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Balance between the cell viability and death in 3D

"Till death do us part"

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Abstract

Cell death is a phenomenon, frequently perceived as an absolute event for cell, tissue and the organ. However, the rising popularity and complexity of such 3D multicellular 'tissue building blocks' as heterocellular spheroids, organoids, and 'assembloids' prompts to revise the definition and quantification of cell viability and death. It raises several questions on the overall viability of all the cells within 3D volume and on choosing the appropriate, continuous, and non-destructive viability assay enabling for a single-cell analysis. In this review, we look at cell viability and cell death modalities with attention to the intrinsic features of such 3D models as spheroids, organoids, and bioprints. Furthermore, we look at emerging and promising methodologies, which can help define and understand the balance between cell viability and death in dynamic and complex 3D environments. We conclude that the recent innovations in biofabrication, biosensor probe development, and fluorescence microscopy can help answer these questions.

Keywords

Bioprint; Cell death; Cell viability; Microscopy; Multicellular spheroids; Organoids

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1. Introduction

As a major tool for *in vitro* physiological studies, cell culture provides insight into tissue development and disease mechanisms, benefitting areas of drug discovery, cancer, and regenerative medicine. 'Classical' two-dimensional (2D) monolayer cell cultures provide low cost, simplicity, and versatility. 3D cell and tissue cultures can bridge the gap between 2D cultures and intravital experiments, as they mimic organ-specific tissue microarchitecture and microenvironment in a relatively simplified setting [1]. 3D cultures efficiently implement the 3Rs principle and minimize animal experimentation [2]. They are expected to become widely accepted in preclinical, drug screening studies and tissue repair applications after improving their standardization [3, 4]. However, the selection of the quality control parameters is not always optimal, and the intrinsic differences and capacity of the used 3D models are not always appreciated. Standardization, not often correctly applied classification, complexity, and variability can become confusing, especially in the understanding of the overall viability of 3D tissue constructs. In this review, we look at existing methodologies which can help define cell viability and death in spheroids, organoids in bioprints and outline their intrinsic differences.

2. Types of 3D tissue models

Based on the architecture and production method, 3D tissue models can be grouped into the three major categories: (a) *spheroids*, based on rather a physically-driven cell assembly process, (b) *organoids* as self-organized 3D structures recapitulating features of the tissue, often consisting of stem cell niche and differentiated compartments, and (c) 3D constructs made of dispersed single cells or multicellular aggregates confined to the *scaffolds and bioprints* within natural and artificial extracellular matrices.

Spheroids are typically formed by incubation in a low adhesive environment and compactization mediated by E-cadherin [5]. Most cell types, such as mesenchymal stem cells (MSCs), adult stem cells (ASCs), induced pluripotent stem cells (iPSCs) and cancer cell lines can produce spheroids [6]. Subsequently, spheroids made of more than one cell type are referred to as 'heterocellular' spheroids. Several techniques such as micro-patterned surfaces and non-adhesive matrices can produce spheroids of different size, viability, differentiation capacity, and therefore the response to chemical and instructive signals [7]. Spheroids vary due to the differences in formation method, cell type, cell number, and culturing conditions [8]. Typical spheroid diameters are in range of 100~700 μm, leading to the 'classical' size-dependent diffusion gradients of glucose, ATP, and molecular oxygen (O_2) and subsequent formation of heterogeneous cell layers and necrotic core [9]. Tumor spheroids can display the presence of 'stable' three-layer concentric structures including necrotic core, quiescent and proliferating areas[10]. The necrotic core makes them similar to *in vivo* solid tumors [11] with the density of extracellular collagen, tissue stiffness, and cell invasive behavior [12, 13]. This prompts their use as an alternative to the *in vivo* experiments in testing drug therapy efficiency [14] and cancer cell invasion [15].

Spheroid cultures can furthermore help investigate the fusion and functional integration of multiple cell types. In the case of assembloids, such interactions are useful in tissue engineering and neurodevelopmental disease modeling studies, such

as Timothy syndrome [16], macular degeneration [17], regeneration of tooth tissue [18] and SARS-CoV-2 neuropathology infection models [19]. Here, the viability of cells in the core rather than necrosis is more important for successful application.
Organoids. In contrast to spheroids, organoids display higher complexity in the organization and cellular composition. The term ’organoid’ describes 3D constructs composed of mesenchymal and epithelial cells [20]. However, even epithelial tissue-derived organoids demonstrate much higher complexity than spheroids. The functional compartment organization, self-sustained stem cell niche, amplification zone and differentiated cells, high-level self-organized cellular diversity of epithelial organoids make them physiologically relevant for mimicking normal and diseased tissues. Organoids from various organs and tissue types can be produced using iPSCs [21], ASCs [22], embryonic progenitors [23], or tissue fragments [24]. This results in the same basic intrinsic patterning as the original tissue and produces a more accurate ‘tissue-like’ organization [3]. Recent advances in cancer modeling, using patient-derived tumor tissues, enable establishing tumoroids and tumor-like organoids. Such models recapitulate the tumor heterogeneity, patient-specific drug response, and disease-specific genomic and proteomic tumor profiles [25].

