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MICROFLUIDIC LIVE-IMAGING WITH CELLVIEWER TECHNOLOGY TO PERFORM BIOTECHNOLOGICAL TASKS

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***Abstract*— Cells grown in a monolayer tend to flatten in the lower part of the plate adhering to and spreading in the horizontal plane without expanding in the vertical dimension. The result is that cells grown in 2D have a forced apex-basal polarity. Microfluidic Live-Imaging with CellViewer technology is an ideal solution to observe the maintenance of a cell in excellent health, trying to bridge the gap between the 2D and 3D model. In this work we propose to test the system on a single isolated Jurkat cell in the microfluidic cartridge and record the timelapse for 4 hours. After adaptive autofocus, when sliding inside the cartridge chamber, the single cell is tracked under the action of the optics and the 3D rotation was experimentally successfully achieved. Then a single cell viability assessment was used using MitoGreen-dye a fluorescence marker selectively permeable to live cells. ImageJ software was used to: calculate the diameter of a single cell, create fluorescence intensity graphs along a straight line passing through the cell, visualize spatial fluorescence intensity distribution in 3D.**

***Keywords*—** Microfluidics; Biotechnology; Live-Imaging; Biomedical; Biomedical Enineering;

I. INTRODUCTION

The traditional 2D culture systems growth alone or in co-culture on plates, in which experiments supported by in vitro imaging are conducted for different functional, pharmacological, toxicological, and even clinical applications; they have long been widely used and already known for the nature of their cost and high repeatability. However, 2D culture systems cannot reach a stage of 3D organization equal to in vivo, due to the disadvantages associated with the lack of specific tissue architecture, mechanical-biochemical signals, cell-cells and extracellular matrix[1]–[3].

One issue with conventional 2D cell culture systems is the inadequate quality and quantity of Extracellular Matrix (ECM), which is fundamental to the support of the structure by facilitating communication between the different cell populations embedded in the matrix by imparting mechanical properties to the tissues[4]. Cells in 2D culture are not surrounded by ECM and therefore are different from the structure of an in vivo cell system, as they cannot: migrate, polarize, differentiate in response to[5]–[8]. Despite their proven value in biomedical research, 2D models cannot support differentiated and cell-specific functions in tissues or accurately predict in vivo tissue functions and drug and biological modulator activities [9]–[12]. These limitations have led to a growing interest in the development of more complex models, such as those that incorporate multiple cell types or involve cell modeling, and in three-dimensional (3D) models, which better represent the spatial and chemical complexity of living tissues[13]–[15]. The methods and timing of the research are expensive, the in vitro and ex vivo activity phase is of enormous importance to have information that can follow the next steps. Most of the research carried out has turned its attention to traditional and 3D methods, the analysis of these results is part of the economics of research. The optimization of available resources and the use of new approaches show new scenarios regarding the potential of laboratory research and the possibility of improving the quality of data and information.

A. 3D models

Some 3D models provide great results in representing tissue structures in the physiological field compared to two-dimensional 2D cell culture[6], [15]–[18]. The fabrics have a hierarchical structure that contains micro-architecture features

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that can be studied on many length scales. These include the subcellular/cellular scale (1–10 μm), which affects cellular function; the multicellular scale (10–100 μm), which determines the type and degree of intercellular interactions; and the tissue scale (100–1000 μm), which correspond[3], [19]. Deciphering population heterogeneities is a long-standing goal in cellular biology. At the level of single cells, such heterogeneities are usually observed at the genomic, transcriptomic or phenotypic levels[20]. In general, spheroids, self-organizing and heterogeneous cell aggregates up to 400–500 μm in size, are used for research, resulting from the suspension or adhesion on the single-cell jamb or co-culture of more than[21]. Spheroidal models have advantages derived from their geometry and the possibility of developing effects in co-culture and sustainability generally long-term, as they mimic optimal cell-cell-cell-ECM physiological interactions, reproducibility, the similarity in protein-gene expression profiles The use of these models is not transferable to cell types, as 3D spheroids of these cells tend to disintegrate or take unpredictable forms[21]. To avoid unpredictable and not currently useful developments, various types of scaffolds are manufactured and applied tools that control the development and structuring of spheroids' uniform dimensions[22].

