Published in final edited form as:

Biomaterials. 2020 October ; 255: 120189. doi:10.1016/j.biomaterials.2020.120189.

Towards systems tissue engineering: elucidating the dynamics, spatial coordination, and individual cells driving emergent behaviors

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Abstract

Biomaterial systems have enabled the *in vitro* production of complex, emergent tissue behaviors that were not possible with conventional two-dimensional culture systems, allowing for analysis of both normal development and disease processes. We propose that the path towards developing the design parameters for biomaterial systems lies with identifying the molecular drivers of emergent behavior through leveraging technological advances in systems biology, including single cell omics, genetic engineering, and high content imaging. This growing research opportunity at the intersection of the fields of tissue engineering and systems biology – systems tissue engineering – can uniquely interrogate the mechanisms by which complex tissue behaviors emerge with the potential to capture the contribution of i) dynamic regulation of tissue development and dysregulation, ii) single cell heterogeneity and the function of rare cell types, and iii) the spatial distribution and structure of individual cells and cell types within a tissue. By leveraging advances in both biological and materials data science, systems tissue engineering can facilitate the identification of biomaterial design parameters that will accelerate basic science discovery and translation.

1. Introduction

Native tissues are composed of multiple cell types that reside within a complex, continuously changing three-dimensional microenvironment consisting of numerous inputs, including extracellular matrix (ECM), soluble factors, mechanical forces, and cell-cell contacts, all of which combine to drive collective tissue function [1, 2]. By mimicking and reproducing these input parameters, biomaterial and microsystems technologies [3, 4] can create diverse emergent tissue behaviors that would not otherwise be possible with conventional two-dimensional (2D) culture systems. By engineering input parameters across three categories i) physical (ECM stiffness and nonlinear elasticity, interstitial and

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

microvascular flows, gradients), ii) chemical (soluble factors and ECM ligands), and iii) cellular (cell sources, cell types, and genetic manipulations) across 3D space and time, biomaterial microsystems can guide the emergence of tissues with diverse behaviors. These responses are exemplified by vasculogenic microcapillary networks [5–7], beating cardiomyocyte microtissues [8, 9], functional skeletal muscle [10, 11], and stem cell-derived tissue organoids [12, 13]. The tunable nature of biomaterial microsystems uniquely allows for the engineering of microenvironments to interrogate the contribution of specific inputs on the resulting cell and tissue behavior. In vitro biomaterial microsystems therefore have incredible potential as a tool for molecularly dissecting mechanisms behind tissue behaviors including tissue formation, disease initiation and progression, and the mechanism of action for therapeutic compounds [3, 14]. Biological systems have complex dynamic, multi-level (genetic, epigenetic, metabolic, protein interactions, intercellular signaling, etc.) regulatory networks making it challenging to identify any one molecular mechanism in isolation by which tissue behaviors emerge. Fully leveraging the potential of biomaterial microsystem platforms will require an analysis framework that can connect and explain the effects of design inputs on emergent cell and tissue behavior via the complex network of intracellular and intercellular molecular regulations.

The path towards developing improved microsystems and material platforms will likely involve the ability to apply tools from systems biology to the analysis of tissue dynamics and structure. Historically, the identification of biomaterial design parameters has ranged from reductionist approaches to high throughput screening systems, which have frequently involved a focused number of cell types or cellular responses. Materials data science approaches that integrate these experimental approaches with machine learning and artificial intelligence strategies [15–17] are emerging as a powerful tool in biomaterials development [18, 19]. Nevertheless, the approaches have often failed to fully capture the complex multivariate biology present in vivo [20], and more recent approaches have sought to capture increased complexity through co-culture to multi-culture systems, 2D and/or three dimensional (3D) culture across multi-well plates, and microphysiological systems that integrate multiple organ systems. Native *in vivo* tissues are increasingly analyzed with emerging molecular tools that provide measurements of thousands/millions of factors in a single experiment. These tools, including single cell RNA sequencing, have revealed the vast heterogeneity in molecular phenotype between cells of the same classical type even within the same tissue compartment [21, 22]. The large data sets generated have the potential to identify the interconnected responses inherent to spatially coordinated biological systems, the role of rare cells and behaviors within the complex systems, and the dynamic nature of the response networks. *Descriptive* single cell sequencing, no matter how detailed, cannot, in isolation, identify the dynamic cellular functions that drive tissue development and disease. In order to move from *descriptive* studies towards *explanatory and predictive* models, we need a better link between systems level molecular phenotypes and the underlying dynamic microenvironment-driven cell and tissue functions. A systems-level approach is necessary to identify nodes and times within the interconnected network of interactions that drive and control emergent tissue behaviors (Figure 1).

