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Thoughts on scaffolds[†]

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Scaffolds are instrumental in the engineering of functional tissues, and therefore have been an intense area of interests within the regenerative biology and medicine areas of research and development. Many approaches exist for creating scaffolds with either natural or synthetic advanced materials, which are subsequently coupled with cells and other materials, and microintegrated with the aid of a bioreactor, finally forming a functional three-dimensional tissue. Although many advances have been made over the years, none of these have truly been successful as postulated by literature for either biomedical or clinical utility. For e.g. generated reconstructs, have many limitations, such as poor cell infiltration throughout the entire depth of the scaffold, to the associated cost and time for generating functional reconstructs mimicking native tissue. These and other roadblocks have truly limited the use of scaffolds as tissue engineering biomaterials/building blocks in regenerative medicine. However, these previously faced obstacles have recently been overcome with new scaffolding

technologies unearthed and pioneered in 2005, which demonstrate the ability to directly handle large quantities of multiple cell types with both a biopolymer and other advanced materials for simultaneously forming a three-dimensional living reconstruct mimicking native tissues. These recently discovered platform biotechnologies will truly have significant ramifications to the engineering of a three-dimensional tissue and for regenerative medicine in general as these platforms are versatile.

1. Introduction

As clinical medicine advances, rapidly with substantial achievements, the estimated lifespan of humans have significantly increased. Although these advancements are noteworthy they bring with them associated obstacles faced as our bodies age, thus having to tackle new challenges such as aging/dysfunctional tissues and organs. Addressing tissue and organ transplantation has been successful to some degree but has associated issues such as the limited availability in tissues and organs from donor pools to the recipient, to post-transplantation requiring lifelong immunosuppression, giving rise to a wide range of significant side effects. Therefore, tissue and organ shortage could be met by the reconstruction and development of synthetic tissues where a natural or synthetically developed scaffold is fully cellularised with the patient's own cells. Thus, eliminating the need for donor tissues/organs and the need for life long immunosuppression. Consequently, we find ourselves today at this stage of the challenge seeking new methods, tools and materials for generating synthetic tissues for addressing our wellbeing. The author also notes that reconstructing a whole organ such a dense (heart or liver etc) or hollow (trachea) one is a challenge of unimaginable magnitude/complexity and therefore the author focusses on tissue reconstruction and development for the patching of aging/damaged organs, which in itself is a task of mammoth proportion. An interesting thought is that organ replacement being complex does not end with the associated biological and medical issues but also has the association of

whether the transplanted organ is conditioned for the recipient's anatomical structure and dimensions – which is also an aspect requiring careful consideration prior to any transplantation. Therefore, this endeavor has seen the coming of cross fertile collaborations for coupling the life sciences with the physical sciences. Molecular, cellular and developmental biologists have achieved great advancements where they have increased our know-how into these respective fields, with the particular achievement of the discovery of induced pluripotent stem (iPs) cells, [1] which provides many opportunities for cellular based therapies to the reconstruction and development of tissues and organs. Similarly, physical scientists have over many years developed novel direct cell assessing and handling approaches which have seen the development of platform technologies such as flow cytometry. That being said there is much misinterpretation about scaffolds in regenerative biology and medicine. Hence it is only recently that in the endeavor of developing synthetic tissues, some scientists have come to realise that scaffolds take precedence, as they provide the much-needed support that cells require to carry out their function in three-dimensions, from migration, adhesion to communication with its surrounding cells to a whole host of other functions as they do in native tissues and organs. It is well-established that tissues containing limited or lacking such supports have significant effects on their ability to undergo repair, regeneration to rejuvenation. In fact, from a molecular level upwards cells have been shown to change in both phenotype and genotype as a result of lacking a supporting scaffold. [2]

Tissue engineers classically have been chasing the idea that cells coupled with a scaffold would allow the reconstruction of a living tissue. This notion however is an incomplete one as merely coupling cells with a scaffold falls short of creating a true three-dimensional fully microintegrated scaffold. Many such studies elucidating the development of scaffolds alongside their ability to harbor cells with existing and new biomaterials, have shown some interesting advancements. Although these achievements have been commendable, their

adoption into the biomedical sciences and clinical medicine has been seldom due to their inability to have uniform and complete cellularisation throughout the scaffold thickness, to their costs and time for their generation. Therefore, in this critical review the author wishes to highlight clearly the advantages and disadvantages of those scaffolding technologies bearing in mind the end goal, which is to see their utility in either the biomedical and/or clinical arena. The readers are strongly reminded that, the technologies discussed in this review have been carefully chosen based on their promise to possibly generate a fully functional soft and dense reconstruct cost effectively, requiring a minimum number of processing steps, together with the shortest time for recreating a living tissue/organ which mimics a native tissue/organ.

2. Methods of generating scaffolds for engineering functional soft & dense reconstructs

The author simplifies these methods into two categories, namely, in-direct and direct approaches. The in-direct methods referred to here are those approaches that cannot handle the cells with other materials, namely the biopolymer with the addition of other biomolecules etc for directly forming a living structure. Hence these methods have many steps and are time consuming for reaching its end goal. The reader must note at this stage that there are some approaches that are referred to as either scaffold-less or scaffold-free technologies, for tissue engineering. These are at best misleading terms or concepts, as scaffolds are a prerequisite for developing a functional tissue of any kind. [2]

2.1. In-direct methods of scaffolding

These methods of scaffolding, are those approaches having many individual steps for developing the scaffold, subsequently manually seeded with cells, which undergoes many re-seeding steps with increased and cyclic bioreactor times in the hope to encouraging microintegration of the cells and scaffold. There are many methods apart from those

highlighted in this section which have also generated scaffolds by way of in-direct methods, however the author chooses not to highlight them, as they require a large number of processing steps thus implying long periods of time or their inability to reconstruct a three-dimensional fully cellularised architecture both cost effectively and in three-dimensions relevant for human transplantation. The reader should note well that in these in-direct methods architectural changes requires a mold or transfer architecture to be altered/modified and thus implies additional costs with the requirement for changing the deemed final cellularised architecture.

2.1.1 Lithography technologies

Lithography technologies [3] (see **figure 1** for illustration on soft-lithography) have many manifestations but essentially they either explore pre-organized architectures in the form a stamp to transfer molecules from the stamp as a pattern onto a pre-fabricated surface or the writing (dip-pen lithography) which transfers molecules with the aid of a fine edged cantilever, subsequently encouraging cells to adhere and grown on the transferred molecular architecture. The approach is unique in that its ability to call cells to seed onto pre-formed architectures which the cells take shape of and proliferate. However, the re-transfer or stacking of molecules onto the cellular surface created through the previous step for forming the second cellular layer, unfortunately is unachievable as the transferring process has been found to damage and destroy a majority of cells previously adhered. Additionally, the process requires multiple steps and therefore it is both time consuming and laborious while also demanding the maintenance of sterility over the entire process. That being said the technology could be useful for assessing adherent cells individually in conjunction with a laser to understanding basic biology, where cell(s) could be introduced to compounds (in the nano scale <50nm) and analyzed via time-lapse microscopy for assessing a wide range of cellular function in two-dimensions. [4]