The key aspect of ‘organoid culture’ is a ‘re-creation’ and maintenance of an artificial stem cell niche *in vitro* within defined 3D cytoarchitecture, where cell differentiation is regulated by the *physical* (i.e. extracellular matrix composition and mechanical properties) [26, 27], *biochemical* (intrinsic and extrinsic chemical signals, growth factors and their gradients)[28] and *chronobiological* factors (i.e. supporting circadian rhythms) [29, 30]. All these factors regulate live-dead balance, closely recapitulating those phenomena of the tissues they are derived from. These crucial features place organoids apart from the spheroids, where the cell live-dead decisions mostly depend on the *physical* (i.e. mass exchange-mediated) availability of bioenergetic substances (e.g. glucose, glutamine, pyruvate) and *environmental* cues (O_2 and pH). These factors are defined by the spheroid size, culture conditions and intrinsic metabolic characteristics of the cell line (Fig. 1).

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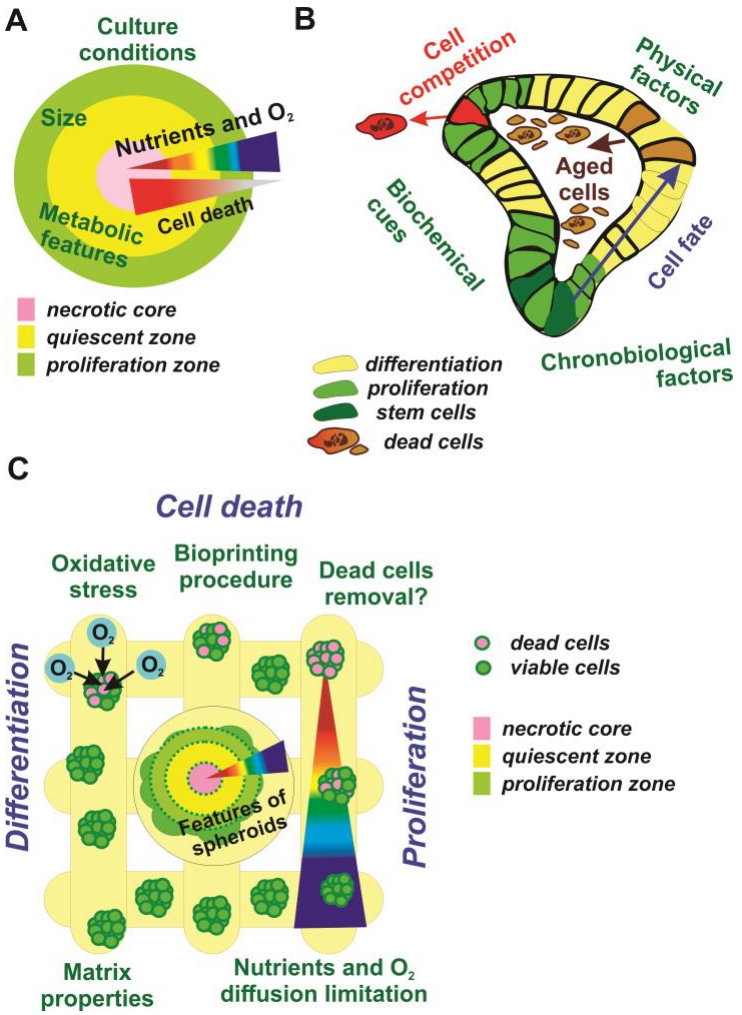

Figure 1. Similarities and differences of cell death and viability in multicellular spheroid (A), organoid (B) and 3D-printed spheroid-based microtissue (C). A: Three main zones in spheroids are indicated by colours. Cell death and proliferation constantly change during spheroid growth and are influenced by their size, internal gradients and external conditions, such as medium composition. Subsequently developed periphery-to-core gradients play the main role in cell fate regulation inside the spheroids. **B**: In a generalised small intestinal organoid the two main domains are present - stem cell niche with the actively proliferating daughter cells, and nonproliferating terminally differentiated cells. These cells follow aging and subsequent 'elimination' by anoikis from the live tissue but often become accumulated within a lumen region. Note that cells can also die due to the competition process within the stem cell niche. Biochemical cues (growth factor gradients, metabolic signalling, nutrient gradients, extracellular matrix and growth medium composition), physical and chronobiological factors (related to the regulation of the cell cycle longevity and circadian rhythms) all influence cell viability and death. Most of the cells in organoid follow the same fate starting from active proliferation of stem cells offspring, differentiation and cell death. **C**: In the classical bioprinted (yellow 'waffle' grid) scaffold-based tissue construct, the balance between cell proliferation, differentiation and death is regulated by multiple intrinsic (spheroid-related factors) and extrinsic factors (matrix properties, biofabrication procedure, biocompatibility of the polymer matrix, nutrient diffusion, local concentration of O_2) under the environment, which is expected to mimic the tissue development conditions, e.g. using a bioreactor.

