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Direct quantification of solute diffusivity in agarose and articular cartilage using correlation spectroscopy

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Abstract

Articular cartilage is an avascular tissue; diffusive transport is critical for its homeostasis. While numerous techniques have been used to quantify diffusivity within porous, hydrated tissues and tissue engineered constructs, these techniques have suffered from issues regarding invasiveness and spatial resolution. In the present study, we implemented and compared two separate correlation spectroscopy techniques, fluorescence correlation spectroscopy (FCS) and raster image correlation spectroscopy (RICS), for the direct, and minimally-invasively quantification of fluorescent solute diffusion in agarose and articular cartilage. Specifically, we quantified the diffusional properties of fluorescein and Alexa Fluor 488-conjugated dextrans (3k & 10k) in aqueous solutions, agarose gels of varying concentration (i.e. 1%, 3%, 5%), and in different zones of juvenile bovine articular cartilage explants (i.e. superficial, middle, and deep). In agarose, properties of solute diffusion obtained via FCS and RICS were inversely related to molecule size, gel concentration, and applied strain. In cartilage, the diffusional properties of solutes were similarly dependent upon solute size, cartilage zone, and compressive strain; findings that agree with work utilizing other quantification techniques. In conclusion, this study established the utility of FCS and RICS as simple and minimally invasive techniques for quantifying microscale solute diffusivity within agarose constructs and articular cartilage explants.

KEY TERMS

solute diffusion; fluorescent correlation spectroscopy; raster image correlation spectroscopy; confocal microscopy; anomalous diffusion

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INTRODUCTION

Articular cartilage is a highly-hydrated tissue whose primary function is to bear load and facilitate low-friction motion between joint surfaces. Articular cartilage is composed primarily of water (~60–85% wet weight), which plays a critical role in the tissue's function.^{3,29} The solid phase of the tissue consists of collagen (15–20%), proteoglycan (~5%), and a small percentage of non-collagenous proteins. Collagen contributes to cartilage's tensile stiffness, while the negatively-charged proteoglycans give it its swelling and water retaining characteristics.²⁹ As a result, collagen and proteoglycan form a highly-hydrated, fiber-reinforced solid matrix that supports the chondrocytes within it.

Being avascular, cartilage relies on diffusion and load-induced convection of interstitial fluid for solute transport.¹⁶ The same is true for engineered cartilaginous tissue.^{22,26} Dynamic loading can enhance advective macro-molecule uptake and transport within cartilage explants¹⁴ and engineered cartilage constructs.^{7,26} However, it is believed that diffusion is the predominant transport mechanism governing small nutrients, waste, and signaling molecules in cartilage. Within porous materials, diffusivity is dependent upon several properties, including material porosity and the size and geometry of the solute.¹⁶ In cartilage, pore size is approximately ~3–6nm,²⁹ depending on tissue composition, structure, hydration, and compaction. Thus, alterations in tissue composition and hydration, including depth- and age-dependent changes in ultrastructure; disease-state; and mechanical loading,²⁹ can influence solute diffusion, and thus chondrocyte metabolism and homeostasis, and tissue health.^{16,26} Indeed, research has shown that diffusivity of small molecular weight solutes (e.g. 3k dextran) are significantly greater in the superficial zone than the middle and deep zones of cartilage, diffusional anisotropy is site and solute specific (anisotropy was observed in the superficial zone for 500k dextrans, but not for a smaller 3k dextran), and mechanical compression leads to decreased solute diffusivity and increased diffusive anisotropy in a strain and site dependent manner.^{19,20,23,32} Such findings are in agreement with presence of substantial depth-wise and site-specific heterogeneity and anisotropy in extracellular matrix composition and structure within articular cartilage.^{19,28}

Various imaging techniques have been used to quantify diffusivity within cartilage. Magnetic resonance techniques, such as diffusion weighted and diffusion tensor MRI, and NMR spectroscopy have been used to quantify diffusion and diffusional anisotropy within cartilage.^{6,13,27} However, they are typically restricted to the study of water or small organic molecules and have limited micro-scale spatial resolution. Adsorption/desorption assays using techniques including optical/fluorescent imaging have also been implemented;^{1,32} however, such assays report bulk diffusion measures and often lack spatial specificity. In contrast, fluorescence recovery after photobleaching (FRAP) is a widely-implemented microscopy technique that can be used to quantify microscale diffusion. Numerous studies have used photobleaching techniques to quantify solute diffusion within engineered cartilage²² and cartilage explants.^{19,20} However, despite its ubiquity of application, diffusion quantification via FRAP is not without limitations. FRAP involves the application of an external perturbation, in the form of photobleaching of concentrated (M) fluorescent solutes, and the use of high laser intensities, which can have phototoxic effects on cells.¹¹ Modeling FRAP data also relies on a number of assumptions, whose validity can affect the derived

transport results. These include: 1) precise knowledge of the geometry of the bleached region of interest (ROI), 2) uniform, isotropic, and instantaneous bleaching of the ROI, and 3) in-plane diffusion.³⁵ Alternatively, several fluorescent-based correlation spectroscopy techniques that can directly quantify solute diffusion in situ while avoiding the limitations of FRAP exist.

