Fraunhofer IPT

White Paper

OCT for 3D cell culture visualization

Introduction

Since decades cell cultures serve a major purpose in a wide variety of research fields. Most of our recent knowledge about the cellular processes within the human body is based on research work instrumenting different kind of cell cultures. Up until 1991 almost all cell based research was done using two dimensional monolayered cell cultures. Since then a constantly growing number of research papers covering the topic of 3D cell cultures has been published. In the year 2000 around 50 scientific papers focusing on the research with three dimensional cell cultures were published, this number increased to over 1700 by 2020.

One reason for this trend is the ability of 3D cell cultures to mimic in vivo conditions better than 2D cell cultures can [1, 2]. This circumstance can have a major effect on the quality and significance of the derived scientific results. In some cases research results of tests with 2D cell cultures can even be misleading regarding the actual in vivo processes [3–5]. In the context of pharmaceutical testing the discrepancy between the results which are achieved by research on 2D cell cultures and those generated by clinical trials is vast, between 88–95 % of all pharmaceuticals in clinical trials never make it to the market, because the results of the testing on 2D cell cultures could not be reproduced by the clinical trials [5, 6]. As 3D cell cultures are capable of recreating the in vivo conditions more realistically researchers hope results generated by 3D cell culture experiments to be more reliable. The fact, that preclinical research results cannot be reproduced in the clinical testing for a large majority of pharmaceuticals also has a major effect on the price of those pharmaceuticals that make it to the market.

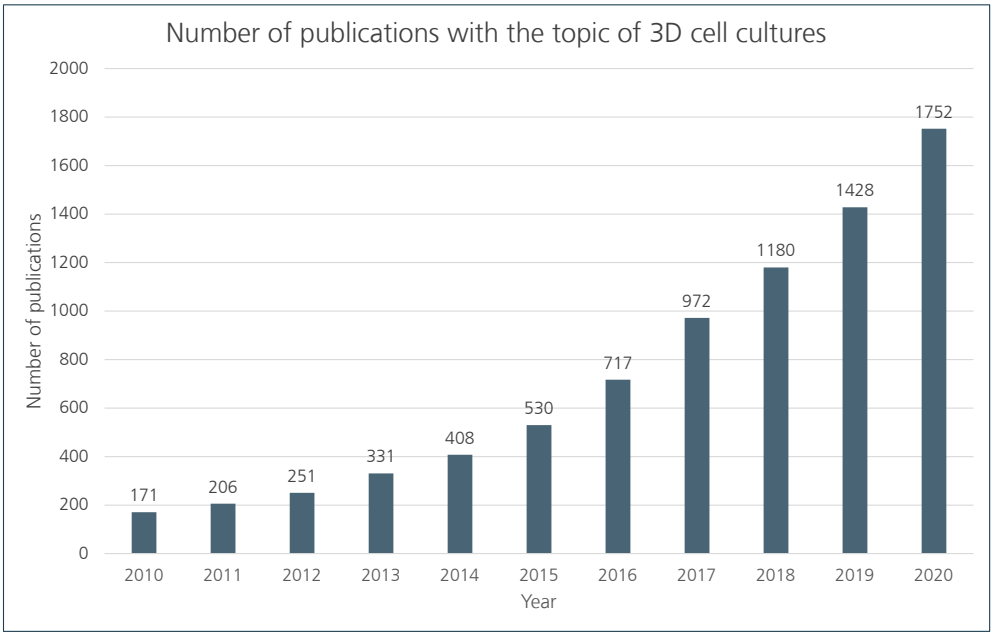

Figure 1: Number of scientific publications with the topic of 3D cell cultures for the years 2010 – 2020[1]

[1]According to a search for scientific, peer reviewed papers on apps.webofknowledge.com literature database on May 12, 2021 using the keywords: 3D cell culture, 3-dimensional cell culture, organoid and tumor spheroid

The mean cost per approved new drug in the US were estimated to be 2558 million USD in the period from the early 2000s to the mid-2010s. 1098 million USD of that amount was spent on the pre-human phase of the development of the pharmaceuticals and 1460 million USD on the clinical phase [6]. Those numbers take into account that only a fraction of the pharmaceuticals which enter the clinical testing make it to the market, which has a major effect on the overall costs on one newly developed drug. If the reliability of the prehuman test phase and thereby the percentage of drugs which enter the clinical testing and in the end eventually make it to the market could be improved, the overall costs for the development of a new pharmaceutical could be drastically reduced.

