Organ printing: computer-aided jet-based 3D tissue engineering

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Tissue engineering technology promises to solve the organ transplantation crisis. However, assembly of vascularized 3D soft organs remains a big challenge. Organ printing, which we define as computer-aided, jet-based 3D tissue-engineering of living human organs, offers a possible solution. Organ printing involves three sequential steps: pre-processing or development of 'blueprints' for organs; processing or actual organ printing; and postprocessing or organ conditioning and accelerated organ maturation. A cell printer that can print gels, single cells and cell aggregates has been developed. Layer-by-layer sequentially placed and solidified thin layers of a thermo-reversible gel could serve as 'printing paper'. Combination of an engineering approach with the developmental biology concept of embryonic tissue fluidity enables the creation of a new rapid prototyping 3D organ printing technology, which will dramatically accelerate and optimize tissue and organ assembly.

'Give us the tools and we will finish the job'
  -Winston Churchill

Although the terms 'tissue engineering' and 'organ printing' were introduced only recently (1987 and 1999 respectively), the study of cell coalescence and tissue assembly has a much longer history and is deeply rooted in developmental biology [1]. The classic work of several generations of outstanding marine and developmental biologists studying cell and tissue coalescence phenomena [2,3], tissue affinity [4], cell adhesion [5] and especially the fluidity of embryonic tissues [6,7] built the biological foundation for modern tissue engineering. In recognition of this, special chapters on developmental biology are included in modern tissue-engineering textbooks [8]. Tissue engineering itself is now often considered to represent a type of applied developmental biology [9]. Organ printing – the application of the principles of rapid prototyping technology (i.e. layer by layer deposition of cells or matrix) – is evolving into a promising approach for engineering new tissues or organs. Here, we show how developmental biology can be applied to organ printing and describe the essential steps and elements of this novel technology. We discuss the challenging technological barriers, the possible strategies to overcome them and estimate the overall feasibility of printing 3D human tissues and organs.

'*Secundum Naturam* or *Contra Naturam*?'

The fact that tissues, such as a blood vessel, can be successfully assembled without any synthetic polymer [10] supports our strong opinion that future progress in the field of tissue engineering will be increasingly based and dependent on the effective application of principles of developmental biology. It is safe to predict that the credo of the next generation of tissue engineers will be '*secundum naturam*' (according to nature) not '*contra natura*' (against nature). Both tissue engineering and developmental biology currently deal with the processes of tissue self-assembly, extracellular matrix deposition and accumulation, and stem cells. We believe that the fusion of these fields could, and will, lead to unprecedented achievements.

Another factor that we believe will accelerate the development of organ printing is time. Tissue engineers, as well as doctors and their patients, do not have the luxury to wait years until engineered tissues and organs become morphologically, biochemically, mechanically and functionally differentiated. Existing tissue technologies do not enable rapid assembling of tissues and organs. The timing issue can be addressed by developmental biology, in which we have learned that embryonic tissues are qualitatively and quantitatively viscoelastic fluids [6,7] with well described flow and fusion behavior. As the work by Thompson *et al.* [8] demonstrates, when embryonic avian heart tubes are initially cut into isolated myocardial 'rings' and placed on a supporting tubular framework in close apposition, they fuse and morph overnight into a single, synchronized, beating heart tube (Fig. 1a,b). The processes involved in this fusion process are still not completely understood, but the nature and time-scale of this phenomenon is inspiring for tissue engineers. Probable candidates for tissue fusion processes include remodeling of the extracellular matrix, cell migration, re-establishment of cell-to-cell contacts or combinations of all these. However, the above simple developmental biological experiment provided us with a powerful insight, which represents a starting point to the proposed concept of organ printing as laid down in present work.

In analogy with the embryonic heart ring fusion experiment, we hypothesize that if cell aggregates are placed in close apposition within a 3D matrix, they

fuse to form a complete disc or tube of tissue. Thus, the intrinsic capacity of closely placed soft tissue fragments or cell aggregates to fuse is the biological foundation on which organ printing technology will be developed (Fig. 1c). Recently, an explanation for the phenomenon of tissue fusion (considered a time-dependent process) has emerged. Using direct quantitative measurements, it was shown that embryonic tissues are viscoelastic fluids [6,7] and, as such, can flow and fuse. Organ printing attempts to apply microfluidic design to cells and cell aggregates triggering biologically relevant phenomena such as fusion. Interestingly, the potential of isolated tissue fragments to regenerate into tissues was originally demonstrated in 1907 by marine biologist Henry von Peters Wilson in his classic studies of coalescence phenomena in dissociated sponges [2] and further supported by Holtfreter [4]. However, it has taken many decades to recognize the technological potential and implications of this fundamental observation for tissue engineering.

