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Three dimensional tumor models for cancer studies

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1. ABSTRACT

It is well recognized that one of the major drawbacks of using traditional two dimensional cultures to model the living systems is inaccurately reflecting the physiological manner in which modulators, nutrients, oxygen, and metabolites are applied and removed. Moreover, the two dimensional culture system poorly reflects how different cell types interact with each other in the same microenvironment. Since the first global development of three dimensional (3D) cell culture techniques in the late 1960s, this last decade has seen an explosion of studies to promote 3D models in the fields of regenerative medicine and cancer. The recent surge of interest in 3D cell culture in cancer research is attributable to the interest in developing closer to real life models. The ability to include various cell types and extracellular components reflect more the physiological conditions of tumor microenvironment. In this short review, we will discuss different approaches of 3D culture system models and techniques with a focus on the 3D interactions of cancer cells with stromal cells in the goal to reevaluate old and develop new therapeutics.

2. INTRODUCTION

Three dimensional (3D) cell culture techniques, pioneered at the beginning of 20th century by Harrisson and Carrel, have been rapidly gaining popularity in recent years (1, 2). In fact, around 90% of all 3D cell culture articles listed on PubMed have been published since 2000. This recent wave of interest and investigation in 3D cell culture, especially in the area of cancer research, is likely attributable to increased recognition of the importance of cellular interactions in the context of the specific microenvironment. The 3D cell culture market is expected to grow to a staggering 3.7 billion US dollars by the year 2021 (3). Although research has come a long way toward improving our understanding of cells, the bulk of this knowledge has come from piecemeal investigations into one signaling pathway or another. This method has produced, to date, a comprehensive picture of the biology of individual cells. However, fundamental gaps remain between our understanding of individual cells and how these cells function collectively in interdependent tissues. In a research climate heavy with the influence of traditional 2D monoculture, understanding the influence of real cell-cell and cell-matrix interactions on general cellular proliferation, differentiation, apoptosis, etc., requires a closer to real life model. Three dimensional models are particularly relevant in the study of interactions between normal and cancerous cells.

Through insights gained by stromal cell and cancer research using two dimensional (2D) models, we now have a better picture of how cells require signals from their environment to differentiate and form functional

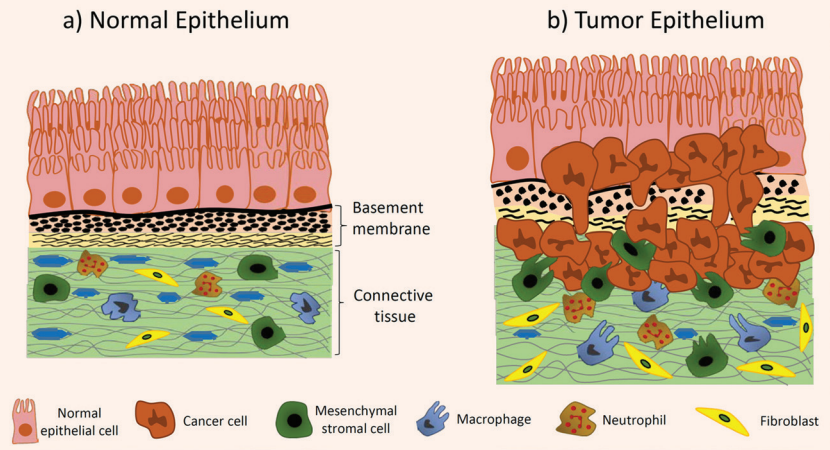

Figure 1. a) Structure of normal epithelium b) Structure of epithelium during epithelial- mesenchymal transition in tumor microenvironment.

tissues. These signals include not only ligands, such as VEGF, Wnt proteins, cytokines, and metabolites, but signals induced through tensile and sheer stresses relayed to cells by their attachments to the extracellular matrix (ECM) (4-7). There are, in fact, numerous studies providing compelling evidence that ECM remodeling is an essential component of the metastatic cascade in cancer (8, 9). Additionally, the distribution of oxygen, nutrients, and signaling molecules in 2D cultures are not the same as in 3D cultures. Indeed, because oxygen has low solubility in tissue culture media, it is sometimes supplied to cells within 3D scaffolds in specially-built perfusion bioreactors (10). This review will focus on the capacity of 3D culture to increase our knowledge of cancer initiation and progression, with the ultimate goal of finding new diagnostics and therapeutics through these techniques. It is compelling to develop and study the tumor 3D structures in order to advance in developing physiologically relevant tumor models.

