# EPR-based Oximetric Imaging: A Combination of Single Point-based Spatial Encoding and $T_1$ Weighting

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## Abstract

**Purpose:** Spin-lattice relaxation time ($T_1$)-weighted time-domain EPR oximetry is reported for in vivo applications using a paramagnetic probe, a trityl-based Oxo71.

**Methods:** The $R_1$ dependence of the trityl probe Oxo71 on $pO_2$ was assessed using single point imaging (SPI) mode of spatial encoding combined with rapid repetition, similar to $T_1$-weighted MRI, where $R_1$ was determined from 22 repetition times ranging from 2.1–40.0 μs at 300 MHz. The $pO_2$ maps of a phantom with three tubes containing 2 mM Oxo71 solutions equilibrated at 0%, 2%, and 5% oxygen were determined by $R_1$ and apparent spin-spin relaxation rate ($R_2$*) simultaneously.

**Results:** The $pO_2$ maps derived from $R_1$ and $R_2$* agreed with the known $pO_2$ levels in the tubes of Oxo71. However, the histograms of $pO_2$ revealed that $R_1$ offers better $pO_2$ resolution than $R_2$* in low $pO_2$ regions. The standard deviations of pixels at 2% $pO_2$ (15.2 mmHg) were about 5 times lower in $R_1$-based estimation than $R_2$*-based estimation (mean ± SD: 13.9 ± 1.77 mmHg and 18.3 ± 8.70 mmHg, respectively). The in vivo $pO_2$ map obtained from $R_1$-based assessment displayed a homogeneous profile in low $pO_2$ regions in tumor xenografts, consistent with previous reports on $R_2$*-based oximetric imaging. The scan time to obtain the $R_1$ map can be significantly reduced using three repetition times ranging from 4.0–12.0 μs.

**Conclusion:** Using the SPI modality, $R_1$-based oximetry imaging with useful spatial and oxygen resolutions for small animals was demonstrated.

**Keywords:** non-invasive in vivo oximetry, tumor tissue oxygen concentration, EPR single point imaging, partial oxygen pressure, spin-lattice relaxation time, triarylmethyl paramagnetic oxygen probe

## Introduction

The importance of EPR imaging (EPRI) stems from the fact that it can be used to quantitatively determine tissue oxygenation, as both the spin-lattice relaxation time ($T_1$) and spin-spin relaxation time ($T_2$) of paramagnetic electrons are shortened in the presence of oxygen. EPRI can be performed in vivo either in the continuous wave mode or in the time domain using pulsed Fourier transform techniques with exogenous paramagnetic probes (1–4). The observed EPR spectral line widths of many paramagnetic systems, such as lithium phthalocyanine (5,6) and trityl radicals (7–9), linearly depend on the partial oxygen pressure ($pO_2$). In fact, the time domain approach to EPR imaging became feasible for in vivo applications after the availability of narrow-line spin probes based on the triphenylmethyl (trityl) radical (7–9).

Recently developed trityl derivatives (8,9) with $T_1$ and $T_2$ of several microseconds are non-toxic at the concentrations required for imaging and have pharmacologic half-lives sufficient for 3-dimensional imaging. Using excitation pulses ~50 ns and low Q resonators, in vivo EPR images were generated using a strategy known as single point imaging (SPI) involving pure phase encoding in the presence of static gradients, and subsequent Fourier transformation (10–20). In order to perform EPR oximetry, the apparent spin-spin relaxation time ($T_2$*-), $T_2$-, or $T_1$-weighted maps can be obtained as in MRI to generate oxygen-dependent quantitative contrast in the images (11-16).

EPR oximetry based on measuring the spectral line width (LW), where $LW = R_2/\gamma_e$ and $\gamma_e$ is the gyromagnetic ratio of the electron, needs correction for self-broadening of the probe due to its own spin-spin interactions, which manifest at concentrations greater than 5 mM (18,21). This self-broadening of the probe cannot be easily deconvolved from the oxygen-dependent broadening in vivo, as it requires quantitative estimation of the spin probe's accumulation in specific tissues, e.g., in kidneys and tumors. Halpern and coworkers reported $R_1$-based EPR oximetry by an inversion recovery electron spin echo (IRESE) sequence and discovered negligible dependence of self-broadening of the spin probe Oxo63 on its concentration (21), providing a means to map in vivo $pO_2$ at greater accuracy for the first time. An analogous technique in MRI using $^{19}F$-labeled tracers has been developed and validated in studying tumor oxygenation (22). In this study, we present another strategy for $R_1$-based EPR oximetry that combines the high resolution capability of SPI by a single pulse sequence (18), and the effect of interpulse delays and $R_1$ on the steady state

magnetization for rapid signal averaging (23–28). This approach is applicable to a wide range of $R_1$ values, is independent of differences in the concentration of the contrast agent as originally demonstrated by Epel and Halpern, and has a lower specific absorption rate (SAR). Oxo71, a deuterated Oxo63 probe, was assessed for: 1) the linear relationship between $R_1$ and $pO_2$, 2) $R_1$ mapping of a phantom by EPR imaging, and 3) in vivo oximetry of a mouse tumor based on a $R_1$ map.

## Theory

Methods for $R_1$ mapping, such as inversion recovery (29), saturation recovery (30) and variable nutation angle (31), are unsuitable for EPR in vivo studies due to high SARs and long scan times. Approaches to the estimation of $R_1$ by the steady-state free precession sequences at short repetition times, and their relative merits and shortcomings, are well documented in the NMR literature (23,24,26). $R_1$-dependent magnetization recovery, i.e., $T_1$-weighted signal intensity, is defined as a function of the TR to estimate $R_1$ by these sequences.

