Biofabrication: A Guide to Technology and Terminology

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Abstract

Biofabrication holds the potential to generate constructs that more closely recapitulate the complexity and heterogeneity of tissues and organs than currently available regenerative medicine therapies. Such constructs can be applied for the regeneration of tissues or as *in vitro* 3D models. As biofabrication is maturing and growing, scientists with different backgrounds are joining this field, underscoring the need for unity regarding the use of terminology. Hence, we believe there is a compelling need to clarify the relationship between different concepts, technologies, and description of biofabrication that are often used interchangeably and/or mixed in the current literature. In doing so, we propose the introduction of a comparative tool to address currently established biofabrication technologies in terms of balance between resolution and speed of fabrication. Our objective is to provide a guide on the terminology used to identify different technologies in the field, which can serve as guidance for the biofabrication community.

What do we mean by biofabrication?

Biofabrication combines principles of engineering, biology, and material science and holds the promise to change the toolbox for many biotechnological disciplines. Recently, in the context of tissue engineering and regenerative medicine applications, the definition of biofabrication as a research field was updated as *"the automated generation of biologically functional products with structural organization from living cells, bioactive molecules,* ***biomaterials****, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes." [1]*. This definition includes the fabrication of scaffolds with hierarchical structural properties or smart surface properties within the realm of bioprinting. It was reasoned that the design of such features would be indispensable to obtain structurally functional biological substitutes. This work provided an overview of the historical evolution and broader meaning of the term, and also specified the research field with a focus on applications in tissue engineering and regenerative medicine, and proposed bioprinting and bioassembly as the two major approaches to biofabrication. Despite this definition and positioning of the field, as well as recent reviews that nicely provide a common framework to the additive manufacturing field at large [2-5], the terminology commonly used especially in recent literature is not clearly defined and lacks consensus. This absence of an agreed and accepted terminology can and partially already does lead to uncertainty or confusion in the description of new approaches and possible misunderstanding as to where a new report fits in relation to previous reports. This could impede the development of the field by making it difficult to correctly map progress in the science and technology of biofabrication.

Here, we intend to follow-up from our previous review updating the definition of biofabrication for tissue engineering and regenerative medicine applications *[1]*. We want to set an overarching terminology framework by clarifying the technologies used within biofabrication strategies, as well as rationalising appropriate terminologies, as an integral communication basis for all the different application fields of biofabrication. We, therefore, feel that a brief review of classical and novel biofabrication approaches, with the aim to point out the differences among them and the limitations

that must still be overcome, is timely. A glossary of the main different terminology clarified in this article is also provided in this article. We also point out potential future research directions where we believe biofabrication may have a major impact, with the objective to collaborate with industry to bring biofabrication strategies to the clinic in a more efficient and consensual manner and to collectively overcome future regulatory and ethical challenges.

Technologies used for Biofabrication

As previously indicated, biofabrication strategies employed for tissue engineering and regenerative medicine can be identified as either bioprinting or bioassembly. Such strategies can be divided into two categories: (i) **scaffold-based** and (ii) **scaffold-free strategies**. In this context, scaffold means a three-dimensional (3D) porous structure that serves as a support for cell adhesion and guides tissue regeneration.

To further classify the various bioprinting and bioassembly technologies in terms of efficiency of fabrication, we introduce the "spatial **Resolution/Time for Manufacturing"** (RTM) ratio as a quantitative characterization of the process underlying a specific technology, considering the ability to produce scaffolds with fine details in a short time as a figure of merit. RTM is defined as:

$$RTM = \frac{Spatial\ Resolution}{Time\ for\ Manufacturing} \stackrel{\text{def}}{=} R \cdot P = \frac{1}{d} \cdot \frac{V}{t}$$

Here, R is the best spatial resolution that can be achieved within the technology and P is the delivery rate of the material being printed or assembled. R is expressed as *the inverse of* the **minimum feature** dimension "*d*": d is measured in meter, thus R is measured in 1/meter; *P* is expressed as volume "*V*" of material (measured in m^3) delivered per unit of time "*t*" (in minutes). As consequence, the physical dimensions of the RTM ratio are square-length/time. In the biofabrication field, the order of magnitude of R and P are 1/µm and mm^3/min respectively: hence, the RTM ratio has to be expressed in 10^{-3} m^2/min for an easier comparison between different technologies. Note that, for each specific technology, *R* and *P* may vary, depending on the material delivered, as well as on the geometry of the scaffold and on its placement in the building chamber.

In the following sections, standard operating procedures are considered (such as commonly used materials, average printing parameters, single or multiple material deposition head) for building a 1 cm side cube of material (= 10^{-6} m^3), lying on one of its faces. Table 1 lists the average RTM ratios for the biofabrication technologies that will be considered in the present work. Figure 1, Key Figure, gives a graphical representation of the distribution of the various biofabrication technologies in the parameter space with axes of minimum feature dimension *d* and delivery rate P: as explained above R and P may vary for each technology, which are represented by circles. In Figure 1, the contour lines of the RTM function are also plotted: technologies along the same contour line have the same RTM. Generally speaking, the higher the value of RTM, the more efficient the process. Most of the technologies are placed along the diagonal of the parameter space "d-P", indicating that higher delivery rates result in lower resolution, and fabricating fine details are in contrast with fast manufacturing. Here, we briefly

outline the most common technologies in each of these strategies in order to illustrate and illuminate their differences, advantages, and limitations.

Scaffold-based strategies. The most commonly used technologies in scaffold-based biofabrication comprise i) **3D printing**, ii) Light-based technologies such as **selective laser sintering** (SLS), **selective laser ablation**, **stereolithography** (SLA) and **two-photon polymerization** (2PP), iii) **fused deposition modelling** (FDM), iv) **wet-spun automated extrusion systems,** v) **3D plotting**, v) **ink-jet printing**, and vi) **electrospinning** (Figure 2). Most of these technologies were originally developed as additive manufacturing technologies for rapid prototyping, but are included as biofabrication strategies when used for biomedical applications.

3D printing. With 3D printing, a jet of binder is directed at a powder-bed to define a pattern controlled by computer-aided design/computer-aided manufacturing (CAD/CAM) software. The solvent binds the powder, thus forming a slice of solid material; subsequently a new layer of powder is laid down and the process is repeated to build the scaffold layer-by-layer [6, 7]. The unbound powder acts as a support for the object during building, allowing the easy fabrication of re-entrant and hollow objects. It can be difficult to remove excess unbound grains and remnants of used solvents/binder. In this respect, it is important to highlight that the term 3D printing should only refer to this specific additive manufacturing technology. This technology allows the fabrication of structures with a lateral resolution controlled by a combination of the powder particle size and the volume that the solvent penetrates by capillary action. Current commercial systems have a feature resolution around 300 μm, which is suboptimal if precise control of cell positioning is required after scaffold fabrication. Its RTM ratio is about $0.1*10^{-3}$ m^2/min, so at the medium level of manufacturing efficiency.

