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DOI: 10.1089/ten.TEB.2016.0322

Principles of the *Kenzan* Method for Robotic Cell Spheroid-Based 3D Bioprinting

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This is the author's manuscript of the article published in final edited form as:
Moldovan, N. I., Hibino, N., & Nakayama, K. (2016). Principles of the Kenzan Method for Robotic Cell Spheroid-Based Three-Dimensional Bioprinting. Tissue Engineering Part B: Reviews, 23(3), 237–244. https://doi.org/10.1089/ten.teb.2016.0322

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Abstract.

Bioprinting is a technology with the prospect to change the way many diseases are treated, by replacing the damaged tissues with live, *de novo* created bio-similar constructs. However, after more than a decade of incubation and many proofs-of-concept, the field is still in its infancy. The current stagnation is the consequence of its early success: the first bioprinters, and most of those which followed, were modified versions of the 3D printers used in additive manufacturing, redesigned for layer-by-layer dispersion of biomaterials. In all variants (inkjet, micro-extrusion or laser-assisted), this approach is material- ('scaffold'-) dependent and energy-intensive, making it hardly compatible with some of the intended biological applications. Instead, the future of bioprinting may benefit from the use of gentler, scaffold-free bio-assembling methods. A substantial body of evidence has accumulated indicating this is possible by use of preformed cell spheroids, which have been assembled in cartilage, bone and cardiac muscle-like constructs. However, a commercial instrument capable to directly and precisely 'print' spheroids has not been available until the invention of the microneedles-based ('Kenzan') spheroid assembling, and the launching in Japan of a bioprinter based on this method. This robotic platform laces spheroids into pre-designed contiguous structures with micron-level precision, using stainless steel micro-needles ("kenzans') as temporary support. These constructs are further cultivated until the spheroids fuse into cellular aggregates and synthesize their own extracellular matrix, thus attaining the needed structural organization and robustness. This novel technology opens wide opportunities for bio-engineering of tissues and organs.

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Introduction.

Bioprinting, a branch of 'additive (bio)manufacturing', has evolved as a technology aiming to include the third coordinate (3D) into its constructs, and thus making them more biologically-meaningful, by adding of multiple 2D layers on top of each other[1]. Apparently, one of the first functional bioprinters was a regular printer refurbished to work with 'bio-matrices'[2]. The concept of 'bioink' is central to the bioprinting technology, known as 'scaffold-assisted' bioprinting, which relies on the use of a soft hydrogel (either alone or containing cells), as cell-supporting matrix[3]. Correspondingly, the versions of this method include 'inkjet bioprinting' (with its variants thermal and piezoelectric, depending on how the hydrogel droplets are produced), 'extrusion bioprinting' (with its pneumatic, piston- and screw-driven variants), and 'laser-assisted bioprinting' (which uses local melting of a polymeric 'ribbon', generating a gel droplet which can be deployed with high speed and precision over the construct)[3].

Since the ability to perform scaffold-dependent bioprinting mostly depends on the embedding material, its properties need to be considered first, rather than those of the cells/tissues to be assembled[4]. For this reason, although a large effort has been devoted to find the appropriate scaffold matrices for bioprinting, and in spite of good proofs-of-concept[2, 5-7], only recently instruments based on this technology became commercially available. Some bioprinting companies use for their production in-house built instruments. Others sell bioprinters at the buyer's own risk, when their claims barely can be backed by actual bioprinted constructs or peer-reviewed publications.

This situation has changed with the invention of a method that does not need exogenous materials, therefore belonging to the biomaterial ('scaffold')-free category[1]. As detailed below, in this method the instrument directly laces together pre-formed cell spheroids containing tens of thousands of cells on support microneedles[8]. Within these spheroids, the cells either have already secreted an extracellular matrix during *in vitro* formation, or do so soon after assembling, thus providing them with robustness and tissue-specific qualities. After an additional *in situ* stage while still attached to their needle support, the spheroids fuse into a compact structure. At this point, the constructs are removed and further cultivated during a 'post-printing maturation' period, until they acquire more of the desired biological qualities. These sequential multiple temporal stages, which are indispensable for the

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progress of this form of bioprinting towards the final construct, recapitulate basic developmental biology (embryological) mechanisms[9], such as spheroid growth[10], intra-spheroid cell motility[11] and layer formation ("cell sorting)'[12].

