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Strategies for improving the physiological relevance of human engineered tissues

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Abstract

This review examines important robust methods for sustained, steady state, *in vitro* culture. To achieve 'physiologically relevant' tissues in vitro additional complexity must be introduced to provide suitable transport, cell signaling, and matrix support for cells in 3D environments to achieve stable readouts of tissue function. Most tissue engineering systems draw conclusions on tissue functions such as responses to toxins, nutrition or drugs based on short term outcomes with *in vitro* cultures (2–14 days). However, short term cultures limit insight with physiological relevance, as the cells and tissues have not reached a steady state.

Keywords

long term culture; tissue engineering; microfluidics; two dimensional culture; three dimensional culture; bioreactors

Defining 'physiological relevance' in tissue engineering approaches

The goal of tissue engineering is to generate living tissue constructs *in vitro* that are morphologically and functionally similar to native tissue. Growing physiologically relevant tissues requires multidisciplinary research where the resulting tissues can be used for the study of human development and disease, to test the efficacy and toxicity of compounds and treatments, and for regenerative medicine applications. In tissue engineering reports, there are many terms that are commonly used to describe outcome measurements of these tissues including: 'physiological relevance', 'mature' and 'stable.' While all of these terms imply that the *in vitro* tissues behave in a similar manner to *in vivo* tissues, they may not describe essential details accurately, unless the terms are properly defined for each case.

To make a general, broad definition, 'physiological relevance' is the characteristic of (or corresponding to) healthy or normal biological functioning. However, in different situations this will mean different things related to tissue engineering. For instance, if the goal of the

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study is to screen drug candidates during preclinical drug development for liver treatment, recreating general cellular functions (oxygen uptake, amino acid metabolism and substrate consumption) and liver-specific functions (drug-metabolizing capacities and the production of liver-specific metabolites) can qualify as physiologically relevant [1]. However, for implantation in a patient suffering from liver failure, the liver will have to additionally contain bile ducts, a functional vascular network and a hepatic microarchitecture, as well as have a substantial regenerative capacity, to be considered physiologically relevant [2].

In the same context, physiologically relevant tissues should contain 'mature' cells specific to the tissue and goal of the study. However, this brings up the question – what is a mature cell? Each tissue contains different cell types that vary depending on the tissue and the state of maturation of that tissue. Therefore, a 'mature cell' can be defined as a cell that exhibits normal biological functions in the 'developed' form of the tissue. 'Developed' in this case refers to the stage of the desired tissue, which can be embryonic, young, aged, diseased, etc. depending on the goals of the study.

After establishing the targeted or required 'mature' status of cells within the tissue it is important to establish when the tissue has become 'stable'. Importantly, having mature cells does not mean the tissue is stable, as the tissue could still be adjusting, expanding, and forming. Therefore, stability can be defined as a tissue that is not changing with time. This can be determined by tracking material properties [3], matrix content [4], or by other markers of function such as secreted proteins [5–8] or endogenous signals [9, 10]. A homeostatic, 'stable' tissue is essential for tissue engineering as a baseline for *in vitro* studies of the efficacy and toxicity of compounds or to maintain phenotype upon implantation for regenerative applications. It is important to note the goals of the study, however, in some disease states, such as tumors, 'stable' tissues would not be the goal.

In this review we describe strategies for improving the physiological relevance of tissue engineered constructs, acknowledging that 'physiological relevance' will vary in definition in different contexts. We will touch upon some of the more common strategies for forming 'stable' biological functions with 'mature' cells that are more in line with *in vivo* function, with a specific focus on the temporal component of culturing engineered tissues *in vitro*.

Strategies for improving the physiological relevance of long term cultures

While the endpoint criteria are specific to the tissue of interest and the desired application common strategies to improve the physiological relevance of tissues (Figure 1) include recapitulating: biological context (such as the extracellular matrix, vascularization and cell types), chemical and mechanical cues (through the use of reagents and bioreactors) and incorporating spatial cues (by culturing cells in 3D). All of these strategies require the optimization of culture conditions in an attempt to form mature, stable tissues.