Well characterized small intestinal organoids provide insight into the mechanisms and the importance of live-dead balance in the intestine. They closely recapitulate crypt-to-villus architecture, where the highly proliferative $Lgr5^+$ crypt base columnar stem cells surrounded by Paneth cells localize strictly in crypts and constantly compete for the limited niche access. During this process, the border stem cells undergo asymmetric cell division in which the committed daughter cells migrate toward the transit-amplifying zone, while the new stem cells remain in the crypt. Their division compensates for the constant loss of differentiated cells at the villus tip-based extrusion zone [31]. *In vivo,* the proliferation, differentiation, and death are controlled by crypt-to-villus gradients of Wnt decrease [32], BMPs increase [33], other growth factors and hormones (e.g. TGF-β and glucagon-like peptide 2), circadian rhythms, innervation, and intestinal microbiota [31]. Crypt base-localized stem cells have a survival advantage, retaining long-term self-renewal potential [34]. Within the organoids, enterocytes die at the villus-like compartments, being shed into the lumen. Their viability is maintained by regular organoid passaging, preventing the accumulation of high dead cell numbers. Morphological features of intestinal epithelium extrusion, death, and shedding are species-specific, with a close resemblance between human and rodent models [35]. Shedding is associated with the redistribution of the tight junction protein zonula occludens 1 (ZO-1) firstly at the apical and then basolateral regions of shedding cells [36]. Reportedly, the enterocytes at the tip of villi die via anoikis, the specific programmed cell death provoked by the loss of anchorage-dependent survival signals from the underlying extracellular matrix [24]. However, no clear data is present on the role of detachment as the initiating stimuli for programmed cell death of enterocytes [31].

Besides, cells within the organoids die due to the cell competition for survival and space between directly interacting cell populations. Depending on the context of mutations and activated pathways, weaker cells are eliminated from the tissue by extrusion and induction of apoptosis or, rarely, the induction of senescence in such cells [37, 38]. Extrusion is more common in epithelial tissues and is important for eliminating newly emerging transformed pre-cancerous cells, as a line of defense against epithelial cancer [39]. This is also used as a quality control mechanism in tissue repair or as a tumor-promoting role [37, 38]. In this context, organoids are essential models to study cell competition *in vitro* [39, 40]. As the mechanism of cell elimination during the competition includes both mechanical and metabolic cues [39] the corresponding analytical approaches should be applied together with studies of cell death and proliferation.

Bioprinted constructs. Biofabrication is an engineering approach, aiming at using small units of live tissue blocks such as aggregates, organoids, biomaterials, and bioactive molecules, to form a biological product, such as a tissue graft or part of the organ. It aims at recapitulating the complexity and heterogeneity of tissues and organs more closely [41] using either bioprinting or bioassembly. Bioprinting refers to the computer-aided process that uses raw materials, e.g. cell aggregates, and biocompatible polymer-based bioinks to print layer-by-layer structures, expected to heal the tissue. Popular techniques include inkjet [42], laser-assisted [43], and extrusion bioprinting [44, 45], which can be used to generate different ‘tissue building blocks’ assembled via a bottom-up approach. In contrast to bioprinting, the bioassembly is a top-down approach in which cells and cell aggregates are seeded in porous scaffolds with a 2D

or 3D organization, allowing them to self-assemble into tissue constructs [46]. Biofabrication deals with single cells, spheroids, and sometimes organoids, making the analysis of their live-dead status complicated and more interdisciplinary.

The complexity of cell death in such systems is related to both the 3D microtissue and the biofabrication procedure itself. The death during and after the biofabrication procedure can be caused by non-optimal bioprinting conditions (e.g. temperature, pH, the potential toxicity of matrix-crosslinking factors), the specific bioink / matrix characteristics (e.g. porosity and O_2 solubility [47-49], presence of O_2-releasing biomaterials [50, 51], mechanical properties and composition) and limited nutrient and growth factors delivery to cells in the mm-size or larger biofabricated constructs. As these constructs can be kept in a long-term culture or be implanted, the viability assessment method should enable for non-destructive long-term monitoring of these constructs in combination with a quantitative readout of O_2 and other key physiological parameters involved in cell death.

3. Cell death modalities in *in vitro* 3D models

In addition to the accidental uncontrollable form of cell death, intensive research over the last two decades highlighted the existence of various pathways of regulated programmed cell death: apoptosis, autophagy-dependent cell death, necroptosis, oxeitosis, NETosis, pyroptosis, ferroptosis, paraptosis, parthanatos [52, 53]. Frequently, these pathways are initiated by specific stress or due to failed adaptation to stress. However, such cell death modalities as apoptosis, autophagy, and anoikis are also involved in non-related to the stress maintenance of tissue and organ homeostasis and are referred to as ‘programmed cell death’ [52]. Many of these cell death modalities, including programmed cell death, were demonstrated in *in vitro* 3D spheroid and organoid models (see Table 1), notably demonstrating the better representation of specific cell death modalities in 3D vs. 2D models [54-56]. Importantly, some cell death modalities, linked to specific tissues and requiring the involvement of specific cell types, can therefore get an advantage from the application of tissue-specific organoid or co-culture models. For instance, application of endometrial organoid co-culture with neutrophiles allowed reconstruction of a primary immune cell response to *Chlamydia* infection and demonstration of NETosis *in vitro* [57]. Schinzel–Giedion midface retraction syndrome cerebral brain organoid model revealed the parthanatos-based neurodegeneration mechanism [58].

3D *in vitro* models not only closely reconstruct the microenvironment of the specific cell death modalities, but also can be principally applied for the evaluation of ‘drug-cell death inducer’ responses and mechanisms of drug-resistance [59],[60]. In some pathophysiological conditions such as cancer, massive apoptotic cell death promotes overall tumor cell survival, facilitating resistance and stimulating neighbor cell proliferation. The “survival messages” are delivered by apoptotic tumor-cell derived extracellular vesicles, which can be phagocytosed by macrophages, parenchymal and neoplastic cells [61, 62]. Recent studies on the modeling of extracellular vesicle-communication with cancer organoids [63, 64], demonstrated the utility of the organoid models to study the mechanics of apoptosis-resistance development in tumors.

