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High frequency ultrasound platform for non-invasive online monitoring of 3D cell cultures

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Abstract: A high-frequency (10-70MHz) ultrasound platform was developed for the study of 3D cellular cultures. The system provides ultrasound images through a continuous monitoring automatically controlled. The experimental methodology proposed is compatible with the sterility and thermal conditions required for cellular studies. This work was focused on the monitorization of S.cerevisiae 3D cultures in a solid agar matrix. Results show how the availability of nutrients and medium inhomogeneities influence the morphological structure and size of the 3D cell colony.

Keywords: Non invasive monitorizacion of cells, high frequency ultrasound, 3D cellular structures.

Introduction

3D cellular cultures have gained significant interest in recent years due to their improved ability to replicate *in vitro* the *in vivo* conditions of tissues and organs, being referred as organoids. Their growing relevance lies, among others, in their potential for drug testing and the development of personalized medicine, as they can be derived from a patient's own cells. [1]. Experimental configurations comprise suspension-based systems, where cells are cultured in Matrigel —a gel that mimics the extracellular matrix by providing structural, biochemical, and biomechanical support- or Organ-on-a-Chip (OoC), which incorporates microfluidic channels to simulate the microenvironment and key functional aspects of living organs at a microscale. More recently, bioprinting has emerged as a promising technique that employs biomaterials such as collagen, Matrigel, and cells to fabricate biological structures for tissue and organ engineering [2]. Despite the advantages of 3D cultures over conventional 2D cultures and animal models, they still have some limitations, such as challenges in recreating the biochemical and physical microenvironment of the human body, lack of innervation, immune cells, and tumor stroma, as well as insufficient vascularization.

In this scenario there is a need to implement monitoring techniques that support controlled growth. Currently, the primary monitoring methods are optical and fluorescence-based techniques, which include conventional optical microscopies such as Widefield Fluorescence Microscopy, Confocal Laser Scanning Microscopy, and Multiphoton Microscopy; as well as super-resolution microscopies such as Stimulated Emission Depletion Microscopy, Single-Molecule Localization Microscopy, and Structured Illumination Microscopy [3]. Other non-fluorescence-based optical techniques are also employed, such as Optical Coherence Tomography (Full-Field OCT, Spectral Domain OCT, and Ultraviolet OCT), as well as Raman spectroscopy and microscopy [4]. Although these techniques are non-invasive, they can be destructive due to prolonged light exposure, leading to progressive fluorescence loss (photobleaching) and cellular damage (phototoxicity). Tissue penetration may also be an issue for some of them.

High frequency ultrasound imaging emerges as another monitoring approach, offering the advantage of continuous, real-time monitoring throughout the growth process without damaging cell arrangements. It enables the study of structural features, morphological changes, mechanical properties such as elasticity, and vascularization at various depths —on the order of millimeters— which is sufficient for the typical dimensions of 3D cultures [5]. Furthermore, ultrasound devices can penetrate opaque materials and have moderate cost, though image analysis requires prior expertise for accurate interpretation.

To explore the potential contributions of ultrasound to this field, a preliminary monitoring study was conducted on a 3D culture of *S. cerevisiae* yeast embedded in a solid agar matrix. The goal of this study is to pave the way for future investigations involving more complex cellular structures, such as the aforementioned organoids.

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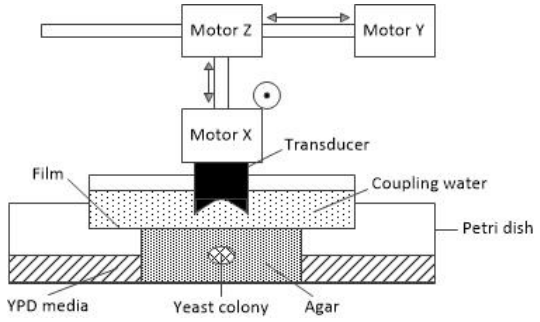

Fig. 1: Detail of the ultrasound probe and coupling with the agar containiong cells.

