Impact of Transcytolemmal Water Exchange on Estimates of Tissue Microstructural Properties Derived from Diffusion MRI

Hua Li,¹,² Xiaoyu Jiang,¹,² Jingping Xie,¹,² John C. Gore,¹,²,³,⁴,⁵,⁶ and Junzhong Xu¹,²,³,⁴,⁵,⁶*

**Purpose:** To investigate the influence of transcytolemmal water exchange on estimates of tissue microstructural parameters derived from diffusion MRI using conventional PGSE and IMPULSED methods.

**Methods:** Computer simulations were performed to incorporate a broad range of intracellular water life times $\tau_{in}$ (50–$\infty$ ms), cell diameters $d$ (5–15 µm), and intrinsic diffusion coefficients $D_{in}$ (0.6–2 µm$^2$/ms) for different values of signal-to-noise ratio (SNR) (10 to 50). For experiments, murine erythroleukemia (MEL) cancer cells were cultured and treated with saponin to selectively change cell membrane permeability. All fitted microstructural parameters from simulations and experiments in vitro were compared with ground-truth values.

**Results:** Simulations showed that, for both PGSE and IMPULSED methods, cell diameter $d$ can be reliably fit with sufficient SNR ($\geq$ 50), whereas intracellular volume fraction $f_{in}$ is intrinsically underestimated due to transcytolemmal water exchange. $D_{in}$ can be reliably fit only with sufficient SNR and using the IMPULSED method with short diffusion times. These results were confirmed with those obtained in the cell culture experiments in vitro.

**Conclusion:** For the sequences and models considered in this study, transcytolemmal water exchange has minor effects on the fittings of $d$ and $D_{in}$ with physiologically relevant membrane permeabilities if the SNR is sufficient ($\geq$ 50), but $f_{in}$ is intrinsically underestimated. Magn Reson Med 77:2239–2249, 2017. © 2016 International Society for Magnetic Resonance in Medicine

**Key words:** diffusion; transcytolemmal water exchange; cell size; intracellular; diffusion time; OGSE; MRI

INTRODUCTION

Diffusion-weighted MRI provides a noninvasive means to probe biological tissue microstructure, and has been used widely for the diagnosis of neurodegenerative diseases and cancer (1). Apparent diffusion coefficients (ADC) obtained from conventional diffusion MRI measurements report the overall averaged diffusion properties of biological tissues. ADC has been found to be sensitive to variations of a variety of tissue properties, including but not limited to cell size (2), cell membrane permeability (3), intra- and extracellular diffusion coefficients (4), and intracellular volume fraction (5). When diffusion times are short enough (such as those obtained using oscillating gradient spin echo (OGSE) sequences at moderately high gradient frequencies (6)), ADC can be sensitive to intracellular microstructural variations such as the sizes of nuclei (7) and organelles (8). Therefore, ADC provides a powerful approach to probe a variety of tissue features from sub- to supracellular scales if appropriate diffusion times are used. However, specific microstructural changes usually cannot be determined directly from ADC measurements alone. In many cases, the detection of specific microstructural parameters such as cell size and intracellular volume fraction may provide additional and sometimes more useful diagnostic information (9). Hence, there is a need to develop quantitative diffusion MRI methods to derive more specific microstructural changes from experimental measurements.

To date, various diffusion MRI methods have been developed for quantitative characterization of tissue microstructure. Q-space imaging provides a means to measure apparent compartment sizes (2), but tends to bias the cell size because of the inability to distinguish contributions from intra- and extracellular spaces (10) and the violation of the short-gradient pulse condition (11). If the transcytolemmal water exchange between intra- and extracellular spaces is slow enough to be ignored, the overall diffusion signals can be assumed to represent simply the sum of signals from each space. This provides an opportunity to decouple intra- and extracellular signals, and thereby to measure specific microstructural parameters. The AxCaliber method, based on the CHARMED model that assumes cylindrical compartments with hindered diffusion in the extracellular space (with a constant diffusion coefficient), has been implemented to measure axon size distributions in animals in vitro (12) and in vivo (13), and humans in vivo (14). The ActiveAx method was developed to measure indices of mean axon diameter instead of a diameter...
distribution for better clinical feasibility (15,16). In addition to these conventional pulse gradient spin echo (PGSE) based methods, double-PGSE (17) and oscillating gradient spin echo (OGSE) (18) sequences have also been developed for quantitative characterization of cell size and intracellular volume fraction. Recently, a temporal diffusion spectroscopy (TDS) based approach (19) has been developed to combine PGSE (long diffusion times) and OGSE (short diffusion times) acquisitions to cover a broader range of effective diffusion times for better quantitative characterization of microstructure at different length scales simultaneously, i.e., from intracellular diffusion effects up to the impact of cell size and spacing. We term this new experimental approach as the imaging microstructural parameters using limited spectrally edited diffusion (IMPULSED) method.