Light-based technologies. SLS uses a laser where the beam of light is selectively directed to a powder-bed, generating local heat and forming patterns of fused material; after its solidification, a new layer of powder is laid down and the process is repeated to build the scaffold layer-by-layer. A variety of thermoplastic polymers [8], metals (in this case SLS is called selective laser melting), ceramics, mixtures of polymer-ceramic [9] and polymer encapsulated ceramic [10] can be used, but the required high temperatures limit the utility of SLS for biofabrication processes. The source materials that are normally used require extra processing to obtain these in powder form with a precise and narrow size granulometry. Selective laser ablation works in the opposite way by ablating of a solid material using a very short time duration laser pulse [11, 12]. If the ablation process is conducted in all of the three directions or if laminated porous films are stacked and bonded on top of each other, a 3D structure can be created. These techniques have proved useful for the fabrication of improved tissue constructs upon seeding the scaffold with cells [13-15]. These technologies do not allow the direct incorporation of pharmaceuticals, biomacromolecules (proteins, growth factors) and cells into the scaffold. Thus, they could be considered biofabrication technologies only when the fabricated scaffolds are designed following hierarchical or smart principles to influence cell activity and achieve functional biological constructs, as previously discussed [1]. In these methods, the resolution of the printed pattern depends on the laser spot size, the thermal conductivity, the surface tension and the absorption of the raw materials, and the grain size. Due to heat conduction, resolution is inevitably larger than the spot size. The RTM is around $1*10^{-3}$ m^2/min for both technologies, which means that these techniques are fast even if the resolution is comparable with that of other methods.

A promising modification of SLS that can release active compounds (e.g. ribonuclease, an exceptionally stable enzyme), is surface selective laser sintering (SSLS) [16], which uses an infrared laser to sinter powder substrates. In this case, the radiation is not absorbed by the polymer particles but by carbon micro-particles spread on the surface of the polymer particles. However, due to several challenges, such as the use of carbon micro particles without proven track record of biocompatibility, the processing of polymers into powder, the lack of extensive studies with unstable biologically active compounds, this technology is not yet ready for the clinic.

SLA, in which light is used to solidify a photosensible resin, has been typically used to produce a negative replica that is filled with ceramic or metallic slurries, and subsequently removed by sintering at high temperatures [17]. Biocompatible and biodegradable photosensitive polymers that can be used in SLA to directly fabricate 3D scaffolds are also being developed and investigated. A few newly developed photosensitive resins are starting to appear in the biomaterials field for this purpose, which have also opened the possibility to use a light projection system instead of a laser source (known as Digital Light Processing, DLP^{TM}) [18, 19]. Some of these also comprise hydrogel formulations, which would then allow the incorporation of cells into the biofabrication process. Despite enhanced resolution (~20-40 μm) with SLA, the biocompatibility of these photopolymers and the photoinitiators used for their cross-linking has to be fully validated. Incorporation of any biological material, including cells, depends on their sensitivity to the light source used [20]. In addition, this method is limited to a cell type that could be incorporated into a hydrogel. Using the data in literature, an RTM ratio of about $0.5*10^{-3}$ m^2/min can be estimated for SLA, which places this method among the more efficient techniques. In case of DLP, the RTM can be increased to $2*10^{-3}$ m^2/min thanks to parallelization of the light beam. An improvement of SLA with respect to spatial resolution is two-photon polymerization (2PP), where a spatially and temporally confined laser beam polymerizes a photosensitive resin [21, 22]. The resolution of this technology can reach the sub-micron scale. Although new photocurable biomaterials with satisfactory biocompatibility are being developed and the speed of this fabrication process has improved, further progress in both materials development and fabrication rate is needed. The RTM is $0.05*10^{-3}$ m^2/min, which means that 2PP has a very high resolution despite taking a long time to manufacture big structures.

Fused deposition modeling. FDM has been extensively used to fabricate custom-made scaffolds and to modulate their mechanical properties for tissue engineering applications, with encouraging results [23-25]. In FDM, molten thermoplastic polymers are extruded into filaments. These filaments are deposited to form a layer and a 3D scaffold is built layer-by-layer. The entire process is controlled by a CAD design. A number of biocompatible thermoplastics have been developed and processed with this technique. However, the majority of the published work has used polycaprolactone as the polymer of choice because of its relatively low melting temperature and its commercial availability in medical grades. Many other extrusion-based tools inspired by FDM have been developed to fabricate 3D scaffolds. These also comprise multi-dispensing systems, such as **3D-fiber deposition** (3DF) and **bioextrusion** that allow depositing different materials at the same time to produce constructs with locally differing physico-chemical properties [4, 23, 26]. The main difference between FDM on one hand, and 3DF and bioextrusion on the other, is that in 3DF and bioextrusion the biomaterials are loaded in a cartridge as pellets or particles instead of being extruded in a filament form. This approach has the advantage of expanding the palette of biomaterials that can be used compared to FDM. An associated disadvantage of 3DF and bioextrusion is the higher susceptibility to thermal degradation due to the long residence times

of the raw material at high temperatures. Although FDM, 3DF, and bioextrusion techniques have considerably improved the quality of tissue engineered constructs with respect to their functional performance [25, 27-29], the high temperatures involved during the fabrication of molten polymers may limit the direct incorporation of biological factors with the technique. A solution could be envisioned if not only metallic [30] or ceramic pastes [31] are employed, but also polymeric pastes that can be processed at room temperature. Alternatively, surface modification techniques could be used to functionalize the fibres and allow grafting of the bioactive agents in specific sites [32]. Additionally, hydrogels that encapsulate biological components could be deposited together with thermoplastic materials, thereby circumventing the limitations [33, 34] imposed by the high temperatures. FDM, 3DF, and bioextrusion have the highest RTM ratio of all methods, around $1*10^{-3}$ m^2/min. Because the post processing phase is practically non-existent, there is a limited need for intervening layers or binders and solvents to remove excess material, as with most other techniques.

Pressure-assisted microsyringe deposition (PAM) and other **wet-spun automated extrusion systems** have been developed to solve the disadvantages associated with high temperatures in FDM, at the same time achieving scaffolds with a higher fiber resolution (~80 μm for FDM vs 10 μm for PAM) [35, 36]. PAM is part of the PAM^2 system, where several working modules can be mounted in parallel on a robotic micro-positioner, for processing at the same time synthetic and natural polymer solutions and living cell suspensions [36, 37]. The main drawback of wet-spun extrusion based technologies is the low vertical dimension (when high resolution is aimed) resulting in a medium RTM ratio of $0.5*10^{-3}$ m^2/min. This implies that thick constructs take more time to be fabricated than with other extrusion technologies. Recent progress by the Lewis group might, however, solve such limitations by fabricating arrays of nozzles that can deposit multiple filaments at the same time [38].