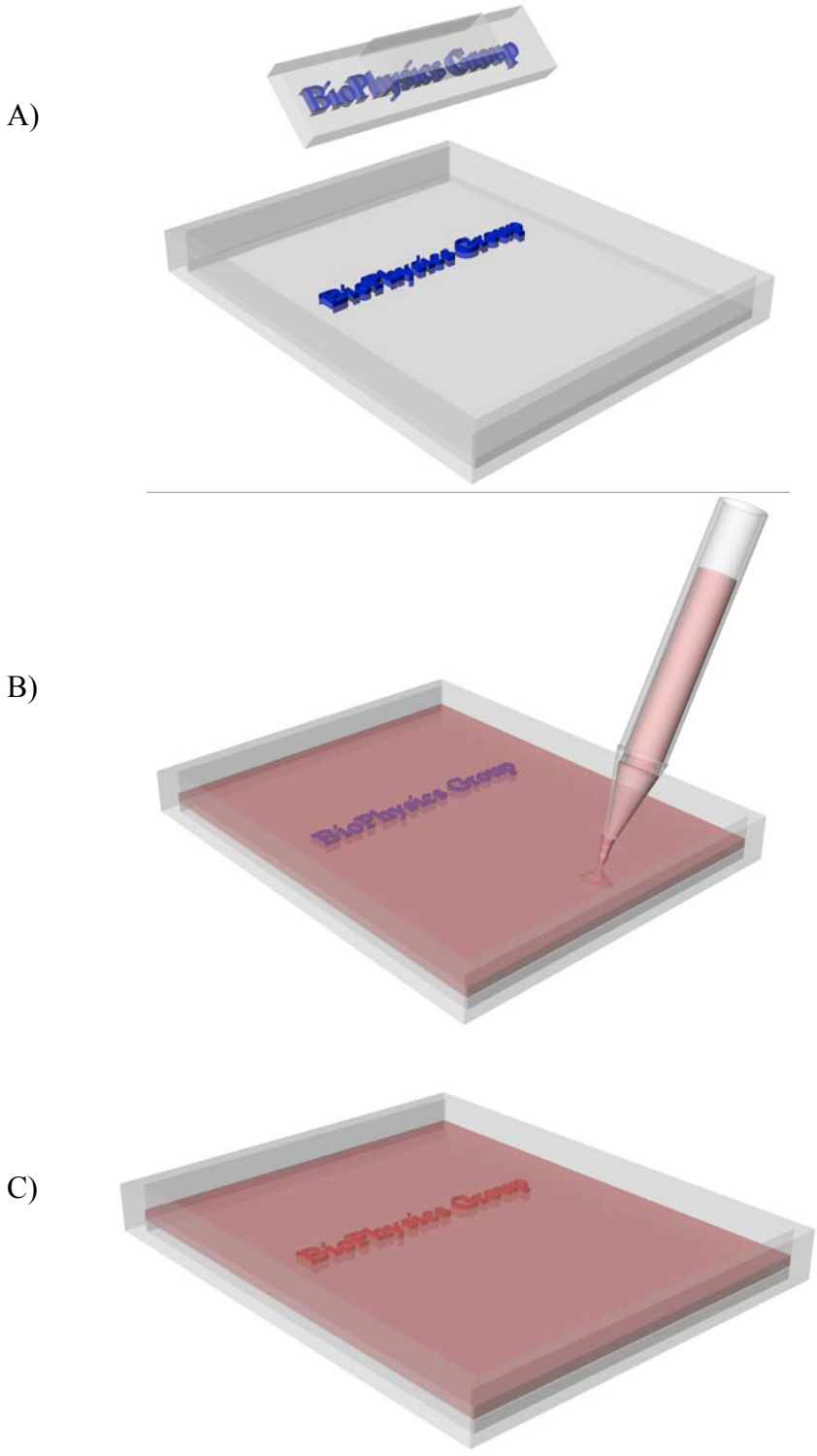


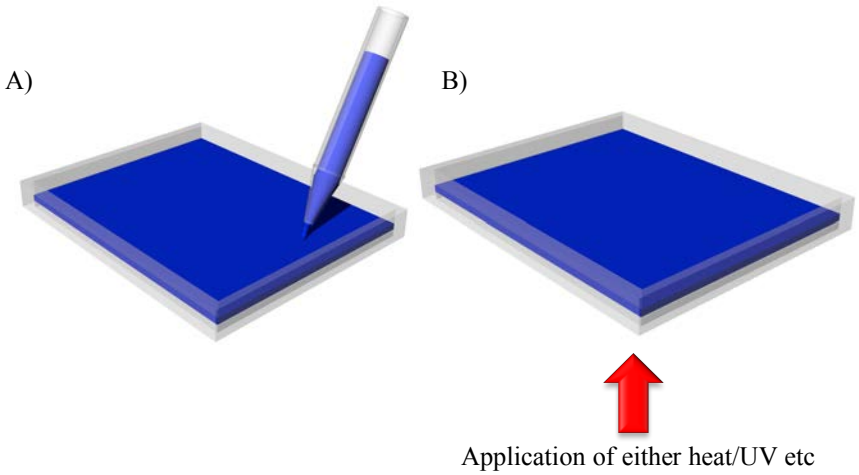
Figure 1. Illustrates the typical stages of exploring soft-lithography A) transferring (by stamping) the molecules in a pre-organized architecture and orientation, B) the introduction of a cell suspension to the transferred molecular architecture and incubation and finally C) the

cells within the suspension migrating and adhering to the transferred molecular architecture. This basic concept is by far followed by a majority of lithography approaches.

Both soft- and dip-pen lithography have undergone some modifications/manifestations where in the case of soft-lithography the stamping approach was used for creating small cell friendly semi-solid blocks made of biocompatible materials, which when introduced and incubated with a cell suspension encouraged cell infiltration into these biopolymer blocks. [5] This however once again is time dependent as cells are required to migrate in their own time, additionally to develop a tissue of practical utility would be near impossible. Similarly, the advancement in dip-pen lithography which writes out the architecture with molecules while the fine cantilever periodically replenishes its molecules at the tip of the pen by moving back and forth from a reservoir holding the molecules has now a continuous feeding tip. [6] This could see cells being deposited onto a substrate, which would be pre-fabricated, the author sees the impracticality, where cellular clogging may take place when cells and a viscous biopolymer is processed within this continuous feed system. Although these lithography technologies are inefficient for cell driven aspect within the biomedical based applications, the technologies have implications to niche areas of research which require accurate protein to DNA and other biomolecular placement for diagnostic and analytical applications. [7] Other lithography family technologies such as stereolithography and photolithography [8] have also undergone exploration for forming cellularised architectures, but these have many limitations such as the inability to have controlled densities of cells compartmentalized in a given permutation and combination with and without the addition of other materials. To finally the processes (lasers generate significant amounts of heat etc) having negative effects on cells during the stage where cells are entrapped by solidifying/crosslinking and compartmentalizing.

2.1.2. Chemical methods

Chemical approaches for developing scaffolds have been by way of exploiting rapidly evaporating solvents with FDA approved biopolymers. Here the biopolymer (which largely is provided as a powder/flakes) is solubilized in a fast evaporating solvent and then cast into the required shape using a mold of some kind, and allowed to either dry in a desiccator or with the addition of reactive chemicals to the application of heat/UV etc. These approaches have been demonstrated to form scaffolds rapidly. [9] However, the process has no control over the generated pore sizes nor control over the pore frame sizes or their uniformity throughout the scaffold (see **figure 2**).



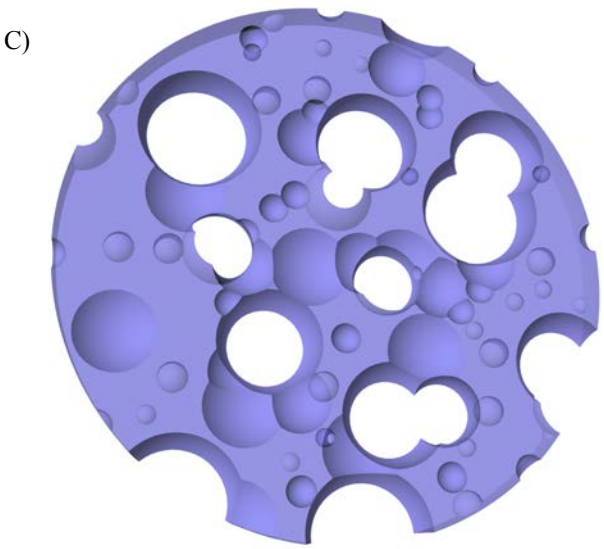


Figure 2. Chemical-based scaffolding approach showing A) biopolymer and/or suspension being placed in the mold, B) the introduction of the cross-linking mechanisms (heat, light or chemical etc) to finally C) the generated biopsy punched scaffold having dimples and voids. Cells could be mixed in with some cell friendly polymer and cast with the aid of a mold. However, cell distribution over the molded architecture would be varied and random as cells would settle down during the crosslinking process.

Scaffolds generated by this approach has been introduced to cells by way of manual seeding and found to encourage cell growth within them. [10] Cells are found to grown in and out of the pores but long term cellular growth has not been reported widely nor has any in-depth in-vivo studies been carried out. A few reasons for this are those fast evaporating solvents/reacting chemicals could leave residual traces which have been found to have triggered cellular death or inflammation in those in-vivo investigations. Addressing this is an issue as residual compounds from those chemicals explored are extremely difficult to fully remove from the generated scaffolds. An interesting aspect the author has come to realize from such scaffolds and others like these, have been that some scientists regard the adhered cells on the inner and outer surfaces in these pores whilst proliferating and stacking themselves on each other, is a tissue in three-dimensions. The author disagrees with this notion that these cells are in three-dimensions. In fact, they are in two-dimensions at best as their plane of adherence is in two-dimensions. Additionally, nutrients much required by cells for maintaining their dynamic metabolisms have limited access to such molecules as they have to pass across barriers/membranes which are part of the scaffold or tightly packed cellular layers. These views are even more so when cells are adhered on pore frames or struts which are larger than the diameter of the cells itself. These limitations have been investigated and have been found to have detrimental effects in-vivo as the cell-adhered architectures have given rise to what is known as foreign body reactions. [11] Hence for these and other reasons

such constructs have been limited in their exploration. Manifestations of this approach have been where friendly biopolymers such as alginate (including those doped with collagen etc) has been mixed with cells and either drawn into fibers using immersed needles in calcium chloride (to other cross-linking solutions), or threads into this cell bearing polymer, which have been found to generate cell-bearing fibers. [12] That being said, such constructs have limited utility in building a three-dimensional architecture without further processing, as the cell bearing constructs are generated as single strands. [13]

2.1.3. 3D printing

This is technology that adds materials when required and thus unlike the previous technologies, significantly reduces material waste during the architectural forming step. [14] An interesting manufacturing concept which brings to the table the ability to eliminate the need for molds. 3D printing could be carried out in many methods, meaning there are many approaches to dispensing or extruding materials for forming a three-dimensional architecture. These would primarily range from ink-jet printing (solenoid, piezoelectric, thermal), to extrusion approaches ranging from thermal sleeve heated systems to those which are screw/plunger driven (**see figure 3**). [15] These approaches in their many manifestations have been shown to generate some complex architectures which are truly magnificent. This printing approach is essentially a 2D materials deposition approach which is used for layering multiple times (or stacking) for creating the third dimension (z-axis). However, these architectures are generated with classical materials, and thus are an ideal approach for creating architectures for the rapid prototyping industry. The 3D printing community has tirelessly struggled however to extend this approach for the last 20 years or so, for directly handling cells (with and without other materials) with a biopolymer for creating a self-standing fully cellularised architecture, requiring no additional assistance during the formation stage. These efforts have been unsuccessful as they are hindered by the extrusion methods

themselves, which stem from these materials handling approaches having direct application of their driving forces applied on the cells while exploring fine bore needles, and thus have seen to cause many cellular damage, clogging of needles to a wide range of other issues which have harm on the cells themselves. [16] Additionally, these explored fine bore needles used in order to form architectures at small scales (in the micrometer) have other ramifications to the manufacturing process. This is a challenge for these processes as the technologies undergo needle wetting effects to those referred to as the barus effect [17] which have negative implications or the generated sizes of either droplets or filaments. Hence the formed droplets/threads which are roughly double the diameter of the needle used, thus on deposition of the material, further spreading occurs on the substrate making manufacturing in the few tens of micrometers a tremendous challenge. [18]

