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Multimodality pH imaging in a mouse dorsal skin fold window chamber model

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Abstract

Upregulate levels of expression and activity of membrane H⁺ ion pumps in cancer cells drives the extracellular pH (pH_e) to values lower than normal. Furthermore, dysregulated pH is indicative of the changes in glycolytic metabolism in tumor cells and has been shown to facilitate extracellular tissue remodeling during metastasis. Therefore, measurement of pH_e could be a useful cancer biomarker for diagnostic and therapy monitoring evaluation.

Multimodality in-vivo imaging of pH_e in tumorous tissue in a mouse dorsal skin fold window chamber (DSFWC) model is described. A custom-made plastic window chamber structure was developed that is compatible with both imaging optical and MR imaging modalities and provides a model system for continuous study of the same tissue microenvironment on multiple imaging platforms over a 3-week period.

For optical imaging of pH_e, SNARF-1 carboxylic acid is injected intravenously into a SCID mouse with an implanted tumor. A ratiometric measurement of the fluorescence signal captured on a confocal microscope reveals the pH_e of the tissue visible within the window chamber. This imaging method was used in a preliminary study to evaluate sodium bicarbonate as a potential drug treatment to reverse tissue acidosis.

For MR imaging of pH_e the chemical exchange saturation transfer (CEST) was used as an alternative way of measuring pH_e in a DSFWC model. ULTRAVIST®, a FDA approved x-ray/CT contrast agent has been shown to have a CEST effect that is pH dependent. A ratiometric analysis of water saturation at 5.6 and 4.2 ppm chemical shift provides a means to estimate the local pH_e.

Keywords

acidosis; pH; cancer; microenvironment; MRI; CEST; fluorescence; in vivo imaging; confocal; window chamber

1. INTRODUCTION

Considerable efforts have been devoted to the development of imaging of cancer biomarkers such as hypoxia^{1,2}, acidosis^{3,4} and upregulation of epithelial growth factors⁵, to name a few. Imaging of cancer biomarkers opens up opportunities for improved cancer detection as well as pre- and post-delivery evaluation of cancer therapies. Moreover, invasive tumor cells have adaptive behaviors that serve to support metastatic growth and manifest themselves as observable physiological changes, which could be used as surrogates to study cancer development and treatment efficacy. For instance, invasive solid tumors often have deficient abnormal vasculature that impedes oxygen delivery and results in localized hypoxia. As an adaptive response, cells tend to switch to anaerobic glycolytic metabolism in which lactic acid is a byproduct. When lactic acid is produced in quantities that exceed the buffering capacity of HCO_3^- , a drop in extracellular pH_e is observed⁶. Interestingly, it has been observed that even at normal oxygen tension, cancer cells favor anaerobic glycolysis, which is known as the Warburg effect⁷. Therefore, acidosis is a common feature in many malignant tumors, and studies have shown that it is a contributing factor in extracellular matrix remodeling, which is a necessary process for metastatic spread⁸. These results have motivated interest in the development of methodologies to image acidosis as well as the effect of possible drug therapies to reverse acidosis.

Thus far, the gold standard for pH measurements in animal models is via pH micro-electrodes. However, this technique can only yield a localized measurement. Optical methods, on the other hand, are more suitable especially for in vivo studies as they give a measurements over an entire field of view and are non-invasive. Nevertheless, unlike measurements with electrodes, optical methods rely on exogenous contrast agents and have sources of errors that can be difficult to account for because they depend on the specific physiological environment of the tissue under study. Therefore, having multiple platforms for in vivo pH imaging, it could allow one to cross-validate experimental results and better understand the biophysical dependences involved in the measurements.

In this paper, we present work on developing two methodologies for measuring pH_e in vivo in a mouse using a dorsal skin fold window chamber (DSFWC) model. The first method utilizes a ratiometric calculation of the fluorescence signals emitted from a pH sensitive agent, 5-(and-6)-Carboxy SNARF®-1, which are captured using a confocal scanning microscope. The second method uses magnetic resonance imaging (MRI) signals to probe the pH dependent chemical exchange saturation effect (CEST) on iopromide, a molecule with two amide groups that each possesses exchangeable protons. The working mechanisms and pros and cons of each methodology are discussed in the paper. Lastly, the optical fluorescence pH imaging method was used for a preliminary study to evaluate sodium bicarbonate administered through a tailvein injection as means to reverse acidosis in the mouse model.

