On the correlation between the water diffusion coefficient and oxygen tension in RIF-1 tumors†

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ABSTRACT: Water diffusion-coefficient mapping was used in conjunction with 19 F inversion-recovery echo-planar imaging (IR-EPI) of a sequestered perfluorocarbon (PFC) emulsion to investigate the spatial correlation between the diffusion coefficient of water and the tissue oxygen tension (pO2) in radiation-induced fibrosarcoma (RIF-1) tumors (n = 11). The diffusion-time-dependent apparent diffusion coefficient, D(t), was determined by acquiring diffusion coefficient maps at 20 different diffusion times. Maps at four representative time points in different regions of the D(t) curve were selected for final analysis. An intravenously administered PFC emulsion, perfluoro-15-crown-5-ether, was used to generate the pO2 maps. D(t) and pO2 data were acquired with the animal breathing either air or carbogen (95% O2 –5 % CO2) to investigate the effects of increased tumor pO2 on D(t). The average increase in tumor pO2 was 22 torr when the breathing gas was changed from air to carbogen. Correlation plots generated from pixel data for D(t) (air breathing) vs D(t) (carbogen breathing) show little deviation from a slope of unity. Correlation plots of D(t) vs pO2 indicate that no correlation is present between these two parameters. This study also confirmed that necrotic tissue was best differentiated from viable tumor tissue based on D(t) maps at long diffusion times. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: oxygen tension mapping; F-19; water diffusion coefficient; RIF-1 tumor

INTRODUCTION

The assessment of tissue oxygen tension (pO2) is an important component in the determination of radiotherapeutic and chemotherapeutic efficacy.1,2 The experimental determination of pO2, however, is a difficult and often invasive procedure involving either electrodes or implanted EPR probes2–4 or exogenously administered compounds.5–8 It would therefore be advantageous to have a non-invasive and more easily measured indicator of the oxygen distribution in tumor tissue. To this end, Dunn et al.9 recently showed that the apparent diffusion coefficient (ADC) of water in chronically hypoxic tumor tissue is directly related to tumor pO2. The existence of a relationship between water ADC and tumor oxygenation would be valuable for differentiating the oxygen status of viable, hypoxic, and necrotic tissue as well as monitoring therapy.

In the initial study by Dunn et al.,9 calculated ADC maps were produced for each of seven RIF-1 tumors at a single diffusion time (15 ms). Oxygen tension measurements were obtained at two locations within each tumor (using EPR of implanted lithium phthalocyanine crystals), corresponding to the positions of the highest and lowest values in the calculated ADC map. The correlation between ADC and pO2 showed a positive trend, i.e., large values of ADC corresponded to large values of pO2. However, the authors noted that such a correlation is restricted to areas where the tumor tissue was chronically hypoxic, but where there was no significant necrosis. The authors hypothesize that such an environment gives rise to impaired osmotic regulation in these cells with ensuing cellular swelling and a concomitant reduction in ADC.

The basis for this hypothesis is similar to that for brain tissue, where cytotoxic edema is thought to be responsible for the decline in water ADC following an acute ischemic insult.10,11

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†Abbreviations used: ADC, apparent diffusion coefficient; D0, the constant value of the ADC measured at long diffusion times; D(t), diffusion-time dependent apparent diffusion coefficient; D(t), diffusion-time dependent apparent diffusion coefficient with the animal breathing air; D(t), diffusion-time dependent apparent diffusion coefficient with the animal breathing carbogen; H and E, Hematoxylin and Eosin; IR-EPI, Inversion Recovery Echo Planar Imaging; M0, proton spin density; PFC, perfluorocarbon; pO2, tissue oxygen tension; pO2, tissue oxygen tension with the animal breathing air; pO2, tissue oxygen tension with the animal breathing carbogen; RIF, radiation induced fibrosarcoma; S/V, ratio of surface area to volume; T, tortuosity.

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In order to fully assess the potential of this method, we have investigated the relationship between water ADC and tumor pO\textsubscript{2} under a wider range of experimental conditions than was employed in the above study. Calculated ADC maps from RIF-1 tumors were compared, on a pixel-by-pixel basis, with tumor pO\textsubscript{2} maps that were obtained from the same location using \textsuperscript{19}F inversion-recovery echo-planar imaging (IR-EPI) of a sequestered perfluorocarbon (PFC) emulsion. This approach ensures that the relationship between ADC and pO\textsubscript{2} can be investigated for the full spectrum of viable, hypoxic and necrotic tumor tissue and will allow us to characterize any limitations that are associated with this method.

The investigation of the relationship between ADC and pO\textsubscript{2} must also take into account the dependence of the water ADC on diffusion time in tumor tissue. In the present work, \(D(t)\) is used to denote the ADC measured at a specific diffusion time, \(t\), while ADC is used to denote the apparent diffusion coefficient without regard to the diffusion time. In all cases the ADC was measured by varying the applied field gradient only. A recent study using RIF-1 tumors\textsuperscript{12} has established that the behavior of \(D(t)\) as \(t\) is changed is dependent upon tumor tissue type. For example, \(D(t)\) values for necrotic tumor tissue are generally large and show little change with diffusion-time, whereas \(D(t)\) values for viable and hypoxic tissue can vary considerably with diffusion time and are generally lower than those for necrotic tissue. Given the potentially confounding effects of time-dependent diffusion on ADC correlations derived using this approach, the effect of the time-dependence of ADC on the correlation between tumor pO\textsubscript{2} and ADC was also investigated.