3. EPITHELIAL TUMOR STRUCTURE

Between 80 - 90% of all cancers arise in the epithelium, which is present throughout the body as a component of the skin tissue, as well as the covering and lining of organs tissues, cavities, and internal passageways (11). Epithelial tissue has a bilayer structure that consists of polarized epithelial cells and ECM rich stroma (Figure 1a). Epithelial cells are very closely packed with almost no gap between cells and are based on a thin membrane-like extracellular matrix. The stroma contains essential structural and nutrient supplement systems, which include blood and lymphatic vessels, immune cells and fibroblasts. Blood vessels do not penetrate beyond the stroma, and nutrients at the apical surface are reached through diffusion. The apical facade of the epithelium protects the tissue by forming a barrier against the external environment. Depending upon the location, the apical layer of epithelium may consist of specialized structures known as cilia or microvilli. These specialized structures have vital functions like absorption, adsorption, secretion or removal of debris. The basal facade of the epithelium is attached to the basal surface through integrins and other matrix adhesion molecules (12). The stroma provides the epithelium with both physical support and ECM. The extracellular matrix is rich in collagen scaffolds and several other structural proteins, which forms the basis for physical support to the epithelial cells (13). The ECM also interacts with epithelial layer by controlling the microenvironment with their ability to bind to various proteins and molecules (12). Epithelial tumors originate from foci and then these cells evolve to proteolytically degrade their basement membrane to become mesenchymal cells by the epithelial-mesenchymal transition program (Figure1b) (7). These now mesenchymal cells enter the blood stream and migrate to farther locations. Malignancies formed in epithelial tissue are known as carcinomas. Carcinomas mainly affect organs or glands that are capable of secretion. Examples include breasts, lungs, prostate, bladder etc. (11). Carcinomas are highly heterogeneous even within the gland/organ type. Each type of neoplasm exhibits distinct histopathological and biological features. For example, according to World Health Organization (WHO) endorsed classification, there are 20 major types and 18 minor types of breast cancers that are prevalent (14). In another example of tumor complexity, efforts to identify the cell origin of the adenocarcinoma of the lungs resulted in unfruitful results due to not only epithelial cell types in the lungs, but also due to heterogeneity even in individual tumors (15). Given the knowledge about the tumor structure, a true to life model of an epithelial tumor would include stromal cells, epithelial cells, immune cells and endothelial cells.

4. MESENCHYMAL TUMOR STRUCTURE

Much of mass of the body is composed of connective tissue or “soft” tissue. Connective tissue is

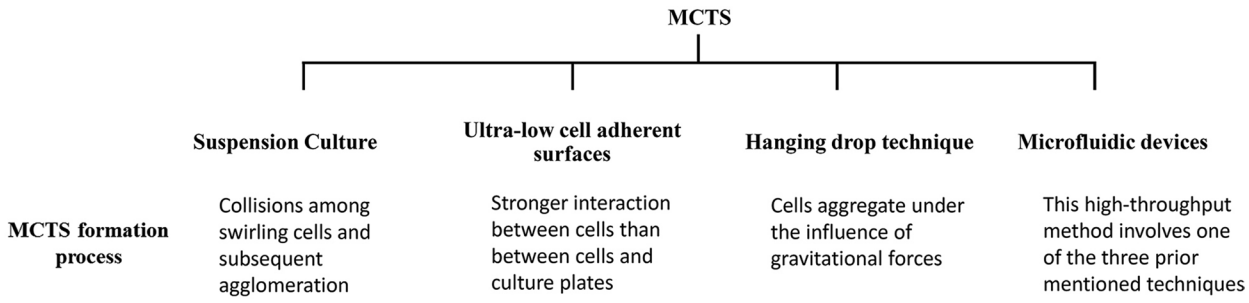

Figure 2. Principle difference among MCTS formation techniques.