Fluorescent correlation spectroscopy (FCS) is an Eulerian technique that relies upon correlation analysis of fluctuations in fluorescence driven by intrinsic physical processes within a small, stationary observation volume.^{12,25} Unlike FRAP, FCS does not rely on external perturbations to study transport, but instead on small, internal particle ‘perturbations’ due to Brownian motion. FCS is a well-developed and highly-sensitive analytical tool for quantifying solute transport. Advantages of FCS include its femtoliter ($\sim 10^{-15}$ L) sized observation volume, well-defined data collection and analysis methodologies, and short experiment times (~ 1 minute). Additionally, FCS does not require high laser powers for data collection, and is performed using low (nanomolar) solute concentrations. Furthermore, FCS can capture the non-linear time dependence of anomalous self-diffusion of particles whose diffusion is hindered by obstacles.⁴ Recently, FCS has been used to quantify solute diffusion in healthy and chemically degraded porcine articular cartilage;²⁴ however, anomalous diffusion was not considered. Nonetheless, FCS is not an image-based technique, thus, individual observations are spatially restricted and cannot be visualized, per se, during collection. Instead, an image-based extension of FCS called raster image correlation spectroscopy (RICS),¹⁰ which exploits the time lags between dynamically captured pixels (observation volumes) in raster-scanned images, allows for quantification of solute diffusion within laser scanning confocal microscope (LSCM) images, and may prove useful for observing and measuring micro-scale solute diffusion in engineered and natural cartilage tissues. However, studies applying RICS to the quantification of solute diffusion within porous, engineered or natural cartilage materials have yet to be performed. The goal of the present study was to apply and compare both FCS and RICS in quantifying the diffusivity of a variety of solutes with sizes relevant to both nutrition and molecular signaling within cartilaginous scaffolds (agarose) and explants tissues (bovine articular cartilage), and to demonstrate their ability to characterize composition and strain, and depth and strain dependencies in diffusivity, respectively, in these materials.

MATERIALS AND METHODS

A. Correlation Spectroscopy Theory and Analysis

In the present study, solute self-diffusivity was quantified via FCS and RICS (Figure 1). The theories underlying FCS and RICS diffusion analysis has been reviewed in the literature^{9,18} and are summarized in the Supplemental Methods.

B. Sample Preparation and Characterization

Agarose Plugs—Agarose was prepared at nominal concentrations of 1, 3, and 5% (wt/vol) in PBS, cast into 1.5mm thick slabs, and solidified at 4°C. Three millimeter diameter plugs ($n = 55$) were prepared using a biopsy punch and equilibrated for 24-hours in 10nM solutions of fluorescein isothiocyanate (389 Da; Sigma-Aldrich, St. Louis, MO, USA),

AlexaFluor488-conjugated 3k dextran (~3,000 Da; Thermo Fisher Scientific, Waltham, MA, USA), or AlexaFluor488-conjugated 10k dextran (~10,000 Da; Thermo Fisher) in PBS. Agarose gels utilized in the present study were characterized in terms of their equilibrium mass, volumetric swelling ratios,³³ and pore size¹⁵ (Supplemental Materials and Methods).

Bovine Articular Cartilage—Intact joints from freshly slaughtered bovine calves (<1-year-old; n = 3) were obtained from a local abattoir (Green Village Packing, Green Village, NJ, USA). Full thickness articular cartilage plugs (3mm diameter) were harvested from the medial femoral condyles within 18 hours of slaughter using a biopsy punch. Joints with visual evidence of injury, damage, or disease were excluded from the study. Plugs (n = 35) were cut into 1mm thick sections and separated into groups corresponding to the superficial (1st section), middle (subsequent section), and deep zones (furthest section absent subchondral bone). Plugs were stained with 2 μ M DAPI in PBS (Thermo Fisher) for 1-hour, followed by 24-hour equilibration in 10nM of fluorescein or 3k dextran in PBS.

C. Imaging

Agarose and articular cartilage plugs were imaged within a custom holder allowing the application of specified strains in unconfined compression between an impermeable glass coverslip and glass slide. Samples were surrounded by dye solutions to maintain hydration and a constant source of solute. Agarose plugs were manually compressed to 0 and 10% strain (larger strains were not applied as gels tended to fracture at strains 20%), while cartilage plugs were compressed to 0, 10, 20, and 30% strain. Samples were allowed to equilibrate for 10- minutes following each compression, a time confirmed to be adequate for these agarose and cartilage plugs to relax to quasi-equilibrium in unconfined compression. Confocal reflectance was used to identify the plug-cover glass interface and position the observation volume/plane ~20- μ m into the sample. DAPI imaging was used to orient the plugs and to allow us to identify interterritorial matrix regions that minimized the presence of chondrocytes within the collected data (Supplemental Figure 1).

FCS and RICS were performed in a paired manner at three locations within each individual plug to obtain an average diffusivity for each sample and technique. All data were collected on a Zeiss LSM780 or LSM880 LSCM (Carl Zeiss, Thornwood, NY, USA), similarly equipped for FCS and RICS. A 488nm Argon laser was used to excite the fluorescent solutes through a 40X water immersion lens (C-Apochromat 40x/1.20 W, Carl Zeiss). A spectral array of GaAsP detectors collected emitted fluorescent light between 490nm and 543nm through a pinhole set to 1 Airy Unit.

Daily, after microscope warmup, the radial (ω_0) and axial (ω_z) waists of the LSCM's point spread function (PSF) were determined by calibration using an aqueous solution of 10nM fluorescein in water. The PSF geometry was established by fitting the FCS or RICS autocorrelation data to diffusion models utilizing a fixed fluorescein diffusion coefficient of 450 μ m²/s.⁸ For FCS, the average PSF geometry was found to be ω_0 = 264.20nm and ω_z = 1324.98nm; for RICS, the average ω_0 = 341.28nm and the ω_z/ω_0 ratio was taken to be 5:1. Subsequently, FCS and RICS analyses were performed with fixed ω_0 and ω_z parameters, while the diffusivity parameters were varied to best-fit to the autocorrelations.

Image collection for both FCS and RICS was optimized based upon published protocols.^{10,24} Each FCS measurement consisted of ten recordings, each 10-seconds long, at a single observation location, encompassing $\sim 10^6$ to 10^8 fluorophore transits. Hardware-based temporal autocorrelation was performed for each 10-second trial, then recorded and visualized using Zen software (Carl Zeiss). If particle aggregation or photobleaching were observed within a 10-second trace (Supplemental Figure 2), that trace was excluded from further analysis. The software package (PyCorrFit)³⁰ was used to construct an average autocorrelation curve from the remaining 10-second trials and to fit this curve to the full form of either the normal (Supplemental Equation 2) or an anomalous (Supplemental Equation 6) diffusion models to obtain average solute diffusion parameters (i.e. normal diffusion coefficient [D], or anomalous diffusion exponent [α] and transport coefficient [γ]).