The major limitation of most scaffold fabrication processes reported in the literature is that each technique is applicable only in particular conditions (e.g. rheology, pressure, temperature, voltage etc.) that restrict the choice of materials. Hydrogels composed of natural polymers (e.g., collagen, gelatin, hyaluronic acid), either in combination with additional biological factors or used alone, are intrinsically biocompatible and biodegradable, and possess biological cues [39]. However, these natural-polymer hydrogels are difficult to process with the techniques discussed so far. As an alternative, scaffolds made of natural biomaterials can be produced by indirect fabrication techniques (e.g. casting a biomaterial into a sacrificial mould realized by additive manufacturing processes). Indirect methods to produce additive manufactured scaffolds have emerged in a number of different approaches with promising results [40]. The development of an alkali soluble photopolymer allowed the use of **indirect additive manufacturing** with hydrogels [41]. In particular, gelatin and collagen scaffolds could be produced by applying indirect SLS manufactured moulds with high resolution and a minimum pore or strut size, on the scale of several tens of micrometers [42, 43]. Indirect methods could be also combined with conventional techniques, such as salt leaching and phase inversion, to fabricate dual-pore scaffolds [44, 45]. Some drawbacks still exist with indirect approaches, including the poor resolution of the additive manufacturing techniques, the casting procedure and the extraction methods. For these reasons the RTM ratio is ~$0.03*10^{-3}$ m^2/min.

Bioplotting and ink-jet bioprinting. Whereas all of the above-mentioned methods have demonstrated different degrees of success in fabricating 3D scaffolds that can accommodate cells that can develop into tissues or proto-tissue structures, most of them are incapable of simultaneously depositing biomaterials

and cells. Therefore, cells need to be separately seeded into the scaffolds produced by these techniques. This limits the flexibility to mimic cell distributions in native tissues particularly when strategies for the regeneration of multiple tissue interfaces or organs are to be developed. Three main set of technologies that have demonstrated the ability to incorporate cells during the process of additive manufacturing into a biomaterial carrier are 3D plotting (or also known as **bioplotting and extrusion bioprinting**), ink-jet bioprinting, and **valve-jet** bioprinting. In bioplotting, the cells are typically encapsulated into a hydrogel carrier biomaterial and extruded by the application of pressure, similarly to wet-spun extrusion based technologies [46-48]. This technique allows the deposition of different cell types in different hydrogel formulations, but is still limited in terms of speed of production and fabrication of constructs with complex shape, mostly due to the lack of optimal hydrogel carriers (also called **bioinks**). The technique also carries some limitations if a stable structure is to be formed. For example, it may be necessary to use a plotting bath containing a fluid of matching density and viscosity to the extruded material to prevent sagging or deformation of the construct immediately after extrusion, or alternatively to use a hydrogel with sufficient viscosity to self-sustain its own weight after processing. An additional approach that has emerged is the extrusion of material into 3D space of another material, in contrast to building a structure from a surface [49-51]. This has been most commonly performed with hydrogels (e.g., continuous or colloidal suspensions) where the material displaces as another material is extruded into it, and has been used to print suspended objects or open-channel structures with the use of sacrificial materials.

In ink-jet bioprinting, cells encapsulated into hydrogel carriers are dispensed in a droplet fashion. By ink-jet bioprinting, it is possible to exquisitely control the number of cells per deposited droplet, thus resulting in a finer control in cell distribution in the fabricated constructs [52, 53]. The development of optimal hydrogels as bioinks for both bioplotting and ink-jet bioprinting remains a challenge and the fluid requirements for both methods are quite different in terms of fluid viscosity and surface tension [54]. The RTM ratio is around $0.5*10^{-3}$ m^2/min for bioplotting due to rapid scaffold production but low resolution, and around $0.1*10^{-3}$ m^2/min for ink-jet bioprinting due to high resolution but also high fabrication time per unit volume. In both technologies the presence of living cells in the bioink limit the RTM ratio: higher extrusion flow and smaller nozzles can induce damages due to shear stress on cell membranes [46-53].

Similar to the ink-jet technique, valve-jet bioprinting is a non-contact, droplet-based method where cells are printed with or without hydrogel carriers [55]. The actuation mechanism of valve-jet is based on pneumatic pressure and ejection of droplets is controlled by solenoid microvalves instead of piezo- or thermal actuators as in ink-jet printing [56]. Currently, the printing resolution (e.g. nano-liter droplet) and throughput (e.g. 1-1000 Hz) of valve-jet bioprinting lie between bioplotting and ink-jet bioprinting, so does the printable fluid viscosity range (up to 100 Pa.s). As the technology is not limited by the nozzle size (like ink-jet printing), the shear stress can be minimized and therefore the technology is amenable to print delicate human pluripotent stem cells [57]. The RTM ratio is therefore around $0.3*10^{-3}$ m^2/min for the valve-jet bioprinting.

Electrospinning. A further promising technique for scaffold fabrication is electrospinning. This technique produces fiber meshes with physical features mimicking those of the native extracellular matrix (ECM). The fiber meshes are created by passing a biomaterial solution through a high voltage electric field near the deposition nozzle. At a defined voltage threshold, which is specific for a defined biomaterial solution,

the surface tension of the biomaterial solution is overcome by the applied electric field, resulting in the formation of an electrohydrodynamic Taylor cone from which fibers are spun and collected on a grounded target plate [58]. Despite being a relatively old technology, originally developed for textile fibre production, this technique is now widely used by the tissue engineering and regenerative medicine community because of the wide range of materials available to the technique and the methods of fibre collection that allow for an expansive spectrum of structures and shapes to be fabricated [59, 60]. An important recent development in the field of electrospinning is the possibility to control the deposition of fibers at a scale of a single fiber. This has been achieved by the so-called near field electrospinning technique for biomaterials in solution and by melt electrospinning writing for molten polymers [61, 62]. Together they constitute the method of electrohydrodynamic writing in which predictable fiber paths are used to direct-write small diameter fibers onto a translating collector. This new electrospinning modality can potentially be used to create scaffold structures that can better mimic the native ECM not only from the physical dimensions of the constituent fibres, but also in terms of structural organization. Although cells have been shown to maintain their viability after the electrospinning process [63, 64], reports describing the possibility of electrospinning cell-laden hydrogels are still limited [65, 66]. The RTM ratio of this technique is currently around $0.1*10^{-3}$ m^2/min.

Scaffold free strategies. Along with the scaffold-based approaches, a number of alternative biofabrication strategies that use biomaterials to provide only structural integrity have been developed (Figure 3). The first examples of these strategies are the works of Forgacs and co-workers who dispensed **cell spheroids** and cylinders into a hydrogel bed using special purpose extrusion bioprinters [67-69]. The hydrogel is used as a support, while a tissue-like structure forms by exploiting the biophysical principles of **tissue liquidity** that governs the fusion of adjacent cell aggregates. In this manner, branched vascular networks [67, 70], nerve grafts [68, 71] and other tissue modules [69] have been successfully fabricated. Additional applications of this technology resulted in commercial products in the form of architecturally and functionally correct human tissue constructs for drug toxicology essays [72]. The principal limitations of this approach are i) the relatively slow fusion of the cell aggregates, which takes typically at least 24-48 hours depending on the cells used and may lead to a somewhat inhomogeneous construct, ii) low spatial resolution, due to the use of micropipettes of relatively large diameter (300 or 500 μm) for the preparation and deposition of gels and cells; and iii) a still limited diffusion of nutrients when large constructs are fabricated. Due to these limitations and to the low resolution of this method, its RTM ratio is less than $0.001*10^{-3}$ m^2/min.

