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Sensitive and automated detection of iron-oxide labeled cells using phase image cross-correlation analysis

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Abstract

Superparamagnetic iron-oxide (SPIO) nanoparticles are increasingly being used to non-invasively track cells, target specific molecules, and monitor gene expression *in vivo*. Contrast changes that are subtle relative to intrinsic sources of contrast present a significant detection challenge. Here we describe a post-processing algorithm, called Phase map cross-correlation Detection and Quantification (PDQ), with the purpose of automating identification and quantification of localized accumulations of SPIO agents. The method is designed to sacrifice little flexibility – it works on previously acquired data and allows the use of conventional high-SNR pulse sequences with no extra scan time. We first investigated the theoretical detection limits of PDQ using a simulated dipole field. This method was then applied to 3D MRI datasets of agarose gel containing isolated dipoles, and *ex vivo* transplanted allogenic rat hearts infiltrated by numerous iron-oxide labeled macrophages as a result of organ rejection. The simulated dipole field showed this method to be robust in very low signal-to-noise ratio images. Analysis of agarose gel and allogenic rat heart show this method can automatically identify and count dipoles while visualizing their biodistribution in 3D renderings. In the heart, this information was used to calculate a quantitative index that may indicate its degree of cellular infiltration.

Keywords

SPIO; MPIO; positive contrast; iron-oxide; superparamagnetic; cell labeling; cell tracking; T₂; T₂-weighted imaging

1. Introduction

The detection of superparamagnetic iron-oxide (SPIO) contrast agents *in vivo* often presents significant challenges. The contrast changes produced by these agents may be subtle relative to other sources of intrinsic contrast, especially if the SPIO biodistribution is not known *a priori*. In this paper we describe a novel post-processing method that can aid in the automated identification of localized accumulations of SPIO agents. This algorithm, called the Phase map cross-correlation Detection and Quantification (PDQ) method, can be used to

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determine the quantity of paramagnetic deposits in tissues. We show that the PDQ method can help to reduce false positives and negatives in the analysis of SPIO-laden tissues.

SPIO agents and larger, micron-sized iron-oxide (MPIO) particles, locally perturb the static magnetic field over length scales on the order of ~ 50 times the particle's diameter [1]. This inhomogeneity causes nearby protons to rapidly dephase, leading to a dramatic reduction in the T_2 and T_2^* relaxation times. T_2^* -weighted images are particularly sensitive to these effects, and often regions of agent accumulation show pronounced hypointensity or complete signal dropout. The development of new *in vivo* imaging methods using SPIO nanoparticles is currently a very active research area. Unmodified SPIO nanoparticles, such as ResovistTM and FeridexTM, are used for liver lesion detection and for distinguishing between normal and cancerous lymph nodes [2]. Modified SPIO particles, for example, conjugated to targeting moieties, such as peptides or antibodies, have been described [3-7]. Other studies have investigated whether SPIOs have detrimental cellular effects [3,8-10], or have improved methods for intracellular SPIO labeling [11,12]. In addition, the empirical detection limits of SPIO-labeled cells have been investigated [9,13,14]. Pilot studies have taken steps towards clinical translation of MRI cell tracking with SPIO agents, for example, demonstrating dendritic cell tracking in human cancer patients undergoing immunotherapy [15].

Often, a key challenge when using SPIO agents is to distinguish the contrast derived from agent accumulation from intrinsic sources of image hypointensity in T_2 - and T_2^* -weighted images. To address this challenge, several imaging methods and pulse sequences have been devised to generate positive contrast images that highlight the presence of SPIOs and other tissue structures that create local magnetic field perturbations due to magnetic susceptibility differences. These techniques include, for example, weighted contrast methods [16,17], spectrally selective excitation [18,19], quantum coherence imaging [20], gradient dephasing [21], and ultrashort TE image subtraction [22]. Effective implementation of positive contrast pulse sequences often requires careful optimization and considerations. For example, positive contrast sequences may require *a priori* estimates of the field disturbance created by SPIO deposits for a particular experiment or require specific imaging hardware [18,21]. Positive contrast methods tend to diminish the signal-to-noise ratio (SNR) per unit scan time compared to conventional magnitude images in order to reap the benefits of positive contrast.

