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Functional ultrasound imaging for assessment of extracellular matrix scaffolds used for liver organoid formation

Ryan C. Gessner [a], Ariel D. Hanson [a,b,1], Steven Feingold [a,1], Avery T. Cashion [a], Ana Corcimaru [b], Bryant T. Wu [a,b], Christopher R. Mullins [e], Stephen R. Aylward [e], Lola M. Reid [b,c,d,**,2], Paul A. Dayton [a,d,*,2]

[a] UNC/NCSU Joint Department of Biomedical Engineering, UNC School of Medicine, Chapel Hill, NC 27599, USA
[b] Department of Cell Biology and Physiology, UNC School of Medicine, Chapel Hill, NC 27599, USA
[c] Program in Molecular Biology and Biotechnology, UNC School of Medicine, Chapel Hill, NC 27599, USA
[d] Lineberger Cancer Center, UNC School of Medicine, Chapel Hill, NC 27599, USA
[e] Kitware, Inc., 101 East Weaver St, Carrboro, NC 27510, USA

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ABSTRACT

A method of 3D functional ultrasound imaging has been developed to enable non-destructive assessment of extracellular matrix scaffolds that have been prepared by decellularization protocols and are intended for recellularization to create organoids. A major challenge in organ decellularization is retaining patent micro-vascular structures crucial for nutrient access and functionality of organoids. The imaging method described here provides statistical distributions of flow rates throughout the tissue volumes, 3D vessel network architecture visualization, characterization of microvessel volumes and sizes, and delineation of matrix from vascular circuits. The imaging protocol was tested on matrix scaffolds that are tissue-specific, but not species-specific, matrix extracts, prepared by a process that preserved >98% of the collagens, collagen-associated matrix components, and matrix-bound growth factors and cytokines. Image-derived data are discussed with respect to assessment of scaffolds followed by proof-of-concept studies in organoid establishment using Hep3B, a human hepatoblast-like cell line. Histology showed that the cells attached to scaffolds with patent vasculature within minutes, achieved engraftment at near 100%, expressed liver-specific functions within 24 h, and yielded evidence of proliferation and increasing differentiation of cells throughout the two weeks of culture studies. This imaging method should prove valuable in analyses of such matrix scaffolds.

1. Introduction

Liver transplantation is the primary treatment for end-stage liver disease [1]. Currently, more than 16,000 adults and children are in need of liver transplants. Unfortunately, the number of livers available for transplantation are in short supply [2]. An alternative to organ transplantation is to support patients using an extracorporeal liver-assist device (LAD). A LAD is a bioreactor comprised of liver cells harvested from donor livers and incorporated into a network of hollow dialysis fibers that mimic blood vessels, which can connect to the patient and thus serve as a bioartificial liver [3,4]. Such bioartificial liver devices provide temporary relief for one to two weeks or until an organ is available for transplantation. They cannot be used longer, since liver cells seeded into all extant forms of bioreactors attach and deposit extracellular matrix and other cellular components onto the hollow fibers, causing “fouling” or clogging of the fibers’ pores, limiting the life span of the device [5].

A more robust alternative is to develop human liver organoids that can be incorporated into a LAD to enable hemodialysis; this provides a more stable and fully functional bioartificial liver in which vascular channels are provided by the native extracellular

* Corresponding author. UNC/NCSU Joint Department of Biomedical Engineering, Lineberger Cancer Center, Chapel Hill, NC 27599-6136, USA.
** Corresponding author. Department of Cell Biology and Physiology, Program in Molecular Biology and Biotechnology, Lineberger Cancer Center, Chapel Hill, NC 27599-7038, USA.
E-mail addresses: gessner.ryan@gmail.com (R.C. Gessner), adhanson@email.unc.edu (A.D. Hanson), sgfeingold@gmail.com (S. Feingold), zackcashion@gmail.com (A.T. Cashion), acorcimaru@gmail.com (A. Corcimaru), btwu10@gmail.com (B.T. Wu), christopher.mullins@kitware.com (C.R. Mullins), stephen.aylward@kitware.com (S.R. Aylward), Lola.M.Reid@gmail.com (L.M. Reid), padayton@bme.unc.edu (P.A. Dayton).
[1] Co-equal second authors.
[2] Co-equal senior authors.

