Holographic tissue engineering using ultrasonic interference patterns

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Abstract

Biological cells in suspension can be organized into ordered structures by the acoustic forces exerted on them by ultrasonic standing wave fields. To date, only simple tissue patterns such as layers have been constructed using this scaffold-less approach to tissue engineering. This is principally due to the use of single-frequency standing waves transmitted from a single source, producing planar nodes and antinodes in the cell suspension. The purpose of this project was to explore the potential of generating complex tissue microstructures using multiple sources, multiple frequencies, and standing-wave cavities of complex geometry. Multi-frequency sources were constructed by stacking piezoelectric elements of different thickness, and by placing sources at opposite ends of the standing-wave cavity, to generate compound standing waves with complex, non-sinusoidal waveforms. Experimental results with microspheres show that such waveforms can be used to custom tailor the tissue pattern, such as the use of square waves to create thinner cell layers as compared to sine waves, or the use of more complex waveforms to create double-layer structures. Multiple sources were also placed at various angles to each other to generate interference patterns in the standing waves. For example, two sources placed orthogonal to each other generated a square lattice of parallel channels. The experiments demonstrate that standing wave patterns can be produced with levels of complexity higher than simple 2D layers. Computer models also show that the holographic approach should be capable of creating tissue patterns with a 3D complexity similar to that of natural biological structures such as alveoli and lobules. Future research will focus on creating actual tissue structures from these results.

Keywords: Tissue engineering
Holographic tissue engineering using ultrasonic interference patterns

1 Introduction

A significant problem in tissue engineering and regeneration is the growth of artificial tissues with complex biological structures. Cells often grow in random close-packed structures in two-dimensional (2D) and three-dimensional (3D) tissue cultures. Natural biological structures, however, are typically comprised of cells that are arranged in convoluted layers, surfaces, tubules, ducts, lobules, and cavities. Examples of such structures include pulmonary alveoli and renal corpuscles. Creating artificial tissues with such complex structures in three dimensions, and that are additionally functional in the human body, is currently one of the biggest challenges in tissue engineering. Tissue templates are therefore used to guide cells into forming complex tissue microstructures.

To date, most templates for tissue engineering have comprised either 2D surfaces or 3D scaffolds to provide a substrate for cell growth. These templates include both artificial materials, such as polymer meshes, and natural materials, such as the extracellular matrix of tissues from animals or human donors from which the cells have been removed. The problems with these methods include the biocompatibility of the substrate material; the geometric limitations of 2D surfaces or 3D scaffolds; the functionality of the artificial tissue if the scaffold or surface is permanent; biochemical and biomechanical issues arising from degradable or bio-absorbable substrates; and the potential for microbial biofilm growth on the surface or scaffold.

Acoustic forces have been used in various forms over the past few decades to manipulate living cells [1]. Acoustic tweezers have been proposed and demonstrated for the manipulation of single cells in microscopy and biological research. Standing-wave acoustic traps have also been developed for similar applications, and the aggregation of cells suspended in a fluid has been demonstrated for very simple standing-wave patterns. The use of acoustic standing waves and forces to selectively separate and manipulate cells is a rapidly growing and maturing field of research, and several groups are developing microfluidic devices that use acoustic standing waves for medical applications such as separating erythrocytes, platelets, or lipid particles in blood. The concentration of living biological cells (erythrocytes) with standing-wave acoustic fields has been demonstrated for extended periods of time (> 15 minutes) without damage to the cells, indicating the potential of this technology for more extensive biomedical applications. Acoustic forces and piezoelectric devices have also been applied to develop a tissue engineering approach that uses inkjet technology. This approach sprays cells onto a substrate in complex patterns to create artificial tissues. However, since the approach still requires a substrate and deposits a 2D layer of cells with each scan, it suffers from many of the same disadvantages as those employing 2D surfaces and 3D templates.

Acoustic standing waves are an active area in biomedical research, particularly in biosensing applications and microfluidic devices, and basic standing-wave patterns have been used to engineer tissues and biomaterials with simple 2D planar and 3D layered geometries [2-9].
Some of these tissue configurations include multilayers of neuro-progenitor cells in fibrin gels and complex cross-patterned band structures of Schwann cells on a 2D planar surface [6,8]. Acoustic levitation and patterning of cells in a cylindrical resonator has also been modeled [9]. However, the use of acoustic fields to generate more complex nonmaterial or virtual templates for tissue engineering has not been previously achieved due to the complexity of the wave field pattern that would have to be created in the culture medium. This paper presents two methods for attaining this higher level of complexity and control in cell suspensions and engineered tissues—modulating the standing wave fields using multiple frequencies that are harmonic with the standing wave chamber (harmonic modulation), and modulating the standing wave fields using multiple transducers at different positions and angular orientations to create complex interference patterns. These interference patterns have the potential to be used as a “virtual” scaffold or hologram for organizing tissue structure, growth, and development from random cell suspensions.