All of the models used to interpret data from these quantitative diffusion methods assume that transcytolemmal water exchange between intra- and extracellular spaces can be ignored. This assumption is usually believed to be true in neural tissues, because the typical diffusion time $t_D$ in PGSE measurements is $<100$ ms, smaller than the intracellular water lifetime $\tau_{in}$ of brain tissues in vivo, reported as $622 \pm 29$ ms (20) or $\sim550$ ms (21). However, some studies have suggested that the “slow” water exchange still significantly affects MRI measurements of nerves in vitro (22) and in vivo (23). Our recent study indicates that the influence of transcytolemmal water exchange on ADC measurements can be ignored only when $t_D$ is at least one order of magnitude smaller than $\tau_{in}$ (24). More complicatedly, transcytolemmal water exchange typically increases significantly, e.g., lesions caused by Parkinson’s (25) and Alzheimer diseases (26), and tumors (27) especially in apoptotic regions (28). This raises concerns about whether the estimates of microstructural parameters are biased by transcytolemmal water exchange. Previous computer simulations have suggested that the estimated intracellular volume fraction may be underestimated in the case of short exchange times (29,30). However, comprehensive studies have not been reported up to date to investigate the influence of transcytolemmal water exchange on the accuracy of fitted microstructural parameters obtained using quantitative diffusion MRI methods.

The present work aims to address these concerns. Diffusion MRI sequences using different diffusion times were investigated. Computer simulations and cell culture studies in vitro were performed, and the cell membrane permeability was selectively changed without affecting other cellular microstructure, to evaluate the precise effect of transcytolemmal water exchange on fitted microstructural parameters. In addition, the influences of signal-to-noise ratio (SNR) and the choices of pre-assigned $D_{in}$ used in the fittings were also studied to evaluate their effects on the fitted parameters.

METHODS

Theory

Diffusion MRI signals were modeled as the sum of signals from intra- and extracellular spaces without water exchange between them, namely,

$$S = f_{in} \cdot S_{in} + (1 - f_{in}) \cdot S_{ex}$$  \[1\]

where $f_{in}$ is the water fraction of intracellular space, and $S_{in}$ and $S_{ex}$ are the signal magnitudes from intra- and extracellular spaces, respectively. Note that $T_2$ relaxation is not considered here. For simplicity, tissues were modeled as tightly packed, spherical cells with an effect mean cell diameter $d$. The analytical expressions to predict diffusion signals of intracellular water restricted by spheres have been reported previously (31). The expression for extracellular water depends on the effective diffusion time, as detailed subsequently. Note that Eq. [1] is used widely for various diffusion methods (15,18,32).

After the diffusion signals were obtained from simulations or experiments, all diffusion MRI signals were fit to Eq. [1]. By comparing the fitted values and ground truth, the effect of transcytolemmal water exchange on quantitative characterization of tissue microstructure can be evaluated. Three diffusion methods—PGSE_I, PGSE_II, and IMPULSED—were investigated.

**PGSE_I and PGSE_II Methods**

The analytical expression for $S_{in}$ using PGSE acquisitions based on the Gaussian phase approximation is given by

$$S_{in}(\text{PGSE}) = S_{0,in} \cdot \exp \left\{ -\sum_k \frac{2B_k \gamma^2 g^2}{\kappa^2 D_{in}^2} \times \left\{ \lambda_k D_{in} \delta - 1 + \exp (-\lambda_k D_{in} \delta) \right\} \right\}$$  \[2\]

where $S_{0,in}$ is the $T_2$-weighted intracellular signal, $\gamma$ is the gyromagnetic ratio, $\delta$ is the gradient separation, $g$ is the gradient strength, $D_{in}$ is the intracellular free diffusion coefficient, and $\lambda_k$ are cell diameter $d$ dependent parameters (19,31).

Previous analyses of PGSE data (12–16) usually assume that the extracellular diffusion is hindered (i.e., extracellular diffusion coefficient is a constant independent of diffusion time), and therefore

$$S_{ex}(\text{PGSE}) = S_{0,ex} \cdot \exp (-b \cdot D_{ex}).$$  \[3\]

Note that the accuracy of this approximation has been questioned previously (33,34). Nevertheless, Eq. [3] was used to describe extracellular diffusion to follow the current, widely used approaches.

