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The time-dependent diffusivity in the abdominal ganglion of *Aplysia californica*: experiments and simulations

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Abstract

The nerve cells of the *Aplysia* are much larger than mammalian neurons. Using the *Aplysia* ganglia to study the relationship between the cellular structure and the diffusion MRI signal can potentially shed light on this relationship for more complex organisms. We measured the dMRI signal of chemically-fixed abdominal ganglia of the *Aplysia* at several diffusion times. At the diffusion times measured and observed at low b-values, the dMRI signal is mono-exponential and can be accurately represented by the parameter ADC (Apparent Diffusion Coefficient). We performed numerical simulations of water diffusion for the large cell neurons in the abdominal ganglia after creating geometrical configurations by segmenting high resolution T₂-weighted (T₂w) images to obtain the cell outline and then incorporating a manually generated nucleus. The results of the numerical simulations validate the claim that water diffusion in the large cell neurons is in the short diffusion time regime at our experimental diffusion times. Then, using the analytical short time approximation (STA) formula for the ADC, we showed that in order to explain the experimentally observed behavior, it is necessary to consider the nucleus and the cytoplasm as two separate diffusion compartments. By using a two compartment STA model, we were able to illustrate the effect of the highly irregular shape of the cell nucleus on the ADC.

1. Introduction

Using diffusion-encoding MRI to get tissue microstructure information in the mammalian brain has been the focus of much experimental and modeling work in recent years (Assaf *et al* 2008, Alexander *et al* 2010, Zhang *et al* 2011, 2012, Burcaw *et al* 2015, Palombo *et al* 2017, 2016, Ning *et al* 2017). Biological quantities of interest include axon diameter (Assaf *et al* 2008, Zhang *et al* 2011, Burcaw *et al* 2015) and orientation (Alexander *et al* 2010, Zhang *et al* 2012), neurite density (Alexander *et al* 2010, Zhang *et al* 2012), and more recently, fiber structure (Palombo *et al* 2017). Experimental protocols that are robust and specific in the identification of these biological quantities have been subject of much research. The experimental parameters that can be varied include the diffusion time and the magnitude and direction of the diffusion-encoding gradients. When the MRI signal is acquired at low gradient magnitudes, the signal contains only information about the Apparent Diffusion Coefficient (ADC).

The incoherent motion of water molecules during the diffusion encoding time causes a signal attenuation from which the ADC can be calculated (Hahn 1950, Stejskal and Tanner 1965, Bihan *et al* 1986). For unrestricted diffusion, the root mean square displacement of molecules is given by $\bar{s} = \sqrt{2D_0 t}$ (Berg 1993, Zhong and Gore 1991) where $d = 1, 2, 3$, for one, two and three dimensions, $D_0$ is the intrinsic diffusion coefficient, and $t$ is the diffusion time. In biological tissue, the diffusion is usually hindered or restricted (e.g. by cell membranes) and the mean square displacement is smaller than in the case of unrestricted diffusion. Intuitively, more hinderance or restriction will occur for more molecules as the diffusion time increases, so we expect that the experimentally determined ADC will decrease with increasing diffusion time (Stejskal and Tanner 1965, Tanner and Stejskal 1968, Grebenkov 2007, Özarslan *et al* 2006).
As a result, the extent of the ADC decrease can be potentially used to gather information about the tissue micro-structure; by acquiring several diffusion weighted images with different diffusion times and fitting the data to a model. For example in (Dietrich et al 2014, Weber et al 2009), the authors determined cell size and membrane distance based on diffusion MRI with multiple diffusion times. Numerous biophysical models have been proposed, usually subdividing the tissue into compartments described by spheres, ellipsoids, cylinders, and the extra-cellular space (Assaf et al 2008, Alexander et al 2010, Zhang et al 2011, Burcaw et al 2015, Fieremans et al 2011, Panagiotaki et al 2012, Jespersen et al 2007). However, it is difficult to connect the geometrical parameters contained in these models to the ground truth values due to the complexity of the brain tissue.

In the mammalian brain, the sizes of neurons and glial cells are on the order of micrometers (Fiala et al 2012), whereas the size of the imaging voxel is on the order of hundreds of micrometers. This makes it extra-ordinarily difficult to disentangle the validation of ADC models from the problem of figuring out the underlying tissue geometry that gave rise to the measured signal. One approach to completely separate the ADC model validation from geometrical uncertainty is the construction and imaging of special phantoms, examples of which include carrot slices (Dietrich et al 2014), spheres filled with a gel in each cell component (Lavdas et al 2013), physical phantoms constructed from resected rat spinal cord (Campbell et al 2005), polyfil fibers wound on a spherical polyamide spindle (Moussavi-Biugui et al 2011), and straight X-crossings of polyester fibers (Pullens et al 2010). In the phantoms, the geometry can be said to be known more or less exactly. However, there is a big gap between the tissue cellular environment and the phantoms. In particular, cells have a nucleus, organelles, and the cells and cell components have a range of sizes and shapes. These effects are certainly present in the imaging of biological tissue and make the direct application of phantom studies results to brain imaging potentially immature.

The precedent considerations motivate our present work of imaging a neural network with much larger components (compared to mammalian cells) for which the geometrical information is easier to ascertain than for the mammalian brain tissue. The advantage of Aplysia’s nervous system is that the cellular structure is relatively simple, with some of the largest cells identifiable in the T2w images we acquire along with the diffusion weighted images. We consider our work an intermediary between the imaging of the mammalian brain tissue and the imaging of specially constructed phantoms. In this sense, we use the Aplysia neural network as a ‘biological phantom’ for dMRI.

