Continuous Production of Acoustically Patterned Cells Within Hydrogel Fibers for Musculoskeletal Tissue Engineering

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Many mammalian tissues have a specific cellular arrangement that enables their unique function. For example, parallel alignment of myofibers enables uniaxial muscle contraction. To engineer structured tissues ex vivo, it is critical to recapitulate this cellular arrangement. Conventional 3D encapsulation often fails to recapitulate this complexity, motivating the need for advanced patterning approaches. In this work, an acoustofluidic device to continuously pattern mammalian cells within hydrogel fibers is engineered. Contactless acoustofluidic forces are used to control the spacing between parallel lines of cells. To enable continuous extrusion of cell-laden hydrogel fibers, a low friction Teflon tube is integrated into the device. A photopolymerizable hydrogel allows triggering gelation externally with light once the cells are under the influence of the acoustic field, setting the patterned cells within the hydrogel fiber. Using this device, the muscle progenitor cells (myoblasts) within the hydrogel are patterned in parallel lines to mimic the structure of skeletal muscle. The increased formation of myotubes and spontaneous twitching of the myotubes in patterned samples are observed. This approach combining continuous fabrication with the tunability of acoustofluidics can create complex 3D tissues to engineer skeletal muscles as well as tendons, ligaments, vascular networks, or combinations thereof in the future.

1. Introduction

Bioengineered tissue mimics are useful for regenerative medicine, drug discovery, and soft robotics.[1–3] A common approach to designing tissue models relies on the encapsulation of cells within 3D biomaterials, which can result in more physiological cell behavior as compared with cells grown on 2D, stiff plastic substrates.[4] Hydrogels have emerged as a versatile biomaterial for 3D encapsulation owing to their tunable mechanical properties and ability to include various biochemical cues to support cell function.[5,4] Hydrogel-based encapsulation has improved the quality of tissue mimics for a range of applications, including cardiovascular, retinal, neural, and musculoskeletal tissues.[7] However, cell encapsulation in 3D hydrogels often results in isotropic cell organization. Yet, many biological tissues—including skeletal muscle, tendon, ligaments, and cardiac tissue—are anisotropic with defined cellular arrangements. Further, the physiologic function can depend on this cellular anisotropy. For example, uniaxial muscle contraction requires coaxial alignment of muscle fibers. Therefore, strategies to organize cells anisotropically in 3D culture would help to engineer the native cellular architecture and function of structured tissues.

Several strategies have been explored to pattern cells in 3D culture. Structural organization has been induced in musculoskeletal tissue models using mechanical and electrical stimuli.[8,9] Techniques such as microfluidics, have also been used to guide network formation in neuronal cells.[10] These efforts demonstrated the utility of recapitulating the structural organization of native tissues. An emerging approach to organizing cells within 3D biomaterials is through the use of acoustic forces, which provides specific advantages as it is label-free and can be scaled while enabling tunable patterning of cells. Acoustofluidics is a non-contact and programmable method for patterning objects, including cells, within a liquid.[11] This phenomenon is based on the force a particle experiences when placed in a strong acoustic field. The scattered acoustic field from the particle interacts with the incident acoustic pressure field, resulting in acoustic radiation forces. The formation of standing waves inside a fluidic cavity can amplify the acoustic pressure due to the high Q-factor and provide control over the
cell positions as cells are driven to the pressure nodes. Acoustofluidic devices have used these features for biological applications, demonstrating the cytocompatibility of acoustofluidic manipulation.[10]

The ability of acoustofluidics to organize cells has motivated the use of different forms of acoustic excitation to form anisotropic cell-laden constructs. Human umbilical vein endothelial cells (HUVECs) and human adipose-derived stem cells (hADSCs) were patterned in coculture with a surface acoustic wave (SAW) device at high resolution (=50 μm) to form vascular networks.[21] Acoustic patterning improved the integration of these vascular constructs with host tissue in mice as compared with non-patterned controls. Bulk acoustic wave (BAW) devices have been used to fabricate skeletal muscle tissue constructs, vascular networks, and cartilage demonstrating tunable control over the cell–cell spacing.[14–16] With BAW devices, the acoustic waves operate over the entire fluidic cavity to produce the desired acoustic field. This enables the direct patterning of larger 3D tissue constructs as compared with SAW devices. In another implementation, scientists have used a vibrating plate device to generate patterns of endothelial cells and mesenchymal stem cells, exhibiting spatial organization similar to Chladni figures using surface waves.[17,18] A similar vibrating plate device was used to pattern cardiomyocytes to produce a 3D model of cardiac tissue.[19] In another recent work, phase-modulated acoustic fields were used to create acoustic holograms for cell aggregation.[20,21] Each of these unique devices demonstrates the advantages of using acoustofluidics for patterning cells. Yet, acoustically patterning cells continuously and in a scalable manner remains a challenge.