2. EXPERIMENTAL SETUP AND MATERIALS

2.1 Dorsal skinfold window chamber model setup

In order for the animal model setup to be compatible with MRI experiments, the window chambers have to be made of non-magnetic materials. Here, the window chamber structures are printed in-house on a Objet Connex300 3-D printer using Objet VeroGray™, a photopolymer that gives a rigid end-product after UV curing.

As depicted in Figure 1, the plastic window chambers are first secured onto a dorsal skinfold of a male SCID mouse before a portion of the top skin layer is surgically removed to expose the underlying tissue. This is followed by implantation of tumor cells from the human prostate cancer cell line, PC-3, which are transfected with green fluorescence protein (GFP). Finally, a 12mm diameter round glass coverslip is fixed over the tissue in the window chamber with a plastic retaining ring. This gives visual access to the site of tumor growth for up to 3 weeks. It should be noted that this is an ectopic animal model and the growth of the tumor is largely restricted to the thin skin fold that is typically less than 2mm thick.

2.2 Fluorescence imaging setup and procedure

All fluorescence images acquired in this work were captured on a Nikon Eclipse E600-C1 confocal scanning microscope. GFP images of the tumor cells are obtained by exciting the tissue with a 488nm Argon laser and collecting the light through a 515/30 nm bandpass emission filter. The mice were anesthetized with 1.5 to 2% concentration of isoflurane at an oxygen flow rate of 1 liter/min. For pH imaging, a cell impermeant fluorescent agent, 5-(and-6)-Carboxy SNARF®-1 (Invitrogen), is used. Its emission spectrum is pH dependent and the ratio of its emission peaks at around 595nm and 640nm varies monotonically with pH over a range from 6–8 pH units. For in vivo pH imaging, 200ul of a 2mM SNARF solution is administered through tailvein injection. SNARF fluorescence is excited using a 543nm HeNe laser. Two images are collected simultaneously through a 595/50nm bandpass filter and a 640nm longpass filter. This is done at 5 minute intervals post SNARF-injection. Subsequent computation of the intensity ratio between the two images with pre-injection background subtraction is used with a pre-established calibration curve to generate a pH map. To minimize measurements errors associated with chromatic aberration and temperature fluctuations^{9,10}, measurements are carried out using an open pinhole of 154um and a temperature controlled environment around the animal. The animal temperature is monitored with a fiber optic rectal probe.

2.3 MRI setup and procedure

MR experiments were done using a 7T Bruker BioSpec MRI machine. As shown in Figure 2, a 12mm diameter ¹H single loop surface coil, which is used for both excitation and receive, is placed directly above the window chamber of a SCID mouse. Similarly, the mice were anesthetized with 1.5 to 2% concentration of isoflurane at an oxygen flow rate of 1 liter/min. ULTRAVIST® 300, a FDA approved X-ray/CT contrast agent, which contains 300 mg of iopromide per milliliter is used as the MRI contrast agent for the CEST experiments. 1.5ml of ULTRAVIST® 300 is given through a bolus subcutaneous injection close to the window chamber to enhance the delivery of the agent to the tumor site. The MRI data

acquisition is based on a CEST-FISP pulse sequence as detailed in reference¹¹. 52 pre-saturation frequencies ranging from -10 to 10 ppm are used to generate the z-spectrum (CEST saturation spectrum) of a chosen region of interest.

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1 pH imaging with fluorescence

The anesthetized animal is placed under the confocal microscope within the temperature control box and the stage is adjusted for focus at with a $1\times$ objective. The setup is not moved during all subsequent data collection, which allows accurate co-registration between GFP images and the pH maps at the same magnification. Figure 3(a) shows a GFP image of the tumor growth in the window chamber at $1\times$. Figure 3(b) shows the pH maps obtained 30 minutes after the SNARF injection and Fig. 3(c) shows the same pH map with the border of the tumor determined from the GFP image. From the tumor growth outline overlaid on the pH map, it is evident that the tumor is more acidic than the surrounding normal tissue. To show the quantitative pH difference between tumorous and normal tissue, two small regions of interest within and outside the tumor region were selected. The average pH values within the two regions of interest are plotted against the time after injection. It is observed that the tumor has an average pH 1.5 units lower than the surrounding tissue. It is also noted that the measurements indicate pH values that are lower than what has been published in the literature for normal tissue. There could be a number of reasons as described in references⁹ and ¹⁰ that could account for the discrepancies. In addition, this is a tumor growing close to the surface of the window chamber and is a model that differs from many studies that use tumors growing deeper inside tissue. However, current results indicate that this methodology is capable of detecting a pH difference between the tumor and normal tissue. Therefore, even though there may be a bias in the absolute measurement of pH, the method may still be a valuable tool for applications such as tumor detection and tracking the pH gradients across tissue during tumor growth.