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matrix components lined by endothelia. “Biomatrix scaffolds”, herein referred to as matrix scaffolds, are a particularly rich form of extracellular matrix extracted from organs or tissue and derived from gentle delipidation and perfusion of high salt buffers to keep all collagens and their associated factors insoluble [15]. The organoids can be formed by preparing matrix scaffolds from decellularized livers and then recellularizing the scaffolds with human cells. These matrix scaffolds can be recellularized in two stages: first, the vascular channels are recellularized with endothelia through which medium and then blood can be perfused; and second, the rest of the matrix is recellularized with a combination of hepatic and mesenchymal stem cell populations that will mature into fully functional liver parenchymal cells along with their mesenchymal cell partners. Current efforts are making use of human hepatic cell lines for human liver organoid formation to establish optimal recellularization protocols. Successful protocols will then be used with freshly isolated human hepatic and mesenchymal stem cell populations and endothelia.

For recellularized scaffolds to yield a human liver organoid able to support patients as a bioartificial liver, cell functions must be comparable to those of normal human livers. Since cell seeding and organoid functionality are directly related to the patency and structure of micro-vascular matrix remnants in the scaffold, there is a crucial need for non-destructive assessment of the structural characteristics of the scaffold, particularly its vascular matrix. Without adequate perfusion, the process of reseeding matrix scaffolds with new cells cannot be accomplished, since this process relies on fluid transport through the matrix remnants of the vascular bed for the delivery of the cells. Also, after cells have been engrafted throughout the scaffolds, their continued functions depend on a long-term delivery of nutrients and oxygen. For this reason, a method to image both the anatomy and flow within the sample in a non-destructive manner is highly desirable.

There are many methods currently employed to image tissue scaffolds, including scanning and transmission electron microscopy (SEM and TEM), optical microscopy [6], magnetic resonance (MR) imaging and microscopy [7], computed tomography (CT) [8], optical coherence tomography (OCT) [9], and Doppler ultrasound [10]. The selection of any one modality will always yield inherent tradeoffs such as cost, invasiveness to the sample, field of view, resolution, acquisition time, and type of information gleaned. From this list, the imaging modalities that can non-invasively image a 3D scaffold with a significant thickness are MR, CT, and ultrasound. MR and CT are widely available in both clinical and research contexts. These modalities have the best field of view, although they require expensive hardware (particularly MR imaging). MR can also require long image acquisition times. On the other hand, CT suffers from poor soft-tissue contrast and can cause radiation damage to cells. Ultrasound has many benefits over MR and CT in that it is real-time, relatively inexpensive, non-invasive, does not use ionizing radiation, and has excellent soft-tissue contrast. In addition, ultrasound is able to assess multiple different qualities of a tissue volume (applicable to both *in vivo* volumes and *in vitro* matrix scaffolds), including tissue structure with standard b-mode [11], mechanical stiffness [12], microvascular perfusion architecture [13], and parametric perfusion rate [14]. One possible challenge hindering ultrasound’s utility for scaffold perfusion assessment to date has likely been the modality’s limited field of view, allowing for freehand visualization of different 2D slices, or small 3D sub-volumes, but traditionally not visualization or quantitation of a large field of view. Our objective in this study was to explore the application of ultrasound to perform 3D visualization and quantification of perfusion throughout a matrix scaffold.

In these studies, we have developed a protocol to enable detailed assessment of vascular structural and functional characteristics within scaffolds in a non-destructive manner. We had two objectives: the first was to explore the application of ultrasound to perform 3D visualization and quantification of perfusion throughout an extracellular matrix scaffold; the second was to demonstrate, using a hepatoblast-like cell line, that the imaging assessments can identify scaffolds that will be successful for creating human liver organoids.

2. Materials and methods

2.1. Decellularization of rat livers

Wistar rats (weights 250–300 g) were obtained from Charles River Laboratories, Wilmington, MA, and housed in animal facilities handled by the University of North Carolina (UNC) Division of Laboratory Animal Management. They were fed ad libitum until used for experiments. All experimental work was approved by and performed in accordance with the UNC Institutional Animal Use and Care Committee guidelines.