Because the Aplysia cells are much larger than the mammalian neural cells, for a given diffusion-encoding sequence, the relevant diffusion time regime is longer for the Aplysia than for mammalian neurons. As a result, it is possible to work with the short diffusion time approximation while using the standard Pulsed Gradient Spin Echo (PGSE) (Stejskal and Tanner 1965) sequence, instead of resorting to more specialized sequences such as Oscillating Gradient (OGSE) (Does et al 2003) sequences.

2. Materials and methods

2.1. Animal model

The neural system of the Aplysia californica consists of five pairs of ganglia: buccal, cerebral, pleural, pedal, and abdominal (Kandel and Kupfermann 1970). The abdominal ganglion was chosen in this imaging study because the cellular network is very well known in terms of position and morphology of single cell neurons and axonal orientation (Conn and Kaczmarek 1989, Musio and Bedini 1990). Moreover, the abdominal ganglion or single neurons from the abdominal ganglion have been investigated using magnetic resonance microscopy (MRM) and diffusion MRM studies (Schoeniger et al 1994, Hsu et al 1997, Grant et al 2001, Lee et al 2015). The abdominal ganglion diagram is shown in figure 1.

In this imaging study, we focused on the large neurons. There are many large neuron cells in the abdominal ganglion with radii of at least 75 μm that are visible by inspection in the high resolution (26 μm isotropic) T2w images. Some of these include neurons L2 to L9, L11, R2 to R8, R14 and R15 (labeled L or R for left or right hemiganglion, e.g. see in (Kupfermann et al 1974)). The single cell neurons with radii smaller than 75μm are not included in this group. We note that the sizes of these identified neurons are not fixed, they vary as a function of the age and the weight of the animal. The large cell neurons contain a nucleus, cytoplasm and are probably surrounded by small satellite (glial) cells (Lee et al 2015). The satellite cells are very small cells, 3μm maximum in radius, without a nucleus (Conn and Kaczmarek 1989, Musio and Bedini 1990, Lee et al 2015).
Several large cell neurons of radii greater than 75 μm (up to 210 μm) that are easily identifiable in the T$_2$w image were selected for this study.

2.2. Sample preparation
Six *Aplysia californica* (National Resource for Aplysia, Miami, FL, USA) were used in this study. The animals were anesthetized by injection of an isotonic magnesium chloride solution (MgCl$_2$, 360 mM; HEPES, 10 mM; pH = 7.5). All chemicals were purchased from Sigma-Aldrich (Lyon, France). The abdominal ganglion was resected and fixed with PFA 4% by immersion for 10 minutes and then washed three times in PBS pH = 7.4. For imaging, the abdominal ganglion was inserted into a 2.0 mm inner diameter (ID) glass capillary filled with fluorinert and then slid inside the transceiver coil.

2.3. Image acquisition
All experiments were performed at room temperature (≈19°C) on a 17.2 T system (Bruker BioSpin, Ettlingen, Germany) equipped with 1.0 T m$^{-1}$ gradients. Radio-frequency transceivers were home-built micro-coils with inner diameters of 2.4 mm, the design of which has been described in (Jelescu et al 2013, Radecki et al 2014).

Typically, a T$_2$w image and five to seven diffusion weighted images were acquired for each sample. The T$_2$w weighted image was acquired using a Rapid Acquisition with Focussed Echoes (RARE) with the following parameters: TR = 1500 ms, TE$_{eff}$ = 48 ms, acceleration factor $A_F$ = 8, isotropic spatial resolution 26 μm, matrix size of 400 × 88 × 88, 8 averages for an acquisition time of 3 hours 14 minutes. The acquisition parameters for the diffusion-weighted images (DP-FISP pulse sequence with bipolar diffusion gradients (Lu et al 2012)) were TE/TR = 1.63/1000 ms, 2 averages, isotropic spatial resolution 52 μm, 3 directions ($x^*$, $y^*$, $z^*$), four samples acquired with seven diffusion encoding times ($δ = 2.5$ ms, $Δ = [5, 7.5, 10, 12, 15, 20, 25]$ ms), one sample acquired with six diffusion encoding times ($δ = 2.5$ ms, $Δ = [5, 10, 12, 15, 20, 25]$ ms) and one sample acquired with five diffusion encoding times ($δ = 2.5$ ms, $Δ = [5, 10, 15, 20, 25]$ ms). All diffusion weighted images were acquired with 8 b-values ([70, 100, 200], · · ·, 700) s mm$^{-2}$), and matrix size of 200 × 44 × 44. The diffusion acquisition time was 2 hours 5 minutes for one diffusion time, 3 directions, 8 b-values. All acquisitions were acquired with a FOV of 10.4 × 2.3 × 2.3 mm$^3$. The average signal-to-noise ratios (SNRs) of the DW images were 31 at $b = 70$ s mm$^{-2}$ and 13 at $b = 700$ s mm$^{-2}$.

2.4. Image analysis
The T$_2$w images were manually co-registered with the diffusion-weighted images. For each of the six imaged ganglia, several three dimensional ROIs were manually segmented slice by slice on the T$_2$w image. We show in figure 2 the T$_2$w image and the physical locations of 3 large cells ROIs from ganglion number 2. In total, we have selected for further analysis 21 ROIs of large cell neurons. The ROIs were manually segmented such that each ROI contains the voxels associated with only one neuron. We selected only the neurons that were clearly indentifiable on the T$_2$w image based on the signal intensity, contrast, and the position within the ganglion (see diagram in figure 1).