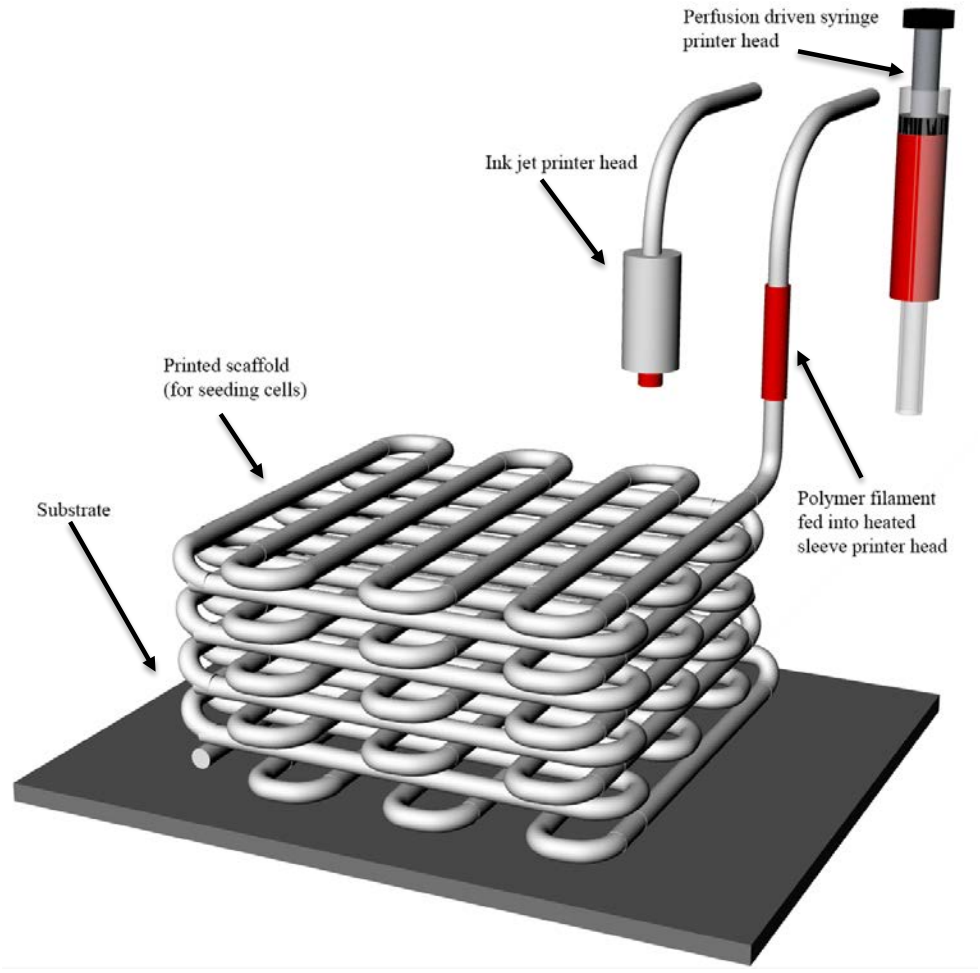


Figure 3. Three-dimensional printing illustrating the three-major materials extrusion methodologies.

A recent addition to the 3D printing portfolio is melt electrospinning writing. [19] This technology works on extruding a polymer melt across two charged electrodes between which a substrate is placed. One electrode holds the melt flowing through it, which is also charged to a high voltage with respect to either a grounded electrode which has in contact or above it a substrate for collecting the extruding polymer melt. The process conceptually is similar to the other 3D extruders with the exception that this process explores an electric field to draw out a polymer filament. One notable advancement this approach overcomes faced by those other 3D printing approaches is that this technology namely, electrospinning explores large bore needles from which fine polymer filaments are drawn out thus avoiding all issues limited by other approaches to the most important which is the ability to form fine polymer filaments extending even to the nano-regime. [20] That being said, the technology much like its other 3D printing cousins cannot handle living cells as the melt temperature could compromise cellular viability. It is important for the reader to note at this stage the technology referred to as continuous liquid interface production (CLIP), which is a renamed and a retrofitted technology, combines chemical methods and three-dimensional printing. CLIP is not a new technology but combines approaches to build 3D architectures layer by layer within a liquid environment, much like other direct write technologies. [21] Hence CLIP and these other direct write technologies can build architectures with classical materials, however this is not the case with living cells as the processes have not yet seen the development of bio-friendly media, where such structures could be developed with cells, or if such media are available maintaining cellular distribution within the developed architecture would be near impossible as the cells would sediment to the based during the structural formation stage. This would

result with the cellular architecture taking a 2D form as opposed to 3D. Additionally these procedures may have negative effects on the direct handling of dense cell populations. [3-21]

2.1.4. Cell sheeting

This technology yields from the advancement of surface technology. [22] The approach works on the principal of having cell culture dishes coated with a specialized polymer, which allows cells to be seeded (**figure 4**), proliferate and reach confluence. Once the given cell type reaches confluency the cell culture dish is brought to a lower temperature wrt incubation temperature, at which stage the cells together with their generated extra cellular matrix peels off as a sheet. The process has been clinically utilized successfully for forming monolayered single cellular sheets for repairing human cornea. [23] Although the author feels this is a neat accomplishment, engineering a dense fully functional multi cellular architecture has yet to be generated for either laboratory or clinical utility. Although not reported, we have found that the technology is laborious for creating a multicellular construct, which could sustain living over a function of time. This results from poor vascularization of the generated 3D construct, by way of stacking multiple cell type sheets, which inhibit vascularization to take form in the z-axes through the staked multiple cellular sheets. Never the less the technology could have potential for the fabrication of singular or multiple cellular type sheets for applications such as skin repair/wound healing.

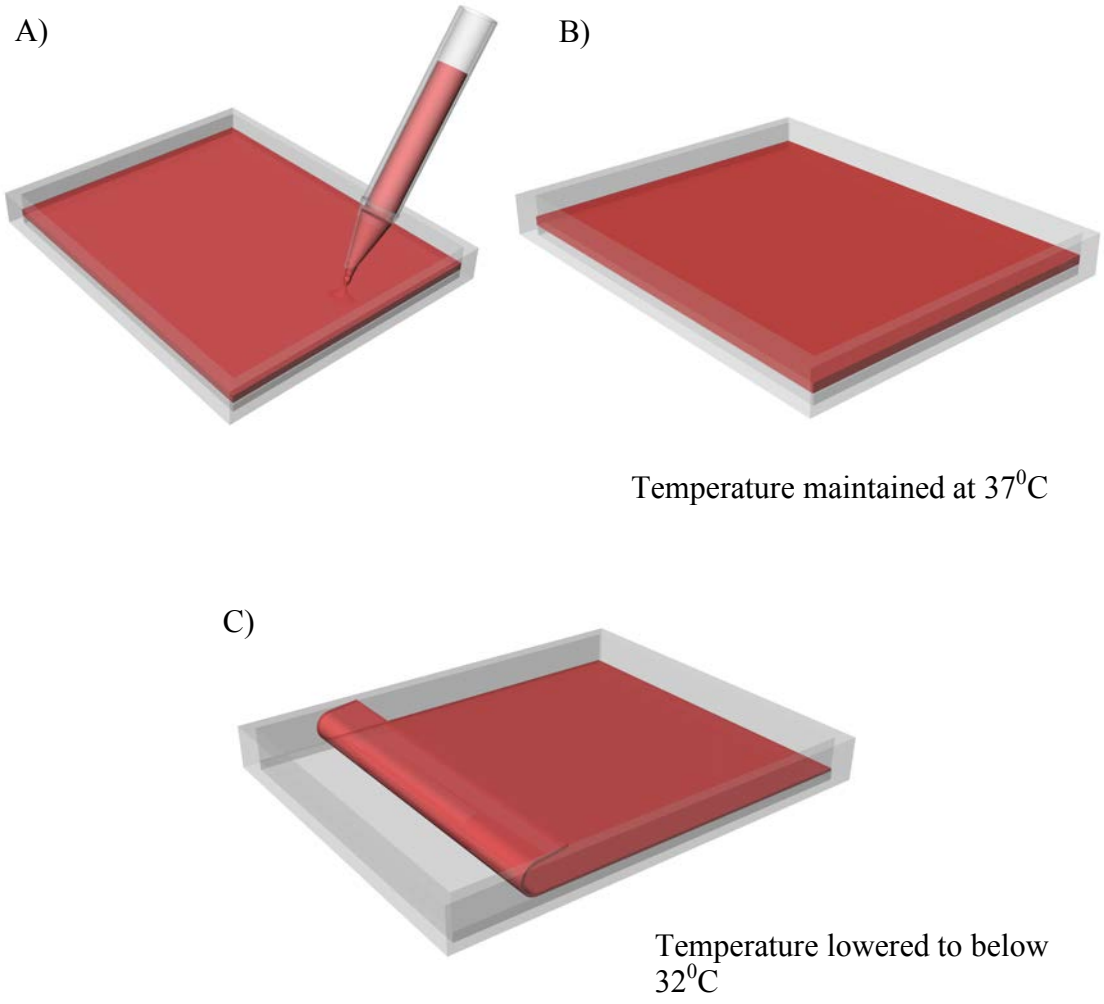


Figure 4. Cell sheeting A) the deposition of a cell suspension on the coated culture dish, B) the filled culture dish containing the cell suspension which would be left till cells are confluent in an incubator to finally C) the peeling off the cellular sheet containing the cells with their generated extracellular matrix at a lower temperature.

2.1.5. Force Spinning

In the past two decades fibers, have taken precedence due to their practical applicability in the real world. Force spinning is a technology reminiscent of the candy floss machine (**figure 5**) which exploits centrifugal forces [24] to extrude fibers from either a fast evaporating polymer suspension or a melt. The technology has some advantage that it does not require any special

equipment or instrumentation, but is hindered by the fact that it cannot generate fibers which could be cross stitched, and requires a drum or removable cylinder like substrate which wraps around as a drum for fiber/scaffold collection. There are many manifestations of this technology retrofitted and renamed but the limitations are yet to be overcome. Interestingly one such manifestation which should provoke readers are where this technology was coupled with electric fields for directing the fibers onto a conveyer belt for making large quantities of scaffold sheets etc. [25] The provoking part here is that these investigators have combined two technologies namely force spinning and electrospinning to do what electrospinning can do alone, without requiring the rotational element thus introducing a waste in energy! There are a few scientists exploring this technology for small scale lab-based scaffold generation but it is hard for the author to see the practicality of this process entering larger scale production through its rotational fiber/scaffold formation approach. A few research groups have tried to incorporate cells into the spinning polymer but have found the polymer to loses a significant proportion of its liquid thus dehydrating the entrapped cells which significantly reduces their viability, to losing cell entrapment during the rapid fiber spinning process.

