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Microscale Diffusion Properties of the Cartilage Pericellular Matrix Measured Using 3D Scanning Microphotolysis

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Abstract

Chondrocytes (cartilage cells) are enclosed within a pericellular matrix (PCM) whose composition and structure differ from those of the extracellular matrix (ECM). Since the PCM surrounds each cell, molecules that interact with the chondrocyte must pass through the pericellular environment. A quantitative understanding of the diffusional properties of the PCM will help elucidate the PCM's regulatory role in controlling transport to and from the chondrocyte. The diffusivity of a fluorescently-labeled 70 kDa dextran was quantified within the PCM of porcine articular cartilage using a newly-developed mathematical model of scanning microphotolysis (SCAMP). SCAMP is a rapid, line photobleaching method that accounts for out-of-plane bleaching attributable to high magnification. Data were analyzed by best-fit comparison to simulations generated using a discretization of the diffusion-reaction equation in conjunction with the microscope-specific three-dimensional excitation and detection profiles. The diffusion coefficient of dextran was significantly lower in the PCM than in the ECM in normal cartilage. In early-stage arthritic tissue, however, no significant differences in diffusivity were detectable. These results support the hypothesis that the diffusivity of the PCM is lower than that of the ECM, presumably due to differences in proteoglycan content, and that osteoarthritic changes in tissue affect the transport properties of the PCM.

Keywords

diffusion; photobleaching; pericellular matrix; articular cartilage; arthritis; FRAP

Introduction

Articular cartilage is the connective tissue that lines surfaces of bones in synovial joints. Cartilage disease, primarily in the form of osteoarthritis, is a major problem, affecting 21.6% of adults in the United States (1). Cartilage is an avascular and alymphatic tissue, suggesting that the primary mode of transport for nutrients, oxygen, waste products, signaling molecules, and matrix macromolecules is by diffusion or convection. Thus, over the course of aging or disease, alterations in tissue properties may influence chondrocyte metabolism by altering the transport of nutrients, signaling molecules, or metabolites. The extracellular matrix (ECM) of articular cartilage is primarily water, making up 60–85% of the tissue's wet weight. The remaining solid matrix is composed of a crosslinked network of type II collagen (15–22% by wet wt.), proteoglycan (4–7% by wet wt.), and lesser amounts of other collagen types (e.g., VI, IX, X) and noncollagenous proteins (2). The aggregating proteoglycan, or aggrecan, in cartilage is composed of a hyaluronic acid backbone to which numerous chondroitin and

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keratan sulfate chains are attached by a link protein. The constituents of articular cartilage are organized in a stratified structure that contributes the unique mechanical behavior of the ECM (3).

Understanding the factors that influence diffusive transport is crucial to determining not only the normal physiological functions of articular cartilage, but also how those functions are altered by disease. In particular, the local structure and composition of the cartilage matrix appear to have significant effects on the rate of diffusion in the tissue. For example, removal of proteoglycans by enzymatic degradation (4) or removal of the entire surface zone (5) increases the bulk tissue diffusion coefficient of 70 kDa dextran. In the surface zone, the diffusion coefficient of 70 kDa dextran is also lower than in the middle and deep zones (6) and is anisotropic (7), potentially due to differences in the composition and orientation of collagen fibers in this zone. Furthermore, proteoglycan concentration has been shown to be inversely correlated with diffusivity (8). Studies of macromolecular diffusion in engineered cartilage show that the accumulation of matrix components, coupled with cell-induced tissue contraction, can significantly decrease tissue diffusivity (9).

The structure and composition of cartilage vary significantly with proximity to the cells, termed “chondrocytes”, which are responsible for maintaining the tissue. The region immediately surrounding the cell, the pericellular matrix (PCM), is characterized by high proteoglycan content, the exclusive presence of type VI collagen in cartilage, and the presence of other molecules such as types II, IX, and XI collagen, fibronectin, aggrecan, and decorin (10–15). In addition to these structural and compositional differences, the PCM exhibits a lower Young’s modulus than the surrounding ECM (16–18). Because of such differences in properties, the presence of the PCM has significant effects on the mechanical environment of the chondrocyte (17,19–22), and it has been hypothesized that the PCM serves as a transducer, or “filter”, of mechanical signals in cartilage (13,23).

