A VERSATILE ULTRASOUND PLATFORM FOR REPRODUCIBLE IN VITRO EXPERIMENTS ON CELLS

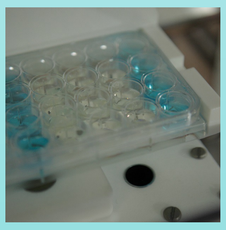
Barbato G[1*]., Cornice J.[2], Zazzeroni F.[2], Papa R.[3], Lucchetti D.[4], Sgambato A.[4,5]

[1]*Inno-Sol SRL – R&D – ROME – Italy;* [2]Dep. Biotechnological and Applied Clinical Sciences (DISCAB), Univ. of L'Aquila, L'Aquila, Italy; [3] Dep. of Public Health & Infectious Diseases, Sapienza University, Rome, Italy; [4]Inst. of General Pathology, University «Cattolica del Sacro Cuore», Roma, Italy; [5]Scientific Direction, «Centro di Riferimento Oncologico della Basilicata» (IRCCS-CROB), Rionero in Vulture, Italy

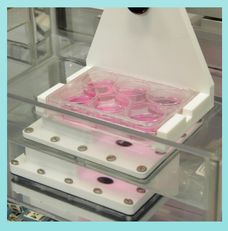
**** presenting author - e-mail: gaetano.barbato@inno-sol.it***

Aim

To perform ultrasound treatments by means of a versatile bench-top US apparatus allowing easy and robust reproducibility using standard set-ups for the cell samples.



SonoWell® enables to perform experiments where cells are kept under controlled non stressful conditions, avoiding the introduction of biased responses within measures, allowing a direct comparison between results obtained with different techniques.



Methods

Sonowell has been designed to use standard plasticware commonly used in molecular biology labs, ensuring the temperature control and sterility conditions.

With SonoWell the researcher is allowed to fix all environmental physical and chemical parameters within different wells and is allowed to vary each one at will.

Simultaneous use of channels (from 1 to 4): same acoustic pressure delivery can be achieved for all transducers since they share the same Near/Far Field plane. This allows comparisons within the same plate of the effect of frequency @ the same acoustic energy delivered on the readout of the cell experiments.

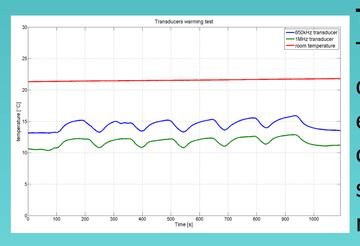
User can decide to use 1, 2 or 4 transducers simultaneously. The frequencies were chosen considering reflection patterns analysis on most common well-plates brands, fixed accordingly to maximize uniform radiation inside the cell culture[1], and can't produce reciprocal interference up to the 5th harmonic.

Plate-Well 3D Motion control: SonoWell controls the Plate motion along the 3D coordinates x,y,z using stepper motors. Plate motion can be along row or column format; Z-axis can be finely tuned to establish positioning of each transducer N/F Field plane below, within or above the cell sample in the wells.

Stable sample temperature: ensured by a thermostated bath for cell culture, continuously degased to avoid both thermal and gas discontinuities

Bakaric M. et al. 2021[2]: « When driven using continuous wave (CW) or quasi-CW excitation with constant input voltage […] the pressure radiated from the transducer(s) increased approximately 0.6% / °C. This was primarily due to an increase in transducer efficiency, which exhibited a relative increase of approximately 0.5% / °C »

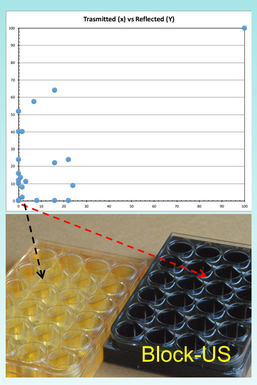
In well plates cumulative heat developed within the transducer by subsequent sonications, ***not adequately dissipated****, will alter its efficiency hence later made sonication will deliver different acoustic energy with respect to the initial ones. Heat dissipated directly through the vibrating membrane into above water will vary the local temperature of the bath altering the speed of US propagation, hence introducing an undetermined element in the experimental set-up.*



Transducers cooling : Transducer temperature rising due to mechanical stress affects efficiency[2,3]. A refrigerated fluid circulation system, ensures sonication response stability and reproducibility up to 100% duty cycle, thus preventing gradients of heat in the thermostated tank which would affect US propagation.

Warming test : 120 s, DC 100%, 5.5 W/cm², ΔT ~3°C.