Based on a recent review[1] commissioned by the journal '*Biofabrication*' to some of the key contributors to the field, the explosive development of this research area is generating terminological dilemmas. This consensus study recommended that not all activities generating 3-dimensional constructs for tissue engineering be named 'Bioprinting'. Instead, depending on the technology, some are more appropriately named 'Bio-assembling', as both are complementary approaches to 'Biofabrication'[1].

Besides the notion of 'scaffold' (which in this context would indicate a supportive material for bioprinting[4, 13-16]), another term with a complex meaning which at times is confusing, is that of 'bioink'. For example, for some authors a 'bioink' is whatever is used for bioprinting: any material, cells, or combination thereof[16-18]. But for many others, including the companies producing them, the 'bioinks' are the embedding bio-materials (the 'scaffolds') for bioprinting.

Anticipating these considerations, the Cyfuse company did not name their *Regenova* robot a '3D bioprinter', but rather a 'Bio 3D-Printer'. Based on the discussion above, this was a good option to signal that there is a difference in methodology from the regular scaffold-based 3D bioprinting. From a commercial standpoint, this designation also makes sense due to the fact that the target user groups are largely the same as with the bioprinting, and because other bio-assembling methods, like the 'bio-pick, place and perfuse' instrument[19], exist although are less known.

1. Limitations of biomaterial-dependent bioprinting.

The reasons for the limitations of 'traditional' bioprinting are derived from several still unsolved problems related to the use of a scaffolding material. One is that the material needs to be supportive for all cells within a construct, and then for the recipient organism, besides being suitable for the bioprinting process *per se*. Apparently, such an universal material is yet to be found, since often each cell type needs

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to be embedded in a different hydrogel[5, 20]. Some of these bioinks are in general proprietary, and thus unsuitable for further optimization by the user, in addition of being expensive.

More importantly, the common printing methods are intrinsically stressful to live cells[21], by exposing them to high shear stress, overheating, and/or toxic compounds generated even from initially cell-friendly materials[22, 23]. Additionally, the constructs based on hydrogels are by necessity *soft*, unless the scaffolds are made more solid upfront, which is possible only for a limited number of tissues, such as bone and cartilage. To deal with this constraint, some research groups incorporate polymeric microfibers within the bio-printed structures, a process called 'hybrid bioprinting'[5, 24, 25]. This provides the needed sturdiness but complicates the other features of a biologically-inspired construct. This is because the native tissue architecture, which always contains a degree of structural randomness, can be hardly implemented by mechanical means. For this reason, even the more recent bioprinted constructs demonstrate a monotonous geometrical design, which only distantly resembles their natural counterparts[5, 6, 20].

It is also notoriously difficult to incorporate in these constructs a vascular system, as necessary components of tissue-engineered organs capable of long-term functionality. When this was attempted within the confines of the current technology, often rudimentary 'channels' were implemented[6, 20]. Some success had the 'organs-on-chip' microfluidic devices[26], but their scaling-up and integration into functional bioprinted constructs need more efforts to succeed. The same issues apply to the innervation of the bioprinted constructs[27].

Furthermore, dealing with individual cells, the material-dependent bioprinting could be slow, because the simplest meaningful structures require millions of cells, which may take a long printing time to be added in droplets even when dispensed through high-speed nozzles. Laser-assisted bioprinting can speed up the process, but it maintains other limitations (such as heating, cell separation, etc.) in the workflow[28]. Also of consideration is that even if printing is performed both gently and fast enough, the 'encapsulation' of the cells within individual droplets isolates them from their neighbors. To overcome this constraint, the cells need to both dissolve their 'cage' and/or to proliferate to the point where they can come in direct contact.

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The issue of post-implantation bio-compatibility will acquire new dimensions with every attempted clinical application, facing a very close scrutiny for the regulatory agencies, such as the FDA. These are justifiably vigilant regarding any materials or substances being either voluntarily or involuntarily incorporated in a bioprinted construct. In particular, the xeno-materials are riskier for constructs from stem/primitive cells, which could be genetically more unstable and at risk of tumor formation in the presence of unusual 'bio-materials'[3].