The saturation by fast repetition (SFR) sequence (28) for $R_1$-weighted in vivo imaging offers a high scan speed at low SAR. The SFR sequence consists of a train of phase-coherent RF pulses of a specific flip angle. In this scheme, when TR is small ($TR < 3T_2$), the signal behavior becomes complex due to the refocusing of the residual transverse magnetization $M_{xy}$ from one pulse by its succeeding pulses, resulting in spin and stimulated echoes (26,27). MRI sequences such as steady-state free precession and gradient recalled echo use this scheme, interleaved with spoiler gradient pulses to destroy the residual transverse magnetization before the next pulse is applied. Such spoiler gradient sequences are not practical for EPRI because of the microsecond time scales required for the gradient settling times, which are in the same time range as the FIDs. In this study, we examined the signal intensities using an SFR sequence of $\pi/2$ pulses by varying TR values from 2.1 to 40 μs. The time-domain signal intensity $S(t_p)$ at a delay time $t_p$ from the $\pi/2$ pulse is approximated to:

$$S(t_p) = M_0 [1-\exp(-R_1 TR)] \exp(-t_p R_2^*) \quad [1]$$

where $M_0$ is equilibrium magnetization. In SPI, image reconstruction is done by monitoring the phase of a single point in the FID at a chosen delay time ($t_p$) following the excitation pulse. The image pixel intensities reflect both $R_1$ and $R_2^*$ effects. Hence it is possible to obtain $pO_2$ maps

based on both $R_1$ and $R_2$* effects from the same data set.

## Methods

### Chemicals

The trityl probe Oxo71, which is the deuterated form of Oxo63 at the methylene moieties indicated by asterisks in Figure 1A, was obtained from GE Health Care (Milwaukee, WI). Gas tanks of argon, air, and mixtures of 2%, 5%, 10% oxygen with nitrogen were procured from local suppliers (Roberts Oxygen, Rockville, MD). Argon and medical air were used for 0% oxygen and 21% oxygen, respectively. The pressure of 21% $O_2$ after equilibration is 760 mmHg × 0.21 = 160 mmHg, which is approximately 0.25 mM $O_2$ at 25 °C.

### Animals

Female C3H Hen MTV mice, supplied by the Frederick Cancer Research Center's Animal Production unit (Frederick, MD, USA), were housed in a climate-controlled and circadian rhythm-adjusted room and allowed food and water *ad libitum*. Squamous cell carcinoma (SCCVII) cells were implanted in the femoral muscle of the right thigh. Approximate tumor size during experimentation was about 10 mm. Experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources (National Research Council, 1996) and were approved by the National Cancer Institute's Animal Care and Use Committee.

### Acquisition of Calibration Data

The spectral and imaging data were scanned on a home built time-domain EPR imager described previously (32) and operating at 300 MHz. The power amplifier used in this study (BT0250-EF-RBB, Tomco) had a rise time and fall time of < 20 ns with a duty cycle of 5%. This allowed a repetition time TR of ~ 2 microseconds using pulse widths of 110 ns for a $\pi/2$ pulse. The $pO_2$ calibration experiments were performed using aqueous solutions of 2 mM Oxo71 equilibrated at five oxygen levels (0%, 2%, 5%, 10% and 21%) and 1, 5, and 10 mM Oxo71 solutions equilibrated at 0% oxygen in separate glass tubes. Oxygen levels were achieved by bubbling the appropriate gas into the sample for about 45 min and maintained by sealing with epoxy. Time-domain EPR signals were recorded by a series of $\pi/2$ pulses separated by TR ranging

from 2.1 to 40 μs.

## Phantom 2D Imaging

Three tubes containing 2 mM Oxo71 solution equilibrated at 0%, 2%, and 5% oxygen were mounted parallel to the magnetic field Y-axis in an equilateral triangular geometry in a plastic holder placed in a resonator of 25 mm diameter (Fig. 1B). The phantom 2D data were acquired in the XZ-plane (cross-section of the tubes) using a 31 × 31 Cartesian grid, $G_{max}$ = 10 mT/m, 5000 signal averages, a sampling dwell time of 5 ns, a flip angle of 90°, TR = 3 - 40 μs, and dead time of 0.25 μs. Calculations and image reconstruction were done by MATLAB (Mathworks Inc., Natick, MA, USA) scripts developed in house.

## In Vivo Imaging

A mouse was anesthetized by 2% isoflurane in medical air, mounted prone on a custom designed holder, maintained at a breathing rate of 60 per min and a core body temperature of 37 ± 1°C by a flow of warm air. The urethra was cannulated using a PE-10 tube. The urine was drained during the experiment. A 30 G needle extended using polyethylene tubing (PE-10) was cannulated into the tail vein. The mouse thigh having the SCCVII tumor was positioned in a 19 mm diameter resonator. Oxo71 solution at 75 mM concentration was administered through tail vein cannulation by giving an initial bolus of 1.125 μmol/g body weight and continuous administration of 0.06 μmol/g/min subsequently to maintain a steady signal level. Images were acquired in the YZ-plane, which corresponds to the sagittal plane on the mouse leg, at 21 × 21 phase encodings in the Cartesian grid, by varying the TR from 2.3 to 40 μs.

## Scan Times

The data acquisition time for a single scan = $TR \times N_{av} \times (N_k + 1) + dT$, where $N_{av}$ is the number of averages, $N_k$ is number of all phase encodings, and $dT$ is about 1 sec, which is sum of the instrument delays before acquisition after changing the configuration or gradient. Background signals were acquired under identical conditions before the data acquisition. For phantom imaging, $N_{av}$ = 5000, $N_k=31^2$, and scan times at TR = 3.4, 8, 12, and 40 μs were 18, 40, 59 and 194 s,

respectively. Approximate $R_2$* may be assessed by a single scan at a specific TR, while three scans at different gradient maxima are necessary to minimize edge artifacts. The scan time for $R_2$* mapping at TR = 40 μs was 194 s and at TR = 12 μs was 59 s. The scan time for three gradient scans performed at TR = 8 μs was 120 s. The scan time for the estimation of $R_1$ from 22 TR values was 24 min and from three TR values at 5, 8, and 12 μs was 125 s. For mice, $N_k = 21^2$ and single scan times at TR = 3.4, 8, 12, and 40 μs were 9, 19, 28, and 90 sec, respectively. The $R_1$ estimation from 23 TR values was 11 min.

