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3D Cell Culture: Techniques, Applications in Healthcare, and Future Challenges

Mukunthan K S
Manipal Institute of Technology, MAHE, Manipal, India, mukunthanselvam@gmail.com

Research Article

3D Cell Culture: Techniques, Applications in Healthcare, and Future Challenges

Jay Rabindra Kumar Samal, Mukunthan K S*

Email: mukunthanselvam@gmail.com

Abstract

Two-dimensional (2D) cell cultures have been the most commonly used technique since cell culture was first introduced. However, these cultures do not accurately represent animal physiology as many factors that play an important role in the growth of animal cells are overlooked. Three-dimensional (3D) cell culture techniques provide higher accuracy in recreating the microenvironment of native tissues. But, 2D cell culture techniques are still preferred for the majority of animal cell cultures as these are well established and relatively inexpensive. Various techniques have been developed for 3D cell culture but these systems are highly complex as the number of parameters has to be considered to achieve the desired functionality. As 3D cell culture is a novel technique, it is not well understood and hence not very easy to handle, which affects their automation and reproducibility thereby increasing their cost. In the present review, we compare the advantages of 3D cell culture techniques over conventional 2D cell cultures, briefly discuss various techniques that have been developed and also have a brief look at the applications of 3D cell culture techniques in healthcare. Finally, we look at the future challenges plaguing the acceptance of this novel technique.

Keywords: 2D cell culture, 3D cell culture, Scaffold, drug delivery, healthcare.

Introduction

Since cell culture was first introduced, flat two-dimensional (2D), surfaces have been the most commonly used substrates for cell growth, based on the assumption that the cellular monolayer so formed provides an accurate representation of animal physiology [1, 2]. This is however not true, as the technique overlooks many factors that play an important role in the growth of animal cells, including communication between adjacent cells of the same as well as different types, communication between cells and the extracellular matrix, and the response of the cells to the dynamic three-dimensional (3D) environment present in vivo [2]. Mammalian cells, when present in vivo, are in a 3D environment, with characteristic chemical and biophysical interactions with their environment as well as with other cells in the surrounding space. Many cell functions like adhesion, migration, and cell proliferation have been found to be influenced by these interactions [3, 4]. It has been observed that 2D cell culture plates, which are commonly used for cell culture, are inadequate in their ability to recreate the environment that may be experienced by the cells in vivo due to the preferential occurrence of certain cellular processes in 3D cell cultures [5]. Thus, 3D cell culture provides higher accuracy in recreating the microenvironment that is experienced by cells in vivo [6].

A plethora of 3D cell culture techniques have been developed, which account for the spatiotemporal microenvironment of the cell to mimic the native tissue [7, 8]. However, these systems are highly

Jay Rabindra Kumar Samal[1], Mukunthan K S[2]

[1] *Department of Biomedical Sciences and Engineering, Tampere University of Technology, PO Box 527, FI-33101 Tampere, Finland.*

[2] *Department of Biotechnology, Manipal Institute of Technology, Manipal Academy of Higher Education, Manipal, Karnataka, India.*

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* Corresponding Author

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complex as several parameters have to be considered for the desired functionality and characteristics to be maintained [**2**].

Advantages of 3D cell culture

3D cell culture provides several advantages over conventional 2D cell culture techniques. The methods that are used for creating 3D cell culture systems offer dynamic control and thus can be manipulated for changing properties of the cellular microenvironment as desired [6]. The ability to manipulate the cellular microenvironment also confers the ability to model disease states in vitro and as a consequence, reduces the need for animal models [9,10]. The effects of drug dosage and parameters for drug delivery are better suited to be studied in 3D cell cultures as multiple layers of cells are formed, which form a natural barrier to drug diffusion, as compared to a cell monolayer in 2D cultures [11]. This technique also allows the growth of co-cultures, with accurate cellular interaction as is found within tissues [12]. Studies have shown that plasma deposition can modify the surface chemistry of the microfibre structure of scaffolds and promote the attachment of cells [13]. The 3D cell culture can have direct application in tissue regeneration as the technique provides conditions that are a requisite for the development of a system comparable to in vivo environment.