The realization of an organoid, pseudo-organ, or neo-organ has in common some processes present in the various stages of development and formation of a living organism. This includes differentiation, proliferation, polarization, adhesion and precisely controlled apoptosis that combined with self-organization and multi-cellular pattern leads to the development of the various districts[22]. The Organoids or Tissues Organs are an in vitro 3D cell cluster derived from stem cells or progenitors and/or donors that spatially organize themselves in a similar way to their counterpart in vivo[23]. In the structuring and organizations of culture systems and especially of co-culture systems cells must maintain an adequate phenotype compatible with the external cellular environment and the duration of this phenomenon must be particularly protracted over time. For adhesion-dependent cells, interactions with the surrounding ECM and neighboring cells define the shape and organization of cells. One of the most surprising differences observed when comparing cell cultures in 2D and 3D is the morphological dissimilarity and their evolution over time. Cells grown in a monolayer tend to flatten on the bottom of the plate dish by adhering and spreading on the horizontal plane without expanding into the vertical dimension. The consequence is that cells grown in 2D have a forced apex-basal polarity. This polarity is probably relevant for certain cell types such as epithelial cells, but it is unnatural for most cells especially those of cubic or multifaceted type. The mesenchyme, if incorporated into a 3D ECM, take on a starry morphology and polarize only by bottom-up during migration[12], [24].

To support and guarantee the functional maintenance of a 3D structure, one must consider the structures and dynamics of regulatory networks, increasingly studied with live-imaging microscopy[25].

However, commercially available technologies that can be used for current laboratory needs are limited, although there is a need to facilitate the acquisition of cellular kinetics with a high spatial and temporal resolution, to elevate visual performance and consequently that of experimentation[6], [26]–[28].

B. **Microfluidic live-imaging**

2D models in Petri dishes allow for collective cell simulation and behaviors related to disease modeling and understanding but the advent of laboratory and organ devices on a chip shows that information obtained from 2D cell cultures on plates differs significantly from results obtained in microfluidic environments as they reflect more biomimetic aspects [30].

2D culture imaging does not allow to fully appreciate the morphology of the cell population and the three-dimensionality of the sample, one of the reasons could be the unappreciated evolutionary changes. The use of imaging is an essential requirement for the study of the structural and functional morphology of the neo-organ, of its positioning / polarization and of cell differentiation, allowing the in vitro modeling of even the most complex organs [31].A new technology based on newly developed microfluidics and imaging techniques can enable the management and identification of the phenotype, the biological activities of the present populations of the present populations without destroying the 3D of an organoid or derived in culture or co-culture of progenitor organs and/or donors who self-organize in space/time like the in vivo[23], [29], [30]. However complete lab-on-a-chip devices that can work with the automated procedure and allows to see the behavioral cities of cells or their alterations to support microfluidic system, have not been prevented in the literature usage[16].

Currently from what can be found in the bibliography, we have found that commercially the only equipment available to perform some specific protocols is CELLviewer[31].

The CELLviewer is a newly conceived and developed multi-technology instrumentation, combining and synchronizing the work of different scientific disciplines in the field of management of both simple and complex 3D culture systems, allows to maintain in the most natural conditions possible the three-dimensional structure, following it over time through high-definition time-lapse microscopy.

II. CELLVIEWER

A. ***What is CELLviewer?***

The CELLviewer, Fig. 1., is a lab-on-a-chip for cells or neo-organs to be managed in the absence of adhesion, designed by CellDynamics, Bologna Italy. This multi-technological system is composed of a hardware tool and disposable parts: microfluidic chips used to insert the sample into them. The device's specific capabilities include environmental control, automatic change of cultural media, the ability to insert individual cells or neo-organs, and perform an optical analysis in the light field, darkfield, and fluorescence microscopy.

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Besides, CELLviewer technology allows through programmable software and automated execution of custom protocols remotely. The CELLviewer is an innovative approach that allows time-lapse monitoring and imaging of suspended 3D models in a dynamic medium, as well as keeping biological units alive and mimicking the environment more naturally than other systems. The CELLviewer system keeps 3D models in suspension, counteracting the gravitational fall of cells, by generating extremely finely controlled micro-currents. In addition to being used to raise the falling sample for gravity, these micro-currents perform two other functions: they allow the biological sample to be moved into the three dimensions in a controlled and custom manner and allow the change of the administration of external stimuli such as drugs, fluorescent probes, growth factors or more. With high-resolution time-lapse fluorescence imaging, either of individual live cells or pseudo-organs has grown inside a suspension, observed, and subsequently placed in a disposable cartridge. The basic system is surrounded by a series of measures that allow you to maintain the environment for cellular life: a disposable cartridge avoids contamination between one analysis and the next; the channels in which we generate currents have dimensions compatible with both spheroids and single cells; The system of changing the culture soil and administering drugs allows an airtight, sterilized connection.