Finally, in order to relate changes in ADC values with changes in tumor oxygenation, time-dependent ADC maps and pO\textsubscript{2} maps were compared for animals breathing either air or carbogen (95% O\textsubscript{2}-5% CO\textsubscript{2}). Breathing carbogen is known to increase the radiosensitivity of hypoxic cells in murine tumors\textsuperscript{13,14} by increasing respiration and cardiac output and, therefore, oxygen delivery.\textsuperscript{15} The pO\textsubscript{2} mapping technique used in these studies has been shown to be sensitive to changes in tumor pO\textsubscript{2} following carbogen breathing\textsuperscript{7} and hence provides a basis for identifying regions of the tumor where corresponding changes in ADC might also be expected.

**BACKGROUND**

NMR diffusion measurements in fluid-filled porous media can provide useful structural information about the sample. The diffusion coefficient of the fluid in the interstitial space varies as a function of the diffusion time because of the interaction of the diffusing molecules with restricting boundaries in the medium. At short diffusion times, only molecules at boundary surfaces are restricted and the value of \(D(t)\), the time-dependent apparent diffusion coefficient, is reduced from \(D_0\) (the bulk diffusion coefficient of the fluid) in direct proportion to the volume of the surface layer of restricted molecules. In this regime, the slope of a plot of \(D(t)\) vs \(t^{1/2}\) is proportional to the ratio of the surface area to pore volume, \(S/V\).\textsuperscript{16,17} A ‘local’ property of the medium (\(D(t)/D_0\)) \((S/V)\sqrt[D_0]{D(t)}/D_0\)) at long diffusion times, \(D(t)\) reaches a constant, diffusion-time-independent value, \(D_{\text{eff}}\), where each molecule has effectively experienced an equivalent portion of the confining medium. In this case, \(D_{\text{eff}}\) is reduced from \(D_0\) in proportion to the tortuosity,\textsuperscript{18-22} \(T\), (i.e. \(D_{\text{eff}} = D_0/T\)), of the connective pathways between pore spaces. Earlier work\textsuperscript{12} has found that using long diffusion times, such that the diffusing water molecules are in the tortuosity regime, is useful for differentiating necrotic from viable tumor tissue. This is the case since the measured ADC is reflecting the effects of restriction on a ‘global’ rather than ‘local’ scale. For a more complete discussion of the application of porous-media theory to biological systems see Ref. 12.

Fluorine-19 NMR spectroscopy and imaging of PFC emulsions have been used extensively to measure tissue oxygenation.\textsuperscript{5-8} The spin–lattice relaxation rate, \(R_1\) \((1/T_2)\), of PFCs is linearly related to the dissolved oxygen concentration\textsuperscript{23,24} and thus allow oxygenation measurements to be performed in any tissue or organ in which the PFC is sequestered. In the case of solid murine tumors, fenestrations in the tumor vasculature allow the intravenously administered PFC emulsions particles to leak into the extravascular space and serve as a non-invasive oxygen probes. Although a number of different PFC molecules have been used for measuring tumor oxygenation,\textsuperscript{5,6,8} more recent candidates, such as perfluoro-15-crown-5-ether,\textsuperscript{7} mitigate most of the problems associated with \textsuperscript{19}F MRI of PFCs. In particular, perfluoro-15-crown-5-ether has a relatively long \(T_2\)-relaxation time compared with previously used PFCs and, since it contains 20 identical fluorine atoms, it has a single resonance which eliminates chemical shift artifacts and J-modulation effects. Fluorine-19 echo-planar imaging (EPI) of perfluoro-15-crown-5-ether permits rapid and sensitive measurements of tumor pO\textsubscript{2}. The methods used in this study have been discussed extensively in Ref. 7.

**MATERIALS AND METHODS**

RIF-1 tumor cells were prepared according to Twentyman et al.\textsuperscript{25} Cells were injected subcutaneously in the lower back of C3H mice \((n = 11)\) and allowed to grow to varying volumes \((0.4 \text{ cc} \text{ to } 1.2 \text{ cc})\). Tumor volumes were determined by using the relation: \(V = (\pi/6)(a \times b \times c)\) where \(a\), \(b\), and \(c\) are the tumor length, width, and height, respectively. When the tumor had reached the desired volume, the tumor-bearing mice were administered a 15
g/kg dose of a 40% (v/v) emulsion of perfluoro-15-crown-5-ether (perfluoro-1, 4, 7, 10, 13-pentaaxacyclopentadecane) (HemaGen/PFC, St Louis, MO) via tail vein injection. Imaging experiments were performed 3–7 days following PFC injection to ensure clearance from the vasculature. Animals were anesthetized during imaging with 1.5% isoflurane delivered in air at 1 L/min. Circulating air at 34°C was used to maintain the animal’s body temperature at 37°C.