In our work, we leveraged the advantages offered by acoustofluidics to engineer a glass capillary-based device to achieve reproducible patterning of cells with tunable spacing within continuously produced hydrogel fibers. This enabled us to pattern cells continuously for increased throughput of 3D organoids or use cases.[25] We performed a frequency analysis to achieve high acoustic radiation forces for the manipulation of cells or particles. In addition, the operating frequency determines the distance between the patterned objects (i.e., pressure nodes of the standing wave). Therefore, it is necessary to characterize the operating frequencies of the device, to provide the user control over particle manipulation and the features of the resulting cell pattern.

Since the structure of the entire device and the operating conditions, such as geometry, material properties, and temperature, have an effect on the resonant frequencies, we performed an admittance measurement to characterize the resonant frequencies of the base device (piezoelectric coupled to a glass capillary filled with water; Figure S1, Supporting Information).[24] To identify suitable operating frequencies for the acoustofluidic device, we first performed a frequency sweep around the identified resonant frequencies and observed the response of 15 μm polystyrene beads to the excitation of the piezoelectric transducer with a frequency modulated sine wave at 10 Vpeak-peak. We observed strong acoustic modes at 2.49, 2.9, and 6.82 MHz, demonstrated by particle accumulation at the pressure nodes (Figure S2, Supporting Information). These modes were reproduced with the same voltage signal (frequency and amplitude) with the addition of the Teflon tube within the glass capillary, resulting in approximate distances of 240, 150, and 75 μm between pressure nodes, respectively (Figure 2a). While many other acoustic modes were observed, the main cell patterning experiments were performed at these operating frequencies (Figure S2, Supporting Information). Since most mammalian cells and the polystyrene tracer particles have positive acoustic contrast, we observed similar acoustic modes when the tracer particles were replaced by cells.

The frequencies at which standing waves arise depend on many factors, including the geometry of the fluidic cavity, the piezoelectric transducer, and other structural factors. Validated numerical models can guide the implementation of acoustofluidic devices and allow for rapid screening of new designs or use cases.[25] We performed a frequency analysis

2. Results and Discussion

To enable acoustic manipulation, we engineered a glass capillary-based BAW device with a piezoelectric transducer attached on the capillary to generate standing acoustic waves (Figure 1a). Acoustic waves originating from the piezoelectric transducers travel through the fluidic cavity and are reflected by the interfaces (glass and free surface), these reflected waves then superimpose with the waves from the piezoelectric transducer to form standing acoustic waves. For acoustic manipulation, the acoustic radiation force acting on the cells should be greater than the viscous drag force applied by the surrounding fluid. To increase the radiation force we operated the acoustofluidic device near its resonant frequencies. To minimize viscous drag and enable rapid movement of the cells, we patterned cells in the unpolymerized hydrogel precursor solution (low viscosity liquid). We then used light as an external trigger to polymerize the gel, fixing the positions of the patterned cells within the hydrogel fiber (Figure 1b). For smooth extrusion, we added a round Teflon tube inside the square glass capillary. This approach enabled continuous patterning of cells, gelation of the hydrogel, and extrusion of cell-laden hydrogel fibers. We demonstrated tunable patterning of polystyrene beads and cells within a liquid using different operating frequencies, verifying the acoustic modes through a numerical model. We used this device to pattern murine muscle cells to form muscle tissue mimics. We observed spontaneous twitching of myotubes in our tissue constructs. The presence of myotube formation was also verified by myosin heavy chain (MyHC) immunocytochemistry (Figure 1c).
to simulate the acoustic pressure field on a 2D cross-section of the device using COMSOL Multiphysics. The simulations predicted similar pressure nodes to those observed experimentally (Figure 2b). In line with the results of the simulations, we also observed that the particles formed coaxial lines by changing the focal plane during the experiment (Video S1, Supporting Information). To understand the effect of the Teflon tube on the acoustic field, we identified acoustic fields at resonant frequencies in the glass capillary with and without the Teflon tube, both experimentally and using numerical analysis (Video S2, Supporting Information). Similar fields were observed in both cases, indicating the minimal effect of the Teflon (Figure S2, Supporting Information). This computational analysis can be used to design bigger or smaller fluidic cavities to either increase the throughput or to improve the spatial resolution and cell spacing. Further, this would allow for the design of additional cell patterning approaches or geometric localization of cells through the use of multiple piezoelectric transducers.

2.2. Teflon Tube Enables Continuous Extrusion of Hydrogel Fibers with Patterned Cells

Retaining the positions of the acoustically patterned cells or particles is important as they can disperse as soon as they are out of the influence of the acoustic field. For this, we used a photo-initiated sol-gel transition of the hydrogel while the patterned objects were still under the influence of the acoustic field. Since light is used as the trigger, we have spatial control over the region of gelation.