In addition to pH maps at $1\times$, it is also possible to obtain pH maps at higher resolution on the microscope. Figure 5 and Figure 6 show the pH maps at $10\times$ and $20\times$ respectively. Similar to what was observed at $1\times$, there is a close correspondence between the tumor and the more acidic region. In vivo measurements of pH at high magnification may be useful for studies trying to evaluate the distribution of pH at the cellular level within the tumor microenvironment.

3.2 pH modulation with sodium bicarbonate

Motivated by numerous studies that show acidosis as a contributing factor in extracellular matrix remodeling and metastasis, there is an interest in finding and evaluating therapeutic agents that can reverse acidosis. In a preliminary study, the fluorescence technique described above was used to evaluate sodium bicarbonate for this purpose. First, SNARF was injected into a mouse and measurements were taken at 5 minute intervals. At 30 minutes after the SNARF injection, when the fluorescence signal had stabilized, a tail vein injection of 200ul of 1M of sodium bicarbonate solution was given. Subsequent fluorescent measurements at 5 minute intervals were obtained until the 70 minute time point. Figure 7 shows a comparison

between the pH maps before and after sodium bicarbonate injection. An overall increase in pH was observed across the whole tissue region. A plot of the average pH measurements of two small region of interest within and outside the tumor indicates that there is an observable jump in pH in both regions immediately after sodium bicarbonate injection. The pH of the more acidic tumor region after the sodium bicarbonate injection increased to a value close to that of the normal tissue prior to injection. This indicates that this is a suitable dosage for temporally reversing acidosis in an animal model and similar protocols will be used for future studies.

3.3 pH imaging with CEST effects

Typically, pH measurement of tissue with a microelectrode is the gold standard for in vivo studies. However, the instrument can only give a very localized measurement and it is difficult to obtain accurate measurements, especially in thin superficial tissues such as occurs in the window chamber. Therefore, it is important to develop an alternative vivo pH imaging methodology that can help cross validate pH measurements on different platforms. MRI is a good pH imaging method to explore and research work is ongoing in this area^{12,13}.

MRI contrast agents are typically based on paramagnetic materials that effect the relaxation rate of water protons. Relatively large quantities of the contrast agent are required to obtain sufficient signal change. On the other hand, the CEST effect relies on protons on the contrast agent that can be saturated at a shifted resonance frequency and exchange with water protons to decrease the measured water signal^{14,15}. Since water is abundantly available in the body and multiple water protons can exchange with the contrast agent during the saturation period, it is possible to obtain contrast with smaller concentrations of the contrast agent and avoid issues of toxicity and perturbing the native tissue environment.

In this study, ULTRAVIST® 300 is used as a CEST agent. It contains iopromide, a molecule that has two sites of exchangeable protons on its two amide groups (see Fig. 9). Their resonant frequencies are shifted by 4.2ppm and 5.6ppm with respect to water. With magnetization saturation at these two frequencies, the water proton signal will be attenuated due to the saturated protons on the amide groups exchanging with the bulk water. The attenuation associated with each exchangeable site is dependent on the actual solute concentration in the tissue, which is difficult to measure or estimate. However, when there are two exchangeable proton sites on the agent, as is for iopromide, the ratio of the signal attenuations becomes independent of solute concentration. Most importantly, this signal ratio is pH dependent and so with proper calibration one can estimate pH by measuring this signal ratio.

