Direct Patterning of Mammalian Cells in Ultrasonic Heptagon Stencil

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***Abstract*- We describe the construction of a ultrasonic device suitable for micro patterning particles and cells for tissue engineering applications. The device is formed by seven transducers shaped into a heptagon cavity. By exciting two and three transducers simultaneously, lines or hexagonal shapes can be formed with beads and cells. Furthermore, phase control of the transducers allows shifting the standing waves and thus patterning at different positions on a surface in a controlled manner. The paper discusses direct patterning of mammalien cells by ultrasound "stencil".**

***Keywords*-** Ultrasound stencil microsystem, acoustic standing waves, patterning cells, sonotweezers

I. INTRODUCTION

Arranging cells in an organised, controlled fashion in order to establish proper intercellular interactions is one of the main challenges in tissue engineering. Current attempts to engineer physiologically functional tissue *in vitro* frequently rely on the use of printing technologies. For instance, soft photolithography stamps have been studied as a strategy for spatially controlling the invasion of cells into the scaffold thus facilitating the generation of organized tissues (Folch and Toner 1998; Zhang, Yan et al. 1999; Wilson and Boland 2003; Charest, Bryant et al. 2004; He, Halberstadt et al. 2004; Khademhosseini, Suh et al. 2004; Iwanaga, Akiyama et al. 2005; Weibel, Lee et al. 2005). While these methods rely on substrate modification for subsequent cell patterning, other approaches try to directly manipulate particles and cells. Ultrasound devices using acoustic standing waves have been investigated towards this goal. In these approaches, acoustic waves generate patterns of high and low acoustic energy, and the microparticles aggregate at the acoustic pressure nodes. Both Standing Acoustic Wave (SAW) and Standing Surface Acoustic Wave (SSAW) devices have been demonstrated to be efficient methods for patterning cells (Shi, Ahmed et al. 2009; Shi, Xiaoyun et al. 2011). However, the systems that are described

generate fixed patterns of acoustic pressure nodes/antinodes and thus do not allow, for example, flexible successive patterning of different types of cells. Finally, optical tweezers (Ashkin, Dziedzic et al. 1987; Grier 2003) also allow manipulating and patterning of micro/nanoscale objects with unprecedented precision. However, optical tweezers have complex optical setups, which are difficult to miniaturize, and are limited in the number of cells that can be manipulated at the same time.

Here, we present a device which operates as an acoustic "stencil", allowing one to pattern cells into geometric patterns that can be positioned at will on the substrate surface. The device consists of a heptagon enclosure (c.a. 2cm) surrounded by ultrasound transducers operating at a frequency of 4 MHz. The acoustic forces responsible for the particle trapping are formed by the acoustic standing waves (interference patterns) generated by activating two or three transducers set at an angle to each other. In these standing waves the particles immobilize at the minima of energy. The acoustic beams, emanating from the transducers, cross within the chamber forming trapping geometries of lines or hexagons, respectively when two or three transducers are simultaneously excited. The patterns of maximum acoustic energy, therefore act as an acoustic stencil since the particles will not immobilize along these patterns, but instead settle along the minima of the acoustic energy. Electronic adjustment of the relative phases of the excitation signals allows the standing wave patterns to be moved at will on the substrate surface. In this paper, the structure of these acoustic devices, termed sonotweezers is discussed in detail as well as their particle and cell patterning capabilities.

II. STRUCTURE AND WORKING MECHANISM

The sonotweezers device was created by bonding NCE51 Noliac Ceramic lead zirconate titanate (PZT) (*E.P. Electronic Components Limited, UK*) plates to a flexible printed circuit board (*Flexible dynamics Ltd, UK*) and folding it into a heptagon. The flexible circuit was designed with holes along the folding edges to facilitate the shaping of the device. Once the circuit is folded, the holes are sealed.

As shown in Fig. 1a, the heptagonal flexible printed circuit board was first sandwiched between two poly(methyl methacrylate) (PMMA) plates to create a sealed unit. Silicone rubber aquarium glue was used as a sealant to ensure biocompatibility of the device. Secondly, the sealed heptagon was mounted on a rigid printed circuit board to allow simple electrical connection to each transducer element.

To reduce these unwanted effects, an agar layer (1.5% Agar in deionised water; *Fisher Scientific UK Ltd, UK*) was deposited at the bottom of the device, thus creating a smaller cavity (0.6 ml of liquid) above the agar that very effectively reduces streaming. Agar has acoustic properties similar to water and does not change the

acoustic wave interference patterns (Zell, Sperl et al. 2007). Fig. 1b shows a schema of the heptagonal chamber with the agar layer.