**Results**

## Calibration Parameters

The relationships (i) LW versus $pO_2$ where LW (μT) = $R_2$*/(28025π), (ii) LW versus [Oxo71], and (iii) $pO_2$ versus longitudinal relaxation rate $R_1$ (= $1/T_1$) were calculated from Oxo71 calibration spectral data. The apparent transverse relaxation rate $R_2$* (= $1/T_2$*) was considered as the asymptotic $R_2$* value at a long TR (Fig. 2A) to avoid echo contributions observed at short TR (Fig. 2B). The linear fit of $R_2$*-based LW to $pO_2$ in the range of 0–21% (Fig. 2C) indicated the following relationship:

$$LW\ (\mu T) = 0.1257\ pO_2\ (mmHg) + 8.786. \quad [2]$$

Eq. [2] was used directly to calculate a $pO_2$ map of the phantom from the LW. However, in vivo studies require correction for the self-broadening of [Oxo71]. The linear fit of LW to [Oxo71] in the range of 1–10 mM at 0% $pO_2$ indicated that:

$$LW\ (\mu T) = 0.5745\ [Oxo71]\ (mM) + 6.733. \quad [3]$$

Therefore, the following equation was used for $pO_2$ estimations from LW in vivo.

$$LW\ (\mu T) = 0.1257\ pO_2\ (mmHg) + 0.5745\ [Oxo71]\ (mM) + 7.637 \quad [4]$$

Next, the $R_1$ value at each $pO_2$ was calculated by fitting the observed spectral peak height $S_p$ as a function of TR (Fig. 2D) according to Eq. [5] below.

$$S_p = M_0\ [1-\exp(-R_1 TR)] \quad [5]$$

$S_p$ depends on the FID profile covered. The peak heights of the observed spectra ($S_p^{obs}$) were obtained by fast Fourier transform of the FID within a constant $t_p$ range beginning right after the dead time and ending just before the intensity rise due to echo. The calculated peak heights ($S_p^{calc}$) were obtained by fast Fourier transform of simulated time-domain signals according to Eq. [1] for

given $R_1$, $R_2$*, TR values, and a scaling factor to account for $M_0$ within the same $t_p$ range. The Nelder-Mead simplex direct search algorithm (33) was used by varying $R_1$ and the scaling factor. The $R_1$ values resulting in the smallest sum of squares of errors between $S_p^{obs}$ and $S_p^{calc}$ were determined. The $S_p$ values shown in Figure 2D were scaled to a maximum of ~1 for each $pO_2$ separately, as the peak heights decrease with increasing $pO_2$ levels. This fitting indicated larger deviations at low TR values (<3 μs) owing to echo contributions. Furthermore, the intensity variation of 21% $pO_2$ was too small to determine $R_1$ accurately with a 300 MHz EPRI scanner. Therefore, we explored the peak height calculation including the spin and stimulated echo contributions according to the procedure described by Gyngell (26). The fitting improved at low TR values as expected (Fig. 2E), but the $R_1$ value at 21% $pO_2$ remained approximately the same as previously estimated by Eq. [1]. It is likely a limitation of the SFR sequence for $R_1$ estimation that a significant fraction of the the signal is lost in dead time. The relationship between $pO_2$ and $R_1$ was found to be linear for $pO_2 \leq 10\%$ (Fig. 2F) according to Eq. [1], as follows.

$$pO_2(\text{mmHg}) = 122.22\ R_1(\text{MHz}) - 20.36 \qquad [6]$$

The advantage of Eq. [1] over the procedure described by Gyngell (26) is its ability to implement the SPI method when the interference from echo signals is negligible.

## Phantom $T_1$-Weighted Imaging

Two-dimensional images of the phantom were scanned by an SFR sequence in the TR range of 3.0–40.0 μs. At low TR values, the echoes led to ghosts at different scales and orientations, superimposed on the original image depicted in Figure 3A. The FOV, orientation, and intensity of a ghost depend on its phase and the time passed since its originating pulse at the current delay time (Figure 3A-3F). For example, at TR = 3 μs and $t_p$ = 1.5, 1.75, and 2.25 μs, the image of original three-tube phantom appears four times, twice in normal orientation and twice in inverted orientation (Fig. 3B–3D). However, only one of those follows the expected intensity decay and phase evolution profile after a π/2 pulse, and the others are removed by fitting. Specific delay times where the interference distorts the image as shown in Figure 3C are to be avoided by suitable choice of delay times ($t_p$). The early delay times were found to have fewer echo signals.

Initially, the $R_2$* map was calculated from the pixel intensities of images at 5 delay times equally spaced from 1.0 to 1.4 μs, since $R_2$* is required to estimate $R_1$ according to Eq. [1]. The

k-space data were preprocessed prior to image reconstruction to correct for any zero shifts and weighted by a tapered cosine window to minimize ringing artifact from the tubes. All the images were reconstructed by scaling to the smallest FOV via chirp-z transformation. Any marginal FOV mismatches arising from inaccurate estimate of dead time were adjusted by recalculating the scaling factors to minimize the errors. Further reduction of ringing artifacts was accomplished by re-gridding of k-space as previously described (17). The pixel intensities $S(t_p)$ were normalized to account for the change in FOV and the spin density ($S_0$, pixel intensity at $t_p = 0$), and $R_2$* maps were calculated by fitting to Eq. [7] as described previously (18).

$$S(t_p) = S_0 \exp(-t_p R_2^*) \qquad [7]$$

The $R_1$ estimation, however, needs only one delay point at different TR values. If the intensities at a specific delay are normalized to the longest delay by which the intensities reach a constant value asymptotically, then the graphs of intensity as a function of TR coincide for all the images at different single point time delays, and the decay due to $R_2$* gets eliminated.