In this paper we describe novel post-processing methods that can aid in the automated identification and quantification of localized accumulations of SPIO agents. The PDQ algorithm can help to reduce false positive and negative results when SPIO deposits are localized, can be used in conjunction with conventional high-SNR imaging pulse sequences, requires no extra scan time and can be applied retrospectively to previously acquired data. A preliminary account of the PDQ method has been described elsewhere [23]. The PDQ method applies an image cross-correlation algorithm to high-resolution MRI data to identify occurrences of the characteristic magnetic susceptibility pattern created by magnetic dipoles in an MRI phase map. A similarity matrix is calculated showing the location and number of dipole patterns, indicative of localized, spheroidal SPIO deposits. We investigated theoretical detection limits using a simulated dipole field, which demonstrated the robustness of the PDQ algorithm in very low SNR images (< 4). At all levels of SNR tested in the simulation, the PDQ method was $\sim 90\%$ accurate. As a further test, three-dimensional (3D) data were acquired in an agarose gel phantom lightly doped with MPIO. When analyzing this homogeneous agarose phantom, the PDQ method found 94% of the dipoles that were identified by visual inspection of MR phase-offset images. Next, we analyzed *ex vivo* 3D MRI data from transplanted allogenic rat heart specimens that were infiltrated with macrophages as a result of organ rejection; the macrophages were *in situ* labeled with MPIO

nanoparticles using techniques described elsewhere [24]. The resulting 3D positive contrast images starkly highlighted labeled cells and other magnetic dipoles. Dipoles were automatically counted by computer and their spatial biodistribution visualized using 3D renderings. In the heart data, this information was used to calculate a quantitative index of MPIO accumulation that potentially reflects the severity of immune cell infiltration (i.e., an “infiltration index”) and the stage of organ rejection. Overall, when analyzing heterogeneous heart tissue, the PDQ method found 79% of the dipoles that were also observed by visual inspection of MR phase-offset images.

2. Background

Raw MRI data are generally recorded in a complex form, where a voxel of intensity, I , has real and imaginary components (i.e. $I = a + bi$, $i^2 = -1$). In conventional MRI, the magnitude image, given by $|I| = \sqrt{a^2 + b^2}$, is typically displayed, and the phase angle information, $I = |I|e^{i\phi}$ where $\phi = \arctan(b/a)$, is typically discarded. Phase MRI maps have been studied extensively [17,25-27]. Generally, a voxel’s phase is proportional to the local magnetic field; in our analysis, we exploit this property to analyze the magnetic field distortions caused by localized spheroidal deposits of paramagnetic agents in tissue. We decompose the phase contributions in each voxel as

$$\phi = \phi_0 + \phi_{INT} + \phi_{MAT} + \phi_{IOX} \quad [-\pi \leq \phi \leq \pi] \quad (1)$$

where ϕ is the measured phase angle, ϕ_0 is the primary phase angle induced by the external magnetic field, ϕ_{INT} is the phase contribution due to the magnetic moments of nearby tissue-fluid interfaces, ϕ_{MAT} is the phase contribution due to the magnetization of a homogeneous tissue or fluid, and ϕ_{IOX} is the phase contribution due to magnetic field perturbations of nearby paramagnetic deposits. The uncertainty in the measured phase angle is given by [28]

$$\sigma_\phi = 1/SNR_{ROI} \quad (2)$$

where σ_ϕ is the standard deviation in the phase angle in a region of interest (ROI), and SNR_{ROI} is the SNR in the conventional magnitude image in the same ROI. In phase images, we define the phase contrast-to-noise ratio (CNR_ϕ) between an ROI and its background as

$$\begin{aligned} CNR_\phi &= (\phi_{ROI} - \phi_{BKG}) / \sigma_{\phi,ROI} \\ &= SNR_{ROI} (\phi_{ROI} - \phi_{BKG}) \end{aligned} \quad (3)$$

where ϕ_{ROI} is the measured phase angle for our ROI and ϕ_{BKG} is the background phase angle near the ROI containing no paramagnetic deposits. The phase map is constrained to the range from $-\pi$ to π and rolls through this range frequently over the image field of view. The $-\pi / +\pi$ boundaries must be removed, and phase-unwrapping algorithms have been previously developed [29,30]; these algorithms determine the multiple of $\pm 2\pi$ that must be added to unwrapped the phase. Modifying Eq. (1) to account for the phase unwrapping correction gives

$$\phi_{Unwrapped} = \phi_0 + \phi_{INT} + \phi_{MAT} + \phi_{IOX} + 2\pi n \quad (4)$$

where $n \in \{\dots, -2, -1, 0, 1, 2, \dots\}$. We assume ϕ_0 , the background magnetic field contribution, is a very low-frequency component of the image compared to all other contributions to

phase. The application of a high-pass filter to the unwrapped phase map (Eq. (4)) therefore eliminates ϕ_0 and results in a phase offset map given by

$$\phi_{\text{Offset}} = \phi_{\text{INT}} + \phi_{\text{MAT}} + \phi_{\text{IOX}} \quad (5)$$