MRI data was acquired using a horizontal bore GE CSI-II 2.0 T/45 cm imaging spectrometer (GE NMR Instruments, Fremont, CA, USA) operating at 80.5 MHz for 19F and 85.5 MHz for 1H and equipped with ±20 G/cm self-shielded gradients. A four-turn, 15-mm diameter solenoidal coil was used for all experiments. Maps of the apparent diffusion coefficient were generated for 20 different diffusion times (from 11.0 ms to 560.5 ms) to delineate the delineate the vasculature. Twenty diffusion-weighted images were acquired for each of the Stejskal–Tanner sequence 26 (K. G. HELMER ET AL. 1998 John Wiley & Sons, Ltd. NMR IN BIOMEDICINE, VOL. 11, 120–130 (1998)) and viable tissue volumes.

Equations

\[
D(t) = k (D(t), t), \quad k = 2/\pi, \quad M = \text{measured signal intensity}, \quad M_0 = \text{signal intensity without an applied diffusion gradient} \]  

(see Ref. 29 for more details). The factor of 2/\pi in the expression for k takes into account the use of half-sine-shaped diffusion-sensitizing gradient pulses. Each image was obtained using either a stimulated-echo variant 27 or a stimulated-echo variant 27 (t_\text{申} = 110–570 ms) or a stimulated-echo variant 27 (t_\text{申} = 85.5–560.5 ms), both employing EPI with a sawtooth data acquisition scheme. 28 Echo times were the same (100 ms) for both sequences to ensure equal T2-relaxation weighting. Diffusion gradients were incremented in 0.6 G/cm steps from 0.6 to 12.0 G/cm for diffusion times less than 100.0 ms. In order to keep the amount of attenuation constant, the initial and incremental gradient values were decreased for diffusion times greater than 100.0 ms. The gradient pulse width, \(\delta\), was 10.0 ms. Coronal EPIs were acquired with FOV = 30 × 30 mm, slice thickness = 3.0 mm, TR = 2 s, NEX = 2 (spin echo) or 4 (stimulated echo), and TE = 100 ms. The EPI data acquisition time was 65.5 ms, the spectral width was ±30 000 Hz, and the digital resolution was 64 × 64 data points. Images were acquired such that the center of the imaging slice coincided with the center of the tumor. Hematoxylin and eosin (H and E) staining of the tumor was performed to identify necrotic regions. Several histological slices were taken from within the imaging slice to check for local differences in necrotic and viable tissue volumes.

\[
\text{In vitro standard curves of } R_1 (\equiv 1/T_1) \text{ vs } \% \text{O}_2 \text{ for the neat perfluoro-15-crown-5-ether were obtained for four different standard gases: 0, 5, 21 and 30 } \% \text{O}_2 \text{ (the balance being N}_2\text{) and four temperatures, T: 27, 32, 37 and 42°C. The gas was bubbled into the PFC for 30 min at the required } \% \text{O}_2 \text{ and a spectroscopic measurement of } T_1 \text{ was made at each of the above four temperatures. Multiple-linear regression was then performed on the data to extract the equation for } \text{pO}_2 \text{ as a function of } R_1 \text{ and T.}
\]

To generate R1 maps from the RIF-1 tumors, 19F images of the sequestered PFC were acquired using slice-selective inversion-recovery echo-planar imaging (IR-EPI) (180°–TI–90°–TE/2–180°–TE/2). Imaging parameters include: FOV = 30 × 30 mm, slice thickness = 3.0 mm, pre-delay = 10 s, acquisition bandwidth of ±70,000 Hz, EPI data acquisition time of 28.6 ms, TE = 70 ms, NEX = 8, pixel resolution of 64 × 64, and seven inversion times of 0.08, 0.20, 0.50, 1.00, 2.00, 4.00 and 8.00 s. The same inversion times and sequence parameters were used for both the calculation and in vivo experiments. Note that the same slice thickness and slice position was used for both the diffusion and R1 maps.

R1 maps were calculated, on a pixel-by-pixel basis, from the 19F IR-EPIs using a Levenberg–Marquardt nonlinear least-squares fitting method. 29 Pixel intensity, \(S(TI)\), was fitted to the equation, \(S(T) = A[1 + B \times \exp(-TI \times R_1)]\), where \(TI\) is the inversion time and A and B are fitting constants. Each R1 map was filtered by: (1) using a diffusion map of the same tumor as a mask in order to fit only those pixels originating from the tumor itself; and (2) excluding pixels in which there was no measured signal in either the air or carbogen data (corresponding to no sequestered PFC). An oxygen tension map was then calculated from the R1 map using the in vivo calibration curves. Histograms of frequency vs \text{pO}_2 were generated by separating the pixel data into bins of 5 torr to display the range of values and to highlight the difference in tumor oxygenation due to the change in breathing gas.