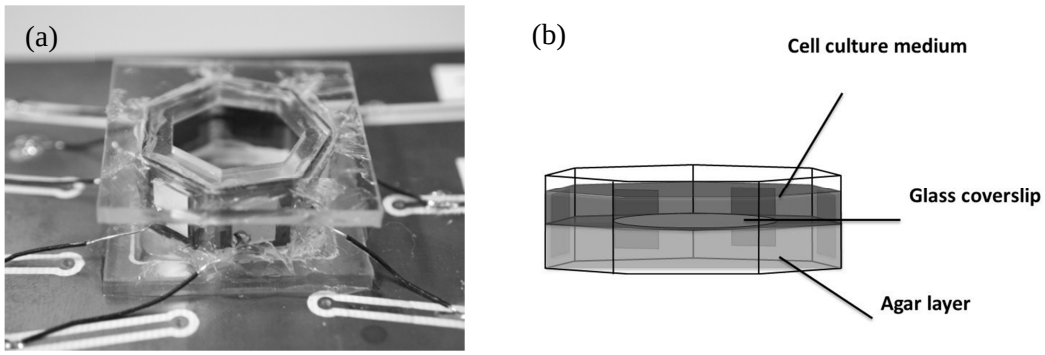


Fig. 1 (a) Device shaped into a heptagon and bonded to a PCB for easy connection of each channel (cell size ~ 2 cm), (b) Schematic of the heptagonal chamber with the agar layer and the cover slip placed in the middle of the cavity.

The PZT transducer plates were 5 mm x 5 mm with a thickness of 0.5 mm. The flexible circuit board was a ribbon of 10 mm width and 72 mm in length, each face of the heptagon being 10 mm long.

Synchronisation between channels was achieved using an arbitrary waveform generator providing four output channels (*TGA12104, Aim and Thurlby Thandar Instruments, UK*) allowing independent control of the amplitude, phase and frequency. The signals from the waveform generators were amplified and electronically matched by high speed buffers, BUF634T (*Texas Instruments, UK*).

This method offers multiple advantages over previous approaches, including a small overall device, compatibility with sterile cell culture, simplicity in experimental setup and control as well as the potential for integration with analytic sensors modules.

The influence and combination of two and three simultaneously excited transducers was investigated and discussed in detail elsewhere (Bernassau, Chun-Kiat et al. 2011). In this paper, each excited transducer was separated by at least one inactive transducer. For the two transducer (1-3) setup, the excited transducers were separated by one inactive transducer (Fig. 2a). In the three transducer (1-3-5) setup, transducer (3) is separated by one inactive transducer on each side (Fig. 2b). In this study Only 180° phase shifts were used to move the micro beads or cells.

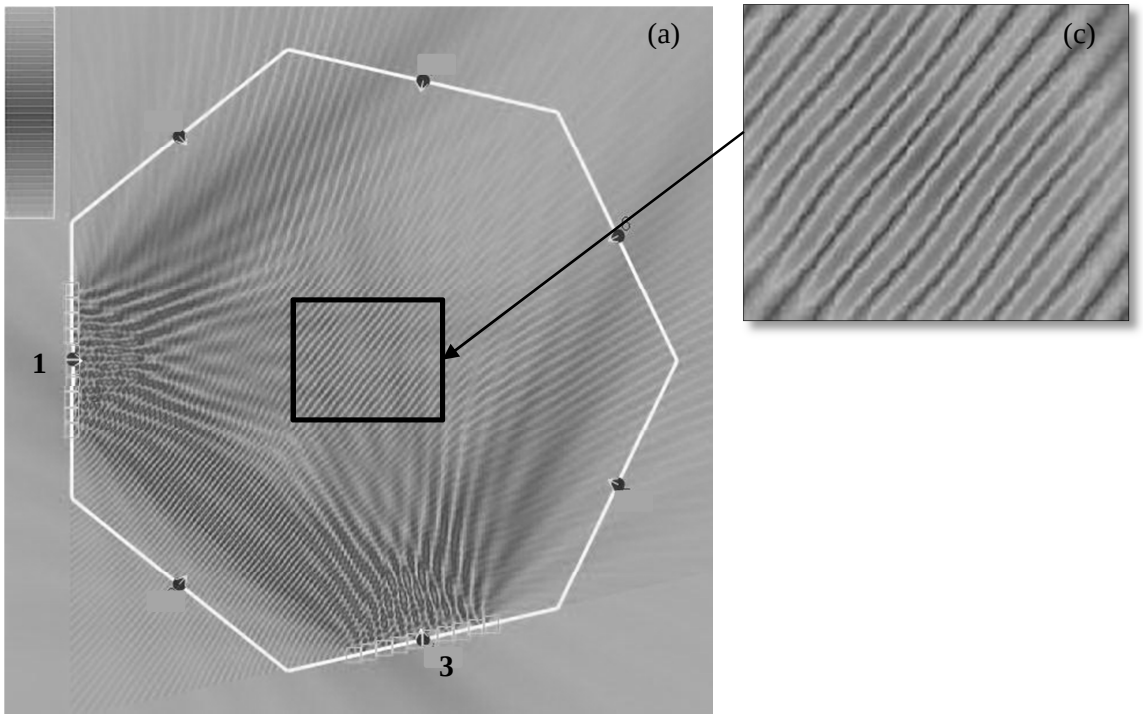
Fig. 2 shows the computer simulation results obtained with two and three transducers excited simultaneously showing the theoretically expected patterns. The program is based on Huygen's principle and simulates the acoustic pressure distribution within the heptagonal cavity. In order to simplify the model, the boundaries were