Within the phase offset map, a spheroid deposit of a paramagnetic or diamagnetic substance will cause a dipolar magnetic field perturbation of the form [31]

$$\phi_{\text{Offset}}(r, \theta) \propto \Delta B_z(r, \theta) = \frac{\Delta\chi B_0}{3} \left(\frac{a}{r}\right)^3 (3\cos^2\theta - 1) \quad (6)$$

where $\Delta\chi$ is the magnetic susceptibility difference between the spheroid and the surrounding material, B_0 is field strength, a is the effective spheroid radius, r is distance from its center, and θ is the angular deviation from the direction of B_0 . The PDQ method analyzes the phase offset images using Eq. (6) as a model function (i.e., template) to locate similar perturbations via an image cross-correlation algorithm. The number of voxels in the template is generally set to a size much smaller than the image, as discussed below. The cross-correlation algorithm systematically overlays the search template onto every template-sized patch across the phase offset image. It then calculates the similarity between the template and each image patch, resulting in a two-dimensional (2D) or three-dimensional (3D) similarity matrix that can be visualized [32]. For 2D specifically, cross-correlating template T with source image S gives the resulting image, R , given by [33]

$$R(u, v) = \frac{\sum_{x,y} [S(x, y) - \bar{S}_{u,v}] [T(x - u, y - v) - \bar{T}]}{\left[\sum_{x,y} [S(x, y) - \bar{S}_{u,v}]^2 \sum_{x,y} [T(x - u, y - v) - \bar{T}]^2 \right]^{1/2}} \quad (7)$$

where $\bar{T}$ is the mean pixel value of the template, $\bar{S}_{u,v}$ is the mean of the image patch that is compared with the template, (u, v) is the template's position on the image, and each summation runs over the template-sized image region that is currently being compared with the template. The result is a similarity matrix "image" containing positive contrast spots that indicate dipoles.

Template size is important; its minimum size should be chosen so that no dipoles are found when the template is cross-correlated with regions devoid of dipoles in the source image. For example, if the template is 3×3 pixels, it will likely detect many 3-pixel wide dipole-like arrangements present in random noise. In samples of tissue that are less-homogeneous in their underlying tissue texture, a larger dipole template may need to be used. The maximum template size should be approximately the same as the largest detectable dipole field in the image. A template any larger would tend to reduce the spatial specificity of cross-correlation detection. This choice of template size for each specific experiment is the only non-automated component of the PDQ method.

For a 2D MR image to be analyzed using this technique, the in-plane orientation of the slice must have a significant parallel component to the applied magnetic field (B_0), as required by Eq. (6). If the slice orientation is orthogonal to the direction of B_0 , the dipolar profile will appear circular in the phase offset image and will not exhibit the lobe pattern needed for the cross-correlation analysis.

The PDQ method can be applied to 3D volumetric data. A 3D cross-correlation analysis using a 3D template will locate dipoles with greater sensitivity and specificity because the template-image similarity calculation is extended into a third dimension. Ideally, the 3D data set should contain near-isotropic voxels. For anisotropic voxels, the cross-correlation template must either be modified to reflect the specific voxel aspect ratio, or the MRI data should be zero-filled to form isotropic voxels prior to analysis.

In summary, the output of PDQ analysis is a two- or three-dimensional image of the similarity matrix that contains distinct multi-pixel regions for each dipole found. From these data one can automatically count the number of dipoles present in an ROI or throughout an entire volume. Additionally, similarity matrix images can be imported into 3D rendering software to facilitate visualization of the dipole distributions within organs or tissues.

3. Materials and methods

3.1 Gel phantom

We acquired MR images of a 2% agarose gel phantom that was lightly doped with 1.6 μm -diameter MPIO particles (Bangs Laboratories, Fishers, IN) to generate localized dipolar field perturbations. Phantoms were imaged using an 11.7 T Bruker AVANCE micro-imaging system using a standard 3D gradient-echo pulse sequence with TE/TR=7/1500 ms and an isotropic resolution of 40 μm . Raw echo data were reconstructed to create a volume phase image. A cost minimization-based phase-unwrapping algorithm was applied to each 2D sagittal section in the 3D volume [34]. Phase unwrapped data were then imported into MATLAB and high-pass filtered with a kernel that excluded the lowest 10% of frequencies. The theoretical dipole phase pattern (Eq. (6)) was used to generate 2D and 3D dipole templates for the cross-correlation analysis. Two different templates having dimensions of 8 and 16 voxels per side were used; these corresponded to image distances of 0.3 mm and 0.7 mm, respectively. When analyzing this relatively homogeneous gel phantom, only template sizes of 3×3 and smaller resulted in a large number of false positives. For templates larger than 3×3 , the number of false positives was independent of template size. MATLAB's 2D normalized cross-correlation function was then applied between the phantom's phase offset image and both templates separately. A 3D implementation was used for 3D normalized cross-correlation analysis, which is similar to Eq. (7), but extended to 3-dimensions.

The similarity matrices resulting from cross-correlation were automatically thresholded to locate each dipole. The threshold was determined by applying the cross-correlation to both the plane orthogonal to B_0 and then to the plane parallel to B_0 . Theoretically, the cross-correlation analysis should find zero dipoles in the orthogonal plane, while the parallel plane analysis should identify many dipolar patterns. Using this property, the similarity matrix cutoff threshold for both images was systematically increased iteratively by the computer until the ratio of dipoles found in the parallel plane, to dipoles found in the orthogonal plane, was maximized. A global maximum was found for this ratio by sampling all threshold values since this relationship had a few local maxima in our analysis. Optionally, adjusting the threshold manually is straightforward by visually inspecting an image because the similarity matrix response to dipoles is robust and highly selective.