mesenchymal in nature, originating from mesodermal layer of the embryo (16). Contrary to epithelial tissue, cells in mesenchymal tissue lack polarity and are able to differentiate into multiple cell types (17). Mesenchymal cells play the pivotal role in tumor metastasis of several kinds of cancers (18). Tumors that originate in mesenchymal cells are called sarcomas. Sarcomas are relatively rare cancers and are observed predominantly in children and young adults (19). Physiologically, sarcomas are aided by stromal cells and ECM with structural support and nutrient supplement. Several reports conclude that ECM has great influence on proliferation, migration, and differentiation in various sarcomas (6, 7, 20). For example, proteins like stathmin I that regulate cell motility increase the metastatic potential of sarcoma cells (21, 22). Enzymes such as matrix metalloproteinases contribute to angiogenesis by unsettling extracellular matrix barriers and enabling endothelial cells migration through the surrounding tissues (5, 23). Sarcomas are less complex compared to carcinomas. However, they are hard to diagnose and are often underrated as benign due to painlessness and its ineffectiveness on overall health (24, 25). Sarcomas develop in bones, joints, and soft tissue, and are predominately observed in children. According to WHO classification, there are 10 major types of soft tissue sarcoma and 9 major types of bone sarcoma, in which both soft tissue and bone sarcomas contain at least one class that is either an undifferentiated or an unclassified sarcoma type (26). Osteosarcoma, Ewing sarcomas and rhabdomyosarcoma are most commonly observed sarcomas among children. Undifferentiated pleomorphic sarcoma, liposarcoma, and leiomyosarcoma are the most common sarcomas in adults (27).

5. TUMOR SPHEROID MODELS

Multicellular tumor spheroids (MCTS) can be obtained by the aggregation and compaction of cell suspension cultured under non-adherent or low-adherent conditions. Tumorospheres are then formed by clonal proliferation in low-adherent conditions (28). Primarily, there are four methods researchers have used to create the MCTS (Figure 2).

5.1. Suspension culture of stromal and cancer cells

In this method, cells are suspended in swirling liquid medium using a rotational motion to resist cell attachment to the culture surface. The swirling cells then form MCTS through collisions and subsequent agglomeration (29, 30). For example, *in vitro* dynamic 3D techniques cultures of Mesenchymal Stem/Stromal Cells (MSC) using spinner flasks and a rotating wall vessel bioreactor have been showed to be beneficial for retaining MSC properties over prolonged period of times (31-33). Spinning bioreactors have been used to generate large production of tumor spheroids to test the efficacy of chemotherapy or immunotherapeutic drugs (34-36).

5.2. Ultra-low cell adherent surfaces

Here, the interactions between cells are stronger than their adhesive forces with tissue culture plates (28). Several studies reported usage of an agarose surface to create MCTS, mainly due to the low cancer cell attachment to agarose (37-39). Commercially available ultralow attachment plates that use a hydrophilic, non-ionic, neutrally charged hydrogel covalently bound to a tissue culture polystyrene surface (Corning, Lowell, MA) have also been extensively used to generate MCTS (40, 41). Researchers have also used extracellular matrix (ECM) and protein-based hydrogel coatings to create MCTS. One such surface that is commonly used is Matrigel, which is obtained from the ECM of mouse sarcoma cells (42). Matrigel does not require single cell suspensions, which are sometimes difficult to obtain. For instance, Young *et al.* cultured cell clusters obtained from LuCap xenografts atop the ultralow attachment plates (43) and Theodoraki *et al.* used spontaneously- formed spheroids of tumor explants sieved for various sizes using cell strainers (44).

5.3. The hanging drop technique

In this method, the cells are suspended in a droplet of medium and are allowed to aggregate into MCTS under the influence of gravitational forces (Figure 3). This technique, used since the last century, is very simple to execute and highly cost-effective. Standard cell culture plates or lower cost bacterial culture plates are alternative choices to generate MCTS as the cells do not attach the

