REVIEW ARTICLE

Digital biomanufacturing supporting vascularization in 3D bioprinting

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Abstract: Synergies in bioprinting are appearing from individual researchers focusing on divergent aspects of the technology. Many are now evolving from simple mono-dimensional operations to model-controlled multi-material, interpenetrating networks using multi-modal deposition techniques. Bioinks are being designed to address numerous critical process parameters. Both the cellular constructs and architectural design for the necessary vascular component in digitally biomanufactured tissue constructs are being addressed. Advances are occurring from the topology of the circuits to the source of the of the biological microvessel components. Instruments monitoring and control of these activates are becoming interconnected. More and higher quality data are being collected and analysis is becoming richer. Information management and model generation is now describing a "process network." This is promising; more efficient use of both locally and imported raw data supporting accelerated strategic as well as tactical decision making. This allows real time optimization of the immediate bioprinting bioprocess based on such high value criteria as instantaneous progress assessment and comparison to previous activities. Finally, operations up- and down-stream of the deposition are being included in a supervisory enterprise control.

Keywords: digital, biomanufacturing, bioprinting, vasculogenesis, microvasculatures, bioinks

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1. Introduction

1.1 Digital Manufacturing

Digital manufacturing promises to increase productivity and robustness in existing processes and facilities, as well as enable the efficient development of difficult, previously unmanageable products or processes[1,2]. It relies upon the comprehensive and real-time controlled interfacing of human and machine sourced information through a centralized system. More than SCADA (supervisory control and data acquisition), it is an embedded interconnection of real-time access to divergent sources of information, and a provider of deep analysis, predictions and process control. Digital manufacturing is a resident and on-line source for continuous optimization of process performance, based on both information available from current operations as well as from previous batches (or time windows). For example, GE's application of the Predix™ cloud-based platform enables powerful handling of rich-data to better support advanced manufacturing platforms (www.ge.com/digital/predix).

2. Digital Biomanufacturing

Digital biomanufacturing is similarly seen as promoting improvements in the manufacturing of biologicals through such initiatives as computer aided design,

enterprise control, and verification[3,4]. Digital bioma-nufacturing (DB) is part of an evolution, one further step in the application of *Industrial Internet of Things* (IIoT). It refers to instruments becoming interconnected, but more than that — it denotes high levels of data analysis, information management and process control being implemented into a "process network". DB promises such value as real-time optimization of the manufacturing process based on such highly valuable criteria as projected product quality and batch profitability.

2.1 Terminology

It is desirable to use a distinct term here to distinguish it because, as in the terms *bioproduction and biopharmacology*, DB addresses the many unique aspects of biologically-based activities. For example, the term digital biomanufacturing may be used to describe the advanced manufacturing practices of many biopharmaceutical entities or vaccines. It is not to be confused with direct digital biomanufacturing processes, such as employed in, e.g., some synthetic biology and 3D bioprinting applications[5]. 3-dimensional bioprinting (3DBP) can therefore be conceived of as one implementation of direct digital biomanufacturing or additive biomanufacturing. 3DBP and bioplotting are also now employing other elements of DB. One example of this is software to support the management of imported digital analytics and imaging files, as well as programs to design, visualize, simulate, and analyze 3D computer models of printed structures[6]. Others include the emerging applications of distributed, closed loop and supervisory control technology to bioprinting. As 3DBP operations move towards the promise of therapeutic applications, these factors will enable more efficient, reproducible and self-adaptive processes.

2.2 Bioinks

As fluids are deposited during 3DBP, the composition of bioinks are very important to the outcome of the printing[7,8]. Precise and universal definitions of most terms in biomedical applications of additive manufacturing are rare[9]. Generally, the term "bioink" refers to a fluid containing living cells (or cell assemblies) and many low and high molecular weight components to be employed in 3DBP. However, there are other usages — some refer to cell-free fluids as a type of bioink. For example, bioinks deposited for ancillary buttressing of the primary product (support bio-

inks), fluids to be removed after leaving a void (sacrificial or fugitive bioinks) and even cell-free matrix solutions intended to be immediately populated with cells post-printing (printed scaffold bioinks). Also, there are printing technologies that either employ optimized de-cellularized natural matrices[10,11], print into polymerization-initiation chemical baths (direct-writing)[12] or whose cell-laden bioinks do not require a scaffold component at all[13].