assumed to be perfectly absorbing. The wave field generated by one transducer, *g(r)*, was modelled as the sum of several simple cylindrical point sources, *f(r)*:

$$f(r) = Ae^{\frac{-\alpha r}{\lambda}} \cos(\omega t - kr + \Phi)$$

$$g(r) = \sum_{i=1}^{n} f(r_i)$$

Where A is the amplitude, α is the damping factor, λ is the wavelength, and Φ is the initial phase in degrees.



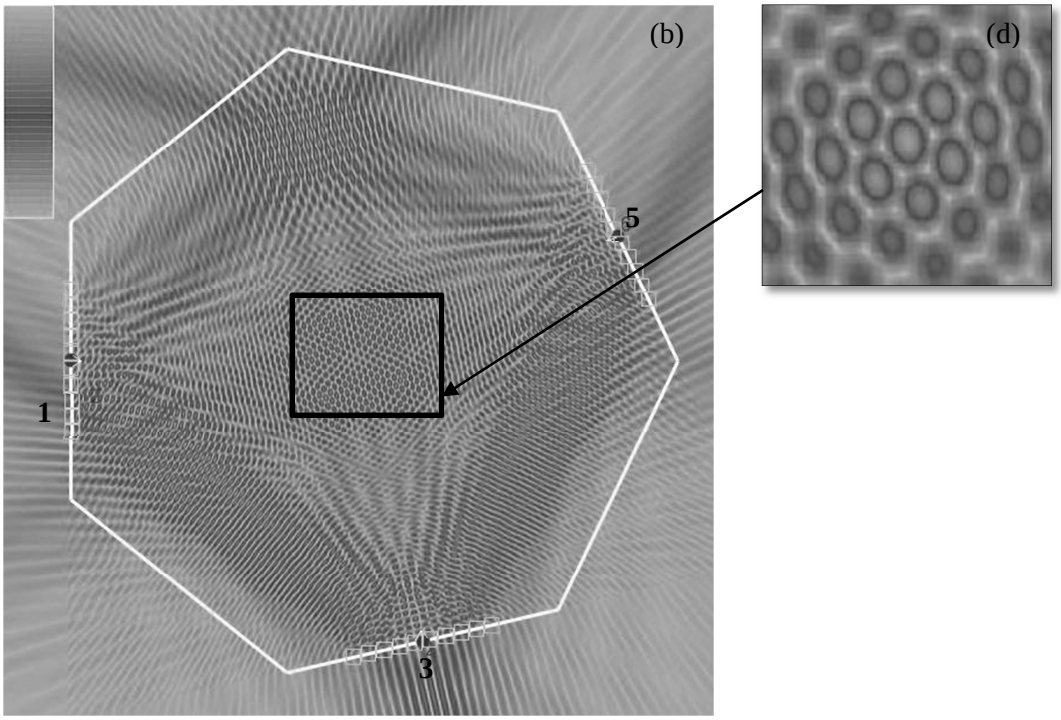


Fig. 2 Simulation results showing the creation of standing waves in the middle of the heptagonal cavity when (a) two (b) three transducers are excited simultaneously. The maxima are dark, and the minima are white; (c), (d) show the central area of (a) and (b) respectively when magnified by 10 times. (c) clearly shows the minima (dark) as edges that are useful for trapping material in lines, whereas (d) shows hexagonal traps.