Altogether, four independent parameters may be fit using this method: $d$, $f_{in}$, $D_{in}$, and $D_{ex}$. We term this combination of measurement and analysis as the PGSE_I method. It has been reported that PGSE acquisitions with relatively long diffusion times have low sensitivity to intracellular features (6,7); therefore, previous studies have assigned $D_{in}$ to an empirical constant value in fittings to reduce the number of free parameters (9,13,15,16). In this case, three free parameters may be fit: $d$, $f_{in}$, and $D_{ex}$, and we term this as the PGSE_II method. Note that the influence of different choices of $D_{in}$ has not been fully investigated before, so we studied this influence on fitted microstructural parameters in the simulations in the present work.
IMPULSED Method

We previously proposed a new experimental IMPULSED method. In this method, PGSE acquisitions are used to obtain data with a long diffusion time $t_D$ (eg, 52 ms), and acquisitions using oscillating gradients with two or more frequencies are used to sample other regions of the diffusion spectrum with shorter diffusion times (where the oscillating frequency $f$ is related to the effective diffusion time as $t_D = 1/(4f)$ for cosine-modulated waveforms). In this way, it covers a broader range of diffusion times and therefore provides a more comprehensive sensitivity to different length scales (19). The analytic expressions for intracellular cosine-modulated OGSE signals have been derived (31) and validated with phantoms (35) previously as

$$S_{in}(OGSE) = S_{0, in} \cdot \exp \left\{ -2\gamma^2 g^2 \sum_k \frac{B_k \lambda_k^2 D_{in}^2}{(\lambda_k^2 D_{in}^2 + 4\pi^2 f^2)^2} \right\}$$

$$\times \left[ \frac{\delta(\lambda_k^2 D_{in}^2 + 4\pi^2 f^2)}{2\lambda_k D_{in}} - 1 + \exp (-\lambda_k D_{in} \delta) + \exp (-\lambda_k D_{in} \Delta (1 - \cosh \lambda_k D_{in} \delta)) \right\}, \quad [4]$$

where $f$ is the diffusion gradient frequency.

When the experimental diffusion times are relatively short, the extracellular diffusion usually cannot be assumed as a $t_D$-independent constant. Because of the complexity of extracellular structure, it is challenging to derive a general analytical equation to describe the diffusion spectra arising from the extracellular space. An empirical model for extracellular diffusion has been proposed recently to describe packed cylindrical axons (36). However, previous studies have found that if only a narrow range of relatively low gradient frequencies is used (such as 0, 40, and 80 Hz used in the current work), the extracellular diffusion coefficient can be approximated as a linear function of gradient frequency (18,33,34,37). Therefore, extracellular diffusion signals can be simplified as

$$S_{ex} = S_{0, ex} \cdot \exp \left[ -b \cdot (D_{ex} + B_{ex} \cdot f) \right] \quad [5]$$

where $D_{ex}$ is the apparent diffusion coefficient of extracellular space measured at very long diffusion times ($f \to 0$), and $B_{ex}$ is the slope of $D_{ex}$ with respect to gradient frequency $f$. $D_{ex}$ is determined primarily by the extracellular tortuosity, and $B_{ex}$ indirectly reflects microstructural information (38). PGSE measurements with a single long diffusion time are used in the IMPULSED method and treated as 0 frequency. Note that this only affects the modeling of extracellular diffusion, whereas the intracellular diffusion of PGSE measurements is diffusion time–dependent, determined by Eq. [2]. Therefore, five independent parameters are fit using the IMPULSED method: $d$, $f_{in}$, $D_{in}$, $B_{ex}$, and $D_{ex}$. The IMPULSED method has been implemented successfully to measure the mean axon diameter (18) and cell size (19), both with validations using light microscopy.

Computer Simulations

A finite difference method was used to simulate diffusion signals obtained using different sequences as shown in (19). The tissue was modeled as tightly packed, spherical cells on a face-centered-cubic lattice (Fig. 2 in (31)) with $f_{in} = 61.8\%$, $D_{in} = 1\, \mu m^2/\mu s$, $D_{ex} = 2\, \mu m^2/\mu s$, and homogenous relaxation times everywhere for simplicity. Eleven different values of cell diameter $d$ evenly distributed from 5 to 20 $\mu m$ were simulated to emulate typical cancer cell sizes, and each $d$ incorporated 16 different intracellular water lifetimes, $\tau_{in}$: 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, 500, and $\infty$ ms. Note that $\tau_{in}$ of rat brain in vivo has been reported as approximately 550 ms (21). The transmembrane water apparent exchange rate has been reported as $2.9 \pm 0.8$ $s^{-1}$ ($\tau_{in} \sim 500$ ms) for viable human brain tumor (27), and tumor cell exchange rate is expected to increase during treatment-induced apoptosis (28). The finite-difference simulations actually incorporated cell membrane permeability $P_m$ instead of $\tau_{in}$ as described previously (39), where $P_m$ is related to $\tau_{in}$ as (40)

$$P_m = \frac{6\tau_{in}}{d - 10D_{in}} \quad [6]$$

All of the other pulse sequence parameters in the simulations were the same as those used in the MR experiments (see subsequently). All simulations (176 combinations of 11 cell diameters and 16 intracellular water lifetimes) took approximately 18.2 h using MATLAB R2015a (MathWorks, Natick, Massachusetts, USA) running on a 64-bit Linux machine with a Xeon 3.30 GHz CPU (Intel, Santa Clara, California, USA).

