Cell selective manipulation with single beam acoustical tweezers.

Michael Baudoin,1, 2 Jean-Louis Thomas,3 Roudy Al Sahely,1 Jean-Claude Gerbedoen,1 Zhixiong Gong,1 Aude Sivery,1 Olivier Bou Matar,1 Nikolay Smagin,1 and Alexis Vlandas†1

1 Univ. Lille, CNRS, Centrale Lille, Yncra ISEN, Univ. Polytechnique Hauts-de-France, UMR 8520 - IEMN, SATT NORD, F-59000 Lille, France.
2 Institut Universitaire de France, 1 rue Descartes, 75005 Paris
3 Sorbonne Université, CNRS, Institut des NanoSciences de Paris, INSPI, F-75005 Paris, France.

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Acoustical tweezers open major prospects in microbiology for cells and microorganisms contactless manipulation, organization and mechanical properties testing. Yet, these tremendous perspectives have so far been hindered by the absence of selectivity of existing acoustical tweezers1–3 and/or their limited resolution restricting their use to large particle manipulation only11–13. Here, we report precise selective contactless manipulation and positioning of human cells in a standard microscopy environment, without altering their viability. The unprecedented selectivity and miniaturization is achieved by combining holography with active materials and fabrication techniques derived from the semiconductor industry to synthesize specific wavefields (called focused acoustical vortices14) designed to produce stiff localized traps. We anticipate this work to be a starting point toward widespread application of acoustical tweezers in fields as diverse as tissue engineering15,16, cells mechanotransduction analysis3,9, neural network study1, or mobile microorganisms imaging17–19, for which precise manipulation and/or controlled application of stresses is mandatory.

I. INTRODUCTION

Contactless tweezers based on optical17–19 and magnetic forces20–22 have been developed in the last decades and have led to tremendous progress in science recognized by several Nobel prizes. Nevertheless, these technologies have stringent limitations when operating on biological matter. Optical tweezers rely on the optical radiation pressure, a force proportional to the intensity of the wave divided by the speed of light. The high value of the latter severely limits the forces that can be applied and imposes the use of high intensity fields. This can lead to deleterious photothermal damages (due to absorption induced heating) and/or photochemical damages (due to excitation of reactive compounds like singlet oxygen23–25 adversely affecting cells integrity. Magnetic tweezers, on the other hand, can only manipulate objects susceptible to magnetic fields and thus require other particles to be pre-tagged with magnetic compounds, a limiting factor for many applications. For biological applications, acoustical tweezers are a prominent technology26–28. They rely on the acoustical radiation force29,30 which -as for their optical counterpart- proportional to the intensity of the wave divided by the wave speed. But, the dramatically lower speed of sound compared to light leads to driving power several orders of magnitude smaller than in optics to apply the same forces (or conversely, forces several orders of magnitude larger at the same driving power). In addition, the innocuity of ultrasounds on cells and tissues below cavitation threshold is largely documented31–37 and demonstrated daily by their widespread use in medical imaging38. Indeed, the frequencies typically used in ultrasound applications (100 kHz to 100 MHz) are far below electronic or molecular excitation resonances thus avoiding adverse effects on cells integrity. Moreover, the weak attenuation of sound in both water and tissues at these frequencies limits absorption induced thermal heating. Finally, almost any type of particles (solid particles, biological tissues, drops) can be trapped without pre-tagging39 and the low speed of sound enables spatial resolution down to micrometric scales even at these comparatively low frequencies.

Nevertheless, the promising capabilities offered by acoustical tweezers have so far been hindered by the lack of selectivity of existing devices and/or their restricted operating frequency limiting their use to large particles only. Yet, the ability to select, move and organize individual microscopic living organisms is of the utmost importance in microbiology for fields at the forefront of current research such as single cell analysis, cell-cell interaction study, or to promote the emergence of disruptive research e.g. on spatially organized co-cultures. In this paper, we unleash the potential of acoustical tweezers by demonstrating individual biological cells manipulation and organization in a standard microscopy environment with miniaturized one-sided acoustical tweezers.

