

Development and validation of a multi-modal customized device to stimulate *in vitro* cell culture systems

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ABSTRACT

This work focuses on the development and validation of a multi-modal stimulation device for *in vitro* cell culture systems. The device was designed to stimulate cells or tissues placed on 12-well culture plates. It is connected to customized software that controls the parameters of photobiomodulation (PBM) and ultrasound stimulation (US) through light-emitting diodes and piezoelectric disks, respectively. A wide range of stimulation protocols can be explored by modulating central frequency or wavelength, power density, and duration. Four different cell lines were used to validate the safety and functionality of the device. Human osteoblasts, chondrocytes, periodontal ligament fibroblasts, and mouse-derived neuronal cells were cultured and stimulated daily with ultrasound (1.0 MHz, 100 mW/cm², 5 min), light (810 nm, 7.5 mW/cm², 5 min) and combined stimuli. After three days, metabolic activity and proliferation were assessed. Different cell types demonstrated distinct biological responses to the stimuli, as osteoblasts and chondrocytes showed increased metabolic activity after combined stimulation or PBM, while the metabolic activity of human fibroblasts or neuronal-like cells was unchanged after three days. This highlights the importance of a rigorous optimization of stimulation protocols according to the target tissue. The safety of the device and its sterilization conditions were demonstrated as there was no cell death or contamination during *in vitro* stimulation. This work features a feasible, safe, and effective multi-modal stimulation device that can provide a wide range of stimulation protocols to better understand their effect on cells or tissues.

1. Introduction

The inefficacy of pharmacological approaches to overcome medical disabilities, such as musculoskeletal and brain disorders, has fostered the scientific community to focus on alternative therapeutic strategies [1,2]. The use of physical stimuli, namely light, electrical, magnetic, mechanical and thermal stimuli have been recognized as promising tools to stimulate tissues and trigger intracellular responses, promoting cell and tissue repair mechanisms [3–5]. Despite their ability to interact with cells and trigger therapeutic responses, distinct stimuli act differently on

the tissues.

Ultrasound (*i.e.*, high-frequency mechanical waves) has been used to mechanically stimulate cells. It is mostly applied in the range of 1.0 and 1.5 MHz frequencies and 30 mW/cm² to 200 mW/cm² intensities, inducing vibrations that propagate through biological tissues [6–9]. These vibrations cause physical disturbances on the surface of the cells which, in turn, activate deformation-sensitive channels and/or transmembrane receptors, giving rise to a chain of biological response events [10]. It has been demonstrated that ultrasound has neuromodulation effects, can promote bone healing and regeneration of soft tissues, such

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as cartilage and periodontal ligament [11].

Optical stimuli in the red and near-infrared range (600 to 1100 nm) and with low intensities have been used for therapeutic purposes as this radiation interacts with the mitochondria and different light-sensitive ion channels, stimulating the bioenergetics of the cell [12,13]. The low-intensity light effect on the tissues is referred as photobiomodulation (PBM), which causes minimal temperature variation in biological tissues, typically below 1 °C [14–17]. In case of significant temperature increase, PBM term cannot be employed. There are other stimulation modalities that use light to increase the temperature, such as photothermal and photodynamic therapies [18]. Recently, a hybrid concept known as thermophotobiomodulation has emerged, describing a synergistic interaction between light exposure and mild, controlled heating that may enhance cellular responses [19]. However, these therapies are not within the scope of this article. The PBM has been applied with a wide range of fluences from 0.4 mJ/cm² to 30 J/cm² [20–22], and intensities from 0.04 to 3500 mW/cm² to stimulate the activity of different cells and tissues [23,24]. It has been reported pain relief and improved physical function, as well as decreased inflammation [20].

The therapeutic effects of PBM and ultrasound stimulation (US) have been documented in the literature for several cell types, being already used at the clinical level [25–30]. However, despite the advances in their applicability and efficiency, the therapeutic potential of these stimulation modalities is yet suboptimal as their parameterization needs to be further explored. In fact, there are still no cell stimulation devices that allow researchers to execute customized protocols and assess different levels of parameters, such as wavelength, frequency, and power density. Also, the majority of the studies describing the application of the abovementioned stimuli in cells use devices designed for clinical stimulation, whose parameterization is unadjusted to stimulate cellular and tissue models. Moreover, the combination of different physical stimuli is yet unexplored, which could theoretically lead to a synergistic therapeutic action.

These limitations have been restricting the operability and optimization of the biological response induced in cells, hindering the

development of novel, safe, and effective therapeutic protocols. In this sense, we recognized the urgent need for the development of a device specifically designed to stimulate *in vitro* models. This would enable a more rigorous and extensive investigation of the effects that the stimuli trigger in cells, as well as a flexible parameterization of the stimuli, according to the type of cells and pathological conditions. It is proposed the development and validation of a multi-stimuli device delivering modulated PBM and US to *in vitro* models, enabling protocol optimization and the study of their effects on distinct cells or tissues.

2. Material and methods

2.1. Design and components

The stimulation device was designed to allow the application of optical and acoustic signals to any type of *in vitro* or *ex vivo* model (e.g., cell lines, primary cultures, bacteria, tissue slices) cultured in commercial 12-well culture plates. The device must be positioned aligned on top of the cell culture plate, allowing the cells in each well to be exposed to direct stimulation, as illustrated in Fig. 1. The stimulation device should be positioned on top of the 12-well culture plate, ensuring that light distribution is confined to each well, without irradiation outside of the well.

The stimulation device is perfectly fitted to the 12-well plate, and the position of both light emitting diodes (LEDs) and piezoelectric transducers is fixed relative to each well (Ø 19.6 mm), ensuring a controlled and reproducible alignment of the electronic components to each well, with constant stimuli propagation distances (both optical and acoustic) in all wells. Also, the piezoelectric transducers are immersed at the center of each well, at an 8-mm distance from cells and with 4.5 mL culture media, providing a consistent propagation distance to the bottom and walls. The LEDs are also positioned in a fixed arrangement relative to each well (at a 12-mm distance from cells). Therefore, this is a controlled setup that minimizes variability and avoids spatial randomness.