Cell death is often linked to normal tissue maintenance. Dying cells can also support survival of neighboring cells by providing necessary resources or affecting their survival strategy through the changes in the microenvironment, metabolism, migration, differentiation, and proliferation [64-66]. For instance, in lung organoids, activation of autophagy does not necessarily result in cell death but controls cell metabolism, supporting tissue regeneration [67]. Therefore, it is important to analyze cell death pathways together with cell metabolism, proliferation, and differentiation in relevant cancer and organoid models for understanding the exact role of the cell death phenomena in relevant physiological context.

Table 1. 3D *in vitro* models recapitulating the regulated cell death modalities.

Cell Death Type	**Definition**	**Examples of the models**
Intrinsic apoptosis	Regulated cell death initiated by perturbed extracellular and intracellular homeostasis, with characteristic massive irreversible mitochondrial outer membrane permeabilization, and executed by caspases, mainly CASP3 [52].	Breast cancer cells (MCF-7) spheroids [68]. *myc*-transfected fibroblast spheroids [69]. Small intestinal organoids [70, 71] [72, 73].
Extrinsic apoptosis	Specific variant of regulated cell death driven by plasma membrane receptors in response to perturbed extracellular microenvironment with CASP8-executed activation cascade, mainly CASP3 (with the optional involvement of mitochondrial outer membrane permeabilization) [52].	
Anoikis	Specific variant of intrinsic apoptosis initiated by the loss of integrin-dependent anchorage [52].	Mouse gastric organoids [74]. Chicken embryo small intestinal organoids [75]. 3D floating-cultures of mesenchymal stem cells and extracellular matrix complexes [76].
Autophagy-dependent cell death	Regulated cell death executed by the autophagy machinery cascade with characteristic increase of lysosomal activity and appearance of double-membrane vacuoles [77] [52].	Breast cancer cells (MCF-7) spheroids [68]. Glioblastoma cell spheroids (U87MG) [78]. Paneth-cells enriched small intestinal organoids [79].

		Tumor spheroids generated from biopsies [54].
Programmed cell death	Regulated cell death, occurs in strictly physiological scenarios triggered by intrinsic cues rather than the perturbations of homeostasis and is not related to the failing adaptation to the stress [52].	Retinal intrinsic cues-triggered apoptosis in retinal organoids [80, 81]. ARTS-mediated apoptosis in stem cell niche in mouse small intestinal organoids [82].
Entotic cell death	Non-apoptotic regulated cell death that originates from actomyosin-dependent cell-in-cell internalization (entosis) and is executed by lysosomes; often in matrix-deprived environment [52].	Selenium-induced death in pancreatic cancer spheroids [83] Breast cancer cells (MCF-7) spheroids [68]. Colon cancer HCT116 spheroids [84].
Necrosis	A common name for death pathway(s) defined by the loss of plasma membrane integrity. These include: necroptosis, ferroptosis and pyroptosis [85]. It is also referred to as an uncontrolled energy-independent form of cell death induced by external injury [62].	Often poorly characterized in details of the exact death pathway. Mouse and human enteroids [86]. Multicellular spheroids [87, 88].
Necroptosis	Regulated cell death modality triggered by perturbations of extra- and intracellular homeostatic cues that critically depend on MLKL, RIPK3, and (at least in some settings) the kinase activity of RIPK1 [52].	Cancer cell spheroids (Caco-2) treated with Alkynyl gold(I) complex [89]. Arctigenin-induced cytotoxicity of human prostate cancer cell spheroids (PC-3) [90].
Oxytosis	Non-apoptotic regulated cell death associated with the depletion of intracellular glutathione leading to ROS accumulation; similar to or the same as ferroptosis [91].	See *Ferroptosis*
Ferroptosis	Iron-dependent regulated cell death under constitutive control by lipid-peroxidase GPX4 [52].	Lung cancer cells spheroids and other cell types [92, 93].
Pyroptosis	Regulated cell death involved in the formation of plasma membrane pores	Colorectal cancer patient-derived

	by members of the gasdermin protein family [52].	organoids [94]. IL-4 and IL-13 treated mouse jejunal organoids [95]. *Salmonella sp.* - triggered intestinal epithelial cell extrusion in intestinal organoids [72].
Paraptosis	A regulated cell death involving non-autophagy extensive cytoplasmic single-membrane vacuole formation as a reaction to the appearance of misfolded endoplasmic reticulum proteins, usually associated with photodamage [96].	Na^+/H^+ exchanger-1 (NHE1) inhibitors-treated cancer cell (MDA-MB-231, MCF-7) spheroids [97]. Cu(II)-complexes induced cancer cell HCT-15 spheroids [98].
Parthanatos	Regulated cell death initiated by PARP1 hyperactivation and the subsequent bioenergetic catastrophe coupled to AIF-dependent and MIF-dependent DNA degradation.	Neurodegeneration models of Schinzel–Giedion midface retraction syndrome cerebral organoids and cortical spheroids [58]. Human organotypic 3D skin models of psoriasis [99]. Metastatic prostate cancer DU145 tumor spheroids [100].
Oxeiptosis	Non-inflammatory caspase-independent and ROS-induced death with apoptosis-like morphology including KEAP1, PGAM5 and AIFM1 [101].	None reported?
NETosis	A ROS-dependent regulated cell death restricted to cells of hematopoietic derivation and associated with NET extrusion [52].	Endometrial organoids co-culture with neutrophils and *Chlamydia trachomatis* [57]. Lung organoid co-culture with neutrophils [102].