We synthesized methacryloyl gelatin (GelMA), a widely used photopolymerizable hydrogel for tissue culture, as the base hydrogel with lithium acylphosphinate (LAP) as the

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Figure 1. Acoustofluidic device for continuous production of patterned cells within hydrogel fibers. a) Cells in a liquid hydrogel precursor solution are patterned using an acoustic field. The patterned cell positions are retained by photopolymerization of the hydrogel. The continuous flow in the device results in the extrusion of the hydrogel fiber with patterned cells. b) Cells can move freely within the liquid hydrogel precursor solution. They are patterned under the influence of the ultrasonic standing wave (black) produced by the piezoelectric transducer attached to the glass capillary. This acoustic field results in acoustic radiation force (red) acting on the cells and moving them to the pressure nodes. To maintain the spatial positions of the patterned cells, the hydrogel is polymerized using an external light trigger. This is done in presence of a photo-initiator, resulting in a chain-growth polymerization forming cross-links (green) between the polymer network (black). c) This method can be used to create functional tissues by patterning cells of interest within hydrogels. Myotubes formed in acoustically patterned constructs (right inset) with nucleus (cyan) and MyHC (red).
We polymerized the gel using an LED light (\(\lambda = 405\), \(I = 10\) mW cm\(^{-2}\)), focused through a microscope objective. The inclusion of the Teflon tube prevented GelMA adhesion to the glass capillary (Figure 1a). We also included Pluronic F-127 (1 or 2 wt%) in the hydrogel formulation to facilitate smooth extrusion of the polymerized hydrogel without affecting cell viability (Figures S5 and S6, and Video S3, Supporting Information).

Having a working protocol for the acoustofluidic patterning device, we demonstrated the tunability of the device by producing cell-laden hydrogel fibers at different frequencies (Video S4, Supporting Information). The rate of production was directly controlled by the flow rate using a syringe pump and was dependent on the gelation kinetics of the hydrogel and the intensity of light. Higher flow rates resulted in incomplete gelation and lower flow rates caused over-gelation, making it difficult to extrude the polymerized hydrogel. Although the Teflon tube weakened the amplitude of the acoustic field, this did not impact the overall production rate, which in our device was limited by the gelation of hydrogel and not the speed of the acoustic alignment. An optimal flow rate of 3 \(\mu\)L min\(^{-1}\) (≈1 mm min\(^{-1}\)) was identified through experiments. Cells patterned in this manner maintained their positions in the hydrogel fibers after extrusion (Figure 3a). To demonstrate the utility of continuous production, fibers up to ≈15 cm in length were produced (Figure S5, Supporting Information). Long fibers were also produced with patterned cells (Video S5, Supporting Information). Since gravity acts in the flow direction, uniform cell density was not maintained in the current device. This can however be tackled with the use of a syringe stirrer attachment on the syringe pump. Since the process of patterning cells was continuous, we were able to tune the patterning during extrusion. This was done by modulating the frequency of operation, which varied the cell–cell spacing. 3T3 fibroblasts were encapsulated in GelMA, the operation frequency was modulated from 2490 to 6820 kHz (Figure 3b, and Video S6, Supporting Information). In principle, this can be done for any of the operating frequencies of the device.

This continuous production of hydrogel fibers offers a unique advantage in various applications. The tissue constructs can be used as “spools” for more complicated textile-based tissue engineering applications. The device can also be used in form of a nozzle for extrusion-based 3D bioprinting. Beyond biomedical applications, this device could also be modified to produce fibers or textiles with anisotropic properties.

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Figure 2. The acoustofluidic device enables tunable positioning via frequency modulation. a) Cross-section of the patterning region of the acoustofluidic device showing the piezoelectric transducer (brown) mounted to the glass capillary (black) with the concentric Teflon tube (green). Upon excitation of the piezoelectric transducer, a standing wave is formed and polystyrene beads (PS beads; grey) or cells move to the pressure nodes (dashed lines) under continuous flow. Optical micrographs of patterned polystyrene beads at identified operating frequencies. The beads form parallel lines inside the device, resulting in defined spacing between the lines of beads. The distance between the lines of cells was estimated by comparing them to the external dimension of the glass tube. The patterning was robust under continuous flow. b) For the numerical simulation, the device cross-section was considered and 2D numerical simulations were performed in COMSOL Multiphysics. Acoustic modes corresponding to similar frequencies to the experimental observations were identified.
2.3. Acoustically Patterned Cells Remained Viable and Spread in the Hydrogel Fibers

Having established a reliable protocol for continuous patterning and extrusion, we performed cell patterning experiments wherein 3T3 fibroblasts were patterned in GelMA. The collected hydrogel fiber was incubated in growth medium and samples were observed on Days 0, 1, 3, and 4. The cells maintained the pattern inside the gels and also spread over time in the gels preferentially in the direction of alignment (Figure 3c).