3D cell cultures are already being used in a variety of different applications in which their unique characteristics are very beneficial, but of course the higher complexity and informational value of 3D cell cultures comes with its own set of challenges. One of the key challenges being, that not the same analysis techniques can be used for 3D cell cultures as are established for 2D cell cultures. Especially the visualization of the 3D cell cultures is complex, time consuming and not ready for an upscaling of the 3D cell culture research. The Fraunhofer Institute for Production Technology IPT sees the optical coherence tomography (OCT) as a key technology to this key challenge of 3D cell cultures and thinks that OCT thereby can support 3D cell cultures in becoming a more realistic alternative to 2D cell cultures and in some cases even animal testing.

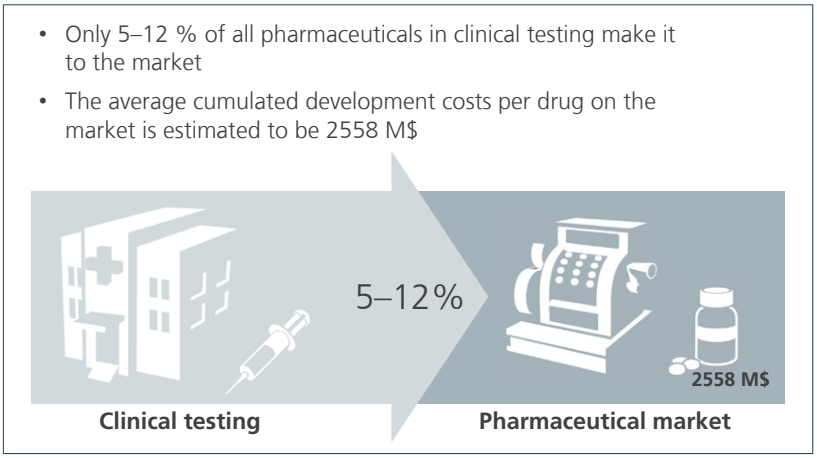

Figure 2: Transition rate and development costs of pharmaceuticals from clinical studies to the market.

What are 3D cell cultures?

3D cell cultures allow cells to grow in all three dimensions forming volumetric shapes, they stand in contrast to 2D cell cultures which are only grown in monolayers forming flat surfaces. 3D cell cultures can be categorized into three categories: 1. scaffold 3D cell cultures where typically a scaffold of hydrogels or inert matrices is used as a framework for the cells to grow on, 2. microfluidic organ-on-a-chip models where cells grow in scaffolds within a microchip which contains channels for liquids to flow through and 3. scaffold-free 3D cell cultures which rely on cells to self-assemble into volumes [7]. Within the scope of this paper we want to focus on the latter kind of 3D cell cultures because they have the most relevance regarding the imaging technologies and most basic research is carried out using scaffold-free 3D cell cultures.

There are two types of scaffold-free 3D cell cultures which need to be distinguished: spheroids and organoids. Multicellular spheroids (MCS) are a merger of a great number of cells of a single or multiple cell lines. MCS consist of the cells themselves and extracellular matrix (ECM) surrounding the cells. The ECM enables the cells to communicate with each other and thereby form a complex communication network. The assembly process of ECMs is a multistage process which starts with a loosely connected cluster of cells and ends with a tightly packed and connected network of cells. MCSs can reach a diameter of about 600 µm and as they grow three different regions within the spheroid itself develop. In the center of the spheroid a necrotic core starts to form, as soon as the spheroid reaches a diameter which does not allow nutrients and oxygen to diffuse to its core anymore. Surrounding the necrotic core lies a zone of quiescent viable cells which then is surrounded by a proliferating zone [8].