Three studies were performed based on simulation-generated data sets:

[Study I] To compare the accuracy of fitted $d$, $f_{in}$, and $D_{in}$ using IMPULSED, PGSE_I, and PGSE_II methods. Note that the PGSE_II method does not provide fitted $D_{in}$. The percentage differences of fitted and ground truth values were evaluated with a broad range of $\tau_{in} 50 - \infty$ ms and $d 5-15$ $\mu m$.

[Study II] To investigate the different choices of predefined $D_{in}$ on the accuracy of fitted $d$ and $f_{in}$ using the PGSE_II method. Only cells with $d = 10$ $\mu m$ were simulated here. Five $D_{in}$ values were investigated: 0.6, 0.8, 1.0 (true value), 1.5, and 2.0 $\mu m^2/\mu s$. Fitted results with different choices of $D_{in}$ were compared with ground truth values.

[Study III] To investigate the influence of SNR on the contrast and stability of the fitted parameters obtained using the PGSE_II and IMPULSED methods. Three SNRs were investigated: 10, 20, and 50, and each with four $\tau_{in}$: 50, 100, 250, and 500 ms. Random noise with targeted SNRs based on the non-diffusion-weighted signals were added to simulated noise-free signals according to the approach described in (41), and then microstructural parameters were fit based on signals with noise. This process was repeated 100 times for each of the 12 combinations of SNR and $\tau_{in}$, and the mean of the 100 fitted values of each microstructural...
parameter \((d, \text{ or } f_{in}, \text{ or } D_{in})\) was calculated and compared with the ground truth values.

Cell Sample Preparation

Murine erythroleukemia (MEL) cancer cells purchased from American Type Culture Collection (Manassas, Virginia, USA) grew under standard culture conditions described previously (24). Large scale of MEL cells were cultured in multiples 150-mm dishes; after spun down and washed with PBS, the cells were fixed with \(4\%\) paraformaldehyde in PBS for approximately 2 h. After fixation, cells were washed and divided into four groups at a cell density of \(4.2\times10^7\) cell/ml, each treated with 0, 0.01, 0.025, and 0.05% (w/v) saponin at room temperature for 30 min to induce various degrees of cell membrane permeability. Note that saponin does not cross cell membranes at low concentration, so it is unlikely to change other cell microstructure (eg, nuclear envelope). For each concentration of saponin treatment, cells were divided approximately evenly into six 0.65ml microtubes (each tube contains approximately 120 million cells). After centrifuge at 6000 g for 2 min, the top fluid was carefully removed and the cell pellet samples were then used for MR experiments. A small, aliquoted sample was spotted on glass slides and imaged directly under phase contrast microscopy for cell diameter estimation.

MR Measurements

All MR diffusion measurements were performed on a Varian 4.7 Tesla MRI spectrometer (Palo Alto, California, USA). The sample temperature was maintained at \(\sim 17^\circ C\) using a cooling water circulation system. The acquisition time was \(\sim 7\) min for measurements of \(\tau_{in}\), and \(9\) min for PGSE and IMPULSED experiments.

Measurements of \(\tau_{in}\)

The intracellular exchange lifetime \(\tau_{in}\) was estimated using constant gradient (CG) experiments (20,40). With a stimulated echo (STEAM) sequence, diffusion weighting was achieved by keeping \(\delta = 10\) ms and varying the gradient separation \(\Delta \) in 30 increments. The minimum \(\Delta \) was 20 ms and the maximum \(\Delta \) was 426, 426, 223, and 121.5 ms for cell samples with saponin concentrations of 0, 0.01, 0.025, and 0.05%, respectively. Diffusion gradients were applied simultaneously on three axes with gradient strength \(g = 5\) g/cm. The experiment was repeated with a lower gradient strength \(g = 0.5\) g/cm to normalize the higher gradient data set, to correct for \(T_1\) relaxation effect during the mixing time. The ADC of the slowly decaying component \((D_{il})\) was determined by linear regression using the last 10 \(b\)-value points. The \(\tau_{in}\) was then estimated as (20,40,42)

\[
\tau_{in} = \frac{1}{g^2 \delta^2 D_{il}}. \tag{[7]}
\]

Note that Eq. [1] is derived from the Karger model based on several assumptions, and its validity is supported by computer simulations (40).

PGSE Experiments

Diffusion signals were measured with PGSE sequences at four different diffusion times \((\delta = 4\) ms, and \(\Delta = 10, 25, 35,\) and \(50\) ms). Diffusion gradients were applied simultaneously on three axes with 11 gradient strengths varying linearly from 0 to 20 g/cm. Other parameters included: repetition time (TR) = 3.5 s; number of excitations = 2; number of dummy scans = 2; receiver bandwidth = 50 kHz; spectral resolution = 390.625 Hz; echo time (TE) = 60 ms.

