Acoustic Holographic Cell Patterning in a Biocompatible Hydrogel

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Acoustophoresis is promising as a rapid, biocompatible, noncontact cell manipulation method, where cells are arranged along the nodes or antinodes of the acoustic field. Typically, the acoustic field is formed in a resonator, which results in highly symmetric regular patterns. However, arbitrary, nonsymmetrically shaped cell assemblies are necessary to obtain the irregular cellular arrangements found in biological tissues. It is shown that arbitrarily shaped cell patterns can be obtained from the complex acoustic field distribution defined by an acoustic hologram. Attenuation of the sound field induces localized acoustic streaming and the resultant convection flow gently delivers the suspended cells to the image plane where they form the designed pattern. It is shown that the process can be implemented in a biocompatible collagen solution, which can then undergo gelation to immobilize the cell pattern inside the viscoelastic matrix. The patterned cells exhibit F-actin-based protrusions, which indicate that the cells grow and thrive within the matrix. Cell viability assays and brightfield imaging after one week confirm cell survival and that the patterns persist. Acoustophoretic cell manipulation by holographic fields thus holds promise for noncontact, long-range, long-term cellular pattern formation, with a wide variety of potential applications in tissue engineering and mechanobiology.

Spatially arranging cells is an essential technique in tissue engineering and a first step in creating biomimetic microstructures and functional cell assemblies.[1,2] Cell cultures with regular patterns of cultured cells are typically used for drug screening, genetic engineering, and in vitro toxicological tests, even though it has been acknowledged that it is generally difficult to properly replicate biological cell function in highly ordered 2D cell cultures.[3] Thus, methods to form more complex arrangements of cells and potentially in 3D is of great importance for a wide variety of cell-based applications. One method is reaggregation of monodispersed primary cells or engineered cell lines in extracellular matrix, which can be used to engineer artificial microtissues in vitro.[4,5] Several methods have been used to pattern cells in hydrogels, including using surface chemistry,[6,7] bioprinting,[8,9] dielectrophoresis,[10,11] and acoustophoresis.[12-14] Surface chemistry and dielectrophoresis methods are based on a prepatterned mold or electrodes, which are not flexible in generating multiple complex images. Bioprinting can generate complex cell patterning by spatially positioning cell-encapsulating materials in a point-by-point regime. However, the long times needed for printing may cause changes between cells extruded at the beginning or at the end of the printing process. A parallel and volumetric method is advantageous as it can provide homogeneous conditions for cell patterning.[15] Acoustophoresis provides good biocompatibility and rapid control over large numbers of cells, and thus is a promising tool for long-range cell patterning.[16,17] Cells can be manipulated with the acoustic radiation force, which is the time-averaged force on a particle suspended in fluid when it is exposed to an acoustic field. If the radiation force is used, then the cells assemble along the nodes or antinodes of the standing acoustic field.[12,18] Here we show that streaming-induced cell patterning with an acoustic hologram can be used as an alternative means to obtain more complex controlled cell assemblies, which better mimic the complex cellular arrangements found in biological tissues.

Acoustic holography is a promising technique for constructing complex pressure and phase distributions. Conventionally, several acoustic emitters are used in acoustic holography, which are individually phase modulated so that a resulting field is formed by superposition.[19] Manipulation and levitation of multiple particles has been shown based on such phased array transducers.[20] However, the simultaneous...
manipulation of a very large number of particles or cells and their arrangement into a complex shape is challenging by this method, since the number of emitters is generally small (<1000). Increasing the number of transmitters also increases the complexity in the electrical driving circuit. Recently, it has been shown that much more complex acoustic fields can be obtained with a static hologram.\cite{21} By encoding the phase information in the topography of a 3D-printed plate, it is possible to generate sophisticated acoustic fields that only require a single transducer. In this case one can easily obtain the equivalent of phase-tuned acoustic emitters spaced at the wavelength of the ultrasound wave, which easily results in the equivalent of more than 10 000 emitters in \( \approx 5 \text{ cm}^2 \) (at a few MHz). Thus, the information content and complexity of the target acoustic field is orders of magnitude higher compared to those obtained with conventional phased arrays. In addition, the acoustic hologram also does not require the usage of complex electronics as only one transmitter is needed. The parallel assembly of polymer particles has been demonstrated using this method.\cite{22} The negative-acoustic-contrast particles, due to acoustic radiation force, aggregated in high acoustic pressure regions. The obtained particle assembly was fixed by a UV light-triggered chemical reaction. This process of directed assembly from a suspension demonstrates that the acoustic hologram can be used to shape objects and composites in ultrasound fields.\cite{23-26} However, the application of the acoustic hologram for cell patterning remains challenging and has not yet been demonstrated. Materially distinct from negative-acoustic-contrast soft polymer particles,\cite{22} cells in culture medium typically show a positive acoustic contrast. One would therefore expect that cells should not aggregate in regions of high acoustic pressure. Moreover, as the acoustic properties of biological cells are similar to that of culture medium, the radiation force is expected to be weak compared to the acoustic radiation force on polymer beads.\cite{27,28}