2.3 Supported Printing Parameters

Therefore, depending upon the specific reference, a bioink must variously support the mechanical and chemical aspects of the particular printing technology(s) employed, structure of the printed assembly, health of the particular cell types employed and post-printing functions[14]. Their specific design and formulation is becoming even more important as the industry is adopting such advances as multi-component bioinks in multi-step 3D printing process and anisotropic matrices[15]. Currently, researchers and printed construct sponsors in 3DBP must develop their own inks. Until quite recently, all the structural material components of bioinks were adopted from other applications. However, some characterized products and bioprinting qualified materials are now becoming commercially available[16,17]. The cell-culture components have been supplied by commercialized culture media formulations and most-often include serum. As applications mature, demand is growing for optimized, serum-free bioinks, and 3DBP-related cell culture media, of consistent quality manufactured in regulated facilities.

2.4 Tunable Fluid Characteristics

Bioinks must provide many distinct features that can be considered as elements of tunable solutions enabling a digital biomanufacturing technology. In 3DBP, this is accomplished by (i) specifically supporting a particular printing technology; (ii) providing a matrix, scaffold or extra-cellular matrix (ECM) for the immediate structural integrity of the printed construct; (iii) supporting the immediate stable culture and robust performance of the living cells within the printed construct (e.g., nutrition, factor and mass-transfer); (iv) enabling required scaffold assembly or polymerization; (v) supporting post-printing cell-attachment, migration or phenotypic progression; (vi) accommodating any required subsequent matrix remodeling, interaction or absorption; and (vii) pro-

viding any product application-specific quality, regulatory or functional requirements.

2.5 Printing Technology

Many technologies supporting digital biomanufacturing are in use today. Chief among them for 3DBP are the laser-assisted, ink-jet, and extrusion (or micro-extrusion) approaches[8]. However, there are other, very creative approaches being explored such as magnetic-based techniques[18]. While there are many overall similarities with these processes, there are some very distinct chemical or mechanical requirements to the bioinks for each. One distinction can be in required physico-mechanical characteristics of the solution — such as its surface tension, conductivity, viscosity, flow characteristics, and any non-Newtonian behavior. Other distinctions pertain to the biologicals, such as consequences of the technique-specific printing pressures, shear or fluid volumes, required cell concentrations, and biocompatibility. A bioink must support both the mechanical and biological requirements of the printing approach adopted.

2.6 Matrix, Scaffold or ECM

Beyond the rheological requirements for the ink in the printing process itself, the post-printing structural characteristics of the bioink are an important aspect of 3DBP. As can be seen from **Vcdrg'3**, many HMW natural and synthetic polymers are employed in 3DBP. Each has unique physical, chemical, and biological properties. Furthermore, the means of controlling their state or and stiffness can have effects on other characteristics of the bioink. The first criteria for such thickening agents or structural elements are that they be "biocompatible". However, this term has different connotations depending upon the application. Characteristics included in various concepts of the term include lack of immunoactivity, cellular toxicity, cell lineage differentiation activity, apoptosis induction, up-or down gene regulation/induction and more. Through the application of cross-linking reagents, light, heat or modulation of supramolecular chemistry, active aspects of a bioink — the viscosity, strength, stiffness, visco-elastic plastic, surface and other characteristics imparted by many matrix components — can be varied. Just what is included in the concept of biocompatibility depends a lot upon the type of cells employed and the final application of the construct. Neither innate nor adaptive immune system activity may be very important in a printed construct

to be employed in an *in vitro* assay. On the other hand, a printed construct that will be matured by a process including immediate removal/exchange of the printed support matrix post-printing may not require much attention to a minor cytotoxicity in the ink. The means of polymerizing the matrix monomer can be significant. These ranges from temperature; to UV, blue or green light; to chemical activators and their effect must be thoroughly examined.

