Mapping Cell Distribution in Amorphous Scaffolds using Confocal Microscopy

Jirun Sun, Nancy J. Lin, Marcus T. Cicerone, and Sheng Lin-Gibson

Polymers Division, National Institute of Standards and Technology, Gaithersburg, MD 20899-8543

INTRODUCTION

One common tissue engineering (TE) approach for regenerating or replacing damaged tissues involves a porous polymeric scaffold that serves as the mechanical framework for cell attachment and proliferation.[1] Cell-material interactions play a vital role in the structure and function of a scaffold and are affected by chemical and physical parameters, as well as the micro-environment surrounding the cells. To reduce the complexity of the cell-material interactions, studies typically have been performed using 2-dimensional (2D) substrates. Consequently, most bioassays and characterization methods have also been developed for the 2D configuration. However, recent work has not been able to demonstrate a direct correlation between results obtained in 2D and in 3-dimensional (3D) models. Several hypotheses for this lack of correlation have been suggested, but experimental methods for characterizing the cell-material interactions in 3D remain elusive, thus making testing these hypotheses difficult.

One technique to evaluate cell-material interactions is to visualize the cells directly on the materials. For 3D scaffolds, confocal laser scanning microscopy is an ideal technique since it is designed to image within a structure rather than only on the surface. However, common synthetic polymers used for preparing scaffolds, including polycaprolactone, poly(lactic acid) and their copolymers, have a maximum imaging depth of only approximately 100 μm. This is insufficient to evaluate the cell distribution in a TE scaffold that may be 1 mm thick or greater. The primary reason for this depth limitation is the scattering that occurs in these semi-crystalline thermoplastics with relatively low glass transition temperatures. The crystalline characteristic is necessary to achieve the required mechanical integrity for supporting tissue regeneration but also results in significant light scattering within the scaffold.

We have recently developed tissue engineering scaffolds based on photopolymerizable amorphous polymers for deep optical imaging.[1,2] These scaffolds are well suited for visible and near-infrared microscopy since they have no crystallinity and therefore minimal scattering. In addition, they exhibit essentially no autofluorescence, and they do not adsorb common fluorescent cell stains. Other advantages of the photopolymerized salt-leaching process include a relatively easy fabrication procedure, absence of toxic solvent, and the ability to tailor the mechanical properties and scaffold architectures to meet specific applications. They also have been shown to promote cell adhesion and growth and are hydrolytically stable over several weeks in cell growth media. The latter property is an important factor for long-term in vitro studies of cell penetration and proliferation.

In this study, we develop quantitative methods for characterizing cell-material interactions using confocal microscopy on optically clear scaffolds. For the first set of parameters, we aim to quantify the cell number and distribution within a 3D scaffold of known structure. Detailed methods for sampling, validation, and quantification will be presented.

METHODS[†]

Reagents. Ethoxylated bisphenol-A dimethacrylate (EBPADMA, degree of ethoxylation $\approx$ 6), was obtained from Esstech Inc. The photoinitiator system of camphorquinone (CQ) and ethyl 4-N,N-dimethylaminobenzoate (4E) was purchased from Aldrich Corp. All reagents were used as received. The resin was activated with a redox photoinitiator system consisting of 0.2 % CQ and 0.8 % 4E (by mass) and stored in the dark until use. Sodium chloride crystals were ground into smaller particles using a mortar and pestle and then separated into defined size ranges (300 μm to 350 μm) using brass sieves (45 mesh to 50 mesh).

Fabrication of EBPADMA tissue engineering scaffolds. Scaffolds were prepared using procedures described in detail elsewhere. Briefly, activated EBPADMA was blended with sieved salt crystals (86 % by mass), packed into a Teflon mold (3.5 mm thick), pressed together between two glass plates, and cured for 5 min per side using a tungsten halogen light (250 W, 120 V). After irradiation, all samples were postcured at 100 $^{\circ}$C for 1 h. The composite samples were soaked in de-ionized water for 5 d with multiple changes of the water to dissolve the salt porogen and leave a porous scaffold (diameter = 8 mm).

Cell seeding and staining of EBPADMA scaffolds. The MC3T3-E1 subclone 4 murine pre-osteoblast cell line was purchased from the American Type Culture Collection. Scaffolds were sterilized using ethylene oxide and hydrated using an ethanol series. Cells of passage 4 to 10 were seeded drop-wise onto the scaffolds at densities of (1 x 10^5 and 5 x 10^5) cells per scaffold. At each time point, the appropriate scaffolds were fixed using 3.7 % (by volume) formaldehyde in phosphate buffered saline (PBS) for 20 min. The cells were then permeablized with 0.5 % (by volume) Triton X-100 for 15 min, blocked with 10 mg/mL bovine serum albumin in PBS, and rinsed with PBS. The cell nuclei were stained with Sytox Green at a 1:5000 dilution in PBS for 30 min, and the actin cytoskeleton was stained for 2 h with Alexa Fluor 546-Phalloidin (Invitrogen) diluted 40-fold in PBS. Samples were rinsed and imaged in PBS.

