NMR shutter-speed elucidates apparent population inversion of $^1$H$_2$O signals due to active transmembrane water cycling

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**Purpose:** The desire to quantitatively discriminate the extra- and intracellular tissue $^1$H$_2$O MR signals has gone hand-in-hand with the continual, historic increase in MRI instrument magnetic field strength [$B_0$]. However, recent studies have indicated extremely valuable, novel metabolic information can be readily accessible at ultra–low $B_0$. The two signals can be distinguished, and the homeostatic activity of the cell membrane sodium/potassium pump ($\text{Na}^+,\text{K}^+,\text{ATPase}$) detected. The mechanism allowing $^1$H$_2$O MRI to do this is the newly discovered active transmembrane water cycling (AWC) phenomenon, which we found using paramagnetic extracellular contrast agents at clinical $B_0$ values. AWC is important because $\text{Na}^+,\text{K}^+,\text{ATPase}$ can be considered biology’s most vital enzyme, and its in vivo steady-state activity has not before been measurable, let alone amenable to mapping with high spatial resolution. Recent reports indicate AWC correlates with neuronal firing rate, with malignant tumor metastatic potential, and inversely with cellular reducing equivalent fraction. We wish to systematize the ways AWC can be precisely measured.

**Methods:** We present a theoretical longitudinal relaxation analysis of considerable scope: it spans the low- and high-field situations.

**Results:** We show the NMR shutter-speed organizing principle is pivotal in understanding how trans–membrane steady–state water exchange kinetics are manifest throughout the range. Our findings illuminate an aspect, apparent population inversion, which is crucial in understanding ultra-low field results.

**Conclusions:** Without an appreciation of apparent population inversion, significant misinterpretations of future data are likely. These could have unfortunate diagnostic consequences.

**Key Words**
active water cycling, apparent population-inversion, shutter-speed
1 | INTRODUCTION

1.1 | Discerning tissue water compartmentalization

The major water compartmentalization in tissue is intra- and extracellular (“inside”/“outside”). In almost all parenchymal tissue, the vascular space comprises a small volume fraction. Thus, in the simplest approximation, this is a two site situation. A reliable way to quantitatively discriminate in vivo $^1$H$_2$O and $^1$H$_2$O$_o$ NMR signals has been a very long quest.

1.2 | Active transmembrane water cycling

Recently, this pursuit has gained much greater importance. It has been discovered that the pseudo-first-order rate constant for homeostatic cellular water efflux ($k_{io}$) has an energetically active component, $k_{io}(a)$, as expressed in Equation 1, and elaborated in Equation 2. The passive component, $k_{io}(p)$, is $\langle A/V \rangle P_W(p)$, where: $\langle A/V \rangle$ represents the voxel average or region of interest average (cell surface area/volume) ratio, and $P_W(p)$ is the diffusive (“passive”) cell membrane water permeability coefficient. In this study, quantities in brackets, $\langle \rangle$, represent voxel or region of interest averages. All symbols and acronyms are defined in the Appendix. It was previously thought $k_{io} = k_{io}(p)$; there was no active component. However, this is not the case: $k_{io}(a)$ is always present, and often dominant. 1-4

$$k_{io} = k_{io}(p) + k_{io}(a)$$ (1)

$$k_{io} = \left( \frac{A}{V} \right) P_W(p) + \left( \frac{x}{[H_2O]} \langle V \rangle \right)^{MR_{NKA}}$$ (2)

the voxel average or region of interest average (cell surface area/volume) ratio, and $P_W(p)$ is the diffusive (“passive”) cell membrane water permeability coefficient. In this study, quantities in brackets, $\langle \rangle$, represent voxel or region of interest averages. All symbols and acronyms are defined in the Appendix. It was previously thought $k_{io} = k_{io}(p)$; there was no active component. However, this is not the case: $k_{io}(a)$ is always present, and often dominant. 1-4 It is elaborated as $\langle x/[H_2O]\langle V \rangle \rangle^{MR_{NKA}}$, where $^{MR_{NKA}}$ is the cellular metabolic rate of the cell membrane Na$^+$,K$^+$-ATPase (NKA) (fmol(ATP)hydrolyzed/cell/s), [H$_2$O] is the intracellular water concentration, and $x$ is the stoichiometric mole ratio of water actively cycled to ATP hydrolyzed by NKA [fmol(H$_2$O)/fmol(ATP)]. Thus, an enzymatic activity generates a membrane permeability. Active transmembrane water cycling (AWC) is a fundamental aspect of water biology not previously described.

This is important because NKA can be considered biology’s most vital enzyme, but its in vivo homeostatic activity has never been measurable or amenable to mapping. 1-4 Significantly, it has been found that $k_{io}$ correlates with metastatic potential in breast 5 and melanoma 6 cell lines, and with neuronal firing in brain tissue. 4 This is a new imaging biomarker with potentially great power.

Previously, the main candidate techniques for $^1$H$_2$O/$^1$H$_2$O$_o$ signal discrimination required the use of an exogenous, extracellular paramagnetic contrast agent (CA$_o$) to increase the $^1$H$_2$O$_o$ $R_{1o}$ ($\equiv 1/T_{1o}$) value selectively. 3,7,8 This will be detailed below. Indeed, studies on model systems 1-4,9,10 in which constant [CA$_o$] ≥ 5 mM can be sustained during complete relaxation recovery measurement, were required to confirm the $\langle x/[H_2O] \langle V \rangle \rangle^{MR_{NKA}}$ term in Equation 2. However, this approach is problematic for in vivo human study. 3

Since the beginning of NMR, there has been a seemingly inexorable march to instruments with higher magnetic field (B$_0$) values. 11 This has been driven by the increased signal/noise ratio and spectral dispersion. However, this trend has not been particularly helpful for the discrimination of $^1$H$_2$O and $^1$H$_2$O$_o$. Even though CA detectability increases slightly with increasing B$_0$, 12 the diminished relaxivity ($r_1$) of approved CAs at current clinical B$_0$ values 13 requires large CA doses. Consequently, safety and environmental regulatory restrictions preclude achieving the high, sustained [CA$_o$] values sufficient for precise $k_{io}$ determination in vivo. 5 Recently, Aime and co-workers have demonstrated CA-free $^1$H$_2$O/$^1$H$_2$O$_o$ discrimination, again in an animal model and cell suspensions, at ultra-low B$_0$ values. 5,10 Here, we present a comprehensive analysis of the fundamental principles spanning the high and low field experiments.