Video 1 shows an image sequence of 54 MR images associated with the 54 saturation frequencies used during the experiment. Each image in the movie has a spatial resolution of approximately 200um. A plot of the normalized intensity variation across the pre-saturation frequency range can be obtained, as illustrated in Figure 10(b), which was obtained from a region of interest within the tumor. It should be noted from Figure 10(b) that the pre-saturation frequencies are not uniformly sampled. A denser sampling occurs around the resonant frequencies of the exchangeable protons to better capture the CEST effects. The raw data are fit to a curve that is a sum of three lorentzian curves (Figure 10(c)). The three

separate lorentzian curves are depicted in Figure 10(d). The lorentzian centered about 0ppm is from the bulk water while the pair of lorentzians centered around 4.2ppm and 5.6ppm are associated with CEST effects. By computing the ratio of this pair of lorentzians against a pre-determined calibration curve, the pH at the region of interest can be derived.

These preliminary results show that it is feasible to use the pH-dependent CEST effect for in vivo pH measurements in the window chamber model. Nevertheless, there are challenges that still need to be addressed in order to obtain reliable estimates of pH. First, it is evident that the CEST dip centered around 5.6ppm is very weak. It is known that, the chemical shift of each exchangeable proton site has to be greater than its exchange rate constant for the attenuation of signal due to CEST effect to be resolved against the water proton signal. As the exchange rate constant becomes comparable to the chemical shift, the lorentzian will start to broaden. As a consequence, as the pH and, thus, exchange rate increases, the CEST dip will gradually disappear. Secondly, there is poor fitting at negative chemical shifts, which might be due to other exchangeable protons on iopromide such as those on the –OH groups. In addition, there could be exchange occurring between iopromide and other biological macromolecules. This could explain why water is not completely saturated at 0ppm. Lastly, the excitation field given by the surface coil might not be uniform. If the tumor growth is large, the non-uniformity might cause inaccuracies in the measurements. To overcome this shortcoming, an alternative would be to use a volume coil for excitation and a surface coil for receiving signals. Continuing work will seek to improve the CEST MRI technique for imaging pH in the window chamber model system and use it to compare with the optical pH measurement technique.

4. CONCLUSION

A multimodality measurement methodology was investigated for in vivo imaging of pH_e in a mouse DSFWC model. We obtained pH maps using the fluorescence signals from the pH-sensitive SNARF contrast agent. This optical technique is capable of imaging the tumor microenvironment at high spatial resolution. The fluorescence method was applied to preliminary investigation using sodium bicarbonate to reverse acidosis in tumor tissue. We also developed and evaluated a MRI method to image pH in the DSFWC model using iopromide, which is a pH-sensitive CEST agent. Preliminary results were obtained but more work is needed to evaluate the precision and accuracy of this MRI technique and then to compare it with the optical approach.

5. BIBLIOGRAPHY

1. Olive PL, Aquino-parsons C, Macphail SH, Liao S, Raleigh JA, Lerman MI, Stanbridge EJ. Carbonic Anhydrase 9 as an Endogenous Marker for Hypoxic Cells in Cervical Cancer Carbonic Anhydrase 9 as an Endogenous Marker for Hypoxic Cells in. *Cancer research*. 2001; 61:8924–8929. [PubMed: 11751418]
2. Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nature reviews Cancer*. 2011; 11(6):393–410. [PubMed: 21606941]
3. Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, Gillies RJ. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer research*. 2006; 66(10):5216–23. [PubMed: 16707446]