Organoids, in contrast to spheroids, cannot be created using any kind of cells, organoids are based on stem cells which are able to differentiate into all sorts of organ specific cell type. The major difference which sets organoids apart from spheroids, is that organoids are able to self-organize [9]. Spheroids are not able to develop any kind of functional regions, organoids however are able to self-organize different functional regions within the organoid itself. Each of these regions is able to consist of a different composition of different organ specific cell types. Organoids can reach sizes of several millimeters in diameter, this development process can take up to several years.

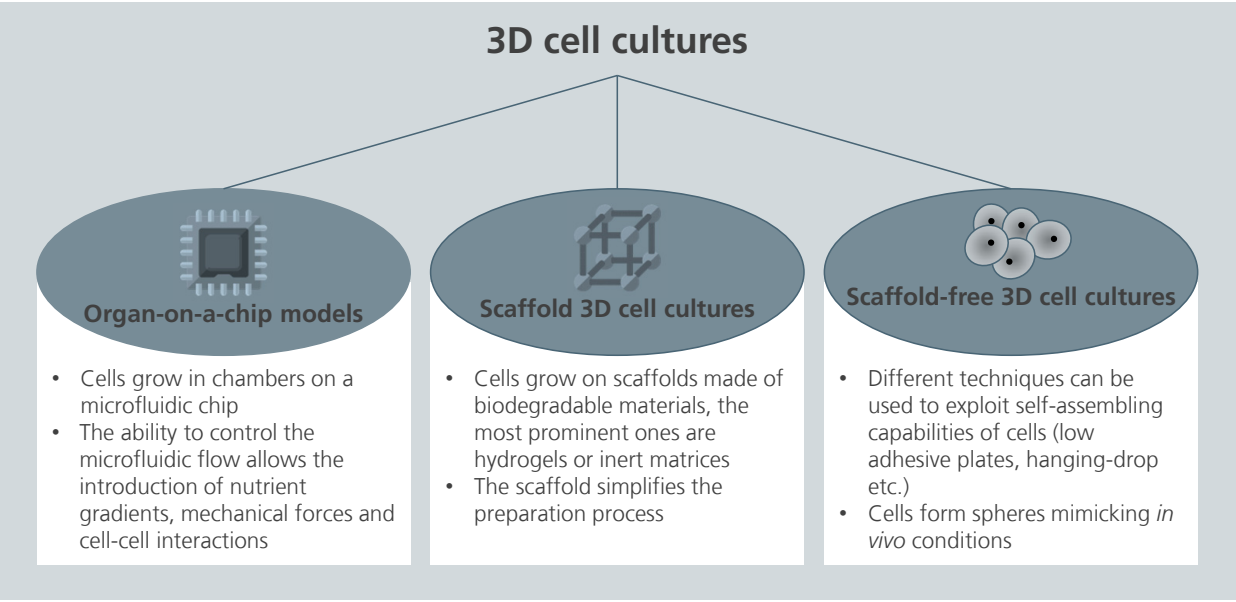

Figure 3: Different categories of 3D cell cultures.

What are spheroids and organoids being used for?

The main research application for spheroids is oncological and pharmaceutical testing. Here, different kind of tumor cell lines are being used in order to derive so called tumor spheroids. With the help of tumor spheroids, the effect newly developed anticancer drugs have on tumor cells is analyzed. As described above, the 3-dimensional nature of spheroids has an important effect on the outcome and the significance of the scientific results. For example it has been shown, that the effect of drugs, which were developed for the treatment of lung cancer, could completely differ between the application on 2D monolayered cell cultures and 3D tumor spheroids [10]. This effect is being traced back to three main differences between 2D and 3D cell cultures: First, the cell-cell and cell-ECM contact is very limited for 2D cell cultures, second, there exists a diffusion gradient of nutrients, waste, oxygen and drugs in 3D cell cultures, which is non-existent in 2D cell cultures and third, 3D cell cultures are able of mimicking the tumors morphology and thereby show a resistance to anticancer drugs in contrary to 2D cell cultures [11]. Spheroids therefore mainly serve the purpose of a more realistic, in vivo mimicking test environment for newly developed drugs. Spheroids are also being used for the drug testing in general and for other smaller research applications, but those areas only play a minor role in the application of spheroids.