The DMRI signals corresponding to the ROIs were processed to compute the experimental ADC using a linear fit of the logarithm of the signal versus the b-value. Even for large cell neurons, there might be, although less pronounced, some anisotropy due to the shape of the cells as well as the shape and position of the nucleus. For these reasons we averaged the ADC in the three directions, $x$, $y$, and $z$, to obtain the mean diffusivity, MD (Le Bihan et al 2001, Mori and Zhang 2009, Basser et al 1994, Kingsley 2006):

$$MD = \frac{ADC_x + ADC_y + ADC_z}{3}.$$  

2.5. Simulations
The dMRI signal is the total transverse water proton magnetization at the echo time (TE). We simulated the transverse water proton magnetization by solving the Bloch-Torrey equation (Torrey 1956)

$$\frac{\partial}{\partial t}M(x, t) = -rf(t)g \cdot xM(x, t) + \nabla \cdot (D^i \nabla M(x, t)), \quad x \in \Omega_l,$$

where $i$ is the imaginary unit, $D^i$ is the intrinsic diffusion coefficient in the geometrical compartment $\Omega_l$, and $γ$ is the gyromagnetic ratio of the proton. The complex-valued magnetization $M(x, t)$ is a function of position $x$ and time $t$, and depends on the diffusion-encoding gradient magnetic field $G(t) = \gamma g \cdot t$. The amplitude and direction information of the diffusion-encoding gradient is contained in the vector $g \in \mathbb{R}^3$ and the time profile of the effective gradient magnetic field is $f(t)$. For the PGSE sequence, the effective time profile is defined by:

$$f(t) = \begin{cases} 1 & 0 < t < \delta, \\ 0 & otherwise, \end{cases}$$

where $δ$ is the duration of the gradient pulses and $Δ$ the delay between the start of the two pulses. The signal is measured at the echo time TE, with $2Δ < TE < 2Δ$.

We solved the above equation subject to impermeable boundary conditions on $\partial \Omega_l$:

$$D^i \nabla M(x, t) \cdot \nu = 0, \quad x \in \partial \Omega_l,$$

where $\nu$ is the outward normal vector and imposed the initial condition:

$$M(x, 0) = 1, \quad x \in \Omega_l,$$

meaning uniform spin density in all $\Omega_l$. The diffusion MRI signal is the integral of magnetization at TE:
The numerical method for solving equations (1)–(3) was adapted from (Li et al 2013).

The $b$-value (Bihan et al 1986), in case of the PGSE sequence is:

$$ b(\mathbf{g}, \delta, \Delta) = \gamma^2 \lvert \mathbf{g} \lvert^2 \delta^2 (\Delta - \delta/3). $$

The ADC can be calculated as:

$$ ADC = - \frac{\partial}{\partial b} \log \left. \frac{S(b)}{S(0)} \right|_{b=0}. $$

The ADC obtained from the numerically simulated dMRI signal was computed in the same way and using the same $b$-values as for experimental data.

In what follows we describe the geometrical configurations used in the simulations. To create computational domains in which to perform numerical simulations of large cell neurons, we segmented the cell outline of one particular large cell neuron. This cell outline (denoted by $\Omega_{\text{cell}}$) was segmented from the anatomical T2w image of the large neuron ROI (see figure 3(a)) and was used for all simulations of large cell neurons. We note that $\Omega_{\text{cell}}$ is a slightly elongated

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Figure 2. T2w image of the abdominal ganglion (Aplysia # 2) and the selected ROIs: (a) 3D representation showing the selected ROIs; (b)-(d) three slices from the T2w image. The scale bar represents 260 $\mu$m.

Figure 3. Geometrical configuration of a large single cell neuron: the cell outline obtained from cell ROI #7 (a); A nucleus with irregular shape was manually generated (b); The nucleus was manually placed inside the cell outline (c); cell nucleus (red) and cytoplasm (green). Different cell sizes were generated by scaling ($R_{\text{eff}} = 60$ $\mu$m (d) and $R_{\text{eff}} = 40$ $\mu$m (e)).
ellipsoid. In addition, we created by hand several examples of irregularly shaped nuclei, \( \Omega_{\text{n}} \), as in figure 3(b). The shape of the nucleus was inspired by the high resolution images in (Lee et al. 2015). The generated \( \Omega_{\text{n}} \) when placed inside \( \Omega_{\text{cell}} \) takes up between 25%-30% volume fraction. Even though not visible in the T_{2w} images, there may be a small volume (up to 5%) of satellite cells (very small cells, 3\( \mu m \) maximum radius, without nucleus (Conn and Kaczmarek 1989, Musio and Bedini 1990, Lee et al. 2015)) surrounding the single cell neurons. However, since the volume of the satellite cells is small and to simplify the study, we did not include the satellite cells in the simulations.

By placing \( \Omega_{\text{n}} \) in \( \Omega_{\text{cell}} \) we obtained several geometrical configurations. To take into account the effect of the size of the cells on the ADC, we simply scaled the reference geometry such that \( \Omega_{\text{cell}} \) had the desired total volume, leading to effective cell radii ranging from 100 \( \mu m \) to 210 \( \mu m \) (figure 3).

The simulated dMRI signal for the large cell neurons can be generated by solving the Bloch-Torrey equations in \( \Omega_{\text{cell}} \) with two compartments: the nucleus \( \Omega_{\text{n}} \) and the cytoplasm \( \Omega_{\text{c}} = \Omega_{\text{cell}} - \Omega_{\text{n}} \). The intrinsic diffusivities in the cytoplasm and nucleus, \( D_{\text{c}} \) and \( D_{\text{n}} \), respectively, were chosen to range from 0.5 \( \mu m^2/\text{ms} \) to 2.4 \( \mu m^2/\text{ms} \) as described in the literature (Lee et al. 2015). The total signal in a large cell neuron is:

\[
S_{\text{cell}} = (1 - \nu_{\text{n}}) S_{\text{c}} + \nu_{\text{n}} S_{\text{n}}
\]

where \( \nu_{\text{n}} \) is the volume fraction of the nucleus, \( S_{\text{c}} \) and \( S_{\text{n}} \) are the signals in the cytoplasm and the nucleus, respectively.

