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PERSPECTIVE

CRYOPRESERVATION OF ORGANOIDS

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Abstract

Organoids represent indispensable opportunities for biomedicine, including drug discovery, cancer biology, regenerative and personalised medicine or tissue and organ transplantation. However, the lack of optimised preservation strategies limits the wide use of organoids in research or clinical fields. In this review, we present a short outline of the recent developments in organoid research and current cryopreservation strategies for organoid systems. While both vitrification and slow controlled freezing have been utilized for the cryopreservation of organoid structures or their precursor components, the controlled-rate slow freezing under protection of Me_2SO remains the most common approach. The application of appropriate pre- or post-treatment strategies, like the addition of Rho-kinase or myosin inhibitors into cell culture or cryopreservation medium, can increase the recovery of complex organoid constructs post-thaw. However, the high complexity of the organoid structure and heterogeneity of cellular composition bring challenges associated with cryoprotectant distribution, distinct response of cells to the solution and freezing-induced injuries. The deficit of adequate quality control methods, which may ensure the assessment of organoid recovery in due term without prolonged re-cultivation process, represents another challenge limiting the reproducibility of current cryobanking technology. In this review, we attempt to assess the current demands and achievements in organoid cryopreservation and highlight the key questions to focus on during the development of organoid preservation technologies.

Keywords: organoids; stem cells; tumour; cryopreservation; vitrification; recovery.

INTRODUCTION

Gradual replacement of animal studies by cell-based model systems is currently widely applied to approach the 3Rs strategy - replacement, reduction and refinement (Directive 2010/63 EU). This long journey started with simple 2D cell cultures and is

currently evolving towards complex 3D environments, which ensure accurate cell-cell and cell-matrix interactions, depiction of cell polarisation and close to natural transport dynamics for nutrients and gases.

The closest resemblance to native tissue/organ structure is considered achievable in organoid cultures. The history of 3D culturing of cell aggregates traces back to the 1950s, but the actual breakthrough in organoid research happened in the 2000s after the discovery that Lgr5-expressing adult intestinal stem cells in Matrigel could self-organize and form cryptvillus structures (1, 2). The discovery of the methods for somatic cell reprogramming into induced pluripotent cells (iPSCs) and the improvement of culture methods for embryonic stem cells (ESCs) played another key role in boosting organoid research (1). Nowadays, the term organoid represents stem cell-derived cultures with the ability to self-assemble into a 3D structure, closely mimicking the natural cell organisation, at least at the micro-scale level (3). The unique, distinguishable features of organoids compared to other 3D culture platforms, including the closest resembling type – spheroids, are the intrinsic self-organisation, natural maturation of established structure and some degree of key tissue-specific functional features (e.g. contraction, nerve impulse conduction, hormonal/paracrine secretion, filtration, etc.). These processes ensure the long-term stability of organoids, while simple spheroid cultures tend to disaggregate and lose regular morphology (4).

Despite much attention recently being paid to organoid generation techniques and evaluating their functional competencies or potential application in various fields of biomedicine, the approaches for biopreservation of these advanced 3D cultures are not fully explored. Cryopreservation is the only strategy to ensure the long-term preservation of biological samples. However, the complexity of organoid systems brings many challenges, which can only be overcome by in-depth cryobiology research and development.

In this review, we present a short outline of the recent developments in organoid research and focus on the current organoid cryopreservation strategies.

ORGANOID TYPES AND GENERATION TECHNIQUES

Organoids can be generated from pluripotent stem cells (ESCs / iPSCs), multipotent organ/tissue-specific adult stem cells (ASCs), or a re-aggregated mixture of primary cells (3). Similarly, to generate the tumour organoids, different types of cells, including cancer cell lines, genetically transformed somatic cells or patient-derived tumour cells, can be applied (5). ESCs, isolated from the inner cell mass of a blastocyst, possess high self-renewal degree and ability to differentiate toward all three germ layers (ectoderm, mesoderm, and endoderm), providing vast potential for organoid research [reviewed in (6)]. However, ethical issues linked to the use of human embryos for ESCs isolation halt widespread application of these cells in biological studies. The discovery of somatic cell reprogramming (7) introduced an alternative source of cells that resemble characteristics of ECSs and have almost unlimited capacities for organoid generation. However, despite the unique plasticity, the iPSC/ESC-based organoids are known to fail to recapitulate gene expression of adult tissues in vivo or can lead to the formation of undesirable cell types (8). Alternatively, the use of less potent post-natal or adult tissue-specific stem cells with partially determined fate can be considered not as a limitation but as an advantage in 3D bioengineering. Here, the patient-specificity of the ASCs can overcome the limited differentiation capacity when organoids are intended for personalised medicine purposes (9). Therefore, the choice of stem cells used for the generation of organoids highly depends on their following application and research goals.