4. Viability modalities

As something counterbalancing cell death, viability is harder to define. Commonly it is assessed via the ratio of live (or dead) cells in relation to the total cell number, using analysis of proliferation or metabolic activity. Subsequently, viability must indeed depend on the 'cell-fate' decisions to either proliferate, differentiate, remain quiescent or die. However, in a more physiological context the exact meaning of 'viability' and the choice of a specific method depends on the model and the goal of the study. Thus, in studies evaluating cytoprotective or cytotoxic effects, the viability would be measured as a number of viable or proliferating cells [103, 104]. However, in more complex studies, where the abilities to differentiate, maintain heterogeneity and 3D organization are important, the multiparametric analyses are needed [73, 105] [106] [107] [108].

Viability in 3D spheroid and organoid systems depends on (but is not limited to) diffusion efficiency of the nutrients, biological waste products and growth factors [109, 110],[111], extracellular matrix mechanical cues [112-114] and capability to provide local growth or differentiation-promoting conditions such as development of Wnt gradient [32]. Spatial organization of cell-cell metabolic interactions with the cells and O_2 gradients can be also viewed as a measure of 3D tissue viability [115, 116],[117, 118]. Physiological hypoxia with O_2 levels, normally <30-85 mmHg play an important role in tissue homeostasis [119]. The extreme hypoxia induces death, through decreased mitochondrial activity, production of reactive oxygen species and nitric oxide. On the other hand, hypoxia is also linked to the activation of hypoxia-inducible factors (HIFs)[120], associated with cell survival and adaptation, switching to available energy sources or even providing a negative feedback loop to reoxygenate the microenvironment.

In contrast to 2D models, 3D spheroids, organoids and microfluidic-based systems help recapitulate physiological nutrient- and O_2-gradients and specific niche-related metabolic profiles. For instance, due to size dictated limitations of waste removal from spheroids, lactate accumulates in specific spheroid niche regions, leading to a localized decrease of oxidative phosphorylation [9] and utilization of the pentose phosphate pathway [121]. Studies of metabolism in small intestinal organoids addressing niche-specific metabolic profiles and interplay between Paneth cells and intestinal stem cell metabolism [115],[122] demonstrate that, in principle, physiological metabolic signaling can be recapitulated in 3D primary tissue cultures. The metabolism in 3D significantly differs from 2D cultures, as was recently demonstrated by metabolic flux analysis of colorectal and pancreatic cancer spheroids [123].

Collectively, 3D *in vitro* models can recapitulate many biological phenomena of cell viability and death in three-dimensional context(s) at different levels of complexity and within a more physiologically relevant cell microenvironment. Importantly, due to the complexity of these models, more advanced multiparametric approaches should be applied to characterize their live-dead phenotype(s) in a physiological context. Below we discuss the available tools to assess this.

5. "Live-dead" measurement approaches

In addition to *spatial* (i.e. distribution and proportion of dead material), organoids, spheroids or bioprints can display the *temporal* aspect of 'live-dead' phenomena. A growing number of physical and microscopy-based methods become available for

investigating the live-dead status in 3D and with temporal resolution. Perhaps the most popular method is fluorescence microscopy utilizing biosensor probes or simply 'biosensors'.

Biosensors enable the monitoring of cell behavior through a combination of biological, chemical, and physical techniques [124, 125]. Table 2 illustrates that some biosensors are endpoint and require cell 'killing' via fixation, lysis, and extraction, while other approaches enable for live cell analysis in a real-time and non-destructive manner. In addition to readout (quantitative, qualitative, semi-quantitative), the method resolution is important: some biosensors measure 'bulk samples', while others can provide single-cell and subcellular resolution.

Table 2. Selected approaches addressing cell viability and death, applicable to 3D tissue models.

What is being measured?	**Technique**	**Method and instances of use in 3D cultures**
Cell mechanical properties	Brillouin spectroscopy	Combination of Brillouin spectrometry, in which monochromatic laser light is inelastically scattered from acoustic waves "phonons", combined with confocal microscopy to measure the longitudinal modulus and the live viscoelastic properties [126, 127].
	Microrheology	Tracking motion of small probes in soft materials to measure elasticity and viscoelasticity in live samples [128].
	Atomic force microscopy	Type of scanning force microscopy in which a cantilever scans over the sample surface causing bending and changing the amount of reflected light into the photodiode. Measures Young's modulus (stiffness) [129].
	Microtweezers	Compressing spheroids between two force-sensing microtweezers containing cantilever tips. Determining Young's modulus by optically tracking tweezers bending with a pattern-matching algorithm [12].
	Raman spectroscopy	A laser-based microscopy technique that relies on the Raman scattering, the inelastic scattering of photons to gain information about the chemical structure, surface properties, molecular interactions, and crystallinity [130]. Works with 3D but normally requires sample fixation [131].

