From single- to double-PFG: Gleaning new microstructural information in complex specimens

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Introduction. Diffusion NMR and MRI have become the most important modalities for non-invasively probing tissue microstructures. To date, the single-pulsed-field-gradient (s-PFG) methodology has been the most widely used both in biomedicine and in porous media MR applications. In neuroscience, diffusion tensor imaging (DTI) has provided an excellent means to study coherently packed anisotropic structures[1], while the q-space approach can be used to extract compartmental dimensions[2]. Notably, when monodisperse compartments are present, the diffusion-diffraction minima are observed at higher q-values, from which accurate compartment dimensions can be extracted[3]. However, when the specimen is characterized by size distributions, the diffusion-diffraction minima vanishes from the E(q) plots. Even when diffusion-diffraction minima are observed, they necessitate the use of strong gradients to reach high q-values. Another limitation of s-PFG is that when anisotropic compartments are randomly oriented, both DTI and q-space MR approaches fail in extracting accurate microstructural information. In such scenarios, even the presence of restricted diffusion is sometimes difficult to infer. Such limitations prevent s-PFG from accurately characterizing, *inter-alia*, grey matter microstructure, which is characterized by randomly oriented anisotropic compartments having a broad size distribution. In white matter, accurate characterization of compartment dimensions requires extremely high q-values, also limiting the applicability of this method.

Double-PFG (d-PFG) MR is emerging as a powerful new tool for studying restricted diffusion, especially where s-PFG is inherently limited. The d-PFG, first proposed by Cory et al. in 1990[4] is an extension of the s-PFG, employing two gradient pairs $\mathbf{G_1}$ and $\mathbf{G_2}$ which are separated by a mixing time (t_m) (Fig. 1A). Another variant of d-PFG was recently introduced, in which the middle gradients are superimposed, yielding t_m=0ms, a desirable property for some applications (Fig 1B). At the high q-regime, zero-crossings, (analogous to diffusion-diffraction minima in s-PFG) were predicted for d-PFG experiments[5], that have extraordinary robustness towards size and orientation distributions. The angular d-PFG experiment, in which the angle ψ between the gradients is varied at a defined q-value, has been predicted to result in a bell-shaped function from which the size of the compartment could be extracted, even at low q-values[6,7], thus obviating the need to reach high q-values in the q-space approach. Recent theoretical studies have provided a general framework for restricted diffusion in multiple-PFG, and especially d-PFG experiments[8,9]. The theory accounts for variation in every experimental parameter in d-PFG, and predicted, *inter-alia*, that d-PFG can provide novel microstructural information: (1) In the high q-regime, the zero-crossings in d-PFG were predicted to persist even in cases of size distributions, where the analogous diffusion-diffraction minima in s-PFG vanish along with the microstructural information they convey; (2) In coherently placed anisotropic compartments, the angular d-PFG was predicted to provide accurate compartment size even at low q-values; (3) When microscopically anisotropic compartments are randomly oriented, the angular d-PFG experiment at t_m=0ms can report on the compartment size, while at finite t_m, the angular experiment was predicted to report on the compartment shape anisotropy (CSA), which enables differentiation between randomly oriented cylinders and ellipsoids, for example

