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Biomimetic Approach to Tissue Engineering

Warren L. Grayson¹, Timothy P. Martens¹, George M. Eng¹, Milica Radisic², and Gordana Vunjak-Novakovic^{1,#}

¹Columbia University, Department of Biomedical Engineering

²University of Toronto, Institute of Biomaterials and Biomedical Engineering

Abstract

The overall goal of tissue engineering is to create functional tissue grafts that can regenerate or replace our defective or worn out tissues and organs. Examples of grafts that are now in pre/clinical studies or clinical use include engineered skin, cartilage, bone, blood vessels, skeletal muscle, bladder, trachea, and myocardium. Engineered tissues are also finding applications as platforms for pharmacological and physiological studies *in vitro*. To fully mobilize the cell's biological potential, a new generation of tissue engineering systems is now being developed to more closely recapitulate the native developmental milieu, and mimic the physiologic mechanisms of transport and signaling. We discuss the interactions between regenerative biology and engineering, in the context of (i) creation of functional tissue grafts for regenerative medicine (where biological input is critical), and (ii) studies of stem cells, development and disease (where engineered tissues can serve as advanced 3D models).

Keywords

Stem cells; scaffold; bioreactor; electrical signals; perfusion

1 “BIOMIMETIC” APPROACH TO TISSUE ENGINEERING

Tissue development and remodeling in living organisms is orchestrated by cascades of regulatory factors interacting at multiple levels, in space and time. Whole animal models certainly provide biologic fidelity (at least within given species), but offer limited control over the local environment, and limited real-time insight. In contrast, traditional cell culture provides significant control over cellular environment along with precise insight into cellular processes, but at the expense of a largely oversimplified 2D experimental context.

In tissue engineering, directing the cells to differentiate at the right time, in the right place, and into the right phenotype, also requires an environment providing the same factors that govern cellular processes *in vivo*. The field is moving away from “flat biology” of cell monolayers in favor of more realistic yet controllable 3D culture environments. We are now getting closer to a new generation of tissue engineering systems that would authentically represent the cell's

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Corresponding author: Gordana Vunjak-Novakovic, Columbia University, Department of Biomedical Engineering, 622 West 168th Street, Vanderbilt Clinic, 12th floor, Room 12-234, New York NY 10032, Tel. 1-212-305-2304, Fax: 1-212-305-4692, E-mail: E-mail: gv2131@columbia.edu.

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environment in a living organism while enabling tight control over the cell environment and real-time insight into cellular and morphogenic events. Along these lines, a “biomimetic” approach to the formation of engineered tissues (Figure 1) directs the assembly of functional tissues using biologically derived design requirements.

2 BIOMATERIAL SCAFFOLDS FOR GUIDING TISSUE ORGANIZATION

Scaffold materials – native or synthetic, permanent or biodegradable – are processed into 3D architectures suitable for cell seeding and cultivation. Material choices are guided by the need to restore cell signaling and to match the mechanical behavior of the tissue being engineered. Scaffolds also serve as “informational templates” to the cells, by implementing patterning, binding of ligands, and sustained release of cytokines [1]. Each tissue poses its own challenges to scaffold design, and we will use cardiac muscle and bone as examples of tissues with distinctly different scaffold requirements (Figure 2).

Cardiac muscle, a dense syncytium of electromechanically coupled cells that transmit force and deformations in multiple directions, requires soft and elastic scaffolds. Examples include hydrogels that enhanced the expression of Nkx2.5, elastomer scaffolds seeded sequentially with cardiac fibroblasts and myocytes that provided an environment for cell differentiation and coupling [2,3]. Also, alignment of scaffold fibers lowered the levels of atrial natriuretic peptide (ANP), indicating a shift towards a more mature cell phenotype [4].

Bone, a tissue containing dense mineralized matrix that can withstand significant compressive loads, requires scaffolds that can provide a framework for mechanical stability along with interconnected large pores for cell infiltration. In one study, silk fibroin scaffold was designed to guide differentiation of human mesenchymal stem cells (hMSCs) into trabecular- or cortical-like mineralized networks [5]. In another study, fully decellularized bone provided “biomimetic” topography, composition, and mechanical properties for osteogenic differentiation of hMSCs [6].

Custom-designed scaffolds also find utility in developing tissue models for drug screening and studies of disease. One particular 3D model of tumor biology was shown to more closely predict cell responses to chemotherapeutic agents than traditional 2D culture [7]. Pioneering work by the Taylor lab [8] has shown that decellularization of intact hearts can produce an ECM-based template which permits perfusion, vascularization, and seeding of myocytes leading to ex vivo pumping function. Cell culture on scaffolds may thus provide new insights into the cellular responses to their environment and the regeneration of tissues, and thereby facilitate application of biological paradigms in tissue engineering.

3 BIOREACTORS FOR ENVIRONMENTAL CONTROL AND PHYSICAL SIGNALLING

3.1 Environmental control of nutrients and metabolites

The critical parameter for cell survival in native and engineered tissues is oxygen (due to its low solubility in plasma and culture medium). In native tissues, oxygen is supplied by vascular network, over short diffusional transport distances and with the utilization of hemoglobin that increases the total oxygen content of blood. In conventional tissue engineering approaches, tissue constructs are just immersed in culture medium. Diffusional transport of oxygen under these conditions can support cell viability within only a ~100 μm thick outer layer, while the construct interior remains hypoxic (Figure 3A,B). To solve this problem, the entire construct can be perfused with culture medium, to supply oxygen to the cells occurs via both diffusion and convection, as in native tissues [9–11].