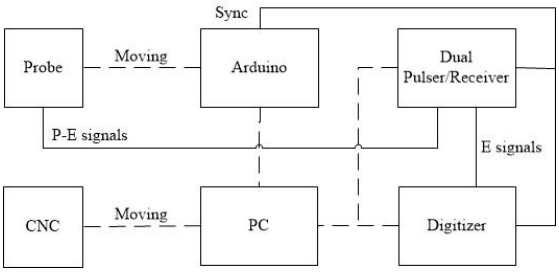

Fig. 2: Block diagram and connections of electronics.

Experimental methodology

A comercial bakery strain of *Sacharomyces cerevisiae* yeast was used for culturing. A small amount of them mixed with 100ml of YPD culture medium (2% glucose, 1% yeast extract and 2% peptone diluted in water) and placed into a culture chamber at 37 °C for 24 hours. From this liquid culture, a small volume was removed with the inoculation loop and gently dabbed on different places on the surface of a Petri dish containing YDP medium with 2% agar concentration, so new yeast colonies grew separately from each other over the agar for several days.

For the ultrasound monitoring assay, a new culture should be grown inside an agar matrix. For this purpose, a new agar with a 0.7% agar content was made under sterile conditions in a laminar flow chamber. Two differents YDP culture media were used for making this agar, once like the previous YPD described above and another using a glucose concentration of 0.3% instead of the usual 2%. The mixture YPD plus agar was heated above 90 °C to make it liquid and then, poured into a polystyrene box with a volume of 7.4 ml. After some minutes, the gel temperature falls down and the viscosity of the agar increases. When its temperature approaches 37 °C, a small yeast colony, 1–2 mm diameter, and about 1 μl volume, was placed inside the gelifying agar using tweezers to push it down until it is 3 mm below the surface. This colony was taken from the Petri dish containing the yeast colonies mentioned at the last paragraph.

Once the new agar is solidified and reached an ambient temperature close to 25 °C, it is removed from the polystyrene box and placed at the center of a new empty Petri dish. Some more YDP medium is added to ensure continuous hydration. Then a sterilyzed transparent plastic film is placed over the agar. This film separates the culture from the water used for coupling the ultrasonic transducer with the cell culture. A wide band (10-70MHz) focalized transducer, from S-Sharp was mounted in a probe able to perform linear scans through a step micromotor (X movement) at 8mm/s speed. The transducer has 10mm curvature radius and 6mm diameter. All the arrangement, ultrasound probe and culture medium (see Fig. 1) was placed over a 3D motorized and thermostatized platform (CNC VEVOR-3018-Pro) which was controlled by a PC and was used for moving the probe in Y and Z directions for getting the 3D dataset. The temperature of the platform was set to 33.5 °C to have 30 °C inside the Petri plate.

Regarding the electronics (Fig. 2), electric wideband pulsed were generated by a dual pulser-receiver (DPR-500, JSR instruments) to excite the ultrasound emission in pulse-echo configuration. Received echoes were sent to a digitizer (Picoscope-6400C), working at 313MHz sampling frequency, which finally transferred data to the control PC. An Arduino Uno microprocessor was used to generate square pulses to drive the internal motor of the probe which makes 8mm length linear scans. This pulses were synchronized with the pulser emission and the digitizer acquisition. Each B-scan was made with 10 μm lateral resolution displacements and an axiallength of 50 μm. To increase tha axial field of view, successive movements in the Z dimension provided a total field depth of 4.5mm in the experiments presented in this work. Finally, Y movements of 50μm were used to register different image slices with a total lateral displacement of 6 mm. This way, the total volume scanned was 8mm x 6mm x 4.5mm, with 1.2pl voxels (0.01mm x 0.05mm x 0.024mm), which is equal to 1.2 pl. 1 hour was needed to acquire a complete image of this volume. Four experiments were made (2 repetitions using 2% glucose medium and other 2 repetitions with 0.3% glucose medium), each lasting 120 hours.

