Quantitative In Vivo Characterization of Intracellular and Extracellular pH Profiles in Heterogeneous Tumors: A Novel Method Enabling Multiparametric pH Analysis

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Abstract

Acid production and transport are currently being studied to identify new targets for efficient cancer treatment, as subpopulations of tumor cells frequently escape conventional therapy owing to their particularly acidic tumor microenvironment. Heterogeneity in intracellular and extracellular tumor pH (pHi, pHe) has been reported, but none of the methods currently available for measuring tissue pH provides quantitative parameters characterizing pH distribution profiles in tissues. To this intent, we present here a multiparametric, noninvasive approach based on in vivo 31P nuclear magnetic resonance (NMR) spectroscopy and its application to mouse tumor xenografts. First, localized 31P NMR spectrum signals of pHi and pHe reporter molecules [inorganic phosphate (Pi) and 3-amino-1-propylphosphonate (3-APP), respectively] were transformed into pH curves using established algorithms. Although Pi is an endogenous compound, 3-APP had to be injected intraperitoneally. Then, we developed algorithms for the calculation of six to eight quantitative pH parameters from the digital points of each pH curve obtained. For this purpose, each pH distribution profile was approximated as a histogram, and intensities were corrected for the nonlinearity between chemical-shift and pH.

Major Findings

For each histogram derived from a Pi or 3-APP resonance, we obtained the following tumor pH profile parameters: weighted mean, weighted median, mode(s), skewness (asymmetry), kurtosis (peakedness), and entropy (smoothness). In addition, relative sizes of tissue volumes defined by characteristic pH ranges were estimated by integration and/or by fitting the curve to multiple modes. Our algorithms and the results obtained for animal models were validated by: (i) computer simulations of 31P NMR resonances and pH profiles; and (ii) comparison with combinations of three or less test solutions at well-defined pH values, containing the pH reporter molecule 3-APP. All calculations were carried out with an Excel spreadsheet, thus avoiding any specialized software or hardware. Consequently, heterogeneous pH and pHe distribution profiles in tumors can be characterized by multiple quantitative parameters derived from classical statistics through histograms obtained from in vivo 31P NMR spectra. This original technique is helpful in analyzing tumor tissue features with increased detail, based on a single experiment also yielding information on underlying energy and phospholipid metabolism.

Introduction

In physiologic tissues, the interplay of metabolism, ion transport, and pH buffering results in efficient pH regulation. The presence of macroscopic and microscopic membrane structures, such as the basement membrane and various cell membranes, permits the coexistence of multiple tissue compartments characterized by different pH values. This highlights the necessity to not only measure average tissue pH values, but also to quantitatively assess pH heterogeneity. Although non-invasive determination of intra- and extracellular pH (pHi and pHe) in mammals, notably by way of nuclear magnetic resonance (NMR) techniques, has become more common in recent years, there is at present no method that provides quantitative parameters specifically characterizing the heterogeneity of pHi and pHe in a given tissue volume. Yet, there is a genuine need for such measurements as a variety of pathologies (cancer and inflammation, among others) are associated with heterogeneous pH regulation (1–3). Even normal activity such as muscle exercise can generate complex tissue pH distributions as a function of biologic characteristics (4). To address this challenge, we have developed a new approach based on

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Quick Guide to Equations and Assumptions

The current standard procedure for deriving a pH value from a pH-sensitive $^31$P nuclear magnetic resonance (NMR) resonance is based on converting the chemical-shift axis of the resonance line into a pH axis. It is generally assumed that, after appropriate intensity correction, (i) the resulting curve adequately reflects the pH within the measured volume, and (ii) the position of the maximum of this pH curve represents the pH. We adopt assumption (i), within certain limits. However, we argue that the maximum of a tissue pH curve often yields a nonrepresentative pH value, because such curves are frequently asymmetric and irregularly shaped. We also contend that a (intracellular or extracellular) pH curve can and should be exploited to quantitatively analyze the respective underlying pH heterogeneity. For a detailed introduction into our algorithms, see Supplementary Section S1.