Our group has utilized gel-cell inoculation and an immediate establishment of the interstitial flow of culture medium through the seeded scaffolds to support physiologically high densities of cultured cells (Figure 3I,J) [9]. In this system, cells are “locked” into the scaffold during a short (10 min) gelation period, and supplied with oxygen at all times during culture. As the culture medium flow rate increases, so does the rate of oxygen supply. At the same time, the flow shear increases as well, creating a non-physiologic situation that can be detrimental to the cells. Recent studies with neonatal cardiomyocytes indicate that the application of interstitial flow at shear stresses higher than 2.4 dyn/cm² resulted in p38 activation and initiation of apoptosis [12].

In native heart, cardiomyocytes are shielded from direct contact with blood by endothelial cells. To mimic the capillary network, cells were cultured on highly porous elastic polymer scaffold [13] with a parallel array of channels (Figure 3C–H). To mimic oxygen supply by hemoglobin, the culture medium was supplemented with a perfluorocarbon (PFC) emulsion (Figure 3H). Since PFC particles are immiscible with the aqueous phase of culture medium, they serve as rechargeable oxygen reservoirs, a mechanism of oxygen supply analogous to that provided by hemoglobin in blood. The combination of medium perfusion through channeled scaffolds and medium supplementation with PFC enabled the cultivation of millimeters thick cardiac constructs with physiologic density of viable cells expressing the molecular and functional cardiac markers [10,11].

3.2 Providing mechanical forces to habitually loaded cells/tissues

In vivo, the changing 3D morphology gives rise to distinct mechanical stimuli that act synergistically with biological factors to regulate tissue development, maturation and remodeling. The iterative interaction between mechanical signals and organizational structure of the tissue results in specific structure-function relationships. For example, the anisotropic mechanical properties of long bones - result of the microscopic lamellar structure - are designed for structural load bearing along the long axis.

From a tissue-engineering perspective, subjecting immature tissues to physiological loading could be a way to acquire the necessary structural and functional characteristics. In most cases, much remains to be learned about the exact nature, levels and regime of application of mechanical signals. Mechanical stimulation also enhances the transport of nutrients to cells within the constructs by inducing convection. Shear stress was shown to have anabolic effect on osteoblasts and to stimulate the expression of bone-specific genes [14], a finding that enabled improved bioreactor designs [15,16]. Similar insights have led to the development of advanced bioreactors with compressive loading of chondrocytes embedded in agarose to form cartilage [17] and cyclic tension to cardiomyocytes in collagen to engineer heart tissue [18]. Notably, the combined use of growth factors and mechanical stimulation synergistically improved the functional properties of engineered cartilage [19].

For bone, it is rather difficult to isolate the effects of shear from those of convective mass transfer. One important study used dextran molecules to obtain medium with different viscosities and thereby vary the shear levels without changing mass transport rates [20]. This approach enabled correlation between matrix deposition and shear stress, without affecting cell proliferation rates and the alkaline phosphatase (AP) activity of the cells.

For cardiac muscle, a classical study performed by Zimmermann and Eschenhagen lab explored the role of mechanical stress on maturation of engineered cardiac tissue [18]. The application of dynamic stretch to neonatal rat cardiomyocytes in ring-shaped collagen gels for just 7 days resulted in mature muscle bundles, well established ultra-structural organization, cell coupling, and force generation.

3.3 Providing electrical signals to excitable cells/tissues

The control of heart contractions is almost entirely self-contained. The propagation of electrical signals across specialized intracellular junctions produces mechanical contractions that pump blood forward. Orderly coupling between electrical pacing signals and macroscopic contractions is therefore crucial for the heart development and function.

The provision of electrical signals was thus expected to also enhance the assembly of engineered cardiac tissues. Cardiac constructs were subjected to supra-threshold electrical signals designed to mimic those in native heart [21]. Over only 8 days *in vitro*, electrical stimulation induced cell alignment and coupling, increased the amplitude of synchronous contractions by 7-fold, and resulted in remarkable ultrastructural organization. Development of conductive and contractile properties of cardiac constructs was concurrent, with strong dependence on the initiation and duration of the applied electrical stimulation (Figure 3).

There are two possible mechanisms of electrical stimulation: via electrical gradients or via electrically induced contraction, and our data [21] as well as data from 2D studies suggest the latter. Interestingly, tissues engineered with the application of electrical field stimulation resemble in many aspects those engineered with mechanical stimulation [18]. Electrical stimulation induced hyperpolarization at the anode end of the cell and depolarization at the cathode end of the cell [22], such that the cells aligned with the electrical field lines were subjected to the largest voltage difference and were likely the first ones to generate action potentials, contract, and couple with other cells via processes at the cells' ends.

The enhanced organization of contractile apparatus by electrical stimulation *in vitro* may parallel some aspects of embryonic development. There is a positive feedback between the electrical stimulation and the progressive development of contractile behavior and tissue ultrastructure. Induction and sensing of stretch appears to be crucial for the development and assembly of sarcomeres. In our experience [23] non-stimulated constructs had thick, diffuse Z-lines (Fig. 3C) similar to those found in the mice knockout for muscle LIM protein, a key Z-line protein involved in the sensing of stretch and signal propagation. It appears that the induction of synchronous contractions by electrical stimulation ensured unaltered calcium handling and maintained the capacity and density of L type Ca^{2+} channels [24].