Recapitulating biological context

Each tissue exhibits variability in the amount and type of extracellular matrix components [11, 12]. Therefore, for the *in vitro* environment the cells should be carefully considered for each tissue to mimic the tissue content and properties. Biomaterial scaffolds predominately

consist of ceramics (examples: hydroxyapatite or tri-calcium phosphate), synthetic polymers (examples: polystyrene, poly-L-lactic acid, polyglycolic acid, poly-D,L-lactic-co-glycolic acid), or natural polymers (examples: collagen, alginate, silk) with varying physicochemical properties, architecture, and degradability [13]. In particular, the porosity, pore dispersal, surface area, mechanical properties, and surface chemistry influence the attachment, migration, proliferation, and production of extracellular matrix by the seeded cells within the scaffold. Additionally, to mimic other aspects of the ECM the process can be aided with a hydrogel (examples: Matrigel, collagen), or the hydrogel can be used as a standalone 3D matrix lacking the structural integrity of a more robust, rigid porous scaffold.

One of the major challenges of generating matrix-rich, dense tissues, however, is the limited mass transfer distances for nutritional supply and waste removal. To address this issue, tissue vasculature (which provides and removes nutrients *in situ*) can be recreated (Box 1). Moreover, to recapitulate the biological context, cellular interactions within a tissue must be considered to help maintain tissue specificity and homeostasis which is fostered through cell-cell signaling. Enhanced differentiation and survival has been achieved in many organ systems by co-culturing relevant cell types, for example: skin [14], neural tissue [15, 16], bone [17], and liver [18]. Co-cultures lead to increased extracellular matrix deposition over mono-cultures, including fibronectin deposits in glomerular tissue [19] and collagen deposition and mineralization in bone tissue constructs [20]. Improved function has been demonstrated by co-cultures including beating cardiomyocytes, which increased fluctuations in intracellular calcium ion concentrations not achieved in mono-cultures [21]. Additionally, proper morphology has been observed in co-cultures for cardiomyocytes [21], endothelial cells [18], and epithelial cells [19] not observed when the cells were cultured individually. Improvements in vascular structures can also be achieved with co-cultures over mono-cultures [17, 18, 22]. While co-culture systems enhance physiological relevance, they increase the complexity of the culture system and require special design considerations. For instance media components, ratios of cell types, and timing of differentiation, need to be optimized to obtain proper tissue formation. Additionally, differential labeling of each cell type is helpful to evaluate cellular interactions and contributions [23].

Finally, the cell types chosen can affect outcomes. Stem cells, primary cells, immortalized cell lines, or modifying the gene expression of cells for a desired phenotype can be considered. Stem cells are undifferentiated cells that have the potential to differentiate into specialized cells [24]. Primary cells on the other hand are already differentiated cells obtained directly from a specific tissue [25]. They have a limited lifespan in culture and eventually undergo senescence and stop proliferating [25]. Immortalized cells in contrast proliferate and evade cellular senescence [25] and are easier to work with, but provide questionable relevance to *in vivo* functions.

Chemical and mechanical cues

Cells require chemical cues, fluid flow and mechanical inputs for proper signaling, nutrient supply and mechanotransduction *in vivo*. Chemical cues such as growth factors drive proliferation, differentiation, or senescence and are highly dependent on cell type. For instance, the TGFβ growth factor family results in diverse and sometimes contradictory roles

in different cellular systems [26, 27]. Therefore, careful consideration for chemical additives is important for each engineered tissue.

Bioreactors play a significant role in enhancing the supply of chemical factors and mechanical signals to engineered tissues. They can improve the quality of engineered tissues, automate and standardize tissue manufacturing, control size and shape, establish proper nutrient and metabolite transport via improved mass transfer, and generate more homogenous cell distributions [28–33]. However, bioreactors must be designed to mimic the mechanical cues the cells experience *in situ*. For instance, articular cartilage is often studied in hydrostatic bioreactors which mimic hydrostatic loading from articulating bones [34], whereas osteogenic differentiation is often studied with perfusion which mimics the interstitial fluid movement through lacunae (caused by bone being loaded in compression and tension) [35]. Additionally, the duration and frequency of the applied stress should be considered, such as fluid flow in bioreactor systems. For cells that experience flow *in situ*, continuous flow is an appropriate choice. As an example, osteoblasts in continuous flow bioreactors increase cell numbers and efficiency of differentiation over static controls [36]. However, sometimes constant flow can lead to low viability and a heterogeneous populations, whereas periodic flow appears to improve results [37]. Periodic flow enables shear sensitive cells (human embryonic stem cells for example [38]) to withstand the flow, and allows for long static incubation periods where secreted factors can accumulate locally. While mechanotransduction is thought to be an important cue driving development of tissue *in vivo* [39], directing the differentiation of cells with biomechanical cues requires optimization of the type of stimulus, the temporal component of the applied stimulus, the insertion of rest (static) periods (if necessary), and the magnitude of the stress itself, which all effect cellular responses [35].