Cell death	TUNEL assay	Quantitative *endpoint* analysis for DNA fragmentation during apoptosis [69].
	Annexin V- Alexa Fluor 488 coupled with cell-impermeable dyes such as propidium iodide, SYTOX dyes (blue, green, red, deep red), or 7-aminoactinomycin staining	Annexin V is a phospholipid-binding protein with a high affinity for phosphatidylserine, which translocates from the inner to outer leaflet of the plasma membrane during apoptosis. Membrane disruption/ permeabilization, during late apoptosis and necrosis, causes DNA intercalation of cell-impermeable dyes[132, 133]. Can be used in both flow cytometry and quantitative 3D live microscopy. SYTOX dyes in combination with activators and inhibitors are popular in studies of cell death, see Table 1.
	Genetically encoded caspase and other cell death targeting FRET biosensor proteins	Förster resonance energy transfer (FRET) between a far-red fluorescent protein (mKate2 or eqFP650) and the infrared fluorescent protein iRFP, connected through a DEVD caspase-3 cleavage site containing linker. Semi-quantitative detection of apoptosis through caspase-3 activation by live fluorescence lifetime imaging microscopy (FLIM)[134, 135]. See also [84, 136] for FRET biosensors.
	Dye-based staining of caspase activity e.g. CellEvent Caspase-3/7 Green detection	Cleavage of fluorogenic peptide substrates conjugated with the four amino-peptide (DEVD), by specific caspases[137, 138]. Quantitative measurement of apoptosis by fluorescence microscopy or flow cytometry.
Viability	Labeling cell proliferation by thymidine analogs (BrdU and EdU)	Thymidine analogs incorporated into nuclear DNA during cell division and visualized either using antibody or click-chemistry (EdU). Typically, endpoint assay for cell proliferation labeling[139, 140]. Live tracing with Hoechst 33342 and BrdU in multiplexed 3D FLIM are also possible[141, 142].
	Replication markers such as Ki67 and PCNA.	*Endpoint* antibody-based immunofluorescence staining technique of nuclear proteins associated with cell proliferation [140, 143].

	Genetically encoded fluorescent indicators, such as Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI) sensors	Two fluorescent proteins fused to cell-cycle-dependent E3 ubiquitin ligases degradation motifs (Cdt1 and Geminin) hereby allow quantitative live visualization during different cell cycle phases. During mid mitosis and G_1-phase, APC/C^{Cdh1} is active resulting in degradation of Geminin, while during S- and G_2-phase SCF^{skp2} is active causing degradation of Cdt1 [144, 145].
	ATP measurement using luciferase-dependent luminescent and FRET assays	Luminescent highly sensitive quantification of ATP, proportional to the number of viable cells. Can be combined with mitochondrial stimulation (FCCP and oligomycin, *endpoint*) to measure overall input in the viability [146]. Can be also performed with microscopy using luciferase and FRET-based biosensors [69, 147].
	MTT / MTS / XTT measurements	Quantitative colorimetric *endpoint* assays utilizing mitochondrial NAD(P)H-dependent dehydrogenases-aided reduction of the reagent into the chromogenic formazan [148, 149]. Works best with bulk sample analysis.
	Cell-permeant dyes and tracers staining different cell compartments such as mitochondria (e.g. MitoTracker Green, tetramethylrhodamine), endosomes, lysosomes, lipid droplets, membrane tension, calcium (Calcein AM green and Fluo-4), and specific cell markers (e.g. SR101).	Broad range of acetoxymethyl (AM) ester-modified dyes that can quantitatively inform on cell calcium or overall viability [150]. Mitochondrial tracers inform on the mitochondrial network structure and polarization (activity and viability) [142, 147] and specific cell markers, e.g. SR101 that visualizes live astrocytes in neural 3D cultures [151]. Most of these dyes are compatible with live microscopy, FLIM and can also help segment functional regions in live organoids [152].
Hypoxia and cell/tissue oxygenation	Redox-sensitive nitroimidazole derivatives such as pimonidazole	Typically provide quantitative endpoint 'snapshot' readout. In a hypoxic environment, the 2-nitroimidazole derivative is reduced and detected using specific antibody [153].
	Fluorescent protein expressed under HIF promotor	Quantitative expression of fluorescent protein upon activation of HIF signaling by fluorescence microscopy or FLIM [154].

	Quenched phosphorescence-based dye conjugates, nanosensors, and tissue engineering scaffolds	Live quantitative imaging of cellular O_2 using PLIM or intensity-based ratiometric microscopy. Can be multiplexed with other dyes and assays and various 3D tissue models: spheroids, neurospheres, organoids, bioprints and scaffold-grown constructs [44, 47, 154-159].
Metabolism and cell bioenergetics	Cellular temperature (intracellular thermometry)	Various dyes and nanosensors enable live quantitative FLIM and confocal microscopy-based assessment of cellular 'heat' generation. Tested with spheroids, where such gradients tend to be linked to mitochondrial function [160, 161].
	Biosensor fluorescent protein-based scaffolds binding collagen and cellulose	Biopolymer-based scaffold materials and hydrogels modified with recombinant protein biosensors, e.g. sensing extracellular pH and Ca^{2+}. Enable for live quantitative FLIM-based multi-parameter assessment of tumor spheroids and intestinal organoids [162, 163].
	Endogenous autofluorescence (NAD(P)H, FAD), exogenous biosensors, and biosensor proteins.	Live quantitative microscopy of cell metabolism based on the two-photon fluorescence of endogenous co-factors such as NADH, NADPH, and FAD using FLIM [110, 147, 164, 165].
	Microplate reader-based assays such as Seahorse extracellular flux (XF) analyser	Quantitative measurement of the bulk 3D tissue models in specialized microplates. It enables probing of the extracellular flux (O_2 and H^+) giving information about oxygen consumption rate and extracellular acidification rate, the key indicators of mitochondrial respiration, glycolysis and ATP production. May have limited utility with bioprints and organoids [147, 164, 166] and is typically an *endpoint* method.

