Optimized Bioprinting of Advanced Tissue Models with the BIO X6

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Abstract

Replicating functional organs and tissues *in vitro* is still a major challenge for scientists around the world. A better understanding of tissue-specific cell processes is needed, especially the cues for vascularization in three dimensions (3D). Biomaterials that support specific cell functions and the access to new advanced 3D bioprinters are essential to the progression of the field. Coupling these components together creates a novel biofabrication solution, permitting the production of highly functional tissue substitutes. CELLINK's bioprinting platform, the BIO X6, with its six printheads and its ability to print hollow tubes with a coaxial needle, is just one part of this leap into fabricating more advanced tissue models. This technical note aims to showcase an example of fabricating advanced models using the BIO X6. In this acellular proof-of-concept study, a five-layer skin model, which comprises extruded dermal layers, coaxial printed vascular channels, precise hair follicle dispensing, and noncontact epidermal coating, was printed using the BIO X6.

Keywords: *BIO X6, bioprinting, biofabrication, bioink, tissue engineering, 3D cell culture, vascularization, coaxial printing, skin tissue model.*

Introduction

Biofabrication of tissue models

Replicating the native tissue *in vitro* is a challenge that many scientists are faced with. 3D bioprinting of cell-laden hydrogels to create tissue models is a relatively new approach with first introduced before the millennium and the development of the first bioprinter system in the early 2000s (Mironov, 2003; Boland, 2006). Tissue engineering and additive manufacturing has evolved significantly since then, including the understanding of cells and their microenvironment, the material development of bioinks, as well as the technology of 3D bioprinters.

Understanding the cells' native environments is critical to recreate tissues and organs for regenerative medicine and transplantation. Adequate translation of the cell behavior *in vivo* to the biofabricated model is important to assure that the function of the tissue is not lost. Each tissue has specific intercellular signaling pathways and dynamic interactions. If the environment is controlled in the right way, cells can be guided toward correct phenotypes and mature into functional tissues (Jakab, 2010). The choice of printing material is crucial to achieve functional tissue models. Bioinks that mimic the extracellular matrix (ECM) of the targeted cell type is a principal factor to consider during the tissue design phase (Rocca, 2018). Both the physical and biological stimuli are important for the cells to attach, proliferate and start producing their own ECM (Zhang, 2017). This includes the stiffness and pore size of the material as well as the incorporation of structural proteins and growth factors. Controlling these cell interactions through tissue-specific bioinks and 3D bioprinting helps bridge the gaps in organ biofabrication.

Even though the field is growing fast, the majority of laboratories are using 3D bioprinters with one or at maximum three printheads to create their models. Using only three different materials or cell types will soon reach its maximum complexity, which is not enough for an advanced and functional tissue model. Facilitating the use of more printheads will be necessary to fabricate different multilayered tissue structures of the human body. CELLINK's BIO X6 3D bioprinter has room for six printheads or toolheads compatible with the Pneumatic, Syringe Pump, Electromagnetic Droplet (EMD), Thermoplastic and Temperature-controlled printheads as well as an HD Camera or Photocuring Toolhead (**Figure 1**). The level of control with six printheads on the BIO X6 enables breakthroughs in tissue engineering and accelerates innovation in the field.

Figure 1. *The BIO X6 3D bioprinter with six printheads controlled by a coupled tablet.*

Vascularization: The main challenge

One hurdle with fabricating thick and large tissue models is the lack of nutrient supply and removal of waste products from the core (Zhang, 2017). Molecular diffusion of oxygen and nutrients is limited to approximately 100 to 200 µm thick layers of tissue (Rouwkema, 2010). To be able to supply the center of thicker constructs with nutrients and oxygen, a network of vascularization needs to be incorporated. In the human body, the network is complex with a gradual decrease in the vasculature size, from the macrovasculature that is up to 2 mm thick down to the capillaries that are 10 to 20 µm (Figure 2) (Jakab, 2010). There have been several attempts to mimic the microstructures using 3D bioprinting (Yu, 2013; Zhang, 2013) as well as investigations of the cues for promoting capillary formation by sprouting of cells (Phelps, 2010; Richardson, 2001), however, there is still room for improvement at mimicking complex networks. Since almost all tissues include some form of blood flow and many organs need a distinct microarchitecture to perform their function, such as capillaries in the nephron system in the kidney (Zhang, 2017), it is evident that this is an area that needs to be developed further.