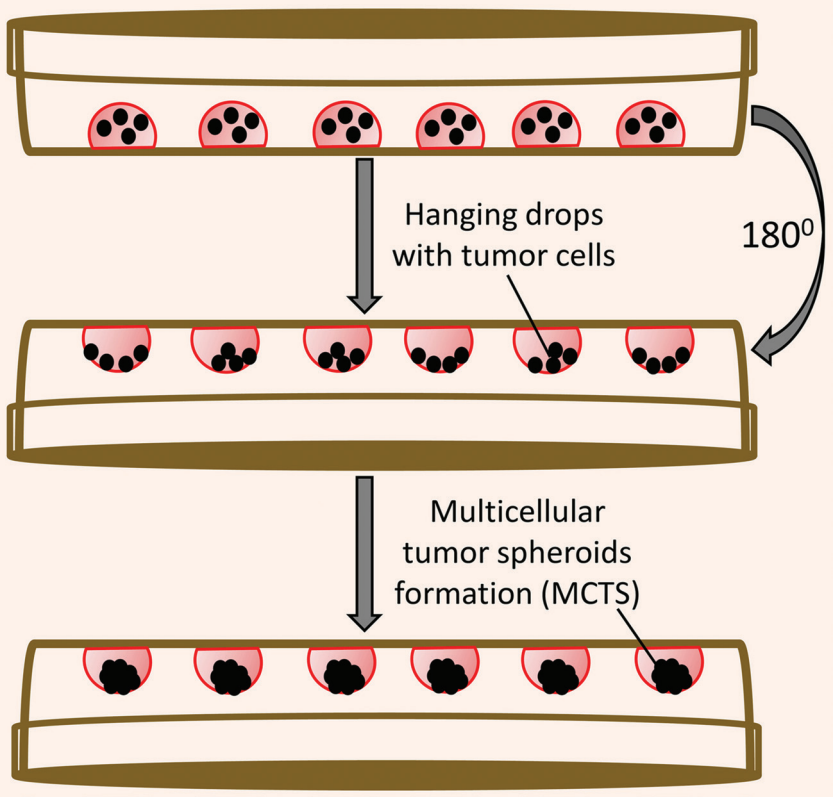

Figure 3. Schematic depicting the principle of hanging drop technique. Dark spots in the center of droplets in (a) and (b) represent cancer cells. Dark mass in the center of droplets in (c) represents well-formed 3D tumor spheroids.

culture surface. One of the major limitations of this technique, and also shared by the two other described above, is an important size variability between MCTS obtained. There are commercial 96 or 384-well plates specially designed to create hanging drops in a more uniform manner using an automated cell culture robot to achieve high throughput drug screening (45). Other commercially available designs have been used to create MCTS of breast cancer cells in a semi-automated process (46).

5.4. Microfluidic devices

Microfluidic technologies involve the manipulation of very small fluid volumes within artificial microsystems (47). In this method, the formation of spheroids is based on hydrodynamic trapping of cells in micro-chambers with controlled geometries. The continuous perfusion of fresh liquid media maintains the compaction of the trapped cells, and the size uniformity of the spheroids can be controlled by perfusion flow rate (48). Each micro-chambers that can house one to a few-hundred cells are usually designed using soft lithography on polydimethylsiloxane (PDMS), an elastomeric material with optical transparency and high gas permeability qualities (49). Several studies have adapted this technology to grow more size controlled spheroids. For example, Sabhachandani *et al.* prepared MCTS of MCF-7 breast cancer cells by encapsulating them in alginate droplets inside a PDMS microfluidic device (50). This device has the capacity to simultaneously culture a thousand MCTS on a chip and can perform drug sensitivity testing in a high throughput manner. Ayuso *et al.* used spheroids of oral squamous carcinoma cells embedded in collagen in a microfluidic device to study their chemotactic response (51). More recently, microfluidics coupled to a flow cytometry device has shown the ability to produce and analyze thousands of spheroids, making this technique suitable for drug screening applications (52). In the coming years, the generalization of 3D printing devices will allow the explosion of microfluidic chip use (53).

Other microfluidic systems have been used for large production of spheroid. For instance, Alessandri *et al*. have developed a microfluidic technique using permeable, elastic, hollow microspheres capsules by co-extrusion of colon carcinoma cells with alginate (54). Similarly, Kim *et al*. obtained a large number of embryonic carcinoma cells spheroids in alginate core-shell microcapsules using a 3D coaxial flow (55).

6. 3D SCAFFOLD-BASED MODELS

To achieve true to life model of cells interactions, other studies have focused on the integration not

only of particular cells but also by addition of multiple components to improve the cellular three-dimensional microenvironment. Several approaches have been used in the construction of 3D scaffold-supported tissue models.

One approach is to use materials with the closest 3D environment already available by using tissue decellularization techniques (56, 57). These promising techniques developed for clinical transplantation applications have not been tested yet to study stromal-cancer interactions but might be really useful once adapted for *in vitro* research studies.