What is organ printing?

Organ printing is a biomedically relevant variant of rapid prototyping technology, which is based on tissue fluidity. Computer-assisted deposition ('printing') of natural materials (cells or matrix) is done one layer at a time until a particular 3D form is achieved. However, recent attempts using rapid prototyping technologies to design solid synthetic scaffolds [12–15] suffer from the inability to precisely place cells or cell aggregates into a printed scaffold. Thus, we believe that organ-printing technology will become increasingly more '*secondum naturam*'. We define organ printing as a rapid prototyping computer-aided 3D printing technology, based on using layer by layer deposition of cell and/or cell aggregates into a 3D gel with sequential maturation of the printed construct into perfused and vascularized living tissue or organ (Figs 2,4). This definition of organ printing includes the many different printer designs and components of the deposition process such as, for example, jet-based cell printers, cell dispensors or bioplotters, the different types of 3D hydrogels and varying cell types.

The procedure of organ printing can be subdivided into three sequential steps: preprocessing, processing and postprocessing. Preprocessing primarily deals with the development of a computer-aided design (CAD) or blueprint of a specific organ. The design can be derived from digitized image reconstruction of a natural organ or tissue. Imaging data can be derived from various modalities including noninvasive scanning of the human body (e.g. MRI or computerized tomography) or a detailed 3D reconstruction of serial sections of specific organs (see [16] for a recent review).

Yet another approach to designing a tissue is based on mathematical modeling using a set of theoretical principles, rules or laws related to spatial organization. One the most impressive recent examples of this technology is called 'constrained

constructed optimization' (CCO), which was developed by Karch *et al.*. [17]. Processing usually refers to actual computer-aided printing or layer-by-layer placement of cells or cell aggregates into a 3D environment using CAD or blueprints. Finally, postprocessing is concerned with the perfusion of printed organs and their biomechanical conditioning to both direct and accelerate organ maturation.

Is organ printing a feasible technology?

To answer this question we define our goal as the successful reduction of complex tasks of organ tissue engineering into a series of simple, testable prototypes and pilot projects. Thus, our testing of the organ-printing hypothesis must include considerations based on results obtained from a series of crucial, well-designed, preliminary experiments. This minimal program must include: development of a printer which can print cells and/or aggregates; demonstration of a procedure for the 'layer by layer', sequential deposition and solidification of a thermo-reversible gel or matrix and demonstration of fusion from closely placed cell aggregates into ring-like or tube-like structure within the gel. Achieving these goals will demonstrate the feasibility of our proposed definition for organ printing technology. To accomplish the first step, a new type of cell printer was developed [18], a device capable of printing single cells, cell aggregates and the supportive, biodegradable, thermosensitive gel according to a computer generated template (Figs. 2,3a). Thermo-reversible gel [19–20] was printed one layer at a time, each on the other with individual layer thickness comparable to the diameter of the cell aggregates (Fig. 2) [21]. In accordance with mathematical predictions [22], it was shown that closely placed cell aggregates and embryonic heart mesenchymal (cushion tissue) fragments could fuse into ring and tube-like structures in 3D gel (Fig. 3). Thus, our pilot experiments strongly indicate the feasibility of organ printing technology.

Why start from the printed tube?

In his seminal work, Danish Nobel Prize Laureate August Krogh [23] demonstrated that vascular density is the most crucial factor for adequate organ perfusion and supply of oxygen and functioning. More recently, Judah Folkman [24,25] introduced a global concept to explain angio-dependency of growth in both tumor and normal tissues. Without adequate vascularization, tissue-engineered organs could not survive and undergo intensive apoptosis and necrosis. Moreover, vascularization is often correctly identifed as a main technological barrier for building 3D human organs [26–28]. The two main strategies proposed for inducing vascularization of tissue-engineered organs are either the incorporation of growth factors into the scaffold to induce angiogenesis after implantation or the pre-seeding of the implant with endothelial cells [27,28]. None of these strategies might prove satisfactory because of the slow rate of vascular tissue remodeling and the complex nature of the highly

branched microvascular networks necessary for maintaining viable cell constructs. Sophisticated attempts to create 'room service-like' branching channels in solid scaffolds to improve the effectiveness of cell seeding fail to consider the key issue of the very nature of organ perfusion [28,29]. Effective organ perfusion is not possible without an endothelialized vascular tree. The proposed printing technology offers a unique opportunity to eventually print a complex branching vascular tree during the overall process of printing a complete organ. Such organs could be immediately perfused after printing (Fig. 4). The lumen of a printed tube could be opened and washed simply by changing the physical characteristics of the gel. Thus, the capacity to print a 3D tube not only is a logical initial step but also a very important indicator of the overall feasibility of proposed organ printing technology.