To evaluate the effect of our patterning process the viability of cells in the patterned sample was compared with the viability of cells in unpatterned (extrusion without acoustics) and 3D bulk (conventional encapsulation) samples. For this, we used myoblasts encapsulated in hydrogels and labelled

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**Figure 3.** Cell patterning and spreading in the hydrogel fibers. a) Analysis of cell positions in unpatterned (left, orange) and patterned (right, green) hydrogel fibers. The extruded fibers were fixed and cell nuclei were stained with DAPI the day after encapsulation. The fluorescence intensity was calculated from the maximum intensity projection of a z-stack (Stack height, 80 μm). The plots were normalized against the total fluorescence in each image. Scale bar, 200 μm. b) Snapshots of the extrusion with dynamic change of operating frequency (Video S6, Supporting Information) 2490 kHz frequency was used at the beginning of the extrusion (right) which was then switched to 6820 kHz. c) 3T3 fibroblasts nucleus (blue) and actin (green) were stained to observe spreading at various time points. Cells spread within the hydrogel and formed aggregates in the direction of alignment. Maximum intensity projection of Z-stack (Stack height, 56 μm) Scale bar, 500 μm.
the cells using a live/dead staining kit. The samples showed similar viability (p-value = 0.206) for each of the conditions (Figure S7, Supporting Information). Further, since temperature rises in acoustic devices can affect cell viability, we measured monitored the temperature of the device continuously for \(\approx 20\) min. The measurement was done using a thermocouple on the piezoelectric transducer which is the source of the heat generation. The temperature remained below 30 °C during the entire measurement (Figure S8, Supporting Information).

4. Experimental Section

Design of Acoustofluidic Device: The acoustofluidic device was designed to enable the following: 1) using acoustic force to manipulate cells, 2) externally triggering the sol-gel transformation of the hydrogel to capture the generated pattern of cells, and 3) continuously producing the hydrogel fiber with encapsulated aligned cells. A piezoelectric transducer \((l_1 \times l_2 \times l_3 = 10 \times 2 \times 1\) mm, PZ26, MEGGITT, Denmark) was mounted on a glass capillary (VitroCom; square; inner dimension, 2 mm; wall thickness, 0.4 mm) using a conductive glue (Hi205 glue, Epoxy technology, Epo-tek) mixed with 5% silver-coated glass particles \((\Omega = 20–27\) μm), to maintain uniform glue thickness. The top and bottom electrodes were connected to a signal generator (AFG 3022B; Tektronix) using insulated copper wires \((l = 20\) mm). To avoid reaching the Curie temperature, conductive silver (LS200N BC, Hans Wolbring GmbH, Germany) was used to connect the wires to the transducer instead of soldering. A standing wave field could be produced inside a glass capillary enabling acoustofluidics. A Teflon tube (outer \(\Omega = 2\) mm, inner \(\Omega = 1.7\) mm; 211928-10, BGB, Sweden) was inserted inside the glass capillary to enable smooth extrusion of the polymerized hydrogel fiber. Since the glass capillary was square and the Teflon tube was round, the gap between the two was filled up with water to avoid acoustic impedance mismatch for the transmission of the acoustic waves.

Methacryloyl Gelatin (GelMA) Hydrogel: To develop this work, two important functions were served by the hydrogel, capturing the cell positions through externally triggered polymerization and providing a cytocompatible scaffold for the cells to proliferate. GelMA was used as it is well known for being cytocompatible and is photopolymerizable. Gelatin was functionalized with the methacrylate group using a previously published protocol.[20] Briefly, methacrylic anhydride (276 685, Sigma-Aldrich) was added to gelatine (Type A, bloom 300, G1890-500G, Sigma-Aldrich) dissolved in heated deionized water for 90 min. Unreacted methacrylic anhydride was removed by centrifuging the solution. The supernatant was diazylized and lyophilized, before use. The obtained gel was characterized by photo-rheology (Figure S3, Supporting Information) and Nuclear magnetic resonance (NMR) spectroscopy (Figure S4, Supporting Information).

Numerical Simulations Using COMSOL Multiphysics: To understand the effect of the Teflon tube on the acoustic field and hence the operating frequencies of our acoustofluidic device, numerical simulations were performed using COMSOL Multiphysics 5.6. A 2D model of the cross-section of the device was built for both glass only and glass-Teflon devices including the piezoelectric transducer and fluid, in both cases (Figure 2b). A frequency-domain study was performed using the following: pressure acoustics (water), solid mechanics (glass, Teflon, and piezoelectric transducer), and electrostatics (piezoelectric transducer). Acoustic-structure boundary and piezoelectric effect were used to link the multiple physics domains. Since our glass capillary was a square, the placement of the piezoelectric transducer by 160 μm from the center was offset and increased the width of the glass capillary by 3.5% to differentiate between orthogonal acoustic modes. The glass capillaries were also rounded off using a fillet of 100 μm radius to avoid singularities at the corners. Standard material properties were used from the COMSOL library, except for the piezoelectric transducer (obtained from the manufacturer MEGGITT, Denmark) and Teflon (used from [31]). The acoustic fields obtained at resonance frequencies were compared to the experimental observations.