The intensities at the first delay time ($t_p = 1.0$ μs ) as a function of TR were chosen to estimate $R_1$ to take advantage of the higher signal to noise ratio. $R_1$ and $M_0$ values were calculated for each pixel separately by fitting to Eq. [1], since $S(t_p)$ and $R_2$* are known. This $M_0$ map is merely a map of scaling factors obtained from the fitting that may have a complex relationship to the equilibrium magnetization, depending on the $pO_2$ map. The background regions containing essentially noise were masked prior to fitting to optimize the computation. The pixels at <5% of the maximum intensity were included in the mask.

The $pO_2$ estimation from LW and $R_1$ using Eqs. [6] and [2] is shown in Figure 4. The $M_0$ and $R_1$ maps were calculated including 22 TR values ranging from 3.4 to 40.0 μs at $t_p = 1.0$ μs, and $S_0$ and $R_2$* were calculated at TR = 40 μs from five $t_p$ values equally spaced from 1.0 to 1.4 μs. In the $M_0$ map (Fig. 4A), the intensities of 0% and 5% $pO_2$ tubes appear to be distinctly different while this difference is smaller in the $S_0$ (TR = 40 μs) map (Fig. 4D). The contrast between the three tubes appears relatively more distinct in the $R_1$ map than in $R_2$* (Fig. 4B and 4E). However, the $pO_2$ maps obtained from both $R_1$ and $R_2$* are in agreement (Fig. 4C and 4F), despite a better SNR of the $pO_2$ map derived from $R_1$. The calculation of the $M_0$ and $R_1$ maps was repeated including only three TR values, 5, 8, and 12 μs (Fig. 5), in order to check the feasibility of increasing the scan speed. These images are in good agreement with those calculated from 22 TR

values (with a mean difference of 0.65 mmHg and 95% pixels within ±5.7 mmHg from the mean, Supplementary Figure 3), indicating the possibility of fast $R_1$ scans using three low TR values approximately in the range of 5-12 μs.

The pixel histograms of 0%, 2%, and 5% oxygenated Oxo71 solutions (Fig. 6) indicate better resolution of $pO_2$ in the maps derived from $R_1$ than from $R_2$*. The $pO_2$ values calculated from the $R_1$ map are more uniform whether 3 or 22 repetition times were used (Fig. 6A and 6B). $pO_2$ values of pixels (mean ± SD) in the regions of 0%, 2%, and 5% $pO_2$ tubes were 0.68 ± 0.20, 1.83 ± 0.23, and 4.95 ± 0.5%, respectively. In contrast, the $R_2$* map gave broader ranges of $pO_2$ values in all three regions, leading to higher overlap of histograms (Fig. 6C and 6D). The mean ± SD of $pO_2$ values for pixels in the regions of 0%, 2%, and 5% $pO_2$ were 1.58 ± 1.02, 2.41 ± 1.14 and 4.72 ± 0.54%, respectively. The phantom study suggests that oximetry by $R_1$ yields not only more reliable $pO_2$ estimates but also better $pO_2$ resolution than the $R_2$* method, despite the linearity of $R_2$* with $pO_2$ over a wider range.

## In Vivo Imaging of Mouse Tumor

The mouse 2D image data were acquired by phase encoding in 21 × 21 Cartesian grid and the images were reconstructed at a 256 × 256 matrix size. A steady infusion of Oxo71 was maintained to provide an adequate signal throughout the data acquisition. The $R_2$* and $S_0$ maps were calculated from five equally spaced delay times from 0.85 to 1.25 μs for 23 TR values ranging from 2.3 to 40 μs. Initially the $M_0$, $R_1$, and $pO_2$ maps were calculated at $t_p$ = 0.85 μs from these data without correction for the variations in the infused Oxo71 probe. Comparison of the pixel intensity versus TR profiles of the phantom with those of the mice revealed differences. In the case of the phantom experiment, smooth profiles asymptotically reached a maximum as expected (Supporting Figure S1A). In the case of the mouse experiment, the profiles were noisy and the pixel intensity continued to increase up to the longest TR, due to continuous infusion of the spin probe (Supporting Figure S1B). The intensity change due to the infused probe appeared to be linearly correlated with TR, since the scan time is directly proportional to the local amount of probe in this case. The rate of increase in the signal due to probe infusion was estimated from TR = 16–40 μs where the signal is expected to reach an asymptotic maximum level (99.8% for $R_1$ = 2.5 μs). Each profile was corrected for this variation and $M_0$, $R_1$, and $pO_2$ maps were recalculated (Fig. 7). Despite the

correction, no appreciable improvement was observed in the $R_1$ maps, indicating the robustness of this method for a large number of TR values. A calculation using only three TR values (4.2, 6, and 12 μs) without applying this correction produced almost identical $M_0$ maps (Fig. 7A and 7D), but the $R_1$ map differed in some regions (Fig. 7B and 7E). However, the $pO_2$ maps obtained from $R_1$ measurement were homogeneous.

## Discussion

This study provides an approach to calculate $pO_2$ from both $R_1$ and $R_2$* methods for the deuterated trityl probe Oxo71, which has a longer $T_2$* than Oxo63. The calibration graph shown in Fig. 2F indicates a linear relationship between $pO_2$ and $R_1$ for $pO_2$ ≤10%. Since the linearity below 10% $pO_2$ is adequate for in vivo studies, extension to higher $pO_2$ levels is not addressed in this study.