Developing such a complex structure as an organoid requires not only specific cell types but also appropriate conditions, ensuring a “niche” microenvironment. Organoid formation can recapitulate both major self-organisation processes during development: cell arrangement and spatially restricted lineage commitment (10). Standard organoid development stages include cell proliferation, early differentiation of single cells or clusters, spheroid formation and following maturation, physical rearrangement, cell sorting, controlled gene expression, and morphogenesis. This process of organoid formation generally takes 2 to 3 months depending on the type and requires different

physical and chemical cues. In vitro, bioactive molecules and cell culture conditions control the maintenance and developmental stage. For example, the initial specification of pluripotent stem cells into endo-, ecto- or mesoderm is driven by activation of Activin A, WNT/BMP4 or Activin A/BMP4 signalling pathways, correspondingly (11). Further steps of tissue-specific differentiation can be achieved by the addition of a specialised cocktail of growth factors leading to changes in cellular phenotype and behaviour.

Numerous successful strategies for organoid generation have been developed so far [reviewed in (1, 4, 12)]. The classical extracellular matrix (ECM)-based approach includes embedding cells into natural or synthetic hydrogel products derived from purified basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (EHS) that should provide a scaffolding structure and mimic the native microenvironment. EHS-based products are commercially available under the trademarks Geltrex®, Matrigel® or Cultrex® Basement Membrane Extract and contain more than 300 biomolecules (13).

In attempts to reduce the complexity and lot-to-lot variations, other natural biopolymers such as type I collagen, fibrin, alginate and hyaluronic acid have been studied as relevant alternatives [reviewed in (5)]. Growing interest has been recently noticed for synthetic hydrogels, including PEG-macromers modified with ECM- and integrin-binding peptides, ensuring proper cell adhesion (14, 15).

Despite its proven efficiency and high popularity, the hydrogel-embedding method does not ensure the formation of organoids from all tissues. This constraint led to the development of alternative techniques including but not limited to: i) hanging drop method (16); ii) air-liquid interface exposure; iii) suspension culture procedure accompanied by the use of spinner flasks or rotating bioreactors; iv) magnetic levitation; v) microfluidic-based approaches or 3D bioprinting [reviewed in (11, 17)]. As a result, by applying the proper technique and signalling cues, various types of 3D organoids can be successfully formed: intestine, brain, heart, liver, kidney, pancreas, optic cup, lung, thyroid and others [reviewed in (3, 5, 11)]

APPLICATION OF ORGANOIDS

As complex and self-organised structures, organoids hold unlimited potential in studying normal organ development and tissue morphogenesis. Although physiological patterns are challenging to be replicated in vitro, some elegant and sophisticated organoid-based models have been established.

The pioneering study of T. Sato and colleagues on intestine organoids consisting of a polarised, columnar epithelium, which was patterned into villus-like structures and crypt-like proliferative zones, confirmed a highly coordinated interaction between different cell types and signalling molecules in organoid morphogenesis (2). These findings boosted this scientific field and encouraged researchers to generate artificial systems for modelling cytoarchitecture and developmental trajectories found in vivo. Recently, E. Gabriel and colleagues presented a protocol for obtaining optic vesicle-containing brain organoids with photosensitising activity suitable for studying vision and light perception (18). Another striking example is a generation of hepatic organoids with a well-organised functional bile canalicular system that makes it possible to analyse cholestasis in vitro (19). Together with gene-editing tools, organoid models represent unique platforms to reveal drivers of diseases with unclear or multiple aetiology and help improve current therapeutic strategies (20). With this approach, numerous disease models have been established, including cystic fibrosis pathology driven by a single gene mutation in CFTR (Cystic Fibrosis Transmembrane Conductance Regulator), Wilson's disease associated with the loss of COMMD1 (Copper Metabolism Domain Containing 1) and even autism spectrum disorder mediated by overexpression of FOXG1 (Forkhead Box G1) (21, 22, 23). It is also worth mentioning the application of organoids for parasitic infections and host-pathogen interaction research [reviewed in (24)].

