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Noninvasive Quantification of Cell Density in Three-Dimensional Gels by MRI

Brian J. Archer¹, Till Überrück², Julia J. Mack³, Khalid Youssef¹, Nanette N. Jarenwattananon⁴, Deniz Rall⁵, Denis Wypysek⁵, Martin Wiese⁵, Bernhard Blümich⁶, Matthias Wessling⁵, M. Luisa Iruela-Arispe³, Louis-S. Bouchard^{1,4}

¹Department of Bioengineering, University of California, Los Angeles

²Institute of Technical and Macromolecular Chemistry, RWTH Aachen University

³Department of Molecular Cell and Developmental Biology, University of California, Los Angeles

⁴Department of Chemistry and Biochemistry, University of California, Los Angeles

⁵Department of Chemical Engineering, RWTH Aachen University

⁶Institute of Technical and Macromolecular Chemistry, RWTH Aachen University

Abstract

Objective: For tissue engineering, there is a need for quantitative methods to map cell density inside three-dimensional (3-D) bioreactors to assess tissue growth over time. The current cell mapping methods in 2-D cultures are based on optical microscopy. However, optical methods fail in 3-D due to increased opacity of the tissue. We present an approach for measuring the density of cells embedded in a hydrogel to generate quantitative maps of cell density in a living, 3-D tissue culture sample.

Methods: Quantification of cell density was obtained by calibrating the ¹H T₂, magnetization transfer (MT) and diffusion- weighted nuclear magnetic resonance (NMR) signals to samples of known cell density. Maps of cell density were generated by weighting NMR images by these parameters post-calibration.

Results: The highest sensitivity weighting arose from MT experiments, which yielded a limit of detection (LOD) of 2.5×10^8 cells/mL/ $\sqrt{\text{Hz}}$ in a 400 MHz (9.4 T) magnet.

Conclusion: This mapping technique provides a noninvasive means of visualizing cell growth within optically opaque bioreactors.

Significance: We anticipate that such readouts of tissue culture growth will provide valuable feedback for controlled cell growth in bioreactors.

Keywords

Tissue engineering; hydrogel; cell growth; bioreactor; cell density; MRI

I. INTRODUCTION

Tissue engineering strives to generate larger, more complex, and increasingly functional substitutes for the clinical purpose of replacing lost or damaged tissue. Additionally, there is a critical need for studying organoid systems in an *ex vivo* setting to determine cell response to controlled stimuli. These approaches typically involve a combination of scaffold, growth factors, and living cells housed in a bioreactor designed to provide suitable culture conditions for tissue growth. Recently, bioreactors have become increasingly specialized by providing dynamic and spatially controlled signals to mimic the dynamic environments present during development [1]–[4]. The tissues and organoids cultured *ex vivo* have increased in size and complexity, to the point where it is difficult to image them with light, such as reloaded decellularized organs and thick bioprinted tissue [5], [6]. More invasive methods such as cross-sectioning and histology are traditionally used to evaluate tissue morphology, but they are not suitable for real-time *in situ* readouts, which are necessary for adaptive control schemes [7].

Noninvasive methods for probing large masses of soft tissue include ultrasonography and magnetic resonance imaging (MRI), two methods that do not require ionizing radiation and can function without contrast agents. In this study, we investigate the utility of contrast agent-free MRI for cell density mapping. MRI provides unparalleled soft tissue contrast in clinical applications, e.g. tumor diagnosis, and has been used to measure hydrodynamic properties of biomaterials [8], [9]. Recent developments in MRI compatible incubators and bioreactors provide additional justification for the development of MRI based, biological measurements [7], [10].

The responses of diffusion-, T_2 -, and magnetization transfer (MT)-weighted nuclear magnetic resonance (NMR) parameters have been used in the past to assess cell density in the context of explanted tumors and brain abscess [11]–[24]. The most popular weighting for generating cell density maps has been diffusion. Correlations were found between the apparent diffusion coefficient (ADC) and cell density in neural [11]–[15], dermal [16], prostatic [17], renal [18], and breast [19], [20] tumors in magnetic fields ranging from 1.5 T to 3 T, but one study by Yoshikawa *et al.* found no correlation between cellularity and ADC in breast cancer at 1.5 T [25]. ADC was found to be correlated to cell density in brain abscess in a 1.5 T magnetic field [21], and using a higher diffusion weighting factor in a 3T field improved the correlation of brain abscess cell density and ADC [22]. MT signal was reported to be inversely correlated to cell density in brain abscess and brain gliomas [23], [24]. T_2 was found to be inversely correlated to cell density in prostate cancer but not gliomas [13], [17], and a study by Roth *et al.* noticed a decreasing trend in T_2 as cells in suspension settled and increased in cell density (significance and goodness-of-fit was not reported) [26]. One study reported a significant positive correlation between T_2 -short and liver cancer cellularity [27]. In all of these previous studies (excluding Roth *et al.*), tumors or abscess were imaged *in vivo* and evaluated for cellularity via histological examination or cell counting.

