Application of a new miniature bioreactor system to generate and test artificial tumor and normal breast ductal tissues.

UWRF Tissue and Cellular Innovation Center
MICROSCOPY INNOVATIONS LLC

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Abstract

During the past decade, the UWRF Tissue and Cellular Innovation Center has been focused on application of natural extra-cellular matrix materials as biomimetic scaffolds for small-scale 3D artificial tissue (AT) and artificial tumor tissue (ATT) modeling. These constructs represent much more physiologically relevant in-vitro models than most standard 2D cultures and are now the focus of a series of studies aimed at developing new models of cancer progression and metastasis. Of particular interest is the modeling of breast adenocarcinoma and monitoring/testing of the progression of these constructs into metastasis-related processes. Although we have been successful in generating ATT's from several cells lines and primary patient tumors, our approach has always been limited by relatively non-standardized culture conditions. These conditions were consistent enough for basic construct characterization and general "proof-of-concept" validation but next-step direct experimentation has been limited by these culture conditions. In late 2013, we partnered with Microscopy Innovations, LLC to begin testing their mPrep capsules as miniature bioreactors in combination with our standard natural matrix/scaffold materials. In these studies, we are working to standardize culture conditions and develop a prototype reactor system approach to study MCF-7 breast adenocarcinoma cell-derived ATT's and their progression toward metastasis. Early loading studies of mPrep capsules with MCF-7, MCF10A non-cancerous ductal cells as well as stromal fibroblast and pre-adipocyte (3T3-Swiss and L1) cell lines have generated substantial tumor and control artificial tissue constructs. Ongoing characterization studies using flow cytometry and Western blot analysis are examining the relative population dimensions of cancer stem cells as well as EMT/MET markers and cell cycle status of both AT/ATT cells and the shed cells appearing in the flow-through effluent of the capsule chambers. To date, this system has shown very significant promise for in-vitro modeling of the complex relationship between tumors or control tissues and the surrounding fluid compartments in which they develop and function in vivo.

Natural de-cellularized 3-D extracellular matrix for in-vitro artificial tissue modeling.

The UWRF Tissue and Cellular Innovation Center (TCIC) is developing new 3D cell culture technologies to model and characterize various developmental and pathologic tissues. A primary focus is the use of de-cellularized extracellular matrix materials from several natural sources, including marine invertebrates, bovine trabecular bone and porcine intestinal sub-mucosa. All of these function as effective matrices for various cells, but the most flexible and therefore our primary material is the marine invertebrate matrix. We have established small-scale artificial tissues (ATs) from avian fetal primary isolates, porcine cardiac stem cells, human embryonic stem cells, approximately 20 ATCC cell lines and several primary patient samples from prostate, lung, colon, brain and breast tumors. In all cases, ATs were established from individual cells or tissue explants and cultured over extended periods of time as continuous "tissue-like" 3D structures. In most cases, tissue survival extended to at least 9 months of continuous culture and in a few cases the cultures have been maintained for more than 2 years. An observation in all these studies using cancer samples or cell lines, is that the 3D environment enhances tissue-specific differentiation. Furthermore, these differentiating cells then organize themselves into larger scale tissue-like structures which utilize most of the available scaffold areas.

Skeletal myoblast-derived AT development on natural 3D scaffolds.

The mouse skeletal myoblast cell line, C2C12 provides an example of tissue-like organizational development with non-cancerous cells on the marine de-cellularized ECM matrix. The bare matrix is shown in Figure 1 below. Matrix fibers actually define an enormous volume of open space into which cells and tissues will grow. Figure 2 illustrates this behavior with C2C12 cells growing out and bridging the openings between fibers with tissue-like structures which, in this case, develop into rudimentary artificial muscle tissues.

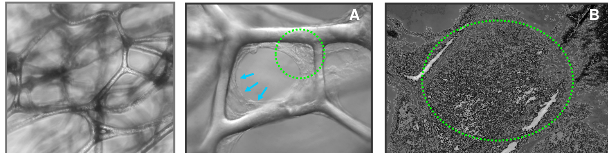


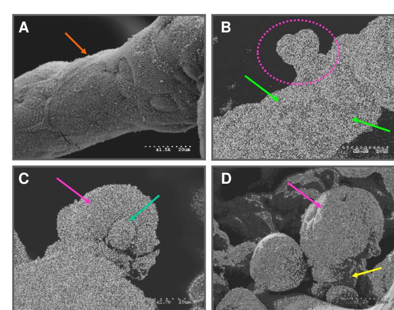
Figure 1: Survey view of natural de-cellularized extra cellular matrix (ECM) scaffold. Note that fiber diameters are between 25-35um, inter-junctional distances are between 150-350um, and open spaces are 150-450um across.

