

Review

# Digital Twins for Tissue Culture Techniques—Concepts, Expectations, and State of the Art

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**Abstract:** Techniques to provide in vitro tissue culture have undergone significant changes during the last decades, and current applications involve interactions of cells and organoids, three-dimensional cell co-cultures, and organ/body-on-chip tools. Efficient computer-aided and mathematical model-based methods are required for efficient and knowledge-driven characterization, optimization, and routine manufacturing of tissue culture systems. As an alternative to purely experimental-driven research, the usage of comprehensive mathematical models as a virtual in silico representation of the tissue culture, namely a digital twin, can be advantageous. Digital twins include the mechanistic of the biological system in the form of diverse mathematical models, which describe the interaction between tissue culture techniques and cell growth, metabolism, and the quality of the tissue. In this review, current concepts, expectations, and the state of the art of digital twins for tissue culture concepts will be highlighted. In general, DT's can be applied along the full process chain and along the product life cycle. Due to the complexity, the focus of this review will be especially on the design, characterization, and operation of the tissue culture techniques.



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**Keywords:** digital twin; tissue engineering; 3D culture; organ-on-a-chip; mathematical model

## 1. Introduction

Tissue culture refers to a micro-structured cell culture under in vitro conditions that take into account the three-dimensionality (3D) and the physiology of the tissue. Areas of application cover basic research (e.g., stem cell research, tumor models), pharmacology and toxicity tests, regenerative medicine (tissue engineering for implantation), as well as in vitro meat, among others. Techniques for tissue cultivation have undergone significant changes during the last decades, and 3D cell culture systems, e.g., for interacting cells and organoids, 3D cell co-cultures, organ/body-on-chip, have become available [1].

As tissue constructs are nowadays applied routinely, e.g., for pharmacological and toxicity tests or for the generation of implantable tissue, the requirements regarding quantity and quality of the generated constructs have significantly increased [2], driven further by quality standards of regulatory bodies such as FDA or EMA [3]. As most protocols and procedures have originally been established in research labs often based on trial and error without defined quality criteria, the transfer to routine, standardized applications is troublesome. Hence, in silico methods such as Digital Twins (DT) have been suggested for a more profound development, characterization, and routine manufacturing of 3D tissue culture systems, comprised under the novel paradigm of “developmental engineering” [4–6]. Furthermore, in silico methods are intensively investigated to replace animal testing, as they are in compliance with the 3R principles (i.e., reduction, refinement, and replacement). [7–10].

According to El Saddik [11], DTs are referred to as “digital replications of living as well as non-living entities that enable data to be seamlessly transmitted between the physical and virtual worlds”. Prominent examples of successful DTs can be found in the Apollo missions or in aircraft construction. In the Industry 4.0 era, DTs are routinely assigned as

the heart of the digital factory and as core concepts of digital transformation strategies. In medicine, under the catchphrase “The Virtual Physiological Human (VPH)”, also referred to as “*in silico* medicine”, the use of individualized, physiology-based computer simulations is targeted in all aspects of prevention, diagnosis, prognostic evaluation, treatment of diseases, and development of biomedical products. While DT-based concepts for production of biopharmaceuticals are already being used in industrial practice or tested by pharmaceutical companies for the development, optimization, and operation of production systems for biopharmaceuticals (recently reviewed in [12,13]), there are so far only a few DT-approaches, mostly for the generation of implantable tissues as Advanced Therapy Medical Products (ATMPs) or tissue-specific test systems for new pharmaceuticals or toxicity tests [5,6,14–19]. This is probably also due to insufficiently precisely formulated expectations and knowledge with regard to the possibilities, but also the limits of model-based DT concepts in a community that works mainly on a data basis.

In the beginning of this review, an overview of tissue culture systems, followed by an introduction to the model-based DT-concepts with a special focus on tissue culture, mainly concerning Tissue Engineering as ATMP and test systems will be presented. Subsequently, the types of mathematical models applied for DT's and various examples for model-based/model-assisted design and operation will be discussed. Finally, conclusions will be drawn concerning the state of the art and future expectation for digital twins for tissue culture concepts.

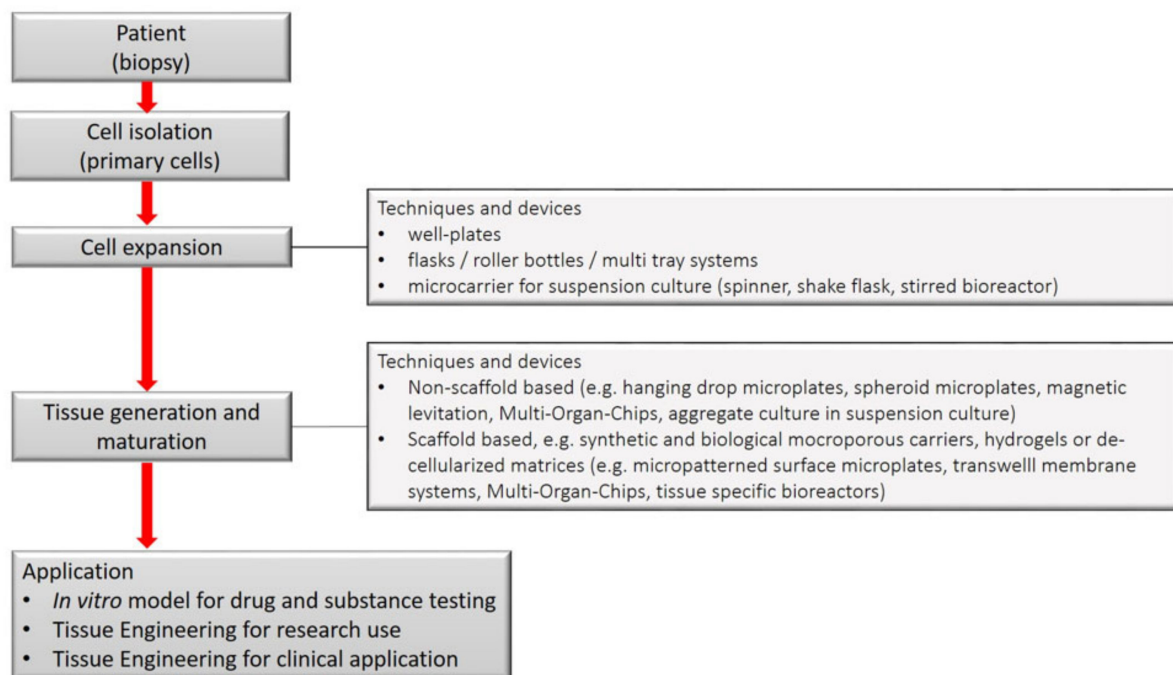
## 2. Overview Tissue Culture Systems

In the following chapter, tissue culture systems are introduced focusing on general aspects first followed by culture systems for cell expansion. Then, different culture systems for tissue generation and maturation are discussed and considerations for their design and operation are reviewed.