2 | METHODS

2.1 | Intrinsic sample or voxel compartmental properties

There are two intrinsic NMR properties of interest: $R_{1i}$ and $R_{1o}$, $R_{1i}$ is the $^1$H$_2$O $R_1$ value. For CA-enhanced MRI, the $^1$H$_2$O$_o$ $R_1$ value is given by Equation 3,

$$R_{1i} = r_{1i} [CA_o] + R_{1o0}$$ (3)

where $r_{1o}$ is the extracellular CA longitudinal relaxivity [CA$_o$], the extracellular CA concentration, and $R_{1o0}$ the $R_1$ value in the absence of CA. In addition to being temperature–dependent, the $R_{1i}$, $r_{1o}$, and $R_{1o0}$ properties are also B$_0$–dependent.

There are two intrinsic cell biology properties of interest: $p_i$ and $k_{io}$. The quantity $p_i$ is the mole fraction (“population”) of water that is intracellular. The intracellular volume fraction, $\nu_i$, is given by Equation 4, where $f_M$ is

$$\nu_i = (1 - f_M) p_i + f_M$$ (4)

the tissue volume fraction inaccessible to mobile aqueous solutes. 3,8 When the vascular fraction is neglected, Equations 5a and 5b obtain, where $p_o$ and $\nu_o$ are the respective extracellular mole and volume fractions.

$$p_o = 1 - p_i$$ (5a)
The pseudo-first-order rate constant for homeostatic cellular water efflux is \( k_{io} \). (The parameter \( k_{io} \) is the reciprocal of the mean intracellular water molecule lifetime, \( 1/\tau_i \). The \( \tau_i \) value is often reported in the literature.) Because we assume a steady-state, \( k_{io} \) is also given by Equation 6, where \( k_{oi} \) is the corresponding influx rate constant. While still temperature-dependent, \( p_i \), \( f_M \), \( k_{io} \), and \( k_{oi} \) do not depend on \( B_0 \) or \( [CA_o] \).

Figure 1 illustrates the isothermal behavior of realistic, representative intrinsic NMR properties over a very large range. The ordinate measures \( R_1 \): the \( R_{1i} \) curves are blue, while the \( R_{1o} \) curves are red. The abscissa is bifurcated: the left side measures an increase in \( \log v_L \), the Larmor resonance frequency (proportional to \( \log B_0 \): the \(^1\)H magnetogyric ratio is 0.023 T/MHz), with \( [CA_o] = 0 \); while the right side measures an increase in \( [CA_o] \), with \( v_L \) constant at 43 MHz (\( B_0 = 1.0 \) T), the largest \( v_L \) reached on the left. The smooth \( R_{1i} \) and

\[
\nu_o = 1 - \nu_i. \tag{5b}
\]

\[
k_{io} = \left( \frac{p_o}{p_i} \right) k_{oi} \tag{6}
\]
$R_{1o}$ curves on the left pass through fitted values for a murine xenograft TS/A breast cancer tumor. They exhibit the familiar dispersive shape [an $R_1$ inflection point near 0.1 MHz (0.002 T)]. The $R_1 B_0$-dependence has long been referred to as NMR dispersion. The $R_{1o}$ values on the right are calculated using $r_{1o} = 3.8 \, s^{-1}(mM)^{-1}$, typical for approved Gd(III) chelates. All magnitudes are for $T = 37^\circ C$.

Figure 2 illustrates the behavior of the intrinsic cell biology properties: we take representative values of $p_i$ (0.8) and $k_{oi}$ (1 s$^{-1}$). The left ordinate measures the $p$ values ($p_i$, blue; $p_o$, red), while the right ordinate measures the overall exchange rate constant, $k$, given by Equation 7.

$$k = k_{io} + k_{oi} \tag{7}$$

the same as for Figure 1. Because these are isothermal plots, these properties exhibit horizontal lines.

### 2.2 Magnetic field-dependence

It has long been known the tissue $^1H_2O$ $R_1$ value increases with decreasing $B_0$ (reviewed in Rooney et al). In the absence of CAo, the $^1H_2O$ longitudinal relaxation mechanism is generally dominated by water intramolecular $^1H$ - $^1H$ magnetic dipole fluctuations at the $v_L$ frequency. As $B_0$ decreases, the $v_L$ value decreases toward the increased probability of experiencing such fluctuations (“spectral density”) found in tissue. In contrast, the $^1H_2O$ $R_1$ value of pure water is not very $B_0$-dependent: the inherent fluctuations have much greater frequency [reciprocal of the molecular rotational (“tumbling”) correlation time constant, $\tau_r^{-1} \approx 10^{12} \, s^{-1}$] than $v_L$, and thus are too fast for efficient relaxation. Therefore, it has long been suspected the inverse $R_1$, $v_L$ relationship is due to the presence of macromolecules in tissue. Macromolecular tumbling is much slower than that of the molecules in pure water. The fluctuation is $\tau_r^{-1} \approx 1.7/MW$, where MW is the macromolecular mass in kDa. (Thus, even a smallish 100 kDa macromolecule has $\tau_r^{-1} \approx 1.7 \times 10^7 \, s^{-1}$.) To our knowledge, there is no simple physical model that predicts the $B_0$-dependence of tissue $^1H_2O$ $R_1$. Any attempt to match data with, say, a superposition of Lorentzian functions requires an empirical distribution of $\tau_r$ values.