Despite the large number of studies published to date investigating the MRI response to cell density *in vivo* for clinical applications, only two studies investigated immobilized cell

cultures in contexts of engineered tissue. In those studies, only diffusion-weighted NMR signals were investigated [28], [29]. Data by Pilatus *et al.* showed a strong correlation between cell density and fraction of intracellular volume measured with diffusion-weighted NMR (not spatially-resolved) measurements in cancer cell lines cultured on solid surfaces, including polystyrene beads and collagen sponges [28]. Anderson *et al.* found a correlation between cell volume fraction and apparent diffusion coefficient in images of hydrogel encapsulated cell pellets, but experiments were run at $\sim 20^\circ\text{C}$ [29]. Compared to physiological conditions used for tissue culture, a lower temperature leads to lower diffusion coefficients [30] and higher diffusion weighted signal (Stejskal-Tanner [31]). Surprisingly, whereas many studies reported a correlation between cell density and NMR signal, none of the studies reported limits of detection (LOD) or quantitation. The LOD figure-of-merit, which is a measure of the level of statistical significance, is necessary in order to correctly interpret results from cell density measurements.

To our knowledge, none of these NMR techniques have been applied to cells encapsulated within a hydrogel under physiological temperature conditions for tissue engineering applications. Such a method for mapping cell density in 3D scaffolds would be of paramount importance in tissue engineering. The extent to which MRI can be used in tissue engineering applications is presently unknown.

In this study, we examined T_2 , MT, and diffusion-weighted ^1H NMR signals as a means to quantify cell concentration in an artificially constructed tissue sample composed of mammalian or yeast cells embedded in an agarose hydrogel. T_1 is not discussed herein because we found it to be less sensitive to cell density. Whereas each weighting (T_2 , MT, diffusion) has its own unique set of influencing factors, they are all used for clinical imaging of soft tissues. For example, in living tissues, the T_2 is known to be significantly reduced for water protons that are immobilized, such as in the case of water molecules interacting with or bound to macromolecule surfaces. In MT experiments, a saturation pulse selectively eliminates net longitudinal magnetization in protons bound to macromolecules, and their depleted magnetic state is transferred to free water protons in the vicinity for readout. In T_2 and MT experiments, the signal intensity typically decreases as cell concentration, and consequently macromolecular content, increases. In excised tumor samples the apparent diffusion coefficient of water has been shown to decrease with increasing cell concentration due to reduced motion in protons that are bound to macromolecules or trapped within intracellular compartments.

Nearly all previous work on the topic has been conducted in the context of clinical imaging as a tool to study tumor composition. The situations encountered in tissue engineering represent a different regime where the cell densities are significantly lower than those of excised tissue. Hence, detection limits are very important. We investigate the efficacy of this technology on yeast and human cell lines encapsulated in gels of varying macromolecular content. By generating quantitative analytical calibration curves for measuring cell concentration from NMR signals, this study paves the way for novel methodologies to map local cell densities in 3D bioreactors for tissue engineering applications.

II. MATERIALS AND METHODS

A. Preparation of NMR Samples

1) Cell Culture and Harvest: Prior to taking NMR measurements, HEK 293 cells (ATCC, CRL-11268) were cultured in a 37 °C, 5% CO₂ incubator in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. The temperature and CO₂ concentration were selected to match conditions used in previously established MRI compatible culture systems [7], [10]. Cells were detached from dish surfaces with trypsin and then neutralized with α DMEM containing 10% FBS. Cells were pelleted by centrifugation at 200 g for 5 min, and the remaining media was aspirated, leaving cell pellets. Quantities of pelleted cells were measured volumetrically with a positive displacement micropipette and diluted in DMEM to obtain a range of cell concentrations.

Dry yeast from *Saccharomyces cerevisiae* (Sigma, YSC2-500 G) were reconstituted in phosphate buffered saline (PBS) immediately prior to use. A range of yeast concentrations was obtained by adjusting the weight % of yeast used.

2) Determining Cell Density in a Pellet: The concentration of HEK 293 cells in a pellet was determined by diluting volumes of cell pellet by 400, 600, or 800-fold in DMEM. The number of cells in these dilutions were counted via hemocytometer and used to determine the original cell concentration in the pellet. Yeast cell concentrations were also determined via hemocytometer counting. Average cell sizes were measured from microscope images of suspended cells on the hemocytometer in ImageJ.