The thresholded similarity matrix was used to generate the final 'pinpoint' images showing the locations of apparent dipoles. The dipoles were counted automatically using a MATLAB (The MathWorks Inc., Natick, MA) connected-region-counting function and volume-rendered in 3D using Amira software (Mercury Computer Systems, Chelmsford, MA). On our 2.4 GHz Intel Pentium 4 platform, the computing time for the 2D/3D cross-correlation takes on the order of minutes for a high-resolution 3D data set, and dipole counting is instantaneous.

Performance of the 3D PDQ method was estimated by a four step process: (i) a multi-slice volume from a central region of the gel phantom (~10% of total volume) was selected to be representative of a 3D PDQ analysis on the entire sample; (ii) dipoles found by visual inspection in the phase image for that volume were tabulated; (iii) the number of dipoles that appeared both in the 3D PDQ pinpoint image and by visual inspection were counted; and (iv) the number of apparent dipoles marked in the pinpoint image that were not detected by visual inspection (i.e., false positives) were also counted.

3.2 Simulations in the low-SNR regime

To investigate how the PDQ algorithm performs with noisy data, it was applied to a synthetic dataset where noise was systematically varied. The synthetic dataset consisted of a phase-offset image containing 40 virtual paramagnetic particles against a uniform diamagnetic background. The 40 particles consisted of 8 dipoles from each of the following template sizes: 3×3, 8×8, 12×12, 16×16, and 20×20. The templates listed corresponded to the unitless susceptibility values 1, 2, 3, 4, and 5, respectively. Without any noise added, the system started with $CNR_{\phi} = 7.0$ for all dipole sizes. To systematically reduce the phase image signal-to-noise ratio, CNR_{ϕ} , Gaussian noise was added to the real and imaginary components of the image's Fourier transform to generate SNR_{ϕ} levels of 0.23, 0.25, 0.27, 0.29, 0.32, 0.35, 0.39, 0.45, 0.5, 0.58, 0.7, 0.88, 1.17, 1.75, and 3.5. At each noise level, a 2D normalized cross-correlation was performed between the simulated image and each of the two templates, sized 8×8 and 16×16 voxels. These two template sizes were chosen because they were slightly smaller and larger, respectively, than the median dipole in the simulated image, and thus it is expected that they will produce a different cross-correlation response. Our aim was to explore the sensitivity to dipole detection for different template sizes, with the systematic addition of noise. A serial application of the cross-correlation analysis was performed; the small template was first applied to obtain a high-sensitivity similarity matrix. Next, the large template's response was used to further reduce false positives and filter out any dipoles that the small template may have detected in random noise. For SNR simulations the resulting similarity matrices for each noise level were not thresholded automatically and a single confidence cutoff threshold for all simulations was determined by eye; this was accomplished by increasing the cutoff until only well-defined peak-valued pixels remained in the original, noiseless image. PDQ performance on the noisy synthetic data using the two-template approach was scored by comparing the resulting pinpoint images with prior knowledge of the simulated dipole locations. This comparison allowed us to extract two important measurements for each level of CNR_{ϕ} . First, we extracted the probability that a pinpoint (single dot) is actually a dipole by calculating $P = (\# \text{ of dots marking a dipole}) / (\text{total } \# \text{ of dots})$. Second, we extracted the probability that any given dipole is found by calculating $P = (\# \text{ dipoles marked by dot}) / (\text{total } \# \text{ of dipoles})$. These probabilities were plotted for every level of CNR_{ϕ} tested. The dual template method was not needed for either the agarose gel phantom analysis or the allograft heart analysis (below). Two templates were tested in the synthetic dataset only to evaluate the PDQ's maximum performance ability when confronted with data sets with exceedingly high noise levels.

3.3 Macrophage-infiltrated allograft rat heart

We investigated intact *ex vivo* rat hearts that had been infiltrated by MPIO-labeled macrophages. The hearts are from an allogeneic heart rejection model, and the details of this model are described by Wu *et al.* [24]. Briefly, for allogeneic transplantation, the heart from a Dark Agouti rat was transplanted to the abdominal region of a Brown Norway rat, whereas, for syngeneic transplantation, the heart from the same strain was used. When MPIO is injected intravenously (*i.v.*), the particles are endocytosed by resident macrophages, effectively labeling these cells *in situ* [24]. Because macrophages play a crucial role in early organ rejection, they will infiltrate engrafted tissues in a number that has been observed to

be proportional to the severity of organ rejection [24]. The MPIO, consisting of 0.9 μm polymer-coated microspheres with a magnetite core (Bangs Laboratories, Fishers, IN), was injected *i.v.* four days after organ transplant. After 24 hours, the rat was sacrificed, perfused, and the intact heart tissues were fixed in a 4% paraformaldehyde and 1% glutaraldehyde solution and then placed in PBS. Data were also acquired in control isogenic rat heart transplants that do not undergo rejection. All animals received humane care in compliance with the Guide for the *Care and Use of Laboratory Animals*, published by the National Institutes of Health, and the animal protocol was approved by our University's Institutional Animal Care and Use Committee.