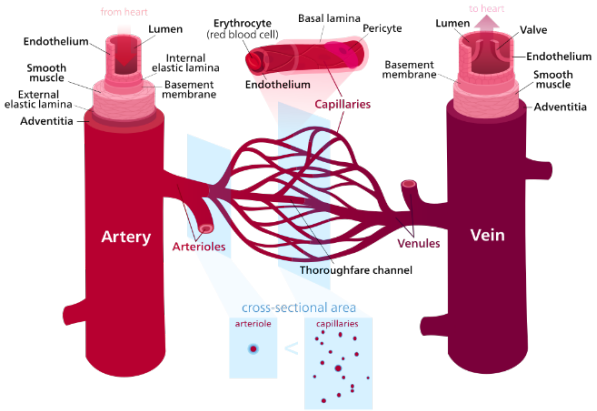

Figure 2. *An illustration of the different types of vessels from large veins to small capillaries (Kelvinsong, 2013).*

Bioengineering functional, complex tissues requires diverse knowledge about cell behavior such as interaction with the environment, communication with other cells and central mechanisms like proliferation, migration, maturation and apoptosis. The biological system is complex and simplifying this in an *in vitro* model has its limitations. Scientists today are faced with challenges on how to layer different cells in bioinks that do not have a clear distinction *in vivo* and how to optimize one medium formulation in a system that requires different growth factors and additives around different cell types. However, the fabrication of full-size tissues and organ substitutes has only just begun and breaking these barriers to replicate the native tissue will lead to a revolution in the field and our society. Can the BIO X6 be the start of this breakthrough?

Bioprinting an advanced skin tissue model

Incorporation of vasculature

Creation of the macrovasculature can be achieved with the BIO X6 and a coaxial needle. The BIO X6 has two independent pressure regulators that are used to simultaneously alter the pressure of the inner core and outer shell of the coaxial needle. After printing and crosslinking of the shell, evacuation of the sacrificial material in the core is an easy way to create hollow tubes. Possible core materials can be Pluronic F-127 or gelatin, which are cooled or heated respectively to liquefy materials after printing (Zhang, 2017). Alginate is a good material for the shell since it is temperature independent and can be *in situ* crosslinked by incorporating calcium chloride ($CaCl_2$) in the core. One alternative approach is to blend the alginate with collagen or ECM material for better attachment of endothelial cells to the lumen.

The evacuation of the channels can be achieved by either letting the liquefied material dissolve in solution or actively evacuating the channel by aspirating the inner core. If the cells have not already been incorporated in the shell material before printing, the cleared channel can be seeded with a cell suspension to endothelialize the lumen. Peristaltic and syringe pumps can be coupled to fabricate tissues for flow and perfusion, or platform rockers can be used to stimulate fluid and nutrient movement.

Creation of layered models

For the creation of intricate models, imagination is the only limitation. For this technical note, an advanced tissue model, an acellular skin tissue model was created (Figure 3). The advanced vascularized skin tissue model includes a hypodermis bottom layer and three layers of support around the vasculature structure (Figure 3A). The channel is created using a coaxial needle with the sacrificial material Pluronic in the core (Figure 3B), which also includes $CaCl_2$ to *in situ* crosslink the alginate in the shell (Figure 3C). Then a dermis layer is printed on top of the channel, which also includes a brim (Figure 3D). Hair follicle droplets (Kunz, 2020) deposited using a needle which first punctures a hole in the dermis and then extrudes the droplet (Figure 3E). Last, an epidermis layer is dispensed on top to secure the dermis brim (Figure 3F).