2. Biomaterial ("scaffold')-free bioprinting

Many of the problems above could be collectively eliminated if a biomaterial-free cell assembling were available. From the early days of this field it has been appreciated the difficulties generated by the use of a 'scaffold', and suggested the most rational alternative: using only cells and the matrix they secrete[15]. This bioprinting approach was vastly explored conceptually[29], and computer modeled[17, 30]. The attempted implementations use 'sacrificial' inorganic materials which permit limited cell-cell interaction, then being removed at a point in the process[15]. For example, Organovo's technology seems to rely on the formation of cell strands temporarily supported by 'fugitive' (sacrificial) hydrogel cylinders or chopped therefrom into shorter fragments, placed in 3D arrangements by a proprietary procedure[31, 32].

Another example, emerging from an academic setting, is the preparation of long 'cellular strands' in alginate tubes[18, 33]. This hydrogel scaffold is then removed and the cell strands re-loaded in a dispensing nozzle for extrusion in a layered 3D arrangement. While not truly 'scaffold free', this method is however a step forward in this direction. Similar cell strands for 3D tissue engineering have been proposed before as micro-patterned or as scaffold-wrapped cell cords[34]. However, the central element of biomaterial-free methods in tissue engineering is the use of cells in bulk, either as spheroids[35, 36], cell sheets[37] or cylindrical[15, 38] cell aggregates, embedded in their own extracellular matrix, and ideally not exposed to xeno-materials (such as hydrogels) at any stage of their preparation. The comparative properties of the two methods are presented in Table I.

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3. Cell spheroids as building blocks for bioprinting

The spheroids as 'building blocks' of a bio-fabricated construct can be endowed with a preemptive internal cellular organization, reminiscent of that of organoids encountered in developmental biology[10]. The pre-formed structures can then be further assembled in larger constructs, operating under the same biological laws as the spheroids, rather than under the constraints of bio-materials.

For example, constructs consisting of about 760 spheroids were made from porcine adipose tissue derived stem cells, each containing 5.0×10^4 autologous cells, and implanted into osteochondral defects (4 mm in diameter and 6 mm in depth) created in the femoral trochlear groove of adult mini-pigs. The histopathology of the implants after 6 months revealed active endochondral ossification underneath the smooth hyaline cartilage. After 12 months, not only the diminishing hyaline cartilage was as thick as the surrounding normal cartilage, but also a massive subchondral bone was present[39].

In another example, a planar construct was made from pulsating spheroids, prepared from three human cell types: cardiomyocytes, endothelial cells and fibroblasts[40]. This construct was surgically applied *in vivo* atop of beating rat hearts. It integrated with the epicardium and connected by anastomosis of the spontaneously formed capillaries with those of the recipient, documented as the recipient's blood abundantly present within the graft. This proof of concept has now opened the way toward testing more complex cardiac patches, and for testing their therapeutic potential.

Another successful example is the magnetic nanobeads-mediated spheroid formation[41-49]. This method (also known as 'magnetic levitation' when the cells are collected on top of the fluid in a tissue culture well rather than on its bottom[41]), is extremely versatile. It has been used in several high-profile studies, being applied for 'bioprinting' of complex structures, such as valves[48], bronchioles[45], or adipose tissue[46]. Similar methods are being actively developed in other settings as well[50, 51]. However, among their common limitations is the difficulty to place and maintain the spheroids in a pre-determined position, or to use spheroids of different compositions and to scale-up to surgically meaningful constructs. For this, the authors introduced the use of additional tools, such as a magnetic 'bio-pen' which manually could bring and keep the spheroids in place [45, 48].

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4. The 'Kenzan' method of bioprinting

To provide the spheroids spatial organization and opportunity to interact and to secrete extracellular matrix, thus obtaining a tissue-specific structural organization and biomechanical robustness one of us (K.N.) invented the micro-needle based method[8, 39, 40]. It was called 'Kenzan' method, after the traditional art Ikebana, where for floral arrangements the stalks are impaled in a dome-shaped metal needle array called 'Kenzan' (in Japanese ken=sword; zan=mountain).