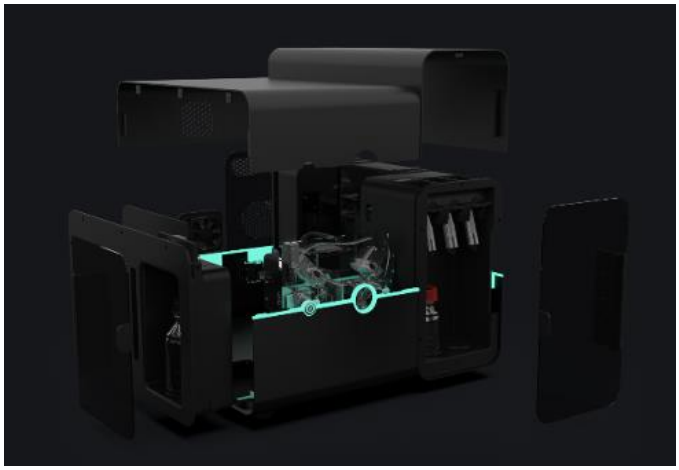

Fig. 1. CELLviewer

The data obtained from CELLviewer allow you to observe and understand spontaneous or induced biological phenomena. To do this, to allow individual cells or various cell populations to be cultured and observed, the tool includes several optical and electronic components.

B. CELLviewer components: Cartridge and Platform

The system mainly consists of an microfluidic integrated platform that combines different technologies, resulting in a hybrid system between an incubator for 3D cell culture and a wide-field microscope for time-lapse, live imaging. The platform is comprised of the hardware and the PC-embedded physical device, working on a single-use patented cartridge Fig. 2.

The cartridge consists of multilayer plasma-bonded PDMS sheets and it is crossed by a complex network of microfluidic channels of different diameters (starting from 0.100 mm diameter to 1 mm diameter), covering a total volume of about 2 mL. It is hosted inside a warming cover in aluminum. Buffer microflows for sample management are generated employing four hydrothermal pumps.

The platform of CELLviewer is a hybrid technology that merges a fluidic module, an optical system, electronics, and software for the system management. The fluidic module is made by motorized rotative valves, selection valves, and pinch valves, and it is intended for the cartridge liquid management. The connection between the cartridge and the hardware system is realized via a motorized clamp connection.

The imaging setup has two cooperating light paths to acquire x-y-z coordinates of a single floating sample in the analysis chamber. Vertical optics has 2.5 X magnification (N.A. = 0.08) objective (Olympus Life Science) and a LD (low definition) camera, while time-lapse imaging is performed on horizontal path, equipped with 20 X (N.A = 0.45) and 40 X (N.A. = 0.60) objectives (Olympus Life Science), mounted on a motorized revolver for agile switching. HD (high definition) camera (Hamamatsu Photonics) acquires up to 30 frames/s in full resolution of 4.0 megapixels with a cooling element built-in, enabling long-term acquisitions. Illumination is based on LED stroboscopic light source that consistently reduces sample photodamage, with a white LED for sample positioning and bright-field imaging and 5 LED for multicolor Epi-fluorescence microscopy. The system is equipped with a 7-filter Pinkel penta-band set (Semrock Inc.), that is designed for imaging of a sample simultaneously labeled with DAPI, FITC, TRITC, Cy5, and Cy7.