Here, we provide a perspective on utilizing recent technological advances in the areas of single cell systems biology, genetic engineering, and high content imaging to link dynamic

single cell microenvironment-driven functions to their molecular drivers within engineered microenvironments. As the field of tissue engineering advances through improvements in biomaterial, cellular, and organoid microsystem technologies, it increasingly bridges the complexity gap between reductionist 2D in vitro cell culture and complex native in vivo tissues (Figure 1). This intermediate complexity allows for the production of realistic models and devices, while allowing for control and interrogation in ways that would not be possible in native tissues. We detail opportunities for application of tools from systems biology to the field of tissue engineering, an intersection we refer to as systems tissue engineering (Figure 1,2) for exploring sources of complexity within engineered microsystems including i) the dynamics of tissue development and dysregulation, ii) emergent heterogeneity and the function of rare cell types through single cell analysis, and iii) the spatial distribution of individual cells controlling structure and function within a tissue.

2. Capturing dynamics within engineered microenvironments

Tissue behaviors emerge from many layers of dynamic cellular processes including cell cycling, migration, and matrix remodeling with time scales ranging from seconds to days, each regulated by subcellular molecular processes including protein-protein interactions and gene expression with timescales from microseconds to hours [23]. Epigenetic changes from these inputs may occur over days or longer guiding long-term cellular processes like cellular differentiation[24, 25]. The dynamic processes are ultimately integrated to regulate cell and tissue behavior. Recording the sequence of molecular regulatory events and the corresponding tissue properties would facilitate the identification of the key pathways that are driving tissue function across the stages of tissue development or disease progression. Biomaterial microsystems provide a means of creating systems that can be dynamically imaged to report on cell differentiation and maturation, and ultimately tissue function. Here we outline emerging methods and opportunities for dissecting dynamic tissue behaviors using biomaterial microsystems.

2.1 Dynamic microenvironments

Engineered 3D hydrogels and microsystems with the ability to analyze dynamics would facilitate the identification of the key drivers of tissue function (Figure 3)[26]. In transitioning cells from 2D tissue culture plastic to 3D culture, particularly within systems that can be remodeled, cells experience dynamic changes in geometry, structure, and composition of their environment. While the biochemical environment can be changed on demand without disrupting adherent cells by changing culture media, more recently, hydrogels have been designed whose biomechanical or biochemical properties can be switched on demand via an external signal, such as light [27, 28]. Cells across multiple material platforms respond with dramatic changes in behavior after altering the mechanical stiffness of the underlying substrate [29–32]. Similarly, material systems that allow for dynamic control of ECM ligands can guide diverse emergent behaviors[33–36]. These systems for modulating the signals on demand are enabling for analysis of dynamic cellular responses. More recent biomaterial designs are supporting fully reversible[37] mechanical or biochemical changes [38], allowing for *in situ* study of the dynamics of microenvironmental memory [39, 40] and its role on cell and tissue function. Furthermore, materials may sense

and respond to their microenvironment, thereby providing biomimetic feedback loops to resident cells [41, 42]. These material systems provide critical tools for probing how dynamic microenvironmental changes control systems-level tissue function.

2.2 Dynamic cell and tissue monitoring with genetic reporters

The accessibility of biomaterial microsystem constructs to imaging is a key feature for analyzing dynamic responses. Optically clear biomaterial culture systems [43, 44] with homogenous microstructure (yet potentially with engineered nanostructures with length-scales less than half the wavelength of the light used for imaging) can transmit light much more efficiently than those containing high refractive index microscale fibers [45, 46] and micron-scale features which scatter light [47]. Light microscopy has long been employed for identifying cell and tissue structures and their dynamics, the spatial position of cells relative to each other, or intracellular distribution of organelles and proteins. Connecting cell and tissue structure and function to dynamic changes in molecular state is uniquely achieved by live cell imaging of genetic reporters.