Owing to the three-dimensionality, spheroid and organoid detection methods are frequently challenged by the sample thickness, presence of multiple cell layers, heterogeneity (including the presence of different ECM or scaffold materials), and their functional features. For instance, the basal membrane barrier function in small intestinal organoids can prevent efficient staining with nanoparticles [167], while high-resolution volumetric immunofluorescence microscopy of organoids often requires fixation and optical pre-clearing[168]. These two examples demonstrate limitations in: (i) choosing the appropriate biosensor and (ii) bringing up additional experimental

destructive treatment setting the model apart from the 'real' and *in vivo* situation. Detection methods can be categorized into destructive and non-destructive methods. The former includes destructive single-cell sequencing, flow cytometry, immunohistochemistry (combined with paraffin embedding and sectioning), or mass spectrometry methods [169]. Most of these methods are time-consuming, labor-intensive, and often lead to the loss of 3D context. To circumvent these issues, live-cell imaging can be used [170]. However, like every staining, it can face disadvantages of the limited probe and dye diffusion, intrinsic tissue transparency, contrast, and temporal/spatial staining [171]. The fluorophore brightness and the optimal light doses can affect the cell function and limit the capability of the method. However, recent innovations in instrumentation, biosensors, multi-parameter microscopy, FRET, FLIM and advances in high-content screening platforms can help addressing these issues [152]. For example, the scission-accelerated fluorophore exchange (SAFE) method allows quenching and completely removes fluorescent signals thereby allowing spatiotemporal multiplexing of immunofluorescence imaging of live cells [172]. Recently, an automated multi-scale 3D imaging platform was created using disposable microfabricated chips, called JeWells. This method helps to obtain high-content 3D images with a throughput of 300 live or fixed stained organoids per hour. Using convolutional neural networks, viability and cell death can be quantified [108]. The high-content analysis already enables for studying single cell-specific 'niche' phenotypes within tumor spheroids and brain organoids [105, 173-175].

The intra- and extracellular *mechanical properties*, such as Young's modulus and stiffness of the ECM become widely appreciated and represent a rapidly evolving area [113]. These properties can influence cell behavior, such as *in vivo* shape, fate, viability, and differentiation [176]. Traditionally used methods typically probe the surface of 3D models, while there is a growing interest towards force- and viscosity-sensitive FLIM biosensors, fluorescent beads and studying cell aggregates within specialized scaffolds providing constrained environments for spheroids and organoids [26, 177, 178].

The main hallmark of *cell death* is the change in cell membrane permeability and is therefore targeted by many detection techniques. The destructive TUNEL assay is widely accepted due to its high sensitivity and ease of use. However, it's expensive and provides false positives from necrotic cells, cells involved in DNA repair, or gene transcription[179]. Annexin-V has the advantage that it can distinguish between early, late apoptotic, and necrotic cells when combined with nucleus-impermeable dyes such as propidium iodide (PI). Recently, Annexin-V fusion proteins containing subunits of the luciferase enzyme NanoBiT were proposed combined with a cell impermeable DNA staining for a non-destructive, real-time kinetics assay[180]. Another traditional fluorescent method, "live/dead" staining utilizes green fluorescent Calcein Green-AM dye together with red-fluorescent ethidium homodimer-1. However, this method often suffers from the capability of the dyes to penetrate across the multiple cell layers ('penetration depth' issue) and non-uniform distribution in spheroids larger than ~200 µm in diameter. The non-invasive, label-free optical coherence tomography (OCT) can help solving these shortcomings [181]. Specific necrosis inhibitors combined with non-specific membrane integrity dyes, such as SYTOX, help visualize ferroptosis[182] and other mechanisms of cell death (see Table 1).

The cell *viability* is classically assessed through the mitochondrial reductase assays (MTT, MTS). More advanced, sensitive, and compatible with the live cell analysis methods are based on luciferase-based ATP measurements (endpoint and kinetic measurements), the use of biosensors specific to pyruvate, lactate, cellular redox, NADH, mitochondrial polarization, and by optical metabolic imaging achieved via two-photon microscopy of endogenous NAD(P)H and FAD [152, 165, 183-185]. *Molecular oxygen (O_2)* is an important complementary parameter for cell death, viability, and mitochondrial activity [186]. This can be quantitatively measured with the help of phosphorescence lifetime imaging microscopy (PLIM), ratiometric fluorescence, or alternative approaches [47, 154]. The semi-quantitative ratiometric approach makes O_2 monitoring possible in endpoint, multiplexed, and kinetic measurements of oxygenation of spheroids and bioprints [48]. These measurements are normally validated by mathematical modeling studies in static and fluidic flow conditions [110, 187].

5.1. Emerging challenges in live-dead assessment of 3D tissues

Experimental approaches utilizing spheroids and organoids as essential bio-ink components continue to drive innovations in tissue engineering and regenerative medicine. Science-fiction fantasies become real, targeting 3D printing of mini-brains [188] and oxygenated neural stem cell-based 3D constructs [44], mimic the space microgravity [189] or engineer the 'cross-kingdom' tissues and communities of plants and plant-derived materials [50, 162, 190, 191].

Even though these novel approaches are very impressive, there are still shortages in the assessment of viability and cell death in 3D cultures. 3D printing of organs is more complex than simply tissue printing, as the complex architecture and microenvironment of the natural organ still need to be considered. Spheroids and organoids are more appropriate than 2D monolayer cultures for mimicking the natural tissue. Nevertheless, many 3D cultures still fail to take the organ cellular properties into account, have different shapes and sizes, lack multi-scale architectures and tissue-tissue-interfaces, and exposure to mechanical cues such as tension, compression, and shear stress [192].