II. ACOUSTICAL TWEEZERS DESIGN

The first experimental evidence of large particles trapping with acoustic waves dates back to the early 20th century.40 Nevertheless, the first demonstration of controlled manipulation of micrometric particles and cells with acoustic waves appeared only one century later with the emergence of microfluidics and high frequency transducers based on interdigitated electrodes.41 In these recent works, trapping relies on the 2D superposition of orthogonal plane standing waves, an efficient solution for the collective motion of particles, but one which precludes...
any selectivity, i.e. the ability to select and move one particle out of a population. Indeed, the multiplicity of nodes and anti-nodes leads to the existence of multiple trapping sites which cannot be moved independently. In addition, multiple transducers or reflectors positioned around the manipulation area are mandatory for the synthesis of standing waves, a condition difficult to fulfill in many experimental configurations.

Selective trapping with single beam requires strong spatial localization and hence tight focusing of the wavefield. In optics, this ability has been achieved with focused progressive waves, a solution also investigated in acoustics. But such wavefields are inadequate in acoustics for most particles of practical interest, since objects with positive contrast factors (such as rigid particles or cells) are attracted to pressure nodes and would be expelled from the focal point of a focused wave. Acoustical vortices provide an elegant solution to this problem. These focused helical progressive waves spin around a central axis wherein the pressure amplitude vanishes, surrounded by a ring of high pressure intensity, which pushes particles toward the central node. Two-dimensional trapping and three-dimensional levitation and trapping have been previously reported at the center of laterally and spherically focused vortices, respectively. However, all these demonstrations were performed on relatively large particles (>300 µm in diameter) using complex arrays of transducers, which are cumbersome, not compatible with standard microscopes, and that cannot be easily miniaturized to trap micrometric particles. Recently, Baudoin et al. demonstrated the selective manipulation of 150 µm particles in a standard microscopy environment with flat, easily integrable, miniaturized tweezers. To reach this goal, they sputtered holographic electrodes at the surface of an active piezoelectric substrate, designed to synthesize a spherically focused acoustical vortex.

Nevertheless, transcending the limits of this technology to achieve selective cells manipulation remained a major scientific and technological challenge. Indeed, the system should be scaled down (frequency up-scaling) by a factor of 10 (since cells have typical size of 10 µm), while increasing dramatically the field intensity, owing to the low acoustic contrast (density, compressibility) between the cells and surrounding liquids. In addition, since the concomitant system’s miniaturization and power increase are known to adversely increase the sources of dissipation, the tweezers had to be specifically designed to prevent detrimental temperature increase and enable damage free manipulation of cells.
First, spherically focused acoustical vortices (Fig. 1A) were chosen to trap the particles. Indeed, the energy concentration resulting from the 3D focalization (Fig. 2F) enables to reach high amplitudes at the focus from remote low power transducers. These spherically focused vortices were synthesized by materializing the hologram of a 45 MHz vortex with metallic electrodes at the surface of an active piezoelectric substrate. The hologram was discretized on two levels resulting in two intertwined spiralling electrodes (Fig. 1D), patterned in a clean room by standard photo-lithography techniques (see Methods section A). The scale reduction compared to our previous generation of acoustical tweezers is illustrated in Fig. 1B. Second, the design of the electrodes was optimized to reduce Joule heating (magnified by the scale reduction) inside the electrodes. To prevent this effect, (i) the thickness of the metallic electrodes was increased by a factor of 2 (400 nm of gold and 40 nm of titanium); (ii) the width of the electrical connections (Fig. 1D) supplying the power to the spirals was significantly increased to prevent any dissipation before the active region; and (iii) two radial electrodes spanning half of the spirals were added as a way to effectively bring power to the driving electrode. Third, a 1.1 mm glass substrate (Fig. 1A, 1C) was glued to the electrodes and placed in between the transducers and the microfluidic chamber wherein the cells are manipulated. This glass substrate has a double function: (i) it enables the fociization of the wave and (ii) it thermally insulates the microfluidic device from the electrodes thanks to the poor thermal conductivity of glass.

