ENGINEERING 3D TISSUE SYSTEMS TO BETTER MIMIC HUMAN BIOLOGY

ABSTRACT

Prioritizing the correlation of measured behavior of *in vitro* cell cultures to *in vivo*, "real life" biology entails embracing technically challenging science. Culturing cells on, or in, a 3D scaffold material is harder than culturing them on flat, optimized plastic. Accommodating the needs of multiple cell or tissue types simultaneously is more difficult than using only one. Perfused systems require pumps and flow paths. Static well plates do not. Yet, the additional challenge of incorporating these and other complexities can be rewarded with *in vitro* relevance to *in vivo* results that is orders of magnitude higher than that of 2D cell culture, and in some cases, surpassing whole organism animal models. The paradigm for *in vitro* research models is shifting to one designed to feature cell interaction circumscribing each cell in all its transecting planes, i.e. a fully 3D micro-environment, over experimental durations spanning weeks. Such experiments will incorporate purposed control of variables such as intensive and extensive scaffold properties, interstitial fluid environment, and cell density (all with consideration of the changes in these living systems over time) towards intentional influence on measurable indicators such as cell shape, interaction (cell-material, cell-solute and cell-cell) and differentiation pathway. The result is higher fidelity *in vitro* recreation of complex, human, *in vivo* biology, useful for more predictive models (drug discovery, personalized medicine) and more productive manufacturing and processing (tissue engineering, regenerative medicine, cell therapy).

INTRODUCTION

The scientific method – hypothesis driven design and execution of an experiment – is great, except when it could kill you (or me). That's why, for example, we go to great lengths to investigate new pharmaceutical agents using proxies of ourselves before testing the hypothesis "this drug candidate has acceptable toxicity" in a human clinical trial. We use a combination of non-living techniques (biochemical assays and *in silico* analysis), *in vitro* models (i.e. cell culture) and animal studies before allowing new compounds to be used in humans. Although our pharmaceutical and regulatory industries are doing the best they can within the current paradigm, to be blunt it's not going very well. According to recent publications, of all drugs that enter clinical trials, only 12% are ultimately approved for use in humans(Paul, Mytelka et al. 2010). Despite best efforts to predict those drug candidates' efficacy and toxicity during preclinical testing using conventional models of "us", 88% of them ultimately fail, mostly for those reasons, when finally put to the test in humans.

We need a new paradigm! The biggest opportunity lies in cell culture – I recently gave a talk to a group of high school STEM whiz kids titled "Your Petri Dish Is So 1887". This ubiquitous scientific container was first published on in

1887 for use with bacteria(Petri 1887), was already part of the background when cells were first widely cultured in the mid-20th century, and remains the predominant paradigm (taking the form of multi-well plates) of cell culture in 2012. The vast majority of human cell types are adhesion dependent, and after fluid transfer into a Petri dish or well plate they attach to the bottom. Once attached, they can proliferate so as to cover the entire bottom surface without stacking to form a confluent, flat monolayer (shorthanded as "2D" cell culture). As evidenced by usage patterns, normal limitations of this experimental mode (the environment is static, diffusion is passive, constant evaporation alters solute concentrations and requires frequent changes, cell numbers plateau at confluence, the cell experiences stimuli largely unrelated to the stimuli it experiences *in vivo*) are viewed as less important than benefits (cells grow well, cost effectiveness, 2D planes are easily imaged with inexpensive microscopes, existing body of data is 2D, granting agencies fund it, high throughput).

SIGNIFICANCE

Few people ask (and fewer answer) a basic question – do the results we get mean anything? Is it at all relevant to the intent of the experiment, which in most cases is to model a process occurring in the human body? Although the base assumption is "yes", in an increasing number of demonstrated cases, the answer to these important questions is actually NO. A quantitative way to measure the "behavior" of a cell in culture is its gene expression. In a beautiful demonstration of asking and answering this basic question, a comparison was made of key gene expression profiles of primary human cancers with comparative immortalized epithelial cells in 2D (Ridky, Chow et al. 2010). Tellingly, the correlation coefficient between the two data sets was 0.0. There are much easier and cheaper ways to obtain data sets with exactly zero correlation to the actual behavior one is trying to characterize, than to conduct 2D cell culture experiments.