3) Cell Encapsulation: Prior to acquiring ¹H spectra, HEK 293 or yeast suspensions were combined in a 1:1 ratio with a liquid solution containing 1.8% low gelling temperature agarose (Sigma, A9045-5G) in PBS at 37 °C. The mixture was immediately added to a 5 mm NMR tube and allowed to gel at room temperature. After gelation, the samples were reheated to 37 °C inside the bore of a Varian 400 MHz (9.4 T) spectrometer equipped with a 10 mm broadband probe and airflow variable temperature (VT) control. This cell encapsulation technique involving the cooling and reheating of cells during agarose gelation is an established procedure known to result in the containment of high viability cells within a gel matrix [32]. The HEK 293 cell densities used for spectroscopy calibrations were 0, 0.7, 1.4, 2.8, 4.2, 5.6, 8.4, 11.3, 16.9, and 22.2 $\times 10^7$ cells/mL, and the yeast cell densities used were 0, 0.2, 0.4, 0.8, 1.6, 2.0, 4.0, 8.0, and 10.0 $\times 10^8$ cells/mL. Each sample was repeated three times using freshly encapsulated cells.

Samples of HEK 293 and yeast cells were prepared for imaging by casting multiple layers of agarose in a 5 mm NMR tube sequentially. Each layer contained a known cell density and was allowed to anneal before the next layer was added. The four cell densities used were 1.4, 2.8, 5.6, and 11.3 $\times 10^7$ cells/mL. Pure agarose was cast above and below the cell laden layers in order to position the cells close to the center of the radio frequency (RF) coil. In the case of yeast imaging, four layers containing 0.63, 1.3, 2.5, and 5.0 $\times 10^8$ cells/mL were cast between layers of acellular agarose. The 5 mm NMR tubes containing layered samples were suspended and imaged in a 40 mm inner diameter RF probe while the temperature was

maintained at 37 °C with heated airflow. A 40 mm probe was used for imaging because it has been shown to be an effective size for accommodating bioreactors and incubation systems [7], [10].

B. NMR Weighting Calibrations

The pulse sequences that were used to calibrate NMR signal to cell density are shown in Fig. 1(a)–(c). T_2 values were obtained from a CPMG experiment (Fig. 1(a)) by integrating the area under the even numbered echoes, normalizing the echo integrals between 0 and 1, and fitting the resulting curve to the exponential decay in (1) for relaxation in the transverse plane:

$$M_{xy}(t) = M_0 e^{-t/T_2} + \beta, \quad (1)$$

where β is a baseline offset, M_{xy} is the transverse magnetization at time t and M_0 is the initial transverse magnetization at $t = 0$.

MT-weighted Z spectra were obtained by applying a train of off-resonance saturation pulses prior to a standard, on-resonance $\pi/2$ (hard) pulse (Fig. 1(b)).

Diffusion-weighted measurements were obtained using a Pulsed Field-Gradient STimulated-Echo (PFG-STE) experiment (Fig. 1(c)). The phase cycling scheme used with this sequence is shown in Table I. The b -value is the diffusion weighting factor:

$$b = \gamma^2 G^2 \delta^2 (\Delta - \delta/3), \quad (2)$$

defined by Stejskal-Tanner [31] in their signal equation

$$\log \left(\frac{S_G}{S_0} \right) = -D\gamma^2 G^2 \delta^2 (\Delta - \delta/3), \quad (3)$$

where D is the diffusion coefficient of water, γ is the gyromagnetic ratio of the ^1H nucleus ($\gamma = 2.675 \times 10^8 \frac{\text{rad}}{\text{s} \cdot \text{T}}$), G is the effective gradient strength of the diffusion weighted gradients measured in T/mm, δ is the duration of a single diffusion weighted gradient in s, and Δ is the diffusion time which is defined as the time between the start of the application of the first diffusion weighting gradient and the start of the second diffusion weighting gradient. S_G stands for signal intensity acquired when using diffusion weighting gradients of effective strength, G . S_0 is the signal intensity acquired without diffusion gradients.

Pulse sequence parameters for all three weightings were chosen to maximize the contrast that results from changes in cell density. Specifically, pulse sequence parameters were arrayed over a range of values, and spectra were acquired at each value in the array. Signal intensities of cell laden gel samples (S) and acellular agarose gel (S_{Ref}) were determined by integrating ^1H peaks from each acquisition. Parameter values that resulted in the largest cell generated signal contrast, defined as $|S - S_{\text{Ref}}|$, per change in cell density were subsequently used for spectroscopy calibrations. The cell densities used for parameter optimization were the same that were used for spectroscopy calibrations. Prior to beginning