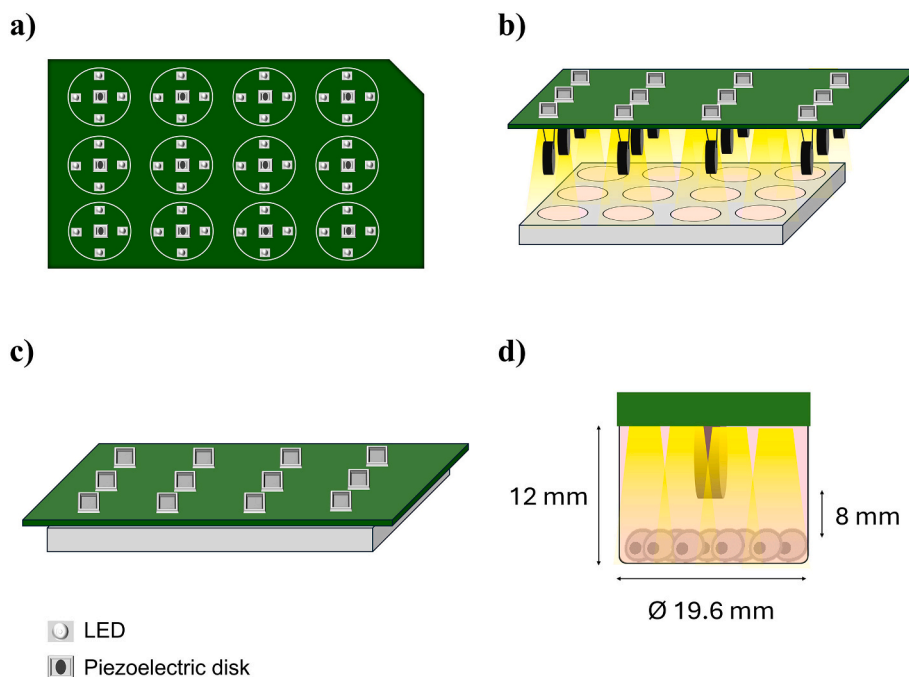


Fig. 1. Schematics of the stimulation plate to be used aligned on top of 12-well culture plate: **a)** upper view, **b)** lateral view, **c)** view of stimulation plate working placed directly on top of 12-well culture and **d)** a close-up of the actuation systems on the well cultured with cells, showing that LEDs are positioned at the top of the well, irradiating the whole well bottom, without light irradiation outside of the well, and showing that the piezoelectric disk is completely immersed in culture medium during stimulation at a 8-mm distance from cells.

2.1.1. Requirements for component selection

For the selection of the stimulation actuators, different factors were considered. At first, the therapeutic window of the physical stimuli was revised, and then, according to the preferred parameters, the actuators were selected based on their geometry, dimensions, and performance.

Among the most representative wavelengths that are employed for PBM biomedical applications are 600, 655, 810, 850, and 940 nm. These wavelengths are commonly explored to stimulate various human tissues, such as cartilage, bone, periodontal ligament, and brain [1,12,13,16,31]. Moreover, the optical set-up should be size-effective to allow the independent irradiation of each well, and suitable for the application of tunable radiation doses. For these reasons, and considering the economic advantage of LEDs, these were selected to provide optical actuation, namely LEDs of 600 nm (Lumileds, USA), 655 nm (Osram GmbH, Austria), 810 nm (Luminus, USA), 850 nm (Würth Elektronik eiSos GmbH & Co. KG, Germany), and 940 nm (Osram GmbH, Austria). The main LEDs' characteristics are exhibited in Supplementary Table S1.

A similar procedure was carried out to select the ultrasonic actuators. Based on the acoustic waves typically employed in therapeutic ultrasound applications [25,29,32,33], the central frequencies of 200, 500, 600 kHz, 1, 1.5, and 2 MHz were selected, and acoustic actuators operating in the range of 100 kHz to 3 MHz were considered. Given that the ultrasonic waves will be delivered through the culture medium in each well, small-size (about 5 mm³) actuators were required to fit the culture wells and to keep a safe distance from the cells. A resonance frequency of 1 MHz and minimum acoustic power density of 5 mW/cm² were also established based on previous cellular studies [4,16,17]. Thus, cylindrical ferroelectric soft piezoelectric discs of PIC255 (modified lead zirconate titanate), of 3-mm diameter and 2-mm thickness, with polarization in the thickness direction, and with silver printed electrodes were purchased from PI Ceramics GmbH (ref 000084902, Lederhose, Germany) (main features on Supplementary Table S2). These piezoelectric discs are appropriate ultrasonic transducers as they present optimal piezoelectric constants for a wide range of frequencies, have reduced dimensions and are commercially available.

2.1.2. Layout and stimulation set-ups

The device was integrated into a PCB (printed circuit board), containing all the electronic components and transducers, externally powered up to 16 V. For the application of the optical stimulus, arrays of four LEDs of the same wavelength (600, 655, 810, 850 or 940 nm) were embedded along the periphery of the culture wells area (one array of one single wavelength per well). The LEDs can be powered at 5 V via micro-USB (up to 500 mA, 2.5 W) or by connecting an external source to one of the connectors on the board (up to 3 A, 15 W). Each line, containing four wells, presented similar array of LEDs of the same wavelength to allow experiments with four replicates under the same irradiation regimen.

For the acoustic actuation with ultrasounds, transducer modules consisting of a plug connected to the piezoelectric disc were coupled to the stimulation device. Piezoelectric transducers can be powered at 5 V via micro-USB or up to 16 V via an alternative set of external connectors. In this configuration, each line can be actuated with an independent signal via an external generator, allowing at least four replicates for each experiment.

The electrical signal to actuate the LEDs and the piezoelectric discs was limited according to the working windows of the electronic components (data available in the component datasheets) to prevent the transducers and the circuits from damage. The electronic circuits of the device were coated with a widely used impermeabilization spray (Plastik Spray 70, KONTAKT CHEMIE, Germany) to ensure that the board or any electronic component would not be damaged in the case of contact with fluids, also avoiding electric short circuits between the electronic parts. Additionally, all the device materials allow sterilization by ultraviolet (UV) radiation and disinfection with 70 % alcohol. This allows stimulation to be performed outside the incubator, inside a

laminar flow chamber, with an external connection to the power supply and the computer (this can also be used as a power supply through a USB connection).