The much more complex nature and structure of organoids compared to spheroids allows for more complex and in-depth research questions. As it is possible to influence in what kind of cell types the stem cells differentiate, by changing and adapting the composition of the media in which the stem cells are immerged, several of so called mini-organs can be generated. This process was demonstrated for instance but non-exclusively for: brain, eyes, kidney, liver, intestine, colon, bladder prostate and pancreas. On top of this widespread set of possibilities one key advantage of organoids is, that they also can be derived from induced pluripotent stem cells and therefore patient specific stem cells can be used, which makes organoids suitable for personalized medicine and the disease mechanic research.

Organoids are being used to study the characteristics and mechanics of infectious diseases, genetic defects and their effect on the organ development and tumor modeling. Furthermore, organoids are being used as a test ground for the research on correcting gene defects using techniques like CRISPR/Cas9 and for the fundamental research of gene function and cell development. A comprehensive overview of the large variety of possibilities organoids open up for researchers in different fields can be found elsewhere [12].

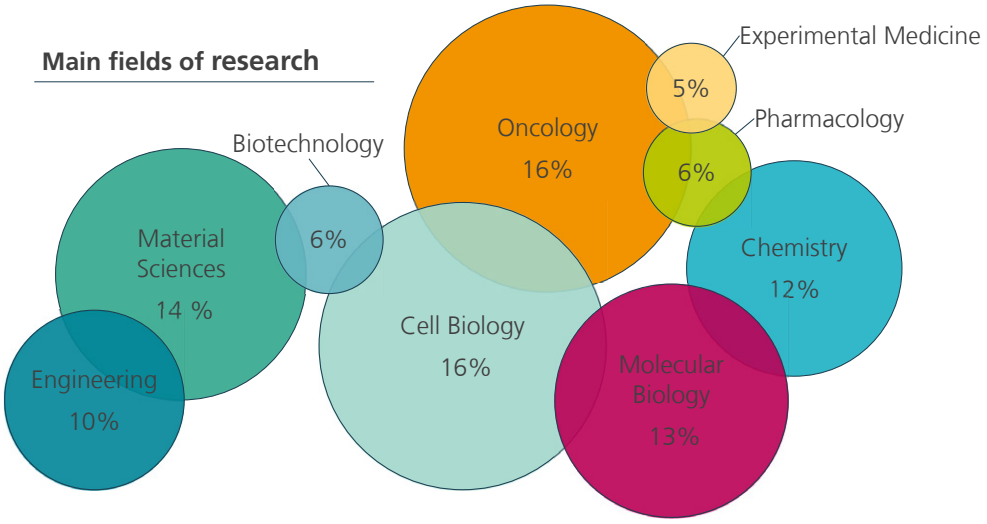

Figure 4: Main fields of research of 3D cell cultures based on the total number of publications.

Which methods can be used for visualizing 3D cell cultures?

Imaging 3D cell cultures is a demanding task as their main components (cells and ECM) both share similar, highly scattering properties and thereby limit the penetration depth for optical imaging modalities quite drastically. 3D cell cultures can reach diameters of about 600 µm in the case of spheroids and > 1 mm in the case of organoids, this means, that most of the informational value of both organoids and spheroids lies below their surface.