Spatial cues

The classic growth of cells on tissue culture plastic and glass 2D surfaces does not reflect the complexity of tissue specific architecture and signaling (biological and mechanical) experienced *in situ*. Many reviews have focused on the striking differences between 2D and 3D culture conditions [40–43], emphasizing the major impact that 3D cultures have on: drug screening outcomes, cell shape, cell-cell interactions, and cellular interactions with their matrix. More complex 3D drug screening *in vitro* not only decreases the use of laboratory animals, but can also improve toxicology screening, as it is more similar to the human *in vivo* condition. Likewise, cell shape in 3D matrices is more similar to their *in vivo* environment resulting in tissue specific signaling not found in 2D cultures. 3D culture also results in proper cell-cell interactions, as opposed to cell overgrowth, contact inhibition, and dedifferentiation often encountered on planar surfaces. Finally, 3D systems improve cellular interactions with their matrix; including cell adhesion, mechanotransduction, force production and cell migration.

Temporal component of tissue engineering

An often overlooked aspect of physiological relevance is the timeframe in which tissue-equivalent structures develop *in vitro*. Short term cultures (2–14 days) often do not result in mature tissues with stable functions or markers [3, 4, 44, 45]. Cultivation time *in vitro* is

required to allow the cells to adjust, expand and form into the targeted tissue-like structures; a process that can take weeks depending on the tissue. For example, chondrocytes require 8 weeks to achieve stable mechanical properties of cartilage [3], while skin can take 12 weeks to reach epidermal homeostasis [4]. Longer time frames in culture enable the cells to secrete their own extracellular matrix and remodel biomaterial scaffolds. However, when cultures are extended for longer periods of time they predictably run into many challenges (Box 2).

Without the proper timeframe the dynamic state of tissues can result in deviations from the goal of the intended studies and targeted outcomes. For instance, if the goal is to test the response or toxicity to a drug then it will be difficult to discern the effect of the drug versus the noise due to the dynamics of the system [44]. However, it should be noted that the dynamic (fluctuating) state of developing tissues *in vitro* over time is different than a dynamic response to an experimental condition, which would be expected in response to drug treatments in the case of pathological studies of a disease. Thus, if these tissues are to be used for drug studies or to create disease models *in vitro*, long culture periods will often be required to enable proper maturation of the tissue.

For clinical applications aimed at restoring tissue function due to organ failure or large tissue defects due to trauma or surgery, long term pre-culturing of tissue constructs may be necessary to generate an adequate mass of differentiated tissue to implant *in vivo*. For instance, to generate fully differentiated cartilaginous tissue in long-term *in vitro* culture, 12 weeks was necessary to maintain phenotype and stable structure when implanting cartilaginous constructs *in vivo* [46]. For bone applications, increasing pre-cultivation time from 24 hours to 14 days before implantation *in vivo* resulted in increased compact connective tissue formation and homogenous host microvessels throughout the scaffolds [47]. Likewise, muscle constructs had significantly increased percentages of vascular volume and myoblast survival over time when pre-cultivation of tissues *in vitro* was extended from 4 to 7 days [48]. For neural applications, pre-culturing neural progenitor cells for a week increased survival of the transplanted cells and resulted in smaller defects compared with no pre-culturing [49]. Based on the range of preconditioning times the required time frame will vary depending on the tissue type linked to the metabolic level of the tissue, along with challenges of integration to native tissues *in vivo* (e.g., re-vascularization, transport limitations).

What element does time play in the development of tissues and their differentiation?