3.4 Microarray platforms utilizing engineered tissues

Microtechnologies based on tissue engineering are becoming powerful tools to manipulate the cellular microenvironment and study cellular responses in real time and in quantitative fashion [25]. Small scale allows high-throughput studies designed to evaluate a large experimental space while utilizing small amounts of cells and materials [26]. One example of this approach is a microarray bioreactor with twelve culture wells on a standard microscope slide format (Figure 6 A,B) [27], designed to accommodate two culture settings: cells attached to a 2D substrate, and cells encapsulated in 3D hydrogel. Both formats allow for controlled perfusion of fluid to the cells, providing strict control of medium composition and hydrodynamic shear that the cells are exposed to. Human embryonic stem cells were cultivated in this system, and assessed for their cardiovascular differentiation potential. Cell viability of cells was greatly enhanced by perfusion (Figure 6E). Another attractive feature of this technology is its compliance with standard imaging formats. Since twelve culture wells were created on a glass slide, light, fluorescence and confocal microscopy can all be used and combined with numerous image analysis options [27].

Microtechnologies can also be utilized to generate small but functional tissue "organoids" for a variety of applications. Form example, cardiomyocytes were patterned on a substrate to produce synchronously contractile organoids that mimic the highly specific organization of

cardiomyocytes *in vivo* (Figure 7 A,B). Over time, the cells functionally couple to each other to create strands of synchronously beating cardiac tissue, with progressively developing contractile function (Figure 7H) and expression of the cardiac differentiation markers (Figure 7 E,F) [28]. Such organoids can find utility in drug screening, electrophysiological studies and fundamental biological research.

4 COMPLEX TISSUES

Tissue engineering has largely utilized “minimalistic” approaches based on homogenous populations of cells provided with suitable environmental conditions to give rise to specific practical outcomes (e.g., mineralized scaffolds or contractile tissues). However, only a few tissue types are derived from single-cell populations. Bone, for example, is in a constant state of flux, based on interactions of osteocytes, osteoblasts and osteoclasts. Both the bone and cardiac tissues are highly vascularized such that the primary cell types are in close contact with other cell phenotypes. Creating complex tissues poses new requirements to the design of *in vitro* model systems, regarding the provision of multiple regulatory signals, mediation of cell-cell interactions and decision-making processes, and the spatial control of cell differentiation and tissue formation. This is the area where inputs from developmental biology are most valuable and most needed. The challenges faced by biologists and engineers are not trivial, and the field may need new tools for regulating micro-environmental conditions towards the formation of complex tissues.

4.1 Engineering osteochondral grafts

Cartilage and bone arise from a common progenitor, the mesenchymal stem cell, and exist in apposition at articular surfaces of synovial joints as well as in the epiphyseal growth plate. Initially, the differences in regulatory factors for chondrogenesis and osteogenesis were addressed by separate cultivation of cartilage and bone constructs, and subsequent joining of the two tissues to stimulate integration [29,30]. In one of the most successful *in vitro* studies, hMSC pellets were press-fit into scaffolds and cultured in chondrogenic medium, followed by the seeding of hMSCs grown in osteogenic medium into the bone portion of the scaffolds. Cartilage-bone integration would likely be facilitated if the two tissues could be formed concurrently, in bioreactors that provide separate supply of bulk medium into the cartilage and bone regions, and separate mechanical loading. Many technical challenges to this truly “biomimetic” approach still need to be overcome, and the biological basis has not been fully established either.

4.2 Engineering vascularized grafts

Tissue engineering has a long-standing focus on the creation of stable vascular networks [31–33], and the vascularization of tissues including muscle [34] and bone [35,36]. Studies investigated co-culture between osteoblasts or osteoprogenitors and endothelial cells resulted in contradictory findings regarding the ability of endothelial cells to induce or inhibit the expression of osteogenic genes. However, even in cases where the endothelial cells have had an inductive effect, concomitant *in vitro* formation of calcium deposition – the hallmark of bone – and blood vessel formation has not been achieved. Angiogenesis and osteogenesis are closely connected and tightly-regulated processes *in vivo* and much needs to be learned about the combinations and timing of the signals – molecular, structural, physical - needed to stimulate the two processes simultaneously *in vitro*.

5 SUMMARY

By faithfully recapitulating the tissue context *in vitro*, we would create realistic experimental models that can be used to better understand developmental and regenerative processes.

Because of the complex and dynamic nature of cell responses to regulatory signals, non-invasive (longitudinal) imaging of engineered constructs during cultivation would greatly advance our ability to study cellular responses to micro-environmental parameters with spatial and temporal specificity. The selection of functional readouts and the creation of appropriate molecular markers, through insights from developmental biology, are critical for developing bioreactor-imaging technologies. On-line imaging would enable us to learn about the combinations and timing of biophysical stimuli that are necessary to direct the fate-making decisions of stem-progenitor cells and guide the formation of tissues with multiple cellular phenotypes. It is rare that the effects of different stimuli – molecular, structural and physical – are additive, and it is necessary to study the biophysical stimulation from a systemic point, and to focus on the entire context. We seem to be getting closer to this goal, through interactions between biology and engineering at multiple levels. While biology provides the fundamental design requirements for tissue engineering, engineered tissues can serve as models for studies of development and disease and thereby foster biological research.

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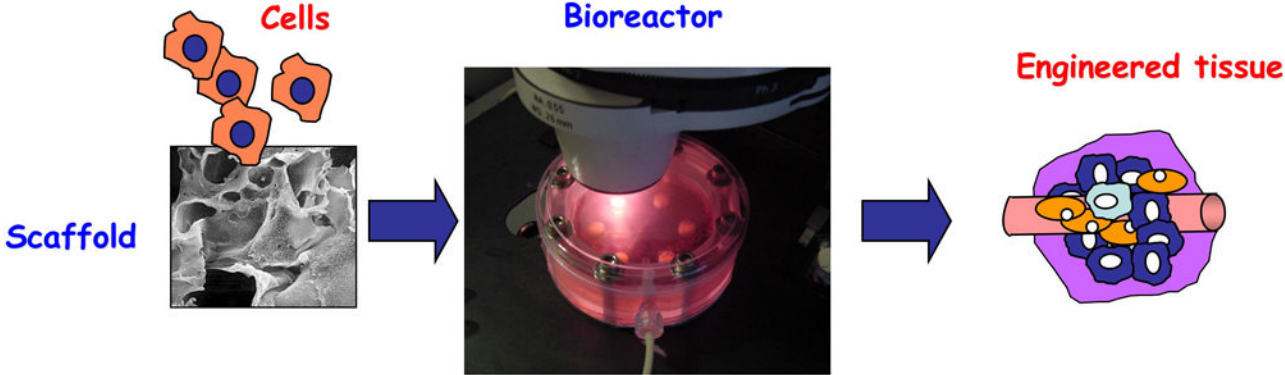
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Oxygen, nutrients, cytokines
 Other cells
 Extracellular matrix
 Physical factors
 Gradients, time profiles