We propose to consider the (digitized) pH curve as an effective pH distribution curve and to approximate it as a histogram in which each digital curve point $k$ corresponds to a histogram bin. From each curve/histogram, we derive the following basic pH parameters:

**Weighted mean pH:**

$$\bar{pH} = \frac{\sum_{k=1}^{m} (pH_k \times W_k)}{\sum_{k=1}^{m} W_k}$$  \hspace{1cm} \text{eq. (1)}$$

where $pH_k$ is the intracellular or extracellular pH value for a given digital point $k$ of the pH curve; $m$ is the total number of curve points used for calculation; and $W_k$ is the scaled weight (ordinate value) of curve point $k$. Scaled weights are weights that have been adjusted to account for the variability of pH intervals between curve points. The nonequidistant character of pH curve points results from the nonlinearity between the chemical-shift and pH scales. Thus, digital curve points that are equidistant on the chemical-shift axis are nonequidistant on the pH axis after point-by-point conversion. Weighted pH mean values directly derived from pH curves without rescaling would overemphasize pH regions represented by relatively “dense” curve points. Scaled weights are also needed for the calculation of the other pH parameters presented later. However, pH modes (curve maxima) have to be obtained directly from the pH curve, because this curve is a representation of the distribution of pH values.

**Weighted median pH:**

$$pH = pH_{k-1} + (pH_k - pH_{k-1}) \times f_{\text{int}}$$  \hspace{1cm} \text{eq. (2)}$$

where $pH_k$ is the pH value of curve point $k$ possessing the cumulative sum $\text{CSUM}(k)$; $pH_{k-1}$ is the pH value of curve point $(k - 1)$ possessing the cumulative sum $\text{CSUM}(k - 1)$; and $f_{\text{int}}$ is an interpolation factor defined as $f_{\text{int}} = (\text{CSUM}(m)/2 - \text{CSUM}(k - 1))/\text{CSUM}(k) - \text{CSUM}(k - 1)$]. Cumulative sums are calculated for scaled weights of curve points. Conventionally, the median is the numerical value separating the higher half of a sample from the lower half or the mean of the two middle values. For a series of weighted values, the location of the “weighted middle” has to be determined by interpolation. This is achieved by the interpolation factor $f_{\text{int}}$ that determines the location of the weighted middle between two adjacent curve points, $k$ and $(k - 1)$, corresponding to the cumulative sums (of scaled weights) that lie just above and below, respectively, the half-sum of the last point of the pH range used. $\text{CSUM}(m)/2$ is defined as the half-sum of a series of $m$ values.

**Skewness of pH distribution:**

$$G_1 = \frac{n}{(n-1)(n-2)} \sum_{k=1}^{m} W_k \left( \frac{pH_k - \bar{pH}}{s} \right)^3$$  \hspace{1cm} \text{eq. (3)}$$

where $s = \sqrt{\sum_{k=1}^{m} W_k (pH_k - \bar{pH})^2}/(n-1)$ is the nominal SD, a parameter analogous to the SD of the mean based on individual observations (= individual contributions to conventional histogram bins). In our algorithm, $n = \sum_{k=1}^{m} W_k$ is a parameter analogous to the total number of individual observations in conventional histograms. Generally, both skewness and kurtosis (see later) characterize the shape of a statistical frequency distribution, i.e., asymmetry and pointedness, respectively. Hence, the absolute value of $n$ is of no importance (as long as it is not too small) as these shape-related pH curve properties only depend on the relative weights of the individual pH curve values and on their deviation from a normal distribution. We verified by computer simulation that skewness and kurtosis asymptotically approach $n$-independent values for $n$ greater than several times the number of digital points $m$.

**Kurtosis of pH distribution:**

$$G_2 = \frac{n(n+1)}{(n-1)(n-2)(n-3)} \sum_{k=1}^{m} W_k \left( \frac{pH_k - \bar{pH}}{s} \right)^4 - \frac{3(n-1)^2}{(n-2)(n-3)}$$  \hspace{1cm} \text{eq. (4)}$$

with parameters being defined as presented above for skewness.
The proposed strategy is based on the circumstance that pH-sensitive magnetic resonance spectroscopy (MRS) signals from heterogeneous tissues represent entire pH distributions (pH profiles), rather than merely providing one "typical" pH, or pH spec.

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to appropriate pH values with HCl and NaOH solutions. Because tissue pH heterogeneity is by far more pronounced and prevalent than pH\textsubscript{c}, heterogeneity, we decided to focus our phantom experiments on 3-APP solutions. However, the validation obtained for 3-APP in this study can be generalized to P\textsubscript{c}, as pH sensitivities and relevant pK\textsubscript{c} values are only slightly different between these two compounds (Supplementary Section S1.1). First, three 245-mmol/L 3-APP solutions were prepared at pH 7.45, 7.00, and 6.50. These solutions were then serially diluted by a factor of 1.5, then 2, between subsequent 31P NMR measurements.