A similar technology was developed by Nakayama and co-workers, who used an ingenious skewering system [73, 74], wherein the cell spheroids are placed on fine metallic needles (i.e. skewers), geometrically positioned as to be consistent with the shape of the desired organ structure (e.g. tubular construct; Figure 4). The novelty in this technology is that the needles prevent the otherwise unavoidable shrinking of the construct upon the fusion of the spheroids (at least in one direction). Others used a tangram-based concept, where different cellular shapes were left to fuse with each other and self-assemble into a macroscopic tissue construct [75-77]. Achieving fully vascularized large constructs with these tissue liquidity-based strategies is still an open challenge. Due to long aggregation time of the cell aggregates, the RTM ratio is slow, less than $0.001*10^{-3}$ m^2/min.

Another approach developed originally by Chrisey and colleagues [78] and later further adopted by Guillemot and colleagues [79] is based on **laser assisted bioprinting** (LAB). This technique has also been known as **laser induced forward transfer** (LIFT) and matrix assisted pulsed laser evaporation (MAPLE). The deposition system is composed of three components: 1) a pulsed laser source, 2) a target from which a biological material is printed, and 3) a receiving substrate that collects the printed material. The target is made of a thin absorbing layer of metal (such as gold or titanium) coated onto a laser transparent support (e.g. glass or a transparent polymer film). Organic materials (molecules or cells) are prepared in a liquid solution (e.g. culture media or a hydrogel precursor), and deposited at the surface of the metal film. The laser pulse induces vaporization of the metal absorbing layer, resulting in the production of a jet of liquid solution which is deposited onto the substrate [80]. The resolution of this system depends on parameters such as the thickness of the bioink layer coated onto the target, the surface tension and the viscosity of the bioink, the wettability of the substrate, the laser fluence, and the air gap between the target and the substrate. LAB has a fairly low RTM ratio around $0.04*10^{-3}$ m^2/min, due to a high resolution and a long fabrication time, which implies that thick constructs need more time for manufacture when compared with other techniques described before, and sometimes the production time of large size structures is not compatible with cell processing times.

A different approach for the biofabrication of tissues and organs consists of bottom-up approaches (Figure 3d-e-f), where micro and nano modules are first engineered and used as building blocks to fabricate the targeted tissues. One of the most successful bottom-up approaches is represented by the cell-sheet engineering method developed by Okano and colleagues, where cells are cultured till reaching confluency on thermo-responsive culture plates, which can easily release the formed cell layers by switching the temperature from 37 °C to room temperature [81]. Larger constructs comprised of multiple layers placed together in a conventional layer-by-layer method have been fabricated and successfully brought to the clinic [82]. With a similar approach, L'Hereux and coworkers developed and brought to the clinic a layer-by-layer approach to fabricate vascular biological grafts from cell sheets [83]. Another classic example is the **micro-masonry concept** pioneered by the Khademhosseini and Demirci labs, among others, where micro-units of cell laden hydrogels are used as regenerative building blocks [84-86]. The modularity of this approach is limitless. Hydrogels of different compositions and embedding different cells can be mixed. Recently, functionalization with DNA segments was also demonstrated, which resulted in a more biologically dynamic recognition of different building blocks during *in vitro* assembly [87]. Furthermore, such blocks can be also precisely positioned using micro-robots [88].

Despite the great flexibility promised by these methods, further studies are needed to effectively demonstrate the fabrication of clinically relevant large vascularized constructs to compensate for the known nutrient diffusion limitations of most hydrogel systems. Similar approaches have been also developed by combining solid micro- and nano-particles with cells, thus offering the advantage of engineering the shape and size of such objects, which can possibly offer further stimuli to direct cell differentiation, particularly when stem cells are used [89, 90]. Their use in combination with cells offers the possibility to impinge on cellular condensation, which results in tissue shrinking, thus offering the opportunity to maintain the dimensionality of large tissue constructs. For bottom-up approaches the RTM ratio is lower than $0.001*10^{-3}$ m^2/min; these low values are due principally to a long time of fabrication due to cell sheets or microunits production and time necessary for the fusion of the different cell sheets or microunits.

Microfluidic technology has also boomed in the recent years to create tissue-on-chip platforms that can recapitulate key functions of targeted tissues and organs. These platforms are typically used in association with a biomaterial formulation, namely hydrogel networks, to culture cells in 3D and study mechanisms behind pathological events and possible treatments. Examples comprise studies on cancer metastasis, lung, liver, intestine, and vessels, among others [91-95]. Further development of these platforms will comprise the integration of sensors to monitor in real-time cell and tissue functionality and other biomaterial formulations to better replicate native ECM of the targeted tissues to be studied. A further advancement in these bio-assembly biofabrication strategies has been reported by the Takeuchi group, where meter long cellular fibers have been created through microfluidic technology and proved to be efficacious in the regeneration of several tissues in preclinical animal models [96]. Cellular fibers are created by encapsulating cell-containing ECM proteins in a pre-gel state in mechanically stable Ca-alginate hydrogel carrier in a co-axial manner, and upon the gelation of the cell containing ECM dissolving the carrier hydrogel. These fibers have also been weaved, thus creating cellular fiber scaffolds [97].

Implications for an integral terminology and future perspectives

Since the pioneering studies that kicked off the research activities in biofabrication [98-101], the field has seen tremendous progress, which includes not only the bioprinting of cells, but also a number of other approaches where cells and biomaterials have been processed to fabricate constructs for applications in tissue engineering and regenerative medicine, and to fabricate *in vitro* models for pharmaceutical screening [102, 103]. In addition, biofabrication can also serve as inspiration for a number of other life science sectors, including food, cosmetic, sensing and diagnostic industries [104-106], by providing new generic biological building blocks. Biofabrication can furthermore be used to describe the manufacture of synthetic biological components including living cells that can be used for actuation and sensing applications [107, 108]; such structures may have applications as *in vivo* devices that do not require on board energy storage.

In this review, we have made a first attempt to define a metric that can compare the fabrication efficiency of the main current biofabrication technologies and usable with new methods as they are being developed. If used in the common range of biofabrication applications (i.e. minimum feature size *d* less than 500 µm), although dimensionally complex, the RTM ratio will objectively classify the continuous optimization and advancement of current biofabrication technologies (see text box on Factors influencing the RTM ratio). A practical example is the recent development of a new SLA technology by the DeSimone group, called continuous liquid interface production (CLIP), which allows creating 3D objects 100 times faster than conventional SLA [109]. Whether this technology could be translated to biomaterials or cell-laden hydrogels remains to be demonstrated but an estimate of its RTM is $5*10^{-3}$ m^2/min. Besides the limitation given by the physical principles behind each technology and by the chemistry of currently available biomaterials, the RTM ratio can be increased by parallelization of printing heads: light-based technologies better than others can exploit this route, and projection SLA (known as Digital Light Processing, DLP™) is a clear and extreme example in this direction [19]. Another technological challenge is the fabrication of complex anatomically shaped constructs. Whereas current

bioprinting technologies can already achieve non-intricate structures, the recent development of new colloidal inks and optimized bathing stages where the bioprinting process takes place could offer new solutions to further increase the degree of complexity in mimicking native organs [50, 51]. In particular, the use of high buoyant density liquids [110], sacrificial fugitive materials [111] or granular gels [50, 112] as media into which depositing bioinks is becoming a new exciting avenue to fabricate larger and more complex constructs. For a more in-depth review on supporting temporary sacrificial materials we refer elsewhere [113].