The final device hence consists of (see Movie M1 in SI, Fig. 1A, 1C, 1E): (i) spiralling holographic transducers generating an acoustical vortex which propagates and focuses inside a glass substrate; (ii) a microfluidic PDMS chamber supported by a glass slide containing cells and placed on top of the substrate, wherein the acoustical vortex creates a trap and (iii) a motorized stage that enables the X,Y displacement of the microfluidic chamber with respect to the trap. The whole transparent setup is integrated in an inverted microscope as depicted in Fig. 1E.

III. CHARACTERIZATION OF THE ACOUSTICAL TRAP

The principle of high frequency acoustical vortices synthesis with these active holograms was assessed through the comparison of numerical predictions obtained from an angular spectrum code and experimental measurements of the acoustic field normal displacement at the surface of the glass slide (XY plane) with a Polytech UHF-120 laser Doppler vibrometer (Fig. 2 A-D). Both the intensity and phase are faithful to the simulations and demonstrate the ability to generate high frequency acoustical vortices. As expected, the wavefield exhibits a central node (corresponding to the phase central singularity) surrounded by a ring of high intensity which constitutes the acoustical trap. The magnitude of the acoustic field (displacement) depends on the driving electrical power and was measured to vary typically between 0.1 nm and 1 nm, at the electrical power used in the manipulation experiments. This corresponds to acoustic powers lying between 20 µW and 2 mW (see Methods section H). The concentration of the acoustic energy through focalization in the propagation plane (XZ) can be seen in Fig. 2E.

An estimation of the lateral force field exerted on a cell of 10 µm radius with density 1100 kg m$^{-3}$ and compressibility $4 \times 10^{-10}$ Pa$^{-1}$ was computed at each point in the manipulation plane of the microfluidic chamber (XY plane, Fig. 2F) with the theoretical formula derived by Sapozhnikov & Bayley. This calculation gives an estimation of the force of the order of 100 pN (see Methods section D for the exact values depending on cells acoustic properties) for an acoustic vibration of 1 nm, corresponding to the upper bound reported for manipulation of objects with optical tweezers.

Finally, the temperature increase due to Joule heating in the electrodes as well as the total temperature increase due to both Joule heating and acoustic wave absorption was measured using an infrared camera to assess potential impact on biological material (See Methods section I). The temperature increase measured after 2 min of manipulation is not significant at the lowest power (displacement amplitude 0.1 nm) while it reaches 4.5 °C at the top of the glass slide and 5.6 °C at the highest power inside a drop of glycerol placed on top of the glass slide and acting as a perfectly absorbing liquid. These measurements indicate that the first source of heat is Joule heating in the electrodes which could be solved by active cooling of the transducer. They also suggest that even at the largest power used in the present experiments, the moderate temperature increase remains compatible with cells manipulation, as assessed in the next section.

