Published in final edited form as:

Anal Chem. 2012 April 17; 84(8): 3833–3837. doi:10.1021/ac203415w.

Electron paramagnetic resonance-based pH mapping using spectral-spatial imaging of sequentially scanned spectra

Shunichi Koda[†], Jonathan Goodwin[†], Valery V. Khramtsov[‡], Hirotada Fujii[§], and Hiroshi Hirata^{*,†}

[†]Division of Bioengineering and Bioinformatics, Graduate School of Information Science and Technology, Hokkaido University, Sapporo 060-0814, Japan

[‡]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Internal Medicine, The Ohio State University, Columbus, Ohio 43210, United States of America

[§]Department of Arts and Sciences, Center for Medical Education, Sapporo Medical University, Sapporo 060-8556, Japan

Abstract

The development of electron paramagnetic resonance (EPR)-based mapping of pH is an important advancement for the field of diagnostic imaging. The ability to accurately quantify pH change *in vivo* and monitor spatial distribution is desirable for the assessment of a number of pathological conditions in the human body as well as the monitoring of treatment response. In this work we introduce a method for EPR-based pH mapping, utilizing a method of spectral-spatial imaging of sequentially scanned spectra to decrease the missing gradient rotation angle, without increasing the spatial field of view. Repeated *in vitro* measurements of pH phantom tubes demonstrated higher precision measurements of the hyperfine coupling constant (HFC) compared to previous EPR-based methods, resulting in mean pH values accurate to less than 0.1 pH across a range of physiologically observed values.

Introduction

One of the key biological parameters in the maintenance of physiological homeostasis in living organisms is pH. Deviation from normal physiological values of pH in the human body is associated with a number of pathological conditions, including cancer,^{1–4} Chronic Obstructive Pulmonary Disease (COPD),⁵ renal failure,^{6,7} cardiac ischemia and brain ischemia.⁸ The ongoing prevalence of such pathological conditions provides ongoing motivation for the development of novel imaging methodologies to aid in the diagnosis and understanding of disease progression. The relationship that often exists between disease and altered pH is one aspect of physiology that can be exploited for diagnostic imaging purposes.

Over the last few decades a number of pH imaging techniques have been developed primarily for measuring pH change in tumors. Broadly speaking these techniques fall into several main imaging modalities: Positron Emission Tomography (PET), Magnetic Resonance Spectroscopy (MRS) and Imaging (MRI), Optical Imaging⁹ and EPR.^{10,11} More recently, a number of advancements have been made using the EPR/MRI hybrid technique, Proton-Electron Double Resonance Imaging (PEDRI), demonstrating pH imaging of

*Corresponding author Division of Bioengineering and Bioinformatics Graduate School of Information Science and Technology Hokkaido University North 14 West 9, Kita-ku, Sapporo 060-0814, Japan Phone and Fax: +81-11-706-6762 hhirata@ist.hokudai.ac.jp.

aqueous samples using variable radio frequency PEDRI,^{12,13} and *in vivo* measurements in tumor,¹⁴ which although displaying high temporal and pH resolution in phantoms, may be somewhat limited in *in vivo* applications due to relatively high RF power dissipation in the imaging sample. A number of nuclear magnetic resonance (NMR) based *in vivo* imaging methods have been developed over the last three decades, however these techniques may also be limited in terms of functional resolution.¹⁵ Currently, the two most established techniques for measuring the pH of tumors and surrounding tissue are fluorescence ratio imaging microscopy and fluorescence lifetime imaging microscopy, which are accomplished by the measurement of emission spectra and lifetimes of fluorophores, respectively.

The relatively narrow range of pH values known to occur between normal and diseased states requires a high precision measurement method, with a resolution capable of differentiating between normal and abnormal pH in the adjacent tissues, which may be particularly relevant for treatment response monitoring.

In this work we outline a pH imaging protocol for 750-MHz continuous-wave (CW) EPR imaging using the pH sensitive spin probe (4-amino-2,2,5,5-tetramethyl-3-imidazoline-1-yloxy - (R1)) (Scheme 1),^{13,16} and an acquisition system that enables *in vitro*, pH measurement in under six minutes with a sensitivity greater than 0.1 pH units. This work provides considerable advancement to previous EPR-based pH imaging studies, due to the relatively fast imaging time,^{10,11,16} and the improved sensitivity to pH due to the method of spectral-spatial imaging of sequentially scanned spectra.

