Engineering multicellular living systems: a Keystone symposia report

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CONCISE REPORT

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Engineering multicellular living systems—a Keystone Symposia report

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Abstract

The ability to engineer complex multicellular systems has enormous potential to inform our understanding of biological processes and disease and alter the drug development process. Engineering living systems to emulate natural processes or to incorporate new functions relies on a detailed understanding of the biochemical, mechanical, and other cues between cells and between cells and their environment that result in the coordinated action of multicellular systems. On April 3–6, 2022, experts in the field met at the Keystone symposium "Engineering Multicellular Living Systems" to discuss recent advances in understanding how cells cooperate within a multicellular system, as well as recent efforts to engineer systems like organ-on-a-chip models, biological robots, and organoids. Given the similarities and common themes, this meeting was held in conjunction with the symposium "Organoids as Tools for Fundamental Discovery and Translation".

KEYWORDS
computational, engineered living, engineered organs, multicellular, systems

INTRODUCTION

Multicellular engineered living systems are designed systems that contain multiple interacting living components and potentially nonliving components as well, such as support structures and microfluidic devices. Designing these systems to emulate natural processes or to incorporate new functions requires a detailed understanding of the biochemical, mechanical, and other cues that dictate cell–cell communication and cell–environment communication, ultimately resulting in the coordinated action of the system. Multicellular engineered living systems run the gamut from organoids—fully biologic entities that rely on internal and external cues to drive self-organization and assembly and are relatively variable and unpredictable—to organs-on-a-chip that can incorporate flow and mechanical stress to mimic biological forces, to highly engineered biorobots able to sense, process signals, and perform mechanical functions. These multicellular engineered living systems have the potential to model diseases in more human-centric, and potentially patient-centric, ways.

On April 3–6, 2022, experts in the field met at the Keystone symposium "Engineering Multicellular Living Systems" to discuss recent advances. This was a joint meeting held concurrently with "Organoids as Tools for Fundamental Discovery and Translation" (see a separate report DOI: 10.1111/nyas.14874). Speakers discussed efforts to engineer human organs, deriving inspiration from human development and nature, using engineered organs as disease models and in drug discovery efforts, and technological advances in areas such as computational modeling of multicellular systems and 3D printing.

KEYNOTE ADDRESS: USING BRAIN ORGANOIDS TO UNDERSTAND DEVELOPMENT AND DISEASE

Paola Arlotta, from Harvard University, opened the meeting with a presentation on using organoids to understand brain development and neurological disorders. Arlotta's group has been instrumental in improving the rigor and reproducibility of organoid systems and in establishing organoids as a key tool for understanding cortical development. Arlotta described some of the mouse embryo work in her lab in collaboration with Aviv Regev's group at the Massachusetts Institute of Technology aimed at understanding how the diverse cell types of the cortex develop. The groups use single-cell RNA sequencing (scRNAseq) to identify the cell types present on each day of embryonic development and then generated a lineage tree that reconstructs the molecular trajectories from early progenitors to terminally differentiated cells.[1] Arlotta's group has investigated the factors involved in cell fate decisions identified *in vivo* using a process known as Perturb-seq to better understand function and impact on cell lineage.[2]

To better understand human brain development, Arlotta's group has developed protocols to reproducibly grow brain organoids from induced pluripotent stem cells (iPSC). The cultured organoids can be

maintained for up to 7 years, rendering them amenable to studying long-term developmental processes. Arlotta showed that cultured organoids recapitulate the cell diversity, as well as some of the structural elements, seen in the human brain.[3,4] More recent work established a protocol based on one originally developed by Yoshiki Sasai's group at RIKEN[5] that reproducibly establishes the cellular diversity of the cerebral cortex from different stem cell lineages.[6] They also showed that each organoid achieves cellular diversity via similar molecular mechanisms,[7] indicating they can be used as model systems to understand development and disease.

Arlotta's group is now using these organoids to better understand neurological disorders. They presented data on organoids containing mutations identified among children with autism. Single-cell methods demonstrated that organoids with mutations in *SUV420H1*, *ARID1B*, or *CHD8* all showed accelerated development of GABAergic neurons. The fact that three distinct mutations converge on a similar cell type and developmental process may hold promise for categorizing types of autism spectrum disorders susceptible to similar treatment modalities. Arlotta hypothesized that the early asynchronous development of GABAergic neurons seen in organoids may result in abnormal cortical circuits years later. Additional data showed that genetic background can modulate the effect of *SUV420H1*, *ARID1B*, or *CHD8* mutation in both a mutation- and phenotype-specific manner, highlighting the potential for using organoids to understand disease in a patient-specific manner.[8]

ENGINEERING PRINCIPLES OF DEVELOPMENTAL BIOLOGY AND REGENERATION

From simple muscular pumps to an engineered heart

One of the long-term goals of biomedical engineering is to engineer entire organs. Efforts to engineer the human heart, for example, have typically focused on matching the anatomy of the organ.