### 2.6. Analytical formula of the ADC in short diffusion time regime

A well-known formula for the ADC in the short diffusion time regime is the following short time approximation (STA) (Mitra et al. 1992, 1993):

\[
ADC_{\text{STA}} = D_{\text{0}} \left( 1 - \frac{4 \sqrt{D_{0}}}{3 \dim \sqrt[\delta]{V}} \sqrt{\Delta S V} \right). \tag{7}
\]

where \( S \) is the surface to volume ratio and \( D_{\text{0}} \) is the intrinsic diffusivity coefficient. In the above formula the pulse duration \( \delta \) is assumed to be very small compared to \( \Delta \). A recent correction to the above formula, taking into account the finite pulse duration \( \delta \) (Schiavi et al. 2016) is the following:

\[
ADC_{\text{STA}} = D_{\text{0}} \left( 1 - \frac{4 \sqrt{D_{0}}}{3 \dim \sqrt[\delta]{V}} C_{\delta, \Delta} S V \right). \tag{8}
\]

where

\[
C_{\delta, \Delta} = \frac{4}{35} \left( \frac{\Delta + \delta^{3/2}}{\Delta} + \left( \frac{\Delta - \delta}{\Delta - \delta/3} - \frac{2(\delta^{3/2} + \Delta^{3/2})}{\delta^{2}(\Delta - \delta/3)} \right) \right)
\]

\[
= \sqrt{\Delta} \left( 1 + \frac{1}{3} \frac{\delta}{\Delta} - \frac{8}{35} \left( \frac{\delta}{\Delta} \right)^{3/2} + \cdots \right). \tag{10}
\]

When \( \delta \ll \Delta \), the value \( C_{\delta, \Delta} \) is approximately \( \sqrt{\Delta} \).

### 3. Results

#### 3.1. Experimental time-dependent ADC

The experimental dMRI signals at multiple diffusion times in the large cell neuron ROIs are shown in figure 4. It is clear that in the range of b-values used here, from 70 to 700 \( s \text{mm}^{-2} \), the logarithm of the signal is a linear function of b-value, meaning that the ADC is sufficient to describe the signal in this range. Higher order effects such as a Kurtosis (Chabert et al. 2005, Frohlich et al. 2006, Jensen et al. 2005, Jensen and Helpern 2010) term need not be considered. Moreover, the signals in the x, y, and z directions do not show significant anisotropy.

The time-dependent ADC measured over the 21 ROIs of large cell neurons are shown in table 1. We found that, when the diffusion time is increased from
5 to 25 ms, the average experimental ADC drops by 9.45% in large cell neurons.

### 3.2. Validation of the STA by numerical simulations in large cell neurons

Assuming a free diffusivity of 2.00 μm²/ms, the average diffusion displacement is between 7.7 and 17.3 μm for the diffusion times between 5 and 25 ms. From the point of view of diffusing water molecules, the diffusion displacement is much smaller than the size of the large cell neurons. Hence, we should be able to apply the mathematical model \( ADC_{STA} \) to the measured ADC in the large cell neurons. Nevertheless, we first used numerical simulations to compare the simulated ADC with \( ADC_{STA} \) (specifically, we used equation (8)).

We constructed several geometrical configurations for the large cell neurons, consisting of a nucleus and surrounding cytoplasm, based on three types of nucleus shapes and positions of the nucleus inside the cell, as shown in figure 5.

To perform simulations, we scaled the configurations 1-4 (figure 5) so that they each have the effective radius of 100 μm. We referred to information from published literature (Lee et al 2015) to choose reasonable biological parameters: \( D_c = 0.67 \mu m^2/\text{ms} \) and \( D_n = 2 \mu m^2/\text{ms} \).

<table>
<thead>
<tr>
<th>( \Delta ) (ms)</th>
<th>Mean (μm²/ms)</th>
<th>SD (μm²/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.974</td>
<td>0.096</td>
</tr>
<tr>
<td>7.5</td>
<td>0.957</td>
<td>0.085</td>
</tr>
<tr>
<td>10</td>
<td>0.938</td>
<td>0.087</td>
</tr>
<tr>
<td>12</td>
<td>0.915</td>
<td>0.080</td>
</tr>
<tr>
<td>15</td>
<td>0.914</td>
<td>0.080</td>
</tr>
<tr>
<td>20</td>
<td>0.897</td>
<td>0.081</td>
</tr>
<tr>
<td>25</td>
<td>0.882</td>
<td>0.087</td>
</tr>
<tr>
<td>Drop</td>
<td>9.45%</td>
<td></td>
</tr>
</tbody>
</table>

We solved the Bloch-Torrey equation for configurations 1-4 in each of the following three domains to obtain the simulated ADC at diffusion times between 5 ms and 25 ms. In figure 6 we show the averaged diffusion time dependent ADC over x, y, and z gradient directions for: a) the nucleus compartment only (only nucleus); b) the cytoplasm compartment only (only cytoplasm); c) a combined compartment including both the nucleus and the cytoplasm with no hindrance to water diffusion between them (no hinderance); d) a combined compartment including the nucleus and the cytoplasm with limited exchange for which we computed the ADC as the weighted average of the

![Figure 5. Generated geometrical configurations using the same cell outline (green), containing three different shapes of nucleus (red) (a, b-c, and d). The positions of nucleus inside the cytoplasm are different in (b and c) but the nucleus shape is the same. The cell outline is the same for the four configurations, and it is generated from the T2w image (large cell ROI #7). The volume fraction of the nucleus in the four configurations is approximately 25%.

| Mean and standard deviations (SD) of experimental ADCs in large cell neurons ROIs (N = 21). The average ADCs were observed to drop by 9.45%. | Table 1. |
ADCs in nucleus and cytoplasm (nucleus+cytoplasm). The close agreement between the numerically simulated ADC and the STA formula is clear for all 3 computational domains in the 4 geometrical configurations. Therefore, we are justified in using the STA formula to compare with the experimental ADC for large cell neurons instead of running further numerical simulations.