IMPULSED Experiments

The IMPULSED measurements used both PGSE and OGSE sequences. For the PGSE measurements, \(\delta = 4\) ms and \(\Delta = 52\) ms. For OGSE experiments, cosine-modulated waveforms were applied with \(\delta/\Delta = 25/30\) ms at two oscillation frequencies \((f = 40\) and \(80\) Hz). TR = 3.5 s and TE = 60 ms for both PGSE and OGSE measurements. Nine \(b\)-values evenly distributed between 0 and \(2\) ms/\(\mu m^2\) were used in both measurements. The maximum gradient strength used in this study was 30 g/cm, which is achievable on, eg, the cutting-the-edge human brain gradient coil (43).

Data Analysis

Diffusion signals obtained in PGSE acquisitions were fit to Eqs. [1–3], and those from the IMPULSED experiments were fit to Eqs. [1], [2], [4], and [5]. Four parameters were fit using the PGSE_I method: \(d, f_{in}, D_{in},\) and \(D_{ex}\); three using PGSE_II by assuming \(D_{ex} = 1\) \(\mu m^2/\text{ms}\); \(d, f_{in},\) and \(D_{ex};\) and five using the IMPULSED method: \(d, f_{in}, D_{in},\) \(\beta_{ex},\) and \(D_{ex}.\) The lsqcurvefit function in MATLAB was used in the optimization with constraints of parameters limited to biophysically meaningful values: \(0 \leq d \leq 30\) \(\mu m,\) \(0 \leq f_{in} \leq 1,\) \(0.1 \leq D_{in} \leq 3\) \(\mu m^2/\text{ms},\) \(0.1 \leq D_{ex},\) \(D_{ex} \leq 3\) \(\mu m^2/\text{ms},\) and \(0 \leq \beta_{ex} \leq 30\) \(\mu m^2.\) To avoid local minima, each fitting was repeated 100 times with randomly generated initial conditions, and the analyses corresponding to the smallest fitting residuals were chosen as the final results.

RESULTS

Simulated Influence of Fitted Parameters on \(\tau_{in}\) and \(d\)

Figure 1 shows the simulated fitting errors of fitted parameters compared with ground-truth values dependent on \(d\) and \(\tau_{in}\) for all three methods. Both IMPULSED and PGSE_II provide accurate estimates of \(d\) with error \(<5\%\) for \(\tau_{in} > 150\) ms and \(d > 7\) \(\mu m,\) whereas the PGSE_I can provide \(<5\%\) errors only when \(\tau_{in} > 350\) ms and \(d\) was between 10 and 16 \(\mu m.\) Consistent with previous computer simulation results (29,30), all three methods significantly underestimated \(f_{in}\), except for IMPULSED and PGSE_II methods when \(\tau_{in}\) is infinitely large. All fitted \(f_{in}\) decreases rapidly with smaller \(\tau_{in}\), indicating that transcytolumal water exchange has a strong influence on fitted \(f_{in}.\) The IMPULSED method provides reasonable fits of \(D_{in}\) with errors \(<10\%\) for most \(\tau_{in}\) and \(d\) values, although the fits with errors \(<5\%\) occurred only when \(d\) was between 7 and 13 \(\mu m.\) The PGSE_I could provide fits of \(D_{in}\) with errors \(<10\%\) only when \(d\) is large (\(\geq 12\))
μm) and τin is long (>400 ms), and those with ≤5% errors occur only at a few combinations of τin and d. We need to reemphasize that Din was empirically predefined in the PGSE_II method, so it cannot be obtained in the fittings. Note that some plots in Figure 1 appear to be noisy; the main reason is that some fitted parameters reached the boundaries of loose-fitting constraints.

Simulated Influence of Predefined Din on PGSE_II Fitting

Figure 2 shows the simulated dependence of the fitted PGSE_II parameters on the choice of predefined Din used in the fittings. Note that only d = 10 μm was simulated here. Except for fast transcytolicmal water exchange (τin < 100 ms), the choices of Din had minor effects on the fittings of PGSE_II. For example, the fitted d were all within 5% difference when the predefined Din was in the range of 0.6–2.0 μm²/ms when τin > 100 ms, indicating that the accuracy of fitted d is not influenced by the choice of Din used in the fittings. fin is consistently underestimated across all τin values, whereas the discrepancy decreases with longer τin. The ground-truth extracellular diffusion coefficient is provided in Figure 2, which is much higher than fitted Dex, which is the extracellular diffusion coefficient at very long diffusion times (ie, determined primarily by the extracellular tortuosity). Note that both the fitted fin and Dex had small dependences on predefined Din: <10% changes occur when Din changes from 0.6 to 2.0 μm²/ms. These results are encouraging. In most cases, Din is unknown. If the influence of Din on the fitted parameters of the PGSE_II method is minor, an empirical value of Din can be implemented and the fitting accuracy is preserved.