With the use of live-cell reporters, fluorescence and luminescence can be employed to capture systems-level dynamics in molecular signaling, with emerging systems enabling large scale analysis. Analytical assays such as RNAseq and proteomics require destructive snapshot measurements, and while these techniques can be performed over a time-course experiment, they must be performed on replicate experiments of unique and distinct sets of cells and in themselves do not allow for direct correlation with tissue function. These limitations can be especially problematic when working with high complexity 3D biomaterial microsystems. Live cell imaging of genetically encoded reporters can uniquely monitor the dynamic signaling within living cells or tissues. High quality reporters have been generated for a variety of intracellular signaling processes, including transcription factors [26, 48–56], microRNA [57–60], protein kinase activity [61–65], metabolism, chromatin organization [66], and protein-protein interactions [67, 68] among others. Genetic reporters are also available for monitoring the dynamic physical state of the cell and extracellular signaling from the microenvironment including hypoxia[69], reactive oxygen species[70–73], membrane potential/ion trafficking[74, 75], metabolism [76, 77], and neurotransmitters engagement[78, 79]. In addition to genetic reporters, chemical biology labeling strategies [80–82] can provide an additional toolset for imaging live-cell dynamics, and the commercialization of chemical probes for many applications including tracking hypoxia[83], reactive oxygen species[84], and organelles and structural features[85–87] within the cell has provided off-the-shelf tools for studying live cell dynamics.

Scaling dynamic genetic reporters to dozens of reporters in a single experiment has been achieved by pre-manufacturing libraries of ready to use reporter transduction agents, such as lentivirus, that can be used to transduce cells in a parallel format [26, 50, 54, 55, 60, 88, 89]. The transduced cells are then cultured and imaged dynamically in high-content array to investigate the dynamic molecular response networks triggered by micro-environmental stimuli. Using microwell plates with each well containing a distinct reporter, the signaling induced by RGD ligand or mechanical stiffness was investigated through identifying the transcriptional response networks of 50 transcription factors (Figure 3)[26]. These studies

identified transcription factors that are specific to ligand density or mechanical stiffness, as well as transcription factors common to both stimuli [26]. Here, bioluminescence imaging of each well reported population level responses, yet was unable to capture cell specific responses.

Recent technological advances have furthered the potency and scalability of genetic reporters for monitoring dynamic cellular processes. Luminescent proteins have been attractive for detecting low levels of transcription factor activity, because the low background imparts a high signal to noise ratio [90, 91]. Advances in luminescent reporter proteins, including improvements in brightness [92] and in red-shifting for better tissue penetration and color multiplexing [93–96], allow them to outperform fluorescent proteins for reporting on whole-tissues or multicellular systems. Pairing the low read noise of Electron Multiplying Charge-Coupled Device (EMCCD) or scientific Complementary Metal-Oxide-Semiconductor (sCMOS) cameras with an appropriate objective and tube lens can enable bioluminescence microscopy [97, 98]. Nonetheless, fluorescent proteins with their much greater brightness still have advantages for single cell and 3D reporter imaging. Advances in commercial DNA synthesis [99, 100] allow for the straightforward and rapid synthesis of the genetic parts, with components assembled into multi-kilobase sequences with robust methods like Gibson Assembly [101]. Further, advances in genetic engineering including the advent of CRISPR cas9 technology [102, 103] and engineering of cas9 variants with improved specificity [104, 105] allows for scalable strategies for reporting on activity of endogenous promoters and enhancers [106–110], or for the insertion of large multi-kilobase [111] genetic cassettes capable of bearing several reporters at a genetic safe harbor site.