Unlike other bioprinters which depend on exogenous materials, the instrument that uses the Kenzan approach, named *Regenova*, and commercialized in Japan by Cyfuse Biomedical, K.K., and in US by Amuza, Inc., relies only on cells to build complex tissue analogues of practically any composition. The cells are first pre-assembled into spheroids, and to provide the spheroids a spatial organization and opportunity to interact and to secrete their matrix, they are robotically 'impaled' in micro-needles as temporary support. The 'kenzans' are made of 160 μm thick stainless steel microneedles, placed at a distance of 500 μm (currently available in 2 formats, of 9x9 or 26x26 needles). Therefore, to come in contact to each other, the spheroids should be about half-millimeter in diameter (400-600 μm), representing aggregates of about 20,000 cells or more, depending on the cell type and the degree of spheroid compaction. The spheroids are pre-formed in, or transferred into, non-adhesive round-bottomed 96 well tissue culture plates, from where they are picked up by a nozzle connected to a mobile arm. The robot is housed in a ventilated hood with one-way air circulation. During the operation, the front window is maintained shut, which permits activity in aseptic conditions.

The main parts of the Regenova platform are:

Plate storage and transport unit; the instrument has two storage magazines, each accommodating up to ten 96-well plates, as well as an operation magazine for plate discharge (Fig. 1A). The plates are automatically taken from the storage magazine, and transported to the printing area.

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Image capture and analysis system. The instrument is equipped with high-quality camera and image analysis software which allows the identification of spheroids in the plates (Fig. 1B), as well as the needle tips (Fig. 1C). This imaging-based identification also provides a pre-printing quality check for the spheroids, those of inappropriate size and/or shape being rejected (the corresponding wells are skipped during printing).

Kenzan holder. The needle array is submerged in a lidless sterile PBS-filled tank, and secured in a holder (Fig. 1D). The mobile nozzle arm carrying a spheroid, contained in a liquid droplet retained by capillarity on its tip (Fig. 1E), is moved on top of the needle array (Fig. 1F), and is lowered over it in a location determined based on the actual needles position, also obtained by imaging. At that point, the negative pressure in the pneumatic system is replaced with a slight positive 'expiration', thereby releasing the spheroid, and the nozzle is transported back to the plate, to pick up another spheroid.

At the bottom of the needle array are two mobile plastic holders (Fig. 1G), which by their sliding permit the separation of the construct from the needle support (the second one helps placing in position the first 'separator' in the needle array for safe re-introduction). The printing process could take 15-20 min per plate, if the spheroids are well prepared. This allows the rapid, large-scale assembling of constructs with multiple pre-designed spheroid layers (Fig. 1H), with as much as 1 cm or more in height (Fig. 1I). Using the same structural fusion of live cell aggregates as in spheroids, these proto-tissue blocks can be further assembled in even larger constructs, when placed and maintained in contiguity during post-printing maturation[8]. The smaller needle array also has a hollow configuration where the central needles and the bottom are missing (Fig. 1G, middle). This permits its connection to a pump, allowing the perfusion with culture medium, during the post-printing maturation of the construct.

Spheroid aspiration and printing unit. The main component of this unit is a nozzle with mouth comparable to a spheroid's size, mounted in a holder. This holder is attached to a mobile arm with micron-precision 3D positioning control. The slight yet tightly controlled depression used to aspire the spheroids is provided by a computer-controlled pump via connectors and a buffer chamber. If the uptake of a spheroid fails twice (e.g. due to attachment on well's bottom or to its inappropriate shape, size or

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sturdiness), the nozzle holder is moved into a cleaning container where it is purged by air expiration of the possible solid contaminants plugging it.

Computer system. This provides the integrated remote control, the diagnostic and malfunction identification. In addition, the structural design is made available through dedicated software. The computer design program called *Bio 3D Designer* is also available offline for convenient pre-printing modeling of the construct geometry (Fig. 1H).