Of importance in these experiments is the difference in \text{pO}_2 measured before and after a change in breathing gas. This difference was characterized using three different measures, each using all (non-zero) pixels in a given \text{pO}_2 map: the mean \text{pO}_2, the median \text{pO}_2, and the weighted-mean \text{pO}_2. Both the mean and the median were calculated since the histograms of \text{pO}_2 frequency were not always normal distributions and the entire distribution was not affected equally by a change in breathing gas. The weighted average of each map was constructed by weighting each pixel \text{pO}_2 by its spin density, \(M_0\), and calculating the mean over all pixels, \(i\), using:

\[
\text{pO}_2 \text{(weighted average)} = \frac{\sum M_0 \text{pO}_2}{\sum M_0}. \tag{1}
\]

Data were acquired first with the animal breathing air, using diffusion-weighted images to generate diffusion maps for each diffusion time. This was followed by the acquisition of the seven IR-EPI 19F images used in the calculation of the \text{pO}_2 map. The breathing gas was then changed to carbogen, and the diffusion and 19F data were again acquired in the same order. The start of data acquisition was approximately 10 min after the change in breathing gas.
RESULTS

Multiple linear regression was used to extract the relationship between dissolved oxygen concentration and $R_1$ and $T$ for four different temperatures and oxygen concentrations from three different trials. The resulting equation was

$$R_1 = 0.711 + 0.026 \times O_2 - 0.010 \times T^2 = 0.998 \quad (2)$$

where $O_2$ is in percent and $T$ is in degrees Celsius. Solving eq. (2) for $pO_2$,

$$pO_2 = 297.4R_1 + 2.940T - 211.4. \quad (3)$$

Equation (3) was used on a pixel-by-pixel basis to transform the $R_1$ maps into $pO_2$ maps. A temperature of $37^\circ C$ was assumed in eq. (3).

The changes seen in the computed $pO_2$ maps, when air is replaced by carbogen as the breathing gas, are presented in Plate 1 for a representative RIF-1 tumor. Plate 1(a) is the map for the air-breathing mouse while Plate 1(c) is the map for the same mouse breathing carbogen. The color scale beside the $pO_2$ map for the carbogen breathing mouse ranges from $-20$ to $80$ torr and is the same for both maps. Note that the majority of the increase in $pO_2$ is evident in the periphery of the tumor where the vascular volume is greater. $30$ The $pO_2$ values were grouped into 5-torr bins and displayed as histograms in Fig. 1(b) (air breathing) and Fig. 1(d) (carbogen breathing). Due to their asymmetric distribution, the median rather than the mean is used as an index for the histograms. Table 1 lists the change in tumor $pO_2$ for each animal using the weighted mean, the unweighted mean, and the median. For the 11 tumors studied here, the average increase in median $pO_2$ value when the breathing gas was changed from air to carbogen was $20 \pm 3$ torr (mean $\pm$ SEM) with a $p$-value of 0.0001. Figure 1 shows a histological slice from the tumor of Plate 1. The colors have been reversed to provide the greatest contrast and hence light areas are regions of viable tissue.

A example of the diffusion data used in this study is presented in Plate 2. The solid line schematically represents the behavior of $D(t)$ as $\sqrt{t}$ is varied. $D(t)$ is plotted vs $\sqrt{t}$ since in that representation the slope of the curve is proportional to $S/V$ for short diffusion times (see Background). Four maps, representative of different regimes along the $D(t)$ curve, were chosen for further

Table 1. Changes in tumor $pO_2$ with a change in breathing gas from air to carbogen for 11 RIF-1 tumors. Numbers are calculated directly from the pixel $pO_2$ values. Weighted means were calculated using eq. (1)

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Difference in weighted means (torr)</th>
<th>Difference in unweighted means (torr)</th>
<th>Difference in medians (torr)</th>
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<tr>
<td>1</td>
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<td>14</td>
<td>13</td>
</tr>
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<td>2</td>
<td>37</td>
<td>37</td>
<td>37</td>
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<td>23</td>
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</tr>
<tr>
<td>11</td>
<td>30</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>20 ± 3</td>
<td>22 ± 3</td>
<td>20 ± 3</td>
</tr>
</tbody>
</table>

Figure 1. Example of the histological slides used to determine necrotic regions for RIF-1 tumors. This slide is for the tumor whose $pO_2$ maps are shown in Plate 1. Light areas indicate viable tissue.

Figure 2. $D(t)$ curves for three pixels taken from the 20 $D(t)$ maps for a single RIF-1 tumor. Solid circles represent data taken from the central part of the tumor, open triangles and closed squares are from pixels in the tumor periphery. The data from the central part of the tumor shows little time dependence, indicative of a smaller degree of restriction than that seen in the periphery. For these data, the effective media regime (the regime in which $D(t)$ has become constant) begins at approximately 100 ms. The value at which $D(t)$ becomes constant is inversely proportional to the tortuosity.
study from the 20 maps calculated. The diffusion times of these four maps were: 11.0, 58.0, 360.5 and 560.5 ms. These maps are representative of: (1) the short time regime (or \( S/V \) regime) in which \( D(t) \) is proportional to \( S/V \) (11.0 ms), (2) the transition regime in which \( D(t) \) switches from the \( S/V \) regime to the effective media regime (58.0 ms), (3) the ‘near’ effective media regime (360.5 ms), and (4) the ‘far’ effective media regime in which the diffusion time is long enough such that all of the tissue is in the effective media regime and \( D(t) \) is proportional to \( 1/T \) (560.5 ms) (see Ref. 12). Single pixel data from a single RIF-1 tumor are presented in Fig. 2 for pixels in necrotic (solid circles) and viable tissue regions (open triangles and shaded squares). Data from 14 diffusion times ranging from 11.0 to 560.5 ms are shown in Fig. 2 (only every other data point is displayed for the short-time data for clarity). The necrotic regions were determined by comparison with histological staining results.