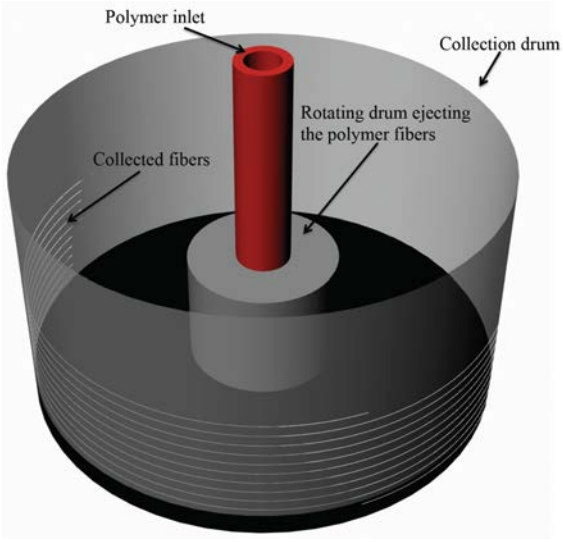


Figure 5. The equipment set-up used for force spinning fibers for generating scaffolds and membranes.

2.1.6. Electrosprays and electrospinning

Electrospraying and electrospinning (**figure 6**) have been around for at least a century. [26] These approaches work on the principal of charging a liquid within a conducting needle, which is placed in an electric field with respect to a grounded or oppositely charged electrode. The potential difference between the electrodes, in stable conditions have been shown to form a liquid cone at the needle exit with a jet emanating from its apex, which either subsequently breaks down into a three-dimensional conical spray plume (electrosprays – generates droplets) or a continuously elongating thread from the jet (electrospinning – generates fibers thus forming scaffolds and membranes). Both these techniques operate at very high voltage, typically in the 1000's of volts. Interestingly these approaches have been shown to generate droplets and fibers in the few nanometers having a wide range of features unrivaled by all other technologies. [27] These features could range from hollow, porous to a wide range of both surface and bulk features having multiple purpose for a given end application. [28] These multi-featured droplets and fibers are generated by way of either coaxial [29] or tri-needle [30] systems to those which are coupled with a wide variety of counter electrodes. [31] The reader should appreciate that, these techniques unlike their rivals explore needles in the several hundreds of micrometers but are able to generate either droplets or fibers in the few nanometers. Hence these features propel these technologies ahead of its competitors for these and many more reasons which will be discussed in detail in this review. A whole host of advanced materials have been processed using these two approaches and found to generate a wide range of architectures having utility for many applications. [32] The reader should also note that these techniques (both electrospraying and electrospinning) have also been developed to print three-dimensional architectures layer by layer. [33] Most notably

electrospinning based three-dimensional printing has a significant disadvantage, where stacked layers cause interference with the electric field with increasing number of layers and results in random deposition. [34] This limitation has been overcome to some extent by the modification of the collection method or substrate. [33] Although this might be a solution it comes with a cost as these substrates are not economical to modify when required. This interference driven limitation has recently been removed by the introduction of electric field focusing plates and rings which have eliminated this obstacle. [35] Captivatingly its sister technology, electrosprays have undergone development to see the unearthing of a true three-dimensional printing technology which required no molds of any kind to act as supports during the forming stage to the most important feature, namely when printing overhangs etc unlike rival technologies. [36] In the biomedical and clinical arena, architectures generated by means of electrospinning has limitations where the generated hollow and/or porous (or other featured) architectures have been found to limit their utility. For e.g. scaffolds generated by way of electrospinning have been found to inhibit cell infiltration throughout the entire thickness of the scaffold, therefore these scaffolds are now being investigated and commercialized for contraception. [37] Proving that the smallest human self-propelling cell (sperm cells) is unable to infiltrate through the fine voids found in generated scaffolds via electrospinning. Moreover, these techniques can directly handle living cells without damaging them form a molecular level upwards!

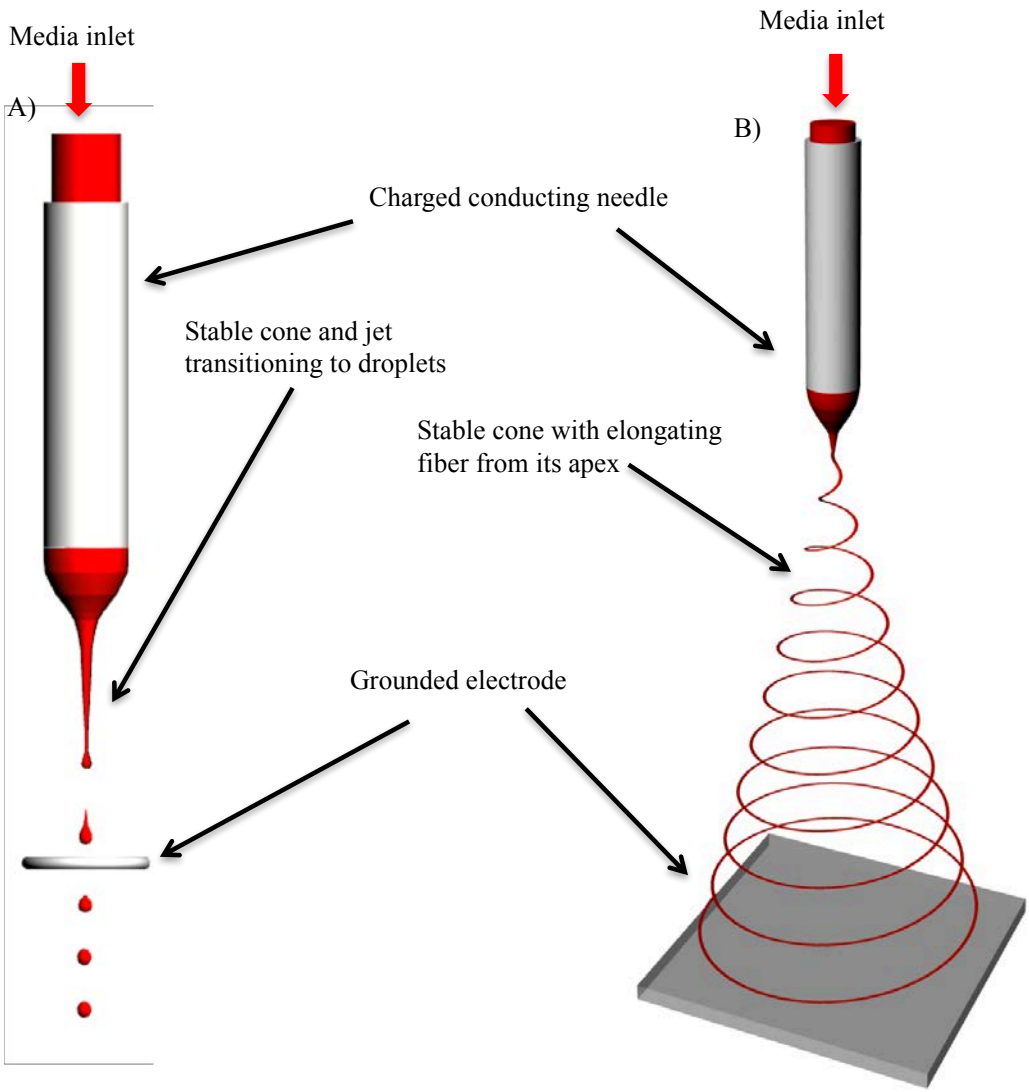


Figure 6. Illustrations of A) electrospraying and B) electrospinning.

2.1.7. Decellularization and recellularisation

This process explores the concept of retaining the backbone structure of a tissue/organ by removing its cellular contents thus leaving behind a near-intact collagen structure. [38] The concept strives to completely remove cells from a tissue or organ thus removing the individual's signature, namely DNA etc. In the process the tissue or organ is sourced either from the donor pool or from an animal source. The procedure (**figure 7**) in brief involves using a biocompatible chemical cocktail to tease out the cells within the tissue or organ of choice thus leaving behind the collagen structure of the tissue/organ. Following cell removal,

the tissue is reintroduced to a new cell source namely the recipients, thus these adult cells are cued to home to their given location within a tissue or organ allowing the reconstruction of the tissue or organ with a new cell source. The process has worked in animals and some human trials have been successful. [39] However, although this is an interesting concept the author feels this approach takes one step forward and three steps back! It is hard to envisage this approach ever entering clinical utility for everyone as it demands all of its tissues and organs from the already oversubscribed donor pool or from an animal source both which demand lifelong immunosuppression, which brings with it many complications. The complications evolve as DNA removal is not complete and the remaining structure is known to lose its integrity due to the cell removal process. [40] This approach could have some application, for example in the generation of a thin non-life threatening tissue. [41]

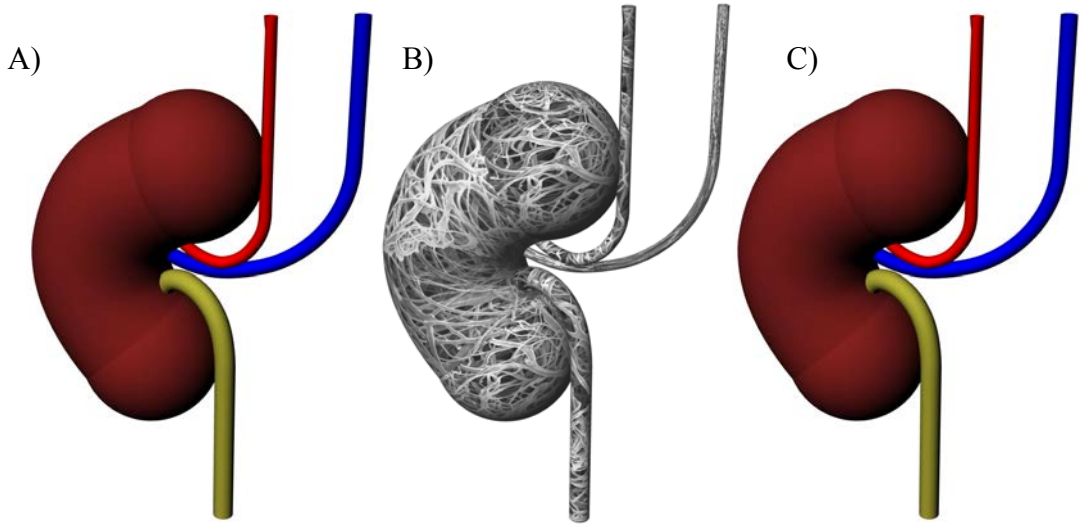


Figure 7. Demonstrates the A) dysfunctional kidney, B) striped of its cells completely, only leave its scaffold backbone. Subsequently panel C) shows cells from the recipient are introduced to the scaffold and these adult cells home to their respective locations in the organ.