### 2.1. General Aspects

Tissue culture can be described as the culture of living cells within micro-assembled devices and supports that create a growth environment mimicking the native tissue as closely as possible [20–22]. Unlike 2D monolayer cell culture, 3D tissue culture is regarded as much more suitable to mimic *in vivo* cells behaviors and organization (morphology and physiology) [23]. The ultimate goal is to create a vital tissue with organ specific structure and functionality considering the complexity of the cellular microenvironment *in vivo* [24–26]. Among others, the following aspects were identified to be of major relevance for tissue culture: Extracellular matrix (ECM) components and stiffness regulating cell differentiation, proliferation, and metabolic cellular functions [2,24–27] and complex cell–cell and cell–matrix interactions (e.g., heterogeneous cell populations including stromal cells, varying cell proliferation zones (quiescent vs. replicating)) [2,28–30]. A heterogeneous cellular microenvironment could be important to reflect *in vivo* conditions with gradients of biomolecular concentrations, e.g., areas of hypoxia, pH gradients, soluble signal gradients, and differential nutrient and metabolic waste transport [2,31,32]. Additionally, flow-induced dynamic effects and mechanical stimuli [33–35] and interactions between multiple organs [36] need to be considered. The required degree of complexity is determined by the specific demands.

The workflow for creating 3D tissue cultures can be divided as shown in Figure 1. In the following, the techniques and devices applied for cell expansion and tissue generation and maturation are briefly discussed, focusing on the required engineering knowledge. Tasks that can be addressed by a mathematical description are discussed in Section 4.



**Figure 1.** Overview of cell culture techniques and devices used along the workflow of tissue generation for different applications.

Devices used for cell expansion and generation of tissue constructs shall be categorized at this point because they are used to some extent for different purposes. First of all, a distinction can be made between static and dynamic cultivation systems. In static systems, the fluid and/or the cellular tissue construct are not moved. The success of static culture is often limited by mass transfer (e.g., supply of oxygen or nutrients or growth factors, removal of waste products), as this relies on a diffusion mechanism only [37]. Furthermore, they are usually not equipped with *on-line* sensors for monitoring of e.g., temperature, pH, or dissolved oxygen.

These drawbacks can be overcome by dynamic cultivation concepts, where cells/tissue constructs are maintained under flow conditions and/or mechanical stimulation. Dynamic culture systems are usually grouped under the term “bioreactor”, a device in which biological or biochemical processes develop under a dynamic, closely monitored, and tightly controlled environment [38–40]. Bioreactors on different scales are applied for cell expansion or generation of 3D tissue constructs.

## 2.2. Culture Systems for Cell Expansion

Cell proliferation is the first step in creating a tissue culture. On a smaller scale, static culture dishes (e.g., well plates, T-flasks, stacked plate systems, mostly single-use) are mostly used for cell expansion [41]. Since these only allow the cell number to be increased by a factor of about 10, multiple subcultures are required, which is considered to be one of the main causes of cell dedifferentiation. As most of these culture systems are operated in an incubator to ensure adequate conditions with respect to temperature, pH, and oxygen supply, it is challenging to monitor and control these parameters. Even if it is well known that oxygen concentration within the body varies for different tissues (e.g., well vascularized tissues with higher oxygen concentration, cartilage tissue with lower oxygen concentration), these effects are hardly considered during practical application. A comprehensive mathematical characterization of the systems would help to detect conditions that are not optimal. [42] Similar to oxygen, cells are exposed to mostly constant nutrient concentrations *in vivo*. However, as medium in culture flasks are usually exchanged batch-wise, cells experience a seesaw behavior of nutrient concentrations causing a detrimental environment to the cells.

As plates and flasks allow for a limited expansion only, scalable bioreactor systems based on matrices to provide cell attachment have been established. These cover hollow fiber bioreactors, bioreactors for macrocarriers (e.g., fixed bed) [43], meander type bioreactors [44], and, in particular, microcarriers (reviewed by [45]) for use in stirred tank reactors, [45] either re-usable or single use. [43,44,46–48] A further alternative is carrier-free cultivation in 3D aggregates or spheroids, or encapsulation, e.g., in alginate or in hydrogels. These techniques are often used to induce a lineage specific differentiation of stem cells. With respect to the expansion of cells, e.g., MSCs, there is conflicting evidence in the literature with regard to the usefulness of these techniques (reviewed by [41]), even if this technique has been successfully adopted for expansion of human pluripotent stem cells [47].

There is strong evidence that the growth and differentiation of tissue cells, especially stem cells, are affected by several types of mechanical forces including stretch, strain, compression, and shear stress, especially in the mostly turbulent environment within a stirred bioreactor. This aspect is discussed more in detail in Section 4.2.

### 2.3. Culture Systems for Tissue Generation and Maturation

#### 2.3.1. Non-Scaffold-Based Organoid Culture

Scaffold-free organoid culture methods rely on the self-aggregation of cells in specialized culture plates, when a surface is not available for cell attachment and cells tend to form cellular spheroids. [1,21,48]. Techniques used to generate spheroids comprise hanging drop microplates, low adhesion plates with ultra-low attachment coating that promotes spheroid formation, micropatterned plates that allow for microfluidic cell culture, and magnetic cell levitation where cells are preloaded with magnetic nanoparticles that are kept floating within an externally applied magnetic field. [2,29] Due to the scaffold-free nature, spheroids can be readily imaged by microscopic techniques, e.g., light, fluorescence, and confocal microscopy. Furthermore, most of the applied techniques are well suited for high-throughput screening [2,21,49].