RICS experiments were performed immediately following each FCS data acquisition in the same sample location. All RICS experiments were conducted at 200–320 $\times$ magnification (40 $\times$ objective zoom times a 5 to 8 $\times$ optical zoom; 50–80nm pixel size). 50 or 100 frames were captured for each test at 256 $\times$ 256- or 512 $\times$ 512-pixels. Pixel dwell time varied from 0.64–12.6 μ s, resulting in line scans between 0.77–7.56ms, and frame rates of 0.52–2.55 fps. RICS images were analyzed using the Globals for Images software, Version 4 (Laboratory for Fluorescence Dynamics, University of California, Irvine, CA). Briefly, moving background subtraction (window size = 4) was performed on each dataset to remove immobile particles.¹⁰ Spatial autocorrelation (Supplemental Equation 8) was then performed on each frame within the dataset to generate a series of autocorrelation surfaces from which an average autocorrelation surface was calculated. This average correlation was then fit to a diffusion model (Supplemental Equation 9) that considered contributions from both raster scanning (Supplemental Equation 10) and particle diffusion (Supplemental Equation 11).

D. Statistical Analysis

Data are presented as mean $\pm$ standard deviation. To determine the influence of the various testing conditions on correlation spectroscopy derived measures of solute diffusion parameters, including solute size, agarose density, cartilage zone, applied strain, and analysis techniques, the following statistical tests were performed. i) One-sample t-tests were used to compare solute diffusion parameters back to the reference of fluorescein in aqueous solution ($D = 450\mu\text{m}^2/\text{s}$). Otherwise, two-way ANOVAs with Tukey's adjustment for multiple comparisons and post-hoc testing were used to compare the effect of ii) solute size and iii) agarose gel density on diffusivity. Paired t-tests were used to compare the effect of v) compressive strain on solute diffusivity in agarose. In cartilage, two-way ANOVAs with Tukey's correction and post hoc tests were used to assess the effect of vi) solute size, vii) cartilage zone, and viii) compressive strain on diffusivity in cartilage. The relationships between paired FCS- and RICS-derived diffusion coefficients were quantified using ix) paired t-tests and x) Pearson Product Moment Correlation. All analyses were performed in GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA); statistical significance was set at $p < 0.05$.

RESULTS

A. Aqueous Diffusion

Using both FCS and RICS, fluorescent solute diffusivity in aqueous solution was assessed (Table 1). All aqueous diffusivities were modeled as normal ($= 1.0$; Figure 2A). Representative FCS and RICS correlation and fit data are illustrated in Supplemental Figures 3A & 5A. For both FCS and RICS, the aqueous diffusivity of fluorescein, set to the reference value of $450\mu\text{m}^2/\text{s}$, was greater than that of 3K ($p<0.0001$, one sample t-test) and 10K dextran ($p<0.0001$) (Figures 2B & C). 3K dextran diffusivity was also greater than that of 10K dextran ($p=0.008$, two-way ANOVA). No significant differences in aqueous diffusivities were observed between FCS and RICS techniques (paired t-test).

B. Agarose

Parameters characterizing the agarose gels utilized in the study, including equilibrium volume swelling ratio, water content, and pore/mesh size (Supplemental Methods and Supplemental Figure 5) are summarized in Table 1. Within agarose plugs, it was observed that FCS-based solute diffusivity was best modeled as anomalous (Table 1, Figure 2A). Inclusion of a freely varying anomalous diffusion exponent (in Supplemental Equation 6) significantly improved FCS curve fits compared to normal ($= 1.0$) diffusion (data not shown; refer to Supplemental Figure 4 for visual comparisons). Anomalous diffusion exponents (α) ranged from 0.98, for fluorescein in 1% agarose (not significantly different from $= 1$, one sample t-test), to 0.88, for 10k dextran in 5% agarose (all other values were significantly different from $= 1$, one sample t-test). Was found to be solute and agarose concentration dependent (9 & 60% of variation, respectively, two-way ANOVA; Figure 2A), and exhibited larger deviations from 1.0 with increasing solute size and gel concentration.

In terms of solute diffusivity, transport and diffusion coefficients (Figures 2B & C) decreased with increasing solute size (81 & 86% of variation explained by solute size for FCS and RICS, respectively, $p<0.0001$, two-way ANOVA) and agarose concentration (8 & 7% of variation, respectively, $p<0.0001$). For the solute and agarose densities investigated, diffusivity was inversely, and linearly, related to gel concentration (Supplemental Figures 5B and 6, Supplemental Table 1). Additionally, application of 10% compressive strain tended to hinder 3k dextran diffusivity (1% agarose, $p=0.07$ & 0.01 for FCS and RICS; 3% agarose, $p=0.22$ for FCS and RICS, paired t test; Figure 3). Overall, for both aqueous solutions and agarose plugs, FCS and RICS quantification techniques were highly reproducible and consistent (Figure 4); exhibiting a very-strong relationship between measurements (Pearson Product Moment Correlation, $R=0.985$, $p<0.0001$) and a linear regression no different from unity ($y=0.9905x$; F-test, $p=0.2912$).

C. Cartilage

Depth- and strain-dependent diffusional properties of fluorescent solutes were quantified in juvenile bovine articular cartilage plugs using FCS and RICS techniques (Tables 2 & 3, Supplemental Figures 4C & 5C). Solute diffusivity in unstrained cartilage, measured by FCS, was best described as anomalous, exhibiting a mean anomalous diffusion exponent (α) of 0.73 ± 0.016 (Figures 5A; refer to Supplemental Figure 3C for visual comparisons).

Diffusional anomaly varied with solute size, but not cartilage zone or compressive strain (solute size = 49% of variation, $p < 0.0001$, & cartilage zone = 1% of variation, $p = 0.7471$, two-way ANOVA; Figures 5A & 6A)

Using FCS and RICS quantification techniques it was observed that cartilage depth (33% and 28% of variation for FCS and RICS, $p = 0.0002$ & $p < 0.0001$, respectively, two-way ANOVA) and solute size (10% and 20% of variation, $p = 0.01$ & 0.001 , respectively) affected diffusivity within unstrained bovine cartilage (Figure 5B & C). For fluorescein, diffusivity was significantly higher in the superficial zone than in the middle and deep zones. In the superficial zone 3k dextran diffusivity was less than that of fluorescein. No additional relationships were observed in the deeper zones.