Kevin Kit Parker from Harvard University described how his group is taking a different biophysics-based approach by first building simple muscular pumps and then adding more complexity and functionality. Over 10 years ago, Parker's group developed a heart-on-a-chip, composed of cardiac muscle tissue on a polymer thin film, that replicates the laminar structure of the heart. The chip can be used to measure contractile performance and diastolic relaxation.[9] Parker's group has also found inspiration in other forms of muscular pumps found in nature—namely jellyfish and stingray. For example, they have reverse-engineered a jellyfish with medusoid constructs made of rat cardiac myocytes that exhibit controllable robust swimming behavior, similar to natural jellyfish.[10] Parker called these efforts a good "training exercise" to learn some of the basic design laws of muscular pumps. His group has also generated tissue-engineered stingray-like tissue from rat cardiac myocytes that can be steered and guided using optogenetics. Parker showed how this system produces undulatory contraction, analogous to how the contractions in the heart move from the bottom up.[11]

Ultimately, to achieve something akin to a human heart, the tissue must be composed of multiple layers and beat autonomously. In the heart, the sinoatrial node—responsible for generating the electrical signal responsible for atrial contraction—is surrounded by a boundary condition, making it structurally and functionally distinct from the rest of the myocardium. Parker's group mimicked this boundary condition in a biohybrid fish and showed that it leads to a spontaneous and self-paced side-to-side contraction that enables the fish to swim without any external control. The system is a contained feedback loop in which muscle contraction–induced bending activates stretch-activated ion channels, resulting in self-sustaining rhythmic contractions.[12] Parker hopes that this work will inform the design of other autonomous systems.

Understanding the rules governing nephron number

The kidney contains approximately one million nephrons, which are responsible for filtering the blood. The final number of nephrons (in humans, for example) appears to be hardwired during development, though there is variability in nephron number among individuals and between kidneys of the same individual. A lower nephron number at birth has been associated with an increased risk of chronic kidney disease (CKD) and hypertension.[13]

Alex J. Hughes from the University of Pennsylvania described work on understanding how the developing kidney maximizes nephron number in an ever-decreasing space. During development, nephrons are generated synchronously with continuous branching of the ureteric epithelium. Hughes created a physics-based model that incorporates parameters such as repulsion between ureteric tubule tip domains, lateral confinement, and radial height to predict tip packing patterns over time. Hughes showed that kidney tips transition through several geometric packing phases, including a frustrated packing state in which tip parent nodes crowd the epithelial surface. Both modeling and experimental data show that this state is resolved by tension in the tubules that pull the parent nodes from the surface. This enables the developing kidney to avoid defects and increase tubule and nephron packing density.[14]

Relationships between tissue form and cell fate in the developing lung

Katharine Goodwin from Celeste Nelson's lab at Princeton University presented work on understanding the molecular and physical mechanisms that drive branching morphogenesis in the developing lung to produce the structure of the bronchial tree. Goodwin showed that the smooth muscle layer that surrounds the developing airway epithelium is critical for shaping bifurcations. Smooth muscle differentiation at the developing bud is required for specifying the bifurcation site. Inhibiting smooth muscle differentiation results in dilated branches, while increasing smooth muscle differentiation results in stunted branches.[15] During her talk, Goodwin showed unpublished data that

expands on their previous work showing how manipulating branch morphology affects epithelial differentiation. These data provide insight into how tissue form impacts patterns of cell fate in the developing airway epithelium.

Microphysiological systems and drug discovery platforms

Several speakers discussed the potential for engineered multicellular living systems to improve the drug development pipeline. The current system relies heavily on nonhuman-based preclinical models that do not always translate well to humans, undoubtedly contributing to the fact that less than 12% of drugs that enter clinical trials are ultimately approved.[16] Engineered multicellular living systems can offer a more human-centric approach to preclinical testing and provide insights on mechanisms of disease, drug safety, and drug efficacy that are not accessible in animal models. In addition, patient-derived systems can inform how different subgroups of patients are likely to respond to a candidate drug, thus streamlining clinical trial recruitment and leading to a more precision medicine-based approach.

Insights from a more mature cardiac model

Many *in vitro* models of the human heart contain only cardiomyocytes and display immature characteristics. **Christine L. Mummery** from Leiden University Medical Center described her group's efforts to develop a more mature model of the heart consisting of cardiac endothelial cells, fibroblasts, and cardiomyocytes cultured in a 3D microtissue; the microtissue contains several ultrastructural features of mature cardiomyocytes, such as aligned sarcomeres. Mummery attributed this mature phenotype to the presence of CX43-mediated gap junctions between cardiomyocytes and fibroblasts, but noted that the presence of the above three cell types likely contributes.[17,18] Mummery showed three examples of how her group is using these cardiac microtissues to model cardiac disease. First, they assessed the impact of mutations in the desmosomal protein PKP2, mutations known to cause arrhythmogenic cardiomyopathy (ACM). Normally, PKP2 and CX43 colocalize at the cell membrane in epicardial cells. In cells with mutated PKP2, trafficking of CX43 to the membrane is impaired. Downregulation of PKP2 in ACM patient human-induced pluripotent stem cell (hiPSC)-derived cardiac fibroblasts in their model recapitulated the phenotypes of ACM, including a decrease in CX43 gap junctions, failure to follow electoral pacing, and arrhythmias. Having all three cell types in the model was, therefore, critical to pinpoint cardiac fibroblasts as an important cellular contributor to ACM.[17]

In the second example, Mummery showed how the maturity of the cardiac microtissue enables the study of the impact of mutations in postnatal isoforms, specifically mutations in the gene *SCN5A*, which expresses sodium voltage-gated channel alpha subunit 5. Mutations in exon 6B, which is expressed only postnatally, leads to Brugada syndrome, a condition characterized by life-threatening arrhythmias. Conventional hiPSC–derived cardiomyocyte systems are phenotypically fetal and do not express the postnatal isoform of SCN5A. Mummery showed that their cardiac microtissue expresses the postnatal isoform of SCN5A, and they have used it to identify mechanisms involved in postnatal splicing.[19]

Finally, Mummery presented unpublished data using the cardiac microtissues to model catecholaminergic polymorphic ventricular tachycardia type 1, in which mutations in *RYR2* disrupt calcium handling. Mummery's group is using this model to generate patient-derived tissues to better understand why some patients respond to current therapies, while others do not, as well as to screen for new therapies.