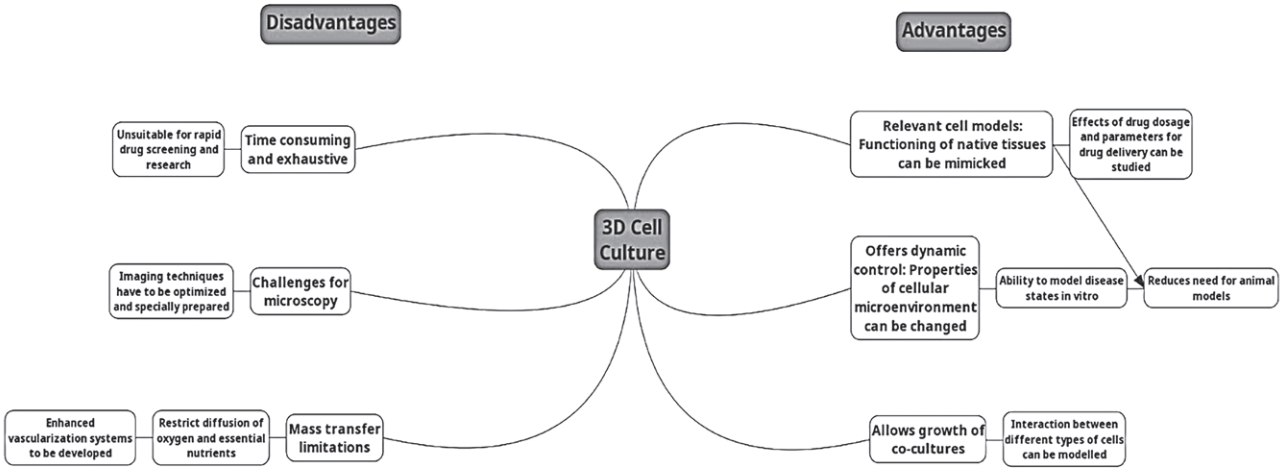

Figure 1: Advantages and Disadvantages of 3D Cell Culture.

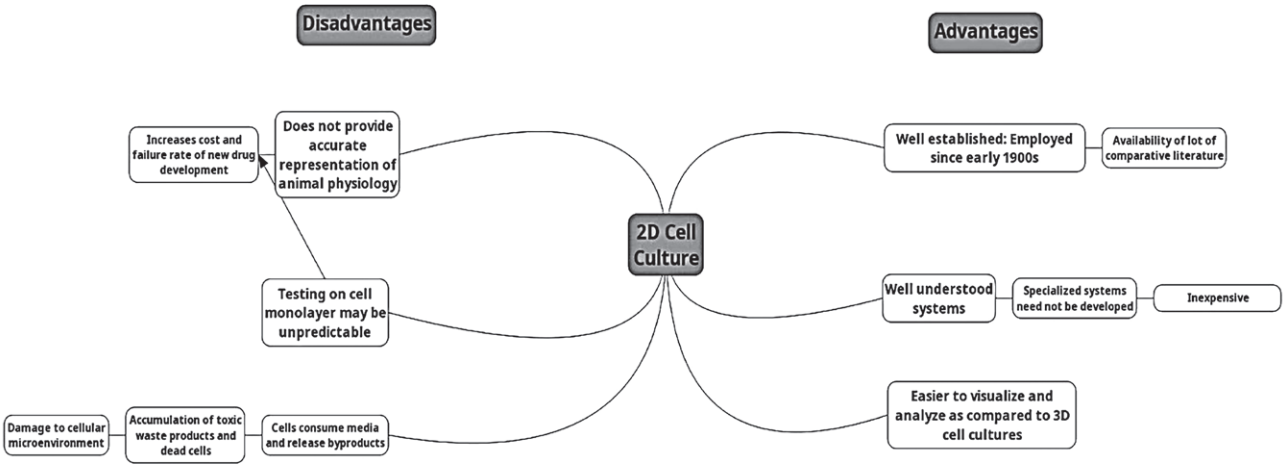

Figure 2: Advantages and Disadvantages of 2D Cell Culture.

Why are 2D cell culture techniques preferred?

The 2D cell culture provides some distinct advantages as a result of which they are still preferred for the majority of cell cultures. These techniques are well established as they have been employed since the early 1900s, resulting in the availability of a lot of comparative literature to compare current results obtained against previous results [14]. These techniques are well understood and thus relatively inexpensive as specialized systems need not be developed [15]. 2D cell cultures are also much easier to visualize and analyse as compared to 3D cell cultures [16].