Since it completely surrounds the cell, the PCM may regulate not only the biomechanical environment of the cell, but also the biochemical environment, by governing the transport of molecules to and from the chondrocyte (12–15). To interact with the chondrocyte surface or be released from the chondrocyte into the ECM, molecules must pass through the pericellular environment. As soluble mediators such as growth factors traverse the PCM, they can be modified or retained (24). Assembly of the mature aggrecan-hyaluronan complex, required to form the ECM, occurs as the aggrecan and hyaluronan molecules move across the PCM after being secreted from the chondrocyte (25). In addition, enzymes that degrade cartilage matrix (metalloproteinases and aggrecanases) must also pass through the PCM before affecting the ECM. Therefore, the rate of molecular diffusion through the PCM may control the proper synthesis, assembly, and function of matrix macromolecules; subsequently, alterations in PCM structure or properties, as may occur with aging or disease (16), may have secondary effects on the cartilage ECM.

To date, there have been few reports on the diffusivity or transport properties of the native PCM. One study has examined diffusion in a reconstituted PCM *in vitro* (26), showing that the presence of a PCM slows diffusion of gold-labeled lipids extending 30–40 nm out from the cell membrane. In other studies, micropipette aspiration was used to measure the biphasic properties of mechanically-extracted chondrons, showing a significant increase in the hydraulic permeability of the PCM with osteoarthritis (18). The native PCM, however, provides unique challenges for diffusion measurements. Because the PCM is typically quite thin (~3 μm thick in porcine chondrons (27)), the techniques historically used to measure diffusion in cartilage (i.e., radiolabel tracer tracking or fluorescence desorption (5,28,29)) cannot be scaled down to perform such measurements within the PCM. Photobleaching techniques such as FRAP (fluorescence recovery after photobleaching) have been widely used at this size scale, but were

developed to measure diffusion in two-dimensional (2D) systems such as cell membranes (30,31). While this technique has recently been extended using 2D numerical simulations to account for tissue inhomogeneity (42), photobleaching in the PCM requires the use of a high numerical aperture lens, which contributes to substantial out-of-plane bleaching that significantly affects the fluorescence intensity measured in the imaging plane and requires three-dimensional (3D) analysis.

In this study, a modified version of the scanning microphotolysis (SCAMP) technique (32) was developed to overcome these challenges of measuring diffusion within the PCM. With this technique, a single diffraction-limited line is rapidly bleached and simultaneously imaged. From these images, the microscale diffusivity was measured within both the PCM and adjacent ECM for both healthy and osteoarthritic cartilage. Due to the higher PCM proteoglycan content, the PCM diffusivity was hypothesized to be lower than that of the ECM; correspondingly, the diffusivity in osteoarthritic tissue was expected to be higher due to the characteristic loss of pericellular proteoglycans.

Methods

General SCAMP Procedure

SCAMP uses a high laser power both to image and to photobleach a line segment, taking advantage of the fast x -direction scanning of the laser scanning confocal microscope (32). The output of this procedure is a single x -line that decreases in intensity over time (Fig. 1). The change in fluorescence intensity is a function of two variables: the rate of photobleaching and the rate of diffusion of the fluorescent molecule of interest. Faster-moving molecules traverse the laser's path more rapidly and so are subjected to the laser for a briefer duration, thereby producing a slower intensity decrease; on the other hand, slower-moving molecules remain in the laser's path and so are more likely to be bleached, yielding a more rapid intensity decrease (Fig. 2). Because the SCAMP procedure measures only the change in intensity during the bleaching period and not the fluorescence recovery period, the experiments can be performed very rapidly (< 50 ms) as compared to typical FRAP experiments (minutes), and bleaching is confined spatially to a relatively small region ($< 37 \mu\text{m}^3$ for the present configuration). To determine the diffusion coefficient and a bleaching rate constant, the intensity-versus-time data are fit to a 3D theoretical diffusion-reaction model that accounts for the out-of-plane bleaching effects (32) (Fig. 3).