2.1.3. Interface and actuation control

The stimulation device includes a microcontroller (PIC32MK1024GPD064, Microchip Technology Inc., USA) with an operating voltage of 3.3 V and a maximum frequency of 30 MHz that allows the parameterization of both the optical and acoustic actuation via a USB connection to computer. A customized software was designed for this purpose, which also allows the user to monitor the performance of the control circuit in real-time.

Optical actuation can be controlled externally from a computer via a USB connection, in which it is possible to vary the current applied to each array of LEDs, the stimulation time, the pulse rate in each row, and the duty cycle (ratio between on and off time) of the irradiation. Based on the programmed current for each array of LEDs, the board applies light stimuli with power densities between 4 and 20 mW/cm², values in line with the literature on photobiomodulation delivered to *in vitro* models [12,13,31,34].

For acoustic actuation, two options are available: i) acoustic waves can be created using an internal signal generator that communicates with the software for the control and monitoring of the actuation of the piezoelectric discs. This circuit is powered by the USB connection (5 V); or ii) for higher voltages, the device has a switch that connects the microcontroller circuit while regulating the voltage externally and independently on each line up to 16 V. The power density for acoustic stimulation was controlled by regulating the driving voltage applied to the piezoelectric transducer, according to the impedance values for each central oscillation frequency [35].

2.2. Optical design and characterization

2.2.1. Spectral analysis

For the selected LEDs (i.e., 600, 655, 810, 850 and 940 nm), their emission spectra were characterized, for each LED individually, using a spectrophotometry top-bench setup with a monochromator (model 74,125, Oriel Newport), a photodiode (S1336-5BQ from Hamamatsu) and a picometer (model 487, Keithley). This procedure was used to measure the current outputted by the photodiode, being the light source each of the LEDs.

2.2.2. Power density analysis

The arrays of LEDs, located in the stimulation device, were characterized in terms of power density (given in mW/cm²) and its dependency on applied current (continuous mode) and actuation time. For both tests, the LEDs arrays, in one well at a time, were placed and aligned, at a 5 mm distance of a photodiode with a 1 × 1 cm² active area, which was coupled to a power meter (model 1918-R, Newport, Wales). A continuous current (ranging from 5 to 150 mA or from 5 to 70 mA, depending on the type of LED) was applied to the LEDs arrays, and a chronometer was used to record the assay time. This optical characterization, conducted in triplicate, aims to predict the emitted power density by each LED according to the applied current.

2.2.3. Transmittance analysis of the culture medium

Using a 12-well plate commonly used in cell culture studies, three photodiodes were placed at the bottom of three wells to quantify the light transmitted through the culture medium, from the top (emitted by the LED arrays) to the well bottom. The LED arrays were actuated with 50 mA. The photodiodes were connected to a transimpedance amplifier (LM324) circuit to convert the current outputted by the photodiodes into a voltage and amplify it. This circuit was connected to a digital oscilloscope (DS1054, Rigor) to measure the voltage transmitted in each well. This study was performed with and without cell culture medium, to assess the effect of the culture medium in the light transmittance from

the top to the bottom of the well, where cells will be cultured. The voltage was measured in three consecutive wells, each containing a photodiode at its bottom. The transmittance was obtained (in percentage) through the ratio between the voltage outputted at each well with culture medium and the voltage at each well without culture medium.

2.3. Acoustic design and characterization

2.3.1. Signal transmission, stability and coating performance

Considering the piezoelectric performance of the acoustic transducers, a preliminary evaluation of the transmission of the acoustic signal through a liquid medium (water) was conducted. This aimed at evaluating the acoustic transmission as a function of actuation frequency and impermeabilization coating materials. For that, two similar piezoelectric discs were placed in a water tank, 2 cm apart. One piezoelectric disc worked as the actuator (input voltage of 5 V), with varying actuation frequency, while the other piezoelectric disc was used as a receptor, connected to an oscilloscope. Two different materials were tested for the impermeabilization of the discs: Plastik Spray 70 (similar to the previously used to protect the electronic circuits), and polydimethylsiloxane (PDMS). To assess the stability of the acoustic signal, the electrical current passing through the piezoelectric disc was measured over 5 min of applying an electric voltage of 5 V.

2.3.2. Impedance analysis

The electrical impedance of piezoelectric disks was measured as a function of the frequency using a frequency response analyzer (CS328A, Cleverscope, New Zealand) when a sinusoidal function (2.83 V) was applied to the piezoelectric disc.

2.3.3. Acoustic propagation through the culture media

One piezoelectric transducer connected to the stimulation device was used to emit the signal and another transducer was placed at the bottom of the 12-well to receive it. The electric voltage at the terminals of the piezoelectric discs was measured. For that, culture wells were filled with 5 mL of media to guarantee the discs were submerged. The tests were run using three different frequencies (i.e., 500 kHz, 1 MHz, and 2 MHz).

2.4. Thermal effect

To assess if the light and acoustic actuation increased medium temperature enough to affect cells, temperature variations were monitored over 5 min using a commercial digital thermometer with a flexible wire probe (DANYU Hardware Tools, China) with a sensitivity of 0.1 °C and accuracy of 1 °C. Each array of LEDs was tested at three different power densities (i.e., 5, 7.5, and 10 mW/cm²). The piezoelectric transducers were assessed when two different signals were applied (i.e., 400 kHz and 1 MHz), also at three power densities (i.e., 100, 150, and 200 mW/cm²), which stimulation parameters are commonly employed in literature [7,10,29]. After 5-min stimulation, the stimulation device was turned off and the wire probe was submerged in the culture medium to record the temperature. The measurements were conducted at room temperature.

2.5. Stimulation effects on cellular viability and proliferation

Preliminary biological tests were performed to ascertain the safety and efficiency of the stimulation device in stimulating *in vitro* models. Cell viability and proliferation assays for stimulated and control (non-stimulated) cells were conducted. Two cell lines (osteoblastic and neuronal-like cells) and primary cultures (chondrocytes and periodontal ligament fibroblasts) were used in this proof of concept.