5.1.1 Fluid mechanics

The shear stress, occurring in the vasculature, influences endothelial cell adhesion, migration, and differentiation [193, 194] and must be considered with many 3D tissue models. Several *in vitro* culture systems modeling migration have been described for mammary glands [195], liver organogenesis [196], and cancer models [197]. The fluidic flow bioreactors and Organ-on-Chip (OoC) platforms can help address tissue mechanics in 3D models. Bioreactors aid the *in vitro* tissue survival before *in vivo* implantation by recapitulating the natural microenvironment by controlling culture medium pH, temperature, fluid pressure, cell shear stress, nutrient supply, and waste removal [198]. However, bioreactor-based screening of key maturation factors is labor- and time-intensive, also requiring special equipment. Alternatively, the *in vitro* tissue spheroid fusion assay can provide a simpler, faster, and thus more cost-effective high-throughput screening of potential 'maturogenic' factors and their overall viability [199]. OoCs often represent 3D multi-chamber channel microfluidic cell culture systems,

being miniaturized *in vitro* models for mimicking the physiological human organ environment [200]. Despite the multitude of such models and the ambition of multi-organ-on-a-chip(s), most of them utilize cell suspensions, 2D or spheroid cultures as the highest level of complexity [201-206]. Recent advances in these methodologies include organoids [207], improved modeling and compatibility with advanced imaging modalities [110], and integration with computational elements [208]. For example, the research focus shifts to improving the design of '4D' tissue engineering scaffold materials and micropatterning technologies to address and direct the microtissue development [114, 209]. The complex multicellular architectures can also be established by force gradients generated via spirals and aster topology cues, thus controlling the self-organization of myoblasts, their differentiation, and steering 3D morphogenesis [210]. Tracking the migration, mechanical forces, and viability can all utilize labeling live-cell tracers and biosensors (Table 2) for advanced analysis using lightsheet fluorescence microscopy [211] or high-content analysis of individual cell phenotypes [173, 212].

5.1.2 Long-term in vivo stability and viability

The *long-term in vivo stability* and *viability* of bioprinted constructs represent another challenge. There are *surgical challenges* (e.g. ischemia, incomplete maturation, host biocompatibility, and vascular network incorporation), and challenges of the *3D culturing and printing*. Vascularization is a prerequisite for maintaining viable grafts and successful tissue incorporation [213]. Currently, there are two main blood vessel incorporation strategies: the controlled release of angiogenic factors [214] and the direct printing of vascularized scaffolds [215]. Challenges of 3D printing include the variable viability of bioprinted tissues influenced by the 3D printing parameters, ranging from 95% (laser-assisted) to 40-80% (extrusion) viability [216]. Computational modeling can help increase cell viability, stability, and reproducibility of the engineered tissue constructs through experimental parameter predictions [217, 218]. Additionally, the stem and cancer cells typically lack control and guidance cues during organoid and spheroid development, resulting in increased variability of spheroid and organoid cultures. Until recently, there was little attention to standardizing these models[159, 219]. So far, such initiatives as MISpheroID consortium[8] and bio-banks address the shortcomings of minimal reporting guidelines [220].

5.1.3. Heterogeneity of metabolism and growth medium composition

Introducing novel detection methods helps to uncover heterogeneity and increase levels of the complexity of the used models. Next to the different zones of cell types, their viability or cellular heterogeneity, 3D cultures can also exhibit *metabolic heterogeneity* [169, 221]. For example, small intestinal organoid culture shows heterogeneous tissue oxygenation within (*trans*-epithelial gradients) and between individual organoids (inter-organoid) could be caused by the metabolic heterogeneity of the original tissue used for the organoid production [159]. Furthermore, a follow-up study using NAD(P)H-FLIM revealed that stem cells can rapidly upregulate oxidative phosphorylation and highlighted the need of controlling the medium composition for a better understanding of cell behavior in the experiment [164]. The selection of the right growth medium is already recognized in cancer microenvironment studies [222]·[223] and hopefully will be soon implemented in the design of advanced organoids, tissue-on-a-chip and bioprinted constructs.

6. Conclusion

Multicellular spheroids, organoids and related bioprinted models can help recapitulate both tissue development and different cell death phenomena, which are often present together in the live tissue. We see that multiple parameters, based on culturing and detection methods, can cause variability in the analysis of live-dead status in 3D cultures. Is your tissue 100% live or 100% dead? We do not believe that there is a method that can help to definitely answer this. However, innovations in live microscopy, non-destructive and multi-parameter imaging can help define and predict the real 3D tissue physiological status. The intrinsic disadvantages of biosensors, such as the cell-dependent staining are being addressed via continued research in chemistry and material sciences, biofabrication methods, and the introduction of novel non-invasive optical imaging techniques, such as near-infrared II light-sheet microscopy [152, 224, 225], potentially using single cell analysis approaches merged with high-content [173, 174] and FLIM-based multiplexing [152, 226, 227]. Essentially, combining mentioned analytical chemistry, biomedical optics and advanced cell biology techniques must be logically integrated with machine learning and advanced modeling approaches [108, 228-232] to further address the viability, reproducibility, and ‘sustainability’ of the popular 3D tissue models.

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Competing interests statement

No competing interests to declare.

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