Modeling neurodegenerative diseases with vascular networks

Roger D. Kamm from MIT described work applying vascular network models to understand neurodegenerative diseases. Kamm's group has been able to grow vascular networks from various types of endothelial and stromal cells, with comparable vascular permeability as *in vivo* systems. These models provide a means to investigate both active and passive transport of small molecules, proteins, and nanoparticles.[20,21] His group has since tailored this model to create a 3D self-organized microvascular model of the blood–brain barrier (BBB) composed of three cell types: iPS-derived endothelial cells, primary pericytes, and primary astrocytes. The model has permeabilities comparable to *in vivo* measurements, as well as ultrastructural and transcriptional features that mimic the BBB.[22,23] Kamm showed how they are using this BBB model to study brain metastasis and drug delivery to the brain. They showed that secretion of CCL2 by astrocytes enhances the extravasation of several tumor cell types, despite decreased permeability of the BBB. Similar to what other speakers described, Kamm's group found that including multiple cell types in the BBB model is critical.[24] In patient-derived xenograft models of glioblastoma, perfusion experiments demonstrated that nanoparticle delivery of the chemotherapy drug cisplatin resulted in increased tumor cell killing than delivery of free cisplatin.[25]

Finally, Kamm showed how his group is using the BBB model to understand the vascular impact of Alzheimer's disease. They developed a monolayer consisting of a neuron channel and an endothelial channel separated by collagen. Systems in which neurons overexpress amyloid beta (Aβ) showed increased vascular permeability and accumulation of Aβ on the endothelial wall.[26] Kamm's group is currently using this model to screen for drugs that will prevent the adverse interaction between Alzheimer's disease neurons and vascular cells.

Developing clinically relevant *in vitro* models to improve drug development

Sandra J. Engle described streamlining drug discovery and development by developing *in vitro* models that can be translated to the clinic. Engle stressed that improving the drug development process will require a stronger human-centric approach. In contrast to this, the standard process that relies on *in vivo* animal models has resulted in

many failures of translation of initially promising results to the clinic. Engle highlighted three key considerations. First, there is a need for physiologically relevant cell systems that use human cells and clinically relevant endpoints. Second, drug screening assays should measure a phenotype that can be consistently measured in *in vitro* cell-based models, *in vivo* preclinical models, and patients. Third, clinical and genetic information combined with knowledge of mechanism of action should be leveraged to select patient populations that are most likely to benefit from a given therapy.

Engle illustrated how these principles could be applied in drug discovery for amyotrophic lateral sclerosis (ALS). She focused on a subset of ALS that is caused by a hexanucleotide repeat in *C9orf72*. iPSC-derived *C9orf72*-mutant motor neurons display several pathologies seen in ALS patients, including decreased levels of C9orf72 protein, toxic RNA aggregates, and toxic dipeptides. These toxic dipeptides can also be measured in the cerebrospinal fluid of patients, thus providing an endpoint that can be translated from preclinical models to clinical studies. Treatment of iPSC-derived motor neurons containing the ALS mutation with an antisense oligonucleotide (ASO) that targets mutant *C9orf72* reduced toxic RNA foci and dipeptide levels without impacting wild-type *C9orf72* mRNA levels. In a transgenic mouse model, the ASO mitigated these cellular phenotypes and improved behavior.[27] Engle stressed that these models are assessing the impact of treatment on a biomarker of disease, namely dipeptides and RNA foci, not on an efficacy marker. Work is ongoing to develop more complex models of the neuromuscular junction based on work from Kamm's group that are amenable to measuring conduction velocity and contractility, which can also be measured in patients.[28]

Accelerating the use of tissue chips in drug development

Danilo A. Tagle from the National Center for Advancing Translational Sciences (NCATS) at the National Institutes of Health (NIH) discussed the center's mission to accelerate the development and utilization of tissue chips for three major applications: safety and toxicity studies, disease modeling and efficacy studies, and clinical trials on chips. Unlike other NIH entities, NCATS is disease agnostic: it is focused on diagnostics and therapeutics across many human diseases and conditions. After its inception in 2012, NCATS's initial focus was to promote the development of tissue chips for safety studies. *In vitro* models are needed both for organs that are susceptible to drug toxicity, like the cardiovascular system, liver, kidney, and GI tract, as well as for areas and disease states that lack good model systems, like rare and pediatric diseases, pregnancy, and neurodevelopment. Of the many projects that NCATS has funded in this regard, Tagle showed an example in which a human liver tissue chip captured signs of liver toxicity upon exposure to an antiviral drug that were not apparent in a rat liver tissue chip.[29]