High-resolution 3D T_2^* -weighted, gradient-echo images of the intact organ were acquired at 11.7 T with TE/TR=8/500 ms and a resolution of $40 \times 40 \times 80 \mu\text{m}$. Before PDQ analysis, data were zero-filled to generate approximately isotropic voxels. After reconstructing and unwrapping the phase images, a 2D cross-correlation analysis was performed on each image slice of the 3D data stack using only the 16×16 template. When analyzing this relatively inhomogeneous heart tissue, template sizes of 8×8 and smaller resulted in a large number of false positive results. For templates larger than 8×8 , the number of false positive results was not found to vary with template size. The slice plane was chosen to be parallel to B_0 . A 3D analysis was also performed, where the confident cutoff threshold of the similarity matrix was automatically determined using the same B_0 -parallel versus B_0 -orthogonal slice comparison as described for the gel phantom (above). Once the threshold parameter was determined computationally, it was applied globally throughout the volume. The dipoles in the tissue volume were counted using a connected-region-counting algorithm in MATLAB. Images were rendered for 3D visualization, and method accuracy and sensitivity were calculated using the same analysis procedure described for the gel phantom (above).

4. Results

4.1 Gel phantom

As an initial evaluation of the PDQ method, we performed 3D imaging studies of an agarose gel phantom doped with MPIO particles to generate localized dipolar field perturbations. A magnitude MR image slice of the phantom (Fig. 1a) shows numerous hypointense spots that are consistent with the presence of MPIO particles, microscopic air bubbles, and perhaps undissolved agarose particles. Figures 1b-c show the phase image of the same slice before and after phase unwrapping, respectively. Applying a high-pass filter to the slice reveals numerous dipolar magnetic field patterns (Fig. 1d). Most dipoles are due to localized paramagnetic entities (e.g., MPIO or air bubble), however a few dipoles appear diamagnetic (Fig. 1d, white arrows). We speculate that these diamagnetic dipoles could possibly be small crystals of undissolved agarose, since these would be more diamagnetic than the aqueous agarose gel.

The templates used for the cross-correlation analysis were calculated using Eq. (6), where Fig. 2a shows a 16×16 voxel 2D template, and Fig. 2b shows a generic 3D version. Figure 3 shows portions of a phase MR image of six different dipolar profiles found in the gel phantom, along with a one-dimensional cross section through the similarity matrix response following a 8×8 and 16×16 template cross-correlation serial analysis. The larger dipole template is insensitive to noise (Fig. 3a). The smaller dipole template is more sensitive to small and weak dipoles (Figs. 3b-c), but is more likely to generate false-positives when cross-correlated against noise. The large 3D template's response alone was used to generate the final PDQ output (Fig. 8a).

We note that cross-correlating either template with the diamagnetic particle (Fig. 3e) resulted in a strong negative response in the similarity matrix; thus, the PDQ method can

provide stringent differentiation between paramagnetic and diamagnetic dipoles, a feature not offered by conventional magnitude images.

A summary of results showing the efficacy of the PDQ method when compared to visual inspection is tabulated in Table 1. The 3D cross-correlation methods showed no statistically significant improvement over a 2D analysis, implying that imperfectly-shaped dipoles in the sample may be a limiting factor when using this method against homogeneous backgrounds such as agarose. Using a representative sample size of 625 mm³ of agarose gel, the dipole density was calculated in Table 1 under the heading “infiltration index.”

Figure 8a shows the 3D-rendered distribution of dipoles using the 3D PDQ method in the gel phantom. Figure 8b shows the 3D-rendered distribution of false positive “dipoles” found by analyzing 2D slices through the volume orthogonal to B_0 at the same threshold; theoretically this field orientation should result in no detectable dipoles, however, several false positives are clearly visible.

4.2 Simulations in the low-SNR regime

To test the robustness of the PDQ method in low SNR images, a simulated dipole field was analyzed. Figure 4a shows the noise-free synthetic phase image, and Figs. 4b-d show the effects of incrementally increasing noise. Smaller (i.e., weaker) dipoles are the first to be dropped from detection with the addition of noise. At $CNR_\phi = 0.3$, 50% of the large dipoles are still detectable by this method. To summarize the effect of noise, Fig. 5 plots the probability of finding a given dipole using the PDQ method in addition to the probability that a “positive” is actually a dipole. The figure shows detection accuracy remains between 85-95% for all values of CNR_ϕ studied. For values of $CNR_\phi > 1.5$, all dipoles are found. Notably, at $CNR_\phi = [0.23, 0.25, 0.29]$, even though the CNR_ϕ is held nearly constant, the probability that a single dot is a dipole varies by ± 0.05 . This variance appears because a different noise field is generated and imposed on the dipole field image for each value of CNR_ϕ . Therefore, two different noise fields can be generated for the same CNR_ϕ value, but the contrast of individual dipoles may vary between the two noise-field images, causing dipole detection probabilities to vary within a small range of values. This likely explains the drop in dipole detection probability at $CNR_\phi = 0.32$ (Fig. 5). The magnitude of this drop is comparable to the intrinsic probability variance of ± 0.05 .