2 Methods

Ultrasonic standing waves were generated using a 30-MHz Stanford Research Systems arbitrary function generator (DS345), a 10-MHz, 100-W RF power amplifier (ENI 240L), a 200-kHz piezoelectric disc (43-mm diameter, 10.5-mm thick, STEMiNC catalog no. SMD43T105F200S), and two 275-kHz piezoceramic blocks (20x15x8 mm, STEMiNC catalog no. SMBLK20W15T84R111). Red polyethylene microspheres (53-63 μm diameter, Cospheric) were mixed with deionized water and surfactant to simulate biological cells and other microparticles. The microspheres facilitated the visualization of the various ultrasonic wave patterns, their ability to levitate microparticles in solution, and their ability to form complex assemblies.

Ultrasonic interference patterns in a 3D fluid medium were simulated using the linear, lossless wave equation for the propagation of sound in fluids. The pressure wave field pattern was calculated using one or more sources of ultrasound and applying boundary conditions on the boundaries of the fluid (test chamber wall or air interface). The boundary conditions set the wave properties necessary for establishing standing waves. Wave field patterns were modeled using simple waveforms (single-frequency sine and cosine waves) and more complex waveforms (multi-frequency waveforms constructed from harmonics such as square waves).

3 Results

3.1 Experiments

An initial experiment was performed to test the hypothesis that modification of an ultrasonic standing wave using odd harmonics to approximate a square wave would spatially focus the nodal regions of the pressure field and confine the microparticles to thinner, more localized layers. Figure 1 displays this concept for standing waves in a column of water, with a simple standing sine wave in Figure 1(a), and a more complex standing wave in Figure 1(b) with higher harmonics creating a square-wave approximation. The node in the square-wave approximation
is more “sharp,” with steeper pressure gradients above and below the node. Therefore, the node region is more localized and well-defined. Note that the standing waves form a node at the water surface due to the lower acoustic impedance of the air (open boundary condition).

Figure 1: Modulating the pressure fields of a standing wave using harmonic frequencies to create a standing wave interference pattern.

Figure 2 shows the experiment conducted to test this hypothesis. A mixture of microspheres in water was confined in an acrylic cylinder, with the air-water surface comprising the upper boundary of the standing wave chamber. A 200-kHz piezoelectric disc was used to generate both a simple standing sine wave, Figure 2(a), and a square-wave approximation, Figure 2(b), in the mixture. As shown in the photographs, the microsphere layers in the sine-wave standing wave pattern [Figure 2(a)] appear more diffuse and thicker than the layers in the square-wave standing pattern [Figure 2(b)]. The layers generated by the square wave also coalesced more quickly and persisted longer than those generated by the pure sine wave. These results indicate that harmonic modulation of the ultrasonic standing waves can be used to shape and modify microparticle assemblies formed by acoustic levitation and standing wave patterns.

Figure 2: Microspheres levitated by (a) a simple sine wave and (b) a square wave.
Further initial experiments were performed with testing the concept that two or more transducers at different positions and angular orientations could be used to generate complex interference patterns in the standing waves, and thus complex coalescent structures in the microparticles. Figure 3 shows results using a square polystyrene chamber and two 275-kHz piezoelectric placed on faces that are 90° to each other. The expectation was that the standing square waves in the chamber would create a cross-hatched node pattern with square channels defined by the nodes. Instead, the microspheres coalesced into a much more complex and cellular pattern, as shown in Figure 3. To exclude the possibility that the chamber’s dimensions were mismatched with the transducer frequency, and thus not conducive to producing standing waves, the frequency of the input signals to the two transducers were tuned across a wide frequency range. However, no significant improvement was observed in the microsphere patterns.

![Figure 3: Standing square waves in a suspension of microspheres created by transducers at 90°.](image)