Characterization of the Acoustofluidic Device: To find the operating frequencies for the cell patterning, admittance analysis and frequency sweeps with tracking particles were performed. SinPhase Analyzer was used for admittance measurement with df = 10 kHz, from 200 kHz to 7 MHz.

Polystyrene tracker particles \((\Omega = 15.49\) μm, Microsphere GmbH, Germany) were used as tracker particles for frequency sweep experiments in the range obtained from admittance analysis. A sine wave with frequency modulation \(V_{\text{peak-peak}} = 10\) V, Frequency Modulation: 5 kHz deviation at the rate of 10 Hz) was produced using a signal generator (AFG 3022B; Tektronix). A signal of 10 V was used unless mentioned.

2.4. Skeletal Myoblasts Show Preferential Myotube Formation and Spontaneous Twitching in the Patterned Regions

Skeletal myoblasts can fuse to form myotubes if the correct biochemical cues are present. Although global cell density makes a negligible difference to the myotube formation, we hypothesized that increasing the local cell density by acoustic patterning could promote cell-cell contact and thereby myotube formation.[23] Primary murine myoblasts were patterned in 4 wt% GelMA (with 1 wt% Pluronics and 0.4 mg mL\(^{-1}\) Matrigel) using various patterning frequencies. The hydrogel fibers with patterned cells were collected in a myoblast culture medium and transferred to a cell incubator. From day 3 on, a low serum differentiation medium was used to encourage myotube formation. From day 7, spontaneous twitching was observed, indicating myotube formation (Video S7, Supporting Information). We confirmed the myogenic nature of the patterned cells by staining for MyHC on day 10. In the unpatterned sample myotubes formed primarily on the surface of the hydrogel fiber, which could be due to the faster cell growth on the surface of the hydrogel compared to the bulk (Figure 4a). However, in the patterned sample, the myotubes could be seen throughout the bulk of the gel within the high-cell density (patterned) regions (Figure 4b,c). Gene expression analysis confirmed the expression of myogenic factors \((\text{MyoD, MyoG, and MyHC})\) in both the patterned and unpatterned samples (Figure S8, Supporting Information). The observed trends in the expression of these canonical transcription factors were consistent with what has been reported previously.[20]

3. Conclusion

We engineered a device for the continuous production of hydrogel fibers with aligned cells. We showed control over cell spacing by changing the operating frequencies. Acoustically patterned cells could spread in our hydrogel and form aggregates. Further, we used this for patterning myoblasts into muscle tissue mimics. Myotubes produced in the patterned cells showed spontaneous contraction. In the future, we aim to explore this technique in an in vivo experiment to test how acoustically patterned tissue mimics compare with native tissue. This technology could be further used in patterning other cell types such as tendons, ligaments, and neurons, amongst others to mimic the anisotropy in the native tissues. The size of the device and continuous extrusion ability can be used to integrate acoustofluidics into 3D bioprinting for an added level of hierarchy.
otherwise. For any signal more than 10 V, a signal amplifier (2100L-1911; MKS Instruments, Germany) was used.

Cell Culture–3T3 Fibroblasts: For initial experiments, NIH 3T3 fibroblasts were cultured on a 150 mm² plastic petri dish. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 61965 026) + 10% fetal bovine solution (FBS, Thermofisher 10 270 106) + 1% penicillin-streptomycin (PenStrep; Gibco, 15140-122) in a CO2 incubator at 37 °C. Cells were passaged every 3 days.

Cell Culture–B6 Murine Myoblasts: Primary murine myoblasts were isolated from C57BL/6J mice following established protocols.[32] The mice were housed at ETH Zürich, tissue collection was performed under a protocol approved by the Zürich Cantonal Veterinarians office (ZH108/18). Tissue culture polystyrene dishes (Ø = 150 mm) were coated with Matrigel (diluted 1:25 in low glucose DMEM, Thermofisher 31 885 023) to improve cell attachment. Myoblasts were cultured on Matrigel-coated dishes in a medium containing equal parts DMEM (Thermofisher 41 966 029) and F-10 medium (Thermofisher 22 390 025) supplemented with 10% horse serum (Thermofisher 16 050 122), 20% fetal bovine serum (FBS; Thermofisher 10 270 106), 1% penicillin-streptomycin (PenStrep; Gibco, 15 140-122), and 10 ng ml⁻¹ basic fibroblast growth factor (bFGF; Bio-Techne 233-FB-500) in a 5% CO2 incubator at 37 °C. Cells were maintained at a 1:5 splitting ratio and passaged every 3–4 days (at ≈ 60%–70% confluence). To induce differentiation, we used a low serum medium consisting of: knockout-DMEM (KO-DMEM; Thermofisher 10 829 018) supplemented with 1% MEM-NEAA (Thermofisher 11 140 035), 1% GlutaMax (Thermofisher 35 050 038), 1% PenStrep (ThermoFisher, 15 140 122), 0.1% beta-mercaptoethanol (Thermofisher 21 985 023), and 2% horse serum (Thermofisher 16 050 122).