MT weighted experiments, the frequency offset of the saturation pulse was optimized. A fifty point array of frequency offset values spaced on a log scale from 0 to 200 MHz was used for optimization (an identical optimization was performed for the range of frequency offsets from 0 to – 200 MHz, but no significant difference was seen in the negative direction). Plots of frequency offset versus signal intensity and frequency offset versus cell generated contrast are displayed in Fig. 2 to highlight the dependence of contrast on frequency offset and show how the optimal parameter value was found. Prior to beginning T_2 weighted spectroscopy calibrations, the echo time (TE) was optimized. TE versus cell generated contrast ($|S - S_{\text{Ref}}|$) was plotted (not shown), and the TE resulting in the largest contrast was selected for calibrations. Because a CPMG sequence was used, signal intensity, S , was found by integrating each echo rather than a full spectrum. A total of 1,500 echoes were acquired at TEs linearly spaced between 0 and 1.4 seconds. Prior to diffusion weighted spectroscopy, both G and δ were optimized to generate the best b -value. The values of δ used were 25, 30, 50, 100, 150, and 200 ms, and at each value of δ , G was arrayed from 0 to 50 Gauss/cm. Spectra acquired at each combination were integrated, and the combination of parameter values resulting in the largest amount of contrast ($|S - S_{\text{Ref}}|$) were used for diffusion weighted calibrations.

NMR signals weighted by T_2 , MT and diffusion parameters vs cell concentration were linearly fit using a weighted least-squares regression. Relevant fitting parameters and their corresponding statistical hypothesis testing probability values (p -values) are reported [33]. The LOD corresponds to the cell concentration at which the weighted NMR signal differs from the acellular agarose NMR signal by exactly three times the average standard deviation:

$$\text{LOD} = \frac{3\bar{\sigma}}{\text{slope}}, \quad (4)$$

where the *slope* is determined from the weighted least-squares regression linear fit and $\bar{\sigma}$ is the average standard deviation of all points used in the corresponding fit.

C. NMR Imaging Protocol

Images of cell density were obtained using a STimulated Echo Multi-Slice (STEMS) sequence modified to generate each of the three types of weightings. T_2 -weighted images were obtained by setting the repetition time (TR) to a long value roughly five times T_1 and the TE to the value optimized in T_2 calibrations. MT-weighted images were generated by applying a train of off-resonance saturation pulses in the beginning of the STEMS sequence, using a long TR, and a short TE. Anti-phase pulse field-gradient pairs were applied after the first and last $\pi/2$ pulses in STEMS to obtain diffusion-weighted contrast. In order to compare the quality of the three weightings over the same scale, each image was normalized between 0 and 1 by applying the following function to every voxel in the image:

$$I_{\text{norm}} = \frac{I - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}}, \quad (5)$$

where I_{norm} is the normalized voxel intensity, I is the pre-normalized voxel intensity, I_{min} is the value of the lowest intensity voxel in the image, and I_{max} is the value of the highest intensity voxel in the image.

Cell density maps were generated from T_2 , MT, and diffusion weighted images using calibration curves relating voxel intensity to cell density. Since images were acquired in a different NMR probe than the one used for spectroscopy measurements, new calibration curves were generated for cell density mapping (not shown). Briefly, regions of interest (ROIs) were drawn within areas of the pre-normalized images containing known cell densities. The same ROIs were used for T_2 , MT, and diffusion weighted images. A calibration curve was fitted to a plot of the mean value of each ROI versus cell density using weighted least-squares regression.

All fits were generated in MATLAB. Exponential fits were generated using MATLAB's nonlinear regression function (fitnlm), which employs the Levenberg-Marquardt algorithm. Linear fits were generated with a nonrobust, weighted linear least squares algorithm (fitlm). The weight of each point was $1/\sigma^2$, where σ is the standard deviation of the measurement.

III. Results

A. HEK 293s

The cell concentration in a pure HEK 293 cell pellet was determined to be $(4.5 \pm 0.9) \times 10^8$ cells/mL. The average diameter of the HEK 293 cells was $12.5 \pm 2.4 \mu\text{m}$. Calibration curves showing the response of T_2 , MT, and diffusion-weighted spectral intensity to cell concentration are seen in Fig. 4. As expected, T_2 -weighted signal decreases with increasing cell concentration. The optimal delay time for generating maximum contrast in T_2 spectroscopy experiments was found to be 140 ms. A single echo with a delay of 140 ms was integrated for each cell concentration to generate the T_2 -weighted calibration curve shown in Fig. 4. The relationship between T_2 -weighted signal and cell density appeared to take the form of an exponential decay. This type of relationship is expected when $1/T_2(C)$ (a function of concentration, C) depends linearly on cell concentration [34]:

$$\frac{1}{T_2}(C) = \frac{1}{T_{2,\text{Ref}}} + \text{const} \times C, \quad (6)$$

where $1/T_{2,\text{Ref}}$ is the transverse relaxation rate in the absence of cells and const is the relaxivity of the cells added to the gel solution. The NMR signal equation for the T_2 -weighted signal acquired at TE ($t = t_E$) is then

$$S = S_0 e^{-\frac{t_E}{T_2(C)}} + \beta = S_0 e^{-\frac{t_E}{T_{2,\text{Ref}}}} e^{-t_E \cdot \text{const} \times C} + \beta. \quad (7)$$