Quantification of diffusional properties in compressed cartilage samples demonstrated that axial strain influenced 3k dextran diffusivity, but not diffusional anomaly (Figure 6). In the superficial zone, 3K dextran diffusivity was significantly higher in unstrained versus strained states by both FCS and RICS (Supplemental Figures 4D & 5). In the middle zone, a similar relationship was observed by RICS. No further trends in diffusivity were apparent amongst higher levels of strain or in the deep zone.

In general, whilst the observed relationships between diffusivity and solute size, cartilage zone, and equilibrium compressive strain were qualitatively similar between FCS and RICS, RICS-derived measures were consistently lower than their FCS-derived measures (14 out of 18 groups exhibited significantly lower RICS measures; Figures 5 & 6, paired t-tests). While paired measures of FCS- and RICS-based solute diffusivity demonstrated strong correlation within cartilage ($r = 0.7751$, $p < 0.0001$; Pearson Product Moment Correlation; Figure 7), the slope of this relationship ($y = 0.7715x$) was significantly less than 1.0 (F-test; $p < 0.0001$), suggesting, on average, a 23% difference (reduction) in diffusional properties determined by RICS.

DISCUSSION

The present study confirmed the ability of fluorescent correlation spectroscopy (FCS) and its imaging extension, raster image correlation spectroscopy (RICS), to quantify solute diffusivity within porous gels and cartilaginous tissues. FCS and RICS are minimally invasive, experimentally straightforward optical techniques for directly quantifying local, microscale solute self-diffusivity *in situ* that don't rely on i) external perturbation to the system, the use of ii) high fluorophore concentrations or iii) intense laser illumination, or iv) *a priori* knowledge of the system under observation. Instead, correlation spectroscopy relies solely upon knowledge of known optical collection parameters, such as the geometry of the observing system's point spread function (FCS and RICS) and the dynamics of its raster imaging pattern (RICS)⁹. Subsequently, quantification of solute diffusivity occurs through the robust application of autocorrelation analyses and curve fitting, and the Gaussian description of Brownian motion.

Our data showed, as expected, that in hydrated porous media, solute diffusivity is influenced by solute size, matrix structure, and matrix compaction, and that both FCS and RICS could

detect these relationships. In agarose gels, measures of solute diffusivity, including diffusional anomaly (α), transport constant (k ; a time invariant diffusion parameter), and diffusion coefficient (D) decreased with increasing solute size, gel concentration, and compressive strain (Figures 2 & 3). These relationships are intuitive as i) larger solutes experience increased hydrodynamic and obstructive effects;² ii) higher concentration agarose gels have decreased water volume fractions and shorter mean free path lengths;² and iii) mechanical compression of agarose reduces pore space, decreases interstitial volume available to the solute, and decreases partition coefficients, phenomena that each hinder solute diffusion⁷. Agarose represents an ideal model to compare measured diffusivities from different fluorescence-based correlation spectroscopy techniques since gel properties are easily controllable and the resultant hydrogels are considerably more homogeneous than cartilage. Furthermore, agarose is a commonly utilized scaffold for engineering cartilage-like tissues.^{7,22,26} The high reproducibility of our FCS and RICS diffusivity measures in agarose (Figure 4), and their alignment with previously published data^{22,31} support the use of both techniques for quantifying solute diffusion within such scaffold materials.

In contrast to agarose gels, articular cartilage is structurally, compositionally, and mechanically heterogeneous.^{28,29} Furthermore, the porosity of cartilage is $\sim 7\text{--}15\times$ smaller than the densest gels tested ($3\text{--}6\text{nm}$ ²⁹ vs. $\sim 46\text{nm}$). Thus, it remained unclear how cartilage's natural heterogeneity and mesh size would influence the ability to quantify solute diffusivity using FCS and RICS. This study demonstrated that both FCS and RICS can be used to study cartilage diffusivity under a variety of conditions, including variable solute sizes, cartilage zones, and compressive strains. Similar to agarose gels, cartilage diffusivity was best described as anomalous when assessed by FCS (Figures 5 & 6). α in cartilage was markedly greater than that of agarose, but did not vary much across the conditions assessed. On the other hand, solute diffusivity in cartilage was significantly, and predictably, reduced compared to those in aqueous solution and agarose gels [data not shown]. Overall, we detected significant effects of solute size, cartilage zone, and compressive strain on diffusivity (Figures 5 & 6). Specifically, we observed fluorescein diffusivity to be higher in the superficial zone than the middle and deep zones, and greater than that of 3k dextran in the superficial zone. Additionally, initial compression of both superficial- and middle-zone cartilage (from 0 to 10% strain) resulted in a reduction in solute (3k dextran) diffusivity. Together, these data reinforce previous findings of cartilage structure,^{19,20} composition (e.g. reduced proteoglycan content in the superficial zone),¹⁹ water content (increased in the superficial zone),⁵ and compaction²⁰ playing a role in regulating diffusion within articular cartilage.

The observation of a markedly reduced correspondence between RICS and FCS-derived diffusional parameters ($R = 0.721$ to 0.775) and significant deviation from a linear regression slope of 1.0 (slope = 0.7751) in cartilage (Figure 7), as compared to agarose ($R = 0.985$, slope = 0.991 ; Figure 4), is of interest. We suspect that this may be attributable to the effect of structural differences among cartilage and agarose on the movement of solutes at the observational length scales of FCS and RICS. FCS data is collected from a stationary ellipsoidal observation volume approximately 250nm in diameter by 1250nm in height, a volume that can easily reside entirely within the dimensions of a given cartilage matrix type (e.g. territorial-interterritorial matrix vs. pericellular matrix).²¹ In contrast, RICS

measurements are acquired from volumes ~1500 times larger ($\sim 0.5 \times 10^{-12}$ L). This observation volume is large enough to encompass both cells and multiple ECM regions. Therefore, it is natural to expect that RICS observations might include material of increased diffusion hindrance, such as the pericellular matrix surrounding chondrocyte, as well as the transitional material between these matrices.²¹ While we attempted to explicitly exclude pericellular and transitional locations in our FCS experiments (we tried to restrict FCS measurements to interterritorial ECM), they could not be avoided in the RICS experiments. Furthermore, cartilage exhibits increased diffusional anomaly compared to agarose (Tables 1–3). Since anomalous diffusion is non-linearly related to time (and thus distance; Supplemental Equation 5) the presence of sub-diffusion (<1.0) could lead to a suppression of RICS diffusion coefficients due to the fact that they are assessed over a larger distance than FCS (several m vs. ~ 0.250 m). However, despite quantitative differences between FCS and RICS-based diffusion magnitudes, the resultant solute size, zone, and strain dependencies were internally consistent, and matched literature observations.^{19,20,32}