We note that the averaged-ADC drop of the combined nucleus and cytoplasm (no hinderance) between $\Delta = 5$ ms and $\Delta = 25$ ms is under 2.5% for all four configurations (both STA and simulation), whereas the weighted average of the STA ADC of the nucleus and cytoplasm compartments with limited exchange (STA: nucleus+cytoplasm) drops by 6.7% for configuration 1, by 5.1% for configurations 2 and 3, and by 4.1% for configuration 4. Moreover, the weighted average of the simulated ADC for this domain (Simulation: nucleus+cytoplasm) drops by 6.8% for configuration 1, by 5.4% for configurations 2 and 3, and by 4.7% for configuration 4. Let us now compare these values with experimental results we described earlier: when the diffusion time is increased from 5 to 25 ms, the average experimental ADC drops by 9.45% in large cell neurons. To achieve the larger ADC drop of the experimental data using numerical simulations, we would need to increase the surface to volume ratios of the two compartments. However, it is extremely difficult to manually adjust the finite element mesh to increase the surface to volume ratios. This requires moving the points of the finite element mesh manually, while satisfying constraints on the compartment volume fractions, the nuclear volume fraction being between 25% and 30% (Jelescu 2013, Lee et al 2015). For this reason, once we have established the validity of the STA formula as a good approximation to the simulated ADC at $R_{\text{eff}} \geq 100\mu$m, we will use the STA formula instead.
of the simulated ADC, since we can easily adjust the surface to volume ratios in the STA formula without constructing a finite element mesh.

We see that the two compartment (nucleus and cytoplasm) with limited exchange computational domain is the best description of the diffusion in the large cell neurons among the four domains considered and that a more irregular nucleus results in a larger ADC drop. We now expand on these ideas using the STA formula.

3.3. Comparing the STA formula with the experimental ADC for large cell neurons

3.3.1. One compartment STA model

If the hinderance to water diffusion between the two compartments can be neglected, then the nucleus and the cytoplasm can be combined into one compartment, with a single diffusion coefficient. This corresponds to the simulations performed in the third computational domain (the combined compartment). From the experimental ADC, by fitting \( \text{ADC} = A C_{\phi, \Delta} + B \) using equation (8), we can find the coefficients \( A \) and \( B \), from which the surface to volume ratio can be obtained:

\[
A = -B \frac{4 \sqrt{D_0}}{3 \ \text{dim} \sqrt{\pi}} \frac{S}{V}, \quad B = D_0
\]  

(11)

If we further assume the shape is a sphere, then

\[
A = -B \frac{4 \sqrt{D_0}}{3 \sqrt{\pi}} \frac{1}{R}, \quad B = D_0
\]  

(12)

from which an estimated cell radius can be obtained. Note that the cell neuron outlines are actually not spherical, and the quantity \( S/V \) depends on the size and the shape of the cell neuron outlines. However, we prefer to use the quantity **effective radius** which is more intuitive and familiar than the quantity \( S/V \).

We denote by

\[
R_{\text{est}} = -D_0 \frac{4 \sqrt{D_0}}{3 \sqrt{\pi}} \frac{1}{A},
\]  

(13)

the estimated cell radius determined by applying the STA formula to the experimental ADC. The idea is to compare the \( R_{\text{est}} \) with the visually obtained effective radius, denoted \( R_{\text{eff}} \) of the 21 large cell ROIs. The results are shown in table 2. It is clear that the cell size is severely underestimated: \( R_{\text{est}} \) is on average only 25% of \( R_{\text{eff}} \). This suggests that a one compartment model is not sufficient to describe the diffusion in the large cell neurons of the \textit{Aplysia}, which is a conclusion we already alluded to in the previous section.

3.3.2. Two compartment STA model

When the cell is made up of different components such as cytoplasm and nucleus, under the short diffusion time and low b-value regime, the water exchange between the cytoplasm and the nucleus can be assumed to be limited enough so that the water exchange does not affect the ADC, then the ABC is the weighted average of the ADC in the individual compartments:

<table>
<thead>
<tr>
<th>ROI#</th>
<th>( R_{\text{eff}} ) (( \mu )m)</th>
<th>( A ) (( \mu )m²/( \mu )s³/2)</th>
<th>( B ) (( \mu )m²/( \mu )s)</th>
<th>( R_{\text{est}} ) (( \mu )m)</th>
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<td>101.3</td>
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ADC_{STA}^{2comp} = (D_n + (1 - f) D_c) \begin{aligned} \left(\frac{S_n}{V} D_n^{2/3} + \frac{S_c}{V} D_c^{2/3}\right) C_{\Delta,\Delta} \end{aligned}

where $f = \frac{V_c}{V}$ is nucleus volume fraction, $D_n$ and $D_c$ are the intrinsic diffusivity coefficients of nucleus and cytoplasm components, $\frac{S_n}{V}$ and $\frac{S_c}{V}$ are the surface by volume ratios for the nucleus and cytoplasm components.