2.5.1. Cell culture

Primary human chondrocytes (P10970; Innoprot®, Spain) and culture medium components (P60137; Innoprot®, Spain) were purchased

from Innoprot®. The chondrocytes were isolated from healthy articular cartilage from the knee joint and cultured in chondrocytes basal medium, supplemented with 5 % fetal bovine serum (FBS), 1 % penicillin/streptomycin and 1 % chondrocyte growth factors. Cells at passage four were used.

Human periodontal ligament fibroblasts (hPLFs) were isolated from human healthy periodontal tissue and purchased from Innoprot®, Spain. They were cultured in DMEM-F12 culture medium supplemented with stable glutamine and supplemented with 1.2 g/L NaHCO₃ (PAN-Biotech GmbH, Germany) with 10 % FBS (Sigma-Aldrich) and 1 % (v/v) penicillin-streptomycin (PAN-Biotech GmbH, Germany). The hPLF cells at passage four were used.

Human fetal osteoblast cell line (hFOB; American Type Culture Collection – ATCC®, USA) was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12) without phenol red (PAN-Biotech GmbH, Germany) containing 2.5 mM L-glutamine (Pan-Biotech®, Germany), 10 % (v/v) fetal bovine serum (FBS; Sigma-Aldrich, USA) and 0.3 mg/mL geneticin (G418; PAN-Biotech GmbH, Germany). The hFOBs at passage eight were used.

Mouse-derived neuroblastoma cells expressing the human Tau P301L mutation (N2a Tau P301L cells, ATCC CCL-131) were grown in DMEM-GlutaMAX (Gibco™, Thermo Fisher Scientific, USA), supplemented with 10 % FBS (Gibco™, Thermo Fisher Scientific, USA), 1 % Anti-Anti (Gibco™, Thermo Fisher Scientific, USA), 1 % Glutamax (Gibco™, Thermo Fisher Scientific, USA), and 5 µL/mL G418 (InvivoGen, USA). The N2a Tau P301L cells were used at passage 11.

Human chondrocytes, fibroblasts, osteoblasts, and mouse-derived neuroblastoma cells were incubated at 37 °C in a humidified 5 % CO₂ atmosphere and the culture medium was changed every two days. Once confluent, cells were detached from the culture flasks using 0.25 % (v/v) trypsin/EDTA (PAN-Biotech GmbH, Germany). The cells were sub-cultured according to the manufacturer's indications and seeded at 20,000 cells/mL in 12-well plates and incubated with 1 mL of culture medium specific for each cell type. The cell seeding was performed one day prior to stimulation.

2.5.2. Stimulation protocol

Cells were exposed to different experimental conditions, as follows:

1. Control with the stimulation device off placed on top;
2. Control without the stimulation device placed on top;
3. PBM using the 810 nm LEDs with 7.5 mW/cm² for 5 min;
4. US using 1.0 MHz with 100 mW/cm² for 5 min;
5. Simultaneous application of PBM and US.

Cells were stimulated for three days, in triplicate, and collected for analysis on the day after the last stimulation session.

2.5.3. Cell viability

Cell viability was assessed using the MTS assay (MTS Cell Proliferation Colorimetric Assay Kit, Abcam, United Kingdom). After the last stimulation session, the MTS reagent was added to the culture medium of each well and blank, according to the manufacturer's indications, for 3 h at 37 °C in a humidified 5 % CO₂ atmosphere. The absorbance of culture medium was measured at 490 nm, in triplicate, using a microplate reader (Biotek Epoch, United States), and the absorbance of the blank was subtracted from the absorbance of the remaining wells.

2.5.4. Cell proliferation

After detaching the cells from the bottom of the wells with 0.25 % (v/v) trypsin/EDTA (PAN-Biotech GmbH, Germany), cells were centrifuged at 300g for 5 min and the cell pellet was resuspended in culture medium and stained with 0.4 % trypan blue (PAN-Biotech GmbH, Germany). The cells were counted on a hemocytometer using the microscope (Kern & Sohn GmbH, Germany). The viability results by MTS were normalized according to the cell number.

2.5.5. Statistical analysis

Statistical analysis was conducted using the 9.0 version of the GraphPad Prism (Boston, Massachusetts, USA) software for Windows. Firstly, the normality of the data was assessed by the D'Agostino & Pearson test. All data was normally distributed and, thus, the One-Way ANOVA test was applied, followed by the Dunnett's test for multiple comparisons. The *p*-value was set at 0.05, and the results were depicted as mean $\pm$ standard deviation.

3. Results

3.1. Optical characterization

The emission spectrum of each LED, in the 400 to 1050 nm optical range, is depicted in Supplementary Fig. S1, as well as its full width at half maximum (FWHM). The spectrum intensity differs significantly between the selected LEDs, being the 600 and 850 nm LEDs the ones that presented the lower intensity spectrum. An array of LEDs was established for each wavelength to increase the output power of the LEDs and minimize the difference between wavelengths. Regarding the FWHM, the LEDs emit at other wavelengths simultaneously. The 810, 850, and 940 nm LEDs showed the highest FWHM, presenting a wider emission spectrum, while the 600 and 655 nm LEDs presented a narrower emission peak with a FWHM of 28 nm. Although a slight shift of the emission peak of LEDs 600, 655 and 940 nm was observed, these variations are small and do not affect the overall performance of the LEDs for the intended application. As expected from each LED's spectral emission (Supplementary Fig. S1), the optical power density of the LED array that is generated in each well is different, depending on the LED used (Fig. 2). By increasing the applied current, the outputted power density also increases, reaching a plateau, where applying higher current values does not induce higher power density values.

The power density decay of each LED array over time is presented in Supplementary Fig. S2. The 600 nm array presented a significant power density decay, decreasing up to 25 % within a 5 min stimulation. The power density of the 655 nm LED array also decreased by up to 5 %. The remaining LED arrays (i.e., 810, 850, and 940 nm) exhibited a minimal decay in the power density, below 5 %, for the whole stimulation time, suggesting that these LEDs present a more stable irradiation throughout time.

From the transmittance spectrum of culture medium, given in Fig. 3a, it is possible to observe a constant light transmission for wavelengths above 600 nm. This indicates that culture medium is responsible for only 10 % of signal attenuation. By analyzing the light interferences between wells (Fig. 3b), a set of interferences and

variations between wells was detected. The short distance between wells caused reflectance and scattering effects in its neighbors' walls, increasing the signal that reached the photodiodes, causing transmittance values higher than 100 % in some LEDs arrays. When the LEDs' array was actuated in consecutive wells at the same time, light transmittance varied about 23 %, 22 %, 20 %, 15 %, and 31 % between consecutive wells for the 600 nm, 655 nm, 810 nm, 850 nm, and 940 nm LEDs array, respectively.