While FCS and RICS are straightforward techniques to implement, several precautions regarding data collection, analysis, and interpretation should be noted. These include: i) accurate calibration of the microscope's point spread function (PSF), ii) use of appropriate fluorescent solutes, iii) knowledge of the spatial limitations of each technique, and iv) appropriate selection of analytical models to describe transport kinetics.

Knowledge of PSF size is required for robust quantification of diffusion kinetics. The geometry and size of the confocal microscope's PSF can be determined two different ways. First, by imaging immobilized point source beads (i.e. smaller than the diffraction-limited resolution of the system) and measuring their reconstructed sizes.³⁴ However, this technique requires specialized reconstruction and analysis software, and is oftentimes difficult to match to the experimental imaging conditions.³⁴ Conversely, calibration of the PSF geometry via the use of a known diffusion standard is a simple and well-accepted method for PSF determination.³⁴ Using this calibration technique, diffusion values are calculated relative to the diffusion coefficient (D) of a calibration standard. In this manner, error in establishing absolute diffusivity is subject to the accuracy of the adopted standard's D and the constancy of the imaging conditions. However, if experiments are calibrated according to the same standard and conditions, the data remain internally consistent. In the present study, calibration was performed daily against fluorescein in aqueous solution at room temperature, for which $D = 450 \mu\text{m}^2/\text{s}$ was established.⁸ The use of this reference standard resulted in diffusion values for the various experimental groups in this study were largely consistent with those previously reported in the literature.^{19,20,22,31}

In all fluorescence-based observation systems, fluorophores should be chosen such that the fluorophore and its observation has minimal impact on the observed specimen. This is easily achieved via FCS and RICS, since nanomolar solute concentrations are required; fluorophore concentrations greater than $\sim 10\text{nM}$ interfere with correlational analyses because fluorescent fluctuations are suppressed in continuously occupied observation volumes. Additionally, a large number of non-reactive, photo-stable fluorophores appropriate for FCS and RICS analysis are available (e.g. dyes, dextrans, proteins, etc.). While fluorescent beads of 'well-defined' size could have been used to inform diffusivity and pore size within

agarose,³¹ the present study focused on quantification of diffusion of molecules relevant to cartilaginous biology. Thus, diffusion quantification was restricted to small molecule fluorescent tracers, including fluorescein (332Da), and dextran dyes (3k and 10kDa). These compounds served as surrogates for molecules with relevance to cartilage nutrition, waste transport, and molecular communication (Table 4). Furthermore, the nano-porous structure of cartilage (~3–6nm)²⁹ precluded the use of engineered beads (typically >10nm) for such studies.

In the present study, analysis of autocorrelation outputs (i.e. fitting) were limited to analytical models of normal (FCS and RICS) or anomalous (FCS), 3D isotropic diffusion of a single solute. When assessed by FCS, normal diffusion was, as expected, an appropriate model for diffusion in aqueous solutions, as well as fluorescein in 1% agarose. However, we confirmed that anomalous FCS diffusion models are more appropriate for quantifying molecular diffusivity of larger solutes in 1% agarose, and necessary for diffusion in higher concentration agarose gels and articular cartilage. One limitation to the study is the present lack of robust analytical approaches for incorporating anomalous diffusion measures within RICS. Furthermore, it is well established that collagen orientation varies throughout the thickness of articular cartilage,²⁹ prior work has shown that such anisotropy can affect solute diffusion in cartilage.^{19,20} While beyond the scope of the present study, FCS and RICS techniques have been used to quantify diffusional anisotropy,³⁸ and future development could incorporate anisotropy in the description of diffusion within cartilaginous tissues.

The spatial limits of FCS and RICS need also be considered when performing confocal correlation spectroscopy. In the present study, observations were restricted to either the stationary, femtoliter FCS observation volume (~250×250×1250-nm ellipsoid) or a dynamically sampled, picoliter RICS observation region (~20×20×1.25 m rectangular cuboid). Being on the order of cartilage cellular constituents, these scales allow for diffusion quantification at resolutions unobtainable with other assays. However, while FCS and RICS are suitable for quantifying microscale level self-diffusion within cartilaginous tissues, they have distinct strengths and weaknesses, and thus appropriate experimental domains. Because of the high (~MHz) sampling rate of FCS, datasets can be collected rapidly (~100-s). However, tissue averaged diffusivity or diffusion mapping can only be collected through repeated data ‘observations’ at different locations within the sample; a process that can be prohibitively time consuming (a 10-point × 10-point mapping of diffusivity would take ~2.75-hours). On the other hand, RICS can be used to acquire tissue averaged data and diffusivity maps (Supplemental Figure 7) in a fraction of the time (approximately tens of minutes), with the added benefit of direct specimen visualization. However, observation times on the order of 10s of minutes may still present concern over phototoxicity. Therefore, we suggest that FCS may be an appropriate tool for the rapid, high-throughput assessment of diffusive transport within ‘live’ cartilaginous tissues, holding promise for the non-invasive and non-destructive, longitudinal, in situ characterization of engineered and regenerated cartilage. Alternatively, RICS-based diffusion quantification may be more appropriate for the detailed quantification of diffusive properties in non-vital specimens, or for end-point quantification. Furthermore, spatial sub-analysis of RICS images should allow for improved 2D microscale mapping of solute diffusivity (see Supplemental Figure 7 for an illustrative example). Lastly, while the present study was

restricted to quantification of solute diffusion at equilibrium, time dependent changes in diffusivity as well as load-induced solute advection can influence nutrient transport within cartilage.^{14,16} Temporal sub-analysis of FCS and RICS data can permit assessment of time-dependent changes in diffusivity under a variety of conditions. Furthermore, FCS and image correlation spectroscopy techniques (such as spatiotemporal image correlation spectroscopy [STICS]) can measure solute advection and may be suitable for quantifying load-induced solute transport within porous materials.