New opportunities for personalised treatment of tumours are offered by tumour organoids that maintain cell-cell interactions, genotype and microenvironment of the primary sample they originate from. However, considering the enormously high heterogeneity of tumours, it is crucial to perform personalised drug response screening and select the prospective treatment options. It was reported

that patient-derived organoids recapitulate the response to treatment in metastatic gastrointestinal cancer patients with high accuracy (25). A series of other studies implicating numerous cancer types confirmed the potential of tumour organoids to act as predictors for clinical treatment response [reviewed in (26)].

Despite the extensive use of organoids for cutting-edge basic science discoveries, their application in regenerative medicine and tissue engineering is still at the early stage. Numerous constraints, including reproducibility and scalability of the organoid generation techniques, potential tumorigenicity of stem cells and incomplete maturation of 3D structures in vitro, limit their clinical application. However, early preclinical results of organoid transplantation showed significant promise for future therapy. For instance, R. Fordham and colleagues applied intestinal organoids to treat a chemically induced colonic injury in mice (27). The authors showed that cells of transplanted organoids contributed to the regeneration of damaged gut epithelium leading to a renewal of crypt-like architecture. Benefits of organoid transplantation have been shown in many other disease models, such as ischemic stroke brain injury, acute liver failure or diabetes (28, 29, 30).

LOW-TEMPERATURE PRESERVATION OF ORGANOIDS

A wide variety of potential areas for practical application of organoid cultures creates a strong demand for their steady supply. Introducing a cryopreservation step into the manufacturing pipeline should help to boost the availability of systems with tissue-like architecture for academic / research, clinical or commercial needs. In addition, cryopreservation of complex organotypic structures during the large-scale culture for more than several weeks provides a feasible solution for controlling the product quality in a reproducible manner (31). Compared to cells in suspension, the 3D organisation, large size and complexity of natural tissue fragments and organoids complicate the freezing approaches. The main challenges to consider during multicellular structure cryopreservation include disruption of organised cell-cell interactions, potential cell cryoinjury from growing ice crystals during the freeze-thawing, different osmotic and cryoprotectant (CPA) tolerance of distinct cell types within the single 3D environment. An in-depth analysis of recent achievements, together with a deep understanding of key cryodamaging factors affecting cryopreservation of 3D tissues, including organoids nature, are required to overcome these obstacles.

Cryopreservation of tissues for subsequent organoid generation

In the past decade, many attempts have been made to cryopreserve isolated tissue fragments for a subsequent organoid generation. Successful formation and subsequent passaging of organoids from cryopreserved biopsies have been shown for malignant and normal tissues (32, 33, 34). According to recent reports, in most cases, the freezing solution contained permeable dimethyl sulfoxide (Me_2SO) in basal cell culture media. Although the number of derived organoids tended to be lower from cryopreserved tissue compared to freshly isolated, the overall success rate of the procedure was comparable (33, 35, 36). A recovery period of 3-7 days after thawing was required to overcome a temporary deceleration of functional activity post cryopreservation (32). The initial growth of organoids formed from frozen samples was slightly delayed, but during the following passaging, the proliferation activity intensified (35). The organoids generated from cryopreserved tumour tissue samples stored at −80°C for more than six months had a similar expression of Ki67, a cell proliferation marker, and cleaved caspase 3, an apoptosis marker, as those derived from fresh tissue (32). In addition, the slow freezing of tissues did not affect the complexity of multilayered morphological architecture specific for organoids of pancreatic, endometrial and gastrointestinal origin (33, 34, 35). The gene expression patterns of cells in organoids formed from cryopreserved and non-cryopreserved tissues were almost identical, and the observed changes were negligible (34, 35). Thus, isolating organoids from frozen tissue samples is a viable option to facilitate their distribution and widespread use.