### 3.2 Computational Models

Although the results shown in Figure 2 validated the harmonic modulation approach, the results shown in Figure 3 were unexpected and non-intuitive. Computational models were therefore used to reconcile these results. Standing wave fields were modeled using solutions for acoustic waves in a rectangular cavity [10]. Unlike the water-air interface for the water column in the levitation experiment (Figures 1 and 2), which is an open boundary condition, the walls of the standing wave chamber (cavity) are rigid. Thus, the normal component of the velocity vanishes at the walls. Application of these boundary conditions yields standing wave solutions comprised of cosine functions for the pressure, with pressure antinodes at the walls (Figure 4). Note that the boundary conditions are conducive to the Fourier synthesis of square waves. The boundary conditions for a rigid-rigid boundary are satisfied by a wave vector of \( k = \frac{m\pi}{L} \), where \( L \) is the width of the chamber and \( n \) is an integer \( (n = 0, 1, 2, \ldots) \). This wave vector is required for the presence of standing waves, but is also compatible with the generation of square waves (where \( n \) is an odd integer) as shown in Figure 4.
Figure 5 shows the node patterns (blue and purple), and thus microparticle coalescence structures, for a square chamber with two transducers at 90° to each other and all boundaries rigid. The approximate square waves in Figures 4(b) and 5(b) were constructed from the fundamental frequency ($f_0$) and first two odd harmonics ($3f_0$ and $5f_0$). In three dimensions, the intersecting nodal planes in Figure 5(b) could be used to engineer thin-walled tissue microstructures with well-defined square channels.

Figure 4: (a) Pure cosine and (b) approximate square-wave standing waves for a chamber with rigid-rigid boundary conditions.

Figure 5: Wave field patterns for (a) pure cosine and (b) approximate square-wave standing waves for a chamber with rigid-rigid boundary conditions and transducers at 90°.

The nodal patterns in Figure 5(b) were the expected result for the experiment shown in Figure 3. However, the experiment in Figure 3 does not have perfect boundary conditions since the transducers do not extend along the entire length of the wall with which they are coupled.
Instead, only a portion of the wall acts as a source for the acoustic waves, thereby resulting in mixed boundary conditions for the standing wave equations. Additionally, the model in Figure 5 assumes no vibrations with a vertical ($z$) component, thus resulting in a 2D solution. However, it is highly likely that some of the transducer vibrations were transmitted through the polystyrene to the bottom of the chamber, therefore creating additional waves propagating vertically that interfered with the 2D horizontal waves.

In addition to modeling standing wave patterns for two transducers mounted at right angles to each other, simulations were also generated for three transducers mounted to produce waves propagating at 30°, 90°, and 150° with respect to the $x$-axis. Figure 6 shows results for rigid boundary conditions for (a) a pure cosine wave and (b) a square-wave approximation ($n = 1, 3, 5$). The nodal patterns display hexagonal symmetry. Again, the approximate square wave with just the fundamental mode and two harmonics produces thin-walled structures at the nodes and a complex channel structure at the antinodes, with small triangular channels interspersed among large hexagonal channels.

![Figure 6: Wave field patterns for (a) pure cosine and (b) approximate square-wave standing waves for a chamber with rigid-rigid boundary conditions and three transducers.](image)

Figure 7(a) shows another model for the three-transducer configuration as above (waves oriented 30°, 90°, and 150° with respect to the $x$-axis) and for simple cosine waves. In this case, however, the standing waves with the 90° orientation were shifted 90° in phase with respect to the other two standing waves. The results resemble a lobular-type microstructure. With the use of harmonic modulation of the wave fields and modulation along the third dimension, such an interference pattern could be refined to create alveolar microstructures as shown Figure 7(b). In Figure 7(b), the red circles represent alveoli and the purple star-shaped structures represent bronchioles.
Conclusions

Ultrasonic standing wave fields can organize suspended biological cells into ordered structures, thereby providing a potential for scaffold-free tissue engineering. However, mostly simple tissue patterns such as layers have been constructed to date due to the use of single-frequency standing waves transmitted from single sources. The objective of this study was to explore the potential of generating complex tissue microstructures using multiple sources, multiple frequencies, and standing-wave cavities of complex geometry. Experimental results with microspheres suspended in water showed that such waveforms can be used to custom tailor the tissue pattern, such as the use of square waves to create thinner cell layers as compared to pure sine waves. However, the ability to generate standing waveforms of arbitrary shape was found to be limited by the boundary conditions of the fluid supporting the waves.

In one experiment, a column of water with a transducer bottom and air interface at the top provided rigid-open boundary conditions, which supported the production of square waves and thin layers. In another experiment, a square chamber with transducers on two adjacent sides provided rigid-rigid boundary conditions. In contrast to the first experiment, the node patterns generated in the square chamber were more complex than those predicted by computer models, indicating that 2D square wave microstructures in a chamber with rigid-rigid boundary conditions may be more challenging to create than originally expected.

The experiments demonstrated that the shape of the standing waveform can be modulated with harmonic frequencies to refine the node patterns and resulting coalescent structure of microparticles. The experiments also stressed the importance of the fluid boundary conditions and standing-wave chamber design. Finally, simulations showed that the holographic approach
has the capability of creating tissue patterns with a complexity similar to that of natural biological structures such as alveoli and lobules.

Acknowledgments
This research was funded by grants from the Western Alliance for Expanding Student Opportunities (Arizona State University, WAESO Award Number S16UR016/S2016ur0024) and Utah Valley University.

References