So far, several publications were based on this technology. For example, live vascular tubes of 2 cm in length and 5 mm in diameter were printed from spheroids prepared from human endothelial cells, smooth muscle cells and fibroblasts[8]. This proto-vessel had enough biomechanical and material resistance to sustain surgical manipulations and suturing. When they were implanted in immuno-deficient rats into abdominal aorta and retrieved after 5 days, these cellular tubes showed maintenance of structural integrity and patency, without thrombosis and displaying a continuous endothelium of donor origin. However, given the low proportion of smooth muscle cells and lack of organized extracellular matrix (i.e., elastic laminae) at the time of printing, all grafts remodeled with enlargement of the lumen area and thinning of the wall[8]. Results with other constructs were also made available as posters at scientific conferences, e.g. tracheal[52] or urethral[53] tubes.

5. Specifics and adjustments of the Kenzan method

Being essentially a spheroid-assembling method, the efficiency and quality of Kenzan bioprinting is directly dependent on that of its building blocks. Below we are summarizing some of the spheroid-related properties of this method.

Spheroid size is determined by the inter-needle distance. The fixed inter-needle distance and the need to put them in contact, makes the size of usable spheroids fall in a relatively narrow range. For this reason, the user has to master the technique of generating optimal spheroids before coming to the printer. While for small-scale, routine constructs this is usually trivial, there are instances when new cell combinations, longer (or too short) incubation times, or tissue culture factors, can make the spheroid

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dimensions unpredictable or sub-optimal. In addition, some constructs may not be made at the scale requested for direct Kenzan assembling. In this case, a solution would be to pre-incorporate the desired cellular structures in 'supporting' spheroids with a generic compositions (such as fibroblasts and endothelial cells, for example).

Related to the size is the need to optimize the time to keep the spheroids in culture for adequate extracellular matrix secretion (see below). This is constrained by the diffusion limit of oxygen (usually 200 μm *in vivo*), or by that of glucose or other nutrients. The spheroid cores could thus be deprived of nutrients if maintained too long in culture. While for more primitive cells hypoxia might be advantageous, for differentiated cells these conditions could be detrimental, making the spheroids fragile at the printing stage and/or during the post-printing maturation.

Another consideration is the localization within the bulk of spheroidal space of cells of an epithelial phenotype. This raises a topological dilemma, because these cells are supposed to stay on a surface. While for endothelial cells this location is less consequential (these cells being capable to easily switch between a cord-like arrangement in pre-capillaries to tubular structures in capillaries), for a *bona-fide* epithelium an intra-spheroid arrangement is less meaningful. As an alternative, these cells can be cultivated on the surface of spheroid by secondary attachment, or on the surface of hydrogel beads.

Spheroids for printing need a balanced cell-cell interaction and extracellular matrix composition. Essential for the spheroids formation are their direct inter-cellular interactions[14]. At the same time, for all subsequent practical applications, a robust extracellular matrix is also crucial. In particular, the stability of the spheroids at the printing stage requires a balance between cell adhesiveness and matrix abundance, which may reduce the strength of direct intercellular adhesive forces by interposition, but gives better material properties. During the spheroid formation these two processes change in opposing directions, adhesiveness decreasing while the extracellular matrix deposition increases. If we add to this that cell survival at the core of the spheroid is also likely to be reduced with time in culture, we have a complex picture of how the spheroids need to be optimized when brought to Kenzan bioprinting.

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Cell distribution within spheroids undergoes a continuous re-arrangement. Also relevant is the actual distribution, proliferation, etc. of cells in heterogeneous spheroids. As extensively shown in developmental biology studies, far from remaining randomly distributed, the cells tend to associate among themselves in more structured arrangements (e.g. layers), by preferentially partnering with those which are similar. Actually, the stronger-interacting cells tend to occupy the core, and the others distribute themselves in concentric layers, in the decreasing order of adhesive strength. This simple process called ‘cell separation’, is one of the fundamental mechanisms driving early development[54]. In larger artificial spheroids, this could be combined with a limited nutrient diffusion, making the cell type that tends to settle at the center to suffer more from a limited nutrient diffusion and to enter apoptosis, which may change in time the cell proportions and thus spheroid properties.