State of the Art

The advent of microfluidics has made it possible to control precisely the spatiotemporal microenvironmental parameters of the cell culture to mimic reality as closely as possible [17, 18]. The existing 3D cell culture techniques can be broadly classified into two categories:

A. *Scaffold-based*

Scaffolds are porous biomaterials that can act as substrates for tissue regeneration [19]. Due to their porous nature, the scaffolds can facilitate the distribution of oxygen, nutrients and removal of wastes and can thus overcome the mass transfer limitations offered by 3D cultures to a certain extent. Cells can proliferate into the pores and migrate within them to eventually adhere [20]. As the cells grow, they interact with each other and turn into structures akin to native tissues [2]. However, the aggregates so formed often have heterogeneous sizes and are referred to as spheroids. These structures can be applied for complex tissue architecture studies [21]. Scaffolds often have fibre structures that provide a large surface area for cell attachment and proliferation [6].

These scaffolds are fabricated by electrospinning of biocompatible polymers, which produces thin nanofibres which can be aligned or random depending on the application [22]. The layout of the scaffold should be similar to the tissue of interest with structure, scale and function being important parameters, which should be reproduced. The scaffold must also be biocompatible to support the growth of cells [2]. In vitro scaffolds are used for 3D cell cultures for application in research such as drug and cosmetic testing, among others while biomedical engineering scaffolds are used for tissue engineering, which can further be bioactive or bioresorbable [23, 24]. Hydrogels, which are hydrophilic polymeric materials are the most used scaffold materials as they mimic the properties of the extracellular matrix and also show stiffness similar to tissues [25]. These gels, being porous, can store factors produced by cells such as growth factors in addition to nutrients. These scaffolds contain high amounts of water and various natural biomolecules such as laminin, collagen, fibrin, or agarose [26]. Different types of polymers can be employed as hydrogels depending on the application desired. Some examples of such polymers include polylactic acid (PLA), polyethylene glycol (PEG), and polyamides. However, the process of solidifying a gel precursor is often complex and this makes the preparation of gels difficult.

Naturally, derived cellulose scaffolds have been developed from apple tissue, which is inexpensive, and obtained from a renewable source that can easily be produced [27]. Bioglass and bioceramics find application as scaffolds in tissue engineering as these are bioresorbable and can improve the regeneration activity of native tissues. Porous metallic surfaces have also been designed to be used as scaffolds as these metals exhibit high fatigue resistance and compressive strengths. Most often, titanium (Ti) and tantalum (Ta) are employed for the fabrication of metallic scaffolds. Apart from natural polymers such as fibrin, hyaluronic

acid, and collagen, composites have been used as scaffolds. These composites are made of two materials with distinct properties to take advantage of the material properties of both components [28].

B. Scaffold-free

These cultures do not rely on solid supports but the spheroids so obtained are smaller and less resistant to shear forces. Three techniques are generally employed for cell culture without solid support [29]. The first of these methods is the forced-floating method, in which well plates are coated with low adhesion polymers. These plates are then filled with cell suspension followed by centrifugation, resulting in a spheroid [20]. In the hanging drop method, a cell suspension is placed inside a micro-well. When the micro-well is inverted cells aggregate and form compact spheroids [30]. Spheroids can also be obtained by placing a cell suspension into a rotating bioreactor. The isolated cells present in the cell suspension will aggregate and a range of spheroids of varying sizes will be obtained [20].