The second area of NCATS's focus is to promote the use of tissue chips for disease modeling and efficacy testing. This represents a large collective effort across the NIH. Funding is awarded in two phases—the first of which is focused on developing and validating the disease models, while the second is demonstrating their utility. Tagle stressed that such chips must ideally recapitulate *in vivo* functions and responses in both normal and disease states, reflect multiorgan pathology and cross talk, capture the diversity of human diseases, and be amenable to real-time functional readouts. In addition, NCATS has supported projects to address the opioid crisis via the 2019 program Tissue Chips to Model Nociception, Addiction, and Overdose and was provided supplemental funding to aid in the development of models for COVID-19 via the 2020 CARES Act. One such project demonstrated the utility of 3D culture over 2D culture when screening drugs for the ability to inhibit SARS-CoV-2 pseudovirus infection.[30] Another area of focus has been to model age-related disorders with tissue chips. Many physiological changes from aging are also observed under microgravity conditions. To study these changes, NCATS is collaborating with NASA, CASIS, and ISS-NL to model age-related diseases under microgravity using tissue chips. Tagle showed some preliminary results on the effects of microgravity on age-related markers in immune cells and cardiac tissues. In addition to the scientific insights, the space program collaboration also stresses the need to automate and miniaturize tissue chip hardware and instrumentation, which may promote broader adoption and use on Earth.

Finally, NCATS's current focus is to support clinical trials on a chip. The goal is to incorporate well-developed, validated tissue chips into rare disease clinical trial frameworks and demonstrate their usefulness in the clinical setting. Tissue chips have the potential to streamline the clinical trial process by helping to select and stratify patient subgroups and may even serve to expand the small patient populations that plague trials of rare diseases. Personalized tissue chips can inform physician decision-making about treatment modifications. Tagle also discussed efforts by industry and regulatory agencies to develop standards and guidelines for these models to facilitate widespread adoption and commercialization.

Accelerating the use of organoids in drug development

Sylvia F. Boj from Hubrecht Organoid Technology (HUB) presented the company's approach to facilitating drug development using patient-derived organoids in preclinical testing. HUB has developed biobanks of several epithelium-based patient-derived organoids of several different tissues. These biobanks provide preclinical models that are linked to patient phenotypes. Boj suggested that the epithelial cell organoids HUB is developing can be instrumental in preclinical lead identification in certain diseases even though they lack the complexity offered from inclusion of multiple cell types or microfluidics. She showed an example in which HUB collaborated with the biopharma Merus, and others in the SuppreSTEM consortium, to screen hundreds of bispecific antibodies to test for tumor cell killing in colorectal carcinoma organoids. The screen eventually led to a single lead compound that entered clinical trials in 2018.[31] The time from drug screening to clinical trials was under 5 years, demonstrating the potential for organoids to reduce the drug development timeline drastically. Boj

also stressed the importance of ensuring that their epithelial-based organoids provide predictive efficacy readouts in a given disease state. Toward that goal, HUB is generating organoids from patient tumors to see if there is a positive correlation between patient and organoid responses to treatment. HUB is also working on adding complexity to the organoid models, for example, by introducing other cell types and improving screening assays to reduce the number of organoids needed to provide reproducible data. Boj foresees the potential to conduct clinical trials in organoids to help identify patients who are most likely to respond to a given treatment.

Engineering a vascularized mini-brain

Alice E. Stanton from Robert Langer and Li-Huei Tsai's labs at MIT presented unpublished work on engineering a vascularized mini-brain tissue. Stanton described the multicellular integrated brain (miBRAIN) model, which incorporates multiple cell types found in the brain in an engineered matrix that mimic aspects of the extracellular matrix (ECM) of brain tissue and enhances neuronal properties and maturation. The miBRAIN contains several features absent in alternative models, including a fully integrated BBB within the neuronal networks, enhanced microglia cell phenotypes, myelination, and biomimetic tissue architecture, and transcriptional signatures indicate that miBRAINs capture enhanced diversity of cell types. Stanton is harnessing this model to address neurodegenerative diseases with the goal of identifying new drug targets and conducting *in vitro* clinical trials.

ADVANCED TECHNOLOGIES FOR ENGINEERING MULTICELLULAR LIVING SYSTEMS: COMPUTATION

Computational modeling is a necessary component of engineering multicellular living systems. Computational approaches enable researchers to uncouple different cues that cannot be tested experimentally in isolation. Several speakers presented their work on using computational modeling to gain a better understanding of how cells coordinate within multicellular systems.

Morphogenesis in the developing forebrain

Yoshihiro Morishita from RIKEN discussed work to reveal the design principles of organ morphogenesis. While much work has been done to understand morphogenesis at a genetic and molecular level, Morishita is interested in understanding it as a physical process to fill the gap between the molecular/cellular scale and tissue-level scale. His lab has done quantitative analyses of tissue and cell dynamics to understand morphogenesis for several types of tissues, including the C-looping of the heart,[32] developing limbs,[33] and teeth.[34] During his talk, Morishita focused on work on the development of the forebrain.[35] During development, morphological changes in the neural tube form the optic cup and lens that eventually form the eye; one of the key molecular players in this process is the sonic hedgehog (SHH), which is expressed in the ventral region of the neural tube. SHH inhibition represses optic vesicle elongation but does not affect the formation of the neural tube itself; deficiency in SHH leads to cyclopia.[36] Morishita showed that there is a critical time window during which disrupting SHH signaling impacts optic vesicle morphogenesis.[37] His group previously computationally reconstructed 3D morphologies of optic vesicle elongation. These results show that optic vesicle elongation is not driven by cell proliferation, suggesting that SHH-dependent collective cell motion drives normal morphogenesis.[35] Morishita showed that SHH regulates anterior–posterior cell polarity in ventral tissues, the direction of which is determined by mechanotransduction. Normally, tissues exhibit cell polarity both in terms of cell shape and phosphorylated myosin light chain localization that is aligned with an external force, and cells move so that the population deforms in a direction orthogonal to the direction of external force. In the absence of SHH signaling, cell polarity is abolished, and cells move randomly, regardless of any external force. This loss of SHH-dependent mechanosensation essentially leaves the cells disoriented.[37]