Wave modes conversion (WMC)[1,4]: Although highly desirable, conducting cellular US stimulation experiments on well-plates pose a number of problems arising from the interfaces of materials crossed by the propagating US wave. Well plates bottom layer is a solid lamina on which the single wells are elevated. Coupling of the US stimulation to the neighbour non-sonicated well is unavoidable since different acoustic waves can propagate in solid material and at the interface liquid/solid and solid liquid for WMC and formation of Raleigh, Stoneley, Scholte and Lamb waves[1,4]. It is however possible to effectively dampen the propagation of some of these waves, reducing their influence onto the neighbouring wells. We introduced into the interwell-spaces a dampening polymer which adsorbs the transverse waves leaking-through, effectively reducing their impact.

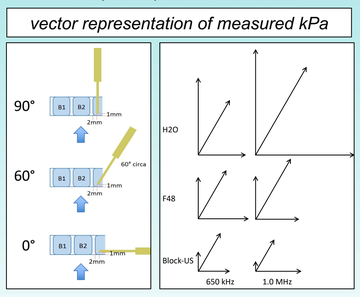


Measuring US transmission and reflection of a series (>50) of polymer compositions, the composition named Block-US was identified. Measuring the side-neighboring well leakage of acoustic pressure using a hydrophone @ 0°, 60°, 90° we estimated the amount of the attenuation of the wave mode conversions propagating in the sample.

inter-well space filler		measured kPa in B2	B3 - 0°	B3 - 60°	B3 - 90°	total fold attenuation
0,65 MHz	H2O	1028,9	46,0	60,9	74,5	1
	F48 AptFlex*	1031,4	48,7	54,2	40,6	1,3
	Block-US	1043,8	30,6	43,2	19,0	1,9
1,00 MHz	H2O	1264,7	95,5	98,3	102,5	1
	F48 AptFlex*	1266,5	42,1	67,4	42,1	1,9
	Block-US	1306,7	44,2	35,1	9,8	3,2

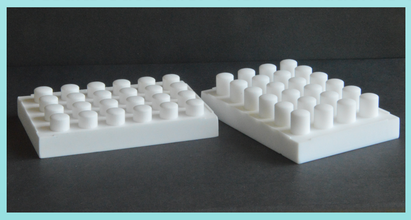
**F48 AptFlex is a product of Precision Acoustics*

vector representation of measured kPa

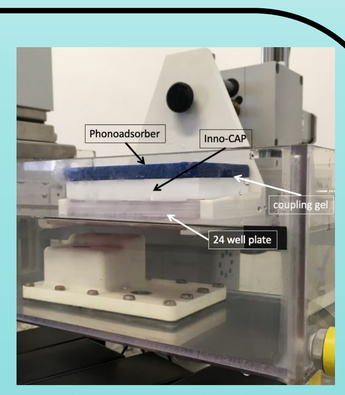


Block-US reduces 1.9 (0.65MHz) and 3.2-fold (1.0MHz) WMC propagation in side-wells

Standing Waves: the formation of standing waves have great on the experimental results outcome. Their formation is desirable when sonoporation is the objective of the experiment, on the contrary in stimulation experiments should be avoided[5].



To minimize standing waves propagating within the cell medium our strategy was to avoid the liquid/air interface by positioning at the interface, slightly immersed (circa ~10μm), **Inno-CAP**: a cap of material which substantially matches the acoustic properties of the medium, and effectively allows US wave propagation to a phono-absorbant material.



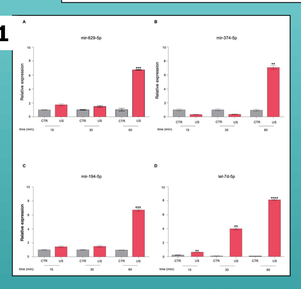
Inno-CAP is made of autoclavable material which would ensure sterility, and its reiterated use, peg height are adjustable to match the height of solution volumes within the well.

Results

Identification of 4 new potential miRNA biomarkers released from prostate cancer cells

Cornice J. et al. *Genes* 2021, 12, 1726-1741

1



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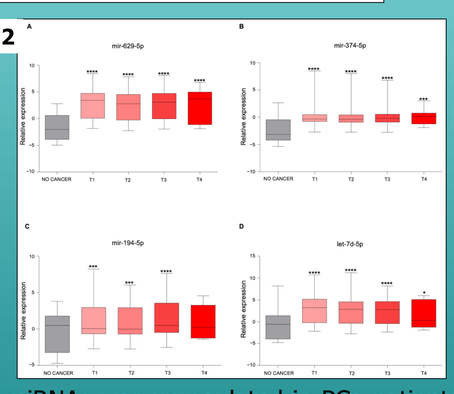
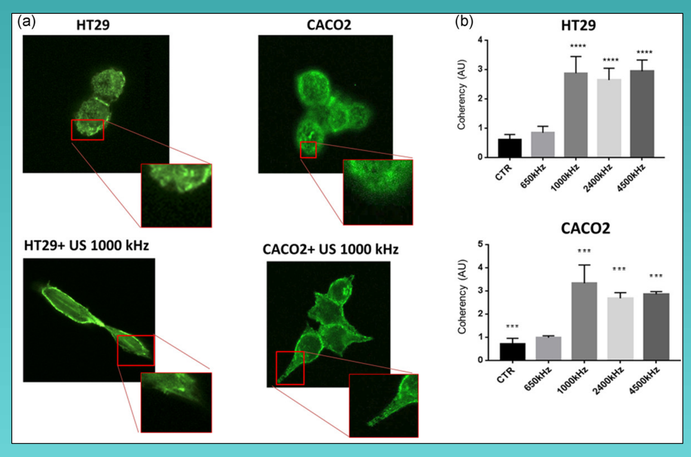