Consequences of spheroids compaction. Inside spheroids the cells move within the limits imposed by the available space, and by the intercellular adhesions. This process, combined with the deposition of extracellular matrix, is beneficial for the ‘healing’ of the holes left behind by the needles. However, if the goal is to print tubes or other hollow structures, this contraction may lead to their premature disappearance, which would need additional stabilization. Also, spheroid compaction may lead to sub-optimal physiological conditions at the core, i.e. reduced oxygen and metabolite diffusion, and from here reduced strength of adhesive forces between cells.

6. Conclusions

Bioprinters conceived so far were mostly adaptations of regular 3D printers for layer-by-layer additive bio-manufacturing, i.e. dispersers of ‘bioinks’ containing or not live cells. Bioinks as droplets or slurries of bio-materials which during printing undergo heating, vibration, extrusion, or other energy-intensive processes, could be hardly bio-compatible with the needs of the contained cells, or with those of the recipient organism. For these reasons, with the exception of several prototypes operated in academic or corporate laboratories and of few commercial instruments, the larger community of investigators still has limited access to efficient bioprinting technology to serve their research needs.

The power of spheroid-based tissue engineering is now materialized in the Kenzan method and the commercial Regenova bioprinter, the instrument capable to put this approach in practice. Besides avoiding the shortcomings of bioinks, another of its benefits is similarity with certain aspects of developmental and tumor biology, routinely ignored or unaccounted for in biomaterial-assisted bioprinting. In spheroids many well-known bio-physical and biological mechanisms are involved, which can be rationally incorporated and more efficiently exploited for tissue engineering purposes.

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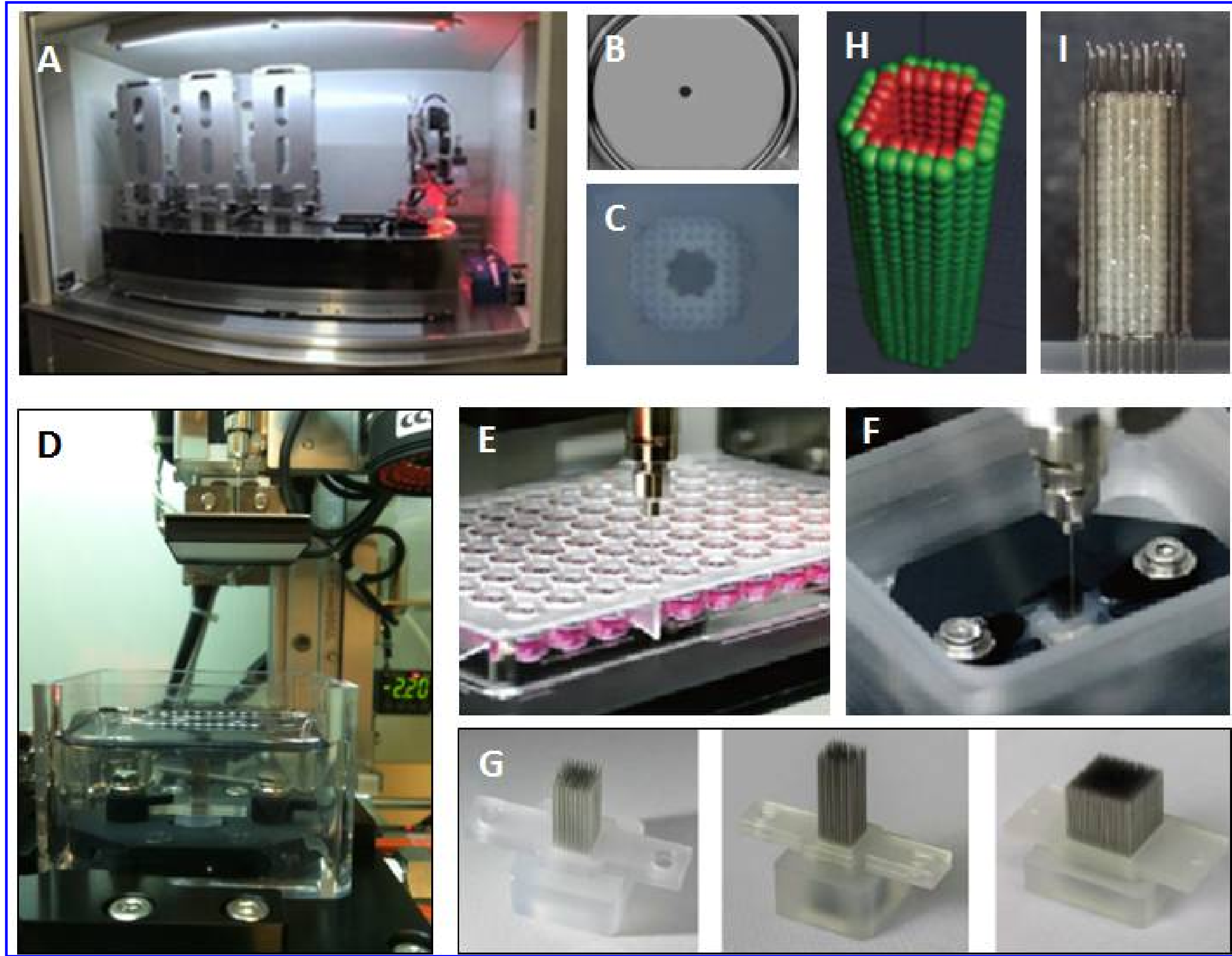