Numerical modeling of the SCAMP Procedure

The SCAMP method was originally developed by Kubitscheck et al. (32), who implemented an analytical solution that involved specific assumptions regarding the physical process of the experiment. This model provided excellent fits to SCAMP experiments for diffusivities less than $2 \mu\text{m}^2\text{s}^{-1}$. In applying this method to measure the diffusivity of larger molecules within the cartilage PCM, however, a number of these assumptions were no longer valid, and a new numerical model was developed to incorporate specific experimental parameters involved in the present testing system.

Measuring larger diffusion coefficients—The basic SCAMP analysis assumes that the x -line is bleached instantaneously, which is a reasonable assumption only if the molecules at one end of the line have not yet diffused away by the time the other end is scanned. This constraint determines the maximum diffusion coefficient that can be measured using SCAMP. In the original description of the method, Kubitscheck et al. (32) estimate that the characteristic diffusion time, $\tau = l^2/6D$ (where l is the length of the line being scanned and D is the diffusion coefficient), should be at least 10 times the line-scan time in order to maintain this assumption. In the previous configuration (32), the upper limit of D was $2 \mu\text{m}^2\text{s}^{-1}$. By scanning a very short

line using a faster, bi-directional microscope, this limit was extended in the present study to $5120 \mu\text{m}^2\text{s}^{-1}$.

Accounting for time spent not bleaching—The confocal microscope used in this study (LSM 510, Zeiss Inc., Thornwood NY) does not scan the laser continuously back and forth across the proscribed 12-pixel line, but rather moves over a longer region than the 12-pixel line (with the laser off) and takes a finite amount of time to reverse direction. Whenever the laser is off, the fluorescence distribution is recovering. This is a significant portion of time; ~300 ms are spent with the laser off for each millisecond with the laser on. Enhancement to the numerical model incorporates this illumination pattern by alternating long and short time steps. During the short time step, bleaching occurs and the process is modeled as diffusion with a consumption term,

$$\frac{\partial C(\vec{x}, t)}{\partial t} = D \frac{\partial^2}{\partial \vec{x}^2} C(\vec{x}, t) - k(x)C(\vec{x}, t). \quad (1)$$

However, during the long time step, the molecules are only diffusing:

$$\frac{\partial C(\vec{x}, t)}{\partial t} = D \frac{\partial^2}{\partial \vec{x}^2} C(\vec{x}, t). \quad (2)$$

Equations 1 and 2, which govern the SCAMP process, were solved by the Douglas-Gunn alternating-direction implicit (ADI) method of discretization (33). The resulting equations (Eqs. 3–8) are provided in the Appendix.

Accounting for data asymmetry—Experimental data showed a consistent asymmetry across the x-line that was not predicted by the original SCAMP model (Fig. 1). This unevenness was caused by the asymmetric nature of the microscope's point-spread function. Thus, to incorporate the microscope's optical asymmetries into the model, the bleach matrix (excitation profile) and point-spread function (detection profile) were derived empirically.

The point-spread function was determined by collecting z-stack images of sub-resolution fluorescent microspheres (Fig. 4) (34). The bleach matrix was measured by photobleaching an immobilized fluorophore, FITC-dextran, in a dehydrated agarose gel. This photobleached region was imaged by z-stack and then 3D-deconvolved from the point-spread function using XCOSM software with the Expectation Maximization algorithm (35–37) to extract the bleach (excitation) matrix (Fig. 5).

Incorporating pre-bleach assumption for first time point—Although the laser nominally bleaches and images simultaneously, the fact that the characteristic asymmetric intensity variation was already present in the first line of each dataset indicated that the collected intensity data were already bleached before the first image was recorded. Thus, the background fluorophore level was estimated from the measured first line of data and a perfectly linear correlation was interpolated from simulated datasets of known background intensity.