3.2. Acoustic characterization

The acoustic transmission, given by the ratio between the emitted and received acoustic signals, is depicted in Fig. S3 from supplementary material. This test was performed using two different configurations of the piezoelectric positioning: one with the lateral surfaces of the piezoelectric discs facing one another, and another with the bases (welding surfaces) facing one another. The second configuration implicated the positioning of the connection wires in between the piezoelectric discs, which comprised a physical obstacle to the acoustic waves, possibly altering the response of the actuators in frequency, and disturbing signal transmission. Thus, for simplification purposes, the configuration having the lateral surfaces facing one another was adopted. Besides, according to the manufacturer, the coupling factor of the piezoelectric discs for radial oscillations (*k_p*, see Supplementary Table S2) is greater than for thickness oscillations (*k_t*), allowing a better acoustic performance.

For both Plastik- and PDMS-coated transducers, the acoustic transmission has main peaks around 350 kHz, which is the result of the resonance frequency of these transducers. While the expected theoretical thickness resonant frequency was 667 kHz, the welding and coating materials may have caused a slight increase in their mass and thus reduced their vibration abilities, decreasing the system's resonance frequency. Besides, there was a slight shift of the resonance frequency between the two impermeabilization materials, and the signal recorded from the piezoelectric discs coated with Plastik showed a peak associated with the piezoelectric antiresonance frequency of the disc (approximately at 300 kHz), which was not observed for the PDMS coating. The plot shows that a significant part of the acoustic signals was attenuated and did not reach the piezoelectric receptor. This attenuation was caused by the acoustic propagation itself, which is a function of the distance between the emission and receptor transducers (Fig. S4, supplementary material).

Although this behavior was observed for both impermeabilization methods, the attenuation was higher for the Plastik-coated piezoelectric transducer. Nevertheless, for both coatings, the piezoelectric discs

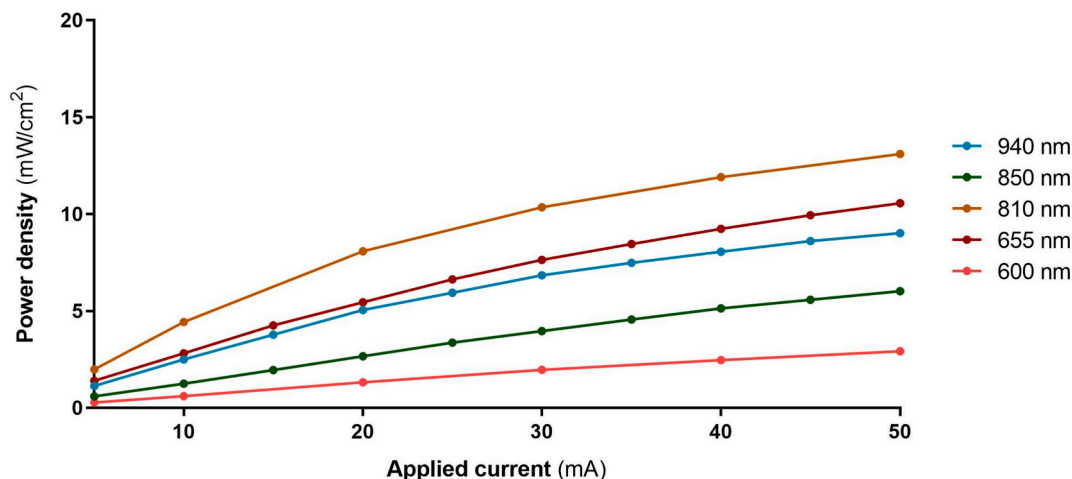


Fig. 2. Optical power density (mW/cm²) of each array of LEDs for input currents up to 50 mA.

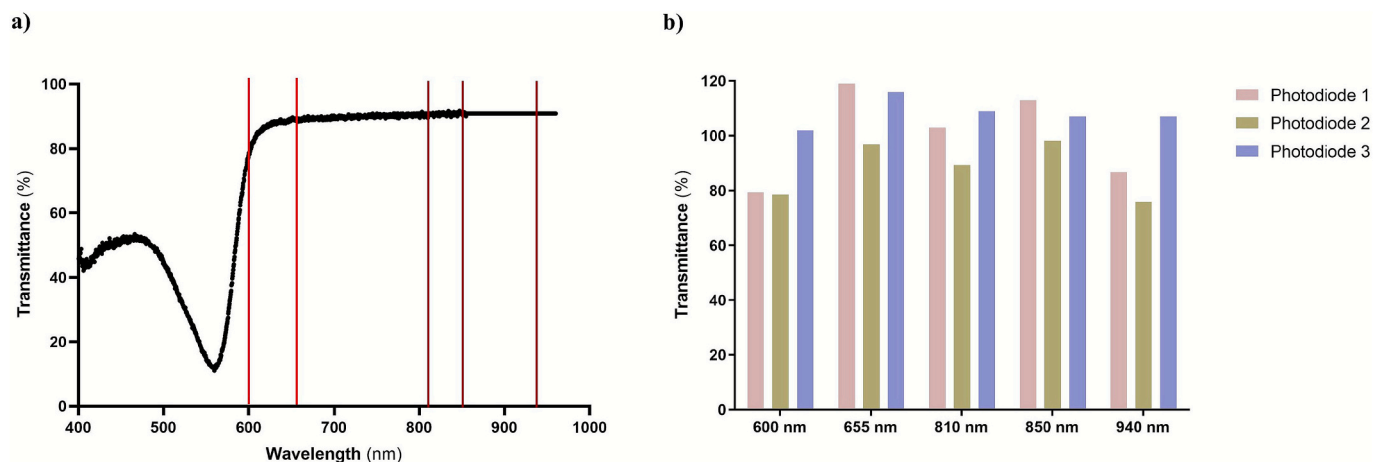


Fig. 3. (A) Transmittance spectrum of culture medium with indication of the wavelengths of the LEDs radiation. (B) Transmittance of the optical signal through the space of the well and culture medium. All photodiodes received LED irradiation, being positioned consecutively in the well plate (the photodiode 1 was placed under the activated LED, the photodiode 2 was placed in the well next to the actuated one, and the photodiode 3 was two wells apart from the first).

responded to the range of frequencies tested (10 kHz to 3 MHz), although they showed more prominent fluctuations between 220 and 500 kHz. In the frequency window of interest, the ability of the piezoelectric discs to produce the desired acoustic signals was validated, as the actuators revealed satisfactory performance indices in this frequency range (minimum transmission ratio of 5.7 %). Despite this, PDMS coating allowed a greater performance for multifrequency actuation, and therefore this was the selected coating to impermeabilize the piezoelectric discs.