Equilibration of 10% oxygen in water yields approximately 0.12 mM oxygen dissolved at 25 °C. In vivo $pO_2$ values in muscle tissues are reported to be around 40 mmHg (34), corresponding to 0.05 mM oxygen at 37 °C. Most tissues have lower $pO_2$ than muscle, except lung and arterial blood. Therefore, the linear range of $pO_2$ versus $R_1$ in this phantom study is adequate for most in vivo studies measuring pathophysiological tissue $pO_2$. The data for SPI-based $R_1$ estimation inherently contain data for $R_2$*-based $pO_2$ mapping, which can estimate higher $pO_2$ ranges if desired.

Phantom imaging experiments suggest that the pixel intensities follow Eq. [5] for a wide range of TR values fairly well at a constant delay time ($t_p$) (Supporting Figure S1A). Three TR values defining the intensity curve were found to be sufficient to produce a reliable $pO_2$ map in vitro, where probe concentrations remain constant with time. Interestingly, the quality of both phantom and mouse images was unaffected by the echoes when suitable delay times and TR values were chosen. The choices of TR and $t_p$ are not critical except for avoiding the overlap of ghosts as shown in Fig. 3C, where the FOVs and phases of the original and ghost images are almost equal (which happens in special cases of TR <5 μs for Oxo71).

The estimation of $R_1$ in vivo by EPRI was previously reported by Halpern and coworkers using the IRESE sequence π-T-π/2-τ-π-τ-echo (21). Subsequently a comparison of various pulse schemes to measure $R_1$ by EPRI was reported by these authors (28) where an inversion recovery

sequence was recommended for reliable spin probe concentration-independent $pO_2$ mapping. An SFR sequence can provide $T_1$-weighted maps from which $pO_2$ can be determined. Though the $R_1$ is not as accurate as the IRESE sequence, it offers faster scan speeds and high spatial resolution at low SAR. This single-pulse experiment is widely used in MRI to estimate $R_1$ (23,24,26). We chose rectangular hard pulses at a flip angle of $90^o$ for this study, where transverse magnetization did not completely decay at TR <3 μs. Optimization of this method using fewer TR values will reduce the scan times to acceptable levels for in vivo imaging, similar to $R_2$*-based SPI oximetry. The phantom studies revealed that the ratio of SDs in $R_2$* versus $R_1$ methods for $pO_2$ <2% is about 5, indicating the possibility of higher resolution by $R_1$ at $pO_2$ values below 2%. In SPI, the one-dimensional spatial resolution Δ is given by $FOV/N = 2\pi/(\gamma_e G^{max} t_p)$ where N is the number of phase encodings, $\gamma_e$ is the gyromagnetic ratio of the electron, $G^{max}$ is the maximum gradient, and $t_p$ is the delay time (20). The $R_2$* computation involves FOV scaling as the $t_p$ value is varied. The spatial resolution is 2 mm for the mouse data at $t_p$ = 0.85 μs (1.7 mm for the phantom at $t_p$=1 μs). However, a spatial resolution of 1.4 mm (1.25 mm for phantom) was realized in the $pO_2$ maps due to extension of k-space dimensions by the regridding necessary for $R_2$* estimation.

Experiments in this study were done with a single maximum-field-gradient configuration, although the multiple maximum-field-gradient configuration was proposed in a previous study (18) to avoid the edge artifacts. The single maximum-field-gradient configuration causes alteration of SPI resolution along with $t_p$ (17). Due to edge artifacts in $R_2$* and $S_0$, some artificially high values unnaturally distributed on the edge of theobject can be obtained. On the other hand, the $R_1$ and the $M_0$ estimations, along with TR, did not give such edge artifacts, since a set of SPIs obtained at the identical $t_p$ have the same resolution. The $R_1$ map was computed at shortest $t_p$ value but at the same resolution applied to $R_2$*. The images were reconstructed at a single $t_p$ value with the best S/N ratio, reducing the uncertainties.

The mouse and phantom images at 5 delay times ($t_p$ = 0.85–1.25 μs) and 3 TR values (TR = 4.2, 6, and 12 μs) are shown respectively in Fig. 8 and Supporting Figure S2 in matrix form for better insights into the data. The $M_0$ and $R_1$ maps calculated at early $t_p$ (0.85 μs in Figure 9 and 1.0 μs in Supporting Figure S2) are shown on the right, and the $S_0$ and $R_2$* calculated for each column are shown in the top two rows. As the FOV changes with $t_p$ but not by TR, the $M_0$ map resembles the images at shortest $t_p$. In contrast, the $S_0$ map depends on the intensity variations with $t_p$ at each

TR. The FID signal in some regions, e.g., those having high $pO_2$ or low intensities, decays to noise faster than the other regions, leading to stronger contrasts in $S_0$ and $R_2$* maps. Further, the relaxation of transverse magnetization varies with TR, leading to differences in the $S_0$ and $R_2$* maps with TR. As a result, the SDs observed in $pO_2$ values based on $R_2$* are inherently higher. In addition, the accuracy of $pO_2$ estimates from $R_2$* depend on the ability to correct for self-broadening accurately. While this is not obvious in the phantom where the concentrations are uniform, it is apparent in mouse imaging when a highly concentrated bolus of the spin probe distributes inhomogeneously in the body via systemic circulation, yielding a wide range of concentrations. This suggests that the use of $R_1$ is superior to $R_2$* for in vivo $pO_2$ studies. The advantage of SPI using an SFP sequence is fast data acquisition at the equivalent scan time of the multigradient approach previously reported for $R_2$*-based oximetry (34).