Determining simulation parameters—The 3D simulation space was $83 \times 53 \times 114$ pixels (x, y, z) based on the proportions of the bleach matrix. This simulation space was selected to be large enough to avoid any edge effects; this was verified by increasing the space size and observing no numerical changes in the simulations. Spatial steps were set to equal the pixel

size on the data derived from the microscope. The time steps were set to mimic actual timing; subdividing time steps further did not change the simulations.

The duration of the experiment balanced a number of conflicting factors. Theoretically, a better fit is achieved for longer experiments. However, shorter experiments are required to keep the bleach region within the confines of the PCM. In addition, computation time increases when simulating and fitting longer datasets. For simulated datasets with typical noise levels added, a fit of 40 time points was found to balance appropriately the goals of keeping the computation time reasonable, the bleach region within the PCM, and the fitting error low (Fig. 6).

To speed computation, simulations were generated for a series of D and k values and saved to a lookup table. This created a searchable library of possible D and k combinations to which data could be quickly compared, while simultaneously sidestepping the earlier strategy of recreating the simulations to fit each new dataset. Implementation of the lookup table reduced from hours to seconds the time required to fit the data.

Data quality was tested by adding different noise levels to datasets created from simulations of known D and k . The new best-fit D and k values were determined for each noisy dataset by comparison to the clean lookup simulations. The signal-to-noise ratio was calculated to describe each dataset. The signal was defined as the change in intensity from the start to the end of the experiment; the noise was the standard deviation of the difference between the best-fit simulation and the data. The error in fit increases significantly with increasing noise, but a signal-to-noise ratio of at least 10 resulted in average errors in the fit below 4% (*data not shown*). Therefore, only experimental data with a signal-to-noise ratio of 10 or greater were used.

Fitting experiment within the PCM—To determine whether a SCAMP experiment would fit within the confines of the PCM, the maximum excursions of the bleached regions were measured at the end of simulated experiments. Considering even the faint bleaching far from the proscribed bleach line is a generous estimate of the bleach region, since molecules at the periphery do not strongly affect the measured intensity. The size of the rectangular prism that enclosed the bleach volume was $3.3 \times 1.9 \times 5.9 \mu\text{m}^3$ (x, y, z) (Fig. 7), which fits within the typical porcine PCM (27).

Experimental Procedure

Tissue was obtained from the femoral condyles of skeletally mature female pigs. The cartilage surface of each joint was graded macroscopically for osteoarthritis using the Collins scale as described by Muehleman et al. (38). Briefly, Grade 0 showed no degenerative changes; Grade 1 showed minimal fibrillation; Grade 2 showed deep fibrillation and fissuring; Grades 3 and 4 showed more extensive fibrillation and erosion. Here, cartilage of Grade 0 was termed healthy ($n=6$ pigs), while cartilage of Grade 1 or 2 ($n=5$ pigs) was considered early-stage osteoarthritic. Cartilage of Grade 3 or 4 was not tested. A full-thickness slice of cartilage was removed from the femoral condyle and incubated overnight in a concentrated solution (25 mg/ml) of FITC-tagged 70 kDa dextran (Molecular Probes/Invitrogen, Eugene, OR).

The cartilage tissue sample was placed in a coverslip chamber, covered with PBS, and immobilized with a custom-built holder. Using the 100x, 1.3-NA oil immersion lens at 6x zoom on a confocal laser scanning microscope (LSM 510, Zeiss), a $1.44\text{-}\mu\text{m}$ wide line (12 pixels) at a depth of $6 \mu\text{m}$ into the tissue was bleached (excitation wavelengths: 458 and 488 nm) forward and backward for a total of 40 passes. As the laser bleached, the emission intensity was also collected (long-pass filter, LP505) and stored as the image for that pass. At each site, 5 to 7 experiments were performed; their intensity values were later averaged together and median-filtered to minimize noise.

The ability of the model to detect changes in the bleaching rate constant, k , was tested by a series of measurements made at increasing depth into the tissue. Since tissue reflects and absorbs some of the laser light, the laser intensity decreases as it penetrates further into the tissue. As the laser intensity decreases, so should the bleaching rate constant. Depth data were log-transformed to maintain normality and satisfy linear regression assumptions.