In conclusion, we have confirmed the ability of FCS, a well-established and commonly used aqueous diffusion characterization technique, to quantify anomalous solute diffusivity within hydrogels and cartilage explants. We have also demonstrated that RICS, an image-based technique, can be used to quantify solute diffusion in hydrated, porous matrix environments in a manner consistent with FCS. Together, FCS and RICS represent simple, rapidly implementable optical techniques for directly quantifying solute diffusion in hydrogels and cartilage at resolutions previously unachievable. As a result, ICS techniques have tremendous potential for facilitating the *in situ* study of diffusion in a variety of applications. These include the benchtop characterization of microscale diffusive properties and solute matrix interactions within cartilaginous tissues during development, aging, disease progression, and therapeutic intervention, as well as studying their correlates with mechanical and compositional properties. In addition, these techniques have the distinct potential to facilitate the minimally-invasive longitudinal quantification of tissue properties and maturation during the engineering and regeneration of cartilaginous tissues.

Further studies are required to determine the relationships between correlation spectroscopy-based measure of diffusion and cartilage function, health and degeneration. It is theorized that proteoglycan loss, an early marker of osteoarthritis, should be associated with overall changes in water content and diffusivity. Indeed, diffusion tensor imaging-based MRI studies have shown usefulness in assessing local variations in articular cartilage microstructure and hydrodynamic status,²⁷ and in identifying tissue changes associated with osteoarthritis.³⁷ Studies in a variety of enzymatically (trypsin)-mediated cartilage degradation studies have identified a range of changes in diffusivity due to experimental proteoglycan loss (e.g. 20–30% increase,³⁹ 0–195% increase,³⁶ 50% decrease to 180%²⁴ increase compared to normal tissue), which were demonstrable by FCS.²⁴ Nonetheless, it is not entirely clear how such experimental proteoglycan manipulation reflects the natural progression of cartilage degeneration. Therefore, studies are needed to determine to what extent changes in optical correlation spectroscopy-derived diffusivity measures can be predictive of natural osteoarthritic disease state and longitudinal osteoarthritic progression. Nevertheless, if successful, correlation spectroscopy-based diffusivity assessment may have the potential, given recent developments in arthroscopic laser scanning confocal microscopy,¹⁷ to be translated to the study of molecular diffusion, transport, function, and disease in intact knees. In this manner, *in vivo* correlation spectroscopy development may be able to inform the direct, clinical evaluation of site-specific cartilage properties and health during routine arthroscopic evaluation and surgery, which could aid in improving treatment and rehabilitation planning following joint injury. Lastly, we note that FCS and RICS-based diffusion quantification are not restricted to articular cartilage, these techniques can be readily adapted to the *in situ* study of diffusion and solute matrix interactions within a

variety of musculoskeletal and non-musculoskeletal tissues, including tendon/ligament, intervertebral disc, muscle, bone, etc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

10k dextran	AlexaFluor® 488-conjugated 10 kilodalton dextran
3k dextran	AlexaFluor® 488-conjugated 3 kilodalton dextran
α	anomalous diffusion exponent
D	Diffusion Coefficient (time invariant parameter)FCS – fluorescent correlation spectroscopy
FRAP	fluorescent recovery after photobleaching
Γ	Transport Coefficient (time invariant parameter)ICS – image correlation spectroscopy
LCSM	laser scanning confocal microscopy
MRI	magnetic resonance imaging
NMR	nuclear magnetic resonance
PSF	point spread function
RICS	raster image correlation spectroscopy
ROI	Region of Interest
θ	radial waist dimension of the microscope point spread function
z	axial waist dimension of the microscope point spread function

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Figure 1. FCS and RICS data collection and analysis

Representative FCS fluorescent intensity trace from a 1-second subset of a diffusion experiment with 10nM fluorescein in aqueous solution (A). Temporal autocorrelation of a 10nM fluorescein FCS dataset and the best fit of this curve to the full form normal-diffusion model (B), including triplet state terms (anomalous diffusion exponent set to 1). RICS image of ‘slowly’ diffusing 200-nm fluorescent spheres in aqueous solution (C) illustrating how slowly diffusing ‘point-source’ particles appear as fluorescent streaks in both the x- and y-directions as a result of the raster-scanning of the spatially over-sampled point spread function. RICS spatial autocorrelation of aqueous 200-nm fluorescent spheres (D); both the height and color of the curve indicate the normalized autocorrelation $[G()]$ magnitude. RICS image of 10nM fluorescein diffusion in aqueous solution (E). RICS spatial autocorrelation of aqueous 10nM fluorescein (F). Note: images C and E, and their corresponding autocorrelation outputs (D and F) were acquired from scans with different pixel- and line-dwell times in order to illustrate RICS data and autocorrelation curves.

Figure 2. Diffusion parameters for fluorescein, 3K dextran, and 10K dextran in aqueous solutions and agarose plugs as determined by FCS and RICS

Anomalous diffusion exponents A; FCS), transport coefficients (B; FCS), and diffusion coefficients (C; RICS) were inversely related to solute size and gel concentration. Data reported as mean $\pm$ SD ($n=5-12$ for each group). Horizontal brackets (—) indicate significant difference in diffusivity between different sized solutes for a given agarose concentration (one sample t-test [against fluorescein at 0%] or two-way ANOVA with Tukey's adjustment for multiple comparisons [all others], $p<0.05$). Numbers (0,1,3,5) indicate significant difference in individual solute diffusivity between indicated agarose concentrations ($p<0.05$; two-way ANOVA). Pound signs (#) indicates significant difference between diffusivity measured by FCS and RICS for a given solute and agarose density ($p<0.05$, paired t-test).