The tremendous opportunity for improvement lies in the fact that cells are living organisms and can respond dynamically to local stimuli provided by and in their environment. **The solution is to provide a different environment with more of the "right" physical, mechanical and biochemical stimuli.** This challenge, when met, will affect much more than *in vitro* modeling of *in vivo* physiology. Aside from the desire to model ourselves and avoid the direct consequences of the scientific method for certain kinds of questions, better *in vitro* systems have enormous implications as both manufacturing methods for implants (tissue engineering and regenerative medicine) and as process steps for cell therapy.

ENGINEERING CELL SHAPE THROUGH MATERIAL INTERACTION

As a living entity, each cell has the potential to sense and respond to physical stimulus at each circumscribing point in all its transecting planes – i.e. its entire surface in three dimensions. When an adhesion dependent cell is presented with a flat surface to which it can favorably attach, it tends to maximize its adhesion and adopts a primarily flat morphology. Cells

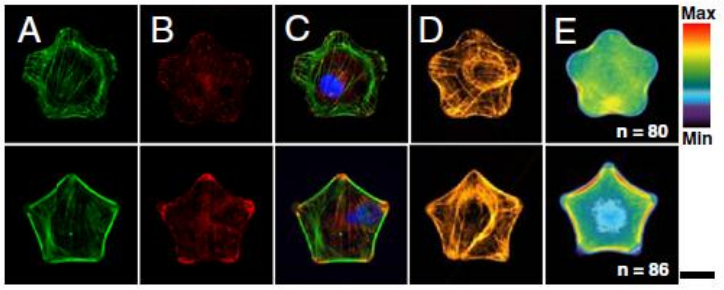
in a 2D paradigm tie up approximately 50% of this interaction capacity with the bottom surface of the well plate, approximately 50% of this capacity interacting with the liquid environment above the flat cell, and a very small amount in lateral cell-cell interactions. Thus, the fundamental value proposition of "3D" cell culture is to provide a microenvironment with the potential for physical interaction is distributed across the entire surface of the cell. This is normally achieved by culturing cells within a scaffold or matrix material, which can span gels, fibers or porous solids among others. Cells in such a 3D culture matrix adopt a more complex morphology (e.g. roughly ellipsoid) which is typically much closer to their morphology in their native state – that of a cell in tissue in a living organism.

Does this matter? Again relying on gene expression as a way to measure cell behavior, significant changes in gene expression profiles, recently genome-wide, of multiple cell types as a result of 3D relative to 2D cell culture conditions have been widely documented. These changes have been shown to be associated with key biological processes like tissue development, cell adhesion, immune system and defense response(Zschenker, Streichert et al. 2012). Thus, cell morphology is fundamentally deterministic of some important aspects such as cell behavior, signal transduction, protein-protein interaction, and responsiveness to external stimuli. This is one of many such studies, and in addition to being different from those observed in 2D, gene expression profiles in 3D are also shown to have much more relevance to those measured *in vivo*(Birgersdotter, Sandberg et al. 2005; Martin, Patrick et al. 2008).

In addition to the value of a 3D microenvironment that more effectively models *in vivo* realities, this form–function relationship is also subject to purposed manipulation towards less "natural" ends. Stem cells' differentiation pathway has been historically controlled by soluble factor interactions, either from a second "feeder" layer cell type or as a result of soluble factors added to the cells' media. Surprisingly, forcing a cell into a particular shape via physical confinement (in the absence of soluble factor manipulation) can also deterministically affect its differentiation pathway(Kilian, Bugarija et al. 2010). Stem cells forced into a variety of geometric shapes (e.g. the stars and flowers shown in Fig 1, top) differentiate down different paths as a result of these forced morphologies.