Experimental section

Spin probe

EPR imaging is based on the measurement of EPR spectra of free radicals, and in the case of this study, the pH sensitive nitroxide molecule R1 ($pK_a=6.1$), was synthesized using the previously described method.¹⁷ The distance between lower field and center field spectral components, referred to as the hyperfine coupling constant (HFC), is altered depending on the pH of the local environment. An example of EPR spectra of R1 with different pH is shown in Fig. 1.

Imaging

To obtain spatial and spectral imaging information, the already well-documented EPR technique of three-dimensional (3D) spectral-spatial imaging is used, whereby the two spatial dimensions are added to one-dimensional spectral data.¹⁸⁻²⁰ This technique, based on the previous two-dimensional spectral-spatial imaging method,²¹ involves data acquisition through a series of gradient projections in accordance with formula (1). θ [degree] is the gradient projection angle; G [T/m] is field gradient; L [cm] is spatial field of view (FOV). B [mT] is magnetic field, representing the spectral width of the image object.

$$\theta = \tan^{-1} (GL/B) \quad (1)$$

Ideally, a maximum angle of 90° is required to obtain full projection data from the object image, however in practice the maximum angle θ is limited by gradient bipolar power supply, which requires an infinitely large positive or negative gradient when $\theta = 90^\circ$. Therefore a reduced value of θ leads to a lower image resolution, as does an increase in the spatial width parameter L , demonstrating a compromise between the parameters. For high-resolution imaging, FOV should be reduced, which requires a reduction in B , the spectral width of the image.

Typically the entire image spectrum, from low field to high field is measured, and halved to give a measure of HFC.²² In this study, we utilized a method for obtaining pH measurements by sequentially measuring only the center field and low field spectral components, thus reducing the spectral width B and the missing angle, without increasing the field of view. The choice of low- and center-field spectral components for HFC measurement is to some degree arbitrary, as the same method may be applied using the center and high field spectral components, and in this case may even demonstrate higher shifts in hyperfine coupling. However, in regards to the effect of reduced FOV and missing angle due to sequential scanning of individual spectra, and the subsequent improvement in spatial resolution and accuracy of pH measurements, the two approaches are equivalent. Both nitrogen hyperfine splitting and g-factor of R1 radical are affected by pH.¹⁶ At low EPR frequency the pH-dependable shifts of the low- and high-field components are determined exclusively by difference in hyperfine constants between protonated and nonprotonated forms of the R1 radical, while contribution of the g-factor in line shift is negligible. Using the low magnetic field spectral component, we firstly reconstruct a spectral-spatial image. This process is repeated for the center field spectral component, thereby providing two images, which are superimposed and subtracted to give the difference in spectral position; equivalent to the HFC value (Fig. 2). By acquiring HFC measurements of known pH samples, as measured with a high precision pH electrode meter, a calibration curve can be constructed (HFC vs pH), to allow for the estimation of pH for unknown samples or image subjects.

EPR imager and image reconstruction

In this work, a previously reported laboratory-built 750-MHz CW-EPR imager was used.^{23,24} After acquiring EPR spectra, three-dimensional (3D) phantom images were individually reconstructed using a method based on a previously reported filtered back-projection (FBP) algorithm.²⁵ The FBP algorithm was executed using FORTRAN (Pro Fortran Version 10.2.1; Absoft Corp., Rochester Hills, MI, USA) with a hamming filter for high frequency noise reduction. During reconstruction of all imaging data, a cutoff threshold of 35 (1/FOV) was applied, as well as imaging threshold of 50% of peak signal. These reconstruction parameters were chosen based on our previous image reconstruction work, where we found these parameters to be most conducive to accurate pH maps acquired using the above-mentioned EPR imaging system. The chosen threshold level is the consequence of the relatively broad absorption peaks and low spatial resolution achieved with this method, which requires a relatively high imaging threshold value in order to achieve pH maps which are both homogenous in pH value as well as representative of the true spatial dimensions of the measured phantoms.