These solutions were transferred to 5-mm NMR tubes (528-PP) from Wilmad, cut to size (length ca. 4 cm) to suit the spatial conditions of the NMR probe described later (see also Supplementary Section S2.1). Up to three of these shortened NMR tubes were inserted into the cap of a shortened 50-ml centrifuge tube (Corning: Fisher Scientific) filled with saline. In addition, a 50 mmol/L 3-APP solution [free acid dissolved in deuterium oxide (D\textsubscript{2}O)] was prepared. This solution was then transferred to (i) a standard size 5-mm NMR tube (528-PP), and (ii) a glass sphere (1 cm diameter).

**Acquisition of 31P NMR spectra and proton images of tumors and phantoms**

Mouse tumors were measured in vivo as described previously (16). For phantom measurements, the spherical phantom, or the centrifuge tube cone filled with saline and with one to three NMR tubes containing 3-APP solution, was placed on the same one-turn 31P surface coil used for in vivo measurements, mounted in the same proton volume coil (16). The NMR spectrometer/imager used for both phantoms and tumors was a BIOSPEC 47/40 system (4.7 T; Bruker). 31P NMR spectra were acquired using a pulse-acquire sequence with proton decoupling. The chemical-shift values of the processed 31P NMR spectra of the 50 mmol/L 3-APP solution in 5-mm NMR tubes was conducted on an AVANCE 400 spectrometer (Bruker) at 9.4 T equipped with a quadrature probe (QNP) by way of a standard pulse-acquire sequence (18–20), with and without proton decoupling.

**Processing of tumor and phantom 31P NMR spectra**

31P NMR free induction decays (FID) were Fourier-transformed after zero-filling and multiplication with appropriate Lorentzian–Gaussian functions (XWINMR software; Bruker). The chemical-shift values of the processed 31P NMR spectra (21) were then converted to pH. After intensity corrections, the resulting datasets served as histograms (22) for the determination of weighted-average pH (mean pH), weighted median pH, skewness, kurtosis, and entropy (23–26). In addition, individual modes were determined. For pH distributions permitting the distinction of two or more characteristic pH ranges, the areas under the individual pH ranges or modes were quantified by two different methods: (i) integration, and (ii) curve fitting using the MDCON (mixed deconvolution) function in Bruker’s TopSpin software. Finally, these evaluation methods were applied to in vivo 31P NMR signals of 3-APP in mouse tumor xenografts (16). High-resolution 31P NMR spectra of 3-APP solutions were processed using Bruker’s TopSpin software. Further NMR processing details, as well as the theoretical background and the algorithms used for the calculation of pH heterogeneity parameters are presented in Supplementary Sections S1 and S2. The EXCEL spreadsheet pH_param_template.xlsx provided by us is, in fact, a computer program; it serves both as an example of our calculations and as a template for use by interested researchers. This EXCEL file can be downloaded using the URL address: http://crmbm.univ-amu.fr/homepage/nlutz/pH_param_template.xlsx. An option to use the results of lineshape deconvolution for better definition of P\textsubscript{c} lines is included in the algorithms implemented in this template.

**In silico calculations**

The purpose of our in silico calculations is 4-fold: (i) to test, based on well-defined Gaussian pH distributions, the validity of our algorithms; (ii) to explore the effects of ppm-to-pH conversion on the symmetric shapes of two distinct Gaussian 3-APP 31P NMR spectral lines; (iii) to study the statistical parameters characterizing the overall pH distributions resulting from the addition, in varying proportions, of the two pH curves generated in (ii); and (iv) to compare simulated, phantom, and in vivo pH heterogeneity parameter values.

As a consequence of the nonlinearity of the ppm-to-pH conversion, a pH lineshape may significantly deviate from its underlying chemical-shift lineshape. We generated two Gaussian curves with a chemical-shift abscissa scaled such that, following ppm-to-pH conversion, the centers of the curves fell upon pH 6.5 and 7.2. These values as well as the associated linewidths were chosen to be close to values commonly found for pH\textsubscript{c} in in vivo experiments. The resulting (asymmetric) pH curves were then used to characterize these simulated pH distributions by means of statistical pH distribution parameters. Conversely, we generated in an EXCEL spreadsheet Gaussian pH curves centered about pH 6.5 and 7.2. On the basis of these curves, we backward simulated the corresponding 3-APP line, and studied the asymmetry effects that (symmetric) Gaussian pH distribution parameters would undergo after conversion to simulated 3-APP resonances. Finally, two computer-generated Gaussian curves were added to model bimodal (27) pH distributions for statistical analysis.