Although in-direct methods could be argued as having some laboratory applications their ability to translate from the biomedical laboratory to the clinic is yet to be seen and realised practically. These issues arise from their inability to generate practical size tissues most relevant to humans alongside their associated processing times/number of steps to the involved costs for reconstructing a tissue/organ which could be transplanted.

The author would like to make aware to the readers at this point that there are other methods currently being investigated for developing tissues/organs, within living animals. These methods although still in their infancy, face issues such as the time taken for the full development and function of a human tissue/organ in comparison to the time required for development and function of an animal tissue/organ. [42]

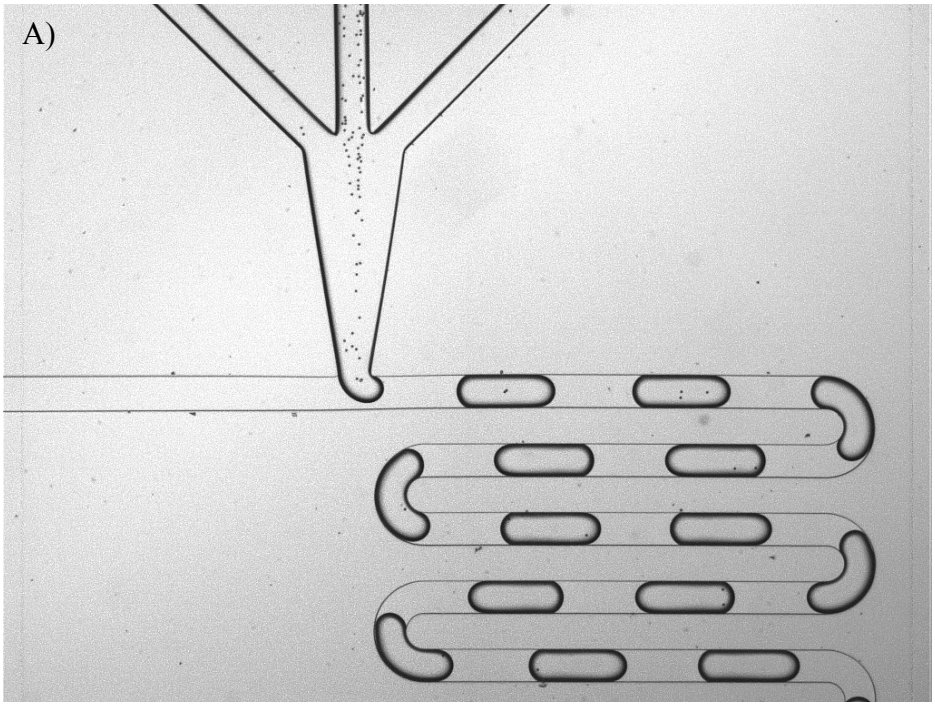
2.2. Direct methods of scaffolding

Direct methods of scaffolding unlike those in-direct methods can handle the cells mixed in with the biopolymer and a wide range of other materials such as proteins etc. The advantages of this over the other category is that these approaches require less steps to generate a structure and less bioreactor time for enabling cells to integrate with the materials and the structure. This also reduces other pit falls such as sterility issues and reduces costs.

2.2.1. Lab on a chip approaches

Lab on a chip also sometimes referred to as microfluidics, is an interesting micro volume liquid handling approach, which has been elucidated to have some attractive features. This technology comes alive when two pieces of polydimethylsiloxane (PDMS) have been fused together. Note that one piece of PDMS is flat on both sides while the other carries the imprinted flow route(s) as channels, having both inputs and outputs punched out through the

entire thickness of that piece of PDMS (**figure 8**). The base and materials which are generally used (above referred to as the PDMS piece having both sides flat) could be replaced with a glass microslide and there are many manifestations of this. Here liquids of various properties are introduced and mixed in precisely controlled volumes and studied for a wide range of applications. [43] This technology has been used for passing cellular suspensions for creating what is referred to as organ on a chip. [44] In addition to this advancement colleagues have also developed this technology to handle cells for cytometry studies and is referred to as fluorescent activated droplet sorting (FADS). [45] Although the technology has many advantages it is limited by its inability to scale up and handle high viscosity liquids to name a few, limiting the technology to the laboratory. Some disadvantages in FADS for e.g. is where the technology cannot handle cell suspensions of high viscosity which are regularly interrogated by its competing approach widely referred to as hydrodynamic focusing which takes place in the cytometer's flow cell. [46] In the context of this review for building cellular architectures as referred to as organ on a chip the technology places cells in two-dimensions at best and stacks them in this configuration rather than in true three-dimensions. [47] We have noted that in some cases this technology is both inefficient and incapable of developing and generating tissues/organs, as the technology either demands the cells to be placed on the base of the chip prior to it being attached to its imprinted architecture thus squeezing and destroying cells out of the imprinted zone. This might not be such an issue with cells under investigation if they were fibroblasts (which are available in abundance), but this would not be the case with those rare cell populations. One way out of this obstacle is to keep the cells flowing through the chip continuously in the hope they will attach and cover the surface area desired but this raises the issue of time and the waste of complete media (media that comprises of all growth factors etc). Therefore, microfluidics limit the yield and have many obstacles to overcome before it could truly move from the laboratory to the clinic.



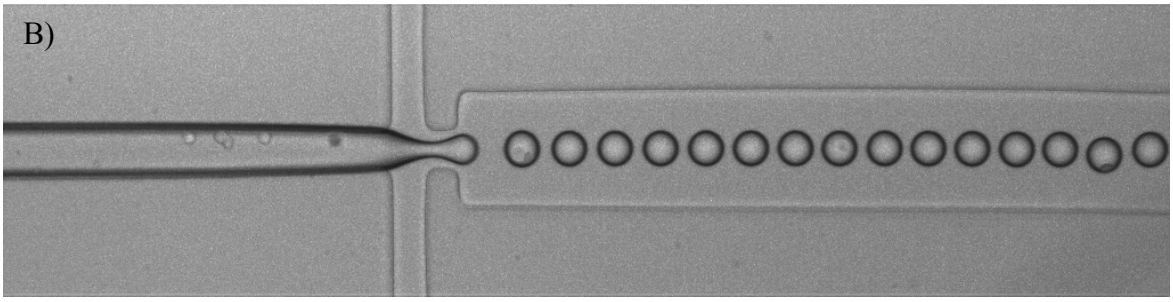


Figure 8. Two architectures amongst many famously investigated in lab chip based investigations. Panel A) depict a typical T-junction and B) demonstrates a flow focusing architecture.

2.2.2. Laser guided cell printing

Laser technology has been in the biological and medical fields for well over half a century. [48] This has come about by the technology widely referred to as flow cytometry which is a florescent activated cell sorting (FACS) technology. [46] Here lasers are used for interrogating the physio chemical nature of single cells, dynamically from a multi-cellular population. FACS is an unrivaled non-evasive single cell analysis technology which enables cells to be identified, sorted, and subsequently reintroduced into the human body, which is one

application amongst many others it has on offer. Laser guided cell writing or printing is where lasers are used as tweezers to pick cells up (or eject cells as a liquid jet etc) and place them where required (**figure 9**). [49] This is a very precise method of handling single cells for precise placement. The approach is uniquely capable of placing cells at different and precise proximities to each other and thus enables development biologists to closely study cellular interactions in either two- or three-dimensions. Unfortunately for the development of a three-dimensional multi-cellular tissue, the technology is limited by its inability to handle multiple cell types simultaneously for generating a true three-dimensional tissue. The reader should note that laser tweezers generate high temperature at their tips and therefore the cells cannot be resident or held in such a harsh environment for long as this would compromise the cell's viability.

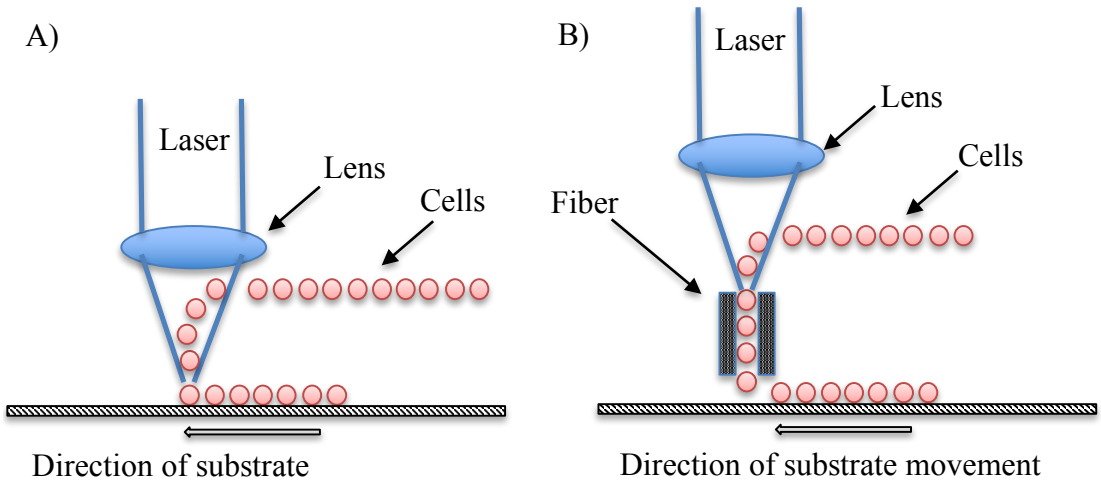


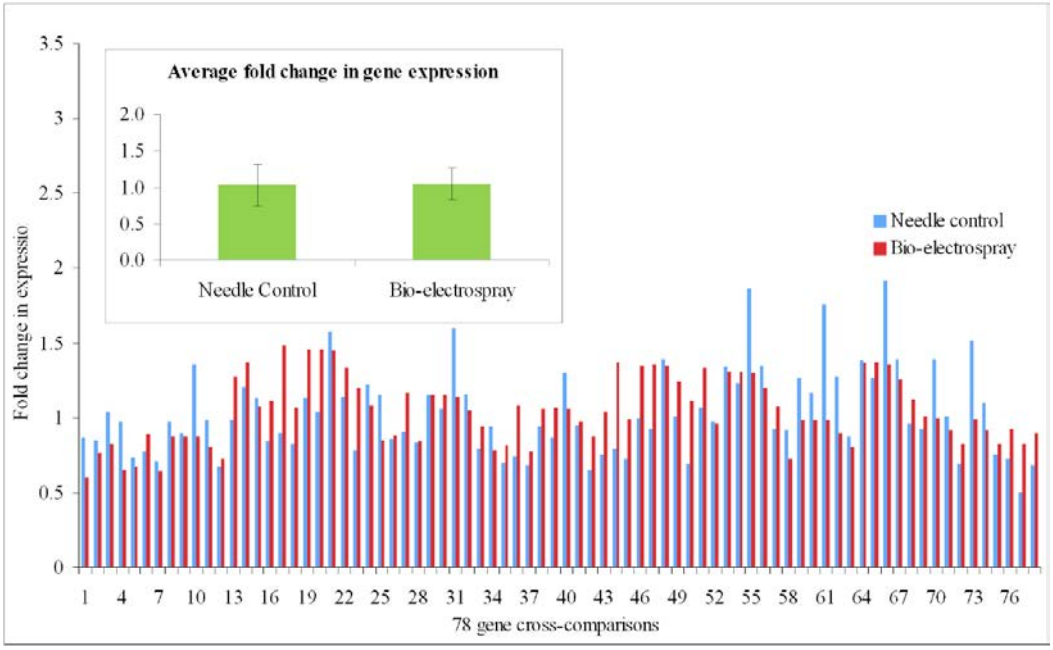
Figure 9. Illustrations showing A) a laser guided cell writing apparatus and B) a modified version which writes with the aid of a hollow fiber.