Figure 3. Influence of equilibrium compressive strain on diffusion parameters for 3k dextran in agarose

Anomalous diffusion exponents (A; FCS), transport coefficients (B; FCS), and diffusion coefficients (C; RICS) in compressed agarose plugs. Data reported as mean $\pm$ SD ($n=6-12$ for each group). When compressed (to 10% strain), the diffusivity of 3k dextran in agarose was significantly reduced. Ampersand (&) indicated significant decrease in diffusivity in strained vs. unstrained agarose; paired t-test, $p<0.05$. Horizontal brackets (—) indicate significant difference in diffusivity between 1% and 3% agarose for a given compressive strain; t-test, $p<0.05$. Pound signs (#) indicates significant difference between diffusivity measured by FCS and RICS for a given solute and agarose density ($p<0.05$, paired t-test).

Figure 4. Relationship between measures of solute diffusion coefficients (RICS) and transport coefficients (FCS) in aqueous solutions and agarose gels

Individual data points for fluorescein (Δ), 3k dextran ($\square$), and 10k dextran ($\circ$) diffusivity in PBS (black symbols), and 1- (green), 3- (blue), and 5% agarose (red) are plotted. 3k dextran in 1% and 3% agarose strained to 10% are shown in solid green and blue squares, respectively. Pearson Product Moment Correlation analysis ($R = 0.985$, $p < 0.0001$) indicate a strong relationship between RICS and FCS diffusivity measurements in agarose ($y = 0.9905x$; solid black line). A one-to-one relationship is indicated by the dashed gray line. The slope of the linear fit was no different than a slope of 1.0 (F-test; $p = 0.2912$).

Figure 5. Comparison of fluorescein and 3K dextran diffusion measures in three different zones of un-compressed bovine calf articular cartilage

Depth dependent difference in anomalous diffusion exponents (A; FCS), transport coefficients (B; FCS), and diffusion coefficients (C; RICS) for fluorescein and 3k dextran in bovine cartilage plugs. Fluorescein exhibited a significant decrease in diffusivity between the superficial, and middle and deep zones (indicated by S, $p < 0.05$, two-way ANOVA, Tukey's post-hoc). A similar, but non-significant relationship was observed for 3k dextran. Significant differences in paired diffusion measures by FCS and RICS (#) were only observed for 3k dextran in the middle and deep zones ($p < 0.05$, paired t-tests). Data reported as mean $\pm$ SD ($n = 5-7$ for each group).

Figure 6. Relationship between 3k dextran diffusion parameters, tissue zone, and applied equilibrium compressive strain in bovine calf articular cartilage

Application of compressive strain did not influence the FCS-derived anomalous diffusion exponent (A). However, compressive strain significantly decreased the transport (B) and diffusion (C) coefficients of 3k dextran within the superficial (FCS) and middle (FCS & RICS), but not the deep zones of cartilage. Data reported as mean $\pm$ SD ($n = 12-14$ for each group). Horizontal brackets (—) indicate significant difference in diffusivity with applied strain for a given cartilage zone ($p < 0.05$, two-way ANOVA). S = significantly different diffusivity compared to the superficial zone for matched strains ($p > 0.05$, two-way ANOVA). Diffusivities as measures by RICS were always observed to be significantly less (~23% less) than those observed via FCS (paired t-tests).

Figure 7. Relationship between measures of solute diffusion coefficients (RICS) and transport coefficients (FCS) in bovine calf articular cartilage

Paired data points are shown for fluorescein (empty symbols) and 3k dextran (solid symbols) in the superficial (Δ), middle ($\square$), and deep ($\circ$) zones of unstrained cartilage. Pearson Product Moment Correlation analysis ($R = 0.7751$, $p < 0.0001$) indicate a significant relationship between RICS and FCS diffusivity measurements. However, this relationship was found to have a slope ($y = 0.7751x$, solid black line) significantly less than 1.0 (F-test; $p < 0.0001$). A one-to-one relationship is indicated by the dashed gray line. This fit indicated that on average RICS-based measures of diffusivity were ~23% less than those of FCS-based measures within cartilage. When all data was compared (unstrained and strained measures included) a similar relationship was observed ($R = 0.721$, $y = 0.6614x$; data not shown).

Table 1

Physical characteristics of different concentration agarose gel constructs, and FCS- and RICS-based quantification of solute diffusivity within agarose gels.

	Aqueous(0%)	1% Agarose	3% Agarose	5% Agarose
Gel Characteristics (n=3 samples/group)				
Eq. Volume Swelling Ratio (Q)	N.A.	70.8±7.3 ^{3,5}	36.8±0.5 ^{1,5}	25.7±0.8 ^{3,5}
Water Content (Q ⁻¹ *100; vol/vol)	"	1.42±0.14 ^{3,5}	2.72±0.01 ^{1,5}	3.89±0.01 ^{3,5}
Approximate Pore (Mesh) Size (nm)	"	126.7	63.6	46.1
Fluorescein (n=8–12 samples/group)				
FCS Anomalous Diffusion Exponent ()	1.0	0.98 0.01 ⁵	0.95 0.07 ⁰	0.92 0.04 ^{0,1}
FCS Transport Coeff. (m ² /s)	450	466.3 24.4 ^{3,5}	409.4 60.5 ^{0,1,5}	228.7 61.1 ^{0,1,3}
RICS Diffusivity (D)	450	455.2 18.0 ^{3,5}	406.4 33.0 ^{0,1,5}	259.6 14.1 ^{0,1,3}
3k Dextran (n=10–12 samples/group)				
FCS Anomalous Diffusion Exponent ()	1.0	0.94 0.02 ^{0,5}	0.92 0.01 ^{0,5}	0.88 0.03 ^{0,1,3}
FCS Transport Coeff. (m ² /s)	165.6 9.8 ^{1,3,5}	130.2 10.8 ⁰	111.2 16.5 ⁰	103.1 24.7 ⁰
RICS Diffusivity (D)	166.3 30.1 ^{3,5}	152.7 23.9 ^{3,5,#}	115.8 19.6 ^{0,1}	96.8 22.8 ^{0,1}
10k Dextran (n=5–8 samples/group)				
FCS Anomalous Diffusion Exponent ()	1.0	0.93 0.04 ^{0,5}	0.90 0.03 ⁰	0.88 0.01 ^{0,1}
FCS Transport Coeff. (m ² /s)	120.6 13.7	106.5 8.8	102.5 12.9	89.0 3.9
RICS Diffusivity (D)	112.9 14.5 ⁵	96.2 11.1	89.9 9.1	83.6 1.9 ^{0,#}