Figure 3 shows an example of the scatter plots for the shortest and longest diffusion times studied of \( D(t) \) for the animal breathing air vs \( D(t) \) measured during carbogen breathing. The solid lines are least-squares fits to the data. The fit parameters are: for \( t_{\text{dif}} = 11.0 \) ms, slope = 0.92 ± 0.02, intercept = −1 ± 4 (\( r = 0.86 \)) and for \( t_{\text{dif}} = 560.5 \) ms, slope = 0.93 ± 0.01, intercept = 6 ± 2 (\( r = 0.95 \)).

Table 2. Fitting parameters for correlation plots of \( D(t) \) for air breathing vs \( D(t) \) for carbogen breathing for RIF-1 tumors. The diffusion time was 560.5 ms.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Intercept</th>
<th>Slope</th>
<th>( r ) value</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>18 ± 2</td>
<td>0.94 ± 0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>16 ± 2</td>
<td>0.93 ± 0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>3</td>
<td>13 ± 1</td>
<td>0.90 ± 0.01</td>
<td>0.99</td>
</tr>
<tr>
<td>4</td>
<td>−35 ± 3</td>
<td>1.15 ± 0.02</td>
<td>0.93</td>
</tr>
<tr>
<td>5</td>
<td>23 ± 6</td>
<td>0.93 ± 0.04</td>
<td>0.77</td>
</tr>
<tr>
<td>6</td>
<td>−28 ± 8</td>
<td>1.17 ± 0.08</td>
<td>0.65</td>
</tr>
<tr>
<td>7</td>
<td>2 ± 2</td>
<td>0.98 ± 0.02</td>
<td>0.95</td>
</tr>
<tr>
<td>8</td>
<td>28 ± 4</td>
<td>0.47 ± 0.04</td>
<td>0.69</td>
</tr>
<tr>
<td>9</td>
<td>−6 ± 1</td>
<td>1.07 ± 0.01</td>
<td>0.98</td>
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<tr>
<td>10</td>
<td>40 ± 6</td>
<td>0.72 ± 0.06</td>
<td>0.53</td>
</tr>
<tr>
<td>11</td>
<td>−50 ± 11</td>
<td>1.5 ± 0.1</td>
<td>0.69</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0 ± 2</td>
<td>0.98 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>
were: slope $= -0.01 \pm 0.01$, intercept $= 23 \pm 1$. Pearson’s $r = 0.54$ (air breathing) and slope $= -0.01 \pm 0.01$, intercept $= 38 \pm 2$, Pearson’s $r = 0.48$ (carbogen breathing).

One of the difficulties with the above presentation is that it is not clear how an individual pixel’s $D(t)$ and $p\text{O}_2$ values change following a change in breathing gas. In order to examine this issue more carefully, two quantities were calculated: $\Delta D(t) = D(t)_c - D(t)_a$, where $D(t)_c$ and $D(t)_a$ are the diffusion coefficients measured with the animal breathing carbogen and air, respectively) and $\Delta p\text{O}_2 = p\text{O}_2c - p\text{O}_2a$, where $p\text{O}_2c$ and $p\text{O}_2a$ are the oxygen tension values measured with the animal breathing carbogen and air, respectively. Shown in Fig. 5(a) is a plot of $\Delta p\text{O}_2$ vs $\Delta D(t)$ for one representative tumor. This plot allows the identification, on a pixel-by-pixel basis, of how a change in $p\text{O}_2$ is reflected as a change in $D(t)$. The shape of the distribution of pixel values can be obtained from projections of the data onto each axis. These projections are shown as histograms in Fig. 5(b) and (c). The distribution for $\Delta D(t)$ is roughly normal and centered around zero, i.e., on average there is no net change in $D(t)$ for a change in breathing gas. The histogram for $p\text{O}_2$, as expected, does reflect an increase with a change to carbogen breathing.

Figure 6 shows the mean (± SEM) of the $\Delta D(t)$ histogram for each animal. Because the diffusion time affects the $D(t)$ data (see Fig. 3) this plot is constructed for each of the four diffusion times: 11.0 (a), 58.0 (b), 360.5 (c), and 560.5 ms (d). The trend of $\Delta D(t)$ in Fig. 6 is the same as in Fig. 3: with increasing diffusion time the distributions become narrower. Note that the distribution of the means for each diffusion time are centered about zero, implying that, on average, for all 11 animals, there is no net change in $D(t)$ with a change in breathing gas.