Nevertheless, from a practical point of view, obtaining a ready-to-use organoid product without the need for a laborious organoid generation procedure seems to be a more attractive option. The organoid biobanks promise a valuable resource for basic science, industry and clinical applications minimising

potential issues related to a typical multistep organoid generation workflow and improving the overall reproducibility of the studies. Large-scale OMICs analyses, advanced functional assays and therapeutic screenings can be performed in the organoid models, thus improving precision medicine and facilitating drug discovery (37, 38).

Slow freezing protocols of organoid cryopreservation

Currently, dominating protocols for all types of three-dimensional organoid assemblies include the use of Me_2SO (7.5-10%) and gradual freezing to −80°C with the following transfer of samples to −150°C or −196°C (39, 40, 41). To improve the penetration of Me_2SO, larger structures are usually cut into small pieces (less than 0.5-mm-diameter) before freezing. During post-thaw recovery, such organoids can successfully reassemble, regaining their initial shape (40, 42). Even though the maintenance of organoid integrity after cryopreservation is an obligatory parameter, it is insufficient to confirm the preservation of functional activity. During the recovery stage, the verification of proliferation potential and retention of an unaltered gene signature are crucial. Positive results confirming structure regrowth and unchanged expression of cell-specific biomarkers have been reported for organoids of different origins, including intestinal, retinal or hepatic (40, 41, 43).

In more than half of published studies utilising the slow freezing technique for organoid cryopreservation, Rho kinase inhibitor Y-27632 has been added to the freezing or thawing/recovery media to improve the cryopreservation outcomes (41, 42, 44). Rho kinases are a family of serine/threonine kinases involved in multiple biological processes, including cell adhesion, motility, division and differentiation (45). Rho kinase inhibitors as additives are widely used for cryopreservation of ESCs and iPSCs. They have been suggested to reduce the dissociation-induced apoptosis by modulation of gap junctions and strengthen adhesive properties. It has been shown that the inclusion of Y-27632 into the freezing medium can increase the organoid recovery after cryopreservation by 2.5-fold compared to untreated organoids, while the addition of ROCK inhibitor after thawing into the recovery medium was slightly less effective and resulted in a 2-fold improvement (46).

In addition to ROCK inhibitors, the benefits of blebbistatin, a highly specific inhibitor of myosin, have been recently proven for retinal organoid cryopreservation. Multicellular systems cryopreserved with 3 M Me_2SO, 0.3 M sucrose and 20 µM blebbistatin had slightly blurry margins immediately after thawing but recovered completely after four days of culture (47). More importantly, the organoids retained populations of retinal progenitors and retinal ganglion cells based on positive immunostaining for $MCM2^+$/beta-tubulin$^+$ or HuD^+, correspondingly. However, the full impact of both ROCK and myosin inhibitors on recovered cells has yet to be realised and detailed research in this area should be done before clinical application is attempted.

Organoids formation and culture are mostly carried out by researchers with specialised training, but using organoids for analysis should be broadly available to all scientific community members because they are indispensable models for biomedical research and preclinical drug discovery. The technology of in-plate cryopreservation of organoids has been recently proposed to ensure their ready-to-use availability (44). Although the cryopreservation procedure is technically complex and requires two different controlled-rate freezers, it ensures a positive outcome in the survival of hepatic, colon and colon tumour organoids in 24- and 96-well plate formats. Cryopreserved and non-cryopreserved tumour organoids had comparable expression levels of metabolism-associated genes *GLUT1* and *PKM2*, proliferation marker *MYC* and specific markers of stem and epithelial cells *LGR5, VIL, MUC2* (44).