3. Capturing the role of single cell heterogeneity in emergent tissue behaviors

Heterogeneity of individual cells within a tissue is known to be of great clinical significance, as illustrated by intratumoral heterogeneity imparting resistance to cancer therapeutics [112]. On a more fundamental level, heterogeneity may facilitate basic tissue functions including fate plasticity and information coding [113]. Dynamic responses that appear continuous at the population scale can be heterogeneous and switch-like at the single cell level [114], indicating there is much to learn about how single cells make functional decision. However, linking systems scale single cell molecular information to the single cell functions within complex biomaterials microsystems requires experimental information linking molecular form to single cell function. New technologies including single cell RNA sequencing [115–117] provide molecular phenotype, and have revolutionized our understanding of the complexity and distribution of single cell phenotypes within *in vivo* tissues. Here, we describe approaches that aim to bridge single cell genomics technologies with high content imaging to capture the contribution of cellular heterogeneity or the function of rare cell populations on collective microtissue function (Figure 2).

3.1 Engineered biomaterial microsystems for single cell analysis

Biomaterial microsystem platforms provide a means by which to elicit and observe single cell functional responses to controlled environments (Figure 4)[46, 118–122]. For example, mature platforms for applying concentration gradients of soluble factors to cells in 3D culture allow observation of heterogeneous single cell chemotaxis responses of individual cells (Figure 4A)[123–126], with some rare cells displaying exceptional chemotaxis. Likewise, manipulation of the mechanical properties and structure of the ECM have allowed for dissection of individual cells exerting forces and migrating in response to the ECM (Figure 4B,D)[45, 46, 120, 127–131]. Single cell force generation can vary by orders of magnitude between cells and microenvironments [46, 132–134]. Using microsystems, the relative role of soluble and cell-cell contact factors in driving behavior has been explored in different cell types (Figure 4E–F)[121, 122] including single NK cell activation [135] and killing of cancer cells (Figure 4F)[122]. Single NK cell cytotoxicity experiments have demonstrated rare NK cells are responsible for the majority of cytotoxic activity against cancer cells[136–140]. Within organoid systems, engineered 3D biomaterial systems have been employed to guide stem cells to differentiate into organoids akin to diverse tissues including intestine [141], optic cup [142], and lung [143]. The stem cells within a colony under a complex process of self-organization and self-sorting [144] according to microenvironmental cues as they differentiate down the various lineages and spatially organize to form 3D organoids [145]. Engineered biomaterials can provide boundary conditions and gradients that break symmetry between individual cells and guide differentiation and organoid development [146]. Across these systems, a vast heterogeneity of single cell behaviors in both time and space has been observed, which motivates the application of single-cell analysis as well as the essential role of dynamic cell behaviors in the overall structure and function of tissues.

3.2 Single cell pseudo-temporal omics analysis

Single cell transcriptomic and multi-omic technologies provide a means to capture a detailed molecular *description* of cell heterogeneity at any given time within engineered microenvironments. Single cell RNA sequencing technology [115–117] has scaled exponentially over the last decade [147] and now has a well-established ecosystem of analysis tools [148–150]. Further, multi-omic technologies provide a means to analyze non-transcriptionally controlled pathways by integrating single cell transcriptomic data with single cell proteomic and chromatin accessibility measurements [151]. CITE-seq [152] and REAP-seq [153] can simultaneously quantify mRNA and extracellular protein content in individual cells by using antibody cocktails barcoded with oligonucleotides which are sequenced along with endogenous mRNA. A complementary tool is the CRISPR loss of function screens, where a library of many guide RNA's can be used to knock down or knock out genes in individual cells, allowing for detailed study of the role of proteins in gene regulatory networks[154–157]. Other sequencing modalities including single cell ATAC-seq [158, 159] and THS-seq [160] can be used to link single cell chromatin accessibility to the state of transcriptome of individual cells. Analysis methods for these genomics datasets provide *descriptive* clustering of cells into categories by their gene expression, but they cannot directly link gene expression to cell behavior without additional information. Information including single cell spatial location and orientation, single cell functional

phenotype, and dynamic changes of individual cells over time are lost when the tissue is processed for sequencing.