**Results**

**Generation of bimodal pH profiles corrected for the nonlinearity between the chemical-shift and pH scales**

The effects of converting the ppm scale of the 3-APP spectrum to a pH scale were first studied for a bimodal pH distribution from a mouse tumor xenograft (Fig. 1A). Most heterogeneous tumors feature irregularly shaped pH distributions rather than strictly bimodal or multimodal patterns (16). However, to validate our method, we primarily focused on bimodal and trimodal pH distributions, because the concept of quantitative pH heterogeneity parameters is best tested and verified on the basis of models representing well-defined pH
distribution functions. Nevertheless, nearly all quantitative parameters suggested in this report are universally applicable to any given distribution of pH values; their use does not depend on the existence of distinct pH modes (see also following paragraph). Our results were first validated by using a phantom consisting of two NMR tubes filled with 3-APP solutions adjusted to pH 6.5 and 7.4 (Fig. 1B and C). Uncorrected ppm-to-pH conversion (Supplementary Eq. S6, with intensity $I_{3-APP}$ as in underlying NMR spectrum) renders the upper part of each pH mode narrower (Fig. 1, bottom row, dotted lines) than it is in the corresponding $^{31}$P NMR spectrum. Middle row, spectra processed with Lorentzian–Gaussian lineshape transformation at GB = 0.01 and LB = −20 Hz (A), or at GB = 0.007 and LB = −25 Hz (B); or with apodization at LB = −15 Hz (C). Bottom row, pH profiles derived from the spectra displayed in the top row. Dotted lines, uncorrected pH curves. Solid lines, pH curves with intensities corrected for the nonlinear relationship between chemical-shift and pH scales. In both tumor tissue and phantom, pH profiles exhibit a bimodal pH distribution pattern, with modes being centered about approximately pH 6.5 (left curve maxima, $pH_{e2}$) and 7.4 (right curve maxima, $pH_{e1}$).

Figure 1. Examples of 3-APP $^{31}$P NMR spectra revealing pH heterogeneity in tissue and in a phantom. A, CCL39 tumor xenograft in a mouse model. B and C, phantom consisting of two NMR tubes filled with 3-APP solutions at pH 6.5 and 7.4. Top row, representative MRI cross-section through phantom or mouse tumor. Shaded areas in image indicate regions saturated by OVS during subsequently conducted localized $^{31}$P NMR spectroscopy. Middle row, spectra processed with Lorentzian–Gaussian lineshape transformation at GB = 0.01 and LB = −20 Hz (A), or at GB = 0.007 and LB = −25 Hz (B); or with apodization at LB = −15 Hz (C). Bottom row, pH profiles derived from the spectra displayed in the top row. Dotted lines, uncorrected pH curves. Solid lines, pH curves with intensities corrected for the nonlinear relationship between chemical-shift and pH scales. In both tumor tissue and phantom, pH profiles exhibit a bimodal pH distribution pattern, with modes being centered about approximately pH 6.5 (left curve maxima, $pH_{e2}$) and 7.4 (right curve maxima, $pH_{e1}$).
(LB) = –25 Hz] were needed for phantom spectra than for tissue spectra (typically GB = 0.01, LB = –20 Hz) because the magnetic-field inhomogeneity is intrinsically lower in phantoms than in tissue. For phantom-derived and computer-simulated pH distributions, the “e” (or “i”) index indicates that the pH modes in question are linked to the chemical-shift of 3-APP (or P_l) signals, and have been chosen with the intention to compare these modes with similar extracellular (or intracellular) pH distribution modes detected in the tumors of 3-APP–injected animals.

31P NMR spectra yield up to eight quantitative pH distribution parameters

It is highly desirable to provide quantitative parameters to characterize tissue heterogeneity with respect to pH. The number of quantitative parameters that can be obtained from a 31P NMR spectroscopy–based analysis of pH heterogeneity is a function of the pH curve shape. The following six parameters can be extracted from virtually any pH curve: (i) pH_{max}, the global maximum of the pH curve (classical 31P NMR method); (ii) pH_e, the weighted-average (mean) pH; (iii) pH_i, the weighted median pH; (iv) skewness; (v) kurtosis; and (vi) entropy (the underlying theory and algorithms are explained in great detail in Supplementary Section S1). For a pH profile that suggests the presence of multiple distinct pH ranges, a characteristic pH value can be obtained for each of these ranges by using methods (i) to (iii). The relative weight of each of these pH ranges can be calculated by (vii) separately integrating the area under the curve for each individual pH range, followed by calculating ratios of these areas. For multimodal pH profiles that are amenable to numerical fitting of analytic curves, (viii) pH values for multiple maxima or modes (pH_{max}, pH_e, etc.), and (ix) areas under individual fitted modes can be obtained as results of the fitting procedure. Alternatively, multiple maxima corresponding to method (viii) can be directly read out by (x) visual inspection or, for more precision, (xi) a software module based on interpolation (such as Bruker’s peak picking routine), whereas the areas for these pH modes can be integrated as described earlier under (vii).