Given the potential clinical application of organoids, the latest trends in the field are driven by the attempts to develop safer protocols. One of the main requirements for cell-based therapeutics is their manufacturing using GMP-compliant xeno-free media, while in most research studies, foetal bovine serum remains the key component for both culture and freezing media. Nevertheless, encouraging results have been recently achieved using commercially available serum-free cryopreservation solutions mFreSR™ or Cryostor® (STEMCELL Technologies) (40, 48, 49). In this case, the viability of intestinal and hepatic organoids ranged within 70-80% after thawing, with the following successful recovery over time (40, 48). The karyotype of organoids maintained stability until passage 40 (40). Considering the

cytotoxicity of Me_2SO, first attempts have been recently made to reduce the cryoprotectant concentrations. Lee and colleagues (50) showed the possibility of reducing the Me_2SO concentration from 10% to 5% without significant changes in cell viability after cryopreservation. There was no discernible difference in gene expression between control (unfrozen) and cryopreserved organoids confirming the suitability of the proposed approach. Minimal negative effect on the structure of ovine abomasum and ileum organoids has been reported after long-term (18 months) low-temperature storage and following seven days of in vitro culture (49). However, in another study, extended storage in liquid nitrogen caused a noticeable time-dependent decrease in the survival rate of cells that should be taken into account during the establishment of standardised cryopreservation protocols for organoids (46).

Vitrification of organoids

Nowadays, vitrification is considered a promising approach to freezing large-sized objects, including oocytes, embryos, isolated tissue fragments or even whole organs (51). This technique allows freezing cells of very different biophysical properties, that being the case in the complex structure of fully developed organoids. In contrast to traditional slow cryopreservation techniques, applying fast cooling rates along with a high concentration of CPA allows the prevention of crystallisation in the extracellular medium. It is suggested that the high concentration of CPA in the vitrification solution promotes rapid penetration and saturation of central areas of organoids, and is therefore more suitable for the preservation of multicellular structures compared to slow freezing. Successful vitrification has been recently shown for cancer and non-cancer organoids (52, 53, 54). The composition of vitrification solutions varies across the studies, with Me_2SO, ethylene glycol and saccharides (sucrose, raffinose, dextran) being the most common CPA. It was shown that the vitrification of human testicular organoids using a standard kit for cryopreservation of human embryos (Vit Kit-Freeze, Irvine Scientific, USA) allowed preservation of up to 95% of viable cells post-thaw. Cells within the organoid recovered and displayed normal phenotype during the subsequent 14 days of culture in standard conditions (54). In line with this study, another group of authors confirmed retention of the delineated interior-interstitial and exterior-seminiferous compartments after vitrification of the same type of organoid, although lower GATA4 immunoreactivity (a transcription factor regulating embryogenesis and, in particular, myofibroblasts differentiation and normal testicular development) and a more diffuse αSMA distribution (a marker of activated myofibroblasts) was noticed (55). These changes suggest an alteration in myogenesis-related pathways after vitrification.

It is worth noting that the use of CPA in high concentrations, common in vitrification, requires careful removal of the CPA immediately after thawing using a stepwise dilution with a washing solution. Removal of vitrification media should be gradual to avoid cell swelling associated with the difference in osmotic concentrations. To date, a wide variety of washing solutions and protocols for CPA removal (varying duration of each dilution step or incubation temperature) has been reported, but all of them are time-consuming and an optimal procedure is yet to be developed. Moreover, the final step of CPA removal procedures usually includes centrifugation, which increases the risk of organoid integrity impairment and contributes to cell damage as an additional stress factor. Q. Liu and coauthors (53) recently proposed a novel in situ cryopreservation technology on the superhydrophobic microwell array chip, eliminating the harvesting and centrifugation steps. Authors showed that after vitrification and thawing of lung cancer organoids on a chip covered with Matrigel®, the CPA could be removed by gentle washing. Assessment of organoids at different time points during recovery revealed spheroid-like morphology similar to the unfrozen counterparts. Cells were able to grow and retained the expression of squamous cell lung cancer markers, including p40, p63, and CK5/6 (53). Despite preliminary encouraging results, thawing of vitrified organoids can be associated with recrystallisation leading to cell injury. In order to minimise the damaging effect, novel technologies for rapid and uniform warming of large objects have been proposed (56). Importantly, cell type-specific CPA cocktails, cooling rates, and effective post-thaw recovery protocols are also required to achieve noticeable progress in establishing banks for ready-to-use organoids.