2.2.3. Bio-electrospraying (BES) and cell electrospinning (CE)

These techniques have evolved from both the basic technologies referred to as electrosprays and electrospinning. [26, 27] As mentioned previously (section 2.1.6), both these technologies work on the same principal. In summary both these approaches charge a flowing liquid within

a needle which is held in an electric field brought about by a potential difference caused by a ground electrode or oppositely charged electrode placed on the opposed side of the charged needle. The electric field draws out the charged liquid towards the counter electrode and either forms a jet which undergoes break up and forms droplets or elongates into a continuous fiber. The former is electrosprays and the latter is electrospinning. In 2005/2006 Jayasinghe et al., [50] started exploring the ability to pass living cells through both these techniques and demonstrated their ability to do so without harming the cells from a molecular level upwards (**figure 10**). [51] The reader should be aware that processed living cells or organisms directly handled are not perturbed from a molecular level upwards, as the current is the effecting parameter in these studies, and are generally in the nanoamperes, whilst the voltage is in the thousands of volts. [52] In electroporation studies the opposite takes places, and therefore is found to damage cells. Those bio-electrospay and cell electrospinning operational conditions are also used in taser gun technology. [53] In a translational standpoint electric fields are used effectively and frequently in the clinic for surgery, through the technology referred to as electrosurgery/electrocuting. [53]

A)



B)

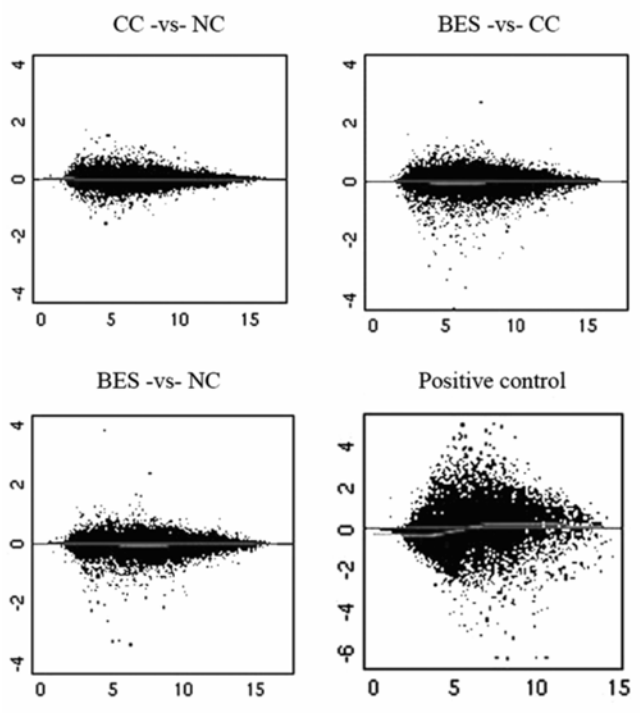


Figure 10. A) Characteristic plots demonstrating gene expression profiles of whole human blood of the needle control, NC and the bio-electrosprayed, BES sample. All genes were cross-compared with all other genes, hence giving 78 sets of data from 13 genes analysed. The red coloured bars represent BES samples and the blue bars represent the NC. The insert graph is the global average change in gene expression relative to the control with standard error bars and B) Gene expression is not altered on post-BES neonatal rat cardiac myocyte. MvA plots generated using bioconductor and gene array results compared between the culture control, CC, needle control, NC and bio-electrosprayed, BES. The vertical axis represents the intensity difference (M) and the horizontal axis the average intensity (A) plotted on a log 2 scale. Ischaemic cardiac tissue is represented by the positive control group with differential expression.

Since then the technologies have not only been used for handling single and multiple dense populations of cells (ranging from mouse and human immortalized, primary, sperm to stem

cells) but have also handle multiple cell suspensions including those whole fertilized embryos etc (**figure 11**). [54]

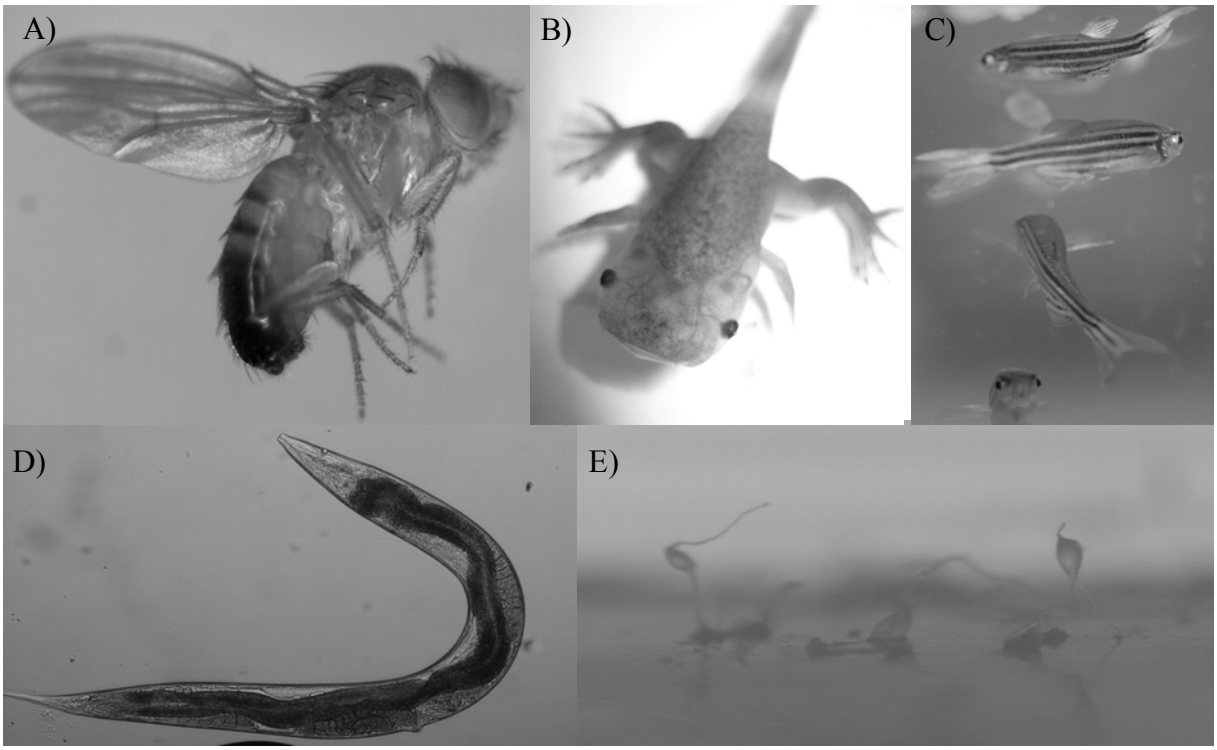
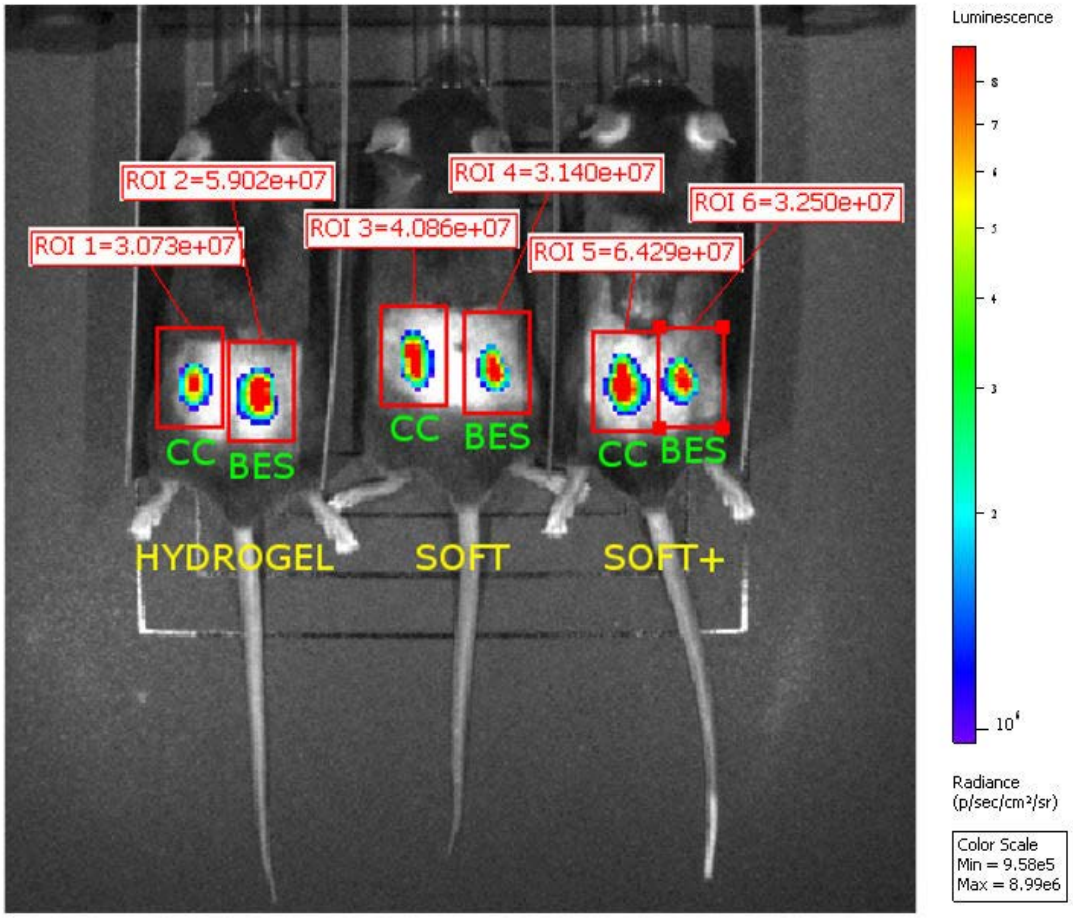


Figure 11. Panels show the model organisms BES and CE, A) *Drosophila melanogaster*, B) *Xenopus tropicalis*, C) *Danio rerio*, D) *Caenorhabditis elegans* and E) *Dictyostelium discoideum*. All the organisms post-treated were observed over a given time course respectively, and seen to develop as expected in comparison to control samples.