Regarding signal stability (Fig. S5, supplementary material), the range of the measured current (2.67×10^{-2} to 4.66×10^{-2} mA) revealed a stable performance of the piezoelectric actuators over time.

From the measured impedance values (Fig. S6, supplementary material), the current in each transducer was calculated for an electric voltage of 5, 10, 15, and 20 V, and the electric power was computed from the current and voltage applied to the piezoelectric disc.

The acoustic power density was calculated considering the coupling factor (provided by the manufacturer, Table S2) and the actuating area (half of the lateral area of the disc). Hence, Fig. 4 presents the acoustic power density outputted by the piezoelectric transducers, as a function of the applied voltage and frequency. It shows that the piezoelectric transducers can apply acoustic signals within the power density ranges reported in the literature (up to 150 mW/cm^2) [7,29,36,37]. As previously described, a maximum electric voltage of 16 V can be applied to the microcontroller of the stimulation device, which transmits the

command to the piezoelectric transducers.

Considering the acoustic transmission analysis (Fig. 5), approximately 50 % of the signal was transmitted from the actuating

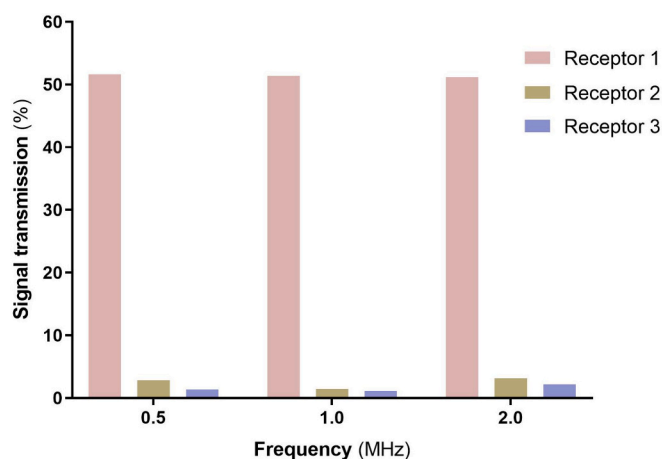


Fig. 5. Acoustic signal transmission from the actuator (in the same well as receptor 1) to receptors 1, 2, and 3, which is increasingly more distant from the actuator.

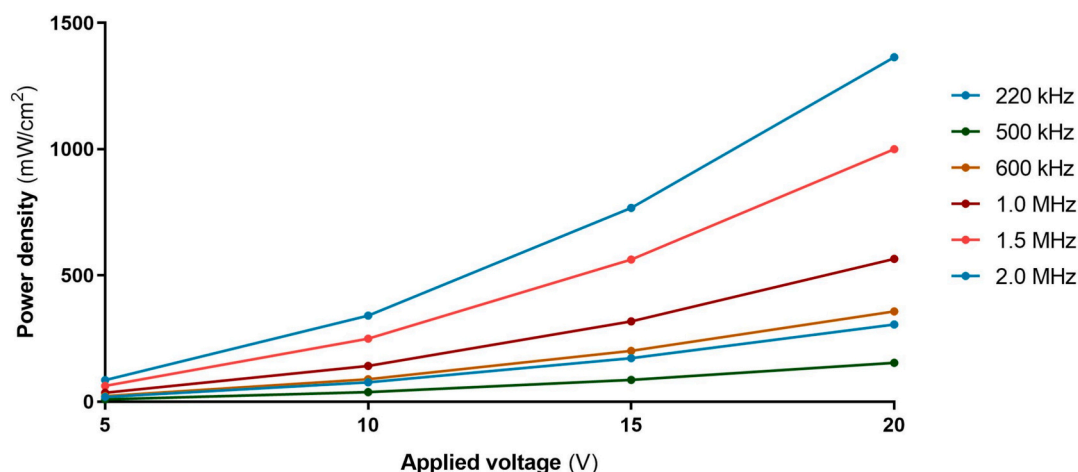


Fig. 4. Acoustic power density outputted by the piezoelectric discs as a function of frequency and applied voltage.

piezoelectric disc to the receiver when both were positioned in the same culture well. To test the interference of the acoustic signal between wells, two additional piezoelectric discs were placed at the bottom of two consecutive wells (one receiver next to the actuated well and another two wells apart from the actuated well), also filled with 5 mL of media. The interference of the acoustic signal between consecutive wells was negligible, thereby indicating that the US perceived at the bottom of each well is not affected by the signal applied in adjacent wells.

3.3. Thermal effect

Supplementary Tables S3 and S4 present the temperature variation for LEDs and ultrasonic transducers during stimulation, respectively. The temperature variation was minimal for PBM and US, increasing up to 0.7 °C only for the 810 nm LEDs with 5 mW/cm². Although temperature increase may alter cell response [38], the registered temperature rise was below 1.0 °C and, thus, it is not expected to influence the cellular response after stimulation.

3.4. Biological effects

Human fetal osteoblasts and mouse-derived neuroblastoma cells (cell lines), as well as human chondrocytes and human periodontal ligament fibroblasts (primary cells) were cultured and stimulated with the device for three days. We tested individual stimulation with the piezoelectric disks and LEDs and both stimuli simultaneously. Throughout the stimulation, cells were constantly observed under an inverted microscope (Kern & Sohn GmbH, Germany), and no alterations in cellular morphology were detected during or after individual or simultaneous stimulation. After three days, metabolic activity was normalized according to the number of cells (Fig. 6).