Spectral-spatial imaging parameters

The scanning parameters for EPR spectral-spatial imaging were as follows: scanning field 1.0 mT, magnetic maximum field gradient 0.16 T/m, maximum angle 83.0 degree, FOV 50.9 mm, magnetic field modulation 0.15 mT, modulation frequency 90 kHz, scan time 0.5 s, time constant 0.3 ms, applied microwave power 2.51 mW, with 272 projections. The scanning protocol was repeated for both the center field spectrum and low field spectrum to allow measurement of HFC, giving a total image acquisition time of 340 sec. In determining the appropriate imaging parameters, one underlying factor was to develop a methodology that could potentially be applied in *in vivo* situations, where reductive reactions necessitate an imaging protocol, which is sufficiently fast so as to obtain the required imaging data prior to significant signal reduction, and which also has sufficient spectral resolution, to obtain accurate and repeatable measures of HFC. With these factors in mind, a scan time of 340 seconds, utilizing 272 gradient projections was found to be a sufficient balance between these requirements. For assessment of reproducibility and to obtain mean HFC values for

calibration curve data points, the scanning protocol was repeated four times for all phantom measurements.

pH phantom preparation

The solutions of the spin probe R1 were prepared in Dulbecco's PBS buffer solution (Dainippon Pharmaceutical Co., Ltd). pH values of the samples were adjusted with 1 M hydrochloric acid or sodium hydroxide, and controlled by electrode pH meter (CyberScan pH 1500, Eutech Instruments, Singapore) with a pH sensitivity of ± 0.002 .

Construction of calibration curve

For calibration curve measurements, pH phantoms were prepared using seven tubes of 5 mm diameter, which were filled with a 1 mM spin probe solution in phosphate. Each phantom tube was scanned individually, four times to obtain mean HFC values, which were then used to construct a non-linear calibration curve, relating HFC and pH. Previous work investigating the HFC dependency on pH for the R1 spin probe ($pK_a=6.1$), has been approximated using the Henderson-Hasselbalch equation.¹⁶ In this work however, we focused primarily on pH values found in the range of normal and pathological states in the human body. For this reason, and to avoid any inaccuracies in pH estimation that may result due to systematic differences between imaging systems, we chose to construct our own calibration curve based on HFC values acquired using our own system, which allow pH estimation only within the pH range of 6.15 to 7.56.

Results and Discussion

Calibration curve

In agreement with previous published work using R1 spin probe,^{16,26,27} we measured a non-linear relationship between HFC and pH in phantoms with solutions ranging from 6.15 to 7.56 pH. The acquired measurements provided sufficient data to form a calibration curve, as shown in Fig. 3, where the error bars represent the standard deviation of mean HFC values for four independent measurements of each phantom.

Accuracy and Reproducibility of pH measurements

The accuracy of pH measurements obtained through the use of the calculated calibration curve, was tested by acquiring HFC measurements from three additional pH test phantoms. To minimize the effects of field inhomogeneity, which exists within the main field of the EPR magnet (approximately 30 ppm), the three phantoms were imaged individually. This was to make use of the fact that the center of resonator is the most homogeneous region of the main field, and so by imaging each phantom individually rather than all together, and placing it in the center of the resonator, any inconsistencies between repeat scans, resulting from field inhomogeneity is minimized. The obtained estimates are shown in Table 1, as well as in Fig. 4, where data points represent mean pH values of four repeat measurements for each of the three test phantoms. In Fig. 5, the mean pH maps of the three test phantoms are shown, displaying both good homogeneity and accurate representation of cross-sectional area of the phantoms.

The tendency for an increase in standard deviation of repeat measurements with increasing pH values can be attributed to the decreasing gradient of the calibration curve (Fig. 3) at higher pH values, and not the variability in measured HFC values, which was found to be consistent across all pH samples. The normal variability of HFC measurements resulting from factors such as sample heating due to RF absorption and background noise, will result in a relatively larger variability in derived pH values for measurements corresponding to the upper portion of the calibration curve, than would the equivalent level of HFC variability for

lower pH phantom measurements. The increased variability of pH estimates at higher values of pH results in a reduction of accuracy of measurements. However, as shown in Table 1, even for the highest value of pH, the average of four measurements demonstrated an accuracy of less than 0.1 pH when compared to the electrode pH value. Achieving this level of accuracy with this imaging method also depends on the stability of magnetic field scans. However, due to the non-linear relationship between HFC and pH, the obtainable accuracy and reproducibility of measurements for a given magnetic field scan stability specification, will vary according to the HFC value being investigated, whereby values above the pH range of 7.1 will require greater field scan stability than a lower pH value, to achieve the same level of accuracy and repeatability. By using the calibration data obtained in this study, we can estimate that for a single pH measurement of pH phantom with value 6.2 ± 0.1 a stability of equal or less than $7 \mu\text{T}$ is required, whereas the equivalent level of stability required for a single measurement at a pH value of 7.44 is equal or less than $1 \mu\text{T}$. This effect can be visualized in Fig. 4, where repeat measurements of pH phantom of 7.44 shows higher variability between repeat measurements than the lower value pH phantoms. This inherent difficulty in obtaining high accuracy measurements in the pH range above 7.1 is one motivation for obtaining repeat measurements whenever possible.