It can be seen in Fig. 2 that the most regular standing wave patterns are situated in the middle of the heptagonal cavity, at the intersection of the travelling waves and the experiments follow this behaviour. For two active transducers a linear pattern of nodes and antinodes is formed (Fig. 2c). For three active transducers, the nodes adopt a pattern with a hexagonal shape (Fig. 2d). It will be shown that the particles and the cells accumulate at the acoustic nodes of minimum acoustic energy.

III. RESULTS AND DISCUSSION

In the original device, substantial particle movement within the acoustic nodes was observed in the liquid, probably due to streaming effects. For example, the particles would travel along but within the lines in which they are trapped (2 transducers case). This adverse phenomenon was ascribed to the large amount of fluid that the cavity contains (~ 2ml of liquid) and to the unbalanced acoustic energy of the travelling waves generated by the transducers that are at angle with each other. Under these conditions, it was difficult to pattern the entities in a stable manner.

A. Patterning of micro polystyrene beads

Polystyrene particles were first used in a proof-of-concept experiment to ascertain and optimize the trapping capabilities of the heptagonal device. All experiments were performed using 10 μm diameter polystyrene beads (*Polysciences Europe, Germany*). The transducers were excited with 8 V_{pp} at a frequency of 4 MHz. In all experiments particles were reproducibly trapped by exciting either two or three transducers simultaneously and the position of the particles could be controlled by shifting the phase of one of the transducers relative to the others.

When two transducers are simultaneously excited, the nodes created are along lines that bisect the angle formed by the two transducers. The distance d between the nodes can be calculated by $d = \lambda/2\sin(\theta/2)$, where θ is the angle formed by the normal to the planes of the two sides with the active transducers. In the case presented in the paper, θ is 105° and λ = 375 μm.

Fig. 3 shows the results when two transducers (1-3) are excited simultaneously. The particles align along lines with a separation distance of 236 μm. Fig. 3b shows the effect of a 180° phase shift of transducer 1: the particles move towards transducer 3 by about 118 μm. With the same combination, if the phase is in transducer 3 the particles moved towards transducer 1.

With three transducers (1-3-5), the particles cluster around acoustic nodes of minimum energy forming a clear hexagonal pattern (Fig. 3c). When the phase of one of the three transducers is shifted, the nodes and antinodes of acoustic energy are displaced, and the clustered particles follow accordingly. Fig. 3d shows the overlay of two photographs of the respective particle pattern with the phase of transducer 1 shifted by 180°. The red hexagon shape represents the agglomerated particles at phase equals 0° and the blue one represents the particles trapped when the phase of transducer 1 has been shifted by 180°.

Therefore, in this heptagonal device, particles can be trapped and displaced in a reproducible and predictable manner in two dimensional patterns. The patterns can be displaced while at the same time retaining their relative positions, simply by shifting the phase of one of the transducers.