Conclusion

Organ printing, or computer-aided layer-by-layer assembly of biological tissues and organs, is currently feasible, fast-evolving and predicted to be a major technology in tissue engineering. Organ printing uses the principle of cellular self-assembly into tissues [30] similar to the way embryonic-like tissues sort and fuse into functional forms dictated by the rules laid out in developmental biology. Besides their obvious application for organ transplantation, 3D perfused, vascularized, printed human tissues (or structural-functional units of human organs) could become popular screening assays for drug discovery and testing and further biomedical research. It is safe to predict that in the 21st century, cell and organ printers will be as broadly used as biomedical research tools as was the electron microscope in the 20th century.

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Fig. 1. Fusion of embryonic myocardial ring. Myocardium rings were cut from Stage 15–16 HH chick ventricle, containing only myocardium, endocardium and some intervening matrix. Isolated rings beat steadily for several days; (a) adjacent apposed rings fused overnight and (b) beat as one. (c). Schematic representation of principle of organ printing technology: placing of cell aggregates layer by layer in solidifying thermo-reversible gel with sequential cell aggregate fusion and morphing into 3D tube.
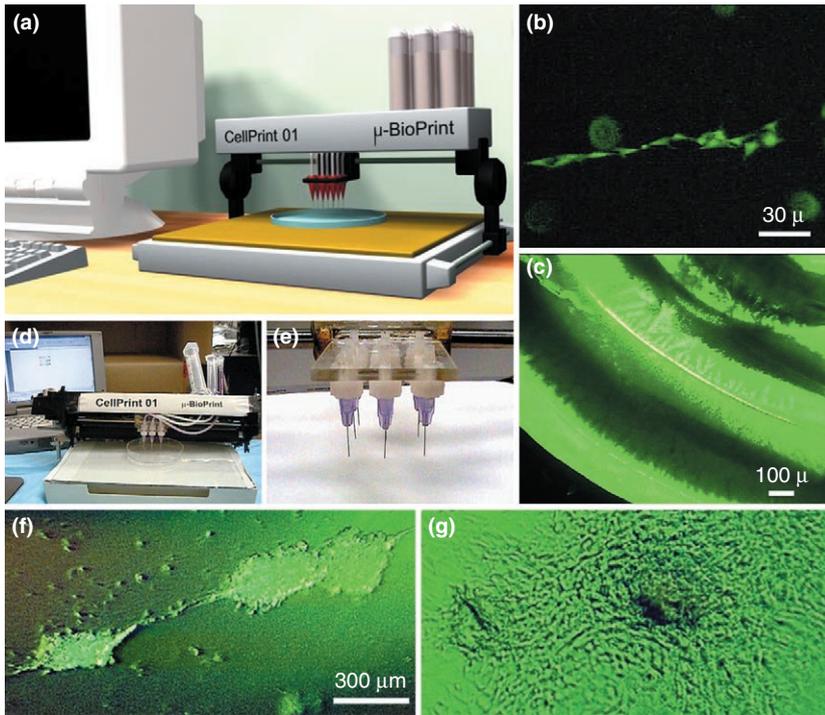
Fig. 2. Cell printer and images of printed cells and tissue constructs. (a) Computer aided design-based presentation of model of cell printer. (b) Bovine aortic endothelial cells were printed in 50-micron size drops in a line. After 72 h the cells attached to the Matrigel support and maintained their respective positions. (c) Cross-section of the p(NIPA-co-DMAEA) gel showing the thickness of each sequentially placed layer. (d) Picture of the real cell printer and part of the print head with nine nozzles. (e). The