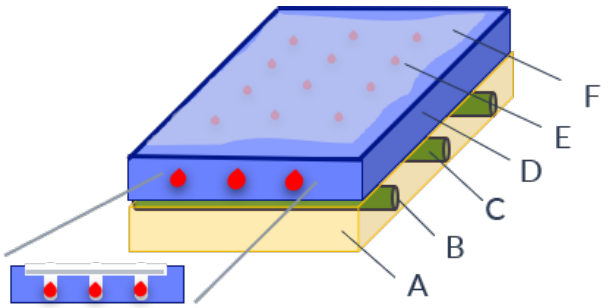

Figure 3. *Illustration of the different skin layers. **A)** Hypodermis printed with GelXA FIBRIN, **B)** inner core of Pluronic with $CaCl_2$, **C)** vascular shell printed with alginate, **D)** dermis printed with CELLINK Bioink, **E)** deposition of hair follicle droplets into the dermis, and **F)** seeding of epidermis.*

A G-code command script was generated for printing the different layers with designated printheads in specific patterns and infill densities as well as the serpentine path of the coaxial needle. With the BIO X6, one has the freedom to choose which parameters are set in G-code and what can be altered by the interface, such as pressure, printing speed and preflow volumes. The model in Figure 3 was printed using the BIO X6 with settings according to Table 1. In this experiment, the model was printed without cells, but the tissue-specific cell types can be incorporated in the representative bioink. The parameters in Table 1 are suggested starting settings that can be varied depending on ambient temperatures and humidity.

Table 1. *BIO X6 printer setup for fabricating a multilayered skin structure with printhead and nozzle/needle selections, as well as used materials and correlating pressures/extrusion and tissue representative.*

Slot	Printhead	Nozzle/Needle	Material	Pressure/Extrusion	Tissue
Printhead 1	Coaxial	Inner 14G coaxial needle	Pluronic 35% with 2 mM $CaCl_2$	200 kPa	Sacrificial
Printhead 2	Coaxial	Outer 18G coaxial needle	Alginate 2%	200 kPa	Vasculature
Printhead 3	Temperature-controlled	22G nozzle	GelXA FIBRIN	40 kPa	Hypodermis
Printhead 4	Pneumatic	22G nozzle	CELLINK Bioink	27 kPa	Dermis
Printhead 5	Syringe Pump	25G 0.5" needle	Cell medium	4 µL (7 µL/s extrusion speed)	Hair follicles
Printhead 6	EMD	300 µm valve, threaded nozzle	Cell medium	10 kPa (5 ms opening time and 200 ms cycle time)	Epidermis

The BIO X6 was set up with a variety of printheads according to Figure 4. All materials could have been printed at room temperature; however, for the best printability, the GelXA FIBRIN was printed at 24°C in the Temperature-controlled Printhead. The printbed was precooled to 10°C to stabilize the GelXA FIBRIN upon deposition and to limit gravitational distortions. The needles and nozzles were calibrated to the same point on the Petri dish, then briefly pressurized to check dispensing functions with bioinks before initiating printing.

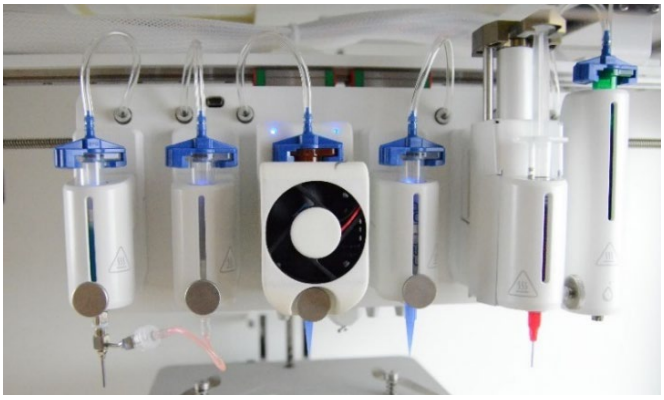

Figure 4. *Image of printhead configuration used with Pneumatic Printheads for the coaxial needle in Slots 1 and 2. A Temperature-controlled Printhead in Slot 3, a Pneumatic Printhead in Slot 4, a Syringe Pump Printhead in Slot 5, and an EMD Printhead in Slot 6.*

Each skin layer was successfully printed, illustrated in Figure 5. An alginate vascular channel (Figure 5B) was printed within the GelXA FIBRIN hypodermal layer (Figure 5A). The CELLINK Bioink dermal layer (Figure 5C) was printed on top of the channel and includes a brim to hold the liquid epidermis in place. The follicle droplets (Figure 5D) were dispensed into the dermis layer with precise depth and symmetry. Last, the epidermis (Figure 5E) was seeded on top of the tissue design through non-contact EMD printing. The GelXA FIBRIN layer can be photo-crosslinked after dispensing, and the complete model can be crosslinked using the Crosslinking Agent containing both $CaCl_2$ and thrombin (Figure 5F).

