

From 3D Bioprinters to a fully integrated Organ Biofabrication Line

V E Passamai, J A Dernowsek, J Nogueira, V Lara, F Vilalba, V A Mironov, R A Rezende and J V da Silva

Divisão de Tecnologias Tridimensionais (DT3D)/Centro de Tecnologia da Informação Renato Archer (CTI). Rodovia Dom Pedro I (SP-65), km 143,6 - Amaraís - Campinas, SP, Brazil.

E-mails: rodrigo.rezende@cti.gov.br; veronica.passamai@cti.gov.br

Abstract. About 30 years ago, the 3D printing technique appeared. From that time on, engineers in medical science field started to look at 3D printing as a partner. Firstly, biocompatible and biodegradable 3D structures for cell seeding called “scaffolds” were fabricated for *in vitro* and *in vivo* animal trials. The advances proved to be of great importance, but, the use of scaffolds faces some limitations, such as low homogeneity and low density of cell aggregates. In the last decade, 3D bioprinting technology emerged as a promising approach to overcome these limitations and as one potential solution to the challenge of organ fabrication, to obtain very similar 3D human tissues, not only for transplantation, but also for drug discovery, disease research and to decrease the usage of animals in laboratory experimentation. 3D bioprinting allowed the fabrication of 3D alive structures with higher and controllable cell density and homogeneity. Other advantage of biofabrication is that the tissue constructs are solid scaffold-free. This paper presents the 3D bioprinting technology; equipment development, stages and components of a complex Organ Bioprinting Line (OBL) and the importance of developing a Virtual OBL.

1. Introduction

3D bioprinting or organ printing can be defined as the use of material transfer processes for patterning and assembling biologically relevant materials (molecules, cells, tissues, and biodegradable biomaterials) with a prescribed organization to accomplish one or more biological functions [1].

Since the inception of organ printing concept as a computer-aided, jet-based 3D tissue-engineering of living human organs [2], this technique has undergone in a progressive development [3-5]. It gradually gained recognition as a reasonable bottom-up solid scaffold-free alternative to the traditional scaffold fabrication approach in tissue engineering (TE), [6].

3D bioprinting has already been used for the generation of several tissues, including multilayered skin, bone, vascular grafts, tracheal splints, heart tissue and cartilaginous structures. Applications of these constructs include the developing of high-throughput 3D bioprinted tissue models for research, drug discovery and toxicology [7]. The field is moving forward, there is a plethora of research being done on bioprinting technology and its potential as a source for tissue grafts and full organ transplants [8]. The increasing number and broadening geography of participants, the emergence of new exciting bioprinting technologies, and the attraction of young investigators indicates the strong growth potential of this emerging field [9].

Research groups in several countries as China, United State, South Korea, The Netherlands and Singapore, besides others, have been investing strongly in the bioprinting area. Singapore, for example, created the Singapore Centre for 3D Printing at the NTU (Nanyang Technological University), which is applying 3D printing for living solutions, among other applications [10]. Researchers from the Wyss Institute for Biologically Inspired Engineering at Harvard University are



searching in bioprinting area, for example, they have enabled the creation of tissue constructs with small blood vessels and multiple cell types, marking important progress toward the printing of living tissues [11]. The University of California, San Diego, has the Chen Laboratory, where the Chen group is interested in developing 3D bioprinting techniques with a micro or nanoscale printing resolution [12]. Concerning Brazil, in 2008 the Brazilian Institute of Biofabrication (INCT-BIOFABRIS) was created by CNPq (National Counsel of Technological and Scientific Development) with the affiliation of many research groups, including UNICAMP (State University of Campinas), USP (University of Sao Paulo), INT (National Technological Institute), UFRGS (Federal University of Rio Grande do Sul) and CTI (Information Technology Center Renato Archer). CTI created, among other groups, the bioprinting research group that develop professional workforce in this emerging field of medical science and provides strong impulse for the ascension of Brazil as a recognized player in bioprinting research.

2. Developments in 3D bioprinting area

There are several companies producing 3D tissues, biomaterial constructs and developing research in the bioprinting area, as shown in table 1.

Table 1. Companies that produce 3D tissues and biomaterials constructs related to 3D bioprinting.