Spheroids, and in particular multicellular spheroids, recapitulate tissue specific cell-cell contacts or cell-matrix interactions. Therefore, they can mimic gradients with respect to oxygen, nutrients, waste products, or pH as well as diffusion of regulatory molecules. Therefore, they have been extensively used for e.g., modelling solid tumor growth and metastasis studies as well as in therapeutic studies [48,50–52].

#### 2.3.2. Scaffold-Based 3D Cell Culture

Scaffold-based culture technologies provide a physical support mimicking key properties of the extracellular matrix for cells to attach, aggregate migrate, and fill the interstices within the scaffold to form 3D, organotypic cultures. Scaffolds range from simple mechanical structures to ECM-like matrices. In the best case, the scaffold provides an organotypic environment [2,38,53,54]. Depending on the specific application, physical factors such as porosity, stiffness, and stability in culture as well as biological properties such as cell compatibility or adherence have to be considered [2]. Furthermore, growth factors, hormones, or other biologically active molecules can be encapsulated to enhance cell proliferation or to promote a specific cell phenotype [2]. Scaffold-based culture is mainly intended for use in more sophisticated culture devices such as organ-chips or specific bioreactor systems (see below).

Scaffolds can be of biological origin or synthetic. If intended for transplantation, they should be biocompatible and biodegradable [2]. Three individual groups of biomaterials, inorganic/ceramic materials (mainly used for bone regeneration), synthetic polymers and natural polymers as well as composites are therefore used in the fabrication of scaffolds for tissue engineering (for review see [38,54,55]).

A special variant of scaffolds are hydrogels, networks formed from dilute polymer chains with given structure and properties, obtained either by intermolecular or by interfibrillar crosslinks. Hydrogels may come from natural sources (e.g., collagen, fibrin or

Matrigel) or can be synthetic, with the possibility of mixing different hydrogel materials to obtain hybrid hydrogels possessing new physical and biological properties. Hydrogels can be functionalized with defined adhesive moieties, include proteolytic sites, and encapsulate growth factors [2,54,56].

### 2.3.3. Microfluidic 3D Cell Culture

Microfluidic 3D cell culture techniques (“organ-on-a-chip” (OC) or “multi-organ-on-a-chip” (MOC)) intend to contribute an additional level of complexity by introducing a controlled perfusive flow aspect to the cellular environment, allowing for continuous nutrition and oxygen introduction as well as waste removal through culture medium [20,57,58]. As microfluidic techniques provide structures in the micrometer range, the typical length scale of *in vivo* microenvironment, it is an ideal tool for recreating the cellular microenvironment. Furthermore, on-line sensors (e.g., temperature, pH, dissolved oxygen, impedance, and fluorescence) can be integrated for monitoring the physiological state of the culture. These techniques allow for the mechanistic investigation of the dynamics of multi-organ interactions, various disease models, or drug activities. [59] Successfully established OC and MOC systems comprise skin, blood vessel, liver, gastrointestinal tract, blood–brain-barrier, lung, kidney, and heart equivalents, among others, as well as combinations thereof ([60–68], reviewed by [36]). Due to the higher complexity, OC- and MOC-techniques allow for high-content but medium-throughput-investigations.

### 2.3.4. Tissue-Specific Bioreactors

Whereas microfluidic techniques are a powerful tool for drug and substance testing, they can hardly be applied for the generation of tissue-intended implants and organ-support. Here the complex task is to recreate the required *in vivo* microenvironment on a scale appropriate for implantation by providing optimal conditions for co-culture of cells of different phenotypes, cell–cell interactions, vascularization, shear and mechano-stimulation, etc. [37,69–75]. Furthermore, any scaffold used for cell seeding has to be biodegradable with respect to later implantation.

These bioreactors used in this respect should enable the control of environmental conditions such as oxygen tension, pH, temperature, and shear stress, as well as allowing aseptic operation (e.g., feeding and sampling). Furthermore, a bioreactor system should allow for automated processing steps, especially during the routine manufacture of tissues for clinical application. Furthermore, specific key criteria for tissue constructs based on cells and scaffolds must also be met, including the proliferation of cells, the seeding of cells onto macroporous scaffolds, nutrient (particularly oxygen) supply within the resulting tissue, and mechanical stimulation of the developing tissues. Various studies have shown that mechanical stimulation (e.g., mechanical compression, hydrodynamic pressure, and fluid flow, which are important modulators of cell physiology) can have a positive impact on tissue formation, particularly in the context of musculoskeletal tissue engineering, cartilage formation, and cardiovascular tissues [76].

Historically, most of these techniques were introduced before the invention of microfluidic techniques. First developments focused on culture systems developed mainly for cultivation of mammalian cells for production of biopharmaceuticals, which were adapted for cultivation of tissue cells in three-dimensional structures (e.g., spinner and shake flasks, membrane-based systems such as hollow-fiber reactors, or fluidized- and fixed-bed reactors). Because these systems are capable of supporting culture of large 3D cell agglomerates, but hardly mimic the *in vivo* microenvironment, numerous culture systems are designed especially for tissue engineering mimicking the special demands of a three-dimensional tissue (including mechano-stimulation) have been developed. Unfortunately, the majority of these have been custom-made, with only very few having been commercialized [69,76].

A great step toward tissue-like structures and vascularization was the invention of decellularization techniques, a process to isolate the extracellular matrix (ECM) of a tissue

from its inhabiting cells, leaving an ECM scaffold of the original tissue, which can be used in artificial organ and tissue regeneration (for review see [69]).

#### 2.4. Considerations for Design and Operation

For appropriate design and operation of 3D tissue culture, not only is a profound biological understanding of the tissue/organ of interest required, but also an extensive „engineering“ knowledge comprising design of special culture systems (bioreactors) (mass transfer, shear effects, etc.), design of special materials for cell immobilization (biocompatible, biodegradable), mass transfer problems (oxygen and nutrient supply, removal of toxic metabolites), as well as control and cultivation strategies (including Operator Training Systems).