Moving toward a *predictive* understanding of cellular heterogeneity and tissue function will require a means of linking and analyzing snapshot *descriptions* of single cell molecular phenotypes across time. Pseudo-temporal analysis of scRNAseq datasets[151], with methods such as pioneering work Monocle [149], achieve this goal by creating single cell trajectories across dynamic processes like differentiation [149, 150, 161–163] and the cell cycle[162]. Here, cells are harvested at several time points during a dynamic process such as cellular differentiation from replicate experiments, and scRNAseq or another omics method is conducted on the cells harvested at each timepoint. The transcriptome of each individual cell across replicate experiments harvested at distinct time points is then ordered on a trajectory of the biological process occurring over time, with this ordering referred to as pseudotime. The ordering and branchpoints of graph networks produced across pseudotime are then used to predict critical molecular regulators and decision points. While original algorithms allowed for only linear trajectories, newer methods support cycling and circular paths [151]. These methods are well suited for the study of dynamic heterogeneous cellular responses in the microenvironment of biomaterial microsystems. While dynamic information can be captured by pseudo-temporal ordering, this ordering is obtained from the unique transcriptome of individual cells obtained from replicate experiments sequenced at multiple times; each individual cell is measured only once, and essential dynamic and spatial information is inherently lost in this process.

3.3 Connecting tissue function to molecular drivers with live single cell imaging

Directly linking dynamic single cell function to dynamic single cell molecular phenotypes requires observation of individual live cells over time. Modern high content live cell imaging assays can track morphology and biomarkers of many thousands of individual cells across time as they respond to stimuli from their microenvironment [164, 165]. In order to provide information on molecular phenotype, cell lines or progenitor cells [166–168] can be produced containing genetic reporter elements using fluorescent or luminescent proteins.

An automated live cell microscope equipped with an incubated stage can image individual cells across many wells of a microtiter plate over time, recording changes in single cell behavior simultaneously with changes in single cell reporter activity. Image analysis codes are then applied to link, correlate, and interrogate the order of changes in dynamic single cell functions with dynamic single cell molecular phenotype. For example, high content imaging of genetic reporters has been employed to screen the effects of thousands of drugs on cell function [169], and to elucidate the single cell toxicity pathways activated by these drugs [170–172] (Figure 5). Here, genetic reporters can be included in one or more cellular subsets of the microenvironment to facilitate imaging and record their specific contribution to the emergent behavior. These methodologies are directly applicable to the study of cells on planar 2D interfaces within biomaterials where multiple cell types or variation in the local microenvironment such as material or soluble gradients are present. We note that the dynamic physical movements and behaviors of individual cells residing within biomaterial microsystems are also routinely recorded to connect material microenvironment to cell

function. Integration dynamic imaging of genetic reporters within such systems completes the linkage from microenvironment to function to molecular drivers within individual cells.

For single cell studies, the process by which reporters are inserted must be considered to account for heterogeneity and bias in reporter function between individual cells. Viral and physical methods may result in integration of the reporter gene at different loci [173] or maintenance of the vector in the nucleus extrachromosomally [174] at variable copy number, which may necessitate production of a clonal cell line to improve consistency of reporter function between cells. Even so, bias may still exist in the reporter function of integrated clones from any adjacent genetic regulatory element. Insertion of reporters at so-called safe-harbor sites, such as the commonly used AAVS1 locus in humans, which are far from any other known genetic regulatory elements, is thought to reduce bias from adjacent regulatory elements [175, 176]. Considering these limitations, techniques such as those using CRISPR cas9[106–110] that can knock-in reporters *in situ* are especially powerful for capturing the full genetic regulatory context in single cells.

A staged discovery strategy may be necessary for incorporating single cell genomics and high content imaging of genetic reporters to efficiently link single cell functions to their molecular phenotypes. Here, an initial round of single cell genomics and pseudo-temporal analysis would be used to identify regulators stratifying the dataset. Then high content imaging on a more focused set of live cell genetic reporters can connect dynamic microenvironment driven functions to the larger systems-level molecular phenotypes. These methods can be computationally integrated with targets, with subsequent validation, such as through the use of high-throughput loss of function screening.