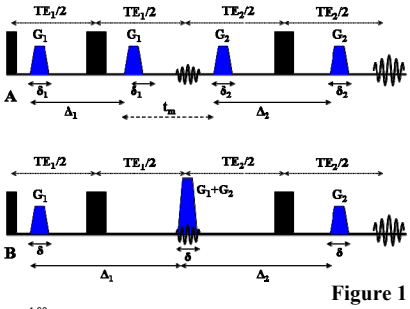

Figure 1

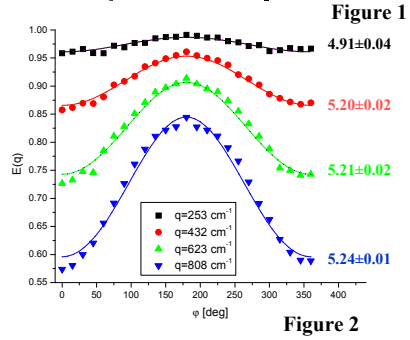

Figure 2

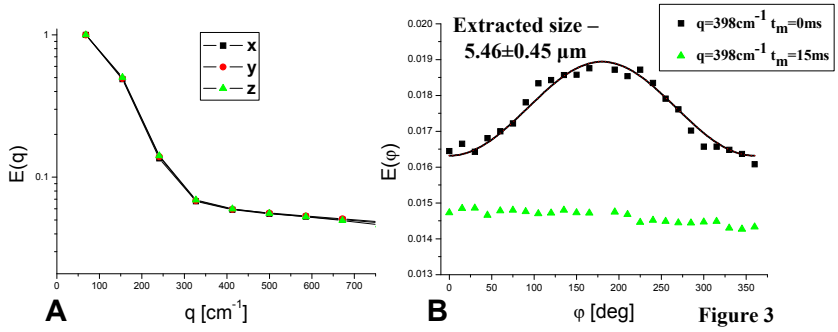

Figure 3

Objectives. Here we experimentally test the new theoretical predictions for d-PFG using phantoms in which the ground-truth is known *a-priori*, and subsequently assess its potential to overcome the inherent limitations of s-PFG in biological settings using cells and isolated neuronal tissues.

Methods. The d-PFG experiments were first performed on phantoms consisting of microcapillaries of well defined nominal inner diameter (ID) and shape[10]. To assess the validity of the theoretical predictions regarding the robustness of zero-crossings to variation in compartment size distribution, both s- and d-PFG experiments were performed on size distribution phantoms which were prepared by mixing water-filled microcapillaries of various sizes. To test the accuracy of the theory to extract small compartmental dimensions, the angular d-PFG experiments were performed as described previously in (10). To challenge our models to provide accurate size and shape in biological cells, we used fixed yeast cells (spherical) and cyanobacteria (elongated) in angular d-PFG experiments. The mean cell size and standard deviation were compared to the same parameters extracted from light microscopy. Finally, the angular d-PFG experiment was performed on optic and sciatic nerves.

Results. The zero-crossings in d-PFG experiments were recently observed experimentally for the first time[11]. We find that indeed the zero-crossings persist in size distribution phantoms, while diffusion-diffraction minima vanish from the s-PFG signal decay due to the size distribution (data not shown). The ability of d-PFG to extract accurate compartmental dimensions using low q-values and weak (clinically relevant) gradients (as low as 15 G/cm) is shown in Figure 2. Here, microcapillaries with nominal ID=5±1µm were used, and the angular dependence can clearly be seen experimentally (symbols). The agreement with the theoretical fit (solid lines) is excellent, and the size that was extracted was consistent with the nominal ID[10] (results are presented adjacent to the plots in Fig 2). Figure 3 shows results from fixed yeast cells. The s-PFG does not provide the size and shape of the cells, yielding an isotropic signal decay (Figure 3A); however, the theoretical fit of the angular d-PFG experiment at t_m=0ms (Figure 3B solid lines) reveals that the compartment size is 5.46±0.45 µm, in excellent agreement with the size extracted from microscopy – 5.32±0.83 µm. Moreover, the loss of angular dependence at longer mixing time (Figure 3B, green symbols) implies that the yeast are spherical, which is consistent with the light microscopy results. Conversely, we show that in randomly oriented locally anisotropic cyanobacteria, the angular dependence at long t_m is not lost, and is consistent with compartment shape anisotropy[9] (data not shown). Preliminary results from optic and sciatic nerves reveal different angular dependencies which are most likely due to the different compartment size and shape within the nerves.

Conclusions. The d-PFG methodology provides novel microstructural information that cannot be obtained using s-PFG both in coherently packed and randomly oriented compartments. Therefore, this methodology may provide a new source of contrast in diffusion MRI. We have validated the new theoretical framework and extracted novel microstructural information both in phantoms in which the ground truth is known *a-priori*, and in biological cells and tissues. Future studies will focus on d-PFG imaging and its ability to provide new contrasts in the grey matter and in pathological conditions.

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