Even if it is common sense that effects of such engineering parameters on biological cultures are worth to be thoroughly investigated, so far, they have been addressed in only a few studies. The combined consideration of biological and engineering effects especially can be improved. A solution is provided by emerging mathematical modelling and computational tools, e.g., for analyzing oxygen, carbon dioxide, and nutrient and metabolism waste material transports. Discovering the optimal engineering parameters is crucial not only to reduce the cost and time of experiments, but also to enhance efficacy and functionality of the tissue construct. [22,77]

### 3. Digital Twins: Concept and Advantages for Tissue Cultures

Historically, a DT was defined as a computational entity of a machine (e.g., airplane, spacecraft, car manufacturing) used as an engineering handle to deal with the increased complexity of these machines (reviewed in [78,79]). Universally applied definitions are still scarce and not sufficient, since DTs are differently defined in industry sectors. Barricelli et al. (2019) investigated the evolvement of the DT concept in literature and defined them rather broad:

*“DTs can be defined as (physical and/or virtual) machines or computer-based models that are simulating, emulating, mirroring, or “twinning” the life of a physical entity, which may be an object, a process, a human, or a human-related feature.” [78].*

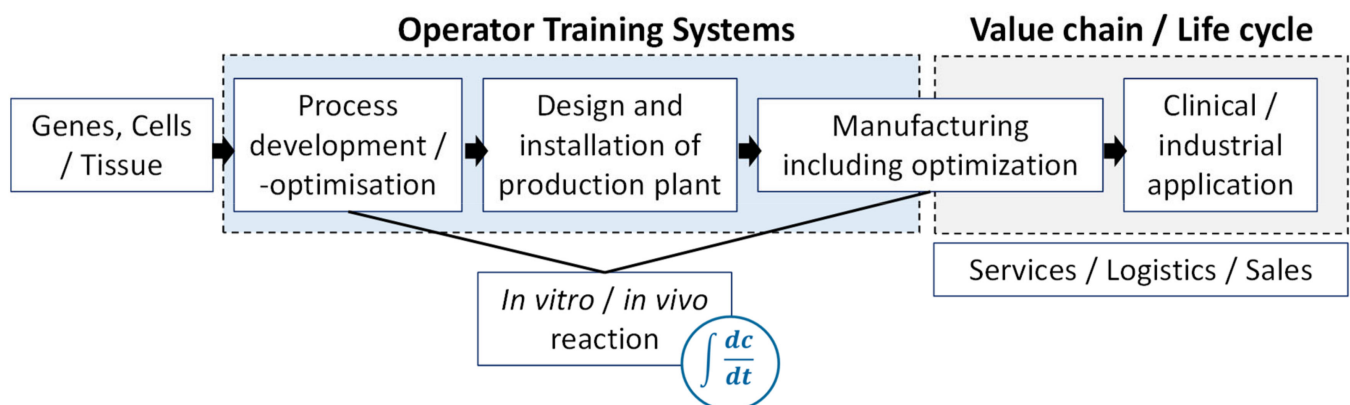
Most of the recent efforts were made in the field of mechanical engineering and aircraft design, since the object of investigation is well known and based on physical laws (e.g., material specifications, mechanical properties). Moreover, modern design processes of machines and manufacturing sides are made using computer-aided design software, which can be the basis for a DT in the respective field. A major overcome obstacle to implement DTs was the continuous increase in computational power, enabling Big Data methods, such as Artificial Intelligence (AI) or data mining techniques.

In the past years, the DT concept is more and more discussed in medical-related fields, such as the design and optimization of manufacturing processes for biopharmaceuticals, the development of personalized medicine, and for the characterization, design, and optimizing of 3D tissue culture systems [15,80–83]. Cell culture is more suitable for high-throughput screening than using laboratory animals, even if the interactions between the organs in an organism are not considered. There are indications that organ-like behavior of the cell culture leads to more meaningful results when the mode of action of a new drug is to be tested and conclusions about its pharmacokinetics and pharmacodynamics have to be drawn [84,85]. Additionally, there are various 3D organ models for the respective entry point and place of action of the substance, for example for the skin, liver, pancreas, or the lungs [86]. Meanwhile, it is common sense that proper development, utilization, and data interpretation in the above-mentioned applications requires *in silico* mathematical modelling to address the tremendous biological and technical complexity. A transient from a technology-driven science-focused field toward a patient-driven, manufacturing-focused one can be seen, driven by innovations at the interface between biology and technology, including robust biological building blocks, precise biomanufacturing technologies, advanced analytical tools, and *in silico* models [15].

The main challenge in tissue culture is the translation of biological knowledge on complex cell and tissue behavior into a predictive and robust engineering process including proper analytical tools and predictive control methods. This involves the quantification and optimizing of tissue engineering production processes and the investigation of the influence of the cultivation environment/environmental conditions *in vitro* on the quality of the cultivated tissue, especially an increasing understanding of the pathophysiology, design treatment strategies, and an appropriate quality control. [87] In the author's opinion, a DT-based framework is seen as a knowledge-based process development and engineering strategy, for which the main advantages are:

1. A comprehensive summary of knowledge of the investigated experimental system into a computational system, with the linkage of physical laws and metabolic understanding to design and evaluate culture systems, also at different scales. This increases the level of knowledge about the process steadily through reiteration processes.
2. Increasing understanding of the process and its influence on cell growth, phenotyping, epigenetic criteria, prognostic markers, etc., in many process steps.
3. Usage of next-generation computational tools to improve the understanding of the cultivation concepts, e.g., optimizers, learning algorithms.
4. Decrease of the development cost for experimental design to define fast and efficient cell expansion, resulting in accelerated time to clinic.
5. Support of regulatory documentation within serial/routine manufacturing to treat individual patients with customized medications.
6. Evolving of the DT during the lifecycle of the medical product, including the prediction of defects and capturing platform knowledge and transferability along the product life cycle with donor-specific manufacturing processes.
7. Evaluation, screening, and virtual testing of new configurations/settings prior to experiments.

It should be noticed that the benefits of the application of DTs heavily rely on their intended use and that the above-described points are only valid for the design, characterization, and operation of the culture system and the respective cultivation process. As can be seen in Figure 2, potential areas of the application of DTs can be found over the whole lifecycle of tissue engineered products.