3.4 High content single cell imaging for 3D systems

Most cells live within a fully 3D rather than a 2D planar geometric context, and 3D cell culture is necessary for obtaining some complex phenotypic responses [177, 178]. However, 3D systems present unique challenges for high content imaging experiments including the requirement to acquire and process large 3D datasets, noise and bias from imaging through hydrogel or ECM material, and the requirements for highly specialized imaging systems for optimal performance. Conventional imaging systems including wide-field and confocal point scanning have substantial limitations for dynamic imaging of 3D microenvironments including phototoxicity and photobleaching as they illuminate through the whole sample [179]. Light sheet microscopy systems provide the unique ability to illuminate only the current imaging plane, greatly reducing phototoxicity in 3D imaging studies. Recent refinements including the advent of lattice light sheet microscopy [180] and the subsequent integration of aberration-correction adaptive optics [181] may further enhance performance. However, conventional light sheet microscopy requires complicated mounting schemes and small samples owing to the requirements of 2 orthogonal light paths. Recent work toward open top light sheet microscopy [182–184] and oblique plane microscopy [185] would allow for plate-based multi-well imaging enabling truly high content 3D imaging studies. For more on the current state of high-content cell imaging we refer the reader to an excellent review [164].

3.5 Tools and opportunities for machine learning in single cell image analysis

Accessible and accurate methods for automated image analysis tasks are needed to enable truly high-content experimental strategies. A mature array of proven tools and techniques for each step of the high-content 2D and 3D imaging workflows, from image acquisition, to segmentation, to downstream analysis, are available [164, 186–190]. Further, analysis methods new to cellular images that incorporate machine learning strategies can outperform classical methods, providing an opportunity to improve the power of high-content assays [191]. These strategies include both supervised, where annotated datasets are provided to train the algorithm, and unsupervised strategies [192]. Deep learning-based approaches to biological image analysis [193, 194], such as those that utilize artificial neural networks, show promise for both complex applications-specific tasks, such as detecting the location of early metastases in whole animal images [195] and common cell counting, detection, and morphology measurement tasks from noisy bright-field, phase-contrast, and fluorescence microscopy images [196]. For an assessment of the recent progress, challenges, and opportunities in applying deep learning to cell image analysis, an excellent review is available [197].

More generally, connecting the material design properties to emergent cell behaviors may be enabled through integration with materials data science. Materials data science involves techniques such as building databases of material properties, high throughput screening, meta-analysis, and molecular simulation [15–17] which is complementary [18, 19], to the detailed cellular and molecular analyses proposed herein for systems tissue engineering. This integration of data from multiple levels can provide a more comprehensive systems design approach that integrates the material properties, the mechanistic cellular and molecular responses, and ultimately function for the intended application.

4 Capturing spatial information driving tissue behavior

The spatial distribution of cells and features within and between niches in a tissue are integral for controlling collective tissue behavior and function [198–200]. Coordinated behaviors within tissues may at least in part ultimately emerge from changes in gene expression within subsets of spatially defined cells [201]. Cells communicate and provide spatial signals to neighbors through concentration gradients [202] and waves [203], cell-cell contacts, ECM remodeling, and mechanical forces [204, 205]. Spatial patterning produced by biomaterial microsystems can be used to recreate complex tissue architectures that would not be otherwise possible (Figure 6) [143, 206–209].

For example, geometric micropatterning of cell adhesion [210, 211] can drive a complex multi-cellular emergent behavior, such as where cancer cell stemness is concentrated at the geometric edges and vertices of colonies (Figure 4C) [119]. Patterning of gene delivery has been employed to control concentration profiles that spatially oriented neurite extension [212, 213]. Similarly, microfluidic generated concentration gradients [214–216] and material stiffness gradients [132, 217, 218] induce chemotaxis and durotaxis respectively in diverse cell types. Patterning of mechanical interfaces guide development of microtissues including the spontaneous polarized development of amnion-like tissue from stem cells [219]. We expect the use of biomaterial microsystems for control of spatial patterning of the tissue

microenvironments paired with emerging spatially resolved sequencing [220–222] will further resolve the mechanisms and role of spatial signaling networks in tissue behaviors.