By applying (1) to the reference sample:

$$S_{\text{Ref}} = S_{0,\text{Ref}} e^{-\frac{t_E}{T_{2,\text{Ref}}}} + \beta_{\text{Ref}} \quad (8)$$

we can write our signal contrast as:

$$S - S_{\text{Ref}} = S_0 e^{\frac{-tE}{T_{2,\text{Ref}}}} e^{-tE \cdot \text{const} \times C} + \beta - S_{0,\text{Ref}} e^{-tE/T_{2,\text{Ref}}} + \beta_{\text{Ref}} \quad (9)$$

where β and β_{Ref} are baseline offsets of cell and acellular reference samples, respectively. Because $T_{2,\text{Ref}}$ is independent of cell concentration, we abbreviate $S'_0 = S_0 e^{-tE/T_{2,\text{Ref}}}$ and $\beta' = \beta - S_{0,\text{Ref}} e^{-tE/T_{2,\text{Ref}}} + \beta_{\text{Ref}}$, so that

$$S - S_{\text{Ref}} = S'_0 e^{-tE \cdot \text{const} \times C} + \beta'. \quad (10)$$

Thus, there is an exponential dependence of T_2 weighted contrast on cell density. The baseline offset, β' , can be found from fitting an exponential regression to the data. Correcting for this offset and taking the natural log of both sides leads to a linear relationship between $\log[S(t_E)]$ and C :

$$\log[S - S_{\text{Ref}} - \beta'] = \text{slope} \times C + \text{intercept}, \quad (11)$$

which shows that a plot of the logarithm of baseline corrected signal vs concentration follows a straight line. None of the previous studies on the response of T_2 to cell density report this very simple relationship [13], [17], [26], [27]. After correcting for the baseline offset, β' , which was determined to be 0.0864, the resulting linear fit had a coefficient of determination (R^2) of 0.95. The slope of the fit was found to be -7.1×10^{-9} (p -value, 1.3×10^{-8}). The LOD for the linearized T_2 -weighted spectroscopy calibration curve was determined to be 9.0×10^7 cells/mL. A single CPMG echo-train acquisition took 48.7 s. Thus, the LOD per root bandwidth was 1.1×10^9 cells/mL/ $\sqrt{\text{Hz}}$.

The MT-weighted results also produced an inverse correlation between signal intensity and cell concentration. An offset frequency of 3,500 Hz was found to generate the highest degree of contrast. A single spectrum at this offset was integrated to generate the MT-calibration curve. Given the equation for magnetization transfer ratio (MTR):

$$\text{MTR} = 1 - \frac{S}{S_0} \quad (12)$$

we can express $S - S_{\text{Ref}}$ as:

$$S - S_{\text{Ref}} = S_0 - S_0 \times \text{MTR} - S_{0,\text{Ref}} + S_{0,\text{Ref}} \times \text{MTR}_{\text{Ref}}, \quad (13)$$

where S and S_{Ref} are the signal intensities of cell laden samples and the acellular reference sample following the application of an off-resonance saturation pulse, S_0 and $S_{0,\text{Ref}}$ are the signal intensities of cell laden samples and the acellular reference sample following the application of an off-resonance saturation pulse, S_0 and $S_{0,\text{Ref}}$ are the signal intensities of the cell and reference samples without an off resonance pulse, and MTR_{Ref} is the MT ratio of the reference sample at the same frequency offset used for cell samples. Combining this with the established positive linear dependence of MTR on cell concentration ($\text{MTR}(C) = \text{MTR}_{\text{Ref}} + \text{const} \times C$) [23], [24], we can determine the dependence of $S - S_{\text{Ref}}$ on C :

$$S - S_{\text{Ref}} = S_0 - S_0 \times (\text{MTR}_{\text{Ref}} + \text{const} \times C) - S_{0, \text{Ref}} + S_{0, \text{Ref}} \times \text{MTR}_{\text{Ref}}, \quad (14)$$

which can be simplified to the linear dependence:

$$S - S_{\text{Ref}} = -S_0 \cdot \text{const} \times C + S'_0, \quad (15)$$

where *const* is a positive real number and S'_0 is used to lump all terms on which there is no concentration dependence:

$$S'_0 = S_0 \times (1 - \text{MTR}_{\text{Ref}}) - S_{0, \text{Ref}} \times (1 + \text{MTR}_{\text{Ref}}).$$

The fitted data were linear ($R^2 = 0.97$), matching the model. The fitted slope was equal to 1.5×10^{-9} ($p = 2.7 \times 10^{-10}$). The MT weighted LOD was determined to be 2.7×10^7 cells/mL. Given a single scan time of 28.3 s (including a delay to account for T_1 relaxation) LOD per root bandwidth is 2.5×10^8 cells/mL/ $\sqrt{\text{Hz}}$.