Unfortunately, effectively engineering the 3D microenvironment is not as simple as providing physical interactions in three dimensions. Topography and mechanical stiffness are among biophysical cues within a 3D context that affect cell function. This is proven out via both the addition of 3D topography (e.g. grooves, pillars, posts, pyramids, pits) to an otherwise flat surface via microfabrication techniques (wherein the cell is cultured <u>on</u> the material) and by incorporating controlled topography internally and culturing the cell <u>in</u> the material(Nikkhah, Edalat et al. 2012); notably topography can also induce effects which determine stem cell differentiation pathways(Kumar, Waters et al. 2012). Mechanical stiffness also

affects cell behavior and function, as exemplified by the presence of an "edge effect" within 3D gel scaffolds – tension within the gel decreases with increasing distance from the container surface. When focal adhesions of cells embedded in a 3D collagen gel were characterized, the number per cell was affected by the distance between the cell and the surface containing the matrix(Fraley, Feng et al. 2011). The authors were able to loosely qualify 2D (cell on surface), 2.5D (cell partially on surface), "3D near" (cell within 250 μm of surface) and "3D far" regions (Figure 1, right) based on the number of focal adhesions per cell.



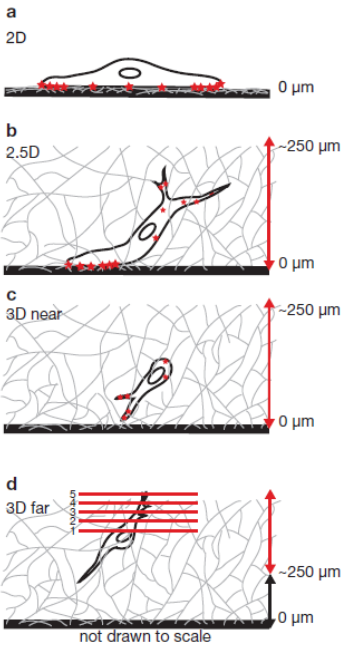


Figure 1 (Top) Immunofluorescent images and fluorescent heatmaps of cells in flower and star shapes demonstrating differential cell response to nuanced physical constraints ultimately influencing differentiation pathway. (Right) Schematic representation of focal adhesion visualization in live HT-1080 cells cultured at increasing distances (a-d) from the dish bottom, characterizing edge effect in 3D matrix.

ENGINEERING THE SOLUBLE ENVIRONMENT

In addition to the interaction of a given cell with the materials and other cells surrounding it, the soluble environment also has a profound effect on cell behavior. At the most basic level, it is through the soluble environment that cells are provided with nutrients, perform basic functions such as respiration, and release waste. Interference with these basic needs over time compromises the viability of the cell culture. Cells cultured on a 2D surface have nearly 50% of their surface area interacting with the soluble environment and simple, passive diffusion is more than sufficient to enable these processes. With frequent media changes to compensate for evaporation and for constant depletion of nutrients and generation of wastes, compromising the viability of 2D cell cultures due to insufficient soluble environment interaction is rarely a concern.

However, inherent in these soluble environment interactions is a cyclic change in the pH of the media and a "feast to famine" dynamic with respect to nutrient access, resulting from frequent replacement of cell media. Media pH in typical 2D

cell culture decreases as the time after the media change increases(Wu and Kuo 2011), and these differing pH levels have been shown to have consequences on cell function(Wu, Urban et al. 2007). The removal of "spent" media, containing relatively fewer nutrients and increased waste, also removes non-waste excretions (e.g. proteins) which may be directly related to the observable phenomenon that cells in 2D culture do not typically stack and are found as mono-layers when confluent. Beginning with cells in a typical 2D culture environment but using a specialized bioreactor which allowed nutrient and waste exchange but preserved insoluble extracellular matrix secretions, mineralizing, collagenous tissue up to 150 μm thick and featuring up to 6 cell layers were created (Dhurjati, Liu et al. 2006).