Figure 2 illustrates two main types of AT growth with C2C12. Note process extension from stationary cells around scaffold corners or junctions (blue arrows) and extension of sheet-like features across open areas (green circles). In panel "A", one corner is forming a sheet-like area while across from it there is also a newly formed "bridging" structure (blue arrows). These collective cellular activities generate large-scale muscle-like sheets and columns of tissue which show evidence of morphological and biochemical differentiation.

Natural 3D scaffolding fosters differentiation and tissue-like development of complex Artificial Tumor Tissues (ATT).

To develop a new analytic and discovery toolset for basic and translational cancer research, the TCIC has recently focused efforts on the MCF-7 breast adenocarcinoma cell-line and related MCF10A "normal" control ductal epithelium. Both cell lines successfully generate ATT's or AT's. Interestingly, MCF-7 cells also produce ductal tissue-like features, including hollow ascinar structures with associated branching support structures.

Figures 3a-d illustrate the tissue-like structural details of MCF-7 ATTs grown on 3D scaffolds for more than 1.5 months. Panel "A" shows a region of relatively smooth and flat cells surrounding the matrix scaffold fiber. Cells are clearly epithelial-like and are covered with a blanket of microvillus structures (orange arrow). Panel "B" shows a region in which cells are individually far more rounded (green arrows). This layer has also sprouted a pair of cells "standing" away from the surface (purple circle). Our interpretation is that these cells represent the starting-point in development of a new acinar structure. Panel "C" illustrates an acinar feature (purple arrow) with a characteristic pinched cell at it's center (blue arrow). Panel "D" shows another larger round structure which may have developed a luminal cavity. There is also evidence here of secondary acinar-like budding on the side of the larger cell mass. The entire feature is also tethered to the scaffold by a narrower pedestal (yellow arrow). These panels appear to illustrate the process of differentiation and the development of relatively normal tissue-like features from a population of ductal carcinoma cells when grown in 3D culture.



Miniature bioreactor system to standardize 3D AT/ATT culture.

Although our efforts to employ natural scaffolding materials for 3D culture of small-scale artificial tissues have successfully illustrated the effectiveness of this toolset for basic and applied cancer research, there are a number of technical issues and limitations to the application of this approach more broadly in a clinical context. To date, our efforts have utilized conventional 6 well plates for the growth of AT's or ATT's and 96 well plates for loading the scaffolds. While effective to some degree, such culture systems do not mimic physiologic tissue conditions and are extremely limited in scalability. Although our matrix approach worked to generate tissue-like structures, it has been very difficult to design workable experiments beyond simple characterization studies, due to our lack of control of the tumor microenviroment.

We feel that an answer to this problem is provided by a novel micro-capsule product called the mPrep System. Each mPrep capsule can function as a flow-through bioreactor which can be readily coupled with our prior small scale 3D AT/ATT development. These capsules are widely used in electron microscopy specimen preparation and multiple applications in 3D cell culture. With the mPrep micro-bioreactor system we can standardize culture conditions for our natural scaffold-based ATT's and open the way for development of a new 3D culture toolset that is expected to give us extremely fine control on numerous parameters and enable the next step in our 3D culture applications.

Each mPrep capsule can become an individual and independent incubator chamber. These bioreactor chambers have direct inflow and outflow which allows media to pass through the AT or ATT in a manner analogous to interstitial fluid or blood. This provides for the fine regulation of fluid flow as well as nutrient, pH and gas gradients within developing 3D constructs. In addition, this arrangement enables the introduction of test substances such as therapeutic agents, cytokines, hormones or other bioactive compounds into the environment of the developing tissue construct. It is expected that this technology will enable the TCIC to enhance and expand our already robust 3D scaffold culture capacity with precisely controllable physiologic conditions as well as a unique ability to capture individual tumor effluents in high concentration. Essentially modeling tumor interstitial fluid flow. Once this overall bioreactor system has been fully developed and tested, the "Histo-Genesis" system is expected to allow for implementation of new and powerful translational medicine applications of 3D ATT cultures.

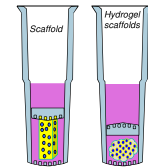


Figure 4 shows that mPrep capsules can be used to entrap many types of tissues, scaffolds or other specimens. Examples shown here are a solid 3D preformed scaffold (our current method) and a loaded "in-place" cellularized hydrogel matrix (currently planned next phase).