4.3 Macrophage-infiltrated allograft rat heart

The PDQ method was found to be effective in detecting and quantifying magnetic dipoles in heterogeneous tissue, many of which represent MPIO-labeled cells and cell clusters. These experiments used an allogenic rat heart transplant that was excised and fixed following acute organ rejection. Prior to sacrificing the rat, its macrophages, destined to participate in the organ rejection, were labeled *in situ* with MPIO [24]. Figure 6 shows T_2^* -weighted magnitude slices (left column) and the corresponding phase offset images (right column) through the rejecting heart viewed from both orthogonal and parallel planes to B_0 (Figs. 6a-b, respectively). The characteristic 2D dipole impressions are scattered throughout the heart slice parallel to B_0 in the phase images (bottom, Fig. 6b), but are mostly absent in the slice orthogonal to B_0 (top, Fig. 6a). This property was used to automatically threshold the cross-correlation similarity matrix results; the PDQ algorithm minimizes false positives by choosing a similarity matrix threshold such that minimal dipoles appear in the B_0 -orthogonal slices, while simultaneously maximizing the number of dipoles detected in the B_0 -parallel slices.

Figures 7a-b show allograft tissue with numerous macrophages infiltrating deep tissue as expected for this heart transplant model. Figure 7a shows putative macrophages labeled by

2D PDQ analysis (red dots) after analyzing a single 2D tissue section. Figure 7b shows the same slice after applying 3D PDQ analysis. A comparison between these 2D and 3D results indicate that more dipoles are detected in the 3D analysis at the expense of only a few additional false positives. Thus, the additional redundancy provided by neighboring image slices improves the overall performance of the 3D PDQ method. As a control, the 3D PDQ method was applied to the heart isograft samples (Fig. 7c). As expected for this isogenic transplant, fewer dipoles are detected in tissue (Table 1). Histological assays of the transplanted tissues examining macrophage infiltration are qualitatively consistent with the results of the PDQ analysis for both the allograft and isograft tissues [24].

Unlike the dipoles detected in the gel phantom that appear mostly punctate after PDQ analysis (Fig. 8a), the 3D-rendered PDQ output for the entire allograft heart volume (Fig. 8c) often displays irregular and linear shapes, possibly indicating clusters of labeled macrophages, intra- or peri-vascular macrophage deposits, or perhaps residual blood in vessels.

Table 1 shows the accuracy and sensitivity of the PDQ method, compared to visual inspection, when applied to the allograft and isograft heart tissue using representative sample sizes of 314 mm³ and 288 mm³ respectively. In the allograft heart, the dipole density, or the infiltration index, was calculated to be 7.36 dipoles/mm³ (Table 1) and may represent the magnitude of macrophage infiltration in the heart tissue at this post-operational stage. A fraction of the dipoles counted are expected to be false positives (~15%, see Methods). However, we note that since continuous regions in the PDQ output, such as due to blood vessels or edge artifact, will only be counted as a single dipole, thus these artifacts represent a small contribution to the total dipole count.

5. Discussion

The PDQ method can be used to generate positive contrast images that highlight individual SPIO-labeled cells, cell clusters, and other magnetic dipoles present in tissues. We test these methods in 3D using gel phantoms doped with isolated paramagnetic dipoles and apply the methods to simulated data to determine how much noise one can introduce before the method fails. Finally, we test our methods in heterogeneous tissue; a 3D dataset from an *ex-vivo* allograft rat heart infiltrated by numerous MPIO-labeled macrophages. The heart was part of a study that aims to develop a non-invasive MRI alternative to biopsy, which is the current gold standard for diagnosing and staging rejection after organ transplantation [24]. We demonstrate that it is feasible to derive quantitative markers based on the density of dipoles present in tissue, and we speculate that the infiltration index may be a useful biomarker for quantifying the degree of cellular infiltration into tissue and thus the extent of organ rejection.

The PDQ method relies on the generation of phase maps from an acquired MRI dataset. Previously MRI phase maps have been successfully used to quantify magnetic field inhomogeneity [35], enhance contrast among tissues with different susceptibilities [17], quantify iron in the brain [25], classify chemical shifts [36], enhance vascular contrast [26,37], identify magnetic particles based on how they appear [38], and undistort MR images [29].