Due to the density of both the matrix and other cells, a particular cell cultured in a 3D scaffold has the potential for severely compromised soluble environment interactions resulting in cell necrosis, particularly in the middle of the construct. The window for effective density management is significantly smaller if the *in vitro* model relies on the passive diffusion that occurs with use of 3D scaffolds in static multi-well plates. For this reason, perfusion culture systems or bioreactors may be used to minimize or alleviate these effects, as well as to stabilize the soluble environment by avoiding feast-to-famine and maintaining pH. Compared with static conditions, perfusion cell culture has been shown to affect culture morphology and organization(Tomei, Siegert et al. 2009), increase key enzyme activity(Goldstein, Juarez et al. 2001), increase mineral deposition and production of protein and cytokines (Mercille and Massie 1999; Gomes, Sikavitsas et al. 2003), increase cell penetration into and distribution throughout the scaffold(Goldstein, Juarez et al. 2001; Gomes, Sikavitsas et al. 2003; Cimetta, Flaibani et al. 2007), increase cell viability especially at the center of cell-scaffold constructs (Mercille and Massie 1999; Cimetta, Flaibani et al. 2007) and to extend optimum cell viability and ultimately the effective duration of the culture experiment.

INCORPORATING BIOLOGICAL SYSTEMS EFFECTS WITH MULTIPLE CELL TYPES

Consideration of a particular cell's interactions with other cells is essential to increasing the correlation of its *in vitro* function and behavior to an *in vivo* organism. These interactions can take the form of direct cell-to-cell contact or of soluble factor interactions mediated by the soluble environment. Our complex human biology relies on both modes of interaction. Intercellular interactions that occur in culture of cells of the same type (monocultures) have been implicitly included in the previous themes discussed; indeed they are at least partially responsible for the morphological changes and functional benefits described. However, a second type of intercellular interaction can be modeled via co-culture of different types of cells, which can occur in the same culture chamber and create direct cell-to-cell contacts (a mixed co-culture) or can occur in multiple, separate chambers having a connected soluble environment through the exchange of soluble factors (a segregated

co-culture). A co-culture and the multi-cellular biological feedback loop it represents are necessary to reproduce many complex *in vivo* effects, which is not surprising given the many interacting physiological systems that combine to result in our complex human biology.

Co-culture provides yet another opportunity to engineer greater relevance into an *in vitro* model. As previously described, stem cell differentiation pathways are one of the best known multiple cell type interactions, whereby the differentiation of stem cell "A" is directed (or suppressed) by the presence of soluble factors from cell "B". Rivaling and perhaps surpassing stem cell co-cultures for scientific activity are cancer co-cultures, particularly cancer-stroma co-cultures – it is increasingly being demonstrated that the incorporation of a second cell type materially impacts cancer cells in culture(Khodarev, Yu et al. 2003), and boosts their relevance to the *in vivo* pathology(Chung, Baseman et al. 2005; Mahadevan and Von Hoff 2007).

LAYERING COMPLEXITY, PRACTICALLY

Incorporation of any of these themes (3D matrix microenvironment; actively stabilized soluble environment, mixed and segregated co-cultures) into an *in vitro* system represents an increase in complexity compared to standard 2D cell culture. Increased complexity is often associated with increased cost and time and decreased efficiency (often measured by throughput). These negative consequences are perhaps the largest reason these promising innovations have not achieved the wide utilization and rapid commercial uptake they were initially expected to. Thus, we find ourselves in a paradigm where correlation of *in vitro* models to *in vivo* results is poor, 88% of drug candidates fail in clinical trials, and where each successful drug costs approximately $1B to develop and launch(Deloitte 2011).