The protocol for decellularizing livers to produce matrix scaffolds has been described previously [15]. Images of tissue in the process of decellularization are given in the online supplement Fig. S1, and results using this protocol are compared to results using other decellularization protocols (Table S1). Male rats were anesthetized with Ketamine-Xylazine, and their abdominal cavity opened. The portal vein was cannulated with a 20-gauge catheter to provide a perfusion inlet to the vasculature of the liver, and the vena cava was transected to provide an outlet for perfusion. The liver was removed from the abdominal cavity and placed in a perfusion bioreactor. The blood was removed by flushing the liver with 300 ml of serum-free DMEM/F12 (Gibco, Grand Island, NY). A delipidation buffer, comprised of 36 U/L of phospholipase A2 in 1% sodium deoxycholate (Fisher, Pittsburgh, PA) was used to remove plasma and nuclear membranes, and was perfused through the liver for ~30 min (up to an hour) or until the tissue became transparent.

This was followed by perfusion for 90 min with a high salt buffer (NaCL). Solubility constants for known collagen types in liver are such that 3.4 M NaCL is adequate to keep them all in an insoluble state, along with any matrix components and cytokine/growth factors bound to the collagens or the collagen-bound matrix components. The liver was rinsed for 15 min with serum-free DMEM/F12 to eliminate the delipidation buffer and then followed by perfusion with 100 ml of DNase (1 mg per 100 ml; Fisher, Pittsburgh, PA) and RNase (5 mg per 100 ml; Sigma Aldrich, St. Louis, MO) to remove any residual contaminants of nucleic acids from the scaffold. The final step was to rinse the scaffolds with serum-free DMEM/F12 for 1 h to eliminate any residual salt or nucleases. Images are provided in Fig. S1. The decellularized liver scaffolds were stored overnight at 4 °C and perfused with serum-free DME/F12 basal media at 3 ml/min via a peristaltic pump (Masterflex, Cole–Parmer, Vernon Hills, IL) before the imaging study was performed. Prior to an imaging study, the scaffold was transferred from the perfusion bioreactor into the sample imaging chamber (Fig. 1). When in the sample imaging chamber, perfusion was maintained at 4 ml/min through the matrix scaffold remnant of the portal vein via the same peristaltic pump.

2.2. Contrast imaging

An overview of the image data processing workflow is provided (Fig. 2). Flash replenishment imaging was performed using an Acuson Sequoia 512 equipped with a 15L8 transducer (Siemens Medical Solutions USA Inc, Mountain View, CA). The “CPS Capture” software algorithm was used to measure perfusion time. The 3D images of the liver matrix scaffold were acquired by scanning the transducer in the elevational direction using a linear stage and motion controller (UTS150PP and ESP300, Newport, Irvine, CA) interfaced through LabVIEW (National Instruments, Austin, TX) as described by Feingold et al. [14]. Perfusion images were parametrically mapped to contrast arrival times between 1 and 10 s. These images were stored in DICOM format with JPEG compression and analyzed offline in MATLAB (Mathworks, Natick, MA). Perfusion times within the regions of interest were assessed.

Acoustic angiography was performed on a prototype dual frequency probe [16] with imaging parameters previously described [13]. The imaging system was a VisualSonics Vevo770 (Toronto, ON, Canada), with pulses emitted at 4 MHz at 1.23 MPa, and echoes received on a 30 MHz transducer with 100% bandwidth after being passed through a 15 MHz high pass filter to remove non-contrast signal. Three-dimensional images were acquired with the VisualSonics 1D linear motion stage with inter-frame distance of 100 μm to yield nearly isotropic voxels. Images were acquired with a frame rate of 2 Hz, with 5 frames averaged at each location. High resolution b-mode images were also acquired with the Vevo770 system using the same imaging parameters, except the transmit frequency changed to 30 MHz. After imaging, data was exported from the ultrasound system as 8 bit uncompressed AVIs. The microvessels were then segmented from these images using an algorithm originally designed for human magnetic resonance angiography images,