Company/Location	Product
Inspheero/Schlieren, Switzerland	3D microtissue models: liver, pancreatic, tumor. 3D cell culture products and assay kits.
3D BioMatrix/Michigan, USA	Perfecta3D Hanging Drop Plates
Rainbow Biosciences and 3D Nano Biosciences/Florida & Texas, USA	BiO Assay, spheroid formation magnetic levitation printer system.
ACEA Biosciences/San Diego, USA	RTCA plates, Impedance-based systems for cell-based assays.
n3D Biosciences/Houston, USA	Plates and Bioprinting Kit for magnetized spheroids.

The production and commercialization of 3D bioprinting technology and organ bioprinting is already an ongoing process. There are several companies producing 3D bioprinters, some of them are: EnvisionTEC, Germany; RegenHu, Switzerland; Sciperio/nScript, USA; Organovo, USA; 3D Bioprinting Solutions Laboratory, Russia; Cyfuse Biomedical K.K., Japan; Bio 3D Technologies, Singapore; GeSiM, Germany; OxSyBio, UK; Aspect Biosystems, Canada; DigiLab, USA, Advanced Solutions Life Sciences, USA; MicroFab Technologies, USA; Seraph Robotics, USA [13].

Particularly, NovoGen MMX Bioprinter (Organovo) has two robotically controlled precision print heads: one for placing the bioink, material containing thousands of cells, other for placing hydrogel scaffolds, or a support matrix (figure 1. a) [14]. Regenova Bioprinter (Cyfuse Biomedical K.K.) has a distinct technique for obtaining 3D tissue constructs; it assembles spheroids containing cell aggregates into a three-dimensional shape by placing them in needle arrays according to the pre-designed 3D data (figure 1. b) [15]. 3D Bioprinting Solutions Laboratory has the Fabion Bioprinter, which is supplied with its own software and has a regular cartesian design allowing it to move in all directions. It also has five nozzles that can pump spheroids and extrude hydrogel and biogel in successive layers with a very high resolution (figure 1. c) [16]. BioFactory (RegenHu) is a high end, versatile and cell friendly three dimensional bio-manufacturing device that allows researchers to pattern cells, biomolecules and a range of soft and rigid materials in desirable 3D composite structures, mimicking tissue models (figure 1. d) [17].

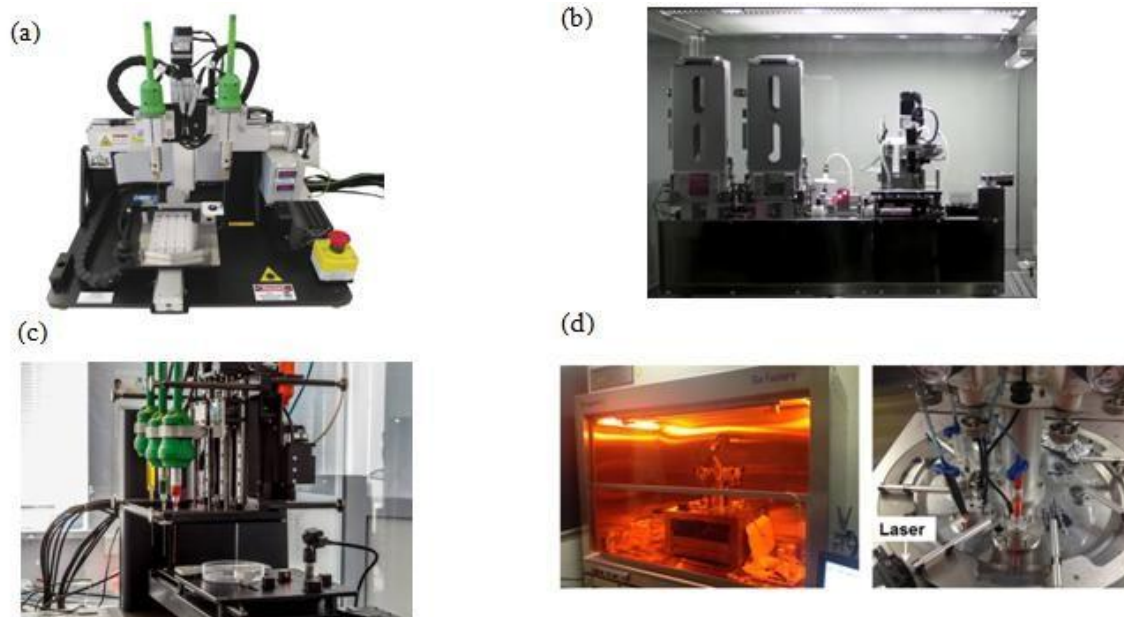


Figure 1. (a) NovoGen MMX bioprinter (Organovo, CA, USA). (b) Regenova bioprinter (Cyfuse Biomedical, Tokio, Japan). (c) Fabion bioprinter (3D Bioprinting Solutions Laboratory, Moscow, Russia). (d) BioFactory bioprinter (RegenHU, Switzerland).