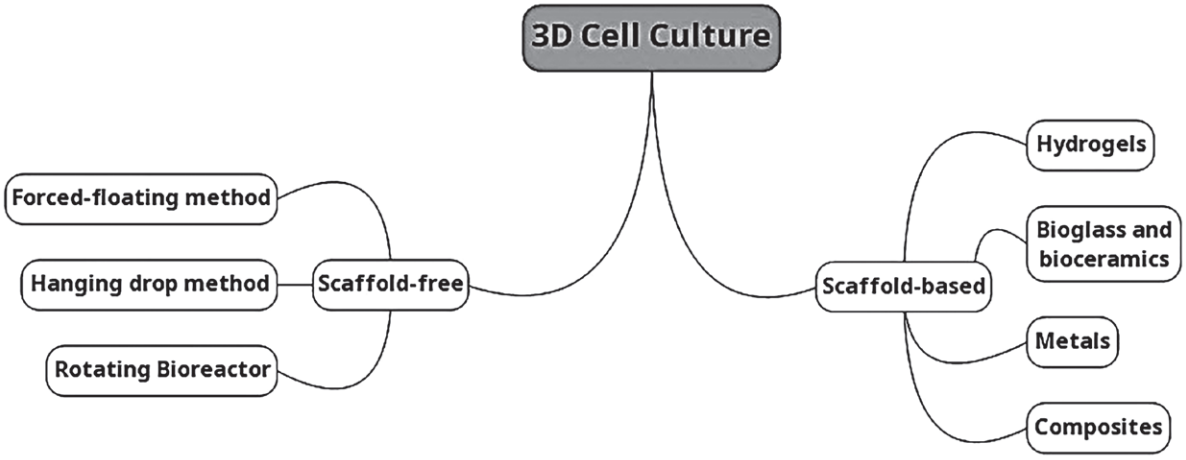

Figure 3: Mind map showing the existing techniques for 3D Cell Culture.

3D cell culture in Healthcare

3D cell culture systems find application over four broad categories in the healthcare setting, which include the regeneration of cells, development of organ models, the study of stem cells, and drug delivery and discovery studies (Figure 5). Recent examples for each of these categories have been briefly discussed in the following section.

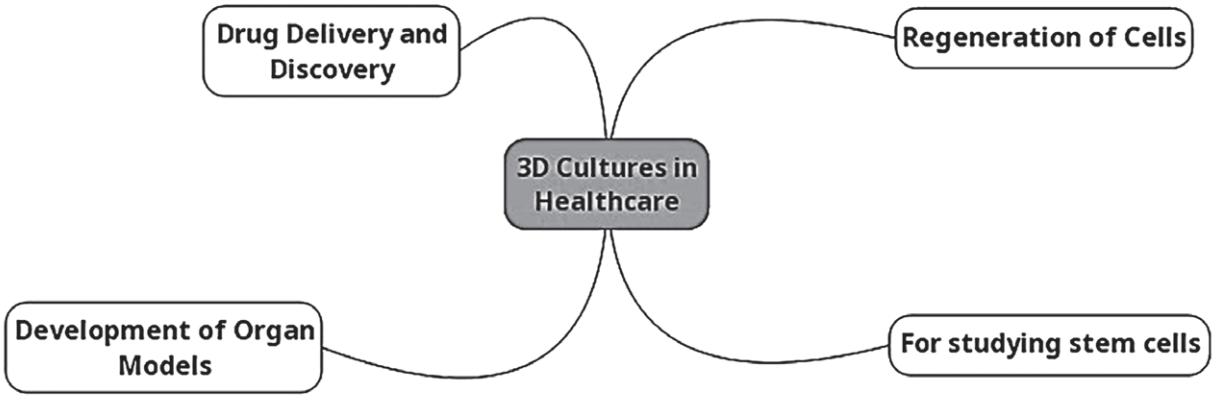

Figure 4: Application of 3D cultures in the healthcare setting.

A. For regeneration of cells

Cells that are regenerated in conventional 2D cultures suffer from the drawback of having different features as compared to cells in vivo and cannot be readily transplanted to the patient. This drawback has been overcome by using 3D cultures which can accurately represent in vivo conditions and provide physiologically relevant cells. Three-dimensional (3D) cell culture models have been employed to study nerve regeneration using stem cell and neuron co-cultures [31]. Tissue-engineered skin and oral mucosa, which can be used in reconstructive surgery procedures also use 3D cell culture for tissue-engineered models [32]. Human articular chondrocytes have been encapsulated in 3D hydrogels for cartilage regeneration [33]. The periodontal ligament, which supports the teeth, has been grown in a 3D construct that can be transplanted onto the site of loss in the teeth and also can be used to study the effect of mechanical loads on these supporting structures in vitro [34].