Four different cell types and sources were stimulated, demonstrating

distinct biological responses to the stimuli. Human osteoblasts had significantly increased metabolic activity after three days of combined stimulation compared to untreated cells ($p < 0.0001$, 95 % CI: -0.03 to -0.02). Individual acoustic or light irradiation did not affect osteoblasts' activity in comparison to the control, but the combined stimulus significantly altered their response. Human chondrocytes showed augmented metabolic activity after light irradiation compared to control ($p = 0.013$, 95 % CI: -0.01 to -0.001), with the combined stimuli resulting in a similar effect on chondrocytes. As opposed, the metabolic activity of human fibroblasts and mouse-derived neuroblastoma cells was not influenced by the individual or combined stimuli.

4. Discussion

This study features the design, development and validation of a multi-stimuli device delivering optical and acoustic stimulation to *in vitro* culture systems. Optical and acoustic stimuli were selected as they possess different cellular mechanisms of action and, thus, can trigger distinct bioeffects. While light triggers the mitochondria and/or light-sensitive ion channels (*i.e.*, TRPV ion channel) [39], acoustic waves induce mechanical deformations at cell surface, opening the mechano-sensitive ion channels (*i.e.*, PIEZO ion channel) [40]. In this sense, both stimuli could be combined to potentiate the biological effects on cells.

This stimulation device was tested in different cell cultures, with animal and human sources, to assess its safety and effectiveness in inducing cellular effects. Four different cell types and sources were stimulated, demonstrating distinct biological responses to the stimuli. These differences in biological responses can be explained not only by the distinct cells used but also by the single combination of parameters that were used as a proof-of-concept. Light was applied with 810 nm LED with 7.5 mW/cm² for 5 min, while ultrasound was employed at 1.0 MHz with 100 mW/cm² for 5 min. This highlights the importance of a

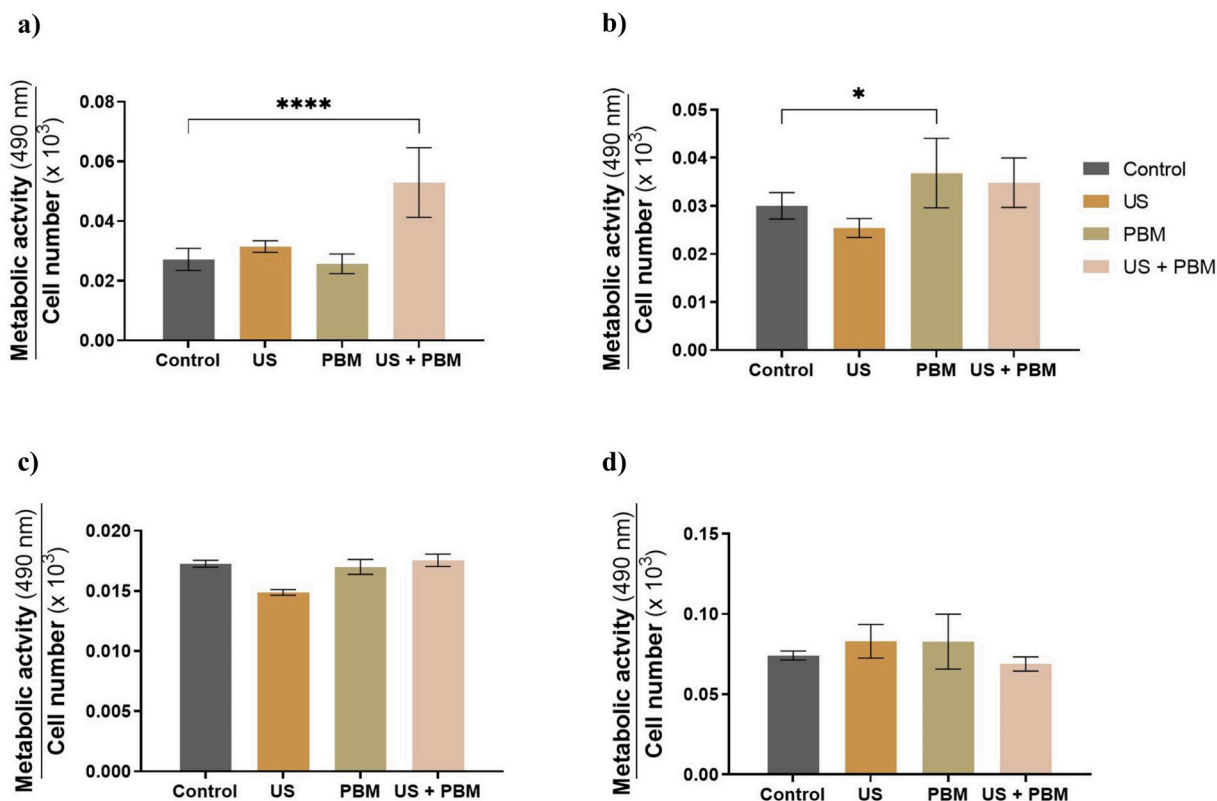


Fig. 6. Metabolic activity of untreated, stimulated with ultrasound, stimulated with LEDs and stimulated with combined stimuli for (A) human osteoblasts, (B) human chondrocytes, (C) human periodontal ligament fibroblasts and (D) mouse-derived neuroblastoma cells. One-way ANOVA was used to determine statistical differences compared to untreated cells, with significance denoted as $p^* < 0.05$.

rigorous optimization of the stimulation protocols considering the target tissue or pathological condition. A variety of combinations of parameters can be further studied with this stimulation device, which will induce diverse responses in different cells. This multi-modal stimulation device was only tested with cell culture, but it is also compatible with specimens of biological tissues with dimensions that fit 12-well plates. The safety of the device was confirmed as there was no cell death for any cell type. The sterilization conditions of this device are compatible with the cell culture environment since no cell contamination occurred during the stimulation procedure.