**Figure 2.** Potential areas for Digital Twins (DTs) in tissue culture.

Starting with the genes, cells, or the tissue, the expansion process is developed and optimized, and the manufacturing plant is designed, qualified, and installed accordingly. Then, the process in the respective scale needs to be optimized and scale-up needs to be validated [88,89]. The DTs act as an engineering tool for process design and optimization and to design special culture systems considering e.g., mass transfer and shear effects. Furthermore, tailored materials for cell immobilization considering mass transfer problems could be focused on (e.g., biodegradability, oxygen supply). To address such tasks, the DT

should be able to describe all or parts of the mechanical integrity including the microstructure and the biostability and biochemical activity of the cultured tissue. Furthermore, different cellular properties, like cell differentiation, proliferation, metabolism, and cell and surface interactions might be considered by the DT.

Within these steps, an operator training system (OTS) including a DT and capturing the main in vivo and in vitro reactions is beneficial (see list of advantages above) [90,91]. OTSs offer the possibility to train future reactor operators and bioprocess engineers in a very practical way without carrying out the real process. Furthermore, they offer enormous potential for the development of control strategies. After manufacturing of the tissue culture, a DT can assist in the value chain to support the delivery and sales up to the clinical or industrial application [12,13].

#### 4. Mathematical Models as Core of a DT

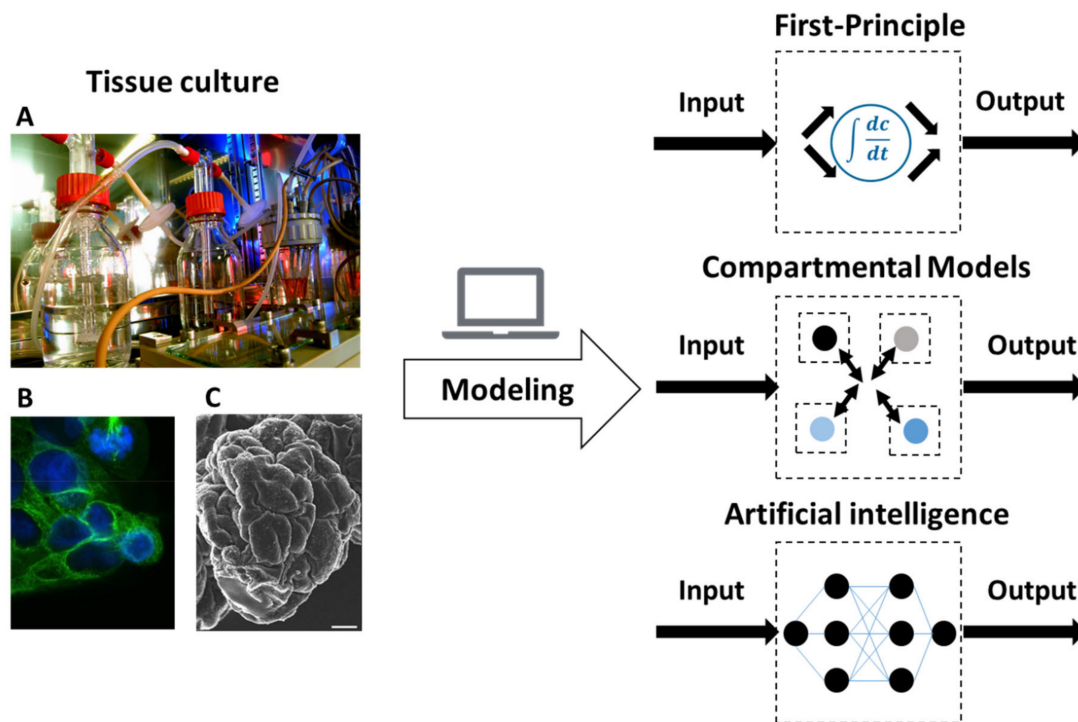
The general difference of DTs is the structure of the underlying algorithms and mathematical models (i.e., modelling paradigm), which are specific to their intended use [92]. No off-the-shelf software tool is so far commercially available since the complexity of the used mathematical model rely on the different number of measures, the available data, and the computational power. Exemplarily, the investigation of fluid flows in 3D cultivation systems by computational fluid dynamics (CFD) requires different models than the automated and computer-guided screening of drugs including big data analytics [93,94]. The type and the degree of complexity of the core mathematical model(s) are key for the function and usability of a DT. In this way, the model should describe the real phenomena as accurately as needed with a clear focus on simplicity and adaptability. The model evolves over time, e.g., starting with a simple structure and well-known effects and adapting stepwise to newly available data and new observations [83,95,96]. In the beginning, the parameters considered should have biological significance and should be determinable by standard experiments to avoid falls conclusions and compromise between the accuracy of the model and the required experimental effort for the determination of the parameters needs to be agreed on for each application. Table 1 summarizes references for specific tasks addressed by mathematical modelling. Specific examples of tissue culture systems for drug/substance testing are discussed in the next sections.

**Table 1.** References for specific tasks addressed by mathematical modelling.

Tasks Addressed by Mathematical Modelling	References
General Reviews	[97–116]
Cell growth, morphology, heterogeneity, dynamics	[117–128]
Cell kinetics	[129–132]
Systems biology	[133,134]
Cell handling, seeding, culture conditions	[135–138]
Penetration, permeation, diffusion	[139–159]
Scaffolds-fluid flow, shear, mass transfer	[112,160–171]
Bioreactor-fluid flow, shear, mass transfer	[45,99,172–185]
Mechanical stimulation	[186–193]
Image-based modelling	[194–198]
Tissue specific effects	[199–206]
Pharmacology	[207–210]
Handling of models	[96,211]
Model-assisted experimental design	[88,92,212–215]



In the following, different model classes are introduced and their potential application in DTs is highlighted in a general manner. In the author's opinion, the most prominent model classes are First-Principles or mechanistic models, Compartmental Models, and Artificial Intelligence, as schematically shown in Figure 3.



**Figure 3.** Overview about established model classes as part of a DT for tissue culture systems. **A:** Flow chamber bioreactor **B:** Labelled L929 cells **C:** Porcine Chondrocytes on Cytodex 3 (Pictures kindly provided by Mr. Roman Jupitz (A), Dr.-Ing. Grit Blume (B), Mrs. Christiane Schaffer, all Hamburg University of Technology).