Effects of RF heating

One of the systematic inaccuracies, inherent to CW-EPR of *in vitro* samples is the effect of sample heating due to RF absorption. Previous experiments in our group investigating image sample temperature increases during spectral spatial imaging, found increases on the surface of the phantom of up to 3 degrees Celsius after approximately 25 minutes imaging; a time approximately equal to that required for acquiring four repeated spectral spatial phantom images. This temperature increase corresponded to an increase in HFC of up to $8 \mu\text{T}$, resulting in an overestimation in pH of up to 0.3. Therefore in this study, to minimize heating effects in phantoms, an electric fan was positioned in near proximity to the EPR system to ensure a continuous air flow through the resonator during image acquisition, maintaining the temperature change during the EPR scanning period to less than 1 degree Celsius.

Despite efforts to maintain the temperature inside the resonator at a constant temperature, an additional consequence of any small increase leads to a small but measureable increase in the center field spectral position. It is therefore necessary to perform spectral spatial imaging on both low field and center field position, in order to accurately measure HFC. Further efforts to stabilize the spectrometer from temperature related fluctuations, may potentially reduce the current image acquisition time by half by imaging only the low field spectrum, and a single spectroscopic measurement of center field spectral position to enable HFC calculation.

Advantages of EPR approach to pH measurement

As previously noted, a number of alternative imaging approaches have been developed for the purpose of pH measurement and visualization. While each of these methods offers a viable approach to pH imaging, there are a number of advantages to using the approach outlined in this study:

- i. In the conventional approach of spectral-spatial EPR imaging, either the lower and center or lower and higher absorption peaks are recorded simultaneously. This leads to a larger missing angle and larger FOV, resulting in a degradation of functional resolution of pH. By obtaining independent measurements of absorption peaks, as described in this work, a reduction in both missing angle and FOV can be achieved.

- ii. Variable radio frequency PEDRI (VFR-PEDRI) is a promising technique, allowing rapid (6.8 s) 2D pH mapping.¹² However, the utilization of the Overhauser effect leads to a sensitivity of the signal enhancement factor to EPR absorption line width. Therefore, line width variance can have a direct affect on the robustness of pH mapping. This sensitivity becomes less relevant when using spectral spatial imaging to measure HFC, which is independent of line width variance.
- iii. MRI techniques such as those utilizing Dynamic Nuclear Polarization are perhaps the most promising techniques for pH measurement in a clinical environment. In particular, the use of hyperpolarized ¹³C-labeled bicarbonate has been demonstrated as perhaps the most viable candidate for *in vivo* pH mapping in humans.²⁸ While the standard deviation of measured pH value with MRI of hyperpolarized ¹³C-labeled bicarbonate is less than 0.1 pH units, the technique is limited by a number of practical requirements, namely the requirement of fast image acquisition time (1-2 min) due to the signal decay of ¹³C nuclei, and the additional facilities that are required for the hyperpolarization process. Despite our EPR method outlined in this paper being applicable primarily for small animal studies, we have demonstrated a similar level of functional resolution to ¹³C-labeled bicarbonate MRI studies, without the requirement of hyperpolarization.
- iv. PET imaging has also been demonstrated in small animals studies as a potentially viable technique for the assessment of pH in a broad range of disease states,³ however, the requirement of radioisotope labeled molecules for signal generation is an obvious disadvantage when compared to non-ionizing imaging techniques that offer a similar level of functional resolution.
- v. Optics/fluorescence-based pH imaging has been well documented, and has been demonstrated as a viable method for 3D *in vivo* small animal imaging.^{9,29} However, these techniques may be limited in their application due to limited penetration depth due to attenuation and photon scattering in biological tissues, as well as potentially non-repeatable pharmacokinetic behavior; issues which may be less relevant for small animal imaging using the method outlined in this paper.