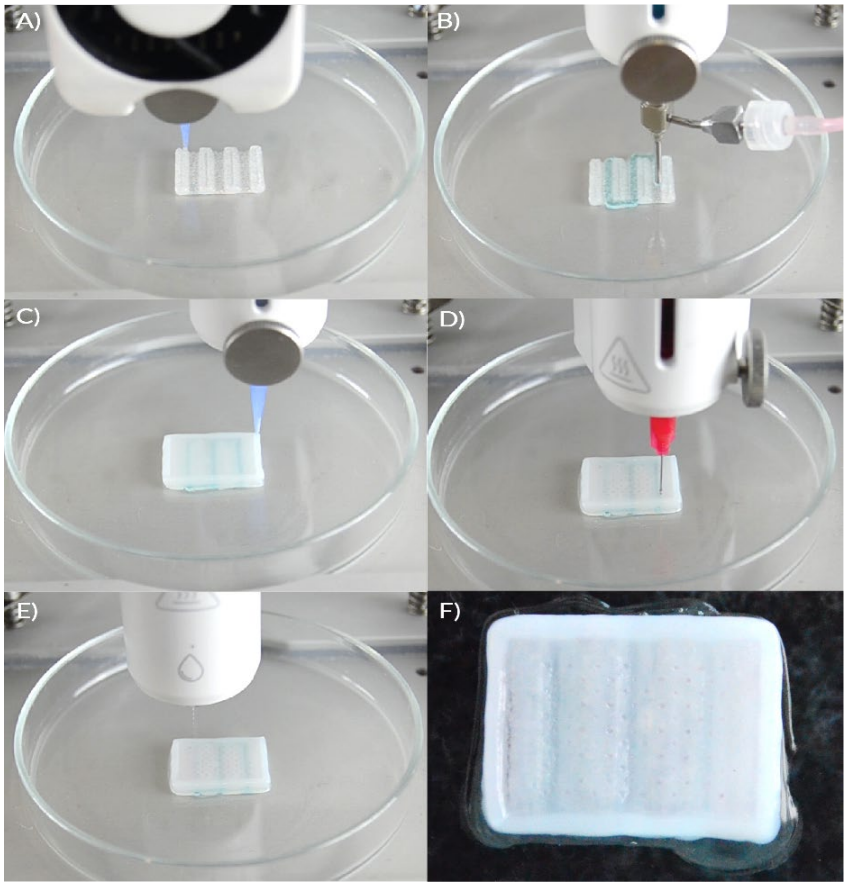

Figure 5. *Images of all five printing layers in the skin model. **A)** GelXA FIBRIN as scaffold for hypodermis, **B)** coaxial vascular tube with sacrificial blue Pluronic in the core and alginate in the shell, **C)** CELLINK Bioink as dermis, **D)** deposition of follicle droplets down into the dermis layer, **E)** EMD printing of the epidermis suspension, and **F)** final printed construct.*

Future experiments, including those with cells, can be adapted with adipocytes together with human umbilical vein endothelial cells (HUVECs) in the GelXA FIBRIN hypodermis, HUVECs in the vascular channel, fibroblasts in the CELLINK Bioink dermis, hair follicle cells in the droplets, and keratinocytes and melanocytes in the epidermis. With these defined cell types, the bioinks could be further tailored to contain the corresponding laminins, ECM materials or growth factors for a fully optimized model. This technical note demonstrates the versatility of the BIO X6 when using all six printheads. It also illustrates the need and importance of advanced bioprinting systems when modeling multicellular tissue replicates.

Summary

- The BIO X6 is a versatile 3D bioprinter with the ability to customize printheads in various combinations and has a user-friendly software that enables complex automated tissue production.
- The BIO X6 supports printing with coaxial needles for the generation of hollow tubes and vascular networks in tissue designs.
- The proof-of-concept skin fabrication study shows the flexibility of six different printheads and the potential for generating advanced tissue models.

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