B. For developing organ models

As the cells developed in 3D cultures resemble in vivo conditions not only in the properties and in features of the cells themselves but the shape of the culture, they serve as effective tools for the development of organ models in vitro. Organs on a chip, which can accurately mimic living organs, have also been developed. An organ on a chip refers to a miniature model of a human organ on a microfluidic chip and is created using microfabrication techniques such as soft lithography [17]. These chips have emerged as important and efficient tools for drug delivery studies and can be applied in drug metabolism studies [35]. Several organs on a chip model have been developed in the recent past, including lung [36, 37], heart [38], muscle [39], and skin [40] among others. Organoids of human urinary tract tissue have been established using 3D cell culture techniques. These organoids can be used to study disease models and the interaction between urothelial and stromal cells [41]. Human hair follicle organ culture models have been developed with potential use in cutaneous biology and dermatology. These models can be used to study and visualize the interaction between epithelial, mesenchymal, and neuroectodermal cells, which are involved in the development of the hair follicle [42]. Zebrafish larvae have been used to develop 3D cell culture models of the heart, termed as Zebrafish Heart Aggregates (ZFHAs). The cardiac tissue developed was found to beat rhythmically for more than eight days. This active cell system can have applications in future studies of cardiac regeneration, drug toxicity testing, and tissue engineering [43].

C. For studying stem cells

Stem cells have enormous potential in healthcare studies as they can be used to model human development and serve as a source of cells for cell regeneration and organ development in vitro. Hence, studying and developing these cells, especially in conditions resembling the in vivo environment is important. Protocols involving 3D cell cultures have been developed to investigate the differentiation of embryonic stem cells into osteoblasts, with potential application in bone regeneration. The osteoblasts cultured can be used for bone regeneration and to treat bone diseases [44]. 3D matrix gels have been used to study the interaction between brain tumour stem cells and endothelial cells to propose that brain cancer stem cells are maintained within vascular niches. The 3D nature of the construct helped in emulating in vivo conditions of vascularity and cell-cell interaction [45]. Human embryonic stem cells were differentiated into hepatocytes in vitro in both 2D and 3D cell cultures. It was observed that the 3D collagen scaffolds gave rise to cells with morphological features and gene

expression characteristic to that of hepatocytes found in vivo. Thus, 3D cultures can serve as a source of hepatocytes and are used for the treatment of disorders affecting the liver [46].

Adult mesenchymal stem cells have been differentiated into adult cartilage tissue using 3D porous silk scaffolds. It was observed that the spatial arrangement of the cartilage cells in the scaffold resembled the native cartilage tissue. The silk-based scaffold developed was biodegradable, had optimal structural and mechanical properties, and had high biocompatibility [47]. The development of hydrogels has been reported for the expansion and differentiation of human pluripotent stem cells. This 3D system enabled the long-term expansion of multiple cells lines and directed differentiation of the stem cells into different lineages. As the system is scalable, and efficient and human pluripotent stem cells can form any cell type of the human body, these systems can be used for biological studies as well as for the development of commercial products [48]. Oligodendrocyte precursor cells have been generated from human pluripotent stem cells using a 3D hydrogel. These cells can be used for the treatment of demyelinating diseases [49]. 3D cellular microarray platforms have been developed that can enable rapid visualization of stem cell fate. The cellular mechanisms involved in stem cell fate can thus be investigated and can be used to direct cellular responses [50, 51].

D. For drug delivery and discovery

Toxicity testing using 3D cell cultures reflects the true physiological response to toxic compounds than conventional 2D cell cultures [52]. HepG2 liver cells were cultured using both 2D and 3D techniques and it was observed that cells in 3D cultures could cope better with cytotoxic agents as compared to their 2D counterparts. The cells grown in 3D cultures showed lower susceptibility to the cytotoxin, maintained the structural integrity, and also showed greater viability, at levels comparable to in vivo conditions [52]. A high-throughput Matrigel-based 3D drug screening method for testing drug sensitivities in JIMT-1 breast cancer cells was developed and compared to 2D cell cultures. It was concluded that 3D cultures showed better comparability to in vivo conditions and thus should be preferred for drug screening studies [53]. Multicellular spheroid models are the most commonly used 3D cell culture techniques and have emerged as ideal techniques for high throughput screening assays for the efficacy of in vivo antitumor agents [54]. A 384 welled hanging drop array involving spheroid culture has been developed for high throughput drug screening. It was observed that the spheroids produced distinct responses which were markedly different from the cells in conventional 2D culture and more closely related to physiological conditions, making this system an efficient way to replicate in vivo conditions in vitro [55].