3. Organ Biofabrication Line

Nowadays there are many researches and developments on robotic bioprinters, focusing in precision placement of spheroids, new biomaterials, cell-laden biomaterials and rapid prototyping technologies for 3D tissue-like structures with a resulting high density [8]. From an engineering point of view, the successful clinical translation of organ printing technology will depend not only on the development of a single tool or device, like a robotic bioprinter, but rather on the entire complex of related technologies and their seamless integration, i.e., an Organ Biofabrication Line (OBL) [18]. The design and development of a fully integrated OBL, or development of a series of integrated automated robotic tools, is imperative for the commercial translation of the organ printing technology [18]. Figure 2 shows a virtual design of an OBL that was designed at Three-dimensional Technologies Division (DT3D) – CTI [19].

The additive manufacturing OBL requires the development or employing of special equipment: clinical cell sorter, stem cell propagation bioreactor, cell differentiator, tissue spheroid bio-fabricator, tissue spheroids encapsulator, robotic bioprinter, and perfusion bioreactor [19].



Figure 2. Organ Biofabrication Line (OBL). Virtual manufacturing is an important technological advance to predict the development of complex products from many areas, as it is a human organ [19].

The development of this integrated line of automated robotic tools at industrial scale requires the interaction of three principal research fields: Engineering, Biology and Material Science (figure 3), including a complex multidisciplinary approach and close research and development collaboration of mechanical engineers, experts in rapid prototyping technology, computers scientists, biomedical engineers, chemical engineers, material scientists with biologists and tissue engineers [20].

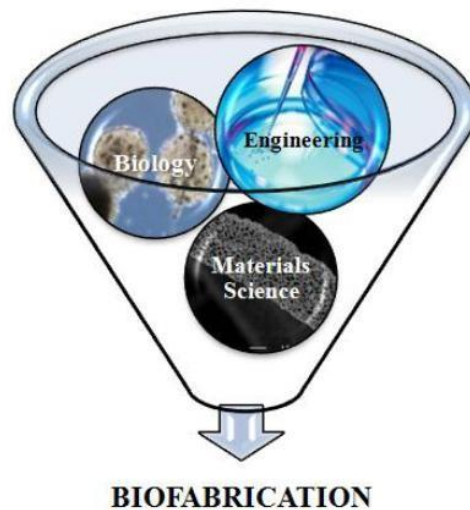


Figure 3. Research fields of the biofabrication area. Adapted from [20].

3.1 Bioprinting stages

Bioprinting is normally splitted into three parts: pre-processing, processing and post-processing, as can be seen in figure 4 [18].

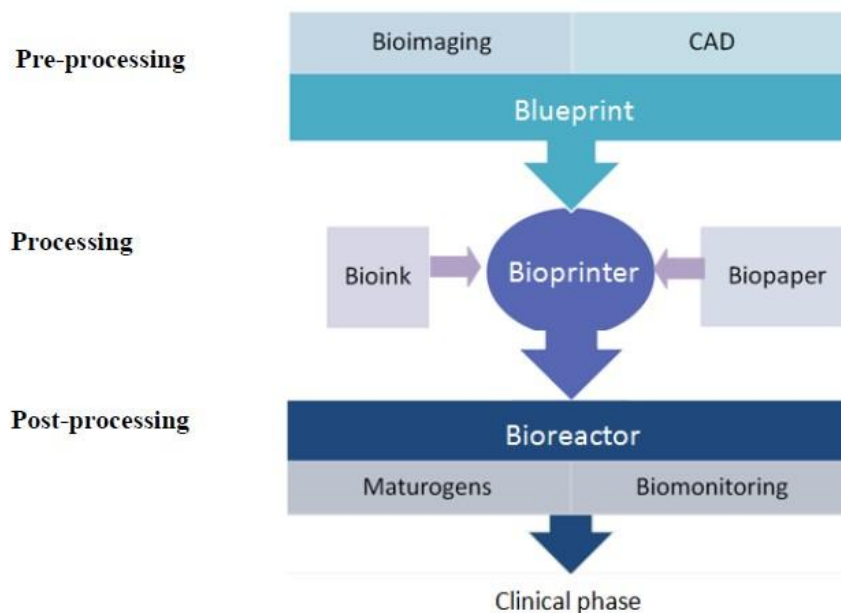


Figure 4. Three main steps in organ printing technology. Adapted from [18].