In vivo cues
 (biologic requirements)



Environmental control
 Cell co-culture
 Biomaterial scaffold
 Flow, loading, electrical signals
 Immobilized and released factors

In vitro cues
 (engineering design)

Figure 1. Developmental biology and tissue engineering
In vivo, the progression of tissue development and remodeling depends on the interaction of time and space gradients of multiple factors that are not entirely known. The “biomimetic” approach to tissue engineering aims to utilize these same factors through the design of biomaterial scaffolds (providing structural, mechanical and logistic templates for cell attachment and tissue formation) and bioreactors (providing environmental control, exchange of nutrients and metabolites, and the molecular and physical regulatory signals). This way, the biological requirements inspire the design of tissue engineering systems, whereas tissue engineering provides controllable models of high fidelity for regenerative biology studies.

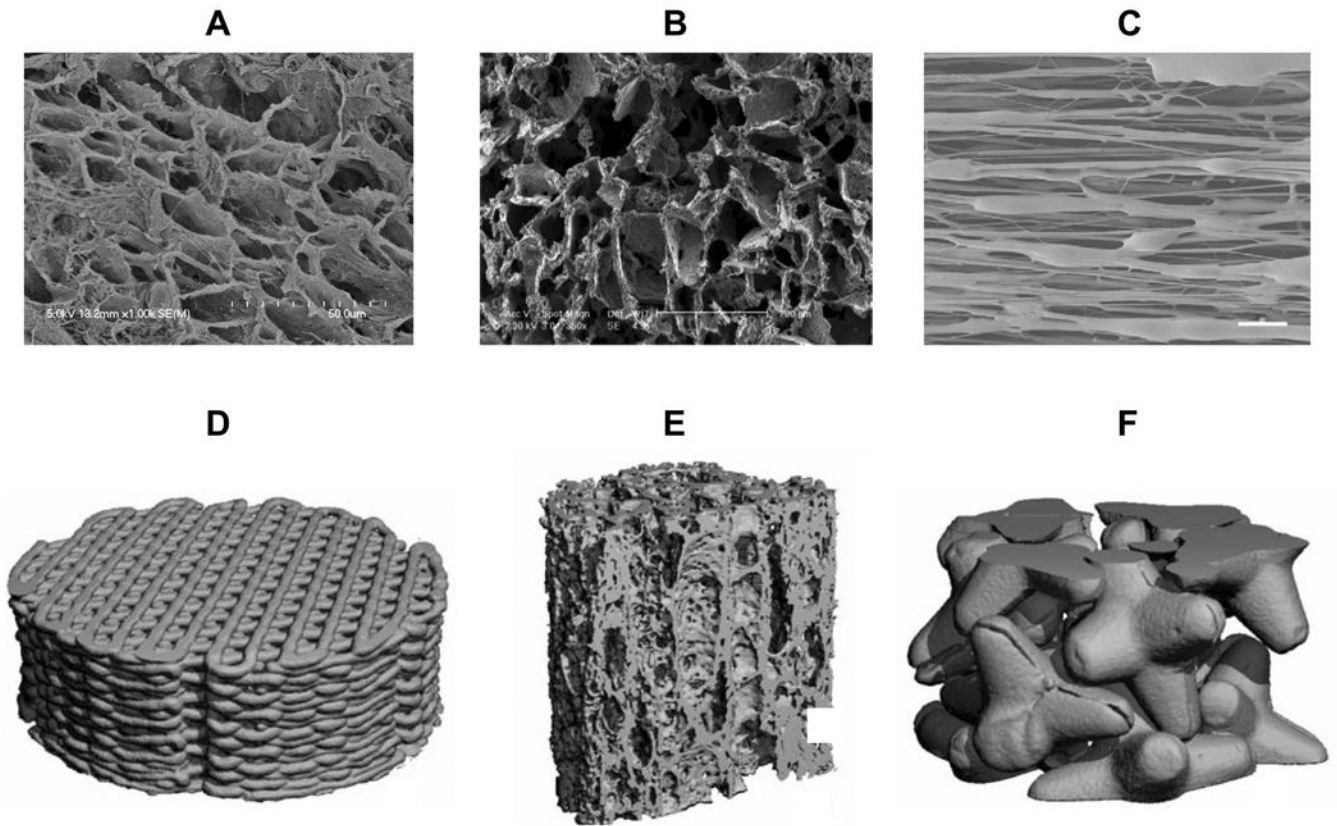


Figure 2. Biomaterial scaffolds as templates for cell attachment and tissue formation

Examples of native and synthetic scaffolds for cardiac tissue engineering (shown as scanning electron micrographs) include **A** decellularized human cardiac tissue and **B** synthetic poly (glycerol sebacate) scaffold, with great similarities in structure and pore distribution, and **C** aligned electrospun, biodegradable polyurethane [4]. Scaffolds for bone tissue engineering (shown as microcomputer tomography images include **D** composite PCL/TCP scaffold produced using fused deposition modeling, **E** poly(L-lactide-co-DL-lactide) scaffold with anisotropic pore structure designed to encourage tissue ingrowth, and **F** jacks-shaped ceramic particles intended to fill bone defects while providing pores for cell and vascular ingrowth [37].