It has been shown by inversion recovery methods that $R_1$ is linearly proportional to $pO_2$ in the range of 0-21% for Oxo63 (21). In this work, although the range of $pO_2$ assessed by $R_2$* includes 0-21%, the $R_1$-based method limits the $pO_2$ range to 0-10% (Fig. 2). This limitation arises from the availability of the FID signal after the dead time at a flip angle of $\pi/2$. Recall that the smaller flip angle limits the change in z-magnetization (Mz) and hence the accuracy of $R_1$ assessment. The best $R_1$ assessment is accomplished when $pO_2$ is close to 0% and scans include both low and high TR values to define the intensity versus TR profile adequately. About 12 TR values from 4.2 to 40 μs were found to be adequate for accurate $pO_2$ estimation by the $R_1$ method. Calibration experiments on individual Oxo71 solutions suggest that it is possible to attain a linear relationship between $pO_2$ and $R_1$ for $pO_2$ <10%. The slope and intercept of eq. [6] are 122.22 and -20.35, with standard errors of ±1.96, and ±0.96, respectively. These values indicate that $pO_2$ can be determined at a resolution of 0.6 mmHg at 0% and ~3 mmHg at 10%. The sensitivities of both $R_1$ and $R_2$* methods were compared with the $pO_2$ levels estimated from phantom images. The $R_1$ and $R_2$* methods estimated 0% $pO_2$ as 5 and 12 mmHg respectively. At 2% and 5%, the $pO_2$ estimations are closer to the expected values. The slope of observed versus expected $pO_2$ in the 0-5% range is closer to 1 for $R_1$ (slope = 0.88) than $R_2$* (slope = 0.71), indicating that $pO_2$ estimated by $R_1$ is more specific and more sensitive than the estimate by $R_2$*. $R_1$ values estimated by three TR points indicated a lower slope of 0.84, pointing to a loss of sensitivity. Although SDs of histograms are expected to decrease with increasing the number of TR points, no apparant improvement was

observed by increasing to 5 TR points. About 12 TR points (phantom scan time = 14 min, supplementary Figure 5) were found to be adequate to obtain SDs similar to those of Fig. 6A. In order to perform 3D imaging in vivo, the scan time is to be reduced to under 10 minutes by constraining severall parameters such as $N_{av}$, matrix size, partial k-space acquisition and leaving out long TR values.

## Conclusion

This study demonstrated the feasibility of mapping spin-lattice relaxation times based on SPI data collection and their use to determine spatially resolved oxygen levels in vivo. The scan time can be reduced to match conventional $R_2$* measurements by using a combination of relatively short TR data sets and further optimization. The single pulse sequence with variable TR is an attractive approach to evaluate $R_1$ for in vivo oximetry due its low SAR. The $R_1$ estimation by SPI simultaneously determines $R_2$*. The fact that the $R_1$ method offers a more accurate $pO_2$ estimation with very little dependence on spin probe concentration, coupled with the intrinsic line width-independent high resolution and the relatively large effective uniform excitation band width, makes this a highly practical approach for small animal EPR oximetric imaging.

## References

1. Epel B, Sundramoorthy S V., Barth ED, Mailer C, Halpern HJ. Comparison of 250 MHz electron spin echo and continuous wave oxygen EPR imaging methods for in vivo applications. Med. Phys. 2011;38:2045–2052.

2. Subramanian S, Matsumoto K, Mitchell JB, Krishna MC. Radio frequency continuous-wave and time-domain EPR imaging and Overhauser-enhanced magnetic resonance imaging of small animals: instrumental developments and comparison of relative merits for functional imaging. NMR Biomed. 2004;17:263–294.

3. Subramanian S, Mitchell J, Krishna M. Time-domain radio frequency EPR imaging. Biol. Magn. Reson. 2003;18:153–197.

4. Eaton G, Eaton S, Ohno K. EPR imaging and in vivo EPR. CRC Press:Boca Raton;1991.

5. Ilangovan G, Zweier JL, Kuppusamy P. Electrochemical Preparation and EPR Studies of Lithium Phthalocyanine. Part 2: Particle-Size-Dependent Line Broadening by Molecular Oxygen and Its Implications as an Oximetry Probe. J. Phys. Chem. B 2000;104:9404–9410.

6. Liu KJ, Gast P, Moussavi M, Norby SW, Vahidi N, Walczak T, Wu M, Swartz HM. Lithium phthalocyanine: a probe for electron paramagnetic resonance oximetry in viable biological systems. Proc. Natl. Acad. Sci. U. S. A. 1993;90:5438–5442.

7. Reddy TJ, Iwama T, Halpern HJ, Rawal VH. General Synthesis of Persistent Trityl Radicals for EPR Imaging of Biological Systems. J. Org. Chem. 2002;67:4635–4639.

8. Yong L, Harbridge J, Quine RW, Rinard GA, Eaton SS, Eaton GR, Mailer C, Barth E, Halpern HJ. Electron Spin Relaxation of Triarylmethyl Radicals in Fluid Solution. J. Magn. Reson. 2001;152:156–161.

9. Ardenkjær-Larsen JH, Laursen I, Leunbach I, Ehnholm G, Wistrand L-G, Petersson JS, Golman K. EPR and DNP Properties of Certain Novel Single Electron Contrast Agents Intended for Oximetric Imaging. J. Magn. Reson. 1998;133:1–12.

10. Halse M, Rioux J, Romanzetti S, Kaffanke J, MacMillan B, Mastikhin I, Shah NJ, Aubanel E, Balcom BJ. Centric scan SPRITE magnetic resonance imaging: optimization of SNR, resolution, and relaxation time mapping. J. Magn. Reson. 2004;169:102–117.