SCAMP experiments were performed in pairs: for each PCM experiment, another was carried out in the adjacent ECM in the same sample. The PCM experimental sites were chosen on the surface- or deep-zone sides of middle-zone cells, since these PCM areas tended to be thickest. The diffusion coefficient was determined for each test site by fitting the experimental data to a pre-calculated entry in the look-up table by minimizing the sum of the squares of error. A signal-to-noise ratio was then calculated as the maximum change in intensity over time divided by the standard deviation of the noise that remained once the best-fit simulation was subtracted out; datasets were used only if their signal-to-noise ratio was greater than 10.

All data processing, fits, and simulations were performed in Matlab (The Mathworks, Natick, MA). Statistics were computed with Statistica (Statsoft, Tulsa, OK). A repeated-measures analysis of variance (ANOVA) with paired PCM and ECM measurements was used to determine the effects of the matrix and the tissue health (based on the Collins grade, healthy or early-stage arthritic).

Results

Overall, the SCAMP model provided excellent fits to the experimental data, with a mean correlation of $R^2=0.92$ (Figs. 1 and 3). The depth series confirmed that the bleaching rate constant decreases significantly with increasing natural log of depth (Fig. 8). Importantly, however, the diffusion coefficients measured through this depth series did not vary significantly, indicating that the model is able to discriminate between changes in D and k .

Values were obtained for diffusion coefficients in the PCM and ECM of healthy and early-stage osteoarthritic tissue samples. The mean ($\pm$ standard error) diffusion coefficient of 70 kDa dextran in the middle zone of healthy porcine cartilage was $23\pm2\ \mu\text{m}^2\text{s}^{-1}$ in the ECM and $19\pm2\ \mu\text{m}^2\text{s}^{-1}$ in the PCM. In early-stage arthritic tissue samples, the diffusivity of the ECM was $23\pm2\ \mu\text{m}^2\text{s}^{-1}$ and that of the PCM was $23\pm2\ \mu\text{m}^2\text{s}^{-1}$ (Fig. 9). The repeated-measures ANOVA showed a significant matrix-by-health interaction ($p=0.008$) and a borderline-significant matrix effect ($p=0.057$). In healthy tissue, the diffusivity of the PCM was significantly lower than that of the ECM (Tukey HSD post-hoc $p=0.018$, Fig. 9). In the arthritic samples, there was no difference between diffusivity in the PCM and ECM (Tukey HSD post-hoc $p=0.91$). That the repeated-measures ANOVA showed a borderline-significant effect of matrix ($p=0.057$), however, suggests that the PCM-ECM difference may still persist in some of the arthritic samples.

Discussion

Our adaptation of the SCAMP technique can be used to measure site-specific diffusivity in a relatively small volume of tissue. The mean diffusion coefficient measured in the ECM, $23\ \mu\text{m}^2\text{s}^{-1}$, is in good agreement with values measured previously using other techniques ($31\ \mu\text{m}^2\text{s}^{-1}$ using fluorescence recovery after photobleaching and $40\ \mu\text{m}^2\text{s}^{-1}$ using radiotracer tracking (4,6,39)). Using the SCAMP method to probe the small volume of the PCM, we have shown that the diffusivity of the PCM in normal porcine articular cartilage is lower than that of the adjacent ECM. Furthermore, we found that there is no difference in diffusivity between the PCM and ECM of early-stage osteoarthritic tissue. These findings suggest that loss of the normal diffusion properties of the PCM may be an early event in cartilage degeneration.