Table 1. Lists of common bioink and 3DBP culture media component ingredients[7,19,20]

Structural matrix elements	Cell culture elements
Agarose	Animal sera
Alginate	Sera fractions
Carrageenan	Hydrolysates
Cellulose	Cell and tissue extracts
Chitosan	Amino acids, nucleotides
Collagen	(poly) peptides
Chondroitin Sulfate	Defined proteins
Decellularized ECM[1]	Non-protein nitrogens
Dextran	Sugars, carbohydrates
Elastin	Sterol and acyl lipids
Fibrin	A, B, C, and E Vitamins
Gelatin	Enzyme activities
Gellan Gum	Metals (trace elements)
HAMA[2]	Cytokines, factors
Matrigel	Peptide hormones
Methacrylated CS[3]	Steroid hormones
Methylcellulose	Transport agents
PPF[4]	Detoxifying agents
PHEM[5]	Antiapoptotics
PEGDA[6]	Protease inhibitors
PEG / PEO[7]	Shear-force reducers
PGLCS[8], PVA[9], Pluronics	Antibiotics
PLA[10], PGLRA[11], PGLYA[12]	Antimycotics
Polyacrylamide	Acid/base/buffers
Polycaprolactone	Antibiotics
Silk fibroin	Shear protectants
HMW structures of above	Viscosity enhancers

1. ECM: Extra cellular matrix
2. HAMA: Hyaluronic acid methacrylate
3. CS: Chondroitin Sulfate
4. PPF: Polypropylene fumarate
5. PHEM: Polyhydroxyethylmethacrylate
6. PEGDA: Polyethylene glycol diacrylate
7. PEG/PEO: Polyethylene glycol/oxide
8. PGLCS: Polyglycerol sebacate
9. PVA: Polyvinyl alcohol
10. PLA: Polylactic acid
11. PGLRA: Polyglycerolic acid
12. PGLYA: Polyglycolic acid

3. Accommodating Newest Approaches

Newer methods being promoted today include the use of hybrid multicomponent bioinks, deposited in a multi-step and even multi-mode 3D printing process. This will obviously demand a higher level of process monitoring, equipment integration, and process control. Co-deposition of two or more bioink streams can integrate desirable physical properties from each constituent component and exhibit complex phase behavior[15]. It is notable that both the effect of the matrix upon a cell type or implantation environment as well as the inclusion of high complements of cells upon the properties of the gel or matrix itself must be considered. Surprisingly, the exact nature of the reticulation of many matrix monomers (actually homo- or hetero-oligomeric complexes), as well the matrices' effects upon the biological nature of the printed construct, are only becoming understood. For example, the "functionalization" of substrate components, including the introduction of soluble cell-binding inducers to matrix components has demonstrated enhanced cell adhesion and spreading[17]. Peptide gels with simple composition and tunable physical properties have been developed to facilitate targeted differentiation[21]. Post printing perfusion with growth or differentiation factors can drive multipotent cells to a desired lineage[22]. Immediate nutrient mass-transport has been facilitated by both anisotropic matrix environments guiding cellular alignment and microchannel architectures as well as the engineering of inherently high isotropic matrix porosity[23]. Finally, issues have arisen in the application of even elegantly designed approaches — such as the observation of an inverse relationship between printed cell density and robust functionality in immediate scaffold development. Generally, therefore, each application must be studied on its own.

3.1 Cultured Cell-support

When cells are included in a printing operation, maintenance of their viability and state must be considered. Suspensions of robust eukaryotic cells can survive for short periods of time in poorly controlled environments in simple buffered salt solutions. However, for optimized performance and in extended pre- or post-printing incubations, many of the common ingredients of modern cell culture media must be supplied either within the bioink formulation, or made available immediately post printing. Thus, while there are mechanical properties to be considered (such as in protecting from shear-stress and/or establishing cellular orientation) in cell-support, the biomolecular characteristics are also important.

3.2 Maintaining Robust Viability

All printing processes involve an ink being formulated, stored for significantly variable periods of time before or during the printing process, pumped through a nozzle or applied to a dispensing or culture plate, and followed by the actual integration into the progressing construct. There, the cells remain in the media, buffer or bioink until at least the remainder of the construct is completed. From that point onwards, the ambient environment of cells in the nascent construct may or may not be altered during a post-printing "maturation". Throughout these stages the cell needs must be supported, and any of these activities can affect the cells viability, differentiation, adhesion, state, functionality and up-or down regulation such as specifically inducing apoptosis.

4. Major Considerations

When cells are included in a printing operation, maintenance of their viability and state of health must be considered. Suspensions of robust eukaryotic cells can survive for short periods of time in poorly controlled environments in simple buffered salt solutions. However, for optimized performance, especially over extended print periods including pre- or post- printing staging operations, many factors must be considered (Vcdrg'4).

4.1 Biomolecular Characteristics

First, while there are mechanical properties to be considered in cell-support (such as protecting from shear-stress and/or establishing cellular orientation), the biomolecular characteristics are also important. Many of the common ingredients of modern cell culture media must be supplied either within the bioink formulation, or made available immediately post printing. Furthermore, most nascent constructs may be altered during a post-printing "maturation" operation. Characteristics of the cells ambient fluid media throughout this are important.