Simulated Influence of SNR

The influence of SNR on fitted d using the IMPULSED and PGSE_II methods is shown in Figure 3. For each real
more accurate and precise fittings compared with the 

\[ \frac{1}{2} \]

fittings at SNR contrast, both diffusion methods provided more reliable values) acquired using the IMPULSED method compared at least partially the result of fewer data points (28 \( b \)-values) with different noises at the same SNR. Hence, 100 fitted value, the fittings were repeated 100 times and each 

dependent on different choices of predefined \( D_m \) used in the fittings.

\( d \) value, the fittings were repeated 100 times and each with different noises at the same SNR. Hence, 100 fitted \( d \) values were obtained (green +) for each real \( d \), and the mean of the repetitions (black \( \times \)) are also shown in Figure 3 to indicate the fitting accuracy. For both IMPULSED and PGSE_II methods at low SNR = 20, there was a broad range of fitted \( d \) values for each real \( d \), indicating that the precision was reduced significantly by noise. By contrast, at SNR = 50, \( d \) could be reliably fit using the IMPULSED or PGSE_II methods despite different \( \tau_m \). The mean values of all fitted results indicate the accuracy of fittings. At SNR = 20, the PGSE_II method provided relatively more accurate fittings compared with the IMPULSED method, whereas the latter overestimated \( d \) for all ground-truth \( d \) and \( \tau_m \) values. Note that this is at least partially the result of fewer data points (28 \( b \)-values) acquired using the IMPULSED method compared with the 45 \( b \)-values used in the PGSE_II method. By contrast, both diffusion methods provided more reliable fittings at SNR = 50. The IMPULSED method provided more accurate and precise fittings compared with the PGSE_II method, especially for \( d \) 5–10 \( \mu \)m. The fitted results showed slightly less variation with higher \( \tau_m \) and SNR = 50, indicating that transcytolemmal water exchange has a minor influence on the fittings of \( d \) when SNR is sufficient. For SNR = 20, the influence of \( \tau_m \) on \( d \) fittings was more significant, especially for the IMPULSED method. Based on these findings, we can conclude that when SNR is low (eg, 20 in the current simulation), the transcytolemmal water exchange does affect fitted \( d \), and higher \( \tau_m \) (slower water exchange) and higher SNR would lead to more accurate estimates of \( d \).

Figure 4 shows the simulated influence of SNR and \( \tau_m \) on fitted \( D_m \) values using the IMPULSED method. SNR significantly affects the accuracy of fitted \( D_m \) values. For SNR = \( \infty \), \( D_m \) can be fit well with <10% errors except when \( d \leq 6 \) \( \mu \)m. This is consistent with previous findings that \( D_m \) cannot be reliably fit when the restriction dimension is too small and the highest gradient frequency used is limited (35). For SNR = 10, \( D_m \) tends to be overestimated with large variations. For SNR = 20, better estimates of \( D_m \) were achieved when \( \tau_m > 250 \) ms and real \( d \geq 10 \) \( \mu \)m. For SNR = 50, it appears that \( D_m \) were all fit reasonably well with most errors <20%, except for \( \tau_m = 50 \) ms. This suggests that the accuracy and precision of \( D_m \) fitting using the IMPULSED approach were limited primarily by SNR when transcytolemmal water exchange is not too fast (eg, \( \tau_m > 100 \) ms). Recall that the PGSE_II method is incapable of providing any \( D_m \) information, whereas the PGSE_I method estimates \( D_m \) poorly even with the noise-free signals (see Fig. 1). This suggests an advantage of using the IMPULSED method to quantitatively characterize tissues in practice.

![FIG. 2. Simulated parameters obtained using the PGSE_II method dependent on different choices of predefined \( D_m \) used in the fittings.](image)

**Experimental Results of In Vitro Studies**

**Cell Size and \( \tau_m \)**

Based on the light microscopy experiments and the analysis approach described previously (19), the MEL cell diameter was measured as 11.34 ± 1.68 \( \mu \)m, consistent with the previously reported 11.74 ± 1.30 \( \mu \)m (19). The volume-weighted cell diameter \( d \) was then calculated as 12.11 \( \mu \)m, used as the ground-truth value in Figure 5. The CG experiments provided \( \tau_m \) values of 161.8 ± 9.4, 157.8 ± 8.9, 106.6 ± 4.3, and 59.4 ± 3.7 ms for 0, 0.01, 0.025, and 0.05% concentration of saponin, respectively.