The PDQ method can potentially improve the detection of spheroid deposits of paramagnetic agents in several ways. Like other positive contrast methods, this approach enhances the ability to detect and differentiate paramagnetic agents from intrinsic sources of hypointensity such as regions of short T₂ and T₂^{*}, low proton density, and susceptibility artifacts across interfaces. It can also improve the ability to detect smaller, weaker, or

partial-volume deposits of contrast agent, and decrease the number of false negatives. Importantly, we show that the PDQ method remains robust when applied to low SNR images.

When analyzing heterogeneous tissues such as the heart, the PDQ method has several potential pitfalls and challenges. As expected, more false negatives and positives are found in heterogeneous tissues compared to gel phantoms. Importantly, the PDQ method is less effective when applied to distributions of SPIO having non-spherical shapes spanning multiple voxels. For example if labeled cells are too dense in tissue, their characteristic imprints on the phase image begin to overlap, reducing PDQ method accuracy. Ideally, paramagnetic disturbances should be localized and punctate in appearance. This requirement is due to the fact that we assume that SPIO-loaded cells or cell clusters behave as ideal point magnetic dipoles. Equation (6) generates valid templates when cells or cell clusters are on the order of the voxel-size or smaller. The PDQ method can be modified to consider arbitrary, non-spherical distributions of SPIO agent. This modification would require the replacement of the phase-offset template generated using Eq. (6) with one that models the magneto-static field of a specific paramagnetic geometry of interest. One way to generate these new composite templates would be to convolve Eq. (6) with a 2D image that represents the distribution of multiple spherical paramagnetic deposits.

Another potential pitfall of applying the PDQ method to tissues is that blood vessels are often detected when they curve parallel to B_0 . The detection of blood vessels may be reduced or eliminated by raising the similarity matrix cutoff threshold, but one risks eliminating the weakest dipoles present in the tissue. Alternatively, a 3D PDQ analysis gives improved blood vessel discrimination; vessels within 2D images have cross-sections that may be indistinguishable from iron-oxide labeled cells.

The choice of MR image acquisition parameters may affect how well the PDQ method performs. The method is expected to detect more dipoles as the image CNR increases. PDQ is also expected to detect dipoles with greater specificity as image resolution increases, since a larger, more detailed cross-correlation template can be used. At lower magnetic field strengths, high-resolution images may be difficult to achieve and 2D slice thickness may also be larger. As the slice thickness increases so does partial-volume, thereby reducing the impact of a dipole's magnetic field disturbance in the phase map. The PDQ method works ideally with a slice thickness no greater than the extent to which a dipole's magnetic field perturbation is significant versus the background noise.

In addition to *ex vivo* examination of tissues, the PDQ method may be applicable to *in vivo* studies. Future studies will investigate the efficacy of the PDQ method *in vivo*, although we suspect that reduced scan times and lower resolutions will require higher agent magnetic susceptibility or concentration to remain as effective as we have demonstrated in *ex vivo* samples. Additionally, short-T2 tissues (e.g., liver) may require higher agent relaxivity or concentrations since sub-voxel dephasing occurs rapidly in these organs, thereby reducing the CNR available for accurate dipole detection.

The PDQ method becomes useful for numerous applications where paramagnetic deposits are quantified and visualized in 3D. For example, in the heart rejection model, the number and spatial distribution of cellular infiltrates provides information about the degree to which the heart allograft is undergoing organ rejection [24]. The number of infiltrating macrophages is related to post-operative day (POD). Macrophages progress from pericardium to endocardium in the heart's left ventricle, reaching different distances from the heart's edge as the POD increases [24]. *In vivo* longitudinal studies using PDQ analysis could potentially yield information about both macrophage quantity, expressed as an

infiltration index, as well as the cell distribution, which could potentially be invaluable for scoring acute rejection progression *in vivo*. Future studies will focus on rigorously validating the use of the infiltration index in *in vivo* rejection models. As an additional example, detecting SPIO-labeled stem cells or neuronal precursors that have been transplanted into the brain would be an ideal application since brain parenchyma is a relatively uniform background. We note that previous studies have reported the detection of very small numbers of SPIO-labeled cells, or even individual MPIO particles in gel phantom and mouse embryos [9,14,39].

In conclusion, we demonstrate that the PDQ method has the potential to improve detectability of localized SPIO deposits in tissue. Because the PDQ method is computerized, it offers consistency and reproducibility and can analyze intricate magnetic susceptibility signal patterns. In addition to providing qualitative and quantitative readouts, the PDQ algorithm may also eliminate the need for time-consuming, manual cell counting and interpreting the subtle changes in grayscale contrast that indicate the presence of labeled cells throughout multiple slices or large organs.