4.2 Environmental Parameters

Second, depending upon the cells, bioink formulation and printing style, it can be critical to control environmental parameters during these steps. These can

Table 2. Considerations in the development of a bioink and 3DBP culture media

- Cell-specific metabolites/factors
- Printing-specific rheology values
- Application-specific matrix elements
- Specifically control or inhibit apoptosis
- Support or inhibit further differentiation
- Co-culturing and tissue environment effects
- Address altered cell metabolism rates and flux
  - Existing media formulations are optimized for
    - rapidly dividing cultures
    - low-density culture
  - There may be complex gradients
    - moving from culture expansion to printing
    - moving from 3D culture to *kp'xkxq* placement
- Material sourcing, qualification, QA and regulatory
- Unique matrix and matrix-active component effects
  - ECM / glycans / saccharides / polyesters / poloxamers
  - Supramolecular chemistry support / control
  - Spontaneous intra- and inter-molecular self-assembly
    - Concentration, ion types, pH
  - Involve multiple linkage types
    - hydrophobic, SS/disulfide bridge
    - Can be assisted
    - Hofmeister series
  - Can be inhibited
    - HAPs DTT, carbonate
    - Must be protected
    - bonds are reversible
  - Reported factor sequestration/binding
- Active and passive rheology effects
  - Additives modulating osmolality and density
  - Additives modulating viscosity and surface tension
  - Deposition in plastic flow, rapid elastic response
  - Consequences of flow rates, nozzle size and hydrodynamic forces
- Print matrix-specific stresses
  - Unusual light, temperatures and pressures
  - Unusual gelling agents, polymerizers, crosslinkers
- Serum-free, xeno-free and protein-free ideal
  - Can consider FBS and animal protein-based formula
  - Regulatory, risk, cost and consistency considerations
- Heightened buffering/antioxidant demands
  - Variable mass-transfer rates & environment
  - Often at high air interface-to-medium ratios
- High plastic mass-to-medium volume ratio
  - Sorption of lipophilic vitamins/lipids/sterols
  - Heightened leachable and particulates concerns

also affect the cell's viability, differentiation, state, functionality and up- or down-gene regulation. One way of providing some degree of environmental control is in specifically engineered dedicated housing immediately surrounding the printer (Advanced Solutions, www.advancedsolutionsonline.com). Another approach is to house the entire printing assembly inside a modular isolator that actively controls such environmental parameters as temperature, CO_2 and humidity. Such equipment also prevents contamination from both exogenous microbes and aerosols generated during the printing process. Randy Yerden, CEO, BioSpherix (www.biospherix.com) recently observed, "Modern cytocentric isolators can aseptically and safely accommodate bioprinters of any dimension, as well as ancillary equipment — plus control critical cell parameters at optimum CO_2 and O_2 levels during printing".

4.3 Many Cellular Requirements

Bioinks may be required to support (for various durations) the stable culture of stem cells, co-culture of diverse differentiated cells, vasculogenesis or other cellular or tissue functions. The cellular requirements can include primary metabolites/factors optimized to the cell populations being printed or unique requirements due to the nature of the (pre- and post-) printing environment. In some applications, a formulation may be required to support 3D high-density culture in a specialized environment achieved post-printing. This includes post-deposition matrix crosslinking or polymerization forces or chemistries. The type and level of cell growth, attachment and other culture factors may be adjusted to accommodate the different demands or function placed upon the cells post-printing, or due to the factor-sequestration by some printing matrices. An increased or different buffering pH chemistry may be required due to the pre- and intra-printing ambient gas mixture. Accommodation of such printing-specific stresses as hydrodynamic or dehydration forces are especially important as the process progresses from the common product development-supporting serum-containing media to a more regulatory-friendly serum-free formulation. As the types of stresses induced by the printing process are known to induce apoptosis or differentiation in some process cell complements, ingredients known to inhibit these undesired responses may be included. As the concept of 4D bioprinting progresses, formulations to either promote or inhibit post-printing differentiation will likely be more strongly considered. Finally, due to the nature of the disposable bioink storage and print-

ing materials, accommodating (or ameliorating) heightened leachables contamination as well as sorption of lipophilic vitamins or lipids may be considered.