In the interpretation of pH curves derived from 31P NMR spectra, spectral line broadening due to magnetic-field inhomogeneity, spectral processing (filtering) and phosphorus-proton J coupling (for 3-APP), and, to a much lesser extent, T_2 processes should be taken into account. In fact, the standard error and width of a 31P NMR–derived pH curve, or of an individual mode within a pH curve, would somewhat overstate the pH range actually present in the sample; for this reason, they are not included in the list of parameters above. Obviously, kurtosis, a measure of the peakedness of a distribution, represents the true pH distribution function more faithfully to the extent that the influence of line shape effects unrelated to pH can be minimized as described in Supplementary Sections S1.2.1 and S1.2.11.

Quantification of pH heterogeneity by statistical parameters was studied on the basis of pH distributions in vivo (mouse tumor models), in vitro (phantom models), and in silico (computer models). In vivo and in vitro results are presented in the following paragraphs, whereas findings of computer simulations are provided in Supplementary Tables S2 and S3 and in Supplementary Figs. S2E and S2F and S4.

Quantification of unimodal pH distributions

We first tested our algorithms for a unimodal pH distribution in a phantom containing a 3-APP solution at pH 7.00. Owing to the perfect pH homogeneity in this sample, the resulting pH distribution curve was very symmetric (Fig. 2A). As a consequence, weighted mean (pH_e), weighted median (pH_i), and mode (pH_{max}) had identical values (Table 1A); compare also with computer-simulated results presented in Supplementary Table S2. The small values obtained for skewness (G1) and kurtosis (G2) in this example suggest a nearly Gaussian pH curve. In fact, the 31P NMR lineshape obtained from a homogeneous 3-APP solution after strong Gaussian filtering has a considerable Gaussian character, and its symmetry is largely preserved in ppm-to-pH conversion if pH approximates the pK_a of 3-APP. Also, the entropy (H) of this pH distribution, indicating its smoothness, was smaller for this example than for any other example studied in this study. The unimodal pH_e distribution in a relatively homogeneous mouse tumor (Fig. 2E) was more asymmetric than the pH distribution in Fig. 2A, the left tail (low pH_e) being heavier than the right tail (high pH_e). Therefore, pH_e < pH_i < pH_{max} and the pH_e distribution showed a negative skew (G1; Table 1E). Although this pH_e distribution was more leptokurtic (increased G2) than that of Table 1A, it also had a higher entropy reflecting a more even distribution. In tumors, the intracellular pH is generally more homogeneous than the extracellular pH (16); pH_i values are distributed over a considerably smaller range than pH_e values. Bi- or multimodal pH_i distributions are rare (Fig. 3A), whereas most tumors exhibit more or less asymmetric unimodal pH_e distributions (Fig. 3B), occasionally presenting a shoulder (Fig. 3C and D). The narrowest unimodal pH_e distribution (Fig. 3B) was the most leptokurtic and the least smooth pH_e distribution (Table 2B). Owing to its high asymmetry reflected by its skewness, the differences between pH_i, pH_e, and pH_{max} were rather large. In summary, all six quantitative parameters applicable to unimodal distributions describe the behavior of the underlying phantom and tissue samples very well, for both pH_e and pH_i.

The number of distinct pH environments in a sample can be modeled by phantom 31P NMR experiments

In addition to the universally accessible parameters described in the preceding paragraph, some 31P NMR–based pH profiles reveal a finite number of distinct pH environments. Note that no assumptions are made with respect to the size and spatial distribution of the underlying tissue volume elements. The capability of 3-APP 31P NMR spectra to reveal multimodal pH heterogeneity was tested by studying phantoms that contained one to three compartments filled with 3-APP solutions of varying pH. As a starting point, a nondecoupled high-resolution 31P NMR spectrum of an 3-APP solution was obtained (Fig. 4A). Then, a 3-APP solution contained in a spherical phantom was measured in a small-animal NMR spectrometer/imager. Under these conditions, the 31P NMR
peaks were broadened such that only five broad 3-APP peaks could be distinguished (Fig. 4B). Subsequently, a glass sphere phantom similar to the phantom used for Fig. 4B (with pH adjusted to pH 7.0) was imaged (Fig. 4C). The quality of the 3-APP spectrum from the selected volume presented in Fig. 4C was comparable with the quality of the spectrum shown