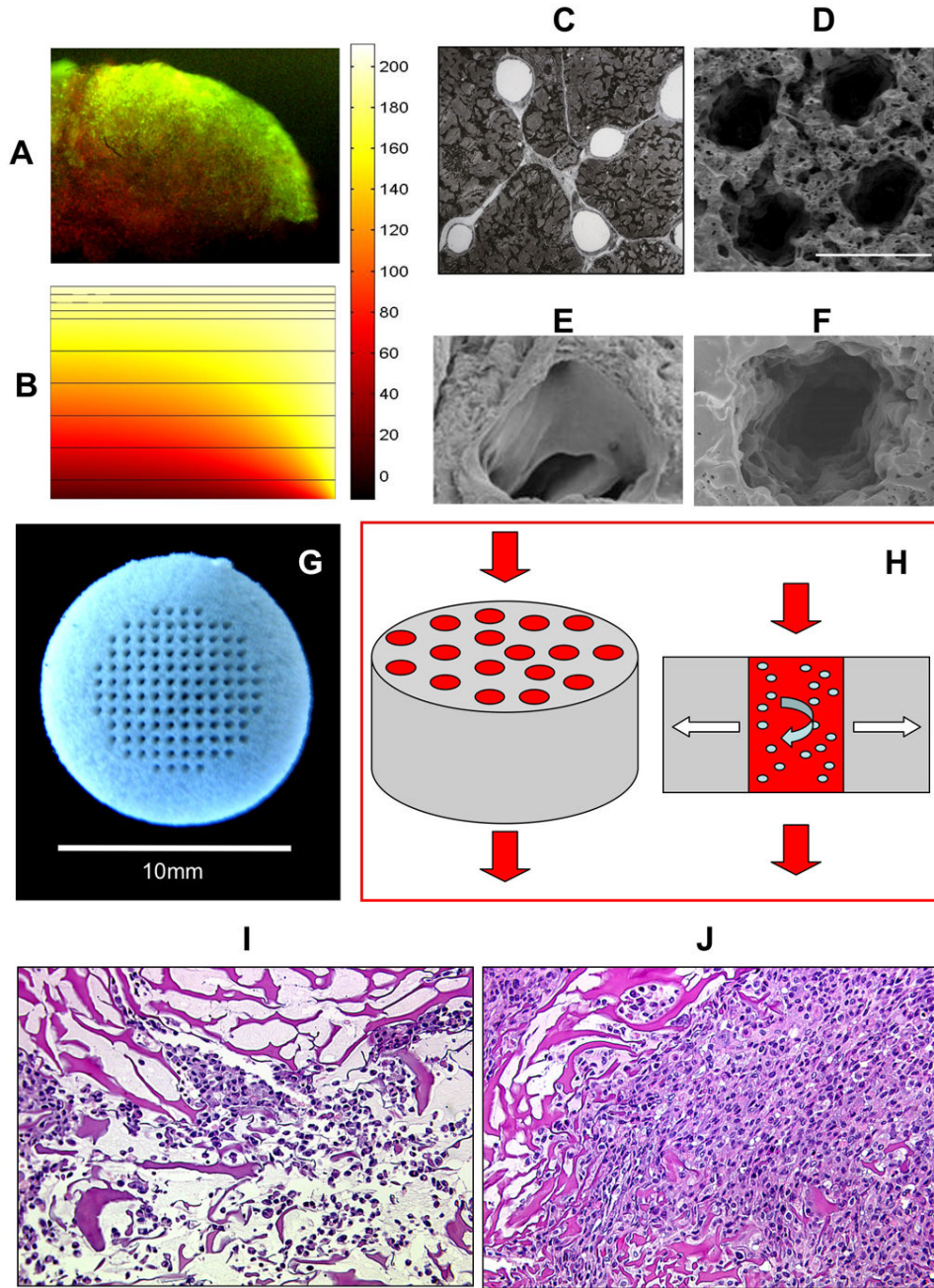


Figure 3. Environmental control of cells in bioreactors via medium perfusion

A) Live (green) and dead (red) staining of engineered cardiac tissue cultivated under static conditions B) Mathematical model of oxygen concentration in an engineered cardiac tissue. The model indicates that when oxygen is supplied by diffusion alone the interior of the construct remains hypoxic (one half of construct cross-section shown) C) Capillary network in the native myocardium D) Poly (glycerol sebacate) (PGS) scaffold with a channel array E) One capillary in the native myocardium F) One channel in the PGS scaffold G) Channel array in the PGS scaffold for perfusion cultivation H) Oxygen supply in a channeled engineered tissue using culture medium with PFC oxygen carriers. H&E staining of a collagen scaffold seeded with C2C12 myoblasts under I) static conditions or J) interstitial culture medium flow [9–11].