printer is connected to a PtdCho via a bidirectional parallel cable together with 9 jets extent of mixing. We use HPGL2 format to send the printing information to the printer. Specifically, the printer nozzle selection (1–9), the X, Y, and Z coordinates to print a dot are transmitted to the printer, which is controlled by a Microchip PIC 16F877. Endothelial cell aggregates 'printed' on collagen before (f) and after their fusion (g).
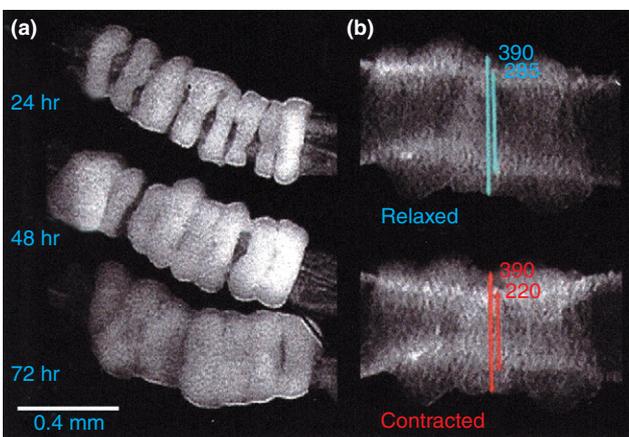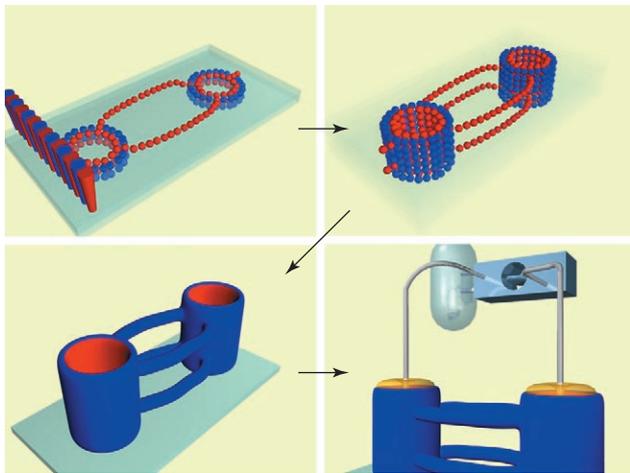
Fig. 3. (a) Printed bagel-like ring that consists of several layers of sequentially (layer-by-layer) deposited collagen type 1 gel. (b) Manually printed living tube with radial branches from the chick 27stage HH embryonic heart cushion tissue placed in 3D collagen type 1 gel. Tube was formed as a result of fusion of three sequential rings. Every ring consists of 16–18 closed placed and fused embryonic cushion tissue explants. Image was taken after 24 h incubation in M199 medium with 10% of chicken serum plus ITS (insulin transferrin-selenium). (c,d) Mathematical model of cell aggregate behavior when implanted in a 3D model gel. (c) Eight aggregates each containing 123 cells before fusion. (d) Fused disc. (e,f) Fusion of aggregates of Chinese Hamster Ovary (CHO) cells implanted into RGD containing thermo-reversible gel and genetically labeled with green fluorescent protein. (e) Ten aggregates (containing ~5,000 cells) before fusion. (f) Final disc-like configuration after fusion. Note strong correlation between mathematical model and experimental data.
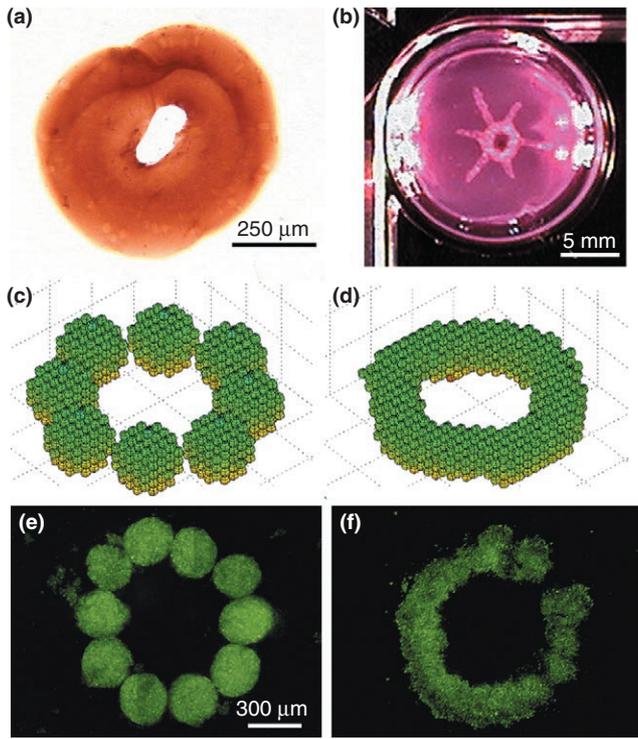
Fig. 4. Printing, assembly and perfusion of an elementary printed 3D vascular unit; schematic representation. Endothelial cells aggregates are shown in red. Smooth muscle cells aggregates are shown in blue. After printing and fusion of cell aggregates the gel is removed from the lumen and large vessels are perfused with perfusion bioreactor.