**Microstructural Parameters of Cell Samples**

Figure 5 summarizes all fitted microstructural parameters of cultured MEL cells with four different cell membrane permeabilities. The IMPULSED and PGSE_II methods provide accurate fittings of \( d \) independent of permeability over a broad range of \( \tau_m \) (59.40–161.80 ms), whereas fitted \( f_m \) decreased rapidly with \( \tau_m \). This is consistent with the simulated results shown in Figures 1 and 3 that, if SNR is not too low (eg, >50) and \( \tau_m \) is not too small (eg, >50 ms), transcytolemmal water exchange has a minor influence on the estimations of \( d \) over a broad range of \( \tau_m \) values, whereas estimations of \( f_m \) could be significantly biased by fast transcytolemmal
water exchange (i.e., higher \( \tau_{in} \) values). \( D_{in} \) estimated by
the IMPULSED method showed approximately no dependence over the \( \tau_{in} \)
range of 106.58–161.80 ms, but decreased to 0.69 \( \mu m^2/\text{ms} \) when \( \tau_{in} \) decreased to 59.40 ms. This suggests that although \( D_{in} \)
is slightly more susceptible to transcytolic water exchange compared with \( D_{in} \)
can still be reliably estimated if \( \tau_{in} \) is not too small (e.g., >100 ms). \( D_{in} \)
fitted from the PGSE method were approximately constant over the \( \tau_{in} \)
range of 106.58 to 161.80 ms. However, as suggested by the simulations
shown in Figure 1, the PGSE method intrinsically underestimates \( D_{in} \) even with noise-free signals, and hence \( D_{in} \) obtained using the PGSE method may not be reliable. All
other fitted parameters, \( D_{ex} \) from the PGSE_I and PGSE_II methods, and \( k_{ex} \) and \( D_{ex0} \) from the IMPULSED
method, showed dependence on \( \tau_{in} \).

**DISCUSSION**

The present work investigated the influence of transcytolic water exchange on the quantitative characterization of tissue microstructure using diffusion MRI methods. Despite the significant variations of \( \tau_{in} \) present in simulations and in vitro experiments, all diffusion signal data sets were fit to currently widely used models that assume no water exchange between intra- and

![FIG. 3. Simulated influence of SNR on fitted \( d \) using the IMPULSED and PGSE_II methods. For each real \( d \), the fittings were repeated 100 times (green +) each with different noises but at the same SNR level; the mean of the repetitions (black ×) is also shown. The red lines represent the identity lines.](image-url)
extracellular spaces. The results suggest that fitted $d$ is relatively insensitive to $\tau_m$ with reasonable SNR in both PGSE_II and IMPULSED experiments, whereas $f_m$ is significantly underestimated for all diffusion methods tested. $D_m$ can be reliably fit only using IMPULSED experiments when SNR is sufficient (eg, 50). These findings may better assist the interpretation of quantitative characterization of tissue microstructure in tumors, in which the transcytolemmal water exchange is often much faster than normal tissue and cannot be ignored (27,28). For example, a previous study using the IMPULSED method provided $d$ and $f_m$ maps of an MDA-MBA-231 triple-negative breast cancer xenograft in vivo (44). Although $d$ was fit reasonably well and confirmed with histology, $f_m$ was significantly underestimated (<50%) in most regions. Based on the findings of the present work, the fitting accuracy of these in vivo findings can be explained by the enhanced transcytolemmal water exchange in tumors. To prove this, the transcytolemmal water exchange needs to be mapped using a method such as filter exchange imaging (FEXI) (27,45) in addition to quantitative diffusion MRI measurements.

It is possible to incorporate transcytolemmal water exchange into quantitative diffusion models, and hence fit all microstructural parameters—including $\tau_m$—simultaneously. The two-exchanging-component system describing diffusion measurements has been thoroughly investigated by Karger et al by assuming two freely diffusing components (46), and was later improved to include restrictions (ie, $d$ and $D_m$) on the intracellular water component (40,42,47). However, this method requires very high b-values (eg, 580 ms/µm²), as was used in rat brain in vivo (20). Stanisz et al performed comprehensive fittings to measure cell size, volume fraction, and membrane permeability simultaneously in bovine optic nerves ex vivo (48). However, high b-values

FIG. 4. Simulated influence of SNR and $\tau_m$ on fitted $D_m$ values using the IMPULSED method. Meanings of symbols and lines are the same as in Figure 3.
$\sim 40 \text{ ms}/\mu\text{m}^2$, high-gradient strength $140 \text{ g/cm}$, and rise time $\sim 500 \mu\text{s}$ were used, which are not achievable in practical imaging. Therefore, one major disadvantage of combining transcytolic water exchange in quantitative diffusion models is that these methods would become complicated and demand very high SNR and long scanning time. This will significantly limit the feasibility of translating these methods into clinical practice. Diffusion MRI usually suffers low SNR and long scanning time in practice; therefore, it is desirable to keep quantitative models simple with fewer free parameters. Partially for this reason, the PGSE II method was able to provide more accurate fits of $d$ compared with the PGSE I method, because PGSE II has fewer free parameters.