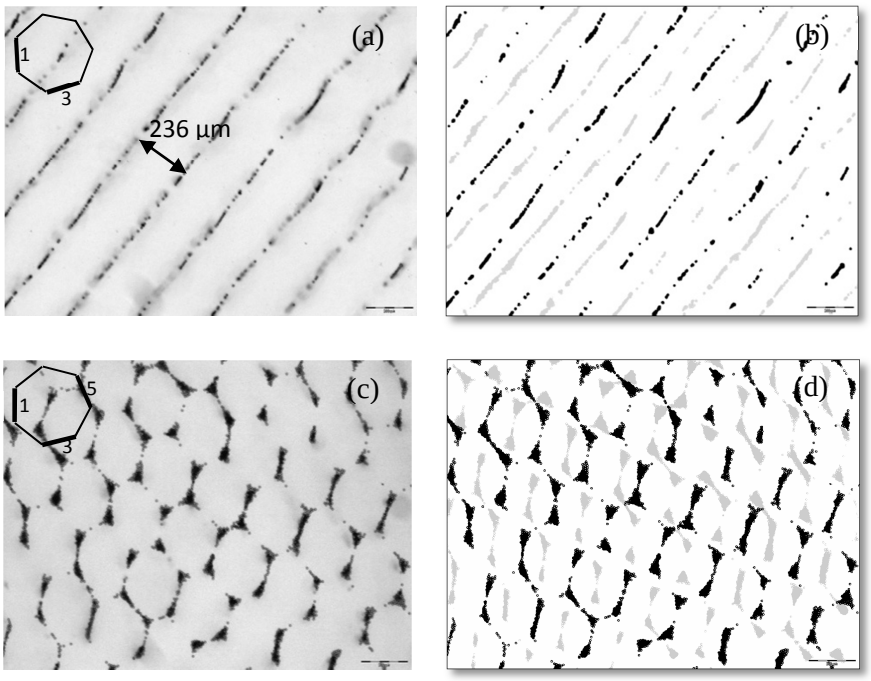


Fig. 3(a) Micrograph showing trapped 10 µm polystyrene particles with transducers 1 and 3 active (inset), the distance between minima is 236 µm. Scale bar = 200 µm. (b) Overlay of two micrographs taken at two different relative phase shifts of transducer 1 and transducer 3 (black = 0°, grey = 180°) showing the movement of the particles by 118 µm, (c) Micrograph of particles clustered in a hexagonal arrangement when transducers 1-3-5 were active, scale bar = 200µm, (d) Overlay of two micrographs taken at two different relative phase shifts of transducer 1 with respect to transducer 3 and 5 (black = 0 °, grey = 180 °), showing the movement of the particles. For (b) and (d) the microscope and sample are kept in a fixed position. ImageJ was used to create a transparent background for the purpose overlay.

B. Patterning of cells

We further examined if the SonoTweezers technique can be readily used to pattern mammalian cells such as Madin-Darby Canine Kidney (MDCK) cells. In this context, the ability to maintain a sterile environment is a critical factor. It is also important to demonstrate that the cells keep their viability and were not damaged e.g. by the ultrasonic forces, or streaming, or excessive heat during the patterning process. Either two or three transducers were excited simultaneously at 8 V_{pp} and 4 V_{pp} respectively.

The heat generated by the ultrasound transducers can be a problem for cell viability as overheating will affect cell metabolism and their capacity to develop. We have investigated the heat generated by two and three transducers excited simultaneously over 90 min when the agar filled the cavity to the ¾ of the height of the heptagon. Fig. 4 shows the temperature over time. The starting temperature was 25.3 °C (room temperature) and

increased slowly to 26 °C. It can be noticed that the temperature after 20 min stays stable and does not exceed 26 °C.

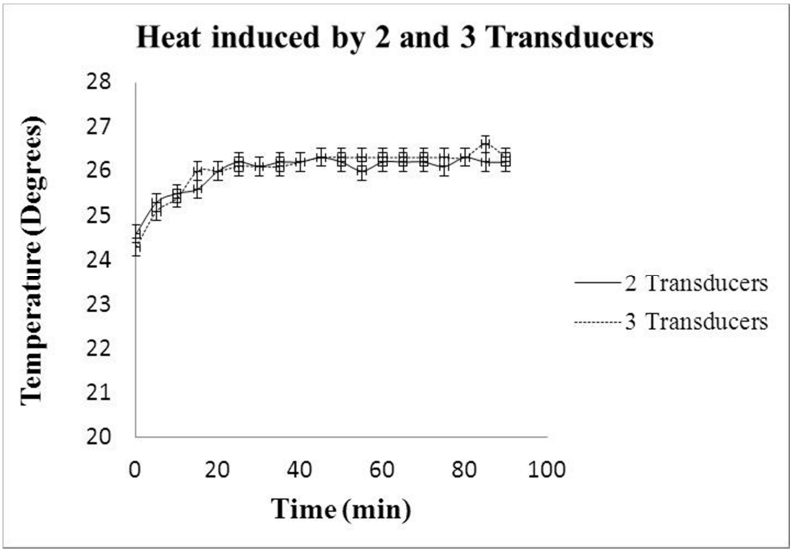


Fig. 4 Evolution of the temperature of the liquid over time.

We can conclude that under the experimental conditions used, that allow a good cell trapping and alignment, the heat generated by the transducer will be small and will not affect the cell viability.