Our modifications to the original SCAMP technique (32) allow for increased applicability of the method. By implementing the asymmetrical time step, the revised SCAMP method accounts for time spent not bleaching, an issue on commercial microscopes. This also allows measurement of larger diffusion coefficients. Our revised technique also incorporates any asymmetries in the point-spread function of the microscope by utilizing the measured point-spread function rather than an idealized, theoretical one. Finally, our modifications also address the ambiguity in the first bleached line and extrapolate back to an appropriate initial unbleached condition. It is possible that the SCAMP technique could be further modified to measure diffusional anisotropy (e.g. (7,40,41)). For example, if the scanned line in SCAMP is sufficiently long and narrow, the measured diffusivity will be dominated by the effects of diffusion across the thickness of the line (i.e., one pixel in our case), thus creating a largely one-dimensional D measurement.

Only one previous study has measured diffusion in the PCM, by quantifying motion of diffusion of gold-tagged lipids tethered to the membranes of isolated keratocytes that were synthesizing a new PCM in culture (26). This study showed that the presence of a PCM can decrease diffusivity by about half as compared to cells with no newly formed, encompassing matrix. Our study, however, is the first to measure the diffusion of a freely mobile molecule in the native chondrocyte PCM *in situ*. Similar to the Lee et al. study (26), our values for diffusivity in the PCM are less than would be expected with no matrix present (i.e., free solution) (43).

The difference in diffusivity between PCM and ECM of healthy tissue is likely attributable to the differences in the structure and composition of the PCM, which contains finer collagen fibers and a higher concentration of proteoglycans relative to the ECM (11). A study in the ECM has shown that diffusivity is inversely correlated with proteoglycan concentration (8). The PCM is also characterized by a network of type VI collagen, which is not present in the ECM of the cartilage under normal circumstances. SCAMP measurements showed a high degree of variability within both the PCM and ECM, which is potentially attributable to inhomogeneities that may be present in the small regions over which the measurement is taken. Similar variability has been observed previously in tests of the biomechanical properties of the PCM (16–18).

Although the data presented here show a significantly lower diffusivity in the PCM as compared to the ECM, these differences in diffusion coefficients are relatively small and unlikely to be limiting to the transport of nutrients or signaling molecules, particularly in the presence of significant convective transport (44–47). Nonetheless, the differences between ECM and PCM properties provide further evidence of the differences in matrix structure between these tissue compartments, as well as the relationships between structure, composition, and properties of cartilage.

While normal cartilage showed a significant difference in diffusivity between the PCM and ECM, the differences were not present in early-stage osteoarthritic samples due to increased D in the PCM of osteoarthritic tissue. This finding is consistent with the previous report of increased hydraulic permeability in the PCM of osteoarthritic cartilage (16). The fact that changes in diffusivity were detected in the PCM, but not ECM, in early osteoarthritis suggests that initial tissue degradation is likely mediated by the chondrocyte's release of enzymes that first affect the pericellular region. These findings are also in general agreement with previous reports of swelling and enlargement of the PCM in osteoarthritic cartilage (48). Taken together, these studies suggest that an early step in the development of osteoarthritis may be the chondrocyte-mediated degradation of the PCM (11,49). An increase in PCM diffusivity may alter the modification or retention of growth factors or chondrocyte-released matrix macromolecules, or perhaps influence the transport rate of growth factors or cytokines to the chondrocyte. Further quantitative understanding of the diffusional properties of the PCM could

help elucidate the regulatory role of this tissue region in controlling molecular transport to and from the chondrocyte.

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Appendix

Appendix

The diffusion-reaction equations (Eqs. 1–2) were solved as follows (Eqs. 3–8). The short time step includes both bleaching and diffusion components:

Step 1, x implicit:

$$\begin{aligned}
 & C_{i,j,k}^{t+1/3} - \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^{t+1/3} - 2C_{i,j,k}^{t+1/3} + C_{i+1,j,k}^{t+1/3}) + \frac{k_{ijk}\Delta t}{2} (C_{i,j,k}^{t+1/3}) \\
 & = C_{i,j,k}^t + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^t - 2C_{i,j,k}^t + C_{i+1,j,k}^t) + \frac{D\Delta t}{\Delta y^2} (C_{i,j-1,k}^t - 2C_{i,j,k}^t + C_{i,j+1,k}^t) \\
 & + \frac{D\Delta t}{\Delta z^2} (C_{i,j,k-1}^t - 2C_{i,j,k}^t + C_{i,j,k+1}^t) - \frac{k_{ijk}\Delta t}{2} (C_{i,j,k}^t)
 \end{aligned} \tag{3}$$

