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Anisotropic Solute Diffusion Tensor in Porcine TMJ Discs Measured by FRAP with Spatial Fourier Analysis

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Abstract

A new method solely based on spatial Fourier analysis (SFA) was developed to completely determine a two dimensional (2D) anisotropic diffusion tensor in fibrous tissues using fluorescence recovery after photobleaching (FRAP). The accuracy and robustness of this method was validated by using computer simulated FRAP experiments. This method was applied to determine the region-dependent anisotropic diffusion tensor in porcine temporomandibular joint (TMJ) discs. The average characteristic diffusivity of 4kDa FITC-Dextran across the disc was $26.05 \pm 4.32 \mu\text{m}^2/\text{s}$ which is about 16% of its diffusivity in water. In the anteroposterior direction, the anterior region ($30.99 \pm 5.93 \mu\text{m}^2/\text{s}$) had significantly higher characteristic diffusivity than the intermediate region ($20.49 \pm 5.38 \mu\text{m}^2/\text{s}$) and posterior region ($20.97 \pm 2.46 \mu\text{m}^2/\text{s}$). The ratio of the two principal diffusivities represents the anisotropy of the diffusion and ranged between 0.45~0.51 (1.0=isotropic). Our results indicated that the solute diffusion in TMJ discs is inhomogenous and anisotropic. These findings suggested that diffusive transport in the TMJ disc is dependent upon tissue composition (e.g., water content) and structure (e.g., collagen orientation). This study provides a new method to quantitatively investigate the relationship between solute transport properties and tissue composition and structure.

Keywords

Fluorescence recovery after photobleaching (FRAP); Spatial Fourier analysis (SFA); Confocal laser scanning microscopy (CLSM); Anisotropic diffusion tensor; Temporomandibular joint (TMJ) disc

INTRODUCTION

The temporomandibular joint (TMJ) is a synovial, bilateral joint with unique morphology and function. The TMJ disc, a fibrocartilaginous tissue, is a major component of jaw function by providing stress distribution and lubrication in the joint.^{23, 24} Disc derangement (e.g., dislocation of the disc) is a common clinical finding in patients with TMJ disorders (affecting more than 10 million Americans).³¹ It has been suggested that degenerative processes predispose the disc to displacement and result in significant changes in disc morphology, biochemistry, function, and material properties.^{31, 32}

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The TMJ disc has a distinctive extracellular matrix (ECM) composition when compared to hyaline cartilage and other fibrocartilaginous tissues [e.g., the intervertebral disc (IVD)]. The TMJ disc is comprised primarily of water with a significant amount of collagen (mainly type I) and a very small amount of proteoglycan.^{4, 11, 21, 22, 29} The TMJ disc is a large avascular structure with human discs being wider mediolaterally than anteroposteriorly (approximately 19×13 mm).²⁸ The nutrients required by disc cells for maintaining healthy matrix are supplied by synovial fluid at the margins of the disc, as well as from nearby blood vessels at the posterior bilaminar zone connection.⁷ Diffusive transport of nutrient solutes through the ECM plays a key role in maintaining the normal function of TMJ discs while the dysfunctional transport of those solutes is believed to contribute to the degeneration of the disc over time. Solute diffusivities in articular cartilage and IVD have been measured using a variety of techniques, including magnetic resonance imaging (MRI),^{10, 12} tracking the net movement of radiolabeled solutes,^{33, 34} and fluorescence²⁷ or radiotracer desorption.^{17, 18} However, to our knowledge the solute diffusivities in the TMJ disc have not been determined. Moreover, differences in biochemical composition and structure distinguish the disc into three regions: the anterior band, intermediate zone, and posterior band.²⁸ Collagen fibers in the anterior and posterior bands run primarily in the mediolateral direction, while in the intermediate zone these fibers run predominately in an anteroposterior direction.²⁰ Studies have shown that the mechanical properties of the TMJ disc are region-dependent and anisotropic.^{7, 15} Therefore, it is reasonable to hypothesize that the solute diffusion in the TMJ disc is inhomogeneous and anisotropic. The objective of this study was to determine the region-dependent anisotropic diffusion tensor in TMJ discs using a new fluorescence recovery after photobleaching (FRAP) technique coupled with spatial Fourier analysis (SFA).