The extra- and intracellular tissue spaces both contain macromolecules, and one could not know for certain if extra- or intracellular macromolecules, or both, dominated the effect. One avoids this uncertainty by writing Equation 8 for iron-free tissue. In this expression: $r_{1M}$ is the tissue macromolecular relaxivity, and $R_{1H_2O}$ is the pure water $R_1$ at physiological temperature. (An extra term can be added if a tissue contains a sufficient amount of paramagnetic iron.) The greater $r_{1M}$ decrease with increasing $B_0$ compared with that of $r_{1o}$ is the cause of the aforementioned slight CA detectability increase.

However, Ruggiero and co-workers used a Matrigel phantom as a model for extracellular space. They found the $R_{1o}$

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**FIGURE 2** The non-dependences of the stipulated intrinsic compartmental mole fractions (“populations”), $p$, and intercompartmental exchange rate constant, $k$ (Equation 7), on: (left) the $B_0$ and (right) the [CAo]. The abscissa is the same as in Figure 1. The intracellular $p_i$ (blue) and extracellular $p_o$ (red) populations are measured on the left ordinate, while $k$ is measured on the right ordinate.
value, so approximated, exhibits only a very slight NMR dispersion, as seen in Figure 1 (left). This supports a long-held suspicion that the $R_1$, $B_0$-dependence is dominated by an increasing $R_{1i}$ value with decreasing $B_0$. This is attributed to sites for water molecules that are characterized as “buried” within macromolecules (H2O), and the surmise that such sites are more abundant in intracellular than in extracellular macromolecules. Water molecules in such sites can more fully experience the slower macromolecule rotation, and thus particularly effective slow intramolecular $^1$H–$^1$H fluctuations. Such macromolecules are endogenous, intracellular contrast agents, CAis. Although their concentrations do not increase with decreasing $B_0$, their relaxivities do. (Relaxivities and concentrations always appear together as products, as in Equation 3.) Nonetheless, the miniscule number of H2O molecules are still in rapid exchange (rate constant $> 10^4$ s$^{-1}$) with the vastly greater number of all other H2O molecules, certainly as compared with the $k_{io}$ magnitude. They could even use the Grothuss proton hopping mechanism. In any case, the cytoplasm is “well–mixed.” This is depicted in the Figure 3 cartoon. Some very large heterogeneous compartments (e.g., Xenopus oocyte [〈V〉 = 840 nL] cytoplasms) can exhibit inhomogeneous $^1$H2O resonances. However, most tissue cell (V) values range from hundreds of fL to a few pL. In such small cells, even a conservatively small diffusion coefficient leads to good water mixing in any NMR experimental time period.

The steady-state transmembrane water molecule exchange process, $k = k_{io} + k_{oi}$, is rate-limiting; i.e., slower than essentially all other water molecule interaction kinetics in tissue. And, this has been the source of considerable confusion in the in vivo MRI literature. If the exchange kinetics were exceedingly slow, or exceedingly fast, the interpretation of experimental $^1$H2O $R_1$ data would be straightforward. But slow and fast are rather misleading adjectives. Figure 2 shows the $k$ does not depend on $B_0$ or [CAo]. This is why we introduced the concept of the longitudinal NMR relaxation shutter-speed, $k_1$, the absolute value function defined in Equation 9. The comparison of $k$ with $k_1$

$$k_1 \equiv |R_{1i} - R_{1o}|$$

(9)
determines the “exchange condition” of the tissue $^1$H2O MR signal. If $k$ is insufficiently greater than $k_1$, a “slow” condition obtains: but if it is sufficiently larger, the system is in a “fast” condition. In experimental terms, a slow condition means the longitudinal relaxation is non-monoexponential, and a fast condition means the relaxation is monoexponential. The $k_{io}$ measurement precision depends on the extent $k_1$ exceeds $k$. Obviously, the greater the $k$ value, the larger the $k_1$ required.

As Figures 1 and 4 indicate, in biological tissue it is $k_1$ that can be manipulated by the investigator [by means of $B_0$ and/or [CAo]], usually not $k$ (almost all in vivo studies are isothermal). Because $k$ does not change, the exchange reaction does not go “faster” or “slower.” Using these terms is meaningful only if one thinks of $k_1$ changes as “warping” time. Thus, a slow condition is more profitably understood as a “large-shutter-speed” (LSS) condition; and a fast condition as a “small-shutter-speed” (SSS) condition. These distinctions are important because the nature of the exchange condition strongly influences the correct interpretation of the experimental result. This is described by the well-known Bloch–McConnell-Woessner (BMW) Rate Law Equations, which elaborate the phenomenological signal equation.

**FIGURE 3** A stylized cartoon depiction of the steady-state water exchange processes that dominate the tissue $^1$H2O MR signal longitudinal relaxation at ultra-low-field. The trans-cytolemmal process has a $k_{io}$ (Equation 2) and a $k_{oi}$ (Equation 6) rate constants. The exchange of water out of and into macromolecular buried sites, H2O, is much faster than $k = k_{io} + k_{oi}$. For the considerations here, cytoplasmic water is “well-mixed.” This figure was prepared with the help of Gangxu Han.
2.2.1 Apparent sample or voxel compartmental properties

The experimental relaxation decay is often fitted with an empirical bi–exponential expression, with apparent fast and slowly relaxing components that are not coupled by molecular exchange, Equation 10, where: $S$ can be a recovery time–course signal or a multi–pulse MR steady–state signal, $S_0$ is the Boltzmann signal, $p_{\text{fast}}$ and $p_{\text{slow}}$ the apparent populations, and $S_{\text{fast}}'$ and $S_{\text{slow}}'$ are fractional recovery or longitudinal and transverse relaxation saturation factor functions (running from 1 to 0). When transverse relaxation cannot be ignored, the saturation factor functions can be complicated. These are left implicit in Equations 11 and 12,

$$ S'/S_0 = p_{\text{fast}}' S_{\text{fast}}' + p_{\text{slow}}' S_{\text{slow}}' $$

(10)

signal, $S_0$ is the Boltzmann signal, $p'_{\text{fast}}$ and $p'_{\text{slow}}$ the apparent populations, and $S'_{\text{fast}}$ and $S'_{\text{slow}}$ are fractional recovery or longitudinal and transverse relaxation saturation factor functions (running from 1 to 0). When transverse relaxation cannot be ignored, the saturation factor functions can be complicated. These are left implicit in Equations 11 and 12,

$$ S_{\text{slow}}' = f \left( \text{time}, \alpha, TR, R'_{1,\text{slow}}, TE, R'_{2,\text{slow}} \right) $$

(12)

where time is the recovery period, and $\alpha$, TR, and TE are the steady-state acquisition pulse flip angle, repetition time, and echo time, respectively. (We have presented examples of recovery and longitudinal steady-state functions.) Each apparent component is defined to have single $R'_{1}$ ($R'_{1,\text{fast}}$, $R'_{1,\text{slow}}$) and complicated $R'_{2}$ ($R'_{2,\text{fast}}$, $R'_{2,\text{slow}}$) values.