Diffusion-weighted signal intensity appeared to be exponentially correlated with cell density. Given the inverse linear dependence of ADC on cell density [16]–[18], [20]–[22], which can be stated as

$$D(C) = D_{\text{Ref}} - \text{const} \times C, \quad (16)$$

and the Stejskal-Tanner equation (3), the relationship between diffusion weighted signal and cell concentration becomes

$$S_G = S_0 e^{-(D_{\text{Ref}} - \text{const} \times C) \cdot b}. \quad (17)$$

Subtracting the reference and simplifying leads to

$$S_G - S_{G, \text{ref}} = S_0 e^{-D_{\text{ref}} \cdot b} e^{b \cdot \text{const} \times C} - S_{0, \text{ref}} e^{-D_{\text{ref}} \cdot b} = D_0 e^{b \cdot \text{const} \times C} + \beta, \quad (18)$$

where $D_0 = S_0 e^{-D_{\text{Ref}} \cdot b}$, $\beta = -S_{0, \text{Ref}} e^{-D_{\text{Ref}} \cdot b}$, and *const* is a positive real number. This can be linearized by correcting for the offset and taking the natural log of both sides:

$$\log[S_G - S_{G, \text{Ref}} - \beta] = \text{slope} \times C + \text{intercept}. \quad (19)$$

A *b*-value of 3,500 s/mm² and diffusion time of 0.10 s were found to generate the highest degree of contrast in diffusion-weighted NMR experiments. Prior to linearization, the offset was determined from a weighted, least-squares exponential regression to be -0.0160 . The linearized diffusion data had a fitted slope of 4.9×10^{-9} ($p = 1.1 \times 10^{-11}$), ($R^2 = 0.99$). The LOD was determined to be 2.1×10^7 cells/mL. The phase cycling scheme used in these diffusion-weighted measurements, resulted in a longer acquisition time of 104 s. The associated LOD per root bandwidth is 3.7×10^8 cells/mL/ $\sqrt{\text{Hz}}$.

Images of NMR tubes containing distinct bands of known cell densities in agarose gels are shown in Fig. 5. The bands contain cell densities of 1.4, 2.8, 5.6, and 11.3×10^7 cells/mL. A TE of 130 ms and TR of 25 s were used for T_2 -weighted imaging due to a slight improvement in image quality over the 140 ms delay that had been optimized in spectroscopy calibrations. The 3,500 Hz saturation offset value obtained from spectroscopy optimization experiments was used for MT-weighted imaging. The parameters optimized in diffusion weighted spectroscopy were found to be inadequate for diffusion-weighted imaging. The parameters did not work for imaging because the diffusion weighted signal per voxel was too weak at the given b -value. Instead, a diffusion time of 20 ms and b -value of 800 s/mm^2 were found to generate sufficient signal while retaining enough contrast between layers. Reducing the b -value further improved signal intensity, but lessened the degree of contrast between the layers.

T_2 and MT-weighted images were able to resolve the four different cell density gels distinctly from each other and from the background agarose signal. The diffusion-weighted images were able to resolve only the two highest cell concentrations, but the lower cell concentration layers could not be distinguished from the background. It can be seen that the diffusion-weighted spectra, especially at the lower end of the cell concentration range, have a low signal-to-noise ratio (Fig. 6). These spectra were generated from the entire sample volume in the RF region in the NMR probe. The HEK 293 cell density images were acquired with a resolution of 128×128 pixels (images shown in Fig. 5 are cropped) and are of 2 mm-thick slices. The total volume per voxel was only 0.043 mm^3 , which does not generate enough signal to be detected at low cell concentrations.

B. Yeast

Yeast measurements show that similar correlations of signal intensity are observed for cell types across kingdoms (Fig. 7). It was determined that a 1 % (w/w) yeast solution corresponds to $(2.0 \pm 0.7) \times 10^8$ cells/mL. The average diameter of the yeast cells was $5.2 \pm 0.9 \text{ }\mu\text{m}$. The exponential offset was found to be 0.0247. The linearized T_2 -weighted slope was found to be -7.2×10^{-10} ($p = 1.8 \times 10^{-9}$) ($R^2 = 0.98$), and the LOD per root bandwidth was 3.3×10^9 cells/mL/ $\sqrt{\text{Hz}}$.

The linear MT fit generated a slope of -1.8×10^{-10} ($p = 5.4 \times 10^{-13}$) ($R^2 = 0.997$). The corresponding MT LOD was 3.8×10^9 cells/mL/ $\sqrt{\text{Hz}}$.

The linearized diffusion fit generated a slope of 2.2×10^{-9} ($p = 5.0 \times 10^{-11}$) ($R^2 = 0.994$). The offset from the exponential fit was -3.6×10^{-4} . The associated diffusion LOD per root bandwidth was 2.7×10^9 cells/mL/ $\sqrt{\text{Hz}}$.