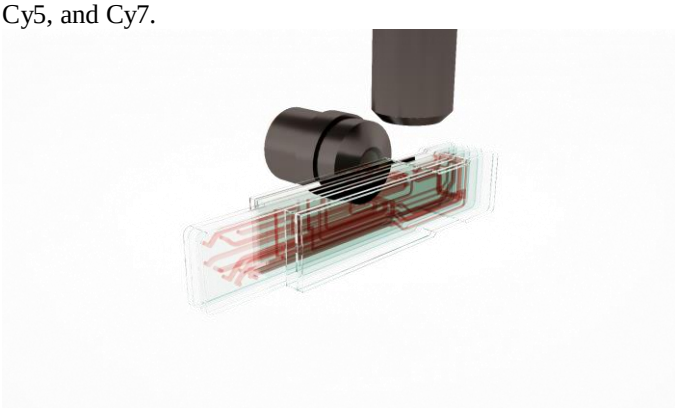

Fig. 2. Cartridge

The central core of the microfluidic circuit includes three parallel analysis chambers with a rectangular shape and a volume of 3 µL each. Each analysis chamber rests on the lower side of the microfluidic circuit for sample load and medium culture replacement with drug solution or fresh swab. Four

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micrometric channels reach the upper side of every chamber to deliver buffer microflows that counteract gravity force, preserving the sample in suspension, while keeping it in focus position for time-lapse, live imaging.

C. What we do with CELLviewer

For the validation of CELLviewer's ability to grow biological samples, a single cell 3D rotation and the viability assessment was used using MitoGreen-dye a fluorescence marker selectively permeable to live cells. It is possible to stain cells with dyes targeting mitochondria to understand the status of the cells such as apoptotic, high energy or mitochondria membranepotential (MMP) lost, and so forth MitoGreen is a fluorescent mitochondrial dye that showed green staining around the nuclear region and within the edges of the plasma membrane, which conceptually overlaps the mitochondrial region, contributing to the study of normal cell function in physiological states. MitoGreen-dye is a cell-permeating, fluorescent green lipophilic dye selective for live cell mitochondria when used at low concentrations. Mitochondria are deeply involved in both cellular life and death mechanisms. In addition to serving as the main source of ATP production, mitochondria also function as one of the main buffers for calcium, which regulates the activities of enzymes. Furthermore, ROS generated by the electron transport chains of mitochondria can cause oxidative damage to cells. For these reasons, staining of mitochondria with fluorescent dyes, antibodies or naturally fluorescent molecules contributes to the study of their structure and function in normal physiological and pathophysiological states. The CELLviewer system enables high-content time-lapse fluorescence imaging of live cells grown in suspension within a disposable cartridge while dispensing drug solutions to the sample chamber to visualize their biological effects at the level of single cell. Following this application note[32], the single Jurkat cells stained with MitoGreen are isolated inside the disposable CELLviewer cartridge and subjected to time-lapse in both the Bright-field and GFP channels. MitoGreen is spectrally similar to FITC, making it excitable at 488 nm. MitoTracker dyes can be applied to measure total mitochondria mass or to study the changes in mitochondria mass following desired treatments. MitoTracker Green FM from Cell Signaling and fromThermoFisher Scientific are the examples of commercially available MitoTracker assays. MitoTracker Green dye stains the mitochondria of living cells but it is not dependent on the MMP MitoTracker Green is not compatible with fixation and the signal can be obtained at excitation and emission wavelengths of 490 and 516 nm, respectively [33].

III. EXPERIMENT AND RESULT

The Jurkat cells were grown at 37 c and 5% CO2 in RPMI 1640 soil, supplemented with 2 mM of L-glutamine, 10% FBS, 100 units/mL of penicillin, and 100 mg/mL of streptomycin. Before the experiments, Jurkat were washed and suspended at final concentration of 5 x 105 cells/ml in FBS culture soil at 5%.

Then, the sample was incubated for 20 minutes in the dark at 37 degrees C with MitoGreen 200 mM (PromoKine, PromoCell). After incubation, the cells were centrifuged at 2000 rpm for 5 minutes to remove excess MitoGreen and resuspended in culture soil at 5% to the CELLviewer work concentration of 5 x 103 cells/ml. The sample is then piped inside a 50ml Falcon tube closed with a 50ml CELLviewer DOCK. After isolation and fluid adaptive autofocus, CELLviewer automatically captures sample images in the Brightfield channel and GFP channel at 0.5 fps with 20X magnification.

A. Single live-cell imaging tracking

Single-cell imaging is used to study cell heterogeneity in cancer line cases. However, direct monitoring of many individual initial cells with morphological change over time has so far not been technically feasible[34], [35]. Single-cell tracking in 3D space is possible and combined with subsequent biochemical analyses of individually tracked cells, keeping their identity traceable with the CELLviewer system. Single-cell 2D tracking can more easily integrate subsequent biochemical analyses and act as surrogate measurements for the 3D situation[23]. On this basis, the 3D rotation of single cells was successfully achieved experimentally in figure Fig. 3.