Modeling how cell–cell interactions affect differentiation

Melissa L. Kemp from the Georgia Institute of Technology discussed her group's approach to computationally modeling the multicellular organization of iPSCs. Kemp's group uses agent-based platforms to model organoids and multicellular systems.[38] During her talk, Kemp focused on their work to model the effect of cell–cell interactions on the loss of pluripotency. Computational modeling of embryonic cells revealed the development of distinct spatiotemporal patterns as cells differentiate. The patterns could be explained by a set of rules that combine baseline stochasticity with intercellular communication, such as how the differentiation state of a cell affects the state of its neighbors as well as paracrine signaling to model the effects of more distal neighbors. Kemp's group devised a method to quantify these patterns that enables them to compare computational and experimental results. She showed that the spatiotemporal patterns of cell differentiation observed experimentally were largely reproduced computationally.[39,40] Kemp's group has also modeled the effect of intercellular transport via gap junctions on cell fate. Photobleaching experiments showed that the diffusibility of small molecules between neighboring cells varied according to the cell cycle, consistent with the observation that the gap junction protein CX43 shows higher sequestration in the cytoplasm in mitotic cells. High CX43 expression is also associated with loss of OCT4 expression or loss of pluripotency. Using a model that incorporates these experimentally determined intercellular transport rates and asynchronous cell division, Kemp showed that they could model the intracellular buildup of a small molecule through a cell population; accumulation of this molecule is followed by waves of differentiation, similar to what is seen experimentally with a small molecule that induces differentiation, like retinoic acid. Introducing intracellular perturbations affected the timing of differentiation, but

the general patterning was robust to these changes.[41] Kemp proposed that controlling CX43 heterogeneity in colonies may be exploited to drive lineage specification.

Dennis A. Norfleet, a graduate student in Kemp's lab, presented unpublished work on the role of bioelectricity as a signal in spatial patterning for morphogenesis and regeneration. Evidence from animal models supports the idea that bioelectricity can serve as a parallel signal to biochemical and biomechanical forms of communication. Bioelectricity has been shown to play a role in morphogenesis in several animal models.[42] Norfleet is using the computational modeling platform Bioelectric Tissue Simulation Engine developed in Michal Levin's group at Tufts University[43] to model intercellular ion flux with a goal of not only understanding how ion gradients and bioelectric signals propagate through a cell population but also to use that understanding to harness bioelectricity as a novel control mechanism for morphogenesis and differentiation.

Mathematical modeling of *M. tuberculosis* dynamics in the granuloma

Elebeoba E. May from the University of Houston presented work on using computational modeling to understand infection and how the dynamics of infection and host factors, e.g., comorbidities, impact disease outcomes. May's group has developed models relevant to *F. tularensis* infection and *E. coli* biofilm formation. During her talk, May focused on *M. tuberculosis*. One of the hallmarks of *M. tuberculosis* infection is the formation of a multicellular structure called a granuloma. The formation of a granuloma is a determining factor of how effectively the host can eliminate the pathogen. A granuloma can also act as a refuge for latent *M. tuberculosis*, which can be reactivated if the granuloma dissolves. *In vitro* data from the Wayne nonreplicating persistence model[44] showed that *M. tuberculosis* can metabolically adapt to slow oxygen depletion, but that rapid oxygen depletion, such as what might occur upon robust immune response and concomitant formation of a granuloma, causes pathogen death. May's group has developed mathematical models to describe the relationship between the host immune response, the change in the bacteria's microenvironment as a result of being enclosed in the granuloma, and bacterial survival. The model leverages *M. tuberculosis* experimental data to show how environmental changes affect gene expression and subsequent metabolic changes. May showed that their model was able to predict the transcription and metabolic responses during slow and rapid oxygen depletion. May's group has expanded on this model, leveraging previous work from Denise Kirschner's lab,[45] to develop an integrated multiscale model that considers the impact of the microenvironment on both the bacterium and the host. They showed that this model captures how the dynamics of the immune response and granuloma organization mediate oxygen availability.[46] Finally, May described their efforts to develop *ex vivo* platforms of the early stages of granuloma formation to acquire more robust cellular-scale data and improve their *in silico* predictions.[47]