Images of low gel temperature agarose gels containing bands of various yeast concentration were acquired (Fig. 8). The MT weighted image produces the best results in which all four layers are distinct and distinguishable from the background. The lower cell concentrations in the T_2 -weighted image blend together, but all layers are distinct from the background. In the diffusion-weighted image, the cell laden bands show an increase in signal from the

background, but the signal to noise ratio is much lower than in the T_2 and MT weighted images.

IV. DISCUSSION

T_2 and MT weighted signals were found to decrease in response to increasing cell density whereas the diffusion weighted signal increased with increasing cell density. The decrease in T_2 and MT weighted signals with increasing cell concentration can be attributed to the increase in macromolecular content as cell density increases. Macromolecular content increases with increasing cell density since the intracellular environment is densely packed with macromolecules at a much higher concentration than the surrounding agarose gel environment used in this study [35].

T_2 is affected by mobility. Water molecules interacting with macromolecules are less mobile and experience reduced relaxation times. Therefore, as macromolecular concentration increases, the transverse relaxation time is reduced for an increasing fraction of water molecules in the sample. The reduced relaxation time results in less signal at TE (hence, weaker T_2 -weighted signal).

MT is a direct measure of macromolecular content through the selective saturation of hydrogen nuclei in water molecules interacting with macromolecules prior to a standard 90° excitation pulse. [34] Since macromolecular content increases with cell density, the fraction of water interacting with macromolecules also increases leading to more signal suppression due to higher numbers of nuclei being selectively saturated.

The slopes of the T_2 and MT fits are 9.9 and 8.3 times larger for HEK 293 cells than yeast cells, respectively. These curves are calibrated to cell number, but macromolecular content scales differently for each cell type. HEK 293 cells were measured to be 13.6 times the volume of our yeast cells using light microscopy. Given estimates of 23% and 35% macromolecular content for HEK 293 and yeast cells, respectively [36], [37], HEK 293 cells have about 8.9 times the macromolecular content per cell, which closely corresponds to the difference in slopes for both of these weightings.

Our diffusion data are consistent with many studies that have shown the diffusion-weighted signal to be linearly proportional to cell density across several tissue types. [11]–[22] The increase in diffusion weighted signal is due to a decrease in diffusivity according to (3). The decrease in water's diffusivity is a result of restriction of molecular motion. A fraction of intracellular water experiences restricted motion due to the presence of cell membranes, which act as barriers, reducing water transport. As cell density increases, the fraction of restricted water scales proportionally for a given cell type, but the fraction of restricted water is not identical to the fraction of intracellular water in the total sample volume. Given their sizes and water content estimates of 77% and 65% for HEK 293 and yeast cells respectively [36], [37], HEK 293 cells have about 16 times as much total intracellular water content as yeast. However, the slope of the HEK 293 calibration plot is larger than the slope of the yeast calibration plot by a factor of two, suggesting that per cell, twice as much water experiences restricted diffusion in HEK 293s versus yeast. This is because the restriction of

intracellular water motion due to membranes only affects a fraction of intracellular water. [26] Intracellular water that is far away from membranes will have a higher diffusivity than water that is close to cell boundaries. Since the yeast diameter is about 42% of the HEK 293 diameter, a larger fraction of the intracellular water in yeast will experience restricted motion due to membrane proximity.

Compared to cell density correlations reported in previous studies, the correlations here had less variability and better coefficients of determination [11]–[24], [26], [28], [29]. This can be attributed to the fact that a more controlled environment is maintained when cells are encapsulated in hydrogels for tissue engineering than when tumors and abscess are formed *in vivo* with variations in their extracellular matrix composition and cell type. In this study we used NMR tubes to contain cell laden gels, but the imaging technique can be applied to any MRI compatible bioreactor as long as perfusion is halted during acquisition

V. CONCLUSION

In this study, we generated maps of cell density in a hydrogel scaffold using T_2 , MT, and diffusion-weighted MRI. We have verified that these three weightings have a clear functional dependence on cell concentration and can be calibrated to measure exact cell densities in thick or opaque media. This is especially useful for monitoring cultures in bioreactors in which local cell density evolves over time due to proliferation and migration.

We found the three weightings to have comparable detection limits when using NMR spectroscopy pulse sequences. However, when imaging lower cell concentrations, the diffusion-weighted signal is too weak compared to T_2 and MT-weighted signals, in terms of resolving cell concentrations below 5.6×10^7 cells/mL within a reasonable time. Our results suggest that despite the popularity of ADC mapping in clinical settings, T_2 and MT-weighted images may be more suitable for tissue engineering applications in which lower cell concentrations are present. The higher variability of T_2 measurements, both in spectroscopy and imaging experiments suggests that MT is the better option for probing small changes in cell concentration, such as the regime of low cell concentrations.