Conclusions

In this work we demonstrated a technique for *in vitro* measurement of a pH sensitive spin probe using spectral-spatial imaging of sequentially scanned spectra. This technique utilizes an imaging system considerably faster than previously reported EPR-based pH measurement techniques^{10,11,16,27,30} with the additional benefit of a reduced missing gradient rotation angle due to the sequentially scanned rather than simultaneously scanned center and low field spectra, and a reduced FOV, which results in improved image resolution with a high level of accuracy, as demonstrated by the proximity of data points in Fig. 4 to the line of equality with pH values measured by glass electrode. Additionally, repeated measurements gave mean pH values accurate to less than 0.1 pH units. In future work, we plan to extend this methodology to the *in vivo* study of pH in tumor-bearing mice. However, despite previous studies which utilized the R1 probe for *in vivo* pH imaging of small animals,¹⁶ there now exist alternative nitroxide spin probes with higher linearity over the normal physiological range, and greater *in vivo* stability than the R1 spin probe,¹⁴ which may be a more viable candidate for future *in vivo* pH mapping studies.

Acknowledgments

This work was supported by grants from Japan Society for the Promotion of Science (21360193 and the NEXT Program LR002 to H.H.). The support from the Global COE program in Graduate School of Information Science

and Technology at Hokkaido University is appreciated, as well NIH grant EB014542-01A1 through The Ohio State University.

References

- (1). Hashim AI, Zhang X, Wojtkowiak JW, Martinez GV, Gillies RJ. NMR Biomed. 2011
- (2). Stubbs M, McSheehy PM, Griffiths JR, Bashford CL. Mol Med Today. 2000; 6:15. [PubMed: 10637570]
- (3). Vavere AL, Biddlecombe GB, Spees WM, Garbow JR, Wijesinghe D, Andreev OA, Engelman DM, Reshetnyak YK, Lewis JS. Cancer Res. 2009; 69:4510. [PubMed: 19417132]
- (4). Gatenby RA, Gillies RJ. Nat Rev Cancer. 2004; 4:891. [PubMed: 15516961]
- (5). Bott J, Carroll MP, Conway JH, Keilty SEJ, Ward EM, Brown AM, Paul EA, Elliott MW, Godfrey RC, Wedzicha JA, Moxham J. Lancet. 1993; 341:1555. [PubMed: 8099639]
- (6). Kraut JA, Madias NE. Pediatr Nephrol. 2011; 26:19. [PubMed: 20526632]
- (7). Kopple JD, Kalantar-Zadeh K, Mehrotra R. Kidney Int Suppl. 2005:S21. [PubMed: 15882309]
- (8). Rehncrona S. Ann Emerg Med. 1985; 14:770. [PubMed: 3927794]
- (9). Zhang X, Lin Y, Gillies RJ. J Nucl Med. 2010; 51:1167. [PubMed: 20660380]
- (10). Kroll C, Herrmann W, Stosser R, Borchert HH, Mader K. Pharmaceut Res. 2001; 18:525.
- (11). Sotgiu A, Mader K, Placidi G, Colacicchi S, Ursini CL, Alecci M. Phys Med Biol. 1998; 43:1921. [PubMed: 9703055]
- (12). Efimova OV, Sun Z, Petryakov S, Kesselring E, Caia GL, Johnson D, Zweier JL, Khramtsov VV, Samouilov A. J Magn Reson. 2011; 209:227. [PubMed: 21320790]
- (13). Khramtsov VV, Caia GL, Shet K, Kesselring E, Petryakov S, Zweier JL, Samouilov A. J Magn Reson. 2010; 202:267. [PubMed: 20007019]
- (14). Bobko AA, Eubank TD, Voorhees JL, Efimova OV, Kirilyuk IA, Petrayakov S, Trofimov DG, Marsh CB, Zweier JL, Grigor'ev IA, Samouilov A, Khramtsov VV. Magn Reson Med. 2011 doi: 10.1002/mrm.23196.
- (15). Gillies RJ, Raghunand N, Garcia-Martin ML, Gatenby RA. IEEE Eng Med Biol Mag. 2004; 23:57. [PubMed: 15565800]
- (16). Khramtsov VV. Curr Org Chem. 2005; 9:909.
- (17). Volodarsky, LB.; Grigor'ev, IA. Synthesis of heterocycling nitroxides. In: Volodarsky, LB., editor. Imidazoline Nitroxides. Vol. vol. 1. CRC Press; Boca Raton, Fla: 1988. p. 5-28.
- (18). Eaton SS, M. MM, S. ES, Stemp DA, R. EG. Chem Phys Lett. 1987; 142:567.
- (19). Maltempo MM, Eaton SS, Eaton GR. J Magn Reson. 1989; 85:303.
- (20). Guanglong H, A. SR, Chzhan M, Samouilov A, Kuppusamy P. Proc Natl Acad Sci USA. 1999; 96:4586. [PubMed: 10200306]
- (21). Maltempo MM, Eaton SS, Eaton GR. J Magn Reson. 1986; 72:449.
- (22). Potapenko DI, Foster MA, Lurie DJ, Kirilyuk IA, Hutchison JM, Grigor'ev IA, Bagryanskaya EG, Khramtsov VV. J Magn Reson. 2006; 182:1. [PubMed: 16798033]
- (23). Sato-Akaba H, Fujii H, Hirata H. Rev Sci Instrum. 2008; 79:123701. [PubMed: 19123563]
- (24). Sato-Akaba H, Kuwahara Y, Fujii H, Hirata H. Anal Chem. 2009; 81:7501. [PubMed: 19645455]
- (25). Liang, ZP.; Lauterbur, PC. Principles of Magnetic Resonance Imaging: A Signal Processing Perspective. IEEE Press; Piscataway, NJ: 2000. p. 207
- (26). Khramtsov VV, Grigor'ev IA, Foster MA, Lurie DJ, Nicholson I. Cell Mol Biol. 2000; 46:1361. [PubMed: 11156481]
- (27). Khramtsov VV, Grigor'ev IA, Lurie DJ, Foster MA, Zweier JL, Kuppusamy P. Spectrosc-Int J. 2004; 18:213.
- (28). Gallagher FA, Kettunen MI, Day SE, Hu DE, Ardenkjaer-Larsen JH, Zandt R, Jensen PR, Karlsson M, Golman K, Lerche MH, Brindle KM. Nature. 2008; 453:940. [PubMed: 18509335]
- (29). Wang L, Li C. J. Mater. Chem. 2011; 21:15862.
- (30). Mader K, Nitschke S, Stosser R, Borchert HH, Domb A. Polymer. 1997; 38:4785.