FRAP is a versatile and widely used tool for the determination of local diffusion properties within solutions, tissues, cells, and membranes.¹⁹ Due to the high spatial resolution, FRAP offers the possibility to microscopically examine a specific region of a sample. For instance, the site-specific (i.e., inhomogeneous) solute diffusion in articular cartilage has been previously studied using FRAP.^{9, 16} The basic principle of FRAP is based on photobleaching the fluorescence of molecular probes and analyzing the recovery of the bleached area.^{1, 13, 25} To date, most FRAP analyses are based on the method proposed by Axelrod *et al.* for isotropic diffusion.¹ Another approach for FRAP analysis is based on the spatial Fourier analysis of the recovery images and has the advantage of potential detection of anisotropic diffusion.^{3, 14, 26, 30, 35, 38, 39} Tsay and Jacobson, using SFA of FRAP images, developed a method for the determination of a two-dimensional (2D) diffusion tensor along the fixed coordinate system.³⁸ Most recently, Travascio *et al.* reported a method for calculating an anisotropic diffusion tensor based on two independent analyses of FRAP images: the Fourier transform (FT) and the Karhunen-Loeve transform (KLT).³⁷ The KLT analysis of FRAP images could determine the principal direction of the diffusion tensor. By combining the FT and KLT analyses, all components of the anisotropic diffusion tensor can be solved for in a single FRAP experiment. This method has been used to characterize anisotropic diffusion in knee meniscus and intervertebral disc tissues.^{36, 37} In this paper, we propose a new method solely based on the SFA to completely determine the anisotropic diffusion tensor, avoiding all of the limitations imposed by KLT analysis.

In this paper, we first discuss the theoretical basis of this new analysis. Next, we apply this analysis to computer simulated anisotropic FRAP experiments to validate the method, as well as make a comparison to the method proposed by Travascio *et al.* in terms of accuracy and robustness. And finally, we apply this analysis to characterize anisotropic solute diffusion in different regions of the TMJ disc using the new FRAP technique.

THEORY

Diffusional transport is governed by Fick's second law and in two dimensions, the partial differential equation becomes:⁶

$$\frac{\partial}{\partial t} C(x, y, t) = \nabla \cdot \{ \mathbf{D} [\nabla C(x, y, t)] \} \quad (1)$$

where (x, y) are the spatial coordinates of the imaging system and t is the time. The function $C(x, y, t)$ represents the spatial concentration distribution of solutes in the x - y plane and $\mathbf{D}$ is the diffusion tensor which is assumed symmetric. Within the small observation field of FRAP experiments, it is assumed that the diffusion tensor ($\mathbf{D}$) is independent of position and time. Therefore, Equation (1) could be rewritten as:³⁰

$$\frac{\partial}{\partial t} C(x, y, t) = D_{xx} \frac{\partial^2 C(x, y, t)}{\partial x^2} + 2D_{xy} \frac{\partial^2 C(x, y, t)}{\partial x \partial y} + D_{yy} \frac{\partial^2 C(x, y, t)}{\partial y^2} \quad (2)$$

where D_{xx} , D_{xy} , and D_{yy} are components of diffusion coefficient tensor ($\mathbf{D}$) in the (x, y) coordinate system.

Given that $\tilde{C}(u, v, t) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} C(x, y, t) e^{-i2\pi(ux+vy)} dx dy$ is the Fourier transform of $C(x, y, t)$, with an arbitrary initial condition and a boundary condition that $C(x, y, t)$ is a constant as $(x, y) \rightarrow \pm\infty$, Equation (2) can be solved by using 2D SFA in the frequency domain:^{3, 30, 35, 37, 38}

$$\tilde{C}(u, v, t) = \tilde{C}(u, v, 0) e^{-4\pi^2(u^2+v^2)D(u,v)t} \quad (3)$$

where $\tilde{C}(u, v, 0)$ is the initial solute concentration and $D(u, v)$ represents the diffusion coefficient in Fourier space (with frequencies u and v) and is defined as:

$$D(u, v) = \frac{u^2 D_{xx} + 2uv D_{xy} + v^2 D_{yy}}{u^2 + v^2} \quad (4)$$

The function $D(u, v)$ can be determined by curve fitting the light intensity of a time series of fluorescence recovery images (in the Fourier space) to Equation (3).

The average of $D(u, v)$ over the arc of a circumference (Integral curve L : $u^2 + v^2 = a^2$, a is an arbitrary constant, see Figure 1) over an angle range from α to β can be given as:

$$D_{\alpha, \beta} = \frac{\int_{L: u^2+v^2=a^2} D(u, v) ds}{\int_{L: u^2+v^2=a^2} ds} = \frac{1}{\beta - \alpha} \int_{\alpha}^{\beta} (D_{xx} \cos^2 \xi + 2D_{xy} \sin \xi \cos \xi + D_{yy} \sin^2 \xi) d\xi \quad (5)$$