Step 2, y implicit:

$$\begin{aligned}
 & C_{i,j,k}^{t+2/3} - \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^{t+2/3} - 2C_{i,j,k}^{t+2/3} + C_{i,j+1,k}^{t+2/3}) + \frac{k_{ijk}\Delta t}{2} (C_{i,j,k}^{t+2/3}) \\
 & = C_{i,j,k}^t + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^t - 2C_{i,j,k}^t + C_{i+1,j,k}^t) + \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^t - 2C_{i,j,k}^t + C_{i,j+1,k}^t) \\
 & + \frac{D\Delta t}{\Delta z^2} (C_{i,j,k-1}^t - 2C_{i,j,k}^t + C_{i,j,k+1}^t) + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^{t+1/3} - 2C_{i,j,k}^{t+1/3} + C_{i+1,j,k}^{t+1/3}) \\
 & - \frac{k_{ijk}\Delta t}{2} (C_{i,j,k}^{t+1/3})
 \end{aligned} \tag{4}$$

Step 3, z implicit:

$$\begin{aligned}
 & C_{i,j,k}^{t+1} - \frac{D\Delta t}{2\Delta z^2} (C_{i,j,k-1}^{t+1} - 2C_{i,j,k}^{t+1} + C_{i,j,k+1}^{t+1}) + \frac{k_{ijk}\Delta t}{2} (C_{i,j,k}^{t+1}) \\
 &= C_{i,j,k}^t + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^t - 2C_{i,j,k}^t + C_{i+1,j,k}^t) + \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^t - 2C_{i,j,k}^t + C_{i,j+1,k}^t) \\
 &+ \frac{D\Delta t}{2\Delta z^2} (C_{i,j,k-1}^t - 2C_{i,j,k}^t + C_{i,j,k+1}^t) + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^{t+1/3} - 2C_{i,j,k}^{t+1/3} + C_{i+1,j,k}^{t+1/3}) \\
 &+ \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^{t+2/3} - 2C_{i,j,k}^{t+2/3} + C_{i,j+1,k}^{t+2/3}) - \frac{k_{ijk}\Delta t}{2} (C_{i,j,k}^{t+2/3})
 \end{aligned} \tag{5}$$

The long time step, during which the laser is off, includes only the diffusion component: Step 1, x implicit:

$$\begin{aligned}
 & C_{i,j,k}^{t+1/3} - \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^{t+1/3} - 2C_{i,j,k}^{t+1/3} + C_{i+1,j,k}^{t+1/3}) \\
 &= C_{i,j,k}^t + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^t - 2C_{i,j,k}^t + C_{i+1,j,k}^t) + \frac{D\Delta t}{\Delta y^2} (C_{i,j-1,k}^t - 2C_{i,j,k}^t + C_{i,j+1,k}^t) \\
 &+ \frac{D\Delta t}{\Delta z^2} (C_{i,j,k-1}^t - 2C_{i,j,k}^t + C_{i,j,k+1}^t)
 \end{aligned} \tag{6}$$

Step 2, y implicit:

$$\begin{aligned}
 & C_{i,j,k}^{t+2/3} - \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^{t+2/3} - 2C_{i,j,k}^{t+2/3} + C_{i,j+1,k}^{t+2/3}) \\
 &= C_{i,j,k}^t + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^t - 2C_{i,j,k}^t + C_{i+1,j,k}^t) + \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^t - 2C_{i,j,k}^t + C_{i,j+1,k}^t) \\
 &+ \frac{D\Delta t}{\Delta z^2} (C_{i,j,k-1}^t - 2C_{i,j,k}^t + C_{i,j,k+1}^t) + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^{t+1/3} - 2C_{i,j,k}^{t+1/3} + C_{i+1,j,k}^{t+1/3})
 \end{aligned} \tag{7}$$

