processing involved the conversion of signal data to the asymmetric magnetization transfer (MTR asym) and the GCE, which was defined as the change in the area under the MTR asym curve between 0.75 and 4 p.p.m.

ASL. We acquired ASL data using a FAIR Look-Locker sequence with a single-slice spoiled gradient echo readout34. Perfusion maps were calculated36 by assuming a blood-partition constant of 0.9 and a longitudinal relaxation time of blood of 1,900 ms37.

Dynamic contrast-enhanced MRI. Mice were administered a 50 mM solution of Gd-DTPA (Magnevist, Schering, Berlin) in saline as a bolus i.p. (0.2 mmol per kg body weight). Before injection, we estimated the baseline longitudinal relaxation rate by fitting a three-parameter model to the multiple-inversion recovery Look-Locker data. Gradient-echo image data were acquired for 5 min to measure the baseline signal intensity and then for another 60 min after injection of the contrast agent. The AUC for gadolinium was then calculated.

[18F]FDG autoradiography. Between 10 and 15 MBq of [18F]FDG was administered i.p. in 0.3 ml of saline. [18F]FDG was allowed to circulate for 60 min to monitor the conditions in the glucocEST experiments, at which point tumors were rapidly excised. We maintained the orientation of the tumors, and after excision the tumors were snap frozen using liquid nitrogen-cooled isopentane and cut in half along the longest axial diameter corresponding to the MRI imaging slice. Standard curves for the quantification of [18F]FDG activity were produced by spotting serial dilutions of the administered dose (1%, 0.1%, 0.01% or 0.001%).

[U-13C]glucose NMR spectroscopy of tumor fragments. [U-13C]glucose (Sigma-Aldrich, Gillingham, UK) was administered i.p. to mice following the in vivo glucoCEST protocol on the benchtop. At 60 min after injection, tumors were freeze clamped, resected and stored at ~80 °C. Samples were separated into the aqueous and organic phases (Supplementary Methods) and freeze dried, and 1H-decoupled 13CNMR spectra of the aqueous phase were acquired using a 500 MHz Bruker DRX spectrometer (Bruker, Karlsruhe, Germany). 13C free-induction decays were processed with a 3-Hz line broadening before Fourier transform. Spectra were analyzed using MestReC (Mestrelab, Santiago de Compostela, Spain) and in-house software written in the Interactive Data Language (IDL, Boulder, California) to fit Lorentzian lineshapes.

Immunofluorescence and histochemical analyses. Pimonidazole was administered i.p. (60 mg per kg body weight) and allowed to circulate for 30 min before the mice were euthanized to measure tumor hypoxia with fluorescence microscopy. Hoechst 33342 was administered i.v. (10 mg per kg body weight) and allowed to circulate for 30 min before tumors were rapidly resected and cut in half. One half was snap frozen in isopentane cooled with liquid nitrogen, and the other half was fixed in formaldehyde. Frozen tumor tissue was sectioned at 10 µm with the tumor orientation maintained for comparison with the MRI scans. Sections were viewed using an Axioimager microscope (Carl Zeiss, UK) at ×10 magnification. Perfusion was viewed using a ultraviolet filter (365-nm excitation), and hypoxia was viewed using a FITC filter (450- to 490-nm excitation). The percentages of tumor hypoxia and perfusion were quantified by defining the autofluorescence thresholds38,39.

Statistical analyses. Mann-Whitney U test was used throughout to assess differences in the magnitudes of samples from two measurements; Spearman’s p was used to assess correlation. P < 0.05 was taken to imply significance.

Additional methods. Detailed methodology is described in the Supplementary Methods.

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Corrigendum: *In vivo* imaging of glucose uptake and metabolism in tumors

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In the version of this article initially published online, the water molecules in Figure 1a had two oxygens and one hydrogen, rather than two hydrogens and one oxygen. The errors have been corrected for all versions of this article.