In this study, we show that cells can also be patterned inside a biocompatible hydrogel with the acoustic hologram. The cells can be assembled in designed 2D patterns and the supporting collagen solution undergoes gelation to immobilize the cell patterns into a 3D matrix. Afterward, the cell pattern can be transferred into an incubator for further cultivation. Viability assays after 7 days show that cells inside the patterns remain viable, which permits their use in subsequent applications and as a cell culture.

Figure 1 shows the general scheme of acoustic holographic cell patterning. Initially, the cells are randomly dispersed in the hydrogel medium (Figure 1a). Then an acoustic field with a complex shape is projected into the cell suspension. Since the acoustic waves are attenuated, their nonlinear propagation induces streaming flows,\cite{29} which in turn cause convection. These flows impart forces upon the cells, causing them to move and aggregate at the high acoustic pressure zones (Figure 1b). After assembly, the collagen solution undergoes gelation to immobilize the cell patterns, which can be then cultivated in an incubator (Figure 1c).

When acoustic waves propagate through fluid media, the absorption of the acoustic wave can result in an unequal distribution of Reynolds stresses, which is equivalent to a net body force imparted onto the fluid. A time-average flow, known as the acoustic streaming flow, is produced as a result of the net force on the fluid. Particles suspended in the fluid are dragged by the flows. The propagation direction and amplitude of the acoustic wave, respectively, determine the direction and magnitude of the net force and thus the nature of the particle movement.\cite{27,30} Compared with the direct manipulation of cells solely based on the radiation force, where cells move toward grid-shape acoustic pressure nodes or antinodes in a standing acoustic field,\cite{12,31} cell manipulation applying both acoustic radiation force and acoustic streaming can be beneficial in generating more complex-shaped cell patterns.\cite{27,32} Moreover, since the acoustic properties of biological cells and the culture medium are very similar, the radiation force on cells is generally weak compared with that on polymer beads.\cite{27,28} Here we instead show that the complex-shaped streaming flow emanating from an acoustic hologram can instead be used to build complex multicellular assemblies from a suspension of single cells.

Figure 2a shows the schematic for the cell patterning using the acoustic hologram. A transducer (88-0518, Olympus) generates a 5 MHz acoustic plane wave propagating along the positive Z-axis through the water it is immersed in. A Petri dish containing single cells suspended in a cell culture medium is placed in direct contact with a water surface to provide acoustic coupling through the bottom wall. A 3D-printed acoustic hologram is put in contact with the transducer \((z = 0 \text{ mm})\) and both are positioned below the Petri dish such that a planar acoustic image forms in the culture medium \((z = 20 \text{ mm})\). The measured acoustic pressure in the image plane and the phase distribution of the hologram are shown in Figure 2b,c, respectively. The acoustic field is scanned by a 0.5 mm tip diameter hydrophone (Precision Acoustics Ltd, UK) at the target plane \((z = 20 \text{ mm})\). To ensure that the hydrophone tip is immersed in the culture
medium, the liquid–air interface is moved further away from the image plane (to \( z = 21 \) mm) by using more culture medium than is used in the cell patterning experiments. Interference between the incident acoustic wave and its reflection returning from the fluid–air interface leads to the formation of pressure nodes and antinodes along the Z-coordinate. Since the acoustic Rayleigh stress difference is proportional to the acoustic intensity, streaming flows are generated in the regions of high acoustic pressure and point in the direction of the Z-axis. These acoustic streaming flows lead to recirculating convection fields that transport suspended cells to the regions of high acoustic amplitude, where they are trapped in the pressure nodes and further aggregate, gradually forming the designed cell patterns as shown in Figure 2d. Typically, the cell patterns are formed within 2–3 min. This process is recorded as shown in Videos S1 and S2 of the Supporting Information. The inset of Figure 2d shows that, adjacent to the high-cell-concentration pattern area, there are low-cell-concentration areas (darker regions), indicating that cells originally found in these regions are transported to and concentrated in the patterned areas.