At the *in vitro* level, researchers have developed particular scaffold materials ranging from natural biomaterials (e.g., collagen, fibrin, hyaluronic acid, gelatin, matrigel, or alginate) (58-62) to synthetic biomaterials (e.g., polymers such as polycaprolactone or polyethylene glycol, and inorganic materials such as titanium or ceramic-based materials) (63-66). Natural biomaterials have the advantages to be biocompatible but are also biodegradable, which can cause problems in the study of stromal and cancer cells interactions (67). These problems do not occur with synthetic scaffolds but they may lack sites for proper cellular adhesion. In addition, 3D cells aggregates may be difficult to recover for further *in vitro* studies (68).

While using a scaffold to encapsulate cells can put diffusion limits on nutrient and waste flow, researchers have used the various scaffold properties such as chemistry, porosity, and stiffness to generate favorable culture performance. For instance, Liang *et al.* compared soft collagen gel to stiff collagen-PEG gels and found that the scaffold stiffness suppressed tumor malignancy in hepatocellular carcinoma cells (69). The elasticity and stiffness of scaffold material can be controlled by changing the concentrations of gelating agents. Ulrich *et al.* developed a strategy to improve the elasticity of weak collagen gels by two orders of magnitude by incorporating the agarose, without disrupting the fiber architecture (70). For example, a recent reports suggests the possibility of using nanofiber based scaffolds (71), which improve the survival and differentiation of mouse embryonic stem cells.

Many reviews reported advantages and disadvantages between the multiple types of 3D scaffold (72-75), and it appears that combinations of different approaches, including with non-scaffold technologies, are the most promising (Table 1).

7. FUTURE PERSPECTIVES FOR 3D CULTURE METHODS

Regardless of the methods used to generate 3D cells aggregates, there are a few mandatory requirements to accurately replicate the *in vivo* environment *in vitro*. Essentially, in varying levels of complexity, these models seek to recreate as closely as possible the real cellular microenvironment by integrating multiple cell types, blood and lymphatic vessels or mimics, and extracellular matrix components (76). Immortalized and primary *in vitro* cultures have now been derived from a wide range of cancers and are currently used in numerous cell biology studies. Newer methods for 3D cultures of these cells will range from bottom-up to top-down methods, including but not limited to: 1) decellularization of natural tissue, followed by recellularization with the desired cell types, 2) gradual layer-by-layer cell growth in culture dishes, 3) micropatterning and microfluidics technologies, and 4) 3D printing. The micropatterning and microfluidics technologies offer a great prospect of standardized MCTS generation for mass production. It will enable scientists to employ high-throughput screening technologies including using more sophisticated MCTS co-culture models, which more closely reflect to the reality of tumor tissues composed of tumor and various stromal cell types (77-79). For example, a stereolithography-based 3D printer using hydroxyapatite nanoparticles suspended in hydrogel is able to create a geometrically optimized matrix to mimic the 3D environment of bones (80). Similarly, 3D bioprinting offers unlimited possibilities to arrange different cell types and ECM-based biomaterials in a normal anatomical arrangement to create a more *in vivo*-like culture performance (81) as shown in Figure 4.

8. DRUG SENSITIVITY PATTERNS

Recent developments in tissue culture technology made it possible to culture patient derived cells to obtain tumor spheroids without losing the original tumor's properties in terms of genotype and phenotype (82-84). These developments may soon replace the traditional 2D cell culture protocols and will establish themselves as standard techniques for culturing cancer cells. Establishment of 3D cell culture as standard technique provides an exciting prospect in drug screening for specific types of tumors for specific patients, which will be a giant leap towards the development of personalized medicine. Nevertheless, these developments also grant different challenges. For example, traditional cytotoxicity assays are developed and optimized for 2D cell culture models and may not serve the purpose in 3D models.

3D cell culture is currently used in anticancer studies, cytotoxicity studies, drug discovery experiments and biosensor/bioassay applications (84-86). 3D cell culture models are superior models and better mimic the actual tumor microenvironment and pathological conditions. For example, 2D cell culture cells are grown in a single layer spread on a plastic surface. When a potential treatment model is tested, cells are prone to death at lower concentrations of chemotherapeutic agents or under low intensity radiation (87-89). The