The auxiliary variable ξ is a function of the frequencies u and v :^{35, 37, 38}

$$\xi = \tan^{-1}(v/u), \quad (\alpha \leq \xi \leq \beta) \quad (6)$$

Based on Equation (5), the average of $D(u, v)$ over the arc from 0 to π can be related to the components of the diffusion tensor $\mathbf{D}$ as:^{35, 37}

$$D_{0, \pi} = \frac{D_{xx} + D_{yy}}{2} = \frac{\text{tr}(\mathbf{D})}{2} \quad (7)$$

Similarly, the average of $D(u,v)$ over the arc from 0 to $\pi/2$ can be calculated as:³⁷

$$D_{0,\frac{\pi}{2}} = \frac{D_{xx} + D_{yy}}{2} + \frac{2D_{xy}}{\pi} = \frac{\text{tr}(\mathbf{D})}{2} + \frac{2D_{xy}}{\pi} \quad (8)$$

The average of $D(u,v)$ over the arc from 0 to $\frac{\pi}{4}$ can be calculated as:

$$D_{0,\frac{\pi}{4}} = \frac{D_{xx} + D_{yy}}{2} + \left(\frac{D_{xx} - D_{yy}}{\pi} \right) + \frac{2D_{xy}}{\pi} = \frac{\text{tr}(\mathbf{D})}{2} + \left(\frac{D_{xx} - D_{yy}}{\pi} \right) + \frac{2D_{xy}}{\pi} \quad (9)$$

According to Equations (7), (8) and (9), all components of the diffusion tensor (i.e., D_{xx} , D_{yy} and D_{xy}) can be obtained and expressed as the functions of $D_{0,\frac{\pi}{4}}$, $D_{0,\frac{\pi}{2}}$ and $D_{0,\pi}$:

$$\begin{cases} D_{xx} = D_{0,\pi} + \frac{\pi(D_{0,\frac{\pi}{4}} - D_{0,\frac{\pi}{2}})}{2} \\ D_{yy} = D_{0,\pi} - \frac{\pi(D_{0,\frac{\pi}{4}} - D_{0,\frac{\pi}{2}})}{2} \\ D_{xy} = \frac{\pi}{2}(D_{0,\frac{\pi}{2}} - D_{0,\pi}) \end{cases} \quad (10)$$

Using all components of the diffusion tensor two principal diffusivities (eigenvalues) of the 2D diffusion tensor can be calculated by:

$$\begin{cases} D_{\text{Eig_Min}} = \frac{D_{xx} + D_{yy} - \sqrt{(D_{xx} - D_{yy})^2 + 4D_{xy}^2}}{2} \\ D_{\text{Eig_Max}} = \frac{D_{xx} + D_{yy} + \sqrt{(D_{xx} - D_{yy})^2 + 4D_{xy}^2}}{2} \end{cases} \quad (11)$$

where $D_{\text{Eig_Min}}$ is the relatively smaller of the two eigenvalues and $D_{\text{Eig_Max}}$ is the relatively larger one. The ratio of $D_{\text{Eig_Min}}$ to $D_{\text{Eig_Max}}$ can be used to represent the anisotropy of the diffusion.

MATERIALS AND METHODS

Computer Simulation of Anisotropic FRAP Experiment

The 2D anisotropic diffusion process of the fluorescent molecule recovery after photobleaching was simulated using finite element analysis software (COMSOL Multiphysics 3.4, COMSOL Inc., Burlington, MA). At the boundaries of the sample (i.e., simulation domain, $4 \times 4 \text{ mm}^2$), the concentration of the fluorescent molecules satisfied the constancy assumption ($C = C^*$). Initially, the concentration distribution of fluorescent molecules was assumed to be uniform ($C = C^*$) outside the photobleached spot and zero within the photobleached area. The diameter of the bleached area was set to $26.71 \mu\text{m}$ according to the optimum value of 8 for the ratio of the imaging frame size ($213.68 \mu\text{m}$) to the initial diameter of the bleached spot as suggested by Travascio and Gu.³⁷ The mesh used in the simulation experiments included 8,784 quadratic Lagrange triangular elements. The convergence criterion for the solution was the relative error tolerance of less than 0.001. The simulation was stopped when the concentration in the bleached spot reached 95% of C^* . For data analysis purposes, an image sequence containing 101 frames (0.6s/frame) representing the FRAP experiment was extracted from the central region of the simulation domain and digitalized to $128 \text{ pixels} \times 128 \text{ pixels}$ (Figure 2a). The frame size was set to $213.68 \times 213.68 \mu\text{m}^2$, which was used in the following TMJ disc FRAP experiment.