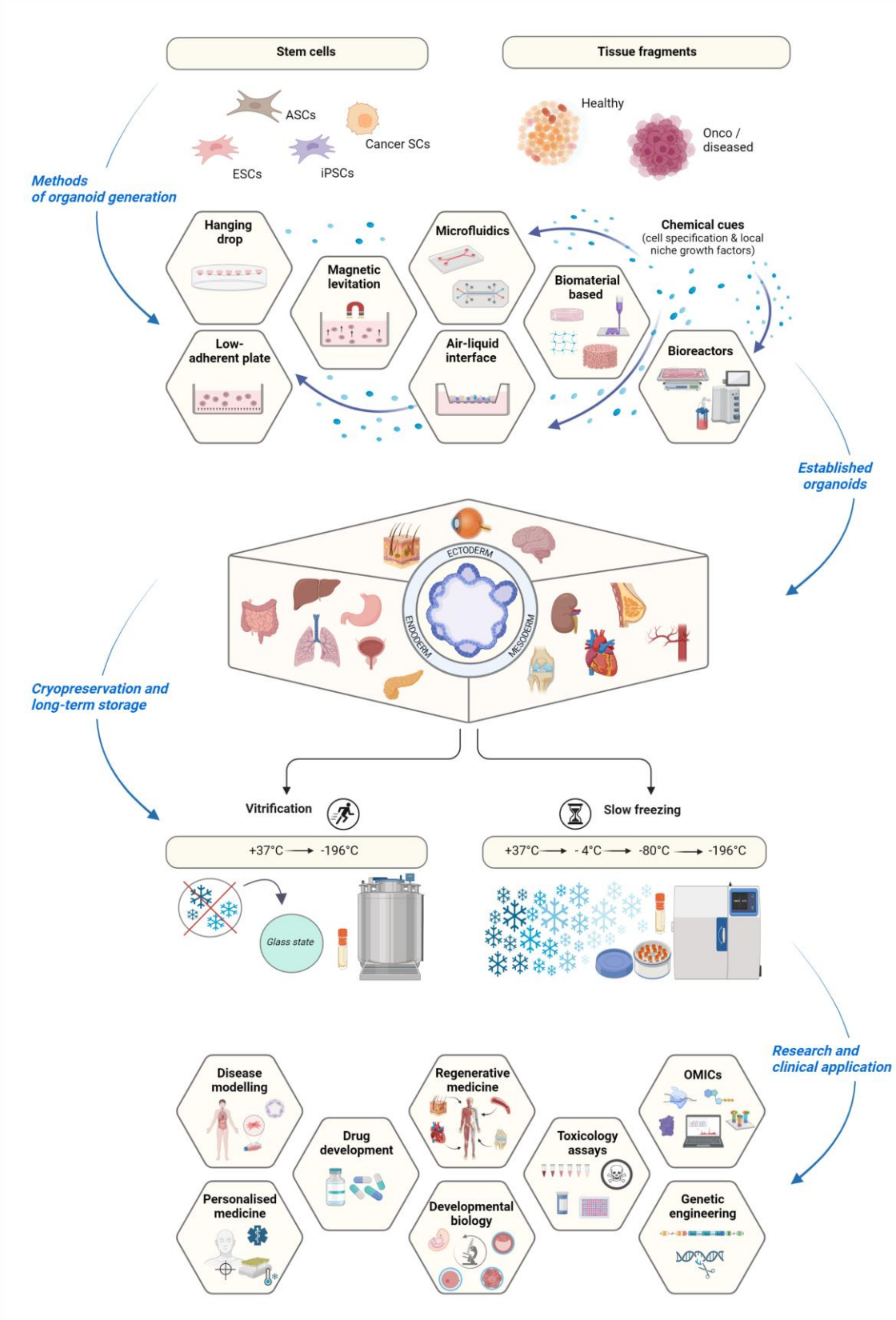

Figure 1. The general organoid research pipeline. This figure was created with BioRender.com

FINAL REMARKS AND FUTURE PROSPECTIVE

In this mini-review, we have discussed the current state of organoid research, focusing on the approaches used for organoid cryopreservation and post-thaw recovery. The simplified general pipeline of organoid research is summarised in Figure 1. The significant timing of organoid generation, which may reach up to several months, limits their wide application. Effective preservation strategies are needed to expand organoid use in research and industry. It is worth mentioning that, to date, more than 15 relatively small "living biobanks" of tumour organoids have been established (12). In contrast, only a few examples of such biobanks for non-tumour organoids have been reported. Yet, the limited availability of the samples, transportation issues and limited organoid maintenance duration stimulate the search for strategies, ensuring large-scale prolonged preservation of organoids for subsequent applications. The low-temperature preservation approaches may overcome these challenges and impulse progress in drug discovery, disease modelling and treatment by ensuring on-demand availability of the 3D organoids with reproducible features.