Confocal Microscopy. Fluorescently labeled polystyrene beads having an average diameter of 10 μm and absorbance/emission wavelengths of 505/515 nm were purchased from Invitrogen. The beads were embedded in the scaffolds by gently shaking the scaffolds in a bead solution (3.6 X 10^5 beads/mL) overnight.

Confocal optical microscopy analysis of the cultured and stained scaffolds was carried out using a Zeiss LSM 510 confocal laser scanning microscope with 1 Airy-disk-unit pinholes in reflectance mode. 5X and 10X magnification objectives were used to cover a broad field of view ($\approx$ 3.2 mm^2 and 0.8 mm^2 for the 5X and 10X objectives, respectively) and encompassed several pores in a single image. A water immersion 40X objective was used to evaluate the cell morphology. Scaffold structure was imaged using reflection of a 488 nm laser. Nuclei stained with Sytox Green were visualized using the 488 laser and a bandpass emission filter (505 nm to 550 nm). Cytoskeletal actin labeled with phalloidin was imaged using a 555 nm laser line and a 560 nm long pass emission filter.

The ImagePro Plus image software was used to quantify the number of nuclei per image. Tiled images were imported into the software and the total nuclei were counted. The standard uncertainty associated with these measurements is less than 5 %.

RESULTS AND DISCUSSION

Two control experiments were carried out to optimize the experimental conditions and to validate the ability of confocal microscopy to evaluate cells on scaffolds. First, to optimize the measurement configuration of the confocal microscope, cells cultured on a 2D surface (tissue culture polystyrene, TCPS) were examined (Figure 1). Using these samples, the lasers, filters, and other optical parameters were adjusted to optimize image quality. The configuration was stored and applied in the subsequent imaging of the scaffolds, with variation only in the laser output and offset gain. A second set of experiments was performed to calibrate the 10X air and 40X water immersion objectives using fluorescently labeled polystyrene beads that were dispersed within the scaffold. The bead diameter measured in the x-y plane at different depths was the same as the value provided by the vendor (10 μm) and was consistent throughout the segments of the z-axis probed. Beads captured with a 40X water immersion objective are shown in Figure 1.

[†] Certain equipment, instruments or materials are identified in this paper in order to adequately specify the experimental details. Such identification does not imply recommendation by the National Institute of Standards and Technology nor does it imply the materials are necessarily the best available for the purpose.
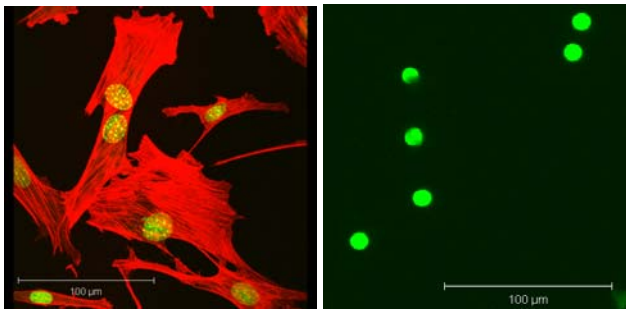
Figure 1. Left: Cells on a TCPS surface imaged using a 40X water immersion objective. Red indicates actin filaments labeled by Alexa Fluor 546-phalloidin, and green indicates nuclei stained by Sytox Green. Right: Fluorescently labeled polystyrene beads imaged using a 40X water immersion objective.

The purpose of this study was to establish quantitative methods to determine and describe the spatial distribution of cells in 3D scaffolds using confocal microscopy. Using the amorphous scaffolds, it was possible to image cells from the surface to approximately 1 mm into the scaffold. Two different imaging methods were used to obtain the spatial distribution of the cells with this capability.