The quantities $\frac{S_n}{V}$ and $\frac{S_c}{V}$ are related to the shape and size of the nucleus and the cell outline. The outline of the large cell neurons can be seen on the T2w images to be spheroid or somewhat ellipsoid. Recall that the volume fraction of the nucleus is $f$. It is easy to show that if the nucleus and the large cell neuron outline are spheres, then

$$\frac{S_n}{V} = 3 f^{2/3}, \quad \text{and} \quad \frac{S_c}{V} = \frac{S_n}{V} + 3 \frac{1}{R_{\text{eff}}}.$$ (15)

The shape of the nucleus inside the cell cannot be seen on the T2w images. Therefore, we referred to the information from published literature (Lee et al 2015) to manually construct a three-dimensional configuration consisting of an irregularly shaped nucleus placed inside an ellipsoid (see Configuration 1, figure 5(a)). For this configuration, we numerically computed the surface to volume ratios:

$$\frac{S_n}{V} = 8.119 f^{2/3}, \quad \text{and} \quad \frac{S_c}{V} = \frac{S_n}{V} + 4.539 \frac{1}{R_{\text{eff}}}.$$ (16)

We then manually constructed another irregularly shaped nucleus (Configuration 2, figure 5(b)) and numerically computed the surface to volume ratios:

$$\frac{S_n}{V} = 6.053 f^{2/3}, \quad \text{and} \quad \frac{S_c}{V} = \frac{S_n}{V} + 4.539 \frac{1}{R_{\text{eff}}}.$$ (17)

For Configuration 3 (see figure 5(c)), we simply moved the nucleus with respect to Configuration 2, therefore, the above surface to volume ratios stay the same. Finally, a third nucleus shape (essentially an ellipsoid) was constructed manually (Configuration 4, figure 5(d)) and the numerically computed ratios are:

$$\frac{S_n}{V} = 4.589 f^{2/3}, \quad \text{and} \quad \frac{S_c}{V} = \frac{S_n}{V} + 4.539 \frac{1}{R_{\text{eff}}}.$$ (18)

It is easy to show that the shapes of the large neuron cell outline and the nucleus affect the surface to volume ratios through the parameters $Q_1$ and $Q_2$:

$$\frac{S_n}{V} = Q_1 f^{2/3}, \quad \text{and} \quad \frac{S_c}{V} = \frac{S_n}{V} + Q_2 \frac{1}{R_{\text{eff}}}.$$ (19)

The parameter $Q$ indicate the non-smoothness of the shape, for a sphere, $Q = 3$, for the ellipsoids we generated, $Q \approx 4.5$, the more irregular the shape, the higher the $Q$. The nucleus shape that corresponds the best to the literature reports (Lee et al 2015) about *Aplysia* neurons is in Configuration 1, where $Q = 8.1$.

Now, we state the two compartment STA model:

$$ADC_{STA}^{2comp} = -\frac{4}{3 \text{dim} \sqrt{\pi}} \left[ Q_1 f^{2/3} (D_n^{2/3} + D_c^{2/3}) + Q_2 D_c^{2/3} \frac{1}{R_{\text{eff}}} C_{\Delta,\Delta} \right. $$

$$+ \left. [f D_n + (1 - f) D_c] \right],$$ (20)

which we will apply to the experimental ADC. This can be stated as a linear regression problem:

$$y = \tilde{c}_0 - \tilde{c}_c \tilde{x},$$ (21)

where the dependent variable $y = ADC_{STA}^{2comp}$ is the diffusion time-dependant ADC, and the independant variable is $\tilde{x} = \frac{1}{R_{\text{eff}}} C_{\Delta,\Delta}.$

First we combined the data from 21 ROIs and the multiple diffusion times to solve one regression problem and obtained the following fitted values:

$$\tilde{c}_0 = [f D_n + (1 - f) D_c] = 0.98043,$$ (22)

$$\tilde{c}_c = -\frac{4}{3 \text{dim} \sqrt{\pi}} \left[ Q_1 f^{2/3} (D_n^{2/3} + D_c^{2/3}) + Q_2 D_c^{2/3} \right] = 1.9026,$$ (23)

where the numerical fitting is shown in figure 7(a).

From equation (22) we plot the relationship between $D_n$, $D_c$ and $f$ for plausible choices of $f$ (between 0.15 and 0.35, see figure 7(b)). We see an inverse relationship between $D_n$ and $D_c$ and this inverse relationship is steeper at higher $f$. This also helps us bound reasonable values of $D_n$, since $D_c$ should not be too small.

In the following, we will limit $D_n$ to the range $D_n \in [1, 2.4] \mu m^2/\mu s$. We plot the relationship between $Q_1$ and $\{D_n, Q_2 \}$, where $D_n \in [1, 2.4] \mu m^2/\mu s$, $Q_2 \in [3, 10]$. We recall that $Q_2 = 3$ implies a spherical shape of the cell outline, and $Q_2 = 4.5$ implies an ellipsoid shape. The higher the value, the more irregular the shape. We choose two values of $f$ ($f = 0.15$ and $f = 0.35$) subject to the constraints in equations (22)–(23). The resulting values for $Q_1$ are shown in contour plots in Figs. 7(c)–(d).

Since the large cell neuron outline has an ellipsoid shape, we can assume that $Q_2 \approx 4.5$. When $f = 0.15$ (figure 7(c)), looking at the line $Q_2 = 4.5$, we see the range for $Q_1$ is between 4 and 5, which is too low, given the information from the literature on the shape of the nucleus (much more irregular than an ellipsoid). At $f = 0.35$ (figure 7(d)), the range of $Q_1$ is even lower, less than 4. Therefore, it is clear that there are some inaccuracies in the above fitting procedure. We
suspect that this is due to the noise in the measured ADCs as well as to errors in the estimated radii of the cells (which was done manually).