Effect of Pluronics on Extrusion and Cell Viability: To study the effect of adding Pluronics to GelMA, 2 wt% Pluronics F-127 (P2443, Sigma-Aldrich) was added to 5 wt% GelMA, with 0.1 wt% lithium acylphosphinate (LAP). This solution was made in PBS and no cells or particles were used for these experiments. For control 5 wt% GelMA with 0.1 wt% LAP was used. Both the precursor solutions were extruded using the same flow rate (5 μL min⁻¹) and similar light conditions (λ = 365 nm, intensity = 10 mW cm⁻²).

Figure 4. Hydrogel fibers with skeletal myoblasts at day 10. All images show the same orientation with the cells patterned along the horizontal. Cells have been stained for nucleus (cyan) and MyHC (red). a) In the unpatterned samples, MyHC+ cells were only observed on the surface of the hydrogel samples. Maximum intensity projection of Z-stack (Stack height, 112 μm). b) In the patterned samples (f = 2900 kHz), MyHC+ cells were present throughout the construct samples. Maximum intensity projection of Z-stack (Stack height, 112 μm). c) Myotubes were observed in the regions of higher localized cell density. Maximum intensity projection of Z-stack ([top] Stack height, 52 μm, [bottom] Stack height, 42 μm). Scale bar, 200 μm.
Table 1. Experimental parameters, in all experiments the same light settings ($\lambda = 405$ nm, $\approx 10$ mW cm$^{-2}$), flow rates (3 $\mu$L min$^{-1}$), and initial polymerization time (60 s) were used.

| Experiment                                      | Hydrogel formulation       | Cells (density)                       | Frequency, amplitude (peak to peak)\(^{a}\) |
|-------------------------------------------------|----------------------------|--------------------------------------|---------------------------------------------|
| Cell patterning and spreading                    | 4 wt% GelMA, 1 wt% Pluronic | NIH 3T3 fibroblasts (2.5 $\times$ 10$^6$ mL$^{-1}$) | 2900 kHz, 15 V                               |
| Frequency switching                              | 4 wt% GelMA, 1 wt% Pluronic | NIH 3T3 fibroblasts (2.5 $\times$ 10$^6$ mL$^{-1}$) | 6820 and 2490 kHz, 10 V                      |
| Quantification of alignment                      | 4 wt% GelMA, 1 wt% Pluronic | C57BL/6J murine myoblasts (2.5 $\times$ 10$^6$ mL$^{-1}$) | No signal; 2900 kHz, 15 V                   |
| Skeletal myoblasts patterning\(^{b}\)            | 4 wt% GelMA, 1 wt% Pluronic | C57BL/6J murine myoblasts (2.5 $\times$ 10$^6$ mL$^{-1}$) | No signal; 2900 kHz, 15 V                   |
| Live/Dead analysis to evaluate effect of patterning on cells | 4 wt% GelMA, 1 wt% Pluronic | C57BL/6J murine myoblasts (2.5 $\times$ 10$^6$ mL$^{-1}$) | No signal; 2900 kHz, 15 V                   |
| Gene analysis                                    | 4 wt% GelMA, 1 wt% Pluronic | C57BL/6J murine myoblasts (2.5 $\times$ 10$^6$ mL$^{-1}$) | No signal; 2900 kHz, 15 V                   |

\(^{a}\)Four different hydrogel samples were prepared. Three with various signal frequencies applied and one as an unpatterned control; \(^{b}\)For all the experiments, sine wave with frequency modulation (5 kHz deviation at the rate of 10 Hz) was used.

To ensure cell viability was not affected by the addition of Pluronic, 10 $\mu$L bulk gels were prepared with encapsulated 3T3 Fibroblasts ($1 \times 10^6$ cells mL$^{-1}$) in two gel formulations, 5 wt% GelMA, 0.1 wt% LAP, with or without 2 wt% Pluronic. The gels were polymerized between two Sigmacote-coated glass plates separated by a 1 mm silicone spacer, using light ($\lambda = 365$ nm, intensity = 20 mW cm$^{-2}$, $t = 45$ s). The hydrogel disks were removed carefully from the glass surface and transferred immediately to the culture medium. The medium was refreshed 30 min after gel formation and every alternating day after that. The samples were cultured in 500 $\mu$L cell culture medium with 1% WST-8 solution for 200 min and the absorbance of the medium was then measured in triplicates with 100 $\mu$L medium each using manufacturer’s protocol.