Three groups of anisotropic diffusion were investigated in the simulation experiments and there were seven pre-defined diffusion tensors in each group. In the first group, a base

diffusion tensor of $\mathbf{D}_0 = \begin{bmatrix} 10 & 0 \\ 0 & 15 \end{bmatrix} \mu m^2/s$ was selected and rotated at seven different angles $\theta_i, i = 0 \sim 6$ ($\theta_0 \sim 6 = 0, \pi/8, \pi/6, \pi/4, \pi/3, 3\pi/8$, and $\pi/2$) using a rotation matrix $\mathbf{R}_i$ defined as:

$$\mathbf{R}_i = \begin{bmatrix} \cos \theta_i & -\sin \theta_i \\ \sin \theta_i & \cos \theta_i \end{bmatrix} \quad (12)$$

The seven pre-defined diffusion tensors, see Figure 2b, were calculated by:

$$\mathbf{D}_i = \mathbf{R}_i^T \mathbf{D}_0 \mathbf{R}_i \quad (i=0 \sim 6) \quad (13)$$

In the second and third group, the base diffusion tensors were $\mathbf{D}_0 = \begin{bmatrix} 10 & 0 \\ 0 & 20 \end{bmatrix} \mu m^2/s$ and $\mathbf{D}_0 = \begin{bmatrix} 10 & 0 \\ 0 & 30 \end{bmatrix} \mu m^2/s$, respectively. The computer simulations of these three groups allowed investigation into the effects of anisotropy and orientation of the diffusion tensors on the accuracy of the new SFA based FRAP data analysis.

To study the effect of experimental noise on this anisotropic FRAP data analysis photon noise was added to the ideal simulated FRAP images. Although photon noise has a Poisson distribution, for large photon fluxes (e.g., during FRAP experiments) Gaussian noise represents a close approximation of Poisson noise.⁵ Similarly to previous studies,^{37, 38, 39} different magnitudes of Gaussian noise were added to the purely simulated recovery images to estimate how image noise affected the analysis. The magnitude of the Gaussian noise was characterized by its standard deviation, σ . Two magnitudes of spatial Gaussian noise (i.e., $\sigma = 5$ and 10) were generated by ImageJ software (Version 1.39f, by Wayne Rasband, National Institutes of Health, USA) and were then superimposed onto the simulated recovery images. For each level of σ investigated, ten FRAP experiments were simulated.

FRAP Experiment on Porcine TMJ Disc

Nine fresh porcine TMJ discs from five young adult pigs (~8-10 months) were used for testing. Razor blades were used to prepare tissue slices from the intermediate, posterior, anterior, lateral, and medial regions of the discs (Figure 3). One tissue slice (about $4 \times 4 \text{ mm}^2$) was obtained from each region. The final specimens with $\sim 650 \mu\text{m}$ thickness were obtained from each tissue slice using a microtome (SM24000, Leica Microsystems GmbH, Wetzlar, Germany) with a freezing stage (Model BFS-30, Physitemp, Clifton, NJ). The tissue slice was microtomed on both inferior and superior surfaces to remove the natural concave shape of the tissue to maintain a flat cartilage surface for the FRAP experiment. Diffusion tensors for FITC-dextran molecules with a molecular weight of 4kDa (FD4, λ_{ex} 490 nm; λ_{em} 520 nm, Sigma-Aldrich®, St. Louis, MO, USA) were measured with FRAP in the horizontal X-Y plane in Figure 3 (parallel to the surface of the TMJ disc). Correspondingly, the three components (i.e., D_{xx} , D_{yy} and D_{xy}) and two principal diffusivities ($D_{\text{Eig_Min}}$ and $D_{\text{Eig_Max}}$) of the anisotropic diffusion tensor were obtained from the FRAP experiment.

Specimens were immersed in FITC-Dextran solution (0.1mM) for 48 hours (tissue swelling was not significant), to allow the concentration distribution of fluorescent solutes to reach equilibrium throughout the tissue. The FRAP experiment was performed on a Leica TCS-SP2 Confocal Microscope System (Leica Microsystems, Inc., Exton, PA) at room

temperature (22°C). The specimen was photobleached using an Ar-488nm laser to create a circular bleached spot with a diameter about 27µm. A Multi-Layer Bleaching (MLB) protocol was used to ensure a 2D fluorescent recovery in the X-Y plane by minimizing the diffusion in the Z-direction along the thickness of the specimen.³⁵ The depth of the bleaching spot was about 70µm from the surface into the specimen and fluorescence recovery was observed on the focal plan at 7µm beneath the surface of the specimen. A HC PlanAPO 20×/0.7NA dry objective (Leica Microsystems, Inc., Exton, PA) was used to collect fluorescent recovery images of 128 × 128 pixels (213.68 × 213.68µm²). For each experiment, 100 frames of recovery images, plus 5 images prior to bleaching, were acquired at a rate of 0.6s per frame. To minimize the contribution of the fluorescence emission of the background, pre-bleaching images were averaged and then subtracted from the post-bleaching image series.