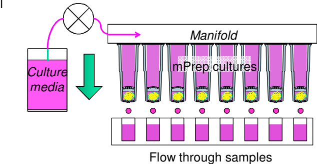


Figure 5 presents the basic concept for the mPrep-based "Histo-Genesis" system. Capsules are connected into a manifold system to feed media and/or treatment substances individually into each unit (arrow shows flow direction). Media that has passed through culture bioreactors is then captured for individualized analysis. Arrow indicates overall flow patterns.

Prototype tests demonstrate that mPrep capsules can be used as bioreactors to develop "living biopsy" samples.

To explore the potential and test the concept of an mPrep-based "Histo-Genesis" system, we have tested the enclosed culture of normal and cancer cell-line based artificial tissues. In our first studies; successful loading trials were conducted with stromal 3T3-L1 and 3T3-Swiss lines as well as artificial tumor tissue studies with MCF-7 adenocarcinoma and MCF10A breast ductal cell lines. In each case, we successfully loaded, established and grew significant ATs or ATTs for up to one month. Outlined below is a representation of those early studies focused on MCF-7 breast cancer constructs.

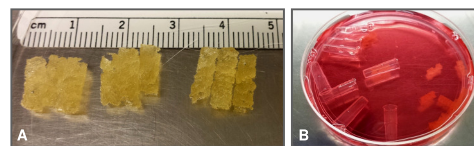
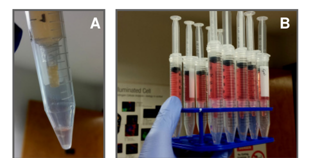


Figure 6a-b (left) shows the preparation of 3D scaffold materials cut to ~1 cm long cylinders in panel "A" and the capsules/scaffolds being soaked in preparation for actual loading in panel "B". Once the units are prepared, suspended cells are drawn into the capsules from below.

Figures 7a-b (right) illustrate a loading experiment in progress. Panel "A" depicts an individual mPrep capsule loaded with scaffold and ~1-5 x 10^6 cells. Once loaded these suspended cells are allowed to adhere for 24-48 hours and then excess or dead cells are removed by flow-through. For preliminary experiments, cells are fed every 24 hours by simple manual activation of flow-through with one complete volume change (~200ul). Panel "B" shows a set of test tumor and control ATT's and AT's respectively, in the early phase "Histo-Genesis" apparatus. Assembled and loaded units are then incubated at 37°C and the media within the syringe reservoir changed when it becomes depleted or shifts substantially in pH as determined by phenol red indicator.



"Histo-Genesis" system effectively supports AT and ATT development and culture.

This series of early phase "Histo-Genesis" prototype tests successfully showed that mPrep capsules can indeed be used to generate and maintain both normal ATs and neoplastic ATTs.

Figure 8 (left) presents a representative sample of MCF-7 ATT generated and cultured for 22 days within an mPrep early phase prototype "Histo-Genesis" system. Tumor nodules or colonies are clearly seen covering one end of the scaffold and to a lesser degree at the other end as well (green circles). Between this robust growth, in the central region, the yellow arrow shows areas where scaffold fibers are sheathed with cells, but less frequently form the nodular structures seen at the ends of the scaffolds. This entire structure is approximately 1 cm in length (blue bar) and had been further cultured for three days following removal from the capsule at the time of this image.

Early mPrep-based ATTs grow and expand in similar manner to those grown on control scaffolds.

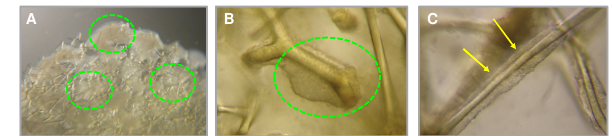
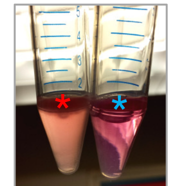


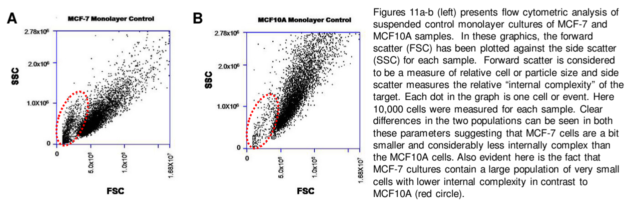
Figure 9a-c (above) details the growth of MCF-7 cells/tissues on the ATT shown in figure 8. Panel "A" illustrates a low magnification view of upper most end of the scaffold where colonies or nodules (green circles) have begun to grow into each other forming larger scale "tissues". Panel "B" illustrates one such colony formed near cut ends of scaffold material. These nodules commonly form near the ends of the scaffolds in mPrep chambers, unlike 6 well dish samples. Although nodules are also seen in more central regions at cross-over points between multiple fibers as well. Panel "C" illustrates an area in which tissue has formed a cylindrical cell layer a few cells deep on long scaffold fibers with no evidence of colonies or nodule formation.