To compensate for the error and uncertainty in the ADCs and the cell radii, we next solved the linear regression problem for each ROI separately:

\[ y = c_0 + c_1 x \]

where the dependent variable \( y = \text{ADC}_2\text{comp} \) is the diffusion time-dependent ADC, and the independent variable is \( x = C_{\text{eff}} \Delta \). This way, we obtain 21 values of \( c_0 \) and 21 values of \( c_1 \). The fitted values will be then:

\[ c_i^j = \left[ fD_n + (1 - f)D_c \right], \quad (25) \]

where we expect inaccuracies in \( R_{\text{eff}}^j \). Therefore, we set the fitting error of \( c_j^i \) (equation (25)) to zero and obtain \( D_c^j \) as a function of the continuous variables \( f \) and \( D_n^j \):

\[ D_c^j(f, D_n^j) = \frac{c_j^i - fD_n^j}{1 - f}. \]

On the other hand, we will not set equation (26) to zero, because we expect the fitting errors (normalized below),

\[ E_{\text{err}}^i(f, Q_1, D_n^j, Q_2) = \frac{4}{3 \dim \sqrt{\pi} c_i^j R_{\text{eff}}^j} \left[ Q_1 f^{2/3} \left(D_n^j\right)^{2/3} + D_c^j(f, D_n^j, c_i^j)^{2/3} + Q_2 1 - 1 \right] \]

\[ i = 1, \ldots, 21, \]

will not reach zero due to the inaccuracies in \( R_{\text{eff}}^j \) (and the ADC). Knowing that the physically plausible values of \( f \) should stay in the range \( 0.15 \leq f \leq 0.35 \), we plot in figure 8 the \( c_1^i \) fitting error, \( E_{\text{err}}^i(f, Q_1, D_n^j, Q_2) \), as a function of \( D_n^j, D_c^j \) and \( f \) from equation (22). c-d) \( Q_1 \) as a function of \( D_n^j \) and \( Q_2 \), based on the best fit \( c_0^i \) and \( c_1^i \) for all the ROIs and all diffusion times.

On the other hand, we will not set equation (26) to zero, because we expect the fitting errors (normalized below),

\[ Q_n^{\text{min}}(D_n^j, Q_2) = \min_{Q_1} E_{\text{total}}(Q_1, D_n^j, Q_2), \]

\[ E_{\text{total}}(Q_1, D_n^j, Q_2) \equiv \sum_{i=1}^{21} E^i(Q_1, D_n^j, Q_2), \quad (27) \]

where the averaged (over \( f \in [0.15,0.35] \)) fitting error for each ROI is defined as:

![Figure 7](image-url)
The solution to the minimization problem in equation (27) depends on the parameters $D_n$ and $Q_2$, but not on $f$ and $D_c$. We make this clear by using the notation $Q_{1,2}^{\text{min}}(D_n, Q_2)$.

In figure 9(a) we show the total fitting error $E^{\text{total}}(Q_1, D_n, Q_2)$ for one particular choice of $D_n$ and $Q_2$, and for this choice, $Q_{1,2}^{\text{min}}(D_n, Q_2) = 7.8$, resulting in the total fitting error $E^{\text{total}}(Q_1, D_n, Q_2) \approx 0.294$. We then do an exhaustive search in $D_n \in [1, 2.4] \mu m^2/\text{ms}$ and $Q_2 \in [3, 10]$. We show in figure 9(b) the contour plot for the value of $Q_{1,2}^{\text{min}}(D_n, Q_2)$. We found no solutions when $D_n \leq 1.4 \mu m^2/\text{ms}$ and we found the range of $Q_{1,2}^{\text{min}}(D_n, Q_2)$ to be between 5 and 9 given the ranges $D_n \in [1.4, 2.4] \mu m^2/\text{ms}$ and $Q_2 \in [3, 10]$. If we take as plausible the value of $Q_2$ to be between 4 and 5 (indicating an ellipsoid) and the value of $D_n$ to be between 1.4\mu m^2/\text{ms}$ and 1.8 \mu m^2/\text{ms}$, we get a range of between 7.5 and 9 for $Q_{1,2}^{\text{min}}(D_n, Q_2)$, indicating a very irregular shaped nucleus, in agreement with the literature (Lee et al 2015). We show in figure 9(c) the contour plot of the $D_c / D_n$ ratio as a function of $D_n$ and $Q_2$. For plausible values of $Q_2$, between 4 and 5, and of $D_n$, between 1.4\mu m^2/\text{ms}$ and 1.8 \mu m^2/\text{ms}$, this ratio is between 0.45 and 0.65, again in agreement with the literature.

Finally, in figure 9(d) we plot the mean values and the standard deviations of $E(Q_{1,2}^{\text{min}}, D_n, Q_2)$ for each ROI $i$ over the ranges of $D_n \in [1.4, 2.4] \mu m^2/\text{ms}$ and $Q_2 \in [3, 10]$ searched. This shows that the obtained minimum fitting error in each ROI is stable across the searched space of $D_n$ and $Q_2$.

The fact that we found no solutions for $D_n < 1.4 \mu m^2/\text{ms}$ means that $D_n \geq 1.4 \mu m^2/\text{ms}$, which is a range of $D_n$ that is consistent with the literature reports on the nucleus ADCs in Aplysia neurons (Schoeniger et al 1994, Grant et al 2001). The nucleus diffusivity is known to be high compared to the cytoplasm diffusivity. It was not obvious from the outset that we would be able to pinpoint $D_n$ to be no smaller than 1.4\mu m^2/\text{ms}$. This came out merely from insisting on the nucleus shape parameter $Q_1$ to be between 3 and 10.

In addition, because we know that big cell neurons are certainly ellipsoid in shape, we were able to constrain the possible values of $Q_2$. This constraint in turn allowed us to conclude that $Q_1$ is between 7.5 and 9, which is a reasonable range for an irregular nucleus.
shape. Without formulating the linear regression problem for each ROI separately, as we described above, the obtained $Q_1$ would be less than 5, which is much too low to be true.

4. Discussion

To study the diffusion time dependence of the dMRI signal and evaluate if measurements at multiple diffusion times can give additional information about the tissue microstructure, we imaged the Aplysia abdominal ganglia at high resolution and several diffusion times. Given the very long experimental times, we used chemically-fixed samples. However, one should keep in mind that the aldehyde fixatives used may significantly alter tissue MRI properties. Shepherd and colleagues reported in (Shepherd et al 2009) that the rat cortical slices fixed by immersion in 4% formaldehyde solution demonstrated 21% and 81% reductions in tissue $T_1$ and $T_2$, respectively. By washing fixed tissues with PBS to remove free formaldehyde solution $T_2$ can be recovered. In addition, the membrane permeability was increased after fixation with 4% formaldehyde (Shepherd et al 2009). However, we find it a worthwhile trade-off since cell (sub-)structures are mostly preserved in the fixation process and this allows us to obtain high resolution data at multiple diffusion times for which in-vivo or fresh tissue imaging would not be possible.