Images and Data Processing

For both computer simulated and experimental FRAP tests, the image processing was completed using custom written codes in MATLAB (MATLAB 7.0, The MathWorks Inc., Natick, MA). Based on Equation (3), the code performed a 2-dimensional Fourier Transform and nonlinear curve-fit to yield solutions for $D(u,v)$, which was the diffusion coefficient in the frequency domain. Based on Equation (5), averaging $D(u, v)$ on 'Ring 4' over different angle ranges generated $D_{0,\pi}$, $D_{0,\frac{\pi}{2}}$ and $D_{0,\frac{\pi}{4}}$. The selection of Ring 4 was suggested by previous studies,³⁷ and the frequency couples (u,v) for Ring 4 are shown in Figure 1. Using Equation (10), the code calculated all components of the diffusion tensor: D_{xx} , D_{yy} and D_{xy} . Finally, the calculation of the principal diffusivities, $D_{\text{Eig_Min}}$ and $D_{\text{Eig_Max}}$, were completed according to Equation (11). The average values, as well as the ratio of the two principal diffusivities for each specimen were determined.

Statistical Analysis

An independent t-test was performed on the principal diffusivities, $D_{\text{Eig_Min}}$ and $D_{\text{Eig_Max}}$, to examine the anisotropy of solute diffusion in each disc region. One-way ANOVA and Tukey's post hoc tests were performed on the average diffusivities to examine the regional effect on the diffusion rate in porcine TMJ discs. This effect was also examined through the ratio of the principal diffusivities. SPSS 16.0 software (SPSS Inc., Chicago, IL) was used for all statistical analysis and significant differences were reported at p-values < 0.05.

RESULTS

Validation Using Computer Simulated FRAP Experiments

2D anisotropic FRAP experiments were simulated by using pre-defined components of the anisotropic diffusion tensor. Numerically simulated FRAP digital images were transformed into the frequency domain using SFA. The principal diffusivities of the anisotropic diffusion process were calculated based on our proposed method. The accuracy of the new method was assessed by the relative error (ϵ) which is defined as:³⁷

$$\epsilon = \frac{|\text{Pre-defined Value} - \text{Calculated Value}|}{\text{Pre-defined Value}} \times 100 (\%) \quad (14)$$

The effects of anisotropic ratio (D_{0xx}/D_{0yy}) and diffusion tensor orientation (θ) on the accuracy of the new method were evaluated. The relative errors (ϵ) of the two principal diffusivities for three different anisotropic ratios ($D_{0xx}:D_{0yy}=1:1.5$, $1:2.0$ and $1:3.0$) with seven diffusion tensor orientations ($\theta = 0 \sim \pi/2$) are shown in Figure 4. Overall, the relative errors of $D_{\text{Eig_Min}}$ were less than 1.5% for all simulated cases (Figure 4a), while the relative

errors of $D_{\text{Eig_Max}}$ were less than 1% (Figure 4b). This result indicated that the accuracy of the new method was not significantly sensitive to $D_{0xx}:D_{0yy}$ and θ . The highest accuracy was achieved when the diffusion tensors were oriented in alignment with the axes of the coordinate system (i.e., $\theta = 0$ or $\pi/2$). The accuracy slightly decreased when the orientation of the diffusion tensor deviated from the axes of the coordinate system. The accuracy of this method increased when the anisotropic ratio ($D_{0xx}:D_{0yy}$) decreased.

The effects of imaging noise (σ) on the accuracy of the new method were also investigated for the cases of $D_{0xx}:D_{0yy}=1:1.5, 1:2.0$ and $1:3.0$, with $\theta = \pi/4$. The typical FRAP images with and without spatial Gaussian noise ($\sigma = 0, 5, 10$) as well as the corresponding curve-fits to determine $D(u, v)$ are shown in Figure 5. The relative errors of the two principal diffusivities increased with increasing σ . The average relative errors were less than 8% at $\sigma = 5$, while the average values were less than 12.5% at $\sigma = 10$ (Figure 6). In this study, the accuracy of the new method was not sensitive to the anisotropic ratio.

FRAP Experiments on Porcine TMJ Discs

The two principal diffusivities of the 2D anisotropic diffusion tensor of 4kDa FTIC-Dextran in the five regions of porcine TMJ disc were determined using our new method and listed in Table 1. The characteristic diffusivity defined as $(D_{\text{Eig_Min}} + D_{\text{Eig_Max}})/2$ was calculated to represent the average solute diffusion rate in each disc region. The ratio of $D_{\text{Eig_Min}}$ to $D_{\text{Eig_Max}}$ was also calculated to evaluate the anisotropy of solute diffusion in the disc.