^{0,1,3,5} = Significantly different from aqueous (0%) condition, 1% agarose, 3% agarose, or 5% agarose, respectively, for a given solute. One sample t-tests for comparison to fixed D [450 m²/s] or [1.0] values, or ordinary two-way ANOVAs with Tukey's adjustment for multiple comparisons and post hoc testing, as appropriate.

= FCS-derived transport and RICS –derived diffusion coefficients differed significantly for given solute and agarose 31 concentration, paired t-test.

Table 2

Quantification of cartilage depth-dependent diffusivity of fluorescein and 3k dextran in unstrained cartilage by FCS and RICS.

	Fluorescein	3k Dextran
Superficial Zone (n=15)		
FCS Anomalous Diffusion Exponent ()	0.62 0.08 ^{3k}	0.79 0.04 ^F
FCS Diffusivity (m^2/s)	115.8 34.7 ^{3k,M,D}	59.1 39.9 ^F
RICS Diffusivity (D m^2/s)	89.8 31.3 ^{3k,M,D}	32.3 16.7 ^F
Middle Zone		
FCS Anomalous Diffusion Exponent ()	0.64 0.05 ^{3k}	0.77 0.08 ^F
FCS Diffusivity (m^2/s)	47.7 20.6 ^S	39.3 10.2
RICS Diffusivity (D m^2/s)	35.8 8.7 ^S	25.2 7.4 [#]
Deep Zone		
FCS Anomalous Diffusion Exponent ()	0.68 0.09	0.77 0.06
FCS Diffusivity (m^2/s)	47.4 20.5 ^S	45.0 14.6
RICS Diffusivity (D m^2/s)	33.4 9.2 ^S	31.5 4.3 [#]

^{3k,F} = Significantly different from respective diffusion parameter between 3k dextran or fluorescein, respectively, for a given cartilage zone. Two-way ANOVA with Tukey's adjustment for multiple comparisons and post hoc testing.

^{S,M,D} = Significantly different from Superficial, Middle, or Deep zone of cartilage, respectively, for a given solute. Two-way ANOVA with Tukey's adjustment for multiple comparisons and post hoc testing.

[#] = FCS-derived transport and RICS –derived diffusion coefficients differed significantly for given solute and agarose 32concentration, paired t-test.

Table 3

FCS- and RICS-based quantification of 3k-dextran solute diffusivity within the different zones of strained and unstrained articular cartilage.

		0% Strain	10% Strain	20% Strain	30% Strain
Superficial Zone (n=15 samples/group)					
FCS Anomalous Diffusion Exponent ()		0.73 0.06	0.74 0.08	0.74 0.08	0.75 0.11
FCS Diffusivity (D or m^2/s)		62.8 31.8 ^{0,20,30,M,D}	45.1 19.6 ⁰	38.1 13.1 ⁰	31.4 99.8 ⁰
RICS Diffusivity (D)		34.7 13.9 ^{0,20,30,#}	24.2 6.9 ^{0,#}	23.3 6.5 ^{0,#}	20.8 7.0 ^{0,#}
Middle Zone (n=12 samples/group)					
FCS Anomalous Diffusion Exponent ()		0.74 0.07	0.75 0.08	0.74 0.09	0.76 0.07
FCS Diffusivity (D or m^2/s)		38.6 7.4 ^S	34.7 11.0	36.7 13.4	30.4 8.0
RICS Diffusivity (D)		43.2 10.0 ^{0,20,30,#}	36.0 11.1 ^{0,#}	34.4 11.9 ^{0,#}	32.4 7.5 ^{0,#}
Deep Zone (n=14 samples/group)					
FCS Anomalous Diffusion Exponent ()		0.70 0.10	0.70 0.13	0.74 0.07	0.74 0.8
FCS Diffusivity (D or m^2/s)		43.0 12.4 ^S	36.7 11.0	39.3 13.0	34.7 21.9
RICS Diffusivity (D)		31.2 12.5 [#]	26.6 6.6 [#]	28.1 15.5 [#]	22.5 7.0 [#]

0,10,20,30 = significantly different from respective diffusion parameter in the unstrained (0%), 10%, 20%, or 30% equilibrium strained condition, respectively, for 3k dextran in a given articular cartilage zone. Two-way ANOVAs with Tukey's post hoc test, with Sidak's adjustment for multiple comparisons.

= FCS-derived transport and RICS –derived diffusion coefficients differed significantly for cartilage zone and compressive strain, paired t-test.

Table 4

Molecules with relevance to cartilage biology and signaling.

Molecular Class	Molecule	Molecular Weight
Nutrients and Waste	Amino Acids (AAs)	75–204 Da
	Lactic Acid	90 Da
	Glucose	180 Da
Signaling Molecules	Prostaglandin E2 (PGE ₂)	352 Da
	Adenosine Triphosphate (ATP)	507 Da
Signaling Peptides/Proteins	Insulin	5k Da
	Insulin-like Growth Factor-1 (IGF-1)	7.5k Da
	Fibroblast-like Growth Factor(s) (FGFs)	7–38k Da
	Tumor Necrosis Factor Alpha (TNF-)	17k Da
	Interleukin-6 (IL-6)	21k Da
	Bone Morphogenetic Proteins (BMPs)	26k Da
	Transforming Growth Factor Beta (TGF-)	25k Da