**DISCUSSION**

Diffusion-coefficient mapping has been shown to be a useful tool in distinguishing pathology from normal tissue in many applications.\(^{10-12}\) By exploiting the structural changes that often accompany pathology, ADC mapping can aid both in its visualization and in the determination of tissue types. Fluorine-19 NMR of sequestered PFC emulsions has been shown to be a rapid and quantitative method of mapping tumor $p\text{O}_2$ distributions in murine tumors.\(^{6,7}\) Together, these two methods allow for a unique view of tumor tissue oxygenation and a method for testing any possible relationship between ADC and p$\text{O}_2$.

In agreement with a previous study using perfluoro-15-crown-5-ether\(^7\) and studies using other PFCs,\(^{23,24,31-33}\) the $R_1$ relaxation rate was found to be linearly related to dissolved oxygen concentration and temperature. The relationship, given in eq. (3), was used to calculate in vitro $p\text{O}_2$ maps on a pixel-by-pixel basis. Examples of these maps are shown in Plate 1. These maps have the same slice thickness as the ADC maps and thus are an improvement over the projection images previously obtained using this PFC in Ref. 7. The $p\text{O}_2$ values for the air breathing animal [Plate 1 (a) and (b)] are the largest in the periphery of the tumor where the vasculature is presumably intact. The lowest $p\text{O}_2$ values were found in the center of the tumor, a region which displayed evidence of necrosis (as determined from the

\(t_{\text{dif}} = 560.5 \text{ ms}\)

**Figure 4.** Scatter plots for air and carbogen breathing showing the correlation between $D(t)$ and $p\text{O}_2$ for the longest diffusion time for a single RIF-1 tumor. These data confirm that carbogen breathing affects only the well vascularized tumor periphery [associated with the lower $D(t)$ values] and to a smaller extent the tissue in the necrotic center of the tumor [associated with higher $D(t)$ values].
A histological slice for the tumor in Plate 1 is shown in Fig. 1. The colors have been reversed for the greatest contrast, and hence light areas correspond to viable tissue. Note that the viable tissue areas correspond well to the most well-oxygenated regions in Plate 1.

The distribution of pO$_2$ values is determined by the final location of the PFC within the tumor. The PFC is delivered to the tumor through fenestrations in the vasculature, so the distribution of pO$_2$ values found in these experiments will be weighted towards higher pO$_2$ values. This is due to the fact that, to reach less well-perfused or necrotic regions, the PFC will either have diffuse to those regions, or an initially well-perfused region may become hypoxic as the tumor grows during the time allowed for the PFC to clear the vasculature. None of the pO$_2$ maps in this study exhibited regions in which there was no signal from the PFC. This is most likely a result of the volume averaging inherent in the 3 mm slice thickness used in these measurements.

The range of pO$_2$ values in the histograms in Plate 1 is taken from $-20$ to $80$ torr. The precision of the $R_1$ measurement resulted a precision in pO$_2$ of $\pm 5$ torr, consistent with earlier studies. The occurrence of negative pO$_2$ values is most likely due to the assumption that the tumor temperature is $37^\circ$C. In this experiment the animals body temperature is maintained by a flow of $37^\circ$C air initially, reduced to $34^\circ$C after 10 min. This reduction is necessary to prevent hyperthermia in the animal. Because the tumor is located on the back of the animal and has a large surface area, it is likely that the tumor temperature is not equal to the core temperature, and is somewhere between $34^\circ$C and $37^\circ$C. In addition, the compromised circulation between body and tumor impedes a major source of heat equilibration in the body. According to eq. (3), a reduction in tumor temperature results in a reduction in pO$_2$, by approximately $3$ torr /°C. The negative pO$_2$ values are consistent with the precision of these experiments ($\pm 5$ torr) and a tumor temperature decreased from the core temperature. This offset in pO$_2$ is, however, of little consequence in the present experiments because any correlation between ADC and pO$_2$ would be independent of the offset. In addition, since the measurement performed with carbogen breathing are compared with those in the same animal breathing air any offset in pO$_2$ will be canceled when differences are taken.

The range of positive pO$_2$ values found in this experiment is consistent with needle electrode measurements performed by Terris et al., which found values up to 60 torr in RIF-1 tumors when subjects were breathing air.

Hypoxic cells are thought to play an important role in the resistance of solid tumors to radiotherapy and chemotherapy. Carbogen breathing is known to increase the radiosensitivity of these hypoxic cells in murine tumors and to increase the pO$_2$ only in particular locations in the tumor. In this study, carbogen breathing was used to change the tissue oxygenation in order to explore any concomitant changes in $D(t)$. By changing the oxygen tension distribution in the tumor, an additional test can be made as to the correlation between $D(t)$ and pO$_2$. For example, if $D(t)$ and pO$_2$ appears to be correlated in a particular region, but $D(t)$ does not increase as pO$_2$ increases, this correlation can be determined to be false or coincidental. Data for carbogen breathing presented in Plate 1(c) shows that the largest increases in pO$_2$ are confined to the periphery. It is in this region that any correlation between $D(t)$ and pO$_2$ would be expected as it includes viable, as well as hypoxic, tissue. The necrotic regions are not expected to have much variation in the value of $D(t)$ or pO$_2$, and therefore any correlation might be weaker in these regions.