#### 4.1. First-Principle or Mechanistic Models

These models are based on fundamental physical/chemical laws and a sound understanding of the investigated system [96,216]. Therefore, they have mostly based on well-known material or energy balances and derived from this first assumption/proposition, i.e., first principles. These balances can then be mathematically transferred and adapted to the modeled system (e.g., mass input into a system equals mass in the system plus mass out) and different other material characteristics can be added. The main advantage of these models is the extrapolation capacity. Since the underlying relationships are well-known from scratch, future states can be predicted beforehand and model parameters are meaningful. Besides physical laws, chemical and biochemical reactions belong to this model class [212].

In the field of medical engineering, first principle-derived models are used to describe the biological functions of tissue and single cells. Hence, the complexity of biological systems is very large with thousands of reactions involved, which are not understood in every detail, and first principle/mechanistic models can then be used to describe known growth characteristics (e.g., cell growth based on consumed glucose). Most published models consider the “bio”-phase using heuristically derived linear/non-linear mathematical expressions, which are distinguished according to the considered complexity of the model (engineering approach, reviewed in [92]). Among other classes, the distinction in segregated and non-segregated models gaining more importance in the last years. In non-segregated models, the biological system is considered equally with the same physiological, morphological, and genetic identity [92,96]. Novel efforts were made to explain segregated biological classes describing individual cellular behavior, such as different cell cycle phases,

their interaction, and impact on cell growth and cell culture quality in systems biology (i.e., population-balanced modeling) [128,217].

#### 4.2. Compartmental Models

In Compartment Models, the investigated system is separated into individual parts, i.e., compartments, with defined sub-models, their shape, and their interactions [218]. The most prominent example is Computational Fluid Dynamic (CFD) simulations predicting fluid flow behavior in 3D shapes and constructions. In the beginning, the geometry of the object is defined with a computer system and the fluid volume is split into discrete cells/elements, known as mesh. Different forms and shapes of the mesh elements (e.g., cubes) can be used and meshing is not limited to just one form so that also combinations can be used.

Next, the physical model is defined. Mostly, the core of CFD is the Navier–Stokes fluid momentum balance describing the fluid motion, enthalpy, heat radiation, and energy conversion (please see [219] for more information). Then, the boundary and initial conditions need to be defined. Exemplarily, the fluid movement at the wall and temperature changes are mostly neglected, which strongly reduces the balance equations. Next, the simulations are initiated, and the data are examined and post-processed afterward. It should be noticed that meshing and definition of boundary conditions heavily involve heuristics and expert knowledge, and mistakes may lead to questionable outcomes [220]. Using CFD, the following phenomena could be investigated and used to design and understand 3D cell expansion processes:

1. Heterogeneous distribution of components, such as growth factors and oxygen.
2. Gradients in the applied system, e.g., pH gradients.
3. Whole system hydrodynamic characteristics, such as average fluid flow.
4. Local-distributed hydrodynamic characteristics, e.g., shear stress.
5. Stationary and time-resolved fluid flow characteristics.

The disadvantages are the high computational power required to solve complex geometries and the strong dependency of the quality of prediction on the mesh and boundary conditions. CFD is especially important for tissue engineering constructs, in which a set of complex phenomena such as hydrodynamics, nutrient transfer, cell growth, and matrix deposition have to be taken into account. Parameters such as fluid velocity, oxygen tension, and stress, which are difficult to determine experimentally, can be derived from CFD simulations and related to first-principle kinetics (e.g., segregated metabolic responses) [219].

#### 4.3. Artificial Intelligence

Artificial Intelligence is broadly seen as theoretical and practical computer systems, which perform with human-like intelligent behavior without human intervention [221]. Mostly, two branches are distinguished: Virtual AI and physical AI. In virtual AI, mathematical algorithms are used for data analysis and computational learning, known as Machine Learning. Common classes of Machine learning are supervised, unsupervised, and reinforcement learning [222]. Supervised learning uses previous examples and a pre-defined output value/function to train the computer system. Model parameter estimation based on experimental data is a common example, where the input is the experimental data, and the algorithms predict the model parameters to fit the data. As output, the goodness of regression is calculated (i.e., reward, judgment, output, error function) and sent back to the algorithm for training [79]. Unsupervised Machine Learning targets the identification of hidden structures/clusters in data without a reward function commonly used in cluster analysis and pattern recognition applications. Exemplarily, Lopez et al. applied unsupervised machine learning to cluster patients based on genetic properties and clinical outcomes to classify and identify high-risk patients [93]. In Reinforcement Machine learning, the algorithms learn based on a cumulative reward function, which can include positive and/or negative feedback, like training tricks to an animal. The agent (i.e.,

a self-guiding computer program) is allowed to perform its own actions to maximize the reward and is organizing itself to do so. It can be used to discover adaptive personalized therapies and to control robots in robotic-assisted surgery [223–226].

#### 4.4. Hybrid Approaches

In hybrid modelling, different model sub-classes (e.g., mentioned above) are summarized into one combined model. Mostly, the structured understanding obtained in first-principle or mechanistic models are the backbone of a hybrid model describing the main investigated phenomena [227]. Effects, which cannot be expressed by the mechanistic equations or which are not understood so far, are then incorporated by data-driven machine learning tools or artificial intelligence [228]. Therefore, the model can be used to describe complex systems in real time applications (e.g., model-based online control of cell expansion) or as an intermediate step in obtaining a systematic understanding of the modelled system.

### 5. Efforts toward “Digital Twin” Concepts for Tissue Culture

As summarized in Table 1, numerous studies have been performed using mathematical models for tissue models, especially CFD simulations. It would be beyond the scope of this review to discuss all this work in detail. The intention of the following section is to show that the complexity of the transition from modelling to DT is fluent and includes an increasing complexity. Therefore, examples were selected, which show on the one hand the potential, and on the other hand, the still-existing limitations of model assisted/DT-concepts for 3D tissue culture.