However, in the vast majority of in vivo MR experiments, there is molecular exchange (steady–state) between the populations, and this makes the situation completely different from the empirical bi–exponential description. This is true even if, to simplify the subsequent derivation, we set $S'_{\text{fast}} = S'_{\text{slow}} = 1$. (As we will see below, however, this is probably, and importantly, not true for in vivo experiments, where multipulse acquisition, with incomplete recovery, is required for imaging purposes.) For nonimaging studies of model systems, $S$ can be measured with $\alpha = 90^\circ$ and complete recovery (large TR). Thus, the longitudinal saturation components in Equations 11 and 12 can be dealt with.

![FIGURE 4 The dependences of the stipulated MR shutter-speed, $k_1$ (Equation 9), and intercompartmental exchange rate constant, $k$ (Equation 7), on (left) the $B_0$ and (right) the $[CA_o]$. The abscissa is the same as in Figure 1, and the ordinate the same as on the right in Figure 2. The vanished shutter-speed VSS condition is indicated. The rate constant $k_{\text{in}}$ can be determined with precision only when $k_1 >> k$, the large shutter-speed regime LSSR period]
With these caveats, the apparent longitudinal relaxation rate constants, \( R_1' \), and longitudinally “fully relaxed” (“unsaturated”) apparent mole fractions, \( p' \), are expressed, in the isochronous \((v_{L0} = v_{Li})\) BMW two-site-exchange (2SX) exchange Equations 13 through 16, as functions of the intrinsic system parameters.\(^7\),\(^18\),\(^22\),\(^23\)

\[
R'_{1,\text{fast}} = \frac{1}{2} \left[ (R_{1i} + R_{1o}) + k \right] + \frac{1}{2} \left\{ \left[ (R_{1i} - R_{1o}) + (k_{oi} - k_{io}) \right]^2 + 4k_{oi}k_{io} \right\}^{1/2}
\]

(13)

\[
R'_{1,\text{slow}} = \frac{1}{2} \left[ (R_{1i} + R_{1o}) + k \right] - \frac{1}{2} \left\{ \left[ (R_{1i} - R_{1o}) + (k_{oi} - k_{io}) \right]^2 + 4k_{oi}k_{io} \right\}^{1/2}
\]

(14)

\[
p'_{\text{fast}} = \frac{1}{2} - \frac{1}{2} \left\{ \frac{(R_{1i} - R_{1o})(p_{io} - p_{i}) + k}{\left[ (R_{1i} - R_{1o}) + (k_{oi} - k_{io}) \right]^2 + 4k_{oi}k_{io}} \right\}^{1/2}
\]

(15)

\[
p'_{\text{slow}} = \frac{1}{2} + \frac{1}{2} \left\{ \frac{(R_{1i} - R_{1o})(p_{io} - p_{i}) + k}{\left[ (R_{1i} - R_{1o}) + (k_{oi} - k_{io}) \right]^2 + 4k_{oi}k_{io}} \right\}^{1/2}
\]

(16)

(These equations correct typographical errors in Equations 6 and 7 of Li et al.\(^7\). The term that was printed as \( p_i(1 - p_i)/\tau_i \) in Equation 6 should have been \( p_i/[(1 - p_i)\tau_i] \), and the square root should have been of the Equation 7 denominator.) Each apparent parameter has contributions from both analogous intrinsic parameters.

3 | RESULTS

It is very important to recognize the experimentally measured relaxation rate constants and mole fractions \((R_1'\) and \( p'\)) are not the same as the system \( R_1 \) and \( p \) values that are desired. We input the Figures 1 and 2 intrinsic parameter values into Equations 13-16 to “reverse engineer” typical parameters from experiments. This is illustrated in Figures 5 and 6, where expected \( R_1' \) and \( p' \) values, respectively, are plotted with abscissae identical to that of Figures 1, 2, and 4. The Figure 5 ordinate is identical to that of Figure 1, while the Figure 6 ordinate is identical to the Figure 2 left ordinate. The empirical labels are given outside the right ordinates: \( R_1'_{\text{fast}} \) (Equation 13) and \( R_1'_{\text{slow}} \) (Equation 14) for Figure 5, and \( p'_{\text{fast}} \) (Equation 15) and \( p'_{\text{slow}} \) (Equation 16) for Figure 6. In Figure 1, the \( R_{1i} \) and \( R_{1o} \) values cross (point): \( R_{1i,cross} = R_{1o,cross} = 1.1 \, s^{-1} \) at \([\text{CA}_0]\) = 0.17 mM. This would be the experimental result if there was no exchange: \( k = 0 \), the no-exchange-limit (NXL). However,
when exchange kinetics are finite this crossing is avoided (Figure 5). (See also Figure 3 of Bai et al.\textsuperscript{24} and Figure 4 of Labadie et al.\textsuperscript{25}) This result is a property of the “mixing” inherent in any coupled differential equations, such as those giving rise to Equations 13-16.