With the development of new bioprinting processes, it is nowadays possible to manufacture tissues with different levels of complexity (Figure 5 a-c). This is achieved by heterogeneous combination of different cells and bioinks. The degree of complexity needed to mimic and eventually replace a tissue and ultimately an organ has started to be considered for constructs tested *in vitro* [114, 115] and *in vivo [116]*. With initial minor functional outcomes, a further understanding on the level of mimicry and complexity necessary to achieve optimal functional tissue or organ replacement is expected as the field matures.

In the perspective of commercial scale production, other standards of quality should be taken into consideration, such as: (i) accuracy, or how closely a manufacturing machine's output conforms to a tolerance within a specified dimensional range, and (ii) repeatability, which captures the equipment's capability to produce consistent output, time after time. These parameters are needed to reduce the extrinsic variability of the advanced tissue models, due to micro-environmental properties, limiting the intrinsic variability related to the cells themselves [40]. Quality control requires a consensus on metrology: limiting the discussion to geometrical consideration, a least squares fit of a point cloud representing the scaffold (e.g. from a μCT scan) to its CAD model can give a measure of the fabrication error [117, 118]. Interestingly, additive manufacturing processes allow for in-process inspection of the internal structure of a part. Furthermore, the accuracy over time (long term stability) is directly related to the off-the-shelf availability and, in a more prosaic way, to shipping and storing methods [119].

Finally, new hydrogels need to be developed that are able to maintain at the same time cell viability and activity, and the physical shape of the final printed construct [120-122]. The dynamic behavior of native ECM is an appealing feature that could be incorporated into new bioinks in future biofabrication strategies. In this respect, what is nowadays called 4D printing could allow such integration where the use of stimuli responsive materials allows for a spatio-temporal change of a 3D object [123, 124]. Whether we are really witnessing 4D printing, a process that should be defined as a programmed temporal shape change occurring during the 3D manufacturing itself, or not is still to be clarified in the field. We advocate for cautious use of the term 4D printing, as all reports so far published in literature show 3D objects that can change shape after the 3D manufacturing process. Nonetheless, these time-morphing 3D objects are certainly an exciting new development of conventional additive manufacturing, which would be thrilling to see to be translated into novel biofabrication strategies (see also outstanding questions).

Concluding remarks and recommended guidelines

As the biofabrication community expands and the applications of this technology grow, it is important to establish a set of definitions and terminology that will help normalize discussion and reports of developments in the field. Some attempts have been already made in the case of 3D scaffolds, for example, with the National Institute for Standards and Technology of the United States of America adopting 3D scaffolds fabricated by additive manufacturing technologies as standards for 3D cell culture [125]. Further standards could be sought into the realm of advanced manufacturing, as recently pointed out by Hutmacher and co-workers, who coined the term “additive biomanufacturing” when standard norms like ASTM or ISO are applied to the biofabrication field [126]. In this context it is important to note that biomanufacturing means the use of living organisms to manufacture a product. In a recent review, the term has been defined more precisely as "a type of manufacturing that utilizes biological systems (e.g., living microorganisms, resting cells, plants, animals, tissues, enzymes, or in vitro synthetic (enzymatic) systems) to produce commercially important value-added biomolecules for use in the agricultural, food, energy, material, and pharmaceutical industries" [127]. Therefore, we wish to stress that, according to the most recent definition of biofabrication [1], “additive biomanufacturing” is a sub-field of biofabrication.

Here, we propose a clarification and classification of the different biofabrication terminologies in current use. In this respect, we recommend that the term 3D printing no longer be used as a general term for all additive manufacturing technologies applied to biofabrication strategies, as 3D printing represents just one such technology as previously described. Rather, we recommend using the name of that specific technology used to create a biofabricated construct, as outlined in this article. When referring to more general biofabrication strategies in tissue engineering and regenerative medicine, we recommend using the two general terms bioprinting or bioassembly. As we previously defined [1], bioprinting refers to the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce **bio-engineered structures**; bioassembly refers to the fabrication of hierarchical constructs with a prescribed 2D or 3D organization through automated assembly of pre-formed cell-containing fabrication units generated via cell-driven self-organization or through preparation of hybrid cell-material building blocks. We advise the members of the community to adopt this terminology approach in their new studies and that the media will report advances in the field with the correct language, instead of broadly using only the term 3D printing.

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Glossary of main terminology to be used in the biofabrication field.

Bio-engineered structures: Biological constructs engineered by using in a pre-defined manner cells, biomaterials, and/or biological factors alone or in combination with each other.
Bioink: Formulation of material(s) and biological molecules / cells processed using bioprinting technologies.
Biomaterials: A material that is used as (part of) a medical device or an advanced therapy medicinal product to replace, restore, or regenerate a tissue or organ and its function.
Cell spheroid: A cluster of cells, with a spherical shape, typically formed by allowing cell suspension to settle into droplet of media.
Electrospinning: A material processing technology that uses high electrical voltage to fabricate fine fibers from polymer solution or molten polymer. Fibers are deposited onto a collector, with a random or defined alignment.
Fabrication rate: Rate of fabrication of a scaffold or of a bioprinted construct using a biofabrication technology. In the RTM, it can be calculated as the time to fabricate 10^{-6} m^3 (1 cm^3)cube, lying on one of its faces.
Fused deposition modelling / 3D-fiber deposition / bioextrusion: Additive manufacturing technologies that can be used for bioprinting, in which a thermoplastic material, in shape of filament or pellet, is hot-extrused and deposited to form a layer of solid material. Using a layer-by-layer approach a 3D scaffold or a 3D construct is built.
In-gel printing: 3D plotting approach, in which the bioink is extruded into a self-healing gel substrate, which provides mechanical support.
Indirect additive manufacturing: A biofabrication approach which uses an additive manufactured mold in which a bioink is casted, injected or compressed.
Ink-jet and valve-jet bioprinting: Printing systems able to bioprint constructs in a layer-by-layer manner by ejecting bioinks in the form of droplets via the nozzle head. Droplet ejection is controlled either by piezo- or thermal-actuators (ink-jet), or by solenoid microvalves (valve-jet).
Laser Induced Forward Transfer (LIFT) / Laser assisted bioprinting (LAB): A bioprinting technique which uses laser pulses to deposit a bioink from a donor slide onto a substrate.
Microfluidic technology: A technology based on geometrically constrained minute volume transport in micro-channels. This technology can also be used to fabricate strands of hydrogels, suitable as building blocks for successive assembling processes.
Micro-mansory concept: Biofabrication approach in which of microunits of cell laden hydrogels are used as regenerative building blocks.
Minimum feature width: Smallest detail that can be fabricated using a biofabrication technology.
3D plotting / bioplotting / robotic dispensing /extrusion bioprinting: Bioprinting technologies that dispense continuous filament of hydrogel materials, extruded through a nozzles using a piston, or a screwing system, or pneumatic pressure as driving force.
Pressure-assisted microsyringe deposition (PAM) / wet-spun automated extrusion systems: Additive manufacturing technologies that can be used for bioprinting based on the extrusion of polymers solved in volatile solvents. The quick evaporation of the solvent allows the shape retention of the 2D pattern deposited by the 3D micropositioner. With a layer-by-layer approach a 3D scaffold can be fabricated.