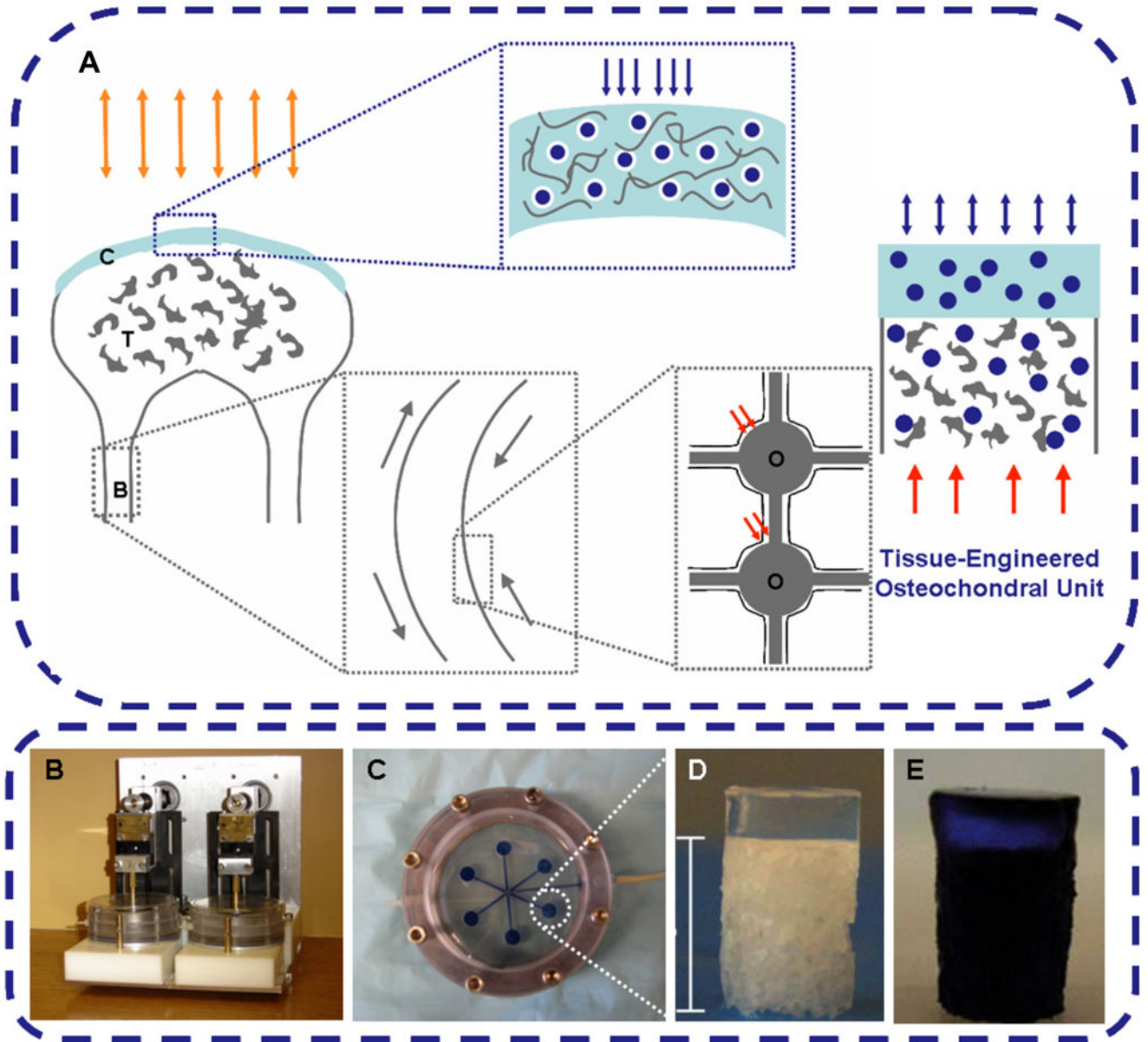


Figure 4. Perfusion and loading for engineering of osteochondral tissues

A Translation of mechanical forces in an articular joint involves compressive loading (orange arrows) of cartilage **C** and bone (**B**). In the cartilage region (blue box), the loads (blue arrows) are transferred to chondrocytes through compression of the ECM resulting in mechanical straining of cells (blue circles). In the bone region (grey box), the compressive loads cause bending (grey arrows) and oscillatory fluid flow around the osteocytes (**O**) trapped in lacunae (red arrows). **B–E** Tissue engineering bioreactors can be designed to provide these same mechanical stimuli – dynamic compression of the cartilage region (blue arrows), and perfusion of the bone region (red arrows). Bioreactors used to impart dynamic compression (via steel platens) to cartilage regions (**B**) and interstitial fluid flow through the bone regions (six osteochondral plugs are shown), **C**. **D** In both cases, osteochondral plugs are made from

decellularized bovine trabecular bone (bone scaffold) interfaced with hydrogel (cartilage scaffold). **E** Blue dye traces flow patterns in the constructs.