At the smallest $B_0$ (0.0002 T; $v_L = 0.01$ MHz) and largest $[CA_o]$ (4 mM) values simulated, when $k_1$ is maximally different from $k$ (Figure 4), the $R_{1,\text{fast}}$ and $R_{1,\text{slow}}$ values are just approaching the LSS limit (LSSL) condition ($k_1 \gg k$): $R_{1,\text{fast}} = R_{1i} + k_{io}$ (28 + 1 = 29 s$^{-1}$) on the left, and $R_{1,\text{fast}} = R_{1o} + k_{oi}$ (15 + 4 = 19 s$^{-1}$) on the right; $R_{1,\text{slow}} = R_{1o} + k_{oi}$ (2 + 4 = 6 s$^{-1}$) on the left, and $R_{1,\text{slow}} = R_{1i} + k_{io}$ (1 + 1 = 2 s$^{-1}$) on the right (Figure 5). However, more importantly, the $p'$ values (Figure 6) have clearly not yet reached the LSSL $p'$ values ($p_i = 0.8$, $p_o = 0.2$) (Figure 2). For in vivo human studies, it is currently not very practical to work near the earth’s field (~0.0001 T),\textsuperscript{26} and it is essentially disallowed to achieve $[CA_o]$ of even transiently 3 mM.\textsuperscript{27} Thus, realistic clinical MR examinations are constrained to never even approach the LSSL condition. The shutter-speed cannot be increased sufficiently.

Therefore, one must account for steady-state transcytolic water exchange kinetics [$k$] if one wishes to extract accurate system $p_i$ and $p_o$ values. This has been experimentally demonstrated for myocardium,\textsuperscript{28} where $p_o$ (extracellular volume fraction, ECF) is an extremely important biomarker.\textsuperscript{5}

Changes in $p_i$ and $p_o$ report tissue edema (a net intercompartmental water transfer) because the mean cell volume ($V$) = $v_i/p$, where $p$ is the cell (number) density (e.g., cells/$\mu$L): the relationship between $v_i$ and $p_i$ is given in Equation 4. Assuming either $k = \infty$ (as has frequently been done), or $k = 0$, is incorrect.

### 3.1 | Relaxation exponentiality

Figure 6 is very informative. It is unproductive to consider only the empirical $R_{1,\text{fast}}, R_{1,\text{slow}}, p_{1,\text{fast}}, p_{1,\text{slow}}$ parameters. What are important are the intrinsic parameters with which they correlate. Because we have simulated over such a wide range, we can assign segments of the apparent curves correctly (blue for $R_{1,\text{fast}}$ and $p_{1,\text{fast}}$; red for $R_{1,\text{slow}}$ and $p_{1,\text{slow}}$). When $k_1$ approaches zero, the faster relaxing apparent component vanishes ($p'_{1,\text{fast}} \to 0$; Figure 3 of Bai et al.\textsuperscript{24} Figure 2 of Vétek et al.\textsuperscript{29}), and the experimental relaxation time-course becomes monoexponential ($R_1'$ is single-valued). When $k_1$ is actually zero, the vanished shutter-speed (VSS, Figures 1, 4-6) condition, $R_1'$ is given by Equation 17.\textsuperscript{30}

\[
R_1' = p_o R_{1o} + p_i R_{1i} = R_{1,\text{cross}}.
\]

**FIGURE 6** With the intrinsic parameters from Figures 1 and 2, we calculated the dependences of the expected (longitudinally) fully relaxed apparent compartmental populations, $p'$ ($p_{\text{slow}}'$, Equation 16; $p_{\text{fast}}'$, Equation 15) on (left) the $B_0$ and (right) the $[CA_o]$. The $p_i$ and $p_o$ population segments are colored blue and red, respectively. The colors switch when the curves pass through the VSS condition. The abscissa is the same as in Figure 1, and the ordinate the same as on the left in Figure 2. An apparent population equality APE point is indicated, as is the region of apparent population inversion API, along with a conservatively small monoexponential relaxation regime. Unfortunately, current clinical MRI protocols fall within this latter region: the system is constrained to the vast shutter-speed “wasteland” exhibited in Figure 4.
The signal arises from all the water: the \( k_{io} \) parameter does not enter the equation, and thus is intrinsically indeterminate.

Practically speaking, however, it is hard to experimentally detect a small minority component even when it is present. We draw a horizontal line at \( p' = 0.1 \) in Figure 6. It strikes the blue \( p'_f \) curve on the left at \( v_L \approx 0.7 \left( v_L \approx 4.3 \text{ MHz; } B_0 \approx 0.1 \text{ T} \) and the red \( p'_{o} \) curve on the right at \( [CA_o] \approx 2.9 \text{ mM} \). Because all current human MR instruments have \( B_0 > 0.1 \text{ T} \) and (as suggested above) \( [CA_o] \) values > 3 mM cannot be sustained, clinical \(^1\text{H}_2\text{O} \) data are constrained to exhibit apparent monoexponential longitudinal relaxation (single-valued \( R'_f \)).

The accessible shutter speeds are too small (Figure 4): clinical MRI is trapped in a vast shutter-speed “wasteland.” This regime indicated is conservatively small: it can be experimentally difficult to detect a minority component even with \( p' \) somewhat greater than 0.1.

To extract \( k_{io} \) and \( p_i \) when the relaxation is effectively monoexponential, when one is in the wasteland, one must vary \( [CA_o] \) (as pharmocokinetically, after a bolus injection) or – now - \( B_0 \), and take advantage, to the extent possible, of the nonlinear \( R'_{1,slow} \) – \( [CA_o] \)- or \( B_0 \)-dependence in this regime (Figure 5). Neglecting the \( p'_{fast} \) (\( R'_{1,fast} \)) contribution is the most common shutter-speed (dynamic-contrast-enhanced) DCE–MRI version [fast-exchange-regime-allowed (FXR-a)].\(^{1,7,8,17,27,31}\) One can see (Figure 6) the \( p'_{fast} \) term is mostly vanished by the exchange effect. However, precision can be poor, and \( k_{io} \) indeterminate in circumstances of insufficient CA extravasation, negligibly small \( [CA_o] \), as in the normal-appearing brain.\(^3\)