Figure 1. Main components of the *Regenova* bioprinting platform. A. Aseptic hood containing the assembling line: a plate storage magazine (far left), followed by two feeding magazines and a plate transportation line towards the mobile arm (far right) and an imaging system (red light). **B.** Cell spheroid imaged within its feeding plate's well. **C**. Top view of a completed cell construct and the tips of supporting micro-needles. **D.** Fluid-immersed Kenzan holder. **E.** Nozzle aspirating a spheroid. **F**. Nozzle depositing a spheroid onto a needle. **G**. Three types of Kenzans (with 9x9 regular and hollow, and with 26x26 needles); note the needle-perforated plastic bases. **H**. A virtual double-layered tube created with the 'Bio 3D Designer' program. **I**. An actual spheroid constructs awaiting post-printing maturation. Images courtesy of Cyfuse Biomedical K.K.

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	BIOMATERIAL-DEPENDENT		BIOMATERIAL-FREE	
	Attributes	**Comments**	**Attributes**	**Comments**
OBJECT CONFIGURATION	Direct image input via CAD	Similar to 3D printing	Approximate	Larger ‘voxel’ size, limited resolution
STRUCTURAL COHESION (‘glue’)	Obtained by non-universal, sometimes proprietary and/or expensive bio-inks	New biological bio-inks emerging (e.g. collagen or fibrin based)	Cells produce their own matrix; constructs are dependent on cell type and quality	Matrix deposition can be unpredictable or insufficient
BIOMECHANICS	Hydrogels are essentially soft; hardening can be cell-damaging	‘Hybrid’ bioprinting as alternative: incorporation of a second (fibrillar) biomaterial	Construct biomechanics less predictable and controllable	Hybrid versions are also likely to be developed
EFFICIENCY	Substantial cell death, for a variety of method-specific reasons	Milder methods are being tested (e.g. laser-assisted bioprinting)	Less or no cell damage Cell-type dependent	By using large spheroids, speed can become comparable or even higher than laser-assisted bioprinting
CELLULAR CROSS-TALK	Material-limited inter-cellular communication (‘encapsulation’)	Not a problem for matrix-rich tissues such as bone, cartilage	Direct cellular interactions	Optional addition of hydrogels into or between spheroids still possible
TISSUE STRUCTURE	Simplistic cellular architecture	Biomaterial dissolution allows more spontaneous cell rearrangements	Follows developmental principles	Incorporation of endothelial cells in spheroids may promote micro-vascularization
BIO-COMPATIBILITY	Cytotoxicity possible, foreign-body reactions likely	Less serious if biological bio-inks are used	Patient-specific cells: MSC, iPSC	Possibly fully autologous constructs
COMMON TECHNICAL PROBLEMS	Nozzle clogging	Limited to ink-jet and micro-extrusion methods	Time of pre-printing preparations	Post-printing maturation time comparable between the two approaches
SCALABILITY	Excellent	***Good for large, cell-homogenous, matrix-rich tissues***	More limited	***Recommended for small, cell-heterogeneous, matrix-poor tissues***