Different *in vitro* tissue platforms offer variable utility for “long term culture” (Box 3). Additionally, the differentiation of cells is affected by the length of culture. Stem cells are an attractive option to form patient-specific tissue engineered constructs and have been successfully incorporated in many long term tissue models (40 days [50], 45 days [45], 50 days [51], 12 weeks [46]). However, stem cells can be unstable in long term culture. For example, chondrogenic [3, 52, 53] and osteogenic advantages (6 weeks, [36]) for primary chondrocytes and osteoblasts, respectively, over mesenchymal stem cells (MSCs), was reported in long term culture. MSCs lagged in chondrogenesis, had decreased viability, generated tissues with lower mechanical properties, and had decreased matrix production compared to the primary chondrocytes, ultimately limiting their utility for in functional

cartilage repair (112 days [3], 70 days [52]). Furthermore, human MSCs had lower differentiation potential in long term culture (6 weeks) than neonatal and adult chondrocytes, with donor age being an additional factor; adult chondrocytes maintained phenotype better in long term culture compared to neonatal cells [53]. The contrast between primary cells and immortalized cell lines is also evident in longer culture periods. For instance, in a 3D model immortalized fibroblasts proliferated excessively and displayed heterogeneous and random increases in thickness of 3D layers, while normal human primary dermal fibroblasts demonstrated consistent DNA levels and maintained consistent thickness for layered structures [54]. Primary cells on the other hand have the the disadvantage of donor-to-donor variability [1] and therefore are not well characterized, making it more difficult to compare outcomes between experiments. Additionally, these cells are often time-consuming to handle, difficult to obtain in large numbers, dedifferentiate in culture, and require highly specific media and supplements. The highly variable nature of tissue platforms and cell types makes non-destructive data point monitoring invaluable in evaluating suitable culture times to establish mature tissues (Box 4).

Concluding remarks and future perspectives

To achieve the goals of tissue engineering a multidisciplinary approach that integrates engineering and biological methodologies is necessary. As more tissue engineering approaches are explored, it will be important to consider the appropriate time frame needed to reach the desired outcomes, which will be specific to each tissue and project goal (e.g., toxicity, drug response, disease formation, nutritional impact, etc.). Long term cultures will not always be practical or the most appropriate choice, therefore, choosing the right tissue platform to address the appropriate questions is essential. Microfluidic systems offer short term options and can address many *in vitro* challenges of interest (Supplementary Tables 1), including applications where portability, small scale approaches are needed and acute responses to toxicants or drugs are sought. However, long term sustained 3D *in vitro* cultures will be critical for tissue engineering of larger tissue constructs for regeneration and complex organ level studies, as well as to assess chronic drug outcomes, disease development and many related topics (Supplementary Tables 2 and 3). With proper timeframes, stable tissues can be formed and studied to match project goals. To achieve long term cultures, more research into combining micro-fabrication techniques with 3D systems will improve the longevity and usefulness of microfluidic systems. In larger scale applications, perfused vasculature will need to be combined with 3D matrices and other bioreactor technologies, including tracking with nondestructive analysis techniques. Co-culturing of multiple cell types will also be needed to generate more relevant tissues. All aspects of these needs will require continued advances to bring these systems to maturity to help focus making this is an area rich in opportunities for innovation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Enhance physiological relevance by using bioreactors, 3D co-cultures, vascularization
- Long term culture may be required to generate stable, physiologically relevant tissues
- Nondestructive data points are essential in long term culture for monitoring the same construct

Box 1 – Recreating tissue vasculature

Vasculature can be achieved by creating interconnected, endothelial lined, channels [55–58] or by allowing cells to re-create their own microvasculature [17, 18, 22, 59]. The crosstalk between endothelial cells and perivascular/stromal cells [22] and bulk organ cells such as osteoblasts [17] or hepatocytes [18], can be used to control the formation and function of blood vessels with intact barrier functions and 3D architectures and biochemical markers similar to *in vivo* vasculature. Moreover, comparing different mural cells, mechanical matrix characteristics, and bioactive components demonstrated that there is a dynamic interplay between these factors and the resulting endothelial network assembled in cultures [59]. While these systems provide increased physiological relevance they are more complex and require optimization of different media components. Additional design concerns also should be considered such as the importance of lymphatic drainage in vascularized systems [60] and vascular stabilizing and destabilizing mechanical stresses [61].