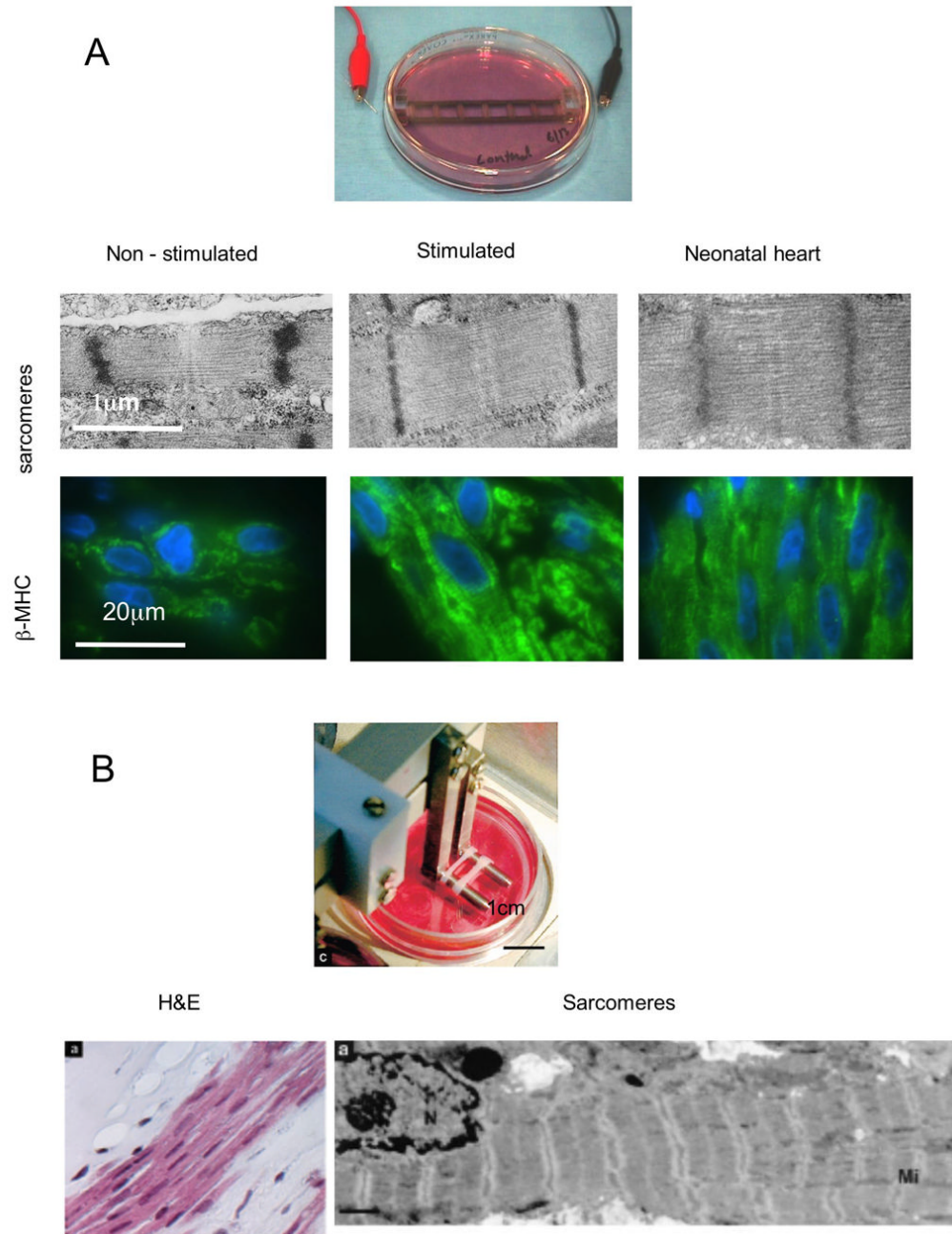


Figure 5. Electrical and mechanical stimulation for engineering of cardiac tissues

A Cardiac-like electrical field stimulation applied to cardiac cells inoculated via hydrogel into collagen sponges [21]. **B** Cyclic mechanical stretch was applied to cardiac cells inoculated in collagen gel [18]. In both cases, stimulation enhanced cell differentiation, alignment and coupling, and resulted in a remarkably well-developed contractile apparatus.