![Figure 2](image)

Figure 2. Series of pH profiles derived from 3-APP $^{31}$P NMR resonances of phantoms and mouse tumors for varying numbers of characteristic pH$_e$ values. A–D, typical shapes of pH profiles of 3-APP phantoms with one to three characteristic pH$_e$ values. E–H, typical shapes of pH$_e$ profiles from 3-APP–injected mouse tumors with one to three characteristic pH$_e$ values. Vertical dashed lines in pH curves indicate the borderline between distinct pH regions within each pH profile used for the integration of separate areas for the pH$_{h1}$, pH$_{h2}$, and pH$_{h3}$ regions. I–L, representative MRI cross-sections of the tumors presenting the pH profiles given in E–H. Shaded areas in images indicate regions saturated by OVS during subsequently conducted localized $^{31}$P NMR spectroscopy.

| Table 1. Parameters characterizing pH$_e$ heterogeneity, as obtained for well-defined test samples (A–D) and tumors (E–H), presented in Fig. 2A–D and E–H, respectively |
|---|---|---|---|---|---|---|---|
| A | B | C | D | E | F | G | H |
| Weighted average (mean), pH$_e$ | 7.02 | 7.15 | 7.19 | 7.03 | 6.76 | 7.06 | 6.71 | 6.95 |
| Weighted median, pH$_e$ | 7.02 | 7.32 | 7.30 | 6.99 | 6.79 | 7.03 | 6.61 | 6.89 |
| Mode, pH$_{h1}$ | 7.02 | 7.48 | 7.38 | 7.42 | — | 7.29 | 7.18 | 7.6$^a$ |
| Mode, pH$_{h2}$ | — | 6.59 | 6.56 | 6.64 | 6.83 | 6.72 | 6.48 | 6.72 |
| Mode, pH$_{h3}$ | — | — | — | 6.99 | — | — | — | 7.20 |
| Area ratios pH$_{h2}$ vs. pH$_{h1}$ (integrated) | — | 0.75 | 0.38 | 1.06 | — | 0.97 | 1.85 | 7.42 |
| Area ratios pH$_{h2}$ vs. pH$_{h3}$ (deconvolved) | — | 0.72 | 0.37 | 1.05 | — | 0.99 | 1.98 | 4.02$^a$ |
| Skewness, G1 | 0.15 | 0.01 | −0.44 | 0.41 | −0.57 | 0.39 | 0.34 | 0.58 |
| Kurtosis, G2 | 0.13 | −0.78 | −0.22 | 0.33 | 0.49 | −0.19 | −0.38 | −0.38 |
| Entropy, H | 3.95 | 4.81 | 5.00 | 5.09 | 5.86 | 5.05 | 6.14 | 4.66 |

$^a$Estimated values; five modes were needed for reasonable fit; fitted areas were combined for comparison with integrated areas.
in Fig. 4B. However, major filtering had been applied to increase the final linewidth of the pH curve to a value close to what is achievable in vivo, such that the multiplet was no longer resolved (Fig. 4F). Next, phantoms containing two and three NMR tubes were imaged (Fig. 4D and E, respectively). These tubes were immersed in saline, and contained 3-APP solutions at about pH 6.50 and 7.45 (Fig. 4D), and at about pH 6.50, 7.00, and 7.45 (Fig. 4E). All 3-APP concentrations were kept at roughly the same order of magnitude to prevent the bases of larger peaks from hiding considerably smaller peaks. The corresponding spectra exhibited two (Fig. 4G) and three (Fig. 4H) components (modes), respectively. This measurement series shows that under ideal conditions, up to three distinct regions of similar size can be identified on the basis of differing pH values, for a pH range close to physiologic values. See Supplementary Section S1.2.8 for further details.