Flow-through effluent in early "Histo-Genesis" prototype experiments demonstrates the versatility and effectiveness of this system.

One of the more interesting features of the "Histo-Genesis" miniature bioreactor system is the captured flow-through effluent. This fluid essentially represents in-vivo tumor-specific interstitial fluid and as such provides us with an enormously useful and powerful window on the tumor and it's current state of growth as well as potential malignancy.

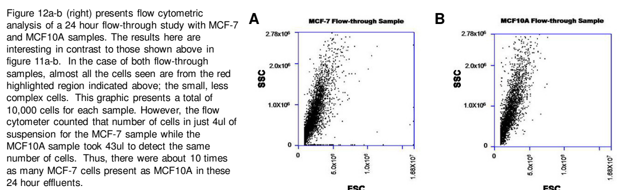
Figure 10 (right) shows the results of a 24 hour flow-through study with MCF-7 and MCF10A ATs/ATTs with 6 tubes of each sample type being pooled to generate ~1 ml of sample. The red star indicates the pooled sample from MCF-7 cultures after 5 days and the blue arrow indicates the same pooled sample from the control breast ductal cell line MCF10A. The tumor derived sample is basically turbid with shed, suspended cells and spheroids, while the normal control sample has perhaps at least 10 times fewer cells and appears relatively clear.





Figures 11a-b (left) presents flow cytometric analysis of suspended control monolayer cultures of MCF-7 and MCF10A samples. In these graphics, the forward scatter (FSC) has been plotted against the side scatter (SSC) for each sample. Forward scatter is considered to be a measure of relative cell or particle size and side scatter measures the relative "internal complexity" of the target. Each dot in the graph is one cell or event. Here 10,000 cells were measured for each sample. Clear differences in the two populations can be seen in both these parameters suggesting that MCF-7 cells are a bit smaller and considerably less internally complex than the MCF10A cells. Also evident here is the fact that MCF-7 cultures contain a large population of very small cells with lower internal complexity in contrast to MCF10A (red circle).

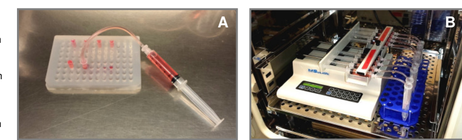
Figure 12a-b (right) presents flow cytometric analysis of a 24 hour flow-through study with MCF-7 and MCF10A samples. The results here are interesting in contrast to those shown above in figure 11a-b. In the case of both flow-through samples, almost all the cells seen are from the red highlighted region indicated above; the small, less complex cells. This graphic presents a total of 10,000 cells for each sample. However, the flow cytometer counted that number of cells in just 4ul of suspension for the MCF-7 sample while the MCF10A sample took 43ul to detect the same number of cells. Thus, there were about 10 times as many MCF-7 cells present as MCF10A in these 24 hour effluents.



Evolution of the "Histo-Genesis" bioreactors generates an automated delivery system which provides constant media flow-though.

Over the course of more than two years, the concept of the "Histo-Genesis" miniature bioreactor system has grown and undergone a series of evolutionary steps based on engineering issues and driven by the biological necessities of maintaining artificial tissues under near-physiological conditions. After establishing that the concept works with our simple phase one system, we have now moved to an automated syringe pump format which supplies constant media changeover and effluent capture.

Figure 13a-b (right) shows the arrangement of the newest phase IV "Histo-Genesis" system which is based on an automated syringe pump system. In panel "A", the capsules are loaded with pre-established ATs or ATTs and then mounted on syringes. Panel "B" shows these units in place on the pump system within the incubator unit.



Summary and future directions.

In this poster, we have presented a brief overview of 3D AT and ATT generation, development and study at the TCIC as well as an introduction to the new mPrep "Histo-Genesis" system concept with preliminary data. To date, we have shown that the "Histo-Genesis" system is capable of providing a critically controlled microenvironment which will enable us to proceed with development of a new translational medicine toolset called the "living biopsy." This concept provides for the establishment of personalized and patient specific ATT at the time of biopsy or surgical resection of a tumor and testing/analysis of this material to provide clinicians with a unique biological perspective on each case of caner that occurs. We expect this approach to greatly enhance therapeutic design and so to improve individual patient outcomes.

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