Using orthogonal differentiation to introduce complexity into organoids

Aric Lu from Jennifer Lewis's group at Harvard University presented work on orthogonally induced differentiation to introduce missing cell types into organoids and to program organoids and tissues. Lu focused primarily on brain organoids, which often lack endothelial cells and vascularization. To introduce endothelial cells into the brain organoids, Lu devised a protocol in which iPSCs are modified to express an inducible transcription factor that drives differentiation toward an endothelial phenotype (called iEndo cells), and then cocultured these with wild-type iPSCs. The resulting programmable organoids contain all the cell types that would normally be present in organoids generated from wild-type cells, in addition to the cell type induced by the transcription factor. Lu used this protocol to create organoids with wild-type iPSCs, iEndo cells, and iNeuron cells, which contain an inducible transcription factor that drives neuron differentiation. Lu showed that the resulting organoids contain a persistent vascular network that permeates them. Using bioprinting techniques, Lu has created different patterns of inducible cells within developing organoids and showed that they maintain their spatial arrangement, thus providing a means to create more sophisticated tissue patterns within organoids.[48]

ADVANCED TECHNOLOGIES FOR ENGINEERING MULTICELLULAR LIVING SYSTEMS

Leveraging 3D bioprinting to produce walking/crawling biomachines

Rashid Bashir from the University of Illinois showed how his group uses 3D bioprinting to develop 3D cellular machines and biorobots. Other speakers during the meeting are also developing cell-driven machines, such as the autonomous fish[12] and stingray-[11] described above by Parker. Bashir's group is interested in building walking/crawling machines powered by skeletal muscle cells with an aim of developing autonomous machines that have neuromuscular control. Approximately 10 years ago, they developed a miniature cantilever-like structure formed of hydrogels and contractible cardiomyocytes via 3D printing; asymmetry between the two arms of the cantilever enables the structure to spontaneously walk.[49] Since then, Bashir's group has worked on different types of structures, including a design inspired by the muscle–tendon–bone complex powered by the actuation of a skeletal muscle strip.[50] They are using 3D-printed scaffolds that allow the formation of muscle strips and rings as basic units to fabricate more complex machines.[50,51] For example, they have built a muscle–ring optogenetic biobot that shows directional location upon stimulation with light.[51] Bashir's group has worked on optimizing and scaling these biobots to increase myotube alignment and number; for example, differentiating myoblasts into myotubes before seeding them produces more force. They are now able to create structures several centimeters in length as well as structures that can turn and be steered with an asymmetric electric field.[52,53] Bashir also showed that they

have designed a 3D neural tissue with electrically active neurons[54] and that stimulating cells during neurogenesis and synaptogenesis can affect network synchronicity.[55] The aim is to leverage these insights to control the level of connectivity within resulting networks within an autonomous biological robot.

Tailoring 3D printing for biological materials

Bioprinting has several inherent challenges compared to 3D printing with plastics. Biological materials are soft and deformable; it can be difficult, therefore, to control the shape of these materials when printing them in air.

Adam W. Feinberg from Carnegie Mellon University described his group's approach to 3D bioprinting termed Freeform Reversible Embedding of Suspended Hydrogels (FRESH).[56] In FRESH, gellable bioinks are printed into a support material that is later melted away. In this way, the bioink is uniformly supported throughout the printing process. FRESH is compatible with a range of bioinks, including alginate, cell slurries, collagen, fibrin, and hyaluronic acid, and support materials, such as gelatin, alginate, agarose, and cell slurries. Structures can also be printed via different print directions in 3D and are not limited to the layer-by-layer printing path normally used in 3D printing, which is important for recreating complex tissue architectures.[57] Feinberg hopes to ultimately use this technology to build entire organs. Toward that goal, his group has built functional components such as collagen heart valves and a contractile human ventricle made of cardiomyocytes printed onto collagen support. Feinberg showed that the printed ventricle recapitulates some functions of the heart—e.g., cardiomyocytes show alignment, and the ventricle exhibits spontaneous and synchronized-paced contractions.[58] They have also printed a 3D full-scale model of the human heart out of alginate, demonstrating the feasibility of large-scale 3D bioprinting to recapitulate organ structures. These alginate models may have applications in surgical training and planning.[59]

Improving bioinks for 3D printing

Sarah M. Hull from Sarah Heilshorn's lab at Stanford University presented work on developing novel bioinks to broaden the utility of 3D bioprinting. Bioinks are generally a mixture of cells and a polymer. After printing, the polymer is often crosslinked to stabilize the structure. The crosslinking strategy is specific to the polymer used. Common crosslinking approaches include light, pH, and salts. These methods can have off-target reactivity that may hinder cell function and viability. To address these concerns, Hull developed a bioink system termed UNIversal Orthogonal Network (UNION) bioprinting. UNION is a diffusion-based system that uses click chemistry to crosslink the bioink after it is printed into a support bath. UNION has many desirable features of an ideal bioink and is compatible with any water-soluble polymer that is amenable to conjugation chemistry, noncytotoxic, and extrudable into a support bath. Hull has made bioink materials that are both mechanically and biochemically customizable and showed how multiple inks can be crosslinked together to create more complex structures.[60] Hull envisions that this strategy will enable multimaterial and multicellular bioprinting of complex mimics of *in vivo* architectures. Toward that goal, Hull's group has used UNION to print corneas and is testing the feasibility of transplanting them into animal models.