Fig. 3. Single 3D live-cell

As is possible to see, when flowed inside the cartridge chamber, the individual cell is tracked under the action of the optics. Under the effect of self-adaption and levitation, a single cell can maintain a stable spatial position.

B. Mitogreen in live 3D single cells

Jurkat (ATCC) Cells
Colture medium RPMI (Gibco, Life Technologies, Thermo Fisher Scientific)
MitoGreen (PromoKine, PromoCell)
CELLviewer imaging system and disposable cartridge
CELLviewer 50 ml DOCK

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After single Jurkat cell isolation in the microfluidic cartridge and fluid adaptive autofocus, CELLviewer automatically captures time-lapse imaged sample for 4 hours in the GFP channel, in Fig. 4., and BrightField channel.

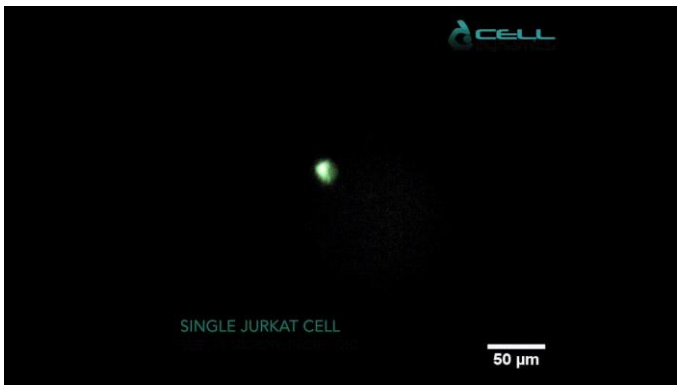


Fig. 4. Mitogreen in live 3D single 3D cell

MitoGreen and resuspended in culture soil at 5% to the CELLviewer work concentration of 5 x 103 cells/ml. The sample is then piped inside a 50ml Falcon tube closed with a 50ml CELLviewer DOCK. It's showed green staining around the nuclear region and within the edges of the plasma membrane.

ImageJ software was used for image analysis using the Measure function to: calculate the diameter of a single cell; Plot profile plugin to create fluorescence intensity graphics along a straight line that passes through the cell; 3D surface plot plug-in to display in 3D the distribution of the intensity of spatial fluorescence. As we can see in Fig. 5. with the Plot profile and 3D surface plot , MitoGreen is a fluorescent mitochondrial dye that showed green staining around the nuclear region and within the edges of the plasma membrane, which conceptually overlaps the mitochondrial region, contributing to the study of normal cell function in physiological states.

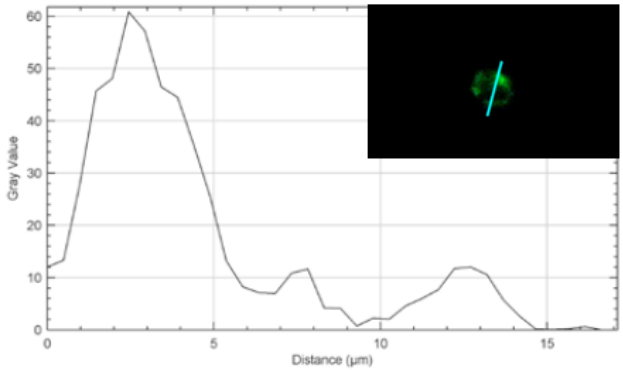
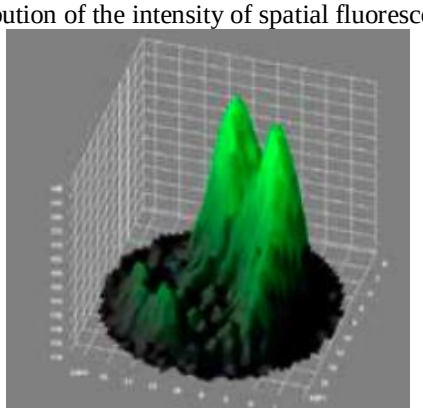


Fig. 5. ImageJ software

Plot profile plugin is used to create fluorescence intensity graphics along a straight line that passes through the cell.