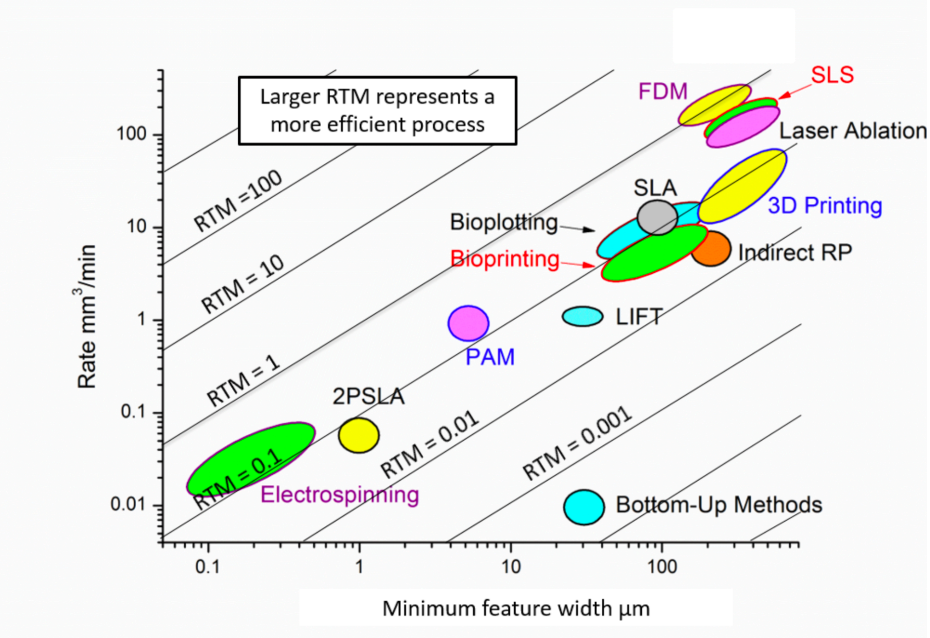
3D printing: An additive manufacturing technology that can be used for bioprinting, in which a jet of binder is directed at a powder-bed to define a pattern. The solvent binds the powder forming a slice of solid material; subsequently a new layer of powder is laid down and the process is repeated to build the scaffold layer-by-layer.
Resolution/time of manufacturing (RTM) ratio: Figure of merit to classify the performance of biofabrication technologies, defined as ratio of spatial resolution over the time required for biofabricating a bio-engineered structure; a larger RTM represents a more efficient process.
Scaffold-based strategy: A biofabrication approach where a biomaterial is used to create a cell-laden scaffold or an acellular scaffold with hierarchical ad/or smart surface properties able to steer cell activity and regenerate a targeted tissue.
Scaffold-free strategy: A Biofabrication approach where a biomaterial is eventually only used as a sacrificial template or support for cells to be deposited and let organize themselves onto it for the regeneration of a targeted tissue. This approach comprises also the deposition of cells and/or biomolecules only with no biomaterial support.
Selective laser sintering: An additive manufacturing technology that can be used for bioprinting, which uses a laser whose beam of light is selectively directed to a powder-bed, generating local heat and forming patterns of fused material; after its solidification, a new layer of powder is laid down and the process is repeated to build the scaffold layer-by-layer.
Selective laser ablation: An additive manufacturing technology that can be used for bioprinting, in which a solid material is abladed using a very short time duration laser pulse. If the ablation process is conducted in all of the three directions or if laminated porous films are stacked and bonded on top of each other, a 3D structure can be created.
Stereolithography: An additive manufacturing technology that can be used for bioprinting, in which light is used to cure a photosensible resin. Although different irradiation approaches, the various stereolithographic systems use a layer-by-layer approach: the energy delivered by the light is sufficient to solidify a certain thickness of the exposed resin and join this layer with the previous one.
Two-photon polymerization: Laser based technique uses near-infrared ultrashort-pulsed laser to excite in a precise and confine space molecules (photoinitiators) to a two-photon state triggering the polymerization of monomers in solution. This was the first technique that allowed the manufacturing of true 3D nano-/ micro-structures without supports.
Tissue liquidity: The notion, introduced by Malcolm Steinberg, that tissues or multicellular aggregates composed of motile and adhesive cells have properties analogous to liquids, evidenced by the fact that irregular tissue fragments spontaneously round up into spheroids and two fragments composed of different cell types mutually envelope each other. Such tissues can be quantified in terms of apparent tissue surface tension.

Table 1. Summary of main features and limitations of biofabrication techniques.

Technique	RTM ratio (10^{-3} m^2/min)	minimum feature width (μm = 10^{-6} m)	Limitations	References
3D™ Printing	Medium (~0.1)	~ 300	Presence of polymeric grains and of excess solvent.	[6, 7]
Selective Laser Sintering	Medium to high (~1)	< 400	Presence of polymeric grains; limited to non-thermo-labile materials.	[8-10]
Laser Ablation	Medium to high (~1)	< 400	Thermo-labile materials (cells and proteins) can be damaged during scaffold fabrication.	[12, 15]
Stereolithography	Medium (~0.5)	~ 30-70	Use of photo sensitive polymers and initiators, which may be toxic.	[18, 20]
2-Photon Polymerization	Medium (~0.05)	< 1	Use of photo sensitive polymers and initiators, which may be toxic.	[21, 22]
Digital Light Processing	Medium to high (~2)	~ 70 - 100	Use of photo sensitive polymers and initiators, which may be toxic.	[19]
Fused Deposition Modeling	Medium to high (~1)	~ 200	Limited use with thermo-labile materials. Evident layered structure.	[23-25]
PAM & wet spun technologies [2]	Medium (~0.5)	~ 20	Limited range of material available and low vertical dimension processing time increasing with increasing the number of material heads used.	[35, 36]
Indirect Additive Manufacturing	Low (~0.03)	~ 200	Limited mould materials.	[40-42]
Bioplotting	Medium (~0.5)	~ 100	Need a self-sustaining gel (bioink) showing high degree of shear thinning.	[46-48]
Ink-jet Bioprinting	Medium (~0.1)	~ 100	Limited range of gels (bioinks) available; inks must be of low viscosity.	[52, 100]
Valve-jet Bioprinting	Medium (~0.3)	~ 200	Delivery rate not sufficient for building clinically relevant	[56, 57]

			constructs	
Electrospinning	Medium (~0.1)	< 1	Difficult to realise thick scaffolds.	
Laser Assisted Bioassembly	Low (~0.04)	~ 30	Difficult to realise thick scaffold; limited range of gels available.	[78, 79]
Bottom-up approaches	Low to Very Low (<0.001)	~ 30	Long-time of fabrication due to microunits production and construct maturation.	[81, 82]

Figures



Border			
State of matter	Liquid		
	Gel and slurry		
	Solid		
	Powder		
Infill			
Fabrication strategy	Major and active role of biomaterials in the printing process	Solid color	3D printing
			Light-based
			FDM
			Pam and wet-spun
			Bioplotting
			Inkjet and valve jet bioprinting
			Electrospinning
			Indirect am
	Biomaterials for temporary structural integrity	Dashed infill	

Key Figure. Figure 1: Distribution of various biofabrication technologies according to their minimum feature width (x- axis) and printing rate (y-axis): The contour lines represents the RTM ratio, a figure of merit for classifying the biofabrication technologies according to their efficiency, taking into account resolution and fabrication throughput. A larger ratio represents a more efficient process.