These measurements were made on Days 2, 4, and 6. Six samples for each condition were used. Paired, two-tailed student’s- t-test was used to compare samples from the same day.

Effect of Acoustofluidics on Cell Viability: To access the effect of acoustofluidics on the cell viability three encapsulation conditions were considered: 1) 3D bulk hydrogel (conventional encapsulation), 2) unpatterned cells (only extrusion without acoustics), and 3) patterned cells (with extrusion and acoustics). All samples had the same formulation of hydrogel (4 wt% GelMA, 1 wt% Pluronic, $\approx 0.4$ mg mL$^{-1}$ Matrigel) and cell density (C57BL/6J murine myoblasts, $2.5 \times 10^6$ mL$^{-1}$). For the 3D bulk hydrogel, 50 $\mu$L samples were prepared between two Sigmacote-coated glass plates separated by a 1 mm silicone spacer, using light ($\lambda = 405$ nm, intensity = 20 mW cm$^{-2}$, $t = 45$ s). For the patterned and unpatterned samples, experimental conditions could be found in Table 1. Live/Dead staining (LIVE/DEAD viability kit, Thermofisher L3224) was used to quantify membrane integrity as a surrogate marker of cell viability. Three images were evaluated for each sample manually. One-way ANOVA was used to compare the three samples.

Temperature Measurement of the Device: Since temperature rise can lower the cell viability, the temperature of the device was measured during operation. The origin of temperature rises in acoustofluidic devices is mostly due to the transducer and hence the temperature change of the transducer was measured during operation. During an active experiment, a continuous flow of the gel and a layer of water between the glass and Teflon layers could provide protection to the cells from any temperature rises. But for this measurement the device was not filled with water, and a higher voltage (20 Vpp) compared to 15 Vpp was also used which was the highest that was used during cell experiments. A resistance-based thermometer (pt100) with Arduino Uno was used for digital readout of the temperature. This device was operated for $\approx 20$ min, monitoring the temperature every 2 s.

Cell Patterning in Hydrogel Fibers: For patterning cells in a hydrogel, cells were suspended in a hydrogel precursor solution and a syringe pump (neMESYS V2, Cetoni) was used to control the flow rate of this solution. To reduce sedimentation of the cells the syringe pump and the device pointed downwards. 1 mL syringe (BD 1 mL Syringe Luer-Lok) with a blunt needle (Sterican MIX, 18 G, Braun) was used to load the hydrogel precursor solution. The exact formulation of the hydrogel precursor depended on the experiment. Cells were used in all experiments except extrusion trials where the cell solution volume was replaced by PBS.

Once the cell and hydrogel solution entered the acoustic device the signal to the piezoelectric transducer was switched on at one of the operating frequencies using similar frequency modulation to the one described in the previous section. The solution was allowed to flow to the end of the channel. To enable a continuous production of hydrogel fiber, it is important to have a hydrogel plug in the end of the channel to ensure that the uncured hydrogel precursor has enough time to gel. At the start of the fiber production, a hydrogel plug of $\approx 10$ mm was created by polymerizing the hydrogel precursor while stopping the flow in fluidic cavity. The time of polymerization, and the subsequent flow rate depended on gel formulation and intensity of light used. All experimental parameters like frequency and amplitude of the voltage applied onto the piezoelectric transducer, gel formulation and additives, cell type and density, for various experiments in this paper can be found in Table 1. Unless otherwise stated, same light settings ($\lambda = 405$ nm, $\approx 10$ mW cm$^{-2}$), flow rates (3 $\mu$L min$^{-1}$), and initial polymerization time (60 s) were used. In all experiments, 25% of the volume of hydrogel precursor is made up of cell growth medium to supply nutrients to the cells while being patterned. All the stated gel wt% and cell densities refer to their final values in the samples.

Skeletal Myoblasts Patterning: For experiments with skeletal myoblasts, the cells were suspended in 4 wt% GelMA, with 1 wt% Pluronic, $0.4$ mg mL$^{-1}$ Matrigel, and 0.1 wt% LAP used as a photoinitiator. Myoblasts suspended in the hydrogel precursor were patterned using the acoustofluidic device. Four different hydrogel samples were prepared using different acoustic parameters. The frequencies of 2490, 2900, and 6820 kHz for three samples respectively were applied, with no voltage signal applied for fourth sample. A higher voltage (15 V, peak to peak) for the lower frequencies (2490 and 2900 kHz) was also applied as the acoustic force scales directly with the frequency, making higher voltage unnecessary for the highest frequency applied.