Developing biomaterials to understand the microvasculature

Anjelica L. Gonzalez from Yale University discussed work creating human biomimetic materials, with a focus on microvasculature. The microvasculature is made up of endothelial cells and pericytes, the latter a stromal cell that guides the function and permeability of the microvasculature. Gonzalez's group is interested in understanding how inflammation and its resolution, or lack thereof, is mediated by the migration of cells like neutrophils into tissues through the microvasculature. They have isolated the microvasculature from organs, including the placenta, skin, lung, and retina, to understand its structure and complexity. She described how pericytes that line the microvasculature deposit thin sheets of ECM of various stiffness. Gonzalez's group has created various ECM mimics to imitate these native basement membranes. Control over the mechanics, composition, and architecture of these ECM mimics modulates the morphology of the pericytes around the microvascular. Her group is using this model to study disease. For example, she described a model of Sweet syndrome, a skin disease in which neutrophils are recruited into the interstitial tissue through the vascular wall. Gonzalez showed that the basement membrane in the microvasculature is thickened, causing pericytes to migrate away from the microvascular wall. Gonzalez put forth a model in which the endothelial cells of the vasculature regulate the barrier function of the pericytes via secretion of macrophage migration inhibitory factor, which ultimately inhibits pericyte barrier function and increases neutrophil transmigration. She stressed that it is important to consider the role of pericytes and their contribution to immune cell migration in inflammation. Gonzalez's work shows how multicellular models can be leveraged to understand how different cell types work together to impact other cells and to incorporate cell–basement membrane interactions. Her group is also evaluating neutrophil function in the basement membrane to understand which types of neutrophils enter the interstitial space and how they interact with the vascular wall.

PhASE-ExM: Super-resolution organoid imaging

Michael R. Blatchley from Kristi Anseth's lab at the University of Colorado Boulder presented work on a new method for expansion microscopy of organoids called phototransfer by allyl sulfide exchange-expansion microscopy (PhASE-ExM).[61] The complexity inherent in organoids makes them difficult to image. Conventional methods, such as 2D sectioning, often make it difficult to visualize the 3D context and

the ECM of the organoids. Methods are also limited to the resolution limitations of confocal microscopy. Expansion microscopy (ExM) was originally developed by Ed Boyden at MIT to address these limitations. ExM enables super-resolution imaging with standard confocal microscopy by physically enlarging and optically clearing biological samples.[62–64] However, there is generally a lack of control over the expansion factor, and ExM methods have not been previously used to image cells grown in synthetic hydrogels. Blatchley has built upon this technique by creating new tunable hydrogel formulations that polymerize in the presence of light, making ExM more user-friendly, enabling long-term storage of precursor solutions and opening up the possibility of using this method for thick samples, such as organoids, and for high-throughput applications. The method holds potential for widespread use among laboratories with a broad range of research interests, as it can be used for any type of sample, as long as the 3D cell culture platform can be degraded.

BIOENGINEERING OF ORGANOIDS

More mature kidney organoids to model disease

Núria Montserrat Puldio from the Institute for Bioengineering of Catalonia discussed her work on creating more mature, complex kidney organoids from hPSCs. Previous protocols to generate kidney organoids developed by Ryuji Morizane generated organoids containing hundreds of nephrons that resembles the human fetal kidney from the first trimester of development.[65] Montserrat Puldio showed that generating progenitors early in the differentiation timeframe and forcing cell–cell contact produced more highly differentiated, mature kidney organoids. Her protocol consists of first generating intermediate mesoderm progenitor cells and inducing them to differentiate into nephron progenitor cells. Bulk RNAseq data showed that the organoids resembled the fetal kidney during the second trimester. Montserrat Puldio showed the importance of the microenvironment for endothelial cell organization. Engrafting the organoids onto the vascular network of the chick chorioallantoic membrane (CAM) resulted in vascularized organoids with a better-organized endothelium. Growing the organoids on hydrogels that mimic the mechanical properties of the CAM promoted cell differentiation and the development of nephron-like structures.[66] Montserrat Puldio's group is continuing to enhance vascularization and explore the impact of cell–cell interactions to produce kidney organoids in a more robust, scalable manner.

Montserrat Puldio also presented data on using the kidney organoids to model disease, specifically to understand how SARS-CoV-2 infects cells. Kidney cells express the main receptor for viral entry, ACE2. A human soluble recombinant form of ACE2, hrsACE2, has been used as a therapeutic for SARS-CoV-1, acting as a decoy. Montserrat Puldio's group showed that hrsACE2 can inhibit viral infection in kidney organoids.[67–69] They are now using their platform to test other therapies as well as to model COVID-19 pathophysiology. Finally, Montserrat Puldio showed how they are using their kidney organoids to understand the link between SARS-CoV-2 infection and diabetes. Exposing kidney organoids to oscillatory glucose conditions can mimic a diabetic-like microenvironment.[70] The kidney organoids exhibit early hallmarks of the diabetic kidney, including increased ACE2 expression, which has been observed in mouse models of diabetes.[71,72] Montserrat Puldio shows that this diabetic milieu increased susceptibility to SARS-CoV-2 infection in the human kidney organoids due to both metabolic rewiring and increased ACE2 expression. Similar results were seen in kidney cells from diabetic patients.[70]

Using kidney organoids to identify therapeutic targets

Ryuji Morizane from Harvard Medical School presented work using a vascularized kidney organoid model to understand the mechanisms involved in different types of kidney disease and injury and to identify druggable targets. Morizane has developed a protocol to create nephron organoids from hPSCs.[73] Using these, he showed that single exposure to the chemotherapeutic cisplatin results in reversible damage to proximal tubule cells, similar to what is observed in acute kidney injury. In contrast, repeated exposure to cisplatin results in irreversible damage to the proximal tubules and morphological changes reminiscent of CKD—that is, the organoids become fibrotic, shrink, and have fewer tubules. Morizane's group has focused on the impact of cisplatin on DNA damage repair (DDR) genes, as initial cisplatin-related injury induces upregulation of DDR genes involved in homology-directed repair (HRD), whereas repeated exposure results in downregulation of these genes. Activating HDR genes in the CKD model reduced fibrosis and preserved tubular structures. Morizane also showed that HDR gene expression was reduced in the kidney in patients with CKD.[74] Together, these data suggest that HDR pathways may be a therapeutic target for CKD.