#### 5.1. Expansion of Stem and Immune Cells

A decisive step for the therapeutic use of stem and immune cells is the increase in the number of cells. In some cases, expansion factors in the range of 500–1000 are required here without affecting the differentiation behavior of the cells [229]. As already mentioned in Section 2.2, a widely applied method for the expansion of these mostly adherent cells is cultivation on microcarriers in suspension reactors, e.g., stirred reactors. However, pilot and industrial-scale reactors (e.g., starting from 2 L up to a few m<sup>3</sup>) are characterized by very heterogeneous flow conditions and concentration environmental gradients, such as inhomogeneous distributions of dissolved oxygen or pH [230–233]. There is strong evidence that the growth and differentiation of tissue cells, especially stem cells, are affected by several types of mechanical forces including stretch, strain, compression, and shear stress [41]. Stem cells react to flow effects, mainly caused by shear stress, not only through changes in growth behavior up to cell death, but also, among other, by a shear induced differentiation. Flow-induced shear stress can be both stimulating and harmful to the cells. A large number of studies have dealt with the question of how a targeted “lineage”-specific differentiation of stem cells can be induced [234–237]. For the expansion of stem cells, an undesired “pre-differentiation” during cultivation is to be regarded as disadvantageous. This requires the least possible variation of the lineage-specific differentiation, while the genetic and epigenetic stability of the cells must be kept under control [238]. To ensure this, the micro-environment that affects the cells must be characterized in a bioreactor and kept as similar as possible during the “up-scaling” of the process [239,240].

In the complex, mostly turbulent environment e.g., in a stirred bioreactor, the local shear rate varies within the vessel and it therefore is very difficult to associate cellular effects (cell damage, differentiation, etc.) with the magnitude of the prevailing shear rate or the associated shear stress.

Flow-induced shear stress can be detrimental or stimulating to the behavior of tissue cells. Cell damage in microcarrier cultures was studied very intensively for permanent or established cell lines in the 1980s and 1990s. Especially for stem cells, it was shown that these cells are affected by shear stress below damaging levels. With respect to cell expansion, preferably without significant unwanted differentiation, a narrow band of

shear stress levels is essential. Turbulent flow in stirred bioreactors, however, represents a scenario that is not characterized by a narrow band of shear stress levels. The effect of broadband stresses associated with the turbulence in stirred bioreactors on preservation of the physiological state of stem cells is still poorly understood. Although these can be easily described using modern CFD methods, a reliable link between flow-related data and cellular effects is still missing, especially a detailed examination of the micro-environment affecting the cells (flow, oxygen concentration, etc.) (reviewed by [41]).

A further severe problem with the expansion of stem cells on microcarriers can be seen in the formation of aggregates that can be several millimeters in size (Note: Diameter of the microcarrier is approximately 200  $\mu\text{m}$ ) [45]. It is obvious that cells in these aggregates are only very poorly supplied mainly with oxygen, so that, in extreme cases, necrotic zones develop with massive cell death. In addition, the influence of large oxygen gradients on the physiological state of the cells has to be considered [241]. Oxygen limitation in stem cell aggregates in stirred suspension cultures is described by Wu et al. [242] using a time-dependent reaction-diffusion model that was linked to a “population balance” model. Again, a link to cellular effects is still missing.

### 5.2. Bioreactors for Liver Tissue Engineering

Using the example of bioreactors intended as bioartificial liver to develop enhanced bioartificial liver support systems to study liver cell metabolism or as an alternative to animal tests for drug screening or toxicity assessment in vitro [243–245], it can be shown how mathematical modelling can help to improve understanding, design, and operation of the system. The ideal biological liver substitute should perform most or all of the liver-specific detoxification, synthetic, and biotransformation functions, many of which are hardly or not at all known. The design of liver-bioreactors poses special technical challenges with respect to liver cell physical-chemical requirements and the scale, as the liver is a highly structured organ with many distinct cell sub-populations and a sophisticated network of blood vessels. Specific phenomena such as “liver zonation”, apparently regulated by gradients of oxygen, hormones, and ECM composition, and the structure function relationship, among others.

Typically, liver constructs are engineered in vitro by culturing liver (or liver-like) cells in/on synthetic scaffolds, which provide the template for cell adhesion, re-arrangement, proliferation, and development. To meet the specifications discussed above, it has been suggested to pattern the biological substitutes after the liver micro-architecture. This comprehensive approach has been realized for organs-on-a-chip’s (see chapter 2.3) on a small scale. However, as for extracorporeal liver support systems, at least 500g cells are supposed to be required, and most culture techniques used in this respect focus just on maintaining the hepatic functions relevant to stop the progression of acute liver failure [42,246]. Most bioreactors used as a bioartificial liver support system are membrane bioreactors (for review compare [55,245–247]), mainly to prevent a cellular immune response. These mostly polymeric permselective membranes act as selective barriers that compartmentalize the cells in an immuno-privileged compartment (i.e., the cell compartment) by preventing immune-competent cells and proteins from crossing their wall, yet they are freely permeable to the nutrients and metabolites necessary for cell survival and metabolism. Engineering challenges addressed by mathematical modelling comprise mainly nutrient and oxygen supply to cells, and the control of biochemical signals gradients and concentrations. Special attention is made to the transport of oxygen, often limiting cell functions and tissue growth in vitro. Just by using mass transfer equations for the mostly diffusive external, transmembrane, and internal transport, linked to cell metabolism, optimal conditions with respect to the composition of gas phase, membrane properties (especially membrane thickness), flow velocities, an internal distance of membranes, etc., it is possible to provide an optimal design of the complex bioreactor system, as shown by the groups of Gerlach and Catapano [246].

### 5.3. Permeation and Diffusion in Cultivated 3D Skin Models

Characterization of native skin or cultured 3D skin models with respect to permeability plays an important role in the development and testing of pharmaceuticals and cosmetics. Extensive efforts have been dedicated to determining the key parameters describing permeability and diffusion. Whereas respective methods are well established for native skin biopsies [248], only few are available for 3D skin models. The need to evaluate skin permeation, test cosmetic products, and screen topically applied compounds is evident. Since animal experiments are under massive debate, ethical and regulatory issues, but also severe differences between animal and human data, pushed the development and commercialization of diverse *in vitro* skin models [249]. Human skin equivalents (HSEs) can be categorized into two main groups: The epidermis-only and full-thickness models. Even if huge progress has been made over the years and a number of skin models are commercially available [250], the barrier function between artificial and human skin can especially differ, so permeation and diffusion investigation in this area are indispensable.