The minor influence of predefined $D_{in}$ on the fitting accuracy of the PGSE II method is encouraging. $D_{in}$ is usually unknown in practice. Previous studies assigned $D_{in}$ empirical values in the fittings (9,13,15,16), but the exact influence of different choices of empirical $D_{in}$ values on fitted results has not been fully investigated. The current study suggests that the influence of $D_{in}$ can be ignored if assigned $D_{in}$ is in a reasonable range (between $-40\%$ and $+100\%$ difference in the present work) when $\tau_{in}$ is not too short ($>100 \text{ ms}$ shown in Fig. 2). Because this influence is minor on fitted results, it is recommended to use the PGSE II method other than the PGSE I method, because of its better accuracy and stability (see Fig. 1). Note that neither PGSE I nor PGSE II methods used in the present work can reliably fit $D_{in}$, presumably because of the relatively long diffusion times used. By incorporating PGSE acquisitions with a long diffusion time ($\Delta = 50 \text{ ms}$) and OGSE acquisitions with short diffusion times ($40$ and $80 \text{ Hz}$, corresponding to $t_D$ of $6.25$ and $3.125 \text{ ms}$), the IMPULSED method is capable of providing a more comprehensive way to fit both intracellular $D_{in}$ and cellular dimension $d$ simultaneously. Note that the IMPULSED method used in the current work incorporates three different $t_D$ values, and fits five parameters, whereas the PGSE II method incorporates four $t_D$ values and fits three parameters. The fitted $d$ using the IMPULSED method with fewer data points is more prone to noise than the PGSE II method shown in Figure 3. We need to emphasize that it is possible to include more acquisitions with additional diffusion times in the IMPULSED method to enhance the sensitivity to $d$, but this in turn will increase the total scanning time. Therefore, it is desirable to optimize the IMPULSED method for specific applications in vivo.

Two relatively low frequencies ($40$ and $80 \text{ Hz}$) were used in the current work. These two low frequencies correspond to $t_D$ of $6.25$ and $3.125 \text{ ms}$, and root-mean-square displacements of $3.5$ and $2.5 \mu\text{m}$ if the diffusion coefficient is $1 \mu\text{m}^2/\text{ms}$. This is already much smaller than typical cancer cell size (eg, $11.34 \mu\text{m}$ in the present work), which enables the feasibility to probe $D_{in}$. Note

![Graphs showing fitted microstructural parameters versus $\tau_{in}$ using three diffusion methods. Error bars in each subfigure denote across-sample STD. $d$ range indicates histology-derived mean cell diameter $\pm$ STD of all cells, and mean $d$ is the volume-weighted cell diameter.](attachment:fig5.png)
that the fitted $D_{in}$ represents an averaged intracellular diffusion property, and even smaller intracellular microstructure such as nuclear size and organelles may not be probed by the frequencies used in the present work (8). To be sensitive to smaller sizes (~2 μm), much higher frequencies (~250 Hz) would be required (18,38).

To ensure translatability of our results, maximum gradient strengths were limited to <30 g/cm, which is achievable on the cutting-the-edge human gradient coils (43). Note that even with typical maximum gradient strength 8 g/cm, OGSE has been implemented on human scanners and shows promising potential to probe microstructure of human brain in vivo (49,50) and may assist in the interpretation of ADC data in stroke patients (51). However, the lowest frequency achievable is limited by echo time, so OGSE acquisitions have reduced sensitivity to larger scale dimensions. Combined with PGSE acquisitions with long diffusion times, sensitivity to both intracellular and supracellular scales can be achieved. In return, both $d$ and $D_{in}$ can be fit, which provides more microstructural information than $d$ alone, as provided by the PGSE II method. Note that higher gradient performance can improve the estimates of the fitting results (52). This is particular true for the IMPULSED method, which uses OGSE acquisitions (53).

**CONCLUSIONS**

Both computer simulations and in vitro cell studies suggest that for PGSE methods with relatively long diffusion times, empirically assigning $D_{in}$ as a constant in fittings significantly increases the accuracy of other fit parameters. Both IMPULSED and PGSE II methods provide accurate fits of mean cell diameter $d$ independent of transcytolic and transcytomental water exchange with sufficient SNR (eg, 50), whereas intracellular volume fraction $f_{in}$ was intrinsically underestimated in both methods and decreased rapidly with shorter $t_{1p}$. The IMPULSED method is capable of estimating intracellular diffusion coefficient $D_{in}$ when appropriate ranges of $d$ (7–15 μm), $t_{1p} > 100$ ms and sufficient SNR (eg, 50) were satisfied, whereas $D_{in}$ cannot be estimated reliably using the PGSE methods even for noise-free data. These results can assist in better interpretation of diffusion data for quantitative characterization of, eg, tumors, where transcytometric and transcytomental water exchange cannot be ignored.

**REFERENCES**