We neglect potential non-monoexponential contributions from vascular \(^1\text{H}_2\text{O} \) or magnetization transfer (MT) from macromolecular \(^1\text{H} \) resonances.\(^{32}\) The model study systems are avascular, or effectively so. In vivo, the vascular contribution is generally limited to the initial portion of the DCE time-course.\(^8\) Also, the low \( \alpha, \) on–resonance RF pulse in a 3D distribution is generally limited to the initial portion of the DCE time-course.\(^8\) Also, the low \( \alpha, \) on–resonance RF pulse in a 3D imaging acquisition is generally not very MT sensitive.\(^{33,35}\)

The contention that \( k_{io} \) can never be accessed by DCE-MRI is predicated on the supposed longitudinally fully relaxed \( p'_{fast} \) and \( p'_{slow} \) contributions.\(^{36}\) The longitudinal saturation expressions (the \( \alpha, \) TR, \( R'_{1,fast} \), and \( R'_{1,slow} \) functions of Equations 11 and 12 have been assessed (Figure S1 of Li et al\(^{36}\); Figure 6 of Buckley\(^{36}\)). If not unity, these weight \( p'_{fast} \) disproportionately relative to \( p'_{slow} \). That is, they make \( S'_{fast} \) and \( S'_{slow} \) each less than unity, but \( S'_{fast} > S'_{slow} \). However, taking this into account and forcing data fittings with the fully longitudinally relaxed biexponential expressions (Equations 10-16) has been found to introduce unacceptable systematic errors, cause \( k_{io} \) to become artificially indeterminate,\(^7,31\) and sometimes to make fittings poorer.\(^36\) When the two components cannot be experimentally discriminated and separately fitted, the \( R'_{1,fast} \) and \( R'_{1,slow} \) \( B_0 \)- and \( [CA_o] \)-dependences tend to counteract one another (the “avoided crossing,” Figure 5), and thus reduce \( k_{io} \) influence (Equations 13 and 14). It seems the \( p'_{slow} \) contribution is disproportionately (essentially exclusively) acquired. So, we consider the transverse saturation expressions (the TE, \( R'_{2,fast} \), and \( R'_{2,slow} \) functions of Equations 11 and 12). In DCE, TR is often < 5 ms, so TE must be very small. Although the saturation expressions can be very complicated,\(^{33,35}\) an infinitely small TE is equivalent to \( R'_{2,fast} = R'_{2,slow} = 0 \). We have proposed, however, it is quite plausible that \( R'_{2,fast} \) and \( R'_{2,slow} \) are sufficiently nonzero due to magnetic susceptibility gradients.\(^7,31\)

During the bolus CA passage, there are significant paramagnetic CA concentration gradients across capillary walls and cell membranes. Plasma [CA] can exceed 5 mM immediately upon CA arrival in the tissue.\(^{27,36}\) Susceptibility gradients due to such concentration differences have been shown to significantly increase \( R' \) values.\(^{37}\) The fully relaxed \( p'_{fast} \) contribution has already been rendered much smaller than \( p'_{slow} \) by exchange (it vanishes in the VSS condition), Figure 6. Thus, even if \( R'_{2,fast} \) and \( R'_{2,slow} \) are equally elevated, it is easy to imagine the fortuitous consequence that \( p'_{fast} \) is completely saturated (“quenched”), leaving partially saturated \( p'_{slow} \) as the meaningful component. Whatever the mechanism, considerable experimental evidence has accumulated that \( k_{io} \) can be usefully estimated in many DCE–MRI experiments (see the Discussion section).

A truly noninvasive diffusion-weighted imaging (DWI) analysis that does not require a CA or a shutter–speed shows considerable promise in determining \( k_i \).\(^3\) This novel DWI approach works at clinical \( B_0 \) values, seems to measure large \( k_{io} \) values with more precision, and allows separation of the irreducible \( v_i \) cell biology factors, \( \rho \) and \( \langle V \rangle \). These pathology-related properties are very important in their own right, and in discriminating the 2 Equation two terms to access \( x^i \text{MR}_{\text{NKA}} \) itself: \( \rho \) and \( \langle V \rangle \) are not accessible with DCE-MRI.\(^3\)

### 3.2 Apparent Population Inversion

Somewhere below 0.1 T, one can begin to detect non-monoexponential \( T_1 \) relaxation caused by \( k_{io} \).\(^5,10\) Figure 6 exhibits further interesting features. When the field value is small enough to observe apparent biexponential relaxation, but not yet as small as ~0.01 T, the apparent minority component does not extrapolate to the true minority component, \( p_0 \) (red), but to the true majority component, \( p_i \) (blue). Between \( B_0 \approx 0.01 \text{ T and the VSS, there is an apparent population inversion (API). This has been noted previously (Figure 4 of Lee and Springer\(^{38}\)). If one conducts only an empirical biexponential analysis (Equations 10-12) of such experimental data, one would find the minority component (blue \( p' \) in Figure 6) has the faster relaxation (blue \( R' \) in Figure 5). If the apparent minority \( p' \) value is near 0.2, as is quite likely, one would be tempted to incorrectly assign it to \( ^1\text{H}_2\text{O}_o \) or any population other than \( ^1\text{H}_2\text{O}_o \), because \( p_i \) is commonly understood to be
near 0.8. This is a common problem with the inappropriate application of an empirical biexponential analysis to data that do not have an intrinsic biexponential nature.\(^{39}\) The condition of apparent population equality (APE) (\(p_i' = p_o' = 0.5\); log \(v_L \approx -0.35\) in Figure 6; \(v_L = 0.43\) MHz, \(B_0 = 0.01\) T), and thus API, occurs only when the true majority component (\(p_i\) here) has the faster relaxation (\(R_1i\) on the left), and at the point given by Equation 18 (derived from Equations 15 and 16), where the \(\kappa_i\) argument (\(\arg \kappa_i\)) is \((R_1i - R_1o)\).

\[
\arg \kappa_{i, APE} = \kappa / (p_o - p_i).
\] (18)

4 | DISCUSSION

4.1 | Why does API happen?