4.1 Spatially resolved omics within engineered microsystems

The most conceptually straightforward approach to link single cell microenvironment-driven function to a systems-level molecular phenotype is to isolate and index individual cells from culture after observing their behavior and function. Single cell isolation [223, 224] can be achieved by physically picking cells using a micromanipulation system, with microfluidic systems [225], or by culturing cells in isolation in microwells. However, current systems for cell picking by micromanipulation remain low throughput and labor intensive to operate [213, 223, 224]. This approach can however be effective for experiments with low cell numbers, as cells can be directly picked from complex multicellular microenvironments. Isolated culture approaches in contrast can be scaled to 10's or 1,000's of individual cells using microfluidic technology[225], but an inherent limitation is that cells will not receive the cell-cell contacts and soluble factors from other cells in their microenvironment. In one isolation culture approach, researchers used an ECM coated Fluidigm C1 chip to isolate individual cells into microwells then applied fluorescence microscopy to track NF- κ B transcription factor reporter activity over time after lipopolysaccharide stimulation[226]. The chip was then used to create single cell cDNA libraries for single cell RNA sequencing providing both a history of dynamic NF- κ B activity and transcriptome data for each individual cell. This capacity to link a history of dynamic functional behavior to the full transcriptome within the same individual cell is unique to the physical separation approach and allows for a direct link between microenvironment driven function and molecular phenotype.

Emerging methods to link spatial position and gene expression include MERFISH[220], seqFISH[221], and STARmap[222], which can detect from 100's [220, 221] to up to 1000[222] genes per individual cell *in situ* within fixed tissue samples. This *in situ* approach would allow for dynamic monitoring of the sample before fixation to connect past behaviors of each cell to the molecular phenotype and spatial information in the sample at the time of fixation. However, current methods remain complex, time consuming, and low throughput, requiring dedicated facilities and expertise [227–229]. In STARmap, DNA nanoballs are produced locally at the site of each individual cell and entrapped within a 3D DNA hydrogel. Advances in nanoparticle and hydrogel technologies will be expected to facilitate improvements in the *in situ* sequencing technology. Even with all of the spatial information preserved, *in situ* spatial genomics remains a destructive snapshot technique and cannot give information about dynamic cell signaling networks.

4.2 Biomaterials and microsystems for spatial interrogation and control

Continuing advances in biomaterial microsystems uniquely allow for spatial control of cells to interrogate the role of spatial organization on tissue function. Synthetic hydrogel chemistries for 3D cell culture allow for high-resolution patterning of degradation [29], mechanical stiffness/crosslinking (Figure 6A)[206], and protein ligands(Figure 6B)[33, 207, 230] using photolithography methods. Just as materials can be patterned with light, optogenetic technology, where light and photosensitive proteins are used to control gene

expression [231, 232], can be used to photo-pattern gene expression within inhabitant cells of a microtissue (Figure 6C) [208]. Through the application of 3D multi-phasic gel-in-gel material systems, cell can be patterned, and material characteristic can be controlled at both the microscale and the mesoscale (multi-microenvironment scale) to drive desired behaviors [233–235]. Application of computerized automated systems for 3D printing of these multi-phasic gels can allow for complex spatial patterning of several biological inks containing multiple cells and/or materials and/or voids in 3D space (Figure 6D) [209, 236, 237]. And patterning systems have also recently extended into the temporal domain with one system describing a method to simultaneously pattern 3 proteins, with spatial and temporal control, within a biomaterial (Figure 6B) [207]. An alternative approach with the ability to recapitulate true in vivo morphologies ex vivo is the decellularization of tissues into a vascularized ECM biomaterial that retains much of its native 3D spatial structure followed by subsequent recellularization by perfusion and ECM guided emergent behavior [238]. Integration of either intact decellularized ECM or decellularized ECM reconstituted into hydrogels is a promising strategy for studying tissue-specific behaviors within engineered microsystems [239, 240]. We anticipate that continuing advances [43, 241] in the spatiotemporal control of biomaterial microsystems will enable mechanistic studies of the spatial coordination and feedback loops driving tissue behaviors.