3D surface plot plug-in is used to display in 3D the distribution of the intensity of spatial fluorescence .



IV. CONCLUSION

Staining of mitochondria with fluorescent dyes, antibodies or fluorescent molecules can greatly facilitate studies of their function and distribution and the viability of cells in healthy and diseased individuals. The preliminary experience conducted with CELLviewer indicates that this equipment responds to the needs of individual operators as it consists of a synthesis of different integrated tools, which works both with manual and automated control. A microfluidic system has been developed and demonstrated that the 3D model can locate the 3D model spatially, it's possible to carry out experiments in direct time in terms of physiology, toxicology and clinical pharmacology.
The entire automated system allows full autonomy and protocol management thanks to the software making the operator free to conduct other work, thus increasing the productivity of his project. In summary, the proposed microfluidic technology can serve as a new platform approach, which has the potential to advance studies at the cellular level[36].

V. DISCUSSION

Compared to other devices in the literature[16], [21], [26], [29], [37] the CELLviewer is able in addition to obtaining detailed images of current cellular morphology with resolution and high-quality data; it is possible to carry out experiments in direct time in terms of: physiology, toxicology, and clinical pharmacology. The entire automated system allows full autonomy and protocol management thanks to the software making the operator free to conduct other work, thus increasing the productivity of his project. A microfluidic system has been developed and demonstrated that the 3D model can be spatially located the 3D model. The Epi-Fluorescence mitochondrial imaging was conducted to guide the configuration. Moreover, by utilizing time-lapse imaging of cells can be achieved, the evolution of cells and their 3D morphology can be acquired. In summary, the proposed microfluidic technology can serve as a new platform approach, which has the potential to advance studies at the cellular level. Given the basic importance of the research economy, the maximization of the times and ways of

producing information is a close and obligatory landing. The preliminary experience conducted with CELLviewer indicates that this equipment responds to the needs of individual operators as it consists of a synthesis of different integrated tools, which works both with manual and automated control. This kind of control system will facilitate the search dynamics even remotely, through software and on different devices, allowing the operator to simultaneously perform other activities that require his physical presence while ensuring maximum quality of the processes implemented. The live-imaging of 3D models in Microfluidics represents an optimal solution in observing the maintaining optimal health of cells while achieving the gap between the 2D model[38]. Recent advances in the design, prototyping, and production of microfluidic systems have enabled new ways of addressing disease study, with the advent of lab-on-a-chip technologies integrating different laboratory operations into individual microfluidic networks and advancing the understanding of cell-cell and cell-biomaterial interactions, with the engineering of organ-on-a-chip devices that mimic the response whole organs and systems using multichannel cell culture chips. These models are beginning to replace the most common cell culture systems, mainly Petri dishes, as the multichannel structure provides cells with a 2D or 3D-like environment to current in vivo configurations. Despite the progress of the last decade in the field of bioengineering, mainly concerning the success of these organ-on-chip systems as relevant research tools for the study of complex pathologies sustainably and economically, there is room for performance optimization. The development and use of devices capable of mimicking in vivo eukaryotic cells is a complex problem in itself, to understand the behavior and interactions with the extracellular environment capable of mimicking in vivo performance by advancing the research of the disease, constitutes a long-sought goal and an ever-present research challenge. Biomedical research on cellular systems of different complexities has evolved over the years towards 3D in which 2D has been deemed no longer enough.

The fields of biotecnological and biomedical research continually moves towards a system that identifies as appropriate, a model capable of representing the in vivo counterpart, striving to validate new approaches with better results than the classic 2D monolayers in more physiological environments. Depending on the circumstances, there are different and often multiple reasons that differ between 2D and 3D where the cell cycle of a single cell alters or that the behavior of cell populations varies in space-time within clusters. The organization, composition, and several structures are among the best-understood signals that are integrated by the cell to regulate many key tasks including survival, differentiation, proliferation, migration, and polarization[39]. Inevitably, the study of biology and cell function in vitro requires stripping the cells of this native cell-cell and multi-cell interactions and their introduction into an environment of suspension and specific adhesion to the culture system. A better understanding of 3D model will help further optimize in vitro systems for the study of cell and tissue functions, as well as better translation to new therapeutic approaches.

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