The curves in Figures 5 and 6 are generated from the BMW 2SX Equations 13-16. On their RHSs, the shutter-speed argument \((R_1i - R_1o)\) appears in many places, and it plays a pivotal role. It is the only factor that changes sign from the left to the right of Figures 5 and 6: being positive on the left, and negative on the right. As a consequence, it is always the population with the apparent faster relaxation that vanishes as the system approaches the VSS condition (two vertical dashed lines in Figures 5 and 6), which is where \(\arg \kappa_1\) changes sign. This behavior can be seen graphically in simulated (Figure 3 of Lee and Springer\(^{38}\)) and experimental (Figure S1 of Zhang et al\(^{9}\)) decay curves, and can be derived from Equations 15 and 16. Thus, if the VSS is approached from the left in Figure 6, by increasing \(B_0\), it is \(p_i'\) that goes to zero. On the other hand, if the VSS is approached from the right, by decreasing \([CA_o]\), it is \(p_o'\) that goes to zero. Our simulations here are all for \(k = 5\) s\(^{-1}\). However, the rate of vanishing does depend on \(k\): all other parameters held fixed, the greater the \(k\) the more shallow the vanishing.\(^{24}\) As a corollary, when a system passes through the VSS condition, the assignments of the apparent relaxation rate constants (\(R'_i, \bar{s}\)) and populations (\(p'_s\))

| \(k_{io}\) increases with: | \(k_{io}\) decreases with: |
|---------------------------|---------------------------|
| Increased NKA pump expression | Ouabain NKA pump inhibition |
| Increasing cytoplasmic ATP | Increasing [\(K_o^+\)] at sufficient [\(K_o^+\)] to cause membrane depolarization |
| Increasing \([K_o^+\)] (at low \([K_o^+\])\, with an NKA Michaelis-Menten signature | WZB117 glucose uptake inhibition |
| Hypoxia | \(O_2 \rightarrow N_2\) switch |
| Cisplatin-induced apoptosis | Extracellular tetrodotoxin voltage-gated sodium channel inhibition |
| Xenograft tumor apoptotic regions | Extracellular AP5 plus DNQX post-excitatory neuronal activity inhibition |
| Human brain metastasis radiosurgery | Glutamine deprivation |
|                          | Hypertension in myocardium |
|                          | Chemotherapy of human breast tumors |
|                          | Phosphatase activation breast tumor therapy |

| \(k_{io}\) correlates with: |
|-------------------------|
| Tumor metastatic potential |
| Neuronal firing |
| Oxidative phosphorylation rate |
| \(O_2\) consumption rate |
| Head and neck cancer mortality |
| \(^{18}\)Fuorou-2-deoxy-D-glucose breast tumor uptake |

ATP, adenosine triphosphate; \(k_{io}\), water efflux k (1/\(\tau_i\); NKA, \(Na^+,K^+-ATPase\) (sodium pump).

*For yeast, pump is PMA1, inhibitor is ebselen.

#Employed shutter-speed (\(\kappa_i\)) dynamic-contrast-enhanced-MRI.

\(^{(2R)}\)Amino-5-phosphonovaleric acid plus 6,7-dinitroquinoxaline-2,3-dione.

Indirect.
must be switched; from blue to red and vice versa in Figure 6. This can be observed in experimental data (Figure 3 of Zhang et al).\(^{40}\)

The deviation of an observed p’ from its corresponding inherent compartmental p does have a physical basis. There are subcompartmental spin populations with different diffusion (phase diagram)\(^{41}\) and/or exchange\(^{31}\) histories. Thus, these can have different \(R_1\) values, but surely they exist in continua.\(^{42}\) Given these complications, the emphasis must remain on the inherent p values, which enjoy the well-mixed attribute, and these can be extracted only with 2SX analyses of experimental data. Furthermore, this is also the only way to determine \(k_{io}\) with a shutter-speed experiment.

### 4.2 Implications

Many results indicate the new metabolic \(k_{io}\) biomarker can be very powerful. As befitting the crucial NKA role in intermediary metabolism, \(k_{io}\) has been reported responsive to several different metabolic alterations (Table 1). For instance, entries a, d, l, and t suggest that \(k_{io}\) reports from ground zero of the oncogenic transformation: it may increase because of the very ion transporter up-regulation that triggers K-Ras/rapidly accelerated fibrosarcoma kinase/mitogen-activated protein kinase signaled uncontrolled cell proliferation.\(^3\) This suggests its potential for early cancer detection. These consequences are surely due to the MR\(^2\)NKA contribution to \(k_{io}(a)\). Because of its vital nature, MR\(^2\)NKA is likely to be altered in most, if not all, pathologies. Another example is systemic multiple sclerosis.\(^{52}\)

The Table 1 entries arising from DCE-MRI estimation are clearly marked with a superscript #. It is important to note that entries d and t show DCE-MRI results that have been validated with more precise ultra-low field model experiments not subject to the DCE uncertainties.

Damadian’s early ex vivo 0.6 T \(^1\)H\(_2\)O MR study to discriminate malignant and normal tissue\(^{55}\) was cited by Lauterbur\(^{56}\) as part of the motivation for developing MRI.\(^{57}\) The fact that subsequent research showed sensitivity and specificity are insufficient for robust cancer detection has been one of the major drivers for the seemingly inexorable increase in \(B_0\) strength (moving to the right in the figures) in clinical MRI.\(^{11,58}\) So, particularly the fact that Ruggiero and co-workers’ results (although nonimaging) were obtained at very small \(B_0\) values, with a fast field cycling study (modest detection \(B_0\)) of an in vivo murine xenographic tumor model,\(^3\) will stimulate renewed interest in ultra-low field MRI (moving to the left in the figures). It is exciting to find AWC is the molecular process that dominates the ultra-low field \(^1\)H\(_2\)O signal.

Ruggiero and co-workers’ finding\(^5\) that it is mainly the \(k_{io}\) increase with malignancy that leads to decreasing \(R_1/p\) provides an explanation for Damadian’s classic observation of the latter phenomenon.\(^{55}\) It suggests his ex vivo NMR acquisitions within five min of rat euthanasia were soon enough to retain most of the in vivo cellular ATP. Also, the increase of \(k_{io}\) with concomitant \(p\) increase in malignancy\(^5\) is consistent with metabolic competition between cancer cells.\(^3\) The greater the cell density, \(p\), the slower the NKA activity per cell.