These continued efforts have seen generated tissues by way of either BES or CE being transplanted into mouse/rat to sheep and pig models. [55] These studies are the first of their kind for any such cell handling approach to be tested in animal models for assessing their efficacy and their true potential (**figure 12**). These platform technologies have also undergone development for modelling human tuberculosis [56] and a wide range of other human diseases which show the true applicability and flexibility of these platforms.

A)


ROI 1=3.073e+07
ROI 2=5.902e+07
ROI 3=4.086e+07
ROI 4=3.140e+07
ROI 5=6.429e+07
ROI 6=3.250e+07
CC
BES
CC
BES
CC
BES
HYDROGEL
SOFT
SOFT+
Luminescence
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 9.58e5
Max = 8.99e6

B)

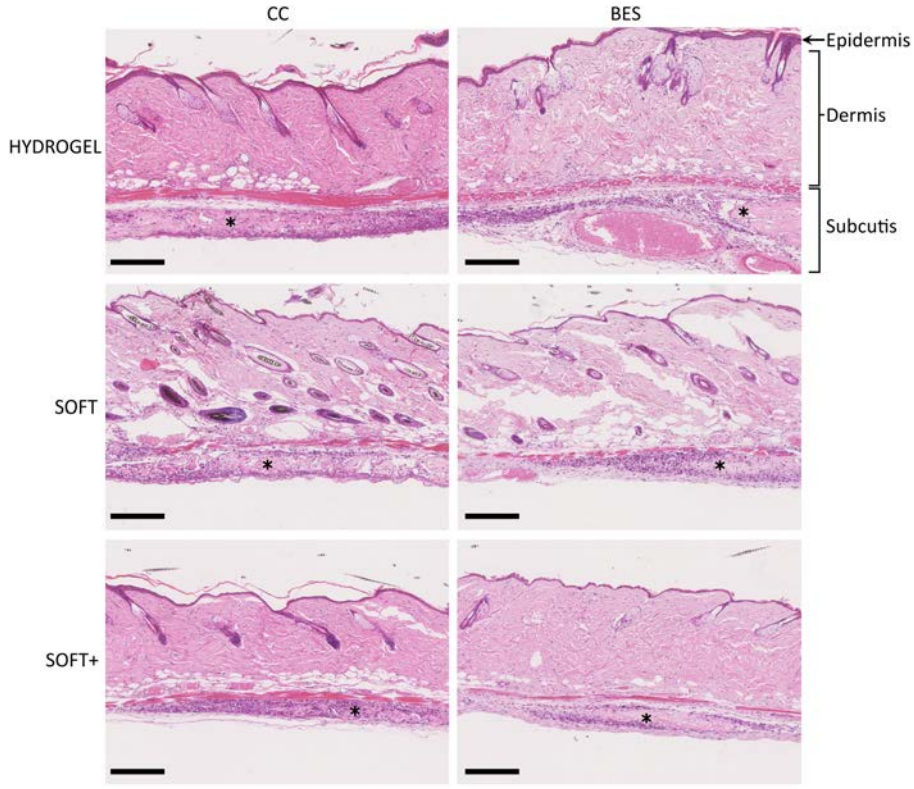

CC
BES
HYDROGEL
SOFT
SOFT+
Epidermis
Dermis
Subcutis

Figure 12. A) Bioluminescent imaging of transplanted macrophage bearing matricies. IC-21-Luc macrophages (CC or BES) were mixed with matrices (Hydrogel, Soft, or Soft+) and subcutaneously injected into the dorsal flanks of C57Bl/6 mice (with Culture Control, CC on the left flank and bio-electrosprayed, BES on the right). Following intraperitoneal injection of d-luciferin, macrophage bioluminescence was detected using an IVIS Lumina II imaging system. Representative image from day 1 post-implantation, indicating the peak detectable radiance in identically sized regions of interest. The cell electrospun, CE results were very similar to the BES implants, B) Histological analysis of the macrophage bearing matrices implantation site. Four days post-implantation, skin was harvested from each dorsal flank, and processed for histology. The various matriciess (indicated by *) were discernible within the subcutis. Cells with the morphological appearance of macrophages were visible surrounding the matrices, with a scattering of cells within the matrix. Scale bar: 200 μm.

2.2.4. Aerodynamically assisted bio-jetting (AABJ) and threading (AABT)

This is jetting process which explores a pressure field through air flow, within a chamber with respect to the surrounding atmosphere, for drawing liquids of varied viscosities through an exit orifice (**figure 13**). [57] Much like in the case of both electrosprays and electrospinning the viscosity of the liquid determines the formation of either droplets or continuous threads (aerodynamically assisted jets/AAJ and threads/AAT respectively). [58] The process similar to both electrosprays and electrospinning have been explored for processing a wide range of materials and suspensions, which have been shown as a competing non-electric field driven process for handling a wide range of materials. [59] An interesting thought the reader should note is that these non-electric field driven processes are idea for handling those materials which are highly conducting which cannot be handled by either electrosprays or electrospinning as they would give rise to discharging or the damaging of those conducting

molecules. Both AAJ and AAT have been explored for directly handling a wide range of living cells and organisms with other materials and have been found to have no detrimental effects brought on the cells or organisms. [60] Since discovering the ability for both AAJ and AAT to handle living cells etc, the technologies have been explored for generating living constructs which have been transferred into small animals which have shown no rejection, and therefore shows their capacity to contribute to the development of tissues and/or organs. [54, 60] These platform technologies now are referred to as aerodynamically assisted bio-jets and bio-threads (AABJ/AABT respectively). These aerodynamically assisted processes have also undergone coupling with electric fields for applications in controlled deposition to the use of these modified systems for printing (controlled volumes of materials) and building architectures as those generated in 3D printing. [57, 63] There are manifestations (pressure assisted cell spinning and pressure driven cell spinning) of these processes which have also been investigated and shown to possess the ability to handle living cells and organisms. [61] AABJ at present is undergoing intense investigation as a sheathless flow cell technology for its entry into flow cytometry and FACS. [62]

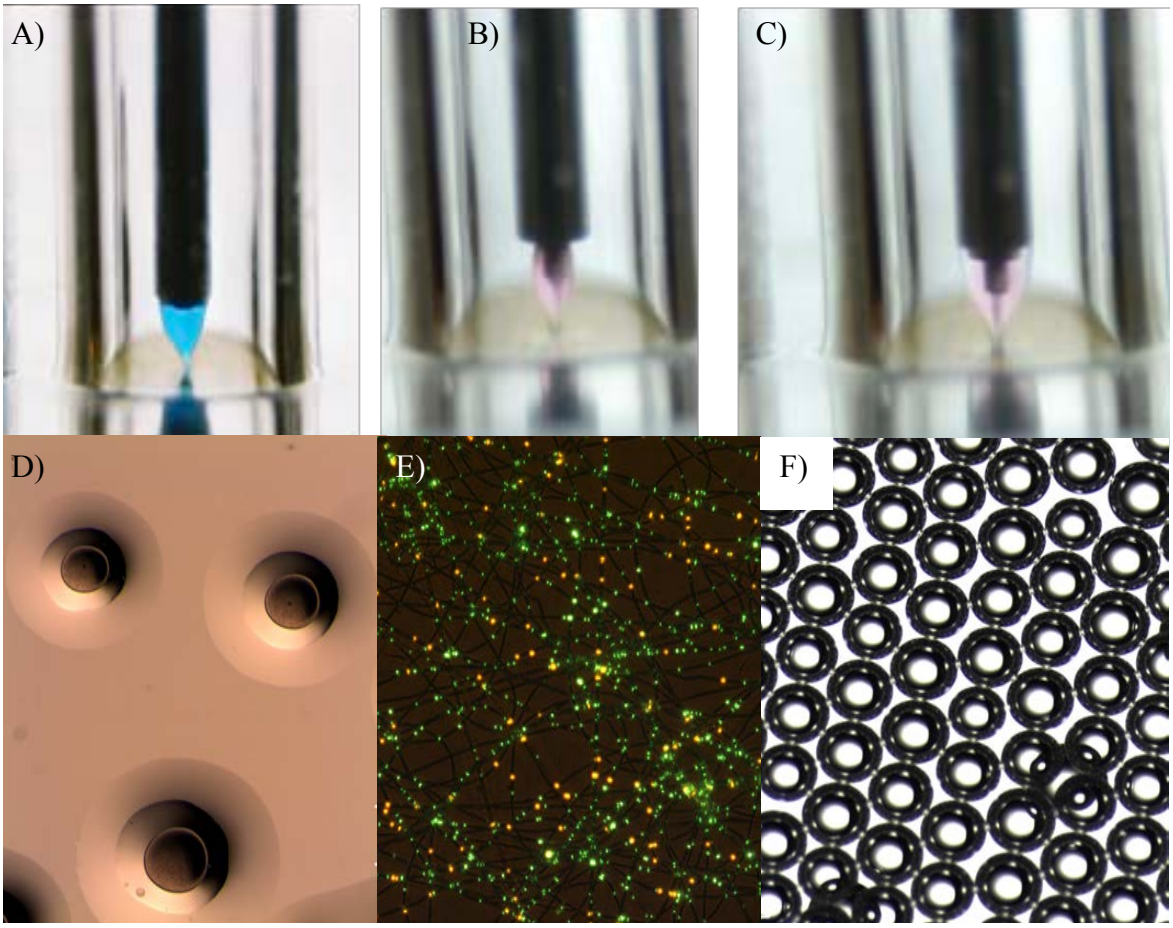


Figure 13. Representative digital images of the A) single, B) coaxial and C) triaxial needle systems explored in the aerodynamically assisted jetting and threading devices. Panel D) shows the residues generated and collected with the triaxial needle configuration, E) cell encapsulated scaffold generated by way of a coaxial aerodynamically assisted bio-threading device to finally, F) microbubbles generated using a single AAJ device.