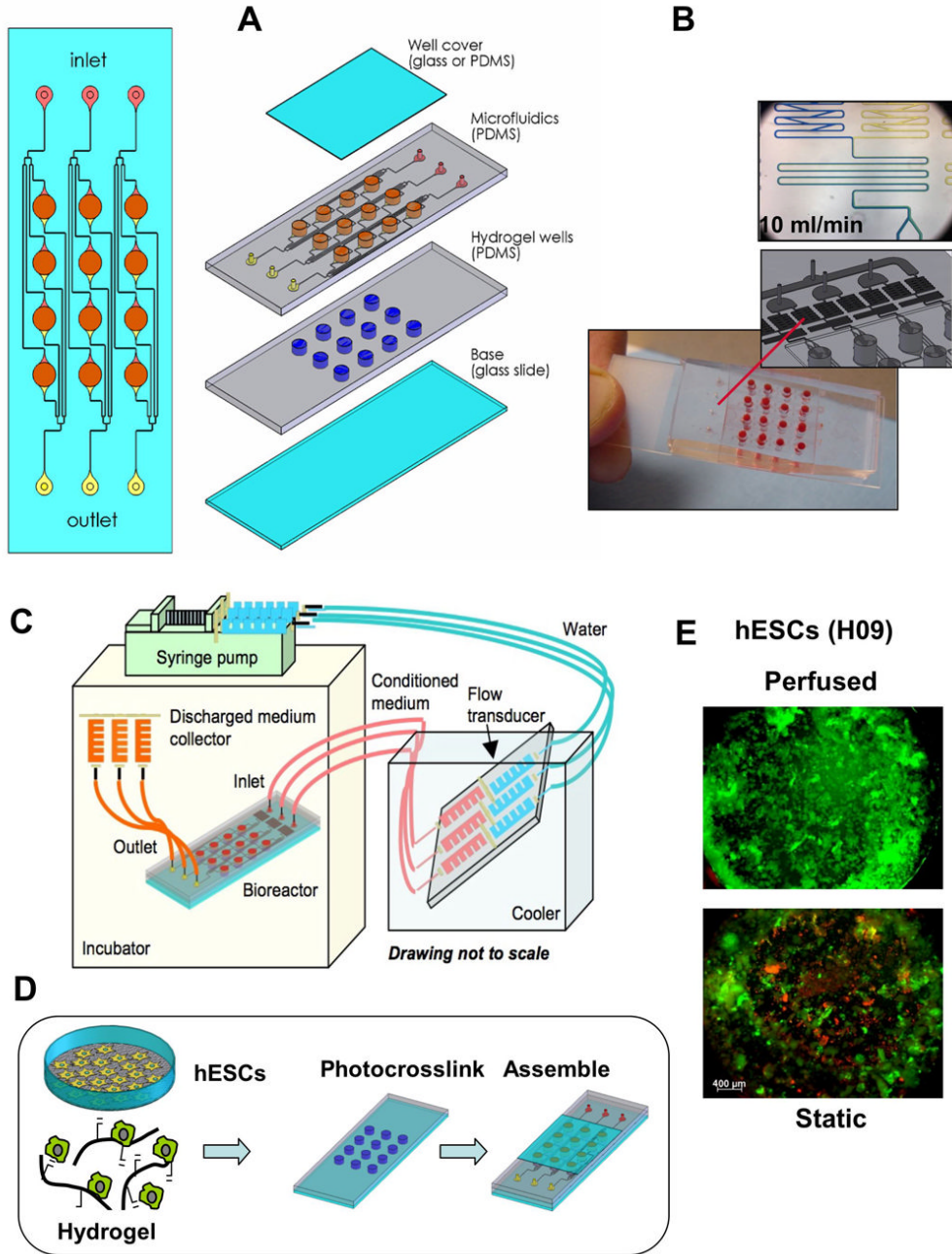


Figure 6. Microarray bioreactors

A An array of 3×4 independent culture wells (3.5 mm in diameter) that are independently perfused with culture medium and can be used for either 2D or 3D cell culture. This microarray bioreactor has a size of a microscope slide, and is assembled from layers of poly (dimethylsiloxane) (PDMS) and glass attached *via* plasma treatment. **B** Microfluidic “mixers” are used to supplement medium with a desired concentration of molecular factors. **C** Bioreactor setup in the incubator. **D** Assembly process for photoencapsulation of hESC. **E** Perfusion enhanced hESC viability [27].

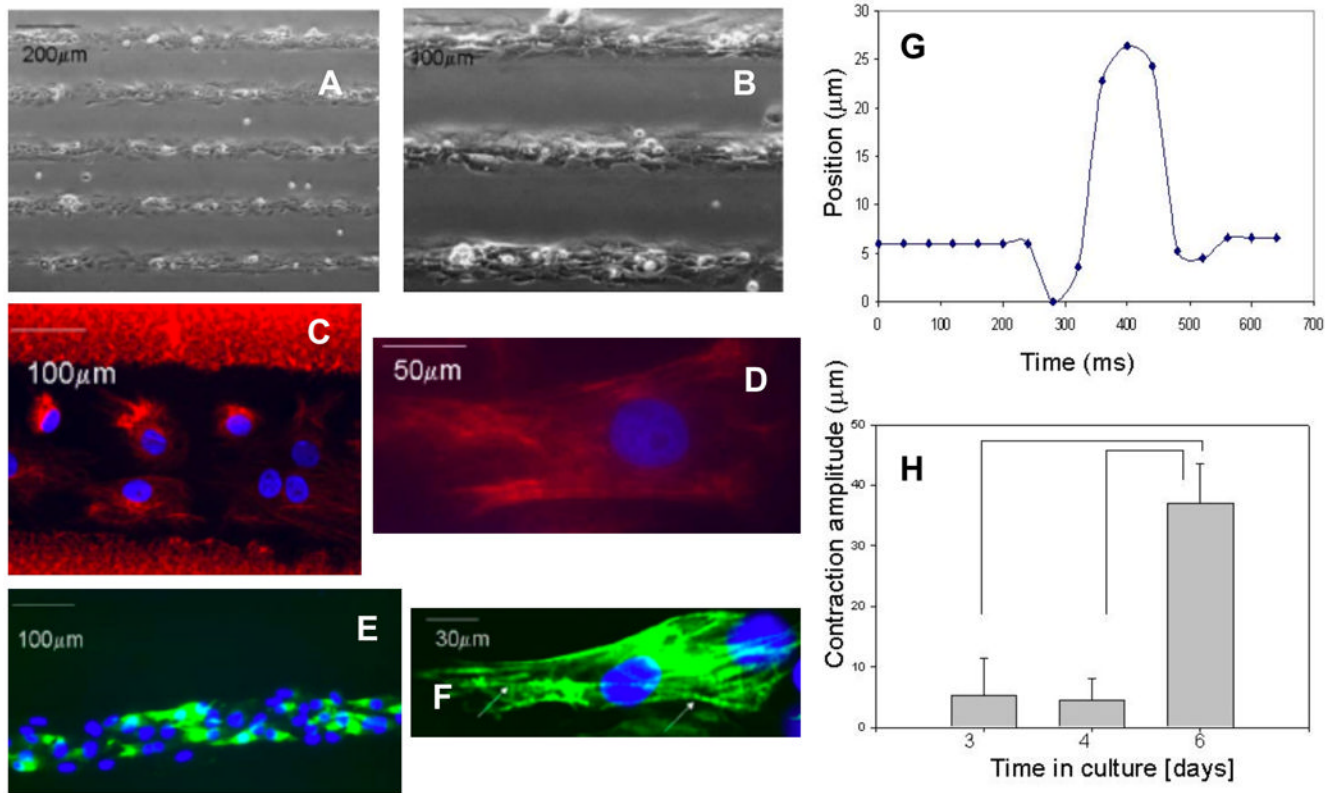


Figure 7. Small tissue organoids for high-throughput research

A,B Patterned surfaces were used to form contractile cardiac organoids over 8 days of culture, starting from isolated cardiac myocytes. **C,D** Immunofluorescent stain for intermediate filament vimentin (red). **E,F** Cardiac troponin I (green) and contractile apparatus (arrows). Nuclei are counterstained with DAPI (blue). **G** Contraction profile of a single organoid determined by on-line imaging. **H** Contraction amplitude of organoids increased with time of culture [28,38].