IV. CELLS MANIPULATION, POSITIONING AND VIABILITY

Cell manipulation is demonstrated in a microfluidic device integrated in a standard inverted microscope (Fig. 3) to illustrate the fact that our approach can be easily transposed to standard microbiology experiments. The device is composed of a thin glass slide treated to prevent cell adhesion and a PDMS chamber of controlled height (38 µm). The cells are loaded by placing a drop of the cell suspension (10-20 µL) on the glass surface using a micro-pipette and carefully lowering the chamber on top of the drop. The position of the vortex core is spotted with four triangular marks deposited at the surface of the glass substrate. Using a XY positioning system it is thereafter possible to align the tweezers center to any cell present in the chamber. Upon activation of the AC driv-
FIG. 2. Acoustic field and radiation forces. A-D. Numerical predictions (A and B) and experimental measurements (C and D) with a UHF-120 Polytec laser Doppler vibrometer of the normalized modulus (A and C) and phase (C and D) of the acoustic normal displacement at the surface of the glass slide (XY plane). In the experiments presented in this paper, the amplitude of the vibrations (displacement) lies typically between 0.1 nm and 1 nm depending on the electrical power applied to the transducers. E. Simulated evolution of the amplitude of the acoustic field in the propagation plane (XZ). This simulation illustrates the concentration of the acoustic energy through focalization. F. Normalized magnitude and distribution of acoustic forces. Left: the white arrows show the convergence of the force field toward the center of the beam but also that the first ring is repulsive for particles located outside the trap. Right: Magnitude of the lateral force along the green dashed line plotted on the left figure. When the force is negative, the particle is pushed toward the center of the acoustic vortex, while when it is positive it is pushed outward. Zero values correspond to static equilibrium positions. The magnitude of the maximum trapping force computed with the code varies between 30 pN and 650 pN (see Methods section D) for vibration amplitude of 1 nm (acoustic power of 2 mW) depending on the exact cells acoustic properties

ing signal, a cell situated inside the vortex core is nearly instantaneously trapped.

The first demonstration of the selective nature of our tweezers is showcased by our ability to pick up a single cell (breast cancer cell MDA-MB-231, $7 \pm 1 \mu m$ in radius) amongst a collection of cells and move it along a slalom course where other free to move cells act as poles (see Fig. 3A and Movie M2). Then a second cell initially serving as a slalom marker, is moved to prove that it was free (Movie M2). The precise displacement can be performed in any direction as demonstrated by the square motion of a cell around another (Fig. 3B, Movie M3). Displacement can be performed even in the presence of other cells without any risk of "coalescence" as the first ring acts as a barrier. As can be seen in Fig. 3C, the radius of the first repulsive ring is typically 40 $\mu m$. The second ring of much weaker intensity can also slightly affect free cells at large power.

One of the key ability enabled by acoustical tweezers is the capture, positioning and release of cells at precise locations. As an illustration, a total of 10 individual MDA cells were therefore positioned to spell the letter "A" and "T" of "Acoustical Tweezers" (Fig. 3C). The total manipulation time to achieve these results was kept under 10 min (less than 2 min per cell). All the operations represented in Fig. 3 were performed with acoustic vibration displacements < 0.5 nm. Finally, we performed some experiments to test the displacement speed obtained with our tweezers. We obtained a linear speed of 0.4 mm s$^{-1}$ for vibration amplitude of about 0.6 nm (corresponding to an acoustic power of 800 $\mu W$). As a comparison, this speed corresponds to the upper bound reported by Keloth et al with optical tweezers, obtained here with a power 35 times weaker and no photoxicity. Furthermore, unlike with optical tweezers, it is still possible to increase this speed with acoustical tweezers by increasing the actuation power and improving the thermal management of the device, as most of the dissipated power comes from the transducer and not from the direct absorption by the medium.

As described in the introduction, one of the main gains which can be expected from transitioning from optical to acoustic tweezers is the absence of deleterious effects of the latter when manipulating live cells. The short-term and long-term viability was investigated using a fluores-
FIG. 3. A. Stack of images illustrating the selective manipulation of a human breast cancer cell (MDA-MB-231) of radius $7 \pm 1 \mu m$ between other cells. The blue dotted line and green continuous line show respectively the future and past path followed by the cell. (See also Movie M2) B. Image illustrating the square relative motion of a trapped cell "1" of $7 \pm 1 \mu m$ (located in the center of the picture) around another cell "2" obtained by superimposing the images of the two cells in the frame of reference of the trapped cell (see also Movie M3). In this frame of reference, the successive positions of cell "2" form a square. For the sake of clarity other cells appearing in the field of view have been removed. C. Manipulation of 10 MDA cells (average radius $9 \mu m$ to form the letters "A" and "T" of "Acoustical Tweezers". Note that in these pictures the focus is voluntarily left under-focused to improve contrast of the cells.