We have acquired and analyzed the diffusion time-dependent dMRI data in the Aplysia neuronal network for large cell neurons. The time-dependent ADC at diffusion times ranging from $\Delta = 5$ ms to $\Delta = 25$ ms were presented. In the spirit of using the Aplysia as a biological phantom for diffusion MRI we conducted an analytical and numerical study in the case of the large cell neurons. The cell outline of these large neurons can be seen in the $T_2$-weighted images, giving a reliable (but still approximate) estimate of the cell size. We chose to sum up the signal values over all the pixels of a manually generated ROI that contains one individual neuron, instead of using it at pixel level, in order to approach the experimental conditions in mammalian neuro-imaging where the cells are contained entirely in one voxel. By retaining only one cell in the ROI, we eliminate much of the uncertainty regarding the geometry of the diffusion medium being imaged. We know that the signal comes from the segmented cell and we know the size of the segmented cell up to some level of error. This is what we mean by a 'biological phantom'.

This phantom retains much of the complexity of the cellular make-up (nucleus, cytoplasm) that is also
relevant to mammalian neuro-imaging while reducing the uncertainty about the cellular size. For example, we addressed the question of whether the nucleus and the cytoplasm should be modeled as two separate diffusion compartments, at least in the short diffusion time regime which is the case for large cell neurons in our experiments. We showed that indeed, two compartment diffusion fits the data better than combining them into a single diffusion compartment. Using the one compartment STA model resulted in a serious under-estimation of the cell size. This is relevant for mammalian imaging as the neurons have similar cellular make-up as the *Aplysia*, only smaller in size. Thus, for short time diffusion imaging (using oscillating gradients, for example), our results suggest that some modeling error could be due to the presence of the nucleus, since often diffusion in the soma is modeled by one compartment diffusion for mammalian neuro-imaging studies. Supported by numerical simulations and after taking into account the noise in the experimental ADC as well as the uncertainty in cell size due to manual segmentation, we established that a two compartment STA model is adequate to explain the behavior of the experimentally obtained ADC. This result also suggests that dMRI may be potentially used to probe the shape of the cell nucleus, which could be indicative of certain cellular abnormalities (Webster et al 2009).

Additionally, we showed that even if one has some information regarding the geometry of the cells being imaged, as we do for the *Aplysia* neurons, it is important to account for the uncertainty in the prior knowledge (in our case, the size of the cells from manual segmentation). We propose fitting the intended model individually to each neuron instead of using averaged quantities. While this is somewhat counter-intuitive since one would have assumed that averaging over all the large cell neurons would reduce noise in the model fitting procedure, we showed that this is not the case for the fitting of the two compartment short-time ADC model, and we suspect this is due to the error in the estimated cell size from the segmentation procedure.

We produced numerical simulations that showed the two compartment STA model is a valid approximation during the diffusion times ranging from \( \Delta = 5 \text{ ms} \) to \( \Delta = 25 \text{ ms} \) for large cells (\( R_{\text{eff}} \gg 100\mu\text{m} \)). The use of the STA formula where one can tune the volume fractions of the nuclear and cytoplasmic compartments as well as their surface to volume ratios increases the types of geometries that are accessible to modeling, in particular, those geometries for which the generation of finite element meshes would be very difficult.

In our work, we considered low b-values at multiple diffusion times to capture the large length scale geometrical structure information about the large neurons of the Aplysia. At the smaller length scales of mammalian cells, it would be important to consider higher b-values and possibly fitting non-linear models such as in (Ingo et al 2014, Gatto et al 2019).

In this study, we modeled the cellular and nuclear membranes as impermeable interfaces and represented the diffusion in each compartment by a diffusion coefficient, \( D_c \) and \( D_m \) respectively. This is a simplification. Knowledge about the intra-cellular and intra-nuclear structures may be incorporated into the model if they become available. For example, the neurofilamentary structure of the giant axon of the squid was studied in (Beaulieu and Allen 1994) and found to result in an anisotropic diffusion ratio of \( 1.2 \pm 0.1 \).

Membrane permeability, for example, may start to play a role at higher b-values and longer diffusion times beyond the regime where the STA model is valid. In the future, the intracellular vesicular and membrane-based organelles may be considered as well. We mention that the influence of different intracellular proteins and cell pathologies in mammalian cells have been studied and reviewed in (Colvin et al 2011, Harkins et al 2009, Matsumoto et al 2009, Sotak 2004).

Given the importance of the morphological features of the cellular nucleus in this study, the addition of higher magnification histology and nuclear counterstaining with 3D reconstruction could help to obtain better ground truth information of the real geometrical contribution of the neuronal nucleus to the dMRI signal, and would be a useful future direction to explore.

5. Conclusions

We have acquired and analyzed the diffusion time-dependent dMRI data in the *Aplysia* neuronal network for large cell neurons. We found that it is not sufficient to approximate the ADC using a one compartment short time approximation (STA) model. Using the one compartment STA model resulted in a serious under-estimation of the cell size. Supported by numerical simulations and by taking into account the noise in the experimental ADC as well as the uncertainty in cell size due to the segmentation procedure, we established that a two compartment STA model is adequate to explain the behavior of the experimentally obtained ADC.

Acknowledgments

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Animal experiments

All animal experiments were conducted in strict accordance with the recommendations and guidelines of the European Union and the French National Committee.
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