4. Rofstad EK, Mathiesen B, Kindem K, Galappathi K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer research*. 2006; 66(13):6699–707. [PubMed: 16818644]
5. Chaffer CL, Weinberg RA. A Perspective on Cancer. *Science*. 2011; 331:1559–1564. [PubMed: 21436443]
6. Kemp G. Lactate accumulation, proton buffering, and pH change in ischemically exercising muscle. *American journal of physiology Regulatory, integrative and comparative physiology*. 2005; 289(3):R895–901. author reply R904–910.
7. Warburg O. Injuring of Respiration the Origin of Cancer Cells. *Science*. 1956; 123(3191):309–314. [PubMed: 13298683]
8. Srivastava J, Barreiro G, Groscurth S, Gingras AR, Goult BT, Critchley DR, Kelly MJS, Jacobson MP, Barber DL. Structural model and functional significance of pH-dependent talin – actin binding for focal adhesion remodeling. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(38):14436–14441. [PubMed: 18780792]
9. Lin Y, Wu T-Y, Gmitro AF. Error analysis of ratiometric imaging of extracellular pH in a window chamber model. *Journal of biomedical optics*. 2012; 17(4):046004. (1–7). [PubMed: 22559682]
10. Lin Y, Gmitro AF. Errors in confocal fluorescence ratiometric imaging microscopy due to chromatic aberration. *Applied optics*. 2011; 50(1):95–102. [PubMed: 21221166]
11. Shah T, Lu L, Dell KM, Pagel MD, Griswold MA, Flask CA. CEST-FISP: a novel technique for rapid chemical exchange saturation transfer MRI at 7 T. *Magnetic resonance in medicine: official journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine*. 2011; 65(2):432–7.
12. Martinez GV, Zhang X, García-Martín ML, Morse DL, Woods M, Sherry AD, Gillies RJ. Imaging the extracellular pH of tumors by MRI after injection of a single cocktail of T1 and T2 contrast agents. *NMR in biomedicine*. 2011; 24(10):1380–91. [PubMed: 21604311]
13. Gallagher FA, Kettunen MI, Day SE, Hu D-E, Ardenkjaer-Larsen JH, Zandt Rin’T, Jensen PR, Karlsson M, Golman K. Magnetic resonance imaging of pH in vivo using hyperpolarized ¹³C-labelled bicarbonate. *Nature*. 2008; 453(7197):940–3. [PubMed: 18509335]
14. Ward KM, Aletras AH, Balaban RS. A new class of contrast agents for MRI based on proton chemical exchange dependent saturation transfer (CEST). *Journal of magnetic resonance*. 2000; 143(1):79–87. [PubMed: 10698648]
15. Ward KM, Balaban RS. Determination of pH using water protons and chemical exchange dependent saturation transfer (CEST). *Magnetic resonance in medicine: official journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine*. 2000; 44(5):799–802.

Figure 1.

(a) Schematic of the cross section of the DSFWC. (b) Picture of a mouse with a plastic custom-made DSFWC sutured onto its dorsal skinfold. (c) The underlying blood vasculature and tumor growth can be visualized and studied for up to 3 weeks in the window chamber.

Figure 2.

A 12mm single loop surface coil is placed close above the plastic DSFWC of the mouse. The DSFWC is fixed onto a plastic mouse holder which prevents motion artifacts during MRI.

Figure 3.

All images are from the same window chamber taken at $1\times$. (a) GFP image of the PC-3 cells. (b) pH map at 30mins after injection of SNARE. (c) A pH map with a contour of the GFP image overlaid on it indicates close correlation between the more acidic region and the tumor site.

Figure 4.

A plot of the average pH measurements of the sampled region of interest of the tumorous and normal tissue over a 45 minute period after SNARF injection.

Figure 5.
(a) is a GFP image taken at 10 $\times$, (b) is the corresponding pH map.

Figure 6.
(a) is a GFP image taken at 20 $\times$, (b) is the corresponding pH map.

Figure 7.

(a) shows a pH map prior to the sodium bicarbonate injection while (b) is the pH map 15 minutes after tail vein injection of 200ul of 1M sodium bicarbonate. A comparison between the two indicates that there is an overall increase in pH across the tissue.

Figure 8.

A plot of the average pH measurements of the sampled tumor and normal tissue regions over a 70 minute period after SNARF injection. 200ul of 1M sodium bicarbonate was administered at the 30minute time point post SNARF injection and there is an observable increase in pH due to the sodium bicarbonate injection.

Figure 9.

The chemical structure of iopromide, the CEST active ingredient in ULTRAVIST[®]. It has two sites of exchangeable protons on the amide groups, which resonate at 4.2 and 5.6ppm shift from water protons.

Figure 10.

(a) A MRI image of the DSFWC. (b) The z-spectrum associated with a small region of interest within the tumor shows the water signal measured as a function of the saturation frequencies applied. (c) The raw data (dotted) are fitted with a sum of 3 lorentzian curves (solid). The individual lorentzian curves are shown in (d). The lorentzian curve centered about 0ppm is associated with the bulk water and the 2 lorentzian curves centered about 4.2 and 5.6ppm are associated with the CEST effects due to iopromide.

Video 1.

A movie of a 54 image sequence of the water signal in the DSFWC obtained as 54 RF pre-saturation frequencies ranging from -100 ppm to 100 ppm are applied. <http://dx.doi.org/doi.10.1117/12.2005472.1>