3.1.1 Pre-processing. It is the design stage. It involves the development of computer-aided design (CAD) or a “blueprint” of a 3D human tissue or organ, based on the use of clinical images and special additive manufacturing. This stage relates to the transformation of virtual reality into physical reality. Magnetic resonance imaging (MRI), computed tomography (CT) and micro-CT are already approaching the desirable level of resolution. Computer-aided reconstructions of human microanatomy and serial histological sections are highly necessary to determinate specific depositions of tissue spheroids and to obtain precise microstructures [21].

InVesalius is an example of a 3D anatomy reconstruction software, developed in CTI (<http://svn.softwarepublico.gov.br/trac/invesalius>). It uses CT or MRI data for the virtual representation of 3D human body parts (figure 5. a). Another method to develop a blueprint of tissue structures has been developed by the company Uformia from Norway (<http://www.uformia.no>), a CTI’s partner, using mathematical functions to generate a digital representation (figure 5. b).

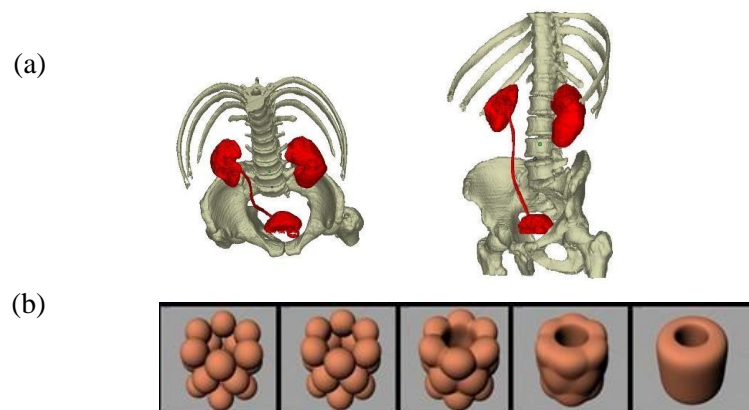


Figure 5. Blueprint examples. (a) Virtual representation of kidneys and a part of skeletal human structure (InVesalius public software for 3D medical imaging manipulation). (b) Generation of a digital representation of tissue spheroids fusion. (Courtesy of Uformia, Norway).

Mathematical modeling of anatomical and histological structures for the design of a blueprint is a powerful tool on the pre-processing stage. One of the most important principles of developmental biology, which inspired numerous discrete cell models, is the differential adhesion hypothesis (DAH). This principle leads to a close analogy between true liquids and living tissues made of adhesive and motile cells, such as most embryonic and some artificial tissues [22]. Also, there is the Monte Carlo Method, a convenient way for studying energetically driven conformational changes of a system. It is a large collection of computational algorithms that involves the usage of random numbers [23]. This and other methods are used in computational modeling, supporting the design of several software to create simulations of biocomplexity problems. Some examples are: CompuCell3D (<http://www.compuCell3d.org>), CellSys (<http://ms.izbi.uni-leipzig.de/index.php/software/cellsys2010>) and TSim - Tissue Simulator (<http://www.tsimsoftware.com>).

The required clinical image information is not a problem to the creation of CAD human organs, the challenge is the way to transform this accumulated knowledge of human anatomy and histology into a viable blueprint with necessary and sufficient instructions for a robotic printer to be able to print a human organ [24].

3.1.2 Processing. It is the production stage. The computer-aided robotic bioprinting process includes the preparation of a “bioink” or self-assembled tissue spheroids, development of a “biopaper” or

processable and biocompatible hydrogel and the usage of a “robotic bioprinter” or computer controlled robotic precise dispenser [21].