Results

Fig. 3 and Fig. 4 shows 3D and 2D representations respectively, of the evolution of a yeast culture over time. In particular, these images belong to the first culture developed with a glucose content 2% in the agar. The 3D representation provides information about the growth towards different directions while 2D b-

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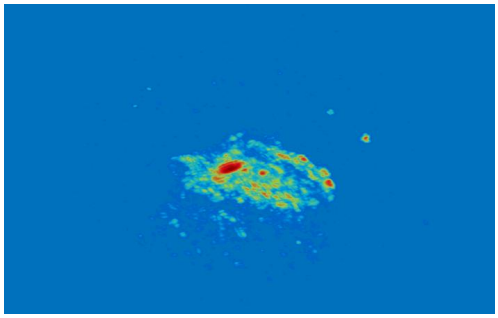
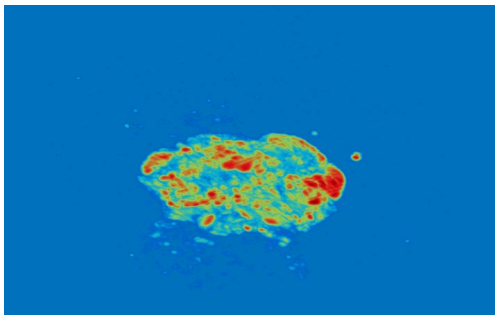
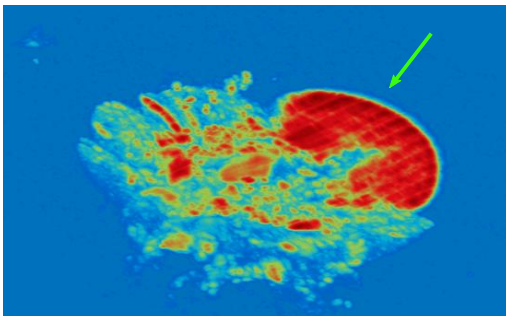


(a) Culture time: 1 hour

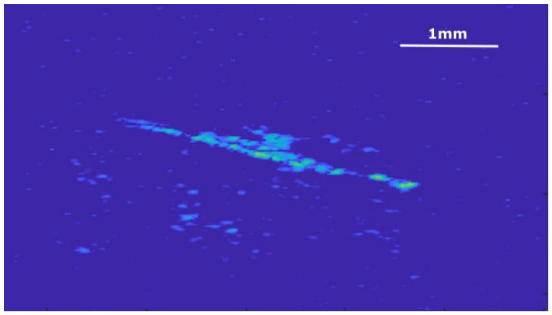


(b) Culture time: 35 hours

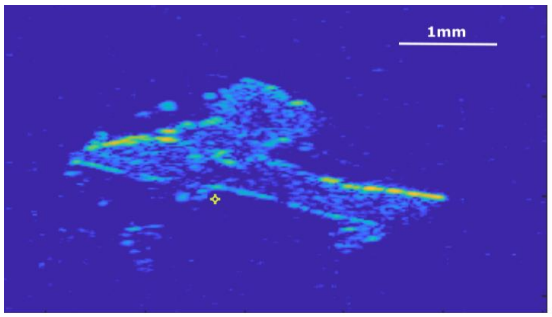


(c) Culture time: 131 hours

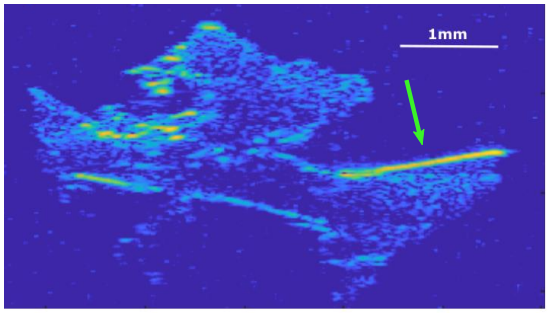
Fig. 3: 3D plot of the yeast colony at different culturing times. The green arrow in (c) marks an apparent crack in the medium.



(a) Culture time: 1 hour



(b) Culture time: 35 hours



(c) Culture time: 131 hours

Fig. 4: 2D section of the yeast colony at different culturing times with scale dimensions. The green arrow in (c) marks an apparent crack in the medium.

scans complete this information by showing the echoic features of the inner parts of the colony.