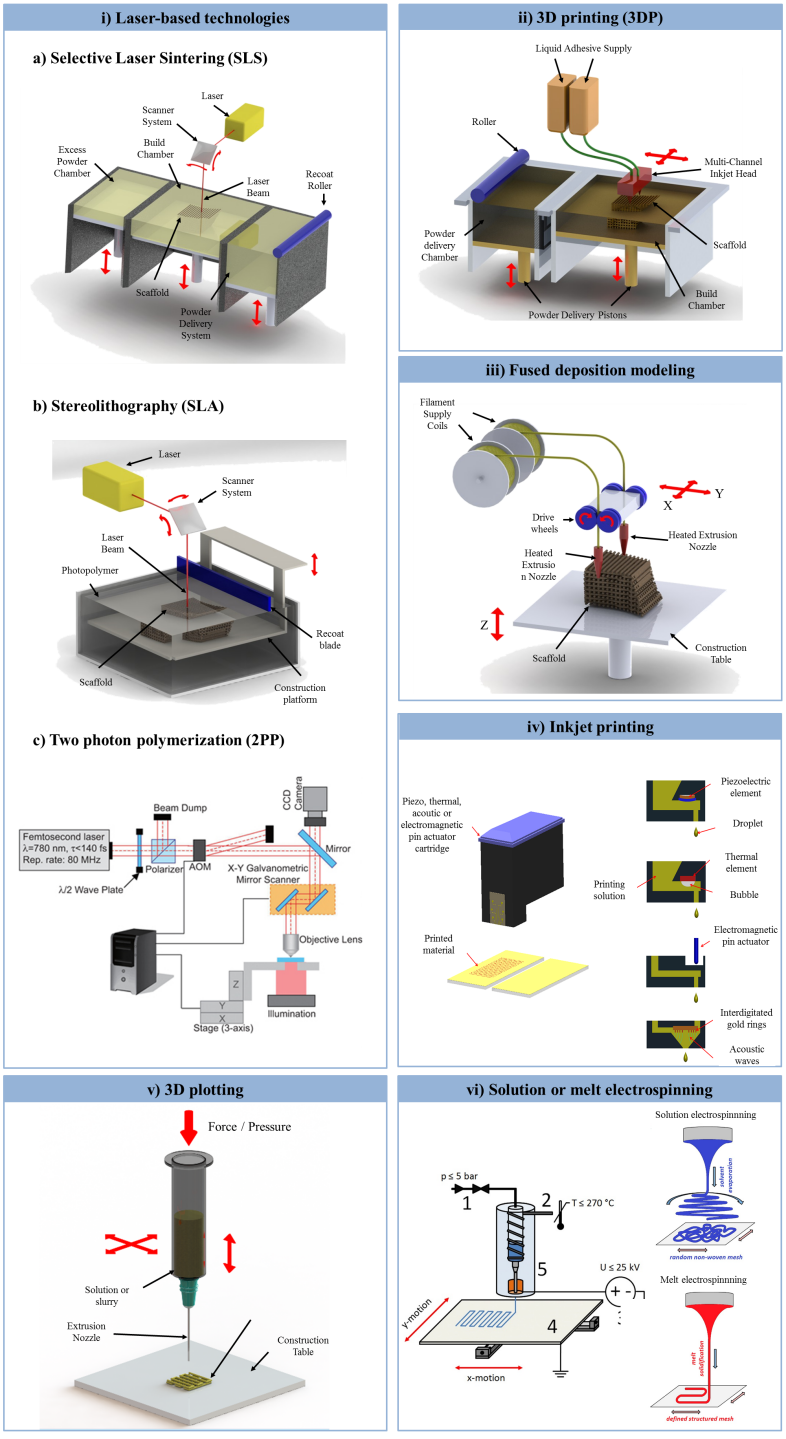


Figure 2. Most commonly used technologies in scaffold based biofabrication: i) Laser-based technologies (selective laser sintering, stereolithography, two-photon polymerization); ii) 3D printing; iii) fused deposition modelling, iv) ink-jet printing; v) 3D plotting; vi) solution and melt electrospinning. Adapted from [128] with permission.