The bioink is a material made from living cells that behaves much like a liquid; the bioprinter will dispense it, in order to create the desired shape [25]. The search of bioinks for usage in tissue printing applications should have a good biocompatibility and tailored viscosity transitions for effective printing and continuous handling. For an extrusion-based process, the bioink should show shear thinning to allow extrusion through a needle and also immediate cessation of flow, upon deposition on the substrate, to retain its plotted shape. Such material would allow the fabrication of complex structures with high resolution and the final mechanical properties of the printed construct should also be sufficient for manipulation and transportation to the next stage [26, 27].

Simple calculations based on the size and volume of tissue constructs and human organs indicates that in order to bioprint a human organ of desirable size, it will be necessary to develop technology for a scalable biofabrication of millions of tissue spheroids. Thus, scalable tissue spheroid biofabrication must be considered as one of critically important technologies enabling organ printing. There are many different technologies for tissue spheroid fabrication, eg. hanging drop method, use of external forces, microarrays, etc (figure 6) [27].

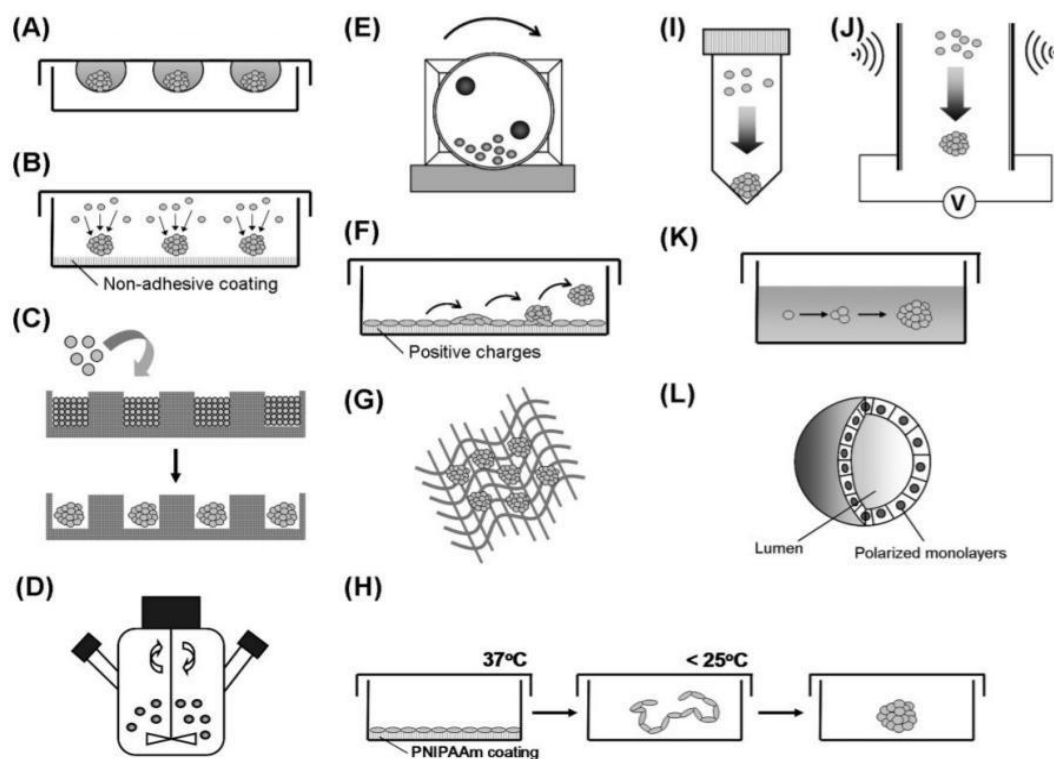


Figure 6. Spheroids fabrication methods [27]. Methods for multicellular spheroids generation. (A) Hanging-drop culture. (B) Single cell culture on nonadhesive surface. (C) Micromolding techniques. (D) Spinner flask culture. (E) Rotary cell culture systems. (F) Hepatocyte self-assembly on Primaria dishes. (G) Porous 3-D scaffolds. (H) The use of Poly(N-isopropylacrylamide)-based cell sheets. (I) Centrifugation pellet culture. (J) Electric, magnetic or acoustic force cell aggregation enhancement. (K) Monoclonal growth of tumor spheroids. (L) Polarized epithelial cysts.