4.4 Application-specific Factors

Depending upon the application, a number of additional manufacturing aspects may need to be considered. For tissues, tissue-mimics or other structures related to either cell and tissue therapies or in IVD applications, the quality and regulatory implications of the bioink must be examined, as has been begun for printed medical devices[24]. For these applications especially, the nature and number of particulates from the disposable components of the printing path could be significant. The composition of biopapers, or a matrix upon which the ink is applied during printing, is another consideration in some applications. An interesting new development for applications employing human pluripotent stem cells is the announcement by GE Healthcare that a serum-derived protein supplement in a completely defined, xeno-free medium can support stable culture of human pluripotent stem cells on untreated matrix[25].

4.5 Vascularization

The importance of including a vascular component in digitally biomanufactured tissue constructs as both a means to provide perfusion to a tissue and impart relevant functionality (as the vasculature also contributes to tissue function) is well appreciated. The ability to establish and maintain a functional microcirculation *in vitro* significantly impacts a broad array of biomedical arenas[21,22]. In virtually every discussion concerning the building of tissue replacements, the critical importance of having a microvasculature integrated into the tissue construct is stressed[23–25]. In cellular assay platforms, the presence of a perfused vasculature in combination with the target parenchyma cell is considered to improve the utility of the assay beyond having just parenchyma cells[26]. Significantly, the smaller elements of the vasculature, the microvasculature, pose unique challenges in a biomanufacturing process. A stereotypical microvessel is comprised of multiple cell types (endothelial cells, smooth muscle/contractile cells, perivascular mesenchymal cells, and immune cells) assembled in a very structured way critical to the microvessel's function[26]. In addition, many individual microvessels are needed (perhaps thousands in some applications) to assemble and effective perfusion circuit. Finally, the organiza-

tion or topology of the microvessels in the perfusion network impacts overall performance[27]. Thus, the incorporation of a vascular supply into a manufactured tissue construct must address the formation of each of the numerous, complex individual microvessels and their integration into a perfusion circuit[28] matched to the needs of the tissue parenchyma.

4.6 Angiogenesis and Vasculogenesis

New microvessels arise from either angiogenic sprouts of existing, parent microvessels or the *de novo* assembly of vascular cells into the microvessels called vasculogenesis[27]. A variety of cell types and strategies have been employed to derive microvessels. These include the use of endothelial cells, both macrovascular and microvascular, endothelial progenitor cells (EPCs), perivascular cell precursors, mesenchymal and hematopoietic stem cells (MSCs and HSCs), and smooth muscle cells incorporated into the construct either alone or in combination. Adipose stromal vascular fraction (SVF) cells show particularly robust vasculogenic activity, perhaps because all of the cell types necessary to forming microvasculatures are present within the isolate[20,29]. Angiogenesis-based strategies include pre-packaging endothelial cells in clusters or aggregates, from which neovessels sprout[30], or the use of intact microvessel fragments as a source of parent microvessels from which neovessels arise via angiogenesis[28].

4.7 Post Printing Cues

In all cases, the newly formed microvessels (or neovessels) are immature in form and function, requiring hemodynamic cues to drive subsequent maturation[31]. This vascular maturation, of both the individual neovessels and network, depends on substantial remodeling and adaptation activities, as the neovessels specify into arterioles, capillaries, and venules and integrate into a contiguous network. Therefore, consideration of bioinks amenable to successful fabrication of vasculatures in a digital biomanufacturing process should support not only vascular cell viability, but also promote individual neovessel assembly and permit adaptation to a mature microvasculature.

5. Vascular Compatible Bioinks

Nearly all the bioinks used with non-vascular cells will also support vascular cells. Furthermore, leveraging the potent self-assembly capabilities intrinsic to vascular cells, these bioinks readily enable formation

of neovessels, whether by angiogenesis or vasculogenesis. This is true of those materials in which vascular cells are incorporated at the time of fabrication (i.e., a hydrogel) or rigid scaffolds that are made and subsequently seeded with vascular cells or vascular elements[32,33]. Moreover, many of the native matrices used as bioinks have intrinsic pro-angiogenic activity such as tumor matrix and hyaluronic acid gels[34,35]. Of course, many strategies have doped bioinks with angiogenic factors either to drive vasculogenesis/angiogenesis from embedded vascular precursors and/or recruit vessel ingrowth into the construct via angiogenesis. The different materials used promote vascular adaptation to different degrees with softer, native matrices being the most favored. Rigid scaffolds do support vascular adaptation, however, this relies on the spaces between the rigid elements, where the neovessels reside, being filled with a softer material.