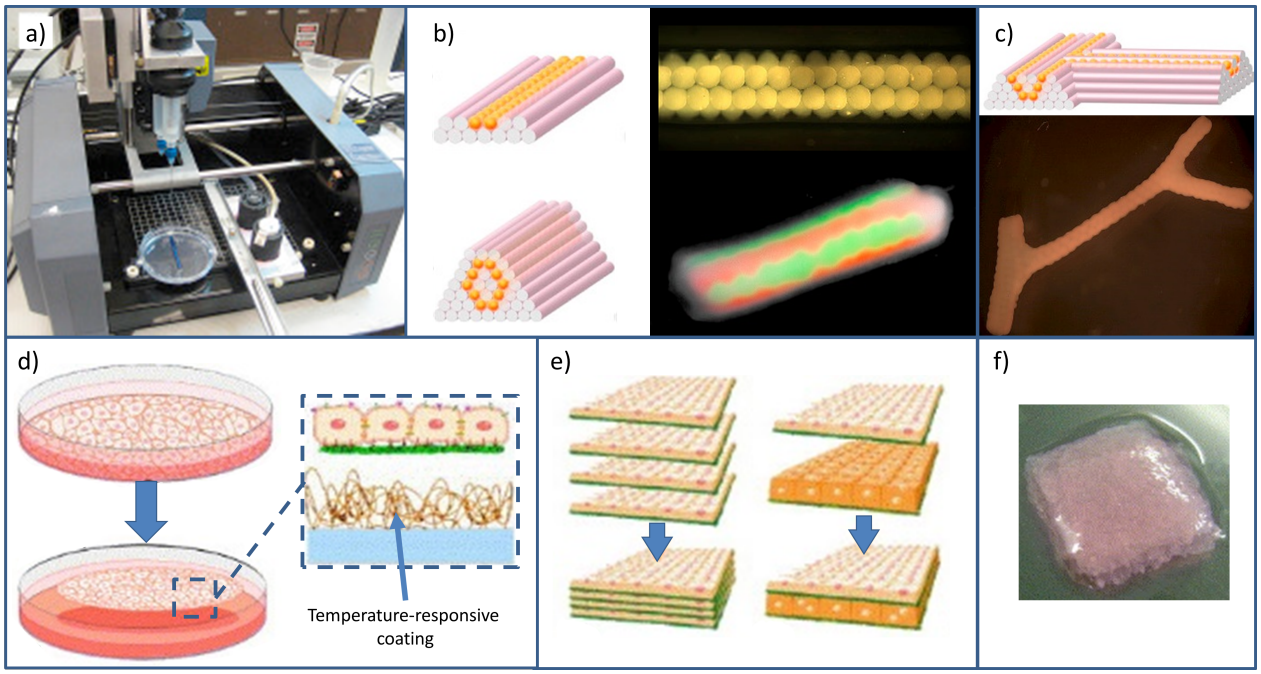


Figure 3. Examples of scaffold free approaches: extrusion bioprinter used to deposit cell aggregates as spheroids and/or cylinders (a); deposition strategy for fabricating a straight (b) or branched (c) hollow shape; representation of cells culture on thermos-responsive culture plates (d) and cell-sheet stacking (e) as steps for obtaining a complex structure (f) in a cell-sheet biofabrication method. Adapted from [129] and [81] with permission.

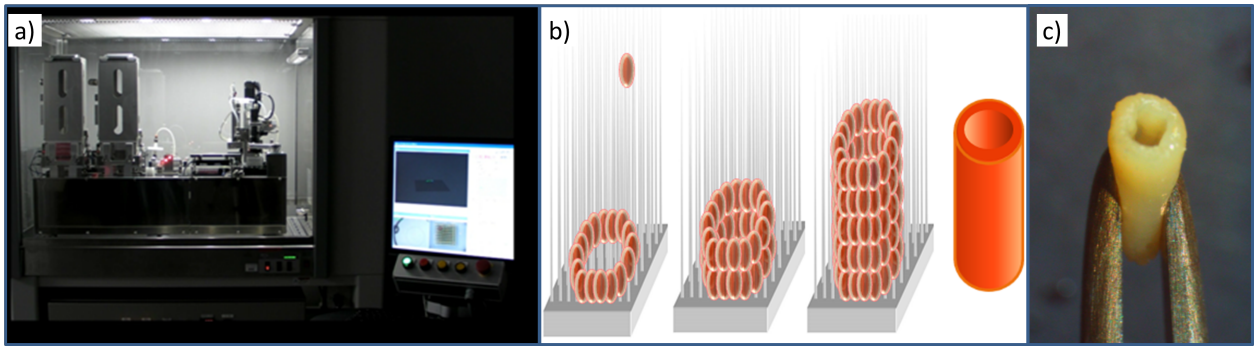


Figure 4. Scaffold free approach based on skewering of tissue spheroids: custom made device for automatic skewering (a); staking strategy for obtaining a hollow shape (b); scaffold free vascular graft generated from mesenchymal stem cells (c). Adapted from [74] with permission.

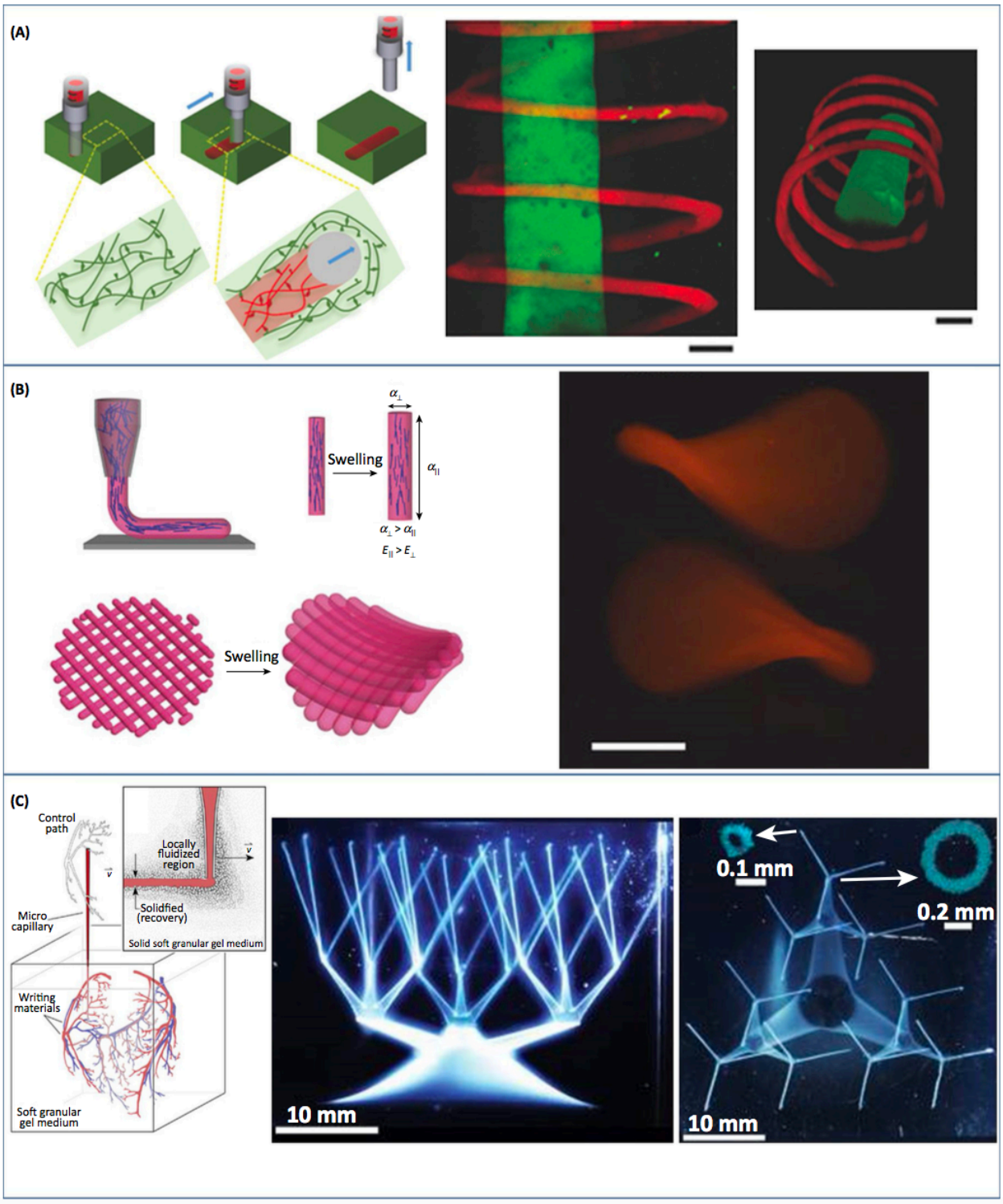


Figure 5. Examples of complex heterogeneous bioprinted structures. (a) bioprinting of shear-thinning supramolecular hydrogels into self-healing support gels allowing to manufacture continuously in 3D space while patterning of multiple inks and cells; (b) example of 4D printing stimuli responsive materials

that allow spatio-temporal changes (e.g. by swelling); (c) example of hierarchically branched tubular networks printed in granular gel printing. Adapted from with permission