In the research [28], a comparison of 600 tissue spheroids fabricated by the micromolded method with 600 spheroid generated by the conventional hanging drop method was developed. The analyses showed that the use of micromolded recessions in a non-adhesive hydrogel, combined with automated cell seeding, is a reliable method for scalable robotic fabrication of uniform-sized tissue spheroids, but uniform-sized is one of several issues to be considered [28]. To choose the best method for scalable tissue spheroid fabrication there are several aspects to be evaluated: well-controlled spheroid size, spheroid velocity formation, co-culture of different cell types, easy to scale up, good preservation of viability, rapid cell aggregation stimuli, high level of cell density, etc. An important aspect is that spheroids have to be capable of being placed in special reservoirs suitable for be used as cartridges. Taking into account all these factors, it must be chosen the most appropriate method and make adaptations to obtain an efficient automated process for scalable tissue spheroid fabrication [27, 28]. After obtaining the microtissues with a high cell density level, it is important the encapsulation of these structures. In this situation, encapsulate tissue spheroids is a way to prevent preliminary and undesirable tissue spheroid fusion before the effective printing. Beside this, the encapsulation process turns them into reinforced structures, protecting the internal live environment. Biomaterials or hydrogels used should provide a lubrication effect for the deposition stage and be permissible for tissue fusion during the post-processing [29].

Once spheroids are obtained and encapsulated, they have to be transported and loaded into adequate “biocartridges” [30]. Then, using blueprint information, the bioprinter will deposit the bioink in well-defined topological patterns into biopaper sheets [31]. In the next step, the construct will be transferred into a bioreactor and spheroids will fuse. The biopaper, a hydrogel, can be removed after construction if required; in this context, it only provides a temporary support for the deposited bioink particles. Consequently, the biopaper is clearly different from solid scaffolds used in classical scaffold-based tissue engineering and in most cases it is desired to be biodegradable, to remove it more easily [32].

3.1.3 Post-processing: The bioprinted outcome is not a functional tissue construct immediately suitable for human implantation [19]. To transform the 3D tissues and organs into functional structures they must undergo accelerated tissue maturation inside a bioreactor [8]. Being a bioreactor an essential component of classic tissue engineering, they were initially used as tools for enhancing cell seeding on solid scaffolds and for providing mechanical conditioning of tubular tissue engineered constructs. The bioreactor is also a container for keeping tissue constructs in a wet and controlled environment and operating conditions (pH, temperature, gas concentrations, nutrient supply and waste removal, physical stimuli supply), and thus maintaining their viability [24]. Those chemical and physical factors that enable accelerated tissue maturation, can be defined as maturogenic factors or simply maturogens. Also, the bioreactor serves as a packing and transportation device for mature tissue engineered constructs. Computational simulations and in situ assays are crucial in this stage. Computational fluid dynamic software packets have been increasingly developed during the past decade and are powerful tools to calculate flow fields, shear stresses and mass transport within and around 3D constructs, including the bioreactor environment [32].

Post-processing is probably the most crucial step in organ printing procedures. An effective tissue maturation will require the development of new bioreactors. Further discovery and experimental validation of potential maturogenic factors are important tasks in the developing of equipment for acceleration of tissue maturation [18].

Strategies for non-invasive and non-destructive biomonitoring, of bioprinted tissue maturation, are still not properly addressed questions in the organ printing technique. It is safe to predict that with a progress in the development of bioprinting equipment, the role of non-destructive and non-invasive methods for estimation of tissue maturation and functionality will only increase. Without the development of effective methods of pre-implantation and post-implantation “quality control” of

bioprinted organs functionality, maturation, and integrity, their approval for clinical use and implantation by regulatory agencies will be practically impossible [21].

The research [33] presented three perfusion circuits in a type of a perfusion bioreactor (figure 7): one perfusion system provides wet environment around the printed constructs; second perfusion system is designed for intravascular perfusion of matured build in vascular tree; and, finally, the third perfusion circuit is designed for enabling the temporal interstitial flow through removable temporal porous minitubes (needles). These removable porous tubes also provide temporal support and serve as some sort of non-biodegradable but removable supporting structure. The distance between these tubes as well as their porosity, must be designed based on mathematical modeling and computer simulation [33].