Alternatively, as an artificial substitute to support high-density cell growth, ‘pseudovascularization’ through hollow fiber membrane bioreactors has shown promising long term results [1, 62]. The hollow fibers act as the blood vessels transferring culture medium throughout the tissue engineered constructs while the bulk system can be extracellular matrix or other scaffold materials with the seeded cells. These fibers can also be designed to degrade [63] in order to allow remodeling once the tissue has been implanted. While this method is simpler than re-creating endothelial based vascular structures and supports diffusion of nutrients, it does not recreate the cellular interactions between vascular cells and the bulk organ.

Box 2 – Challenges of extended culture periods

In vitro tissue cultures inevitably encounter challenges when they are extended for longer periods of time. Cells often proliferate excessively and become over confluent, altering signaling patterns from contact inhibition [64]. Extended time in culture can also cause cells to de-differentiate [65, 66]. Viability is another issue, as longer term culture increases the risk of contamination [67]. Additionally, necrotic centers can develop, as cells proliferate on the edge of constructs while the cells in the center have a reduced nutrient supply and can become hypoxic [68]. This is especially apparent with metabolically active cardiac [69], hepatic [5, 70], and pancreatic [71] cells, and larger tissue constructs (> 1 mm depending on the diffusion coefficient of the tissue [72]). Hypoxic environments also effect tissue formation, such as with cartilage, intervertebral disc, and cornea, where matrix production is highly dependent on oxygen tension [73]. Long term cultures also increase the resources needed to maintain cultures. Moreover, long term monitoring of the same tissue construct at multiple time points is essential to decrease heterogeneous results.

Box 3 - Different tissue engineering platforms for long term culture

2D microfluidic platforms have been proposed for cellular scale tissue engineering approaches, including components of tissues where planar surfaces are relevant (i.e., tight junctions, endothelialization, barrier functions), since they have a high surface area to volume ratio, result in low consumption of reagents, have increased sensitivity of measurements, and display high spatio-temporal control [74, 75]. The small size of microfluidic devices makes them well suited for portable applications such as toxicity sensors [76]. Long term 2D tissue engineered systems have been cultured in microfluidic platforms (Supplementary Table 1) including intestine [77], liver [78, 79], skeletal muscle [80, 81], vasculature [82] and neural networks [83]. However, cultures in 2D microfluidic devices are functional for approximately 11 days (an average for the studies in Supplementary Table 1).

Three dimensional tissue engineering approaches are another platform [40–43], and have been explored combining the advantages of microfluidic manufacturing with increased tissue complexity [84–89]. When microfluidic technology was combined with 3D cultures there is an improvement in culture time achieved over 2D microfluidic conditions (an average 19 days for the studies in Supplementary Table 1). For instance, 3D artificial liver modular devices extended hepatocyte culture for 90 days, meeting criteria for FDA toxicity testing [5, 6]. As platforms are developed further, 3D microfluidic devices should become increasingly useful as a bridge between microfluidic technologies and large scale three dimensional tissue cultures.

Finally, the longest duration systems (Figure i) have been demonstrated in larger 3D tissue engineered systems (averaging the static platforms and 3D bioreactor platforms summarized in Supplementary Tables 2 and 3, respectively, indicates approximately 8 weeks for the average culture period). 3D bioreactor systems have increased cell viability, tissue organization, and the distribution of cells throughout 3D matrices at extended time points of cultivation (Supplementary Table 3), including : (direct) perfusion bioreactors (6 weeks, [36]), (indirect) hollow fiber perfusion bioreactors (3 weeks, [1]), perfusion bioreactors with mechanical stimulation (67 days, [90]), rotating wall vessel bioreactors (9 weeks, [91]), spinner flasks (6 months, [7]), orbital shakers (16 weeks, [3]) and cyclic hydrostatic pressure (21 days, [92]).