3D cell culture chips in tandem with microfluidic tools can prove to be an effective tool for drug toxicity testing. Microfluidic 3D hepatocyte cultures could be developed to test for drug hepatotoxicity. The engineering of the3D microenvironment made it possible to maintain the spatiotemporal conditions close to those found in vivo [56]. Co-cultures of osteoblast and endothelial cells have been grown on 3D biomaterials to evaluate the biocompatibility of novel biomaterials that have been proposed for bone regeneration [57]. Cell-material interactions involving studies with respect to cytotoxicity and internalization of nanomaterials are more accurately represented when 3D cell cultures are used. These systems can help in understanding and controlling the interactions between nanomaterials and cells [58]. 3D cell culture approaches have been

widely applied in the recent past for identifying and evaluating potential drug molecules, as these are more clinically relevant as compared to conventional 2D cultures. These approaches are even more applicable for the development of inexpensive and efficient models for anti-cancer drug screening [20]. Spheroids of cancer cells were developed and used as a model to evaluate various chemotherapy protocols. It was suggested that spheroid cultures were more useful as compared to 2D cultures in evaluating drug efficacy and chemotherapy combinations due to their resemblance to in vivo conditions [59].

Factors affecting 3D constructs

A. *Effect of matrix composition*

It has been found that the strength, as well as the composition of the matrix, is important in the development of the cells that can be grown on the matrix. High collagen concentrations can inhibit the differentiation of embryoid bodies by inhibiting apoptosis. Fibronectin, on the other hand, stimulates the differentiation and vascularization of endothelial cells. It has been suggested that the biochemical properties of the scaffold can be modulated to influence stem cell patterning [60].

B. *Effect of matrix stiffness*

Matrix stiffness can affect the differentiation of mesenchymal stem cells (MSCs). Thixotropic gels were used for the study and different phenotypes of neural, myogenic, or osteogenic cells were obtained with varying liquefaction stress. It was also observed that immobilization of cell adhesion peptides led to an increase in differentiation as well as the proliferation of MSCs [61].

C. *Effect of scaffold structure*

Varying scaffold structures can induce different and unique gene expressions in bone marrow stromal cells. The effect of scaffold structure on stem cell fate is more pronounced than the effect of scaffold composition. It has been suggested that the efficiency of scaffolds can be optimized by engineering the scaffold to force cells to differentiate into desired morphologies and fates [62].

Imaging techniques for 3D cultures

Most of the imaging techniques for 2D cultures involve the transmission of light through the sample. However, these techniques cannot be readily employed for 3D cultures as the samples are often too thick for light to be transmitted through. Confocal microscopy can be used to obtain high-resolution images of thicker samples but is limited to thicknesses of 100 μm. Multiphoton microscopy (MPM) can be used to obtain images of samples of thicknesses up to 1 mm. However, MPM is limited to fluorescence imaging and thus requires fluorescent markers or auto-fluorescent samples, which may not be feasible for all samples. Optical coherence tomography (OCT) is a suitable imaging technique for samples that are several mm thick. The type of culture and cells employed, the features of interest, and the cost of the imaging technique often play an important role in the choice between various imaging techniques [63].

Future challenges

The scaffolds and substrates developed for 3D cell culture may incorporate compounds from viruses and animal sources and thus could be a hindrance for cell cultures. These culture systems are also prone to contamination. Many of the techniques currently employed are time-consuming and exhaustive, thus unsuitable for rapid drug screening and research. The use of these techniques brings forth further technical challenges for microscopy. Well-established imaging technology exists for analysing 2D cell culture systems. However, imaging techniques for 3D cell cultures will have to be optimized and often specially prepared for the specific experimental setup [16].

The diffusion of oxygen and essential nutrients are restricted by mass transfer limitations as the cells

forming the outermost layers serve as a natural barrier for the cells present in the innermost layers [11]. Enhanced vascularization systems will have to be developed to overcome this drawback. Although these techniques are better suited for culturing animal cells, they have only recently gained prominence and due to their novelty, the underlying phenomenon and related implications have not been completely grasped [12]. Hence, it is still not very easy to handle these techniques. While these techniques could be inexpensive in the future and could skip testing on animals, the development of reproducible applications and automation can prove to be extremely costly [12].

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