Prior to the cell patterning experiments, the device was sterilized by rinsing briefly in 70% ethanol followed by three washes with sterile water. A 13 mm glass cover slip, coated with Poly-L-lysine (PLL, Sigma-Aldrich, Gillingham, UK) to improve adhesion, was placed in the middle of the cavity on top of the agar layer. Finally, 0.5 mL of cell culture medium (Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 100U Penicillin + 0.1 mg/ml Streptomycin) were added. A PMMA lid with a hole in the centre of the heptagon was placed on top of the device to maintain sterile conditions and to make sure that the cells added to the medium arrived close to the centre of the cavity, where the standing wave patterns formed.

For cell patterning, 200 µl of cell suspension at a concentration of 10^6 cells/ml were introduced into the SonoTweezers. Fig. 5 shows photographs of the MDCK cells patterned with two and three excited transducers. It can be easily seen that the patterns are identical to those formed with polystyrene beads. The cells agglomerated at the nodes of minimum acoustic energy.

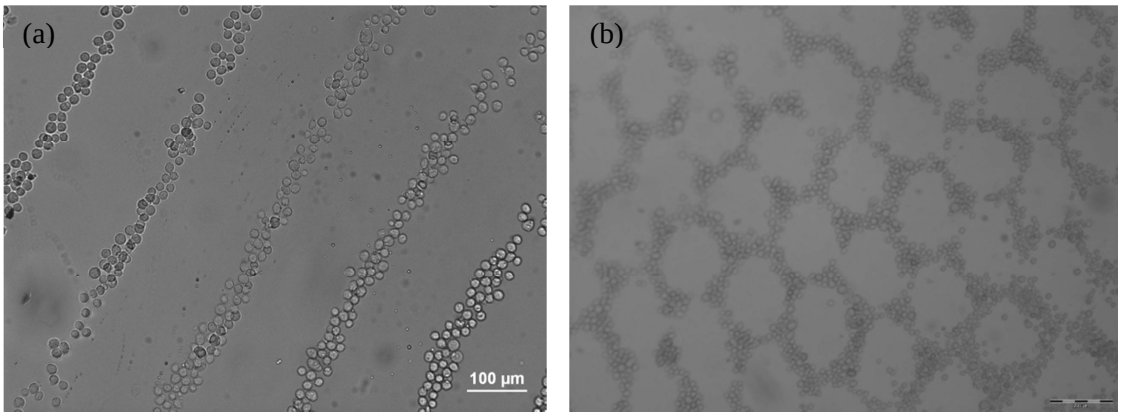


Fig. 5 Photographs of MDCK cells patterned with (a) two (b) three transducers. Scale bar (a) 100 µm, (b) 200µm.

In order to check the viability of the process with regards to possible cell patterning by successive seeding into acoustically defined distinct places, we also performed a phase shift of 180° on transducer 1 with a 1-3 combination on the patterned cells. A first layer of cells was introduced and patterned as mentioned above. After allowing the cells to adhere to the PLL glass coverslip for 1 hour a 180° phase shift was performed. Fig. 6 shows photographs of the patterned cells after letting them adhere for 1 h under ultrasound (a), and after 10 min with a 180° phase shift on transducer 1 (b) and then 5 min after adding new cells (c). It can be seen that after the 180° phase shift only a very small amount of cells have escaped their initial position, while the majority of cells remained well attached to the substrate. To quantify the displacement of the cells after 10 min of 180° phase shift from their initial position, Particle Image Velocimetry (PIV) software was used (*JPIV*, (Vennemann 2007)). Fig. 7 shows the displacement of the MDCK cells after 10 min 180° phase shift. We observe that most of the cells moved by less than 5 pixels from their initial position. A possible explanation for the cells being moved could be that they had been forced on top of others, during the initial patterning, preventing attachment to the cover slip surface. However, we cannot exclude the possibility that a small subset of cells had been affected by the exposure to ultrasound thereby which could have prevented them from adhering to the substrate.

After establishing that the initial pattern was stable after phase shifting transducer 1, we applied a second batch of cells (at the same concentration as before). As expected these cells were trapped at the shifted acoustic pressure nodes and settled in-between the first batch of cells as illustrated in figure 6 (c). This result exemplifies the general usefulness of the device for cell engineering applications that require controlled deposition of cells onto a substrate.