The average characteristic diffusivity across the disc was $26.05 \pm 4.32 \mu\text{m}^2/\text{s}$. Regional differences in characteristic diffusivity were observed in the anteroposterior and mediolateral directions (Figure 7a). In the anteroposterior direction, the anterior region ($30.99 \pm 5.93 \mu\text{m}^2/\text{s}$) had significantly higher characteristic diffusivity than the intermediate region ($20.49 \pm 5.38 \mu\text{m}^2/\text{s}$, $p < 0.0003$) and posterior region ($20.97 \pm 2.46 \mu\text{m}^2/\text{s}$, $p < 0.00005$). In the mediolateral direction, the medial region ($26.51 \pm 6.30 \mu\text{m}^2/\text{s}$) and lateral region ($26.71 \pm 5.50 \mu\text{m}^2/\text{s}$) had higher characteristic diffusivities than the intermediate region, although this trend was not statistically significant.

Figure 7b showed that there was a significant difference between $D_{\text{Eig_Min}}$ and $D_{\text{Eig_Max}}$ in each disc region ($p < 0.001$). The ratio of $D_{\text{Eig_Min}}$ to $D_{\text{Eig_Max}}$ was approximately 0.45~0.51 (with 1.0 indicating isotropic) across the disc without significant regional differences (Figure 7c). These results indicated that the diffusion of 4kDa FTIC-Dextran was highly anisotropic in the porcine TMJ disc.

DISCUSSION

In this study, a new method solely based on the SFA of FRAP images was developed to completely determine the anisotropic diffusion tensor in hydrated soft tissues. This method was used to determine the region-dependent anisotropic diffusion tensor in porcine TMJ discs.

The accuracy and robustness of our new method was validated by the computer simulated FRAP experiments. Our method demonstrated several key advantages over the previous studies on the SFA based FRAP methods for anisotropic diffusion. Most of all, it makes possible to deal with any orientation of the diffusion tensor ($\theta = 0-\pi/2$). The complete diffusion tensor, including both diagonal and off-diagonal components, can be determined using this new method.

Compared to the method recently reported by Travascio *et al.*,³⁷ our method is more accurate and robust. In their method, an anisotropic diffusion tensor was determined based

on two independent analyses of FRAP images: the Fourier transform and the Karhunen-Loeve transform. The KLT analysis of FRAP images can determine the orientation of the diffusion tensor and by combining the FT and KLT analyses, Travascio's method can determine all components of the anisotropic diffusion tensor in a single FRAP experiment. In this study, we proposed a method solely based on the SFA to completely determine the anisotropic diffusion tensor which avoids all of the limitations imposed by KLT analysis. For example, due to the limitations of the KLT the Travascio's method is not applicable when the orientation of the diffusion tensor coincides with the fixed coordinate system (i.e., $\theta = 0$ or $\pi/2$).³⁷ More importantly, the major limitation of their method, as recognized by the authors, is the robustness to deal with the imaging noise which is unavoidable in real experiments. In their simulation results the average relative errors reached 40% at $\sigma = 10$ noise level, while the average values reached 20% at $\sigma = 5$ level.³⁷ Under the same noise conditions the average relative errors of our method were less than 12.5% at $\sigma = 10$ level and less than 8% at $\sigma = 5$ level. It is apparent that our new method is more robust against the noise and our method only needs one image analysis (i.e., the SFA) resulting in fewer numerical errors for the determination of the diffusion tensor. In our simulation without imaging noise, the relative errors of $D_{\text{Eig_Min}}$ were less than 1.5% (Figure 4a) while under the similar conditions, the relative errors reached 14% in simulations performed by Travascio *et al.*³⁷ In the above accuracy comparison, it needs to be noted that the sizes of the bleaching spot and imaging frame in this study were only about half the values of those parameters used in the study by Travascio *et al.*³⁷ This implies that the imaging sampling might also affect the precision of the results.

This study represents the first measurement of solute diffusion in TMJ disc tissues. The average diffusivity of 4kDa FITC-Dextran in porcine TMJ discs ($26 \mu\text{m}^2/\text{s}$) was approximately 16% of the diffusivity in water ($162 \mu\text{m}^2/\text{s}$, based on the Stokes-Einstein equation with the hydraulic radius of 1.4nm) and was comparable to the diffusivity of the similar sized solute (3kDa FITC-Dextran) in porcine articular cartilage ($75 \mu\text{m}^2/\text{s}$) measured by FRAP.¹⁶ Most recently, Benavides *et al.* reported that there is a region-dependency of water diffusion in porcine TMJ disc using diffusion tensor MRI.² In the anteroposterior direction, the mean water diffusivity was higher in the anterior and posterior bands compared with the intermediate zone. In the mediolateral direction, the mean water diffusivity was higher in the medial and lateral aspects than in the center. Our study of 4kDa FITC-Dextran diffusion showed exactly the same trends.