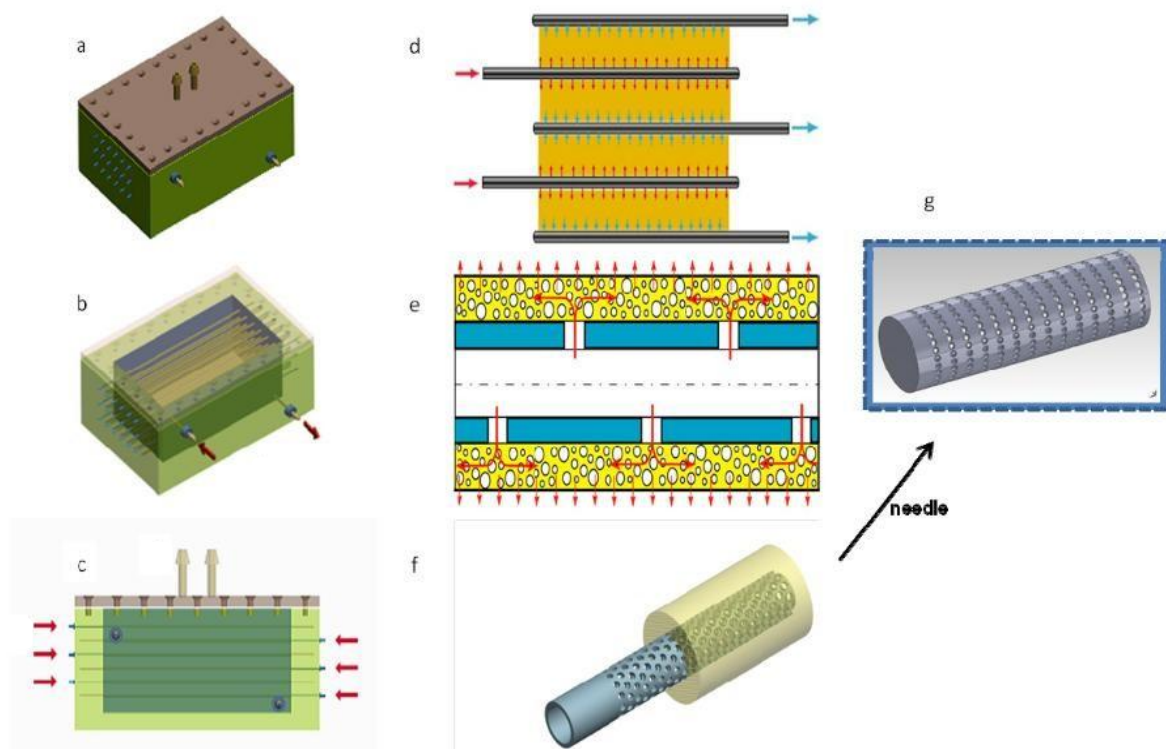


Figure 7. a) Triple perfusion bioreactor chamber. b) Inlet and outlet for extra-construct perfusion circuit. c) Temporally removable porous tubes for interstitial intra-construct. d) Fluid flow direction. e) Longitudinal section removable porous tube. f) Computer-aided design (CAD) of a temporally removable porous tube. g) CAD of a general needle. [33]

4. Conclusions and future perspectives of an OBL

Organ printing is a rapidly emerging technology that promises to transform tissue engineering into a commercially successful biomedical industry. It is an interdisciplinary field involving many science areas, among biology, computer, physics, materials and engineering. This computer-aided layer-by-layer additive biofabrication uses aggregates of thousands pre-sorted cells, microtissues called spheroids, as building blocks for originating a 3D bioprinted living structure.

It is obvious that organ printing cannot be only produced with just a robotic bioprinter, it would be like assuming that intrinsic biological structures fabrication is a very simple process, whereas the reality is quite the opposite. It requires a set of specific equipments and a controlled aseptic area. A future OBL has to be specifically well designed and studied, being that the reason why design and

computer simulation of a previous virtual biofabrication line will simplify and improve the process. It must combine all possible visual information of machines, devices, human resources and processes, as well as it must include *in silico* assays about tissue spheroids formation, spheroids fusion, cell viability, tissue and organ development in each corresponding stage. Virtual OBL must integrate essential tools like CAD, computer simulation and modelling, complex mathematical modelling, new specific software for biological simulations, special equipments and information technologies in general.

5. Acknowledgements

To São Paulo Research Foundation (FAPESP), Brazil; The Nacional Council for Scientific Technological Development (CNPq), Brazil, and The Brazilian Institute of Biofabrication (INCT-Biofabris).

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