However, there are strong indications that we are moving into a new era of *in vitro* models. A first is that commercial examples of each of these individual innovations have become increasingly commonplace. With biologically derived gel matrices having led the way, there are now many commercially available scaffolds specifically marketed for 3D cell culture. Most commonly used in multi-well plates to maintain throughput but simultaneously limited by its static format, these scaffolds are primed for layering the additional complexities of a stabilized, actively perfused soluble environment and clever utilization of co-culture, potentially with multiple matrices matched to cell type. Basic co-culture has become widespread through the use of inserts fitted into the wells of multi-well plates and more recently via mixed co-cultures made possible by microfabrication techniques. Limitations for these first iterations include the static nature of multi-well plate culture and (for inserts) a narrow range of materials suitable as these membranes, but they have established important baselines that will be expanded with integration of more and better 3D physical and soluble microenvironments. Finally,

early bioreactor systems have demonstrated the clear benefits of perfusion but have suffered from having high per experiment costs, a requirement for atypical cell culture equipment and low throughput.

These first examples of successfully integrating a single innovation theme that acceptably increases complexity (i.e. is worth the trade-off) have laid the foundation for "layered complexity" approaches that may break new ground in adoption and utilization. In the United States, recent NIH and DARPA grant solicitations themed around modeling three and ten (respectively) interacting physiological systems were awarded to microfluidics "lab-on-a-chip" submissions (Figure 2, left). The microfluidics approach embraces perfusion systems at a micrometer scale (the scale of the cells themselves), while layering the complexity of co-cultures at various points in the fluidic channels and utilizing 3D matrix environments. Technical challenges include scaffold loading and recovery, "edge effects" of matrices, management of cell/matrix density over time, and successful maintenance of constant flow in channels with small dimensions. Another approach that successfully achieves the desired layered complexity may be thought of as "meso-fluidic", where culture chamber dimensions are on the scale of millimeters rather than micrometers (Figure 2, right). These approaches inherit the benefits of more traditional bioreactors, including the highest cell viability over time, best potential to model complexity and broadest incorporation and recovery of diverse 3D scaffold materials but also successfully layer the complexity of both mixed and segregated co-cultures and more controlled management of the soluble environment through smaller flow circuit volumes. Although cost may be mitigated to the extent that these smaller bioreactor systems can utilize the cost structure of 2D cell culture processes, lower throughput in meso-fluidic systems remains a tradeoff.

Figure 2: Examples of microfluidics (left, Wyss Institute) and meso-fluidics (right, KIYATEC Inc.) "layered complexity" *in vitro* systems. Both are perfusion based and incorporate 3D microenvironments and co-culture interactions but differ in scale, manufacturing techniques, cost and throughput.

Other non-technical factors are aligning with the emergence of such "layered complexity" technological approaches. The economic and political environments have changed such that there is an increased focus on the societal value derived from the expenditure of granting agencies' (and ultimately the public's) research monies. It is becoming less and less

acceptable to fund and conduct research which can be demonstrated to have a low, or zero, correlation to the real biology being modeled when alternatives with higher correlation exist, even though they are more complex. Funding agencies have been increasingly supportive of initiatives that mandate the incorporation of these layered complexities. Contractions within the global pharmaceutical industry have placed renewed emphasis on finding and adopting new approaches that drive down development costs as well as point towards new understanding of complex biology (and new targets, mechanisms and pathways). Successful regenerative medicine and cell therapy business models have emerged and heightened the demand for improved cGMP invitro manufacturing and quality control processes. And finally, increasing societal impetus (particularly in the EU) is also driving broader and faster adoption of more complex *in vitro* models and techniques showing good promise to refine, reduce and replace the use of animals. The combination of these technical, economic and political factors results in unprecedented opportunity and marshaled support to usher in a new paradigm for *in vitro* models.

FINANCIAL DISCLOSURE

Dr. Gevaert is the CEO of, and owns stock in, KIYATEC Inc., a company focused on *in vitro* models with higher correlation to *in vivo* results via convenient and cost effective, perfused, multi-cell or tissue 3D cell-based assays.

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