**The sensitivity of in vivo $^{31}$P NMR spectroscopy in identifying multiple distinct pH environments can be modeled by phantom experiments**

The ability of 3-APP $^{31}$P NMR spectra to identify distinct sample regions by their pH values not only depends on the number of such regions and on the pH differences between these regions, but also on the relative intensities of the pH modes associated with these regions. We mimicked variations in relative volumes within a tissue region by varying the 3-APP concentrations.
Table 2. Parameters characterizing pH, heterogeneity in tumors

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<th>C</th>
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<td>—</td>
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<td>Area ratios (deconvolved) $pH_{g2}$ vs. $pH_{g1}$</td>
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$a$Estimated values. Data (A–D) are based on $^{31}$P NMR signals of P$_i$ obtained from tumors presented in Fig. 3A–D.

concentration in one of the two NMR tubes of the phantom used. The two pH values chosen were about pH 6.50 and 7.45. The acidic solution was diluted to approximately half the concentration of the alkaline solution. Then, serial dilutions were prepared for the acidic solution as described in the Materials and Methods, and a $^{31}$P NMR spectrum was acquired after each dilution step. The qualitative results of these measurements are presented here, whereas the quantitative results after each dilution step. The qualitative results of these measurements are presented here, whereas the quantitative results after each dilution step.

Quantification of bimodal and multimodal pH distributions

Two basic forms of bimodal pH distributions were studied in phantoms and mouse tumors. In the first type, both modes resulted in maxima of similar heights (Fig. 2B and F); in the second type, one mode was significantly more pronounced than the other (Fig. 2C and G). Although both modes in Fig. 2B were of comparable height, the distribution pattern was not symmetric as the areas under the two peaks were different; the pH$_{mode}$ area was roughly three quarters of the pH$_{g1}$ area (Table 1B). Almost identical area ratios were measured by two different methods: (i) integration based on the weights of digital curve points, and (ii) deconvolution based on fitted Gaussian/ Lorentzian functions (29).

The pH distribution asymmetry for the phantom described in Table 1B leads to a difference of 0.17 pH units between pH$_c$ and pH$_e$. The pH$_{mode}$ and pH$_{g2}$ modes were well separated because of the large difference of approximately 0.9 pH units (Fig. 2B). In contrast, the two modes in the tumor pH$_c$ distribution (Fig. 2F) exhibited more overlap as their pH difference was only approximately 0.6 pH units (Table 1F). Because in the latter example, the areas under the two modes were almost identical (ratio close to unity), the pH$_c$ distribution was nearly symmetrical and, as a consequence, the difference between pH$_e$ and pH$_c$ was virtually negligible (Table 1F). For the examples plotted in Fig. 2C and G, the overlapping modes were characterized by similar area ratios; pH$_{mode}$/pH$_{g1}$ ≈ 0.4 for the former (Table 1C), and pH$_{mode}$/pH$_{g2}$ ≈ 0.5 for the latter (Table 1G). As was to be expected from these asymmetries, pH$_{mode}$ was greater than pH$_{g1}$ (Table 1C), and smaller than pH$_{g2}$ (Table 1G), respectively, by the same amount (~0.1 pH units). The superimpositions of the two pH curve modes were comparable because the differences between pH$_{mode}$ and pH$_{g2}$ were similar (~0.8 and 0.7 pH units, respectively). Although these relative area ratios represent relative tissue volumes, pH heterogeneity should not be confused with morphologic heterogeneity. An example of the latter is illustrated in in vivo images obtained by a T$_1$-weighted MRI sequence, for all tumors whose pH profiles are presented in this report (Fig. 5). It is obvious from these cross-sections that the glycolysis-deficient variant, CCL39/gly$^{-}$ (Fig. 5A and B), showed less morphologic heterogeneity, notably less necrosis, than most wild-type tumors, CCL39 (Fig. 5C–F). Nonetheless, even relatively homogeneous appearing tumors may produce clearly heterogeneous pH profiles (Figs. 2G and 3A, corresponding to Fig. 5G and B, respectively).

The concepts of skewness, kurtosis, and entropy are mostly used to characterize unimodal probability distributions (30). However, they can also reveal global characteristics of pH profiles with more than one mode. For instance, the nearly symmetric modes displayed in Fig. 2B result in vanishing skewness (Fig. 2B), whereas the strong asymmetries indicated by modes of unequal peak heights (Fig. 2C and G) resulted in negative and positive $G_1$ values as shown in Table 1C and G, respectively. Two examples of trimodal pH$_c$ distributions were analyzed. The three modes from our phantom could be quantified relatively easily (Fig. 2D). However, in vivo situations amenable to precise quantification of three separate modes are rare; we present here an example (Fig. 2H) in which one mode (pH$_{mode}$) appears as a shoulder on another mode (pH$_{g3}$). Further details about the evaluation of bi- and multimodal pH...
distributions are given in Supplementary Section S1.2.10. A flowchart displaying all basic steps required for our pH parameter calculations is given in Supplementary Fig. S6.