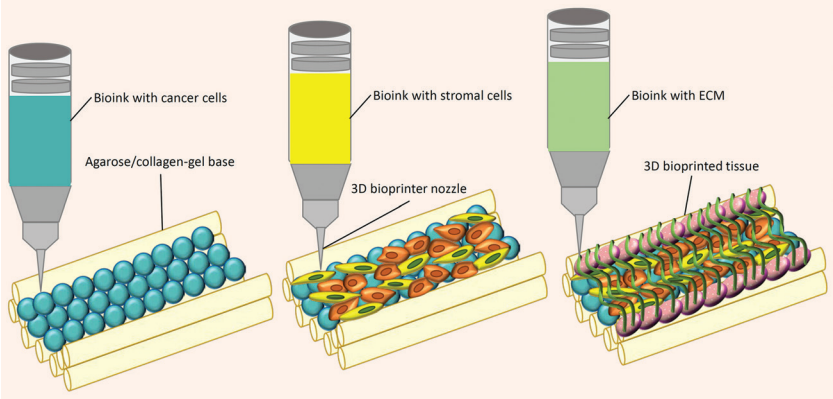

Figure 4. A 3D bioprinter prints cell aggregates in precise patterns to yield anything from simple 3D cell spheroid to whole tissue. 3D bioprinting also makes it possible mimic tumor microenvironment that is closely comparable to real tumor microenvironment.

Table 1. Advantages of 3D vs. 2D cell culture models

3D culture	2D culture
Superior model and closely represents the tumor microenvironment	Less relevant with tumor cell monolayers
ECM interacts with cells by providing structural, nutrient, and communicative support	Cells are in contact with ECM and with plastic on one surface
Co-culture model mimics tumor microenvironment with multiple cells layers	Co-culture model incapable to create a tumor microenvironment
Tumor drug distribution and drug dosage can be studied more precisely	Drug distribution cannot be studied on a monolayer
Cell-cell interactions are similar like in tissues *in vivo*	Limited cell-cell interactions

reason for this kind of observation is that the drug/radiation has to penetrate just a single layer in order to be effective against cancer cells. Whereas, MCTS grown in 3D model are multilayered structures and may not likely be killed at similar concentrations. This phenomenon may be attributed to the inefficiency of the low doses of drug or radiation, which may not be able to penetrate the core of the tumor, a close mimic of the real tumor conditions. Similarly, differences in cellular compaction has been observed in cancer cells spheroids model generated by suspension culture, ultra-low attachment or hanging drops techniques, which led to an increased chemotherapeutic resistance (90). Hence, MCTS/3D model serves as valid targets for developing personalized medicine/drug screening/drug discovery experiments.

9. CAVEATS

Though 3D scaffold based models boast close resemblance to real tumor micro-environment, it is not a 100% match to real tumor environment for obvious reasons (anatomical complexity, physiological context, etc.). Apart from that, 3D models also suffer from severe reproducibility problems. Oftentimes, it is observed that the size of spheroids is not uniform (86, 91, 92). Pipetting errors or non-homogenous cellular resuspension can lead to important size difference. In addition, the presence of any small particles in the culturing media could modify cells aggregation and the fate of the spheroid shape (93). This non-uniformity in the spheroid size leads to inequivalent distribution of nutrients, differences in microenvironment, and inequivalent drug/radiation exposure. 3D scaffolds also demand a much more carefully controlled environment in terms of temperature and pH, which is laborious (94-97). Post culture process is another laborious task involved and automated solutions are yet to be improvised. It is also difficult to analyze 3D models using the most common biology lab tool the microscope in which typical light penetration depth is around 100 µm (98). Insufficient penetrability of light makes it tricky to analyze the cells in the inner layers of the spheroids, causing some well-established, cost effective techniques like MTT, Trypan blue assay ineffective in the analysis of 3D models.

3D cell culture undoubtedly has improved the efficiency of non *in vivo* assays by closely mimicking the tumor microenvironment. Scientists are able to obtain more meaningful data before they enter into any mouse or human models and were successful in eliminating those drug candidates which are only effective against less relevant 2D cell culture models. However, 3D culture models still are impeded by some reproducibility problems, and lack of automation makes it a tedious technique. Addressing these problems will greatly enhance the capability of the technique itself and high throughput screening as well.

10. ACKNOWLEDGEMENTS

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Abbreviations: 3D: three dimensional, 2D: two dimensional, ECM: extracellular matrix, MCTS: multicellular tumor spheroids, MSC: mesenchymal stromal/stem cells, MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide, PDMS: polydimethylsiloxane, PEG: polyethylene glycol WHO: world health organization

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