Step 3, z implicit:

$$\begin{aligned}
 & C_{i,j,k}^{t+1} - \frac{D\Delta t}{2\Delta z^2} (C_{i,j,k-1}^{t+1} - 2C_{i,j,k}^{t+1} + C_{i,j,k+1}^{t+1}) \\
 &= C_{i,j,k}^t + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^t - 2C_{i,j,k}^t + C_{i+1,j,k}^t) + \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^t - 2C_{i,j,k}^t + C_{i,j+1,k}^t) \\
 &+ \frac{D\Delta t}{2\Delta z^2} (C_{i,j,k-1}^t - 2C_{i,j,k}^t + C_{i,j,k+1}^t) + \frac{D\Delta t}{2\Delta x^2} (C_{i-1,j,k}^{t+1/3} - 2C_{i,j,k}^{t+1/3} + C_{i+1,j,k}^{t+1/3}) \\
 &+ \frac{D\Delta t}{2\Delta y^2} (C_{i,j-1,k}^{t+2/3} - 2C_{i,j,k}^{t+2/3} + C_{i,j+1,k}^{t+2/3})
 \end{aligned} \tag{8}$$

FIGURE 1.

The bleached line's intensity decreases over the course of a SCAMP experiment. Throughout this timeframe, the intensities are similar for an experimental dataset (top) and its corresponding theoretical simulation (bottom).

FIGURE 2. Average fluorescence intensity across a SCAMP line is plotted for a range of diffusion coefficients, D ($\text{length}^2\text{s}^{-1}$), and bleaching rate constants, k . Higher diffusion coefficients and lower bleaching rate constants lead to slower bleaching.

FIGURE 3.

Average line intensity decreases over time due to photobleaching in SCAMP experiments. The simulation provides excellent fits to the data ($R^2=0.98$).

FIGURE 4.

The point-spread function was measured by assembling z-stack images of sub-resolution fluorescent microspheres. x - y is the imaging plane. The clear asymmetry varies greatly from the theoretical point-spread function, which would show rotational symmetry along the z -axis. An intensity of 1.0 represents a voxel maximally detectable by the microscope; an intensity of 0.0 represents a voxel minimally detectable by the microscope.

FIGURE 5.

The bleach (excitation) matrix was determined by photobleaching along a 12-pixel line in an agarose gel which contained immobilized fluorophore, and then imaging the matrix at very low laser power. x - y is the imaging plane; x is the line-scan direction. A higher intensity corresponds to a greater degree of bleaching that occurs at a given point in space.

FIGURE 6.

Percent error in the estimate of D decreases as more time points are fit and as the level of noise in the dataset decreases. The datasets being fit were simulations of known D to which random noise with standard deviation of 2 (SD2) or 3 (SD3) had been added. (n=11)

FIGURE 7.

SCAMP experiments fit within the PCM. A DIC image (A), fluorescence image stained for type VI collagen (B), and overlay of the two (C) show a porcine chondrocyte with the surrounding PCM defined by the presence of type VI collagen (staining as described in Youn et al. (27)). A porcine chondrocyte is typically 14 μm in diameter and surrounded by a 2- μm -thick PCM. When positioned strategically, a box containing the maximal excursion of a bleached region (3.3 $\mu\text{m} \times 1.9 \mu\text{m} \times 5.7 \mu\text{m}$) fits within this PCM (D).

FIGURE 8.

The bleaching rate constant, k (s^{-1}), decreases significantly with increasing depth into the tissue (top, linear regression $p=0.00005$), while the diffusion coefficient, D , does not change significantly (bottom, linear regression $p=0.1$). The line and equation (top) show the significant fit of the bleaching rate constant versus $\ln(\text{depth})$ ($R^2 = 0.44$).

FIGURE 9.

Mean (+sem) diffusion coefficients were calculated for the ECM (dark bars) and PCM (light bars) of normal and early-stage-arthritic porcine cartilage. Normal tissue has a significantly lower diffusion coefficient in the PCM than in the adjacent ECM (*p=0.018).