The results of this study indicated that the diffusion rates of uncharged dextran molecules vary by region in the TMJ disc. The anterior region had higher characteristic diffusivity than the intermediate region and posterior region in the anteroposterior direction, while the medial and lateral regions had higher characteristic diffusivities than the intermediate region in the mediolateral direction. Previous studies showed that solute diffusivity in cartilaginous tissues depended on tissue water content in a manner that a decrease in the solute diffusivity occurs as tissue water content decreased,^{9, 35, 37} and these findings were also observed in our study. For the porcine TMJ disc, it has been reported that along the anteroposterior axis, the anterior band ($74.5 \pm 2.9\%$) had higher water content than the intermediate zone ($73.7 \pm 3.1\%$) and posterior band ($70.1 \pm 4.0\%$), while along the mediolateral axis, the medial region ($75.3 \pm 2.1\%$) had a higher water content than the central ($71.3 \pm 3.7\%$) and lateral ($71.3 \pm 4.1\%$) regions.⁸ The correlation between the diffusivity and tissue water content suggests that the diffusive transport in porcine TMJ discs is dependent on tissue composition. The inhomogeneous distribution of tissue components results in region-dependent diffusivity in TMJ disc tissues.

The results of this study also demonstrated that solute diffusive transport in porcine TMJ discs is highly anisotropic with the ratio of $D_{\text{Eig_Min}}$ to $D_{\text{Eig_Max}}$ around 0.45~0.51 across

the disc. Studies on meniscus and annulus fibrosus showed that the anisotropic solute diffusion in fibrocartilage is due to the orientation of collagen fibers.^{36, 37} The orientation of the largest principal component ($D_{\text{Eig_Max}}$) of the diffusion tensor is parallel to the direction of the bundles of collagen fibers, while the smallest principal component ($D_{\text{Eig_Min}}$) is perpendicular to the fibers. Inside the porcine TMJ disc, Detamore *et al.* reported that collagen fibers were well organized and had a ring-like structure around the periphery and were oriented anteroposteriorly through the intermediate zone.⁸ Our results suggested that the diffusive transport in TMJ disc is not only dependent upon tissue composition but also tissue structure, especially collagen structure.

In this study, we measured diffusivities of 4kDa FITC-Dextran (hydraulic radius equivalent to insulin)¹⁶ to examine the region-dependent anisotropic diffusion in porcine TMJ discs. However, it is important to note that the dextran molecules have a linear structure that may not duplicate the molecular structure of other physiologically relevant molecules in the TMJ disc, which may be more globular in structure. Pluen *et al.* have shown that flexible macromolecules have greater mobility than similarly sized globular molecules in a random fiber matrix.²⁶ It is also important to note that the diffusion tensor was determined in the horizontal plane in this study. The diffusion in the vertical direction (i.e., superior-inferior direction) could be the main pathway for nutrient transport through the TMJ disc. Therefore, in order to fully understand solute transport in the TMJ disc, it is necessary to determine the 3D diffusion tensor in each disc region and correlate it to tissue composition and structure.

In summary, a new method solely based on the SFA has been developed to determine the 2D anisotropic solute diffusion tensor in the fibrous tissues using FRAP. The accuracy and robustness of this method was validated using computer simulated FRAP experiments. The new method was applied to determine the 2D diffusion tensor of 4kDa FITC-Dextran in the porcine TMJ discs. It was determined that the diffusion of this solute in TMJ discs was inhomogeneous and anisotropic. These findings suggested that diffusive transport in TMJ disc is dependent upon tissue composition (e.g., water content) and structure (e.g., collagen orientation). This study provides a new method to quantitatively investigate the relationship between transport properties and tissue composition and structure. The obtained transport properties are crucial for future development of numerical models studying nutritional supply within the TMJ disc.

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Figure 1.