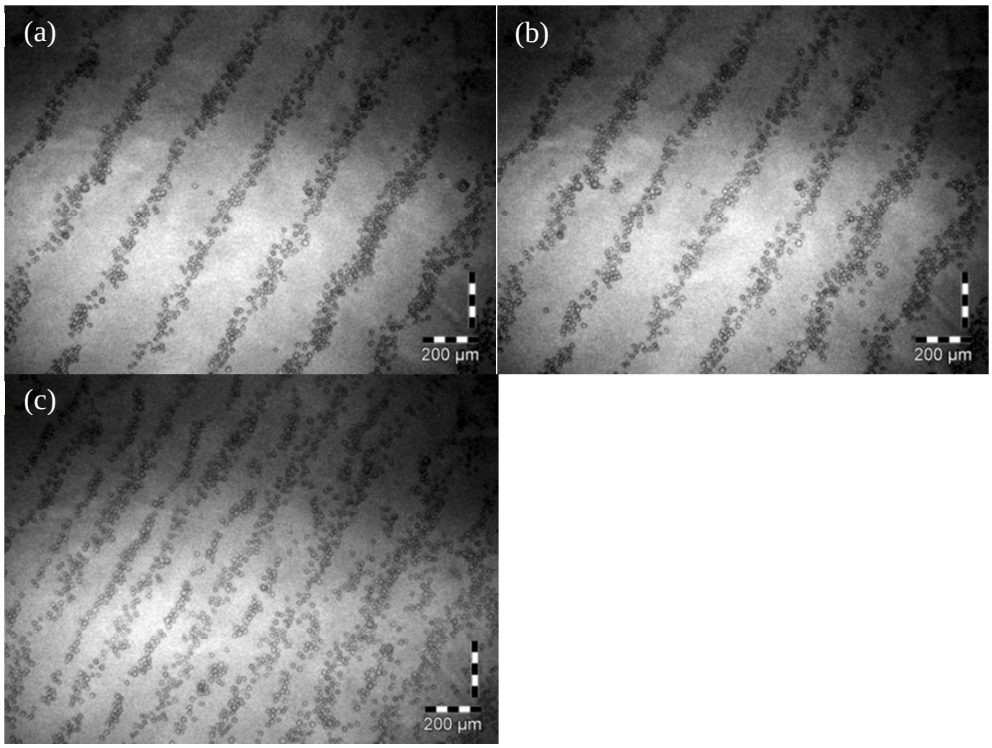


Fig. 6 Photographs of MDCK cells patterned with two transducers (a) after 1hour with the ultrasound switch on , (b), (c) after 10 min with a 180° phase shift on transducer 1, (c) with a second batch of cells added after the phase shift.

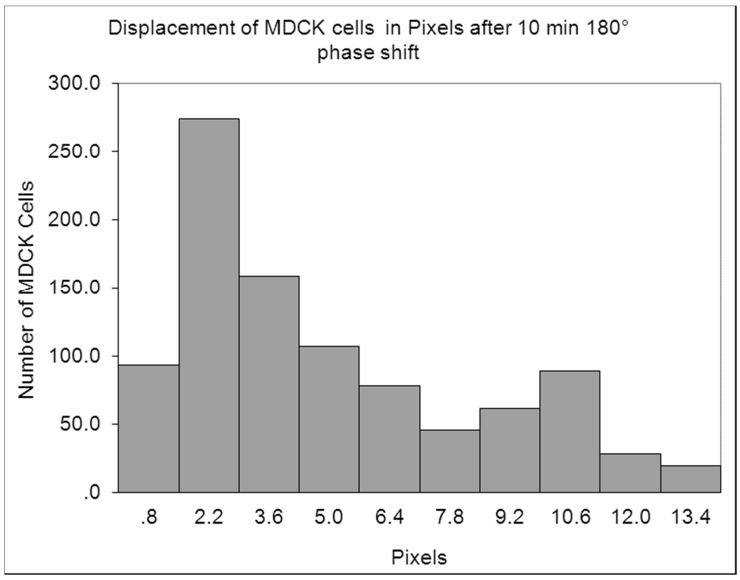


Fig. 7 Histogram showing the MDCK cells movement from their initial position and after 10 min of excitation at a phase shift of 180°.

IV. CONCLUSION

In this paper we presented a new acoustic device that can successively pattern particles and cells in a controlled fashion by electronic phase shifts. In effect the device is acting like a spatio-temporal acoustic stencil preventing

cells from setting in the areas on high acoustic energy. This device offers multiple advantages over previous approaches, including small device size, general biocompatibility and sterile cell culture capability, simplicity in experimental setup and control and the potential for integration with other analytic sensor modules.

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