5 Conclusion

The integration of biomaterial technologies with genomics and high content imaging offers the opportunity to molecularly dissect normal and abnormal tissue responses, and ultimately to develop the design principles for biomaterial systems that direct cellular processes and tissue formation. This combination of techniques offers the opportunity to describe the complex spatio-temporal processes at the cellular scale, while also capturing the heterogeneity of cellular responses, with applications in basic science, drug development, and tissue engineering [143, 242, 243]. Given the complexity of emergent tissue behaviors, the path toward designing improved biomaterial microsystems will likely involve the application of systems-level experimental workflows that can elucidate the role of dynamics, spatial coordination, and rare cells in driving tissue functions (Figure 2). This overview of systems level analysis and the currently available tools highlights opportunities for innovation in the biomaterial platforms and their integration with computational algorithms that can process the data and identify the design parameters, which will advance the understanding of biomaterial function and potentially enhance the ultimate pace of translation.

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Figure 1. Systems tissue engineering for study of complex microenvironments.

A. The systems tissue engineering paradigm operates in a space spanning from the study of thousands of unique simplified microenvironments to just a few highly complex microenvironments. **B.** Systems tissue engineering measures complexity using tools from systems biology within high complexity microenvironments created using tissue engineering.

Figure 2:

Strategies for linking single cell functions to their molecular phenotype within engineered microenvironments.

Material-generated behavior

Dynamic transcriptional response

Figure 3. Monitoring dynamic transcriptional responses to engineered biomaterial.

Human foreskin fibroblasts bearing 50 transcription factor activity genetic reporters were cultured on polyethylene glycol (PEG) hydrogels with tunable stiffness and RGD ligand density. The fibroblasts exhibit distinct dynamic changes in transcription factor activity networks in response to material stiffness and material RGD ligand density. Adapted with permission from [26].

Figure 4: Recording dynamic single cell behaviors within engineered microenvironments.

A. Cancer cell chemotaxis up a linear concentration gradient within a 3D collagen matrix. Adapted with permission from [118] **B.** Cancer cell traction force generation and matrix alignment within a 3D collagen matrix. Adapted with permission from [46] **C.** Cancer cell stemness marker expression depends on proximity to edges and vertices in geometry. Adapted with permission from [119] **D.** 3D methacrylated dextran (DexMA) hydrogel crosslinking controls endothelial cell density and multicellularity in angiogenic sprouting. Adapted with permission from [120] **E.** Local collectivity of T cells within microwells

promotes memory differentiation. Adapted with permission from [121] F. Microsystems with isolated or connected channels allow for study of soluble mediators driving single NK cell killing kinetics of cancer cells. Adapted with permission from [122].

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Figure 5. Case study: High-content live-cell imaging of drug-induced liver toxicity.

A. Human hepatoma HepG2 cell lines expressing various stress response pathway genetic reporters on bacterial artificial chromosomes were imaged in high-content live-cell format for 24 hours after addition of different drugs to culture. Adapted with permission from [171].

B. Automated image analysis was conducted to segment each cell nucleus and cytoplasm and record single-cell stress response from reporters. Reproduced with permission from [170].

C. Unsupervised hierarchical clustering was applied to infer the means and kinetics by which each drug compound and dose induced cellular stress. Reproduced with permission from [171].

Figure 6. Spatial patterning of biomaterial microsystems.

A. Application of photolithography techniques to a UV-reactive acrylated hyaluronic acid (AHA) hydrogels can create intricate spatial material patterns (red) that guide inhabitant cell behavior (green). Adapted with permission from [206]. **B.** Photopatterning of UV-reactive polyethylene glycol (PEG) hydrogel for reversible 3D patterning of several protein ligands within the same material. [207] **C.** Expressing optogenetic proteins paired with gene regulatory circuits in cells can be used to photopattern gene expression within tissue constructs [208]. **D.** Advances in 3D printing including stereolithography techniques can

create intricate 3D tissue topologies. A vascularized 3D printed tissue construct containing hepatocyte aggregates exhibits functionality as measured by albumin promoter activity after implantation into mice with chronic liver injury. Adapted with permission from [209].