To analyze the mechanism that underlies the aggregation of cells due to streaming, a single focus spot located at the liquid–air interface was projected in the Petri dish using the hologram shown in Figure 3a. The time-lapse image in Figure 3b shows the transport of dispersed cells due to streaming flows in the X–Y plane. The incident acoustic beam propagates along the +Z-direction and interferes with the wave reflected at the fluid–air interface. The resulting trapping sites correspond to minima of the Gor’kov potential traps (shown in Figure 3c). The cells are therefore not only pushed together in the X–Y plane (particles labeled with “1” in Figure 3c), but also trapped vertically at the center of the acoustic beam (particles labeled with “2” in Figure 3c) in a balance between gravitational force, buoyant force, Stokes drag force, and acoustic radiation force. A detailed force analysis around the trapping site is shown in Figure S2 of the Supporting Information. The resultant cell aggregation process is also shown in Video S3 of the Supporting Information.

To form a 3D matrix around the assembled cell patterns, neutralized collagen type I is added to the cell culture medium. The medium is first prepared on ice and the low temperature ensures that the collagen I solution is viscous. It is then added to the cell suspension prior to the acoustic experiment. As the medium warms over the course of the experiment (≈30 min), the solution spontaneously undergoes gelation, forming a viscoelastic 3D matrix with a physiological stiffness comparable to that of the cell pattern, effectively immobilizing it. Once this occurs, the Petri dish with the cell pattern is transferred to an incubator for culturing and cell assays. Cell aggregation rates in the acoustic field were estimated for different collagen fractions using particle image velocimetry (Figure 3d). While the cell aggregation rate decreases as the collagen fraction increases, all collagen formulations permit full cell aggregation prior to hydrogel gelation. Acoustic patterning is therefore possible in hydrogels with different collagen fractions, which permits control over the mechanical properties of the 3D matrix—an important factor for instance in the field of mechanobiology.

The ability of the acoustic hologram to quickly pattern cells in a noncontact, long-range fashion was tested using HCT-116 colon cancer cells, which are ideal for this system due to their fast doubling times, strong cadherin-based cell–cell attachments, and low levels of single cell migration in confining microenvironments. Bright-field mosaic microscopy allows for direct visualization of the acoustic pattern at both 1 day postpatterning (Figure 4a) and 7 days postpatterning (Figure 4b). To obtain information on the ability of the patterned cells to interact mechanically with the 3D hydrogel matrix, the live cell dye SiR-actin was used to directly visualize F-actin bundles in living cells. Figure 4c shows strong enrichment of F-actin signals in the patterned regions.
analysis of fluorescence contrast is shown in Figure S3 of the Supporting Information. An in situ observation of the cell spreading process (conducted after three days of cultivation) is shown in Figure S4 of the Supporting Information. Finally, to determine if the patterned cells survive in the dense collagen environment over longer time periods, Cyto-dye was used for imaging in the 3D hydrogel. Strong green fluorescence signal in the patterned region (Figure 4d) proves that cells survive in the acoustically patterned assemblies in the 3D hydrogels—here, for at least a week in a collagen hydrogel with physiologically relevant stiffness. The viability ratio is estimated by screening captured microscopic photographs of the samples via a published cell statistic code[38] after labeling the cells with Cyto-dye. The data indicate no significant differences between samples with or without acoustic pattern as shown in Figure S5 of the Supporting Information.