The first approach combined multiple x-y images collected using the low magnification objective at adjacent locations to determine the cell distribution in the x-y plane over the entire scaffold. The information gathered by this approach is restricted to one 2D slice. Figure 2 (left) illustrates a tile image of the entire scaffold surface consisting of 36 images (6x6 images obtained using a 5x objective). To determine the spatial distribution as a function of depth, this procedure was repeated at different sample depths by lowering the objective in 100 μm intervals to approximately 1 mm below the top surface of the scaffold. By turning the scaffold upside down, the cell distribution on the other side of the scaffold could also be obtained. The depth was the traveling distance of the objective and was longer than the actual distance by a factor $\approx$ 1.35 (refractive index of water). For a 3.5-mm thick scaffold, this approach is limited to collecting data from the two surfaces. Sectioning the scaffold into two halves and scanning the newly exposed surface (Figure 2, right) provides the information needed to calculate the relative cell distribution from top to bottom. Using the tile technique to image the surface and the interior allows for the cell distribution over the entire scaffold to be deduced.
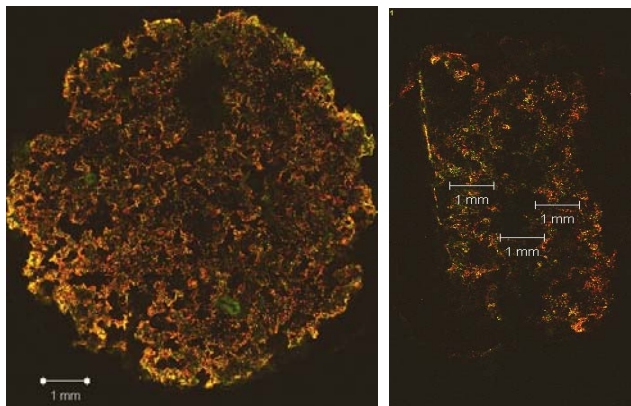

Figure 2. Tile images of cells on scaffolds, from the top surface (left image, an output of 6X6=36 images) and from the cut surface in the center (right image, an output of 3x6=18 images).

Images of the cell nuclei collected using the tile technique were quantified to determine the cell number as a function of depth. The results for a representative scaffold seeded with 500,000 cells and cultured for 24 h are shown in Figure 3. The cell number decreased significantly as a function of depth, demonstrating that the cell distribution is not uniform at 24 h. Our studies have shown that the

initial cell distribution depends on the seeding protocol and the number of cells seeded.
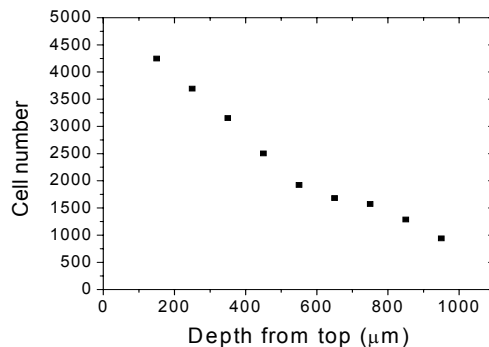

Figure 3. Number of cells / 50 mm^2 as a function of depth in a representative scaffold. The depth is the traveling distance of the objective from the top of the scaffold.

The second approach determines the cell number, density, and morphology by collecting and analyzing a series of precisely positioned stacks of 2D images. This approach provides spatially rich information, particularly with regard to the cell-cell interactions. Two sample images are illustrated in Figure 4. By sampling a series of 3D volumes at specific locations on the scaffold, it is possible to obtain the spatial distribution of the cells over the entire scaffold.
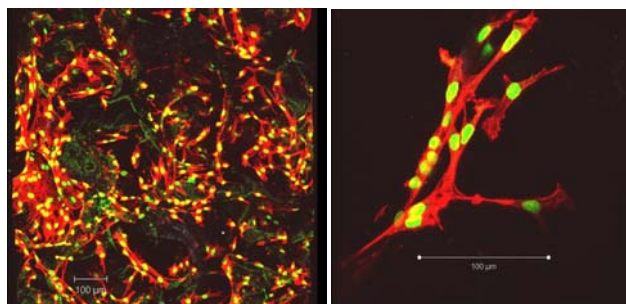

Figure 4. Left: Projection view of 137 2D images (captured using a 10X objective) with an interval of 5.27 µm; Right: Projection view of 215 2D images (captured using a 40X water immersion objective) with an interval of 0.75 µm between images.

CONCLUSIONS

An amorphous, biocompatible dimethacrylate scaffold affords method development for deep optical microscopy. In 3D space, cell number and spatial distribution are key to the scaffold performance. Two methods for quantitatively obtaining cell spatial distribution using these scaffolds. One of these method is based on collecting 2D slices of the entire scaffold as a function of depth, and the other involves sampling multiple 3D volumes over various locations on the scaffold. Detailed comparison between results obtained by these two methods will be presented.

REFERENCES
1. Landis, F.A.; Stephens, J.S.; Cooper, J.A; Cicerone, M.T.; Lin-Gibson, S. *Biomacromolecules* **2006**, *7(6),* 1751.
2. Lin-Gibson, S.; Cooper, J.A.; Landis, F.A., Cicerone, M.T. *Biomacromolecules*, **2007**, DOI: 10.1021/bm061139q, in press.