A related issue is the determination of the diffusion time which yields optimal differentiation between viable, hypoxic, and necrotic tissue. By optimizing the diffusion time, the dynamic range of $D(t)$ can be maximized and

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Figure 5. Plot of $\Delta$pO$_2$ vs $\Delta$ADC (a) and the projections on the $\Delta$ADC (b) and $\Delta$ADC (c) axes for a single Rif-1 tumor. Each point represents one pixel. The differences are constructed by subtracting the value of the parameter with air breathing from the value with carbogen breathing.
clearer evaluation of the correlation between $D(t)$ and $pO_2$ will result. The ADC of water molecules diffusing in RIF-1 tumors has been shown to be time dependent. In the present experiment, this is shown schematically in Plate 2 and with single pixel data in Fig. 2. In Plate 2, large values dominate the $D(t)$ map at short diffusion times, both in the central regions and the periphery. As the diffusion time is increased, the majority of pixels in the periphery show a decline in $D(t)$. In the central region, however, the diffusion coefficient shows only a small decline with increasing diffusion time. This indicates how $D(t)$ maps can be used to differentiate between areas of different tumor tissue types: $D(t)$ maps can be acquired for a range of diffusion times and the time dependent behavior can indicate the different regions. The difference between time-dependent behaviors is maximized at long diffusion times and it is at these diffusion times, therefore, that the most differentiation between necrotic and viable tumor tissue will be apparent.

The time-dependent behavior of $D(t)$ is shown more clearly in the single-pixel data presented in Fig. 2 which were acquired from the central region, which includes necrotic tissue (solid circles), and from two different regions in the periphery (solid squares and open triangles). The pixels in the tumor periphery are clearly time dependent, while the data from the pixel in the central core shows little change over the range of diffusion times indicating that the latter environment contains fewer restrictions. The data in Plate 2 and Fig. 2 indicate that the maximum contrast between regions of different tissue types is found for diffusion times greater than approximately 100 ms. These data show the motivation behind the diffusion times chosen for further study: the data taken for a diffusion times of 11.0 and 58.0 ms will not have the optimal contrast when compared with the data obtained at 360.5 and 560.5 ms.

In order to illustrate the effects of diffusion time on the correlation plots of $D(t)$ for air breathing vs $D(t)$ for carbogen breathing, the extreme cases (diffusion times of 11.0 and 560.5 ms) are presented in Fig. 3. It is clear from the two plots that the diffusion time influences the spread in the data. The fit parameters are: for $t_{diff} = 11.0$ ms, slope = 0.93 ± 0.02, intercept = −1 ± 4 ($r = 0.86$) and for $t_{diff} = 560.5$ ms, slope = 0.93 ± 0.01, intercept = 6 ± 2 ($r = 0.95$). The increase in the correlation coefficient with diffusion time is consistent with the idea that the measured diffusion coefficient, at longer diffusion times, reflects the longer scale structure of the sample and not simply the local variations. It may be argued that the shortest diffusion time within the effective-media regime would be the best choice for analysis since that would minimize the averaging over different tissue types. The

Figure 6. Plots of $\Delta$ADC vs RIF-1 tumor number at each of the four diffusion times investigated: 11.0 ms (a), 58.0 ms (b), 360.5 ms (c), and 560.5 ms (d). Each data point represents the mean ± SEM of all pixels containing a measurable amount of perfluorocarbon.

longest diffusion time of 560.5 ms was used for analysis, however, since it corresponds to a diffusion length of 33 μm, roughly an order of magnitude smaller than the pixel length of 470 μm (the voxel size is 470 μm × 470 μm × 3 mm) and therefore, the partial volume averaging arising from the pixel size will dominate the effects due to the diffusion time. In addition, the fact that the slope is close to unity and intercepts are close to zero implies that carbogen breathing has little, if any, effect on \( D(t) \).

Table 2 presents the slope and intercept values for each of the measured tumors as well as the average for each parameter. The small average intercept (0 ± 2, mean ± SEM) as well as the average slope near unity (0.98 ± 0.01, mean ± SEM) implies that the change in \( D(t) \) brought about by carbogen breathing is a small effect at most. This data has also been analyzed using the restricted maximum likelihood (REML) method\(^{35} \) which iteratively estimates the random variances of the slopes and intercepts of these data for each animal. The variances are used as weights for the original data points (pixel values) in a weighted least-squares fit. The REML analysis gave, for the entire data set, slope = 0.97 ± 0.03 and intercept = 1 ± 2. The \( p \)-value for the intercept being different from zero was 0.72 and the \( p \)-value for the slope being different from one was 0.30 and, therefore, neither value was statistically different from the null result of slope = 1.00 and intercept = 0. This result is interesting in that, while it may be expected that the correlation between \( D(t) \) and \( pO_2 \) is rather weak in the viable tissue that is already well-oxygenated, there is no population of pixels that exhibits a large shift in \( D(t) \) (see Figs 3, 5 and 6). This restricts the usefulness of using \( D(t) \) as a clinical indicator of \( pO_2 \), since the sensitivity to relatively small \( pO_2 \) changes seems to be low. While a number of animals individually displayed statistically significant deviations from the null result, these deviations did not correlate with other parameters measured in this study such as tumor volume. Therefore, the results for all animals taken together are reported.