cent viability assay as well as post exposure cell observation. A first set of experiments was thus conducted to address the short-term viability of MDA cells. The cells were captured for 2 min in the vortex at maximum power (amplitude 0.9 nm) to mimic a standard positioning sequence and observed for any sign of damage during manipulation and for 30 min afterwards. During manipulation, no increase of fluorescence was observed suggesting that the sound field does not induce membrane permeabilization which often correlates with viability decrease. After the tweezers were switched off, the cell did not display any increase of fluorescence and remained at an intensity well under the dead cells found nearby ($5 \times$ to $10 \times$ lower, see SI). This strongly supports that short-term damages produced by the acoustical tweezers is minimal.

It is however known that damages experienced by a cell can lead to its death for hours afterwards. To assess the long-term impact of cell manipulation using acoustical tweezers, we performed a viability assay overnight. The MDA cells were seeded at 60 ($\%$) confluence ratio in two glass devices with no surface treatment and left to re-adhere for 5h. Nine cells located at different positions in the two different microfluidic chambers were exposed to the tweezers of acoustic vortex at maximum power for 2 min each. An observation of the cells was performed after 19h (half the population doubling rate of MDA cells) to compare their viability with a control region of the device (see Fig. 4A). No extra mortality was observed in the illuminated region (dead/live cell ratio of 3%) compared to the statistics performed on the overall device (dead/live cell ratio of 5%). This likely indicates that the dead cells are depositing randomly and that the tweezers do not provoke extra mortality. We also studied in detail the fate of the nine illuminated individual cells (see Fig. 4B-E). All the cells exposed to the acoustic field (the green circle indicates the extension of the first ring of the vortex) and their immediate neighbours were alive and showed no difference compared to the nearby cells.

V. CONCLUSION AND OUTLOOKS

In this work, cell harmless selective manipulation has been demonstrated through the capture and precise positioning of individual cells amongst a collection in a standard microscopy environment. Both short-term and long-term viability of manipulated cells have been evaluated, showing no impact on cells integrity. This opens widespread perspectives for biological applications wherein precise organization of cells or microorganisms is a requisite. Furthermore, the radiation force which can be applied and the speed at which cells can be moved are at the state of the art currently known for optical tweezers, while requiring two orders of magnitude less wave power and with no deleterious effect such as phototoxicity. Engineering optimization of these tweezers to limit Joule heating will hence enable the application of stresses several orders of magnitude larger than with
optical tweezers without altering cells viability, a promising path for acoustic spectroscopy\textsuperscript{28}, cell adhesion\textsuperscript{53} or cell mechanotransduction\textsuperscript{11–13} investigation. In addition, new abilities could be progressively added to these tweezers: The focused vortex structure used for selective particle trapping in this paper is also known to exhibit 3D trapping capabilities\textsuperscript{3,5}. This function was not investigated here owing to the confined nature of the microchamber but could closely follow this work. Synchronized vortices could also be used to assemble multiple particles as recently suggested by Gong & Baudoin\textsuperscript{54}. This would enable to investigate tissue engineering and envision 3D cell printing. Finally, the most thrilling and challenging perspective to this work might be the future development of Spatial Ultrasound Modulators (analogies to Spatial Light Modulator in optics), designed to manipulate and assemble many objects simultaneously. While such revolution is on the way for large particles manipulation in air\textsuperscript{55–57}, it would constitute a major breakthrough at the microscopic scale in liquids wherein the actuation frequencies are 3 orders of magnitude larger. The present work hence constitutes the cornerstone towards widespread applications of acoustical tweezers for biological applications.

\* Corresponding author: michael.baudoin@univ-lille.fr; http://films-lab.univ-lille1.fr/michael
\* Corresponding author: alexis.vlandas@tiemn.fr
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