11. Hallac RR, Zhou H, Pidikiti R, Song K, Stojadinovic S, Zhao D, Solberg T, Peschke P, and Mason RP. Correlations of noninvasive BOLD and TOLD MRI with pO2 and relevance to tumor

radiation response. Magn. Reson. Med. 2014; 71: 1863-1873.
12. Winter JD, Akens MK, and Cheng H-LM. Quantitative MRI assessment of VX2 tumour oxygenation changes in response to hyperoxia and hypercapnia. Phys. Med. Biol. 2011; 56: 1225.
13. Safronova MM, Colliez F, Magat J, Joudiou N, Jordan BF, Raftopoulos C, Gallez B, and Duprez T. Mapping of global R1 and R2*values versus lipids R1 values as potential markers of hypoxia in human glial tumors: A feasibility study. Magn. Reson. Imaging. 2016; 34: 105-113.
14. Mason RP, Shukla HP, and Antich PP. In vivo oxygen tension and temperature: Simultaneous determination using 19F spectroscopy of perfluorocarbon. Magn. Reson. Med. 1993; 29: 296-302.
15. Guo Q, Mattrey RF, Guclu C, Buxton RB, and Nalcioglu O. Dynamic measurements of pO2 by T1 and T2 of 19F with PFOB. Paper presented at: Society of Magnetic Resonance in Medicine 11th Annual Meeting1992; Berlin.
16. Thomas SR, Pratt RG, Millard RW, Samaratunga RC, Shiferaw Y, and Clark LC. Evaluation of the influence of the aqueous phase bioconstituent environment on the F-19 T1 of perfluorocarbon blood substitute emulsions,. Radiology. 1991; 18:159.
17. Jang H, Subramanian S, Devasahayam N, Saito K, Matsumoto S, Krishna MC, McMillan AB. Single Acquisition Quantitative Single-Point Electron Paramagnetic Resonance Imaging. Magn. Reson. Med. 2013;70:1173–1181.
18. Matsumoto K, Subramanian S, Devasahayam N, Aravalluvan T, Murugesan R, Cook JA, Mitchell JB, Krishna MC. Electron paramagnetic resonance imaging of tumor hypoxia: Enhanced spatial and temporal resolution for in vivo pO2 determination. Magn. Reson. Med. 2006;55:1157–1163.
19. Devasahayam N, Murugesan R, Yamada K, Reijnders K, Mitchell JB, Subramanian S, Krishna MC, Cook JA. Evaluation of a high-speed signal-averager for sensitivity enhancement in radio frequency Fourier transform electron paramagnetic resonance imaging. Rev. Sci. Instrum. 2002;73:3920–3925.
20. Subramanian S, Devasahayam N, Murugesan R, Yamada K, Cook J, Taube A, Mitchell JB, Lohman JAB, Krishna MC. Single-point (constant-time) imaging in radiofrequency Fourier transform electron paramagnetic resonance. Magn. Reson. Med. 2002;48:370–379.

21. Epel B, Bowman MK, Mailer C, Halpern HJ. Absolute oxygen R 1e imaging in vivo with pulse electron paramagnetic resonance. Magn. Reson. Med. 2014;72:362–368.
22. Zhao D, Jiang L, and Mason RP. Measuring Changes in Tumor Oxygenation. Methods Enzymol. 2004; 386: 378-418.
23. Deoni SCL, Rutt BK, Peters TM. Rapid combinedT1 andT2 mapping using gradient recalled acquisition in the steady state. Magn. Reson. Med. 2003;49:515–526.
24. Spencer RG., Fishbein KW. Measurement of Spin–Lattice Relaxation Times and Concentrations in Systems with Chemical Exchange Using the One-Pulse Sequence: Breakdown of the Ernst Model for Partial Saturation in Nuclear Magnetic Resonance Spectroscopy. J. Magn. Reson. 2000;142:120–135.
25. Ernst RR, Anderson WA. Application of Fourier Transform Spectroscopy to Magnetic Resonance. Rev. Sci. Instrum. 1966;37:93–102.
26. Gyngell ML. The steady-state signals in short-repetition-time sequences. J. Magn. Reson. (1969). 1989;81:474–483.
27. Carr HY. Steady-State Free Precession in Nuclear Magnetic Resonance. Phys. Rev. 1958;112:1693–1701.
28. Epel B, Halpern HJ. Comparison of pulse sequences for R1-based electron paramagnetic resonance oxygen imaging. J. Magn. Reson. 2015;254:56–61.
29. Bydder GM, Young IR. MR imaging: clinical use of the inversion recovery sequence. J. Comput. Assist. Tomogr. 1985;9:659–675.
30. Bydder GM, Young IR. Clinical use of the partial saturation and saturation recovery sequences in MR imaging. J. Comput. Assist. Tomogr. 1984;9:1020–1032.
31. Gupta RK. A new look at the method of variable nutation angle for the measurement of spin-lattice relaxation times using fourier transform NMR. J. Magn. Reson. 1977;25:231–235.
32. Matsumoto S, Utsumi H, Aravalluvan T, Matsumoto K, Matsumoto A, Devasahayam N, Sowers AL, Mitchell JB, Subramanian S, Krishna MC. Influence of protonT1 on oxymetry using Overhauser enhanced magnetic resonance imaging. Magn. Reson. Med. 2005;54:213–217.
33. Nelder J, Mead R. Errata. Comput. J. 1965;8:27–27.
34. Matsumoto A, Matsumoto S, Sowers AL, Koscielniak JW, Trigg NJ, Kuppusamy P, Mitchell JB, Subramanian S, Krishna MC, Matsumoto K. Absolute oxygen tension (pO2) in murine fatty

and muscle tissue as determined by EPR. Magn. Reson. Med. 2005;54:1530–1535.

**Figure Legends**

Figure 1. A. The structure of triarylmethyl probe Oxo71. The asterisks indicate deuterated methylene moieties. B. Schematic (left) and cross-section (right) of the three-tube phantom showing the tubes filled with spin probe solution as filled circles.

Figure 2. Dependence of $R_1$ on $pO_2$ for Oxo71. A. Phantom imaging FID signals acquired at TR = 12 μs. B. Steady state free precession FID profiles observed in phantom imaging at TR = 2.1 μs. Note the beginning of echoes at the short TR. The echo is truncated by the TR. C. Linear dependence of line width calculated from $R_2$* on $pO_2$. D. The spectral peak heights normalized to $M_0 = 1$ as a function of TR in the range 2.1–40 μs at 0%, 2%, 5%, and 10% $pO_2$ of a 2mM Oxo71 solution. Observed data are shown by the symbol × and continuous lines indicate the fit to Eq. [5]. Note the larger deviations at low TR values. E. The deviations are reduced at low TR if the echo contributions are included in the fit. F. Linear dependence of $pO_2$ on $R_1$.