Box 4 – Non-invasive methods to characterize cells are necessary for long term cultures

Nondestructive data points are essential in long term cultures to monitor the same construct throughout growth to help determine when tissues have reached homeostasis. Non-invasive monitoring of tissues helps to control the number of samples required to track the progress of differentiation and maturation of the tissue. Nondestructive outcomes include tracking secreted factors, morphological change in the cells and matrices, metabolic state and many related outcomes. Choosing which secreted factors are appropriate depends on the tissue or organ system being evaluated and the goals of the study. For instance, to monitor hepatic function in long term culture (90 days) supernatant samples were collected to analyze albumin secretion and urea synthesis [5, 6]. On the other hand, adipose differentiation was analyzed over a 6 month period by secretion of leptin and glycerol [7], while bone differentiation over a 46 day period was assessed by the presence of osteocalcin in the supernatant [8]. Additionally, glucose consumption (4 weeks [93], 10 weeks [94]), and lactate released [94] in the culture medium were non-invasive methods used to track cell metabolism over long culture periods.

Non-invasive imaging techniques can also be utilized to track tissue differentiation. 2D microfluidic systems are relatively easy to monitor, as monolayers of cells are often cultured directly on a glass cover slide. This approach can be reproduced with 3D scaffolds, for instance, by entrapping a porous polymer matrix in polydimethylsiloxane and bonding it to a glass coverslip for visualization with a confocal microscope [95]. This platform could be paired with non-destructive dyes or endogenous markers. Noninvasive magnetic resonance microscopy was used to track changes in collagen structure and mineral content to monitor in a 9 week study [62]. Morphological, biochemical, and tissue organizational parameters can be acquired from endogenous sources of contrasts, such as signals emitted in the two photon spectra. As an example, metabolic activity was tracked via two photon fluorescence imaging linked to adipose tissue engineered systems over a 6 month period to quantify redox ratios [9] and the formation of lipid droplets in a 9 week study of 3D adipose tissue [10]. Additionally, micro-fabrication techniques can enhance non-destructive data point collection. To help with tracking over time, devices can register time-lapse images of the same imaging area [96]. Multiple electrode arrays have also been formed on a chip for studying neural activity over a 4 week period [97].