Although there is no statistical correlation between \( D(t) \) and \( pO_2 \), as the data presented in Fig. 4 shows, there are a number of interesting relationships between the data in different regions of these plots. The scatter plots emphasize that the greatest change in \( pO_2 \) is for pixels with the lowest values of \( D(t) \), pixels that were identified with reasonably well-vascularized, non-necrotic tissue in Plate 1 and Fig. 1. The pixels with the highest values of \( D(t) \) (identified as necrotic tissue by H and E staining) show little if any change in \( pO_2 \) with a change in breathing gas. There has been no attempt made to make a linear fit to these data for each animal as the slope with air breathing is highly dependent upon tumor size and, therefore, necrotic fraction. The change in slope with breathing gas is also highly variable for the same reason, since only the \( pO_2 \) of viable tissue is affected by a switch to carbogen breathing. To illustrate the degree of correlation that is obtained from these plots, the data in Fig. 4 were subjected to a linear least-squares fit (\( pO_2 = \text{intercept} + \text{slope} \times \text{ADC} \)) with the results shown as a solid line in both cases. For the animal breathing air, the intercept = 23 ± 1 and slope = −0.012 ± 0.009 (Pearson’s \( r \) = 0.54), while for carbogen breathing, the intercept = 38 ± 2 and slope = −0.014 ± 0.012 (\( r \) = 0.48).

The presentation of the correlation plots in Fig. 4 only addresses the question of what the gross correlation is between \( D(t) \) and \( pO_2 \) over the entire tumor slice. There is no way to determine from Fig. 4 how each individual point is affected by the change in breathing gas. A complementary view of these data is presented in Fig. 5. This plot, of \( \Delta pO_2 \) vs \( \Delta D(t) \) for a representative tumor [Fig. 5(a)], shows both the change in \( D(t) \) and the change in \( pO_2 \) for each pixel. This plot should reveal any correlation in the data that is obscured in Fig. 4 for example, are small positive changes in \( D(t) \) correlated with large changes in \( pO_2 \)? Figure 7(a) shows no such hidden correlation. The projections on each axis of Fig. 5(a) [shown as histograms in Fig. 7(b) and (c)] show the gross behavior of each parameter and it can be clearly seen that, while there is a net shift in \( pO_2 \), there is no such shift in \( D(t) \). Figure 6 shows that the distribution of the \( \Delta D(t) \) means over all animals and diffusion times is centered around zero change.

The data suggests the following picture: hypoxic regions in tumors become necrotic as the tumor volume increases and cells become further removed from the tumor vasculature. As necrosis proceeds, cell membranes rupture and the resulting debris is subsequently degraded by autolysis or heterolysis. It would be expected that water diffusing in necrotic regions would experience fewer restrictions than their counterparts diffusing in viable tissue and higher ADCs are therefore expected for necrotic regions. Consequently, for lower ADCs (corresponding to viable tumor tissue) greater values of \( pO_2 \) are expected. Lower (or zero) \( pO_2 \) values are associated with necrotic tissue which have correspondingly higher ADC values.

In contrast to the above results, Dunn et al.\(^{39} \) found a positive correlation between ADC and \( pO_2 \) in a study combining NMR ADC maps with oxygen tension measured using EPR of implanted LiPc crystals. Oxygen tension measurements were performed in the region of the pixels with the highest and lowest values on an ADC map. The results in that study were assumed to hold only for non-necrotic regions. In the present work, all pixels (with sequestered PFC) are included in the analysis and, presumably include viable, hypoxic, and necrotic tissue. With reference to Fig. 4 it can be seen that even when the pixels at the highest ADC values (\( > 1.4 \times 10^{-5} \text{ cm}^2/\text{s} \) and corresponding to necrotic tissue) are excluded, the correlation between \( D(t) \) and \( pO_2 \) is still non-existent. In addition, given the broad spread in the pixel data, there are many possible choices of two ADC values at the extreme ends of the range which would demonstrate a
positive correlation between ADC and pO2. Unfortunately, this approach does not capture the complexity of the data and can lead to erroneous conclusions.

In summary, this study demonstrates the absence of correlation between $D(t)$ and pO2 and RIF-1 tumors when viable, hypoxic, and necrotic tissue are all included. Furthermore, excluding necrotic tissue data still results in no correlation between tumor water ADC and pO2 as measured using PFCs. However, this method, which combines both $D(t)$ and pO2 measurements, may be useful in following treatment regimens and for establishing treatment efficacy in a non-invasive manner. Changes in tumor tissue viability and oxygen status can be imaged through the use of sequestered PFCs and necrotic tissue can be separated from viable using $D(t)$ maps. This study also demonstrates that the best $D(t)$ contrast between necrotic and non-necrotic tissue is achieved at long diffusion times ($>\sim 100$ ms in the RIF-1 model).

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