5.1 Synthetic Channels

An alternate approach to incorporating a perfusion supply involves creating channels through a matrix within which vascular cells (usually endothelial cells) are seeded onto the channel walls, thereby fabricating a simple vessel-like element. Connecting the channels to each other results in a perfusable network of endothelial cell-lined channels serving to provide a means fluid flow through the construct. The endothelial cell lining adds a biological dynamic to the channels by functionalizing the fluid-tissue interface as a regulated exchange barrier. However, adaptation into more native-like microvasculatures is limited as the channel topology is fixed and vascular remodeling, even with the addition of other vascular cells is constrained. Cellularized channel systems are usually made either by soft photolithographic methods or 3D bioprinted sacrificial reverse molds[36].

5.2 Combining Approaches

The latest efforts at establishing a microcirculation *in vitro* seeks to combine the microfluidic endothelial cell-lined channel platform with a native, derived microvasculature. Here, the channels serve as a perfusion source which, when contiguously connected to a neighboring microvasculature, help to drive the formation of a microcirculation. Often, vascular cells are used to form the initial, native microvasculature to be connected to the channel system[37]. In contrast, Advanced Solutions Life Sciences is using isolated microvessels to form the native microcirculation[36].

5.3 Biomanufacture of Vascularized Systems

With the promise of these *in vitro* microcirculations, exciting new opportunities arise for building more native-like tissue models and mimics for use in the laboratory (and eventually tissue replacement). However, these vascularization advances raise new biomanufacturing challenges as the complexity of the systems rise. For example, individual cell types within systems such as endothelial cells, other vascular cells, targeted parenchymal cells (e.g., hepatocytes, tumor cells), and tissue-specific stromal cells all have unique media and microenvironmental requirements that must be coordinated to support the construct as a whole. Also, new biofabrication strategies addressing when and how to integrate vasculatures with parenchyma cells and other cells types, including staged incubation steps, need to be developed. While 3D bioprinting is a key fabrication approach, the successful strategies in the future will undoubtedly include other fabrication methods. Related to this, organizing manufacturing workflows becomes paramount as different fabrication steps are staged through the entire manufacturing process. While these practices are common to non-biological manufacturing programs, their applications to biomanufacturing have yet to be comprehensively implemented (Figure 1). However, new tools enabling these broader biomanufacturing activities with living systems are emerging[36] and groups are beginning to develop the concepts and methods needed to build complex tissues.
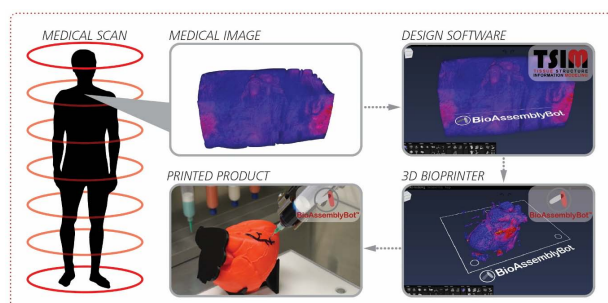

Figure 1. Example work flow (arrows) for digital biomanufacturing. A medical scan of a patient (an MRI of the chest) is imported directly into a commercially available prototyping software (TSIM®, Advanced Solutions Life Science). The biological content (i.e., the structure to be fabricated) is extracted from the image set to produce a 3D digital prototype which is then used to print the physical version in a contour-printing capable robotic arm printer (BioAssembly Bot®, Advanced Solutions Life Science). The process entails a spectrum of technologies including image processing, *in silico* model generation, biology, clinical data, 3D prototyping, robotics, biomaterials, and cell biology.

6. Conclusion

Bioprinting of vascularized tissues has demanded a harmonization of diverse technologies, equipment and materials[38]. Multi-matrix material bioinks are being developed meeting each structural, biologic and regulatory requirement[39]. Multimodal printers can deposit with high speed, mass and resolution[40]. Novel algorithms and software package guide the deposition of neovessels of various sources printed into synthetic networks designed to mature into a contiguous network of arterioles, capillaries, and venules. Digital biomanufacturing promises continuity and optimization of tissue printing operations by insuring real-time access to the required information through high-demand calculations upon rich, timely data.

Conflict of Interest and Funding

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