6. Conclusions

Emerging cellular-molecular MRI applications increasingly utilize paramagnetic contrast agents. These applications will benefit from methods that offer improved sensitivity to localized agent concentrations by increasing specificity and reducing false positives and negatives. The cross-correlation method presented here generates positive contrast images that can be overlaid onto conventional magnitude images to quickly highlight contrast agent deposits that exhibit a dipole impression in MRI phase images. We demonstrate the method's sensitivity and robustness at low SNR and how it is easily extended to count thousands of dipoles automatically and visualize their distributions in 3D renderings. Like other positive contrast methods it helps differentiate dark areas due to SPIO from other intrinsic sources of hypointensity, but unlike these methods it requires no prior knowledge of agent concentration or distribution, no special imaging pulse sequences, no extra scan time, and can be applied retrospectively to previously acquired data.

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Figure 1.

Various representations of the same MR image of a gel phantom containing a mixture of MPIO particles, air bubbles, and undissolved agarose crystals. The image types displayed include- **a:** magnitude image, **b:** phase image, **c:** unwrapped phase image, and **d:** phase offset image. Arrows indicate diamagnetic dipoles. Each dipole in the magnitude image (**a**) appears as a dark spot against the background while those in the phase offset image (**d**) have a clear dipolar impression.

Figure 2. Representative dipole templates used for **a:** 2D and **b:** 3D cross-correlation analysis. For both templates, dark areas are the negative phase offset and bright areas are the positive phase offset. Templates were generated computationally using Eq. (6).

Figure 3. Normalized cross-correlation analysis applied to various dipole impressions found in the gel phantom phase offset images. The gray and black lines are the maximum similarity when an 8×8 and 16×16 template, respectively, are cross-correlated with- **a**: region of noise, **b-d**: paramagnetic objects of various strengths, and **e**: unidentified diamagnetic object. The larger dipole template is impervious to noise (**a**), while the smaller dipole template detects noise but is more sensitive to small, weak dipoles (**b-c**). Note that cross-correlating the template with the diamagnetic object (**e**) results in a strong negative response.

Figure 4. Synthetic dipole field images (left column) and the corresponding results of cross-correlation analysis with varying amounts of noise (right column). Each dot indicates the location of a dipole. The same simulated image is analyzed at CNR_{ϕ} **a:** 7.0, **b:** 0.7, **c:** 0.35, and **d:** 0.23. As noise increases, small dipoles drop from detection, but the largest dipoles remain detectable to the highest values of noise. At noise levels where dipoles are nearly undetectable by eye, the algorithm is able to locate half of dipoles present.

Figure 5.

Plot of (i) probability of finding a given dipole in the synthetic dipole field when using PDQ analysis and (ii) probability that a dot in the similarity matrix image is a dipole. Both probabilities are plotted versus CNR_{ϕ} . At $CNR_{\phi}=0.3$, 50% of the large dipoles are still detectable by this method. Detection accuracy remains between 85-95% for all noise values studied. For values of $CNR_{\phi} > 1.7$ (not shown) all dipoles are found.

Figure 6.

Magnitude images (left column) and phase offset images (right column) of the same heart allograft tissue sample when viewed from **a**: a plane orthogonal to B_0 and **b**: a plane parallel to B_0 . As predicted by Eq. (6), phase impressions matching the 2D dipole template are scattered throughout the slice parallel to B_0 (**b**), but are absent in the slice orthogonal to B_0 (**a**).

Figure 7. MPIO-labeled macrophages infiltrating heart tissue detected by **a:** 2D and **b:** 3D PDQ analysis on heart allograft tissue and **c:** 3D PDQ analysis on heart isograft control. Each red dot marks a dipole found. The white arrow shows an example of artifact highlighted by the PDQ method, which is presumed to be a blood vessel. Dipoles found in the allograft are dispersed throughout the myocardium, while the isograft control has a lower dipole density in tissue.

Figure 8.

3D renderings of PDQ-detected dipoles. Shown is the gel phantom analyzed with **a:** B_0 -parallel slices and **b:** B_0 -orthogonal slices. Panel (c) shows the 3D PDQ analysis for the allograft rat heart infiltrated by MPIO-labeled macrophages. Gel and heart volumes are outlined in translucent blue, while dipoles are rendered as white spots. The arrow denotes dipoles found in a typical tissue slice. All dipole marks in the gel phantom appear spherical, but a fraction of marked areas in the heart tissue have linear shapes and may indicate curvilinear distributions of labeled macrophages or blood vessels with trajectory components parallel to B_0 .

Table 1

Results applying 3D PDQ to the gel phantom and allograft and isograft heart tissues. Visual inspection was performed by manually searching for dipolar patterns in MRI phase-offset images. 'Dipoles missed' represents the fraction of dipoles found by visual inspection that were missed by PDQ. The infiltration index represents the number of dipoles detected per unit tissue volume.

Dataset	Dipoles found by visual inspection	Dipoles found by PDQ	Dipoles missed	Infiltration Index (dipoles/mm ³)
Gel Phantom	50	47	6%	12.8
Heart Allograft	29	23	21%	7.36
Heart Isograft	31	28	10%	3.22