Morizane's group is also modeling genetic kidney diseases using a vascularized kidney organoid developed in collaboration with Jennifer Lewis's group at Harvard University. The platform uses a perfusion chip to apply fluidic stress to the organoid, which enhances organoid vascularization. Morizane showed that this fluidic stress is also imperative to properly model the genetic disease ARPKD. Previous static organoid models of ARPKD do not faithfully recapitulate the cystogenesis seen in human disease. Introducing fluidic stress, however, resulted in clinically relevant phenotypes, including dilatation of distal nephrons and cyst formation. Transcriptomics analysis of this kidney-on-a-chip identified hundreds of previously unidentified targets that are differentially expressed in ARPKD. Morizane's group has evaluated whether inhibition of these targets affects cyst formation in the ARPKD model, possibly providing insights into pathways to target in patients.[75]

Understanding early cell fate and the emergence of complex structures

Todd C. McDevitt, currently at Sana Biotechnology, presented work from his previous group at the Gladstone Institutes. McDevitt focused

on two stories that illustrate the early cell fate commitment and organizational events that occur before the organization of complex structures. In the first, McDevitt showed how his lab has developed an organoid model of the neural tube extension. Exposing human neural organoids to exogenous WNT induced robust uniaxial extension of the organoids, which otherwise remain spheroid. Single-cell analysis of the elongating organoids revealed two primary populations, neural and mesodermal lineages. They also observed anterior–posterior patterning of *HOX* expression that correlated with the expression of the genes in the developing spinal cord. For example, *HOXA1*, which is associated with the hindbrain, was expressed in the anterior regions of the organoid, while *HOXC9*, which is associated with the thoracic region, was expressed in the posterior region. In contrast, spheroid neural tube organoids do not express posterior *HOX* genes. The organoids also exhibited posterior accumulation and persistence of NMPs, which contribute to elongation. Inhibiting the WNT pathway in these NMPs via silencing of TBXT inhibited WNT-mediated uniaxial elongation, resulting in the protrusion of multiple epithelial buds from the spheroids. McDevitt's group is now using this model to better understand the early events that dictate cell organization and patterning in the developing CNS.[76]

During the second part of his presentation, McDevitt focused on efforts to create cardiac organoids. Cardiac development is dependent on contributions from surrounding tissues and multiple cell sources that have been difficult to recapitulate *in vitro*. McDevitt's group has developed a protocol to introduce cellular complexity and influence the commitment of an epicardial population within cardiac organoids by growing them in a multilineage medium. These multilineage organoids mimic human fetal development, demonstrating a persistent epicardial layer around the periphery. Over time, however, some of these organoids acquire a population of noncardiac cells, which were found by scRNAseq to be gut cells. Subsequent experiments showed that variation in the presence of an endoderm population in the early progenitor population determined whether the organoids would eventually develop gut cells. McDevitt's group identified factors associated with gut-enriched organoids and cardiac-enriched organoids and showed that the crosstalk between the mesoderm and endoderm is key to the ultimate fate of these multilineage organoids. McDevitt hopes that this model will provide insights into how distinct tissues coemerge from separate germ layers and a better understanding of multitissue interactions.[77]

Improving organoid maturation with synthetic biology

Deepak Mishra from Ron Weiss's lab at MIT discussed work using synthetic biology to enhance organoid maturation. Conceptually, Weiss' lab foresees introducing synthetic gene circuits into iPSCs cells that result in the cells sensing their state to direct different gene expression patterns based on that state. Weiss's group has developed a 3D liver organoid model based on the transient, inducible, heterogeneous expression of *GATA6*. Inducing a pulse of *GATA6* expression results in the rapid emergence of all three germ layers and a vascularized tissue that spontaneously develops a liver-like structure.[78] Mishra is working on understanding the spatial and temporal development of different cell lineages within this model. He also presented unpublished work on introducing conditional synthetic gene circuits based on a programmable RNA regulation platform developed in the Weiss lab[79] to induce hepatocyte maturation.

Delivering human organoids into a damaged intestine

Adriana Mulero-Russe from Andres Garcia's lab at Georgia Tech presented work on improving the delivery of intestinal organoids into humans to repair damaged mucosal lining caused by diseases like IBD. Previous work in the lab showed that human intestinal organoids can engraft and repair intestinal tissue after a small biopsy wound in mice. Wound healing was more effective when the organoid was delivered in the context of a biomaterial.[80] Mulero-Russe is working with Michael Helmrath at Cincinnati Children's Hospital, who previously showed that fragmented organoids can engraft in a mouse model of extensive intestinal wounds, but efficiency is low.[81] Mulero-Russe presented unpublished work on developing a hydrogel to enhance engraftment efficiency.

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COMPETING INTERESTS

The authors declare no competing interests.

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