There are two established approaches for imaging 3D cell cultures: imaging intact volumetric cell cultures and imaging sections of cell cultures. The sectional imaging of cell cultures is done by cutting them into thin slices (< 10 µm) using a microtome and visualizing every slice by its own using a conventional microscope or a fluorescence microscope which enables sub-micron resolution. In order to cut such thin slices it is necessary to freeze or fixate the cell cultures. The cutting and handling process can induce permanent changes to the 3D cell cultures and thereby be the reason for a difference between the actual structure and the visualized structure [13, 14]. Additionally the preparation, cutting, imaging and processing of the single-slice-data is cause to an enormous time effort. For the imaging of complete intact 3D cell cultures three different technologies are being used: (fluorescent) confocal laser scanning microscopy, multiphoton microscopy and (fluorescent) light-sheet microscopy. While all three of these technologies work in different ways, they all share the same limitation of penetration depth, the subsurface information of the 3D cell cultures is lost due to multiple scattering. In order to use any of these techniques for the visualization of 3D cell cultures it is necessary to make them more transparent for the used wavelength of light. There are several different approaches to make 3D cell cultures more transparent, all of them can be summarized under the term "optical clearing". Its aim is to homogenize or match the refractive index of all components of the 3D cell culture and thereby limit the internal scattering to a minimum. Optical clearing was originally used for the visualization of tissue samples but is being more and more established in the visualization of 3D cell cultures [15]. A visualization of the inner structures of 3D cell cultures without either cutting them into thin slices or optically clearing them is not possible with standard methods. The process of optical clearing is always based on the usage of a solvent, the chemical processes which are caused by the solvent can introduce alterations in the 3D cell culture. These alterations can be swelling, shrinkage or the quenching of fluorescent characteristics, further the procedure of optical clearing is in general time consuming and can be demanding in its application. A highly detailed review of different clearing methods and their advantages and disadvantages can be found elsewhere [16]. Summarizing it can be stated, that the visualizing of 3D cell cultures by either cutting them into thin slices or optically clearing them is: time consuming, involves several different steps which can be technically demanding and give rise to mistakes, makes it necessary to handle the cultures outside their medium and have a potential influence on the informational value of the measurements. Further none of the established visualization methods is able of a non-invasive imag-ing of the 3D cell cultures, which in face of the long and time consuming generation of 3D cell cultures is another significant drawback. These limitations play a major role in why 3D cell cultures are not being used more often and more frequently [1]. A comparison of the established visualization methods for 3D cell cultures, their advantages, disadvantages and key characteristics is given in Table 1.

Visualization methods					
	Sectional imaging		Visualization of whole 3D cell cultures		
Noninvasive	No		No		
Clearing necessary	No		Yes		
Thin slicing needed	Yes		No		
Possible sample alterations	Swelling, shrinkage and mechanical alterations		Swelling, shrinkage and quenching of fluorescent characteristics		
Imaging technologies	Microscopy	Laser scanning microscopy	Light-sheet fluorescence microscopy	Two-photon microscopy	Confocal laser-scanning microscopy
Penetration depth	< 1 µm		> 1 cm *	≈ 1 mm *	≈ 100 µm *
Resolution	< 1 µm		≈ 1 µm	< 1 µm	
Acquisition time	Very high**	Moderate**	High	High	Very high

* stated penetration depth is only reachable for cleared 3D cell cultures, ** acquisition time relates to the imaging of one single thin slice (< 10 µm thickness)

Table 1: Comparison of the current standard methods for the visualization of 3D cell cultures.

An alternative approach: optical coherence tomography

A possible alternative to the current standard procedures for the visualization of three dimensional cell cultures is optical coherence tomography (OCT). OCT is an imaging modality which originates from the field of ophthalmology, it is based on low coherence interferometry and is able to generate high resolved cross-sectional images. It is thereby possible to receive both topographical and tomographical information in one measurement. Resolutions of up to 1–2 µm can be achieved in the axial direction and 10–15 µm in the lateral direction [17]. OCT works completely contactless and non-invasive and is thereby capable of measuring 3D cell cultures, without the need of any handling processes, medium changes or preparation steps. Because of that it is possible to use OCT for a longitudinal in vitro monitoring of 3D cell cultures and their growth.

Several different research groups worldwide have demonstrated, that OCT is capable of visualizing 3D cell cultures and thereby potentially could replace the current standard methods. In 2019 Tsai et al. used OCT to monitor the growth of tumor spheroids and quantify their size, shape and inner structure [18]. One year later the same group used OCT to monitor the effectiveness of an anti-cancer drug and its dose by quantifying the effect the drug had on the dimensions of the cell cultures [19]. Huang et al. have also demonstrated the use of OCT for the longitudinal monitoring of tumor spheroids with a size of up to 600 µm in diameter, they used both morphological and physiological markers for the quantitative valuation of the 3D cell cultures [20]. In further studies, they were able to demonstrate the automated measurement of 3D cell cultures in a 96 well plate [21]. Both Jung et al. and Hari et al. have successfully used OCT Systems to quantify the cell viability and the size of the necrotic regions within spheroids [22, 23]. The toxicity and drug effect was also the central focus of the study of Yang et al. in 2020, they assessed the effect of anti-cancer drugs, which are designed for the use against breast cancer, not on spheroids but on mammary epithelial organoids [24]. Also using organoids Capowski et al. used OCT to ensure uniformity of retinal organoids which are being used to produce mature human retina out of human pluripotent stem cells [25]. Liu et al. used OCT to visualize and monitor human nasal epithelial organoids and in particular quantify the ciliary beat frequency [26].