In our review, we attempted to outline the currently used CPA compositions, cooling regimens or bioactive additives applied to reach the demanded outcomes. Both major cryopreservation approaches (vitrification and slow controlled freezing) have been proved to be suitable for organoid preservation. Interestingly, the majority of the published reports demonstrate successful cryopreservation by indirect parameters, such as reassembling or regaining functional properties after a significant post-thaw recultivation period, while the primary evaluation is usually limited by cell viability measurement. In order to reach the on-demand availability of the organoids for basic and translational research, it is necessary to establish a broad spectrum of validated methods ensuring the thorough evaluation of the functional activity of organoids in due terms post preservation. Moreover, since organoids are multicellular structures, additional focus should be placed on evaluating the response of distinct cell types to the freeze-thawing process. The obtained data will provide unique information for the further improvement of cryopreservation protocols, establishing new conditions or non-toxic cryoprotective cocktails. It is already clear that new creative approaches are required to minimise cell damage induced by freezing complex multicellular systems. Promising results have been shown for intestinal organoids encapsulated into core-shell alginate hydrogel (57). The core-shell structure promoted a better recovery of cells within the organoids, which underwent slow freezing, most likely by preventing intracellular ice formation and reducing mechanical damage during post-thaw centrifugation. Interestingly, the encapsulation approach was initially proposed for spheroids representing a simpler structure than organoids, being composed of a single cell type (58). Therefore, cell spheroids can be considered affordable primary model systems in the development of cutting-edge methods for organoid cryopreservation. With advances in understanding key parameters affecting cells during freezing, it becomes possible to unravel complex physical dynamics that occur during cryopreservation using quantitative modelling methods (59, 60). Appropriate processes simulation can potentially help identify minimal times needed for equilibration and saturation with CPA and select the cooling and plunge temperature while considering the individual differences in membrane permeability and size of individual cells or multicellular constructs. The establishment of permeability coefficients within the 3D spheroids/organoids will help optimise freezing regimens and improve cryopreservation outcomes. By combining the experimental data and mathematical physics equations, it has been shown that the permeability coefficients for Me_2SO differ for cells in suspension and spheroids. Here, the lowest calculated values have been reported for the inner layers of 3D structures (61), which can be related to cell-to-cell interactions and synthesis of the intercellular matrix, affecting the diffusion of water molecules and CPA. For more sophisticated systems like organoids, further advancement of proposed models describing cell/structure behaviour during freeze-thawing is essential.

It should be noted that there is a high demand in organoids for performing mass-scale 3D assays in 96 or even 384 multi-well plate format (62). Considering significant time- and labour-investments required for organoid development, it would be beneficial to separate processes of 3D culture production and their

following use in testing. One of the promising strategies capable of addressing this challenge is the cryopreservation of a spatial arrangement of various cells and the further application of bioprinting to form organoids from these immature freeze-thawed multicellular fragments. Although being non-trivial and arduous, a combination of cryopreservation and bioprinting has already demonstrated successful examples (62, 63). Cryopreserved spheroids of fibroblasts were printed onto a needle array and used to fabricate a scaffold-free tubular construct (prototype of blood vessel, diaphragm, and conduits for nerve regeneration) (63). The feasibility of bioprinting of in-plate cryopreserved colonoids using GelMA polymer as a bioink has been recently shown; analysis of obtained structures 48 h post-print showed high cell viability and reproducibility (62).

The multicellular organoid systems represent unlimited opportunities in current research and therapy. The strategies for effective preservation of organoids may bring indispensable benefits with great potential in various fields of biomedicine, including drug discovery, regenerative and personalised medicine or tissue and organ transplantation.

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