3. Possible translational applications

3.1. Cell expansion and cryopreservation

Cell expansion and preservation for later use is common practice in regenerative biology and medicine. Classical approaches to cell expansion has been where cells have been isolated from given native tissue and expanded in sterile flasks maintained in an incubator within a given complete media. This approach has allowed cell expansion in practical quantities but have also had the negative effect of their expansion being either slowed and/or not successful as the cells are expanding over a 2D space. Also, the process is not economical as the foot print of these flasks are relatively large and therefore demands large numbers of incubators etc. Some of these issues have been addressed through the multi-platform cell culture flask which has many platforms of which cells could expand on within the same flask. [63] Although this is a step in the right direction cells are yet expanding in the 2D! With the ability to form either encapsulated or entrapped cells within either beads or scaffolds such as those generated using either BES/CE and AABJ/AABT, the foot print of those architectures could be smaller and yet the cells would have access to those nutrients etc in true 3D. In our hands, we have explored both these techniques for this purpose with the expansion of primary human cell types and found that a much higher percentage of cells are recovered over the same time when compared with those expanded using conventional methods. This results as the cells are in 3D and hence have access to nutrients in 3D. Furthermore having control over the pore sizes of these cell-bearing beads and fibers nutrient/molecular gating could be enabled. Similar, cell bearing architectures have been frozen down to -80^{0}C and have been thawed and assessed for their percentage of recovery, viability and functionality in contrast to those classical approaches. These studies have demonstrated a greater yield of cells recovered from these 3D architectures generated by way of these techniques (BES/CE and AABJ/AABT) in comparison to those recovered from classical methods over the same time frame of freezing. This is a direct result of the processes allowing the controlled compartmentalization of cells

within a given layer (either as a multi compartmentalized bead or multicore fiber/scaffold) of liquid/polymer which would both control the cells experiencing the lowering of the temperature during the freezing process, to the cells gentling getting accustomed to the increasing of temperature, within these architectures allowing the cells to thaw during the cell recovery process.

3.2. Reverse engineering natural scaffolds an alternative to decellularisation and recellularisation

The author introduced and discussed earlier the method referred to as decellularisation and recellularisation. This technology although appearing to have some significance still demands a majority of its tissues and/or organs from the donor pool, which is oversubscribed, or from a regulated animal house for sourcing its tissues and organs. Once these tissues or organs are sourced, the cells are teased out of these tissues/organs, using bio-friendly chemical cocktails which have been shown to compromise the decellularised scaffold. Many other unknowns regarding the cell-free scaffold have yet to be answered, such as residual DNA which may cause negative effects on the repopulated cells and those other side effects which may arise due to the residual DNA etc. Cell electrospinning and aerodynamically assisted bio-threading have been shown to generate architectures which have been transplanted into animal models and have shown no negative effects. Thus, it is plausible to generate a three-dimensional human tissue which could be incubated for complete integration and maintained over a function of time. Such tissues could later be decellularised and recellularised with the recipient's cells. Thus, avoiding the need to source tissues and organs from either the donor pool or an animal source, which would completely remove the need for life long immunosuppression to the patient. Additionally, the required number of steps for generating a recellularised functional 3D reconstruct would take much less time to those approaches followed by the conventional methods of decellularisation and recellularisation. This stems

from the fact that those architectures generated via either CE/AABT have the cells already integrated with the biopolymer and other biomolecules during the reconstruction stage.

3.3. Development of functional three-dimensional model tissue

Although there said to be many methods to developing three-dimensional fully functional tissues, these approaches have many steps which are laborious and require long time frames amongst other obstacles, which need to be overcome to generate a 3D tissue. These methods have many limitations and at best are in 2D and not in 3D. This is not the case for those reconstructs generated by way of BES/CE and AABJ/AABT, which have shown to hold cells in 3D to one another whilst maintaining their dynamic metabolisms and allowing cells to carry out all their expected cellular behavior. In fact, reconstructs generated by either of these processes (BES/CE and AABJ/AABT) have been explored to assess their ability to model human tuberculosis, showing great promise when compared to those approaches used to model the same disease in 2D or in animal models. These studies pave the way for these approaches to entire and promote the ability to carry out a wide range of biological studies for high through-put screening to the development of vaccines and drugs to their discovery, to finally carrying out humane research, which avoids sacrificing large numbers of animal models previously explored to carry out these very investigations.

3.4. Tissue reconstruction for patching damaged and/or ageing tissues/organs to cell therapy

The author previously stated that although there are many approaches to developing a tissue, the methods discussed in this review present those that are best placed to do so bearing in mind the time taken to generate a tissue with the least number of steps. There is a huge perception that 3D printing can develop a 3D fully functional tissue, which in this review has been shown to not be the case. This is due to its inability for those many manifestations of 3D printing to handle multi-cellular densely populated cell suspensions, with a biopolymer and

other materials such as proteins etc. In this instance BES/CE and AABJ/AABT overcome these limitations faced by its rival technologies as the needles used for accommodating the cellular suspensions etc are large in their bore diameter in comparison to any other direct cell handling approaches, yet are able to generate single cellular sized residues without the need for exploring prefabricated substrates. Additionally, the processing speed of these techniques are far greater than any rival technology and have been biologically, chemically, and physically fully assessed in comparison to control cells and whole organisms. Thus, establishing these approaches as the front running flexible platform biotechnologies in the endeavor for reconstructing a fully functional 3D tissue. Tissues reconstructed by way of these approaches (BES/CE and AABJ/AABT) have undergone intense in-vivo studies demonstrating the ability to develop tissues for transplantation. Such reconstructs can not only be explored for tissue and organ repair, rejuvenation and replacement but could also be used as localized cell therapy approaches for delivering a whole host of biological therapies (experimental and medical cells and/or genes).

4. Conclusion

This critical review set out to introduce the many scaffolding approaches said to have the ability to significantly contribute to the field of regenerative biology and medicine. As there are many such technologies for handling living cells the author carefully chose which approaches to highlighted based on their perceived progress highlighted in the literature. That being said the author also clarified at the onset of this review that the rules for choosing the approaches highlighted in this review were solely based on the ability to build a true three-dimensional tissue in practical dimensions, at low costs and with the least number of processing steps and time for reconstructing a living architecture. The author did so categorizing the many methods into two sets, namely which were established as the direct and in-direct methods of developing a tissue. The in-direct methods discussed lithography approaches, chemical methods, 3D printing, cell sheeting, force spinning, electrosprays/electrospinning and finally decellularization and recellularization. All these methods were discussed in detailed but shared one common obstacle which were that they had many laborious processing stages involved in order to see the generation of a fully cellularised scaffold. Although these approaches are not capable of entering the clinic for mass utility as a result of their practicality, the techniques were shown to have some utility in small-scale in niches areas. An important feature the in-direct scaffolding methods lacked was their inability to handle the cells with the biopolymer and other biomolecules such as proteins etc for the direct formation of a living tissue. Instead they required many steps in order to reconstruct a tissue which at the end had too many steps which introduces many other complications such as sterility and their inability to place cells in true three-dimensions. In contrasts, direct methods of scaffolding were able to handle all the constituents of a living tissue directly which could be deposited for the reconstruction of a living architecture. Nonetheless the many approaches discussed in this category had other limitations where the cell densities needed to be controlled together with, in some cases their inability like in the

category of in-direct methods for not possessing the ability for placing cells in true three-dimensions. In this category, we discussed lab on chip, laser guided cell writing, bio-electrosprays/cell electrospinning, and finally aerodynamically assisted bio-jetting and threading.

The author argued, the most favorable processes were bio-electrosprays/cell electrospinning, and aerodynamically assisted bio-jetting and threading. These were due to their ability to handle all the required materials with living cells to form large quantities of cell bearing beads or scaffold without perturbing the cells from a molecular level upwards. Interestingly, in the literature processes such as lab on chip, laser guided cell writing and 3D printing of cells etc have been discussed but have limited details reported on the cell viability using well established biological interrogating approaches such as flow cytometry to other molecular level analysis (karyotyping, gene micro arrays, RT-PCR or even RNA–Seq). This leaves the author with much skepticism in regards to those results discussing cellular viability and well-being. Additionally, apart from bio-electrosprays/cell electrospinning, and aerodynamically assisted bio-jetting and threading no other approach had undergone rigorous in-vivo testing, hence this left the author in a difficult position as it make comparison difficult.

At present bio-electrosprays and cell electrospinning are undergoing their checks through preclinical trials and it is hoped that these studies will bring out further their applicability to the real world and see them enter the clinic in the not so distant future. In parallel, these approaches are being regularly used in the biomedical laboratories for developing biological models, for understanding basic biology to the testing of a wide range of compounds and molecules to the development of these approaches for many other applications. It is hoped that these approaches together with their non-electrified driven technologies will enter phase I clinical studies soon.

Finally, this critical review set out to elucidate to the readers the advantages and disadvantages of those many approaches said to have promise in the regenerative biology and medicinal areas of research and development. The author hopes these thoughts discussed in this review provokes the readers to think outside the envelope and possibly see the arguments raised in this review and their real applicability for translation to either the biomedical laboratory and/or the clinic.

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Scaffolds are pivotal in the development of synthetic tissues for a wide range of applications in the biomedical and clinical sciences. Recent developments in the field of bioscaffolds are reviewed and discussed.

Keyword: scaffolds, bio-electrospraying and cell electrospinning, three-dimensional tissues, biological models, in-vitro and in-vivo studies

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Thoughts on scaffolds