Figure 1. EPR spectrum. Hyper-fine coupling (HFC) measured as the distance between low- and center-field spectral components, represents the parameter sensitive to the pH of the local environment.

Figure 2.
Concept of pH imaging using spectral spatial imaging to measure hyper-fine coupling.

Figure 3.

Calibration curve relating measured HFC with pH ($pK_a=6.1$). Data points cover the range of normal and acidic pH values typical of those seen in biological systems.

Figure 4. EPR measured pH vs electrode pH. Graph shows accuracy and precision of pH values obtained using EPR acquired HFC values and the previously constructed calibration curve. Each data point represents the mean of four separate measurements of a single phantom, which was scanned individually with the standard deviation shown with error bars.

Figure 5.

Two-dimensional pH maps generated from 3D reconstructed images of three pH phantoms. The displayed images have a FOV of 25.45 mm with 256×256 pixels, which were cropped from images with a FOV of 50.9 mm, and 512×512 pixels to remove empty image space. Each image represents the mean image from four separate measurements of a single phantom, with each phantom scanned individually.

Scheme 1.

Molecular structure of pH sensitive spin probe (4-amino-2,2,5,5-tetramethyl-3-imidazoline-1-yloxy (R1)).

Table 1

Measured pH values for three separately scanned pH phantoms, with mean and standard deviation of four repeat measurements, and comparison of mean with electrode pH values.

pH	1st image	2nd image	3rd image	4th image	Image mean	Mean pH – electrode pH
6.32	6.34 ± 0.02	6.25 ± 0.09	6.32 ± 0.02	6.38 ± 0.02	6.32	0.00
6.68	6.64 ± 0.01	6.50 ± 0.17	6.70 ± 0.03	6.53 ± 0.09	6.60	-0.08
7.44	7.17 ± 0.07	7.44 ± 0.15	7.57 ± 0.08	7.28 ± 0.10	7.37	-0.07