Figure 3. The effect of spin and stimulated echoes at short repetition times for a phantom containing three tubes. A-C. TR = 3 μs and delay times of 1.0, 1.5, and 2.25 μs, respectively. D-E. Delay time = 1.75 μs and TR values of 3, 5, and 12 μs, respectively. The crosshairs are not part of the map. Notice multiple ghost 3-tube shapes of different sizes and orientations at TR = 3μs in C and D where two overlapping images are in normal orientation and two more are in inverted orientation at lower scales. These begin to disappear at TR = 5 μs and are fully absent at TR = 12 μs.

Figure 4. Phantom images calculated using data acquired at 22 TR values (3.4, 3.8, 4.2, 4.6, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10, 11, 12, 14, 16, 20, 25, 30, 35, and 40 μs). Scan time = 23 min. A-C. $M_0$, $R_1$, and $pO_2$ maps, respectively. D-F. Spin density, $R_2$*, and $pO_2$ maps calculated from 5 delay times of 1.0–1.4 μs at TR = 40 μs. Scan time = 3.2 min.

Figure 5. Phantom images calculated from signal intensities at 3 TR values (5, 8, and 12 μs). Scan time = 2 min. A-C. $M_0$, $R_1$, and $pO_2$ maps, respectively. D-F. Spin density, $R_2$*, and $pO_2$ maps calculated from 5 delay times of 1.0–1.4 μs at TR = 12 μs. Scan time = 1 min.

Figure 6. Histograms of $pO_2$ maps in the regions of tubes at $pO_2$ = 0%, 2%, and 5%. A. Calculated using $R_1$ derived from 22 TR values. B. Calculated using $R_1$ derived from 3 TR values. C. Calculated using $R_2$* derived from 5 points at TR = 40 μs. D. Calculated using $R_2$* derived from 5 points at TR = 12 μs. Scan time = 27 s.

Figure 7. In vivo $R_1$-based mapping of a mouse bearing a SCCVII tumor. A-C. $M_0$, $R_1$, and $pO_2$ maps calculated from signal intensities measured at 23 TR values (2.3, 3.2, 3.4, 3.8, 4.2, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10, 11, 12, 14, 16, 20, 25, 30, 35, and 40 μs). Scan time = 10.8 min. D-F. $M_0$, $R_1$, and $pO_2$ maps calculated using three TR values (4.2, 6, and 12 μs). Scan time = 52 s. The background is masked in all maps. A region of interest covering high $M_0$ is indicated by the dashed contour line in all the maps to allow comparisons.

Figure 8. Overview of $R_1$ and $R_2$* determination methods. Single point images $S(t_p)$ of a mouse leg bearing a SCCVII tumor calculated at TR = 4.2, 6, and 12 μs and $t_p$ = 0.85, 0.95, 1.05, 1.15 and 1.25 μs are shown as a matrix. The images in the columns are used to calculate $R_2$* and $S_0$ maps. The images in the rows are used to calculate $R_1$ and $M_0$ maps. The images at longest TR (column at 12 μs) and shortest $t_p$ (row at 0.85 μs) provide relatively better signal to noise.

Figure 9. *Left*: $R_2$* and $S_0$ calculated from single point image intensities $S(t_p)$ at TR = 4.2, 6, and 12 μs and $t_p$ = 0.85, 0.95, 1.05, 1.15 and 1.25 μs of a mouse leg bearing a SCCVII tumor. *Bottom right*: $M_0$ and $R_1$ calculated at $t_p$ = 0.85 μs. *Top right*: The $pO_2$ maps calculated from $R_2$* at TR= 12 μs and from $R_1$ calculated at $t_p$=0.85 μs. The relationship between $R_2$* and $pO_2$ is: $pO_2$ (mmHg) = 7.957 LW (μT) - 4.571 [oxo71] (mM) - 60.765. LW (μT) = 0.1257 $pO_2$ (mmHg) + 8.786, and LW (μT) = 0.5745 [oxo71] (mM) + 6.733, so the relationship between $R_1$ and $pO_2$ is $pO_2$ (mmHg) = 122.21 $R_1$(MHz) - 20.36.

Supporting Figure S1

The pixel intensity profiles as a function of TR. A. Phantom data in the 0% $pO_2$ region. B and C. In vivo data of a mouse tumor for the top 1000 pixels, before and

after probe abundance correction, respectively.

Supporting Figure S2

Overview of $R_1$ and $R_2$* determination methods. Single point images of a three-tube phantom calculated at TR = 5, 8, and 12 μs and $t_p$ = 1.0, 1.1, 1.2, 1.3 and 1.4 μs are shown in the black box. The rows above the box are the $R_2$* and $S_0$ maps calculated at the corresponding TR. Right, top row: The $pO_2$ map calculated from $R_2$* at TR= 12 μs and the $pO_2$ map calculated from $R_1$. Right, bottom row: $M_0$ and $R_1$ maps calculated at $t_p$=1.0 μs are indicated by the red arrow.

Supporting Figure S3

Bland-Altman plot comparing pixel to pixel $pO_2$ values of the phantom calculated using (*a*) 22 TR values and (*b*) 3 TR values. The ordinate is (*a*) – (*b*) and the abscissa is their mean.

Supporting Figure S4

Relationship between $R_1$ determined by SFR sequence and $R_2$* of Oxo71 as the $pO_2$ is varied from 0 – 10%.

Supporting Figure S5

A. Phantom $pO_2$ map calculated using data acquired at 12 TR values (4.2, 4.6, 5, 6, 7, 8, 10, 14, 20, 25, 30 and 35 μs). B. Histograms of $pO_2$ in the regions of tubes at $pO_2$ = 0%, 2%, and 5%. Scan time = 14 min.