Basic research of optical stimulation on cells is often conducted by applying lasers or LEDs directly to cultured cells [41–46]. This multi-stimuli device comprised LEDs instead of laser diodes. The selected LEDs possessed a small emission spectrum, allowing stimulation under defined wavelengths. The LEDs light beams are wide but confined to the well, with minimal light transmission to adjacent wells, being suitable for *in vitro* stimulation. Although LEDs are known to induce temperature increase during stimulation as opposed to lasers [47], the multi-stimuli device caused only a minimal temperature rise, below 1 °C, which demonstrates the feasibility of using LEDs to stimulate *in vitro* culture systems. In addition, the observed biological effects prove that LEDs can also elicit biological responses in cells, supporting their use in this device. Since LEDs are cost-effective when compared to lasers, the inclusion of LEDs in this device makes it more affordable and accessible. In case of acoustic stimulation, the ultrasonic transducer can be applied in direct contact to the culture well, immersed in culture media (similar to this device) or immersed in a water bath [10].

So far, there is no stimulation device that allows the application of both stimulus on cells (individual or combined), which depending on the target tissue, may result in an augmented biological response compared to individual stimulus, as it was already demonstrated. Also, those stimulation devices do not offer the possibility of adjusting a wide range of stimulation parameters. For US, the device allows working with central frequencies from 0.2 to 2.0 MHz, and power densities from 60 mW/cm² to 250 mW/cm². For each LED, power densities between 4 and 17 mW/cm² could be tested. For both stimuli, pulse frequencies from 1 Hz to 50 Hz can be employed, as well as any duty cycle or duration. Therefore, this device could be used to stimulate distinct cells from distinct tissues (e.g., periodontal ligament, cartilage, bone, brain), allowing a targeted optimization. Although the multi-stimuli device was specifically designed to 12-well plates, it can be easily scalable to 6- and 24-well plates by adjusting the positioning of the electronic components. Another important feature of the multi-stimuli device is its flexibility to incorporate alternative optical and acoustic components. The LED arrays can be easily customized to include other wavelengths of potential interest, such as 780 nm [48,49], enabling stimulation at different spectral ranges depending on the intended cellular or tissue targets. Similarly, others piezoelectric transducers can be applied to operate at different central frequencies and actuation patterns [26,50,51], which may allow the modulation of distinct mechanotransduction pathways. This versatility broadens the applicability of the device, supporting its use in the extensive protocol optimization toward cellular responses for diverse tissues and experimental conditions.

Despite the promising results of the multi-stimuli device, some limitations should be acknowledged. For other well plates, such as 48- and 96-well plates, the device cannot be adapted since the well area of those culture plates is not sufficient to accommodate the LEDs array and piezoelectric transducer. Also, this study identified a pronounced power decay for the 600 nm LED, likely due to suboptimal thermal management. Future work should consider improved heat dissipation or replacing this emitter with a more thermally stable LED to ensure consistent performance. The biological models tested were restricted to four cell types from animal and human sources, which may not fully represent the diversity of tissues where optical and acoustic stimulation could be applied. In addition, the biological effects of stimulation are likely to vary according to the specific cell type, physiological state, and

microenvironmental conditions. Therefore, an in-depth protocol optimization is required to better comprehend the effect on cells behavior, which can be possible by using this device. An interesting direction for future research is the use of the stimulation device under mild and controlled heating conditions, known as thermophotobiomodulation, as a potential strategy to enhance cellular stimulation efficiency through the synergistic effects of light and heat.

There is little clinical evidence that supports light and therapeutic ultrasound application into clinical practice due to the inconsistent findings in the literature about their efficacy [13,25,26,29,52–55]. This clinical research employs commercially available devices that apply therapeutic ultrasound or light to relieve pain, decrease inflammation, and treat multiple disorders. However, there is no information on what the stimulation protocols are based on. This also contributes to the low understanding of the beneficial effects of both stimuli. In this sense, these stimulation devices offer the possibility of performing an extensive optimization of these stimuli in targeted cells, which findings could be used in the future to improve the current devices' efficacy or develop new and more customized devices to regenerate human tissues.

The stimulation device herein proposed is a customized, feasible, and promising strategy to stimulate *in vitro* models with light and ultrasound. It can be personalized by adjusting the number of LEDs in the arrays, the wavelengths of LEDs, and the type of piezoelectric transducers to allow stimulation in other central frequencies, being also cost-effective since it contains economical components. The device is feasible as it was demonstrated that it can effectively increase cellular metabolism depending on the type of cells and tissues for a single combination of parameters, without causing a significant temperature increase. Since a wide range of parameters can be combined, its potential to induce biological responses in different cells rises. Given the manifold possibilities, this multi-modal stimulation device holds a promising approach to stimulate *in vitro* cultures of distinct cells and tissues and to extensively test and optimize stimulation protocols for both individual and combined stimulation, paving the way for their effectiveness in the clinical context.

5. Conclusion

The current work describes the design, characterization, calibration, and biological validation of a multi-modal stimulation device that allows the application of individual and simultaneous stimuli to *in vitro* models. The device is connected to a software that allows complete control and monitoring of the optical and ultrasonic actuation, enabling the development of optimized stimulation protocols for multiple biomedical applications. Preliminary biological studies including viability and proliferation assessments showed the effectiveness, feasibility, and safety of the device to deliver a wide variety of stimuli to different cell lines and primary cultures, including osteoblasts, chondrocytes, periodontal ligament fibroblasts, and neuronal-like cells. Depending on the type of cells and their genetic profile (unmutated cells or models of disease), the stimuli provided by this device produced different biological responses, since some of the stimulated cells exhibited a significant increase in cell viability and proliferation compared to non-stimulated cells, while in other cases the stimuli applied individually produced a greater impact on cell metabolic activity than the other stimulation conditions. These results demonstrate the feasibility and safety of this stimulation device, as well as its effectiveness in interacting with cellular metabolism. By using this device to test different combinations of stimulation parameters and addressing their impact on specific biological pathways, researchers will be able to optimize the stimulation protocols and therefore move forward toward clinical applicability and success.

CRediT authorship contribution statement

Sofia Oliveira: Writing – review & editing, Writing – original draft,

Visualization, Methodology, Investigation, Conceptualization. **Fran-cisca Monteiro**: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Susana O. Catarino**: Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Betina B. Hinckel**: Writing – review & editing, Supervision, Project administration, Conceptualization. **Ioannis Sotiropoulos**: Writing – review & editing, Supervision, Project administration, Conceptualization. **Ana Leal**: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Filipe S. Silva**: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Óscar Carvalho**: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

No competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jphotobiol.2025.113306>.

Data availability

Data will be made available on request.

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