We treated a panel of prostate cancer (PCa) cell lines (LNCaP and DU145) with US at 4 frequencies and 3 acoustic pressures we profiled the release of miRNAs in the extracellular space. We provide evidence that at 1 MHz and I_{SPTA} 300mW/cm² US-mediated an amplified release of miRNAs from both androgen-dependent (AD) and -independent (AI) PCa cells occurs (1).

We identified 4 PCa-related miRNAs, whose levels in LNCaP and DU145 supernatants were significantly increased following US treatment: mir-629-5p, mir-374-5p, mir-194-5p, and let-7d-5p. We further analyzed a publicly available dataset of PCa (2), showing that the serum expression of the novel miRNAs was upregulated in PCa patients compared to controls, thus confirming their clinical relevance. Also their presence is already detectable in the early stages of disease, representing thus potentially valuable new Biomarkers tool for PCa diagnosis at early stage.

LIPUS induced changes in malignant phenotype of CRC cells

Lucchetti D. et al. *J. Cellular Physiology* 2020, 235, 5363-5377

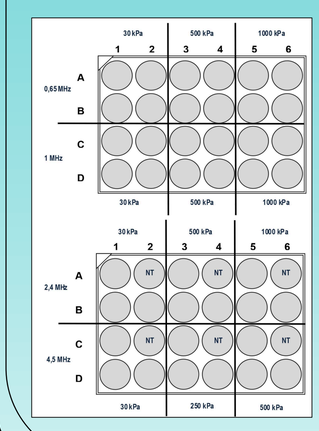


We used confocal microscopy to assess the polarization of CRC cells HT29 and CACO2 structure to verify whether LIPUS might affect actin cytoskeleton. While untreated cells displayed a dense network of the actin fibers spreading diffusely throughout the cytoplasm, following LIPUS treatment stress fibers increased and were massively anchored to the cells cortex traversing the cytoplasm in different directions resulting in a high polarized and organized cell structure. To evaluate differences in cytoskeleton organization actin fiber coherency and filopodia-like protrusions were considered. The coherency is calculated from the structure tensor of each images pixel is bounded between 0 (isotropic areas) and 1 (highly oriented structures).

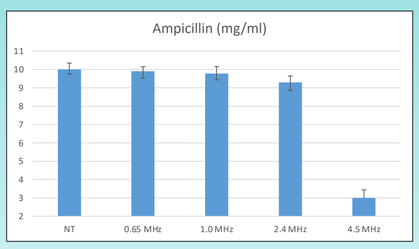
LIPUS induced important actin filaments reorganization with increased coherency indicating a higher organization of cytoskeleton compared with control cells (a) at frequencies higher than 1MHz (b).

UltraSound influence MIC of antibiotics on multiresistant bacterial cells

Selan L. et al. New Microbiologica 2019, 42, 52-54



The widespread use of antibiotics has resulted in a growing problem of antimicrobial resistance in community and hospital settings. The increasing outbreak of various multidrug-resistant (MDR) Staphylococcus aureus strains has resulted in treatment difficulties, which imposes a burden on health-care systems and intensifies the need for new antimicrobial agents. We tested if a co-treatment of US at several acoustic pressures was affecting the Minimal Inhibitory Concentration (MIC) of ampicillin antibiotic on USA300 a multiresistant S. aureus strain which is normally resistant to this drug.



Only at higher acoustic pressures (1 Mpa) a slight statistically non-significant effect was visible for 0.65-2.4 MHz frequencies. On the contrary, at 250 and 500 kPa 4.5MHz MIC resulted inhibited, with the latter pressure resulting in 1 log MIC decrease.

Conclusions

SonoWell®, used in conjunction with Inno-CAP and Block-US (products manufactured by Inno-Sol srl, Italy), enables to perform robust and reproducible experiments on cells, reducing the values of the standard deviation and in doing so the observed effect becomes statistically significant, whilst using all standard plate-wells commonly available in biological labs.

References

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3) Richard C. et al. *Ultrasonics*, 42, 417-424, (2004)
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