A number of methods for the determination of diffusion and permeability parameters have been developed for large biopsies (e.g., the Franz diffusion cell, Fluorescence recovery after photobleaching (FRAP), Fourier-transform-infrared (FTIR) spectroscopy, tape stripping [251]). These together provide a detailed characterization of diffusion and permeation effects within the skin. However, most of them can hardly be adapted to skin tissue models used in drug and substance testing. Here, usually small culture devices, e.g., 12- or 96-well plates or even Multi-organ-chips are preferred, as they allow for handling of a large number of samples in parallel or investigation of multi-organ interactions. Furthermore, most of the methods mentioned above require treatment of the sample in one or the other way. Therefore, they do not allow to determine the time-depending changes of diffusion and permeation *in vitro*.

Hsu et al. [148,251,252] developed an alternative method based on skin tissue cultures in a membrane insert system. By this, the permeation coefficient of substances, especially permeation of substances with a high molecular weight, through a skin-tissue barrier can be determined. The diffusion coefficient is estimated via parameter optimization performed in COMSOL Multiphysics. This method can be used to investigate changes in the permeation behavior of the skin model during the cultivation or it can also be adapted for other systems, which use membrane insert Systems, e.g., Multi-Organ-Chips [156]. Furthermore, different diffusion mechanisms, e.g., diffusion described by Fick's law or abnormal diffusion, can be included. Furthermore, the simulation is an attractive tool to support the experiments. On the one hand, it can be used to understand physical phenomena and to reduce experimental effort. On the other hand, it is modular and can be integrated into a more complex system to support permeation studies.

### 5.4. An *in Silico* Strategy for 3D Tumor Models

Identification of new drug targets and biomarker profiles for innovative, personalized new treatment strategies against cancer require an in-depth understanding of signaling networks and their changes upon drug response. Tissue culture techniques offer tumor models mimicking the tumor microenvironment and providing multiple read-out options to reflect the clinical situation. The group of Walles et al. [253,254] combined a 3D *in vitro* model for lung carcinoma based on a decellularized tissue matrix to provide a complex microenvironment for cell growth and an *in silico* model to identify dependencies in signaling networks involved in proliferation, apoptosis, and invasion. The combined *in vitro* and *in silico* model represents allow to identify biomarker profiles for targeted therapies.

### 5.5. Digital Strategies from Bench to Bedside

Digital, model-based strategies have already been developed for a variety of tissue engineering processes. This is illustrated using the example of the extensive work of the working group of Geris et al. (reviewed in [14,115]). Using skeletal tissue engineering as a case study, a number of mathematical models at various stages of use between bench

and bedside are discussed and ranging from pure data-driven models to models built on known mechanisms and first principles. Generally, in all these models, the aim is firstly to understand the biological process at hand and secondly to design strategies *in silico* to enhance the desired *in vitro* or *in vivo* behavior. The shown examples cover the cellular regulation of the growth plate, a system approach to the design of new biomaterials, 3D neotissue growth modelling for understanding and optimization of the bioreactor process, and on-line monitoring techniques to determine critical quality attributes. This comprehensive approach underpins that the tools toward a digital transformation of development and manufacturing of tissue engineering products as ATMP are at hand and the main goal for the future will be to link these in a comprehensive, digital strategy.

## 6. Conclusions

In this contribution, the concepts, expectations, and the state of the art of DTs were reviewed for tissue culture techniques with a special focus on their design, characterization, and operation. In the authors' opinion, mathematical modelling and its incorporation into a digital twin has the strong potential to consider a high number of different data and knowledge sources and to use these to gain an improved understanding of tissue culture techniques, especially for ATMPs for human use as well as for drug and toxicity studies [12,13]. This is featured by new capabilities via digitalization and "Internet of things" (IoT). The possibility to access, sort, and incorporate a vast amount of information and experience, not only on the same system but also on related systems and processes, will provide new efficiencies for tissue culture techniques. A digital twin-assisted process development framework enables knowledge-based and donor specific test systems and/or manufacturing processes for ATMPs.

This digital transformation will be stimulated with novel sensors, enhancement of computational power and AI algorithms, as well as versatile data management approaches [216]. Data can be gathered along the full development and application chain and along the product life cycle. Upon that, models can be generated, even automatically [212,217]. With carefully planned experiments, enhanced quality analytics, and an in-depth determination of critical process parameters, mathematical models (factor analysis, etc.) will help to make development more efficient and faster and predict properties that could endanger the safety of the end product as well as make changes dynamically to mitigate those risks. The models can be integrated into process control systems and as knowledge management tools in quality systems and can be adapted as a platform to novel tasks. By this, a general approach for a science and cultivation process-based cell expansion and tissue manufacturing system with decreased costs and accelerated time to clinic can be industrially implemented.

However, there is much to be done to make this vision possible. An integrated data to knowledge approach, integrated in process and software platforms along both the process chains (e.g., for ATMP's) and the product life cycle is needed. To conduct this ambition, the following enablers are required: Monitoring and Process Analytical Technology (PAT), Knowledge Management, Data Integrity, Data Science, and mathematical models to achieve process and platform understanding [89,255,256]. A particular challenge compared to production of biopharmaceuticals is seen in the fact that case-specific, personalized processing is required, especially for ATMPs. Beside these technical challenges, researchers and scientists are required who are trained in an interdisciplinary and inter-sectoral manner, covering classical biochemical engineering, real industrial applications in ATMP cell cultivation, integrated processing, digitalization solutions, and advanced analytics.

Further research is needed to closely link the real-world tissue cultures and the respective virtual DT's to regulatory guidelines, especially the new Good Manufacturing Guideline (GMP) on ATMP with the new concept, that initial development data are required "to be used for subsequent phases of development" (ICH Q10). Even though these tools are partly established for production of biopharmaceuticals and encouraged by the FDA, they have hardly been applied yet to manufacturing of ATMPs. A first approach in this respect was suggested [257]. The digital twin approach can fit into existing regulatory

guidelines, but it is likely that both industry and government agencies will insist on gaining experience and confidence with this radically new approach for designing and controlling bioprocesses [258]. Taken together, an embedded framework of which the DT is the core element will improve tissue culture techniques significantly in the future.

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