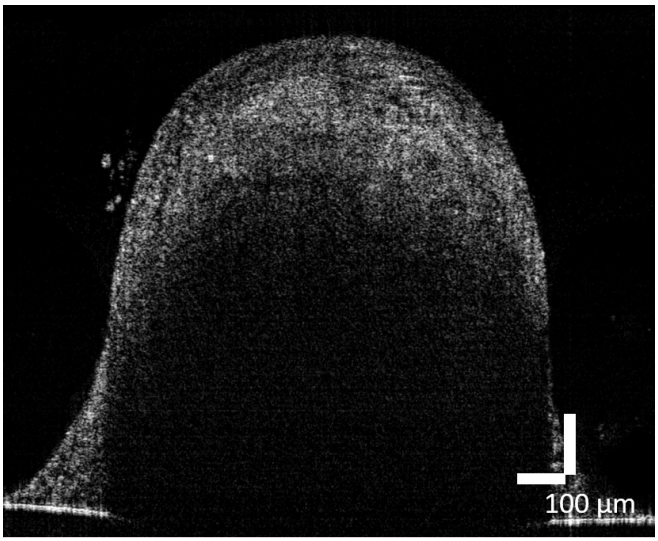

Figure 5: Cross-sectional OCT-Scan of a large spheroid, revealing its subsurface structures.

What needs to be worked on?

Of course, OCT as an optical measurement system is also limited in its capabilities. OCT comes with its own challenges which stem from its working principles. Those limitations are in some cases different and in some cases similar to those of confocal laser scanning microscopy, multiphoton microscopy and light-sheet microscopy. OCT shares its major limitation with all techniques of microscopy: penetration depth. Even though in comparison to microscopy the penetration depth of OCT, which lies for the use with 3D cell cultures in the range of 500 µm, is very high, it still is a central constraint for OCT and its application on 3D cell cultures. The effect of the limited penetration depth of OCT can be seen in Figure 5 where an OCT scan of a spheroid with a diameter of about one millimeter is shown, towards the middle of the cell culture the signal strength constantly decreases. The limited penetration depth is also the reason for the fact that most literature, which can be found on the use of OCT with 3D cell cultures, evolves around tumor spheroids. The maximum size of tumor spheroids is much smaller in comparison to organoids, which can reach diameters of several millimeters, this is why longitudinal monitoring of organoids by OCT can be difficult.

Fraunhofer IPT identified several limitations and challenges which we believe are the reason for the fact, that even though OCT shows an enormous potential to enable a much faster and cost effective visualization and monitoring of 3D cell cultures, it still is a niche application. Penetration depth as described above is one of those limitations, the Fraunhofer IPT is working on ways to expand this limitation. As the penetration depth is limited by scattering and therefor is a physical limitation, it is not possible to overcome the limited penetration depth entirely, but there are possible approaches to bypass its limits in some extend. Two of those approaches are: the axial registration of multiple OCT scans, which were acquisitioned under different angles and directions and the use of longer NIR-wavelengths. Besides a higher penetration depth it is necessary to realize a flexible and adaptive automation of the OCT system itself in order to make OCT a real alternative for the use with 3D cell cultures and in the laboratory environment in general. By doing that it will be possible to acquisition OCT scans of a large quantity of samples all within different sample holders. First results towards an automated OCT system were already demonstrated but all of them are specialized on the use with well plates and are not flexible enough for a use in different laboratory scenarios. Ultimately the goal must be to prepare the OCT technology for the laboratory of tomorrow which will be fully automated and therefor requires digital and automated subcomponents.

Fraunhofer IPT is working towards this goal because we are convinced, that with the right adaptations and innovations OCT is capable of supporting the research on 3D cell cultures in a way no other imaging modality is capable of and thereby enable the assessment of important research questions in different fields.

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DOI 10.24406/ipt-n-643638