Discussion

This report describes the first method providing quantitative heterogeneity parameters that characterize the statistical distribution of tissue pH values. The approach presented here can be extended to further statistical parameters describing the shape of pH distributions (pH profiles). In fact, besides skewness, kurtosis, and entropy, statistics provides a number of parameters that characterize distribution functions, each one presenting specific advantages and disadvantages (31). An original characteristic of our approach is that it provides multiple quantitative parameters describing global features of pH heterogeneity within a selected tissue volume. These parameters describe details about the exact shape of pH distributions. None of the current methods designed to assess spatial pH differences in tissues in vivo provides such parameters. For instance, fluorescence imaging microscopy using a pH-sensitive fluorophore (32) is able to detect pH variations with submillimeter resolution (1, 33), but as an optical method this approach is restricted to tissue surfaces. Proton-based NMR methods such as chemical-shift imaging (CSI) of exogenous pH markers are well established (34, 35); however, their applications have essentially been limited to distinguishing pH values in a viable tumor rim from those of tumor tissue close to necrosis in two-dimensional pH charts. More recent pH mapping techniques based on pH-sensitive relaxation (36), chemical exchange saturation transfer (CEST;
ref. 37), or hyperpolarized $^{13}$C CSI (38) offer somewhat better spatial resolution than $^1$H CSI. However, besides being rather intricate, requiring special pulse sequences, and/or lacking validated robustness, these techniques do not offer quantitative parameters characterizing the distribution of pH values. They may thus be considered complementary to the $^{31}$P NMR method presented here. Our technique results in pH profiles indistinguishably taking into account both macroscopic and microscopic pH heterogeneity; therefore, the detection of pH heterogeneity is not limited to differences between tissue regions above a critical size. In contrast, magnetic resonance images are voxel-based, and for this reason pH differences existing within a voxel (i.e., between tissue regions smaller than a voxel) are averaged and cannot be represented by image-based pH maps.

The method presented here allows simultaneous noninvasive characterization of complex in vivo pH$_{t}$ and pH$_{e}$ distributions. It opens important perspectives in many areas of biomedical research, either in animal models or in humans. Both intra- and extracellular pH heterogeneity can be determined simultaneously in animal models, based on the $^{31}$P NMR resonance of endogenous P$_i$, and exogenous 3-APP, respectively. As for other measured biologic quantities, the pH parameters suggested here need to be evaluated in comparisons between groups of animals or humans to determine their respective roles in biologic research. Although at the present time human applications are limited to intracellular pH analysis (based on the P$_i$ signal), future development of extracellular phosphonated pH markers that are safe for human use should lead to an extension of this method to patient studies. In addition, the pH profiles presented in this report can be obtained with spatial resolution in cases in which the use of appropriate $^{31}$P NMR sequences (e.g., CSI) is feasible. Finally, metabolic events frequently coupled with pH changes [notably hypoxia-induced energy (39) and phospholipid metabolism (40)] can be analyzed from the very same $^{31}$P NMR spectrum obtained for pH analysis, and can be directly correlated with pH heterogeneity to analyze the metabolic

![Figure 5. MRI cross-sections for mouse tumor xenografts.](image-url)
underpinnings of variations in the pH parameters introduced here. In addition, the $^{31}$P NMR measurement can be combined seamlessly with other in vivo experiments in the same examination for further research into the physiopathologic basis of pH heterogeneity, including both in vivo and in vitro metabolomics (41). For instance, the total pH heterogeneity determined by our new method (i.e., macroscopic and microscopic pH heterogeneity combined) may be used in conjunction with diffusion-weighted MRI results and spatially resolved pH maps to clarify to what extent structures underlying macroscopic diffusion tensor anisotropy (42) and pH variations explain the total pH heterogeneity found in tumors. Histologic microscopy and other ex vivo microscopic methods may reveal microscopic structures potentially contributing to the total tumor pH heterogeneity quantifiable by the method presented here.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Correction: Quantitative In Vivo Characterization of Intracellular and Extracellular pH Profiles in Heterogeneous Tumors: A Novel Method Enabling Multiparametric pH Analysis

In this article (Cancer Res 2013;73:4616–28), which was published in the August 1, 2013 issue of Cancer Research (1), Fig. 4 was incorrectly labeled because of a production error. The online version has been corrected and no longer matches the print version.

Reference


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