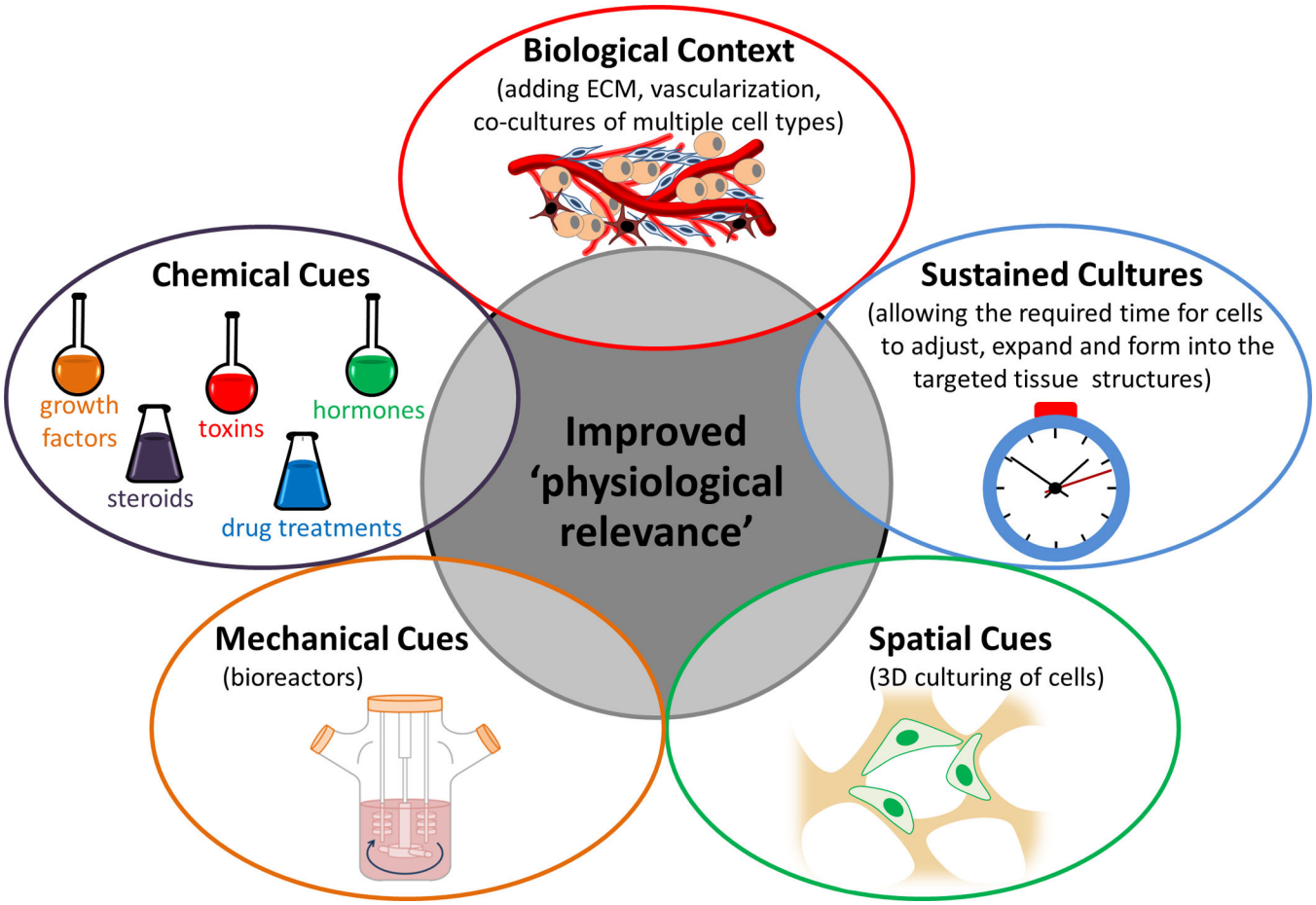

Figure 1.

To improve the 'physiological relevance' of engineered tissues biological context (extracellular matrix, vascularization and cell types), chemical cues, mechanical cues (bioreactors), spatial cues (culturing cells in 3D), and temporal timing of cultures should be considered.

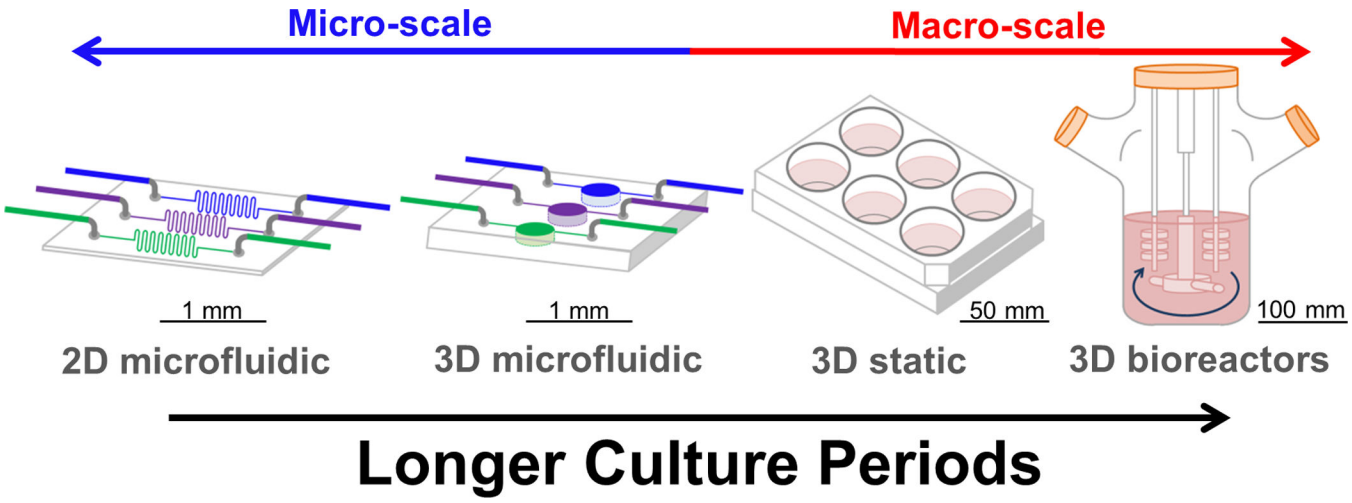

Figure i.

Long term culture in different in vitro platforms. To achieve greater culture duration in vitro; the increased complexity offered by 3D systems over 2D systems, along with larger tissue constructs with enhanced perfusion are required.