In summary, we have demonstrated biocompatible cell patterning using an acoustic hologram. Compared to conventional acoustic patterning, the fields generated by an acoustic hologram permit the assembly of custom-designed, complex-shaped cell patterns. Hydrogel encapsulation enables cell assemblies to be transferred and incubated with good cell viability over long time scales. In principle, the technique can also be used to obtain 3D cell assemblies. This cell assembly method demonstrated herein is fast, simple, and amenable to use in biology laboratories. Ultimately, as noncontact assembly of 3D cell-based structures is an important goal in tissue engineering,[39] regenerative medicine,[40] developmental biology,[41] and mechanobiology,[35] acoustic holographic patterned cell assemblies have the potential to play a powerful role in biomedical research.

Experimental Section

**Numerical Simulation:** A finite element method-based numerical simulation was conducted using COMSOL Multiphysics 5.3 with the acoustic–solid interaction and laminar flow modules. With the assumption that the acoustic wave emanating from the hologram is approximately axisymmetric, a 2D-axisymmetric model of the beam cross-section was considered. The modeling schematic and parameters are respectively shown in Figure S1 and Table S1 of the Supporting Information. The same phase distribution was used as the one generated by the hologram in the experiment for the input wave. The resulting field was calculated and from it the Gor’kov potential distribution $U_G$ was obtained[42]...
where $R$ is the radius of the computation cell, $\langle p^2 \rangle$ and $\langle v^2 \rangle$ are respectively the mean-square fluctuations of the acoustic pressure and velocity, $\rho_c$ and $\rho_m$ are the density of the computation cell and medium, respectively. The body force $F_b$ is a gradient of Gor'kov potential could be calculated as

$$F_b = -\nabla\Phi\rho_c$$

(2)

The body force $F_b$ generated by acoustic attenuation is obtainable by substituting the calculated acoustic field in\(^{[29]}\)

$$F_b = \rho_m \beta V$$

(3)

where $\beta$ is the attenuation coefficient in the fluid medium. Then the streaming profile is calculated with substitution of the body force in the widely known incompressible Navier–Stokes equation. The Stokes drag force is then calculated as

$$F_D = 6\pi\eta RV$$

(4)

where $\mu$ is the dynamic viscosity of the medium, and $V$ is the flow velocity relative to the particle.

**Preparation of the Cell Suspension:** HCT-116 human colon cancer cells were used in all acoustic holographic cell patterning experiments. Immediately prior to cell patterning experiments, the following samples were fully mixed on ice: 150 μL cell culture medium comprising DMEM (31966, Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (100 μg mL\(^{-1}\)) (Life Technologies), 1 mL of collagen solution from bovine skin (C4243, Sigma-Aldrich), 250 μL 0.1 mol L\(^{-1}\) aqueous sodium hydroxide solution and 300 μL of 5 × 10\(^{5}\) mL\(^{-1}\) cell suspension. To prevent premature collagen gelation, all solutions and materials were kept at 4 °C for 1 h prior to mixing. The cooled Petri dish containing the hydrogel slowly warms up when it is placed in contact with the water tank (at room-temperature) for the acoustic experiments and thus undergoes gelation.\(^{[41]}\)

**Cell Microscopy:** A Leica TCS SPS confocal laser scanning microscope with an HCX PL Fluotar 10× objective and LAS AF software were used to image cellular assemblies in the collagen hydrogel. Using the Tile Scan tool, a 14 × 14 tile mosaic was captured at 80 μm z intervals and stitched to visualize the full hydrogel. To verify F-actin expression, the fluorogenic jasplakinolide-based cell permeable dye SiR-actin (Spirochrome) (excitation/emission 652/674 nm) was included in the fluorogenic jasplakinolide-based cell permeable dye SiR-actin. Immediately prior to cell patterning experiments, the following samples were fully mixed on ice: 150 μL cell culture medium comprising DMEM (31966, Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (100 μg mL\(^{-1}\)) (Life Technologies), 1 mL of collagen solution from bovine skin (C4243, Sigma-Aldrich), 250 μL 0.1 mol L\(^{-1}\) aqueous sodium hydroxide solution and 300 μL of 5 × 10\(^{5}\) mL\(^{-1}\) cell suspension. To prevent premature collagen gelation, all solutions and materials were kept at 4 °C for 1 h prior to mixing. The cooled Petri dish containing the hydrogel slowly warms up when it is placed in contact with the water tank (at room-temperature) for the acoustic experiments and thus undergoes gelation.\(^{[41]}\)

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

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**Keywords**

cell patterning, direct assembly, holograms, hydrogels, ultrasound

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