In the figures presented, the volume occupied by cells increases over time. Growth is limited by the semi-solid nature of the agar and the lack of mobility in yeast cells. However, as new cells emerge by gemation, they occupy new regions of agar while seeking nutrients. This causes colony expansion in multiple directions, with irregularities in geometry that vary across the different growths monitored. These deviations from ideal globular growth may stem from local variability in nutrient distribution, statistical differences among individual cells, or mechanical variability in the gel matrix, which may experience linking failures or crack formation. This phenomenon is evident in Fig. 4, where a highly echoic line marked with an arrow (seen as a plane in Fig. 3) breaks the colony's globular structure. This echo could indicate a crack caused by internal pressure from yeast anabolism increasing biomass and/or yeast catabolism producing carbon dioxide that may form bubbles. Further studies are needed to determine the exact origin of these echoes.

Quantifying the echogeneity of the colony may help to evaluate its growth. A common way to measure microorganism concentration in liquid media is by using a spectrophotometer to assess light absorption, which correlates with cell density. Growth curves often begin with a lag phase, where cells adapt to new conditions. If conditions are similar to the previous environment, this phase may be absent (as in our case).

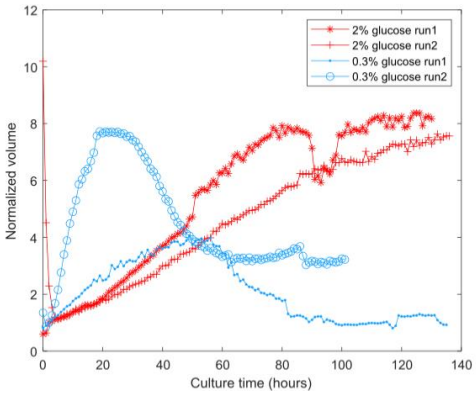


Fig. 5: Evolution of the yeast colony volume along time for 0.3% and 2% glucose content in the culture medium. The volume of the colony is normalized to the initial volume of the colony.

Then, exponential growth occurs as cells replicate, until nutrients decline, slowing growth and leading to a plateau when cell death equals cell formation.

In a parallel way, it is proposed in this work to evaluate the number voxels reaching a given intensity threshold. This number multiplied by the volume of each voxel (1.2 pl) provides the volume of the colony above such amplitude threshold. For this purpose, envelope data were referenced to the maximum and converted to dB. Fig. 5 represent the evolution of the volume of the colony above -30dB, for the two repetitions of the experiment (run 1 and run 2) made for each glucose concentration of the agar (0.3% and 2%). As the volume of the colony was sligthly different at the beggining of each experiment, this volume was normalized by the initial volume of the culture to make the volume increasing comparable between experiments. An initial growth phase was found to be even faster when the amount of glucose was lower. This probably is due to the fact that the starting colonies, which were taken from another agar plate which was cultured along several days, were also adpated to a low glucose availability. However, after some days of growth those colonies growing in the medium with a lower glucose amount exhibit a reflectivity loose between the second and the third growing day. On the contrary, those colonies growing in the medium with 2% glucose concentration did not reach this decaying stage, at least before reaching 130 culturing hours, showing a behavior similar to that described for light absorption in liquid media. The decreasing of reflectivity found for the lower glucose concentration experiments may be a result of four different processes: cell deaths exceeding cell production when the glucose availability is suddenly reduced; a fast carbon dioxide production giving rise to wave reflection (first reflectivity increment) which, afterwards, is slowly dispersed in the medium (reflectivity decreasing); a high local concentration of cells which is followed by a stage of cell spreading in the medium which made many cells not able to produce an echo level reaching the threshold for quantification, and/or a change in the cellular state and physical properties as a result of nutrient depletion: yeast may form spores with a significant smaller size than yeast cells. Further studies should clarify the most determinant yeast processes under the reflectivity evolution registered.

Conclusions

The high frequency ultrasound imaging system proposed was able to monitor 3D yeast growing arrangements in a semi-solid matrix, providing information of structures developed inside such formations. This opens the possibility of using such technology to monitor other 3D cellular arrangements (tissues, organoids...) providing insights about their structural and mechanical features.

Acknowledgements

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