In addition to using this cell imaging technique for studying cell growth and spatial distribution in 3D tissue culture models, the noninvasive aspect of this readout means that it can be applied to the real-time control and optimization of cell growth in bioreactor systems employing adaptive methods to control spatiotemporal patterns of mechanical and chemical stresses. T_2 and MT-weighted MRI can provide useful cell density maps when noninvasive readouts in thick tissue cultures are needed.

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Fig 1. (a)-(c) Timing diagrams for NMR experiments.

Diagrams of NMR pulse sequences used to calibrate ^1H NMR spectra or echo intensity to cell density. Portions of the sequence bracketed in red were repeated n times. (a) A Carr-Purcell-Meiboom-Gill (CPMG) sequence was used to provide T_2 -weighted signals. The number of echoes collected was 2,400. (b) Pulsed, off-resonance irradiation MT sequence provides MT weighting. 400 Gauss pulses were used. (c) A PFG-STE sequence was used to provide diffusion-weighted signals.

Fig. 2. MT Optimization.

(a) MT Z spectra of agarose samples containing range of cell concentrations. S represents the normalized intensity of the signal obtained by integrating the weighted NMR spectrum. (b) MT contrast curves obtained by subtracting an acellular agarose gel sample MT curve from curves of samples containing cells. The difference, $S - S_{\text{ref}}$, represents the signal contrast generated by the presence of cells. (S_{ref}) is the signal intensity of the acellular agarose sample. The frequency offset resulting in the largest signal change in response to changes in cell density was later used for imaging experiments.

Fig. 3. Stimulated Echo Multi-Slice Sequence.

Diagram of a STEMS pulse sequence with optional modifications corresponding to respective weighting. Diffusion weighted (D.W.) gradients are highlighted and bracketed in brown. A pulsed, off-resonance, Gaussian saturation pulse used to generate MT weighted images is bracketed in magenta. Red brackets refer to a portion of the pulse sequence repeated n times, which in this case is the off resonance saturation pulse used for MT weighted imaging. The off resonance saturation pulse for MT was calibrated to 540° (3π). Slice select (S.S.) gradients are highlighted in blue, frequency encoding (F.E.) in green, spoiler gradients in cyan, and phase encode (P.E.) in white.

Fig. 4. Cell Density Calibrations.

From top to bottom: T_2 -weighted, MT-weighted, and diffusion-weighted calibration curves of NMR spectra intensity to HEK 293 cell density. Each point represents the signal generated by agarose alone subtracted from S_{Ref} , the signal generated by a given cell concentration. Error bars represent one standard deviation ($n = 3$).

Fig. 5. HEK 293 Cell Density MRIs.

(a) From left to right: T_2 , MT, and diffusion-weighted MRIs of an NMR tube containing stratified layers of varied HEK 293 cell densities in agarose gels. The fourth image on the far right is a photograph showing optical opacity of the layers. Bottom and top of the tube are filled with acellular agarose gel mixed with DMEM. The phenol red in DMEM gives it a pink color. Four layers from top to bottom contain cell concentrations of 1.4, 2.8, 5.6, and 11.3×10^7 cells/mL. The MRI signal throughout the region is normalized between 0 and 1. The curvature between layers is due to the menisci that form during the gel casting process

in a 4.2 mm inner diameter NMR tube (Wilmad 513-7PP-7). Each layer was cast and annealed sequentially, allowing it to form and retain its meniscus as the agarose annealed.

(b) Cell density maps calculated from T_2 , MT, and diffusion-weighted MRIs using calibration curves relating voxel intensity to cell density.

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Fig. 6. Diffusion Weighted ¹H Spectra.

Several example spectra acquired with a PFG-STE spectroscopy sequence

Fig. 7. Yeast Cell Density Calibrations.

From top to bottom: T_2 , MT, and diffusion-weighted calibration curves of NMR spectra intensity to yeast cell density. Each point represents the signal generated by agarose alone subtracted from S_{Ref} , the signal generated by a given cell concentration. The T_2 and diffusion data were corrected for their respective offsets prior to linearization. Error bars represent one standard deviation ($n = 3$).

Fig. 8. Yeast Images.

(a) T_2 , MT, and diffusion-weighted MRIs of an NMR tube containing stratified layers of various yeast cell densities in 0.6% agarose gel. A reference photograph of the tube is included. Four layers from top to bottom contain yeast cell concentrations of 0.63, 1.3, 2.5, and 5.0×10^8 cells/mL. (b) Cell density maps calculated from weighted images.

TABLE I

PHASE CYCLING SCHEME FOR PFG-STE PULSE SEQUENCE

Pulse	Phase Cycling
1. $\pi/2$	$[0, \pi/2, 0, \pi/2]$
2. $\pi/2$	$[0, 0, \pi/2, \pi/2]$
3. $\pi/2$	$[0, 0, 0, 0]$
Receiver	$[0, \pi/2, \pi/2, 0]$

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