All four samples were collected in the cell growth medium, and incubated in myoblast growth medium. On day 3, a part of each of the samples was used for quantifying the patterning of cells in the...
hydrogel samples. The rest of the samples were incubated in myoblast differentiation medium to initiate myotube formation. The samples were incubated for seven additional days. On day 10, the samples were washed in HBSS +/− twice for 5 min each and then fixed in 4% paraformaldehyde solution (in HBSS +/−) for 45 min on a shaking table. The fixed samples were washed again three times and incubated in a blocking solution (2% donkey serum and 0.5% triton X-100 in PBS) for 1 h at room temperature. The samples were stained for MyHC by incubation first in the primary antibody solution (1 μg mL−1 mouse ant Myersin heavy chain monoclonal antibody in blocking solution; Biotechne MAB4470-SP) overnight at 4 °C. The samples were then washed (0.5% Tween 20 in PBS) for 10 min at room temperature and then incubated in the secondary antibody solution (2.5 μg mL−1 Donkey Anti-Mouse IgG NorthernLightsTM NL557-conjugate; Biotechne, NL007) overnight at 4 °C. The samples were then washed twice with PBS for 5 min at room temperature. Actin (AlexaFluor 488 Phalloidin; A12379, ThermoFisher) and nuclei (DAPI; D9542, Sigma–Aldrich) were stained according to manufacturer’s protocols for ~30 min at room temperature.

**Gene Expression Analysis:** The following samples were prepared for gene analysis: 1) Patterned cells (pat, collected at day 10, 7 days after inducing differentiation), 2) unpatterned cells (unpat, collected at Day 10, 7 days after inducing differentiation), 3) 3D differentiated cells in bulk gels (3D diff, collected at Day 10, 7 days after inducing differentiation), 4) 3D undifferentiated cells in bulk gels (3D undiff, collected at day 3, without differentiation), and 5) 2D differentiated samples (2D, collected when spontaneously twitching myotubes were visible, ~4 days post differentiation). Same gel formulation and cell densities were used for samples 1–4. Samples were lysed and homogenized (2D sample was only lysed) in Trizol (ThermoFisher, 15 596 026) and RNA was isolated from the homogenized lysates using phase separation. cDNA was obtained using a high-capacity cDNA reverse transcription kit (Thermofisher, 4 368 814). 5 ng of cDNA was used for gene expression analysis using PrimeTime™ Gene Expression analysis system (IDT, 1 055 770). The list of primers and probes is given in **Table 2** below. Gene expression results were normalized to the endogenous control (mGapdh) and to the 2D sample.

**Statistical Analysis:** For the data on the effect of Pluronics on cell viability, paired, two-tailed student t-test was used to compare samples (n = 6) from the same day. For the cell viability data from Live/Dead staining, one-way ANOVA was used to compare the three samples (n = 3).

**Table 2.** Sequence of primers and probes used for gene expression analysis.

|            | Fwd                                  | Rev                                  | Probe                                                |
|------------|--------------------------------------|--------------------------------------|------------------------------------------------------|
| mGapdh     | 5′-CTGCACTCATCAGACATCTAG-3′           | 5′-AATGTGAAGTCCGGTTGTCG-3′           | 5′/36-FAM/TCAGGCCTG/EN/CACCCCTGCTG/3IAbKQFQ/-3′      |
| mMyod1     | 5′-GACAACACCCGAACCTTT-3′              | 5′-GCTCTGATGCCAGTGATG-3′             | 5′/36-FAM/AGCACACC/EN/CCTACTACAGTGG/3IAbKQFQ/-3′    |
| mMyh1      | 5′-CTGACTCTTGCTTGATCTCT-3′            | 5′-GGCCAGCTCCAAGTCTGCTCT-3′          | 5′/36-FAM/AGGCCCCAA/EN/TCAGAGTCGAT/GCGGA/3IAbKQFQ/-3′|
| mMyog      | 5′-TGTCGACCCACATCTAGC-3′              | 5′-GACAGTTAAGAGGAGTCAGAGGAT-3′      | 5′/36-FAM/TCCTCACC/EN/AGGACATGATCCT/3IAbKQFQ/-3′    |

**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.

**Author Contributions**

D.V.D. formulated the project, designed and performed the experiments, wrote the paper. P.R. designed the early acoustofluidic devices and formulated the project. O.D. optimized the protocols for the hydrogels. J.Z. and O.B.N. designed muscle cell experiments, gene analysis experiments, and isolated primary myoblasts. C.L. designed and processed gene analysis experiments. V.K. designed and calibrated temperature measurement experiments and setup. M.W.T. formulated the project, designed experiments, supported hydrogel synthesis and cell culture experiments, wrote the paper. J.D. formulated the project, designed experiments, supported acoustofluidic experiments, wrote the paper.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**

3D cultures, acoustofluidics, cell patterning, extrusion printing, muscle tissue engineering

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