Diffusion coefficient $D(u, v)$ in frequency domain (u, v) . The averages of $D(u, v)$ over the arcs from 0 to π , 0 to $\pi/2$ and 0 to $\pi/4$ can be used to determine the components (D_{xx} , D_{yy} and D_{xy}) of the diffusion tensor. On the 'Ring4':

$$\begin{aligned}
 D_{0, \frac{\pi}{4}} &= [D(4, 0) + D(4, 1) + D(4, 2) + D(3, 2) + D(3, 3)] / 5; \\
 D_{0, \frac{\pi}{2}} &= [D(4, 0) + D(4, 1) + D(4, 2) + D(3, 2) + D(3, 3) + D(2, 3) + D(2, 4) + D(1, 4) + D(0, 4)] / 9; \\
 D_{0, \pi} &= [D(4, 0) + D(4, 1) + D(4, 2) + D(3, 2) + D(3, 3) + D(2, 3) + D(2, 4) + D(1, 4) + D(0, 4) + \\
 &\quad D(-1, 4) + D(-2, 4) + D(-2, 3) + D(-3, 3) + D(-3, 2) + D(-4, 2) + D(-4, 1) + D(-4, 0)] / 17
 \end{aligned} \tag{15}$$

(a)

(b)

Figure 2.

(a) The computational domain with the initial and boundary conditions for FRAP simulation experiments. (b) The orientation of pre-defined anisotropic diffusion tensor generated using various rotation angles. θ is the rotation angle from the principal diffusion tensor coordinate (X_0 , Y_0) to the observed diffusion tensor coordinate (X , Y).

Figure 3.

The schematic of FRAP experiments on porcine TMJ discs. Tissue specimens were prepared from the anterior, posterior, intermediate, lateral, and medial regions of the discs. The diffusion tensor of 4kDa FITC-Dextran was measured with FRAP in the horizontal X-Y plane (parallel to the surface of the TMJ disc).

Figure 4. Effects of the orientation (θ) and anisotropic ratio (D_{0xx}/D_{0yy}) of the diffusion tensor on the relative error (ϵ) for the determination of (a) $D_{\text{Eig_Min}}$ and (b) $D_{\text{Eig_Max}}$.

(a)

(b)

Figure 5.

Effect of the imaging noise on the determination of the $D(u, v)$. **(a)** Typical computer simulated FRAP images without and with spatial Gaussian noise ($\sigma = 0, 5, 10$). **(b)** Typical curve-fits to determine $D(4,4)$ for the simulated FRAP image sequences without and with spatial Gaussian noise ($\sigma = 0, 5, 10$).

(a)

(b)

Figure 6. Effect of the imaging noise on the precision of determined diffusion tensors with different anisotropy: **(a)** The relative error (ε) for D_{Eig_Min} and **(b)** the relative error (ε) for D_{Eig_Max} .

Figure 7. The results of statistical analysis of determined diffusion tensors in porcine TMJ disc. **(a)** Characteristic diffusivities $[(D_{\text{Eig_Min}} + D_{\text{Eig_Max}})/2]$ were significantly region-dependent (* $p < 0.05$ compared to Anterior). **(b)** Significant differences between $D_{\text{Eig_Min}}$ and $D_{\text{Eig_Max}}$

were detected in all disc regions (* $p < 0.05$). (c) The ratio of $D_{\text{Eig_Min}}$ to $D_{\text{Eig_Max}}$ was not region-dependent.

Table 1

The results (mean±SD) of the diffusion tensor of 4kDa FTIC-Dextran in the five regions of porcine TMJ discs. $D_{\text{Eig_Min}}$ and $D_{\text{Eig_Max}}$ are the two principal diffusivities of the diffusion tensor. $(D_{\text{Eig_Min}} + D_{\text{Eig_Max}})/2$ is the characteristic diffusivity that represents the averaged solute diffusion rate in each disc region. $D_{\text{Eig_Min}}/D_{\text{Eig_Max}}$ was calculated to evaluate the anisotropy of solute diffusion in each disc region.

	Anterior	Medial	Intermediate	Lateral	Posterior	Average
$D_{\text{Eig_Min}}$ ($\mu\text{m}^2/\text{s}$)	18.32±4.18	17.10±4.72	13.57±3.97	15.61±4.28	13.37±4.15	15.75±1.56
$D_{\text{Eig_Max}}$ ($\mu\text{m}^2/\text{s}$)	43.67±11.39	35.92±8.95	27.40±8.31	37.80±11.95	28.58±5.90	36.35±8.66
$D_{\text{Eig_Min}} + D_{\text{Eig_Max}}/2$ ($\mu\text{m}^2/\text{s}$)	30.99±5.93	26.51±6.30	20.49±5.38	26.71±5.50	20.97±2.46	26.05±4.32
$D_{\text{Eig_Min}}/D_{\text{Eig_Max}}$	0.45±0.17 *	0.48±0.10 *	0.51±0.14 *	0.47±0.24 *	0.49±0.18 *	0.47±0.12

* ($D_{\text{Eig_Max}}$ was significantly larger than $D_{\text{Eig_Min}}$ in each region, $p < 0.05$).