There has been only a small amount of fast field cycling work in human studies: a recent report at 0.06 T has been published.\(^{59}\) However, significant efforts to produce such human-sized instruments are under way. The same is true for “portable” low-field scanners, with possibly \(B_0 < 0.1\) T (Garwood MG, personal communication).\(^{60}\) These could become very valuable metabolic instruments.

Figure 6 illustrates, however, that \(B_0\) values sufficiently small to access the LSSL condition are quite unlikely to be reached. This emphasizes the importance of the BMW 2SX analysis detailed here. Otherwise, the bi-exponential relaxation results that will be obtained can be very easily misinterpreted. This would cause quite unfortunate confusion, and actually represent a medical set-back.

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**APPENDIX: ACRONYMS AND SYMBOLS**

- APE: apparent population equality
- API: apparent population inversion
- ATP: adenosine triphosphate
- (A/V): mean cell area/volume ratio
- AWCI: active water cycling
- α: read pulse flip angle
- B0: main magnetic field
- BMW: Bloch-McConnell-Woessner
- CA: contrast agent
- CAi: intracellular CA
- CAo: extracellular CA
- [CAo]: CAo concentration
- DCE: dynamic-contrast-enhanced
- DWI: diffusion-weighted imaging
- ECF: tissue extracellular volume fraction
- FXL: fast-exchange limit
- FXR: fast-exchange-regime
- fM: tissue macromolecular volume fraction
- H2Obu: buried water
- H2Oi: intracellular water
- H2Oo: extracellular water
- [H2Oi]: H2Oi concentration
- k: steady-state water exchange rate constant
- kio(a): active kio contribution
- kio(p): passive kio contribution
- koi: water influx k
- K-Ras: Kirsten rat sarcoma virus oncogene
- kL: longitudinal MR shutter-speed (SS)
- LSS: large SS condition (formerly, SXR)
- LSSL: large SS limit (formerly, SXL)
- sMRNKA: cellular NKA metabolic rate
- MW: molecular mass
- NKA: Na+,K+-ATPase (sodium pump)
- NXL: no-exchange-limit
- ν0L: Larmor frequency (often ν0)
- νLi: intracellular ν0
- νLo: extracellular ν0
- p: tissue water mole fraction (“population”)
- p0: intrinsic H2O p
- p0: intrinsic H2O p
- p′: apparent p
- p′fast: fast-relaxing component p′ (formerly, aS)

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\[ p'_s \quad H_2O, p' \]
\[ p'_{\text{slow}} \quad \text{slow-relaxing component } p' \quad (\text{formerly, } a_i) \]
\[ p'_{\text{o}} \quad H_2O_o, p' \]
\[ \text{PMA1} \quad \text{plasma membrane } H^+\text{-ATPase} \]
\[ P_W \quad \text{membrane water permeability coefficient} \]
\[ P_W(p) \quad \text{passive } P_W \]
\[ R_1 \quad \text{longitudinal relaxation rate constant} \]
\[ R_{1,\text{cross}} = R_{1i} = R_{1o} \]
\[ R_{1i} \quad \text{intrinsic } ^1H_2O_i R_1 \]
\[ R_{1o} \quad \text{intrinsic } ^1H_2O_o R_1 \]
\[ R_{1o0} \quad R_{1o} \text{ in the absence of } CA_o \]
\[ R'_1 \quad \text{apparent, approximate single } R_1 \text{ value} \]
\[ R'_{1,\text{fast}} \quad \text{fast-relaxing component } R'_1 \quad (\text{formerly, } R_{1S}) \]
\[ R'_{1i} \quad \text{apparent } R_{1i} \]
\[ R'_{1o} \quad \text{apparent } R_{1o} \]
\[ R'_{1,\text{slow}} \quad \text{slow-relaxing component } R'_1 \quad (\text{formerly, } R_{1L}) \]
\[ R_2 \quad \text{transverse relaxation rate constant} \]
\[ R'_2 \quad \text{apparent } R_2 \]
\[ R'_{2,\text{fast}} \quad \text{fast-relaxing component } R'_2 \]
\[ R'_{2,\text{slow}} \quad \text{slow-relaxing component } R'_2 \]
\[ \text{RHS} \quad \text{right-hand side} \]
\[ r_i \quad \text{longitudinal relaxivity} \]
\[ r_{\text{IM}} \quad \text{macromolecular } r_i \]
\[ r_{10} \quad \text{CA}_o r_i \]
\[ \rho \quad \text{cell (number) density} \]
\[ S \quad \text{tissue } ^1H_2O \text{ signal strength} \]
\[ S_0 \quad \text{Boltzmann } S \]
\[ S_{\text{fast}} \quad \text{apparent fast-relaxing saturation factor} \]
\[ S_{\text{slow}} \quad \text{apparent slow-relaxing saturation factor} \]
\[ S_{\text{SS}} \quad \text{shutter-speed } (K_i) \]
\[ S_{\text{SSS}} \quad \text{small SS condition (formerly, } FXR) \]
\[ S_{\text{SXL}} \quad \text{slow-exchange-limit} \]
\[ S_{\text{XLR}} \quad \text{slow-exchange-regime} \]
\[ S_{\text{TE}} \quad \text{pulse sequence magnetization echo time} \]
\[ S_{\text{TR}} \quad \text{pulse sequence repetition time} \]
\[ T_1 \quad \text{longitudinal relaxation time constant} \]
\[ T_{1o} \quad \text{intrinsic } ^1H_2O_o T_1 \]
\[ \tau_i \quad \text{mean } H_2O_i \text{ molecule lifetime (1/k_{io})} \]
\[ \langle V \rangle \quad \text{mean cell volume} \]
\[ V_{\text{SS}} \quad \text{vanished SS condition (formerly, } FXL) \]
\[ V \quad \text{tissue volume fraction} \]
\[ v_i \quad \text{intracellular } v \]
\[ v_0 \quad \text{extracellular } v \quad \text{(formerly, ECF)} \]
\[ 2SX \quad \text{two-site-exchange} \]